microbiotec 09

28|29|30
Novembro '09
Vilamoura
Algarve

Book of Abstracts of MicroBiotec09

ORGANIZATION & SPONSORS

ORGANIZATION

Universidade do Minho
Dep. de Engenharia Biológica

FCT FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA
DEP. CIÊNCIAS DA VIDA

spbt sociedade portuguesa de biotecnologia

Sociedade Portuguesa de Microbiologia

IBB
INSTITUTE FOR BIOTECHNOLOGY AND BIOENGINEERING

CREM
CENTRO DE RECURSOS MICROBIOLÓGICOS

SPONSORS

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Quilaban

Roche

FUNDAÇÃO CALOUSTE GULBENKIAN

FUNDAÇÃO LUSO-AMERICANA

BIO-RAD

ORDEM DOS BIÓLOGOS

Caixa Geral de Depositos

Fisher Scientific

VWR

alfagene

STAB vida
The Home for Gene Technology

ELNOR
EQUIPAMENTOS TÉCNICOS E DE LABORATÓRIO, S.A.

nzytech
genes & enzymes

SARSTEDT

paralab

BRITISH COUNCIL

LIDEL
www.lidel.pt

Baptista Marques

TURISMO DE PORTUGAL algarve

UNIVERSIDADE CATÓLICA PORTUGUESA
FACULDADE DE CIÊNCIAS ECONÓMICAS E EMPRESARIAIS

BOOK OF ABSTRACTS OF MICROBIOTEC09

BOOK OF ABSTRACTS OF MICROBIOTEC09

28-30 NOVEMBER 2009, VILAMOURA, PORTUGAL

ORGANIZED BY

SOCIEDADE PORTUGUESA DE BIOTECNOLOGIA

SOCIEDADE PORTUGUESA DE MICROBIOLOGIA

INSTITUTE FOR BIOTECHNOLOGY AND BIOENGINEERING (UNIVERSIDADE DO MINHO - DEPARTAMENTO DE ENGENHARIA BIOLÓGICA)

CENTRO DE RECURSOS MICROBIOLÓGICOS (UNIVERSIDADE NOVA DE LISBOA, FACULDADE DE CIÊNCIAS E TECNOLOGIA – DEPARTAMENTO DE CIÊNCIAS DA VIDA)

EDITED BY

THE ORGANIZING COMMITTEE OF MICROBIOTEC09

This volume contains abstracts presented at the MICROBIOTEC09 Meeting, held in Vilamoura, Portugal, between November 27th and 30th, 2009.

Title: **Book of Abstracts of MICROBIOTEC09**

Edited by The Organizing Committee of MicroBiotec09 (José A. Teixeira, Ilda Sanches, Eugénio Ferreira, Álvaro Fonseca, António Vicente, Armando Venâncio, Duarte Oliveira, Filomena Martins Pereira, Isabel Couto, Lucília Domingues)

First edition November 2009

ISBN: 978-972-97810-6-3

Cover design: Helena Martins www.helenamartinsdesign.com

Publisher:

Universidade do Minho, Departamento de Engenharia Biológica

Campus de Gualtar, 4710-057 Braga, Portugal

This publication was sponsored by:

FCT Fundação para a Ciência e a Tecnologia

MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Foreword

This book contains the abstracts presented at the 3rd joint meeting of the Portuguese Society of Microbiology and The Portuguese Society of Biotechnology - MicroBiotec09, held in Vilamoura, Portugal, over 3 days, from the 28th to the 30th of November, 2009. MicroBiotec09 comes in the sequence of previous conferences organized by each society, since 1982, date of the I Encontro Nacional de Biotecnologia (Lisbon), till 2005, date of the first joint meeting - MICRO'05 + BIOTEC'05 (Póvoa de Varzim). Following this joint meeting, another - MICRO 07 + BIOTEC 07 + XXIII JPG took place in Lisbon (2007).

MicroBiotec09 is a joint organization of "Sociedade Portuguesa de Biotecnologia", "Sociedade Portuguesa de Microbiologia", Institute for Biotechnology and Bioengineering (Universidade do Minho – Departamento de Engenharia Biológica) and Centro de Recursos Microbiológicos (Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia – Departamento de Ciências da Vida).

MicroBiotec09 brings together both young and established researchers and end users to discuss recent developments in different areas of Biotechnology and Microbiology. The conference program has thus been divided in 8 major sessions: Microbial Physiology, Molecular Biology and Functional Genomics; Cell and Tissue Engineering, Biomaterials and Nanobiotechnologies; Clinical Microbiology and Epidemiology; Environmental Microbiology and Biotechnology; Health and Pharmaceutical Biotechnology; Cellular Microbiology and Pathogenesis; Industrial and Food Microbiology and Biotechnology; Bioinformatics, Comparative Genomics and Evolution.

A special session to celebrate the 200th anniversary of Charles Darwin's birth and the 150th anniversary of the publication of his landmark work "On the Origin of Species by Means of Natural Selection" will also take place.

A total of 295 abstracts are included in the book, consisting of 6 invited lecturers, 10 oral presentations and 44 short oral presentations given in 3 parallel sessions, along with 4 slots for viewing poster presentations. All abstracts have been reviewed and we are grateful to the members of scientific and organizing committees for their evaluations. It was an intensive task since 328 submitted abstracts were received.

It has been an honor for us to contribute to setting up MicroBiotec09 during an intensive period of 6 months. We wish to thank the authors who have contributed to yield a high scientific standard to the program. We are thankful to the sponsors who have contributed decisively to this event. We also extend our gratefulness to all those who, through their dedicated efforts, have assisted us in this task.

On behalf of the Scientific and Organizing Committees we wish you that together with an interesting reading, the scientific program and the social moments organized will be memorable for all.

November 2009

The Organizing Committee

Committee Members

Scientific Committee:

Arsénio Fialho (IST, IBB)

Cecília Leão (UM, ICVS)

Eugénio Ferreira (UM, IBB)

F. Xavier Malcata (UCP, CBQF)

Guilherme Ferreira (UAlg, IBB)

Helena Santos (ITQB/UNL, ITQB)

Hermínia de Lencastre (ITQB/UNL, ITQB)

Ilda Sanches (FCT/UNL, CREM)

Isabel Sá Correia (IST, IBB)

Joaquim Cabral (IST, IBB)

José A. Teixeira (UM, IBB)

José Melo-Cristino (FM/UL, IMM)

Júlio Novais (IST, IBB)

Manuel Carrondo (FCT/UNL, IBET)

Manuel Mota (UM, IBB)

Manuel Santos (UA)

Margarida Casal (UM, CBMA)

Miguel Prazeres (IST, IBB)

Milton Costa (FCT/UC)

Nelson Lima (UM, IBB)

Pedro Moradas Ferreira (UP, IBMC)

Rogério Tenreiro (FC/UL, BioFIG)

Virgílio do Rosário (IHMT/UNL, CMDT-LA)

Organizing Committee:

José A. Teixeira (UM, IBB) - Co-Chair

Ilda Sanches (FCT/UNL, CREM) - Co-Chair

Eugénio Ferreira (UM, IBB) - Co-Chair

Álvaro Fonseca (FCT/UNL, CREM) - Co-Chair

António Vicente (UM, IBB)

Armando Venâncio (UM, IBB)

Duarte Oliveira (FCT/UNL, CREM)

Filomena Martins Pereira (IHMT/UNL, CREM)

Isabel Couto (IHMT/UNL, CREM)

Lucília Domingues (UM, IBB)

Table of Contents

Plenary Sessions

The dynamics and control of intracellular replication of *Salmonella*

David W. Holden

Imperial College London, Department of Microbiology, Faculty of Medicine, South Kensington Campus, London SW7 2AZ

E-mail: d.holden@imperial.ac.uk

Abstract

My laboratory developed signature-tagged mutagenesis as a high throughput technique to identify genes that are required for microbial growth in different environments. Initially we used the *Salmonella*/mouse model of typhoid to establish the feasibility of the method, and in the process discovered genes of the SPI-2-encoded type III secretion system, which is required for intracellular bacterial growth. Since then we have studied the organization of SPI-2, regulation of its genes, and several effector proteins that it delivers across the vacuolar membrane into epithelial cells and macrophages.

Recent work by our group and others has revealed the biochemical functions of some of the effectors, but how they work to promote intracellular growth is still far from understood. In this talk I will discuss the contribution of SPI-2 to bacterial growth and propose a new mechanism for regulation of effector translocation.

Pathogen genomics, the age of discovery

Matthew Holden

The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

E-mail: mh3@sanger.ac.uk

Abstract

The genome of an organism is the template for the components of the cell, which enable it to thrive and survive. Mapping the genome through sequencing allows scientists to explore the biology of an organism in a more holistic manner. New horizons in research have opened up with the development of genomics. Since the first bacterial genome was published in 1995, the complete genomes of nearly one thousand bacteria have been deposited in the public sequence databases. One group of organisms that have been intensively sampled is pathogens. Comparative genomic analysis using these data has provided new insights into the genetic basis of host specificity, pathogenesis and evolution. In this talk I will illustrate this, with examples from a comparison of two closely related streptococci, which has shed light on the emergence of a novel pathogen.

Much of the sequence data currently available has been produced with first generation sequencing technology, i.e. Sanger chemistry and capillary sequencing. With the arrival of second generation sequencing technology platforms in recent years, the scale and scope of sequencing that can be undertaken has dramatically increased. I will describe how the new sequencing technologies are being applied to undertake high-resolution genotyping for large numbers of strains, and therefore investigate bacterial population diversity on an unprecedented scale.

Audacity of Personalized Medicine: Marrying OMICS to PHRs?

Eugene Kolker

Seattle Children's Research Institute

E-mail: eugene.kolker@seattlechildrens.org

Abstract

OMICS Premises: New discovery-based, synthesis and integrative approaches to research are being used to complement traditional, hypothesis-based, reductionist, and subtractive approaches.

OMICS Promises: Widen, accelerate and transform your research capabilities.

Personal Health Records: Shift control over your health into your hands.

This presentation addresses Data Analyses challenges related to OMICS and PHRs and integration thereof.

Meeting the Modern Demands of Applied Life Sciences and Biotechnology: the Concept of Biological Resource Centres

Dagmar Fritze[1,2]

1: DSMZ- German Collection for Microorganisms and Cell Cultures, Inhoffenstrasse 7b, 38124 Braunschweig, Germany
2: President of the European Culture Collections' Organisation

Abstract

High quality research in the life sciences and innovative solutions to global problems requires access to high quality biological materials and associated information. The evolution of the biotechnology industry and its application to agriculture, health, chemical or energy industries ultimately depends upon our ability to harness the potential of biodiversity and all it has to offer: to improve health outcomes, boost the productivity of agriculture and industrial processes, and enhance environmental sustainability.

The EU initiative for a knowledge based bio-economy considers the transformation of knowledge from the life sciences into new, sustainable, ecologically efficient and competitive products as an enormous challenge. The recent OECD Report *The Bioeconomy to 2030: designing a policy agenda* (2009) emphasises that the biological sciences are adding value to a multitude of products and services, thus calling for its own economic sector: the so-called bioeconomy. The expectation is that by 2030 the products of white biotechnology and bioenergy will constitute around a third of the industrial production.

Microbial resource collections consist of laboratory held, living biological material that can be exploited for global research cross cutting the agricultural, food, healthcare and biotechnological sectors. The holdings of service collections are like a cornucopia, a rich source from which can be drawn as demands come up. Being charged with the task of accessioning and supplying living biological material, such service culture collections play a central role between the interests of various user communities. On one side are the providers of living biological material: individual scientists, institutions and countries of origin. On the other side are the various kinds of recipients/users of cultures of microorganisms from academia and industry. Additionally, culture collections also act as mediators of related up-to-date information and technology of relevance not only to academia and industry but also for regulatory bodies.

Providing access to biological material and scientific services while at the same time observing donor countries' rights, intellectual property rights, biosafety and biosecurity aspects poses demanding challenges. Particular issues are the topics 'patenting', 'CBD rights of country of origin, stakeholder rights, access and benefit sharing', 'biosafety / biosecurity'. All three topics relate in the end to the availability of biological material, and to the terms under which a particular biological (microbiological) material is accessible.

BRCs will provide the necessary underpinning of biotechnology, providing the living tools to drive research and bioeconomy. Consequently, Biological Resource Centres are needed around the world, equipped with techniques and expertise to cope with the depth and breadth of emerging biodiversity; providing access to high quality biological material and scientific services while at the same time observing donor countries' rights, intellectual property rights, biosafety and biosecurity aspects. The challenge for biotechnologists and culture collections is to keep abreast of developments in taxonomy and systematics, as well as new methods for the authentication and identification, cultivation and maintenance of cultures. As this will be especially difficult on an individual basis, cooperation and harnessing the power of networking on a national, regional and global level will be the only way forward to achieve sustainable support for the various shades of 'green', 'red' or 'white' biotechnology.

Metabolic and evolutionary engineering of bakers' yeast for bioethanol production

Jack Pronk

Kluyver Centre for Genomics of Industrial Fermentation & Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Email: j.t.pronk@tudelft.nl

Abstract

Yeast-based production of bioethanol from crude hydrolysates of lignocellulosic plant biomass requires fast and efficient, anaerobic fermentation of complex sugar mixtures. In addition to glucose and other readily fermentable sugars, such hydrolysates contain xylose and arabinose, whose conversion by bakers' yeast (*Saccharomyces cerevisiae*) requires metabolic engineering. Indeed, metabolic engineering has yielded *S. cerevisiae* strains with good xylose and arabinose fermentation characteristics are now available. However, their performance during growth on defined sugar mixtures and in real-life hydrolysates is usually less than during growth on single sugars in laboratory media.

Sequential use of carbon sources, which is typically observed during anaerobic fermentation of sugar mixtures by *S. cerevisiae*, is usually interpreted in terms of repression/induction of pathways and kinetic interaction of the substrate (e.g. competition for common transporters). Because the potential involvement of different processes in mixed substrate utilization, evolutionary engineering offers an attractive tool for optimization. A new evolutionary engineering regime, which was designed to apply balanced selective pressure on each of the sugars in a mixture, resulted in fast alcoholic fermentation of glucose-xylose-arabinose mixtures by engineered *S. cerevisiae*.

Expanding the range of sugars that can be used by *S. cerevisiae* is not the only challenge in bioethanol production. In addition to sugars, plant biomass hydrolysates contain compounds that inhibit yeast growth and fermentation. A notable example is acetic acid, which is present in all common feedstocks for bioethanol production. Based on recent research in our laboratory, I will discuss how metabolic engineering can turn the presence of acetic acid into an advantage.

Satellite Session

Support:

Microbes and the tree of life: fulfilling Darwin's dream

Álvaro Fonseca

Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica

E-mail: amrf@fct.unl.pt

Abstract

The idea that all life on Earth shares a common evolutionary history is one of Darwin's outstanding contributions to modern biology. Common descent is often illustrated by genealogical trees that portray the chronological relationships among species, both contemporary and ancestral. The first phylogenetic trees based on morphological, physiological and palaeontological observations were published during Darwin's lifetime, but many of the relationships depicted therein were merely hypothetical and had only little experimental support. Microbes were already present on some of those trees, but the possibility of drawing fairly accurate, all-inclusive phylogenetic trees had to wait until the advent of molecular biology and bioinformatics in the last quarter of the 20th century. Those trees were based on nucleotide sequence comparisons of conserved homologous genes from organisms as different as animals, plants, fungi, protists and bacteria. The so-called universal tree of life led to revolutionary classification proposals, such as that put forth by Carl Woese and co-workers. The initial opposition to their proposal from the biological community was no doubt linked to the rather inconspicuous place of humans and other animals on a highly entangled and diverse tree where microbes are clearly prominent. Darwin had only dreamt about the possibility of reconstructing such a tree, but he was certainly aware of Man's modest placement on its branches. Modern evolutionary biology has now demonstrated without a shadow of a doubt that not only was Darwin's belief entirely correct, but that microbes are the most ancient and diverse living beings on this planet. It's up to microbiologists to ensure that this view of life is evident to all our fellow humans, many of whom haven't quite grasped the deep implications of Darwin's visionary ideas.

Oral Session:
S1 – Microbial Physiology, Molecular Biology and Functional Genomics

Reference

386

Identification of receptors of the quorum sensing signal autoinducer-2

Pereira, Catarina S. (1, 2); Regt, Anna K. (3); Miller, Stephen T. (3); Xavier, Karina Bivar (1,2)

1: Instituto Gulbenkian de Ciencia, Portugal;
2: Instituto de Tecnologia Quimica Biologica, UNL;
3: Swarthmore College, USA

E-mail: kxavier@igc.gulbenkian.pt

Keywords: quorum sensing, cell-cell signal, interspecies signalling

Abstract

Although a variety of bacterial species have been reported to use the inter-species communication signal autoinducer-2 (AI-2) to regulate multiple behaviors, the molecular mechanisms of AI-2 recognition and signal transduction remain poorly understood. To date, two types of AI-2 receptors have been identified: LuxP, present in *Vibrio* spp, and LsrB, first identified in *Salmonella typhimurium*. In *S. typhimurium*, LsrB is the ligand binding protein of a transport system that enables internalization of AI-2. Here, using both sequence analysis and structure prediction, we establish a set of criteria for identifying functional AI-2 receptors. We test our predictions experimentally, assaying key species for their ability to import AI-2 *in vivo* and test their LsrB orthologs for AI-2 binding *in vitro*. Using these experimental approaches, we were able to identify AI-2 receptors in organisms belonging to phylogenetically distinct families such as Enterobacteriaceae, Rhizobiaceae, and Bacillaceae. Phylogenetic analysis of LsrB orthologs strongly suggest that this pattern results from one single origin of the functional LsrB gene in a g-proteobacteria with posterior independent events of lateral gene transfer to the a-proteobacteria and Firmicutes. Finally, we used mutagenesis to show that two AI-2 interacting residues are essential for AI-2 binding ability. These two residues are conserved in the binding site of all the functional AI-2-binding proteins but not in the non-AI-2 binding orthologs. Together, these results strongly support our ability to identify functional LsrB-type AI-2 receptors, an important step in investigations of this inter-species signal.

Reference

354

Altered apoptotic signaling in the yeast model for Batten disease

Osório, Nuno S. (1); Almeida, Agostinho J. (1); Carvalho, Agostinho (2); Leão, Cecília (1); Sousa, Nuno (1); Ludovico, Paula (1); Laranjinha, João (3); Rodrigues, Fernando (1)

1: Life and Health Sciences Research Institute (ICVS), University of Minho, Portugal;
2: Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy;
3: Faculty of Pharmacy and Center for Neurosciences and Cell Biology, University of Coimbra, 3000 Coimbra, Portugal

E-mail: nosorio@ecsaude.uminho.pt

Keywords: Batten, Neuronal Ceroid Lipofuscinosis, Lysosomal Storage Diseases, Model organisms, Oxidative Stress, Nitric Oxide

Abstract

Juvenile neuronal ceroid lipofuscinosis (JNCL), also known as Batten disease, is a fatal recessively inherited neurodegenerative disorder. The major clinical features of this disease are vision loss, seizures and progressive cognitive and motor decline starting in childhood. Mutations in CLN3 are known to cause the disease, allowing the generation of research models in different organism that are powerful tools for Batten disease research. BTN1 is the *Saccharomyces cerevisiae* CLN3 orthologue and is localized to the yeast vacuole. We found that the yeast model for Batten disease obtained by the deletion of BTN1 (btn1-Δ) has increased resistance to oxidative stress. Expression of human CLN3 complemented this btn1-Δ phenotype, and equivalent BTN1/CLN3 mutations correlated with JNCL severity. We show that this phenotype is caused by limited endogenous synthesis of nitric oxide (•NO) in btn1-Δ and that it can be reverted by addition of L-arginine to the medium. This defect in •NO synthesis seems to suppress the signaling required for yeast oxidative stress-induced programmed cell death, thus explaining btn1-Δ phenotype of increased resistance. Subsequently, we have also demonstrated the presence of a defect in •NO synthesis in the brain of a mouse model for Batten disease supporting the significance of our findings. Presently, in an attempt to further elucidate the link between BTN1/CLN3 dysfunction and altered NO signaling, we are evaluating the regulatory interplay between L-arginine and calcium levels in the cytosol and vacuoles of btn1-Δ cells. Our studies indicate that altered •NO synthesis may be present in cells from Batten disease patients and possibly interfere with neurotransmission. To gain further understanding on the basis of the •NO synthesis defect may result in relevant advances in our knowledge of the pathological mechanisms underlying Batten disease.

PTCD/SAU-NEU/70161/2006 FCT

Reference

104

Phage SPP1 entry into *B. subtilis* cells: identification of SPP1 tail proteins involved in the recognition of host receptors

Baptista, Catarina (1); Auzat, Isabelle (2); São-José, Carlos (1); Santos, Mário A. (3); Tavares, Paulo (2)

1: Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal;
2: Unité de Virologie Moléculaire et Structurale, CNRS-INRA, Gif-sur-Yvette, France;
3: Departamento de Biologia Vegetal, Faculdade de Ciências da Universidade de Lisboa, Lisbon, Portugal

E-mail: cbaptista@fm.ul.pt

Keywords: bacteriophage, adsorption, receptor binding protein, receptor, B. subtilis

Abstract

Phage SPP1 targets two different receptors of the *B. subtilis* cell surface during adsorption, the glucosylated teichoic acids of the bacterial cell wall (WTA) and the membrane protein YueB (1). SPP1 interaction with WTA is reversible but it greatly increases the rate of irreversible binding to YueB, which is responsible for the triggering of SPP1 DNA ejection (2). We have recently proposed a mechanism by which fast adsorption to and desorption from WTA allows SPP1 to effectively scan the cellular surface until it is captured by YueB (1). In the work presented here we searched for SPP1 tail proteins or substructures specifically recognizing WTA and YueB.

The available evidence suggested that gp21, one major component of the SPP1 tail tip, could be responsible for YueB recognition (2, 3), although the direct interaction between these two proteins has never been experimentally demonstrated. To that end we used a YueB-affinity matrix to specifically capture tail substructures or proteins. The source of these components were protein extracts produced from infections with mutant phages SPP1*sus31* and SPP1*sus45*, which are defective for the production of the major head (gp13) and major tail proteins (gp17.1), respectively. SPP1*sus31* infection of a non-permissive *B. subtilis* strain produced tails and tail tips, whereas SPP1*sus45* produced heads and tail tips. We show that the YueB-affinity matrix specifically captured a ~135 kDa protein. This molecular weight is compatible with that expected for gp21 (~124 kDa). Analysis by MALDI-TOF mass spectrometry of the trypsinated, gel-excised 135 kDa band revealed gp21 as first hit when using the ProFound tool. These results indicate that gp21 or a gp21-containing substructure is responsible for binding to YueB.

Based on the knowledge obtained in the kinetics of SPP1 adsorption (1) we designed a method to selectively isolate SPP1 mutants impaired in reversible adsorption. Sequencing of gene *21* in several mutants revealed two single amino acid substitutions in the gp21 C-terminal end. These mutations were corrected after recombination with a wild type copy of gene *21*, which restored normal adsorption to the recombinant phages. In conclusion our results strongly suggest that the tail tip protein gp21 is involved in both types of adsorption.

1. Baptista et al., 2008. J. Bacteriol. 190: 4989-4996.
2. São-José et al., 2006. J. Biol. Chem. 281:11464-11470.
3. Plisson et al., 2007. EMBO J. 26: 3720–3728.

Reference

201

Transcription regulation in *Archaea* – characterisation of the virus coded regulator SvtR

Peixeiro, Nuno (1); Guillière, Florence (2); Desnoues, Nicole (1); Guijarro, J.Iñaki (2); Prangishvili, David (1); Sezonov, Guennadi (1)

1: Institut Pasteur, Unité de Biologie Moléculaire du Gène chez les Extrémophiles, F-75015 Paris, France;
2: Institut Pasteur, Unité de RMN des Biomolécules, CNRS URA 2185, F-75015 Paris, France

E-mail: nuno.peixeiro@pasteur.fr

Keywords: *Archaea*, Crenarchaeal Virus, Sulfolobales, Transcription, Transcription Regulation

Abstract

In the *Archaea*, that constitute the third domain of Life, important biological processes, such as replication, reparation and transcription, are performed by specialised proteins resembling the ones used by Eukaryotes. The transcription is assured by a RNA polymerase that, by its composition and activity, represents a simplified version of the eukaryal RNA Polymerase II. The basal transcription machinery is completed with two transcriptional factors with close homologues in the eukaryal world, a TATA-box binding protein (TBP) and a transcription factor B (TFB), that ensure that the RNA polymerase recognizes the promoter. Surprisingly, the transcriptional regulators identified so far within the *Archaea* resemble in structure and, in most cases, in mechanism to regulators from the Bacterial domain.

We have characterised the function and the structure of a small archaeal transcriptional regulator, protein SvtR (*Sulfolobus* virus transcription regulator), coded by the rod-shaped SIRV1 virus. This virus infects the hyperthermophilic archaeon *Sulfolobus islandicus* that thrives at 85 °C in acidic hot springs. The SvtR protein forms a dimer in solution, binds to DNA using its β-sheet and presents a ribbon-helix-helix (RHH) fold, very similar in structure to those of bacterial RHH proteins despite the low sequence similarity. In order to detect all the binding sites on the 32,3 kb SIRV1 linear genome, we designed and performed a global genome-wide search of targets based on a simplified electrophoretic mobility shift assay. Four targets were recognised by the protein, the strongest of which was observed with the promoter of the gene coding for the virion's three tail fibers. SvtR binds to this promoter region 83bp upstream of the gene's TATA-box. When assayed in a host reconstituted *in vitro* transcription system, protein SvtR repressed transcription of both this structural gene and of its own gene.

SvtR is the first deeply characterised viral coded transcriptional regulator from Crenarchaea and belongs to a new group of archaeal transcriptional regulators that are able to regulate genes located distant of the main binding site. This novel type of regulation has never been described in this domain of life.

Reference

351

Oxidative stress during chronological life span in *Saccharomyces cerevisiae*

Mesquita, Ana (1); Silva, Alexandra (1); Sampaio-Marques, Belém (1); Almeida, Bruno (1); Logarinho, Elsa (2); Leão, Cecília (1); Rodrigues, Fernando (1); Ludovico, Paula (1)

1: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal;
2: IBMC, Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal

E-mail: ammesquita@ecsaude.uminho.pt

Keywords: Oxidative stress, chronological life span, *Saccharomyces cerevisiae*

Abstract

Saccharomyces cerevisiae is an accepted model for studying longevity by providing key insights into both genetic and environmental factors that can control aging [1]. Yeast life span can be assessed either by the replicative life span (RLS) of mother cells [2] or by the chronological life span (CLS) of quiescent cells during the stationary phase of growth [3]. During CLS, the response to oxidative stress has been suggested of particular importance, however, a clear cause-effect relationship between oxidative stress and CLS modulation has been difficult to establish. Oxidative stress arises from a significant increase in the concentration of reactive oxygen species (ROS) and/or a decrease in their detoxification mechanisms. In this scenario, mitochondria and peroxisomes play a major role contributing to the balance between ROS generation and the activity of enzymatic and non-enzymatic antioxidant systems that scavenge or reduce ROS concentrations. Our results support, according to previous reports, a protective effect of mitochondria in CLS modulation as demonstrated by the reduction of CLS in *rho0* cells. However, these cells still displayed high levels of intracellular ROS accumulation, suggesting that other ROS sources, namely peroxisomes, may contribute to CLS modulation. The study of the peroxisomes's role on CLS showed that cells with altered peroxisome and peroxisomal catalase function had an extension of CLS, suggesting a negative role for these organelles during CLS. Moreover, confocal microscope analysis showed that aged cells present reduce amounts of peroxisomal catalase located in peroxisomes, suggesting an inefficiently import of this enzyme into this organelle. Thus, contrary to mitochondria, a negative contribution for peroxisome in CLS modulation is suggested.

This work was supported by a grant from FCT - Fundação para a Ciência e Tecnologia, PTDC/AGR-ALI/71460/2006. A.M, A.S., B.S.M. and B.A. have fellowships from FCT, Fundação para a Ciência e Tecnologia (SFRH/BD/32464/2006, SFRH/BD/33125/2007, SFRH/BD/41674/2007 and SFRH/BD/15317/2005; respectively).

[1] Guarente, L. (2000). Sir2 links chromatin silencing, metabolism and aging. Genes Dev 14, 1021-6.
[2] Mortimer, R.K. and Johnston, J.R. (1959). Life span of individual yeast cells. Nature 183, 1751-2.
[3] Longo, V.D. (1999). Mutations in signal transduction proteins increase stress resistance and longevity in yeast, nematodes, fruit flies, and mammalian neuronal cells. Neurobiol Aging 20, 479-86.

Reference

184

A novel regulator of the expression of beta-lactam resistance in *Staphylococcus aureus*

Arede, Pedro (1,2); de Lencastre, Hermínia (2,3); Oliveira, Duarte C. (1,2)

1: Centro de Recursos Microbiologicos, FCT/UNL,Caparica Portugal;
2: Instituto de Tecnologia Quimica e Biologica, FCT/UNL, Oeiras, Portugal;
3: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA.

E-mail: pedroarede@fct.unl.pt

Keywords: MRSA, Antibiotic Resistance, mecA, mecR2

Abstract

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen both in hospitals and in the community. The *mecA* gene, the central genetic element of methicillin resistance, which confers cross-resistance to all b-lactams, is regulated by a sensor-inducer (*mecR1*) and a repressor (*mecI*). However, some molecular details on the induction mechanism remain to be elucidated and the existence of other yet unidentified regulatory genes has been speculated.

Comparative genomic sequence analysis revealed that, in some clinical MRSA strains, a putative third regulatory gene (*mecR2*) might be involved in the control of b-lactam resistance expression. In this study, we performed experiments in order to confirm the role of the putative *mecR2* in the phenotypic expression of broad-spectrum b-lactam resistance.

Experiments

(i) We constructed a *mecR2* null mutant in a prototype MRSA strain by an insertion-deletion strategy. The resistance levels to oxacilin (a methicillin analogue) were evaluated by diffusion disk, microdilution and population-analysis profile. A dramatic decrease in the oxacillin resistance was observed for the *mecR2* mutant when compared to the parental strain.

(ii) The *mecR2* transcription was evaluated by reverse-transcriptase PCR in a set of prototype clinical MRSA strains in the presence and absence of oxacillin. As a control, the transcription of *mecA* was also monitored. In all strains tested, the *mecR2* transcript could be detected in the oxacillin-induced cultures, paralleling the *mecA* induction.

(iii) The putative coding region for MecR2 was cloned in the *E. coli* pET system under the control of an inducible promoter. SDS-PAGE analysis of crude extracts revealed the presence of a band with the predicted size of the recombinant MecR2 in induced cultures, which increased in intensity with the induction time and was absent in non-induced cultures.

Conclusions

The *mecR2* mutant phenotype confirmed that this determinant interferes with the expression of b-lactam resistance, probably at the level of *mecA* transcription. Moreover, we could demonstrate that *mecR2* is transcribed in response to b-lactams in prototype clinical MRSA strains and that it can be expressed in *E. coli*. We purpose that MecR2 may function as anti-repressor or a co-activator of the *mecA* transcription.

Poster Session:
S1 – Microbial Physiology, Molecular Biology and Functional Genomics

Reference

103

Genetic analysis of antibiotic resistance of *Enterococcus* spp. strains recovered from Seagulls of Berlengas natural reserve in Portugal

Radhouani, Hajer; Pinto, Luís; Poeta, Patrícia; Igrejas, Gilberto

University of Trás-os-Montes and Alto Douro, Portugal

E-mail: hradhouani@utad.pt

Keywords: Antimicrobial resistance, *Enterococcus* spp., genes, proteins, VRE

Abstract

Background: *Enterococcus* spp. is intrinsically resistant to many antimicrobials and their ability to acquire resistance to others agents is well identified. The analysis of proteomics technology continues to improve, it can combine information of genomics, proteomics and metabolics in the near future, to obtain a more accurate picture of anti-microbial applications.

Objective: Genomic and proteomic characterization of the antimicrobial resistance of faecal *Enterococcus* spp. strains from seagulls.

Methods: 1. Faecal samples were sampled in Slanetz-Bartley agar plates supplemented with 4 mg/L of vancomycin. 2. Antibiotic susceptibility was tested for 11 antibiotics by the disk diffusion CLSI method. 3. Polymerase chain reaction was used to identify the species and to determine genes encoding antibiotic resistance. 4. Separation of proteins by 1DE (SDS-PAGE). 5. Separation of proteins by 2DE (IEF x SDS-PAGE).

Results: From the 57 seagull samples, 54 faecal samples showed the presence of *enterococcus* isolates (94.7%). The majority of the tetracycline-resistant strains carried *tet* (M) and/or *tet* (L). The *erm* (B) gene was detected in 63.2% of the erythromycin-resistant isolates. The *vat* (D) and *vat* (E) genes were present in 8.3% and 16.7% of the quinupristin/dalfopristin-resistant isolates, respectively. The *ant* (6)-Ia gene was present in 57.1% of the streptomycin-resistant isolates and seven of the nine kanamycin-resistant isolates carried the *aph* (3)´-IIIa gene. The *cat* (A) gene was found in one of the chloramphenicol-resistant isolates. The VRE strains were detected in 6 of the 57 seagull samples (10.5%). All the VRE were *van*A-containing enterococci. The *tet* (M) gene was found in all 5 tetracycline-resistant *van*A strains. The *erm* (B) gene was demonstrated in all the *van*A strains. In our study were observed 15 different protein profiles by monodimensional gel electrophoresis. Profiles of the same species show qualitative variations, which corresponds to a complete correlation between the whole cell protein profiles and results of conventional biochemical tests identification. Our results may reflect the expression of a few membrane proteins involved in antibiotic resistance. This is also in agreement with the results of a preliminary study of protein expression in two *van*A-containing enterococci strains using analytical 2DE.

Conclusion: In this study it has been showed a relatively high diversity of proteins.

Reference

116

Clues to the mechanism of transcriptional regulation of two cell wall genes in *Staphylococcus aureus*

Grilo, Inês Ramos (1); Sobral, Rita Gonçalves (1); Ludovice, Ana Madalena (1, 2); de Lencastre, Hermínia (1, 3); Tomasz, Alexander (3)

1: Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Laboratory of Molecular Genetics, Rua da Quinta Grande nº6, Apt 127, 2780-156 Oeiras, Portugal;
2: Faculdade de Ciências e Tecnologia (FCT) of Universidade Nova de Lisboa, Department of Biotechnology, 2829-515 Monte da Caparica, Portugal;
3: The Rockefeller University, Laboratory of Microbiology, 1230 York Avenue, New York, NY10065, USA

E-mail: inesgrilo@itqb.unl.pt

Keywords: *Staphylococcus aureus*, dcw cluster, transcriptional regulation.

Abstract

In contrast to most bacterial species, in *S. aureus* the genes related to cell wall biosynthesis and cell division are not all organized in a single cluster (*dcw* cluster), but in several smaller islands which are spread throughout the chromosome. One of such mini-clusters is composed by *ddlA* and *murF* genes, the protein products of which are responsible for the dimerization of D-alanine and the addition of the dipeptide to the peptidoglycan muropeptide precursor, respectively. We have previously determined that *ddlA* and *murF* genes, ordered sequentially in the chromosome, are part of the same transcriptional unit. A conditional mutant was constructed in which only the *murF* gene was under the control of the IPTG inducible promoter, while the *ddlA* gene remained under the control of its native promoter (1). Transcription analysis performed for *ddlA* and *murF* independently for the conditional mutant, by RT real-time PCR indicated that *murF* transcription was inhibited or enhanced depending on the inducer concentration. Surprisingly, *ddlA* gene behaved in the same way. Therefore, we aim to explore the mechanism by which *murF* expression regulates the transcription of the whole operon.

For this purpose several approaches were used, including mapping of the cluster promoter by primer extension, characterization of the regulatory role of the regions neighbouring the promoter by activity assays (promoter fusion using *xylE* as reporter gene), gel-shift mobility assays using different cellular extracts (from COL and from the *murF* conditional mutant) and DNA-protein binding strategies using DNA coated streptavidin beads as bait.

The promoter sequence was determined to be distant from the translation initiation codon by more than 200 bp. Promoter fusion results indicate that the inhibition of *murF* transcription does inhibit the expression from the *ddlA-murF* native promoter and that specific regions are implicated in this process, possibly due to the binding of an unknown repressor/activator protein. To identify such protein, parallel DNA-binding protein purification assays are presently being performed, using the 300 bp region surrounding the promoter as bait.

The understanding of the regulation mechanisms behind the transcription of the *ddlA-murF* mini-cluster, may help to elucidate the regulatory linkage between the cell division process and the cell wall biosynthesis pathway.

1 - Sobral et al., 2006. J. Bacteriol. 188 (7): 2543.

Reference

121

Interaction Studies of a conserved membrane-bound complex in SRB: the QmoABC complex from *Desulfovibrio desulfuricans* ATCC 27774

Ramos, Ana Raquel; Venceslau, Sofia S.; Pereira, Inês A.C.

Intituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal

E-mail: arramos@itqb.unl.pt

Keywords: Membrane-bound complex; Sulfate reduction; *Desulfovibrio*; protein interaction; Surface Plasmon Resonance

Abstract

In the anaerobic respiration of sulfate, performed by sulfate reducing bacteria, reduction of the terminal electron acceptor takes place in the cytoplasm. The membrane-associated electron transport chain that feeds electrons to the cytoplasmic reductases is still very poorly characterized. Genome analysis of sulfate-reducing organisms sequenced to date permitted the identification of only two strictly conserved membrane complexes, pointing to an essencial role in sulfate respiration. One of these complexes, the QmoABC was isolated from the membranes of *Desulfovibrio desulfuricans* ATCC 27774 [1]. The complex is composed of three subunits and contains two hemes *b*, two flavin adenine dinucleotide groups and several iron-sulfur centers. The macroscopic redox potencials of the two hemes *b* in the Qmo complex were determined to be -20 and +75mV, which are in a suitable range to be involved in electron transfer from menaquinol to APS.

Interaction experiments performed with Surface Plasmon Resonance support the proposal that QmoABC interacts with APS reductase. Thus, the QmoABC complex provides a link between the menaquinone pool and the cytoplasmatic reduction of sulfate.

[1] Pires, R.H. et al, 2003, BBA 1605, 67-82

Reference

125

Formate metabolism in *Desulfovibrio vulgaris* Hildenborough

Silva, Sofia M. da; Pereira, Inês A. C.

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal

E-mail: sdasilva@itqb.unl.pt

Keywords: Formate, Sulfate-Reducing Bacteria, Formate Dehydrogenases, Hydrogen

Abstract

Desulfovibrio vulgaris Hildenborough (DvH) is a model organism for the study of Sulfate-Reducing Bacteria (SRB). The sulfate respiratory chain is still poorly understood and there seems to be several pathways involved in the energy conservation, such as hydrogen or CO cycling. Hydrogen is an important energy source for SRB in natural habitats, and previous studies showed that growth with H_2 leads to the up-regulation of formate dehydrogenases (Fdh) and pyruvate-formate lyase in DvH [1], suggesting that formate cycling provides an alternative pathway for energy generation.

The DvH genome codes for three different Fdhs. In this work we show that the formate dehydrogenase activity of cells grown in H2/sulfate increases several fold over lactate-grown cells. We isolated the three DvH Fdhs, including FdhABC3, FdhAB, a soluble heterodimeric protein, and the soluble subunits of the membrane-bound Fdh, CfdABCD. The three Fdhs show significant differences in activity profile. The growth conditions, including metal composition of the culture medium, influences the expression profile of the three Fdhs.

1. Pereira, P.M., He, Q., Valente, F.M.A., Xavier, A.V., Zhou, J., Pereira, I.A.C., Louro, R.O., Antonie Van Leeuwenhoek, 2008. 93(4): p. 347-362.

Reference

127

Real-time fluorometric evaluation of P-glycoprotein inhibitors in cancer cells

Spengler, Gabriella (1,2); Viveiros, Miguel (1); Martins, Ana (1,2); Rodrigues, Liliana (1,2); Molnár, Joseph (3); Amaral, Leonard (1,2)

1: Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL), Portugal;
2: Unidade de Parasitologia e Microbiologia Medicas (UPMM), IHMT/UNL;
3: Institute of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Hungary

E-mail: gspengler@ihmt.unl.pt

Keywords: ABC-transporters, P-glycoprotein, fluorometry, flow cytometry

Abstract

In tumour cell lines, multidrug resistance is often associated with an ATP-dependent decrease in cellular drug accumulation related to the over-expression of the members of the ATP-binding cassette (ABC) transporter superfamily. In the course of chemotherapy, further mutations and selection may greatly increase the expression of multidrug transporters, which protect the tumour cells against chemotherapy.
The real-time fluorometry uses the fluorochrome ethidium bromide (EB), which is considered as a common substrate of bacterial efflux pumps. Ethidium bromide has been shown to be particularly suitable to be used as a probe because it emits weak fluorescence in aqueous solution (outside cells) and becomes strongly fluorescent in non-polar and hydrophobic environments. Based on former results with bacterial transporters, we employed this methodology to detect and demonstrate the activity of the eukaryotic ABC-transporter P-glycoprotein (Pgp or ABCB1).
The effect of an agent on the Pgp is normally conducted with the use of flow cytometry. This involves the employment of a fluorochrome substrate such as rhodamine 123 which is extruded by the Pgp transporter and which is increasingly retained if the transporter is inhibited. The aim of the real-time fluorometric method is to easily and accurately detect and quantify the transport of the Pgp substrate EB through the cell membrane, at working concentrations that will not affect cell viability nor perturb cellular function, in order to readily assess efflux activity in neoplastic cells.
In our studies we used L5178 mouse T-cell lymphoma cellstransfected with human *ABCB1* gene and classical MDR modulators such as verapamil and reserpine to standardize the method.
The design of potent and selective P-gp inhibitors requires extensive screeningof compounds of natural or synthetic origin. The fluorometric assay is a new application of the RotorGeneTM 3000 (Corbett Research, Sydney, Australia) real-time thermocycler and provides information about transport kinetics thereby offers a rapid, high-troughput, reproducible, accurate and inexpensive screening of Pgp-inhibitors.

Viveiros M et al (2008) Int J Antimicrob Agents 31: 458-462.
Spengler G et al (2009) In Vivo 23(1):81-87.
Spengler G et al (2009) Anticancer Res 29(6):2173-2177.

Reference

132

Molecular diagnosis of sheep's subclinical mastitis from Baixo Alentejo region

Guerreiro, Olinda Rosa Fragoso das Neves (1); Costa, Manuela (2); Canada, João (2); Duarte, Maria de Fátima (1)

1: Centro de Biotecnologia Agrícola e Agro-Alimentar do Baixo Alentejo e Litoral (CEBAL), Beja, Portugal;
2: Instituto Politécnico de Beja - Escola Superior Agrária, Beja, Portugal

E-mail: olinda.guerreiro@cebal.pt

Keywords: sheep's subclinical mastitis, molecular diagnosis, Baixo Alentejo

Abstract

Mastitis is an important disease, which consists in infection and inflammation of the udder, with great impact in bovine, ovine and caprine, affecting directly the mammary gland, and considered to be one of the major causes of dairy industry losses. A great majority of mastitis have a subclinical presentation without visible signs, or any other symptoms. This subclinical form generally induces milk composition alterations causing an: increase of somatic cell count (SCC), decrease in casein, lactose and fat contents reducing the milk yield and quality. Among the various pathogens causing ovine mastitis, *Staphylococcus aureus* is recognized to be the most frequent pathogen isolated in subclinical cases, however there are others responsible agents, such as *Streptococcus agalactiae, Strept. uberis* and *Strept. dysgalactiae*. The molecular diagnosis by Polymerase Chain Reaction (PCR) technique allows a rapid and efficient answer to the farmers, leading to a more reliable treatment and animal traceability. In this study, were analyzed 89 sheep's raw milk samples from local farms, plus 5 bulk milk from a cheese make factory from Baixo Alentejo. The chemical properties of the different milk samples were analyzed using the Milkoscan (FOSS) determining the protein, fat, lactose, total solids and solids non-fat. Genomic DNA was extracted by cellular lise using Proteinase K, followed by PCR reaction using universal primers. Further, the contaminated samples were studied using specific primers for pathogen identification. Our results demonstrated that none of the chemical milk properties were modified, although we identified by molecular methodology 25 contaminated samples with *Staphylococcus aureus,* whichrepresents 49% off all studies cases, other agents, such as *Strep. uberis*, *Strep. agalactiae* and *Strep. dysgalactiae* were also identified.
The molecular method developed can be easily incorporated as part of routine screening either for individual or milk bulk samples, in the early detection of subclinical infected sheep's.

Fthenakis, G.C.; Jones, J.E.T. (1990). Br. Vet. J., I46: 43-49.
Riffon, R.; Sayasith, K.; Khalil, H.; Dubreuil, P.; Drolet, M.; Lagace, J. (2001). Journal of Clinical Microbiology, Vol. 39 (7), 2584–2589.

Reference

136

Microbial nucleic acids employed in diagnostics, sequencing and phylogenetics are subject to detrimental inhibitors and mutagens

Paterson, Robert Russell Monteith; Lima, Nelson

IBB/Centre for Biological Engineering, University of Minho, 4710-057 Braga, Portugal

E-mail: russell.paterson@deb.uminho.pt

Keywords: nucleic acid, mutagens, inhibitors, isoepoxydon dehydrogenase

Abstract

Sequencing the genomes of microbial species has increased tremendously. Nucleic acids (NA) are used also for diagnostic and phylogenetic analyses of microbes. It is essential that protocols ensure representative NA. The effect of the "spent" growth medium on NA has not been considered. Surprisingly, fungi are grown on media that support inhibitors and mutagens when producing NA for these purposes[1,2,3]. This situation is illogical as these secondary metabolites may affect the structure of NA[2] and/or inhibit PCR polymerases used in PCR[3].

The objective of the work was to highlight how NA analyses could be affected by self produced mutagens and inhibitors. Hence, (a) PCR of the *idh* gene of patulin production in fungi (e.g. *Penicillium expansum*) and (b) interpretation of the scientific literature were employed to determine the seriousness of the situation. Analysis of *idh* was successful for culture dependant PCR (CDP) and culture independent PCR (CIP). A reversible inhibition was observed in CDP presumably from inhibitors in cultures. Inhibition was observed in CIP. In some cases, taxa which were predicted to be positive for *idh* were not, and *vice versa*. A logical interpretation of this was that the gene was mutated by cultural components. In addition, the PCR reaction may have been inhibited and internal amplification controls (IAC) are required.

The conclusions were that it is illogical to grow microbes for NA analysis in a milieu of mutagens and inhibitors. Reports on diagnostic methods and phylogenetic schemes are undermined consequently. Work on *Aspergillus flavus* ismost vulnerable to this criticism, as they produce aflatoxins which are the most carcinogenic natural compounds. Numerous fungi produce inhibitors and mutagens and so the problem is widespread. There may be an equivalent situation for bacteria[2,3]. It is essential to grow microbes in a manner to avoid mutagens and inhibitors. Some recommended procedures would be to grow the cultures for a shorter period, although the ratio of mutagen to NA is important. Analysing cultures immediately upon isolation is preferred. Continuous culture could be used to avoid secondary metabolism. Finally, IAC are required for PCR in general.

1. Paterson, RRM. (2004) Mycol Res, 108, 1431-1437.
2. Paterson, RRM, Lima, N. (2009) J Appl Microbiol, 106, 1070–1080.
3. Paterson RRM. (2007) J Appl Microbiol, 102, 1-10.

Reference

167

Polymerization of *Bacillus subtilis* FtsZ – the importance of using the right pH

Scheffers, Dirk-Jan (1); Gündogdu, Muhammet (2); Kawai, Yoshikazu (2); Errington, Jeff (2); Hamoen, Leendert (2)

1: Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Portugal;
2: Institute for Cell and Molecular Biosciences, Newcastle University, UK

E-mail: djscheffers@itqb.unl.pt

Keywords: cell division, FtsZ, MinC, SepF, polymerization

Abstract

Bacterial cell division starts with the formation of the Z-ring, composed of polymers of the bacterial tubulin homologue FtsZ. *In vitro* studies of FtsZ polymerization are generally carried out in buffers of slightly acidic pH (pH 6.5) which is very conducive for FtsZ polymerization. We have started to perform *in vitro* studies of FtsZ polymerization in the presence of proteins that regulate FtsZ ring formation. Our work has revealed that both a negative regulator (MinC) and a positive regulator (SepF) of FtsZ polymerization in *Bacillus subtilis* are not functional in 'standard', pH 6.5, polymerization buffers. When we increased the pH to physiological levels (pH ~7.5) we found a significant increase in the effect of MinC on FtsZ polymerization. SepF, which is not stable at pH 6.5, is stable at pH 7.5 and forms remarkably large ring structures. The SepF rings are capable of bundling FtsZ polymers in large tubular structures. Our findings stress the importance of buffer choice in the studies of FtsZ polymerization.

Reference

170

One to one amino acid–base pair contacts of AraR, a regulator of sugar utilization in *Bacillus subtilis*

Correia, Isabel (1); Franco, Irina (1); Sá-Nogueira, Isabel (1,2)

1: Instituto Tecnologia Química e Biológica (ITQB-UNL), Oeiras, Portugal;
2: Departamento de Ciências da vida (DCV), Faculdade de Ciências e Tecnologia (FCT-UNL), Monte de Caparica, Portugal.

E-mail: isabelc@itqb.unl.pt

Keywords: Protein-DNA Interactions, Bacterial Transcription Factor, Trancriptional Regulation, Gene expression

Abstract

Protein-DNA interactions play a central role in genetic activity, especially in the regulation of gene expression. Therefore, it is of great importance to clarify the molecular details of protein-DNA interactions, as they form the basis of our knowledge of how this process takes place. To understand the nature of sequence-specific binding in transcription, we characterized the AraR, a key transcription factor in the gene network that regulates the uptake and degradation of carbohydrates in *Bacillus subtilis*. AraR exhibits a mosaic structure comprised by two domains of different evolutionary origins (1). The N-terminal region, responsible for DNA recognition and binding, contains a winged-helix-turn-helix signature of the GntR family of bacterial regulators. The C-terminal domain, which mediates responses to environmental stimuli, displays significant similarity to the LacI/GalR family. AraR binds to palindromic sequences At(T/A)tGTaCGTAcaa(A/T)T in the promoter region of the *ara* genes (1,2). Electrophoretic mobility shift assays (EMSA) was used to determine *in vitro* the dissociation constant (K_d) of the wild-type AraR and mutant proteins with a DNA fragment bearing the two operators (OR_{A1}-OR_{A2}) and the same fragment comprising mutations in the OR_{A1} box. To validate the *in vitro* results an identical strategy was tested *in vivo* by confrontation of the different *araR* alleles and the mutant operator sequences in different *B. subtilis* strains. With this methodology we clarified same aspects of the specific properties of AraR-operator sequence interactions.

This work was supported by grant PPCDT/BIA-MIC/61140/2004 from FCT, POCI 2010 and FEDER to ISN and grant SFRH/BD/29469/2006 from FCT to IC.

(1) Franco I.S., Mota L.J., Soares C.M., and Sá-Nogueira I. (2006). J. Bacteriol.188: 3024-3036.
(2) Franco I.S., Mota L.J., Soares C.M., and Sá-Nogueira I. (2007). Nucleic Acids Res. 35 (14): 4755-66

Reference

190

Influence of Calcium and pH in the accumulation and efflux of EB

Martins, Ana Sofia (1,2); Spengler, Gabriella (1,2); Costa, Susana (1); Viveiros, Miguel (1); Amaral, Leonard (1,2)

1: Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, Portugal;
2: UPMM, Instituto de Higiene e Medicina Tropical, Portugal

E-mail: anam@ihmt.unl.pt

Keywords: *E. coli*, pH, Calcium, Efflux

Abstract

Efflux of antibiotics by *E. coli* AG100 is performed by a variety of transporters, efflux pumps (EP), that ensures the survival of the bacterium in widely diverse media. Because calcium ions (Ca) are extremely important for cell signalling, membrane transport channels and activity of ATPases that provide energy functions, the role of Ca in the extrusion of an efflux pump substrate under conditions that challenge the bacterium was investigated.
Ethidium bromide (EB) is a substrate of bacterial EPs. Accumulation and efflux of EB was followed at 37ºC, under different pH of medium, with and without metabolic energy (glucose), and in the presence and absence of Ca and/or EDTA. The signal of EB inside the cell was automatically detected by a real time method that distinguishes accumulation from efflux.
At pH8 the accumulation of EB is glucose (GLU) dependent and greater than at pH5. At pH8, chlorpromazine (CPZ) augments the retention of EB, especially with the omission of GLU. This retention, at pH8, can be nullified by the addition of Ca. The role of Ca is further illustrated with the addition of the divalent chelator EDTA. The addition of Ca to an EDTA containing medium nullifies the accumulation promoted by EDTA. The simultaneous presence of CPZ and EDTA synergistically increases accumulation. At pH5 the effects of CPZ, EDTA and Ca are minimal.
Ca is needed for a variety of metabolic and energy deriving pathways within the cell that yield protons for activation of ABC type transporters. Our results suggest that there are 2 general types of transporter systems in *E. coli*: one that is dependent upon metabolic energy and is evident at pH8, and another that is demonstrable at pH5 and which consists of 8 or more EPs that include the main EP of this organism, the AcrAB-TolC pump. These latter EPs are dependent upon protons present in the periplasm for their activation. The concentration of periplasmic protons is controlled by the concentration of protons at the surface of the cell that is at least two or more pH units than that of the bulk medium. This is due to their attraction to the lipopolysaccharide layer. Whereas at pH5 the concentration of surface protons is readily maintained, at pH 8 the dissociation of protons from the surface into the bulk medium is great, reducing the availability of protons to the periplasm. Hence, when *E. coli* is challenged by a noxious agent, the extrusion of this agent is made possible at pH greater than 7 by an ABC type transporter.

Reference

249

AraL, a phosphatase in the *Bacillus subtilis* arabinose operon?

Godinho, Lia Marques (1, 2); de Sá-Nogueira, Isabel (1, 2)

1: Microbial Genetics, Instituto Tecnologia Química e Biológica (ITQB-UNL), Oeiras, Portugal;
2: Departamento de Ciências da Vida (DCV), Faculdade de Ciências e Tecnologia (FCT-UNL), Monte de Caparica, Portugal

E-mail: liagodinho@itqb.unl.pt

Keywords: gene function, protein expression, bacterial phosphatase, post-transcriptional regulation

Abstract

The arabinose operon of *Bacillus subtilis araABDLMNPQ-abfA* comprises nine genes and is regulated at the transcriptional level by induction in the presence of arabinose [1]. The first three genes of the arabinose metabolic operon, *araA*, *araB*, and *araD*, encode the enzymes required for the intracellular conversion of L-arabinose into D-xylulose 5-phosphate, which is further catabolized through the pentose phosphate pathway. AraNPQ are components of a putative ABC-type transporter, AraM is a dehydrogenase, and AbfA an arabinofuranosidase. The function of AraL is still unknown, however is currently classified as putative phosphatase belonging to the Haloalkanoic Acid Dehalogenase (HAD) superfamily, subfamily IIA [2]. Members of subfamily IIA usually display low substrate specificity and the boundaries defining physiological substrates are somewhat overlapping [3].

Here, we report the over-production in *Escherichia coli* of a recombinant version of AraL with a His6-tag fused to the C-terminus. The protein was purified to homogeneity and displayed phosphatase activity. Activity towards the substrate p-nitrophenol phosphate (pNPP) was measured at 65ºC, pH 7.5, and using Mg^{2+} as a cofactor. Moreover, by mutagenesis, we show that the level of protein production in *E. coli*, is controlled by a mRNA secondary structure which encompasses the *araL* ribosome binding site. The physiologic role of AraL and the implications of this post-transcriptional regulatory mechanism in *B. subtilis* will be discussed.

[1] Sá-Nogueira, I., T. Nogueira, S. Soares, and H Lencastre. (1997). Microbiology 143:957-969.
[2] Burroughs A. M., Allen, K. N., Dunaway-Mariano D., Aravind, L. (2006) J. Mol. Biol. 361, 1003-1034.
[3] Tremblay, L. W., Dunaway-Mariano, D., Allen, K. N. (2006). Biochemistry 45, 1183-1193.

Reference

252

Genome-wide transcription program involving the Haa1p-regulon in acetic acid-stressed yeast

Mira, Nuno P (1); Keller, Greg (2); Sintra, Eva R (1); Becker, Jorg D (3); Winge, Dennis R (2); Sá-Correia, Isabel (1)

1: Institute for Biotechnology and Bioengineering, Instituto Superior Técnico, Av Rovisco Pais, 1049-001 Lisboa;
2: Biochemistry and Internal Medicine, University of Utah Health Sciences Center, Salt Lake City, UT 84132;
3: Affymetrix Platform, Gulbenkian Institute of Science

E-mail: nuno.mira@ist.utl.pt

Keywords: acetic acid adaptation, acetic acid resistance, genome-wide transcription regulation, weak acids, stress response

Abstract

The transcriptional activator Haa1p controls a recently described weak acid-sensing system essential for *S. cerevisiae* resistance to acetic acid[1]. The transcription of ten documented Haa1p-target genes[2] is activated in response to acetic acid[1] but their protective effect against this acid is much below the effect exerted by Haa1p[1], suggesting that additional gene targets exist. To identify all the genes up-regulated by Haa1p in response to acetic acid a transcriptomic approach was used. The transcript levels of 60% of the acetic acid induced genes (51 out of 85 activated genes) was reduced in D*haa1* mutant indicating that this transcription factor is a key player in reprogramming the transcriptional response to acetic acid. Haa1p-activated genes include transcription factors, multidrug resistance transporters, cell wall proteins, protein kinases and other proteins of poorly characterised function. Mapping of *TPO3* gene promoter, one of Haa1p-targets, led to the identification of a DNA motif that serves as a binding site for Haa1p. A degenerate form of this motif is enriched among Haa1p-activated gene promoters. Several of the Haa1p target genes were found to be implicated in yeast resistance to acetic acid, although at different levels. Results also sustain the idea that increased resistance to acetic acid mediated by Haa1p involves the reduction of the intracellular concentration of the acid.

[1] Fernandes et al., BBRC, 337, 95-103, 2005.
[2] Keller et al., JBC, 276, 38697-702, 2001.

Reference

260

Influence of pH on cellular growth of *Pichia pastoris* KM71H by fed-batch process

Wanderley (4), Marcela Silvestre Outtes (1,2,3); Oliveira, Carla (1); Mussatto, Solange Inês (1); Bruneska, Danyelly (2,3,5); Lima Filho, José Luiz (2,3,4); Domingues, Lucília (1); Teixeira, José Antonio (1)

1: University of Minho, Portugal;
2: Grupo de Prospecção Molecular e Bioinformática (ProspecMol), Brazil;
3: Lab. de Imunopatologia Keizo Asami (LIKA), Brazil;
4: Programa de Pós-graduação em Ciências Biológicas, Brazil;
5: Departamento de Bioquímica, Brazil

E-mail: marcelasow@gmail.com

Keywords: *Pichia pastoris*, Fed-batch fermentation, cellular growth, pH, expression system

Abstract

Pichia pastoris is a methylotrophic yeast that can be genetically engineered to express proteins for industrial use. One of the most important advantages of protein expression in *P. pastoris* is its capability of growing on minimal medium and efficiently secreting heterologous proteins with low secretion levels of endogenous proteins. Operational variables such as pH, temperature, stirring rate, among others, usually affect the microorganism's growth during the fermentation processes. Therefore, the present work aimed to evaluate the influence of pH on cellular growth of *P. pastoris* KM71H by fed-batch process. The fermentation run was carried out in a 1.6 L (total volume) bioreactor, being performed in two phases: In the first stage (24 h), the yeast was batch-cultured in BMGH medium; while in the second stage (72 h), it was cultivated by feed-batch operation with a feeding medium containing 50% glycerol and 12ml/l of trace metal solution. During the overall process, which lasted after 96 h, the aeration and temperature conditions were fixed at 10 ml\L.h, 1.5 vvm and 30°C, respectively. Different pH values were evaluated: 5.0, 5.5 and 6.0. Cellular growth was determined by measuring the fermentation broth UV-spectrophotometric absorbance at 600 nm, which was correlated to a calibration curve (dry weight ´ optical density). Glycerol consumption was detected by HPLC analysis. *P. pastoris* KM71H successfully grew in all the evaluated pH values; but the highest biomass production was observed at pH 5.0 (98.79 g/L). Although *P. pastoris* is reported as being a microorganism able to grow over a wide pH range (from 3 to 7); it was not observed high cell density of *P. pastoris* KM71H strain when cultivated at pHs 5.5 and 6.0. High cellular growth is especially important for proteins secretion, as the concentration of product in the medium is roughly proportional to the concentration of cells in culture. Finally, these results reveal the possibility of obtaining high cell density of *P. pastoris* KM71H by fed-bach cultivation at pH 5.0, which can be a suitable condition for the yeast application in heterologous proteins production. Supported by: CNPq, ISAC-ERASMUS

Reference

265

Digging out Evidences on *Escherichia coli* stringent response from Scientific Literature

Carneiro, Sónia (1); Lourenço, Anália (1); Carreira, Rafael (1,2); Rocha, Miguel (2); Ferreira, Eugénio C. (1); Rocha, Isabel (1)

1: IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Portugal;
2: 2Departament of Informatics / CCTC, University of Minho, Portugal

E-mail: soniacarneiro@deb.uminho.pt

Keywords: *Escherichia coli*, Stringent Response, Literature Mining

Abstract

The study of microbial stress responses in model-organisms like *Escherichia coli* (*E. coli*) is expected to expand general knowledge on these systems. Stress responses take place when cells are subjected to a wide variety of environmental attacks, including nutrient starvation, heat shock and toxic compounds. After a sudden environmental change, all organisms, from the simplest bacterium to the most complex organism, sense and respond by increasing production of a certain class of molecules that buffer them from harm. As a result, organisms have developed and evolved mechanisms to survive stress.

Stringent response, caused by amino acid starvation, has remained a topic of interest during several years due to its role in microbial growth performance. Due to amino acid limitation, the rate of RNA synthesis and other cellular reactions is severely reduced. Experimental work has shown that this response is characterized by the accumulation of guanosine nucleotide, (p)ppGpp, maintained by RelA and SpoT proteins. However, most of the results coming from such research work lay in textual publications in peer-reviewed journals and as such, its computational analysis in view of a systems-level understanding of the phenomenon is not straightforward. The manual curation involved in searching stringent-related information in literature is a labour-intensive endeavour.

In our study of the mechanism of stringent response in *E. coli,* a Literature Mining computational system assisted the process of manual curation. Documents were retrieved from PubMed and an organism-specific dictionary supported automatic recognition of biological entities. Results corroborate that the guanosine nucleotide, RelA and SpoT proteins and the transfer and ribosome ribonucleic acids (tRNA and rRNA, respectively) are key players in the process. However, less reported players, such as the 50S ribosomal subunit protein L11, Fis transcriptional dual regulator and ribosome modulation factor were also identified. Whenever possible, entities were linked to additional database information and classified according to Gene Ontology functional annotation. Besides such immediate information gains, results show that literature mining processes are able to reduce manual curation and, at the same time, support the linkage of literature information with publicly available data. This is important for reconstructing more comprehensive regulatory networks and, ultimately, understanding stress-related mechanisms.

Reference

283

Study of Jen1p homologs by heterologous expression in *Saccharomyces cerevisiae*

Barata, Beatriz (1); Foskolou, Zozefina (1); Bessa, Daniela (2); Pacheco, António (2); Queirós, Odília (2); Casal, Margarida (1); Soares-Silva, Isabel (1)

1: CBMA (Centre of Molecular and Environmental Biology) / Departament of Biology / University of Minho, Campus de Gualtar, Braga, 4710-057, Portugal;
2: Sciences Department and CICS, ISCS-N / CESPU, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal

E-mail: oqueiros@hotmail.com

Keywords: Jen1, permeases, homologs, Saccharomyces

Abstract

In *Saccharomyces cerevisiae* the lactate permease is encoded by the *JEN1* gene (TC 2.A.1.12.2), a member of the Major Facilitator Superfamily (Casal *et al*, 1999). In *Kluyveromyces lactis,* as well as in *Candida albicans,* two homologous genes were described that encode two *JEN1*-like transporters specific for monocarboxylate and dicarboxylate uptake, KlJEN1/KlJEN2, and CaJEN1/CaJEN2 respectively. (Lodi et al., 2004; Queirós *et al,* 2007, Soares-Silva et al 2004). The sequencing of other hemiascomycetes genomes by the Genolevures project has demonstrated the existence of a family of *JEN1* homologs in several yeasts. Some of these yeasts have more than two homologs, but their function is still unclear. Such is the case of the yeast *Debaryomyces hansenii* and the yeast *Yarrowia lipolytica*, with 4 and 6 homologs.In a phylogenetic analysis *D. hansenii* genes are grouped by two in a Jen1 and Jen2 cluster, but the *Y. lipolytica* genes form a completely separate branch. In order to study the importance of these genes we performed their heterologous expression in the strain *S. cerevisiae jen1* D *ady2*D, that presents no activity for a carboxylate permease, using the expression system p416GPD (Soares-Silva et al 2007). With this approach, we intend to functionally characterize the members of the entire yeast Jen permease family members.

Casal, M. et al. (1999) J. Bacteriol. 181, 2620-2623.
Lodi, T. et al. (2004) Gene 339, 111-119.
Queirós, O. et al. (2006) Curr. Genet. 51, 61–169.
Soares-Silva, I. et al. (2004) Mol. Membr. Biol. 21:6, 403 - 411.
Soares-Silva, I. et al. (2007) Mol. Memb. Biol. 24, 464-474

Reference

304

Acetic acid-induced apoptosis in *Saccharomyces cerevisiae* involves global translation impairment

Silva, Alexandra (1); Paredes, João (2); Santos, Manuel (2); Ludovico, Paula (1)

1: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Portugal;
2: Department of Biology and CESAM, University of Aveiro, Portugal

E-mail: xanasilva@ecsaude.uminho.pt

Keywords: Yeast apoptosis; translational machinery; acetic acid stress

Abstract

Global mRNA translation efficiency decreases during the progression of apoptosis in mammalian cells [1]. In yeast, hydrogen peroxide induced apoptosis is also characterized by a sharp decrease in protein synthesis due to phosphorylation of the a-subunit of the translation initiation factor-2 (eIF2a) by the Gcn2 protein kinase (the amino acid control kinase) [2]. We have already shown that the expression of the translation factors eIF4A, eEF1A, eEF2 and eEF3A decreases during acetic acid-induced apoptosis [3], which suggested that translational regulation of gene expression is important during apoptosis. In order to better characterize the activity of eIF2a under different scenarios of yeast apoptosis, we are studying the occurrence of translational alterations during acetic acid induced apoptosis. Polysome profiling analysis showed a global reduction in translational activity, correlated with the phosphorylation of eIF2a by Gcn2 protein kinase. We demonstrate that the latter is a crucial player in the execution of acetic acid-induced apoptosis since a GCN2 deleted strain displayed a more resistant phenotype and a severe reduction of eIF2a phosphorylation. Deletion of TOR1 and GCN4, encoding key players of the sensing of amino acid and the general amino-acid control (GAAC) system, respectively, also increased resistance to acetic acid related with reduced eIF2a phosphorylation. These preliminary results suggest that the efficiency of mRNA translation is an important component of the cellular response to acetic acid-induced apoptosis.

[1] Holcik, M. and Sonenberg, N. (2005). Nat. Rev. Mol. Cell Biol. 6(4):318-327.
[2] Shenton, D., Smirnova, J.B., Selley, J.N., Carrol, K., Hubbard S.J., Pavitt, G.D., Ashe, M.P. and Grant, C.M. (2006). J. Biol. Chem. 281(39):29011-29021.
[3] Almeida, B., Ohlmeier, S., Almeida, A.J., Madeo, F., Leão, C., Rodrigues, F. and Ludovico, P. (2009). Proteomics. 9(3):720-732.

Reference

331

The lactate permease from *Saccharomyces cerevisiae* and the lactose permease from *Escherichia coli* share a common structure that extends to the substrate binding pocket

Soares-Silva, Isabel (1); Diallinas, George (2); Casal, Margarida (1)

1: Molecular and Environmental Biology Centre (CBMA) /Department of Biology, University of Minho, Campus de Gualtar, Braga, 4710-057, Portugal;
2: Faculty of Biology, Department of Botany, University of Athens, Panepistimioupolis, Athens 15781, Greece

E-mail: ijoao@bio.uminho.pt

Keywords: *S. cerevisiae*, lactate permease, structure/function studies

Abstract

The unravelling of the crystal structures of three members of the Major Facilitator Superfamily, as enabled the study of several other permeases at the structure and function level. The LacY permease from *Escherichia coli* is one of the most well characterized permease, thus becoming a "model permease" for structural studies (Guan and Kaback, 2006). The *Saccharomyces cerevisiae* lactate permease is encoded by the *JEN1* gene (TC 2.A.1.12.2), a member of the Major Facilitator Superfamily that belongs to the Sialate:H^+ Symporter (SHS) family (Casal *et al*, 1999). By homology threading of the Jen1p with the LacY permease we were able to obtain a 3D dimensional model, that together with site directed mutagenesis strategies, pointed to the existence of common structure between these two permeases. We have also shown that a highly conserved motif in 7th transmembrane segment (TMS7) is part of the substrate translocation pathway (Soares-Silva et al, 2007). In this work we identify two new functional motifs, one in TMS5 and another in TMS11. The domain in TMS5 was identified by structure/function studies based on phylogenetic molecular comparisons among Jen1p homologues with different specificities, towards monocarboxylate or dicarboxylates, and is critical for distinguishing mono- and di-carboxylate permeases. The conserved aminoacids in TMS11 domain pointed to the importance of this domain that was demonstrated to be involved in substrate binding. Overall we have rationally designed several mutants that modified the transport capacity for lactic acid, and new mutants were able to grow on succinate and malate as sole carbon and energy source. Jen1-GFP fusions have demonstrated that the mutant proteins are correctly targeted to the plasma membrane.
In all three functional motifs identified in Jen1p there are crucial amino acids located at analogous positions in LacY. Thus we propose the existence of a common structure not only at the global level, but also in respect to the fine structure of the substrate binding pocket in members of the MFS family.

This work was supported by Portuguese grant POCI/BIA-BCM/57812/2004. ISS received a fellowship SFRH/BPD/22976/2005.

Casal, M., et al. (1999) J. Bacteriol. 181, 2620-2623.
Guan, L. and Kaback, H. R. (2006) Annu. Rev. Biophys. Biomol. Struct.35:67-91
Soares-Silva, I., et al. (2007) Mol. Membr. Biol. 24, 464-474.

Reference

332

Role of a two-component regulatory system from *Sinorhizobium meliloti* in adhesion to plant roots

Santos, Mário Rui; Medeiros, João; Mata, Márcia; Moreira, Leonilde

IBB- Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Lisboa

E-mail: mario.r.santos@ist.utl.pt

Keywords: *Sinorhizobium meliloti*, Symbiosis, two-component regulatory system

Abstract

Sinorhizobium meliloti establishes a symbiotic nitrogen fixation relationship with the leguminous plant *Medicago sativa*. This symbiotic process involves signaling between the bacteria and the plant which then initiates the development of root nodules, where bacteria undergo endocytosis and become surrounded by a plant membrane. Attachment of *Sinorhizobium* to roots is the early step of the infection process and, except for exopolysaccharides, no other molecules mediating adhesion are known. In other Gram-negative bacteria, adhesion is controlled by the two-component regulatory system CpxA/CpxR. The response regulator CpxR, controls the expression of genes involved in motility, chemotaxis and of the adhesive appendages curli and P pili. The search for the CpxA/CpxR protein homologues in *S. meliloti* predicted proteins resulted in the identification of two proteins encoded by SMb21560 and SMb21561 genes, which showed 31 and 41% identity at the amino acid level with CpxA and CpxR from *Escherichia coli*, respectively. The gene directly downstream of SMb21561 encodes an hypothetical protein similar to CpxP, a periplamic protein controlled by CpxAR and essential to maintain periplasmic homeostasis. In order to understand if SMb21560, SMb21561 and SMb562 have a role in *S. meliloti* adhesion to plant root hairs, deletion mutants for each of the genes were generated. Several phenotypic properties such as biofilms, pili biosynthesis, motility, adhesion to root hairs and nodulation assays are being carried out and data will be presented.

Reference

348

Expression of the *Paracoccidioides brasiliensis* CDC42 homolog in *Saccharomyces cerevisiae* triggers a multiple budding phenotype

Almeida, Agostinho; Xavier, Sandra; Cunha, Cristina; Marques, Maria Belém; Ludovico, Paula; Rodrigues, Fernando

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal

E-mail: jfmafm@hotmail.com

Keywords: *Paracoccidioides brasiliensis*, cdc42, *Saccharomyces cerevisiae*, multiple budding phenotype

Abstract

The dimorphic fungus *Paracoccidioides brasiliensis* is characterized by a multiple-budding yeast that presents a heterogeneous cell growth, both in size and shape. Specific mutations in the Rho-like GTPase gene *CDC42* from *Saccharomyces cerevisiae* result in a multiple budding morphology and loss of controlled polarized growth, resembling what occurs in *P. brasiliensis*. Previous work in our laboratory has suggested that *PbCDC42* plays an important role in the control of cell size, atypical morphology and virulence of the yeast cells. Thus, the goal of this work was to further investigate the particularities that the Pbcdc42p may have and underlie its specific function. To achieve this goal, we have chosen to use *S. cerevisiae* as model for expression of *PbCDC42*. Expression of Pbcdc42p induced formation of both abnormal and multiple budding cells. In addition, our data indicate that Pbcdc42p overrides the spatial and temporal control of cell division in *S. cerevisiae*, but without disrupting DNA replication/segregation. Furthermore, to identify which specific amino acidic residues underlie what seems to be a different function of the Pbcdc42p, we performed site-directed mutagenesis of *ScCDC42*. Using as template for selection a protein sequence alignment of several organisms (*P. brasiliensis, S. cerevisiae*, *Penicillium marneffei, Drosophila melanogaster*, *Coccidioides immitis, Cryptococcus neoformans* and *Homo sapiens*), we chose three specific residues of *PbCDC42* to switch in *ScCDC42*: Ile85Thy (isoleucine/threonine), Ile117Thy (isoleucine/threonine), Ile126Arg (isoleucine/arginine). Ongoing work is directed to the evaluation of the phenotype of *S. cerevisiae* Δ*cdc42* cells harboring the mutagenic proteins. Altogether, our analyses contribute the elucidation of the true function of the pleiotropic Pbcdc42p in *P. brasiliensis*.

Almeida A.J., was supported by a Post-Doc fellowship from FCT (SFRH/BPD/33035/2006).

Reference

349

Cdc42p controls yeast-cell shape and virulence of *Paracoccidioides brasiliensis*

Almeida, Agostinho João (1); Cunha, Cristina (1); Carmona, Jenny (1); Sampaio-Marques, Belém (1); Carvalho, Agostinho (1); Malavazi, Iran (2); Steensma, Yde (3); Johnson, Douglas (4); Leão, Cecília (1); Logarinho, Elsa (1); Goldman, Gustavo (2); Castro, G

1: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;
2: Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil;
3: Institute of Biology, Leiden University, Leiden, The Netherlands;
4: Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, Vermont, USA

E-mail: j_menino@hotmail.com

Keywords: Cdc42, cell division, virulence, *Paracoccidioides brasiliensis*

Abstract

The thermal dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis, one of the most common systemic mycosis in Latin America. While at environmental temperatures it grows as a mold, at 37º C it shifts to the distinctive multiple budding yeast form. Besides its unique budding pattern and cell division, the yeast form of *P. brasiliensis* is also characterized by a polymorphic cell growth, i.e., the existence of mother and bud cells during growth with extreme variations in cell size and shape within the same cellular population, suggesting that these cells may follow, if not a different set of rules, at least a lax control of the establishment and maintenance of polarity during growth. Since the small Rho-like GTPase Cdc42 is a pivotal molecule in establishing and maintaining polarized growth for diverse cell types, as well as during pathogenesis of certain fungi, we evaluated its role during cell growth and virulence of the yeast-form of *P. brasiliensis*. We used antisense technology to knock-down *PbCDC42's* expression in *P. brasiliensis* yeast cells, promoting a decrease in cell size and more homogenous cell growth, altering the typical polymorphism of wild-type cells. Reduced expression levels also lead to increased phagocytosis and decreased virulence in a mouse model of infection. We provide genetic evidences underlying Pbcdc42p as an important protein during host-pathogen interaction and the relevance of the polymorphic nature and cell size in the pathogenesis of *P. brasiliensis*.

Almeida A.J., was supported by a Post-Doc fellowship from FCT (SFRH/BPD/33035/2006).

Reference

350

Paracoccidioides brasiliensis yeast cells do not obey standard rules during cell growth

Almeida, Agostinho João (1); Barros, Diana (1); Hernandez, Orville (2); Oliveira, Pedro (1); McEwen, Juan (2); Ludovico, Paula (1); Rodrigues, Fernando (1)

1: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;
2: Cellular and Immunogenetics Group, Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia

E-mail: j_menino@hotmail.com

Keywords: Cdc42, cell growth, *Paracoccidioides brasiliensis*, cell shape

Abstract

Paracoccidioides brasiliensis, a thermodimorphic fungus, is the etiological agent of paracoccidioidomycosis one of the most frequent systemic mycoses affecting the rural population of Latin America. Its yeast form is generally characterized by a multiple budding phenotype and a polymorphic cell growth, leading to the formation of cells with extreme variations in shape and size. The present work focuses on the analysis of different morphological features of *P. brasiliensis* yeast cells and possible correlations that might exist among them (mother cell size and shape; bud number, size and shape). Furthermore, as a pivotal molecule in important cellular processes in various cell types and organisms, we aimed to evaluate a possible correlation between the expression of the Rho-like GTPase Cdc42 (Pbcdc42p) with the analyzed morphological features. To answer this question, we used real-time PCR (RT-PCR) to determine *PbCDC42* transcript levels and stereomicroscopy (for contour measurement of 150 mother cells and buds) for morphological analysis of exponentially growing batch culture yeast cells from the 3 recently classified distinct lineages (S1, PS2, and PS3), using both clinical and environmental isolates. We show that cell size and cell shape of both the mother and bud cells are very heterogeneous both within the same isolate and among the isolates. Bud cell size per mother cell was also shown to be highly heterogeneous. Regarding bud number per mother cell, most isolates showed no predominance or budding pattern, although strains T10B1 and T15LN1 presented a higher frequency of cells with 1 and more than 5 buds, respectively. Moreover, no associations were detected between mother cell and bud cell size and shape, suggesting that *P. brasiliensis* yeast cells do not obey the critical mass rule between mother and daughter cells that other yeast do (e.g., *S. cerevisiae*). Interestingly, expression of *PbCDC42*, although heterogeneous among the 3 cryptic species, seems to show a negative correlation with bud number in the tested isolates, but not with any of the other analyzed morphological features. Altogether, this study provides a quantitative assessment of morphological traits of *P. brasiliensis* yeast cells, suggesting that *P. brasiliensis* does not follow standard rules during growth.

Almeida A.J., was supported by a Post-Doc fellowship from FCT (SFRH/BPD/33035/2006).

Reference

353

Aconitase as target of degradation during oxidative stress induced apoptosis in *Saccharomyces cerevisiae*

Marques, Helena; Silva, Alexandra; Sampaio-Marques, Belém; Ludovico, Paula

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

E-mail: helenamarques@ecsaude.uminho.pt

Keywords: Aconitase, oxidative stress, apoptosis, Sacharomyces cerevisiae

Abstract

Aconitase, a tricarboxylic acid (TCA) cycle enzyme, has been reported to be partially inactivated during oxidative stress induced by hydrogen peroxide (H_2O_2) [1, 2]. A yeast digestome analysis with an Yca1p-enriched protein extracts, combined with mass spectrometry, identified aconitase as a putative target of Yca1p, or Yca1p-activated proteases, fragmentation during hydrogen peroxide-induced apoptosis [3]. Aco1p cleavage is abrogated when cells are incubated with a protease inhibitor or with Δ*yca1*-enriched protein extracts. The increased resistance of *ACO1* deleted strain, but not of *ACO2* deleted strain, to H_2O_2 treatment when comparing with wild type strain suggests an involvement of Aco1p, but not of Aco2p in H_2O_2-induced apoptosis. Moreover, incubation of cells with fluoracetic acid, a specific inhibitor of aconitase activity, turns them more resistant to H_2O_2-induced apoptosis. Contrarily to GAPDH cleavage dependence of nitric oxide (NO) signaling [3], aconitase cleavage appears to be independent from NO signalling and Δ*aco1* cells still present similar levels of NO. Altogether the results indicate that Aco1p is a target of degradation during H_2O_2-induced apoptosis but the mechanisms underlying its involvement in cell death remain to be elucidated.

A.S. and B.S.M. have fellowships from FCT - Fundação para a Ciência e Tecnologia (SFRH/BD/33125/2007 and SFRH/BD/41674/2007, respectively).

[1] Bulteau, A., Ikeda-Saito, M., Szweda, L.I. Biochemistry, 2003. 42:14846-55.
[2] Chen XJ, Wang X, Butow RA, Proc Natl Acad Sci U S A. 2007 Aug 21;104(34):13738-43.
[3] B. Almeida, S. Buttner, S. Ohlmeier, A. Silva, A. Mesquita, B. Sampaio-Marques, N.S. Osorio, A. Kollau, B. Mayer, C. Leao, J. Laranjinha, F. Rodrigues, F. Madeo, P. Ludovico, J Cell Sci 120 (2007) 3279-3288.

Reference

355

The human TLR4 Asp299Gly polymorphism discriminates between *C. albicans* and non-albicans species

Menino, João Filipe (1); Costa, Pedro (1); Carvalho, Agostinho (1,2); Cunha, Cristina (1,2); Osorio, Nuno (1); Almeida, Agostinho (1); Saraiva, Margarida (1); Pedrosa, Jorge (1); Romani, Luigina (2); Castro, Gil (1); Rodrigues, Fernando (1)

1: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;
2: Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy

E-mail: joaomenino@ecsaude.uminho.pt

Keywords: *Candida albicans*, Asp299Gly, Susceptibility, TLR4, Type-1 IFNs

Abstract

The recognition of pathogenic microorganisms by the innate immune system relies on pattern recognition receptors (PRRs), being the Toll-like receptors a major group of PRRs. Toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, as well as structures from fungal and mycobacterial pathogens. Polymorphisms in this receptor have been associated to functional alterations, mainly concerning the release of cytokines. Recent findings from our group have shown an increased susceptibility, among individuals carrying the Asp299Gly polymorphism (rs4986790), to *Candida* non-*albicans* species comparatively to *Candida albicans* species. Moreover, previous studies have already established an association between this polymorphism and a reduced cytokine response and increased susceptibility to Gram-negative infections and yeasts such as *C. albicans*.
To further elucidate the role of this polymorphism in susceptibility to these type of infections, we have used an *in vitro* model of infection where PBMCs from homozygous wild-type and heterozygous individuals for the Asp299Gly polymorphism were stimulated with either heat-killed *C. albicans* and two non-*albicans* species (*C. glabrata* and *C. krusei*). At different time-points after infection, the cytokine expression profile for TNF-α, IFN-α, IFN-β, IL-10 and IL-1β was assessed by Real-Time PCR. Our data show a biased cytokine expression profile for Type 1-IFNs in PBMCs from heterozygous individuals stimulated with non-*albicans* species, but not with *C. albicans*. These data suggest that the presence of the Asp299Gly polymorphism may impact the outcome of infections with different pathogenic fungi, by modulating the differentiation of the adequate immunological response. The elucidation of TLR4 downstream key players involved in the cellular signalling pathways will be of major interest.

This work was supported by a grant from FCT – Fundação para a Ciência e a Tecnologia, Portugal (FCT: PIC/IC/83313/2007). J.M., A.A. and A.C. have fellowships from FCT.

Reference

363

Mycobacteriophage Ms6 LysB specifically targets the outer membrane of *Mycobacterium smegmatis*

Gil, Filipa (1); Grzegorzewicz, Anna (2); Catalao, Maria João (1); Vital, João (1); McNeil, Michael (2); Pimentel, Madalena (1)

1: Centro de Patogénese Molecular, URIA Faculdade de Farmácia de Lisboa, Portugal;
2: Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins,USA

E-mail: mpimentel@ff.ul.pt

Keywords: Bacteriophage lysis, mycobacteria, lipolytic enzymes

Abstract

In order to accomplish lysis, dsDNA phages produce, at the end of their lyitc cycle an endolysin which targets the peptidoglycan and a holin which controls endolysin function. In addition to these two proteins some bacteriophages encoded additional proteins which increase the efficiency of lysis. The lytic cassette of mycobacteriophage Ms6 encodes a protein, LysB, previously identified as a lipolytic enzyme able to hydrolyze the ester bond in lipase and esterase substrates. In the present work we show that LysB can hydrolyze mycolic acids containing lipids from the mycobacterial cell wall. We were able to demonstrate that LysB release mycolic acids from the mycolyl-arabinogalactan-peptidoglycan complex, by LC-MS analysis. Analysis of the extractable lipids from *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG and M tuberculosis H37Ra, treated with LysB have shown that this protein also targets a trehalose diester of two mycolic acid molecules, a glycolipid with a role in the virulence of pathogenic strains. We propose that LysB participates in lysis by targeting the linkage between the arabinogalactan-peptidoglican layer to the outer membrane and that it also hydrolyses other mycolic acids containing lipids

Reference

367

Cell death induced by ammonium in amino acid-starved cells of *Saccharomyces cerevisiae*

Santos, Júlia Margarida (1); Sousa, Maria João (2); Leão, Cecília (1)

1: Life and Health Sciences Research Institute, University of Minho, Braga, Portugal;
2: Molecular and Environmental Research Centre (CBMA)/ Department of Biology, University of Minho, Braga, Portugal

E-mail: jum.santos@gmail.com

Keywords: ammonium, amino acid-starved cells

Abstract

In the present work, we found that ammonium stimulates cell death in amino acid-deprived auxotrophic cells of *Saccharomyces cerevisiae*, whereas this effect was not observed when the same cells were starved for nitrogen. The toxic effect was not dependent on ammonium metabolization as its analogue methylamine produced an identical outcome. Furthermore, cells starved for nitrogen presented higher levels of glutamine synthase and inhibition of this enzyme did not significantly affect ammonium toxicity. Ammonium-induced cell death was accompanied by an increase in ROS production, chromatin condensation, exposure of phosphatidylserine on the outer surface of the plasma membrane, and PI^+ staining. Cell death was not decreased in the presence of Z-vad-FMK, however, the yeast metacaspase seems to have some role in the process since death was abolished in *yca1* D cells. *rim13*D cells, deleted for the yeast calpain homolog, were also resistance to ammonium-induced cell death, indicating that this protein may play a pro-apoptotic role in the process.
Ammonium-induced cell death is involved in different human disorders that are accompanied by hyperammonemia. However, the precise molecular mechanisms triggering ammonium-induced cell death remain poorly understood. In addition, deprivation of essential amino acids has been employed as a strategy in cancer therapy but resistance is often found. Our results show that ammonium can stimulate cell death in amino acid-deprived cells, opening the possibility to use *S. cerevisiae* as an useful model for the identification of signaling pathways and of new therapeutic targets for these diseases.

This work was developed in the scope of the project PTDC_AGR-ALI-71460/FCT.
JS holds a PhD fellowship (SFRH / BD / 33314 / 2008) from Fundação para a Ciência e a Tecnologia.

Reference

370

Absence of ADP/ATP carrier rescues the apoptotic sensitive phenotype of *Saccharomyces cerevisiae* POR1 deletion strain

Cardoso, Teresa; Pereira, Clara; Pacheco, Andreia; Côrte-Real, Manuela; Sousa, Maria João

CBMA (Centro de Biologia Molecular e Ambiental), Universidade do Minho, Portugal

E-mail: mcortereal@bio.uminho.pt

Keywords: apoptosis, yeast, ADT/ATP carrier, Porin

Abstract

The voltage-dependent anion channel (VDAC) is a mitochondrial outer membrane channel, which allows for the passage of molecules up to 1.1 to 1.5 kDa. In mammalian apoptosis, VDAC is thought to be involved in the formation of the permeability transition pore, together with the adenine nucleotide translocator (ANT). This pore can mediate mitochondrial outer membrane permeabilization (MOMP) which is responsible for the release of apoptogenic factors. Although release of cytochrome *c* in yeast apoptosis has been demonstrated [1], it is not entirely clear how it occurs. Absence of AAC proteins (yeast homologues of ANT) impairs MOMP and cytochrome *c* release, being associated to a higher resistance to acetic acid-induced apoptosis [2]. On the other hand, deletion of *POR1* (coding for yeast major VDAC isoform) resulted in a higher sensitivity to acetic acid. Whether VDAC and AAC can interact and belong or not to the same death pathway is the subject of this study. Herein we have disrupted *POR1* gene in a strain lacking AAC *(Δaac1/2/3*) and assessed its effects in acetic acid-induced death. *Δpor1Δaac1/2/3* mutant*,* unlike *Δpor1* single deletion strain*,* was found to be more resistant than the wild type strain and showed similar resistance as the *Δaac1/2/3* strain. Moreover, the time course appearance of cellular markers (ROS production, chromatin condensation and loss of membrane integrity) in the *Δpor1Δaac1/2/3* mutant was identical to *Δaac1/2/3*. These results suggest both proteins share the same apoptotic pathway and that the pro-survival role of VDAC in yeast cell death is only relevant in the presence of AAC.

[1] Ludovico, P. Rodrigues, F. Almeida, A. Silva, M.T. Barrientos, A. Côrte-Real, M. (2002). Mol. Biol. Cell 13, 2598-2606.
[2] Pereira, C., Camougrand, N., Manon, S. Sousa, M.J. Côrte-Real, M. (2007). Mol Microbiol 66(3):571-82.

Reference

378

Functional and structural characterization of a protein cross-linking and site-specific labeling enzyme

Fernandes, Catarina; Plácido, Diana; Isidro, Anabela; Carrondo, Maria Arménia; Archer, Margarida; Henriques, Adriano O.

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal

E-mail: cat@itqb.unl.pt

Keywords: protein cross-linking, site-specific labeling, assembly, surfaces, surface modification

Abstract

Transglutaminases (TGases) catalyze the acyl transfer reaction between the γ-carboxamide group of a peptide-bound glutamine and a primary amine, often the side chain of a protein-bound lysine. The resulting ξ-(γ-glutamyl)lysil cross-link is highly stable, contributing to the chemical and physical integrity of several biological tissues or structures. Here, we report on the characterization of Tgl, a 28 kDa transglutaminase from *Bacillus subtilis*, produced specifically during the developmental process of sporulation, during which it serves a role in assembly of the spore surface layers. Tgl itself is assembled around the surface of the developing spore. The enzyme was overproduced in *Escherichia coli* in high yield, and could be purified to nearly homogeneity in a single chromatographic step. The purified protein, found to be monomeric in solution, was active in a simple buffer, and capable of conducting protein-protein cross-linking reactions. Moreover, under conditions of excess of a low molecular weight amine donor conjugated to a fluorophor (such as dansyl-cadaverine), Tgl was show to efficiently mediate transfer of the fluorophor to glutamine residues on target proteins, thus resulting in their fluorescent labeling. We show that Tgl functions in catalysis through a papain-like Cys116, His200, and Asn188 triad and hence that Tgl, like other TGases, it belongs to the papain-like superfamily of cysteine proteases. The presence of Asn in the active site is unique to Tgl, and is most likely structurally and mechanistically related to the specificity of the enzyme. We have also solved the crystal structure of Tgl at 1.9 Å resolution in its native form and bound to the inhibitor cystamine. The active site is close to the surface of the molecule, but the critical Cys116 residue is located on the sidewall of a hydrophobic tunnel that transverses the molecule from one side to the other. Preliminary evidence suggests an asymmetric molecule, with acceptor sites for the glutamine- and lysine-donor substrates located on opposite sides of the protein. Tgl is the smallest TGase characterized, and has simple requirements for activity, underscoring its potential in applications ranging from manipulation of protein surfaces and assemblies, to site-specific protein labeling.

Reference

379

Non-classical targeting and regulation of expression of GAPDH1 from *Kluyveromyces marxianus*

Barbosa, Joana (1); Faria, Juliana (1); Silva, Telma (1); Bousbaa, Hassan (1); Queirós, Odília (1); Moradas-Ferreira, Pedro (2,3); Ferreira-da-Silva, Frederico (2); Falcão-Moreira, Roxana (1)

1: Departamento de Ciências e Centro de Investigação em Ciências da Saúde (CICS), Instituto Superior de Ciências da Saúde – Norte / CESPU, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal;
2: Instituto de Biologia Molecular e Celular (IBMC) da Universidade do Porto;
3: Instituto de Ciências Biomédicas Abel Salazar (ICBAS) da Universidade do Porto

E-mail: roxana.moreira@iscsn.cespu.pt

Keywords: glyceraldehyde-3-phosphate dehydrogenase, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, promoter, cell wall proteins,

Abstract

The glyceraldehyde-3-phosphate dehydrogenase isoform 1 from *Kluyveromyces marxianus* (kmGAPDH1p) has demonstrated functional diversity, closely associated with cellular adhesion, thermo-tolerance, glycolitic activity, carbon metabolism and membrane stabilization (2,3). Although it does not contain a N-terminal signal peptide accountable for targeting to the ER (1), it is targeted to the cell wall, where it is accumulated at 40 ºC - a supraoptimal growth temperature (1). Our knowledge with respect to the underlying basic mechanisms which provide this functional diversity of GAPDH, is still very limited. Aiming to study the ones regarding the molecular mechanisms which regulate gene expression, the promoter of kmGAPDH1p gene was cloned and it's sequence studied. The applied strategies and results are shown. The mechanism of kmGAPDH1p secretion can be an alternative mechanism to the known classic secretion route, which exists either in *K. marxianus* and *S. cerevisiae*. In fact, studies done in a *S. cerevisiae* strain transformed with a vector expressing kmGAPDH1p (3) revealed that, in this model, the protein is also redirected to the cell wall, as in wild type *K. marxianus*, despite the absence of the typical N-terminal sequence responsible for the targeting for the secretion pathway. Aiming at depicting the secretion route, the movement of the protein in the cell was tracked by subcelular localization experiments of kmGAPDH1p and kmGAPDH1p fused to an ER retention signal (HDEL). These results will be shown and related with the ones obtained by the immunoprecipitation of biotinylated cell wall proteins of *S.cerevisiae* cells expressing kmGAPDH1p and kmGAPDH1p fused to HDEL sequence. The involvement of the ER in the secretion pathway of kmGAPDH1p will be discussed.

(1) Fernandes, P. A. et al (1995) Yeast 11, 725-733
(2) Falcão Moreira, R. et al (1998) Microbiology 144, 681-688
(3) Falcão Moreira, R. et al (2000) Yeast 16, 231-240

Reference

380

Forespore to mother cell switch in the expression of a zinc anti-sigma factor helps maintaining compartmentalized gene expression during spore development in *Bacillus subtilis*

Serrano, Mónica (1); Moran Jr., Charles P. (2); Henriques, Adriano O. (1)

1: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal;
2: Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta GA, USA

E-mail: serrano@itqb.unl.pt

Keywords: cell type-specific gene expression, sigma factor, anti-sigma factor, cell-cell communication, morphogenesis

Abstract

Spore differentiation in *Bacillus subtilis* occurs in a rod-shaped sporangium divided into a smaller forespore and a larger mother cell. Soon after asymmetric cell division, a phagocytic-like process results in the engulfment of the forespore by the mother cell. The forespore then becomes competent for the assembly of a series of concentric protective layers that will encase the mature spore. These morphological changes are controlled by a series of four compartment-specific RNA polymerase sigma factors. sF and sE, both activated soon after division, control the early stages of development in the forespore and the mother cell, respectively. Following engulfment completion, sF is replaced by sG, whereas sE is substituted by sK. sG is produced under sF control early in development, but remarkably, its activity is only detected following engulfment completion. It is not yet known how is sG kept inactive prior to engulfment completion. However, sG is the target of two anti-sigma factors, SpoIIAB and CsfB. The latter is a novel type of zinc anti-sigma factor initially produced in the forespore under the control of sF. We show that CsfB works by directly interacting with sG in the vicinity of a region of the sigma molecule (region 2.2, conserved among s70-type sigma factors) that interacts with the b´subunit of core RNA polymerase. Mutations in this region of sG make it refractory to CsfB and increase the activity of the sigma factor in vivo. We also present evidence, that neither SpoIIAB nor CsfB *per se* seem to have a determinant role in inhibiting the activity of sG in the forespore prior to engulfment completion, raising the possibility that yet another anti-sigma G factor is present in the forespore. Nevertheless, the two anti-sigma factors may inhibit residual pre-engulfment activity of sG. We further show that following engulfment completion CsfB is eliminated from the forespore and begins to accumulate in the mother cell in a sK-dependent manner. In the mother cell, CsfB concurs with SpoIIAB (and with the LonA protease) to keep the auto-regulatory sG inactive in this compartment. Thus, while re-enforcing the activation of sG in the forespore, the forespore to mother cell switch in the activity of CsfB helps maintaining proper compartmentalization of gene expression, reducing transcriptional noise, and promoting the fidelity of the morphogenetic process of spore development.

Reference

391

RNase II family of enzymes and their relevance in the post-transcriptional control of microbial gene expression

Matos, Rute Gonçalves; Reis, Filipa Pereira; Barbas, Ana; Arraiano, Cecília Maria

Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Oeiras, Portugal

E-mail: rmatos@itqb.unl.pt

Keywords: RNase II, RNase R, Exoribonucleases, Control of Gene Expression

Abstract

In this work we focus on the characterization of RNase II-family members. RNase II is the prototype of a ubiquitous family of highly processive hydrolytic exoribonucleases involved in the maturation, turnover, and quality control of RNA. The members of this family of enzymes can act independently or as a component of the exosome, an essential RNA-degrading multiprotein complex. Therefore, analysis of the molecular biology of RNase II-family of enzymes is extremely relevant for the comprehension of post-transcriptional control of gene expression in microbes, and for the understanding of their microbial physiology.

The resolution of the crystal structure of *Escherichia coli* RNase II showed that this enzyme is constituted by four domains: two N-terminal CSD and one C-terminal S1 domain involved in RNA binding, and a central catalytic RNB domain.

Recent studies showed that RNase R CSD and S1 domains are important for the RNA binding and are the responsibles for the selection of RNAs to be degraded. We also showed that the RNB domain, *per se*, has exoribonucleolytic activity and is able to cleave double stranded substrates (Matos *et al*, 2009).

The active site of the protein is formed by four highly conserved aspartates with different roles in catalysis, with Asp209 being the only critical residue for the activity of the enzyme without affecting the ability to bind to the RNA. The same results were obtained for RNase R. We also identified Tyr253 as the residue responsible for setting the end-product of RNase II and also for other members of the family (Matos *et al*, 2009). We verified that both Glu390 and Tyr313 play an important role in the discrimination of RNA versus DNA. The most interesting finding was that the substitution of Glu542 by Ala turned RNase II into a “super-enzyme”, which is 100-fold more active when compared to the wild type enzyme and is also able to bind to RNA more efficiently (Barbas, A., Matos, R. G. *et al*, 2009). This discovery will have very important implications for the biotechnological applications of recombinant enzymes.

Although *E. coli* RNase II, RNase R and yeast Rrp44p all share the same basic organization, there are important differences in their activities, namely the presence of a second nuclease domain apart from the RNB catalytic domain that confers an endonucleolytic activity to Rrp44p. This activity is located in the PINc domain and confers different properties to the full length protein (Schaeffer, D. *et al*, 2009).

Schaeffer, D., Tsanova, B., Barbas, A., Reis, F. P., Dastidar, E. G., Sanchez-Rotunno, M., Arraiano, C. M. and van Hoof, A. (2009). Nature Structural & Molecular Biology. 16, 56-62

Barbas, A., Matos, R. G., Amblar, M., Lopez-Viñas, E., Gomez-Puertas, P. and Arraiano, C. M. (2009). The Journal of Biological Chemistry. 284, 20486-20498 * contributed equally

Matos, R. G., Barbas, A. and Arraiano, C. M. (2009). Biochemical Journal. In press.

Reference

406

Insights on the biossinthetic pathway of mannosylglucosylglycerate in the deep-branching phylum Planctomycetes

Cunha, Sofia (1); Costa, Joana (1); Santos, Helena (2); da Costa, Milton S. (1, 3)

1: Centro de Neurociências e Biologia Celular, Departamento de Zoologia, Universidade de Coimbra, 3004-517 Coimbra, Portugal;
2: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande 6, Apartado 127, 2780-156 Oeiras, Portugal;
3: Departamento de Bioquímica, Universidade de Coimbra, 3001-401 Coimbra, Portugal

E-mail: sophie.cunha@gmail.com

Keywords: New biosynthetic pathway, Compatible solutes, Planctomyces

Abstract

The vast mainstream of microorganisms is capable of responding, within intrinsic limits, to changes in the salt concentrations in the environment and many, resort to the accumulation of organic solutes to counterbalance external decreases in water availability and internal turgor pressure. These solutes that accumulate to high concentrations and protect cells against water stress were designed compatible solutes (CS) (1). The planctomyces (PL) are an unusual, but widely distributed, group of bacteria which are proving to be increasing relevant in areas of research. Since PL are widespread in terrestrial and aquatic habitats differing in salinity, oxygen availability, trophic levels and temperature (2, 3), and given its particular phylogenetic position, we began to investigate the osmotic stress responses in *Rhodopirellula baltica*. This mesophilic organism accumulates sucrose (SU), trehalose (TR), mannosylglucosylglycerate (MGG), glutamate, and two novels partially identified sugar-derived CS. The sugar-derived osmolyte MGG has only been identified in the termophilic bacterium *Petrotoga myotherma*. In this organism the synthesis of MGG proceeds via a two-step pathway, involving the activities of glucosyl-3-phosphoglycerate synthase (GpgS) and mannosylglucosyl-3-phosphoglycerate synthase (MggA). Homologues for the *P. mobilis* GpgS and MggA were detected in the *R. baltica* genome, suggesting that the same pathway could be active. Indeed, the incubation of *R. baltica* cell-free extract carrying the recombinant GpgS and MggA catalysed the formation of a compound that was dephosphorylated to MGG by alkaline phosphatase. These observations allowed us to partially identify the biochemical pathway leading to the synthesis of MGG in *R. baltica*.

(1) Brown, A. D. 1976. Bacteriol. Rev. 40: 803–846.
(2) Fuerst JA. 2004. World Fed. Cult. Collect. Newsl. 38:1–11.
(3) Costa J, Empadinhas N, Gonçalves L, Lamosa P, Santos H, and da Costa MS. 2006. J Bacteriol. 183:1022-1030.

Reference

407

Dissimilar physiological responses to chromate and dichromate in the hexavalent chromium resistant and reducing strain *Ochrobactrum tritici* 5bvl1

Francisco, Romeu (1); Moreno, António (1,2); Morais, Paula Vasconcelos (1,3)

1: IMAR-CMA, University of Coimbra, 3004-517 Coimbra, Portugal.;
2: Department of Zoology, University of Coimbra, 3004-517 Coimbra, Portugal.;
3: Department of Biochemistry, University of Coimbra, 3001-401 Coimbra, Portugal.

E-mail: pvmorais@ci.uc.pt

Keywords: Chromate, dichromate, Cr(VI) resistance

Abstract

Studies of Cr(VI) toxicity are generally performed using chromate salts in solution, both when studying the effects on prokaryotes and eukaryotes. Some studies on human carcinogenesis and toxicology on bacteria were done using dichromate, but comparison with chromate was never reported before, and dichromate existence was never taken into consideration and usually overlooked.

We therefore performed a comparative study of the effect of dichromate and chromate on the physiology of *O. tritici* strain 5bvl1, a highly Cr(VI)-resistant and reducing microorganism. We demonstrated that the addition of chromate or dichromate sodium salts to medium at neutral pH ended-up in two different solutions with a different balance of chemical species. Cr(VI) was toxic to *O. tritici* strain 5bvl1, as clearly shown on growth, reduction, respiration, glucose uptake assays and by comparing cell morphology. Moreover, the addition of sodium dichromate was always more toxic to cells when compared to chromate and achieved a higher inhibition of every parameter studied.

The toxicity differences between the two Cr(VI) oxyanions described for this strain indicate the possibility of a different impact on the environment, as a function of pH, temperature, and redox potential. This may be of major importance, considering the slight acidity of most of the arable lands.

Reference

410

Expression analysis of genes involved in trehalose pathways in *Rubrobacter xylanophilus*

Nobre, Ana (1); Pereira, Patrícia M. (2); Santos, Manuel A.S. (2); da Costa, Milton S. (1, 3)

1: Centro de Neurociências e Biologia Celular, Departamento de Zoologia, Universidade de Coimbra, 3004-517 Coimbra, Portugal;
2: CESAM - Depart. de Biologia, Universidade de Aveiro, 3810-193 Aveiro, Portugal;
3: Departamento de Bioquímica, Universidade de Coimbra, 3001-401 Coimbra, Portugal

E-mail: anobre@cnc.uc.pt

Keywords: *Rubrobacter xylanophilus*, trehalose, biosynthesis, genes expression

Abstract

Some microorganisms developed specific adaptations to survive and adapt to extraordinarily inhospitable environments. When, for example, a microorganism faces hyperosmotic conditions, the most common strategy to protect cells from dehydration and maintain a positive turgor pressure essential for cell division is the accumulation of compatible solutes [1]. However, besides osmotic adaptation, compatible solutes may serve other roles as they have been shown to stabilize proteins against a variety of stress conditions, namely oxidative stress or high temperatures.

Trehalose is best known for its role as a universal protector molecule, protecting cells and biomolecules from stress imposed by low water activity, heat, oxidation, desiccation and freezing. This nonreducing disaccharide is the primary organic solute in the radiation- and desiccation-resistant bacterium *Rubrobacter xylanophilus*. It is constitutively accumulated at high concentrations and regardless of growth temperature or salinity, in tryptone/yeast extract-based medium [2]. The genes of four pathways for trehalose synthesis were detected in the genome of *R. xylanophilus* and the two systems TPS/TPP and TreT were functionally characterized [3].

In this work, we are analysing the expression of the genes involved in four known pathways of trehalose synthesis, namely *tps*/*tpp*, *treS*, *treY*/*treZ* and *treT*, in *R. xylanophilus*, by real-time PCR, in two different stress conditions.

[1] Empadinhas, N., and M. S. da Costa. 2008. Int. Microbiol. 11:151-161.
[2] Empadinhas, N., V. Mendes, C. Simões, M. S. Santos, A. Mingote, P. Lamosa, H. Santos, and M. S. da Costa. 2007. Extremophiles 11:667-673.
[3] Nobre, A., S. Alarico, C. Fernandes, N. Empadinhas, and M. S. da Costa. 2008. J. Bacteriol. 190:7939-7946.

Reference

412

Unusual heme ligation in the NrfH cytochrome c quinol dehydrogenase

Matos, Daniela; Cunha, Filipa; Rodrigues, Maria Luisa; Archer, Margarida; Pereira, Inês A. C.

Instituto de Tecnologia Química e Biológica, Portugal

E-mail: ipereira@itqb.unl.pt

Keywords: NrfH, quinol dehydrogenase, membrane complex, nitrite reductase NrfHA

Abstract

The NrfH cytochrome *c* is a membrane-anchored tetraheme cytochrome *c* that oxidises the quinone pool and is the physiological partner of the NrfA nitrite reductase. NrfH belongs to a wide family of cytochrome *c* quinol dehydrogenases that play a crucial role in bacterial respiration, by oxidizing menaquinol and transferring electrons to various periplasmic oxidoreductases [1,2]. These cytochromes are very widespread in bacterial respiratory chains. The NrfHA proteins form a bacterial membrane bound complex that reduces nitrite by the oxidation of menaquinol [1,2].

We have determined the structure of the native NrfHA complex [2], and elucidated its menaquinol binding site through the structure of the NrfHA bound to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), which is shown to act as a competitive inhibitor of the NrfH quinol oxidation activity [3]. The menaquinol-binding site is close to heme 1 of NrfH, which has very unusual heme coordination. Here we present further studies of the characterization of the NrfH proteins from two different organisms, *Desulfovibrio vulgaris* a sulfate-reducing *Deltaproteobacterium* and *Wolinella succinogenes* a nitrite-reducing *Epsilonproteobacterium*.

[1] Pereira I.A.C., LeGall J., Xavier A.V. and Teixeira M. (2000). Biochem. Biophys Acta 1481 (1), 119-130
[2] Rodrigues M.L., Oliveira T., Pereira I. A. C. and Archer M. (2006) EMBO J., 25, 5951–5960
[3] Rodrigues M.L., Scott K.A., Sansom M.S.P., Pereira I.A.C. and Archer M. (2008) J. Mol. Biol., 381, 341-50

Oral Session:
S2 – Cell and Tissue Engineering, Biomaterials and Nanobiotechnologies

Reference

171

Nanostructured bioactive templates in polymeric shells

Roque, Ana Cecília (1); Batalha, Íris L. (1); Pina, Ana Sofia (2); Marcos, Ana Sofia (2); Hussain, Abid (2)

1: IBET, Portugal;
2: REQUIMTE/CQFB, FCT-UNL, Portugal

E-mail: cecilia.roque@dq.fct.unl.pt

Keywords: magnetic nanoparticles, biopolymer, antibody, affinity receptor

Abstract

Iron oxide magnetic nanoparticles (MNPs) find applications as drug delivery vehicles, contrast agents in MRI, diagnostic tools and separation aids. Magnetic fluids can be stabilized by the addition of surfactants that also provide favourable surface properties, tailored for specific applications of interest. Also, as MNPs can get close to a target biological entity, their surface can be modified to add affinity and specificity towards desired molecules. Several biopolymers, such as polysaccharides, can be used to create a shell for the encapsulation of the MNPs while increasing their colloidal stability, functionality and biocompatibility. Gum Arabic (GA) is a naturally occurring exudate from *Acacia senegal* and *Acacia seyal* trees, and possesses a highly branched polysaccharide structure. This biopolymer alone is widely used in the food and pharmaceutical industries. Recent studies indicate its potential in controlled drug delivery systems, carriers for the microencapsulation of oils and other bioactive molecules as well as in the stabilization and increased biocompability of nanostructures. In this work we explored the surface modification of MNPs with GA and related biopolymers by different routes and used these materials as a platform for the creation of bioactive magnetic entities. Preliminary *in vitro* studies were also performed with mammalian cell cultures as to assess the effect of these biomaterials on the cellular density and viability. FTIR spectra confirmed the presence of polymers at the surface of coated MNPs. The adsorption isotherm of GA at the surface of bare magnetite followed a Langmuir model (maximum capacity of 1 g GA/g MNP). The diameter of the MNPs magnetic core was between 11-14 nm. DLS measurements registered different size of agglomerates depending on the biopolymer coating method. MNPs to which the biopolymers have been adsorbed affected less the cellular density of the mammalian cell cultures, when compared to bare MNPs. We have further modified the coated MNPs with receptors for biological molecules at high densities. These particles have shown to bind specifically to the target (with maximum capacities of up to 340 mg target/mg MNP and K_a values of 10^5 M^{-1}), while retaining the magnetization properties. These affinity systems find applications in various bioengineering and nanobiotechnology fields.

Reference

246

A multiple emulsion formulation of bacteriophage encapsulated in lipid nanovesicles

Balcão, Victor Manuel (1,2); Azevedo, André Filipe (1); Castro, Luís Miguel (1); Costa, Carla Isabel (1); Santos, Sílvio (2); Matos, Carla Manuela (1); Moutinho, Carla Maria (1,2); Teixeira, José António (2); Azeredo, Joana Cecília (2)

1: Grupo de Investigação em Bioengenharia e Química Biofarmacêutica, Universidade Fernando Pessoa, Rua Carlos da Maia nº 296, P-4200-150 Porto, Portugal.;
2: IBB - Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar 4710-057, Braga, Portugal.

E-mail: vbalcao@ufp.edu.pt

Keywords: Bacteriophages, nanoemulsion, encapsulation

Abstract

The emergence of antibiotic-resistant bacterial strains and the week penetration of antibiotics in bacterial biofilms put an emphasis in the need for safe and effective alternatives for antimicrobial treatments. The application of strictly lytic bacteriophages (or phages) has been proposed as an alternative (or complement) to conventional antibiotics, allowing release of the natural predators of bacteria directly to the site of infection. Probably, the major advantage of phage-based therapy lies in the fact that phages replicate directly in the site of infection, becoming profusely available where they are most needed. When compared to antibiotics, phages present many relevant advantages: (i) permanently high concentrations at the infection site, increasing in the presence of (viable) bacterial host, with elimination occurring only after eradication of the later; (ii) total compatibility with antibiotics; (iii) specificity against target-bacteria; (iv) higher penetration in bacterial biofilms, by inducing production of enzymes that hydrolyze biofilm polymeric matrix; and (v) while bacteria can develop resistance to phages, isolation and large-scale production of new lytic phages is much simpler and economical than developing a new antibiotic. Water-in-oil-in-water (W/O/W) emulsions are examples of multiple emulsions, in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous aqueous phase. Due to their compartimentalized internal structure, multiple emulsions present advantages over simple O/W emulsions for encapsulation, such as the ability to carry both polar and non-polar molecules, and a better control over releasing of therapeutic molecules. In the present research work, the potential of nanoencapsulating a broad lytic spectrum phage able to infect enteric Salmonella and *E. coli* has been investigated. Phage phi-PVP-SE1 was entrapped within W/O/W multiple nanoemulsions, aiming at mimicking the multifunctional design of biology, with several lipid matrices, poloxamers and stabilizing layer compositions. Physicochemical characterization of the optimized phage-encasing nanovesicle formulations encompassed determination of particle size, size distribution and particle charge, via Zeta potential analysis, surface morphology via SEM, encapsulation efficiency, and thermal analysis via DSC. The antimicrobial activity of the nanoemulsions produced was also assessed in vitro, using several microbial strains.

Reference

286

Studying DNA conformation with the bulk acoustic wave sensor

Tomé, Brigitte; Ferreira, Guilherme N.M.

Institute for biotechnology and bioengineering, Portugal

E-mail: brigitte.tome@gmail.com

Keywords: biosensors, DNA conformation, QCM

Abstract

DNA-based sensors represent a very active research field since gene analysis is crucial for the diagnosis of hereditary and infectious diseases, the classification of organisms and in forensic chemistry. More common sensors are based on the hybridization reaction that consists in the immobilization of a nucleic acid probe on the surface of the sensor to recognize the complementary sequence present in the sample solution. In theory, the frequency shift should be maximal with a fully complementary sequence and no frequency shift should be observed with non-complementary DNA. In a recent work, a new model has been proposed that correlates acoustic wave measurements to the conformation of surface‐attached DNA molecules, stating that acoustic ratio measurements can be used to discriminate between DNA of various sizes and shapes independently of the amount of surface bound quantities. Considering this new model, we are using the quartz crystal microbalance to analyze the conformation of DNA. We rely on impedance analysis of the sensor to assess the variations of the model parameters and correlate them to the physical phenomena and chemical properties of the adsorbed media at the surface. In particular the variation of resistance and inductance are measured and correlated with variations of viscoelastic properties and mass of the adsorbed biofilms, respectively. Insights regarding the rigidity of the films are thus obtained which enable the assessment of immobilized DNA bending owing to protein-DNA binding.

Reference

311

AuAg alloy nanoprobes for molecular diagnosis

Dias, Jorge Teixeira (1,2); Almeida, Carina (1); Doria, Gonçalo (1,2); Franco, Ricardo (2); Pereira, Eulália (3); Baptista, Pedro (1)

1: CIGMH/DCV, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal;
2: REQUIMTE/DQ, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal;
3: REQUIMTE/DQ, Faculdade de Ciências, Universidade do Porto, Portugal

E-mail: jorge.t.dias@gmail.com

Keywords: Gold, silver, alloy, nanoparticles, DNA

Abstract

In past years, gold nanoparticles (AuNPs) derivatised with thiol-modified ssDNA oligonucleotides (Au-nanoprobes) have found wide application in the development of highly sensitive and selective diagnostic methods for DNA/RNA [1]. Silver nanoparticles (AgNPs) also present excellent proprieties for biodetection, in particular their extinction coefficient of the surface plasmon band (SPR) being approximately 4 times that of AuNPs with the same diameter. However, existing protocols for AgNPs functionalisation by thiol-modified oligonucleotides are cumbersome and time-consuming [2]. To circumvent this limitation, our approach has been to use gold-silver alloy nanoparticles (AuAgNPs), taking advantage of the ease of derivatisation of AuNPs and the enhanced SPR extinction coefficient of AgNPs.

Here we report the synthesis of AuAgNPs and their derivatisation with thiol-ssDNA oligonucleotides (AuAg-nanoprobes) for application in molecular diagnostics. The AuAg-nanoprobes were used to specifically detect a sequence derived from the RNA polymerase b-subunit gene of *Mycobacterium tuberculosis*, the etiologic agent of human tuberculosis. Complementary targets were detected following a non-cross-linking assay that has been developed by our group for Au-nanoprobes [3]. The assay consists of the spectrophotometric comparison between solutions before and after salt-induced nanoprobe aggregation. The presence of a complementary target prevents AuAg-nanoprobe aggregation and the solution remains orange, while non-complementary targets do not prevent aggregation, resulting in a visible change of color from orange to grey.

[1] Baptista et al., Anal Bioanal Chem, 391 (2008) 943-950;
[2] Graham et al., Anal Chem 80 (2008) 2805-2810;
[3] Baptista et al., Clin Chem, 52 (2006) 1433-1434.

Reference

389

Green synthesis of highly stabilized nanocrystalline gold and silver particles by extremophilic yeasts

Mourato, Ana (1); Gadanho, Mário (2); Lino, Ana (1); Tenreiro, Rogério (2)

1: Universidade de Lisboa, Faculdade de Ciências, Centro de Química e Bioquímica (CQB), Edificio C8, Campus da FCUL, Campo Grande, 1749-016, Lisboa,Portugal;
2: Universidade de Lisboa, Faculdade de Ciências, Centro de Biodiversidade, Genómica Integrativa e Funcional (BioFIG), Ed. ICAT, Campus da FCUL, Campo Grande, 1749-016, Lisboa,Portugal

E-mail: rptenreiro@fc.ul.pt

Keywords: silver nanoparticles, gold nanoparticles, extremophilic yeasts, spectroscopy

Abstract

Nowadays, research in nanotechnology deals with the search of eco-friendly processes for the synthesis of stable nanoparticles (NPs), with well-defined shape and controlled narrow size. Also, due to the great demand for precious metals in the fields of electronics and catalysis, recovery of gold and silver from both primary and secondary sources is of significance. Microbial recovery of precious metals with the formation of NPs is an environmentally friendly alternative to conventional methods, and therefore fulfills both mentioned issues.
In the present study, two yeast strains (Y1 and Y2) isolated from an acid mine drainage in Portugal were used for the production of silver and gold NPs that were subsequently characterized by spectroscopic and microscopic techniques.
For the biosynthesis of NPs, the yeast strains were grown in liquid medium at 22°C with aeration and the cell biomass, collected by centrifugation, was exposed for 24 hours to an aqueous solution of 0.6 mM chloroauric acid ($HAuCl_4$) and 1mM silver nitrate ($AgNO_3$), respectively. Progress in the reaction in both sets of solutions was monitored by UV-vis spectroscopy and NPs characterization was assessed by Transmission Electron Microscopy (TEM), Energy Dispersive Spectroscopy (EDS), X-ray diffraction and Fourier Transform Infrared Spectrophotometry (FT-IR).
The resulting silver and gold NPs displayed controllable structural and optical properties as demonstrated by UV–vis spectroscopic studies, TEM observations and EDX and XRD data. The size of the silver NPs was smaller than 20 nm, independently of the strain used, although there was preferential crystal growth dependence. Gold NPs were obtained by using both strains, but cell lysis was observed for Y1, suggesting that the reduction of metal ion occurred due to interactions both with the cell wall and intracellular binding sites. The size range of the gold NPs was ca. 20 to 100 nm, pointing to a higher reduction rate of Au ions compared to the Ag ions. All NPs were well dispersed, indicating that they are capped by stabilizing agents. The possibility of protein as a stabilizing material in silver NPs has been revealed by FTIR analysis.
Understanding the mechanism of crystalline spherical NPs synthesis at cellular and molecular level by these extremophilic yeasts will be the main focus of subsequent research.

Reference

233

Pimaricin controlled released with smart PNIPAA nanohydrogel

Fuciños, Clara (1); Amado, Isabel Rodríguez (1); Fajardo, Paula (1); Katime, Issa (2); Pastrana, Lorenzo Miguel (1); Rúa, Maria Luisa (1)

1: University of Vigo, Spain;
2: University of Pais Vasco, Spain

E-mail: clarafg@gmail.com

Keywords: nanohydrogel, PNIPA, pimaricin, antibiotic, drug release

Abstract

Pimaricin is a polyene macrolide antibiotic produced by submerged aerobic fermentation of *Streptomyces natalensis* and related species. It is used as a food additive to control the growth of yeasts and moulds on the surface of cheese and other non-sterile products, such as meat and sausages (Vanden Bossche et al., 2003).

In order to desing a suitable delivery which are able to protect the pimaricin from hostile environment as well as to manipulate the pimaricin release utilizing environmental stimuli we propose the use of poly(N-isopropylacylamide) thermosensitive nanogels ($PNIPAA_m$).

This type of polymers have been widely investigated for controlled drug delivery. $PNIPAA_m$ nanogels have a mechanism which allows drugs loaded into PNIPAAm gels to be squeezed out of the polymer when the temperature is raised above the lower critical solution temperature (LCSTs) (Wu et al., 2005) because of the polymer becomes insoluble in water at this temperature (Katime et al., 2003).

The objective of this work was to study the pimaricin release kinetics in a nanogel of PNIPAAm copolymerized with acrylic acid (PNIPA-AA). For this propouse pimaricin was loaded into the nanogel at room temperature (25ºC) overnight. Then the pimaricin-loaded swollen gel samples were placed in a dialysis tubing and was dialyzed in destilled water at two different temperatures, 15 and 37 ºC. Pimaricin concentrations in the release medium were analyzed by spectrophotometry at 319 nm.

The maximum fractional release of pimaricin from nanogel through the dialysis tubing was compared with corresponding value of pimaricin release in nanogel absence to substract the dialysis effect (F_{max}). So, the release profile showed that F_{max} was 0.4. This means that the amount released from nanogel was lower than in a normal pimaricin dialysis process. However, the equilibrium dialysis point was increased 2.3 times. Also, the release changing the temperature from 37 ºC to 15 ºC during 3 cycles showed a maintained released. This was consistent with the acrylic acid effect (Eeckman et al., 2004). Therefore, the PNIPA-AA nanogel was useful to obtain a sustainable release of pimaricin.

Eeckman, F., Moës, A.J., Amighi, K. (2004). International Journal of Pharmaceutics, 273, 109-119.
Katime, I.A., Katime., O., Katime, D. (2003). Los materiales inteligentes del milenio: Los hidrogeles macromoleculares. Síntesis, propiedades y aplicaciones. Servicios editoriades de la Universidad del País Vasco (Bilbao, Spain).
Vanden Bossche, M.E., Rochette, F. (2003). Journal Of Veterinary Pharmacology And Therapeutics, 26, 5-29.
Wu, J-Y., Liu, S-Q., Heng, P.W-S., Yang, Y-Y. (2005).. Journal of Controlled Release, 102, 361-372.

Reference

316

Recombinant Elastin-Like Polymers: from the design towards application

Machado, Raul (1); Araújo, Rita (1,2); Silva, Carla (2); Bessa, Paulo (3,4); Cunha, António M. (5); Teixeira, José António (6); Cavaco-Paulo, Artur (2); Arias, Francisco Javier (7,8); Reis, Rui L. (3,4); Rodríguez-Cabello, José Carlos (7,8); Casal, Margarida (1)

1: CBMA, Dept. Biology, Univ. Minho, Portugal; 2: 2C2T, Dept. Textile Eng., Univ. Minho, Portugal; 3: 3B's Res. Group, Univ. Minho, HQ European Inst. Excellence on Tissue Eng. and Regenerative Medicine, AvePark, Portugal; 4: IBB – Inst. for Biotechnol. Bioeng., Portugal. CBMA, Dept. Biology, Univ. Minho; 5: Inst. for Polymers and Composites, Dept. Polymer Eng., Univ. Minho, Portugal; 6: Dept. Biological Eng., Univ. Minho, Portugal; 7: BIOFORGE, Univ. Valladolid, Valladolid, Spain.; 8: CIBER BBN, Valladolid, Spain

E-mail: raulmachado@bio.uminho.pt

Keywords: elastin-like, nanoparticles, subtilisin, drug delivery, BMP-2

Abstract

With the development of protein engineering and nano(bio)technologies it is now possible to use amino acids to design and produce genetically engineered Protein-Based Polymers (PBPs).
These polymers occur in a wide range of biological systems, fulfilling precise functional roles. Its properties are due to the presence of short repeating sequences contained in the fibrous proteins, such as mammalian elastin. Elastin-Like Polymers (ELPs) are biopolymers based on the aminoacid sequence VPGXG (where X is any naturally occurring aminoacid except proline) that reversibly coacervate above a critical temperature (T_t), showing a visible transition phase that can be explored as a purification method. Additionally, the ability of ELPs to self-assemble into nanostructures in response to environmental signals allows them to be explored for controlled drug delivery devices or nanosensors. The polymer poly(VPAVG), a ELP where the central glycine (G) is substituted by a L-alanine (A), was chemically synthesized by Rodríguez-Cabello and co-workers and described by Urry as having thermoplastic properties. These groups reported its characterization, demonstrating its extreme biocompatibility both *in vitro* and *in vivo,* as well as the ability to self-assemble, forming microparticles that can entrap active substances during the self-assembling process.
In the present work a new thermally responsive, biologically synthesized ELP based on the $(VPAVG)_{220}$ sequence was produced with standard molecular genetic tools and, as expected, the polymer displayed an inverse temperature transition (T_t) which could be explored as a purification approach (1). Sequence and purity was confirmed by MALDI TOF and SDS-PAGE analysis and purified polymer was thermally and physically characterized. Due to its self-assembling behaviour near 34 ºC stable spherical microparticles of a ~1 µm diameter were obtained, ready solubilized when a strong undercooling was achieved. By fusing the ELP with Subtilisin E DNA sequence we were able to produce a soluble chimeric protein with improved properties in wool yarn treatment, when compared with the commercial Subtilisin (2). The ELP was also exploited as a drug delivery system for the controlled release of BMP-2 and BMP-14 (3). The ELP system showed a high efficiency of encapsulation with a sustained release for 14 days. The activity of the growth factors was maintained and an increased bioactivity was observed when combining the release of BMP-2 and BMP-14.

1 – Machado, R., Ribeiro, A.J., Padrão, J., Silva, D., Nobre, A., Teixeira, J.A., Arias, F.J., Cunha, A.M., Rodríguez-Cabello, J.C., Casal, M., Journal of NanoResearch, 2009, 6, 133-145.
2 – Araújo, R., Silva, C., Machado, R., Casal, M., Cunha, A.M., Rodríguez-Cabello, J.C., Cavaco-Paulo, A., Biomacromolecules, 2009, 10, 1655-1661.
3 – Bessa, P.C., Machado, R., Nürnberger, S., Dopler, D., Banerjee, A., Cunha, A.M., Rodríguez-Cabello, J.C., Redl, H., van Griensven, M., Reis, R.L., Casal, M., submitted.

Reference

275

An integrated approach for human stem cell expansion and neuronal differentiation in a fully controlled bioreactor

Serra, Margarida; Brito, Catarina; Sousa, Marcos F.Q.; Alves, Paula M.

IBET/ITQB, Portugal

E-mail: anabrito@itqb.unl.pt

Keywords: stirred tank bioreactors, embryonic stem cells, expansion, neuronal differentiation, 3D-aggregates, NT2 cells

Abstract

To fulfill the expectations raised by stem cell therapy it is urgent to develop robust and totally controlled culture systems, specially designed for the production of high numbers of differentiated and well-characterized cells, expanded as fast and pure as possible. In this work, we successfully developed a bioprocess for the expansion of human neurons using fully controlled stirred tank bioreactors. This was accomplished by integrating cell expansion and differentiation in a two-step bioprocess. The human embryonal carcinoma cell line Nera-2/cl.D1 (NT2) was the cellular system used because it is a valuable model for both undifferentiated human embryonic stem (hES) cells and human neuronal differentiation *in vitro.*

Within this context, the expansion of undifferentiated NT2 cells as 3D-aggregates was firstly optimized in spinner vessels; different bioreaction conditions, including cell inoculum concentration and feeding strategy, were studied. The media exchange operation mode with an inoculum concentration of $4x10^5$ cell/mL was the most efficient strategy tested, with high cell viabilities and a 4.5-fold increase in cell concentration achieved within 5 days. These results were further validated in the bioreactor with pH, oxygen and temperature control, where similar culture profile and metabolic performance were obtained. Furthermore, characterization of the expanded population by immunofluorescence microscopy and flow cytometry showed that NT2 cells maintained their pluripotent and undifferentiated phenotype. Neuronal differentiation efficiency was maintained, confirming that the stem cell potential was not compromised in the bioreactor culture.

Finally, the neuronal differentiation step was integrated in the bioreactor process, by addition of retinoic acid when cells were in the middle exponential phase. Neurosphere composition was monitored and neuronal differentiation efficiency evaluated along the culture time. The results show that, when compared to standard methodologies using static conditions, bioreactor cultures allowed a significantly increase of 10-fold in neuronal differentiation efficiency while reducing drastically (by 30%) the time required for the differentiation process.

The controlled bioprocess developed herein represents a strong and promising starting point for the development of novel technologies for the production of pluripotent cell derivatives, broadly adaptable to other cell types, including hES and iPS cells.

Poster Session:
S2 – Cell and Tissue Engineering, Biomaterials and Nanobiotechnologies

Reference

152

Erythropoietin production in *Arabidopsis thaliana* cell cultures

Dias, Sónia Marisa; Pires, Ana Sofia; Abranches, Rita

Instituto de Tecnologia Química e Biológica, Plant Cell Biology Laboratory, 2781-901 Oeiras, Portugal

E-mail: s_m_dias@hotmail.com

Keywords: Molecular farming, erithropoietin, *Arabidopsis thaliana*, Medicago truncatula, recombinant protein, suspension cell cultures

Abstract

The large-scale production of recombinant proteins in plant systems, known as Molecular Farming, offers many practical and safety advantages when compared to traditional platforms. Plant cell suspension cultures are an attractive alternative system, as they combine the advantages of both whole plant and cell culture production systems. In this work cell suspension cultures from *Arabidopsis thaliana* are used as a platform to study the expression of the recombinant protein Erythropoietin (rEPO), a human glycosylated hormone which regulates the formation of erythrocytes in mammals.

The main objectives consist on assessing the potential of *Arabidopsis* cell suspension cultures for recombinant protein production and performing an integrative analysis of the results obtained, taking into account cell culture performance in terms of cell line stability, cell viability, yield and product recovery. By comparing these results with the ones obtained from Medicago truncatula, another plant model under study in our laboratory, we will gain further insight on species-specific factors that have an impact on final product quality.

Cell cultures were generated from transgenic *Arabidopsis* plants which express rEPO, either secreted or ER-retained. For both forms we confirmed that the recombinant protein was present either in the culture medium or in the cellular extracts. Several strategies were addressed to purify EPO from cell cultures of *Arabidopsis* and *Medicago*, including ion-change and affinity chromatography, and we have achieved partial purification of EPO. We have also observed the morphology of the cells and analysed the viability of the cultures using light and fluorescence microscopy. We compared cultures of both plant species and performed several staining procedures using neutral red and acridine orange fluorescent dyes. Arabidopsis cultures showed smaller cells than those of Medicago and more compact clusters. In Medicago cells the vacuolar compartment took up most of the cellular volume and the cytoplasmatic volume was reduced.

Future work involves the optimization of *Arabidopsis* cell cultures to maximize accumulation of EPO and designing a better approach for EPO purification. These results will contribute to the establishment of new plant-based platforms for production of high valuable molecules.

Gonzalez-Melendi P, Pires AS and Abranches R. (2009). Functional Plant Biology 36, 431-441.
Pires AS, Cabral MG, Fevereiro P, Stoger E and Abranches R. (2008). Biotechnology Journal 3, 916-923.
Abranches, R.; Marcel, S.; Arcalis, E.; Altmann, F.; Fevereiro, P. and Stoger, E. (2005). Journal of Biotechnology 120, 121-134.

Reference

194

Antimicrobial activity of methylene blue and toluidine blue O covalently bound to a modified silicone polymer surface

Piccirillo, Clara (1,2); Perni, Stefano (3); Gil-Thomas, Jesus (4); Prokopovich, Polina (5); Wilson, Michael (3); Pratten, Jonathan (3); Parkin, Ivan P. (2)

1: Escola Superior de Biotecnologia, Universidade Catolica Portuguesa, Portugal;
2: Materials Chemistry Research Centre, Chemistry Department, University College London, United Kingdom;
3: Eastman Dental Institute, University College London, United Kingdom;
4: Departemento di Quimica Organica, Universitat de Valencia, Spain;
5: Wolfson School of Mechanical and Manufacturing Engineering, Loughborough University, United Kingdom

E-mail: cpiccirillo@esb.ucp.pt

Keywords: LAAAs, antimicrobial activity, silicone, surface modification

Abstract

Catheters are medical devices widely used in hospital for the delivery of fluid from and to the body and for drug administration; they are normally made of polymeric materials, such as silicone or polyurethane. However, a problem often associated with their use is the growth of bacteria colonies on their surface, leading eventually to the formation of a biofilm. This can cause infections, normally referred to as Catheters Related Infections (CRIs). CRIs can affect the health of patients, in some cases they also be lethal; furthermore, they can be very expensive to treat [1].

Several approaches have been studied to eliminate or reduce the CRIs; one possibility is the use of Light Activated Antibacterial Agents (LAAAs). LAAAs are compounds that, if irradiated, produce active species, such as singlet oxygen, radicals, etc. They can react with the bacteria, killing them. This procedure is known as Photo Dynamic Therapy (PDT).

Previous work described the use of dyes such as Methylene Blue (MB) or Toloudine Blue O (TBO), included within the silicone or polyurethane matrix; a remarkable decrease in the viable bacteria number on the surface of the polymers was observed [2,3].

In this paper we report about MB and TBO covalently bounded to a silicone surface; because of its chemical inertness, the surface had to be activated/modified. UV spectra confirmed the presence of very small amount of dyes bounded to the silicone surface. The materials were tested on bacteria such as *Staphylococcus epidermidis* and *Escherichia coli*. The results show that they have very high antimicrobial activity, despite the very low concentration of the LAAAs; therefore they could be potentially used in hospital environment, to reduce the extent of CRIs.

[1] B. Trautner and R. Darouiche, Arch. Int. Med., 2004, 4, 842–850.
[2] S. Perni, C. Piccirillo, J. R. Pratten, P. Prokopovich, W. Chrzanowski, I. P. Parkin and M. Wilson, Biomaterials, 2009, 30(1), 89–93.
[3] S. Perni, P. Prokopovich, C. Piccirillo, J. R. Pratten, I. P. Parkin and M. Wilson, J. Mater. Chem., 2009, 19, 2715–2723.

Reference

200

In Vitro Adherence of *Staphylococcus aureus* to Polyurethane and Polypropylene Threads Used in Facial Rejuvenation

Leite, Bruna Arruda (1); Pizzolitto, Elisabeth Loshchagin (1,2); Pizzolitto, Antonio Carlos (2)

1: University of São Paulo – USP, São Carlos/SP, Brazil;
2: Sao Paulo State University-UNESP, Araraquara/SP, Brazil

E-mail: bru_biomedica@hotmail.com

Keywords: Biofilm, biomaterials, polymers, bacteria adherence, face lift

Abstract

Staphylococcus aureus is frequently implicated as one of the aetiological agents of biomaterial-associated infections. The bacterial biofilm formation on synthetic and natural polymers plays an important role in industry and modern medicine, and makes it difficult to treat infections caused by colonized foreign bodies. The face lift is a cosmetic surgery that can make the face appear younger. Skin reinforcement with special golden threads is being used for correction of ptotic alterations of the facial skin. The aim of the present study was to assess bacterial attachment on polypropylene and polyurethane (derived from the castor oil plant - *Ricinus communis*) threads using culture methods and scanning electron microscopy (SEM). *Staphylococcus aureus* ATCC 25923 was used in this study. A 200 µL of this cell suspension (10^8 cfu/ml) was used to inoculate 15 mL of Mueller Hinton broth. The threads of 1.0 cm in length were inserted, one by one, into separated Falcon tubes containing Mueller Hinton broth inoculated with the *S. aureus* suspension, and incubated at 37°C under constant agitation. After 90 minutes, 4, 24, 48, 72 and 120 hours polypropylene and polyurethane threads were removed, rinsed and introduced into glass tubes containing 5.0 mL of sterile physiological saline solution, sonicated and vortexed. The supernatant was diluted three-fold and 0.1 mL of a 10^{-3} dilution was seeded onto trypticase soy agar. After incubation at 37ºC for 24h the viable cells were counted. Statistical analysis included calculation of arithmetic means and the results were shown in terms of cfu/mL. The attachment of *S. aureus* to polypropylene and polyurethane threads occurred rapidly. SEM observations showed singly and in pairs cocci, chains of three or four cells and irregular “grape-like” clusters. The viable cell counts on polyurethane threads after 90 minutes, at 4, 24, 48, 72 and 120 hours showed $5.0x10^4$; $1.1x10^6$; $3.0x10^6$; $3.9x10^6$; $3.8x10^6$; $6.0x10^6$ and on polypropylene showed $9.0x10^4$; $6.8x10^5$; $7.7x10^6$; $2.7x10^7$; $2.7x10^7$; $2.7x10^7$. This study showed that *S. aureus* attached both to polypropylene and polyurethane threads and formed biofilm, as demonstrated by SEM. In conclusion both surfaces are susceptible to bacterial attachment and, the consequence can be an involvement of biomaterials in post-operative complications.

Reference

266

Monitoring mammalian cell adhesion with the quartz crystal microbalance

Da Silva, Ana Carina; Tomé, Brigitte; Ferreira, Guilherme N.M.

Institute for Biotechnology and Bioengineering (IBB), Portugal

E-mail: anadsilva@ist.utl.pt

Keywords: Quartz crystal microbalance; Biosensor; Cell adhesion, Fibroblats; Epithelial cells.

Abstract

Cell culture has become an increasingly important research platform in several scientific areas. For the *in vitro* survivor and proliferation of a cell culture, anchorage-dependent cells must adhere to a tissue culture surface and spread. The quartz crystal microbalance (QCM) is being used by us as a technique to study, in real-time and non-invasively, the ability of different mammalian cell lines (HEK 293T and HeLa (epithelial cells) and COS-7 cells (fibroblasts)) to attach and spread on the bare gold sensor's surface, in the presence and in the absence of serum proteins. Initially, serum-free or serum-supplemented medium was injected in the QCM's chamber and frequency and resistance signals were allowed to stabilize. Cells were injected and processes of cell adhesion/spreading were followed over several days, through the monitoring of the resonance frequency and resistance. In serum free-medium, cell injection induced a decrease in frequency for all cell types, meaning that cells are adsorbing at the sensor's surface. Cells reach the sensor's surface during the first hour, so the remaining changes in frequency shift are due to viscoelastic alterations that cells promote at cell-surface boundary. These alterations are also responsible for the increase of resistance observed during experiments. For COS-7 cells, a considerable difference is notice for the initial resistance values, meaning that important viscoelastic phenomena are occurring at this stage. Once fibroblasts are the major cell involved in the synthesis and homeostatic maintenance of the extracellular matrix, being able to synthesize and secrete most ECM components, those differences could be related to this high metabolic activity of COS-7 cells. Next, experiments where performed in serum-supplemented conditions, to analyze the adhesion behaviour in the presence of a complex and pre-established serum-proteins layer. It was observed that all cells behave differently in the presence and in the absence of serum. In the case of HEK 293T almost no viscoelastic alterations are detected and for HeLa and COS-7 a more rigid structure was probably formed in the presence of serum proteins. The presence of cells at sensor's surface was confirmed by fluorescence microscopy. QCM has proven to be suitable to study in real-time and non-invasively the adhesion of cells onto gold surfaces, allowing the distinction of different adhesion processes and detecting different adhesion behaviour between different cell lines.

Reference

268

Bio-synthesis of nanosized metal sulphide semiconductors

Pinto da Costa, Joao Miguel (1); Santos, Erika Silva (1); Monteiro, Olinda Coelho (2); Costa, Maria Clara (1)

1: Universidade do Algarve, Portugal;
2: Universidade de Lisboa, Portugal

E-mail: jcosta@ualg.pt

Keywords: Sulphate-reducing bacteria, acid mine drainage, bioremediation, nanoparticles.

Abstract

Acid mine drainage (AMD) is a serious and widespread environmental problem that occurs when sulphide minerals are exposed to oxygen and water. This problem is quite relevant in Portugal, where extensive sulphide mining activities played an important role until recent times. Several approaches are reported for AMD treatment, but the use of sulphate-reducing bacteria (SRB) has been identified as the most cost-effective (Barnes *et al.*, 1998). This process, however, generates an excess of sulphide and the elimination of this excess and the disposal of the metal sulphides are also problems that need to be solved. Thus, the use of H_2S to produce nanocrystalline materials is a welcomed approach to convert potentially harmful wastes in useful products for innovative applications. This can be regarded as an integrated green solution not only to eliminate wastes, but also in generating products potentially useful, namely in water decontamination, as semiconductor nanomaterials can be used as catalysts in photo assisted decomposition of organic pollutants. (Wang *et al*, 1998; Chatterjee and Dasgupta, 2005). The final goal of this project is the synthesis of morphologically well-defined metal sulphide semiconductor nanoparticles using by-products of a sulphate-reducing biological process that was developed for the treatment of acid mine drainage (Barros *et al*, 2008), using a specific bacterial consortium of *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis* species (Martins *et al*, 2009). The synthesis of the nanomaterials can preferentially take place *in situ* or the by-products of the sulphate reducing-bacteria activity (sulphide and/or metal precipitates) can be moved to a different environment to produce nanomaterials. Preliminary experiments have shown that semiconductor nanocrystals can be obtained by adding biologically generated sulphide to aqueous solutions containing heavy metals under specific experimental conditions, that include the use – or not – of substrates. Moreover, the nanoparticles produced show morphological characteristics which depend on the reaction conditions, allowing chemical control on size dependant functionalities. The properties of the biological nanosulphides, analyzed by TEM and X-ray diffraction, are being compared with those obtained by a chemical route. Future work will include the possibility of integrating this synthesis with the bioremediation process.

D. Chatterjee, S. Dasgupta, J. Photochem. Photobiol., C 6 (2005) 186–205.
L.J. Barnes, F.J., Janssen, P.J.H., Scheeren, J.H., Versteegh, R.O., Koch, Trans. Inst. Min. Metall. (Sect C: Mineral Process. Extr. Metall.), 101, (1998) 181-187
M. Martins, M. L. Faleiro, R. J. Barros, A. R. Veríssimo, M. A. Barreiros, M. C. Costa, J. Hazardous Materials (2008).
R.J. Barros, C. Jesus, M. Martins, M.C. Costa, Process Biochemistry (2008) Submitted.
T. Wang, H. Wang, P. Xu, X. Zhao, Y. Liu, S. Chao, Thin Sol. Films, 334 (1-2) (1998) 103-108

Reference

288

Detection of HIV-1 virion infectivity factor using an amorphous silicon photodiode

Vistas, Cláudia A. R. (1); Conde, João Pedro (2); Ferreira, Guilherme N. M. (1)

1: Institute for Biotechnology and Bioengineering, Portugal;
2: INESC Microsistemas e Nanotecnologias, Lisbon, Portugal

E-mail: claudia.vistas@ist.utl.pt

Keywords: Optical biosensor, fluorescence, photodiode, quantum dot, protein detection

Abstract

An integrated platform based on a hydrogenated amorphous silicon (a-Si:H) p-i-nphotodiode is used to optically detect the HIV-1 Vif (virion infectivity factor) labeled with semiconductor quantum dots (QDs). Anti‑HIV‑1 Vif single chain fragment antibodies (scFv-4BL) were used and immobilized in two different ways: 4BL was covalently linked to the surface of a glass slide or was conjugated to magnetic beads (MBs). The Vif protein was conjugated to the CdSe/ZnS core-shell QDs and this complex was put in contact with the 4BL antibody immobilized in order to carry out the recognition reactions. The signal was measured by putting the glass slide, where the recognition reactions occurred, or by putting a drop of the complex MBs-4BL/Vif-QDs, which was easily isolated by common magnet separation, on top of the photodetector. The QDs used were excited at wavelength less than 400 nm resulting in the emission of visible light at 600 nm. An amorphous silicon-carbon (a‑SiC:H) filter, integrated in the chip, cuts the excitation light while allowing the transmission of the emission light to the a‑Si:H layer producing a photoresponse. The detection limit of the present device was of the order of 0.01 pmol for the QD600 in solution (corresponding to a concentration of 10^{-9} M). The HIV-1 Vif labeled with QDs was successfully detected in both procedures: in the 4BL immobilized on the surface of the glass slide it was possible to detect QDs at a surface density of ~0.6 pmol/cm^2; in the 4BL immobilized on the MBs 0.03 pmol of QDs in solution could be detected by fluorescence using the a-Si:H photodiode.

Reference

312

Gold nanoprobes – A new approach to differentiate between *Mycobacterium tuberculosis* complex members

Costa, Pedro (1,2); Inácio, João (1); Amaro, Ana (1); Botelho, Ana (1); Baptista, Pedro (2)

1: Laboratório Nacional de Investigação Veterinária, Lisboa, Portugal;
2: CIGMH/DCV, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal

E-mail: pedro.costa@fct.unl.pt

Keywords: *Mycobacterium tuberculosis* complex, Mycobacterium bovis, *Mycobacterium tuberculosis*, Gold nanoprobes

Abstract

Members of the *Mycobacterium tuberculosis* complex (MTC) are causative agents of human and animal tuberculosis. This complex encompasses several phylogenetically-related species, namely *Mycobacterium tuberculosis*, the main etiological agent of human tuberculosis, and *Mycobacterium bovis*, the causative agent of bovine tuberculosis, a relevant worldwide zoonosis with high impact in host health and economy. Clear epidemiological evaluation towards appropriate and effective disease control requires unambiguous differentiation between MTC members. Routine diagnosis has been increasingly relying on the molecular identification of these pathogens. We have used a novel approach for the sensitive, specific and fast identification of MTC members using *gyrB* locus targeted thiol-linked oligonucleotide-modified gold nanoparticles. This gold-nanoprobe strategy relies on the colorimetric differentiation of specific DNA sequences induced by differential aggregation profiles in presence and/or absence of specific target hybridization. Three gold nanoprobes were successfully developed for the specific identification of MTC members, *M. bovis* and *M. tuberculosis*, respectively. This novel nanodiagnostic approach allows rapid differentiation of the etiological agents, making it suitable for the routine diagnosis of tuberculosis in veterinary and human health settings.

Reference

313

RNA Profiling with Gold Nanoparticles

Conde, João (1); Santos, Margarida (1); Rosa, João (1,2); Severino, Paulo (3); Tavares, Silvia (4); Baptista, Pedro (1)

1: CIGMH/DCV, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa,Portugal, Portugal;
2: REQUIMTE/DQ, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal;
3: CEDOC/DI, Faculdade de Ciências Médicas, Universidade Nova de Lisboa,Portugal;
4: CBAA/DBEB, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

E-mail: conde.bio@gmail.com

Keywords: Nanoparticles, Gold nanoparticles, RNA detection and quantification, Nanotechnology

Abstract

The use of gold nanoparticles derivatised with thiol modified oligonucleotides (Au-nanoprobes) has lead to a new era of molecular nanodiagnostics with great potential in biomolecular identification. Based on the differential non-cross-linking aggregation of these Au-nanoprobes we were able to develop a colorimetric method for the detection of gene expression[1], pathogens[2] and single base mutations/single nucleotide polymorphisms (SNP) [3]. Detection is achieved by colour comparison between solutions containing the Au-nanoprobe with either a complementary or a non-complementary target sequence, upon increasing ionic strength. The presence of a complementary target prevents aggregation upon salt addition and the solution remains red; conversely, the non-complementary targets do not prevent Au-nanoprobe aggregation, resulting in a colour change from red to blue.

Here, we demonstrate the applicability of this method to different RNA analysis situations: i) identification and quantification of mRNA (33 to 170 fmol/mL); ii) detection and quantification of miRNA in spiked mixtures (20 to 80 fmol/mL); iii) detection of aberrant mRNA derived from the human *BCR-ABL* fusion gene and to differentiate the fusion product from the normal *BCR* and *ABL* sequences; and iv) detection of transcripts from the proto-oncogene *c-Myc* and the tumour suppressor gene *p53*.

[1] P. Baptista, G. Doria. D. Henriques, E. Pereira, R. Franco, J. Biotechnol. 119 (2005) 111-117.
[2] P. Baptista, M. Koziol-Montewka, J. Paluch-Oles, G. Doria, R. Franco, Clin. Chem. 52 (2006) 1433-1434.
[3] G. Doria, R. Franco, P. Baptista, IET Nanobiotechnol., 1 (2007) 53-57.

Reference

315

Use of Au-nanoprobes for the detection of SNPs associated with antibiotic resistance in *Mycobacterium tuberculosis*

Veigas, Bruno (1); Perdigão, João (2); Portugal, Isabel (2); Couto, Isabel (3,4); Viveiros, Miguel (3); Baptista, Pedro (1)

1: CIGMH/DCV, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal;
2: Centro de Patogénese Molecular/URIA, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal;
3: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL);
4: Centro de Recursos Microbiológicos (CREM), Universidade Nova de Lisboa

E-mail: brunoveigas@me.com

Keywords: *Mycobacterium tuberculosis*, Au-nanoprobes, antibiotic resistance

Abstract

Tuberculosis (TB) is one of the leading causes of infection in humans, causing high mobility and mortality all over the world. The rate of new cases of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) continue to increase [1], and since these infections are very difficult to manage [2], they constitute a serious health problem. In most cases, anti-TB drug resistance has been related to mutations in several loci within the pathogen's genome. The development of fast, cheap and simple screening methodologies would be of paramount relevance for the early detection of these mutations, essential for the timely and effective diagnosis and management of M/XDR-TB patients.

The use of gold nanoparticles derivatised with thiol modified oligonucleotides (Au-nanoprobes) has lead to new approaches in molecular diagnostics - nanodiagnostics. Based on the differential non-cross-linking aggregation of Au-nanoprobes we were able to develop a colorimetric method for the detection of specific sequences and applied this approaches to studies of gene expression, pathogen identification and single base mutations/single nucleotide polymorphisms (SNP) discrimination [3,4]. This gold-nanoprobe strategy relies on the colorimetric differentiation of specific DNA sequences induced by differential aggregation profiles in presence and/or absence of specific target hybridization - the presence of complementary target prevents aggregation and the solution remains red; non-complementary/mismatched targets do not prevent Au-nanoprobe aggregation resulting in a visible change of color from red to blue.

Here we report on the synthesis and characterisation of Au-nanoprobes for the specific identification of SNPs within the beta subunit of the RNA polymerase (*rpo*-beta locus), responsible for over 95% of rifampicin resistant TB cases.

1. World Health Organization. Anti-tuberculosis drug resistance in the world: report No. 4. Geneva, Switzerland: WHO; 2008.
2. Martins M, Viveiros M, Couto I, Amaral L. Int J Tuberc Lung Dis. 2009 13:569-73.
3. Pedro V. Baptista, Maria Koziol-Montewka, Jolanta Paluch-Oles, Gonçalo Doria, Ricardo Franco, Clinical Chemistry 52, No. 7, 2006
4. Gonçalo Doria, Ricardo Franco and Pedro Baptista. IET Nanobiotechnology, 1(4): 53-57 (2007).

Reference

318

Ultrasonic fragmentation of DNA and its importance in sample treatment for detection of specific nucleotide sequences

Larguinho, Miguel Ângelo Rodrigues (1); Santos, Hugo Miguel (2); Doria, Gonçalo (1,2); Capelo, José Luis (2,3); Baptista, Pedro Viana (1)

1: CIGMH/DCV, Faculdade de Ciências e Tecnologia - Universidade Nova de Lisboa, Portugal;
2: REQUIMTE/DQ, Faculdade de Ciências e Tecnologia - Universidade Nova de Lisboa, Portugal;
3: BioScope Group, Nutrition and Bromatology Area, Faculty of Science, University of Vigo, Spain

E-mail: miguel.larguinho@gmail.com

Keywords: Ultrasound, sonoreactor, DNA, gold nanoprobes, nanodiagnostics

Abstract

DNA based diagnosis usually requires sample pre-treatment for making the target molecule/region available for bio-recognition. The sample preparation step should be fast, cheap, allowing for high-throughput and avoid cross-contamination. Sample preparation methods for DNA assaying are commonly based on molecular biology techniques (e.g. restriction digestion, PCR, etc), which improve hybridization kinetics but are expensive and require complex setups. More recently, the use of ultrasounds has provided an alternative to circumvent common limitations of these techniques, yielding DNA fragments compatible with most downstream applications [1].

Here, we present the comparison between different ultrasound platforms [2] for DNA sample preparation in terms of effective fragmentation of DNA (plasmid and genomic). After careful evaluation of the attained results, an optimized protocol was developed so as to prepare DNA samples for subsequent use in a molecular detection method based on use of gold-nanoprobes [3].

L.T. Mann, U.J. Krull, Biosens. Bioelectr. 20 (2004) 945-955.
H.M. Santos, J.L. Capelo, Talanta 73 (2007) 795-802.
P. Baptista, G. Doria, D. Henriques, E. Pereira, R. Franco, Journal Biotechn. 119 (2005) 111-117.

Oral Session:
S3 – Clinical Microbiology and Epidemiology

Reference

426

Genética e epidemiologia do vírus A(H1N1) 2009

Froes, Filipe

Hospital Pulido Valente, Lisboa

Abstract

A origem da estirpe pandémica de vírus influenza A (H1N1) 2009 é o resultado de várias recombinações de segmentos de RNA de diferentes origens animais ocorridas em vasos misturadores suínos. A origem por mecanismo de recombinação (shift antigénico) difere do mecanismo de mutação (drift antigénico) da gripe sazonal e condiciona importantes diferenças na epidemiologia e apresentação clínica da infecção.

Assim, por ser um microrganismo novo para o sistema imunológico do Homem, não existe imunidade de grupo o que explica o aumento da taxa de ataque até 30% da população, cerca de 3 a 6 vezes mais do que em relação à gripe sazonal, e a ocorrência de actividade gripal nos meses de Verão, com os estabelecimentos escolares fechados. De igual modo, constata-se uma maior incidência nos grupos etários mais jovens, verificando-se que cerca de 80% dos casos confirmados apresentam idade inferior aos 30 anos. Há, igualmente, uma maior incidência de formas graves de doença nas grávidas, asmáticos e obesos, provavelmente resultante de diferentes interacções do vírus com hospedeiros susceptíveis.

Se estamos perante a primeira pandemia do século XXI, também pela primeira vez na história da humanidade nunca tanta informação e conhecimento estiveram disponíveis para os profissionais de saúde e população, em geral. Mas este conhecimento representa uma responsabilidade acrescida de prepararmo-nos mais adequadamente e sobretudo de fazermos melhor.

Reference

140

Chlamydia trachomatis and *Neisseria gonorrhoeae* infections in pregnant adolescents: Prevalence and association with maternal and fetal morbidity

Costa, João Borges (1); Matos, Cristina (2); Lourenço, Isabel (2); Alves, Maria José (2); Pereira, Filomena Martins (1)

1: Instituto de Higiene e Medicina Tropical, Portugal;
2: Maternidade Alfredo da Costa

E-mail: joaobc77@hotmail.com

Keywords: Chlamydia trachomatis, Neisseria gonorrhoeae, pregnant adolescents

Abstract

Background: Pregnant teenagers have higher prevalence of many sexually transmitted infections (STI). Portugal is the country in Western Europe with the second highest incidence of pregnancy in adolescents but data on STI in this group is not known.

Objectives: To assess the prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in a population of pregnant adolescents from the two main obstetric hospitals in Lisbon and search for significant with maternal/fetal morbidity.

Methods: A total of 204 pregnant were interviewed and tested for *C. trachomatis* and *N. gonorrhoeae* in first-void urine/cervical swabs by polymerase chain reaction, targeting the cryptic plasmid in the first bacteria and the *cppb* gene in the second. Data was analysed with the program SPSS, with a 5% level of exact significance.

Results: The prevalence of *C. trachomatis* was 11.8% (24/204) and of *N. gonorrhoeae* was 4,9% (10/204). No positive serologies were found for syphilis and human immunodeficiency virus infection and trichomonosis was diagnosed in two adolescents. The birth was eutoccic in 73.5% (150/204) and the morbidity in the mother (defined as fever during or after delivery or chorioamnionitis) was 3.4% (7/204). Preterm birth occurred in 16.7% (34/204) and low birth weight in 9.8% (20/204). Statistical analysis showed significant associations for infection with *N. gonorrhoeae* and maternal morbidity ($\chi 2 = 22.4$; $p = 0.03$) and for infections with *C. trachomatis* and/or *N. gonorrhoeae* and low birth weight ($\chi 2 = 6.25$; $p = 0.021$).

Discussion/conclusions: STI can cause spontaneous abortion, preterm birth and low birth weight. However, the proportion of these events related to *C. trachomatis/N. gonorrhoeae* infections may differ among different age groups and countries, with conflicting data being published. In this study, the prevalence of these infections was found to be similar to other countries in this age group and to our knowledge, no other studies targeting associations between these infections and morbidity in adolescence pregnancy were previously performed in Portugal. Our study reinforces the importance of screening for STI, namely *C. trachomatis*, which are not routinely looked for in Portugal, despite the recommendations of American/European guidelines, not only aiming to stop transmission of this infection from pregnant adolescents to their neonates, as also as an opportunity for councelling in view of preventing future infections with these or other STI agents.

Reference

269

Longitudinal colonization by Group A streptococci during 2000-2004 in Portugal

Pires, Renato (1); Alves, Carolina (1); Esteves, Filipe (1); Rolo, Dora (1); Almeida, João Manuel Feio (1); Ramos, Teresa (2); Gameiro, Clotilde (2); Andrade, Filomena (2); Lopes, Ana (2); Romeiro, Luísa (2); Vaz, Fátima (2); Morais, Ana (2); Brito-Avô, António (2); Gonçalo-Marques, José (3); Santos-Sanches, Ilda (1)

1: Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (FCT/UNL), Caparica, Portugal;
2: Centro de Saúde de Oeiras, Oeiras, Portugal;
3: Hospital de Santa Maria, Lisbon, Portugal

E-mail: rhmp@fct.unl.pt

Keywords: Group A streptococci, longitudinal colonization, carrier, clones, emm type

Abstract

Group A streptococci (GAS) colonize the throat or skin and are responsible for a number of suppurative infections and nonsuppurative sequelae. Children are considered a major reservoir of GAS and also a reported target population for pharyngitis, as well as for suppurative and nonsuppurative complications caused by GAS. They may also represent the pool from which adults acquire severe invasive disease.

A 4-year longitudinal study was conducted between 2000 and 2004 aimed to elucidate the epidemiology of asymptomatic colonization by GAS. Oropharyngeal samples were taken in three collection periods - October, February and May - of each year, from different populations in Oeiras area: children (0-6 yrs) in 8 day-care centers (DCCs), children and adolescents (7-16 yrs) in 3 schools and adults (DCC and school staff and household). Bacterial identification was carried out by standard methods. GAS recovered from recurrent carriers - defined as colonized more than once during 2 to 12 sampling periods - were typed by pulsed-field gel electrophoresis (PFGE) and by sequencing of the *emm* gene encoding the M surface protein (*emm* typing).

A total of 5,494 samples were recovered from 1,729 individuals. The total number of GAS isolates was 626 (carriage rate of 11.4%). We further analyzed 243 (38.8%) isolates from 113 (6.5%) recurrent carriers. Two children from DCCs were colonized 4 times and 111 individuals were colonized 2 or 3 times during the 12 sampling periods. Forty-seven PFGE types and twenty-four *emm* types were detected (among the 243 isolates). Six PFGE types (or clones) accounted for 54.3% of the isolates (n=132): PFGE.AB/*emm*12 (n=34); PFGE.X/*emm*1 (n=31); PFGE.AP/*emm*12 (n=27); PFGE.BG/*emm*3 (n=18); PFGE.AO/*emm*4 (n=5)/other *emm* types (n=6); PFGE.BE/*emm*89 (n=10). A total of 54 individuals (47.8%) were carriers of *emm*12 isolates at least once, although of different PFGE types. Strain replacement was observed in 78.8% of the carriers (n=89), while 21.2% (n=24) were carriers of a same strain (14 individuals carried the *emm*12 strain).

In conclusion, isolates of *emm* type 12 seem to be successful colonizers as they were identified as widely disseminated among epidemiological related and unrelated carriers and were persistent overtime. It may have clinical relevance since association between *emm*12 and GAS pharyngitis or paediatric invasive GAS infections has been reported in Portugal.

Reference

158

Exploring the relationship of strains characterized by sequence based typing methods using goeBURST algorithm

Francisco, Alexandre P. (1,3); Bugalho, Miguel (1,3); Vaz, Cátia (2,4); Melo-Cristino, José (5); Ramirez, Mário (5); Carriço, João André (1,5)

1: KDBIO, Inst. Eng. Sistemas e Computadores – ID em Lisboa, Rua Alves Redol 9, 1000-029 Lisboa, Portugal;
2: SAT, Inst. Eng. Sistemas e Computadores – ID em Lisboa, Rua Alves Redol 9, 1000-029 Lisboa, Portugal;
3: Instituto Superior Técnico, Universidade Técnica de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal;
4: DEETC, Instituto Sup. Eng. de Lisboa, R. Conselheiro Emídio Navarro, nº. 1, 1900-Lisboa, PORTUGAL;
5: Instituto de Microbiologia,Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal

E-mail: jcarrico@fm.ul.pt

Keywords: MLST, Sequence-based microbial typing methods, Data visualization,

Abstract

With the decreasing DNA sequencing costs, sequence-based typing methods are rapidly becoming the gold standard for epidemiological surveillance with the data generated being also used to study microbial population genetics. Methods such as Multi-locus Sequence Typing (MLST), Single Nucleotide Polymorphism analysis and Multi-locus Variable Number of Tandem Repeats Analysis (MLVA), provide the reproducibility and comparability of results needed for a global scale bacterial population analysis, while having enough discriminatory power to be used in local epidemiological studies. All these methods provide a strain characterization profile that can be coded into a numeric or character sequence (allelic profile). The similarity between strains is then accessed based on the number of differences between those profiles. For a more comprehensive analysis of the possible patterns of evolutionary descent for MLST data, a set of rules were proposed and implemented in the eBURST algorithm (Feil, 2004). These rules allow the division of a data set into several clusters of related strains, dubbed clonal complexes, by implementing a simple model of clonal expansion and diversification. Within each clonal complex, the rules identify which links between STs correspond to the most probable pattern of descent. We recently identified a global optimum solution to the eBURST algorithm: goeBURST (Francisco, 2009). This algorithm uses a graphic matroid approach to find the optimal forest for a dataset based on a pre-defined set of rules, correcting some assignments of the eBURST algorithm that violated those rules.

In order to visualize the goeBURST results we have created stand-alone java interface that allows users to submit their MLST data, and visualize the resulting clonal complex assignment as a navigable graph. Subsets of data can also be selected to represent a certain feature, such as an antibiotic resistance pattern. Other important features are: file upload of relevant information for the strains under analysis and allowing to visually explore the results of queries, link confidence assessment, as well as the ability to configure the rules for the tie-break levels needed for the generation of the graphic matroid, thus allowing the use of data from any sequence-based typing method based on allelic profiles.

This software provides a providing a standard data analysis method for these sequence-based methodologies and is available at http://goeburst.phyloviz.net/.

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. Journal of Bacteriology 2004, 186(5):1518-1530
Francisco AP, Bugalho M, Ramirez M, Carrico JA., BMC Bioinformatics 2009, 10:152doi:10.1186/1471-2105-10-152

Reference

382

Identification of a novel virulence-associated adhesin cluster unique to epidemic strains of ET-12 lineage of *Burkholderia cenocepacia*

Mil-Homens, Dalila; Fialho, Arsénio M

Instituto Superior Técnico, Portugal

E-mail: dalilamil-homens@ist.utl.pt

Keywords: *Burkholderia cenocepacia*, ET12 lineage, virulence factor, trimeric autotransporter adhesions.

Abstract

Burkholderia cenocepacia is an important opportunistic pathogen causing serious chronic infections in patients with cystic fibrosis (CF). Adaptation of *B. cenocepacia* to the CF airways may play an important role in colonization and persistence of the infection. The bacteria sense the environment and respond with an arsenal of virulence factors, among which are the denominated trimeric autotransporter adhesins (TAA). We have identified a cluster of TAAs located downstream to the cci island that is a marker for virulent *B. cenocepacia* strains. This cluster represents a prototype of a two-component phosphorelay system and is organized with one sensor histidine kinase, two response regulators, four TAAs, one outer membrane protein and two hypothetical proteins. We have determined the genetic organization of the cluster and demonstrated that it is specific of the epidemic ET-12 lineage of *B. cenocepacia*. Environmental factors such as osmolarity, oxygen limited conditions and oxidative stress significantly enhance the expression levels of the adhesin cluster at the transcriptional level. These changes in expression occurred preferentially for cells grown under static conditions and required the presence of extracellular matrix (ECM) proteins, such as fibronectin, collagen type I and laminin. Knock-out mutants of *B. cenocepacia* K-56 BCAM0219 and BCAM0224 TAAs and BCAM0218 histidine kinase were constructed and assayed for adherence to extracellular matrix (ECM) proteins, biofilm formation, swimming and swarming. We also tested for their virulence in the infection model *Galleria mellonella* (the wax larvae) caterpillar. All the mutants showed an attenuated virulence effect comparing to the wild type, and the BCAM0224 TAA mutant leads to a really higher larvae survivance, demonstrating that is involved in virulence to the host. In conclusion, we identified in the genome of *B. cenocepacia*, a novel and unique adhesin cluster that played an important role in pathogenicity.

Poster Session:
S3 – Clinical Microbiology and Epidemiology

Reference

114

Effect of one dose of the 7-valent pneumococcal conjugate vaccine (PCV7) on single and multiple colonization

Frazão, Nelson (1, 2); Sá-Leão, Raquel (1, 3); Lencastre, Hermínia (1, 2)

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa (ITQB/UNL), Oeiras, Portugal;
2: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA;
3: Centro de Matemática e Aplicações Fundamentais (CMAF), Universidade de Lisboa, Lisboa, Portugal

E-mail: nfrazao@itqb.unl.pt

Keywords: Streptococcus pneumoniae, PCV7, single colonization, multiple colonization

Abstract

Streptococcus pneumoniae is an important pathogen that frequently colonizes the nasopharynx of young children. The capsule is the main virulence factor. PCV7 targets seven capsular types most of which are commonly carried. The effect of PCV7 on colonization is traditionally evaluated by serotyping one pneumococcal isolate per carrier. We aimed to assess the effect of one dose of PCV7 on single and multiple pneumococcal colonization.

Nasopharyngeal samples of 95 healthy children aged up to 24 months attending day care centers in Lisbon, were obtained in May and June 2001. The vaccinated group (77 children) received a single dose of PCV7 in May immediately after sampling whereas the control group (18 children) did not receive the vaccine. Up to 10 pneumococcal isolates were studied per child at each sampling period. Overall, 1,287 isolates were serotyped and representatives were typed by pulsed-field gel electrophoresis.

In the vaccinated group, from May to June, among single carriers, vaccine serotypes (VT) decreased from 60% (271 isolates) to 41% (189 isolates), and non vaccine serotypes (NVT) increased from 40% (179 isolates) to 59% (274 isolates) ($p<0.001$). Among multiple carriers, VT decreased from 45% (29 isolates) to 19% (11 isolates), and NVT increased from 55% (36 isolates) to 81% (48 isolates) ($p=0.002$).

In the control no statistically significant changes were observed. From May to June, among single carriers, VT increased from 64% (70 isolates) to 73% (80 isolates), and NVT decreased from 36% (40 isolates) to 27% (30 isolates) ($p=0.147$). Among multiple carriers, VT increased from 95% (19 isolates) to 100% (10 isolates), and NVT decreased from 5% (1 isolate) to 0% ($p=1$).

To understand the ecological mechanism(s) responsible for the vaccine's effect we analyzed the colonization pattern changes in both vaccinated and control groups. In the vaccinated group only 35% of the pneumococcal isolates acquired *de novo* were VT contrasting with 70% in the control ($p<0.001$).

To our knowledge this is the first study analyzing the effect of one dose of PCV7 in single and multiple colonization. We concluded that one month after vaccination with a single dose, PCV7 led to a serotype replacement phenomenon in single and multiple carriers and this was mainly accomplished by prevention of de novo acquisition of VT.

Reference

124

Early detection of bacterial DNA based on *secY*, *lipL21* and *lipL32* genes in human leptospirosis

Carreira, Teresa (1,2); Nunes, Monica (1,2); Vieira, Maria Luísa (1,2); Collares-Pereira, Margarida (1,2,3)

1: Unidade de Leptospirose e Borreliose de Lyme, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa;
2: Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa;
3: Unidade de Genética e Patologia Moleculares, Hospital do Divino Espírito Santo de Ponta Delgada, EPE

E-mail: tcarreira@ihmt.unl.pt

Keywords: human leptospirosis; acute phase; molecular diagnosis

Abstract

Leptospirosis, which has a particular high incidence in the Azores (about 10 times greater) when compared to mainland Portugal, is now identified as one of the emerging infectious diseases worldwide. The early laboratory diagnosis of human leptospirosis is important because the severe infections can have a fatal outcome. The available serological techniques, including the reference microscopic agglutination test (MAT), lack on sensitivity in the first days of the disease. In order to surpass this limitation, some attempts have been made with PCR-based assays using conventional primers designed on the *Leptospira* genome, except for *L. kirschneri* (e.g. G1-G2 for *secY*) [1], or in genes such as *lipL*32[2] and *lipL*21[3] which encode OMP's (Outer Membrane Proteins) known for their immunogenic role during the infection. The DNA sequence of these last two genes was found to be conserved amongst all pathogenic *Leptospira* and absent in non-pathogenic species.

The present work aimed at the optimization of the leptospirosis molecular diagnosis (PCR-based) in the acute clinical stage, using the referred genes as an alternative approach to the serological MAT diagnosis.

A total of 161 sera obtained in the first (median=5) days of disease, from Portuguese inpatients with a leptospirosis clinical suspicion, were submitted to three assays with the following primers: G1-G2, *lipL32* and *lipL*21. A battery of reference *Leptospira* serovars (pathogenic and non-pathogenic) was evaluated to determine the primers' sensitivity.

The results obtained (so far) showed the presence of *Leptospira* DNA in 116 (72.0%) and in nine (6.3%) serum samples with *lipL*32 and the conventional primers (G1-G2), respectively. The evaluation of *lipL21* is still ongoing, but preliminary results with the reference serovars, already indicate a lower sensitivity than with *lipL32.* When analysed by MAT, the tested sera showed agglutinins anti-*L. interrogans* sensu lato in only 37 (22.9%) samples.

These findings confirm that the PCR-based diagnosis was significantly (p<0,001) more sensitive than the reference serological approach, in the acute phase of leptospirosis. Furthermore, the *lipL32* nested-PCR seems to be a reliable diagnostic option, especially in the absence of specific antibodies.

1. Gravekamp et al. (1993). J Gen Microbiol, 139: 1691-1700.
2. Jouglard et al. (2006). Can J Microbiol, 52: 747-752.
3. Cheema et al. (2007). Indian J Exp Biol, 45: 568-573

Reference

133

Development of a multi-region hybridization assay (MHABG0214) for the identification of subtypes B, G and recombinant forms CRF02_AG and CRF14_BG of the human immunodeficiency virus type 1 (HIV-1) circulating in Portugal

Freitas, Ferdinando Bernardino; Esteves, Aida; Piedade, João; Parreira, Ricardo

Instituto de Higiene e Medicina Tropical, Portugal

E-mail: ferdinando@ihmt.unl.pt

Keywords: HIV-1; Multi-region hybridization assay; Molecular epidemiology; Portugal

Abstract

Since most methods used for the genetic characterization of HIV-1 are based on the analysis of specific regions, they only provide a fragmented view of the viral genome, and often fail to identify recombinant viruses. The most efficient method for HIV-1 genetic characterization is still full-genome sequencing, but the associated costs and low throughput preclude it from being routinely used for the analysis of large numbers of viral strains. In this context, the aim of our study was the development of a new multi-region hybridization assay (MHA_{BG0214}) for fast, relatively inexpensive, specific and high throughput genotyping of the major HIV-1 forms circulating in Portugal, where the epidemic, though characterized by a plethora of highly diverse strains, is dominated by subtypes B, G and CRF02_AG and CRF14_BG circulating recombinant viruses.

In its conventional real-time PCR format, MHA relies on the amplification by PCR of several regions (usually ≥5) scattered along the HIV-1 genome with so called universal primers. The obtained amplicons are then distributed to several second-round amplification reactions, each carried out with inner universal primers and a subtype-specific TaqMan probe. Based on full alignments of representative HIV-1 reference sequences, we designed universal and subtype-specific primers/probes for the amplification of 7 different regions of the viral genome: the Gag, Protease, Reverse Transcriptase, Integrase, Vpu, Gp120 and Gp41 coding sequences. Optimization of reaction conditions, established using 7 HIV-1 references, then served as a starting point for the analysis of 50 HIV-1 strains circulating in Portugal, using either proviral DNA immobilized on FTA™ cards or viral RNA extracted from plasma samples. Universal and subtype-restrictive amplification conditions were set for 6 of the 7 targeted sections of the viral genome, the integrase coding region consistently revealing ambiguous subtyping results. MHA_{BG0214} was implemented using a real-time PCR-based approach, with amplicon detection dependent on the use of either SYBR Green I (all regions) or a TaqMan probe (Gp41). Alternatively, a technically less demanding strategy based on conventional PCR and agarose gel analysis of reaction products was also developed. Regardless of the experimental schemes used, preliminary data disclosed high specificity of the obtained subtyping results for all regions of the HIV-1 genome analysed.

Reference

134

Prevalence of infection and genetic analysis of GB virus C in a population group comprising a large number of intravenous drug users (IDUs)

Branco, Cristina Vieira; Esteves, Aida; Piedade, João; Parreira, Ricardo

Instituto de Higiene e Medicina Tropical, Portugal

E-mail: cbranco@ihmt.unl.pt

Keywords: GBV-C/HGV, intravenous drug users, E2 genotyping, phylogenetic analysis

Abstract

GB virus C (GBV-C) (also known as hepatitis G virus or HGV) is a tentative member of the *Flaviviridae* family, closely related to the hepatitis C virus (HCV). Although initially regarded as a potential cause of hepatitis, more recent studies have found no evidence for association between GBV-C and human disease. GBV-C is very common among individuals infected with the human immunodeficiency virus (HIV) and/or HCV, and among IDUs, most probably due to similar transmission routes. Due to the scarcity of information regarding GBV-C infection in Portugal, we decided to estimate its prevalence in a group of 214 individuals from the Lisbon metropolitan area, including a large number of IDUs (70.6%), and to carry out a genetic characterization of the circulating viral strains.

GBV-C viremia was assessed by nested PCR amplification of a section of the conserved 5'-untranslated region using total RNA extracted from plasma samples. The genetic characterization of GBV-C strains was carried out on a randomly selected group of 44 viral strains, focusing on the analysis of a genomic PCR fragment comprising most of the E2 surface glycoprotein coding region.

The overall prevalence of GBV-C infection was 40.6%. Among those with GBV-C viremia, monoinfection was detected only in 23.0% of the samples, while coinfection with HIV, HCV, or HIV/HCV was revealed in 19.5%, 16.1%, and 41.4% of the population, respectively. Statistically significant associations were detected between GBV-C viremia and the age (≥25 years) or the use of intravenous drugs. Phylogenetic analysis of the E2 region, established segregation of Portuguese GBV-C strains with genotypes 1 (G1, n=10) and 2 (G2, n=34) reference sequences, irrespective of the analytic method used (Neighbor Joining, Maximum Likelihood or a Bayesian Markov chain Monte Carlo approach). Unexpectedly, 15 strains assigned to G2 were shown to form a separate cluster in all phylogenetic trees. This group of sequences, designated G2*, was characterized by the overall genetic conservation (7.8% diversity) and the presence of specific amino acid polymorphisms (Q134, H258, and F182), when compared to its sister clade (G2). However, different from what the phylogenetic tree topologies might, at first, suggest, analysis of the distributions of intra and inter genotype pairwise genetic distances did not support the appointment of G2* as a new GBV-C genotype, but rather as a geographic subcluster within the G2 radiation.

Reference

137

Low prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage among young children in Portugal

Tavares, Débora A. (1); Sá-Leão, Raquel (1, 2); Miragaia, Maria (1); de Lencastre, Hermínia (1, 3)

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa (UNL), Oeiras, Portugal;
2: Centro de Matemática e Aplicações Fundamentais (CMAF), Universidade de Lisboa, Lisboa, Portugal;
3: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA

E-mail: datavares@itqb.unl.pt

Keywords: *Staphylococcus aureus*, methicillin-resistant, community-acquired, carriage, children

Abstract

Staphylococcus aureus is a natural nasal human colonizer and a major nosocomial pathogen. During the last decade, an increasing incidence of pediatric infections due to community-acquired MRSA (CA-MRSA), including children with no identifiable risk factors, has been documented worldwide, suggesting that children may constitute a reservoir of MRSA in the community.

In this study, that is part of a larger one on nasopharyngeal ecology, we aimed to evaluate the prevalence of MRSA colonizing young children and compare results with those obtained in a study conducted by us a decade ago, when this prevalence was less than 0.5%.

Nasopharyngeal samples were obtained from 2,100 children, aged up to 6 years old, attending day-care centers (DCCs) in Oeiras, in 2006, 2007, and 2009, and in Montemor-o-Novo, in 2009. Swabs were streaked in mannitol salt agar and isolates that were mannitol- and coagulase-positive were considered *S. aureus*. Isolates were tested for susceptibility to 12 antibiotics with the disk-diffusion method according to CLSI guidelines. MICs to oxacillin were determined for oxacillin-resistant isolates by E-test and the *mecA* gene was screened by PCR. MRSA isolates were further characterized by PFGE, *spa*, and SCC*mec* typing.

Seventeen percent of the children (n=366) were colonized with *S. aureus*. Resistance rates were 88% for penicillin, 14% to erythromycin, 6% to clindamycin, 2% to tetracycline, and <1% to oxacillin, rifampicin, ciprofloxacin, and SXT. None of the isolates was resistant to vancomycin, linezolid, quinupristin/dalfopristin or gentamicin. Three MRSA (0.14% of 2,100 samples) were isolated. The carriers were girls (two aged 3 and one aged 5 years), attending different DCCs in Oeiras in 2006. The MRSA strains had properties of CA-MRSA, such as low-level resistance to oxacillin and limited resistance to non ß-lactams. Two strains had a PFGE pattern related to that of USA700 (ST72-SCC*mec*IV) and carried SCC*mec* IVa and IVc. The other strain was, by PFGE, related to USA300 (ST8-SCC*mec*IV) but carried SCC*mec* VI, which was first described in a nosocomial MRSA clone identified in Portugal. USA700 has been associated to community and health-care settings; USA300 is a major epidemic CA-MRSA clone in the USA and is also spreading in Europe.

We conclude that the prevalence of CA-MRSA in this group remains extremely low. Given the current concern about CA-MRSA, continuous surveillance in this and other risk groups is mandatory.

Reference

142

Epidemiology of hepatitis C virus among HIV co-infected individuals in the Lisbon urban area

Rocha, Mª Raquel (1); Parreira, Ricardo (1,2); Piedade, João (1,2); Esteves, Aida (1,2)

1: Unidade de Ensino e Investigação de Virologia, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal;
2: Unidade de Parasitologia e Microbiologia Médicas, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal

E-mail: airamraquel@iol.pt

Keywords: HCV subtypes, HIV co-infection, intravenous drug users, Lisbon

Abstract

Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are major health concerns worldwide. Due to shared modes of transmission, co-infection with both viruses is rather common. HIV infected patients with chronic hepatitis C progress faster towards liver disease and are more impervious to current HCV therapy. Moreover, the sustained virological response rate is dramatically influenced by the HCV genotype in co-infected patients. With this in mind, and given that HIV-positive people with a history of injection drug use are frequently co-infected with HCV, we decided to conduct a retrospective study to determine the prevalence of HCV infection and HCV subtype distribution in a group of HIV infected intravenous drug users (IDUs).

Plasma specimens (n=66), collected between 1998 and 2001 from patients being treated for HIV infection, were tested for HCV antibodies (ImmunoFlow™ HCV rapid test, Core Diagnostics, UK) and HCV RNA (RT-PCR amplification of the conserved 5'UTR). In order to identify HCV subtypes and detect potential recombinants, two additional genomic regions (C-E1 and NS5B) were amplified, sequenced, and subjected to phylogenetic analysis.

On the whole, 57 samples were positive for HCV antibodies (86.4%), and in 53 of these amplification of the 5'UTR demonstrated active HCV infection. Unexpectedly, 40% (n=4) of the seronegative samples showed amplification of viral RNA. Sample collection prior to seroconversion and/or lack of antibodies due to advanced immunosupression might account for this result. Phylogenetic analysis revealed predominance of IDU-related subtypes mostly (1a, 45.6%; 3a, 21.1%; 4d, 15.8%; and 4a, 8.8%). One sample demonstrated subtype discordance for the two regions analysed, suggesting infection by a putative intergenotypic 4a/4d recombinant or a mixed infection. Since recombination between HCV is a rare event, clonal analysis of both amplicons will elucidate this issue.

Curiously, while description of genotype 4 circulation is quite recent in Europe, its prevalence in this Portuguese subpopulation was already high (25.0%) one decade ago. Furthermore, the combined high prevalence of HCV genotypes 1 and 4 (70.2%) in the group of HIV-infected IDUs under study, and the fact that only about 20% of HIV-positive patients infected with these genotypes respond to therapy, strengthens the urgent need of effective measures to fight both viruses.

Reference

143

High prevalence of hepatitis C virus genotype 4 among intravenous drug users in Lisbon

Calado, Rita Diogo Almeida (1); Parreira, Ricardo (1,2); Piedade, João (1,2); Esteves, Aida (1,2)

1: Unidade de Ensino e investigação de Virologia, Instituto de Higiene e Medicina Tropical, Portugal, Universidade Nova de Lisboa, Lisboa;
2: Unidade de Parasitologia e Microbiologia Médicas, Instituto de Higiene e Medicina Tropical, Portugal, Universidade Nova de Lisboa, Lisboa

E-mail: rcalado@ihmt.unl.pt

Keywords: HCV, subtypes, intravenous drug users, Lisbon

Abstract

Hepatitis C virus (HCV) affects 170 million people worldwide and has become a major cause of liver cancer and cirrhosis. In developed countries, intravenous drug use is the predominant mode of transmission. Although recent studies demonstrate a high prevalence of HCV in Western Europe intravenous drug users (IDUs), data concerning prevalence of infection and subtype distribution in Portugal is scarce, with only five Portuguese HCV sequences available in international data bases.

Our goal was to characterize the epidemiology of HCV infection, including estimation of prevalence and genotype distribution, in IDUs from Lisbon.

Blood samples from 135 attendees of a drug treatment centre were collected (March 2008 to February 2009) and tested for the presence of HCV antibodies. To detect active HCV infection, viral RNA was extracted from seropositive plasma and subjected to RT-PCR amplification of the 5' UTR. For subtyping, amplification of the C/E1 and NS5B coding regions, direct sequencing of amplicons, and phylogenetic analysis were carried out.

A total of 82 (60.7%) individuals were positive for HCV antibodies (mean age 40.0 years, 76.8% male). In 67 (81.7%), the presence of HCV RNA was detected and amplicons for the C/E1 and NS5B regions were successfully obtained. Phylogenetic analysis showed that the HCV strains belonged to subtypes 1a (49.2%), 1b (6.0%), 3a (22.4%), 4a (13.4%), and 4d (9.0%). Concordance between the subtypes of the two regions analysed was observed for all the samples. This result suggests the absence of recombinant viruses, in agreement with the low level of recombination described for HCV. The predominance of subtypes 1a and 3a among IDUs has been widely described in Europe, while the presence of genotype 4, related with drug consumption and immigration, was only recently reported for several Southern European countries, but with lower rate values. Bearing in mind that 1) the HCV genotype is one of the most important factors affecting the response to antiviral treatment, 2) genotypes 1 and 4 present the lowest rates of sustained virological response to current therapy, and 3) HCV can spread from drug users to the general population, the combined high rate of genotypes 1 and 4 found in this IDU group (77.6%) strengths the need for molecular epidemiology studies, targeting not only specific groups but also the general population. This will allow a better evaluation of the burden of hepatitis C in Portugal.

Reference

146

Incidence and relatedness of *Pseudomonas aeruginosa* isolates producers of VIM-2 from two central hospitals in Portugal

Reis, Teresa (1); Pereira, Sónia (1); Ribeiro, Graça (3); Vital, Clementina (3); Alves, Ana Florinda (2); Cardoso, Olga Maria (1)

1: University of Coimbra, Portugal;
2: Lab Microbiology, Centro Hospitalar de Coimbra, Portugal;
3: Lab Clinic Pathology, Hospitais da Universidade de Coimbra, Portugal

E-mail: ocardoso@ci.uc.pt

Keywords: *Pseudomonas aeruginosa*, metallo-beta-lactamases, VIM-2, RAPD

Abstract

Objective: *Pseudomonas aeruginosa* (PA), an opportunistic human pathogen with a high antibiotic-resistance capacity, is one of the leading causes of nosocomial infections. The aims of this study were to identify metallo-b-lactamases (MBLs) in imipenem (IP) resistant isolates obtained from two central hospitals in central Portugal and to characterize those isolates by random amplification of polymorphic DNA (RAPD).

Methods: 91 IP resistant isolates were collected from Centro Hospitalar de Coimbra (CHC) [Pneumology (P), Neurosurgery (N), Infectious (I), Medicine (M) wards, Paediatric Hospital (PH) and Hospital of Pombal (HP)] from June 2007 to June 2008 and 31 from Hospitais da Universidade de Coimbra (HUC) [Neurotraumatology (NT), Surgery III (S), Hepatic Transplant (HT), Cardiology (C), Medicine (MI), Orthopedy (O), Infectious (IC), and Emergency (ER) wards] from January 2008 to June 2008. Double combined disk test was used for MBLs screening. Minimal inhibitory concentrations (MICs) were determined by E-test method. PCR was done to research bla_{VIM}, bla_{IMP}, bla_{GIM}, and bla_{SPM}. PCR products were sequenced and analysed. DNA was amplified using the primer 5'-AGCGGGCCAA-3'. We assigned a letter for each different RAPD profile.

Results: Double combined disk test was positive in 32 isolates from CHC and 18 of HUC. Presence of bla_{VIM} was positive in 12 isolates from CHC and 15 from HUC. Other MBLs tested were not observed. 27 isolates (54%) showed bla_{VIM-2} by DNA sequencing. Among strains with VIM-2, MICs revealed that aztreonam inhibited 81.5%, piperacillin 66.7%, ceftazidime 25.9% and meropenem 22.2%. RAPD generated 14 different patterns (A to O). 7 patterns were from HUC (A to G) with pattern A as the most prevalent, disseminated in 3 wards: NT, S and HT (7 strains). Other patterns were represented by 1 or 2 strains. Patterns H to O belonged to CHC, where pattern H was predominant (5 strains), appearing in different wards of PH. Other profiles were constituted by 1 or 2 strains. Identical RAPD patterns between two hospitals were not seen.

Conclusions: Monitoring of PA MBLs producers should become a standard aspect on local and global surveillance systems. Prevalence of VIM-2-encoding genes was mainly due to a gene spread and, in a lesser extent, to clonal dissemination, therefore possibility of VIM-2 spreading in Gram negative pathogens can emerge as a great problem in clinical settings and underscores the need for these resistant determinants surveillance.

Reference

148

Genetic diversity and complexity of anti-retroviral drug resistance in HIV-1 strains circulating among intravenous drug users in the Greater Lisbon

Videira e Castro, Sandra (1); Venenno, Teresa (1,2); Esteves, Aida (1,2); Parreira, Ricardo (1,2); Pádua, Elizabeth (3); Piedade, João (1,2)

1: Unidade de Ensino e Investigação de Virologia, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa;
2: Unidade de Microbiologia e Parasitologia Médicas, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa;
3: Laboratório de Referência da SIDA, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa

E-mail: scastro@ihmt.unl.pt

Keywords: HIV-1; pol gene; genetic diversity; drug resistance; intravenous drug users.

Abstract

The genetic diversity of HIV-1 is recognized as a potential problem for the diagnosis and treatment of HIV/AIDS. Even though a number of useful anti-HIV-1 drugs have been approved for use, the development of resistance drives forward anti-retroviral drug research. HIV-1 *pol* encodes for three essential enzymes in the viral replication cycle: protease (PR), reverse transcriptase (RT) and integrase (IN). Despite being good drug targets, these enzymes have been associated with the rapid emergence of drug resistance. So, considering that sequence information is useful for anti-HIV drug research, the aims of this study were:
§ To evaluate the genetic diversity of PR, RT and IN coding sequences in HIV-1 strains circulating among intravenous drug users (IDUs) from the Greater Lisbon;
§ To identify mutations associated to increasing levels of resistance to currently licensed anti-retroviral drugs.
Blood samples were collected between 1998 and 2009 from 51 HIV-1 seropositive IDUs (71% male, mean age 37.5 yrs.). RNA extracted from plasma was amplified by nested PCR, after an *in vitro* reverse transcription step to cDNA, to originate PR, RT and IN amplicons of 460, 650 and 906 bp, respectively. DNA sequences (n=124), generated by sequencing of PCR products or plasmid clones, were edited using BioEdit Sequence Alignment Editor and subsequently submitted to preliminary subtype characterization, performed with NCBI HIV Genotyping and REGA HIV-1 Subtyping Tools. On the whole, HIV-1 non-B subtypes were identified in 62.9% (78/124) of the sequences obtained: PR [15 B, 1 F1, 18 G, 5 CRF02_AG, 2 unique recombinant forms (URFs); n=41]; RT [18 B, 1 F1, 20 G, 3 URFs; n=42]; and IN [13 B, 1 F(CRF12_BF), 7 G, 4 G(CRF02_AG), 10 G(CRF14_BG), 6 URFs; n=41]. These results show that this HIV-1 epidemic is dominated by subtypes G and B and their recombinant forms. It is also significant that one third of all the sequences (41/124) seem to be derived from inter-genotype recombinants. Confirmation of these preliminary results by an in-depth phylogenetic analysis is ongoing and will be presented. Finally, it has been speculated that differences in amino acid residues of HIV-1 proteins from distinct subtypes can modulate their function *in vivo* and this is of particular interest in connection to the drug susceptibility profile of the virus. Patterns corresponding to PR, RT and IN resistance–associated mutations will be investigated via the Stanford Genotypic Resistance Interpretation Algorithm.

Reference

163

Should we worry about "minor" pathogens in bovine mastitis?

Bexiga, Ricardo (1); Carneiro, Carla (1); Cruz, António (2); Oliveira, Manuela (1); Vilela, Cristina Lobo (1)

1: Faculdade de Medicina Veterinária, Portugal;
2: Escola Tecnológica de Gestão Industrial

E-mail: adrbexiga@fmv.utl.pt

Keywords: Bovine, mastitis, staphylococci, corynebacterium

Abstract

Non-aureus staphylococci (NAS) and *Corynebacterium bovis* have been reported as the most frequently isolated pathogens from bovine intramammary infections (IMIs) in several studies. Despite that, practitioners seldom focus on them when solving udder health problems. Somatic Cell Count (SCC) is an indicator for IMMs, reflecting the degree of inflammatory response at udder level. Farmers are paid according to the SCC of milk produced.

This study was performed on 4 dairy farms where quarter milk sampling was performed according to National Mastitis Council protocols, every 4 weeks on each farm for 48 weeks. On the initial visit, 12 cows were randomly selected from those that developed subclinical mastitis, detected by the raise of SCC above the 200.000 cells/ml threshold. On subsequent visits, cows from which NAS had been isolated were resampled; other animals were selected according to the above criteria to totalise 12 cows. Identification of NAS was performed by biochemical profile (ID 32 Staph, bioMérieux). Presumptive identification of *C. bovis* was made by growth in trypticase soy agar (TSA) and TSA supplemented with 1% tween 80. Quarter milk samples' SCC were also determined (Fossomatic).

We followed 221 IMIs due to NAS. The bacteriological spontaneous cure rate after one NAS isolation (1 positive sample followed by 2 negative in successive months) was 19.8%, after 2 isolations was 6.7% and after 3 was 3.0%. Bacteriological cure rates differed between NAS species: 28% for *S. epidermidis* and 11% for *S. chromogenes*, the 2 most frequently isolated species. The geometric mean SCC for quarters with IMIs was low: 116.221 cells/ml for NAS (106.478 cells/ml for *S. epidermidis*, 150.229 for *S. chromogenes*, 129.991 for *S. simulans*, 73.438 for *S. intermedius*) and 118.518 cells/ml for *C. bovis*, while samples that yielded no bacterial growth had 38.173 cells/ml. Previous studies have shown that NAS are frequently resistant to antimicrobials used for mastitis treatment. Multiresistant strains and methicillin-resistant *S. epidermidis* have been identified. Bacteriologic self-cure rates of IMIs due to NAS were low, which means that infections tend to be long-lasting if no antimicrobial treatment is performed. Despite that, the impact on SCC tended to be low and similar to IMIs due to *C. bovis*. It should be clarified if these microrganisms truly cause mastitis, i.e. infection of the mammary gland or if they simply colonize the teat, leading to a low impact on milk quality.

Reference

165

Antioxidant and Antimicrobial Properties of Portuguese Propolis

Guimarães, Marco Rafael Machado (1); Carvalho, Maria João (1); Carvalho, Luis (1); Cunha, Ana (2); Aguiar, Cristina Alexandra Almeida (3); Ferreira, Ana Margarida V. Duarte (1)

1: Centro de Química, Universidade de Trás-os-Montes e Alto Douro, 5001-801 Vila Real;
2: Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB), pólo Universidade do Minho, Campus de Gualtar 4710-057 Braga;
3: Centro de Biologia Molecular e Ambiental (CBMA), Universidade do Minho, Campus de Gualtar 4710-057 Braga

E-mail: marcogms10@gmail.com

Keywords: Propolis; antimicrobial; antioxidant

Abstract

Propolis is a resin collected by honeybees from the bud and bark of certain trees and plants and it is stored inside their hives. It has been used in folk medicine from ancient times in many countries. It has been reported to possess various biological activities, such as antibacterial, antifungal, antiviral, anti-inflammatory, anticancer, antitumoral and, more recently, allelopathic properties.

Propolis usually contains a variety of chemical compounds such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino acids, being its composition qualitatively and quantitatively variable with the vegetation of the area where it is collected. Due to geographical differences, propolis from Europe, South America and Asia have different chemical composition and also distinct biological activities.

In this study, we investigated the *in vitro* antioxidant and antimicrobial activities of different extracts obtained from different colored samples of portuguese propolis (sample 1-Vila Real, 2-Vila Nova de Cerveira and 3-Leiria). Propolis samples were collected as crude materials and were extracted with ethanol at room temperature. The ethanol suspension was filtered and the filtrate was concentrated to give the ethanolic extract of propolis (EEP). The EEP was further fractioned with hexane and chloroform solvents. The fractions obtained, HEX-Fr and CHLO-Fr, and the residual ethanolic extract were concentrated. Total polyphenol and flavonoid contents in EEP, HEX-Fr and $CHCl_3$-Fr were determined by Folin–Ciocalteu and aluminum chloride colorimetric methods..

Active free radicals, together with other factors, are responsible for cellular ageing and many conditions such as cardiovascular diseases, cancer, diabetes, arthritis, Parkinson and Alzheimer diseases. Several reports have proved the usefulness of the free-radical scavenging properties of antioxidants and their general benefits to human health but the development of more effective antioxidants of natural origin are desired. An assay system for evaluating antioxidant activities of propolis extracts - the free radical-scavenging activity on 2,2¢-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation – was also used in this work

Antimicrobial properties were screened by a disk diffusion assay against selected Gram-positive/Gram negative bacteria as well as yeasts indicator strains. Ethanolic extracts showed greater bioactivity than for propolis fractions.

Reference

168

Dissemination of specific *Staphylococcus epidermidis* clones in the community

Rolo, Joana (1); Miragaia, Maria (1); de Lencastre, Hermínia (2)

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Oeiras, Portugal;
2: Laboratory of Microbiology, The Rockefeller University, New York, New York, USA

E-mail: jrolo@itqb.unl.pt

Keywords: Community, *S. epidermidis*, SCCmec

Abstract

Staphylococcus epidermidis is one of the major nosocomial human pathogens. The great majority of hospital-associated *S. epidermidis* isolates belong to a single clonal lineage (CC2) that evolves rapidly by frequent acquisition of mobile genetic elements, like the staphylococcal cassette chromosome *mec* (SCC*mec*) carrying the determinant of methicillin resistance, *mecA*. Mobility of the cassette is achieved by site-specific recombinases called *ccr* (chromosomal cassette recombinases).

Despite the importance of *S. epidermidis* as a pathogen, little is known about the epidemiology of this species in the community. In this study, we aim to compare the population structure of *S. epidermidis* in the community and hospital settings. To achieve this aim, we studied two bacterial collections isolated from nasal swabs in the same geographic region of Portugal: a collection of 192 *S. epidermidis* isolates obtained from healthy young adults during four sampling periods (1996-1999) and a collection of 94 isolates collected during a two-year period (2000-2001) from inpatients in a military hospital. Species identification was performed by internal-transcribed spacer PCR (ITS-PCR). The genetic background of *S. epidermidis* isolates was analyzed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). *mecA* and *ccr* genes were detected by PCR. SCC*mec* typing and subtyping was achieved by multiplex PCR assays.

We identified 18 methicillin resistant *S. epidermidis* (MRSE) in the community setting (9%). A high genetic diversity was found in this collection, which comprised 48 PFGE clusters; however, only 33% of strains carried *ccr* genes. In contrast, 80% of hospital isolates were MRSE classified into 19 PFGE clusters with a high number of strains (87%) carrying *ccr* genes. Most of the PFGE clusters defined were specific of hospital or community, but we also found a major PFGE cluster among methicillin susceptible strains that was identified in both environments. Despite the differences observed, almost all strains studied belonged to CC2 and SCC*mec* IVa was the most frequently carried element among MRSE.

Our results indicate that community and nosocomial *S. epidermidis* have evolved to adapt differently from a common genetic background. *S. epidermidis* adaptation to hospital environment seems to be associated to a lower genetic diversity and higher acquisition rate of SCC elements.

Reference

172

The polyphasic approach in the diagnosis of human Lyme borreliosis

Fernandes-Marta, Filipa Margarida (1); Nunes, Mónica (1,2); Carreira, Teresa (1,2); Vieira, Maria Luísa (1,2); Collares-Pereira, Margarida (1,2,3)

1: Unidade de Leptospirose e Borreliose de Lyme, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa;
2: Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa;
3: Unidade de Genética e Patologia Moleculares, Hospital do Divino Espírito Santo de Ponta Delgada, EPE

E-mail: filipa.marta@gmail.com

Keywords: Lyme Borreliosis, laboratory diagnosis, polyphasic approach

Abstract

Lyme borreliosis (LB) is an emerging vector-borne disease with worldwide distribution, particularly in the temperate zone. This chronic multi-systemic infectious disorder is caused by a diverse group of zoonotic bacteria which are transmitted by hard ticks among vertebrate hosts (e.g., small mammals, birds and lizards). In Europe, the main tick-vector is *Ixodes ricinus* which is implicated in the human transmission of diverse pathogenic genospecies namely *Borrelia burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, and *B. lusitaniae*[1]. The first three species are usually associated with arthritis, chronic skin disorders and neurological manifestations, respectively; however, LB can mimic a large number of other illnesses[2]. The diagnosis of LB is generally based on clinical manifestations, history of tick bite/contacts and serological tests, which may be falsely negative in patients with early disease, but quite reliable for diagnosing chronic stages.

This work aimed at the application of a polyphasic laboratory approach, in order to search the presence of spirochetal DNA through a nested-PCR [targeting the 5S (*rrf*) - 23S (*rrl*) intergenic spacer], besides the usual demonstration of specific antibodies, using the screening indirect-immunofluorescent assay (IFA - with a polyvalent conjugate) and the confirmatory western blotting (WB - IgM and IgG).

The results obtained with all assayed serum samples from 125 patients with a clinical suspicion of LB showed the following IFA reactivities: 55 (44%) positives (at 1:256) and 21 (17%) non-conclusive (at 1:128). From the 62 (82%) out of 76 IFA positive/doubtful sera, 24 (39%) evidenced a positive immunoblot with at least one of the conjugates (n_{IgM}=9, n_{IgG}=11) or with both ($n_{IgM,G}$=4). *Borrelia* DNA was detected in 21 (17%) out of the 125 studied patients and only eleven (18%) showed also a positive WB result.

The molecular findings seen in this study empathized the importance of using a PCR-based technique to search for borrelial DNA in suspected LB patients, together with specific serology, aiming at the successful diagnosis and clinical treatment of a very debilitating disease, also in the Portuguese population.

1. Collares-Pereira et al. (2004). J Clin Microbiol, 42: 1316-1318.
2. Steere et al. (2001). N Eng J Med, 345: 115-125.

Reference

189

Application of benzo[a]phenoxazinium chlorides in *Candida albicans* inactivation by photodynamic therapy

Pereira, João Paulo Campos (1); Lopes, Marlene Alexandra da Silva (1); Gonçalves, Maria Sameiro Torres (2); Coutinho, Paulo José Gomes (3); Belo, Isabel Maria Pires (1)

1: Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Gualtar, 4710-057 Braga, Portugal;
2: Centre of Chemistry, University of Minho, Gualtar, 4710-057 Braga, Portugal;
3: Centre of Physics, University of Minho, Gualtar, 4710-057 Braga, Portugal

E-mail: jpc_pereira@sapo.pt

Keywords: Photodynamic therapy, *Candida albicans*, Benzo[a]phenoxazinium chlorides

Abstract

The photodynamic therapy is a non-invasive model with a substantial potential in antimicrobial therapy. The treatment may be a fast way to control and reduce microbial burden of localized infections that are resistant to the standard antibiotic regimens. This technique results of the interaction between photons of visible light, of appropriate wavelength, and a photosensitizer in the presence of oxygen, that combine to produce cytotoxic species[1].

Along this study, the potential photodynamic action in *Candida albicans* was assessed. Three benzo[*a*]phenoxazinium chlorides, namely *N*-(5-amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)- *N*-ethylethanaminium chloride (Nile Blue, **1a**) and its derivatives, *N*-ethyl-*N*-[5-(3-hydroxypropylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride (**1b**) and *N*-[5-(3-hydroxypropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride (**1c**) previously synthesized[2] were used as photosensitizers. In an earlier stage, the optimized times of the photosensitizers absorption by cells were determined through absorbance and fluorescence methods. Cells were exposed to radiation at a wavelength of visible light for 20 minutes in two different ways, first they were washed in PBS before being exposed to radiation, and then they were irradiated with benzo[*a*]phenoxazine solution. The cellular viability was determined using the method of reduction XTT and colony counting.

From all compounds tested, the benzo[*a*]phenoxazine **1c** reveled to be the most effective in *Candida albicans* inactivation. An inactivation rate of 85.3% for XTT method and 67.1% for the colony counting method was obtained when 4×10^7 cells in 200 µM solution of **1c** were exposed to photodynamic therapy, without washing the cells. For all benzo[*a*]phenoxazinium chlorides studied, it was obvious that the method of irradiation without cell washing achieved the higher cell inactivation for *Candida albicans*. It was also evident that Nile Blue (**1a**) only begins its inactivation of this strain when concentrations higher than 300 µM of this benzo[*a*]phenoxazine are used to inactive 4×10^7 cells/mL. Compound **1b** originated an intermediary inactivation, with better results than Nile Blue (**1a**), but less effectiveness than **1c**. This work indicates that the Nile Blue derivatives have a great potential of clinical application for *Candida albicans* infections treatment.

1. Donnelly, R. F.; McCarron, P. A.; Tunney, M. M. Microbiol. Res. 2008, 163, 1-12.
2. Frade, V. H. J. ; Coutinho, P. J. G. ; Moura, J. C. V. P. ; Gonçalves, M. S. T. Tetrahedron 2007, 63, 1654-1663.

Reference

192

Allelic variability of the β-lactamase (*bla*) locus in *Staphylococcus aureus*

Milheiriço, Catarina (1); Portelinha, Ana (2); de Lencastre, Hermínia (1,3); Oliveira, Duarte C. (1,2)

1: Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Oeiras, Portugal;
2: Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal;
3: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA

E-mail: cicm@itqb.unl.pt

Keywords: MSSA, MRSA, β-lactamase, β-lactam resistance

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of nosocomial infections and has recently emerged as a community acquired pathogen. Resistance to β-lactam antibiotics in *S. aureus* can be mediated by the production of β-lactamases encoded by the *blaZ* gene, which encodes for resistance to penicillins only, or by the acquisition of an extra penicillin-binding protein – PBP2A, the gene product of *mecA*, which encodes for cross-resistance to all β-lactams. Interestingly, despite the cross-resistance provided by the *mecA* gene, the great majority of contemporary clinical MSRA strains is still positive for the *bla* locus. Until now, few studies have addressed the allelic variability of the *bla* locus among *S. aureus* strains. In this study, the allelic variability of the *bla* locus in a representative collection of epidemic MRSA clones and unrelated methicillin-susceptible *S. aureus* (MSSA) strains was evaluated, in an attempt to make evolutionary correlations between *bla* types, resistance phenotypes and genetic lineages.
The allelic variation on the *bla* locus was evaluated by DNA sequencing of internal fragments of 533bp for *blaZ* gene in 54 MRSA and 33 MSSA strains and, for a subset of 52 strains, by DNA sequencing of internal fragments for *blaZ* regulatory genes (484 bp for *blaI* and 537 bp for *blaR1*). The β-lactamase activity was also evaluated in 27 MRSA and 13 MSSA strains, by spectrophotometric analysis of the degradation of the chromogenic substrate CENTA.
Thirteen different *blaZ* allotypes, nine *blaI* allotypes and twelve *blaR1* allotypes were detected in our collection. No correlation could be established between the *blaZ* allotype and MRSA lineages. In contrast to the genetic lineages, which are much more diverse among MSSA than MRSA, the *bla* locus was found to be more variable among MRSA strains. Only one single nucleotide polymorphism (SNP) was found to originate a nonsense mutation within *blaZ*. CENTA degradation rates were found to be proportional to growth rates and could not be correlated with *bla* allotypes.
Alltogether, the results of this study suggest that the evolution of the *bla* locus did not parallel the evolution of the MRSA lineages. Interestingly, in spite of the presence of *mecA* in MRSA strains, it seems that there is a selective pressure to keep the *bla* locus functional.

Reference

197

Molecular characterization and antimicrobial susceptibility profiles in *Streptococcus agalactiae*: association of erythromycin resistance with subtype III-1 genetic clone family

Florindo, Carlos; Viegas, Silvia; Paulino, Albertina; Rodrigues, Emanuel; Gomes, João Paulo; Borrego, Maria José

Instituto Nacional de Saúde Dr. Ricardo Jorge, Portugal

E-mail: carlos.florindo@insa.min-saude.pt

Keywords: *Streptococcus agalactiae*, colonization, macrolide resistance, MLST

Abstract

Streptococcus agalactiae (*S. agalactiae*) is the predominant bacterial cause of invasive perinatal infections. We aimed to study the *S. agalactiae* carriage rate among Portuguese reproductive-aged women, the prevalence of antibiotic resistance and the molecular characterization of the clinical isolates. Vaginal and rectal swabs were collected from 4269 women (2005-2007). *S. agalactiae* isolates were subjected to capsular typing by molecular methods, and were evaluated for antimicrobial susceptibility and the respective genetic determinants. Multilocus sequence typing (MLST) was used to analyse the genetic relatedness among isolates presenting resistance to erythromycin. To our knowledge, this is the first report of *S. agalactiae* colonization rate in Portugal performed according to CDC guidelines. *S. agalactiae* was found in 6.2% of women, where capsular genotypes III (35%), V (33%), Ia (16%) and II (10%) were the most frequent. All isolates were susceptible to penicillin and vancomycin, whereas resistance to clindamycin and erythromycin was detected in 10% and 19% of isolates, respectively. For the latter, 53% displayed the constitutive MLSB phenotype (conferring high-level resistance to macrolides), 42% had the inducible MLSB, and M phenotype accounted for 5% of isolates. *erm* methylase genes were exclusively associated with MLSB phenotype isolates, whereas the M phenotype was due to the presence of *mefA*. Also, we found an association between erythromycin resistance and subtype III-1/ST-19 genetic clone family. In conclusion, considering the association of genetic lineages expressing type III capsule with macrolide resistance, and its literature reported relation with invasive infection in infants, epidemiological surveillance of this genotype is advisable.

Reference

199

Biofilm formation by *Staphylococcus epidermidis* in bacterial communities

Oliveira, Manuela (1); Ribeiro, Inês (2); Bexiga, Ricardo (1); Vilela, Cristina Lobo (1)

1: Faculdade de Medicina Veterinária de Lisboa, Portugal;
2: Escola Superior de Biotecnologia / AESBUC-ETGI, Sintra, Portugal

E-mail: clvilela@fmv.utl.pt

Keywords: biofilm, *Staphylococcus epidermidis*, mastitis

Abstract

Staphylococcus epidermidis is a recognised mastitis pathogen, frequently detected in dairy herds, often responsible for subclinical infections and chronic bovine mastitis. The formation of capsule and slime or of multicellular complexes known as biofilms by these bacteria has been described as putative virulence factors, eventually leading to infection persistence. During the course of intramammary infections, biofilm structures may facilitate staphylococciadherence and colonisation of the mammary epithelium, contributing to the evasion of immunological defences and hampering pathogen eradication.

To evaluate staphylococci biofilm-forming ability in the presence of other bacteria frequently isolated from infected bovine udders, mixed bacterial suspensions of a biofilm-positive *Staphylococcus epidermidis* strain (ATCC 35984) and a mastitis isolate were performed. The mastitis isolates tested were *Streptococcus agalactiae, Streptococcus uberis; Escherichia coli* and *Corynebacterium* sp. After incubation to allow biofilm formation, a FISH protocol was applied, with the following steps: fixation with paraformaldehyde 4% in PBS (2 h); dehydration using ethanol serial concentrations (50%, 80% and 96%, 3 min); permeabilization with 0,01 mg/ml lysostaphin (4 min); hybridization with 10µl of hybridization buffer (0,9 M NaCl, 20 mM Tris-HCl, 0,01% SDS and 5 ng/µl of a 16S rRNA fluorescent probe, Sta) (3 h, 45 ºC); stringency washes with washing buffer (0,9 M NaCl, 20 mM Tris-HCl and 0,1% SDS) (15 min, 45 ºC); and observation under fluorescence microscopy in the x1000 amplification (Objective HCX PLAN APD) in a Leica DMR microscope.

Co-culture with *Streptococcus agalactiae* and *Streptococcus uberis* did not influence staphylococci biofilm-forming ability, while *E. coli* and *Corynebacterium* spp. impaired biofilm formation by *Staphylococcus epidermidis*. Glucose metabolization by bacteria belonging to *Corynebacterium* genus and *E. coli* could have contributed to the difference observed, since this cabohydrate is essential for biofilm formation in staphylococci.

Further studies are required, aiming at better simulating *in vivo* conditions, thus providing a better model for biofilm formation in the udder.

Reference

202

Identification of clinical isolates *Trichophyton rubrum* using a rapid and accurate mass spectral analysis (MALDI-TOF ICMS)

Pereira, Leonel (1); Dias, Nicolina (1,2); Santos, Cledir (1); Lima, Nelson (1)

1: IBB - Biological Engeneering Centre, Braga, Portugal;
2: CITS - Centro de Investigação em Tecnologias da Saúde, Gandra, Paredes, Portugal

E-mail: cledir.santos@deb.uminho.pt

Keywords: Clinical Mycology, Dermatophytes, MALDI-TOF ICMS

Abstract

Dermatophytes are keratinolytic fungi that are responsible for the commonest dermatologic condition called “ringworm” in man. The affinity for keratinized tissues by dermatophytes, implies in most of the cases, that the infection remains restricted to the nonliving cornified layers of the skin, nails, and hair [1]. Among dermatophytes, the species *Trichophyton rubrum* is of particular clinical interest for man because is the most common agent of human dermatophytoses. Macro and micro-morphological examination combined to physiological analysis of primary isolates grown in selective culture media are still the most used methods in routine laboratory. Besides their low specificity, an accurate diagnosis may take 3 to 4 weeks to be achieved Modern identification methods involve molecular biology by using PCR technology based on differential sequence elements. It is gradually becoming clearer that microbial identification and authentication requires a polyphasic approach to generate quality data which are accurate and useful [2]. Microbial mass spectral analysis has been progressively more incorporated to the polyphasic approach to improve the accuracy of the microbial identification issue. Matrix Assisted Laser Desorption Ionization Time of Flight Intact Cell Mass Spectroscopy (MALDI-TOF ICMS) is becoming an alternative to DNA-dependent methods so it has been already successfully applied to the rapid identification and classification of microorganisms [3].The aim of this work was to test the applicability of MALDI-TOF ICMS for identifying clinical isolates of *T. rubrum.* In this study twenty clinical isolates of *T. rubrum* were grown on Sabouraud culture medium. Plates were incubated for 7 days at 25 ºC. All the isolates were identified both macroscopically and microscopically. From the same plate, a tiny sample (about 50 mg) was transferred to stainless steel templates. A 0.5 ml of dihydroxy-benzoic acid (DHB) matrix solution was added to the sample and air dried. Peak lists of individual samples were compared with the superspectra database generating a ranked list of matching spectra from SARAMIS software. All strains were accurately and consistently identified as *T. rubrum* by MALDI-TOF ICMS combined to SARAMIS database analysis. Spectral mass analysis proven to be a rapid method, as the analysis took only a few minutes to perform with the benefit of any laborious sample preparation procedures or any expensive chemical reagent was needed.

[1] Rippon, JW. (1988) Saunders (W.B.) Co Ltd. Philadelphia, USA.
[2] Keys, C.J., Dare, D.J., Sutton, H., Wells, G., Lunt, M., McKenna, T., McDowall, M., Shah, H.N. (2004) Infect Genet Evol, 4, 221-242.
[3] Santos, C., Paterson, R.M.R., Venâncio, A., Lima, N. J. Appl. Microbiol (In Press).

Reference

214

Effect of essential oils in *Leishmania tropica* and *Leishmania major* Promastigotes

Machado, M (1,2); Cavaleiro, C (1); Salgueiro, L (1); Sousa, M C (1)

1: Faculdade de Farmácia,/CEF, Universidade de Coimbra, 3000 Coimbra, Portugal;
2: Departamento de Farmácia, Escola Superior de Saúde do Vale do Ave/CITS/IPSN-CESPU, 4760 Vila Nova de Famalicão, Portugal

E-mail: mcsousa@ci.uc.pt

Keywords: Leishmaniasis, treatment, plant extracts, essential oils, MTT

Abstract

Protozoan parasites of the genus *Leishmania* cause visceral, cutaneous and mucosal diseases in humans, which are collectively referred as leishmaniasis. Cutaneous leishmaniasis is promoted by *L. major* and *L. tropica*. The treatment of choice is still based on the use of pentavalent antimony, in spite of its toxicity, side effects, rate of relapse, cost, length of the treatment and resistance. One of the main reasons that a good single drug has not yet emerged is due to the fact that the parasites are protected inside macrophages and, hence the compounds that are toxic to the parasite are also toxic to the cell host. Screening plant extracts for anti-*Leishmania* activity is a valuable research option, counter-parting screening of synthetic compounds. Plant extracts offer a huge diversity of compositions and constituents, most of them commercially unavailable and structurally difficult to synthesize. Such compounds easily diffuse across cell membranes and consequently gain advantage in what concerns to interactions with intracellular targets. Essential oils were obtained by hydrodistillation from fresh plant material and analysed by GC and GC-MS. Constituents were identified from their retention indices on two different phases GC columns (polydimethylsiloxane and polyethyleneglycol) and from their mass spectra, which were compared with reference data. The antileishmanial screening test was performed on culture promastigotes of *L. tropica* and *L. major* incubated in growth medium, HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% fetal heat inactivated fetal bovine serum (HFBS). Promastigotes at logarithmic phase culture (10^6 cells/ml) were incubated 24/48 h at 26 ºC with different concentrations of essential oils. The viability of promastigotes was assessed by the tetrazolium-dye (MTT) colorimetric method. The experiments were performed in triplicate and in at least six independent assays. Results were expressed as concentrations that inhibit parasite viability by 50% (IC_{50}). The tested essential oils, namely *Cymbopogon citratus*, *Lavandula luisirei*, *Thymus capitelatus* and *Junyperus oxycedrus* revealed a strong activity against promastigotes with IC_{50} values ranging from 16 µg /ml to 52 µg/ml. None of the tested essential oils had cytotoxic effects on mammalian cells lines. Future studies will continue for the development of new agents against leishmaniasis from these essential oils, namely their natural constituents.

Reference

236

Molecular epidemiology of macrolide and/or tetracycline resistant *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *dysgalactiae*, and *Streptococcus uberis* from bovine mastitis

Rato, Márcia Alexandra (1); Bexiga, Ricardo (2); Nunes, Sandro Filipe (3); Cavaco, Lina Maria (2,4); Vilela, Cristina Lobo (2); Santos-Sanches, Ilda (1)

1: Centro de Recursos Microbiológicos, Dep. Ciências da Vida, Faculdade de Ciências e Tecnologia/ Universidade Nova de Lisboa, Caparica, Portugal;
2: Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária/ Universidade Técnica de Lisboa, Lisbon, Portugal;
3: Presently at Cambridge Infectious Diseases Consortium, Cambridge University, Cambridge, UK;
4: National Food Institute, Faculty of Life Sciences/ Technical University of Denmark, Copenhagen, Denmark

E-mail: alexandra.marcia@gmail.com

Keywords: Veterinary streptococci, PFGE, macrolide resistance

Abstract

Streptococcus agalactiae (Group B Streptococcus, GBS), *S. dysgalactiae* subsp. *dysgalactiae* (Group C Streptococcus, GCS) and *S. uberis* are bovine mastitis pathogens. Antibiotic resistance remains a growing concern, justifying a continuous local surveillance among mastitis pathogens. Additionally, current knowledge on the molecular features of field isolates from Portugal is virtually unknown with the exception of subclinical mastitis- associated *S. uberis* (1). The aim of the present work was to evaluate the clonality of field isolates of GBS, GCS and *S. uberis*, and macrolides and tetracycline resistance patterns proved valuable for effective mastitis control in dairy herds.

Field isolates of GBS (n=32), GCS (n=18) and *S. uberis* (n=30) from 12 herds in Portugal were characterized by pulsed field gel electrophoresis (PFGE) with computer-assisted DNA-band analysis. Resistance to macrolides (erythromycin-E), lincosamides (pirlimycin-PRL), tetracycline-TET, and the constitutive macrolide-lincosamide resistance phenotype (cMLS) was evaluated by disk diffusion. Resistance genes (*mef*A; *erm*A; *erm*B; *lin*B; *tet*M; *tet*O; *tet*T; *tet*S; *tet*Q; *tet*K; *tet*W; *tet*L) were screened by PCR.

A total of three PFGE clusters comprised 72% of the GBS and four PFGE clusters comprised 53% of the *S. uberis* and all were found to be herd-specific. Two GCS strains from different farms had identical PFGE patterns. Co-resistance to E and PRL (18%-27%) and to TET (60%-100%) was observed in all species. Resistance to PRL and susceptibility to E (LSA phenotype) was found in GCS and *S. uberis*. Diverse genotypes were found: *erm*B/*tet*O/*tet*K in GBS; *erm*B/*tet*O or *lin*B/*tet*M in GCS; *erm*B/*tet*O or *lin*B/*tet*S in *S. uberis*.

The results suggested contagious and environmental sources for *S. uberis* and GCS infections and extensive cow-to-cow transmission of GBS. The LSA phenotype, previously described only in human GBS (2) and *S. uberis* (3), was found in this study in bovine GCS and was associated with the *lin*B gene. Linkage of several antimicrobial resistance genes in unique strains and clones suggests vertical and horizontal gene dissemination.

(1) Rato M.G. et al. 2008. J Dairy Sci. 91:4542.
(2) Malbruny et al. 2004. J Antimicrob Chemother. 54:1040.
(3) Petinaki et al. 2008. Antimicrob Agents Chemother. 52:626.

Reference

242

Prevalence and clonality of MRSA in the Atlantic Azores islands: establishment of the epidemic EMRSA-15 clone

Conceição, Teresa (1); Tavares, Ana (1); Miragaia, Maria (1); Hyde, Karyne (2); de Lencastre, Hermínia (1,3)

1: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal;
2: Hospital do Divino Espírito Santo, Ponta Delgada, Açores;
3: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA

E-mail: teresagc@itqb.unl.pt

Keywords: MRSA, Azores islands, EMRSA-15

Abstract

The prevalence of nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) in continental Portugal is currently close to 45%, mainly due to the spread of three major pandemic clones: EMRSA-15 (ST22-IV); New York/ Japan (ST5-II) and Brazilian (ST239-III). Regarding the Azores archipelago, nothing is known concerning MRSA epidemiology. In the present study, we identify the MRSA clonal types currently circulating in an Azores central hospital, and compare them with the major MRSA clones circulating in continental Portugal and in Europe.

Between January 2007 and February 2008 a total of 108 nonduplicateMRSA isolates were collected from both inpatients and outpatients attending the Hospital do Divino Espírito Santo, Ponta Delgada, Azores. Resistance profiles were determined for all isolates by the VITEK system and molecular characterization was performed by pulsed-field gel electrophoresis (PFGE). Representatives of all PFGE types were further characterized by *spa* typing, multilocus sequence typing (MLST) and staphylococcal chromosome cassette *mec* (SCC*mec*) typing. The presence of the Panton-Valentine leukocidine (PVL) genes was detected by PCR.

The 108 MRSA isolates were assigned to four different PFGE types (A to D). The overwhelming majority of the isolates (87%, n=94) were characterized by PFGE type A, *spa* type t032, ST22, and SCC*mec* IVh, characteristic of the EMRSA-15 clone. PFGE type B included 11% (n=12) of the isolates distributed into four subtypes and was associated to ST5. Subtypes B1 and B2 comprised strains with *spa* types t062, t311, t2049 and t2724, and SCC*mec* VI, while subtypes B3 and B4 shared *spa* type t002 and SCC*mec* IVc, which are both features typical of the Pediatric clone, previously reported as a minor clone in Portuguese hospitals. Interestingly, three isolates with subtype B1 carried the PVL. PFGE type C included a single isolate and was related to the highly epidemic Berlin clone (*spa* type t004, ST45-IVa), and PFGE type D was associated to *spa* type t1839, ST1339 and SCC*mec* type V variant, which is usually seen among community acquired MRSA.

In this study, we provided the first description of the population structure of MRSA in the Azores islands, which is in line with recent reports from continental Portugal and European hospitals. The regular personal exchange between islands and the continent combined to the high percentage of emigration to North America, may explain the dominance and spread of the EMRSA-15 and Pediatric clones.

Reference

251

Identification of the first Community-Associated Methicillin-Resistant *Staphyloccus aureus* (CA-MRSA) in Portugal

Tavares, Ana (1); Miragaia, Maria (1); Mato, Rosario (1); Rolo, Joana (1); Melo-Cristino, José (2); Lito, Luís M. (2); Gonçalves, Maria Luisa (3); Martins, Filomena (4); Gonçalves, Elsa (4); Paixão, Paulo (5); Marto, Natália (5); Francisco, Sandra (6); de

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica (ITQB), Oeiras, Portugal;
2: Serviço de Patologia Clínica do Centro Hospitalar Lisboa Norte, EPE, Lisboa, Portugal;
3: Laboratório de Patologia Clínica, Microbiologia, Hospital dos SAMS, Lisboa, Portugal;
4: Centro Hospitalar de Lisboa Ocidental, Laboratório de Microbiologia do Hospital de S. Francisco Xavier, Lisboa, Portugal;
5: Laboratório de Patologia Clínica, Hospital da Luz, Lisboa, Portugal;
6: Serviço de Patologia Clínica, Hospital da Força Aérea, Lisboa, Portugal;
7: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA

E-mail: atavares@itqb.unl.pt

Keywords: CA-MRSA, PVL, Portugal

Abstract

MRSA were traditionally confined to hospitals (HA-MRSA). However, in the last decade CA-MRSA emerged worldwide as a pathogen causing serious infections in the community. Recent data suggest that CA-MRSA isolates are different from HA-MRSA having distinctive genetic backgrounds and a high virulence potential. The presence of the Panton-Valentine leukocidin (PVL) has been strongly associated with CA-MRSA. In spite of the clinical relevance of CA-MRSA, no information exists on its prevalence in Portugal. In the present study we aim to monitor the presence of CA-MRSA as well as to characterize the CA-MRSA clones circulating in the community in Portugal.

From January to April 2009, 48 *S. aureus* isolates and the corresponding questionnaires, were sent to our laboratory at ITQB by five hospitals from Lisbon. Isolates were characterized by pulsed-field gel electrophoresis (PFGE) and the type and subtype of staphylococcal chromosomal cassette *mec* (SCC*mec*) determined by multiplex PCR. Clonal lineages were predicted by the combination of PFGE and SCC*mec* typing results, using a representative database. The PVL genes were detected by PCR. For the PVL positive isolates the *spa* type was determined by sequencing. Additionally 21 specific staphylococcal virulence genes were detected by PCR, namely: enterotoxin genes, toxic-shock syndrome toxin gene, exfoliative toxin genes, *lukE–lukD* leukocidin genes, the class F *lukM* leukocidin gene, gamma and β hemolysin genes.

From the 48 isolates, 37 were MRSA and 11 were MSSA. Three out of the 48 isolates carried the PVL genes of which two were CA-MRSA associated to clone USA300 with the *spa* type t008 and SCC*mec* IVa. One PVL positive strain was a CA-MSSA (methicillin-susceptible) with an atypical PFGE pattern and *spa* type3644. The two CA-MRSA isolates shared the same virulence genes, namely *sel*, *lukE–lukD*, *hlg* and *hlgv* genes. The CA-MSSA isolate carried the virulence genes *etd*, *sel*, *sei*, *seg*, *lukE–lukD*, *hlg* and *hlgv*.

We report here the first three cases of CA-MRSA/MSSA infections in Portugal. The CA-MRSA strains causing infections belonged to the epidemic USA300, a predominant and high transmissible clone in the United States that recently started to spread in Europe. The follow-up of these studies will enable to evaluate the prevalence and extension of dissemination of this and other clones in different regions of Portugal.

Reference

259

Discrimination of *Burkholderia cepacia* complex clinical isolates using Fourier Transform Infrared Spectroscopy and chemometric approaches

Coutinho, Carla P.S. (1,2); Sá-Correia, I. (2); Lopes, J. A. (1)

1: REQUIMTE, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal;
2: Institute for Biotechnology and Bioengineering, CEBQ, Instituto Superior Técnico, Lisboa, Portugal

E-mail: carla.coutinho@ff.up.pt

Keywords: *Burkholderia cepacia* complex (Bcc), bacterial strain discrimination, FTIR spectroscopy, Antimicrobial susceptibility

Abstract

Bacteria of the *Burkholderia cepacia* complex (Bcc) are opportunist pathogens and a risk factor for increased morbidity and premature mortality among cystic fibrosis (CF) patients. The species *B. cenocepacia* and *B. multivorans* were predominantly recovered from different CF populations, although *B. cepacia* has been frequently isolated in Portugal [1]. Bcc bacteria are inherently resistant to most of the known antimicrobials. However, phenotypic clonal variants can be isolated during chronic infection, in particular variants with increased antimicrobial resistance, following aggressive antibiotic therapy [2].

This work reports the development of a methodology envisaging the eventual classification or discrimination of Bcc clinical isolates, at the species level and at the ribopattern level, and of clonal isolates with variable phenotypes, using Fourier Transform Infrared (FTIR) spectroscopy. This vibrational spectroscopic technique is a potential alternative to conventional methods for rapid identification and typing of bacteria, since it captures information about their molecular composition.

A set of 185 Bcc isolates (*B. cepacia*, *B. cenocepacia-recA* lineages III-A and III-B-, *B. multivorans, B. stabilis)*, collected at Hospital de Sta. Maria and moleculary characterized [1], were analyzed by FTIR and chemometrics. Reference strains (10) were used to validate the method. The optimal spectral window for discrimination was the 900-1500cm^{-1} region (proteins, polysaccharides, phospholipids/DNA/RNA) [3]. The discrimination at the species level led to misclassification error rates up to 30%. For *B. cepacia* and *B. cenocepacia* ribopatterns, misclassification error rates of 4% were obtained. Sequential clonal isolates, with the same ribopattern but variable resistance to antibiotics and other different relevant phenotypes (A. Pinto-de-Oliveira, unpublished results), were discriminated [3]. In conclusion, FTIR spectroscopy and chemometrics open the door to the differentiation of sequential clonal Bcc isolates with an altered chemical composition, presumably as the result of expression of adaptation mechanisms to the CF lung environment, in particular to aggressive antibiotic therapy.

Acknowledgments –This work was supported by FEDER and FCT (PTDC/SAU-MII/69591/2006 and SFRH/BD/32729/2006). INFARMED provided the FTIR spectrometer and Prof. P.Vandamme (Ghent Univ.) the reference strains, under the EuroCareCF (LSHM-CT-2005-018932) activities.

[1]- Cunha M.V., Pinto-de-Oliveira A., Meirinhos-Soares L., Salgado M.J., Melo-Cristino J., Correia S., Barreto C., Sá-Correia I. (2007) J Clin Microbiol 45:1628–33

[2]- Leitão J.H., Sousa S.A., Cunha M.V., Salgado M.J., Melo-Cristino, Barreto M.C., Sá-Correia I. (2008) Eur J Clin Microbiol Inf Dis 27:1101–11

[3]-Coutinho C.P., Sá-Correia I., Lopes J.A. (in press) Anal. Bioanal. Chem., DOI 10.1007/s00216-009-2908-4.

Reference

270

Importance of efflux systems on the resistance to fluoroquinolones by *Staphylococcus aureus*

Falcão, Celeste (1,2); Costa, Sofia Santos (1,3); Viveiros, Miguel (1,4); Machado, Diana (1); Martins, Marta (1,5); Melo-Cristino, José (6); Amaral, Leonard (1,4,5); Couto, Isabel (1,3)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: Centro Hospitalar Lisboa Norte E.P.E.;
3: Centro de Recursos Microbiológicos (CREM), UNL;
4: COST ACTION BM0701 (ATENS);
5: UPMM; IHMT, UNL;
6: Centro Hospitalar Lisboa Norte E.P.E., Instituto de Microbiologia, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa

E-mail: falcao.celeste@gmail.com

Keywords: *Staphylococcus aureus*, efflux pumps, fluoroquinolone resistance

Abstract

Staphylococcus aureus is one of the most important clinical pathogens for which several mechanisms of resistance to antimicrobial compounds have been described. However, antimicrobial resistance mediated by efflux systems is still poorly characterized for *S. aureus,* despite the description of several efflux pumps in this species, which confer resistance to different types of compounds, including antibiotics, biocides and dyes, such as ethidium bromide (EtBr).

In this work, we screened and characterized the efflux systems present in a collection of 52 clinical isolates of *S. aureus* resistant to fluoroquinolones, in order to understand the role played by efflux systems in the resistance to this class of antibiotics.

The efflux activity was evaluated by the EtBr-agar cartwheel method and by the determination of the minimum inhibitory concentrations (MICs) for different substrates of efflux pumps, including fluoroquinolones, in the presence and absence of efflux pumps inhibitors. The genes coding for eight efflux pumps were screened by PCR, and the expression of six of them assessed by qRT-PCR. The possible presence of mutations that confer resistance to fluoroquinolones was investigated by sequencing the QRDR regions of *gyrA* and *grlA* genes. Finally, a representative group of isolates was typed by PFGE.

The application of these different methodologies detected efflux activity in 12 of the 52 isolates screened, and correlated this activity with over-expression of several efflux pumps and increased resistance to fluoroquinolones. The inhibition of these efflux systems did not result in the total reversion of the resistance phenotype to susceptibility, yet it implied a significant decrease in the resistance levels to these antibiotics, regardless of the type(s) of mutation(s) found in *grlA* and / or *gyrA* genes. The mutations found in *grlA* and / or *gyrA* genes accounted for the remaining level of resistance to fluoroquinolones that was not efflux mediated.

Thus, the results obtained in this work do not exclude the importance of these mutations in resistance to fluoroquinolones in *S. aureus*, but underline the contribution of efflux systems for the emergence of high-level resistance to these drugs and even to the emergence of multidrug resistant *S. aureus* in hospitals.

Reference

271

Genetic diversity of the *Staphylococcus aureus* efflux pump gene *norA*

Costa, Sofia Santos (1,2); Viveiros, Miguel (1,3); Falcão, Celeste (1,4); Melo-Cristino, José (5); Amaral, Leonard (1,3,6); Couto, Isabel (1,2)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: Centro de Recursos Microbiológicos (CREM), UNL;
3: COST ACTION BM0701 (ATENS);
4: Centro Hospitalar Lisboa Norte E.P.E.;
5: Centro Hospitalar Lisboa Norte E.P.E, Instituto de Microbiologia, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa;
6: UPMM; IHMT, UNL.

E-mail: scosta@ihmt.unl.pt

Keywords: *Staphylococcus aureus*, efflux pumps, multidrug resistance

Abstract

Multidrug resistance efflux pumps are capable of extruding multiple antimicrobial compounds from the cell, contributing to the development of resistance phenotypes. In *Staphylococcus aureus* one of the most well studied efflux pumps is NorA, responsible for reduced susceptibility to fluoroquinolones, dyes and biocides. Early studies showed that its encoding gene, *norA*, has some variability, with two alleles described, *norAI* and *norAII*. However, the relevance of each type of *norA* allele in the clinical setting, in terms of distribution and relation with resistance phenotypes is scarcely documented. In our study, the promoter region and structural *norA* gene sequences were analyzed in ten *S. aureus* isolates, including the reference strain ATCC25923 and nine clinical isolates, in order to ascertain the prevalence of each *norA* allele and establish a correlation between the *norA* alleles and susceptibility phenotypes to different antimicrobial compounds.

Eight clinical isolates carried the *norAI* allele, whereas *norAII* was present in two strains, one clinical isolate and ATCC25923. Of the 8 isolates carrying *norAI*, seven showed three types of alterations, namely an insertion downstream of the -10 consensus promoter sequence and the mutations Gly147Ser and Gly291Asp. The remaining isolate showed no alteration when compared to the originally described allele. The two strains carrying the *norAII* allele showed a high number of mutations when compared to the *norAII* allele described earlier, of which only one resulted in an aminoacid substitution, Asn200Asp. qRT-PCR analysis detected n*orA* over-expression for only 2 out of 7 isolates tested, each one carrying a different type of *norA* allele. Minimum inhibitory concentration (MIC) determinations showed no direct correlation between the type of *norA* allele and the strain susceptibility profile towards different substrates, namely fluoroquinolones, dyes and biocides.

This study evidenced the higher prevalence of the *norAI* allele among the strains studied and the occurrence of multiple alterations at the nucleotide level, which were more specific for *norAI*, suggesting that this may be the more significant allele in terms of multidrug resistance development. Further characterization of *norA* diversity should clarify the importance of the different NorA variants and their interplay with the remaining *S. aureus* efflux pumps.

Reference

274

New method for the rapid screening of efflux pump activity in multi-drug resistant clinical strains

Martins, Marta (1,2); Viveiros, Miguel (1,3); Couto, Isabel (1,4); Santos Costa, Sofia (1,4); Pacheco, Teresa (5); Amaral, Leonard (1,2,3)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: UPMM (IHMT, UNL);
3: COST ACTION BM0701 (ATENS);
4: Centro de Recursos Microbiológicos (CREM), UNL;
5: Laboratório de Microbiologia, Hospital Egas Moniz, Lisboa, Portugal

E-mail: mmartins@ihmt.unl.pt

Keywords: Multi-drug resistance, efflux activity, efflux pumps, ethidium bromide, screening method

Abstract

Multi-drug resistance (MDR) among bacterial clinical isolates has become a major concern. Because MDR can be due to the over-expression of efflux pumps, the clinical laboratory needs methods for the timely identification of efflux-mediated MDR phenotypes.
We have developed a simple, instrument free method for the rapid screening of efflux-mediated MDR in bacteria, the EtBr-agar cartwheel method. This method uses agar plates containing varying concentrations of EtBr - a common substrate of efflux pumps - that are swabbed with up to 12 bacterial cultures. The higher the concentration of EtBr needed to produce fluorescence of the bacterial mass, the greater the efflux capacity of those cells.
In this work, we applied this method to screen for efflux activity among both reference and clinical isolates of Gram-negative and Gram-positive pathogens, namely *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella* Typhimurium and *Salmonella* Enteritidis, *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium*. The 42 clinical isolates studied, all showing a MDR phenotype, originated from hospitals in Portugal, France and Denmark. Identification and antibiotic susceptibility were provided by the collaborating hospital microbiology laboratories. Antibiotic susceptibility data was confirmed by the Kirby-Bauer method, following the CLSI guidelines.
Bacterial cultures swabbed in EtBr-agar plates fluoresced at different EtBr concentrations, depending on their capacity to efflux this substrate. All the species showed efflux activity, however, some demonstrated higher fluorescence values such as strains of *E. coli*, *Enterobacter aerogenes* and *Enterococcus faecium*. In almost all cases the efflux activity could be correlated with the decrease of minimum inhibitory concentration values of the antibiotics in the presence of efflux pump inhibitors.
Our results show that the EtBr-agar cartwheel method can be useful for the detection of efflux activity among clinical isolates of both Gram-negative and Gram-positive bacteria, which results in a MDR phenotype. Due to its simplicity, it can be easily adapted to the routine clinical laboratory, affording the simultaneous evaluation of 12 bacterial strains in a single plate.

Reference

280

Insights in non-*Candida albicans* Candida biofilms

Henriques, Mariana; Martins, Margarida; Negri, Melyssa; Silva, Sónia; Azeredo, Joana; Oliveira, Rosário

Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal

E-mail: mcrh@deb.uminho.pt

Keywords: Non-*Candida albicans* Candida spp, Biofilms, Extracellular Enzymes, Antifungal Resistance, Extracellular alcohols

Abstract

The number of infections caused by *Candida* spp has greatly increased in the past years, which has been attributed to an increase in the number of AIDS and immunocompromised patients, in the elderly population and the more frequent use of indwelling medical devices. Most Candidiasis have been attributed to *Candida albicans*, however, recently, non-*Candida albicans Candida* (NCAC) spp, as *C. parapsilosis*, *C. glabrata* and *C. tropicalis*, have been identified as common pathogens. Furthermore, Candida biofilm formation has important clinical repercussions due to their inherent tolerance to antifungal therapy and ability to withstand host immune defenses. Consequently, it is of utmost importance to understand the physiology and virulence of NCAC spp biofilms. Thus, the main aim of this work is to present some insights in *C. parapsilosis*, *C. glabrata* and *C. tropicalis* biofilms, through (i) biofilm characterization (structure and matrix composition); (ii) evaluation of antifungal agents tolerance and (iii) determination of putative virulence factors (extracellular enzymes and extracellular alcohols).

SEM observation of *Candida* spp biofilms revealed that biofilm architecture was neither species nor strain dependent. However, *C. glabrata* biofilms, which presented lower biomass, formed, generally, a more compact and thick structure than *C. tropicalis* and *C. parapsilosis* ones. Regarding matrix composition, *C. glabrata* presented, in general, higher amounts of proteins and polysaccharides, in opposition to *C. tropicalis* that presented lower amounts of both components. Biofilms antifungal resistance tests revealed that *C. glabrata* biofilms present high resistance to fluconazole and itraconazole, in comparison with the other NCAC spp biofilms.

With respect to putative virulence factors, the production of extracellular enzymes, namely proteases and phospholipases was also evaluated in *C. tropicalis* but there were no differences in the levels of enzymes production by biofilm and planktonic cells. Regarding the extracellular alcohols, it was found that *C. parapsilosis* and *C. tropicalis* produce farnesol, 1-dodecanol, 2-phenylethanol and isoamyl alcohol (already described for *C. albicans*). Furthermore, the latter was produced by *C. tropicalis* in a higher amount than by *C. parapsilosis*.

Overall, with this extensive research work it was possible to describe and relate several virulence features of NCAC spp with their putative virulence and invasiveness of human epithelia.

Reference

285

Colonization and Infection by Group B Streptococci in Pregnant Women and Newborns from Santarém, Portugal

Rodrigues, Filipe Borges (1); Prieto, Emília (2); Rato, Márcia (3); Pires, Renato (3); Sanches, Ilda Santos (3); Pereira, Filomena Martins (2)

1: Hospital Distrital de Santarém, Portugal;
2: Centro de Recursos Microbiológicos, Unidade de DST, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa;
3: Centro de Recursos Microbiológicos, Dep. Ciências da Vida. Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

E-mail: filipe.rodrigues84@gmail.com

Keywords: GBS; colonization, antibiotic resistance, prevention, clonality

Abstract

Group B streptococci (GBS) remain a major cause of mortality and morbidity in the newborn, thus it is critical to know the mechanism(s) of pathogenesis as well as the prevalence and the features of circulating strains, such as the capsular serotypes, clonality and antimicrobial resistance traits.
This study aimed to analyse the GBS vaginal/anal colonization in 270 pregnant women with 35 to 37 weeks of gestation followed at the Hosp. Distrital de Santarém from November 2007 to June 2008 and all cases of GBS infection in the same period.
All GBS [n=72 colonization isolates and 3 invasive isolates: two from newborns with late onset disease (LOD) and one from an adult with an urinary tract infection (UTI)] were classified into serotypes based on the capsular polysaccharide antigens. Antimicrobial susceptibility and resistance phenotypes to macrolides (M) or to macrolides-lincosamides-streptograminB (MLS) were evaluated by agar disc diffusion. Macrolide resistance genes were searched for by PCR. Clonality of isolates was assessed by pulsed-field gel electrophoresis (PFGE).
The prevalence of colonization was 21,1%. The colonization isolates were of capsular serotypes Ia (26,3%), III (19,3%), V (17,55%), IV (15,8%), II (10,5) and Ib (8,8%). The LOD isolates were Ia and III and the UTI isolate was III. All isolates were susceptible to ofloxacin, penicillin and vancomycin and most were susceptible to macrolides (90%) and lincosamides (91,7%). Resistance to tetracycline was high (80%). LOD isolates were tetracycline-resistant and the UTI isolate was susceptible to all antibiotics. Among the macrolides and/or lincosamides (Lin) resistant isolates (n=8), the constitutive MLS isolates (n=2) carried the *erm* (B) gene and inducible MLS isolates (n=2) carried the *erm* (A) gene. One of the M isolates carried *erm* (A) and the remaining two M isolates, as well as one Lin isolate, were negative for the *erm* genes and *mef* (A). The GBS were polyclonal (26 PFGE patterns among 27 isolates tested) and no relationship was found between clonality, invasiveness, serotypes and phenotypes of antibiotic-resistance.
In conclusion, the high prevalence of colonization by GBS among these pregnant women, and the carriage of antimicrobial resistant strains emphasize the importance of prevention by detecting GBS at the end of pregnancy and selecting appropriate antibiotics for intrapartum prophylaxis. The identification of serotypes is also of importance, considering the use of *GBS* conjugate *vaccine*s.

Reference

293

Mutational adaptation in *Mycobacterium tuberculosis*: the emergence of multidrug resistance

Machado, Diana (1); Couto, Isabel (1,2); Amaral, Leonard (1,3,4); Viveiros, Miguel (1,4)

1: Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: Centro de Recursos Microbiológicos (CREM), UNL;
3: UPMM (IHMT, UNL);
4: COST ACTION BM0701 (ATENS)

E-mail: dianamachado@ihmt.unl.pt

Keywords: Tuberculosis, *Mycobacterium tuberculosis*; multidrug resistance; mutational adaptation; phenotypic adaptation.

Abstract

Multidrug resistant tuberculosis (MDRTB) is caused by organisms with high-level resistance to both isoniazid (INH) and rifampicin (RIF), the main antibacillary used in tuberculosis therapy. Resistance to INH is mainly due to mutations in *katG* or *inhA* genes, whereas resistance to RIF results from point mutations in the *rpoB* gene. These mutations are independent and are required to occur concurrently for the organism to become multidrug resistant (MDR). Monoresistance to RIF is rare and is almost always accompanied by INH resistance. In this work we intended to understand the mechanism by which MDR develops during the course of the 6 month therapy regimen, using the *M. tuberculosis* H37Rv reference strain and a clinical strain monoresistant to RIF. These strains were characterized phenotypically by antibiotic susceptibility testing (AST) to the five first line drugs and MICs determination to INH and RIF using the BACTEC™ MGIT™ 960 system, equipped with the Epicenter V5.53A software and the TB eXIST module. Genotypic characterization of *katG*, *inhA* and *rpoB* genes was carried out by PCR and reverse hybridization protocols. For the antibiotic exposure process, both strains were constantly subjected to the critical concentrations of INH and RIF, independently, for approximately 6 months. The H37Rv strain exposed to RIF took about 30 days to reach full growth and this time-frame was extended in subsequent passages. When exposed to INH, the strain took 14-16 days to reach full growth in the first passage, and 3-4 days in subsequent passages. The clinical strain showed a similar behavior towards INH exposition. On the other hand, when exposed to RIF, this strain grew rapidly, as expected, since it carries a mutation in the *rpoB* gene. After the 6 months of constant exposure to RIF or INH both strains were subjected to an AST that showed that the clinical strain became MDR whereas H37Rv became resistant to INH only. Genotypic analysis detected no alteration in the *rpoB* and *inhA* genes of H37Rv strain but the entire *katG* gene was deleted. The clinical strain showed no genetic alteration at the end of the INH exposure process although it became clinically INH resistant, suggesting the involvement of efflux pump over-expression.

Overall, these results illustrate different strategies by which *M. tuberculosis* strains respond when exposed to clinically relevant concentrations of the same antibiotic, all of which may ultimately result in the emergence of MDRTB.

Reference

294

The role played by efflux pumps in macrolide resistance in *Mycobacterium avium* complex

Rodrigues, Liliana (1,2); Sampaio, Daniela (1); Couto, Isabel (1,3); Machado, Diana (1); Kern, Winfried V. (4,5); Amaral, Leonard (1,2,5); Viveiros, Miguel (1,5)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL), Lisboa, Portugal;
2: UPMM, IHMT/UNL;
3: Centro de Recursos Microbiológicos (CREM), UNL;
4: Center for Infectious Diseases and Travel Medicine, University Hospital, Freiburg, Germany;
5: COST ACTION BM0701 (ATENS)

E-mail: lilianarodrigues@ihmt.unl.pt

Keywords: *Mycobacterium avium* complex; efflux pumps; macrolide resistance

Abstract

Mycobacterium avium complex (MAC), comprising *M. avium* and *M. intracellulare*, is clinically important since it can cause severe infections in AIDS patients and other immunocompromised individuals. Therapy of MAC infections is problematic due to the intrinsic resistance of these bacteria to many of the available antimicrobial drugs. The use of the macrolides clarithromycin and azithromycin has improved the outcome of MAC infections, but therapeutic failure is still a major problem. We have recently shown that efflux pumps of MAC play an important role on this resistance phenotype. In fact, increased activity of efflux pumps is known to contribute to a multidrug resistance phenotype by extruding a wide variety of chemically and structurally unrelated compounds from the cell, preventing them from reaching their cellular targets. Thus, the characterization of such efflux pumps is crucial for the design of new antimycobacterial therapeutic strategies. In this work, we have studied the efflux pump activity in MAC clinical strains by a real-time fluorometric method and evaluated the contribution of active efflux to the resistance to macrolides.

Two MAC reference strains (*M. avium* ATCC25291^T and *M. intracellulare* ATCC13950^T) and eighteen clinical strains isolated from AIDS patients were evaluated for macrolide resistance in the presence and absence of the efflux pump inhibitors (EPIs) thioridazine, chlorpromazine and verapamil. The efflux activity of these strains was assessed by a real-time fluorometric method that detects extrusion of ethidium bromide, a known efflux pump substrate.

The results to be presented show that: (i) resistance to clarithromycin was significantly reduced in the presence of thioridazine, chlorpromazine and verapamil; (ii) the same EPIs decreased the efflux of ethidium bromide from MAC cells, as demonstrated by fluorometric analysis; and (iii) the intracellular retention of [^{14}C]-erythromycin increased in the presence of these inhibitors, providing further evidence that active efflux contributes to MAC resistance to macrolides.

In conclusion, this study demonstrates that efflux pumps play an important role in MAC resistance to antibiotics, particularly to macrolides, and supports the use of EPIs, such as thioridazine, as helper compounds in antimycobacterial therapy.

Reference

297

The effect of efflux pumps inhibitors on the transport of ethidium bromide and antimicrobials across *Mycobacterium smegmatis* cell wall

Ramos, Jorge (1); Rodrigues, Liliana (1,2); Couto, Isabel (1,3); Amaral, Leonard (1,2,4); Viveiros, Miguel (1,4)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: UPMM (IHMT, UNL);
3: Centro de Recursos Microbiológicos (CREM), UNL;
4: COST ACTION BM0701 (ATENS)

E-mail: jramos@ihmt.unl.pt

Keywords: *Mycobacterium smegmatis*, efflux pump inhibitors, LfrA, MspA, antimicrobials

Abstract

Active efflux systems and reduced cell wall permeability are considered to be the main causes of mycobacterial intrinsic resistance to many antimicrobials. In this study we have compared the *M. smegmatis* wild-type strain mc^{2}155 with mutants for LfrA and MspA, the main efflux pump and porin of *M. smegmatis*, respectively, for their ability to extrude ethidium bromide (EtBr), a known efflux pump substrate, in the presence or absence of efflux pump inhibitors (EPI) and correlated these results with the ability of the EPI to reduce the susceptibility of *M. smegmatis* to several antimicrobials.

The methods used for this purpose were: (i) a 96-well microplate screening assay with the mycobacterial cells grown in Middlebrook 7H9 with 10% of OADC in presence of increasing concentrations of EtBr and different concentrations of the EPI; and (ii) a semi-automated fluorimetric method that detects EtBr efflux on a real time basis during a period of 60 to 90 minutes. The EPI employed were chlorpromazine, thioridazine, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil. The efflux activity detected for each strain by these two methods was then correlated with resistance to several antibiotics (ATB), by the determination of their minimal inhibitory concentrations (MIC) in the presence or absence of the EPI. The ATB tested were streptomycin (STR), isoniazid (INH), rifampicin (RIF), ethambutol (ETB), amikacin (AMK), ciprofloxacin (CIP), clarithromycin (CLR) and erythromycin (ERY).

The results obtained show that, in the absence of the major porin of *M. smegmatis*, MspA, the accumulation of EtBr decreased and the cells became more resistant to several ATB. When the major *M. smegmatis* efflux pump, LfrA, is deleted the mutant strain showed increased accumulation of EtBr and increased susceptibility to EtBr, INH, RIF, ETB and CIP. Reduction of the MIC for STR, RIF, AMK, CIP, CLT and ERY was observed in the presence of the EPI tested, with the exception of CCCP.

These results put in evidence that MspA is an important channel for entrance of quaternary ammonium compounds (QAC) and ATB into the cells and that active efflux *via* the LfrA pump is involved in low-level resistance to several ATB and QAC in *M. smegmatis*. The methodologies and results developed and obtained with this model non-pathogenic mycobacteria will be used for the evaluation of the same EPI synergetic activity on the susceptibility of multi-drug resistant strains of *Mycobacterium tuberculosis* to RIF and INH.

Reference

298

High prevalence of SCCmec types V and VI among non-*S. epidermidis* coagulase-negative staphylococci species

Bouchami, Ons (1, 2); Miragaia, Maria (1); Oliveira, Duarte Carvalho (3); Ben Hassen, Assia (2); de Lencastre, Hermínia (1,4)

1: Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal;
2: Bone Marrow Transplant Center of Tunisia, Tunis, Tunisia;
3: Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Caparica, Portugal;
4: Laboratory of Microbiology, The Rockefeller University, New York, NY, USA

E-mail: onsbouchami@itqb.unl.pt

Keywords: CNS, methicillin resistance, SCCmec

Abstract

Methicillin-resistant coagulase-negative staphylococci (MRCNS) are the most important pathogens associated to intravenous catheter-related infections. Methicillin-resistance is mediated by the *mecA* gene, which is carried by the staphylococcal cassette chromosome *mec* (SCC*mec*). Mobility of SCC*mec* is promoted by site-specific recombinases (*ccr*). Up until now eight different SCC*mec* structures have been described in *Staphylococcus aureus*. Whereas SCC*mec* type IV is the most common in *Staphylococcus epidermidis*, little is known regarding the SCC*mec* structures carried by non-*S. epidermidis* CNS species. In this study, we aimed to assess the distribution of SCC*mec* structures among *non-S. epidermidis* MRCNS.

Ninety-two MRCNS isolates were collected from different clinical specimens at the bone marrow transplant centre in Tunisia, during 2002 and 2004, comprising *Staphylococcus haemolyticus* (45 isolates), *Staphylococcus hominis* (38), *Staphylococcus sciuri* (3), *Staphylococcus warneri* (2), *Staphylococcus xylosus* (2), *Staphylococcus cohnii* (1), and *Staphylococcus lugdunensis* (1). Species identification was confirmed by internal-transcribed spacer PCR (ITS-PCR). The presence of *mecA* and *ccr* genes was confirmed by PCR. SCC*mec* typing was performed by multiplex PCR, and by PCR amplification of the *ccr* and *mec* complexes. The genetic background of the isolates was characterized by pulsed-field gel electrophoresis (PFGE).

Among the 92 *mecA*-positive isolates, SCC*mec* types V (14%) and VI (7.6%) were the most prevalent, and were almost exclusively associated to *S. haemolyticus* and *S. hominis* species, respectively. SCC*mec* types II – IV were present in 5.4% of the isolates (2, 1, 2 isolates, respectively). Thirty-eight-isolates (41%) could not be typed. Interestingly, 25 out of 38 *S. hominis* isolates carried new combinations of *mec* complex class A and previously known *ccr* allotypes. Moreover, a high number of non-typeable SCC*mec* (31 out of 38), were found among *S. haemolyticus* species. The 45 *S. haemolyticus* isolates were classified into 11 PFGE types and 38 subtypes and the 38 *S. hominis* isolates were classified into 10 PFGE types and 36 subtypes, revealing high genetic diversity. No correlation was found between PFGE and SCC*mec* types, suggesting a high rate of SCC*mec* acquisition.

Our results suggest that *S. haemolyticus* and *S. hominis*, might be the origin and reservoirs of SCC*mec* type V and VI for other more virulent staphylococcal species, such as *S. aureus*.

Reference

302

Identification of non-tuberculous mycobacteria by molecular methods: comparison of three in-house protocols

Cerca, Pedro (1); Machado, Diana (1); Viveiros, Miguel (1,2); Amaral, Leonard (1,2,3); Couto, Isabel (1,4)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: COST ACTION BM0701 (ATENS);
3: UPMM; IHMT, UNL;
4: Centro de Recursos Microbiológicos (CREM), UNL

E-mail: pcerca@ihmt.unl.pt

Keywords: non-tuberculous mycobacteria (NTM), molecular identification methods, Internal Transcribed Spacer (ITS), hsp65, restriction enzyme analysis (REA)

Abstract

Although *Mycobacterium tuberculosis*, the etiologic agent of human tuberculosis is the main cause of mycobacteriosis in Man other, non-tuberculous mycobacteria (NTM), may also cause infection in humans, particularly in imunocompromised patients. NTM identification by conventional biochemical and culture methods is time consuming. The crucial role of the laboratory in establishing accurate diagnosis demands faster identification methods.
In this study we compared three molecular methods for the identification of NTM based on *restriction enzyme analysis* (REA) of: (i) the 16S-23S rRNA Internal Transcribed Spacer (ITS) region with *BsuRI*, *HhaI* and *TaqI*, (ii) the *hsp65* gene with *BstEII* and *BsuRI*, and (iii) the ITS and its flanking regions with *HhaI*. For this purpose, we evaluated a collection of 30 clinical NTM isolates, 2 NTM reference strains, corresponding to 11 different species, previously identified at our laboratory using the GenoType® Mycobacterium CM/AS. Among the three methods tested, the one based in the analysis of the ITS region provided the best results, identifying correctly 19 of the 32 isolates, belonging the NTM species most frequently isolated in our laboratory, namely *M. avium, M. gordonae*, *M. kansasii*, *M. chelonae, M. fortuitum, M. abscessus* and *M. xenopi*. However, it could not distinguish between *M. intracellulare* and *M. scrofulaceum*. The *hsp65*-REA also identified the most frequent NTM species, identifying correctly 18 of the 32 isolates tested. However, it showed a lower reproducibility, mainly related to incomplete *BsuRI* restriction. Finally, the method based on the ITS and flanking regions showed the lowest identification performance, with only 12 out of the 32 isolates correctly identified. Most of the unidentified isolates showed no amplification with the primers used in this last method.
In conclusion, from the three methods tested, the one based on the analysis of the ITS region provided the best results and showed higher reproducibility. However, the need to use several restriction enzymes to achieve a final identification delays delivery of results and increases the cost of these methodologies. Although several studies refer the usefulness of these in-house methods for the rapid and inexpensive identification of NTM, our experience shows that their application requires careful optimization and that their applicability depends largely on the NTM species diversity on each laboratory.

Reference

306

ATPase inhibitors as new efflux pump inhibitors of *Escherichia coli*

Costa, Susana (1); Martins, Ana (1,2); Spengler, Gabriella (1,2); Amaral, Leonard (1,2,3); Viveiros, Miguel (1,3)

1: Unidade de Micobactérias, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT, UNL), Portugal;
2: UPMM (IHMT, UNL);
3: COST ACTION BM0701 (ATENS).

E-mail: susanacosta@netcabo.pt

Keywords: Efflux pump inhibitors, AcrAB, ATPase, *Escherichia coli*.

Abstract

Antibiotic resistance in Gram-negative bacteria can be increased by extrusion of the antibiotic through efflux systems. In *Escherichia coli*, the major efflux system is the AcrAB efflux pump (EP) that extrudes many drugs with the assistance of other EPs, mainly driven by energy coming from the proton motive force (PMF).

In this study we have studied the role of other energy sources on the efflux activity of *E. coli* using three well characterized strains: (i) the parental wild-type *E. coli* K-12 AG100 which has intact and functional AcrAB; (ii) the *E. coli* K-12 AG100A whose genes that code for the AcrAB system have been deleted; (iii) the *E. coli* $AG100_{TET}$ that was adapted to increasing concentrations of tetracycline and over-expresses the AcrAB system and other EPs.

It was used a semi-automated fluorimetric method that detects the efflux of the fluorochrome ethidium bromide (EB), on a real time basis at 37ºC under different environmental conditions: (i) energy source, (ii) pH, (iii) presence and absence of known inhibitors of energy biosynthetic pathways at concentrations that do not affect the cellular viability. The compound 8-hydroxyquinoline, was used as inhibitor of the electron transport chain, sodium azide and sodium orthovanadate (Na_3VO_4) as inhibitors of ATPase and carbonyl cyanide m-chlorophenylhydrazone (CCCP), chlorhexidine and 2,4-dinitrophenol (DNP) as un-couplers of oxidative phosphorylation.

The results obtained show, for all strains, that glucose and pH affects the accumulation and efflux of EB. The retention of EB via inhibition of the efflux activity by AG100 was significantly increased in the presence of Na_3VO_4, DNP and CCCP. These inhibitory effects were reduced in presence of glucose or low pH. From all the inhibitors tested, Na_3VO_4 was the one that revealed the highest inhibitory activity which was reversed by the presence of glucose in the medium. Similar results were obtained for the AG100A revealing that these effects are not specific of the AcrAB system. With respect to the $AG100_{TET}$ significant increase of EB accumulation was observed when Na_3VO_4, DNP and CCCP were added to the assay. This effect was reduced in presence of glucose or low pH.

This study shows the dependence of the efflux activity of *E. coli* on energy from the hydrolysis of ATP by the ATPases, besides the already known dependence on the oxidative phosphorylation, to maintain the PMF of the cells. Na_3VO_4 has potential to be a new broad range efflux-pump inhibitor.

Reference

309

Clonal spread of serotype 6C pneumococci causing invasive disease in the Barcelona area, Spain (1997-2008)

Rolo, Dora (1,2); Fenoll, Asunción (3); Ardanuy, Carmen (1,2); Calatayud, Laura (1,2); Alegre, Montserrat (1,2); Cubero, Meritxell (1,2); Martín, Rogelio (1); Liñares, Josefina (1,2)

1: Hospital Universitari de Bellvitge, IDIBELL, Barcelona, Spain;
2: CIBERES, ISCIII, Madrid, Spain;
3: Centro Nacional de Microbiologia, ISCIII, Madrid, Spain

E-mail: dbispo@idibell.cat

Keywords: Streptococcus pneumoniae, 6C serotype, clones, invasive disease

Abstract

Streptococcus pneumoniae is an important human pathogen causing pneumonia and meningitis. After the introduction of the 7-valent conjugate pneumococcal vaccine (PCV7) in children, the rates of serotype 6A (Ser6A) increased in several countries. This fact was related to the emergence of a new serotype, serotype 6C (Ser6C). In Spain, PCV7 was introduced in 2001 and the current estimated uptake is nearly 50%. Among the invasive pneumococci received at the Spanish Pneumococcal Reference Laboratory from 10 Hospitals of the Barcelona area, the Ser6A rate was 2.6% in the pre-PCV7 period (1997-2000), 5.4% in the early-PCV7 period (2001-2004) and 3.3% in the late-PCV7 period (2005-2008).

The aims of this study were: 1) To analyze the proportion of Ser6C among invasive Ser6A pneumococci in the Barcelona area. 2) To analyze trends in Ser6C throughout 1997-2008 period.

During the study period 225 invasive Ser6A pneumococci were detected by Quellung reaction, of them 169 isolates available for this study. Ser6C was screened by PCR. Antibiotic susceptibility was studied by microdilution. Ser6C pneumococci were analyzed by Pulsed Field Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST).

Thirty (17.8%) of 169 Ser6A pneumococci were serotype 6C. The rate of Ser6C decreased from 14.3% (5/35) in the pre-PCV7 period to 13.0% (7/54) in the early-PCV7 period (p=1.000), and increased to 22.5% (18/80) in the late-PCV7 period (p=0.311). Ser6C pneumococci were mainly isolated from men (n=20). Fifteen Ser6C pneumococci were isolated from 15-64 years old patients, thirteen from ³ 65 years old adults and only two isolates were recovered from £ 15 years old children. The Ser6C pneumococci were isolated from blood (n=22), CSF (n=3), pleural fluid (n=3) and ascitic fluid (n=2). Although four genotypes were found by PFGE, one accounted for 80% of Ser6C pneumococci and was related to ST224 after MLST analysis. The distribution of this clone was as follows: 3 isolates in the pre-PCV7 period, 3 in the early-PCV7 period and 14 in the late-PCV7 period. Pneumococci belonging to this clone had Penicillin MICs of 0.12 mg/L whereas the remaining isolates were susceptible to all antibiotics tested.

The increase of Ser6A among invasive pneumococci recovered in the Barcelona area was associated to the spread of a clone (ST224) related to the new Ser6C. Further studies are needed in order to know the impact of PCV-13 vaccine on this new serotype.

Reference

324

Binary biofilm behaviour: sum of the synergistic and antagonistic interactions

Machado, Idalina Maia; Lopes, Susana Patrícia; Pereira, Maria Olívia

IBB-DEB, Portugal

E-mail: idalina@deb.uminho.pt

Keywords: binary biofilms, microbial interactions, bacterial resistance

Abstract

Biofilms can be defined as multicellular communities, embedded in a polymeric matrix, attached to a surface, where bacteria may show different phenotypic features that favour survival in hostile environments. Biofilms are colonizers of every surface being the leading cause of hospital infections and source of pathogenic bacteria. Several species can coexist in biofilms being its behaviour the sum of synergistic and antagonistic interactions, as they can produce metabolites that can interfere negatively or positively with growth and biofilm formation.In this work, the interactions between *S.aureus*, *S.sciuri*, *P.aeruginosa*, *P.fluorescens*, and *E.coli*, in single and binary biofilms, were examined. Biofilms were characterized by total mass, through crystal violet, metabolic activity, through XTT method, and number of cells.Single biofilms formed by *E. coli* and *S. sciuri* had the highest values of biomass. However, *S.sciuri* biofilms are less active holding the smallest number of cells. These results emphasised that each bacteria has an intrinsic sessile formation behaviour that leads to biofilms with different phenotypic features that might be on the basis of distinct answers to antimicrobials in a disinfection scenario. The biofilms resulting from the combination of *P. aeruginosa* with, respectively, *E.coli* and *S.aureus,* leads to binary biofilms with clearly more mass but less activity than the singles ones, being however the number of the binary biofilm-grown cells higher. The same behaviour was also observed for the binary biofilm formed by *P. fluorescens* and *S. sciuri*. It can be said that the growth of these species in a mixed biofilm clearly favours the sessile growth of each species, which may be disadvantageous in terms of sanitation. The number of cells does not seem to be proportional with metabolic activity, being the formation of binary biofilms favourable for some bacterial species and adverse for other. The reduced activity showed by binary biofilms can be explained by the fact that bacteria growing alone do not face cell-to-cell signalling stress. These data seem to indicate that the presence of *Pseudomonas* favoured the sessile growth of the other species, which may impair its eradication. In fact, the resulting binary biofilms seems to have cells in a latent state embedded in a matrix with more quantity of exopolymers that will shield bacteria from stress factors and make the access of antimicrobials more difficult.

Reference

339

Comparison of the prevalence and clonal structure of high-level gentamicin and glycopeptide-resistant *Enterococcus faecium* collected in two Lisbon hospitals during 2007-2008

Portela, Raquel (1); Miranda, Daniela (1); Ferreira, Teresa (2); Sancho, Luisa (3); Sardinha, Teresa (3); Mato, Rosario (1)

1: Laboratory of Molecular Genetics. Instituto de Tecnologia Química e Biológica. Universidade Nova de Lisboa (ITQB/UNL). Oeiras, Portugal;
2: Serviço de Patologia Clínica do Centro Hospitalar de Lisboa Centro, EPE. Lisboa, Portugal;
3: Serviço de Patologia Clínica, Hospital Fernando Fonseca. Amadora, Portugal

E-mail: rportela@itqb.unl.pt

Keywords: *Enterococcus faecium*, multi-resistant, clonal structure, Lisbon hospital

Abstract

Portugal is one of the European countries with rates of nosocomial vancomycin-resistant *Enterococcus faecium*-Efm >20%. Intra- and interhospital dissemination of particular strains was identified in Northern Portuguese hospitals.
The aim of this study was to evaluate and compare the prevalence and clonal structure of high-level gentamicin-HLGR and glycopeptide-GR resistant Efm isolates recovered from clinical samples during 2007-08 from two Lisbon hospitals: HospA, includes large neonatology and paediatric services, and HospB, has an important haematological ward, without paediatric ward.
The VITEK2 system and disk diffusion method were used for microbial identification and antimicrobial susceptibility testing. The *vanA*, *aac(6')-aph(2")* genes and *asaI*, *cylA*, *gelE*, *hyl* and *esp* virulence determinants were detected by PCR or Multiplex-PCR. PFGE with *Sma*I restriction was used for clonal assessment. GR isolates were tested by multilocus sequence typing (MLST).
During 2007-08 the prevalence of HLGR and GR was 27% and 10% in HospA (n= 116 isolates), and 45% and 15% in HospB (n= 85 isolates).
The 25 HLGR isolates of HospA were of 18 PFGE types and the 29 isolates of HospB were of 13 PFGE types. A majority of pulsed types (14 and 9 from each institution) were of single isolates. Only one PFGE was detected in both years in HospB and two PFGE types were common to isolates of both hospitals. The frequency of the *esp* and *hyl* genes was 55% and 61% in isolates of HospA and 74% and 39% in isolates of HospB.
The 6 HLGR/GR isolates of HospA were of 3 PFGE types and the 9 HLGR/GR isolates of HospB were of 6 PFGE types. Any of these patterns were detected in both years or found at both hospitals. Different PFGE patterns-virulent profiles associations were identified: **d**-*hyl*$^{+/-}$-*esp*$^{+/-}$, **g**, **af**, **s**-*hyl*, **x**-*hyl-esp*, **cc** *hyl-esp*; **k**-*asa-esp-gelE*, **i**-*esp*. Isolates belong to PFGE **af** and **g** were ST 280 while Efm PFGE **x**, **cc** were ST 17 and ST 18 respectively.
The 90% of the GR Efm (10 isolates) recovered in both years and institutions were PFGE **d**-*hyl* (except 2 strains)-ST125.
In conclusions each hospital has a specific population of HLGR and HLGR+GR Efm probably due to the particularity of each institution. A continuous replacement of clonal types was observed, except for GR clones which remain stable, overtime, in both institutions. The diversity of genetic lineages found, in contrast with a previous dominance of ST-17 and ST-18 lineages, suggest that extensive genetic events may have occurred.

Reference

359

Erysipelothrix rhusiopathiae in a stranded harbour porpoise (*Phocoena phocoena*) in Portugal

Carneiro, Carla (1); Oliveira, Manuela (1); Bexiga, Ricardo (1); Sequeira, Marina (2); Afonso, Fernando (1); Vilela, Cristina (1)

1: Faculdade de Medicina Veterinária - UTL, Portugal;
2: Instituto da Conservação da Natureza e da Biodiversidade, Portugal

E-mail: amcarlacarneiro@fmv.utl.pt

Keywords: *Eerysipelothrix rhusiopathiae*, harbour porpoise, isolation

Abstract

The natural equilibrium between the host and its environment may be challenged by microorganisms, even those considered harmless. This unbalance becomes patent when immunological barriers are threatened and normal microbiota may express and/or disseminate virulence factors. Environmental microorganisms may, under host stress circumstances, trigger zoonotic infections with impact upon public health. *Erysipelothrix rhusiopathiae* is an ubiquitous Gram-positive, rod shaped bacterium that may persist for long periods in the environment, including marine locations. It has been isolated from many species of wild and domestic mammals and birds, as well as reptiles, amphibians and fish. It is also considered an occupational pathogen, being the aetiological agent of erysipeloid, a skin disease in men. In animals, it may cause erysipelas in swine, birds and, more recently described, in free-ranging or captive fish-eating cetaceans.

Every year, several marine mammals strand along the Portuguese coast. On April 16, 2009, a stranded harbour porpoise (*Phocoena phocoena*) was submitted for post-mortem examination to investigate factors that might have contributed to its death. Necropsy included gross and histological examination. Routine bacteriology techniques were performed. Generalized congestion, small haemorrhages in the lungs and the presence of several macrophages in air spaces were observed. These macrophages were foamy and some contained eosinophillic granules in the cytoplasm. *E. rhusiopathiae* was isolated in pure culture aseptically collected samples from the lung, liver, spleen and kidney. Identification was based on the morphology and biochemical profile of isolates. According to the pathological findings, no typical lesions were associated with the evidence of *E. rhusiopathiae* in this animal, suggesting an acute septicaemic form.

Post-mortem examination may provide useful information for the health maintenance in captive marine animals as well as for the health of wild populations. The potential for transmission of *E. rhusiopathiae* to humans should not be underestimated. Little is known about the pathogenic potential of *E. rhusiopathiae* strains present in wild marine mammals, therefore special precautions should be taken when handling live or dead marine mammals. Moreover, information on *E. rhusiopathiae* animal infections may help inferring the level of exposure to this microorganism and the risk of disease transmission to contacting people.

Reference

374

PFGE typing methodology and proteomic analysis for assessing *Bacillus pumilus* diversity

Branquinho, Fabiana Raquel (1); Amorim, Maria Manuela (2); Freitas, Ana Raquel (1); Pintado, Maria Manuela (2); Peixe, Luísa (1)

1: REQUIMTE. Serviço de Microbiologia. Faculdade de Farmácia da Universidade do Porto. Porto. Portugal;
2: CBQF – Escola Superior de Biotecnologia – Universidade Católica. Porto. Portugal

E-mail: fraquelb@gmail.com

Keywords: *Bacillus pumilus*, Pulse Field Gel Electrophoresis (PFGE), Dodecyl Sulfate Polycrylamide Gel Electrophoresis (SDS-Page)

Abstract

The ability to discriminate *Bacillus pumilus* isolates using fast and reliable methods is relevant for epidemiology studies of infections thereof and also to assess the contamination origin of different products such as medicines and cosmetics. In this study we optimized a *Pulsed Field Gel Electrophoresis* (PFGE) typing methodology and compared its discriminatory ability with a proteomic analysis by *Dodecyl Sulfate Polycrylamide Gel Electrophoresis* (SDS-Page).

Twenty *B. pumilus* isolates were analyzed, recovered from 3 medicines samples (n=10), 3 cosmetic products (n=6), and 3 animal sources (n=3). *Bacillus pumilus* ATCC 14884 was also included in the study. Identification of bacterial isolates was performed by Gram stain, Mini API and sequencing of rDNA 16S. PFGE method [27h (3.5-15s)] following macrorestriction with *Apa* I was performed. Supernatants of *B. pumilus* cultures (grown overnight on TSB at 30 ºC) were resolved by SDS-Page following the procedure of *Schägger and Jagow* [(1)]. Dendrograms were produced from PFGE and SDS-Page band profiles using band-based cluster analysis with the Dice coefficient and unweighted-pair group method with arithmetic mean algorithm using InfoQuest™ FP version 5.4 software. All *B. pumilus* isolates, showed to be typeable by PFGE with *Apa* I restriction enzyme. According to the banding pattern analysis obtained by PFGE, the strains were classified into 14 pulsotypes. Dendogram analysis revealed a 59,6% level of similarity for all the isolates. Four clusters at similarity levels greater than 90% were observed, being grouped in one of them all the animal isolates (G_1, G_2 and G_3) and in two of them isolates M_{17} and M_{18}; M_8, M_{12} and M_{16}, recovered from a medicine sample for human use. Based in SDS-Page patterning, results revealed a 33,3% level of similarity for all the isolates. With a similarity level greater than ≥78%, three clusters comprising isolates G_1, G_2 and G_3; $ATCC_4$, M_7, M_9 and M_{13}; M_8, M_{12} and M_{16}, were observed. Remarkably, consistent strain discrimination was obtained with both methods, except in the isolates M_{17} and M_{18} which showed a high similarity profile in PFGE, and in the cluster comprising the isolates $ATCC_4$, M_7, M_9 and M_{13} that revealed a high similarity profile in SDS-Page.

Thus, PFGE with *Apa* I for DNA macrorestriction and SDS-Page were found to be highly discriminatory methods for *Bacillus pumilus* typing.

(1) Schägger, H. and G. von Jagow. 1987. Anal. Biochem. 166:368-379.

Reference

395

Arrival of *Klebsiella pneumoniae* producing KPC carbapenemase at Hospital Santa Maria, Lisboa

Machado, Paula (1); Silva, Alexandra (1); Lito, Luis (2); Melo-Cristino, José (3); Duarte, Aida (1)

1: Faculdade de Farmácia, Universidade de Lisboa;
2: Centro Hospitalar Lisboa Norte - Hospital de Santa Maria, Lisboa;
3: Faculdade de Medicina, Universidade de Lisboa

E-mail: aduarte@ff.ul.pt

Keywords: *Klebsiella pneumoniae*, Carbapenemase, KPC-3

Abstract

Klebsiella pneumoniae isolates harboring KPC enzymes have been identified in many geographical areas since 2001. KPC-type carbapenemases are increasingly prevalent in USA and Israel and are an emerging concern in South America, China and Europe. Numerous problems exist in the detection and treatment of patients with such isolates.

The aim of this study was to characterize the extended-spectrum beta-lactamases (ESBLs) in *K. pneumoniae* 2354FF isolate showing high-level imipenem and meropenem resistance. MICs were determined by Etest and isolates were screened for ESBLs, metallo-beta-lactamases (MBLs) and class A carbapenemase-producing phenotypes. The presence of bla_{SHV} and $bla_{TEM}bla_{KPC}$ genes and Tn4401-like elements were screened by specific PCRs and DNA sequence analysis. M13 fingerprinting typing method was used to compare the isolate with other *K. pneumoniae* isolates found in this hospital with resistance to carbapenems. The MIC for imipenem was >32 mg/L, unchanged by EDTA. The beta-lactamase activity against imipenem and meropenem was inhibited in the presence of clavulanic acid. The strain was also resistant to extended-spectrum cephalosporins, aztreonam and gentamicin (MICs >/= 32 mg/L). *K. pneumoniae* 2354FF produced KPC-3 carbapenemase, with the bla_{KPC-3} gene located within a Tn4401-like element, which is part of a conjugative plasmid. In several bla_{KPC}-produing isolates the transposon consists of a transposase, a resolvase, and two putative insertion sequences (IS) elements, IS*Kpn6* and IS*Kpn7* located downstream and upstream of bla_{KPC} gene, respectively. In pKP2354 the IS*Kpn7* element is not present. M13 fingerprinting analysis revealed that this genotype had not been detected in the hospital previously. This is the first description of the identification of carbapenem-resistant *K. pneumoniae* isolate harboring KPC beta-lactamases at Hospital de Santa Maria, Lisboa. This beta-lactamase is likely located on a transposon that is part of a conjugative plasmid and thus has a very high potential for dissemination. Our findings should alert medical authorities to implement stringent methods for the detection and spread control of emerging KPC carbapenemases in Portugal.

Reference

401

CTX-M-15: Clonal dissemination and horizontal spread among *Klebsiella pneumoniae* and *Escherichia coli* isolates from bacteremia during 7 years at Hospital de Santa Maria

Caneiras, Catia (1); Lito, Luis (2); Melo Cristino, Jose (3); Duarte, Aida (1)

1: Faculdade de Farmácia da Universidade de Lisboa;
2: Centro Hospitalar Lisboa Norte, Hospital de Santa Maria;
3: Faculdade de Medicina da Universidade de Lisboa, Instituto De Medicina Molecular

E-mail: aduarte@ff.ul.pt

Keywords: CTX-M-15, Bacteremia, *Klebsiella pneumoniae*, *Escherichia coli*

Abstract

The isolation of *Escherichia coli* and *Klebsiella* spp multidrug-producing Extended Spectrum b-Lactamase (ESBL) in severe invasive infections such as bacteraemia, is a major therapeutic challenge. To increase knowledge of these microorganisms, it was made the characterization of the genetic determinants of resistance and virulence of isolates obtained during 7 years (2000-2007) at Hospital de Santa Maria. Susceptibilities to antimicrobial agents were determined and the genetic context of $bla_{\text{CTX-M-type}}$gene was examined by PCR. The isolates were compared by M13 fingerprinting. Plasmid replicons were determined using the PCR-based replicon typing scheme described by Carattoli et al (2005) with specific primers for 18 plasmid replicons. Phylogenetic group and virulence genes were detected by PCR with specific primers. Sequencing confirmed the isolates resistant to cefotaxime, ceftazidime and ciprofloxacin produced the $bla_{\text{CTX-M-15}}$ gene downstream of IS*Ecp1.* The *Klebsiella* spp. has demonstrated a great genetic variability, in contrast with the existence of a predominant clone in the isolates of *E. coli*, which persists in different wards at hospital during 5 years. These isolates CTX-M15 producers belonged mostly to phylogenetic group B2, and also in group D, A and B1.Among the *E.coli* isolates the most frequent plasmid incompatibility group was Inc F. Other combinations involving F replicon were found, namely A/C, N and Y plasmid replicons. In *K. pneumoniae* isolates were found only the IncHI1 plasmid incompatibility group. A superior ability of horizontal spread of *E. coli* was demonstrated when compared with *Klebsiella* spp. (2.04 replicons/isolate Vs 0.59 replicons/isolate). The *E.coli* persistent clone showed the pathogenicity islands IV536, ICFT073 and IICFT073 and the virulence patterns mostly found were *fimH, ecpA, iucC* of the nine virulence factors investigated. So far the EcpA pilus only described in entero-haemorrhagic *E.coli* O157:H7 appears in this work always associated with the fimbriae FimH. The combined action between these virulence factors seems allow the adhesion and invasion in the bladder epithelium or in the enterocyte acting as a primary cause of bacteremia. Despite the critical role of plasmids in horizontal gene transfer, this study has suggested that the $bla_{\text{CTX-M-15}}$ genes cannot be readily transmitted from *E. coli* to *K. pneumoniae*.

Oral Session:
S4 – Environmental Microbiology and Biotechnology

Reference

358

Anaerobic LCFA degradation: the role of syntrophic and non-syntrophic bacteria

Sousa, Diana Z. (1); Cavaleiro, Ana J. (1); Pereira, M. Alcina (1); Alves, Joana I. (1); Smidt, Hauke (2); Stams, Alfons J. M. (2); Alves, M. Madalena (1)

1: IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, University of Minho, Braga, Portugal;
2: Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands

E-mail: dianasousa@deb.uminho.pt

Keywords: LCFA, biogas, syntrophy

Abstract

Syntrophic relationships are the key for biodegradation in methanogenic bioreactors. We review our work on the ecological and physiological features of syntrophic communities involved in the degradation of saturated and unsaturated long-chain fatty acids (LCFA), as well as their potential application to convert lipids/fats-containing waste to biogas. DGGE fingerprinting and sequencing showed the importance of *Syntrophomonas* closely related bacteria during batch and continuous degradation of unsaturated and saturated LCFA[1,2]. An obligatory syntrophic LCFA degrader – *Syntrophomonas zehnderi* – was isolated (in co-culture with *Methanobacterium formicicum*) from an oleate enrichment culture[3]. Oleate is an unsaturated LCFA with 18 carbon atoms and is one of the most common LCFA in wastewaters. The capability of degrading unsaturated LCFA is not widespread within *Syntrophomonas* genus. From the 11 *Syntrophomonas* species or subspecies that can use fatty acids only 3 are able to use oleate[4]. Enrichment cultures on oleate (unsaturated LCFA) and palmitate (saturated LCFA) resulted in distinct bacterial communities, which could be correlated with differences in the chain saturation[4]. Communities enriched on oleate could also degrade palmitate, but the opposite was not the case. The principle pathway of LCFA degradation is through ß-oxidation, but the initial steps in the conversion of unsaturated LCFA are unclear. We hypothesize that LCFA chain saturation might be a non-syntrophic process, i.e. unsaturated LCFA can function as electron donors and acceptors, as protons released in a first ß-oxidation step could be used to hydrogenate the unsaturated hydrocarbon chain. To test this, oleate was continuously fed to 2 bioreactors containing methanogenic-active and methanogenic-inhibited anaerobic sludge. Palmitate accumulated in both methanogenic-active and methanogenic-inhibited bioreactors up to a concentration of 2 mM and 8 mM, respectively. These results suggest the occurrence of a non-syntrophic step during the degradation of unsaturated LCFA in anaerobic bioreactors. Genome sequence of S. *zehnderi* (currently ongoing at DOE-JGI) will possibly allow to get more insights into the biochemical pathways of unsaturated LCFA degradation.

Acknowledgment: Financial support from Portuguese Science Foundation (FCT) and European Social Fund (ESF) through the PhD grants SFRH/BD/8726/2002 and SFRH/BD/24256/2005.

1 Sousa DZ, Pereira MA, Smidt H, Stams AJM & Alves MM (2007) FEMS Microbiol Ecol 60: 252-5
2 Sousa DZ, Pereira MA, Stams AJM, Alves MM & Smidt H (2007) Appl Environ Microbiol 73: 1054-64
3 Sousa DZ, Smidt H, Alves MM & Stams AJM (2007) Int J Syst Evol Microbiol 57: 609-15
4 Sousa DZ, Alves JI, Alves MM, Smidt H & Stams AJM (2009) Environ Microbiol 11: 68-80

Reference

254

Adaptive responses to phenol induced stress at the level of *Pseudomonas putida* KT2440 membrane proteome

Roma-Rodrigues, Catarina (1); Santos, Pedro M. (1); Benndorf, Dirk (2); Rapp, Erdmann (2); Sá-Correia, Isabel (1)

1: IBB – Institute for Biotechnology and Bioengineering/Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais 1049-001 Lisboa, Portugal;
2: Bioprocess Engineering, Otto von Guericke University, Universitätsplatz 2, 39106 Magdeburg, Germany

E-mail: catromar@ist.utl.pt

Keywords: stress response, phenol, membrane proteome, Pseudomonas

Abstract

In order to gain insights into the global mechanisms underlying phenol toxicity and tolerance in *Pseudomonas*, our research group has explored an expression proteomics approach, but such experimental strategy was focused on soluble proteins [1, 2]. In the present study we examined the effects of phenol induced stress on *Pseudomonas putida* KT2440 membrane proteome. When these bacterial cells are exposed to 800 mg/L of phenol under the experimental conditions tested, a growth arrest, of about 4 - 5 hours, is observed [1]. Cell samples used in proteomic analysis were collected during the period of growth arrest (1h after phenol addition) and when growth was resumed (after 5h of phenol addition) and the total membrane fraction was separated from the bulk extract by ultracentrifugation. The total membrane fraction was subsequently separated in the inner membrane and the outer membrane fractions, using a sucrose gradient ultracentrifugation. Proteins from membrane samples were separated by two-dimensional gel electrophoresis (2-DE) and identified by mass spectrometry. Expression proteomic analysis revealed that the adaptive response of the membrane proteome of *P. putida* KT2440 to phenol results in an increased abundance of proteins involved in peptidoglycan biosynthesis and in electron transport, while the abundance of ferric siderophore receptors and of the porins OprB, OprG and OprQ was decreased. Results also suggest that phenol export may be mediated by the solvent efflux pumps TtgABC and Ttg2A2B2C, whose content of the periplasmic (TtgA and Ttg2A) and outer membrane (TtgC and Ttg2C) components reached a 35-fold increase. The identification of the targets of phenol toxicity and of the mechanisms that allow *P. putida* KT2440 to thrive under phenol stress will allow more rationale strategies for the development of bacteria with higher solvent tolerance for applications in bioremediation and whole-cell biotransformations in media with organic solvents.

[1] Santos PM, Benndorf D, Sá-Correia I. (2004) Proteomics 4:2640-2652.
[2] Santos PM, Roma V, Benndorf D, Von Bergen M, Harms H, Sá-Correia I. (2007) OMICS: a journal of integrative biology 11: 233-251

Reference

329

Resistance to herbicides and other chemical stresses of agricultural interest: role of plant Major Facilitator Superfamily transporters

Remy, Estelle (1); Lacerda, Rafaela (1); Cabrito, Tânia (2); Teixeira, Miguel (2); Sá-Correia, Isabel (2); Duque, Paula (1)

1: Instituto Gulbenkian de Ciência, Portugal;
2: Instituto Superior Técnico, Portugal

E-mail: eremy@igc.gulbenkian.pt

Keywords: xenobiotics, resistance, MFS transporters, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*

Abstract

In recent decades, the extensive use of herbicides to provide crop protection has led to the emergence of multiple resistant weeds worldwide, thus challenging agricultural practices. It is therefore of crucial importance to understand the molecular basis of herbicide resistance, and more globally of Multiple Drug Resistance (MDR), the simultaneous acquisition of resistance to a wide range of unrelated cytotoxic compounds (Sá-Correia *et al*., 2009). *Saccharomyces cerevisiae* has been intensively used to unravel the molecular mechanisms underlying MDR in more complex organisms. In particular, yeast has been used as a model to elucidate the detoxification processes leading to tolerance to one of the most widely used herbicides worldwide, the 2,4-dichlorophenoxyacetic acid (2,4-D) (Teixeira et al., 2007). One yeast plasma membrane MDR transporter belonging to the Major Facilitator Family (MFS), Tpo1p, is a determinant of 2,4-D resistance and is postulated to be involved in its active direct extrusion from the cell (Teixeira *et al*., 2002). Based on the role played by Tpo1p in yeast adaptation to 2,4-D, and in view of the substantial conservation of molecular pathways among eukaryotic organisms, we have developed a strategy combining a candidate approach in *Arabidopsis thaliana*, the key model organism in plant biology, with a heterologous expression approach in *S. cerevisiae,* with the main goal of evaluating the role played by plant MFS transporters in 2,4-D resistance in particular and MDR in general. In a preliminary screen, nine *A. thaliana* genes encoding MFS transporters exhibiting homology with yeast *TPO1* have been selected. Three of these show significantly increased expression upon exposure to 2,4-D, rendering them excellent candidates for functional analysis both in yeast and *A. thaliana*. One of these plant MFS transporters, At5g13750, has already been found to confer increased resistance to 2,4-D in yeast, in addition to other xenobiotic compounds, such as IAA (indole-3-acetic acid), Tl^{3+} and Al^{3+} (Cabrito *et al*., 2009). Phenotypical analyses of corresponding mutants in *A. thaliana* strongly suggest that At5g13750 also confers resistance to 2,4-D and IAA in plants. In addition, At5g13750 confers sensitivity to Pb^{2+} and Co^{2+}. These results confirm the effectiveness of the strategy employed and support our working hypothesis that plant MFS transporters play a role in 2,4-D resistance and MDR in general.

Reference

209

Molecular analysis of phosphorus removing-related populations in membrane bioreactors

Silva, Ana Filipa (1); Carvalho, Gilda (3); Reis, Maria Ascenção (2); Crespo, Maria Teresa (1)

1: IBET/ITQB, UNL, 2781-901 Oeiras Portugal;
2: REQUIMTE/CQFB, Chemistry Department, FCT, UNL, 2829-516 Caparica;
3: IBET/ITQB, UNL, 2781-901 Oeiras, Portugal and REQUIMTE/CQFB, Chemistry Department, FCT, UNL, 2829-516 Caparica

E-mail: filipasilva@itqb.unl.pt

Keywords: membrane bioreactors, activated sludge, PAO, EPS, FISH

Abstract

Membrane bioreactors (MBR) for wastewater treatment have numerous advantages in comparison to activated sludge systems, such as their small footprint and high quality effluent. The introduction of membranes in activated sludge systems likely affects the bacterial community profile, in view of the higher sludge retention time and sludge concentration normally operated in these systems; the lower oxygen transfer rate due to higher biomass concentration; the selectivity of the membrane as a physical barrier able to retain macromolecules in the membrane tank.

The bacteria responsible for biological nutrient removal are some of the microorganisms whose presence is crucial for municipal wastewater treatment. It is generally acknowledged that the long sludge ages normally operated in MBRs support slow growing organisms such as nitrifiers. Nevertheless, the presence of polyphosphate accumulating organisms (PAOs) in MBRs has seldom been investigated, despite the proved effectiveness of MBRs for enhanced biological phosphorus removal (EBPR).

In this work, the microbial community structure of several pilot- and full-scale MBRs was evaluated concerning the phosphorus removing-related populations. Fluorescence *in situ* hybridisation (FISH) analysis was carried out on samples collected at the membrane tank of four full-scale and four pilot MBR plants. Most of the analysed plants contained an anoxic and an aerobic zone. Only two plants contained also an anaerobic compartment for phosphorus removal.

FISH quantification analysis showed a relatively high abundance of PAOs in all plants, with levels at times even higher than those observed in the two analysed MBR plants that contained a dedicated anaerobic compartment. These results suggest that MBRs, along with the other known advantages, can also support the growth of phosphorus removing bacteria even without an anaerobic compartment.

The fact that PAOs tend to grow in MBRs even when these were not designed for EBPR could be related to the high floc compactness and density of external polymeric substances (EPS), which were observed in most examined samples. This is possibly related to the filtration of EPS by the membrane and high sludge compactation in the cake layer, which added to the higher biomass concentration might enhance the creation of anaerobic zones within the flocs.

-

Reference

330

Persistence of inoculated ectomycorrhizal fungi and succession dynamics in field transplanted *Quercus rubra* seedlings

Sousa, Nadine R.; Franco, Albina R.; Oliveira, Rui S.; Castro, Paula M.L.

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

E-mail: nrsousa@esb.ucp.pt

Keywords: Ectomycorrhiza, ectomycorrhizal fungal communities, field trial, plant growth performance, Quercus rubra

Abstract

Previous studies have shown that ectomycorrhizal (ECM) fungi have a positive effect on the establishment of nursery grown seedlings and may influence their survival on the first years. ECM fungi often mitigate stress factors by increasing seedling capacity to capture nutrients therefore contributing to successful plant establishment. However, due to the high specificity of ECM fungi, an increased effort is needed to fully understand their role . We investigated tree establishment performance of pre-inoculated *Quercus rubra* seedlings on a reforestation site, monitoring ECM fungal persistence and succession dynamics between selected ECM fungi and native fungal community. Nursery grown *Q. rubra* seedlings inoculated with a mixture of ECM fungi (*Cenococcum geophilum*, *Hebeloma crustuliniforme*, *H. mesophaeum*, *H. velutipes*, *Paxillus involutus*, *Scleroderma citrinum*), were transplanted to Serra da Cabreira, Northern Portugal. Non-inoculated control oak seedlings were also transplanted. Two years after planting, the presence of the inoculated ECM fungi was assessed using ITS-DGGE. Results showed that ECM fungi enhanced the establishment and improved the growth performance of out planted oak seedlings. *C. geophilum* was significantly associated with inoculated saplings but other ECM fungi species were also found, suggesting that ECM fungal community and plant establishment performance could be related. Ordination analysis demonstrated that ECM fungal communities of inoculated and non-inoculated samples are significantly different. Inoculation with selected ECM fungi at nursery stage improved field growth performance of *Q. rubra* seedlings. Further monitoring is needed to increase knowledge on the persistence of ECM fungal communities and their succession dynamics. The present work reinforces the role of ECM fungi as a successful biotechnological tool aiding reforestation projects.

Reference

345

Diversity, virulence and biocontrol of *Xanthomonas campestris* affecting *Brassicaceae* plants in Portugal

Cruz, Joana (1); Eloy, Madalena (2); Pedro, Luis (3); Tenreiro, Rogério (1); Cruz, Leonor (1,2)

1: Universidade de Lisboa, Faculdade de Ciências, Center for Biodiversity Integrative and Functional Genomics (BioFIG), Ed. ICAT, Campus da FCUL, Campo Grande, 1749-016 Lisboa, Portugal;
2: Instituto Nacional de Recursos Biológicos, Unidade de Investigação de Protecção de Plantas, Tapada da Ajuda 1349-018, Lisboa; Portugal.;
3: Universidade de Lisboa, Faculdade de Ciências, Instituto de Biotecnologia e Bioengenharia, Centro de Biotecnologia Vegetal, C2, Campo Grande, 1749-016 Lisboa, Portugal.

E-mail: joana.ccruz@gmail.com

Keywords: Brassica sp., black rot, genomic fingerprinting, pathogenicity, bioactive compounds

Abstract

Xanthomonas sp. belongs to the Y subdivision of Proteobacteria and includes 27 species of plant pathogens some of which are important quarantine organisms like *X. fragariae* and several pathovars of *X. axonopodis*, as well as some non regulated species like *X. campestris* (*Xc*). *Xc* pv. *campestris* is considered the causative agent of black rot disease of Portuguese cabbage and other cultivated and spontaneous Brassicaceae plants, causing significant losses. Genomic characterization of *Xc* strains obtained from these hosts in Portugal has not yet been addressed and the control of black rot of crucifers is still hindered, since the use of antibiotics is not allowed and copper compounds are inefficient. In this study a set of 121 *Xanthomonas* spp. strains obtained in Portugal and from international reference collections previously identified by biochemical and pathogenicity tests were characterized using BOX-, ERIC-, and MSP-PCR genomic fingerprinting techniques, and their genomic profiles analysed using hierarquical numerical analysis. Further differential race assessment was performed by inoculating all 34 isolates of *Xc* onto six different cultivars and lines of *Brassica* sp. plantlets under environmental controlled conditions. The use of essential oils obtained from aromatic/medicinal plants known for their antimicrobial activity was tested on the control of *Xc*. Essential oils obtained by hidrodistillation from *Origanum vulgare*, *Syzygium aromaticum*, *Mentha pulegium* and *Thymus mastichina* were tested *in vitro* using the agar plate diffusion method against eight selected strains. The genomic fingerprinting profiles obtained permitted to typify and to allocate different xanthomonads into distinct clusters. In the composite dendrogram 32 isolates of *Xc* obtained from Brassicaceae plants with black rot symptoms were included in a unique megaclutser with similarity levels higher than 38%. The only exceptions were from two isolates obtained from *Rorippa nasturtium-aquaticum* and from *Ranunculus* sp. showing atypical profiles. Race determination displayed unexpected virulence patterns revealing that *Xc* pv. *campestris* is not the only causative agent of black rot of crucifers in Portugal. The antibacterial activities of oregano and clove essential oils were respectively 2,4 and 1,8 times higher than streptomycin, suggesting their potential as sources of bioactive compounds alternative to traditional black rot control methods.

Reference

409

Hymenobacter flocculans sp. nov., and *Hymenobacter puteal* sp. nov. two new species of the genus *Hymenobacter* isolated from an uranium mine

Chung, Ana Paula (1); Lopes, André (1); Nobre, M. Fernanda (2,3); da Costa, Milton S. (2,4); Morais, Paula Vasconcelos (1,4)

1: IMAR-CMA, University of Coimbra, 3004-517 Coimbra, Portugal;
2: Center for Neuroscience, University of Coimbra, 3004-517 Coimbra, Portugal;
3: Department of Zoology, University of Coimbra, 3004-517 Coimbra, Portugal;
4: Department of Biochemistry, University of Coimbra, 3001-401 Coimbra, Portugal

E-mail: pvmorais@ci.uc.pt

Keywords: Hymenobacter, uranium mine

Abstract

Two red-pink pigmented strains designated A2-50A^T and A2-91^T, with optimum growth temperature of about 25°C and an optimum pH for growth between 6.0 and 6.5, were recovered from Poço das Cobras, one of the steps of the water treatment plant in Urgeiriça mine (Central, Portugal). Phylogenetic analysis of the 16S rRNA gene sequences showed that these organisms represented two distinct branches within the family *Flexibacteraceae* most closely related to the members of genus *Hymenobacter*. The two strains shared about 95.6% of similarity in the 16S rRNA gene sequence and about 88.2 to 92.3% with other members of genus *Hymenobacter*. The two new isolates could be distinguished from each other by differences in growth temperature range, differences in carbon source assimilations and fatty acid composition. The relative amounts of the fatty acids identified as summed feature 4 (17:1 anteiso B and/or iso I) was higher in strain A2-91A^T, and the unsaturated fatty acid 16:1 ω5c was present in lower proportions when compared to strain A2-50A^T. On the basis of the phylogenetic analysis and physiological and biochemical characteristics we propose the name *Hymenobacter flocculans* for the new species represented by strain A2-50A^T and *Hymenobacter puteal*for the other species represented by strain A2-91^T.

Poster Session:
S4 – Environmental Microbiology and Biotechnology

Reference

108

Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups

Poeta, Patrícia Alexandra (1); Radhouani, Hajer (1); Pinto, Luís (1); Gonçalves, Alexandre (1); Rodrigues, Jorge (1); Estepa, Vanessa (2); Torres, Carmen (2); Igrejas, Gilberto (1)

1: University of Trás-os-Montes and Alto Douro, Portugal;
2: University of La Rioja, Logroño, Spain

E-mail: ppoeta@utad.pt

Keywords: Antibiotic resistance, wild boars, ESBL-producing *E. coli*, β-lactamases, phylogenetic groups.

Abstract

Background: β-lactams are among the most clinically important antimicrobial agents in both human and veterinary medicine. However, resistance to these antibiotics has been increasingly observed in bacteria, including those of animal origin. Antibiotic resistance of ESBL-positive faecal *Escherichia coli* isolates of wild animals has been studied by our research group in Portugal [5,6] allowing the scientific community for a better understanding of the problem of antibiotic resistance in the wildlife and the consequences of this aspect in different ecosystems.

Objective: To detect ESBL-containing *E. coli* isolatesin faecal samples of wild boars in Portugal, to characterize the phylogenetic groups of these isolates and the type of ESBL encoding genes they harbor.

Methods and results : ESBL-producing *E. coli* isolates have been isolated from eight of seventy seven faecal samples (10.4%) of wild boars in Portugal. The ESBL types identified by PCR and sequencing were $bla_{\text{CTX-M-1}}$ (6 isolates) and $bla_{\text{CTX-M-1}}$ + $bla_{\text{TEM1-b}}$ (2 isolates). Further resistance genes detected included *tet* (A) or *tet* (B) (in three tetracycline-resistant isolates), *aad*A (in three streptomycin-resistant isolates), *cml*A (in one chloramphenicol-resistant isolate), *sul*1 and/or *sul*2 and/or *sul*3 (in all sulfonamide-resistant isolates). The *intI*1 gene encoding class 1 integrase was detected in all ESBL-producing *E. coli* isolates. One isolate also carried the *intI*2 gene, encoding class 2 integrase. The ESBL-producing *E. coli* isolates could be assigned to phylogenetic groups B1 (3 isolates), B2 (3 isolates) or A (2 isolates). Amino acid change in GyrA protein (Ser83Leu or Asp87Tyr) was detected in three nalidixic acid-resistant and ciprofloxacin-susceptible isolates. Two amino acid changes in GyrA (Ser83Leu + Asp87Asn) and one in ParC (Ser80Ile) were identified in two nalidixic acid- and ciprofloxacin-resistant isolates.

Conclusion: As evidenced by this study wild boars could be a reservoir of antimicrobial resistance genes.

Bush, K. 2008. 1987-2006. Clin. Microbiol. Infect. 14 (Suppl 1), 134-43.
Coque, T. M., Baquero, F., Canton, R. 2008. Euro. Surveill. 20, 13(47).
Paterson, D. L., Bonomo, R. A. 2005. Clin. Microbiol. Rev. 18, 657-686.
Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Rojo-Bezares, B., Jouini, A., Zarazaga, M., Rodríguez, J., Torres, C. 2008. J. Antimicrob. Chemother. 59, 1311-1312.
Poeta, P., Radhouani, H., Igrejas, G., Gonçalves, A., Carvalho, C., Rodrigues, J., Vinué, L., Somalo, S., Torres, C., 2008. Appl. Environ. Microbiol. 23, 439–7441.

Reference

113

Exploiting *S. cerevisiae*- and *C. elegans*-based eukaryotic screening bioassays to assess the potential toxicity of xenobiotics

Gil, Fátima N (1); Costa, Catarina P (1); Ramos, Christian G (1); Leitão, Jorge H (1); Martins, Lígia O (3); Becker, Jorg D (2); Viegas, Cristina A (1)

1: IBB - Institute for Biotechnology and Bioengineering, CEBQ, Instituto Superior Técnico, Lisboa, Portugal;
2: Gene Expression Unit, Instituto Gulbenkian de Ciência, Oeiras, Portugal;
3: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal

E-mail: fatima.gil@ist.utl.pt

Keywords: Toxicity bioassays; eukaryotic models; environmental biomonitoring; transcriptomics.

Abstract

Short-term bioassays, using simple eukaryotic models, are required to rapidly assess and warn potential toxic effects of xenobiotics in environmental samples. In addition, they can be used for the preliminary screening of chemicals toxicity, eventually followed by more complex and expensive testing. To contribute to the development of cost-effective and non-animal alternative toxicity screening methods, we compared two small-scale bioassays to assess the toxicity of pesticides and of synthetic dyes. One is based on their inhibitory effects on the growth curve of the model yeast *S. cerevisiae,* using microtiter plate susceptibility assays [1, 2]. The other measures their effects on the reproduction of age-synchronized worms of *C. elegans*, a free-living soil invertebrate that has recognized relevance in soil and aquatic ecotoxicology. Data on the relative toxicities determined for pesticides from different chemical families and for diverse azo- and anthraquinonic dyes will be presented, and their correlation with ecotoxicity data reported in the literature will be discussed.

With the aim to identify molecular biomarkers that may be useful for environmental biomonitoring of herbicide toxicity using the yeast model, whole-genome DNA microarrays are being used to examine the global expression profiles occurring in yeast cells in response to sub-lethal levels of herbicides. Cells exposure to a 20%-inhibitory concentration of alachlor led to 97 genes/ORFs with statistically significant higher levels of transcripts than the control cells (FC > 1.5), while 34 were down-regulated (FC < -1.5). A total of 15 genes whose expression was modified between ±2.5- and 46-fold and are within functional categories statistically over-represented in our data-sets when compared to the yeast genome, were selected as possible candidates as biomarkers of alachlor exposure. Determination of the dose-response and exposure time-dependency of the expression of these particular genes is under way, in order to get a clearer picture of their possible relevance to diagnose potentially adverse effects of the herbicide in environmental samples.

Acknowledgments: to FEDER and FCT (contracts PTDC/AMB/64230/2006 and PTDC/BIO/72108/2006)

[1] Papaefthimiou, C., Cabral M.G., Mixailidou, C., Viegas, C.A., Sá-Correia, I., Theophilidis, G., 2004. Environ. Toxicol. Chem., 23: 1211-1218;
[2] Pereira, L, Coelho, A.V., Viegas, C.A., dos Santos, M.M.C., Robalo, M.P., Martins, L.O., 2009, J Biotechnol., 139: 68-77.

Reference

118

An integrated approach involving chemical and ecotoxicological evaluation of soils spiked with herbicidal commercial formulations containing atrazine, before and after the implementation of a bioremediation process

Costa, Catarina P (1); Chelinho, Sónia (2); Moreira-Santos, Matilde (2); Viana, Paula (3); André, Sandra (3); Lima, Débora B (1); Ribeiro, Rui (2); Fialho, Arsénio M (1); Sousa, J Paulo (2); Viegas, Cristina A (1)

1: IBB-Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Lisboa;
2: IMAR – Instituto do Mar - CIC, Departamento de Zoologia, Universidade de Coimbra, Coimbra;
3: APA-Agência Portuguesa do Ambiente, Amadora

E-mail: cristina.viegas@ist.utl.pt

Keywords: atrazine biodegradation, Pseudomonas sp. ADP, ecotoxicological assessment, bioremediation efficacy

Abstract

s-Triazine herbicides are used worldwide and relatively persistent in soil. Atrazine and its dealkylated metabolites are among the most frequently detected pesticides on water compartments due to run-off and leaching events. Concerns regarding their impact on human and ecosystems health have promoted search for efficient bioremediation strategies for atrazine polluted environments. *Pseudomonas* sp. ADP is the best characterized atrazine-mineralizing bacteria. A previous study, based on mineralization assays carried out in small closed microcosms spiked with ^{14}C-labelled atrazine, showed that bioaugmentation with *Pseudomonas* sp. ADP combined with soil amendment with organic acids could be a promising approach to cleanup soil polluted with high herbicide concentrations [1]. We examined this potential bioremediation strategy in larger open soil microscoms for optimization under more realistic conditions. A crop soil from Central Portugal was spiked with herbicidal commercial formulations containing atrazine as single active ingredient [2] or mixed with s-metolachlor. The doses tested were 10-, 20- or 200-fold higher than the recommended dose for agricultural application, mimicking over-use and spill scenarios. We provide evidences, based solely on chemical analysis of soil samples, that one bacterial inoculation or several successive inoculations plus biostimulation, depending on the initial level of soil contamination with the herbicidal commercial formulations, allow rapid (in up to 1 wk) removal of atrazine from soil (> 98% of initial) [2]. To shed light on the possible ecological impact due to application of the bioremediation treatments in atrazine-contaminated land, soil samples and the respective leachates and eluates obtained from bioremediated and non-bioremediated soil were also tested for toxicity over standard soil and aquatic test species. Data is presented integrating atrazine biodegradation in soil microcosms as well as the efficacy of soil cleanup based on the ecotoxicological evaluation. Results to be presented stress the importance of monitoring ecotoxicity before and after the implementation of a bioremediation approach to obtain a more realistic glimpse of its potential ecological impact.

Acknowledgments: to FEDER and FCT, Portugal (contracts PPCDT/AMB/56039/2004 and PTDC/AMB/64230/2006 and grants to SC and MMS)

[1] Silva E, Fialho AM, Sá-Correia I, Burns RG & Shaw LJ., Environm. Sci. Technol. (2004), 38: 632-637
[2] Lima D, Viana P, André S, Chelinho S, Costa C, Ribeiro R, Sousa JP, Fialho AM & Viegas CA, Chemosphere (2009) 74:187-192.

Reference

123

Genotyping of a large number of Azorean rodent-field *Leptospira* isolates using a polymerase chain reaction-based assay

Nunes, Mónica (1,2); Gonçalves, Ana Teresa (1); Carreira, Teresa (1,2); Vieira, Maria Luísa (1,2); Collares-Pereira, Margarida (1,2,3)

1: Unidade de Leptospirose e Borreliose de Lyme, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa;
2: Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa;
3: Unidade de Genética e Patologia Moleculares, Hospital do Divino Espírito Santo de Ponta Delgada, EPE

E-mail: monicanunes@ihmt.unl.pt

Keywords: Leptospirosis, Azores, rodent isolates, genotyping

Abstract

Leptospirosis, a re-emergent infectious disease caused by pathogenic *Leptospira*, is a relevant public health problem in the Azores, showing the highest incidence rate (about 10 times greater) when compared to mainland Portugal[1]. Contact with rodent urine is the major human source for spirochete transmission. Typically, leptospires were classified according to antigenic determinants through time consuming cross agglutination absorption protocols but, more recently, a molecular classification has been described with the proposal of at least 16 genomic species[2]. Although providing useful taxonomic information, this reliable approach is still dependent of the established serological classification with which epidemiologists are more familiar. Hence, serovar and serogroup assignations will continue to be used.

The aim of this study (under the USA Scientific Cooperative Agreement, No. 58‑4001‑3-F185) was to investigate the usefulness of a polymerase chain reaction‑based assay which amplifies repetitive DNA elements present within bacterial genomes, as a first tool for genotyping a large number of *Leptospira* kidney isolates obtained from 1,856 live-trapped rodents (2004-2006) in São Miguel (n_{SM}=988) and Terceira (n_T=868) Islands.

So far, DNA from 620 out of 1,102 positive cultures (225^+ / 587^+ from São Miguel and 395^+ / 515^+ from Terceira) was amplified using the single primer iRep1[3]. The production of distinct and reproducible molecular fingerprints was firstly generated with reference serovars for almost all pathogenic serogroups. A percentage of “representative” isolates per animal species and habitat was also sequenced (16S rRNA fragment) to confirm the results obtained.

Two genomic species were identified in both islands, *L. borgpetersenii* serogroup Ballum and *L. interrogans* sensu stricto serogroup Icterohaemorrhagiae, with a similar distribution pattern regarding the infected rodent species. The house-mouse (*Mus musculus*) was the major *Leptospira* carrier for Ballum isolates (73.7%; 392/532), whereas Icterohaemorrhagiae was only maintained by the two rat species (*Rattus rattus* and *R. norvegicus*). In spite of the attempt to increase the discriminatory power of this molecular classification, it was not possible to distinguish the identified bacterial species at the serovar level. In conclusion, this modern approach provided a simple and rapid typing of *Leptospira sp*., at the serogroup status, being an important tool in large epidemiological surveys.

(1) Vieira et al. (2006) Int J Infect Dis 10:378-386.
(2) Adler & Moctezuma (2009) Vet Microbiol, doi: 10.1016/j.vetmic.2009.03.012.
(3) Romero & Yasuda (2006) Mem Inst Oswaldo Cruz, 101(4): 373-378.

Reference

129

Isolation, characterization and bioactivity of bative *Bacillus thuringiensis* strains from São Miguel Island

Teixeira, Mário Brum; Duarte, Ana Judite; Ferreira, Ricardo Gamboa; Toubarro, Duarte; Cabral, Carla; Oliveira, Luisa; Simões, Nelson

Universidade dos Açores, Portugal

E-mail: marimmt@hotmail.com

Keywords: *Bacillus thuringiensis*; 2DE; insecticidal crystal proteins

Abstract

Bacillus thuringiensis (Bt) is an entomopathogenic bacterium largely used in biological control of insects. The interest for searching new isolates of this bacterium is mainly due to the interest in the identification of new bioactive proteins expressed in the crystal, because they could provide alternatives to control resistant insects. In a survey for *B.t.* conducted in the Island of São Miguel we found about 180 isolates with bipiramidal shape, among which 163 displayed associated embedded body, visualized by Phase contrast microscopy. It is accepted that new crystal isoforms should be good sources for these new bioactive proteins. Thus we compare one isolate with an embedded body in the crystal (S11D) with a normal bipyramidal crystal isolate (S27E). We tested S27E and S11D against *Ephestia kuehniella* (*Ek*), *Popillia japonica* (*Pj*), *Mythimna unipuncta* (*Mu*) and *Phyllocnistis citrella* (*Pc*) larvae. Both isolates were highly toxic against Lepidoptera, but S27E showed a significantly higher activity than S11D. LC50 of S27E against *Ek and Mu larvae* was 56 µg/ml and 104 µg/ml, respectively. S11D showed LC50 of158 µg/ml and 122 µg/ml against the same insects. Against the coleopteran *Pj* larvae theisolate S11D caused the highest mortality. These data suggested the presence of an unexpected protein with activity against coleopteran. Thus we compare both isolates for cry proteins and genes. By MS/MS analysis of the crystal proteins and by PCR we detected in both isolates *cry1* and *cry2* genes, which are the usual cry genes in bipiramidal crystals. However we detected by 2DE analysis different proteins between the two types of crystals. These new proteins are being analysed by MS/MS and we are using degenerate primers to identify by PCR potential new genes in the embedded body of S11D.

Reference

130

Phenotypic and genetic Diversity of *Bacillus thuringiensis* strains isolated in Corvo Island

Duarte, Ana Judite; Cabral, Carla; Oliveira, Luísa; Simões, Nelson

University of Azores, Portugal

E-mail: anaju_duarte@hotmail.com

Keywords: *Bacillus thuringiensis*; insecticidal crystal proteins, Cry, Rep-PCR.

Abstract

This study aimed to find native strains of *Bacillus thuringiensis* (*Bt*) that are toxic to some insect pests. *Bt* strains were isolated from soil samples, and identified based on biochemical analysis and in the parasporal inclusion morphology. Bipiramidal and spheric inclusion types were identified in these isolates.

Genetic diversity was analyzed based on Rep-PCR patterns. Dendogram show a great genetic variability among isolates from this small island, however 6 clusters of 2 to 4 isolates were obtained. In order to determine toxicity of these isolates, 5 different pests were used: 3 Lepidoptera, *Ephestia kuehniella* (*Ek*), *Mythimna unipuncta* (*Mu*), and *Phyllocnistis citrella* (*Pc*) Larva; 1 Coleoptera, *Popillia japonica* (*Pj*) larva; and 1 Diptera, *Ceratites capitata* (*Cc*) adult. Only one isolate, Cr13D, demonstrate a high toxic activity for Lepidoptera species. Results showed Lc50 of 65, 294 and 347 µg/ml for Ek, Mu and Pc, respectively and no insecticidal was observed in Pj and Cc.

Cr13D isolate were secrened for the insecticidal family genes *cry1*, *cry2*, *cry4*, *cry7*, *cry8*, *cry9A*, *cry10*, *cry11*, *cry17Aa*, *cry27Aa*, *cry5*, *cry12*, *cry14*, *cry21A*, and for citotoxidal genes *cyt1A* and *cyt2* by PCR amplification. This isolate amplified product to *cry1 and cry2* family genes, with typical activity against Lepidoptera and no amplification was observed for the cry encoding toxins against Coleoptera and Diptera.

The highly active *Bt* isolate Cr13D tested in this work appeared promising for new bioinsecticide formulations and maybe even useful to obtain genetically modified resistant plants.

Reference

139

Influence of glycerol on xylose reductase and xylitol dehydrogenase activities of *Candida guilliermondii* during batch fermerntation of sugarcane bagasse hemicellulosic hydrolysate

Arruda, Priscila Vaz de (1); Chaud, Luciana Cristina Silveira (1); Duque, Tales Esteves (1); Corrêa, Rozelle Campos (2); Rodrigues, Rita de Cássia Lacerda Brambilla (1); Felipe, Maria das Graças de Almeida (1)

1: Engineering School of Lorena, Brazil;
2: University of Taubaté

E-mail: mgafelipe@debiq.eel.usp.br

Keywords: glycerol, xylose reductase, xylitol dehydrogenase, *Candida guilliermondii*, sugarcane bagasse hemicellulosic hydrolysate

Abstract

Xylitol is a sugar alcohol that has beneficial health properties such as for dental caries and respiratory infection prevention. In *Candida guilliermondii* the xylose metabolism starts with the reduction of xylose to xylitol catalyzed by NADPH-dependent xylose reductase (XR). Xylitol is oxidized to xylulose by NAD(P+)-dependent xylitol dehydrogenase (XDH) and xylulose is then phosphorylated to xylulose-5-phosphate by xylulokinase, which can be converted in pyruvate through a connection between pentose phosphate and glycolytic pathways. Some researches have showed that during the xylose-to-xylitol bioconversion, glycerol is formed in low concentrations. Glycerol has been known to play fundamental role in several vital physiological processes. In particular, the formation of this compatible solute as a byproduct has been regarded as a response to the cellular stress provoked by the environmental conditions inflicted on the microorganism; additionally, it can maintain cytosolic cell redox balance through NAD^+ regeneration. In this work the effect of glycerol on xylose-to-xylitol bioconversion by *C. guilliermondii* was examined by its addition (1.0 g/l) to treated sugarcane hemicellulosic hydrolysate containing (g/l): xylose (55.9); glucose (4.72), arabinose (4.76), acetic acid (4.67), furfural (0.012), hydroxymethylfurfural (0.015) and total phenolic compounds (3.25). The experiments were performed in a stirred tank bioreactor, initial pH 5.5, K_La $20h^{-1}$, 30ºC, 96h. Control experiments (without glycerol addition) was also evaluated. According to the results the presence of glycerol favored the xylose-to-xylitol bioconversion, mainly the xylitol productivity. It was verified that the XR (0.5302 $U/mg_{protein}$) and XDH (0.6497 $U/mg_{protein}$) activities were favored by the glycerol addition, corresponding to the increase of 19.5% and 12.6%, respectively, in relation to the medium without glycerol. In the control experiments the glycerol (byproduct) was produced since the first 6h. These facts could be associated to the NAD^+ regeneration through glycerol formation, due to assimilation of xylose to this byproduct formation. Additionally, the glycerol formation reduces the availability of NADH, and the storing of NAD^+, cofactor required by xylitol dehydrogenase enzyme, could have increased the xylitol to xylulose conversion, to the detriment of its accumulation and subsequent excretion to the medium.

Acknowledgements: FAPESP; CNPq and CAPES

Reference

144

Analysis of production and purification of a mutant amidase from L10 strain of *P. aeruginosa*

Arteiro, José M. (1, 2); Martins, M. Rosário (1,3); Candeias, M. Fátima (1,3); Salvador, Cátia (1); Figueiredo, Inês (1); Karmali, Amin (4); Caldeira, A. Teresa (1,2)

1: University of Évora, Portugal;
2: CQE, University of Évora;
3: ICAAM, University of Évora;
4: CIEQB, ISEL

E-mail: jmsa@uevora.pt

Keywords: Mutant amidase; *Pseudomonas aeruginosa*; Amidase purification

Abstract

Microbial amidase (acylamide amidohydrolase, EC 3.5.1.4) is an intracellular enzyme that catalyses the hydrolysis of amides producing the corresponding acid and ammonium ions. Amidase has a great biotechnological application; it has been used in neuro-biochemistry, plant physiology, medicine, detoxification of industrial effluents containing toxic amides and in food and detergent industry (Cravatt *et al*, 1996; Fournaud *et al*, 1998).

The present work is concerned with the production and purification of a mutant amidase from L10 strain of *P. aeruginosa*, obtained from a wild-type ATCC 8602. The cultures were performed in a bioreactor during 32 h at 37ºC, 250 rpm. Cells were collected by centrifugation and broken by sonication. Amidase activity was measured by a direct potentiometric method, using an ion-selective electrode (ISE) to quantify the ammonium released by acetamide hydrolyse, used as subtract. Amidase activity was maximal at 25 h of culture, in the stationary phase, showing that amidase produced is a secondary metabolite.

Purification of the amidase was performed by affinity chromatography on epoxy-activated Sepharose 6B-acetamide column. Chromatogram at 280 nm showed three peaks. Amidase, confirmed by amidase activity, corresponds to peak 2, eluted at 80 mL. The active fractions were analysed by SDS-PAGE and compared with commercial amidase from *P. aeruginosa* (Sigma A 6691) to test its purification. Mw was determined by native-PAGE and by size-exclusion chromatography (SEC) coupled to UV-HPLC.

Further works are in progress to immobilize whole cells and purified amidase, having in account the development of a biosensor.

This work was supported by FCT, PTDC/AGR/AAM/73460/2006.

Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A., Gilula, N.B. (1996). *Nature*, 384, 83-87.
Fournaud, D., Vaysse, L., Dubreucq, E., Arnaud, A., Galzy, P. (1998). *Journal of Molecular catalysis B: Enzymatic,* 5, 207-211.

Reference

145

Submerse culture of endophytic microorganism: Quantification of the bioactivity and extract stability

Serrano, Nadja Fernanda Gonzaga (1,2); Dubreuil, J. Daniel (2); Hokka, Carlos Osamu (1); Sousa, Cristina Paiva (1)

1: Federal University of Sao Carlos, Brazil;
2: Université de Montreal

E-mail: nadjaserrano@gmail.com

Keywords: submerse culture, endophytic microorganism, bioactivity, extract stability

Abstract

Endophytic microorganisms that colonize internal tissues of plants represent an important source of bioactive natural products with chemical structure and biological activity diversities. These endophytic bacteria live without causing any negative effects and in turn produce bioactive compounds that are relatively unstudied as potential sources of novel natural products for medical and commercial exploitation. Brazilian tropical savannah trees are likely to be excellent specimens to search for endophytic bacteria. An endophytic microorganism isolated from leaves of *Prunus* spp. (Brazilian tropical savannah tree at São Carlos-SP, Brazil) was screened and evaluated for its antimicrobial activity against important pathogenic bacteria (Ratti *et al*., 2008). The genetic identification of the endophytic isolated was done using 16S RNAr analysis. The endophytic bacterium was cultivated in ISP2 broth (28 °C, 180 rpm) and the stationary phase culture (48-120 h) was centrifuged (10,000 rpm, 20 min). Fresh brut extract was obtained by filtration (0.22 µm membrane) of cell-free supernatant. The bioassays were conducted using well diffusion assay against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. In order to exploit the chemical nature of the bioactive molecules, the 10-fold-concentrated brut extract (speed-vac) was submitted to enzymatic, thermic and pH variation treatments. The endophytic microorganism isolated was identified as *Paenibacillus polymyxa* and it was given the strain designation RNC-D. Fresh brut extract showed maximal antimicrobial potency of 800 $AU.mL^{-1}$ against *E. coli* (48 hours culture) and *S. aureus* (120 hours culture). The extract kept bioactivity against *S. aureus* and *E. coli* after to be submitted to proteases assay, pH variation from 2.0 to 9.0 and thermic treatment (-80, -20, 0, 4ºC, room temperature, 37, 60, 80, 100 ºC during 1 hour and 121ºC/15 minutes). This research will be continued to evaluate the antimicrobial spectrum of the isolated molecules contained on the extract produced by *P. polymyxa* RNC-D. At the same time, we intend to elucidate the chemical structure of the novel isolated substances. It has already become highlighted that an enormous potential of endophytic microorganisms, its products and utilitarian discovery in this field holds exciting promise.

Ratti, RP., Serrano, NFG., Hokka, CO.Sousa, C.P. (2008). J. Venom. Anim. Toxins incl. Trop. Dis., 14(2): 294-302.

Reference

160

Looking for circulating viruses in *Alectoris rufa*

Dias, Maryline Marques; Dias, Deodália; Caeiro, Maria Filomena

Faculdade de Ciências da Universidade de Lisboa, Portugal

E-mail: maryline.dias@gmail.com

Keywords: Alectoris rufa , bird viruses, cinegetic specie, epidemiologic vigilance.

Abstract

Partridges of the *Alectoris* genus (Order Galliformes) constitute a group of birds with economic and game relevance for some of the southern European countries. In Portugal, *Alectoris rufa* is the only autochthones species present throughout the whole territory and it is important at economic, social, scientific and game levels. Apart from natural populations, populations in captivity are also to be considered since they represent a significant proportion of the local and national economy.

Relatively little is known about *A. rufa* pathogenic agents like parasites and viruses. Until now, the pathogenic viruses related to bird species are members of the virus Families *Flaviviridae*, *Paramyxoviridae*, *Coronaviridae*, *Orthomyxoviridae* (with single stranded RNA genomes) and *Poxviridae* (with a double stranded DNA genome).

This work aims at the gathering of as much information as possible about the viruses associated to populations of this species. In this study, cloacae and oral swab samples from partridges in captivity were collected and conceded by veterinaries from *Direcção Geral dos Recursos Florestais*. Nucleic acids extracted with Trisure (Bioline) were further subjected to PCR with Phire Hot Start DNA polymerase (Finnzymes) and to RT-PCR with One-Step RT-PCR kit (Qiagen). Partridge's *beta actin* DNA and mRNA sequences amplification was performed to validate the quality of the nucleic acid samples as well as positive controls for PCR and RT-PCR experiments; the selected primers were based on *Gallus gallus* published sequences. *A. rufa* PCR and RT-PCR products were 1300 bp and 650 bp band sizes respectively.

The presence of virus sequences from the already referred virus families was checked in the samples, by amplification of highly conserved regions with primers selected from the literature.

Positive results were achieved when searching for Poxvirus, Paramyxovirus and Coronavirus sequences. PCR products amplified with primers for Poxvirus were only achieved once and, probably, constitute false positives. RT-PCR products amplified with primers for Paramyxovirus were not yet subjected to sequencing. RT-PCR products with the expected size (300 bp) were amplified with primers for Coronavirus; 15 samples in 20 analyzed were positive. Four of the obtained products were sequenced and matched with sequences from other avian Coronavirus. This result allowed concluding that Coronaviruses are probably circulating among partridges in captivity. .

Reference

164

Insights into the mechanisms of toxicity and tolerance to the thiol-reactive agricultural fungicide mancozeb in yeast, as suggested by a chemogenomic approach

Dias, Paulo Jorge (1); Teixeira, Miguel Cacho (1); Telo, João Paulo (2); Sá-Correia, Isabel (1)

1: IBB - Institute for Biotechnology and Bioengineering, CEBQ/IST, Portugal;
2: Centro de Química Estrutural, IST, Portugal

E-mail: pjdias@ist.utl.pt

Keywords: *Saccharomyces cerevisiae*, chemogenomics, mancozeb, phytopathogenic fungi, fungicide resistance

Abstract

Saccharomyces cerevisiae was used to uncover the mechanisms underlying resistance and toxicity of the agricultural fungicide mancozeb, linked to the development of cancer and Parkinson's disease. Genome-wide screening of a yeast deletion mutant collection revealed 286 genes required for mancozeb resistance. The most significant GO terms enriched in this dataset are associated to transcriptional machinery, vacuolar organization and biogenesis, intracellular trafficking and cellular pH regulation. Clustering based on physical and genetic interactions further highlighted the role of oxidative stress response, protein degradation and carbohydrate/energy metabolism in mancozeb stress tolerance. Genes involved in multidrug resistance and the PDR related transcription factors Yap1p, Yrr1p and Rpn4p, recently involved in mancozeb response and tolerance [1], were also identified as determinants of yeast resistance to mancozeb. Remarkably, Yap1p is the documented regulator of more than 90% of the genes encoding mancozeb-induced proteins, as revealed by expression proteomic analysis [2].

Mancozeb was found to act in yeast as a thiol-reactive compound, but not as a free radical or ROS inducer, leading to massive oxidation of protein cysteins. This is consistent with the requirement of genes involved in glutathione metabolism and in protein regeneration or degradation to provide cell protection against mancozeb and with the registered increased expression of such proteins in mancozeb stressed cells [2].

The identification of *Botrytis cinerea* homologs of yeast mancozeb resistance determinants is expected to guide studies on mancozeb resistance in phytopathogenic fungi. Moreover, the generated networks of protein-protein associations of yeast mancozeb resistance determinants and their human orthologs share a high degree of similarity suggesting that the indications from this global analysis may increase the understanding of mancozeb toxicity and adaptation mechanisms in humans.

[1] Teixeira MC, Dias PJ, Simões T, Sá-Correia I (2008) Biochemical Biophysical Research Communications, 367: 249-255.
[2] Santos PM, Simões T, Sá-Correia I (2009) Proteomics, 9: 657-670.

Reference

177

Chromobacterium violaceum as a potential biosurfactant-producing microorganism

Antunes, Adriana Almeida (1); Campos-Takaki, Galba Maria (2); Teixeira, José Antonio Couto (3); Rodrigues, Ligia Raquel (3)

1: Center of Biological Sciences, Federal University of Pernambuco, Brazil;
2: Center for Research in Environmental Sciences, Catholic University of Pernambuco, Brazil;
3: Institute of Biotechnology and Bioengineering, Departament of Biological Engineering, University of Minho, Portugal

E-mail: adri_antunes@yahoo.com.br

Keywords: Chromobacterium violaceum; biosurfactant production; surface tension

Abstract

Chromobacterium violaceum is a Gram-negative bacterium found in the soil and water in tropical and sub-tropical environments. Its complete genome sequence revealed wide varying alternative pathways for energy generation, complex and extensive systems for stress adaptation, motility and widespread utilization of quorum sensing for control of its inducible systems. Biosurfactants are amphiphilic compounds produced by microorganisms, both intra and extracellularly, that reduce surface and interfacial tensions.
In this work, *C. violaceum* UCP 1552 isolated from the contaminated area of Pernambuco, was used. Biosurfactant production was carried out in 500mL Erlenmeyer flasks containing 250mL of LB medium [tryptone – 10g/L, yeast extract – 5g/L, sodium chloride – 5g/L] plus 5g/L glucose and 1.6g/L soy oil, at 150 rpm and 30°C. Samples were collected at different fermentation times (from 0 to 188h) to evaluate cellular growth, glucose consumption and biosurfactant production (by reduction of surface tension and emulsification index determination).

Biomass growth was observed during the first 96h and afterwards the cells entered the stationary phase. Moreover, glucose was consumed in the first 30h. Surface tension of the fermentation broth free of *C. violaceum* cells recovered after 188h was found to be 32 mN/m. The highest emulsification index was observed for 12h experiment, being 56 and 59% for sunflower oil and n-hexadecane, respectively.
Results gathered in this study reveal the *C. violaceum* potential as a biosurfactant-producer opening novel perspectives for its application in the environmental area.

Financial support: CAPES

Reference

198

Fungal decolourisation of textile dyes in liquid medium under alkaline conditions

Ottoni, Cristiane Angelica; Santos, Cledir; Lima, Nelson

Universidade do Minho, Portugal

E-mail: c.a.ottoni@hotmail.com

Keywords: white rot fungi (WRF), dye decolourisation,ligninolytic enzymes, alkaline condiction, Tramestes versicolor

Abstract

Dyes have been extensively used in a broad range of industries, especially in dyeing textiles which generate large amounts of alkaline effluents. In recent years there has been an increasing interest in white rot fungi (WRF) which were found to be able to degrade many xenobiotic compounds including such dyes. WRF produce all or some of the extracellular ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lcc) which are responsible for the degradation of pollutants.

The purpose of the present assay was to screen WRF for degradation of textile dyes in liquid medium under alkaline conditions.

Fourdifferent WRF strains, *Trametes versicolor* MUM94.04 MUM04.100 MUM 04.101 and *Phanerochaete chrysosporium* MUM94.15 obtained from the Micoteca da Universidade do Minho (MUM) culture collection were used. Decolourisations of Poly R-478 and Reactive Black 5 (RB5) at 0.1 gL^{-1} concentration were carried out in liquid medium containing Yeast Nitrogen Base supplemented with 5 gL^{-1} saccharose. The effect of pH in a range from 8 to 10 was studied. The samples were incubated at 30 ºC with shake (150 rpm) during 7 days. On days 1, 3, 5 and 7 the decolourisation, saccharose and enzymatic activities (LiP, MnP, Lcc, glioxal oxidase (GLOX) and proteases) were assessed using absorbance, HPLC and colorimetric methods, respectively. The fungal biomass was also evaluated by dry-weight method.

The four strains decolourised more efficiently RB5 than Poly R-478. Concerning RB5, MUM94.04, MUM04.100 and MUM94.15 yielded best results rising 75% of decolourisation at pH 9.5. Among the ligninolytic enzymes produced by MUM94.04 and MUM04.100 Lcc had the highest activity. Activities of LiP, MnP, GLOX and proteases were also quantified. For *P. chrysosporium* MUM 94.15 very low LiP, MnP,activities were detected at pH 8.5-10.0 in the samples although the decolourisation was similar to that observed in *T. versicolor* MUM94.04 and MUM04.100.

The results showed that increase alkaline conditions turn the fungal decolourisation more strictly. Mechanisms of dyes degradation for each strain are now under studied.

Reference

206

Olive mill wastewater: a suitable medium for lipase production by yeasts

Pereira, Carina; Gonçalves, Cristiana; Belo, Isabel

IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar 4710-057, Braga, Portugal

E-mail: carina.duraes@deb.uminho.pt

Keywords: olive mill wastewaters, *Yarrowia lipolytica*, Candida cylindracea, lipase, bioreactor.

Abstract

The olive oil consumed in the world is mainly produced in the Mediterranean countries, with Portugal as one of the ten major producers. Olive oil production results on a large amount of liquid waste, which represents a critical environmental problem. The quality and quantity of olive mill wastewater (OMW) constituents are dependent of many factors, such as olives type and maturity, climatic conditions and region of origin, cultivation methods, and technology used for oil extraction[1].The most important organic compounds of OMW include sugars, tannins, polyphenols, polyalcohols, pectins and lipids, with chemical oxygen demand (COD) up to 220 $g.L^{-1}$. Previous work has shown that OMW can be used as culture medium for different lipolytic yeast strains[2,3]. The aim of this work is the optimization of lipase production and the wastewater degradation by two lipolytic yeast strains, *Yarrowia lipolytica* W29 and *Candida cylindracea* CBS 7869. OMW collected from different 3-phase continuous olive mills were used and characterized chemical and biochemically. OMW based medium composed by non diluted and centrifuged (3000 rpm, 10 min) OMW, was supplemented with ammonium chloride, yeast extract, and phosphate buffer (pH 7.2, 0.1 M). Batch cultures were conducted in a 2-L bioreactor (Biolab, B.BRAUN) at different values of pH, aeration and agitation rates. Both strains were able to grow on OMW based medium and to consume sugars and COD. Comparing the performance of both strains at pH 7.2 with agitation and aeration rates of 400 rpm and 1.5 $L.min^{-1}$, respectively, *Candida cylindracea* revealed to be the most efficient strain for lipase production. These results confirm that the yeast strains used have a great potential for OMW valorization by its use as culture medium for biomass and enzymes production. Further work is been conducted in order to optimize the overall lipase production process.

The authors acknowledge the financial support provided by ‘‘Fundação para a Ciência e Tecnologia” (Project PTDC/AMB/ 69379/2006; Grant SFRH/BD/27915/2006).

1 Roig A., Cayuela M.L., Sánchez-Monedero M.A., (2006),Waste Manage, 26, 960-969.
2 Gonçalves C., Lopes M., Ferreira J. P., Belo I., (2009), Bioresource Technol, 100 (15), WasteEng2008, 3759-3763.
3 Lopes M., Araújo C., Aguedo M., Gomes N., Gonçalves C., Teixeira J.A. and Belo I., (2009), J Chem Technol Biotechnol, 84 (4), 533-537.

Reference

208

Detection of fungi in untreated water sources

Fernandes, Diana (1); Domingues, Michel (1); Pereira, Vanessa (1); Basílio, Carmo (1); Crespo, Maria Teresa (1); San Romão, Maria Vitória (1,2)

1: Instituto de Biologia Experimental e Tecnológica (IBET) and Instituto de Tecnologia Química e Biológica (ITQB) - Universidade Nova de Lisboa (UNL), Av. da República, Estação Agronómica Nacional, 2780-157 Oeiras, Portugal;
2: L-INIA, Ex Estação Vitivinícola Nacional, Quinta de Almoinha, 2565-191 Dois Portos, Portugal

E-mail: tcrespo@itqb.unl.pt

Keywords: occurrence, fungi, water sources, DGGE, identification

Abstract

Despite their wide occurrence, little attention has been given to the presence of fungi in aquatic environments.
A comparison between different isolation techniques and cultural media, described for the detection of fungi, using different water sources - surface water, spring water, and groundwater - revealed that the best technique to adopt for the analysis of fungi is the membrane filtration using dichloran rose bengal chloroamphenicol as cultural media.
The occurrence and significance of fungi and indicator bacteria (total coliforms and *Escherichia coli*) are reported after more than a year of routine sampling. As expected, the groundwater samples showed consistently significantly lower levels of total coliforms, *E. coli*, and fungi compared to the surface and spring water samples.
The results obtained in the temporal analysis of the population present in different water sources using ITS gene amplification and denaturing gradient gel electrophoresis showed that different species were expected to occur in the different matrices.
The most frequently detected fungi isolates in each matrix were therefore identified at the species level, based on morphology and other phenotypic characters (1). The results of this research show that fungi occur widely in drinking water sources. The genera *Aspergillus, Cladosporium, Penicillium,* and *Candida* were often detected.

Acknowledgments
We thank Professor Robert Samson (from CBS) for valuable discussions and Lara Silva, Paula Alves and Catarina Dourado for technical assistance. Vanessa J. Pereira and Carmo M. Basílio thank Fundação para a Ciência e a Tecnologia for the grants BPD/26990/2006 and BD/19264/2004. Financial support from Fundação para a Ciência e a Tecnologia (PTDC/AMB/66024/2006) and the EEA Financial Mechanism, Empresa Portuguesa das Águas Livres (EPAL), Município de Almada, and IBET (through project PT0012), is gratefully acknowledged. We thank Idexx Laboratories through Iberlab and Imunoreage for the Quanty Tray Sealer 220.

Pereira, V.J., Basílio, M.C., Fernandes, D., Domingues, M., Paiva, J.M., Benoliel, M.J., Crespo, M.T., San Romão, M.V. 2009. Water Research, In Press.

Reference

211

Characterization of mercury reducing microbial cultures acclimatized in different carbon sources

Almeida, Bárbara (1); Carvalho, Gilda (1,2); Fradinho, Joana (2); Oehmen, Adrian (2); Reis, Maria Ascenção (2); Crespo, Teresa (1)

1: IBET/ITQB, Portugal;
2: REQUIMTE/CQFB, Chemistry Department FCT, UNL, 2829-516 Caparica

E-mail: balmeida@itqb.unl.pt

Keywords: mercury bioremediation; mixed microbial cultures; merA gene; DGGE; FISH

Abstract

Mercury is an extremely toxic metal that is widely present in the environment due to both geological and industrial sources. Some bacteria are able to withstand mercury environments through a detoxification mechanism, based on the reduction of ionic mercury (Hg^{2+}) into elemental mercury (Hg^{0}) through a mercuric reductase enzyme (*merA*). Since most wastewater treatment plants employ biological processes in the system, the biological removal of mercury can be a potentially viable alternative instead of using chemical or physical approaches. The feed composition in wastewater treatment systems plays an important role on the microbial selection and consequently on the treatment efficiency. This study aimed at the characterization of microbial mixed cultures capacity to reduce mercury when fed with different carbon sources.Sludge from a known mercury contaminated site in the Tagus River (Lisbon, Portugal) was enriched with different carbon sources, glucose, ethanol and acetate, where the mercury removal performance and microbial community of these mixed cultures were assessed. The mercury removal performance of the enriched biomass was carefully monitored throughout the study and correlated with the microbial population diversity in each sludge. The microbial communities were characterized through classical isolation techniques and culture-independent molecular methods, including fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE). Phylogenetic and functional diversity of the isolates was assessed through sequence analysis of their 16S rDNA and *merA* gene, respectively. Additionally, quantitative real time PCR was performed in order to measure the copy number of *merA* gene present in the different reactors throughout the study. Results showed that the carbon source played a major role both in the microbial community structure and mercury reducing performance. Glucose and ethanol seemed to enrich similar groups of microorganisms that carry out mercury reduction more efficiently. Acetate showed the worst performance, associated to a substantially different microbial community.

Acknowledgments
Fundação para a Ciência e Tecnologia (FCT) through the projects PTDC/AMB/65702/2006 and PPCDT/AMB/57356/2004 the post-doc grants SFRH/BPD/30800/2006 and SFRH/BPD/20862/2004.

Reference

221

UV-Induced bacterial inactivation: Towards a global model of UV-mediated toxicity in bacteria inhabiting the surface water layers

Santos, Ana L.; Lopes, Silvia; Henriques, Isabel; Gomes, Newton C. M.; Almeida, Adelaide; Correia, António; Cunha, Angela

CESAM & Department of Biology, Universidade de Aveiro, Portugal

E-mail: alsantos@ua.pt

Keywords: UV radiation, reactive oxygen species, scavenger, cytotoxicity, genotoxicity

Abstract

Heterotrophic bacteria play a central role in carbon and energy fluxes in ecosystems, but their small size render them highly susceptible to the effects of UV radiation (UVR) that can be either directly mediated by photochemical reactions at the DNA-level or indirectly by UV-induced reactive oxygen species (ROS). However, the contribution of the direct and indirect mechanisms to UV cyto- and genotoxicity in bacteria is unknown.

To estimate the contribution of the direct and indirect effects of UVR, cell suspensions of bacterial isolates from Ria de Aveiro (Portugal) were exposed to UVR. Cytotoxic effects were assessed by monitoring bacterial viability, while genotoxic effects were evaluated by fluorometric analysis of DNA unwinding. Intracellular ROS generation was assessed with the fluorometric probe DCFH-DA. The involvement of different ROS in UV damage was assessed by irradiating bacterial suspensions in the presence of ROS scavengers, namely, the generalist scavenger N-acetyl-L-cysteine and the specific scavengers ascorbic acid (peroxide and hydroxyl radicals scavenging), mannitol (hydroxyl free radical scavenger), histidine and sodium azide (singlet oxygen scavengers).

UV exposure resulted in a 2-6 logs reduction in bacterial viability and a 2-30% increase in intracellular ROS generation, accompanied by a substantial formation of DNA strand breaks (6-90%), widely variable among the bacteria tested. Ascorbic acid was the most effective scavenger, suggesting a role of peroxides in UV-induced damage. Scavenger presence was accompanied by an increase in bacterial viability, demonstrating ROS contribution to UV-induced cytotoxic effects. Variable levels of ROS removal with different scavengers suggest that the type of ROS involved in UV-induced damage might diverge among different bacteria.

Differences between intracellular ROS generation with H_2O_2 (peroxide inducer) alone and with UVR suggest that, besides peroxides, other ROS might be formed upon UV-exposure. Variable methylene blue-mediated increases in ROS levels by photodynamic action suggest that the contribution of singlet oxygen to UV-indirect effects is also unequal among bacteria.

These results indicate that different intracellular ROS might be involved in UV-induced effects on bacteria, potentially playing an important role in UVR cyto- and genotoxic effects that might add to the effects of extracellular ROS generated by organic matter phototransformation, with unknown impacts for aquatic biogeochemistry.

Reference

231

Modelling of *Bacillus amyloliquefaciens* CCMI 1051 cultures using artificial intelligence based tools

Caldeira, A. Teresa (1, 2); Vicente, Henrique (1, 2); Neves, José (3); Arteiro, J. Maria (1, 2); Roseiro, J. Carlos (4)

1: Universidade de Évora, Departamento de Química, Rua Romão Ramalho nº 59, 7000-671 Évora, Portugal.;
2: CQE, Évora, Portugal;
3: Universidade do Minho, Departamento de Informática, Braga, Portugal;
4: Instituto Nacional de Engenharia, Tecnologia e Inovação, Departamento de Biotecnologia, Azinhaga dos Lameiros, 22, 1648-038 Lisboa, Portugal

E-mail: atc@uevora.pt

Keywords: Bacillus amiloliquefaciens.; Spore Formation; Anti-fungal Activity; Neural Networks

Abstract

It is well known that *Bacillus* species produce a wide variety of metabolites with interesting biological activities, namely antibiotic compounds as iturinic lipopeptides, being the aspartic acid a favourable nitrogen source for iturinic compouns production by *B. subtilis* and by *B. amyloliquefaciens*. The incubation time is another factor to be considered on antibiotic production.

On the other hand, Artificial Neural Networks (ANN) are widely accepted as a tool that offers an alternative way to tackle complex problems. They can learn from examples, are fault tolerant in the sense that they are able to handle noisy and incomplete data, are able to deal with non-linear problems, and once trained can perform prediction and generalization at high speed.

The prediction of Bacillus sporulation (BS) and antifungal activity of compounds (AFA), from incubation time of cultures (IT) and from aspartic acid concentration (AA) is a complex and highly nonlinear problem for which there are no known methods to predict them directly and accurately.

The aim of this study is to optimize the production of antifungal compounds in B. *amyloliquefaciens CCMI 1051 cultures* using ANN. The database to be used contains anti-fungal data of cultures with different IT (1-9 days) using AA (0.4-5.6 g/L) as nitrogen source [1].

In order to obtain the best prediction of the AFA and BS, different network structures and architectures have to be elaborated. The optimum number of hidden layers and the optimum number of nodes in each of these will be found by trial and error. The model being depicted above was in meam time accomplished, and the results obtained with it appointed that the maximum AFA is achieved with 2.6 g/L of aspartic acid on day 9. However, with AA of 4.8 g/L a similar maximum value of activity is obtained for incubation time over 6 days. The model shows a dual behaviour for AFA, depending of the IT. When the IT is higher than 5 days the AFA versus AA shows a pronounced sigmoid profile, converging to a common maximum value of AFA. On the other hand, for IT lower than 5 days mentioned profile is ill-defined and the common converging point isn't observed.

The conclusion is that the use of ANNs show to be a potent computational tool that must be present in any intelligent predictive task applied to Bacillus cultures, evidencing nitrogen source as key factor to be considered in these kind of problems, where the time of incubation plays a role in secondary production of active compounds.

[1] Caldeira A.T., Savluchinske-Feio S., Arteiro Santos J.M., Coelho, A.V., Roseiro J.C (2008) Journal of Applied Microbiology, 104, 808–816.

Reference

245

Quantification of fungal biomass in *Penicillium brevicompactum* biofilms by image analysis

Siqueira, Virginia M.; Lima, Nelson

IBB–Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

E-mail: nelson@iec.uminho.pt

Keywords: Biomass, Fungal biofilms, Image analysis

Abstract

Biofilms are microbial communities consisting of microorganisms surrounded by an extracellular polymeric matrix attached to a surface. The filamentous fungi (ff) are excellent colonizers of surfaces, forming biofilms in potable water distribution systems and are directly linked to problems of quality control and public health. The mycological culture-dependent techniques are indirect and time consuming, been of great importance the implementation and improvement of methodologies that involves characteristics such as sensitivity, selectivity, reproducibility and shorter time of analysis. The measurement of biomass in a biofilm is required for numerous activities related to the study and prediction of biofilm behavior, including, for example, making comparisons between different disinfectants or antimicrobial agents for control of biofilm.
The aim of this work is the development of a new technique for measuring fungal biomass in biofilms using image analysis.
Penicillium brevicompactum (MUM 05.17) was chosen as a fungal model as it is the most commonly ff isolated from tap water (Gonçalves *et. al.*, 2006). The biofilm formation was made with slide glasses in contact with sterilized tap water into a Petri dish under 25 ^{o}C during 10-14 days. The biofilm growth was confirmed when small dots of mycelium were observed on the surface of the slide. Calcofluor White M2R was used as staining for the visualization of fungal cells walls. After staining the microscope slides with biofilms of *P. brevicompactum* were observed under an Axioskope epifluorescent microscope (Carl Zeiss, Germany) using UV light equipped with 40x/0.30 and 10x/0.65 objectives. The images were acquired with a color camera Zeiss AxioCam HRc using the software Zeiss Axiovision to calculate the hyphal length. The others measures were made based on the value of fungal density and average diameter of 5µm as described by Gaspar *et. al.* (2001).
P. brevicompactum was able to grow in water and form biofilms on glass slides under the conditions described here. Calcofluor staining technique was easy to perform with quick fungal wall detection. The images generated were adequate to assess the measurements using the software Zeiss Axiovision. From the data generated and to validate this image analysis technique fungal dry-weight and ergosterol content are now used.

Acknowledgement: Virgínia M de Siqueira is supported by the grant SFRH/BD/43719/2008 from FCT.

Gaspar, M.L.; Cabello, M.N.; Pollero, R.; Aon, M.A. Current Microbiology, 42:339-344, 2001.
Gonçalves, A.B.; Paterson, R.M.R.; Lima, N. International Journal of Hygiene and Environmental Health, 209:257-264, 2006.

Reference

250

Functional characterization of AraNPQ, a *Bacillus subtilis* ABC-type sugar transport system

Ferreira, Mário José Rodrigues (1, 2); de Sá-Nogueira, Isabel (1, 2)

1: Microbial Genetics, Instituto Tecnologia Química e Biológica (ITQB-UNL), Oeiras, Portugal;
2: Departamento de Ciências da Vida (DCV), Faculdade de Ciências e Tecnologia (FCT-UNL), Monte de Caparica, Portugal

E-mail: marioferreira@itqb.unl.pt

Keywords: Hemicellulases, arabinosyl oligomers, microbial ABC transporters

Abstract

The plant cell wall is structurally complex and biologically recalcitrant. Microorganisms, in particular saprotrophs, play a fundamental role in the decomposition processes of plant biomass and secrete numerous polysaccharide-degrading enzymes that attack cellulose, hemicellulose, and pectin. Mobilization of plant biomass for chemical and fuel production is a major biotechnological challenge of the 21st century and the application of polysaccharide-hydrolyzing enzymes in biomass saccharification is promising.

Bacillus subtilis possesses an enzymatic consortium that enables the release of arabinosyl oligomers and arabinose from hemicellulose arabinans and arabinose-containing polysaccharides. In our current working model, arabinan degradation is accomplished by at least two extracellular hemicellulases, AbnA and Abn2. The resulting products, arabinose and arabinooligosaccharides, are transported by different systems. Arabinose enters the cell mainly through the AraE permease and the uptake of arabinose oligomers occurs most likely via AraNPQ, a putative ABC type transporter, belonging to the metabolic operon *araABDLMNPQ-abfA* [1, 2]. To ascertain the involvement of AraNPQ in the uptake of arabinose oligomers and/or mixed oligomers we constructed a chromosomal *araN* in-frame deletion. The physiological effect of this mutation in the utilization of arabinan, arabinooligosaccharides and arabinose-containing polysaccharides as the sole carbon and energy source was determined. The results indicated that AraNPQ is responsible for the uptake of α-1,5-arabinosyl oligomers.

In this transport system, AraN is the sugar-binding protein and AraPQ the transmembrane proteins, however no ATPase was found in the operon. In *B. subtilis* two putative ATPases, MsmX and YurJ, were suggested to energize several systems, including AraNPQ [3]. We inactivated the *msmX* gene and found that its product is essential for the functionality of AraNPQ. Taken together, these results represent the first characterization of an ABC-type transporter devoted to the uptake of arabinose oligomers, and the existence in *B. subtilis* of one ATPase (MsmX) interacting with different ABC transport systems.

This work was supported by grant PPCDT/AGR/60236/2004 from FCT, POCI 2010 and FEDER to ISN.

[1] J. M. Inácio and I. de Sá-Nogueira, (2008) J. Bacteriol. 190: 4272 – 4280;
[2] J. M. Inácio, I. L. Correia and I. de Sá-Nogueira, (2008) Microbiology 154: 2719 – 2729;
[3] Y. Quentin, G. Fichant and F. Denizot, (1999) J. Mol. Biol. 287: 467 – 484.

Reference

257

Trametes versicolor cultures with agro-industrial wastes: Production and biological activity of protein-bound polysaccharides

Martins, M. Rosário (1,2); Caldeira, A.Teresa (1,3); Salvador, Cátia (1); Heber, Mauro (1); Semedo, Catarina (1); Candeias, M.Fátima (1,2); Karmali, Amin (4); Arteiro, José M. (1,3)

1: University of Évora, R. Romão Ramalho, 59, 7002 - 554 Évora, Portugal;
2: ICAAM, University of Évora, Apartado 94, 7002-554 Évora, Portugal;
3: CQE, University of Évora, R. Romão Ramalho, 59, 7000 - 671 Évora, Portugal;
4: CIEQB, ISEL, 1950-062 Lisboa

E-mail: mrm@uevora.pt

Keywords: *Trametes versicolor*, protein-bound polysaccharides, agro-industrial waste

Abstract

Several species of mushrooms have been valued as edible and medicinal resources. Polysaccharide mushroom-derived have shown medicinal effects such as blood pressure and cholesterol lowering, liver protection, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral and anti-tumoral activities.

Most of the studies relate the isolation of polysaccharides from mycelium (IPS), however few studies refer the isolation of polysaccharides from supernatant (EPS) of *T. versicolor* cultures.

The aim of this study was to select an appropriate agro-industrial waste, as carbon source, in *T. versicolor* cultures for the production of protein-bound polysaccharides and to evaluate the biological activity of produced complexes.

The cultures were prepared in shake-flask and the different agro-industrial wastes were added one by one to the basal medium. The agro-industrial wastes used were: tomato pomace, beet wastes, rice peel, wheat straw and sunflower. Flasks were incubated at 28°C, for 20 days, at 150 rpm and the production of the IPS and EPS was followed. The highest polysaccharide concentration was achieved with sunflower wastes at the 14^{th} day of culture.

EPS and IPS were extracted from sunflower cultures. Antioxidant properties were evaluated by radical scavenging activity using DPPH method and lipid peroxidation inhibition capacity of the complexes was determined by erythrocytes hemolysis. Superoxide dismutase and catalase activities of the complexes were measured. Both EPS and IPS complexes presented antioxidant activity, however EPS activity is higher.

High resolution techniques were used for extraction and purification of these complexes. Average molecular weights (Mw) of polysaccharides were determined using size-exclusion chromatography (SEC) coupled to UV/RI-HPLC. Four main compounds were isolated from EPS (1KDa<Mw<17KDa) and three from IPS (1KDa<Mw<100KDa).

Antioxidant properties of EPS and IPS produced by *T. versicolor* in sunflower cultures suggest that they can be used as an important source of bioactive compounds with potential medicinal value. Further studies are in progress to evaluate the bioactivity of the isolated compounds.

Reference

258

Continuous process of biodegradation of the organic matter in textile effluent using adapted mixed cultures

dos Santos Oliveira, Ivy (1); Borges Silva, Messias (2); da Cruz Pradella, José Geraldo (1); Lamas Samanamud, Gisella Rossana (2); Almeida Loures, Carla Cristina (2); de Souza, André Luiz (2); Cobra Guimarães, Oswaldo Luiz (2); Silvério da Silva, Silvio (

1: University of São Paulo, Brazil;
2: University of São Paulo, EEL/ Lorena, Brazil

E-mail: ivyso@dequi.eel.usp.br

Keywords: biological treatment, mixed culture, microbiology of activated sludge, advanced oxidation processes, textile effluent

Abstract

Aiming to the treatment of textile industry effluents Advanced Oxidation Processes (AOP) were used associating biological treatment with immobilized cells in fluidized bed reactor. Four experiments were peformed according to an ortogonal arrangement of Taguchi L_4 (2^3) where control variables were aeration flow of 100 and 300 mL/min, minimum and maximum respectively, rate of dilution of 0,01 and 0,08 h^{-1} and the use or not of pre-treatment with AOP (Reagent of Fenton, pH=3 ,0 and T=25°C). Zeolites spheres as support and a mixed culture of microorganisms originated from activated sludge of sewer treatment adapted for the treatment of the textile residue were used. The predominant species were of the phylum Rotifera, protozoa that have a crucial role in depurating environments under organic pollution. The experiments performed aimed to verify the reduction percentage of organic matter expressed in chemical oxygen demand (COD), total organic carbon (TOC), color and turbidity. The experiments were performed in fluidized bed reactor with capacity of 2,2L and a microorganism suspension volume equal to 10% of the volume of the medium was used as inoculum to a constant temperature of 30ºC and pH=7,0. During the first 24 hours the experiment was performed in a batch process for the production of biofilm. After that period the continuous phase began by feeding the bioreator with textile effluent. For the two experiments performed with *in natura* effluent the stationary state was reached in approximately 11 days with the COD reduction efficiency of 87,18% minimum dilution flow and 74,38% for maximum. The curve behavior of both COD and TOC were quite similar with approximately 85% of reduction. In the two experiments performed with the pre-treated effluent with AOP the stationary state was reached in about 5 days for the experiment using the flow of minimum dilution and 1 and a half day for the experiment using maximum dilution flow, being the COD reduction percentage of approximately 88,80% in the minimum flow and 81,20% in the maximum flow. The TOC curve behavior in both cases were quite similar showing a reduction percentage of 80%. The results obtained confirmed the potential use of aerobic biorreactors with immobilized cells in zeolite, formed by adapted cultures to the biodegradation of the present organic matter in the textile effluent. The dilution flow was shown quite significant in the determination of hydraulic retention time in the reactor.

Reference

289

Improved image analysis procedures for monitoring activated sludge systems with filamentous bulking

Mesquita, Daniela P. (1); Amaral, António L. (2); Ferreira, Eugénio C. (1)

1: Universidade do Minho, Portugal;
2: Instituto Politécnico de Coimbra, Coimbra

E-mail: daniela@deb.uminho.pt

Keywords: activated sludge, filamentous bulking, image analysis

Abstract

Activated sludge systems are frequently used in wastewater treatment plant. This process is the most suitable and studied system. However, several problems are being always detected, such as filamentous bulking. Filamentous bulking is typically caused by an overabundance of filamentous organisms that interfere with the settling and compaction process. This phenomenon can be studied and related with settling parameters by automated image analysis using different microscopy acquisitions. However, by using these standard image analysis procedures some relevant information about the state of the sludge is enclosed. Conventional routines, using monochrome images are not suitable to detect the filamentous bacteria which are gram-positive or gram-negative. Moreover, the traditional image acquisition methodologies are not capable to detect both viable and damaged bacteria present within the sludge. Presently, the gram-stain evaluation is performed by visual inspection and manual counting using a microscope which is a tedious procedure. Also, to overcome the lack of viability information, an epifluorescence staining method composed with two nucleic acid-binding stains can be used.
For this study, a lab-scale activated sludge reactor was monitored during 100 days through image analysis information and the operational parameters were modified inducing filamentous bulking. Morphological changes were investigated by using new acquisition methods such as epifluorescence staining LIVE/DEAD® BacLight™ Bacterial Viability Kit, the LIVE *Bac*Light™ Bacterial Gram Stain Kit and the traditional bright field. The overall results revealed an improvement of the sludge morphological characterisation, combining these new image analysis procedures with the conventional routines.

Reference

290

Nitrate removal from landfill leachate in an anoxic rotating biological reactor

Cortez, Susana; Teixeira, Pilar; Oliveira, Rosário; Mota, Manuel

IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Portugal

E-mail: susana_cortez@deb.uminho.pt

Keywords: Anoxic rotating biological contactor, landfill leachate, nitrate removal

Abstract

Nitrogen pollutants in wastewater are usually removed by biological methods such as autotrophic nitrification and heterotrophic denitrification.
The aim of this work was to study the denitrification process in an anoxic rotating biological contactor (RBC) for the final treatment of a landfill leachate with very high nitrate load.
The reactor was inoculated with acclimatized activated sludge and operated in a continuous mode with a hydraulic retention time of 10 h, using acetate as external carbon source. Two experiments were performed: one with a carbon to nitrogen ratio (C/N) of 2 and increasing nitrate load (from 100 to 560 mg $N\text{-}NO_3^-$/L); in the other the C/N ratio was varied while nitrate load was kept constant (220 mg $N\text{-}NO_3^-$/L).
In the first experiment the average removal efficiency of $N\text{-}NO_3^-$ was about 96 % and no nitrite accumulation was verified, highlighting the RBC high efficiency. Moreover, N_2 was the most abundant gaseous product indicating a complete denitrification process. An overall COD removal efficiency of 69% was attained. However, this does not comply with the maximum discharge limit (125 mg O_2/L).
To reduce COD in the effluent, in the second experiment lower C/N ratios (C/N=1.2 and 1.5) at constant nitrate load were tested. This change resulted in lower nitrate removal efficiencies while COD effluent values were still high. C/N was then increased again to the value used in the first experiment (C/N=2) and that caused an increase in nitrate removal. This puts to evidence that the organic matter present in the leachate is non-biodegradable. Thus the process depends deeply on the supplementary carbon added.
Based on the present results, it can be concluded that the tested anoxic RBC is a potential and convenient process for the removal of nitrate from landfill leachates with high nitrate loads.

Reference

292

Site-directed mutagenesis studies on a key protein of an environmentally friendly bacterium

Lourenço, Sílvia P. (1); Morgado, Leonor (1); Londer, Yuri Y. (2); Pokkuluri, P. Raj (2); Schiffer, Marianne (2); Salgueiro, Carlos A. (1)

1: Requimte, CQFB, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (FCT/UNL), Portugal;
2: Biosciences Division, Argonne National Laboratory, Argonne, Illinois 60439, USA

E-mail: csalgueiro@dq.fct.unl.pt

Keywords: Geobacter, metal bioremediation, electron transfer

Abstract

Geobacter species, in particular *Geobacter sulfurreducens* (*Gs*) have many applications in the bioremediation of toxic metal contaminated soils and in biological electricity production from waste organic matter [1]. Some of these toxic metals, such as U(VI), Tc(VII), Cr(VI) and Fe(III), are insoluble, being reduced outside the cell [2,3]. In order to accomplish this function, *Gs* has an electron transfer chain, which guides the electrons from the cytoplasm through the periplasm to the cell exterior. Earlier studies on *Gs* mutants revealed the important role played by the periplasmic triheme cytochrome PpcA in *Gs* bioremediation, since *Gs* cells with the gene encoding for PpcA knocked-out had their ability to reduce toxic metals severely affected [4]. In order to understand the functional mechanism of this protein, PpcA mutants were produced by site-directed mutagenesis and their redox properties studied by visible and NMR spectroscopies. Some of these mutants showed important alterations of their redox properties, providing new clues to a better understanding of the functional mechanisms of PpcA.

Acknowledgments: This work is supported by project PTDC/QUI /70180/2006 from Fundação para a Ciência e Tecnologia (Portugal). LM was supported by Fundação para a Ciência e a Tecnologia grant SFRH/BD/37415/2007.

[1] D.R. Lovley (2006) Nature Reviews Microbiology 4, 497-508.
[2] D.R. Lovley (2003) Nature Reviews|Microbiology 1, 36-44.
[3] G. Reguera, K.D. McCarthy, T. Mehta, J.S. Nicoll, M.T. Tuominen, and D.R. Lovley. (2005) Nature 435,1098-1101.
[4] J.R. Lloyd, C. Leang, A.L. Hodges Myerson, M.V. Coppi, S. Cuifo, B. Methe, S.J. Sandler, D.R. Lovley (2003) Biochem J 369, 153-161.

Reference

296

Heavy-metal resistance of *Marinobacter aquaeolei*

Nóbrega, Franklin Luzia (1); Almeida, Maria Gabriela (1); Devreese, Bart (2); Moura, Isabel (1); Pauleta, Sofia Rocha (1)

1: REQUIMTE, CQFB, Dep. Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal;
2: Laboratory for Protein Biochemistry and Biomolecular Engineering (L-ProBE), Ghent University, 9000 Ghent, Belgium

E-mail: franklin.nobrega@dq.fct.unl.pt

Keywords: heavy metal resistance, copper stress, proteomics, *Marinobacter aquaeolei* 617

Abstract

Bioaccumulation and biotransformation of metals by bacteria, together with selection of resistant strains in metal-polluted environments is widely reported but the successful use of one strain to the bioremediation of different heavy metals is yet to be found [1,2]. The study of these microorganisms is important to understand the mechanism of toxicity of these heavy metals in man and other forms of life [3].

In the present work, the effect of cadmium, cobalt and copper ions in the growth of a marine bacterium, *Marinobacter aquaeolei* 617, was studied. We have found that the minimum inhibitory concentration (MIC) of these ions that impair *M. aquaeolei* 617 aerobic growth was 200 µM, 4 - 8 mM, and 1.6 mM for cadmium, cobalt and copper ions, respectively. These results established this bacterium as a cadmium, cobalt and copper resistant strain. The effect of these heavy metals in the growth of *M. aquaeolei* 617 was determined using solid artificial sea water (ASW) plates and liquid ASW medium, supplemented with lactate and yeast extract as carbon source, in which the growth was followed during 24 to 48 h.

In liquid medium, in respect to copper and cadmium ion stress, there is no lag phase with increased $CuSO_4$ and $CdCl_2$ concentration in the medium, but the growth rate decreases until it reaches null values at MIC. Nevertheless, the OD_{600nm} at the stationary phase in the presence of either 1.5 mM $CuSO_4$ or 190 µM $CdCl_2$, for copper and cadmium ion stress, respectively is identical to the one of the control growth. In the case of induced cobalt stress, the growth profile presents a considerable lag phase with the increase in $CoCl_2$ concentration but the OD_{600nm} of the stationary phase is also identical to the one of the control growth.

Under the scope of copper resistance, we have carried out an analysis of the proteome profile of the periplasmic fraction of *M. aquaeolei* strain 617 grown aerobically in the presence of 1.0 mM $CuSO_4$, using two-dimensional gel electrophoresis and mass spectrometry. The preliminary comparative analysis of the periplasmic protein profile of *M. aquaeolei* strain 617 shows that there are around 23 protein spots that appeared upregulated. The MALDI TOF-TOF MS analysis enabled us to identify a copper-binding protein that might be involved in the copper resistance of *M. aquaeolei* strain 617. This copper binding protein was heterologously expressed in *E. coli* and a biochemical characterization was carried out.

[1] Silver, S. and Phung, L.T., Annu. Rev. Microbiol. (1996) 50:753–89
[2] Silver, S. and Phung, L.T., J. Ind. Microbiol. Biotechnol. (2005) 32:587–605
[3] Nies, D. H., Appl. Microbiol. Biotechnol. (1999) 51: 730-750

Reference

301

Propolis from Azores: evaluation of allelopathic activities using an in vitro culture plant model

Paulo, Jorge Fernando Ferreira de Sousa (1); Ferreira, Ana Margarida (2); Carvalho, Maria João (2); Carvalho, Luis (2); Fernandes-Ferreira, Manuel (1,4); Aguiar, Cristina (1,3); Cunha, Ana (1,4)

1: Departamento de Biologia, Universidade do Minho, Campus de Gualtar 4710-057 Braga;
2: Centro de Química, Universidade de Trás-os-Montes e Alto Douro, 5001-801 Vila Real;
3: Centro de Biologia Molecular e Ambiental (CBMA), Universidade do Minho, Campus de Gualtar 4710-057 Braga;
4: Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB), polo Universidade do Minho, Campus de Gualtar 4710-057 Braga

E-mail: jorgescppaulo@gmail.com

Keywords: Propolis, bioactivities, flax, growth, PSII maximum quantum yield

Abstract

Propolis is a complex misture of natural origin produced by honeybees (*Apis melifera*) from collected plant exsudates, which main ecological function is to protect the hive from infections. Its composition is very diverse and origin-dependent: it includes polyphenols, waxes, resins, balsams, aromatic acids, sugars, volatile oils and pollen grains rich in inorganic minerals. Several biological properties have been reported for this product, such as, antitumor, anti-inflammatory, antioxidant, antimicrobial, among others. More recently, allelopathic effects were also reported. The aim of this study was to evaluate propolis effects on plant physiology and development in order to assess its potential as a source of new bioherbicides.

Two propolis samples (4 and 5) from Angra do Heroísmo (Azores) were collected and extracted with ethanol. The total ethanolic extracts (EE) were further fractionated with *n*-hexane and chloroform. The extracts and fractions thus obtained were tested by incorporation in MS basal culture medium, using *in vitro* cultures of flax (*Linum usitatissimum* L.) as the biological model.

The allelopathic activities were analyzed both with the flax germination model, by growth and F_v/F_M analyses in *in vitro* grown seedlings, and with the undifferentiated cell model, determining chlorophyll and water contents, growth and morphogenic capacity in flax *callus* cultures. Propolis extracts and fractions exerted differential effects at the physiological and developmental levels. EE4 induced very significant root and hypocotyl growth inhibition in a dose-dependent manner. This effect was detected only with the respective *n*-hexane fraction. F_v/F_M results highlighted the common dual nature of plant extracts and other natural products: protective effects, in this case against high light stress, and inhibitory effects at higher concentrations. With *calli* cultures, both extracts demonstrated similar activities reducing cell proliferation (dry weight) and cell water content. However, in relation to total chlorophyll content, propolis EE presented stimulatory effects. Concerning morphogenic capacity, EE5 inhibited the differentiation of shoots and roots. Among the fractions tested, that of *n*-hexane from sample 4 stood out as the most active, seeming a promising target for further studies on propolis bioactive compounds.

Reference

307

Growth inhibition of *Saccharomyces cerevisiae* UE-ME3 by diuron correlates with a decrease of antioxidant responses

Pinheiro, Marta (1); Alves-Pereira, Isabel (1,2); Ferreira, Rui (1,2)

1: Escola de Ciências e Tecnologia, Departamento de Química, Universidade de Évora, Portugal;
2: Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Portugal

E-mail: raf@uevora.pt

Keywords: *Saccharomyces cerevisiae*, diuron, catalase

Abstract

Diuron, N'-(3,4-dichlorophenyl)-N,N-dimethyl-urea, is a systemic and non-selective herbicide, derived from urea, mainly used to control weeds on hard surfaces such as, roads and railway tracks, and to control weeds in crops, such as, pear and apple trees, forestry, and wheat. It kills plants by blocking electron transport at photosystem II thus inhibiting photosynthesis. According this we suspect that this herbicide can be an oxidative stress inductor. Diuron is persistent and contaminates groundwater, sediment and soil. Several reports indicate that diuron-treated soils can continue to be toxic to plants at least three years after initial treatment. In waters, this herbicide is stable to hydrolysis only breaking down very slowly under neutral conditions. Diuron breaks down more rapidly when microorganisms are present. It is toxic to mammals but juveniles are more susceptible than adults being classified as likely carcinogen. Although is of low toxicity to birds is moderately toxic to fish. Given above, we are trying to assess as diuron affects growth and antioxidant response of *Saccharomyces cerevisiae* UE-ME_3, a wine wild-type yeast (Alentejo-Portugal) belonging to the Enology laboratory collection of University of Évora. The cells (10^6 cells ml^{-1}) at mid-exponential phase were inoculated in YEPD medium with 2% (w/v) glucose and incubated during 72 h in a water bath with orbital stirring, at 28ºC, in the absence or presence of 5, 25, 50 and 75 µM diuron. Samples from each treatment were used for wet weight determination and to obtain 12000 *g* sediment and respective supernatant which were used for determination of CAT A and CAT T activities, according to Beers and Sizer (1952), as markers of antioxidant response. The results show that diuron cause a linear decrease of biomass production (r = 0.9725), occurring a decline of 52% in wet weight of yeast cells growing in presence of 75 µM diuron in culture medium. In other hand, it was observed a decrease of CAT A, and a linear decrease (r = 0.9808) of CAT T activities, occurring a decline of 84% and 38 % in the yeast cells growing in same conditions. These results appoint us diuron as a growth inhibitor of wine wild-type yeast *Saccharomyces cerevisiae* UE-ME_3, in level range assayed, response which appears correlates with a decrease of catalases activities.

Beers RF, Sizer JW (1952) J Biol Chem 195:133–138.

Reference

308

TiO2-NP increase triacylglycerols contents, lipid peroxidation and glutathione conjugates metabolism of *Saccharomyces cerevisiae* UE-ME3

Capela-Pires, Joana (1); Alves-Pereira, Isabel (1,2); Ferreira, Rui (1,2)

1: Escola de Ciências e Tecnologia, Departamento de Química, Universidade de Évora, Portugal;
2: Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Portugal

E-mail: iap@uevora.pt

Keywords: TiO2-NP, Saccharomyces cervisiae, γ-GT

Abstract

Titanium dioxide (TiO_2), the naturally occurring oxide of titanium is the most commercially used form of Ti nanoparticles (Ti-NP). Previous studies had established that these nanoparticles generate free radicals, both acellularly, specially through photocatalytic activity, and intracellularly. Moreover other reports also demonstrate that TiO_2-NP is able to generate elevated amounts of free radicals, which induced indirect genotoxicity mainly by DNA-adduct. Because of enlarged use of engineered nanoparticles and the increased pressure to commercialise this growing technology, there is an increasing interest to evaluate metabolic effects of NP in living cells, as well as, to develop a deeper understanding of the nature, fate and behaviour of nanoparticles in the environment. Though it is often tempting to consider NP as simple molecules, they are in fact complex mixtures where high surface area is an important component. Abiotic factors such as pH, ionic strength and the presence of organic matter will alter aggregation chemistry; and are expected to influence toxicity. Data on biological effects show that NPs can be toxic to bacteria, algae, invertebrates and fish species, as well as mammals. However, much of the ecotoxicological data is limited to species used in regulatory testing and freshwater organism. Data on bacteria, yeast, terrestrial and marine species is particularly lacking. Consequently the main objectives of this work was to determine de effects of TiO_2-NP in γ-glutamyltransferase (γ-GT) activity, as well as, triacylglycerols (TG) and TBAR products contents of wild-type wine *Saccharomyces cervisiae* (Alentejo-Portugal), belonging to the Enology laboratory collection of University of Évora. The cells (10^6 cells ml^{-1}) at mid-exponential phase were inoculated in solid YEPD medium with 2% (w/v) glucose and incubated during 72 h, at 28ºC, in the absence or presence of 0.1, 0.5, 1 and 5 µg/ml TiO_2-NP prepared by sonication. Samples from each treatment were used to determine TG and TBAR products contents, and to obtain the post-mitochondrial supernatant, which were used for γ-GT activity determination according to (Szasz, 1976).The results shown that TiO_2-NP induce an increase of TG, and TBAR products contents as well as an increase of γ-GT activity.

Szasz, G. (1976) Clinical Chemistry, 22, 2051-2055.

Reference

317

Effect of nitrogen limitation on starch accumulation in *Chlorella vulgaris*

Dragone, Giuliano Marcelo; Fernandes, Bruno Daniel; Abreu, Ana Paula; Vicente, António Augusto; Teixeira, José António

University of Minho, Portugal

E-mail: gdragone@deb.uminho.pt

Keywords: Microalgae, *Chlorella vulgaris*, starch, urea, nitrogen limitation

Abstract

Due to the limited stocks of fossil fuels and the production of greenhouse gas carbon dioxide on their combustion alternative sources of energy are being investigated. Recently, microalgae have received much attention as a renewable energy resource because these photosynthetic microorganisms can convert sunlight, water and CO_2 into potential biofuels (1). The microalga *Chlorella vulgaris*, particularly, has been considered as a potential raw material for bioethanol production because it can accumulates high levels of starch when grown under optimized culture conditions (2).

The aim of the present work was to study the effect of nitrogen limitation on starch production by the microalgae *C. vulgaris*.

C. vulgaris CCAP 211/1e (P12 strain) was obtained from the Culture Collection of Algal Laboratory, Institute of Botany, Academy of Sciences of the Czech Republic. The original (nitrogen sufficient) growth medium based on the elementary composition of algal biomass had the following initial composition (mg l^{-1}): 1,100 $(NH_2)_2CO$, 237 KH_2PO_4, 204 $MgSO_4 \cdot 7H_2O$, 40 $C_{10}H_{12}O_8N_2NaFe$, 88 $CaCl_2$, 0.83 H_3BO_3, 0.95 $CuSO_4 \cdot 5H_2O$, 3.3 $MnCl_2 \cdot 4H_2O$, 0.17 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.7 $ZnSO_4 \cdot 7H_2O$, 0.6 $CoSO_4 \cdot 7H_2O$, and 0.014 NH_4VO_3 in distilled water (3). Nitrogen limited growth medium was formulated by omitting urea from the original growth medium. The microalgae were grown in 1 l Schott flasks with 0.4 l of medium. Cultures were maintained at 30ºC under continuous, cool white fluorescent lamps. Light intensity was approximately 100 mmol m^{-2} s^{-1} at the surface of the photobioreactors. The concentration of suspended algal biomass was determined by optical density measurement at 700 nm. Starch content in the microalgae was determined colorimetrically by the anthrone reaction.

The results showed that starch accumulation in *C. vulgaris* was strongly related to nitrogen concentration. Under nitrogen limited growth conditions, starch constituted 36% of the algal biomass after 118 h of cultivation, whereas nitrogen sufficient microalgae contained 8% of their dry weight as starch. On the other hand, nitrogen sufficient condition led to an increase in biomass concentration, with the highest biomass concentration of 2.06 g l^{-1}. It can be concluded that accumulation of starch is enhanced in nitrogen limited cultures of *C. vulgaris* P12.

(1) Chisti, Y. Biotechnology Advances, 25, 294–306, 2007.
(2) Hirano, A., Ueda, R., Hirayama, S., Ogushi, Y. Energy, 22, 137–142, 1997.
(3) Douskova, I., Doucha, J., Livansky, K., Machat, J., Novak, P., Umysova, D., Zachleder, V., Vitova, M. Applied Microbiology and Biotechnology, 82, 179-185, 2009.

Reference

321

Production, isolation and chemical characterization of the exopolysaccharide from the white-rot fungus *Trametes versicolor*

Sousa Pereira, Susana R.; Coimbra, Manuel A.; Xavier, Ana Maria R. Barreto

QOPNA, CICECO, Departamento de Química, Universidade de Aveiro, Portugal

E-mail: susana.raquel@ua.pt

Keywords: Tramentes versicolor, exopolysaccharide, extraction, solubility, beta-glucan

Abstract

Trametes versicolor is a white-rot fungus with oxidative potentialities for pulp and paper industry. This fungus produces an exopolysaccharide (EPS), a (1→3)-β-glucan, with claimed anticancer, antimicrobial and immunomodulatory activities. The aim of this study was to produce, isolate, solubilise and characterise chemically the EPS produced by *T. versicolor*. The fungus was produced by a submerged-culture fermentation and the EPS was fractionated according to its solubility in ethanol solutions. Neutral monosaccharides and uronic acids composition of the isolated fractions were performed. The average molecular weight and the composing glycosidic linkages were also investigated. Nuclear magnetic resonance spectroscopy (NMR) was performed to one of the samples. The fraction which precipitated in 80% ethanol was composed by 95% of neutral sugars and glucose was the predominant one (98%), with traces of arabinose and xylose. In spite the fact that the different studies cited in literature refer that this polysaccharide is soluble in water, its solubility was not verified, even when the suspension was heated at 80ºC. However, this EPS is soluble (3,3 mg/mL) in NaOH alkali solutions and has resistance to acid hydrolysis. Since the EPS is soluble in the culture medium but becomes insoluble in water after ethanol precipitation, its recovery was attempted by evaporation of the culture medium. As the solution has been concentrated, a precipitate rich in glucose, with a composition identical to the former, was obtained. The remaining soluble fraction was composed by 29% neutral sugars. Mannose was the main sugar (52%), followed by 28% glucose, 15% galactose and 5% of other sugars. The analysis by NMR showed that the sample contained high concentration of compounds from the yeast extract component of the culture medium (α-mannans from mannoproteins). This study allowed to observe that the EPS produced in the liquid medium by *Trametes versicolor* is, when separated from the medium, insoluble in water but soluble in alkali solutions. Its average molecular weight is 225 kDa. Glycosidic-linkage analysis by methylation revealed that the main glycosidic linkages were α(1,4) and β(1,3)-glucose. This EPS is different from the polysaccharides found in literature, like Polysaccharopeptide (PSP) or Polysaccharopeptide Krestin (PSK), among others.

Reference

323

Tree vaccination for pine wilt disease: resistance induction in *Pinus* spp. by inoculation with an avirulent nematode strain

Franco, Albina C.R.; Roriz, Mariana; Santos, Carla; Rodrigues, Rui; Rodrigues, Ana C.; Vasconcelos, Marta W.

Escola Superior de Biotecnologia, Portugal

E-mail: arfranco@esb.ucp.pt

Keywords: Bursaphelenchus xylophilus, pine wilt disease, induced resistence

Abstract

Bursaphelenchus xylophilus (Steiner and Buhrer) Niclke is a pine wood nematode (PNW) responsible for causing pine wilt disease (PWD) (especially on *Pinus pinaster* trees), detected through the observation of external symptoms (i.e. chlorosis of the needles). It is well known that plants have a sophisticated system for overcoming infection by a wide range of pathogens, which can be induced immediately, producing a systematic defence or a local response to the virulent agent. Previous studies have demonstrated the induction of *P. thunbergii* tree resistance by inoculating plants with avirulent PWN strains, suggesting that the creation of a biological control on PNW is possible ("Vaccination"). Moreover, this induced resistance can also provide an experimental system to clarify physiological interactions between the PNW and pine trees. The resistance induction to pine wilt disease was assessed on young pine seedlings, under sterile conditions. Subsequent inoculations were induced by (i) avirulent isolate of *B. xylophilus* (C14-5) and (ii) virulent nematodes strains, on different *Pinus* spp. seedlings. The resistance mechanisms of PWN were assessed and the effect of seedling inoculation was determined in terms of the expression of several *Pinus* genes potentially involved in the disease response. Finally, PWN progression was also monitored to confirm successful seedling inoculation, and the possibility for full tree nursery seedlings resistance induction will be addressed.

Reference

327

Activated carbon as a redox mediator on azo dye reduction: influence of surface chemistry and pH

Pereira, Raquel (1); Pereira, Luciana (1); Pereira, Fernando (2); van der Zee, Frank (1); Alves, Madalena (1)

1: IBB-Instituto Biotecnologia e Bioengenharia, Centro Eng. Biológica, Universidade do Minho;
2: Laboratório de Catálise e Materiais, Dep. Eng. Química, Faculdade Eng. Universidade do Porto

E-mail: lucianapereira@deb.uminho.pt

Keywords: azo dyes, dye reduction, activated carbon, redox mediator

Abstract

Azo dyes account for about 60-70% of all dyes in textile, food, pharmaceutical, leather, cosmetics and paper industries and are the most common synthetic colorants released into the environment, causing undesirable colouring of surface waters, as well as toxicity and mutagenicity problems. Their removal is a major concern when treating dyeing-processing wastewaters. Under anaerobic conditions, azo dyes are non-specifically reduced, a fortuitous but often slow process. Acceleration can be achieved by using electron-shuttling compounds that speed up the reaction by acting as redox mediators. Activated carbon (AC) has been shown a feasible redox mediator. [1] Moreover, it was shown that the surface chemistry of AC plays a key role in dye adsorption performance. [2] In this study, the effect of modified AC on anaerobic chemical dye reduction was assayed. The surface chemistry of AC was selectively modified, without changing significantly its textural properties, by means of chemical oxidation using 6 M HNO_3 (AC with acidic surface properties) and thermal treatments (900 ºC) under a flow of H_2 or N_2 (AC with basic surface properties). Oxidation with 5% O_2 (in N_2) was also performed leading not only to surface chemistry changes (acidic properties), but also in the textural properties. Characterization of AC samples was also done. Four azo dyes from different classes, Acid Orange 7, Reactive Red 2, Mordant Yellow 10 and Direct Blue 71, were tested and at different pH values, 5, 7 and 9.

Batch experiments in the presence of low amounts of AC, demonstrated an increase of the first-order reduction rate constants for all the studied azo dyes as compared with assays without AC. The reduction of AO7 and MY10 with all the treated AC was highly dependent on the pH, with optimum rates at pH 5 and 7, respectively. Higher rates of RR2 and DB71 reduction were obtained at pH 5. The best decolourisation results were obtained with basic AC samples (AC_N_2 and AC_H_2). Comparing the rates of single dyes, MY10 was the faster reduced (12 ± 2.3 d^{-1}) and RR2 the slowest (1.3 ± 0.1 d^{-1}). In fact, MY10 was almost completely decolourised in 1 day. Colour removal of 80% was obtained for DB71 at a rate of 5.6 ± 0.3 d^{-1}. AO7 and RR2 were the most resistant to degradation (~ 60 %).

[1] van der Zee et al., 2003. Environ. Sci. Technol.: 37, 402-408.
[2] Pereira et al., 2003. Carbon 41: 811-821.
[3] Faria et al., 2005. Water research: 39, 1461-1470.

Reference

328

Microbiologic assessment of indoor air quality in hospitals

Graça, Joana (1, 2); Pinto, Claúdia (3); Lopes, Ariana (4); Gonçalves, Sérgio (4); Santos, Isabel (3); Nicolau, Ana (1); Lima, Nélson (3)

1: IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Portugal;
2: Biotempo – Consultoria em Biotecnologia, Lda., Portugal;
3: Micoteca da Universidade do Minho, IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Portugal;
4: Centro Hospitalar do Alto Minho, Portugal

E-mail: joanaccg@iol.pt

Keywords: air quality, hospital, health affections, biologic risks, airborne microorganisms, bioload

Abstract

Air quality in hospital environment has become an important issue in modern society and its assessment appears to be a fundamental tool on diseases diagnostic and prevention. The most common airborne microorganisms include bacteria and fungi species, which are responsible for multiple health affections like allergies and respiratory diseases.
Throughout one year, a study was developed in order to quantify the bacterial and fungal load present in the indoor air samples collected on different facilities within three Portuguese hospitals of medium to large dimension in the North of Portugal. With this research we aim to provide helpful information relatively to the identification and prevention of hospital associated biologic risks and consequently to define and implement an appropriate overall methodology to its control.
Air samples were collected twice monthly in each of the hospitals over a period of eleven months, using a Merck MAS-100 air sampler and 90 mm Petri dishes containing Dichloran Rose Bengal Chloranphenicol Agar (DRBC) and Chocolate-Polyvitex Agar to collect viable fungi and bacteria particles, respectively. A volume of 100 L were collected in neonatology and intensive care unit, whereas samples of 50 L were collected in the internal medicine, the emergency room and the main entrance hall, as well as in the outside. All the samples were taken in duplicate. The plates were incubated 3-5 days at 25 ºC for fungi particles and 2 days at 37 ºC for bacteria particles, after which colony forming units (CFU) were counted and identified.
Despite of all the differences between the hospitals (location, size, services and facilities) the results have shown a prevailing presence of *Cladosporium*, *Penicillium, Aspergillus* and *Staphylococcus* species. Also, the lower bioload levels were finding in more confined areas within the hospital, like neonatology and intensive care unit. On the remaining facilities more than 80% of the samples revealed contamination levels higher than the maximum reference level of 500 CFU/m^3 imposed by Portuguese legislation on D.L. 79/2006, 4th April. Inclusively, inner contamination revealed to be higher than in the outside which suggests the existence of dissemination agents on the inside of the buildings.
This study represents a contribution on the assessment of the main biological risks inherent to exposure of patients and hospital workers and is fundamental to apply preventive measures to minimize the exposure risks.

Reference

336

Production of microalgal biomass through the dairy industry effluents

Queiroz, Maria Isabel (1); Silva-Manetti, Adriana (1); Santos, Gabriele (1); Limberger, Fernanda (1); Jacob-Lopes, Eduardo (2)

1: FURG-Universidade Federal do Rio Grande, Brazil;
2: UFPEL-Universidade Federal de Pelotas

E-mail: biotecnofurg@yahoo.com.br

Keywords: Dairy effluent, production of biomass microalgal

Abstract

The rapid population growth, combined with the limitation of cultivable land and famine are barriers to be broken. An option that has been much studied to alleviate these problems is the production microalgal biomass grown in alternative culture medium, because when it comes to synthetic medium, the high cost of nutrients can be a production limiting factor. In the case of cultivation in alternative culture medium like industrial waste, the limiting factors for biomass production are restricted to light, temperature and agitation of the culture. Accordingly, various species of microalgae, including cyanobacteria, chlorophytes and diatoms have been used in the production of biomass and cellular compounds that can be applied widely as intermediate inputs and final products of processes related to bioenergy, food and pharmaceuticals. Thus, the objective of the study was to verify the production of biomass from microscopic cyanobacteria *Aphanothece microscopica* Nägeli grown at different temperatures in the dairy industry effluent. The effluent was collected monthly, for a period of 6 months, from the Cooperativa Sul Rio Grandense de Laticínios, Pelotas, RS, Brazil, and characterized according to pH, COD, total nitrogen and phosphorus in accordance with the methodology described in Standard Methods for the Examination of Water and Waste Water. For the conduction of the experiments of the effluent, the pH was adjusted to 7.6 and inoculated with 200mg.L^{-1} of microscopic *Aphanothece microscopica* Nägeli cells in the logarithmic phase of growth. The experimental conditions used were 30°C and 20ºC, C/N ratio of 20 and absence of light. The results demonstrate the potential use of wastewater to produce biomass microalgal, with productivity at 30ºC and 20ºC of up to 70.4mg/Lh and 160.2mg/Lh, respectively. The use of wastewater from milk processing presented a potential for cultivation at 30ºC and 20ºC, considering the maximum cell density parameters (1045mg.L^{-1}; 841mg.L^{-1}), maximum specific growth rate (0.25h^{-1}; 0.36h^{-1}), generation time (2.06h, 1.92h) and residence time to achieve the maximum cell concentration (12h; 4h), respectively. The values obtained for the coefficients of conversion of organic matter in biomass, 0.56$mg_{biomass}$/mg_{COD} (30ºC) e 0.89$mg_{biomass}$/mg_{COD} (20ºC) which suggests a high consumption of organic matter with low conversion to biomass.

Reference

337

Evaluation of biodegradability potential of sugarcane bagasse hemicellulosic hydrolysate toxic compounds by *Issatchenkia occidentalis* CCTCC M 206097 and *Issatchenkia orientalis* CCTCC M 206098 yeasts

Fonseca, Bruno Guedes (1); Baratella, Bruno Fernandes (1); Rodrigues, Rita de Cássia Lacerda Brambilla (1); Hou-Rui2, Zhang (2); da Silva, Sílvio Silvério (1)

1: Universidade de São Paulo, Brazil;
2: The Chinese Academy of Sciences

E-mail: guedes@debiq.eel.usp.br

Keywords: Sugarcane hemicellulosic hydrolysate, toxic compounds, biodetoxification, microaerobic, Issatchenkia

Abstract

Sugarcane bagasse can be fractioned in cellulose, hemicellulose and lignin. Mild acid hydrolysis produces a hemicellulosic hydrolysate rich in fermentable sugars and limited in fermentative activity inhibitors. Physical or chemical detoxification methods to overcome the negative effect of hydrolysate inhibitors are costly, produce wastes and can't reach the same fermentability of synthetic medium. In contrast, biological detoxification could represent an improvement in hydrolysate treatments by reducing waste, volume and sugar loss. This work evaluated the biodegradability of toxic compounds in the sugarcane hemicellulosic hydrolysate by the yeasts *Issatchenkia occidentalis* CCTCC M 206097 and *Issatchenkia orientalis* CCTCC M 206098. The yeasts inocula were obtained by previous cell growth in semi-defined medium (containing glucose as carbon source). Biodegradability experiments were conducted in 125 mL Erlenmeyer flasks containing 50 mL of hydrolysate (51.50 g/L xylose, 1.81 g/L glucose, 0,016 g/L furfural, 0016 g/L HMF, 0.30 g/L ferulic acid, and 0.60 g/L syringaldehyde) and initial pH 5.50. All media were supplemented with 5.0 g/L $(NH_4)_2SO_4$, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, 0.1 g/L NaCl, 0.2 g/L yeast extract, and 2.0 g/L urea and incubated at 200 rpm and 30 °C for 48 h. Sugars and other compounds were quantified by HPLC. In 24 h the results showed total furfural removal from hydrolysate for both yeasts. Also, total and partial (84%) removal of HMF by *I. orientalis* and *I. occidentalis,* respectively. The biodegradability of syringaldehyde and ferulic acid were 70.0% and 76.7% by *I. occidentalis* and 16.7% and 17.0% by *I. orientalis*, respectively. However, *I. orientalis* used xylose as a carbon source (10%), while *I. occidentalis* didn't grow in the hydrolysate, even in microaerobic condition. Therefore, the biodegradability of the furan and phenolic compounds by yeast *Issatchenkia* was possible in a single stage and by selectively removing the inhibitors from sugars in the sugarcane hemicellulosic hydrolysate.
The results showed the potential of these yeast strains in the biodegradability of the inhibitors present in sugarcane bagasse hydrolysate, which can be used in fermentative processes.

Acknowledgements: CNPq.

Reference

338

Molecular profiling of microbial communities in anaerobic bioreactors treating oleic acid rich wastewater

Salvador, AF; Cavaleiro, AJ; Pereira, MA; Alves, MM

IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal

E-mail: alcina@deb.uminho.pt

Keywords: Oleic acid, microbial community, methane, 16S rRNA gene

Abstract

Anaerobic treatment of long-chain fatty acids (LCFA) rich effluents, such as dairy effluents, can result in a high methane production. LCFA are degraded by syntrophic bacteria to hydrogen and carbon dioxide and also to acetate, which are consumed by methanogenic *archaea* to produce methane. A better knowledge of the microbial communities involved in the process is of utmost importance, because it can contribute to improve bioreactors performance and enhance methane production efficiency. In this work, the composition and dynamics of an anaerobic microbial community was studied during the high rate operation of a bioreactor treating a synthetic wastewater made up of sodium oleate and skim milk. The bioreactor was operated with a step feeding during 213 days and in continuous thereafter, with organic loading rates from 5 to 31 kg COD m^{-3} day^{-1}. Reactor performance improved during the step feeding start-up, achieving a maximum methane yield of 91 % and a COD removal efficiency of 97 % (Cavaleiro et al., 2009). Accumulation of LCFA onto the sludge was observed only during the first 62 days of operation. During the continuous period COD removal averaged 99 % and a maximum methane yield of 98 % was reported. Fifteen biomass samples were collected during the 665 days of operation. Total DNA was extracted and 16S rRNA gene was amplified for DGGE fingerprint analysis and for cloning/sequencing analysis. According to DGGE profiles, shifts in microbial composition were more evident during the first 100 days of the step feeding start-up. Dominant bacterial and archaeal ribotypes prevailed in the system, even when working in continuous high-rate mode. Archaeal 16S rRNA gene sequences showed higher identity to those of *Methanobacterium* and *Methanosaeta* genera. On the other hand, bacterial 16S rRNA gene sequences were most similar to those of uncultured bacteria and were assigned to phylum Firmicutes, Cloroflexi and Bacteroidetes. In conclusion, the microbial community developed during the start-up of the bioreactor was able to efficiently convert high oleate loads to biomethane, and remained stable until the end of the experiment.

Acknowledgments: Financial support from Fundação para a Ciência e a Tecnologia through the projects PTDC/BIO/69745/2006, POCTI/CTA/46328/2002 and the PhD grants SFRH/BD/24256/2005 and SFRH/BD/48960/2008.

Cavaleiro AJ, Salvador AF, Alves JI, Alves M. 2009. Environ Sci Technol. 43(8), 2931-2936.

Reference

342

Microflora variations in live *Cancer pagurus*

Pedro, Sónia; Costa, Sara; Teixeira, Bárbara; Marques, António

INRB, I.P./ IPIMAR, Portugal

E-mail: spedro@ipimar.pt

Keywords: microflora, Cancer pagurus, crab

Abstract

A comparative trial of the microflora of *Cancer pagurus* was conducted in order to evaluate the effect of different variables in the level and type of contamination. Populations of aerobic heterotrophic bacteria present were estimated using spread plate dilution technique. Proteolytic and chitinolytic bacteria were assessed as separate physiological groups of heterotrophic bacteria.

The sex of animals had an effect in the microbial contamination, with 92 % of males presenting higher levels of total bacteria than females. Among males total bacteria levels ranged from approximately 10^2 to 10^5 cfu/g whereas among females those levels ranged from non-detected to approximately 10^4 cfu/g. Concerning tissue microbiological contamination, the data obtained showed that 67 % and 83 % of muscle samples presented higher levels of total and proteolytic bacteria, respectively, than viscera. In muscle, total bacteria levels ranged from approximately 10^3 to 10^5 cfu/g, while proteolytic bacteria ranged from non-detected to approximately 10^4 cfu/g. In viscera the levels of total bacteria showed a wider range, with counts from 10 to 10^5 cfu/g (Fig. 1). Proteolytic bacteria levels ranged from non-detected to approximately 10^3 cfu/g. Where proteolytic bacteria were isolated, this physiological group contributed to 15-40 % of muscle total counts and to 17-48 % of viscera total counts. Differences in the level of chitinolytic bacteria were also observed between tissues (muscle *vs.* viscera), with muscle samples presenting higher contamination than viscera, the former with levels ranging from non-detected to 10^3 cfu/g. No clear trends were observed in the microbiological levels among animals captured at different seasons, origins or presenting different body conditions. The results support that abundance and composition of the microflora in live *Cancer pagurus* change according to tissue and sex.

Authors acknowledge EU project COLL-CT-2006-030421 CRUSTASEA for funding.

Reference

343

Contribution of the PROTOFILWW project to the knowledge of the activated sludge ecology: uncommon microfauna species

Moura, Claúdia (1); Neto, Marta Martins (1); Santos, Liliana (1); Mina, Isabel (2); Lima, Nelson (1); Mota, Manuel (1); Nicolau, Ana (1)

1: IBB – Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, Braga 4710-057, Portugal;
2: Centro de Fisiologia Molecular e Biotecnologia de Plantas, Universidade do Minho, Campus de Gualtar, Braga 4710-057, Portugal

E-mail: martaneto@deb.uminho.pt

Keywords: Activated-sludge, Microfauna, Protozoa, Waste water tretament performance

Abstract

Activated-sludge processes represent a component of the largest biotechnology in the world, gaining increased importance as a consequence of the expansion of the human population and the impact of human activities in the water quality. The importance and the role of the protozoa and little metazoa communities in the purification process of activated-sludge plants are well established. Even though, contributions to this field of knowledge are still scarce: very few studies have established reliable relationships between the protozoa and/or small metazoan communities and the operational conditions or physical-chemical parameters of the wastewater treatment plants (WWTP). The correlations between the plant performance or the operational conditions and the abundance of certain species have also been studied, leading to the development of a number of methodologies to assess the activated-sludge plant performance, being the Sludge Biotic Index (SBI), conceived by Madoni in 1994, the best known method to evaluate the performance of the WWTP. The present investigation being carried on aims at the identification of the microfauna (protozoa plus small metazoa) on 37 activated-sludge WWTP, of different regions of Portugal, operating under different environmental conditions. Data base on the prevalence, the abundance and the distribution of protists and little metazoa in activated-sludge systems will be presented. These project will also contribute to the systematic use of these methods when evaluating the performance of the biological treatment of wastewater, namely activated-sludge processes. Each of the 37 WWTP is being studied four times a year allowing for the identification of the causes of malfunctioning. In a different perspective, this will contribute for the answer of questions such as: Why do different microfauna species appear in similar conditions? Do the WWTP tend to maintain the same populations or do these change often? Are the changes cyclic or occasional? Do the less common species appear due to the systematic sampling or are they characteristic of certain emvoronmental conditions? Do they indicate geographical differences? Do the uncommon species appear in specific extreme conditions? Can these uncommon species can play a central role in the characterization of Portuguese WWTP?

Acknowledgments: Fundação para a Ciência e a Tecnologia for financial support of the project PTDC/AMB/68393/2006.

Reference

344

Purification and characterization of PAzo, an azoreductase from *Pseudomonas putida* MET94

Mendes, Sónia; Pereira, Luciana; Martins, Lígia O.

Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal

E-mail: smendes@itqb.unl.pt

Keywords: Azoreductase, flavoproteins, enzyme characterization, heterologous expression, dye degradation

Abstract

Azo dyes account for about 50% of all dyes in the textile, food, pharmaceutical, leather, cosmetics and paper industries and are, along with anthraquinonic dyes, the most common synthetic colorants released into the environment. Dye removal from wastewaters with traditional physicochemical processes, such as coagulation, adsorption and oxidation with ozone is expensive, can generate large volumes of sludge and usually require the addition of environmental hazardous chemical additives. On the other hand, most of the synthetic dyes are xenobiotic compounds which are poorly removed by the use of conventional biological treatments. Over the last years, we have focused our attention into environmental biocatalysis studies, with special emphasis in the use of bacterial laccases for the biotransformation of synthetic dyes (1,2). In the present study, *Pseudomonas putida* MET94 was selected among 84 bacterial strains as the most active synthetic dye degrader. This strain showed significant decolourization over a wide range of structurally diverse azo dyes after growth in complex liquid culture medium. Dye decolourisation was shown to be growth-associated, occurring only under anaerobic growth conditions. Kinetic assays performed with resting whole-cell systems and with cell-free extracts showed that the enzymatic activity is oxygen-sensitive and NADPH-dependent. The native azoreductase enzyme (PAzo) was purified and characterized showing a typical signature of a FMN-dependent flavoprotein. The purified enzyme is a homodimer with a molecular mass around 40 kDa. The corresponding *pAzo* gene was cloned and expressed and *Escherichia coli* and the recombinant enzyme biochemically and kinetically characterized. This work is expected to allow the future design, by protein engineering techniques, of optimised microorganisms targeted to the bioremediation of wastewaters containing dyes.

This work was supported by FP6-NMP2-CT-2004-505899 and PTDC/BIO/72108/2006 grants. L. Pereira holds a Post-Doc fellowship (SFRH/BPD/20744/2004) from Fundação para a Ciência e Tecnologia (FCT), Portugal. Special thanks are due to Diana Mateus and Catarina Pimenta for the initiation of this project and André T. Fernandes for assistance with the molecular biology and fluorescence studies.

(1) Pereira et al. 2009 J. Biotechnol. 139:68-77
(2) Pereira et al 2009 Adv Synth Catalysis, in press

Reference

346

Bacterial community structure of biohydrogen production process in extreme thermophilic conditions (70ºC)

Abreu, AA (1, 2); Alves, JI (1); Pereira, MA (1); Karakashev, D (2); Angelidaki, I (2); Alves, MM (1)

1: Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal;
2: Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet 115, DK-2800, Kgs Lyngby, Denmark

E-mail: angela_abreu@deb.uminho.pt

Keywords: Biohydrogen, dark fermentation, extreme thermophilic conditions

Abstract

The search for new energies is a priority and hydrogen is one of the most promising alternatives to fossil fuels, since water is the only product of oxidation. The attention on dark fermentation has increased recently due to the fact that H_2 can be generated continuously at high rate from renewable organic materials [1]. Fermentation under extreme-thermophilic conditions (70ºC) promote better pathogenic destruction, better thermodynamics conditions and less contamination with methanogenic organisms, comparing with mesophilic and thermophilic systems [2]. In this study, two different granular systems were investigated in order to get insight into the structure of the bacterial communities involved in H_2 production under extreme-thermophilic conditions. Heat treated methanogenic granules (HTG) and engineered heat treated methanogenic granules (EHTG) were individually inoculated in two EGSB reactors, fed with arabinose and glucose (1:1 (w/w)) at a final concentration of 5gCOD l^{-1}. EHTG were obtained by contact of granules with an enriched H_2-producing culture in batch mode for 3 days. The EHTG system showed more stable and efficient H_2 production achieving a maximum production rate of 2.7lH_2 $l^{-1}d^{-1}$ and a conversion of 175mlH_2g^{-1}substrate. In the HTG system no steady state was achieved and only a transient H_2 production was observed with two maximum peaks of 0.8 and 1.5lH_2 $l^{-1}d^{-1}$. Granular samples collected during the experiment as well as the enriched H_2-producing culture were analyzed by molecular ecology techniques, such as PCR-DGGE, cloning and sequencing. The dominant bacterial ribotypes found in the EHTG system DGGE profiles were closely related to *Clostridium* sp., *Sporolactobacillus* sp., *Bacillus* sp., *Klebsiella* sp. and *Thermoanaerobacterium* sp.. *Thermoanaerobacter*-like organism corresponded to dominant DGGE bands in the enriched culture but could not be found in the continuous EHTG system. Nevertheless, the contact of HTG with this culture contributed to the development of a stable and efficient hydrogen production during the EHTG system operation. The presence of active hydrogen producers in the EHTG system during the reactor start-up, seems to have created favourable conditions for the development of an efficient H_2-producing bacteria community. This seems to be the case of organisms affiliated to *Clostridium* sp. and *Klebsiella* sp. that corresponded to dominant bands in the EHTG system DGGE profiles, but were not detected in the HTG system.

[1] Kotsopoulos TA, Zeng RJ, Angelidaki I. 2006. Biotechnology and Bioengineering 94(2):296-302.
[2] Das D, Veziroglu TN. Int. J. Hydrogen Energy 2001; 26: 13-28

Reference

347

Enzymatic transformation of lignin-based compounds

Rosado, Tânia; Durão, Paulo; Martins, Lígia O

Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal

E-mail: tisrosado@itqb.unl.pt

Keywords: laccases, lignin, redox mediators, biotransformation, renewable polymers

Abstract

Laccases are oxidoreductases belonging to the multicopper oxidase family of enzymes. Their catalytic centres consist of three structurally and functionally distinct copper sites; T1 copper ("blue copper") is a mononuclear centre involved in the substrate oxidation, whereas T2 and T3 form a trinuclear centre involved in the dioxygen reduction to water. These enzymes are useful biocatalysts for diverse biotechnological applications, owning to their relative non-specific oxidation capacity, the lack of requirement for co-factors and the use of readily available oxygen as an electron acceptor. Laccases are both involved in lignin biosynthesis and biodegradation. It is expected these enzymes, as well as other lignolytic oxidoreductases, could be useful for efficient and eco-friendly transformation of lignins, introducing new properties or inducing condensation reactions among monomers or oligomers. Lignin, a heterogeneous aromatic polymer, most commonly derived from wood, is highly recalcitrant towards degradation but its chemical nature (including aromatic and aliphatic moieties) makes it an interesting alternative source for aromatic chemicals. Lignin has surfactant properties and it is already used as additives in concrete, dyes and other products. Lignin is a natural adhesive in wood and this characteristic can be exploited in the eco-manufacturing of fibreboards substituting formaldehyde. It should be pointed out that the extended use of renewable plant polymers will decrease the industrial dependence from petrochemical sources. In the present study, we focused on research related with bioconversions involving the recombinant CotA-laccase from *Bacillus subtilis* (1) for the potential development of new lignin-based high value products. We are following a multidisciplinary approach to study the enzymatic homo and hetero coupling of lignin model compounds. The effect of redox natural mediators (methyl syringate, acetosyrongone, syringaldehyde) and other reaction parameters such as pH and temperature, are being studied on the oxidation by CotA-laccase of lignin model compounds, either phenolic or non-phenolic.

This work was supported by a project grant from European Union (BIORENEW, FP6-2004-NMP-NI-4/026456). P. Durão holds a Ph.D. (SFRH/BD/40696/2007) from Fundação para a Ciência e Tecnologia, Portugal.

(1) Martins et al. 2002 J. Biol. Chem. 277:18849-18859

Reference

357

Enhancing methane production from fat by bioaugmenting *Syntrophomonas zehnderi* to anaerobic sludge

Cavaleiro, AJ; Sousa, DZ; Alves, MM

IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal

E-mail: acavaleiro@deb.uminho.pt

Keywords: LCFA, bioaugmentation, *Syntrophomonas zehnderi*, methane

Abstract

Long-chain fatty acids (LCFA) are commonly present in fatty-wastewaters and can be used for biogas production in bioreactors. The conversion of these compounds to methane is strongly dependent on the synchronized activity of acetogenic syntrophic bacteria and methanogenic archaea. Bioaugmentation of anaerobic sludge with LCFA-degrading bacteria can be strategically used to enhance methane production from fat. *Syntrophomonas zehnderi* is an obligate syntrophic bacterium that is able to degrade saturated and unsaturated fatty acids with 4 to 18 carbons[1]. This feature makes it potentially suitable as a bioaugmenting strain to enhance degradation of the wide range of LCFA present in wastewater.

In this work, the potential of bioaugmenting anaerobic sludge with *S. zehnderi* as a means of improving methane production from oleate was evaluated. *S. zehnderi* was pre-grown in a bicarbonate-buffered anaerobic medium supplemented with oleate, at 37 ºC. Two sets of bottles were then prepared, with and without sepiolite, a solid microcarrier. The microcarrier was used to investigate a potential increase in the microbial kinetics properties during LCFA degradation[2]. In each set of bottles, a non acclimated granular sludge was bioaugmented with the co-culture and fed with 1 mM sodium oleate. Blank assays (without oleate) and control assays (with inactivated co-culture) were also prepared. Inactivation of the co-culture was performed by heat treatment (121 ºC, 40 min, 2x). Methane, LCFA and volatile fatty acids were monitored during the assay by GC and HPLC.

Oleate could be degraded by bioaugmented and non-bioaugmented sludges, as verified by GC analysis at the end of the experiment. However, methane production in bioaugmented assays was faster, either with or without microcarrier. For the assays with sepiolite, about 71% of the initial substrate could be accounted for the methane measured after 12 days of incubation in bioaugmented sludge; a much lower methane yield, i.e. 13%, was observed in non-bioaugmented bottles. A high methane recovery, i.e. 92%, was also achieved in bioaugmented assays without microcarrier, though only after 32 days of incubation. The potential of bioaugmenting anaerobic sludge with *S. zehnderi* for efficient LCFA conversion to methane was shown, as the presented results evidence an improvement in the kinetics of the process and methane recovery from oleate.

Acknowledgment: Financial support from FCT through the grant SFRH/BD/24256/2005.

(1) Sousa DZ, Smidt H, Alves MM & Stams AJM (2007) Int J Syst Evol Microbiol, 57: 609 – 615.
(2) Alves MM, Pereira MA, Novais JM, Polanco F & Mota M (1999) Water Environ Res, 71: 209 – 217.

Reference

361

Enzymatic Biotransformation of Azo and Anthraquinonic Dyes

Pereira, Luciana (1); Mendes, Sónia (1); Coelho, Ana V. (1); Viegas, Cristina A. (2); Robalo, Maria Paula (3, 4); Martins, Lígia O. (1)

1: Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Portugal;
2: IBB-Instituto de Biotecnologia e Bioengenharia, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Portugal;
3: Departamento de Engenharia Química, Instituto Superior de Engenharia de Lisboa, Portugal;
4: Centro de Química Estrutural, Complexo I, Instituto Superior Técnico, Portugal

E-mail: lmartins@itqb.unl.pt

Keywords: anthraquinonic dyes, azo dyes, biotransformation, biocatalysis, laccase, radical coupling

Abstract

Color is usually the first contaminant to be recognized in a wastewater, as very small amounts of synthetic dyes (10-15 $mg.L^{-1}$) are highly visible, affecting the aesthetic merit, transparency and gas solubility of water bodies. Around 10^6 tons of synthetic dyes are produced annually, of which 1–1.5 10^5 tons are released to the environment. Dyes are in general stable organic pollutants, persisting in the environment and concerns have been raised that such artificial compounds may be xenobiotic. Therefore the development of methods for their degradation has been increasingly explored. Redox biocatalysts are highly sought after because of the selectivity, controllability and economy of their reactions. The understanding of the molecular mechanisms of dye transformation is important, not only for development of efficient cleaning-up processes, but also for the search for harmless dyeing compounds to be synthesized by green chemistry processes. Laccases are oxidoreductases that have a great potential in various biotechnological processes mainly due to their high non-specific oxidation capacity, the lack of a requirement for cofactors, and the use of readily available oxygen as an electron acceptor. Laccases constitute a large subfamily of the multicopper oxidase family of enzymes and catalyze the four-electron reduction of oxygen to water by the sequential one-electron uptake from a suitable reducing substrate. We have used recombinant CotA-laccase, a bacterial thermoactive and intrinsically thermostable enzyme from *Bacillus subtilis*, extensively studied at the biochemical and structural level, which has the predictable robustness for biotechnological applications (1). We found that this bacterial enzyme does not require the addition of redox mediators for the decolourisation of a wide range of structurally different dyes and presents optimal activity in the alkaline pH range, distinctive features when compared with fungal laccases (2). The enzymatic oxidation of the azo dye Sudan Orange G and of the antraquinonic dye Acid Blue 62 was studied in more detail using a multidisciplinar approach that combined enzymology, electrochemistry, mass spectrometry, nuclear magnetic resonance and microbiology in order to get mechanistic insight that could help us to understand the enzymatic biotransformation process and could also guide us to further develop by using protein engineering tools a useful enzymatic technology (2).

(1) Martins et al 2002 JBC, 277:18849-59; Enguita et al. 2003 JBC, 278: 19416-25; Durão et al 2008 Biochem J 412: 339-346.
(2) Pereira et al. 2009 J Biotechnol. 139:68-77; Pereira et al 2009 Adv Synth Catalysis, in press

Reference

368

Characterization of a new family of heme-based sensor proteins: impact on environment adaptability of *Geobacter sulfurreducens* cells

Fernandes, Ana P. (1); Pessanha, Miguel (2); Gouveia, Zélia (2); Todorovic, Smilja (2); Londer, Yuri Y. (3); Pokkuluri, P. Raj (3); Schiffer, Marianne (3); Salgueiro, Carlos A. (1)

1: Requimte, CQFB, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (FCT/UNL), Portugal;
2: ITQB, Universidade Nova de Lisboa, Av. da República EAN, 2780-157 Oeiras, Portugal;
3: Biosciences Division, Argonne National Laboratory, Argonne, Illinois 60439, USA

E-mail: csalgueiro@dq.fct.unl.pt

Keywords: Geobacter, metal bioremediation, sensor proteins

Abstract

Geobacter sulfurreducens (*Gs*) has the ability to couple the anaerobic oxidation of organic compounds with the reduction of several metals. Among the metals this bacterium can reduce some are relatively innocuous, as it is the case of Fe^{3+}, but others are extremely toxic (Cr^{6+}) or radioactive (U^{4+}). In response to environmental conditions, such as the presence of these metals, bacteria act through the signals detected by protein sensory systems, which enable regulation of gene expression and control of chemotaxis.

The family of *Geobacteraceae* predominates in many subsurface locations, where cells have to find adequate anaerobic environments in order to survive. Thus sensing redox potential is crucial for their survival. In *Gs* cells, ten of these signal transducers have conserved sensor domains containing *c*-type hemes, which are involved in the signal sensing from the surrounding medium. This family of proteins has sensory and chemotactic signatures, which may be of particular relevance to environmental bioremediation of metals.

Two of these proteins, encoded by genes *gsu0582* and *gsu0935*, are methyl-accepting chemotaxis proteins with similar predicted topologies: an N-terminal tail in the cytoplasm, followed by a transmembrane helix, a periplasmic sensor domain, another transmembrane helix, and cytoplasmic domains consisting of a HAMP domain followed by a methyl-accepting chemotaxis protein domain [1, 2]. In order to bring about the sensing properties and ligand selectivity by these sensor proteins, spectroscopic studies were carried out. The results of these studies and the ligand binding mechanisms will be presented.

Acknowledgments: This work is supported by project PTDC/BIA-PRO/74498/2006 from Fundação para a Ciência e Tecnologia (Portugal).

[1] Londer, Y. Y., Dementieva, I. S., D'Ausilio, C. A., Pokkuluri, P. R., Schiffer, M. (2006). FEMS Microbiol. Lett. 258: 173-181.

[2] Pokkuluri, P. R., Pessanha, M., Londer, Y. Y., Wood, S. J., Duke, N. E. C., Wilton, R., Catarino, T., Salgueiro, C. A., Schiffer, M. (2008). J. Mol. Biol. 377: 1498-1517.

Reference

369

Use of Immobilised Bacterial Systems for Heavy Metal Removal

Pires, Carlos (1, 2); Guerreiro, Antonio (1); Magan, Naresh (1); Castro, Paula (2)

1: Cranfield Health, Cranfield University, U. K.;
2: Escola Superior de Biotecnologia, U.C.P. Portugal

E-mail: cmpires@mail.esb.ucp.pt

Keywords: Contaminated Soils, Polymers, Bacterial Heavy Metal Resistance and Uptake

Abstract

Recently the bacterial diversity of sediments collected from an industrially polluted site in Estarreja, Portugal, was investigated. Those sediments present high levels of contamination, namely by heavy metals. During the bacterial diversity survey three strains (strains EC30, 1C2 and 1ZP4) capable of growing in high concentrations of Cadmium and Zinc (>250 ppm for both metals) were isolated. Phenotypic characterization, phylogenetic analysis by sequencing the 16S rRNA genes and metal tolerance tests were performed with the selected isolates. 16S rRNA gene sequence similarity showed that strains EC30, 1C2 and 1ZP4 were related with some genera within the families *Alcaligenaceae, Bulkholderiaceae* and *Sphingobacteriaceae*, respectively. An immobilisation strategy was developed in order to remove heavy metals from waste water streams using the isolated bacterial strains. Bacterial cells were immobilised on different polymeric supports (natural alginate and pectate) and porous synthetic cross-linked polymers. Synthetic polymers were produced with weak alkaline monomers in order to promote bacterial adhesion. Different inoculum/support ratios and strains were tested either with 100 ppm solution of Cd or Zn and with a 100 ppm mixture of each metal. Heavy metal removal/uptake was affected by polymer type, inoculum support ratio and bacterial strain. Overall, bacterial immobilisation on synthetic polymers resulted in higher heavy metal removal, compared with the other two polymers. Regardless of polymer type and concentration, increasing the bacterial inoculum level increased heavy metal removal. The maximum removal rate was achieved when strain EC30 and 1ZP4 were used (>70 % removal for Zn slightly lower for Cd). The optimum pH range for metal removal was 6.0-7.0, for all polymer/bacterial strain tested. Current research is focusing in examining the kinetics of metal uptake by these highly tolerant strains, in free and immobilised systems.

Reference

381

Soil biological activities and soil atmosphere gas exchanges

Cruz, Cristina (1); Rosa, Ana Paula (1); Chaves, Sandra (2); Tenreiro, Rogério (2); Martins-Loução, Maria Amélia (1)

1: Faculdade de Ciências da Universidade de Lisboa, CBA,Portugal;
2: Faculdade de Ciências da Universidade de Lisboa, BioFIG,Portugal

E-mail: ccruz@fc.ul.pt

Keywords: soil carbon, soil nitrogen, soil water content, Mediterranean, Soil atmosphere gas exchange

Abstract

Ecosystem components interact to determine the overall response of the system to changing climate and atmospheric composition, as well as feedback to the atmosphere to mitigate or exacerbate any future climate change. The exchange of greenhouse gases between the biosphere and the atmosphere is largely controlled by external drivers - climate, wet and dry deposition, land use, etc., and will be significantly affected by the predicted future changes in these drivers. Well-documented future drivers include changes in soil N status and C sequestration, with consequent changes in nutrient cycling characteristics. The impacts of driver changes across European ecosystems, particularly under Mediterranean conditions are poorly understood and knowledge has been hampered by a lack of integrated system-level studies.

The aim of this work was to correlate greenhouse gas fluxes between soil and atmosphere in a Mediterranean-type ecosystem with soil biological activity under various availabilities of water, carbon and nitrogen.

The study site is located in Alcochete, 50 km East of Lisbon. The region has a typical Mediterranean climate: hot and dry summers and moderated cool humid winters. Annual precipitation is 600 mm. The soil is sandy and poor in nutrients and organic matter. The gradient of nitrogen availability at the site is created by a point source of ammonia (a barn with 200 cows). Soil was collected, and the concentration of carbon and nitrogen manipulated by addition of known concentrations of urea and glucose, at two water availabilities (50 and 25% of soil pore space). Methane, carbon dioxide, ammonium and nitrous oxide (N_2O) emissions were simultaneously assessed using static chambers. CH_4 and N_2O were determined by gas chromatography, using a flame ionization detector and electron capture detector.

The data obtained so far show that when soil carbon and nitrogen contents are very low, soil biological activity responds to relatively small increments in the availability of these two factors. When soil carbon content was low, soils were always a sink for atmospheric methane, independently of water availability (25-50%) or temperature (8-22°C).

The results highlight the importance of edaphic and environmental factors in soil biological activity, and the importance of this activity in the gas fluxes between soil and atmosphere, that may have feedback effects on the environment.

Reference

383

Increased activity of the phosphorelay triggers sporulation during growth in a gut strain of *Bacillus subtilis*

Serra, Cláudia; Henriques, Adriano O.

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal

E-mail: serra@itqb.unl.pt

Keywords: gut microbiology, response regulator, response regulator aspartyl phosphatase, bistability

Abstract

Sporulation in *Bacillus subtilis* is generally regarded as a last resource adaptation to extreme nutritional stress, triggered only upon entry into the stationary phase of growth. We have found that a wild (undomesticated) gut isolate of *B. subtilis* forms spores with high efficiency during exponential growth, and hence bypassing the cell density, nutritional, cell cycle and other signals that normally delay sporulation. High-level sporulation during growth occurs because of increased activity of Spo0A, the key regulatory protein which, when activated by phosphorylation governs entry into sporulation. We used fluorescence microscopy to quantify the activity of Spo0A in individual vegetative cells across the population, and found that compared to a laboratory strain, more cells of the gut strain accumulate Spo0A above a certain threshold level which is required for the initiation of sporulation. Moreover, by replacing the auto-regulatory Spo0A promoter by an inducible promoter, we show that normal sporulation can take place at nearly undetectable levels of Spo0A. We infer that Spo0A is also more active per cell of the gut strain, and is not normally the limiting factor for sporulation. We show that the genes for three response regulator aspartyl phosphatases (Rap) are absent from the genome of the gut strain. These phosphatases act on a cascade of regulatory proteins, known as the phosphorelay. The phosphorelay is served by five sensor kinases, that transfer phosphoryl groups to Spo0A via two intermediate proteins, Spo0F, a modified response regulator, and the phosphotransferase Spo0B. The Rap phosphatases act on Spo0F, and negatively regulate the phosphorelay during exponential growth, preventing entry into sporulation. They are however inactivated at the end of growth by a quorum-sensing mechanism involving import of extracellular inhibitory peptides. Increased activity of the phosphorelay caused by the absence of the three Rap proteins thus explains the elevated activity of Spo0A both across the population and per cell of the gut strain, and shows that the activity of the phosphorelay and not the levels of Spo0A (above) is the limiting factor for entry into sporulation. We speculate that in the gut strain, the phosphorelay is tuned to favor high-level sporulation during growth. Because spores but not vegetative cells, survive exposure to bile salts and low pH, this is a characteristic that may favor survival of the population in the gut environment.

Reference

390

GreenPROJECT: a 3-year survey of microbial phytopathogenic agents in turfgrass

McGuire, Daniel; Lemsaddek, Abdelhak; Tenreiro, Rogério

Universidade de Lisboa, Faculdade de Ciências, Centro de Biodiversidade, Genómica Integrativa e Funcional (BioFIG), Edifício ICAT, Campus da FCUL, Campo Grande, 1749-016 Lisboa, Portugal

E-mail: drmcguire@fc.ul.pt

Keywords: Phytopathology, turfgrass, fungi, nematodes

Abstract

Turfgrasses have been recognized for their importance to the quality of life for over 2000 years and serve us in many important ways. Our leisure time is greatly enhanced by a well-maintained turf.

Belonging to the family Poaceae (Gramineae), turf species and cultivars comprise around 7500 species and are remarkably adaptable to various environments. Affected by numerous pathogenic agents, the costs of diseases and efforts to control them are sometimes difficult to evaluate. Therefore, phytopathologists have focused on disease control as a primary research objective.

One of GreenPROJECT's aims is to identify microbial phytopathogenic agents and advise greenkeepers. A direct microscopy-based observation of the injured areas is, in most cases, a suitable tool for fungal or nematode identification, allowing for a rapid diagnosis of the responsible agents.

Over the last three years, 128 fungi (21 genera) and 29 nematodes (13 genera) have been identified as responsible for diseases in 204 samples from golf courses and soccer lawns. Of the 60 fungi observed in 2006, 15 different genera were described, being *Pythium* spp., causal agent of foliar blight, the most frequent. For 2007, a total of 29 fungi of 10 genera where identified, with *Colletotrichum* spp., responsible for anthracnose, as the most common. In 2008, 43 fungi from 16 genera were detected and *Drechslera* spp., causal agent of melting-out, was the most prominent. The seasonal distribution of the fungi identified along this study revealed *Fusarium* spp. and *Pythium* spp. to be the most frequent genera in winter/spring and summer/autumn, respectively.

The number of identified fungi was higher in summer and autumn (46 fungi from 12 genera and 53 fungi from 15 genera, respectively) than in other seasons. Regarding phytopathogenic nematodes, they were responsible for a much smaller number of turf grass pathologies than the fungi, having only been observed 29 isolates from 13 genera in 3 years. The summer season revealed the highest number of phytopathogenic nematodes with a total of 13 from 8 different genera. The highest number of pathogens was observed during the warm seasons of summer and autumn. The increase in humidity coupled to a warm temperature brought on by the early autumn season appears ideal for a rapid growth of fungi.

Reference

393

AQUACHIP - A DNA Chip for the detection of several microorganisms in water samples – Design and preliminary results

Vieira, Helena; Martins, Nelson; Vale, Filipa F.

Faculdade de Engenharia Universidade Católica Portuguesa, Portugal

E-mail: helenamvieira@gmail.com

Keywords: Water quality, DNA chip, AQUACHIP, Environment, microbiology

Abstract

Waterborne pathogens are responsible for several diseases, either due to the consumption of contaminated drinking water or due to the contact with polluted recreational waters. There is an increasing awareness that emergent and viral pathogens should also be monitored for determining water quality. Conventional detection methodologies present several shortcomings, such as reliance in indicator species, low throughput and increasing resources as more species are to be detected. DNA chips have the potential to serve as surveillance systems for the simultaneous detection of pathogens, overcoming these limitations. In the present study, a rapid method for the detection of multiple waterborne pathogens (bacteria and viruses) was developed, using a DNA chip (AQUACHIP®). Species and group specific probes were implemented on a DNA chip, both for mandatory and non-mandatory microorganisms. Considerations regarding the AQUACHIP® design (probe layout, replicates and dilutions), DNA labeling and amplification, and the first tests performed.

The probes were previously developed and validated and are described in detail elsewhere [1]. These are divided in three main groups: probes for the detection of the groups and species of microorganisms that are currently enforced by the Portuguese and European legislation (the coliform bacteria group, *Escherichia coli*, Enterococci and *Clostridium perfringens*); a group of probes for non-mandatory microorganisms, selected according to their impact on public health (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Legionella pneumophila*, *E. coli* O157, *Campylobacter coli* and *C. jejuni*, *Salmonella* spp. and *Shigella* spp.); and two probes for pathogenic virus detection (Hepatitis A virus and Norovirus genogroup I).

The chip was designed in order to maximize the cost-effectiveness, replicability of the results, and to allow the determination of the sensitivity limits of the technology. Additionally, the chip was designed to accommodate future expansion to a greater number of species. Each AQUACHIP® was printed in quadruplicate in glass slides, allowing for the processing of a maximum of four water samples simultaneously; in each AQUACHIP®, three dilutions of nine replicates of each probe were distributed semi-randomly in different zones, to avoid probe location biases. Preliminary results of the application of the chip are presented.

This work was funded by Fundação Calouste Gulbenkian, program Environment and Health 2005.

[1] Vale, F.F., Silva, A.M., Granja, A.T., Vale, M.J. and Vieira, H. Physica Status Solidi (c) doi: 10.1002/pssc.200881703 (2009)

[2] Lee, D.Y., Shannon, K. and Beaudette, L.A., J Microbiol Methods, 65 (2006) 453-467

Reference

396

Xylanase and β-xylosidase production: alternatives for the autohydrolysis liquor application

Michelin, Michele (1,2); Polizeli, Maria de Lourdes T. M. (2); Silva, Daniel P. (1,3); Ruzene, Denise S. (1,3); Vicente, António A. (1); Jorge, João A. (2); Terenzi, Héctor F. (2); Teixeira, José A. (1)

1: IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal;
2: Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, 14040-901 Ribeirão Preto-SP, Brazil;
3: ITP - Instituto de Tecnologia e Pesquisa, Tiradentes University, Campus Farolândia, 49032-490 Aracaju-SE, Brazil

E-mail: silvadp@deb.uminho.pt

Keywords: xylanases, lignocellulosic materials, autohydrolysis processes

Abstract

Research into microbial xylanases production is a required development as these enzymes have several important industrial applications (*e.g*. bioconversion of lignocellulosic waste into their constituent sugars). Treatments of lignocellulosic materials in autohydrolysis processes, under optimized conditions, lead to the solubilization of hemicelluloses (liquid phase, liquor), allowing for the use of fractions remaining (cellulose/lignin) in other bioprocess, reducing industrial costs of the whole raw material. For this reason, wheat straw autohydrolysis liquor may be considered as a fermentation medium adjunct for the production of xylanase and b-xylosidase by strain *Aspergillus ochraceus* in submerged cultivation containing wheat bran as substrate. For the preparation of the liquor, wheat straw was milled, sieved (1.0 mm screen), dried, homogenized and stored. Extraction was done by adding water to the wheat straw sample in a closed and pressurized vessel (solid/liquid ratio of 1:10w/w), and heating the system to 200ºC for 30 min. The obtained liquor (hemicelluloses reach fraction) was separated from the solids by filtration. The hemicelluloses were then precipitated with three volumes of 95% ethanol (20°C/24 h) and dried for yield determination (4.9%), or used directly as liquid substrate. Adams medium was used for cultivation with different carbon sources: 1%w/v birchwood xylan; 0.5%w/v wheat bran; 1%w/v wheat bran; and combination of 1%w/v wheat bran and 10%v/v liquor. The cultivation conditions were 30ºC/100 rpm, for 7 days. Xylanase was assayed by DNS, while b-xylosidase by released *p*-nitrophenolate using 1% birchwood xylan as substrate in citrate-phosphate buffer, pH 6.0 for xylanase determination and 0.25% pnp-b-D-xyloside in citrate-phosphate buffer pH 4.5 for b-xylosidase. One unit of enzymatic activity was defined as the amount that liberated 1 mmol of product per minute on assay conditions. The results showed that the use of the liquor as an alternative substrate to xylanase production allowed for the obtention of values similar to those using 1% wheat bran until 96 h, decreasing after this time. However, in the case of b-xylosidase production, the liquor had a positive effect as, after the first 24 h of cultivation higher activities values were obtained, allowing to conclude on the interest of its use as an alternative substrate for improved b-xylosidase production.

Acknowledgements: FAPESP (Brazil); FCT (Portugal).

Reference

403

Molecular diversity of endophytic microbial community from pine trees with pine wilt disease

Proença, Diogo Neves (1); Francisco, Romeu (1); Santos, Clara Vieira (1); Bidarra, Luis (1); Abrantes, Isabel (1, 2); Morais, Paula Vasconcelos (1, 3)

1: IMAR-CMA, 3004-517 Coimbra, Portugal;
2: Departament of Zoology, FCTUC, University of Coimbra, 3004-517 Coimbra, Portugal;
3: Departament of Biochemestry, FCTUC, University of Coimbra, 3001-401 Coimbra, Portugal

E-mail: diogo.proenca@gmail.com

Keywords: Pine Wilt Disease, DGGE, endophytic microbial community, Bursaphelenchus xylophilus

Abstract

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease (PWD). Native to North-America, was introduced to Japan, has spread into China and Korea and recently in Europe. In Portugal was detected in south 1998 and has spread in 2008. *B. xylophilus* has been thought to be the only pathogen of PWD, however, there has been an increase in claims that bacteria associated with PWN play a role in PWD. On the other hand, many papers described potential uses of plant-associated bacteria as agents stimulating plant growth and plant health and as involved in the control of phytopathogens.

This study aimed to assess differences between the endophytic microbial community structure from infected and non infected *Pinus pinaster*, based on DGGE (Denaturing Gradient Gel Electrophoresis) profiling and related it with bacteria associated with PWN.

The study started by selecting sites from the area where the nematode *B. xylophylus* was first identified in Portugal in 1999 and from the new areas where *Pinus pinaster* trees with symptoms were found. Sample consisted of cross-sections from cut trees.

The dendrograms generated from DGGEfingerprints of endophytic microbial community were characterized by the presence of 4 to7 dominant bands, with microbial complexity varying between symptomatic and asymptomatic trees. Intraindividual variation was low in symptomatic trees, as demonstratedby the homology in cluster analysis which was not observed for asymptomatic trees.

Isolates were grouped into RAPD types. RAPD types including more than one strain were detected. RAPD type representatives were identified by 16S rRNA gene sequence analysis and belonged to two different phylogenetic groups: *Betaproteobacteria* and *Gammaproteobacteria.*

This study shows that the endophytic microbial community is different between pine trees in different health conditions. We show also that different plant species from the same sampling site have different endophytic microbial community. Moreover, this study demonstrated the presence of bacteria associated with PWN.

Reference

408

Microbial diversity of groundwaters and bottled natural mineral waters

França, Luís A.; da Costa, Milton S.

Centro de Neurociências e Biologia Celular, Portugal

E-mail: luisfranca@gmail.com

Keywords: Natural mineral water, Microbial biodiversity, Microbial population dynamics

Abstract

Natural mineral waters are defined as microbiologically wholesome waters, characterized by constancy of chemical composition. Modification of their biological components is prohibited by European law. The lack of knowledge about the flora of the aquifers is limited to our ability to assess microorganisms in subterranean environments from which the microorganisms in bottled water may originate. Also, the methods used to study mineral water microbiology have been chosen based on public health demands in detriment of our need to understand the ecology of the microorganisms in this environment. So far, practically all microbiological data has been obtained using classic culture methods. However, the widespread use of molecular phylogenetic studies, revealed the inadequacy of these methods in providing a reliable picture of the existing biodiversity.

Our major goal was to assess the microbial community diversity of subterranean water and understand the microbial population dynamics after bottling and storage of natural mineral water. The comparison of data from molecular phylogenetic analysis and simultaneous parallel isolation studies will give us a detailed profile on the microbial biodiversity existing in the aquifers, as well as their stability. Samples from groundwater at source and bottled water will be retrieved from two aquifers with different hydrological and physicochemical characteristics.

Samples were collected from borehole head from two aquifers and from the bottles, immediately after bottling and after 6, 15, 30, 60 and 180 days storage. Samples were concentrated by filtration, through variable pore size filters. These were placed on R2A and on diluted R2A agar plates, and incubated at 22 and 37ºC for up to 21 days under aerobic conditions. The heterotrophic bacterial population assessed by colony forming units (CFU) was very low, ranging between 10^{-2} CFU.ml^{-1} in the borehole to 10 CFU.ml^{-1} in bottled water immediately after bottling, but increased very rapidly upon storage and reached maximum levels within 7-15 days (10^{4} CFU.ml^{-1}). Strains were grouped by typing, selected representatives identified by 16S rRNA gene sequence analysis and potential new species will be classified by polyphasic analysis. Further analysis with culture-independent techniques, namely PCR-DGGE to fingerprint the bacterial populations and in situ 16S rRNA gene sequencing, will provide us with new data that will allow an overview of the environments biodiversity.

Reference

411

Thermophilic co-digestion of organic fraction of municipal solid wastes with FOG wastes from a sewage treatment plant: reactor performance and microbial community monitoring

Martín-González, Lucia (1,2); Castro, Rita (1); Pereira, Alcina (1); Alves, Maria Madalena (1); Font, Xavier (2); Vicent, Teresa (2)

1: IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, University of Minho, Braga, Portugal.;
2: Departament d'Enginyeria Química, Universitat Autònoma de Barcelona, Bellaterra, Spain.

E-mail: madalena.alves@deb.uminho.pt

Keywords: anaerobic co-digestion, municipal solid wastes, fat wastes, microbial community, 16S rRNA gene

Abstract

Anaerobic digestion is widely applied to treat the organic fraction of municipal solid wastes (OFMSW). Sewage treatment plant fat/oil and grease wastes (STP-FOGW) have a high theoretical methane potential, a fact that makes them suitable as co-substrate for anaerobic digestion process. Co-digestion of both wastes could be a feasible treatment that would enable valorization of STP-FOGW and also obtain a higher methane yield in the whole anaerobic digestion process. In this work, the performance of a 5l continuous lab-scale reactor was evaluated in two operation periods: when fed only with OFMSW (Period 1) and with a mixture of OFMSW and STP-FOGW at a volatile solids (VS) co-digestion ratio of 6:1 (Period 2). Reactor performance was evaluated in terms of VS reduction, biogas production, methane content in biogas, volatile fatty acids (VFA) and long-chain fatty acids (LCFA) content. In addition, sludge samples were collect in both periods and the microbial structure analyzed through PCR-DGGE of the 16S rRNA gene fragments and cloning/sequencing techniques.

During Period 1 methane yield average value was 0.36 (m3 CH4/ kg VS added) and TS and VS reduction were around 60 and 70%, respectively. As a result of STP-FOGW addition, an enhancement in methane yield was noticed in Period 2, reaching an average value of 0.51 m3 CH4/kg VS added. VS and TS efficiency were not affected by the STP-FOGW addition.

DGGE-fingerprints analyses showed that bacterial community was more affected by the STP-FOGW addition than the archaeal one. The obtained bacterial profiles clustered in two distinct groups, before and after the extended presence of STP-FOGW.

Stable reactor performance was observed during the whole experiment revealing that co-digestion of OFMSW with STP-FW is a feasible treatment option for both wastes.

Oral Session:
S5 – Health and Pharmaceutical Biotechnology

Reference

107

Structural instability in plasmid vectors for DNA vaccination

Oliveira, Pedro H (1); Prather, Kristala J (2); Prazeres, Duarte M (1); Monteiro, Gabriel A (1)

1: IBB, Institute for Biotechnology and Bioengineering, Biological Engineering Research Group, Instituto Superior Técnico, 1049-001 Portugal;
2: Department of Chemical Engineering, Massachusetts Institute of Technology, Room 66-458, Cambridge, Massachusetts 02139, USA

E-mail: pcoliveira@ist.utl.pt

Keywords: plasmid, DNA vaccine, homologous recombination, repeats

Abstract

Upon the development of an effective DNA vaccine, the nature of the DNA in terms of its composition and structure represents a safety concern regarding plasmid stability. Particularly, the presence in pDNA of direct repeats, inverted repeats or hotspots for the insertion of mobile elements are often responsible for the formation of structurally aberrant forms, thus threatening its clinical application. Moreover, the low frequency of some of these events often impairs its own detection.

In the recent years some authors have found practical evidences for structural instability phenomena occurring spontaneously in plasmids used for DNA vaccination (reviewed by Oliveira et al., 2009). These include direct-repeat mediated deletion-formation events in the vector pCIneo (Ribeiro et al., 2008) or IS1-mediated instability in a vaccine against HIV (Prather et al., 2006).

In this work we present data on the influence of different growth parameters (medium composition, temperature shifts, aeration, absence/presence of selective pressure) and stress intensity (antibiotic concentration) on pCIneo deletion-formation. Furthermore, we show that this commercial vector contains additional instability regions, including a structure-dependent hotspot for IS2 insertion (Oliveira et al., submitted). Predictive models for recombination frequency were also developed that take into account repeat and spacer length (Oliveira et al., 2008).

A computational search for direct and inverted repeated regions with high recombination potential performed in a large sample of commercial vectors, led us to conclude that these hotspots are widespread, even in plasmids currently used for DNA vaccine development. Additional lambda-red mediated strain engineering enabled us to obtain safer *Escherichia coli* strains for plasmid propagation.

Altogether, these findings are crucial in understanding not only how pDNA instability can be shaped by stressful environment but also the real extension of potentially threatening hotspots.

Prather, K. J. L., Edmonds, M. C. and Herod, J. W. (2006). Appl. Microbiol. Biotechnol. 73, 815-826.
Ribeiro, S. C., Oliveira, P. H, Prazeres, D. M. F. and Monteiro, G. A., (2008). Mol. Biotechnol. 40, 252-260.
Oliveira, P. H., Lemos, F., Monteiro, G. A. and Prazeres, D. M. F. (2008). Plasmid 60, 159-165.
Oliveira, P. H., Prather, K. J., Prazeres, D. M. F., Monteiro, G. A. (2009). Trends Biotechnol. (accepted).

Reference

360

Genome-wide phenotypic and proteomic analyses in *Saccharomyces cerevisiae* for the identification of novel molecular mechanisms of action and targets of the anti-cancer drug imatinib

dos Santos, Sandra C.; Sintra, Eva R.; Mira, Nuno P.; Sá-Correia, Isabel

IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal

E-mail: ss.santos@ist.utl.pt

Keywords: *Saccharomyces cerevisiae*, imatinib resistance, imatinib targets, vacuolar H+-ATPase, vacuolar pH

Abstract

Imatinib (Glivec®, Novartis) is a selective tyrosine kinase inhibitor of the oncogenic protein Bcr-Abl, the molecular hallmark of chronic myeloid leukaemia (CML). Imatinib (IM) achieved outstanding results and revolutionised the treatment of CML, being also used in the treatment of other malignancies, particularly gastrointestinal stromal tumors. However, drug resistance is an emerging problem, especially in advanced stages of CML.

In this work the yeast *Saccharomyces cerevisiae* was used as a model eukaryotic system to identify and study mechanisms of action of IM, as well as pathways involved in drug resistance. Two distinct genome-wide approaches were employed: (1) a screening of the yeast haploid deletion mutant collection with individual knockouts of most nonessential yeast genes; and (2) an expression proteomics study, in which the yeast cytosol and membrane subproteomes were analysed to identify proteins with altered expression levels in the presence of IM, including phosphorylated proteins. From the systematic mutant screening, 52 genes have emerged as novel determinants of resistance to IM [1]. These genes are involved in DNA repair and transcription control, cell cycle control and differentiation, vesicular transport, protein trafficking, and vacuolar pH homeostasis, a process which is over-represented in our dataset by 13 genes that encode subunits and assembly factors of the yeast vacuolar proton-translocating ATPase (V-ATPase). Additional studies using fluorescence microscopy showed that physiological acidification of the yeast vacuole is severely compromised following IM treatment, in a dose-dependent mechanism. Approximately 80% of the gene dataset has human orthologs, thus establishing a correspondence between the responses to IM in yeast and human cells. The ongoing expression proteomics study has been revealing interesting results that will be presented and confronted with those from the chemical genomic approach.

In conclusion, the eukaryotic model *S. cerevisiae* has been successfully used, for the first time, to identify novel molecular mechanisms of action and targets of the anti-cancer drug IM, most of them having human orthologs. So far the results remarkably suggest that IM might act as an effective inhibitor of V-ATPase function in yeast, identifying V-ATPase activity and vacuolar function as novel IM targets.

[1] dos Santos, SC and Sá-Correia, I. (2009) OMICS: A Journal of Integrative Biology 13(3): 185-198.

Reference

183

Polysaccharide-based nanoparticles for transmucosal protein delivery

Rodrigues, Mafalda; Ferreira, Guilherme N.M.; Grenha, Ana

Institute for Biotechnology and Bioengineering (IBB), Centre for Molecular and Structural Biomedicine (CBME), University of Algarve, Faro, Portugal

E-mail: a38613@ualg.pt

Keywords: nanoparticles, protein delivery, chitosan, carrageenan

Abstract

Many protein-based biomolecules evidence therapeutic potential, but their systemic administration through routes alternative to the parenteral is limited by the development of adequate carriers, which protect encapsulated molecules from harmful environmental conditions. In this sense, the incorporation of bioactive agents into small polymeric carriers, such as nanoparticles, has been gaining popularity in drug delivery, since they can provide a controlled release of the encapsulated drug when compared to conventional forms [1] and also act as vehicles to target specific tissues [1] or cross epithelial barriers [2].

Given their natural origin, polysaccharides frequently compose the matrixes of these carriers, because they easily comply with requisites of biocompatibility, biodegradability and non-toxicity, always mandatory in drug delivery [1,2]. Chitosan (CS) is a cationic polysaccharide which observes these requests and further evidences mucoadhesion capacity [2]. Carrageenan (CRG) is also a polysaccharide, displaying anionic character. Previous studies demonstrated the capacity of these polymers to assemble into nanoparticles (approx. 500 nm), by mild ionic complexation [3].

In this work, a cross-linking agent, tripolyphosphate (TPP), was included in the formulations in order to produce nanoparticles with suitable properties for protein transmucosal delivery. TPP intends to reduce nanoparticles' size, which is expected to increase their intimate contact with mucosal surfaces. Different CS/CRG/TPP mass ratios were tested and the resultant nanoparticles displayed sizes between 170 and 275 nm and a positive ζ-potential within +39 and +65 mV. The developed nanocarriers demonstrated great ability to encapsulate proteins. Bovine serum albumin (BSA) was used as model protein, at theoretical concentration of 30% (w/w) respective to chitosan. Nanoparticles exhibited association efficiencies as high as 54%, with the corresponding loading capacities varying within 17 and 22%. The release profile of the associated model protein from the nanoparticles' is currently being determined. Considering the obtained results, the developed CS/CRG/TPP nanoparticles appear to be a promising protein carrier for transmucosal delivery purposes.

The authors acknowledge the Portuguese Foundation for Science and Technology (FCT) the financial support through the research project PTDC/BIO/69682/2006 and the grant SFRH/BD/46439/2008, and further acknowledge IBB/CBME, LA, FEDER/POCI 2010.

[1] Chiellini, F., Piras, A.M., Errico, C., Chiellini, E. (2008) Nanomedicine 3: 367-393.
[2] Bowman, K., Leong, K.W., (2006) Int. J. Nanomedicine 1: 117 – 128.
[3] Grenha, A., Gomes, M.E., Rodrigues, M., Santo, V.E., Mano, J.F., Neves, N.M., Reis, R.L. (2009) J. Biomed. Mater. Res. A (accepted).

Reference

173

Evaluation of a novel fusion system for soluble protein overexpression in *Escherichia coli*

Costa, Sofia Judite Marques da (1,2); Silva, Pedro (2,3); Almeida, André (2,4); Castro, António (2); Domingues, Lucília (1)

1: Institute for Biotechnology and Bioengineering, Portugal;
2: Instituto Nacional de Saúde Dr. Ricardo Jorge - Centro de Saúde Pública Dr. Gonçalves Ferreira, Porto, Portugal;
3: Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal;
4: Hitag Biotechnology, Lda., Portugal

E-mail: sofia.costa@deb.uminho.pt

Keywords: recombinant protein production, novel fusion system, soluble overexpression, *Escherichia coli*

Abstract

Proteins production requires a successful correlation between expression, solubility and purification steps. As an expression system, *Escherichia coli* combines its low cost and ease of use with rapid expression, being widely used for heterologous protein production. This host cell has however several drawbacks, namely at the expression of insoluble proteins aggregated into inclusion bodies. Many efforts have been made to overcome such problems, including the optimization of expression conditions and the use of solubility fusion tags. The use of fusion tags for protein production remains challenging since none of the available fusion systems work universally with every partner protein. A novel fusion system had been recently discovered and submitted to a patenting process by Hitag Biotechnology, Lda. This fusion system consists of low molecular weight peptides/proteins from recombinant antigens, which have demonstrated to increase soluble protein expression levels in *E. coli*.

This work aims at the evaluation of the effects of two novel fusion tags on soluble protein expression in *E. coli*. Specific primers were designed to amplify and sub-clone gene sequences that encodes for frutalin, *Cryptosporidium parvum* 12kDa protein and *Giardia lamblia* cyst wall protein. These target proteins present therapeutic and diagnostic interests and had shown to be difficult-to-express in *E. coli*. Proteins were first fused to novel tags and than expressed in *E. coli*. Proteins purification was carried out by affinity chromatography, using nickel-NTA columns. Pooled fractions were dialysed against phosphate buffer pH 7.4 and latter analysed by SDS-PAGE. Protein expression levels were determined by Bradford assay.

When fused to novel tags, all target proteins were successfully expressed in *E. coli*. Comparing to the respective non-fused proteins, both novel tags used in this work promoted an increase from three to nine folds on soluble proteins expression levels. The SDS-PAGE analysis confirmed the purity of Ni-NTA pooled fractions, corroborating also these results. Tag1-fusions achieved higher production yields than fusions with Tag2.

In this work, three different target proteins were used to evaluate two novel fusion tags. The soluble overexpression effect offered by this novel fusion system may provide an important advance in recombinant protein expression processes in *E. coli*.

Reference

153

Colonization of *Streptococcus pneumoniae* in the era of seven-valent conjugate pneumococcal vaccine

Nunes, Sónia (1); Sá-Leão, Raquel (1,2); Simões, Alexandra S. (1); Frazão, Nelson (1); Brito-Avô, António (3); de Lencastre, Hermínia (1,4)

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Oeiras, Portugal;
2: Centro de Matemática e Aplicações Fundamentais, Universidade de Lisboa, Lisboa, Portugal;
3: Centro de Saúde de Oeiras, Oeiras, Portugal;
4: Laboratory of Microbiology, The Rockefeller University, New York, USA

E-mail: snunes@itqb.unl.pt

Keywords: Streptococcus pneumoniae, seven-valent pneumococcal conjugate vaccine, antibiotic resistance, vaccine, serotype

Abstract

Streptococcus pneumoniae (Pn) remains one of the most important human pathogens, particularly among young children and the elderly. Since 1996 we have been monitoring changes in the Pn population inhabiting the nasopharynx (NP) of young children (up to 6 years old). In 2001, the seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in the Portuguese market. Although PCV7 is neither included in the National Vaccination Program nor subsidized by the Government, in 2007, approximately 70% of the target population had received at least one dose of PCV7.

The aim of this study was to evaluate the impact of PCV7 on the pneumococcal nasopharyngeal flora among day-care center attendees, and estimate the expected coverage of the novel 10-valent (PCV10) and 13-valent (PCV13) vaccines in this population.

Data on antimicrobial consumption and nasopharyngeal samples were obtained in the pre-(2001), and in two post-vaccination (2006 and 2007) periods. Pneumococci were isolated by routine methods, and antibiotic susceptibility testing and serotyping were performed.

We observed that carriage rates remained high and stable (average 67%) although serotype replacement occurred. In 2007, non-vaccine types (NVT) accounted for 86% of the isolates. Antibiotic use in the month prior to sampling, remained high (+/- 19%) in 2006 and 2007. Antimicrobial resistance rates obtained in 2007 were comparable to those observed before the introduction of PCV7. However, the proportion of NVT resistant to at least one antibiotic increased from 16% in 2001 to 36% in 2007. Out of 286 NVT isolated in 2007, 25 serotypes were recovered, of which ten (n=104 isolates) were associated with antibiotic resistance. The NVT most frequently associated with antibiotic resistance were 19A, 6C, 15A, and 11A. Serotypes 19A and 6A were the most abundant in 2006 and 2007, whereas serotypes 1, 5, and 7F were rarely detected as in other carriage studies worldwide. In 2007, 14% of the serotypes detected were included in PCV10 and 40% in PCV13.

In conclusion, since the introduction of PCV7, dramatic changes have occurred in the pneumococcal nasopharyngeal flora of young children in Portugal. Rates of resistance to antibiotics have not decreased and are now mostly associated to NVT suggesting that the antibiotic pressure is very high. Continuous surveillance is needed to monitor the impact of conjugate vaccines and its coverage as well as intervention strategies aimed to decrease antibiotic consumption.

Poster Session:
S5 – Health and Pharmaceutical Biotechnology

Reference

147

RNA clearance from plasmid-containing lysates by phenyl boronate adsorption

Gomes, A. Gabriela G. N.; Azevedo, Ana M.; Aires-barros, M. Raquel M.; Prazeres, D. Miguel F. T.

Instituto Superior Técnico, Portugal

E-mail: gabriela.gomes@ist.utl.pt

Keywords: Gene therapy, *Escherichia coli*, Phenyl boronate

Abstract

Gene therapy aims to treat or prevent acquired and genetic diseases by employing genes inserted into vector molecules like plasmid DNA (pDNA) or recombinant viruses. Due to safety issues, plasmid vectors have been preferred over viral ones. Alongside with other developments, a range of processes has been proposed to purify pDNA from *Escherichia coli* cells. One of the most abundant impurities in a typical *E. coli* lysate is host RNA (more than 95%). In this work we examine the possibility of using phenyl boronate affinity in batch and fixed bed mode to retain RNA impurities and purify pDNA. Phenyl boronate ligands form complexes with molecules containing two vicinal hydroxyl groups in cis configuration, via the formation of two covalent bonds in a five-member ring complex. This feature can be used to explore one of the few chemical differences between RNA and DNA, i.e. the presence of vicinal 2', 3' *cis*-diol at the 3' end of the RNA molecule [1]. Since the 3'-hydroxyl is absent in DNA, plasmids will not esterify with boronate matrices, making it possible to separate pDNA from RNA. The phenyl boronate chemistry is represented by an equilibria. In strong alkaline conditions phenyl boronate in the trigonal planar form is able to ionize and form a tetrahedral negative form that is usually more stable to interact with cis-diols and produce five ring complexes than the latter form. Nevertheless, the dominant factor to control complex stability is geometric and steric and the tetrahedral boronate is more congested than the trigonal ester. We found that most of the total RNA in an alkaline lysate is adsorbed in batch mode to the phenyl boronate resin in a short time interval (1-5 min). A screening of different adsorption buffers was performed using $MgCl_2$ and glycine (pH 9.0), just MgCl2 and finally just water. Comparative results concerning RNA adsorption in batch and fixed bed with water will be shown.

[1] Xiao-Chuan, L. (2006) Chin. J. Chromatogr. 24, 73-80

Reference

149

Targeting viral infectivity factor (Vif) with a specific antibody: a molecular therapy approach against HIV-1

Soares, Sandra S.; Pedro, Luísa; Ferreira, Guilherme N.M.

Institute for Biotechnology and Bioengineering (IBB), Centre for Molecular and Structural Biomedicine (CBME), Universidade do Algarve, Faro, Portugal

E-mail: sssoares@ualg.pt

Keywords: Viral infectivity factor, HIV-1, scFv4BL antibody, Förster resonance energy transfer, molecular therapy

Abstract

Viral infectivity factor (Vif) is a conserved accessory protein abundantly expressed in the cytoplasm of human immunodeficiency viruses type 1 and 2 (HIV-1 and HIV-2) infected cells, to which key role functions have been attributed during viral infectivity, namely the neutralization of innate inhibitory host defense mechanisms against retroviruses. Therefore, Vif is considered to be an important alternative therapeutic target for inhibition of HIV-1 viral infectivity. Strategies that prevent or limit expression of Vif are expected to be beneficial in the treatment of HIV-1 disease, since cellular antiviral factors may then effectively act against infection. One of these strategies is to use intracellularly expressed antibodies, termed intrabodies, to neutralize Vif's actions. Such single-chain fragment variable (scFv) antibodies were already generated against HIV-1 Vif [1], and shown to bind specifically and selectively to HIV-1 Vif *in vitro* [2-3].

In this work we explore the use of an anti-Vif specific intrabody (scFv4BL) as potential anti-HIV-1 biotherapeutic agent in molecular therapy approach. Fluorescent microscopy and Förster Resonance Energy Transfer (FRET) were used to evaluate intracellular binding of these scFv's to HIV-1 Vif in animal cell cultures (HEK293T). The results show a predominant pattern of co-localization of both proteins in the cytoplasm of mammalian cells within a distance of about 10 nm, suggesting intracellular scFv4BL/Vif specific recognition and interaction.

Moreover, the successfull of Vif recognition by the specific antibody lead us to test the effectiveness of scFv4BL antibody to target Vif-expressing animal cell lines and to neutralize Vif's activity.

Summarizing, this work clearly shows that molecular therapy approaches which deliver Vif intracellular antibodies may represent a new therapeutic strategy for HIV-1 control.

The authors thank to Portuguese Foundation for Science and Technology (FCT) the financial support through the research project PTDC/BIO/69682/2006 and the grants SFRH/BPD/30290/2006 (S.S.S.) and SFRH/BD/36674/2007 (L.P.), and further acknowledge IBB/CBME, LA, FEDER/POCI 2010.

[1] Gonçalves, J., Silva, F., Freitas-Vieira, A., Santa-Marta, M., Malho, R., Yang, X., Gabuzda, D., Barbas III, C.F. (2002) J. Biol. Chem. 277: 32036-32045.

[2] Encarnação, J. M., Rosa, L., Rodrigues, R., Pedro, L., Silva, F.A., Gonçalves, J., Ferreira, G.N.M. (2007) J. Biotechnol. 132: 142-148.

[3] Ferreira, G. N. M., Encarnação, J. M., Rosa, L., Rodrigues, R., Breyner, R., Barrento, S., Pedro, L., Silva, F.A., Gonçalves, J. (2007) Biosens. Bioelectron. 23: 384-392.

Reference

150

New combinatorial strategy to multimerize single-domain antibodies to increase therapeutic potency

Oliveira, Soraia Rafaela Santiago (1, 2); Goncalves, João (1, 2)

1: URIA-CPM Faculdade de Farmácia da Universidade de Lisboa, Portugal;
2: Instituto de Medicina Molecular da Universidade de Lisboa, Portugal

E-mail: soraiaoliveira@ff.ul.pt

Keywords: Single-domain antibodies, multimerization, avidity, affinity

Abstract

Antibodies are very specific natural molecules that recognize and lead to elimination of different types of antigens. In the past 30 years, the scientific community has seen an exponential increase in antibody research. As a result, new technologies regarding the selection, engineering and production of antibodies or antibody-like fragments have emerged. Currently, single-domain antibodies (SDA) are among the most studied and interesting kind of antibody fragments. These SDAs consist in the variable heavy or light chain of an antibody. SDAs combine advantages of antibodies and of small molecules, like high expression and solubility, plasticity of CDR loops and capacity of tissue penetration. But these SDAs have exhibited poor *in vivo* targeting efficiency, probably due to their low binding avidity and fast clearance from blood circulation. The multimerization of these SDAs could overcome these drawbacks and increase the therapeutic potency of these SDAs. Hence, a new strategy that allows the construction and selection of bivalent SDAs that recognizes different epitopes from the same antigen is presented here. This way, we increase both the molecular mass of the antibody providing a greater chance for these molecules to bind to their antigen, and the affinity by avidity. The strategy consists in the construction of bivalent antibody libraries derived from positive clones already selected by Phage Display. The library was constructed using 3 different linkers to favor the recognition of epitopes in different locations of the antigen. This library represents the totality of combinations offered by individual positive SDA clones. As a result, when another Phage Display is made, only those bivalent antibodies that target epitopes in close proximity will be selected. This strategy has already been tested with excellent results, strongly suggesting being a powerful and useful technique that permits selection of bivalent antibodies with higher affinity due to an increase in avidity.

Reference

151

An HIV-1 gp120 loop grafted in a single domain VL antibody is sufficient for CD4 binding

Couto, Andreia Domingos (1,2); Gonçalves, João (1,2)

1: Faculdade de Farmácia de Lisboa, Portugal;
2: IMM, Instituto de Medicina Molecular, Universidade de Lisboa

E-mail: andreiacouto@ff.ul.pt

Keywords: single domain antibodies, HIV-1, CD4, biopharmaceuticals

Abstract

The fusion process of the Human Immunodeficiency Virus type 1 (HIV-1), is mediated by the gp120 surface protein and the gp41 transmembrane protein. To facilitate viral entry, the gp120 glycoprotein must bind to cell-surface CD4, alter its conformation to reveal a site for co-receptor attachment, and trigger conformational rearrangements in the gp41 glycoprotein to mediate fusion of viral and host cell membranes. Therefore the gp120-CD4 interaction is critical for virus-cell fusion. The gp120 region that binds CD4 is the target of the broadly neutralizing antibody B12. The B12 antibody targets gp120 and recognizes a highly conserved epitope overlapping the CD4-binding region of gp120. This antibody is one of the four known human monoclonal antibodies identified that can efficiently neutralize a broad array of primary isolates of HIV-1 in vitro and can protect against viral challenge in vivo.

The goal of this project is to evaluate the potential of CD4 targeting and virus-cell fusion inhibition of the gp120 loop, recognized by the B12 antibody. This will be done using the 23 amino acids loop of gp120, grafted at the CDR1 of a highly stable rabbit single domain VL antibody, designated VL-B12. The potential of this VL-B12 construct is being tested for CD4 binding by ELISA assay against soluble CD4 and by FACS analysis using HeLa cells that constitutively express the CD4 receptor (HeLa-P4) and appears to have a high binding to CD4 in the conditions tested.

Preliminary inhibition assays performed in Jurkat cells, a Human T lymphocyte cell line, in the presence of the VL-B12 construct show no apparent HIV-1 NL4-3 inhibition, indicating that VL-B12 -CD4 binding alone is not sufficient to block virus-cell fusion and that a steric effect due to the presence of a larger molecule may be necessary for HIV-1 fusion inhibition. The VL-B12 will be tested in inhibition assays in interaction with different molecules to further evaluate its HIV-1 inhibition potential.

This VL-B12 appears to be very promising for CD4 targeting and a valuable mediator of biopharmaceuticals.

Reference

155

Anti-microbial activity of N,N'-dioxide quinoxaline and phenazine derivatives

Vieira, M. (1 2 3); Fernandes, R. (1); Ribeiro da Silva, M. D. M. C. (2); Noronha, J. P. (3); Prudêncio, C. (1)

1: Ciências Químicas e das Biomoléculas, Escola Superior de Tecnologia da Saúde, Instituto Politécnico do Porto;
2: Departamento de Química, Faculdade de Ciências, Universidade do Porto;
3: Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

E-mail: mav@estsp.ipp.pt

Keywords: anti-microbial activity, quinoxalines, phenazines

Abstract

The nitrogen heterocyclic organic compounds 1,4 dioxide derivatives, such as quinoxalines and phenazines, have been widely investigated due to the interest for their use as synthetic drugs, with wide application. Several studies have reported their pharmacological activity, particulary as antibacterial agents [1, 2, 3, 4]. It has been established a relation between energetical and structural properties and biological activity, once these compounds present *N* – oxide bonds, increasing their oxidative capacity.

The thermochemical study of seven *N,N´*-dioxides: 2-hidroxiphenazine dioxide (2HF), 2-methyl-3-benzoylquinoxaline-1,4-dioxide (2M3BQNX), 2-methyl-3—benzylquinoxaline-1,4-dioxide (2M3BZQNX), 3-methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC), 3-methyl-N-(2-methylphenyl)-2-quinoxalinecarboxamide-1,4-dioxide (3MN2MNFQNXC),3-amino-2-quinoxalinecarbonitrile-1,4-dioxide (3A2QNXCN) and 2-amino-3-cianoquinoxaline-1,4-dioxide (2A3CNQNX) has been recently developed in order to establish relationships among the energetical, structural and reactivity properties [5, 6, 7].

The present work reports the study of anti-microbial activity for the seven referred compounds against bacteria *Staphylococcus aureus, Escherichia coli* andyeast *Saccharomyces cerevisiae* and *Caloplaca stellata,* with determination of minimal inhibitory concentration (MIC), by means of microdilution by plate method.

[1] V. K. Tandon, D. B. Yadav, H. K. Maurya, A. K. Chaturvedi, P. K. Shukla, BioInorganic and Medicinal Chemistry, 14, 6120-6126, 2006
[2] A. Carta, G. Paglietti, M. E. R. Nikookar, P. Sanna, L. Sechi, S. Zanetti, Eur. J. Med. Chem., 37, 355-366, 2002
[3] A. De Logu, L. H. Palchykovska, V. H. Kostina, A. Sanna, R. Meleddu, L. Chisu, I. V. Alexeeva, A. D. Shved, Int. J. Antimic. Agents, 33, 223-229, 2009
[4] H. J. Lee, J. S. Kim, M. E. Suh, H. J. Park, S. K. Lee, H. K. Rhee, H. J. Kim, E. K. Seo, C. Kim, C. O. Lee, H. Y. Park Choo, Eur. J. Med. Chem., 42, 168-174, 2006
[5] J. R. B. Gomes, M. D. M. C. Ribeiro da Silva, M. A. V. Ribeiro da Silva, Chem. Phys. Letters, 429, 18-22, 2006
[6] M. D. M. C. Ribeiro da Silva, J. R. B. Gomes, J. M. Gonçalves, E. A. Sousa, S. Pandey, W. E. Acree, Jr., J. Org. Chem., 69, 2785-2792, 2004
[7] E. A. Sousa, J. M. Gonçalves, L. Gales, A. M. Damas, P. Gomes, S. Pandey, W. E. Acree, Jr., M. D. M. C. Ribeiro da Silva, J. Phys. Org. Chem., 20 (7), 491-498, 2007

Reference

156

Design of synthetic single domain VL antibodies that inhibit HIV-1 infection through binding to gp41 ectodomain

Santos, Catarina (1,2); Couto, Andreia (1,2); Gonçalves, João (1,2)

1: URIA-CPM Faculdade de Farmácia da Universidade de Lisboa, Portugal;
2: IMM-Instituto de Medicina Molecular, Universidade de Lisboa

E-mail: catarinasnts@yahoo.com

Keywords: single domain VL antibodies, gp41, synthetic library; six-helix bundle, intermediate pre-harpin state, heptad repeat 1, loops CDR1 and CDR3

Abstract

The entry of Human Immunodeficiency Virus type 1 (HIV-1) is mediated by a surface viral glycoprotein (gp120) and a transmembrane viral protein (gp41). Gp120 is responsible for the recognition and binding to the CD4 cellular receptor, while gp41 promotes the fusion process. This process begins with the binding of gp120 to CD4, which induces conformational rearrangements in the gp41 protein and folding it into an intermediate pre-harpin state. The binding of gp120 to its co-receptor induces changes in gp41 conformation that culminate in a trimer-of-harpins (six-helix bundle structure), a more stable conformation. The six-helix bundle is formed by three heptad repeat 2 (HR2) regions that pack in an antiparallel fashion against hydrophobic grooves in the coiled-coil formed by three heptad repeat 1 (HR1) regions. The energy released in the formation of the six-helix bundle triggers the fusion of the viral and cellular membranes. The intermediate pre-harpin state represents a major target in the inhibition of HIV-1, due to the fact that is conserved (among HIV-1 strains) and crucial regions of gp41 are only exposed in this conformation.

The aim of this project is the inhibition of HIV-1 entry, through binding of single domain antibodies to conserved regions of the gp41 ectodomain, like HR1. In this work, a synthetic library of rabbit single domain VL antibodies, grafted in CDR1 and CDR3 was constructed. The CDR1 and CDR3 loops contain twenty two amino acids and twelve amino acids in each loop were randomized with only four amino acids in each position (Ser, Tyr, Ala and Asp). The synthetic library was screened by phage display against the complete gp41 ectodomain and against the HR1 region alone. The selected single domain VL antibodies were tested for binding specificity in ELISA. Preliminary results show that single domain VL antibodies can inhibit HIV-1infectivity at variable levels, depending on expression levels and binding to the gp41 ectodomain and HR1 peptide.

The HR1 region of gp41 presents itself like a good target to the discovery of new drugs and the selected single domain VL antibodies seem to be a promising alternative to the actual fusion inhibitors of HIV-1.

Reference

179

Activity of the N,N'-dioxide quinoxaline derivates on human cells

Fernandes, Rúben (1); Vieira, M. (1,2,3); Ribeiro da Silva, M.D.M.C. (2); Sousa, R. (4); Malheiro, I. (4); Prudêncio, C. (1)

1: Escola Superior de Tecnologia da Saúde, Portugal;
2: Departamento de Química, Faculdade de Ciências, Universidade do Porto;
3: Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa;
4: Laboratório de Citogenética, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto

E-mail: rpf@estsp.ipp.pt

Keywords: quinoxalines, antibacterial, cytotoxicity

Abstract

In the latest years quinoxalines and their poly-functional derivatives have been largely studied for pharmacological and medical purposes. Recently some studies point to their antimicrobial interest.
Our group has been studying the antibacterial properties of N,N'-dioxide quinoxaline derivates, namely quinoxaline N,N-dioxide: quinoxaline 1,4-dioxide (QNX), 2-methylquinoxaline 1,4-dioxide (2METQNX), 6-chloro-2,3-dimethylquinoxaline 1,4-dioxide (2,3METCLQNX) and 3-benzoyl-2-methylquinoxaline 1,4-dioxide (2METBQNX).
In the present work we have studied the activity of some N,N'-dioxide quinoxaline derivates on human lymphocytes. For this purpose, the mitochondrial dehydrogenase activity of the lymphocytes was measured. The principle of the method was the reduction of a tetrazolium salt [2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salts]. The mitochondrial dehydrogenases of viable cells reduce the tetrazolium ring, yielding an orange formazan derivative, which is water soluble and is measured spectrophotometrically. The degree of the N,N'-dioxide quinoxaline derivates cytotoxicity was indicated by the amount of formazan produced.

Reference

180

Optimization of recombinant production of human cytochrome P450 3A4

Rosa, Luis Filipe Morgado (1, 2); Ferreira, Guilherme N. M. (1, 2)

1: IBB, Portugal;
2: CBME, Universidade do Algarve

E-mail: lfmrosa@ualg.pt

Keywords: CYP3A4, Recombinant Production, Bioreactor, Fed-Batch

Abstract

CYP3A4 is a haemic membrane-anchored protein, the most abundant cytochrome expressed in the human liver. It is responsible for the metabolization of approximately 50% of all pharmaceuticals in the market, and its activity results in the hydroxylation of cyclic aliphatic molecules of the therapeutic drugs. It is also responsible for the metabolization of endogenous compounds, such as testosterone. Due to the wide range of suitable substrates, and the in vivo importance of this enzyme, it has been the focus of increased interest in pharmacodynamics and biotechnology, where the application of P450 enzymes for synthetic purposes was enhanced. The use of P450s in biosensors and biochips has great potential in medical diagnostics, environmental monitoring, and food quality control.

In order to maximise recombinant CYP3A4 production, the expression of the protein was optimized in batch and fed batch operation modes in 2L bioreactors using *Escherichia coli*. Results indicate that dissolved oxygen is a key parameter to obtain high levels of active enzyme. The enzyme produced was purified by IMAC and SEC to obtain 15.5 mg per litre of growth medium.

Acknowledgments: The authors acknowledge funding from the Portuguese Foundation for Science and Technology, particularly project POCI/QUI/63166/2004 and the PhD grant SFRH/BD/36294/2007, and IBB/CBME, LA, FEDER 2010.

Reference

182

Evaluation of the potential application of recombinant frutalin in cancer therapeutics

Oliveira, Carla; Peixoto, Joaquim; Teixeira, José A.; Domingues, Lucília

IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Portugal

E-mail: carlaoliveira@deb.uminho.pt

Keywords: Cytotoxicity, Human breast cancer (T47D cell line), Human cervical cancer (HeLa cell line), Lectin, Recombinant frutalin

Abstract

Frutalin is the alpha-D-galactose-binding jacalin-related lectin expressed in breadfruit seeds (*Artocarpus incisa*). This lectin may be used in cancer diagnostics/therapeutics due to its potential ability to recognise specific carbohydrates expressed in cancer cells membranes and/or cells surface receptors. However, the extraction of frutalin from its natural source has several disadvantages, as it is a time-consuming process, with relative low yields, and typically results in a heterogeneous mixture of different natural isoforms. Frutalin isoforms may have distinct biological properties leading to undesired results variability when applied in cancer diagnosis and therapy. In order to overcome these limitations frutalin was expressed and produced in the *Pichia pastoris* expression system [1]. The ability of recombinant frutalin to recognise human tumour cells has already been demonstrated in immunohistochemical studies conducted with human prostate tissues removed from patients by surgery. Moreover, it showed higher capacity than native frutalin to differentiate malign from benign prostate diseases, highlighting its potential diagnostic application, namely as a bio-marker of prostate cancer [2]. In this work, the ability of recombinant and native frutalin to inhibit cancer cells proliferation was studied *in vitro* and the results obtained for each lectin were compared. Recombinant and native frutalin demonstrated a similar considerable potential therapeutic application, as both lectins strongly reduced cancer cells growth and induced their death. Native frutalin inhibited irreversibly the proliferation of HeLa (human breast cancer) and T47D cells (human cervical cancer) in a time- and dose-dependent manner. Recombinant frutalin, which has less carbohydrate-binding affinity than native frutalin, performed identical cytotoxicity on these cells, at similar concentrations. The two lectins also promoted visible morphological changes in cancer cells. These results indicate that the anti-cancer activity of frutalin is not dependent on its sugar-binding affinity. Therefore, recombinant frutalin might be used as a potential anti-cancer agent, in alternative to native frutalin.

[1] Oliveira C, Felix W, Moreira RA, Teixeira JA, Domingues L. (2008). Protein Expr Purif 60: 188-193.
[2] Oliveira C, Teixeira JA, Schmitt F, Domingues L. (2009). Submitted.

Reference

223

Molecular characterization of portuguese patients with Citrullinemia Type I (urea cycle disease)

Nogueira, Célia Teixeira (1); Leitão, Luzia (1,2); Azevedo, Luísa (3); Domingues, Lucília (2); Vilarinho, Laura (1)

1: Genetical Medical Centre Jacinto de Magalhães, I&D Unit, Genetics Department, INSA, Portugal;
2: IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, 4710-057 Braga, Portugal;
3: IPATIMUP

E-mail: celianogueira55@hotmail.com

Keywords: Citrullinemia Type I; Argininosuccinate synthetase; urea cycle disease; hyperammonemia; ASS1

Abstract

Citrullinemia Type I (CTLN1; MIM# 215700) is a rare autossomal recessive disorder caused by a genetic deficiency of the argininosuccinate synthetase enzyme (ASS; MIM# 603470; E.C.6.3.4.5.). ASS is the third enzyme of the urea cycle involved in the synthesis of arginine and catalyses the conversion of citrulline, aspartate and ATP into argininosuccinate, AMP and pyrophosphate in the cytosol of periportal hepatocytes where the urea cycle is localized but is also expressed in most body tissues involved in synthesis of arginine. The incidence of this disorder is estimated to be 1 in 57,000. Biochemically, CTLN1 is characterized by an extreme elevation of citrulline in plasma and urine, low plasma arginine and increased excretion of orotic acid in urine. Clinically, patients with CTLN1 may be associated with neonatal or infantile-onset citrullinemia (classical form) that is the most common clinical presentation, characterized by symptomatic hyperammonemia leading to life-threatening encephalopathy and mental retardation in surviving patients, and early death when untreated. The other form of this disorder is the “mild citrullinemia” when patients present only biochemical but no clinical phenotype.

The objective of this study was to investigate at a molecular level seven CTLN1 portuguese patients from different regions of country, diagnosed in our Center. Three of them were detected in the extended newborn screening program.

Sequence analysis of genomic DNA from these patients was performed to identify disease-causing mutations in the *ASS1* gene.

In the studied patients, seven mutations were identified, including four *missense*, one *nonsense*, one *splicing* and one deletion causing f*rameshift*. Five of these mutations have already been described in the literature (p.R363W, p.G390R, p.G324S, p.F150LfsX8 and p.R344X), while the other two represent novel mutations (p.Y83C, c.174+1G>T). Only one patient was homozygous for the p.G390R mutation, all the others were combined heterozygotes for different mutations in the *ASS1* gene.

In summary, we described the genetic background of seven patients with CTLN1. Moreover, we found two novel mutations on the *ASS1* gene underlining the genetic heterogeneity of ASS deficiency. Our date corroborate the importance of a molecular testing to confirm CTLN1 patients suspected by a metabolic evaluation or detected by extended newborn screening programs and offer accurate prenatal diagnosis to couples at high risk of having affected children.

Reference

224

Piezoelectric sensors for biorecognition analysis

Rodrigues, Rogério M. M.; Ferreira, Guilherme N. M.

IBB - Institute for Biotechnology and Bioengineering, CBME - Centre for Molecular and Structural Biomedicine, University of Algarve Campus de Gambelas 8005-139 Faro Portugal

E-mail: rogerodrigues@gmail.com

Keywords: Piezoelectric, QCM, Biosensors, Immunosensors, Impedance analysis

Abstract

The formation processes of molecular complexes – such as DNA hybridization, antibody-antigen binding, protein-nucleic acids binding, among others – represent important biochemical processes in nature. These specific recognition processes allow their use for the development of microbioanalytical devices and methods capable of specific molecules and complex structures detection, as well as quantitative characterization of the kinetics involved.

In the present work, we use piezoelectric devices, namely quartz crystal microbalance (QCM), for the study of molecular interactions in liquid medium. The piezoelectric transducers allow the detection of biorecognition label free molecules events. Its operation depends on the mechanical vibration of a quartz crystal where its resonance frequency is sensitive to the changes in mass on the surface.

We demonstrate that these devices operating in liquid environments also respond significantly to the properties of solutions and films that can easily lead to incorrect interpretation of results. Impedance spectroscopy methods were used to characterize the surface of the sensor, and to build a RLC equivalent electrical circuit in order to differentiate all the contributions to the final signal of the sensor [1]. Thus, we are able to differentiate between load mass signals and losses of acoustic energy due to viscoelastic effects. These devices can be used as piezoelectric immunosensors to detect HIV-1 Vif accessory protein in solution. For that purpose, anti-Vif recombinant antibodies were purified from *Echerichia coli* and immobilized on the surface of the sensors, through self-assembled monolayers (SAMs) and used as biorecognition material [2]. The data obtained allowed the determination of kinetic constants of antibody-antigen interaction [3]. The results demonstrate the potential applicability of the developed piezoelectric sensors in biorecognition and quantitative analysis.

[1] J. M. Encarnação et al., Biosens. Bioelectron., 2007, 22, 1351-8.
[2] G. N. M. Ferreira et al., Biosens Bioelectron., 2007, 23, 384-92.
[3] J. M. Encarnação et al., J. Biotechnol., 2007, 132, 142-8.

Reference

232

Bacteriostatic synergistic effect between of crude extract of Brazilian trees leaves

Malvezzi, Cristiane Karina (1, 2); Silva, Silvio Silvério (1, 2); Souza, Jacqueline Stella Barbosa (1); Silva, Emily Ane Dionízio (1); Freitas, José Eduardo (1)

1: Faculdades Integradas Teresa D'Avila - FATEA, Brazil;
2: Universidade de São Paulo - USP, Brazil

E-mail: crismalvezzi@yahoo.com

Keywords: antimicrobial activity, medical plants, *Staphylococcus aureus*, synergism, hydroalcoholic extract

Abstract

The use of medicinal plants in the world, and especially in South America, contributes significantly to primary health care. Many plants are used in Brazil in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy. This study aimed to evaluate the antimicrobial activity of hydroalcoholic extract from leaves of *Eugenia uniflora*, *Syzygium cumini* and association between the extracts against *Staphylococcus aureus* (ATCC25923). The antibacterial activities of extracts were determined by macrodilution techniques in BHI broth. The minimum inhibitory concentration (MIC) was determined by measuring the optical density in the spectrophotometer (540 nm) and was defined as the lowest concentration of crude extract that produced an 80% reduction in visible growth compared with control (non inoculated broth). MIC 80% values were different among extracts. *Eugenia uniflora* presented a good activity against the *S. aureus* with MIC of 2 mg/ml and *Syzygium cumini* presented reduction in visible growth, but less than 80%. The association between the extracts of leaves of different plant species showed the highest antibacterial activity with MIC of 0.5 mg/ml. Inhibitory activity on bacterial growth of the association of the hydroalcoholic extract from leaves of Syzygium cumini with Eugenia uniflora was higher when compared with the inhibition of bacterial growth promoted by extracts alone, indicating a bacteriostatic synergistic effect between these two extracts.

Acknowledgments: FAPESP

Reference

235

Bee propolis effect on protection of RBCs membrane integrity

Moreira, Leandro; Rogão, Mónica; Pereira, Ana Paula; Morais, Margarida; Costa, Bruno; Dias, Luís; Dias, Teresa; Estevinho, Leticia

Instituto Politécnico de Bragança, Portugal

E-mail: monica_crsa@hotmail.com

Keywords: Hereditary Spherocytosis, Propolis, RBC, Membrane oxidation

Abstract

Propolis is a resinous substance collected from plants by bees. The propolis composition depends on the surrounding vegetation, the season, and the area from which derive. This hive product usually contains a variety of chemical compounds such as polyphenols (flavonoids, phenolic acids and esters), steroids, and amino acids.

The hereditary spherocytosis (HS) is a congenital hemolytic anemia, with origin in the modification of membrane proteins of erythrocytes, which leads to increased susceptibility to hemolysis and a decrease of the cell over-life. The HS is the most common red blood cell (RBC) membrane disorder in European Caucasians, with a prevalence of roughly 200-300 per 10^6, and to Japanese population 5.7-20.3 per 10^6.

The aim of the present work was to determine if propolis extracts could affected the red cell membrane integrity and comparing the effect of two propolis extracts from different regions (Bornes - Trás-os-Montes; Fundão - Beira Interior).

In this work, two adults were studied, one with the syndrome HS splenectomized and one healthy used as control. Diagnosis of HS was made by clinical features, identification of spherocytes on peripheral blood smears and abnormal osmotic fragility.

The results show that the two propolis extracts affected the erythrocyte membrane fragility in both individuals (control and patient with HS).

There is also a possibility for an oxidative damage of red blood cell membrane in HS, similar to the one recorded in other hemolytic anemia. Indeed, the spherocytes were found to be more sensitive than normal erythrocytes to the action of oxidation inducing drugs. Phenolic compounds of propolis have a large spectrum of pharmaceutical properties, however the more studied was the antioxidant activity. The second aim of the present work was to determine if hemolysis of RBCs could be induced by oxidative stress conditions, and to verify if the propolis can inhibit the hemolysis doing its antioxidant properties. Results show that when RBCs of patient with HS were incubated with 1 mM of H_2O_2, the hemolysis with and without oxidant had different levels. This effect was blunted when the RBCs were incubated with propolis extract, which might indicate that propolis act as free radical scavenger protecting the membrane integrity against oxidative effect.

Reference

248

Fungidal and bacterial activity of N,N-dimethyl-4-(2,2,2-trichloro-1-(phenylamino)ethyl)aniline

Ferraz, Ricardo (1,2); Petrovski, Zeljiko (2); Fernandes, Ruben (1); Noronha, João P. (2); Prudêncio, Cristina (1,2)

1: Ciências Químicas e das Biomoléculas, Escola Superior de Tecnologia da Saúde, Instituto Politécnico do Porto;
2: REQUIMTE/CQFB Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

E-mail: ricardoferraz@eu.ipp.pt

Keywords: organic synthesis, drugs

Abstract

N,N-dimethyl-4-((phenylamino)methyl)aniline **(1**) was prepared by condensation of aniline and 4-(dimethylamino)benzaldehyde [1] *N,N*-dimethyl-4-(2,2,2-trichloro-1-(phenylamino)ethyl)aniline (**2**) was synthesized by trichloromethylation of the imine (*N,N*-dimethyl-4-((phenylimino)methyl)aniline **(1)**) with trichloroacetic anhydride under microwave irradiation [2] (*Sheme 1)*.

The present work reports the study of bacterial and yeast activity for the compound **2**. The bacteria used in this study are *Staphylococcus aureus, Escherichia coli* andtheyeast are *Saccharomyces cerevisiae Candida albican*. The results that we will present are the determination of minimal inhibitory concentration (MIC), by means of microdilution by plate method and the specific growth constants for this microorganism.
Further studies are being performed to determine viability and cellular injury with this drug.

[1] Yu, H.; Fang, Y.; Xia, Y.; Wu, J. Syn Commun., 2006, 36, 2421.
[2] Nongkunsarn, P.; Ramsden, C. A. Tetrahedron, 1997, 53, 3805.

Reference

276

Bacteriophage infection of uropathogenic *Escherichia coli*

Moura, Alexandra (1, 2); Sillankorva, Sanna (1); Oliveira, Dulce (1); Azeredo, Joana (1)

1: Universidade do Minho, Portugal;
2: Escola Superior de Saúde Jean Piaget/Vila Nova de Gaia

E-mail: alexandramoura1@gmail.com

Keywords: Urinary Tract Infections, Antibiotic, Resistance, Bacteriophages

Abstract

Urinary tract infections (UTI) are among the most common bacterial infections affecting a large proportion of the worldwide population, especially women. These infections are mainly caused by *Escherichia coli* that have the ability to adhere and invade the urothelium, causing recurrent infections as a result of the formation of intracellular bacterial communities. This pattern of bacterial growth makes their eradication by antibiotic therapy a serious problem, since penetration of the bacterial biofilm by the antibiotics is not effective, which sheds a renewed interest in finding effective alternatives to such antibiotherapy.

The experimental work described herein aims at determining the lytic spectrum of three different colibacteriophages against clinical isolates of *E. coli*. The bacteriophages used in this work were from DSMZ international collection (T1, T4 and phi X174) and their lytic performance was tested against clinical isolates of *E. coli* obtained from Hospital São Marcos (Portugal), most of them showing resistance to antibiotics. Bacteriophage phi X174 was able to infect 5.80% of the clinical isolates, while bacteriophage T4 showed efficacy against 21.74% when acting upon a pool of 69 (analysed) bacterial strains. Bacteriophage T1 showed the broadest lytic spectrum, by infecting ca. 27.54% of the 69 strains of *E. coli* evaluated, acting even in very resistant ones. Moreover, bacteriophage T1 was found to be stable and active in urine, under in vitro conditions. The results presented herein clearly indicate that bacteriophages might be considered an effective alternative to antibiotics for therapeutic and/or preventive actions against UTI.

Reference

287

Kinetic study of nordihydroguaiaretic acid recovery from *Larrea tridentata* by microwave-assisted extraction

Martins, Silvia Lopes Ferreira (1); Aguilar, Cristóbal Noe (2); De La Garza-Rodriguez, Iliana (2); Mussatto, Solange Inês (1); Teixeira, José António (1)

1: University of Minho, Portugal;
2: Autonomous University of Coahuila, Mexico

E-mail: silviamartins80@gmail.com

Keywords: Nordihydroguaiaretic acid, *Larrea tridentata*, Microwave-assisted extraction

Abstract

Nordihydroguaiaretic acid (NDGA) is a powerful antioxidant that can be found in plants like *Larrea tridentata* (*Zygophyllaceae*), also known as creosote bush, which grows in semidesert areas of Southwestern United States and Northern Mexico [1]. Several studies have demonstrated that NDGA has important biological activities with great interest in the health area, such as antiviral, cancer chemopreventive, and antitumorgenic activities [2]. Extraction of bioactive compounds from plants is conventionally performed using a heat-reflux extraction method. However, different techniques have been developed in order to decrease extraction time and solvent consumption, as well as to increase the extraction yield and enhance the extracts quality [3]. The objective of this study was to develop a microwave-assisted extraction (MAE) method for NDGA recovery from *Larrea tridentata* leaves, and compare the obtained results with those found by using the conventional heat-reflux extraction (HRE).

The MAE method consisted in suspending the leaves in methanol, and the obtained suspensions were irradiated with microwaves at 800 W in a pre-setting procedure where after each period of 1 min the sample was allowed to cool at room temperature. Different methanol concentrations (25, 50, 75 and 100 % v/v) and solid/liquid ratios (1/5, 1/10, 1/20 and 1/30 w/v) were tested. Conventional extraction of NDGA was performed using a heat-reflux system. The extraction temperature for both methods was 70 ± 2°C. NDGA was quantified by HPLC, and samples of the MAE treated material were examined by scanning electron microscopy in order to verify the treatment influence on the structure of the plant.

The extraction time of NDGA from *Larrea tridentata* leaves was significantly reduced (p<0.05) from 18 to 1 min when MAE was used instead of the HRE. In addition, significantly higher (p<0.05) yields of NDGA were obtained by MAE comparing with HRE (3.79 ± 0.65 and 3.42 ± 0.19 %, respectively). The optimum conditions for NDGA extraction consisted in using 50% (v/v) methanol as extraction solvent in a solid/liquid ratio of 1/10 (g plant material/ ml extraction solvent). Scanning electron micrographs demonstrated that the improvement of NDGA extraction by MAE might be related to a greater extent of cell rupture of the plant material. In conclusion, MAE was proved to be a fast and efficient method for NDGA extraction from *Larrea tridentata* leaves comparatively to conventional heat-reflux extraction.

[1] Ross, I.A. (2005). Larrea tridentata. In: Medicinal Plants of the World - Chemical Constituents, Traditional and Modern Medicinal Uses (Volume 3), Ross. I.A. (Ed.). Humana Press, New Jersey, pp. 263-270.
[2] Lambert, J.D., Dorr, R.T., Timmermann, B.N. (2004). Pharmaceutical Biology, 42: 149-158.
[3] Wang, L. and Weller, C.L. (2006). Trends in Food Science & Technology, 17: 300-312.

Reference

325

Bactericidal effect of essential oil of Angolan *Cymbopogon citratus* and its majority component

Soares, Marta Isabel Lopes Rodrigues de Oliveira (1); Silva, Andreia (1); Coutinho, Filipe (1); Vinha, Ana Critina Ferreira (1); Herdeiro, Teresa (1); Catarino, Pedro (1); Estevez, José M. García (2); Iglesias, Raúl (2); Machado, Marisa (3)

1: Escola Superior de Saúde do Vale do Ave, Instituto Politécnico de Saúdo do Norte, Cooperativa Ensino Superior Politécnico e Universitário;
2: Laboratorio de Parasitología, Facultad de Biología, Universidad de Vigo;
3: Faculdade de Farmácia/CEF, Universidade de Coimbra, 3000 Coimbra, Portugal

E-mail: marta.soares@ipsn.cespu.pt

Keywords: Cymbopogon citratus, Óleo essencial, Citral, Actividade antibacteriana, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, Klebsiella penumoniae, Proteus mirabilis

Abstract

Cymbopogon citratus (DC) Stapf. (erva príncipe), Poaceae – Gramineae, originated in India but it is also grown elsewhere in tropical and sub-tropical as Angola, where this is of great importance in the country's traditional medicine. Is consumed as a beverage aromatic and used in traditional by its lemon fragrance. It is also used in traditional medicine of almost all continents and covers a wide range of such indications of disorders digestives, infectious, inflammatory, nerve, as well as other health problems. Its use in traditional medicine covers a wide range of indications by tradition, trust, and lack of economic power. The antimicrobial properties of essential oils arouse interest are an alternative to consumers in the natural additive practice as a source of food conservation. This work wants to analyze qualitative and quantitative composition of the essential oil of *C. citratus* and assess is activity bacterial *in vitro* and is majority component the citral against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883) e *Proteus mirabilis* (ATCC 25933). The essential oil has been obtained by fresh plant hidrodestilation using the apparatus of Clavenger amended in accordance with the European Pharmacopoeia. The essential oil was analyzed by CG and CG-in, and the constituents were identified from its retention in CG index of two columns of different stages, and from mass spectrum, which were compared with reference data. The bacterial activity of *C. citratus* and citral was evaluated by the dissemination in agar. All experiments were carried out in triplicate on at least three independent tests. The essential oil and the citral revealed an injunction of dose-dependent growth, and *S. aureus* more likely that the *S. epidermidis*. Both showed inhibition from concentrations with 5% of oil in *S. aureus* and *S. epidermidis*. It was found differences statistics to the strains taking into account the concentration used. The statistical difference as regards positive controls means that the effect of compounds tested in these strains of *E. coli*, *K. pneumoniae* and *P. mirabilis* is not equivalent or greater, however there is significance ($p<0,001$) compared with the negative control. The results suggest that this essential oil and its majority component have the potential for use against bacterial infections caused by *S. aureus* and *S. epidermidis*.

Reference

340

Use of Cyclodextrins as scavengers of inhibitory by-products in light controlled in vitro synthesis of RNA

V. Pinheiro, André (1,2); Tavares, João (2); Parola, A. Jorge (1); Lima, J. C. (1); Baptista, Pedro V. (2)

1: REQUIMTE/DQ, Faculdade de Ciencias e Tecnologia, Universidade Nova de Lisboa;
2: CIGMH/DCV, Faculdade de Ciencias e Tecnologia, Universidade Nova de Lisboa, Portugal

E-mail: pmvb@fct.unl.pt

Keywords: Coumarin, Cyclodextrin, Caged-nucleotides

Abstract

We recently reported on the light activation of transcription, where caged nucleotides were applied to *in vitro* transcription reaction in order to obtain a light controlled enzymatic synthesis of RNA. In the absence of light no RNA formation was detected due to efficient blocking by the coumarin moiety. After irradiation caged ATP was released with quantitative precision and RNA polymerization was resumed. In this work,
[7-(diethylamino)coumarin-4-yl]methyl](DEACM) was used as a photolabile protecting group to cage ribonucleotides due to its high extinction coefficients in the visible region of the spectrum, fast deprotection kinetics and absence of radical intermediates. However, the DEACM alcohol (DEACM-OH) photoproduct was shown to inhibit the transcription reaction for concentrations higher than 30 μM [1].

In order to overcome inhibition by the DEACM-OH photoproduct, we evaluated the use of molecular scavengers to sequester DEACM-OH formed after irradiation. α-, β- and
g-cyclodextrins were tested for their ability to remove free coumarin molecules from solution through the formation of supra-molecular complexes by determination of association constants of the coumarin-cyclodextrin complex. The influence of cyclodextrins in transcription reaction was also evaluated.

Our results show that the use of cyclodextrins as scavengers led to an increase of DEACM-OH threshold concentration for inhibition leading to an increase of efficiency of the light controlled *in vitro* transcription.

Acknowledgements: This work was supported by Fundação para a Ciência e Tecnologia (REQUIMTE, CIGMH, PTDC/BIO/66514/2006, PTDC/SAU-BEB/66511/2006, FRH/BD/24276/2005 to A.V.P.).

[1]. A. Vidal Pinheiro, P. Baptista, J.C. Lima, Nucleic Acids Res., 2008, 36, e90.

Reference

375

Functional foods and phytotherapeutic: use as agents of plant development and prevention of public health

Silva, Viviana (1); Coutinho, Filipe (1); Soares, Marta Isabel Oliveira (1,2); Iglésias, Raúl (2); Estevez, José Garcia (2); Vinha, Ana Cristina Ferreira (1,2)

1: Escola Superior de Saúde do Vale do Ave, Instituto Politécnico de Saúdo do Norte, Cooperativa Ensino Superior Politécnico e Universitário, Portugal;
2: Laboratorio de Parasitología, Facultad de Biología, Universidad de Vigo, Espanha

E-mail: martaoliveirasoares@hotmail.com

Keywords: Functional foods; phytotherapeutic; public health; antioxidant; plants of green leaf

Abstract

The current way of life may cause the increase of chronic degenerative diseases, whose treatment is not always possible with pharmacological medication. The Diet Therapy and Phytotherapy are on the rise, associated with functional foods phytotherapeutic action. The antioxidant action of bioactive compounds depends on their chemical structures and their concentrations in food. The main objective of this study was to characterize and evaluate fourteen plants of green leaf of national culture in its physical-chemistry, levels of antioxidant compounds and synergistic effects between them, measured by the method of capture of free radical DPPH. The chemical parameters studied were water content, pH, ascorbic acid content and also total carotenoids. Influenced by the antioxidant activity and solvent-extraction technique, two methods of extraction and four kinds of solvents were used. The extracts were used to determine the level of phenolic compounds, the spectrophotometric method, the Folin-Ciocalteau reagent and expressed as gallic acid equivalents. The measurements were in triplicate using the analysis of variance (ANOVA) and Tukey's test, with significance level of 5%. All plants exhibited a positive relationship between the content of bioactive compounds and their antioxidant action ($p < 0.05$) but not in the chemical profile, although there is statistical significance. In evaluating the method extractor, all solvents showed greater efficacy in method I, promoting the relationship between the solvent and extraction time ($p < 0.05$). Methanol is the solvent with a higher rate of extraction followed by ethanol, boiling water and water at room temperature. All extracts exhibited similar values of inhibition obtained by the synthetic antioxidant BHT. The spinach, broccoli, cabbage, hand, watercress and turnip showed high antioxidant action, values above 70%. Antioxidant action with moderate (50-70%) are the extracts of lettuce, Brussels sprouts, lombardy and cauliflower, while the endives, red cabbage and curly kale had weak antioxidant action. All plants showed levels of ascorbic acid, carotenoids and phenolic compounds different. The synergism between the composition and actions of antioxidants was observed, showing the interest in consumption of vegetables in the diet and its action phytotherapeutic the quality of public health.

Oral Session:
S6 – Cellular Microbiology and Pathogenesis

Reference

119

A new enzyme involved in the N-acetylation of muramic acid residues of peptidoglycan in *Staphylococcus aureus*: Impact on growth, oxacillin resistance and lysozyme sensitivity

Figueiredo, Teresa de Almeida (1); Sobral, Rita Gonçalves (1); Ludovice, Ana Madalena (1, 2); Tabei, Keiko (3); Severin, Anatoly (3, 4); De Almeida, João Manuel Feio (2, 5); De Lencastre, Hermínia (1, 4); Tomasz, Alexander (4)

1: Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Laboratory of Molecular Genetics, R. da Quinta Grande nº6, Apt 127, 2780-156 Oeiras, Portugal; 2: Dep. Ciências da Vida, Fac. Ciências e Tecnologia, Univ. Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal; 3: Wyeth Research, Pearl River, New York, 10965, USA; 4: The Rockefeller University, Laboratory of Microbiology, 1230 York Avenue, New York, NY10065, USA; 5: Centro de Recursos Microbiológicos (CREM), Dep. Ciências da Vida, Fac. Ciências e Tecnologia, Univ. Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal

E-mail: talmeida@itqb.unl.pt

Keywords: *Staphylococcus aureus*; cell wall; virulence; Beta-lactam resistance

Abstract

Staphylococcus aureus, a highly successful human pathogen, presents structural characteristics that provide resistance against the host defense system, namely full insensitivity to lysozyme. This widespread antimicrobial compound, naturally present in the host, is a muramidase which cleaves the glycosidic bond between the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues of peptidoglycan. The mechanism of lysozyme resistance in *S. aureus* is still under study although *O*-acetylation of NAM by OatA O-acetyltransferase seems to be at the molecular basis (1).

Through a database search in the genomes of *S. aureus*, SACOL1951 ORF, annotated as a Mur ligase, was found to be present in all sequenced staphylococcal species. SACOL1951 gene was placed under the control of an inducible promoter in the background of MRSA strain COL. The growth rate and oxacillin inhibition halo of the mutant grown with different inducer concentrations, were evaluated. Cell wall of the mutant grown without inducer was analyzed by RP-HPLC and the most significant structures were identified by mass spectrometry. The impact of the conditional mutation on lysozyme resistance was addressed. The SACOL1951 protein domain architecture suggests a role in peptidoglycan biosynthesis because it comprises two domains: the Mur ligase central domain, and a C-terminal domain of unknown function. In the absence of inducer, the growth rate of the mutant decreased significantly and the oxacillin inhibition halo indicated that this gene is important for β-lactam resistance. The peptidoglycan of the mutant showed lack of acetylation of the muramic acid molecule and a significant replacement of isoglutamine of the stem peptide with glutamate. This block in the amidation of glutamate was previously observed in mutants of the *glnRA* operon (2). The absence of *glnRA* point mutations in SACOL1951 conditional mutant suggests that the step of glutamate amidation is subsequent and probably dependent on SACOL1951 enzymatic action. The conditional mutant grown in the absence of inducer showed increased sensitivity to lysozyme. The results indicate that SACOL1951 protein is involved in two structural modifications of *S. aureus* peptidoglycan: the deacetylation of NAM and the amidation of the stem peptide glutamate, which seem to have an important biological role since they affect simultaneously the β-lactam resistance level and the virulence capacity of the strain.

1) Bera, A., et. al. 2005. Mol. Micro. 55: 778-787.
2) Ornelas-Soares, A., et. al. 1993. J. Biol. Chem. 268: 26268-26272.

Reference

303

Modulation of ubiquitinated aggregates by *Salmonella*

Mesquita, Francisco S.; Thomas, Mair; Holden, David W.

Centre for Molecular Microbiology and Infection, Imperial College, United Kingdom

E-mail: f.mesquita06@imperial.ac.uk

Keywords: Salmonella, ubiquitin, deubiquitinase, SseL

Abstract

Intracellular replication of *Salmonella* enterica is modulated by effectors translocated across the *Salmonella*-containing vacuole (SCV) membrane by *Salmonella* pathogenicity island-2 encoded type III secretion system (SPI-2 T3SS). After entering epethelial cells the SCV migrates to a perinuclear region and establishes selective interactions with the host endocytic system. Bacterial effectors delivered to the host cell by T3SS can interact with the host ubiquitin system. These effectors are capable of mimicking the function of their eukaryotic counterparts by acting as E3 ligases or deubiquiting (DUB) enzymes. This allows them to manipulate different host ubiquitin pathways, such as immune signalling, proteasome degradation and cellular trafficking. Here we describe the formation of ubiquitinated aggregates in the vicinity of the bacteria micro-colony in cells infected with *Salmonella*. We also demonstrate that the clearing of these ubiquitinated aggregates is dependent on the DUB activity of the SPI-2 effector SseL. Further characterization of these aggregates revealed that they co-localize with late endocytic markers recruited by *Salmonella* and that they occur in a SPI-2 dependent manner. Finally we also analyse the contribution of other SPI-2 effectors to the formation of ubiquitin aggregates and the types of ubiquitin chains involved.

Reference

376

Control of cell shape by the novel bacterial core morphogenetic component RodZ

Costa, Teresa (1); Alyahya, Anisah (2); Rodrigues, Joana (1); Jacobs-Wagner, Christine (2); O. Henriques, Adriano (1)

1: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Lisboa, Portugal;
2: Department of Molecular, Cellular and Developmental Biology, Yale University, New haven CT, USA

E-mail: teresac@itqb.unl.pt

Keywords: cell shape, cell cycle, actin cytoskeleton, bacterial morphogenesis

Abstract

How cells acquire and maintain shape is a fundamental question in cell biology. The cell wall and cytoskeleton are major determinants of bacterial cell shape, but the molecular mechanisms linking the two systems are unclear. RodZ is a conserved protein, recently found to be a key determinant of cell shape in the Gram-negative organisms *Escherichia coli* and *Caulobacter crescentus*. In addition, overproduction of RodZ in the Gram-positive bacterium *Bacillus subtilis* caused gross alterations in the normal rod shape of the cell. Here, we describe a *rodZ* deletion mutant of *B. subtilis* that forms short rods with increased width, spherical and crescent-shaped cells, thus establishing the requirement for RodZ in the control of cell shape in this organism. We have investigated the localization of RodZ in a merodiploid strain expressing *gfp-rodZ* from the ectopic, non-essential *amyE* locus. A GFP-RodZ fusion localizes in a helical pattern extending along the long axis of the cell, independently of the tubulin homolog FtsZ, and thus independent on cell division. In contrast, assembly of the RodZ-GFP helical structures requires the cytoskeletal actin homolog MreB. Time-lapse microscopy showed that the RodZ helical pattern is maintained during the cell cycle and that the helices are highly dynamic, a behavior also documented for MreB and other cytoskeletal proteins. Moreover, FRAP experiments showed that GFP-RodZ molecules have a very rapid exchange behavior. We also show that the RodZ helical structures collapse under conditions where growth is perturbed, and that helices are also lost from cells entering the stationary phase of growth. Together with the structural organization of RodZ, which consists of an N-terminal cytoplasmic domain, followed by a single transmembrane segment and an extracellular domain, these results support a model in which RodZ has a pivotal role in coupling the activities of the cytoskeleton and of the external cell wall biosynthetic machinery.

Reference

392

Regulating the regulators: studies on the role of RNases and small non-coding RNAs in the post-transcriptional control of gene expression in *Salmonella typhimurium*

Silva, Inês Jesus; Saramago, Margarida; Domingues, Susana; Viegas, Sandra Cristina; Arraiano, Cecília Maria

ITQB/UNL- Instituto de Tecnologia Química e Biológica / Universidade Nova de Lisboa, Portugal

E-mail: ijsilva@itqb.unl.pt

Keywords: small RNA, ribonuclease, Salmonella

Abstract

In recent years, hundreds of functional non-coding RNAs have been identified. These RNAs do not encode proteins but have intrinsic functions as regulators. This group of molecules is generally known as small non-coding RNAs (sRNAs) and can be differently classified according to its way of action over their targets (Viegas, S.C. and Arraiano, C.M. 2008).

Eukaryotic and prokaryotic cells contain a wealth of these small RNAs with determinant roles in the post-transcriptional control of gene expression. In pathogenic bacteria, a large number of sRNAs coordinate adaptation to stress and expression of virulence genes. We have analysed the expression profiles of four sRNAs (MicA, SraL, CsrB and CsrC), conserved among many enterobacteria, in different growing conditions. This has given us valuable information about the conditions of expression of these sRNAs in Salmonella, which are probably related to their function and targets in this bacterium. Namely, the higher levels of these sRNAs in growth conditions under which the expression of the *Salmonella* Pathogenicity Island (SPIs) genes is induced may indicate a relation with virulence functions.

To understand the regulation by sRNAs it is fundamental to study their turnover since this may impact on the regulatory capacity of a sRNA. The specific ribonucleases (RNases) involved in the degradation of these sRNAs should be one of the main factors responsible for these differences (Andrade, J.M. *et al*. 2009). We have constructed *Salmonella typhimurium* mutants for several endo- (RNase E and RNase III) and exo- (RNase II, RNase R and PNPase) ribonucleases and for Poly(A) Polymerase I (PAP I). The processing and stability of the four sRNAs was then analyzed in the RNase mutant strains constructed and cleavage sites were mapped in order to understand the mechanism of decay (Viegas *et al*. 2007).

We also purified several of these main ribonucleases of *Salmonella* and performed *in vitro* activity assays to clarify their role on the sRNAs processing and decay. The characteristics of these RNases were compared to homologous proteins in other human pathogens namely *Escherichia coli* and *Streptococcus pneumoniae*.

In this work we have identified some of the main RNases involved in the post-transcriptional control of each of these sRNAs. Our global results give important information about the expression, processing and turnover of sRNAs, bringing insight into the mechanisms of pathogenesis and sRNA function in *S. typhimurium*.

- Viegas SC and Arraiano CM. Regulating the regulators: How ribonucleases dictate the rules to control small non coding RNAs. RNA Biology 2008; 5(4)
- Andrade J, Pobre V, Silva IJ, Domingues S and Arraiano CM. The role of 3'–5' exonucleases in RNA degradation Prog Mol Biol Transl Sci 2009;85:187-229
- Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vogel J, Arraiano CM. Characterization of the role of ribonucleases in Salmonella small RNA decay. Nucleic Acids Res 2007; 35:7651-64

Reference

187

Bacterial peptidoglycan secondary modifications modulate the *Drosophila* innate immune response

Atilano, Magda (1); Yates, James (1); Ligoxygakis, Petros (2); Filipe, Sergio (1)

1: Instituto de Tecnologia Química e Biológica (ITQB-UNL), Portugal;
2: Department of Biochemistry, University of Oxford,UK

E-mail: atilano@itqb.unl.pt

Keywords: Bacterial peptidoglycan, Teichoic acids, *Staphylococcus aureus*, Innate immunity

Abstract

Both vertebrate and invertebrate organisms are able to detect invading bacteria and mount an immune response aimed at their elimination. The fly model organism, *Drosophila melanogaster*, triggers an innate immune response upon recognition of bacterial peptidoglycan (PGN) by the Peptidoglycan Recognition Proteins (PGRPs). This response consists in the activation of a cascade of events that leads to the production of different antimicrobial peptides (AMPs).

PGN from the pathogenic bacteria *Staphylococcus aureus* can be recognized by the secreted, low molecular weight protein PGRP-SA, triggering an efficient and specific activation of the Toll pathway with the consequent production of drosomycin and other AMPs. PGN is an essential component of bacterial cell wall (CW) and is composed by alternating sugar units, N-acetylglucosamine and N-acetyl muramic acid, cross-linked by short peptides whose composition depends on the bacterial species. The PGN produced by *S. aureus* can present further secondary modifications such as the attachment of additional glycopolymers - the wall teichoic acids (WTA) or the capsular polysaccharides.

In an attempt to understand the impact of PGN secondary modifications on its recognition by the immune system, we constructed *S.aureus* mutants lacking WTA (*ΔtagO*) and purified their CW and PGN. In this work we show that both CW and PGN purified from *S. aureus* unable to produce teichoic acids could bind more efficiently a recombinant PGRP-SA than the equivalent products produced by the parental bacterial strain. Accordingly, injection of CW and PGN purified from *S. aureus tagO* null mutant into live Drosophila flies led to a more efficient activation of the Toll pathway than those products produced by the parental bacterial strain.

Efforts to identify the difference between the PGN of the *S. aureus tagO* null mutant and the parental strain, as purification of PGN implies chemical removal of bound WTA, showed that PGN from the mutant strain was significantly less crosslinked. These results show that the attachment of teichoic acids and an efficient crosslinking contribute to prevent detection of bacterial PGN by the host.

Reference

161

Identification of new cellular proteins essential for HIV-1 replication: the importance of kinases and phosphatases in HIV-1 life cycle

Rato, Sylvie (1,2); Maia, Sara (1,2); Resende, Leonor (1,2); Brito, Paula M (1,2); Pereira, Carina F (1,2); Moita, Catarina (2); Freitas, Rui P (2); Hacohen, Nir (3,4); Moita, Luis Ferreira (2); Gonçalves, João (1,2)

1: URIA-CPM, Faculdade de Farmácia da Universidade de Lisboa, Portugal;
2: IMM, Faculdade de Medicina da Universidade de Lisboa;
3: Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Charlestown, MA, 02129, USA;
4: Broad Institute of Harvard and MIT, Cambridge, MA, 02142, USA

E-mail: sylvierato@ff.ul.pt

Keywords: HIV-1; shRNA; T lymphocytes; druggable proteins; kinases and phosphatases.

Abstract

Progression of HIV-1 infection is largely dependent on the interaction between the host system and the virus. Understanding cellular pathways and proteins involved in the multistep process of HIV-1 infection may result in the discovery of more adapted and effective therapeutic targets.
Kinases and phosphatases are a druggable class of proteins critically involved in the regulation of signal pathways of eukaryotic cells. Here, we focused in discovering kinases and phosphatases that are essential for HIV-1 replication in T-cells and non-deleterious for the cell. For this purpose we used a short-hairpin-RNA (shRNA) library enriched for all human kinases and phosphatases. This library is constructed in a lentiviral vector, allowing transduction of mammalian cells with gene specific shRNA leading to stable and long-term effective gene suppression.
In this work, we developed a shRNA screening in Jurkat T-cells, starting with the transduction of the lentiviral library expressing kinases- and phosphatases-shRNAs and the subsequently challenge with HIV-HSA. Cells resistant to HIV-1 infection were selected by lack of HSA expression and individual clones were isolated after long-term cell culture. We identified new 14 different proteins essential to HIV-1 replication in T- lymphocytes that do not affect cell viability. This group of proteins includes kinases belonging to MAPK, JNK and ERK pathways, a phosphatase involved in pleiotropic cellular functions and proteins implicated in DNA repair.
We focused in the identification of HIV-1 replication steps affected by knock-out expression of these proteins. 3-5 shRNA for each identified gene were re-cloned and replication assays were performed with the most efficient shRNAs. We observed that HIV-1 replication was drastically inhibited in all shRNA clones, being this inhibition progressive and cumulative over seven days of viral growth. We have not detected differences in cell entry and integration, suggesting a role of these proteins during viral transcription/translation. This assumption is supported by results showing that Gag expression was inhibited in HIV-1 resistant T-cells and by shRNA transient assays that show some proteins with role in HIV-1 transcription.
This study brings new insights for the complex interplay of HIV-1/host cell, showing additional knowledge on cellular pathways that are essential for HIV-1 replication but non-important for cell viability and opens new possibilities for antiviral strategies.

Reference

122

The lvfH gene of *Listeria monocytogenes* encodes a novel virulence factor highly activated during infection

Carvalho, Filipe; Camejo, Ana; Ferreira, Pierre; Sousa, Sandra; Cabanes, Didier

IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal

E-mail: filipec@ibmc.up.pt

Keywords: *Listeria monocytogenes*, infection, virulence factor, pathogenesis

Abstract

Listeria monocytogenes is a highly adaptable Gram-positive bacterium that can induce potentially lethal listeriosis in immunocompromised human hosts. As a facultative intracellular pathogen, *L. monocytogenes* is able to invade and replicate inside different eukaryotic cell types, and by spreading from cell to cell, disseminate infection. This process is highly dependent upon the expression of specialized listerial proteins, which act as virulence factors on specific steps of the bacterial infectious cycle. Our recent studies on the *L. monocytogenes* EGDe transcription profile in infected mice revealed several genes that were highly activated throughout the infection timeline and that could be involved in virulence. Among these is *lvfH*, a gene absent from the closely related non-pathogenic *Listeria innocua* genome, which encodes a protein putatively involved in the L-rhamnose biosynthesis pathway. In order to confirm and characterize the role of this gene in *L. monocytogenes* virulence, we generated an *lvfH* deletion mutant and investigated the influence of this mutation on the *Listeria* infectious process. In vitro assays demonstrated that the mutant appeared to be unaffected in its host cell membrane-adhering properties, but was significantly impaired in its ability to invade different mammalian cell lines. Organs of mice intravenously infected with the *lvfH* mutant displayed a significantly decreased bacterial load, as compared to wild type-infected mice. This phenotype was unrelated with an intrinsic growth defect of the mutant, since it presents a growth profile in broth and within murine macrophages similar to the wild type strain. Animal organs infected with an *lvfH*-complemented strain showed infection levels comparable to those observed with the wild type strain, confirming that *lvfH* is required for full virulence in this model. Further work will be focused on the functional characterization of *lvfH* and its integration in the complex mechanisms of *L. monocytogenes* pathogenesis.

Camejo et al. 2009. PLoS Pathog 5(5): e1000449.

Reference

362

The protective IFN-gamma-dependent activation of macrophages induced during infection with *Mycobacterium ulcerans* is impaired by the exotoxin mycolactone

Torrado, Egídio (1); Fraga, Alexandra G. (1); Logarinho, Elsa (1); Martins, Teresa G. (1); Carmona, Jenny A. (1); Gama, José B. (1); Carvalho, Maria A. (2); Proença, Fernanda (2); Castro, António G. (1); Pedrosa, Jorge (1)

1: Life and Health Sciences Research Institute (ICVS). School of Health Sciences, University of Minho. Braga, Portugal;
2: Chemistry Research Center. School of Sciences. University of Minho. Braga, Portugal

E-mail: afraga@ecsaude.uminho.pt

Keywords: M. ulcerans; mycolactone; IFN-gamma

Abstract

Mycobacterium ulcerans is the etiological agent of a necrotizing cutaneous disease, known as Buruli ulcer (BU). The pathology caused by this pathogen is associated with the production of the lipidic exotoxin mycolactone. Following our recent demonstration of an intramacrophage growth phase for *M. ulcerans*, we investigated the biological relevance of interferon (IFN)-γ, as well as the mechanisms activated by this cytokine in *M. ulcerans*-infected macrophages.

We used three different *M. ulcerans* strains selected based on their virulence for mice and the type of mycolactone produced: the low virulent mycolactone-negative strain 5114; the intermediate virulent, mycolactone C-producing strain 94-1327; and the highly virulent, mycolactone D-producing strain 98-912.

IFN-γ-deficient mice showed an increased susceptibility to infection only with strains 5114 and 94-1327, suggesting that this cytokine plays a protective role in infections with the low and intermediate virulent strains of *M. ulcerans*, but not with the highly virulent strain. In line with this, IFN-γ-activated mouse primary bone marrow-derived macrophages controlled the proliferation of the low virulent and the intermediate virulent strains, the latter only at low multiplicities of infection. The effector mechanisms induced by IFN-γ in infected macrophages leading to *M. ulcerans* growth restriction involved both phagosome maturation and acidification, as well as increased nitric oxide production. In agreement, the addition of mycolactone D, purified from cultures of the highly virulent strain, led to a dose-dependent inhibition of phagosome maturation and nitric oxide production in IFN-γ-activated cultured-macrophages infected with the mycolactone-negative strain, resulting in an increased bacterial burden.

Our results suggest that the protection mediated by IFN-γ during the intramacrophage phase of *M. ulcerans* infection depends on the type and amount of mycolactone produced.

Poster Session:
S6 – Cellular Microbiology and Pathogenesis

Reference

138

New tyrosine-phosphorylated proteins involved in *Listeria monocytogenes* cellular infection

Almeida, Maria Teresa; Cabanes, Didier; Sousa, Sandra

Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal

E-mail: srsousa@ibmc.up.pt

Keywords: Cellular infection, *Listeria monocytogenes*, cell signaling pathways

Abstract

Listeria monocytogenes is a human food borne pathogen that may lead, in particular in immunocompromised individuals, to a severe disease characterized by septicemias, meningitis, meningo-encephalitis and abortions. The study of the cell biology of *Listeria* infectious process provided insights in the way bacteria manipulate the host and revealed unsuspected functions of cellular proteins. To cause infection pathogens interfere with crucial host intracellular pathways, different pathogens often hijack the same signaling pathways. In particular, host phosphorylation cascades are preferential targets of infecting bacteria.

In this study, using *L. monocytogenes* as a pathogen model, we showed that eukaryotic cells present a variable protein phosphorylation pattern upon infection. We addressed in particular the tyrosine phosphorylated protein profile triggered by *Listeria* infection and identified the motor protein, Myosin IIA (MyoIIA), differentially tyrosine-phosphorylated in response to *Listeria* uptake.

We demonstrated that MyoIIA is not only tyrosine-phosphorylated over the time of infection, but is also recruited with actin at the bacteria entry site. In addition, we were able to show that the inhibition of MyoIIA activity affected *Listeria* entry into non-phagocytic cells. Surprisingly, the reduction of MyoIIA expression using RNAi techniques resulted in an increased *Listeria* uptake. Together these data point to the role of a novel myosin class in the internalization of *Listeria*, correlating for the first time, myosin posttranslational modifications and *Listeria* infection.

Reference

157

Both central ectodomain and Second Heptad Repeat (HR2) from HIV-2 CCR5/CXCR4-independent human immunodeficiency 2 primary isolates transmembrane glycoproteins affect the dynamics of envelope glycoproteins complex

Santos-Costa, Quirina (1); Parreira, Ricardo (2); Azevedo-Pereira, José Miguel (1)

1: CPM-URIA Faculty of Pharmacy University of Lisbon, Portugal;
2: Unidade de Virologia/Unidade de Parasitologia e Microbiologia Médicas (UPMM), Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa

E-mail: santos_costaq@yahoo.com

Keywords: Env glycoproteins; HIV-2; CCR5; CXCR4; Amino acid motifs; coreceptor usage.

Abstract

Human immunodeficiency virus 2 (HIV-2) infection is characterized by a slower disease progression and lower transmission rates. The molecular features that could be assigned as directly involved in this *in vivo* phenotype remain essentially unknown, and the importance of HIV-2 as a model to understand pathogenicity of HIV infection has been frequently underestimated. The early events of the HIV replication cycle involve the interaction between viral envelope glycoproteins and cellular receptors: the CD4 molecule and a chemokine receptor, usually CCR5 or CXCR4. Despite these chemokine receptors been regarded as the main coreceptors for human immunodeficiency virus 1 (HIV-1) entry into cells, we have previously shown that in some HIV-2 asymptomatic individuals, a viral population exists that is unable to use the main coreceptors described for HIV, namely CCR5 and CXCR4.

The goal of the present study was to investigate whether possible regions in the *env* gene of these viruses might account for this phenotype.

The *env* gene of HIV-2_{MIC97} and HIV-2_{MJC97} was amplified by polymerase chain reaction, using the Expand Long Template PCR System (Roche). Amplified fragments were cloned into the pCR3.1 cloning vector (Invitrogen) and used to transform ultracompetent *Escherichia coli* DH5α-T1 cells (Invitrogen). Doubled-stranded plasmid DNA was purified (Endofree Plasmid Maxi kit – Qiagen). Purified DNA was sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Seven clones of each amplified fragment were sequenced, and a consensus sequence constructed for each individual analysed.

In conclusion, the data generated in this study have shown that HIV-2_{MIC97} and HIV-2_{MJC97} amino acid sequences have differences that could be determinant of the unique phenotype presented by these two primary isolates. The molecular characterization of these *env* genes reveals the existence of remarkable differences in the primary amino acid sequence, particularly in the V1/V2 and C5 region of the surface glycoprotein. Moreover, in the transmembrane glycoprotein some unique sequence signatures could be detected in the central ectodomain and second heptad repeat (HR2). Some of the mutations affect well-conserved residues and, may affect the conformation and/or the dynamics of envelope glycoproteins complex, including the SU-TM association and the modulation of viral entry function.

Santos-Costa Q, Moniz-Pereira J, Mansinho K, Azevedo-Pereira JM. 2009. Virus Res. 2009.
Lin G, Bertolotti-Ciarlet A, Haggarty B, Romano J, Nolan KM, Leslie GJ, Jordan AP, Huang CC, Kwong PD, Doms RW, Hoxie JA. J Virol 2007
McKnight A, Shotton C, Cordell J, Jones I, Simmons G, Clapham PR. J Virol 1996

Reference

185

A screen for chlamydial type III secretion substrates using *Yersinia enterocolitica* as a heterologous system

Almeida, Filipe; Mota, Luís Jaime

Instituto de Tecnologia Química e Biologia, Universidade Nova de Lisboa, Oeiras, Portugal

E-mail: filipebaeta@itqb.unl.pt

Keywords: Pathogenesis, Chlamydia, Type III Secretion, Yersinia, Effectors

Abstract

Chlamydia trachomatis is an important human pathogen (causing sexually transmitted diseases and ocular infections) which belongs to a large group of highly related obligate intracellular bacteria. Chlamydiae are characterised by a developmental cycle comprising two morphologically distinct forms: elementary bodies (EBs), which are capable of invading host cells but are metabolically inert, and reticulate bodies, which are non-infectious but are metabolically active and replicate intracellularly. Chlamydiae are not amenable to genetic manipulation and therefore the knowledge of the mechanisms used by these bacteria to thwart host cells is limited. Yet, chlamydiae are believed to interfere with a wide diversity of host cell processes by using a type III secretion system (T3SS) to deliver several effector proteins into host cells. For example, Tarp and CT694 have been described as chlamydial T3S effectors acting during invasion, or early cycle development. Here, we aimed to identify novel *C. trachomatis* type III secretion (T3S) effectors injected by EBs into host cells. Heterologous T3SSs have been instrumental towards the identification of chlamydial T3S substrates. Therefore, we first tested the suitability of *Yersinia enterocolitica* as a surrogate T3SS. Hybrid proteins comprising the first 10, 20, or 40 amino acids of known chlamydial effectors (IncA and IncC) fused to the N-terminal of mature TEM-1 β-lactamase were expressed and secreted by *Y. enterocolitica* in a T3S-dependent manner, whereas mature TEM-1 alone was expressed but not secreted. These observations confirmed that the *Y. enterocolitica* T3SS can recognise N-terminal T3S signals within chlamydial proteins. Because the amino acid sequence of T3S effectors usually do not display identity to other proteins in the databases, we searched for proteins with no clear function among those that by proteomics studies have been reported to be present on chlamydial EBs. By this procedure, we selected 21 candidate chlamydial T3S effectors. We are currently using *Y. enterocolitica* and TEM-1 hybrids to test for the presence of T3S signals within those 21 proteins and, as additional controls, within three other known chlamydial effectors (Tarp, CT694, and CT695) and within proteins that are unlikely T3S substrates. The results of these ongoing studies will be presented and discussed.

Reference

196

Regulation of intracellular anti-HIV1 host factor APOBEC3G

Ferreira, Luis Filipe (1,2); Guerreiro, Patrícia (1,2); Esteves, João (1,2); Ataíde, Joana (1,2); Gonçalves, João (1,2); Soeiro, Inês (1,2)

1: URIA - CPM, Faculdade de Farmácia, Universidade de Lisboa, Portugal;
2: IMM - Instituto de Medicina Molecular, Universidade de Lisboa, Portugal

E-mail: luisferreira@ff.ul.pt

Keywords: APOBEC3G, HIV-1

Abstract

APOBEC3G (A3G) was the first endogenous protein reported to be able to impair HIV-1 replication. It acts as a cytidine deaminase on the HIV cDNA resulting in unviable virions. To counteract this effect, HIV-1 expresses a protein able to interact with A3G, impairing it–Vif. Recent studies show that A3G expression is raised in patients infected with HIV but presenting a slow disease progression. Other studies showed that it is possible to enhance A3G expression through stimulation of specific cell surface receptors. The aim of this project is to understand the molecular ways by which different T–cell specific receptor stimulation could effectively trigger an increase in A3G expression. For that we are focusing on the A3G promoter and expression from H9 cells, a T-cell line know to be non-permissive to HIV-1 infection.

Several groups have reported phosphorylation of the Akt kinase as a key effect for cell signalling response to specific receptor stimulation and it is used as a control of cell activation. Gp120 is an HIV-1 surface protein that specifically binds to the CD4 receptor. In H9 cells, our results indicate that gp120 stimulation increases the expression of A3G and also the phosphorylation state of Akt. One of the targets of Akt, FoxO3a, also increases its phosphorylated -inactive- state in response to gp120 stimulation. FoxO3a is a member of the Forkhead family of transcription factors that plays a crucial role in controlling cell cycle progression, stress responses and apoptosis.

Using bioinformatic tools we identified a putative promoter area of A3G. To determinate its activity, we cloned it on a vector with a *luc* reporter gene. In the same sequence we also found several possible binding sites for FoxO3a. To determine their importance we made three different 5´end promoter deletions and cloned them on the same reporter vector. To analyse the possibility of FoxO3a's involvement in A3G expression, the produced plasmids were tested in the presence and absence of a constitutively active FoxO3a.

We were also interested in determining which FoxO3a binding site is more relevant for A3G expression regulation. For that we inserted point mutations in each of the possible FoxO3a binding sites. Our preliminary results show that the activity of the A3G promoter raises as the promoter is shortened and loses FoxO3a possible binding sites. From this, we have a strong indication that FoxO3a could be a crucial transcription factor involved in A3G expression regulation.

Reference

212

Importance of C-C chemokine receptor 8 (CCR8) as an alternative coreceptor for HIV-1 and HIV-2

Calado, Ana Marta (1); Espirito Santo, Maria de Fátima (1); Matoso, Paula Cristina (1); Santos-Costa, Quirina (1); Machado, João (2); Antunes, Francisco (3); Maltez, Fernando (2); Rosado, Lino (4); Santos-Ferreira, Maria Odette (1); Pereira, José Miguel A

1: Scholl of Pharmacy, University of Lisbon, Portugal;
2: Serviço de Doenças Infecciosas, Hospital Curry Cabral;
3: Serviço de Doenças Infecciosas, Hospital de Santa Maria;
4: Serviço de Pediatria, Hospital Dona Estefânea

E-mail: mrtcalado@ff.ul.pt

Keywords: HIV-1, HIV-2, coreceptors, CCR5, CXCR4, CCR8, pathogenesis

Abstract

HIV entry into cells occurs by a sequencial process that includes: $CD4^+$ binding, coreceptor (CoR) binding and membrane fusion reaction. CCR5 and CXCR4 are considered to be the most important CoR, although there is a receptor expressed in lymphoid organs and particularly in thymus: CCR8, which has been shown to function as an alternative coreceptor.

Our main goal was to determine the phenotype and to assess the ability of HIV strains to use CCR8 as a CoR. We also tried to establish a correlation between CoR usage and disease stage of sequential samples.

We tested 34 primary HIV isolates (17 HIV-1 and 17 HIV-2) for CoR usage in human osteosarcoma cell line (GHOST) coexpressing CD4 and the CoR CCR5, CXCR4 or CCR8.

Our results showed that 12% of HIV-2 isolates were able to efficiently use CCR5 and CXCR4. Interestingly, CCR8 was used by 36% of the isolates; for HIV-1 isolates we find that 47% of the isolates used both CCR5 and CXCR4 and that CCR8 were used by 41 % of the isolates. We also demonstrated that the percentage of isolates able to use CCR8 and other coreceptor (CCR5 and/or CXCR4) were similar for HIV-1 and HIV-2. Interestingly, for HIV-1, the simultaneous use of CCR5, CCR8 and CXCR4, is observed more frequently than in HIV-2 (35% and 12%, respectively); however, the simultaneous use of CCR5 and CCR8 was used only by HIV-2 isolates (12%).

Regarding sequential samples, it was not possible to establish a correlation between the CoR usage and disease stage. However, for HIV-2_{JTL}, during the asymptomatic stage, the CoR usage was CCR5, changing to dual-tropism R5X4 when it reached the symptomatic stage. When the patient began the treatment, there was a new change of the CoR usage for CCR5. Another interesting isolate was HIV-2_{MLR}, which changed from CCR5 usage to multiple-tropic (CCR5, CXCR4 and CCR8) during the asymptomatic stage.

To conclude, in both HIV-1 and HIV-2, there are some evidences that CCR8 may be important for HIV infection as an alternative CoR and that it can be a possible target for antiviral therapy. Since this CoR is expressed in human monocytes, Th2 lymphocytes and abundantly in thymocytes, it may have implications for HIV pathogenesis and treatment, particularly for neonatal and pediatric patients.

Reference

255

Molecular strategies employed by *Burkholderia cenocepacia* to adapt to the airways of cystic fibrosis patients under antimicrobial therapy, as revealed by transcriptomic analysis and quantitative proteomics

Madeira, Andreia S. (1); Santos, Pedro M. (1); Mira, Nuno P. (1); Pinto-de-Oliveira, Ana (1, 2); Sá-Correia, Isabel (1)

1: IBB- Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisboa, Portugal;
2: Instituto de Microbiologia, Faculdade de Medicina da Universidade de Coimbra, Coimbra, Portugal

E-mail: andreia.madeira@ist.utl.pt

Keywords: Cystic fibrosis, proteomics, microarrays, antibiotic resistance, lung adaptation

Abstract

Cystic fibrosis (CF) patients, chronically infected with bacteria of the *Burkholderia cepacia* complex (Bcc), are subjected to prolonged and aggressive antimicrobial therapy, exposing these intrinsically multi-resistant bacteria to antibiotic selective pressure [1]. In order to elucidate the molecular strategies employed by *B. cenocepacia* to adapt to the airways of CF patients under antimicrobial therapy, the transcriptome and the proteome of isolate *B. cenocepacia* IST4113, obtained from a CF patient after 3 years of chronic infection, was compared with the expression pattern of the first isolate that infected the same patient (IST439). Isolate IST4113 was obtained immediately following a period of intravenous therapy with ceftazidime/gentamycin. This clonal isolate IST4113 exhibits a ribopattern identical to isolate IST439, but shows an increased resistance to trimethoprim/sulfamethoxazole, piperacillin/ ceftazidime and tobramycin as well as other relevant phenotypic differences in the context of bacterial pathogenesis.
Considering the expression proteomics strategy, soluble and membrane protein fractions were separated by 2-D DIGE. A total of 61 proteins whose content was higher and 27 whose content was lower in IST4113 compared with IST439, were registered. The two main functional classes enriched with proteins that showed an increased content are “energy metabolism” and “translation”. Proteins involved in peptidoglycan, folate and nucleotide biosynthesis, in protein folding and in iron uptake, also exhibit a higher concentration in IST4113 compared to IST439, suggesting a possible involvement of these physiological processes on CF lung environment adaptation and/or resistance to the above referred antimicrobials. The transcriptomic approach, focused on the same two isolates, has revealed significant differences on their transcript levels: 194 genes were found to exhibit an increased transcription level whereas 157 exhibited a decreased transcription level in IST4113 compared to IST439. Indications from this microarray analysis will be confronted with those obtained by quantitative proteomics and a model for the mechanisms underlying *B. cenocepacia* adaptation to the CF lungs and antibiotic resistance will be suggested.

Acknowledgements: This work was supported by FEDER and FCT (PTDC/SAU-MII/69591/2006 and SFRH/BD/37012/2007). The microarray analysis was carried out in the context of the Burkholderia array Project BA-009-A, funded by Cystic Fibrosis Foundation Therapeutics Inc., with the support of University of North Carolina – Chapel Hill Center of Bioinformatics and the University of Cardiff.

[1] Leitão J.H. Sousa S.A., Cunha M.V., Salgado M.J., Melo-Cristino J., Barreto M.C., Sá-Correia I. (2008) Eur. J. Clin. Microbiol. Inf. Dis. 27:1101-1111.

Reference

279

CCR5 and CXCR4 inhibitors: Susceptibility study on human immunodeficiency virus type 2 (HIV-2) to neutralization

Espírito Santo, Maria (1); Calado, Ana Marta (1); Santos-Costa, Quirina (1); Dorr, Patrick (2); Azevedo-Pereira, José Miguel (1)

1: CPM-URIA, Faculdade de Farmácia, Universidade de Lisboa;
2: Pfizer Global Research and Development-Sandwich Laboratories

E-mail: espiritosanto@ff.ul.pt

Keywords: HIV-2, coreceptor usage, CCR5 inhibitor, CXCR4 inhibitor

Abstract

Since the emergence of AIDS, many efforts have been made in order to better understand the whole mechanism of viral infection, for HIV.

Despite the potential interest resulting from the study of the mechanisms involved in the interaction of HIV-2 with the host cell, due to its inherently less virulent phenotype in vivo, most information about the coreceptor (CoR) usage and pathogenesis is taken from studies using HIV-1 as a model. As a general consequence, many of the drugs used to treat HIV-1 infection are not effective against HIV-2, because they are used based on existing data for HIV-1.

CoR antagonists (CCR5 and CXCR4 antagonists) belong to a group of entry inhibitors with a novel mechanism of action, acting outside the cell by binding to CoR and preventing its use during initial HIV–cell interaction, unlike the other existing drugs that target HIV proteins. Until now, little is known about HIV-2 susceptibility to these inhibitors.

In this study we determine the susceptibility of HIV-2 to CCR5 and CXCR4 inhibitors using six strains previously isolated and characterized in our laboratory (HIV-2_{MS}; HIV-2_{ALI}; HIV-$2_{ABG.01}$; HIV-2_{MMS}; HIV-$2_{ADC.05}$ and HIV-2_{SAB},). HIV-1_{BaL} was used as control. CCR5 inhibitors used were (TAK-779, MVC, PF-2221753 and mAb2D7). SDF-1alpha and mAb12G5 were used as CXCR4-target inhibitors. Inhibition assays were done using human osteosarcoma cell line (GHOST) coexpressing CD4 and the CoR CCR5 or CXCR4 and viral replication was monitored by Ag p24 detection in culture supernatant.

Our results indicated that TAK-779, MVC and PF-227153, efficiently inhibited all HIV-2 strains (60 to 90% inhibition for a concentration corresponding to the IC50 described for HIV-1_{BaL}). Remarkably, mAb2D7 did not inhibit any of the strains except HIV-2_{ALI} for which the percentage of inhibition was 84%.

We were also able to demonstrate that SDF-1alpha, with a concentration of 5ug/ml had an 86% inhibitory effect, for three of the four strains studied. While for mAb12G5, despite using a concentration of 10ug/ml, we saw a lower inhibitory effect when compared to the SDF-1alpha.

These results enable us to conclude that the CCR5 antagonists under study are more efficient for HIV-2 than for HIV-1_{BaL} and mAb 2D7 does not inhibit strains that were inhibited by other CCR5 inhibitors.

Finally, our findings also suggest that CXCR4 natural ligand is more effective when compared with anti-CXCR4 monoclonal antibody for the inhibition of these strains.

Azevedo Pereira, J. M., Q. Santos-Costa, K. Mansinho, and J. Moniz-Pereira. 2003. Virology 313:136-146.

Dorr Patrick, M. Westby, S. Dobbs, P. Griffin, B. Irvine, M. Macartney, J. Mori, G. Rickett, C. Smith-Burchnell, C. Napier, R. Webster, D. Armour, D. Price, B. Stammen, A. Wood, and Manos Perros. 2005. Maraviroc (UK-427,857),. Antimicrobial Agents and Chemotherapy 49: 4721–4732.

Reference

334

Bioaccumulation of amylose-like glycans by *Helicobacter pylori*

Coimbra, Manuel A. (1); Ferreira, José A. (1); Pires, Cristiana (1); Paulo, Marina (1); Azevedo, Nuno F. (2); Domingues, M. Rosário (1); Vieira, Maria João (2); Monteiro, Mario A. (3)

1: Universidade de Aveiro, Portugal;
2: Universidade do Minho, Portugal;
3: University of Guelph, Canada

E-mail: mac@ua.pt

Keywords: *Helicobacter pylori*, cell-surface polysaccharide, glucan, environmental pressure

Abstract

Helicobacter pylori cell surface is composed of lipopolysaccharides (LPSs) yielding structures homologous to mammalian Lewis *O*-chains blood group antigens. In this study we present evidence that *H. pylori* also expresses/accumulates amylose-like glycans. Bioaccumulation of amylose was found to be enhanced with the subcultivation of the bacterium on agar medium and accompanied by a decrease in the expression of LPS *O*-chains. On the other hand, during exponential growth in F12 liquid medium, an opposite behavior is observed, *i.e.* there is an increase in the overall amount of LPS and decrease in amylose content. As such, this work suggests that under specific environmental conditions, *H. pylori* expresses a phase-variable cell-surface α-(1→4)-Glc moiety.

Reference

365

TLR2 controls IL-17 responses to *Mycobacterium tuberculosis*

Saraiva, Margarida; Cruz, Andrea; Teixeira-Coelho, Maria; Carmona, Jenny A.; Sousa, Carole; Ramos-Pereira, Daniela; Pedrosa, Jorge; Castro, António G.

Life and Health Sciences Research Institute (ICVS). School of Health Sciences, University of Minho. Braga, Portugal, Portugal

E-mail: msaraiva@ecsaude.uminho.pt

Keywords: *Mycobacterium tuberculosis*, TLR2, IL-17

Abstract

Phagocytic cells, such as macrophages and dendritic cells (DC), are the initial targets of *Mycobacterium tuberculosis* and are the first cells to sense the presence of the pathogen in the host due to their expression of several pattern recognition receptors. Activation of macrophages and DC upon *M.tuberculosis* recognition contributes to the launch of microbicidal mechanisms that are important to control intracellular bacterial growth and also to the establishment of the cytokine milieu and of the co-stimulation needed for T cell activation and differentiation. Interleukin-17 (IL-17) is one of the effector cytokines expressed during the infection by *M.tuberculosis*. The initial cells that express IL-17, and that have been suggested to be the main source of this cytokine during *M.tuberculosis* infections, are gd T cells. At later stages of the infection, differentiation of CD4+ Th17 cells also occurs.

In this study, we investigated the role of TLR2 in the development of IL-17 responses to *M.tuberculosis*. We found that, during an in vivo infection with *M.tuberculosis*, absence of TLR2 compromised IL-17 responses in the lung. This early defect in IL-17 responses is most likely due to an impaired stimulation of gd T cells. As the infection progresses, the expression of p19, and thus of IL-23, was diminished in the lung of *M.tuberculosis*-infected TLR2 deficient animals as compared to WT ones. Since IL-23 is a survival factor for IL-17-producing T cells, our data suggest that at later stages of infection, the reduced IL-17 expression in the lung of *M.tuberculosis*-infected TLR2 deficient animals is due to a defect in the maintenance of both IL-17-producing gd T cells and of Th17 cells. Interestingly, the differentiation of Th17 cells was not affected in the draining lymph nodes of mutant animals. The decreased IL-17 expression in the lungs of *M.tuberculosis*- infected TLR2 deficient animals led to a decreased expression of several chemokines, such as MIP-2 and consequently to a deficient recruitment of neutrophils. Moreover, TLR2 deficiency also had implications on granuloma formation. Overall, our data points out to a role of TLR2 in regulating the course of *M.tuberculosis* infection.

Funded by PTDC/SAU-MII/70895/2006. M. Saraiva is a Ciência 2007 fellow.

Reference

366

Control of *Mycobacterium tuberculosis* infection depends on the time of previous exposure to BCG vaccination

Cruz, Andrea (1); Fraga, Alexandra G. (1); Ramos-Pereira, Daniela (1); Saraiva, Ana L. (1); Saraiva, Margarida (1); Correia-Neves, Margarida (1); Cooper, Andrea (2); Pedrosa, Jorge (1); Castro, António G. (1)

1: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;
2: Trudeau Institute, Saranac Lake, NY 12983

E-mail: acruz@ecsaude.uminho.pt

Keywords: *Mycobacterium tuberculosis*, M. bovis BCG vaccination

Abstract

Tuberculosis is responsible for approximately 2 million deaths each year. *Mycobacterium bovis* BCG is the only available vaccine to prevent tuberculosis. The protection conferred by BCG is suggested to last only for 10-20 years. To develop a better vaccine against tuberculosis, it is crucial to improve our understanding on the cellular and molecular mechanisms triggered by BCG.

The development of Th1 cells induced by BCG and the production of IFN-γ are critical to the outcome of infection by mycobacteria. Th17 cells are also induced very rapidly following BCG inoculation and are important for the development of memory responses and during a recall response against *M. tuberculosis*. Since protection conferred by BCG vaccination wanes off over time, we analysed if there are any changes in the characteristics of the immune response at different time-points after BCG vaccination, and how that difference impacts protection against *M. tuberculosis*.

C57BL/6 mice were vaccinated with BCG 21 or 120 days previous to *M. tuberculosis* intranasal infection. The number of Ag85- and ESAT-6- specific T cells producing IFN-γ or IL-17 was quantified by ELISPOT at different days post infection. The protection conferred by BCG was assessed by quantification of the bacterial load in the lung. Our results show that the protection achieved 120 days after BCG vaccination was higher than that observed at a shorter period (21 days). In both cases, protection was associated with a decrease in the number of both Th1 and Th17 cells specific for Ag85 or ESAT-6, as compared to non-vaccinated mice, being the reduction in the number of Th1 cells more pronounced after 120 days of BCG vaccination. Moreover, the presence of effector/memory $CD4^+$ T cells responding to Ag85 influenced the primary response to the ESAT-6, from *M. tuberculosis*, independently of the bacterial load.

Our results show that BCG vaccination induces functional changes on the profile of the T cell response, with an impact on protection. Furthermore, protection induced by BCG did not directly correlate with the number of IFN-γ producing T cells. Further studies are needed to characterize in more detail the phenotype of T cells in this model.

Supported by PTDC/SAU-MII/70895/2006

Oral Session:
S7 – Industrial and Food Microbiology and Biotechnology

Reference

176

New trends in biocatalysis in industrial biotechnology

Fonseca, Luis P.

Instituto de Biotecnologia e Bioengenharia - IBB, Portugal

E-mail: luis.fonseca@ist.utl.pt

Keywords: Biocatalysis, sol-gel, immobilization, Penicillin acylase, Biosensors

Abstract

The recent interests of research in the field of Biocatalysis and Biotransformation focus on the development of new bioprocesses specially new applications in biocatalysis and development of analytical tools for environmental and bioprocess monitoring.

Biocatalysts aimed at industrial applications are often designed in immobilized form, as this provides the required flexibility for different modes of operation or/and reactor configurations, eases process integration and often enhances operational and shelf stability. Suitable manipulation of the immobilization matrix may also contribute to modify enzyme selectivity. Although, plenty of immobilization methodologies and support materials are available for biocatalyst immobilization, only a few are suitable for industrial application, since cost and disposal requirements have also to be considered, as well as toxicity of the materials and methods in some application areas.

Within this scope, several approaches for the production of high performing biocatalysts have been developed or improved in-house. Most of those recently implemented have relied in the use of hydrogels and sol-gel. Particular efforts have also been made as to allow a given methodology to be effective when different enzymes/systems are considered, but also to implement highly reproducible and scalable methodologies. Examples of the work performed include the immobilization of cutinase, penicillin acylase, inulinase and oxi-reductases. The resulting biocatalysts have been characterized for kinetic and operational parameters upon application in relevant bioconversion processes. Future work encompasses the implementation of miniaturized systems for speeding up development.

The design of new biocompatible materials is of crucial importance for biocatalysts in field of Biosensors as it is expected that the properties of the new materials can be a significant and innovative contribution of applicability in relation to traditional screen printed, carbon paste and conducting polymers materials. A new protein-ionic-conducting-based biocompatible materials with tailor-made properties based on Ion Jelly^{R} technology are in development and tested to build a new generation of planar amperometric biosensors.

Reference

175

Monitoring *Listeria monocytogenes* by PCR-DGGE in pediocin-treated maize silage

Amado, Isabel Rodríguez; González, Clara Fuciños; Bernárdez, Paula Fajardo; Guerra, Nelson Pérez; Lorenzo, Pastrana Castro

Universidade de Vigo, Spain

E-mail: sabelara@uvigo.es

Keywords: food safety, *Listeria monocytogenes*, PCR, DGGE, fermentation, bacteriocins

Abstract

Silage quality relies on the achievement of a low pH by lactic acid fermentation and the maintenance of anaerobic conditions, which can inhibit growth of some harmful microorganisms such as *Listeria monocytogenes* (1). LAB inoculation has been used to obtain such inhibition, however, the use of bacteriocin-producing LAB as silage inoculants has not yet been tested, although it is considered a promising alternative (2).

The objective of this work was to reduce the presence of *Listeria monocytogenes* in maize silage using both the adition of lactic acid bacteria and the combination of pediocin -with activity against this pathogen- and lactic acid bacteria resistant to this antimicrobial.

In all experiments 100 g of maize were vacuum-packed in polythene bags and stored for 30 days at 25ºC. A commercial inoculant (11C33, Pioneer Hi-bred Co., Ltd., Iowa, USA) was used and both pediocin and the above mentioned inoculant, to study the combined effect on the presence of *Listeria monocytogenes*. Pediocin was produced by fed-batch fermentation of *Pediococcus acidilactici* (3) and partially purified by diafiltration. *Listeria monocytogenes* strain (CECT 4032) was artificially added to all silages and samples were taken after 1, 2, 5, 8, 16 and 30 days of ensiling.

To follow the presence of *L. monocytogenes*, the DNA of the fermented product for each treatment was extracted using the phenol-chloroform method following PCR amplification of a fragment of the *iap* gene using the primers List-univ. 1 and List-univ. 2 (4). Electrophoresis was performed in a polyacrilamide gel containing a 20-40% urea-formamide denaturing gradient increasing in the direction of the electrophoretic run.

The results obtained showed a single band that comigrated at the same distance of the corresponding marker at all sampling times and in all treatments tested, indicating that there is no other strain of *Listeria* that the one artificially added to the silage. Pediocin treated silages showed a markedly effect on the reduction of *Listeria* presence, which disappeared by day 8, although in Pioneer treated and control silages the band was visible until the end of the fermentation period studied.

These results are encouraging in terms of the use of pediocin as silage additive, as is able to considerably reduce the viability of *L. monocytogenes* regarding untreated silos without altering the microbiota responsible for fermentation.

1. Driehuis, F., Oude Elferink, S. J. W. H. and van Wikselaar, P. G. (2001) Grass Forage Sci. 56: 330–343.
2. Ohmomo, S., Tanaka, O., Kitamoto, H. K. y Cai, Y. (2002), Jpn. Agric. Res. Q. 36: 59–71.
3. Guerra, N. P., Fajardo Bernárdez, P., Torrado Agrasar, A., López Macías, C. and Pastrana Castro, L. (2005), Biotechnol. Appl. Biochem., 42(1):17–23.
4. Cocolin, L., Rantsiou, K., Lacumin, L., Cantoni, C. Comi, G. (2002) Appl. Environ. Microbiol. 68: 6273–6282.

Reference

110

Effect of the application of a galactomannan coating incorporating nisin on the growth of *Listeria monocytogenes* on Ricotta cheese

Martins, Joana T.; Cerqueira, Miguel A.; Souza, Bartolomeu W. S.; Avides, Maria do Carmo; Vicente, António A.

Universidade do Minho, Portugal

E-mail: joanamartins@deb.uminho.pt

Keywords: Edible coating, galactomannan, nisin, *Listeria monocytogenes*, Ricotta cheese

Abstract

Antimicrobial packaging, besides protecting the product from the external environment, inhibits or delays microorganism growth in foods and meets the actual demand of consumers for healthier foods, containing less additives (Lopez-Rubio, Gavara, & Lagaron, 2006).

Cheese is a ready-to-eat type of food that has been associated with foodborne listeriosis. *Listeria monocytogenes* is an important ubiquitous foodborne pathogen which may contaminate foods at pre- and post-harvest stages of production. To overcome this problem bacteriocins could be entrapped in a suitable edible coating applied to food. Nisin is an antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis*. and research studies have revealed its ability to inhibit the growth of some pathogenic bacteria (Sobrino-López, & Martín-Belloso, 2008).

The aim of this study was to evaluate the antimicrobial activity of coatings of galactomannans from *Gleditsia triacanthos* incorporating nisin against *L. monocytogenes* during storage of Ricotta cheese at 4 °C.

Three different treatments were tested: a control with no coating; a sample with coating containing no nisin and a coating with 50 $IU.g^{-1}$ of nisin. Samples of cheese (20 g) were immersed in 0.5 % w/v galactomannan solution containing glycerol (as plasticizer) (1.5 % v/v). To test the effectiveness of the treatments Ricotta cheese samples were surface-inoculated with a solution containing approximately 1×10^6 $CFU.ml^{-1}$ of *L. monocytogenes*. Microbiological and physical-chemical parameters (color change, pH, moisture content and weight loss) were monitored over 28 days for cheese stored at 4 ºC.

Among the three treatments, the combination of coating and nisin showed the best results, followed by the coating containing no nisin. Counts of *L. monocytogenes* were lower ($p<0.05$) in nisin-containing coating that in no-coated cheese. The nisin-containing coating presented a reduction from 5.1 to 4.4 log $CFU.g^{-1}$ after 2 days of storage. For samples coated with nisin, reductions of 2.2 log $CFU.g^{-1}$ were achieved for samples after 7 days of storage.

These results suggest that the application of these coatings could be a potential food packaging solution for the release of nisin in view of the control of *L. monocytogenes* spoilage in cheese.

Lopez-Rubio, A., Gavara, R., & Lagaron, J. M. (2006). Bioactive packaging: turning foods into healthier foods through biomaterials. Trends in Food Science & Technology, 17, 567-575

Sobrino-López, A., & Martín-Belloso, O. (2008). Use of nisin and other bacteriocins for preservation of dairy products. International Dairy Journal, 18, 329–343

Reference

262

Bench-scale production of biopolyesters from waste glycerol

Almeida, Catarina Dias de (1, 2); Cavalheiro, João M. (1); Cesário, Teresa (1); Fonseca, M. Manuela Regalo da (1)

1: Institute for Biotechnology and Bioengineering - Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal;
2: Centro de Investigação Interdisciplinar Egas Moniz, Instituto Superior de Ciências da Saúde Egas Moniz, Quinta da Granja, 2829-511 Monte da Caparica, Portugal

E-mail: catarina.almeida@ist.utl.pt

Keywords: biodiesel, biopolymers, Cupriavidus necator, PHB, glycerol

Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters and the most common type of PHA is the homopolymer poly(3-hydroxybutyrate) (P(3HB)). Certain co-polymers (e.g. P(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB) and the homopolymer P(4HB)) present adequate properties for specific uses, namely in the medical and pharmaceutical areas. It was verified that, when adequately processed, these PHAs are biocompatible. Some microbial strains are able to accumulate these polymers under unbalanced growth conditions. For high scale production, economical evaluation studies led to the conclusion that 48 % of the total production costs was ascribed to the raw materials, in which the carbon source for growth and polymer accumulation could account for 70 % to 80 % of the total cost. Therefore, the carbon source choice is a key factor for PHAs industrial production.
Currently, there are around 120 plants in the EU producing up to 6.1 million t of biodiesel annually (of which 400 thousand t in Portugal) resulting in around 400 000 t of a glycerol rich-phase (GRP) originated as a by-product in the transesterification of oils. A *Cupriavidus necator* strain was chosen to produce PHAs both from GRP and from commercial glycerol as control substrate. Polymer accumulation is triggered by nitrogen limitation. For P(3HB-co-4HB) biosynthesis γ-butyrolactone was used as the co-substrate. High-cell density fed-batch cultures, aiming at attaining high polymer productivities while minimizing energy consumption, are being carried out with control of dissolved oxygen, pH and temperature in bench-scale reactors (stirred tank and air-lift). The performance of the two reactor types is being compared and the consumption of C source and precursor quantified.
The thermal and mechanical properties of the obtained copolymers with different 4HB % monomer incorporation are determined at the Universities of Liège and Strasbourg. The study of cultivation regimes yielding copolymers targeted to specific applications, using GRP as the major carbon source, is currently under way.

ACKNOWLEDGEMENTS- This work is being financed by the EU Integrated Project BIOPRODUCTION (NMP2-CT-2007-026515). C Almeida and JM Cavalheiro are supported by fellowships from FCT, Portugal (SFRH/BPD/26678/2006 and SFRH/BD/45266/2008). We are grateful to Prof. C. Grandfils, Université de Liège, Belgium, for the analytical support.

Reference

253

Global analysis of yeast determinants of resistance to ethanol: the important role of FPS1

Teixeira, Miguel C; Mira, Nuno P; Raposo, Luís R; Sintra, Eva R; de Carvalho, Carla CCR; Lourenço, Artur B; Sá-Correia, Isabel

IBB - Institute for Biotechnology and Bioengineering / CEBQ, IST, Portugal

E-mail: eva.sintra@ist.utl.pt

Keywords: *Saccharomyces cerevisiae*, alcoholic fermentation, ethanol stress, Fps1, chemogenomics

Abstract

The understanding of the molecular basis of yeast resistance to ethanol may guide the design of rational strategies to increase process performance in industrial alcoholic fermentations. In this study, 250 determinants of resistance to ethanol in *S. cerevisiae* were identified through the screening of the yeast disruptome for mutants with differential susceptibility towards ethanol induced stress. The most significant GO terms enriched in this dataset are those associated to intracellular organization, biogenesis and transport, in particular regarding the vacuole, the peroxisome, the endosome and the cytoskeleton, and those associated to the transcriptional machinery. Clustering the proteins encoded by the identified determinants of ethanol resistance by their known physical and genetic interactions highlighted the importance of the vacuolar protein sorting machinery, the V-ATPase complex and the peroxisome protein import machinery. Evidence was obtained showing that vacuolar acidification and increased resistance to the cell wall lytic enzyme β-glucanase occur in response to ethanol induced stress.

Based on the genome-wide results, the particular role of the *FPS1* gene, encoding a plasma membrane aquaglyceroporin which mediates controlled glycerol efflux, in ethanol stress resistance was further investigated. *FPS1* expression contributes to decreased 3H-ethanol accumulation in yeast cells, suggesting that Fps1p may also play a role in maintaining ethanol intracellular level during active fermentation. The effect of *FPS1* expression in the phospholipid and ergosterol composition of yeast membranes prior and upon exposure to ethanol stress was analysed in this context. Interestingly, the over-expression of *FPS1* confirmed the important role of this gene in alcoholic fermentation leading to an increased final ethanol concentration under conditions that lead to high ethanol production.

Reference

188

The influence of the S-layer protein on the resistance of *lactobacilli* to lysozyme

Dias, Rita; Hogg, Tim; Couto, José António

Universidade Católica Portuguesa - Escola Superior de Biotecnologia, Portugal

E-mail: jacouto@esb.ucp.pt

Keywords: lysozyme, lactobacilli, wine, resistance

Abstract

Lysozyme is a widespread enzyme in nature being widely produced commercially from egg white. This enzyme causes degradation of the peptidoglycan cell wall of Gram positive (and, to a lesser extent, Gram negative) bacteria, by cleaving the β (1-4) bond of N-acetylmuramic acid and N-acetylglucosamine (Gerbaux, *et al*. 1995, Prescott, *et al*, 1996). Lysozyme can used in wine production to prevent growth of lactic acid bacteria in order to control the extension of the malolactic fermentation or to avoid spoilage caused by these bacteria.

Previous work in our lab has shown that the response of wine lactic acid bacteria to lysozyme is species and strain dependent. Some lactobacilli strains isolated from Port wine were found to be highly resistant to lysozyme (survival in the presence of 500 to 2000 mg/L). It was observed that the resistant strains produce a crystalline proteic layer (S-layer) which envelops the peptidoglycan. This layer, composed exclusively of proteins or glycoproteins, is thought to have a protective role to environmental factors and can also be an important structure for cell adhesion to surfaces (Yasui, *et al*, 1995).

In this work we investigated the putative protective role of the S-layer against lysozyme action. Several treatments were used to remove the S-layer using LiCl, trypsin, SDS, proteinase K. The treatment with LiCl (5M) was the most effective in the removal of the S-layer as evidenced by SDS-PAGE. LiCl treated populations of *Lactobacillus hilgardii* 35 exposed to lysozyme (2000 mg/L) were found to keep their resistance. However, when ethanol (20% v/v) was added to the assay medium a strong inactivation effect was observed (> 6 log reduction in 30 min). An inactivation effect, but lo a lesser extent, was also found in cells non-treated with LiCl exposed to lysozyme+ethanol. The results show that the S-layer protein may exert a protective effect against the action of lysozyme. It is also seen that ethanol plays a role in the sensitiveness of cells to lysozyme.

Gerbaux V., Villa A., Monamy C. and Bertrand A. (1997) Use of Lysozyme to Inhibit Malolactic Fermentation and to Stabilize Wine After Malolactic Fermentation. Volume 48, Americam Society for Enology and Viticulture.

Prescott, Lansing M., Harley, John P., Klein, Donald A. (1996) Microbiology, third edition, WCB, E.U.A.

Yasui, T., Yoda, K. and Kamyia, T. (1995) Analysis of S-layer proteins of Lactobacillus brevis, FEMS Microbiology Letters, 133, pp. 185-186.

Reference

227

Real-time polymerase chain reaction for the quantitative detection of soybean in meat products

Mafra, Isabel (1,2); S. Amaral, Joana (1,3); Soares, Sónia (1,3); P.P.Oliveira, M. Beatriz (1)

1: REQUIMTE, Serviço de Bromatologia, Faculdade de Farmácia, Universidade do Porto, Portugal;
2: CBQF/Escola Superior de Biotecnologia UCP, Portugal;
3: ESTiG, Instituto Politécnico de Bragança, Campus de Sta. Apolónia, 5301-857 Bragança, Portugal

E-mail: isabel.mafra@ff.up.pt

Keywords: Soybean, food allergens, real-time PCR, TaqMan probe, meat products

Abstract

Soybean protein is reported to be the most widely used vegetable protein in the meat industry. Several characteristics of soybean protein such as the emulsifier properties, preventing the coalescence of fat during heating, and the increased capacity of water-holding improving the texture of the final product are reasons for its generalised use. However, as soybean is included in the group of ingredients potentially allergenic, if not declared, it can be considered a hidden allergen, representing a potential risk to sensitised individuals. Various methods have been proposed for the detection of soybean in food products, mainly based on the analysis of proteins, such as immunological assays, electrophoretic and chromatographic methods. Due to the higher stability of nucleic acids when compared to proteins and to their ubiquity in every type of cells, DNA molecules have been the target compounds for species identification in several recent works [1].

The aim of this work was to develop highly sensitive and fast DNA-based techniques as alternative to the currently used protein-based methods. For that purpose, binary mixtures of soybean protein in pork's meat were prepared. In a previous stage of this project, qualitative PCR techniques were successfully applied in the species-specific PCR detection of soybean lectin gene in Frankfurt type sausages [2]. In the present work, we propose a novel approach for the quantitative detection of soybean in processed meat products by means of real-time PCR coupled with fluorescent TaqMan probes. The assays involved the amplification targeting an eukaryotic DNA fragment with specific primers and probe as reference gene for quantification. The amplification of soybean lectin gene was performed in parallel reactions using specific primers and probe. With the values of cycle threshold (Ct) a calibration standard curve was obtained using the DDCt method, allowing the detection and quantification of soybean protein in pork's meat in the proportions of 0.1% to 50%. The established real-time PCR technique was successfully applied in the confirmation of qualitative PCR results and in the estimation of soybean protein in commercial meat products.

[1] Mafra, I., Ferreira, I.M.P.L.V.O., Oliveira, M.B.P.P. (2008). Food authentication by PCR based methods, Eur. Food Res. Technol. 227:649-665.
[2] Soares, S., Amaral, J.S., Mafra, I., Oliveira, M.B.P.P. (2009). Polymerase chain reaction assay to detect soybean in processed meat products (submitted).

Poster Session:
S7 – Industrial and Food Microbiology and Biotechnology

Reference

109

Fungal communities on cork

Basílio, Maria do Carmo (1); Houbraken, Jos (2); Samson, Robert (2); Frisvad, Jens (3); Barreto Crespo, Maria Teresa (1); San Romão, Maria Vitória (4)

1: ITQB/IBET, Av da República, apt 12, 2781-901. Oeiras, Portugal;
2: CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands;
3: Center for Microbial Biotechnology, Biocentrum-DTU, Technical University of Denmark, Søltofts Plads 221, DK-2800 Kgs. Lyngby, Denmark;
4: INRB-L-INIA- Ex-Estação Vitivinícola Nacional, Quinta de Almoinha, 2565-191, Dois Portos, Portugal

E-mail: vsr@itqb.unl.pt

Keywords: cork, fungal diversity, new species, extrolites,

Abstract

Cork is a natural, renewable and biodegradable material used for many centuries as a wine closure.
The manufacturing of cork stoppers involves a boiling operation followed by a rest stage of 3 – 4 days aiming cork planks flattening and a humidity decrease to the necessary level to allow cork cutting. In that phase the fungi that normally colonized the cork in the cork oak tree and survived the boiling treatment, together with the fungi present in the factory environment completely covers the cork slabs. To study the shift in the cultivable fungal community during several manufacturing stages of cork discs a sampling scheme was planned through the key stages of the process. Fungi were isolated and almost of them identified until species level using phenotypic and molecular methods. The results showed a predominance of *Penicillium* genus followed by *Aspergillus*, *Chrysonilia*, *Mucor* and *Trichoderma* genera. *Penicillium glabrum* was the predominant isolated species, present in all the sampling stages. A new *Penicillium* species was identified and described.
To address the quality of the final product and better characterise the fungal isolates, the extrolites produced by the fungal species that survived the boiling stage were analysed through GC-MS and HPLC using both diode array and fluorescence detectors. The results show that cork medium is not a suitable media for the secondary metabolites production, although some fungi produced some extrolites.

Acknoledgements: FCT for PhD grant BD/19264/2004. Amorim & Irmãos (Coruche, Portugal). This research received support from the SYNTHESYS Project http://www.synthesys.info/ which is financed by European Community Research Infrastructure Action under the FP6 “Structuring the European Research Area” Programme.

Reference

111

Detection of toxigenic isolates of *Aspergillus* in selective medium

Almeida, Inês Filipa (1); Magalhães, Salomé Alexandra (2); Martins, H. Marina (2); Bernardo, Fernando (3)

1: Faculdade de Medicina Veterinária, Universidade Lusofona de humanidades e tecnologia, Portugal;
2: INRB, IP, Laboratório Nacional de Investigação Veterinária;
3: CIISA, Faculdade de Medicina Veterinária

E-mail: ines.almeida@dgv.min-agricultura.pt

Keywords: Mycotoxins, Aflatoxin-production, selective medium

Abstract

Aflatoxins are toxic and carcinogenic metabolites that belong to a class of compounds known as mycotoxins. They are known to be produced by *Aspergillus flavus*, among others species, however, this one is considered to be the most aggressive.
A simple, rapid and reproducible procedure was used this work. The procedure consisted on growing *Aspergillus flavus* on a selective medium, coconut agar modified (CAMM). The essential ingredient in this medium was $ZnSO_4 7H_2O$ (Martins, *et al*, 1989). Forty five samples collected from feed were screening in Cooke Rose Bengal medium to characterize the mycobiota, after that the samples which reveal to be positive for *A.flavus* (32) were grown in a selective medium (cocoa), with the purpose to test aflatoxin ability, therefore the colonies were exposed to ammonium vapour, in order to correlate the colour intensity of *A.flavus* production with the quantification by HPLC. The colony reverse of aflatoxin producing strains of *Aspergillus flavus* turned pink when their cultures were exposed to ammonia vapor. The positive strains were tested for their toxinogenic potential by high liquid chromatography quantification. Inspite of that only eleven strains of *A. flavus* (34.3%) were able to produce aflatoxins with levels ranging between 11.25 and 1346.2 µg/Kg (average= 642.8 µg/. For this reason this method was considered to be useful for rapid screening of aflatoxin-production (Almeida, I, *et al*., 2009).

1. Almeida, I., Martins, H.M., Magalhães,S.,and Bernardo, F., 2009, 3rd Symposium of mycotoxins, 28th April Ghent-Belgium.
2. Martins, H.M., Bernardo, F., and Martins, M.L., 1989. Rev.Ciências Vet. 94:532, 177-181p.

Reference

112

Mycological characterization of Portuguese traditional dry sausages (in modified atmosphere package)

Almeida, Inês Filipa (1); Barreto, António (2); Matos, Teresa (3)

1: Escola Superior de Hotelaria e Turismo do Estoril;
2: CIISA, Faculdade de Medicina Veterinária;
3: Instituto Superior de Agronomia

E-mail: ines.almeida@dgv.min-agricultura.pt

Keywords: Meat Products, Food Safety, Mycobiota, Lipolytic Activity

Abstract

Portuguese traditional meat products exhibit a great commercial variety, as a result of its raw materials, ingredients and processing diversity. Several microorganisms (lipolytic, glucolytic and proteolytic) can develop in dry smoked sausages and undertake changes on the sensorial characteristics, consistency, flavour and general aspect. Considering so, the presence and development of lipolytic yeasts can affect these products quality. Peroxissomal β-oxidation of yeasts results in the production of hydrogen peroxide and free fatty acids due to their incomplete degradation.

Spoilage ecology is affected by a combination of intrinsic and extrinsic factors. Factors such as the use of natural intestines of pork and beef to stuff meat batters, the microrganisms population of the raw meat material, the introduction of spices as formulation ingredients, the smoke, the application of modified atmosphere and the CO_2 concentrations and the storage temperature all have great influence on the micoflora of these types of meat products and also on the growth and survival of spoilage microorganisms during shelf-life period.

A total of twelve random samples of Portuguese traditional dry sausages – “morcelas” (n=4), “chouriços” (n=4), “farinheiras” (n=3) and “linguiça” (n=1), packaged in modified atmospheres, were collected and subjected to mycological evaluation, both in the beginning and at the end of the shelf life of the products.

Yeast colonies were enumerated and each isolated colony was observed microscopically for morphological characterization and identification at genus level. Yeast identification was based in biochemical monitorized conventional kits. Lipolytic activity was tested using Tributyrin Agar. Positive colonies exhibit rise to clear digested zones surrounding the colonies.

In the first study phase (in the beginning of the shelf life), 75.0% of the samples were positive and with 5.9 log CFU/g to 1.8 log CFU/g. In the second study phase (at the end of the shelf life) only 33.3% of the samples were positives (with 5.7log CFU/g to 3.4 log CFU/g). In a general way, there was a reduction of the level of yeasts during the shelf life of these products. The biggest reduction was observed in “morcela” with a decrease of $8x10^5$ CFU/g.

Saccharomyces cerevisae, *Candida pelliculosa* (41%), *C. holmii* (1.32%), *Zygosaccharomyces* spp. (0.86%), *Kloeckera japonica* (0,04%) and *Penicillium* spp were isolated. 98.6% of them revealed lipolytic activity.

Reference

135

Characterization of sakacin G – like bacteriocin produced by *Lactobacillus sakei* ST154CH, strain isolated from salpicão, a traditional pork product from the North-West of Portugal

Todorov, Svetoslav (1); Franco, Bernadette (1); Vaz-Velho, Manuela (2)

1: Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Alimentos e Nutrição Experimental, Laboratório de Microbiologia de Alimentos;
2: Instituto Politécnico de Viana do Castelo, Escola Superior de Tecnologia e Gestão

E-mail: mvazvelho@estg.ipvc.pt

Keywords: bacteriocin, sakacin G, *Lactobacillus sakei*, optimisation, salpicão

Abstract

Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria. Many LAB produce bacteriocins with rather broad spectra of inhibition. Several LAB bacteriocins have been studied and they offer great potential applications in food preservation.

StrainST154Ch, isolated from *salpicão,* a traditional cured/smoked pork product from Portugal, was identified as *Lactobacillus sakei* based on biochemical tests, sugar fermentation reactions (API50CHL), PCR with species-specific primers and 16S rDNA sequencing. Bacteriocin ST154Ch inhibited the growth of *Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae, Lactobacillus rhamnosus, Listeria innocua, Listeria ivanovii* subsp. i*vanovii, Listeria monocytogenes, Pseudomonas* spp., *Staphylococcus aureus, Streptococcus caprinus* and *Streptococcus* sp. The mode of action of the bacteriocin was bactericidal. The peptide is inactivated by proteolytic enzymes, but not when treated with a-amylase, lipase or catalase, Triton X-100, SDS, Tween 20, Tween 80, urea, NaCl and EDTA. The highest bacteriocin production (800 AU/ml) was recorded after 24 h in presence of 10.0, 20.0 or 30.0 g/l D-glucose. Optimal production of the bacteriocin ST154Ch was recorded in the presence of a combination of meat extract and yeast extract or tryptone, meat extract and yeast extract. Presence of only meat extract (20.0 g/l) or tryptone (20.0 g/l) or combination of tryptone and yeast extract (12.5 g/l and 7.5 g/l, respectively) reduced bacteriocin production by 75%. Replacing the KH_2PO_4 by K_2HPO_4 resulted in reduction of bacteriocin ST154Ch activity. Optimal production of bacteriocin ST154Ch was recorded at presence of 10.0 g/l and 20.0 g/l KH_2PO_4. Presence of glycerol, at 5.0 g/l, increased bacteriocin ST154Ch production up to 1 600 AU/ml. Tween 80 (2.0 g/l and 5.0 g/l) added to the MRS medium, increased bacteriocin ST154Ch production up to 1 600 AU/ml. Exclusion of manganese sulphate resulted in total lost of activity of the cell-free supernatant. Exclusion of magnesium sulphate from the media formula increased the bacteriocin production up to 1 600 AU/ml.

Based on the genetic approach, strain ST154Ch harbours associated genetic determinants for production of a variation of the well known sakacin G.

Reference

178

Parallelization in the design and characterization of an immobilized bioconversion system for inulin hydrolysis

Chheub, Vanessa; Cattorini, Stefano; Marques, Marco P.C.; Carvalho, Filipe; Fernandes, Pedro

IBB-CEBQ-IST, Portugal

E-mail: pedro.fernandes@ist.utl.pt

Keywords: miniature bioreactors, microtiter plates, inulin hydrolysis

Abstract

A battery of miniature (1.5 mL) stirred reactors along with shaken 24-well microtiter plates (MTP) were tentatively used for the high throughput characterization of a bioconversion system for inulin hydrolysis. Inulinase immobilized in polyvinyl alcohol (PVA) based beads was used as biocatalyst [1]. Experimental conditions were established which ruled out external mass transfer limitations. Miniature reactors and MTP could thus be effectively used for the characterization of the bioconversion system, through the evaluation of the effect of temperature, pH and initial substrate concentration on the initial reaction rate (IRR) of inulin hydrolysis. IRR pattern upon immobilization was not significantly affected by the combined effects of pH and temperature changes, optimum pH being observed at 4.5 for both free and immobilized enzymes, whereas the optimal temperature increased from 55ºC to 60ºC after immobilization. Diffusion limitations were observed as result of immobilization. Two inulin sources, dahlia tubers and chicory roots, were evaluated as raw materials for fructose syrups, the latter allowing for the highest maximal reaction rate and an apparent higher affinity towards the biocatalyst, as could be evaluated from the (apparent) kinetic constants of the Michaelis-Menten equation. Such behavior could possibly be ascribed to the shorter chain length of the chicory inulin used. Bioconversion runs of 50 gL^{-1}inulin solutions, typically performed in 1 mL reaction volumes during characterization studies, were effectively scaled-up to 10 and 50 mL reaction volumes, consistently allowing for full bioconversion in 24-hour batch runs, following a pattern of product formation common to the three scales. Currently underway is further scaling-up based in data gathered in miniature systems and evaluation of other miniature reactor configurations, which allow for continuous operation in this bioconversion system.

[1] Fernandes P, Marques MPC, Carvalho F, Cabral JMS (2009) J Chem Technol Biotechnol 84, 561–564

Reference

193

Optimization of a fructooligosaccharides purification method using activated charcoal

Nobre, Clarisse (1); Dominguez, Ana (1); Torres, Duarte (1, 2); Rocha, Orlando (2); Rocha, Isabel (1,2); Ferreira, Eugénio Campos (1); Teixeira, José António (1); Rodrigues, Lígia Raquel (1,2)

1: University of Minho, Portugal;
2: Biotempo Lda., Portugal

E-mail: clarissenobre@deb.uminho.pt

Keywords: FOS, Fermentative broth, Activated charcoal

Abstract

Fructooligosaccharides (FOS) have gained large commercial interest due to its beneficial properties in the human health as prebiotics. FOS are produced industrially by fermentative processes. However, the result of such fermentations is a complex mixture containing salts and approximately 50% (w/w) of low molecular weight sugars that have to be eliminated. Among other techniques that have been studied, the adsorption onto activated carbon is still the most suitable one since activated carbon is cheap, has a large surface area and pore volume conducting to a good sorption capacity. Furthermore, this sorbent can be regenerated during desorption with ethanol. Based on the above discussion, in this work the adsorption and desorption characteristics of FOS on activated carbon, using a gradient of ethanol, were optimized. Initially, the activated carbon was loaded with fermentative broth. To remove the non adsorbed sugars, a washing step with pure water was included. Afterwards, the retained sugars were recovered by elution with a gradient of ethanol increased sequentially with specific volumes from 1 to 50% (v/v). Fractions collected at different time points were evaporated and subsequently freeze-dried. This process was found to be very efficient in the demineralization of broth, and it was possible to recover 80% of the initial FOS loaded on the column with 89% of purity. Some of the fractions were found to contain 97% of pure FOS in total sugars. In summary, purification of FOS using an activated charcoal column is a very efficient process yielding high levels of purity from a fermentative broth.

Reference

195

Free radical scavenging capacity and total phenolic content of three microalgae

Padilha, Mária (1,2); Batista, Irineu (1); Mello, Ricardo (1,4); Ramos, Marina (1); Batista, Ana Paula (3); Serralha, Fátima (3); Raymundo, Anabela (3); Sousa, Isabel (4); Gouveia, Luísa (2); Bandarra, Narcisa Maria (1)

1: INRB/L-IPIMAR, U-VPPA, Av. Brasília 1449-006 Lisboa, Portugal;
2: LNEG-DER - Unidade Biomassa, Estrada do Paço do Lumiar, 1649-038 Lisboa, Portugal;
3: Núcleo de Investigação em Engenharia Alimentar e Biotecnologia, Instituto Piaget - ISEIT de Almada, Quinta da Arreinela de Cima, 2800-305 Almada, Portugal;
4: DAIAT – Instituto Superior de Agronomia / Technical University of Lisbon. Tapada da Ajuda, 1349-017 Lisboa, Portugal

E-mail: irineu@ipimar.pt

Keywords: microalgae, antioxidant activity, radical sacavenging, phenolic content

Abstract

Microlgae are considered a rich source of natural antioxidants but their content depends on the species and growing conditions. Thus the antioxidant capacity and total phenolic content of freeze dried *Chlorella vulgaris*, *Isochrysis galbana* and *Diacronema vlkianum* were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the Folin-Ciocalteu method, respectively. The antioxidants were extracted using methanol following different procedures. In a first set of trials, *C. vulgaris* was extracted under the following conditions: (i) stirring for 0.5 to 2 min in a Vortex mixer at room temperature and (ii) keeping at -80 ºC for 3 or 24 hours and two cycles at -80 ºC (1.5 h)/room temperature. The extracts obtained under these conditions did not present substantial differences in their radical scavenging activity and phenolic content. These results led to choosing the most feasible procedure i.e. stirring with a Vortex for 1-2 min. *D. vlkianum* exhibited the highest radical scavenging capacity (RSC) (60.7 mg DPPH/g microalgae) followed by *I. galbana* (12.8 mg DPPH/g microalgae) and *C. vulgaris* (1.8 mg DPPH/g microalgae). The same order was observed for the phenolic content, i.e. 43.4 mg gallic acid/g microalgae for *D. vlkianum*, 35.9 mg gallic acid/g microalgae for *I. galbana* and 5.3 mg galic acid/g microalgae for *C. vulgaris*. The RSC values expressed in terms of phenolic content were 1.40, 0.36 and 0.34 mg DPPH/g phenolic compounds, respectively for *D. vlkianum*, *I. galbana* and *C. vulgaris*. These results suggest that *I. galbana* and *C. vulgaris* have similar type of phenolic compounds responsible for the antioxidant activity exhibited. On the other hand, the value for *D. vlkianum* may indicate the presence of different type of phenolic compounds or a higher amount of antioxidants other than these compounds.

Reference

203

Proton homeostasis of *Saccharomyces cerevisiae* during wine fermentation

Viana, Tiago; Loureiro-Dias, Maria C.; Prista, Catarina

Centro de Botânica Aplicada à Agricultura,ISA-TUL, Portugal

E-mail: tiago.utl@gmail.com

Keywords: *Saccharomyces cerevisiae*, wine fermentation, proton homeostasis, ethanol

Abstract

Wine fermentation is a complex ecological and biochemical process, during which yeast cells are submitted to a number of adverse stress conditions (osmotic pressure, low pH, ethanol, nutrient limitation and starvation, temperature).
This work was part of a project with the broad objective of improving the production/quality of wine, as far as it depends on yeast fermentation. Specifically, the present work aimed to characterize yeast proton homeostasis during must fermentation under experimental conditions simulating real winery conditions for white wine and red wine production.
To accomplish this goal, an industrial strain of *Saccharomyces cerevisiae* (ISA1000), isolated from a commercial starter (FERMIVIN) was used. This strain was grown in white grape must, under experimental conditions simulating a real winery, at 15 and 30ºC, temperatures commonly used for white wine and red wine production, respectively.
Six different growth situations were selected for 15ºC: the beginning, the middle and the final of exponential growth phase, the beginning and the middle stationary growth phase and the end of fermentation (when no sugar was detected by standard methods). For 30ºC, the same situations were chosen, except the first sample point. Results were compared with the same strain grown at the same temperatures in mineral medium, 2% glucose (usual lab conditions).
To evaluate the efficiency of proton homeostasis, intracellular pH, proton extrusion and proton influx through the plasma membrane were measured at both temperatures. The effect of ethanol on proton extrusion and proton influx was also evaluated at the same temperatures as the cells were grown. The most relevant results were obtained at 15ºC, in stationary phase. Under these conditions, *S. cerevisiae* ISA1000 exhibited low pH_i ($pH_i \approx 5$), inability to extrude H^+ and very low H^+ permeability. At 30ºC, at the same fermentation stage, the cells presented low H^+ permeability and low H^+ extrusion capacity, although they had a higher pH_i ($pH_i \approx 6$) than at 15ºC. For both temperatures, the effect of ethanol on H^+ permeability was less evident in stationary phase cells.
These results open new gates for research on yeast performance during late stages of wine fermentations.

Reference

204

Effect of castor oil hydrolysis in the production of gamma-decalactone by the yeast *Yarrowia lipolytica*

Braga, Adelaide; Gomes, Nelma; Teixeira, José A.; Belo, Isabel

IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar 4710-057, Braga, Portugal

E-mail: adelaidebraga1@gmail.com

Keywords: lipases, gamma-decalactone, castor oil, *Yarrowia lipolytica*

Abstract

The production of aromas by biotechnological processes is of great interest due to its increasing acceptability, especially in the food industry, in comparison with similar products obtained by chemical synthesis.

Gamma-decalactone, a peach-like flavour compound, is an example of those products and can be obtained biotechnologically by the biotransformation of ricinoleic acid, the main constituent of castor oil, a natural and non-toxic oil, biodegradable and a renewable resource, obtained from the seed of the castor plant, *Ricinus communis*. That biotransformation can be carried out by various microorganisms, such as the non-conventional yeast *Yarrowia lipolytica*, considered as non-pathogenic and as GRAS by the American Food and Drug Administration. In addition, this microorganism has also the ability to produce lipases that play an important role in the hydrolysis of castor oil.

The purpose of this work is to improve the biotransformation of ricinoleic acid into gamma-decalactone, integrating an enzymatic hydrolysis of castor oil in the process, using commercial lipases and a lipase produced by the yeast. The study of different microbial lipases aimed at producing the best hydrolyzed oil to use as a precursor for the production of gamma-decalactone.

The castor oil hydrolysis essays were conducted in flasks, using four different commercial enzymes: lipase of *Candida rugosa* from Sigma and lipases CALB L®, Lipozyme TL IM® and Lipolase ® 100T from Novozymes. The effect of temperature (37, 55 and 75ºC) and pH (6, 7 and 8) was estimated for each enzyme. Results demonstrated that the highest hydrolysis percentage (95%) was obtained with Lipozyme TL IM® after 54 h of reaction, at 37 ºC and pH 8.

Furthermore, different strategies for gamma-decalactone production in flask experiments were also investigated, namely the addition of previously hydrolyzed castor oil to the culture medium, the addition of an immobilized lipase to the biotransformation medium and finally, inducing lipase production prior to the biotransformation stage. These strategies were compared with the usual biotransformation process (without addition of lipase or previously hydrolyzed oil) and the results obtained will be presented.

Reference

210

Enzymatic production of fructose fatty acid ester using lipases from C. antarctica and porcine pancreatic

Neta, Nair do Amaral Sampaio; Rodrigues, Ligia Raquel; Teixeira, José António

Universidade do Minho, Portugal

E-mail: nairsampaio@ig.com.br

Keywords: Lipase; Fructose ester; Biosurfactants; Esterification

Abstract

The aim of this work was to produce fructose fatty acid ester by enzymatic esterification of a fatty acid (oleic acid or linoleic acid) with fructose, using lipases (CALB) from *Candida antarctica* type B and porcine pancreas. The esterification reaction was conducted at 150 rpm and 40 °C during 72 hours. Equimolar (0.5 mmol) amounts of fructose and fatty acid were mixed with 0.6 ml of ethanol and sodium sulfate anhydrous (0.1 g) was added for the adsorption of the water generated during the reaction. In all experiments, 22.5 mg of lipase were used. A control experiment was performed using the same conditions except for the addition of lipase. Samples were analyzed by thin layer chromatography (TLC), using silica gel plates. The plates were placed in an iodine chamber to develop fatty acid and sugar ester spots. According to the literature an Rf of 0.5 is expected for the fructose fatty acid ester using chloroform/ hexane (1:1, v/ v) as eluting solvent. Four different reaction schemes were studied in this work namely, sample 1 (oleic acid, fructose, *C. antarctica* lipase, sodium sulfate and ethanol), sample 2 (oleic acid, fructose, porcine pancreatic lipase, sodium sulfate and ethanol), sample 3 (linoleic acid, fructose, *C. antarctica* lipase, sodium sulfate and ethanol) and sample 4 (linoleic acid, fructose, porcine pancreatic lipase, sodium sulfate and ethanol). From the TLC assays, the formation of fructose ester was observed for samples 1, 2 and 3. Additionally, the yield of esterification was determined by calculating the amount of residual fatty acid in the reaction mixture, which was determined using a volumetric method, as described elsewhere. Esterification yields of 74.3, 41.4, 63.5 and 11.2 % (v/ v) were determined for samples 1 to 4, respectively. Therefore, it was possible to conclude that the lipase obtained from *C. antarctica* had a better performance than the one obtained from porcine pancreas. Furthermore, the reaction schemes that used oleic acid conducted to higher yields of fructose ester production. These results point out that the enzymatic production of fructose esters is worthwhile and suggest the need for further research.

Reference

213

Microbiological and physicochemical characteristics of industrial cracked green olives´ fermentation

Alves, Maria do Carmo; Cavaco, Teresa; Gaspar, Nelma; Quintas, Célia

Universidade do Algarve, Instituto Superior de Engenharia

E-mail: alves.maria33@gmail.com

Keywords: cracked green olives, microbial growth, fermentation

Abstract

Table olives are a widespread fermented food produced and consumed in Mediterranean areas. In the south of Portugal a common method of production, is to crack green olives of the Manzanilla variety and brine them directly after harvesting. The fruits are kept in the brining solutions until they partially lose their natural bitterness. The main organoleptic property of the final product, which is mostly appreciated by consumers, is a residual bitterness, and as a result of this fact untreated olives are regarded as a typical and natural food.

The present work studied the microbiological and physicochemical changes during the natural fermentation of cracked green olives carried out in a local industry, according to the producers' protocols. Olives were harvested, sorted, cracked, brined and fermented at room temperature, involving two processes: Process A, where olives' brine was changed in the middle of the fermentation process, while in Process B olives were maintained in the same brine. The microbial populations studied were total viable counts, lactic acid bacteria, *Enterobacteriaceae,* yeasts, and moulds. The physicochemical parameters monitored were pH, titratable acidity, reducing sugars, phenolic contents and colour. At the end of the fermentation process, (55 days - Process A and 73 - Process B) yeasts were predominant. A clear decrease of the mould population was observed during both fermentations. No viable counts of *Enterobacteriaceae* where found in Process A and in Process B after 49 and 38 days of fermentation, respectively.

Both ways of processing cracked green table olives (Process A and Process B) affect the acidity levels, the concentrations of organic acids, the level of phenolic compounds and the survival of *Enterobacteriaceae* in the brines,. as well as the organoleptic attributes of the olives. In Process A, the acids produced through microbial activity and the phenolic compounds from the olives were partially removed when the brine was changed. In these conditions the growth of *Enterobacteriaceae* in Process A was inhibited later when compared to Process B. Olives produced under these circumstances acquire the desirable organoleptic characteristics earlier as the partial removal of phenolic compounds causes a quicker debbiterization. Nevertheless, both fermentations gave origin to final products with the expected sensorial attributes where *Enterobacteriaceae* were not isolated according to the methodology used.

Reference

215

Screening of commercial immobilized lipases for the production of structured lipids for dietetic products: activity and batch operational stability tests

Nunes, Patrícia A. (1,2); Pires-Cabral, Paula (1,2); Ferreira-Dias, Suzana (1)

1: Instituto Superior de Agronomia, Centro de Estudos de Engenharia, Universidade Técnica de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal;
2: Instituto Superior de Engenharia, Universidade do Algarve 8005 Faro, Portugal

E-mail: pcabrita@ualg.pt

Keywords: acidolysis; capric acid; commercial lipases; enzymatic modification; olive oil; structured lipids

Abstract

In recent years, the interest in dietary lipids towards prevention, as well as treatment of several diseases, in order to improve health condition, has increased dramatically. These functional structured lipids may be produced by enzymatic modification of fats and oils and used for nutritional, pharmaceutical and medical applications. They consist of novel triacylglycerols (TAGs) obtained by changing the native fatty acid (FA) profiles or by the incorporation of new desired FA in the acylglycerol backbone. As an example of SLs with known benefits in human health are TAGs containing medium chain fatty acids (M) at positions 1 and 3 and a long chain fatty acid (L) at position 2, i.e. MLM type TAG. In fact, these SLs present dietetic interest for their lower caloric value (about 5kcal/g).

This work aimed at the production of TAGs of MLM type. These SLs were obtained by interesterification of olive oil with capric acid catalysed by three different 1,3-selective commercial immobilized lipases from *Thermomyces lanuginosa* (Lipozyme TL IM)*, Rhizomucor miehei* (Lipozyme IM) and *Candida antarctica* (Novozym 435). The reactions were performed at 40ºC in solvent-free media or in *n*-hexane media. A molar ratio olive oil:free fatty acid of 1:2 was used. In general, higher yields were attained in solvent-free media. Under these conditions, the incorporation yields in the TAGs, after 24-h reaction time, were 28.7%, 31.0% and 31.8% for acidolysis catalyzed by *Rhizomucor miehei, Thermomyces lanuginosa* or *Candida antarctica* lipases, respectively.

The operational stability of the three immobilized lipases was evaluated in repeated use along 10 consecutive batches (1batch = 24 h) carried out in solvent-free media, at 40 ºC. Lipozyme IM maintained the initial activity along the reutilization batches. Novozym 435 activity decreased to half of its initial value at the end of the 10th batch, following a series-type inactivation kinetics, and Lipozyme TL IM had lost completely the activity at the end of 8 th batch.

Reference

219

Production of bioethanol from concentrated cheese whey lactose using flocculent *Saccharomyces cerevisiae*

Silva, Ana Carina; Guimarães, Pedro M. R.; Teixeira, José A.; Domingues, Lucília

IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

E-mail: prguimaraes@deb.uminho.pt

Keywords: Cheese whey, lactose fermentation, bioethanol, yeast, flocculation

Abstract

The lactose in cheese whey (the main by-product of dairy industries) is an interesting substrate for fermentation processes, particularly for the production of bulk commodities such as bioethanol, due to the large amounts of whey surplus produced globally. Whey fermentation yields potable ethanol that can be used not only for fuels but also in food and beverage industries. Since most lactose-consuming microorganisms do not present physiological characteristics suitable for ethanol production bioprocesses, the construction of genetically engineered *Saccharomyces cerevisiae* (wild strains are lactose-negative) strains able to ferment lactose has been envisaged.

Our group has constructed a lactose-fermenting *S. cerevisiae* strain expressing the *LAC12* (permease) and *LAC4* (beta-galactosidase) genes of *Kluyveromyces lactis*, using a highly flocculent strain (NCYC869) as host for transformation. The properties of the original recombinant (T1) were further improved using evolutionary engineering approaches, which yielded an evolved strain (T1-E) that fermented lactose faster with higher ethanol yield and with improved flocculation (Guimarães et al., 2008, Appl Environ Microbiol 74: 1748-56).

In shake-flask fermentations with concentrated whey containing 150 g/L initial lactose, the evolved strain was unable to completely consume the lactose (lactose residual > 35 g/L) producing 6% (v/v) ethanol. Supplementation of the whey with 10 g/L of corn steep liquor (CSL) enhanced lactose consumption (residual < 3 g/L) and increased the ethanol titre to 8% obtained after 42 h of fermentation, which corresponds to an ethanol productivity of > 1.5 g/L/h. The evolved strain has further been tested in a 6 L air-lift bioreactor, producing over 6% ethanol from concentrated whey (110-120 g/L lactose) supplemented with 10 g/L CSL. Taking advantage of flocculation, yeast biomass was easily recycled by sedimentation for repeated-batch operation, allowing accumulation of high cell densities in the bioreactor. The yeast biomass was active for over 5 consecutive fermentations during which viability (methylene blue staining) was > 95%. The ability of the evolved recombinant to ferment concentrated cheese whey lactose together with its flocculation characteristics have the potential to greatly enhance the economical viability of whey-to-ethanol conversion processes.

Reference

226

Fungal flora in the wine regions of Vinho Verde (Portugal) and Alvariño (Spain): Incidence of *Botrytis cinerea* and *Penicillium expansum* and subsequent production of off-odours in grape juice

Morales-Valle, Héctor; Paterson, Russell; Lima, Nelson; Venâncio, Armando

Departamento de Enginheria Biológica da Universidade do Minho

E-mail: hmorales@deb.uminho.pt

Keywords: Botrytis cinerea; *Penicillium expansum*; Geosmin; Off-odours

Abstract

Vintage in the wine region of Vinho Verde (North of Portugal) is carried out during the last week of September or even during early October. In the homologue wine region of Alvariño (South of Galicia, Spain), harvesting is carried out earlier. The *Gray mould* - caused by the development of *Botrytis cinerea* - is the most frequent grape disease in these regions and it may be associated to some species of *Penicillium*. This may lead to the production of volatile compounds which may persist during the vinification process.

The purpose of this assay was to assess whether significant differences in the incidence of *B. cinerea* and *Penicillium* spp. (mainly *P. expansum*) exist in the vineyards of both countries. In a second phase, the isolates of *B. cinerea* obtained were tested for their capacity to induce the production volatile compounds.

Grape samples were incubated until fungi were observed and then transferred to new plates for genera identification. Those isolates identified as *Penicillium* spp. were further identified to species level. Each one of the *B. cinerea* isolates were inoculated in grape juice and re-inoculated with *P. expansum*. The samples were analyzed for volatile compounds by Solid Phase Micro-Extraction (SPME) coupled with GC/MS (Boutou *et al*., 2007). The compounds analyzed were geosmin (GEO); methyilisoborneol (MIB); fenchone; fenchol and chloroanisoles.
The vineyards in Portugal registered higher incidence of *Penicillium* spp and specifically of *P. expansum*. About 20% of samples presented peaks at the retention time of GEO. However, although the characteristic ion of GEO was detected (m/z 112), this was not the main ion in the spectrum. All the samples accumulated MIB; 50% accumulated 1-octen-3-ol; 44% accumulated fenchone and 78% accumulated fenchol. One or more tricloroanisols were detected only in 1 to 5 % of the samples.The higher incidence of *P. expansum* in Portuguese vineyards may be due to a later harvesting, when rainfalls are more common. Proper agricultural management which may include the use of weaker rootstocks to advance the ripeness of grape should be considered. There seems to be higher incidence of other fungal metabolites different from GEO that cause off-odours. Actually, MIB was detected in all the samples. Accumulation of GEO may occur in very specific incubation conditions of the fungi responsible.

Boutou, S.; Chatonnet, P. (2007). J. Chromat. A. 1141, 1-9

Reference

228

Analysis of volatile fungal metabolites in grape juice naturally contaminated with *Botrytis cinerea* and *Penicillium expansum*

Morales-Valle, Héctor; Silva, Luis-Carlos; Paterson, Russell; Oliveira, José Maria; Venâncio, Armando; Lima, Nelson

Departamento de Enginheria Biológica da Universidade do Minho

E-mail: hmorales@deb.uminho.pt

Keywords: Botritis cinerea; *Penicillium expansum*; Geosmin; Volatile compounds; SPME; GC/MS;

Abstract

The contamination of grapes by *Botrytis cinerea* is one of the most destructive diseases in vineyards. Besides the injuries caused to the grape and the undesirable oxidation of the botrytized musts, the grapes become more sensitive to the attack of other fungi thus facilitating the production of fungal volatile metabolites that can lead to the presence of off-odours in grape juice and wine. The presence of *Penicillium expansum* in botrytized grape juice is associated to the accumulation of geosmin (GEO) and 2-methylisoborneol (2MIB) in wine which are responsible of earthy odours.

The objective of this assay was to develop a method for the analysis of fungal volatile metabolites in naturally contaminated grape juices. The metabolites to be analyzed were GEO; 2-MIB; Fenchone; Fenchol; 1-octen-3-ol; 2,4,6-trichloroanisole; 2,3,4,6-tetrachloroanisole; 2,4,6-tribromoanisole and pentachloroanisole. For that purpose, a method for analysis of volatile compounds in wine (Boutou *et al.*, 2007) was adapted to grape juice.

Grape juice spiked with standards of the analytes was used during the whole process in order to take into account the matrix effect. The extraction of the compounds was carried out by Solid Phase Micro-extraction (SPME) and the analyses were performed by Gas Chromatography coupled with Mass Spectrometry (GC/MS). The extraction parameters a) time of extraction, b) content of ethanol, c) saturation of NaCl, d) pH and e) temperature were tested. Those conditions that maximized the extraction of GEO and 2MIB were chosen as optimal.

SPME extraction during 30 min at 50 ºC optimized the extraction of GEO and MIB. Also, bigger peaks of both analytes were observed when the samples were saturated with NaCl and in the absence of ethanol. The pH of the sample did not seem to influence when saturated with NaCl and thus, the extractions were performed at grape juice pH. Good calibration curves, repeatability and recovery were obtained for GEO, MIB, fenchone, fenchol and 1-octen-3-ol. However, R^2 for calibration curves of anisoles was lower than 0,900.

This method permits to detect and quantify several volatile metabolites of fungal origin. The optimization of the extraction was performed in the basis of GEO and MIB because the olfactory threshold in wine of this compound is very low (50ng l^{-1}) and so the method should detect very low amounts of it. However, the anisoles can only be detected but not quantified.

Boutou, S.; Chatonnet, P. (2007). J. Chromat. A. 1141, 1-9

Reference

229

Contribution of cereals and breakfast cereals to mineral availability; phytate concentration and expression of mineral transporters

Santos, Ana Rita Basílio; Ribeiro, Beatriz Cardoso; Gomes, Ana; Vasconcelos, Marta

Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Portugal

E-mail: ritasantos305@gmail.com

Keywords: Phytates; Cereal grain; Biovailability; Genetic expression; Mineral transporters

Abstract

In ideal scenery, daily food components should contain all the necessary macro and micronutrients for good health. However, in several countries, many nutritive deficiencies, such as of Fe, Ca, Zn and Cd have been identified. The understanding of which genes are involved in the transport of these mineral nutrients to the edible plant parts is of great importance. Amongst the genes involved in this mechanism are the ferritins, the Zips and the phytosiderophores. Later, when a mineral is ingested, only a fraction is absorbed and used by the organism. Several compounds influence bioavailability, one of which is phytate, the dominant storage of phosphorus in cereals. This work aimed at evaluating phytate concentration in daily diet cereals (Avena sativa, Hordeum vulgare, Secale cereale, Lolium multiflorum, Zea mays, Triticum aestivum and breakfast cereals) as well as to study ferritin, ZIP and iron phytosiderophore gene expression in different plant parts. RT-PCR analysis showed that ferritin was expressed in different wheat tissues, as well as in rye and oat leaf. The ZIP gene was expressed in wheat and barley stems and leaves. Corn and ryegrass leaves expressed the iron/phytosiderophore gene, as well as oat stem and leaf. Bioinformatic analysis showed that the ferritin motif has much more conserved regions than the ZIP motif, suggesting that the ferritin gene, in these cereal grains, suffered smaller evolutionary changes. This statement was also supported by the phylogenetic analysis.

Phytate was determined using the modified WADE reagent method. However, in order to increase the total phytate extraction from cereal grain samples, several parameters were optimized in the extraction protocol: 1) increased time of digestion to 9 and 16h, 2) increased HCl concentration to 0,8M, and 3) modification in the sample:WADE ratio. First and second parameters allowed extracting the maximum phytate amount. With regards to the phytate concentration, rye, oat and corn showed to have the highest phytate concentration, as well as Nestum Honey and Inflated Wheat with Honey, in case of breakfast cereals.

Reference

234

Encapsulation of different probiotic bacteria in fresh cheese

Rodrigues, Francisca Lisboa (1); Andrade, José Carlos (2); Andrade, M. Beatriz Oliveira (1)

1: Faculty of Pharmacy University of Oporto, Portugal;
2: Health Sciences Research Center, Department of Pharmaceutical Sciences, Instituto Superior de Ciências da Saúde, Gandra, Portugal

E-mail: franciscapintolisboa@gmail.com

Keywords: probiotics, encapsulation

Abstract

The most popular food delivery systems for **probiotic** cultures have been freshly fermented or unfermented dairy foods, including milk, yoghurt, ice cream and desserts. The growth in the development of foods containing probiotics is expected to continue. To be successful, manufacturers must consider the effects of the environment of the food during processing and storage to ensure that the concentration of probiotics at the time of consumption provides a therapeutic dose to consumers. In addition, the foods with added probiotics must maintain the characteristic sensory attributes of the traditional food. Numerous techniques, such as **encapsulation**, have been adapted to enhance the viability of probiotic bacteria in the harsh conditions characteristic of many cultured dairy products. Current research on novel probiotic formulations and microencapsulation technologies exploiting biological carrier and barrier materials and systems for enteric release provides promising results.

Because of its higher pH, fat content and more solid consistency, cheese offers certain advantages over fermented milk products in terms of delivering viable probiotics to the human gut and, therefore, has been considered to be an ideal vehicle for probiotic uptake. Additionally, the oxygen level of cheese is remarkably reduced by the cheese microorganisms within a few weeks of storage, which provides an almost anaerobic environment favouring the survival of bifidobacteria. It is a well-established fact that although highly strain dependent, the viability of probiotic strains in cheese is restricted by the presence of salt.

The purpose of this work is to discuss the potential for cheese as an effective vehicle for incorporation of probiotics into the food supply, to discuss the processing conditions necessary to effectively incorporate them into the cheese and to discuss materials and methods for the microencapsulation of lactobacillus and bifidobacterium in fresh cheese..

Reference

237

Contribution to the characterization of the natural fermentation of Medronho (*Arbutus unedo* L.)

Santo, David Miguel Cabrita do Espírito; Cavaco, Teresa; Quintas, Célia; Galego, Ludovina

Universidade do Algarve- Instituto Superior de Engenharia, Portugal

E-mail: disturbencia@hotmail.com

Keywords: *Arbutus unedo*, Medronho, Yeasts, fermentation

Abstract

"Aguardente de Medronho" is a traditional spirit produced in the South of Portugal, much appreciated due to its sensory characteristics. It is manufactured by fermenting the berries of the Mediterranean shrub *Arbutus unedo L* (strawberry tree). Historically this product has been a complement to farmers' income, and on account of that fact, over the last years efforts have been made to improve the quality of the production process and of the final product. The present study investigated the fermentation process and its influence in the quality of the distillate. The evolution of the total viable counts and of the yeast populations during the fermentation process was controlled. The changes of pH, Brix, reducing sugars, acids, ethanol, ethyl acetate and isopentanols were also monitored. Fruits were harvested during the month of November, mixed with water in 500 l stainless steel vessels that were immediately closed and then allowed to ferment over a specific period of time. This process, took place at room temperature, during autumn and winter, relying on the natural microbiota initially present on the fruits and on the surrounding environment. Total viable counts were determined using acidified Plate Count Agar and yeasts were counted on acidified Malt Extract Agar. Sugars and acids were measured by High-performance liquid chromatography (HPLC) and the volatile compounds (ethanol, ethyl acetate and isopentanols) were studied by headspace-solid-phase microextraction-gas chromatography (HS-SPME-GC) with a polyacrylate fiber. During the fermentation period, yeasts grew exponentially reached stationary phase and remained on this phase for the rest of the study. The levels of sugars and acids increased during the first days and decreased thereafter. The content of ethanol, ethyl acetate and isopentanols reached values of 700 g, 2,5 g and 0,7 g per kg of fermented fruit, respectively, as a result of the metabolic microbial activity. The quality parameters of the distillate obtained after the fermentation period were in accordance with both the European Directive (Regulation (EC) Nº 110/2008 of the European Parliament and of the Council of 15 January 2008) and the specific Portuguese "medronho" distillate legislation (Decreto-Lei nº 238/2000 of 26 September 2000).

Reference

239

Integrated strategy to protect stored rice from *fungi*

Matos, Olívia Cruz (2); Magro, Ana (1); Barata, Margarida (3); Carolino, Manuela (3); Bastos, Margarida (4); Mexia, António (5)

1: Instituto de Investigação Científica Tropical. Lisboa. Portugal;
2: Instituto Nacional de Recursos Biológicos, Quinta do Marquês, 2784-505 Oeiras. Portugal;
3: Universidade de Lisboa, Faculdade de Ciências, Centro de Biologia Ambiental, Lisboa, Portugal.;
4: Universidade do Porto, Faculdade de Engenharia (LEPAE), Porto, Portugal;
5: Universidade Técnica de Lisboa, Instituto Superior de Agronomia, Lisboa, Portugal

E-mail: oliviamatos@sapo.pt

Keywords: stored rice, fungal infection, fungus control, plant extracts, natural products

Abstract

Rice (Oryza sativa) is the main food of half of the population of our planet. The growth of fungi together with the eventual occurrence of mycotoxins can be responsible for serious economic losses and public health risks. Knowing the contaminating mycota of different origins is a prerequisite to the establishment of mycotoxin control programs.

Socio-economical and environmental factors conduced to an extreme reduction of rice availability, while the estimated rice production losses increased in all continents what increases the importance of new harmless strategies to the control of fungi affecting stored rice. Natural products from plant origin were screened for the control of main pernicious fungi.

In this work we have collected rice samples from different origins (national and imported) and these samples were analysed for fungal infection. Several fungi were isolated, *Absydia, Alternaria, Aspergillus, Bipolaris, Botrytis, Chaetomium, Curvularia, Cunninghamela, Epicoccum, Fusarium, Geotrichum, Helicoma, Nigrospora, Penicillium, Pyricularia, Rhizopus, Scytalidium, Stemphylium, Sordaria, Trichoconiella, Trichoderma, Trichothecium e Ulocladium.* Some of the fungi isolated are mycotoxins producers.

We also study a way to control the growth of some of these fungi using plant extracts and essencial oils from *Syzyginum aromaticum* and *Laurus nobilis*. Promising results were obtained.

Ana Magro, Margarida Barata, Olívia Matos, Margarida Bastos, Manuela Carolino, António Mexia (2008). Fungos/Fungi. In Contribuição para a protecção do arroz armazenado/ Contribution for integrated management of stored rice pests (Mancini, R., Carvalho, O., Timlick, B. & Adler, C. eds.). Lisboa, Capítulo 1, 1-11.

Ana Magro, Margarida Barata, Olívia Matos, Ana Passarinho, Inês Pires, Alexandra Faro, Manuela Carolino, Margarida Bastos, Arlindo Lima, António Mexia (2008). Detecção e identificação de fungos no arroz armazenado/Detection and identification off fungi in stored rice. In Contribuição para a protecção do arroz armazenado/ Contribution for integrated management of stored rice pests (Mancini, R., Carvalho, O., Timlick, B. & Adler, C. eds.). Lisboa, Capítulo 1, 12-22.

O. Matos, C. Pinto Ricardo, (2006). Screening of plants against fungi affecting crops and stored foods. In Naturally Occurring Bioactive Compounds (Editors M. K. Rai and D. Mares), Elsevier B.V., 137-168.

Reference

240

An industrial brewer's yeast strain in very high gravity wort fermentations

Lima, Luis (1); Brandão, Tiago (2); Ferreira, António (2); Lima, Nelson (1); Teixeira, José (1)

1: Universidade do Minho, Portugal;
2: Unicer Bebidas S.A.

E-mail: luislima@deb.uminho.pt

Keywords: Brewing, High gravity wort, Fermentation

Abstract

In the beer production, the fermentation of worts whose gravity is higher than 18 ºP has been presented as an efficient way to improve the capacity of brewery facility without changes in the brewhouse.
In this work, a brewer yeast strain was submitted to high gravity fermentations to evaluate the influence and the limits of the environmental parameters on the fermentation performance and final product profile. The variables included in this study were the primary fermentation temperature, wort concentration (extract) and yeast pitching rate. Static fermentations were carried out anaerobically in 2 L tall tubes (EBC recommendation for small scale brewing fermentations) with 15, 18 and 22 ºP wort, at constant temperature of 12, 15 and 18 ºC. The yeast was grown aerobically at 27 ºC in 15 ºP wort (saturated with air) and collect by filtration to inoculate by mass at different pitching rates of 12, 18 and 22 million cells/ml. Fermentations were monitored daily measuring the ethanol, extract, pH, temperature, biomass in suspension and viability. At the end of the primary fermentation, the green beer was analysed concerning diacetil, esters and high alcohols by gas chromatography. Fermentation data were statistically treated in order to obtain an optimum approach for the studied parameters. For statistical proposes, the fermentation performance was measured by ethanol produced, apparent extract and the fermentation time.
Although the temperature has a positive effect on the indicators of the fermentation performance, the wort concentration has not a positive effect for all indicators. When the wort has higher concentration at the beginning of the fermentation, as expected, the fermentation takes longer to be finished with more ethanol produced.
Considering that the produced ethanol, the fermentation time and the residual extract have the same level of interest for the brewing process, the statistical analysis showed that the optimal point for the wort concentration, fermentation temperature and pitching rate is 20 ºP, 18 ºC and $20x10^6$ cell/ml, respectively. The optimal conditions were repeated and the beer profile regarding the aroma profile was determined.

Reference

241

Identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section *Flavi* isolated from Portuguese almonds

Rodrigues, Paula (1,2); Kozakiewicz, Zofia (1); Venâncio, Armando (1); Lima, Nelson (1)

1: IBB–Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal;
2: CIMO–Escola Superior Agrária de Bragança, Campus de Santa Apolónia, 5301-855 Bragança, Portugal

E-mail: nelson@iec.uminho.pt

Keywords: Aspergillus; Section Flavi; aflatoxins; cyclopiazonic acid

Abstract

Aspergillus subgenus *Circumdati* section *Flavi*, also referred to as the *A. flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided in two groups of species. One includes the aflatoxigenic species *A. flavus*, *A. parasiticus* and *A. nomius*, which cause serious problems in agricultural commodities, and the other one includes the non-aflatoxigenic species *A. oryzae*, *A. sojae* and *A. tamarii*, traditionally used for production of fermented foods. Differentiating aflatoxigenic from non-aflatoxigenic species and strains in food commodities is of major importance in food quality control. A polyphasic approach consisting of morphological, chemical and molecular characterization was applied to 31 isolates of *Aspergillus* Section *Flavi* originating from Portuguese almonds, with the aim of characterizing and identifying aflatoxigenic and non-aflatoxigenic strains. On the basis of morphological characters, we found two distinct groups among the population under study: 58% were classified as *A. parasiticus* and the remaining 42% were classified as *A. flavus*. Chemical characterization involved the screening of the isolates for aflatoxins B (AFB) and G (AFG), and also for cyclopiazonic acid (CPA), by HPLC. All *A. parasiticus* isolates were strong AFB and AFG producers, but no CPA production was detected. The *A. flavus* isolates showed to be more diversified, with 77% being atoxigenic, whereas 15% produced CPA and low levels of AFB and 8% produced the 3 groups of mycotoxins. Molecularly, two genes of the aflatoxin biosynthetic pathway, *aflD* (=*nor1*) and *aflQ* (=*ord1*= *ordA*) were tested for presence and expression (by PCR and RT-PCR, respectively). The presence of both genes did not correlate with aflatoxigenicity. *aflD* expression was not considered a good marker for differentiating aflatoxigenic from non-aflatoxigenic isolates, but *aflQ* showed a good correlation between expression and aflatoxin-production ability.

Acknowledgements: Paula Rodrigues is supported by the grant SFRH/BD/28332/2006 from FCT.

Rodrigues P, Venâncio A, Kozakiewicz Z, Lima N, 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus Section Flavi isolated from Portuguese almonds. International Journal of Food Microbiology 129: 187-193.

Reference

244

An electronic tongue for beer differentiation

Dias, Luís G. (1); Peres, António M. (2); Barcelos, Tânia P. (1); Morais, Jorge Sá (1); Machado, Adélio A. S. C. (3)

1: CIMO, Escola Superior Agrária, Instituto Politécnico de Bragança, Portugal;
2: LSRE, Escola Superior Agrária, Instituto Politécnico de Bragança, Portugal;
3: LAQUIPAI, Departamento de Química, Faculdade de Ciências da Universidade do Porto, Portugal

E-mail: ldias@ipb.pt

Keywords: Electronic tongue, Beer differentiation, Linear discriminant analysis

Abstract

In this work an electronic tongue, based on a potentiometric solid-state multi-sensor array, with 36 polymeric membranes, was built for developing an analytical tool to apply in process monitoring and quality control. As a first approach, this tool was applied together with a supervised pattern recognition tool to semi-quantitatively differentiate beers with different alcoholic levels.

The multi-sensor array device was built with membranes prepared using PVC, as polymeric matrix, and different combinations of 6 plasticizer compounds and 6 membrane additives. The multi-sensor system includes a reference electrode Ag/AgCl with double junction, being connected to a multiplexer Agilent Data Acquisition/Switch Unit model 34970A. Measurements were performed in a double wall glass cell thermostatized at 25 ºC. All samples were degassed and analysed during a 10-15 minutes period.

The ability of the multi-sensor device to recognise different alcohol levels was initially evaluated using ethanol standard solutions (concentrations varying from 0.1% to 10.0%). The results obtained showed that the response of some of the polymeric membranes varied linearly when the ethanol content increased. Considering these satisfactory results, the device was applied for semi-quantitative alcohol level beer recognition.

Forty-two beers, with ethanol levels varying between 0% to 8%, from different countries, of different brands and colors and prepared from different grains, were purchased in Portuguese commercial markets. The ethanol content of each beer was confirmed by HPLC analysis using a Varian HPLC system with a refraction index detector, equipped with a Supelcogel C-610H column (30cm x 7.8mm ID).

The multi-sensor signal pattern recorded for the beers analysed, together with linear discriminant analysis (LDA) was used to distinguish between 4 groups of beers with different alcoholic levels (A: lower than 1%, B: between 4% and 5%, C: between 5% and 6% and D: between 6% and 8%). A stepwise LDA analysis was performed and it was verified that only the signals of 18 polymeric membranes were included in the final model ($p<0.001$ for Wilks' Lambda test). Three significant discriminant functions were established ($p<0.001$) accounting for 100% of the total variance. The approach had a satisfactory ethanol level recognition performance, as it allowed 95.2% and 73.8% correct classification for original grouped cases and "leaving one-out" cross-validation procedure, respectively.

Acknowledgements: Financial support received from FCT, Lisbon, under research projects POCI/QUI/58706/2004 and PPCDT/QUI/58076/2004 is gratefully acknowledged.

Reference

256

Physiologycal characterization of an industrial strain of *Saccharomyces cerevisiae* during wine fermentation

Prista, Catarina (1); Salvador, Vanessa (1); Viana, Tiago (1); Fareleira, Paula (2); Loureiro, Virgílio (1); Loureiro-Dias, Maria C. (1)

1: Instituto Superior de Agronomia, Portugal;
2: L-Instituto Nacional de Investigação Agrária/ Instituto Nacional de Recursos Biológicos, I.P., Portugal, Portugal

E-mail: cprista@isa.utl.pt

Keywords: *Saccharomyces cerevisiae*,wine fermentation,cell viability,proton homeostasis, sugar uptake

Abstract

Although very many studies have been performed on the physiology of lab *Saccharomyces cerevisiae* strains under lab conditions almost nothing is known on how the industrial strains behave under the stress conditions of winemaking.
A *S. cerevisiae* strain was selected from a commercial starter and used to inoculate white grape must in all the experimental assays. The fermentation process was performed at 15, 25 and 30ºC and was monitored up to the end of the fermentation, when no sugar was detected by standard methods. Several physiologic parameters were evaluated for cells collected at defined physiological stages.
As fermentation proceeds, the OD and the number of viable and biological active cells evolved in a similar way for the 3 assayed temperatures. After 7-8 generations, cells entered stationary phase (the longer phase of the fermentation process). Still, no significant decrease of the number of viable and biologically active cells was observed up to the point where no sugar was detected, after which a reduction of viable cells up to 40-50% was observed. Consistently, significant signs of apoptosis and/or necrosis (30-45% of the cells) were only detected in cells collected after sugar exhaustion.
H^+ extrusion doubled during the exponential phase, decreasing significantly at the end of fermentation. Also, passive H^+ influx was significantly higher in exponential phase cells and decreased significantly in cells entering the stationary phase, even when exposed to ethanol up to 16% (v/v). These differences were more pronounced for lower fermentation temperatures.
Kinetic parameters for in vivo ^{14}C-glucose and ^{14}C-fructose uptake and the time course for substrate consumption (by in vivo ^{13}C-NMR), and enzymatic activity were evaluated. The results were consistent with the good performance of the cells during the fermentation.
Curiously, regardless the fact that cells revealed a “healthy” state up to the end of fermentation, stationary phase cells presented a quite low intracellular pH, reaching values of 5.0 and 5.4 at the end of fermentation performed at 15 and 25ºC, respectively. At 30ºC the intracellular pH value was 5.8 at the same stage. In view of the results obtained for the other physiological parameters evaluated, these intracellular pH values cannot be assigned to a defective metabolic state of the cells.

Reference

261

Influence of culture medium and temperature in the growth and sporulation of *Penicillium expansum*

Prata, Margarida Barros (1,2); Mussatto, Solange Inês (1); Rodrigues, Lígia Raquel (1); Teixeira, José António (1)

1: Universidade do Minho, Departamento de Engenharia Biológica, Portugal;
2: Universidade do Porto, Departamento de Química, Portugal

E-mail: gui_prata@iol.pt

Keywords: P. expansum, growth, sporulation, potato dextrose agar, czapek-dox agar.

Abstract

Microorganisms require nutrients as a source of energy and certain environmental conditions in order to grow and reproduce. In the environment, microbes have adapted to the habitats most suitable for their needs. However, in laboratory these requirements must be met by a culture medium[1].

In the present work, mycelia growth and early spore formation of *Penicillium expansum* (MUM 02.14) at 22 ºC and 27 ºC, in Potato Dextrose Agar (PDA) and CZAPEK-Dox Agar (CZ) media, were evaluated. Both media were prepared according to indications set by the manufacturers (PDA – *BD Difco* ™, and CZ –*Oxoid Ltd.*), autoclaved at 121 ºC for 15 min, and poured in Petri dishes. Twelve plates were prepared: 6 with PDA and 6 with CZ medium. All of them were inoculated with 40 µl of a spore suspension containing 7.55×10^7 spores/ml. Six plates (3 PDA and 3 CZ) were incubated at room temperature (22 ºC), while the remaining were grown at 27 ºC. Growth was assessed every day during eight days and the results were expressed in millimetres of colony diameter per hour. Sporulation was determined by counting in a Neubauer chamber after suspending the spores in 5 ml of 0.1% w/v Tween 80 solution.

Statistical analysis of the data revealed a significant influence of the temperature (at 95% confidence level) on *P. expansum* mycelia growth. Nevertheless, at the same set of temperature there was no difference between both media. On the other hand, the sporulation results showed statistically significant differences when varying both the culture medium and temperature, with CZ being the less suitable medium for *P. expansum* sporulation at both temperatures. PDA medium gave the best sporulation results at 22 ºC (29.16×10^7 spores/ml), which allowed us to conclude that such conditions are the most feasible ones for a rapid growth and sporulation of *P. expansum.*

1- Rani, I., Sohail, M., Akhund, S. and Abro, H., Abrus sucrose agar a new medium for the growth of fungi (2007). Pak. J. Bot., 39: 1883–1885.

Reference

263

Temperature and solids effect on gas-liquid mass transfer

Ferreira, António M. A. (1,2); Ferreira, Cecília (2); Rocha, Fernando (2); Teixeira, José António (1)

1: Centro de Engenharia Biológica da Universidade do Minho, Portugal;
2: Departamento de Engenharia Química, Faculdade de Engenharia da Universidade do Porto, Portugal

E-mail: AFerreira@deb.uminho.pt

Keywords: Bubble column; mass transfer; multiphase reactors; absorption, solids

Abstract

The temperature and solids effect on the mass transfer characteristics in a bubble column was studied experimentally for the systems air/water/polyvinyl chloride (PVC) beads and air/water/expandable polystyrene (EPS) beads. Volumetric liquid side mass transfer coefficient, k_La, was determined under different temperatures (20, 25, 30, 35ºC), solid concentrations (up to 5%), gas flow rates (up to 7.4 mm/s) and solid sizes (549 and 210 µm for PVC beads, and 591 µm for EPS beads).

The results show that the temperature plays an important role on mass transfer phenomena, by increasing k_La and, simultaneously, promoting the solids influence on k_La.

The presence of solids affects negatively k_La being this effect more pronounced for the largest particles (PVC case). In addition, a decrease in k_La occurs when the solid loading increases (observed in both cases, PVC and EPS).

Comparing the PVC and EPS effects on k_La, it can be concluded that physical and chemical properties of solids are important parameters to be taken into account on this kind of studies.

An empirical correlation for k_La on the experimental variables was developed.

Reference

264

Use of immobilized cells in repeated fermentation cycles for an efficient biotechnological production of an alternative sweetener - xylitol

Sarrouh, Boutros Fouad (1); da Silva, Silvio Silvério (1, 2)

1: Engineering School of Lorena - USP, Brazil;
2: Fatea - Faculdades Integradas Teresa D'Ávila, Brazil

E-mail: bsarrouh@yahoo.es

Keywords: repeated batch fermentation, immibilized cells, fluidized bed reactor, hemicellulosic hydrolysate, xylitol

Abstract

Xylitol is a valuable pentitol and has specific health claims in the world market. It is suitable for diabetes, recommended for oral health. On an industrial scale, xylitol is currently produced through chemical reduction of xylose. The chemical process for production of xylitol is expensive because of the high temperature and pressure required for hydrogenation of xylose. Furthermore, extensive steps for separation and purification add to the cost. Alternatively biotechnological production of xylitol could be of economic interest and attractive, as crude hemicellulosic hydrolysate can be used as potential substrates, instead of pure xylose, to reduce the cost of production. However, the concentrations, yields and production rates obtained from fermentation media consisting in lignocellulosic hydrolysate are still the bottlenecks of a large-scale process, although they can be improved by selecting the right fermentation system, operation mode and cultivation conditions. Immobilized cell systems have been traditionally considered as an alternative for increasing the process overall productivity and minimizing production costs. In order to improve xylitol batch production on sugarcane bagasse hemicellulosic hydrolysate, in this work, 5 repeated fermentation cycles were carried out in a three-phase fluidized-bed bioreactor (FBR) using immobilized *Candida guilliermondii* FTI 20037 cells in Ca-alginate beads. According to obtained results, it was found that the immobilized cells could be reused successfully for 5 batch cycles with average xylitol yield (Yp/s) of 0,69±0,034 g/L and average volumetric productivity (Qp) of 0,43±0,14 g/L.h in the bioreactor for 360 h of consecutive fermentation.

Acknowledgment: FAPESP.

Reference

267

Antimicrobial susceptibility of *Listeria monocytogenes* strains derived from food and food-processing bakery plant

Silva, Joana; Eusébio, Cátia; Carneiro, Luisa; Magalhães, Rui; Santos, Isabel; Almeida, Gonçalo; Teixeira, Paula

CBQF/ESBUCP, Portugal

E-mail: jglaranjeira@mail.esb.ucp.pt

Keywords: *Listeria monocytogenes*, antibiotic resistance

Abstract

The susceptibility of 167 strains of *Listeria monocytogenes* isolated from a bakery industry, from food and food-processing environment, to 11 antibiotics was determined by the standard agar dilution methodology. The tested antibiotics were: ampicillin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, nitrofurantoin, penicillin, rifampicin, streptomycin, tetracycline and vancomycin; minimalinhibitory concentrations values were used to classify the strains into sensitive, moderately resistance and resistant.

All the tested isolates were found to be susceptible to ampicillin, chloramphenicol, gentamicin, nitrofurantoin, penicillin, rifampicin, tetracycline streptomycin and vancomycin. In the case of erythromycin, 54 isolates (32%) were susceptible, 68 (41%) displayed moderately resistance and 45 (27%) were resistance. Concerning to ciprofloxacin, a moderate resistance was observed in 12 strains (7%) against 155 strains (93%) that were susceptible.

Generally, this study showed that *L. monocytogenes* strains are susceptible to the antibiotics commonly used in the treatment of listeriosis. Concerning that antibiotic resistance in some *L. monocytogenes* strains has already been described, a continued study of emerging antimicrobial resistance is important to guarantee an effective treatment of human listeriosis.

Reference

272

Selection of natural materials for use on yeast immobilization

Genisheva, Zlatina Asenova; Mussatto, Solange I.; Oliveira, José M.; Teixeira, José A.

IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.

E-mail: zgenisheva@gmail.com

Keywords: immobilization

Abstract

The interest in cell immobilization for alcoholic beverage production has increased in the last decades due to the several advantages that this process presents, including increased productivity, reduced risk of contamination, biocatalyst recycling, rapid product separation and others. As a consequence, it is of great importance to find cheap, abundant, non destructive and food grade purity immobilization supports for a good quality of the final product [1]. In the present work, three different materials were tested as support for yeast immobilization: corn cobs, grape stems and grape seeds.

The support materials were prepared by washing with distilled water and drying at 60 ºC until constant mass. A commercial *Saccharomyces cerevisiae* was the yeast strain used in the experiments. For inoculum preparation, the yeast was cultivated in YPD medium in static conditions at 30˚C for 24h. Fermentation runs were performed in semi-synthetic medium with the following composition (% w/v): glucose 9, yeast extract 0.4, $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.1, and $MgSO_4$ 0.5. The assays were carried out in 500 ml Erlenmeyer flasks containing 200 ml of medium and 2 g of the material carrier. The flasks were statically incubated at 30 ˚C for 60 h. For comparison, assays under the same conditions described above were also performed without support addition. Fermentations were carried out in duplicate, and samples were taken every 12 h for estimation of biomass, glucose consumption and ethanol production. Immobilized cells concentration was determined at the fermentation end, according to Brányik et al. [2].

All the fermentation runs with immobilized cells lasted after 24 h, half of the necessary time for total glucose consumption in medium containing free cells. Among the three evaluated materials, grape seeds gave the highest cells immobilization results (52 mg/g support), while corn cobs gave lower results (46 mg/g support) and no cells adhesion was observed into grape stems. Ethanol production was maximum (47.9 g/l) in the medium containing grape stems; however, cells were totally in the free form in this medium. Cells immobilized in grape seeds gave ethanol production (44.9 g/l) higher than cells immobilized in corn cobs (41.8 g/l). It was thus concluded that grape seeds is a wine-making residue with great potential for use as immobilization support during the ethanol production by *Saccharomyces cerevisiae*.

[1] Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R., Koutinas, A.A. Food Microbiology, 21 (2004) 377-397.
[2] Brányik, T., Vicente, A., Oliveira, R., Teixeira J. Biotechnology and Bioengineering, 88 (2004) 84-93.

Reference

273

Evaluation of phisiological and microbiological tomatoes cultivars (*Lycopersicon esculentum*) in biotechnology science

Vinha, Ana Mendes Ferreira (1,2); Coutinho, Filipe (1); Soares, Marta Oliveira (1); Barros, Raquel (1); Iglesias, Raúl (2); Estevez, José García (2)

1: Escola Superior de Saúde do Vale do Ave, Instituto Politécnico de Saúdo do Norte, Cooperativa Ensino Superior Politécnico e Universitário, Portugal;
2: Laboratorio de Parasitología, Facultad de Biología, Universidad de Vigo, Espanha

E-mail: anavinha33@gmail.com

Keywords: *Lycopersicon esculentum*; Physiological characterization; Microbiologic profile; Biotechnology science.

Abstract

The fruits present a great importance in the feeding human being and in the public health, not only for its nutritional wealth, as well as for the easy digestion, rich in minerals and vitamins levels. The tomato fruit (*Lycopersicon esculentum)* can be characterized as a fruit with a complex matrix where, the nutritional quality is evaluated in terms of macronutrients, vitamins and minerals.

The establishment of standards of quality for foods and its degree of relative specifications to the limits of tolerance, relative to the pathogenic microorganisms, depends on the previous knowledge of the microbiotic flora and its interactions with climacteric conditions. The evaluation of the physical and chemical quality of the fruits allows establishing the quality of the fruit and its commercial standardization. The fruits, in its natural state, are susceptible to microbiologic deterioration. The natural ground, water, air and predators, are some examples of interferents with some meaning in the significant increase of the microflora of vegetables.

The main objective of this study was to evaluate and characterize four tomato cultivars: "*Redondo*", "*Rama*", *Chupeta*" and "*Cereja*", in their physiological and microbiological profile (pH, moisture content, color, water activity, total soluble solid text, citric acid and ascorbic acid content and microorganisms profile). For the statistical analysis of results, using the univariate analysis (ANOVA) and t-Studant with significance level of 5%. All tomatoes cultivars had presented differences ($p<0.05$), wants in the centesimal characterization as microbiological profile. The tomato cultivar "Redondo", more used in food industry, presented high contents of ascorbic acid (27.91 mg/100g) and it was observed the presence of aerobic mesophilus ($1.5x10^3$ a $2.3x10^6$), mainly in Redondo tomatoes. The total coliforms C.F.U. were $7.1x10^4$ observed in Rama cultivar, demonstrating that the botanical aspects influence the nutritional profile of the fruits. Although the absence of information on these national tomatoes cultivars, this study becomes important in the evaluation, characterization and valuation of cultivating them in study, for different areas of the biotechnology, such as: nutrition, industry, dietotherapy and for the agriculture good manufacturing practices.

Sahlin, E.; Savage, G.P.; Lister, C.E. J. Food Composit Anal. 2004; 17(5): 635-647.
Raffo, A.; Leonardi, C.; Fogliano, V.; Ambrosino, P.; Salucci, M.; Gennaro, L.; Bugianasiu, R.O.; Giufrida, F.; Quaglia, G.. J Agric Food Chem. 2002; 50: 6550-6556.
Rebelo, E. Food Technology, 2006, 1(6): 99-106.

Reference

277

Safety issues concerning phage therapy for veterinary applications

Azeredo, Joana (1); Ferreira, Eugénio C. (1); Gannon, Ben (2); Carvalho, Carla (1); Santos, Silvio (1); Victor, Krylov (3)

1: IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal;
2: University of Bristol, Department of Clinical Veterinary Science, Langford, North Somerset, BS40 5DU, United Kingdom;
3: State Institute for Genetics of Industrial Microorganisms, 1st Dorozhnii proezd 1, Moscow, 113545, Russia

E-mail: jazeredo@deb.uminho.pt

Keywords: bacteriophages, safety evaluation, phage-resistant mutants, bacterial toxins

Abstract

Phages have been proposed as natural antimicrobial agents to fight bacterial infections in animals. This is supported by several scientific evidences of the efficacy of phage therapy for veterinary applications. However, there are important safety issues that should be taken into consideration when developing a phage product for veterinary applications. For example it is of utmost importance to guarantee that phages are non-temperate and do not encode bacterial toxins. The phage administration strategy and timing should also be adequate in order to reduce the development of phage-resistant mutants. The present work describes the main strategies used to ensure a safe phage product for veterinary application, based on the results obtained on the scope of the European Project Phagevet-P (Veterinary Phage Therapies as Alternatives to Antibiotics in Poultry Production FP6-2003-Food-2-A:007224).

Reference

281

Genotypic and phenotypic characterization of different probiotic strains of *Lactobacillus* spp. and *Bifidobacterium* spp.

Henriques, Silvino S.; Ribeiro, Guilherme M.; Marques, Maria R.; Vasconcelos, Marta W.; Gomes, Ana M.

UCP - Escola Superior de Biotecnologia, Portugal

E-mail: silvino.henriques@gmail.com

Keywords: Bifidobacterium and Lactobacillus, bile salt hydrolase, linoleic acid isomerase, genotypic analysis, adhesins

Abstract

The probiotics' beneficial effects on human health are well documented. One such benefit is the capacity of certain probiotic strains to decrease serum cholesterol levels in the presence of bile salts. This is done via the activity of the bile salt hydrolase (BSH) enzyme. In the present study, three bile salts were tested: glycocholate, taurocholate and taurodeoxycholate. Glycocholate induced the highest specific activities, and activity was strongly strain dependent: *Bifidobacterium animalis* Bb12 showed the lowest activity, whereas *Lactobacillus acidophilus* L10, *L. pentosus* LMG 10755 and *L. brevis* LMG 6906 showed the highest activity. Another enzyme, conjugated linoleic acid (CLA) isomerase, was object of study because of its beneficial health effects associated with the conversion of linoleic acid into its conjugated forms, which have been associated with anticarcinogenic and antiatherogenic effects in animal models, as well as immunomodulating and fat reduction activities. Both ewe and goat fermented milks induced rising CLA values in the presence of probiotics, in particular by *B. animalis* strains. No significant differences between matrices were detected. Bioinformatic and PCR analysis using orthologous primers were further conducted in order to evaluate the effect of genetic similarities and enzymatic activities. The data suggested a strong evolutionary relationship between sequences, probably due to genetic conservation amongst different probiotic species and strains. Similarities between genotypic and phenotypic analysis were found, but for the most part detection of the gene and/or evolutionary closeness did not correlate with enzymatic activities. PCR of CLA detected the gene on most *Lactobacillus* species tested. Two strains of *B. animalis* and one strain of *L. acidophilus* on which this gene was detected induced CLA production. Other genes related to probiotic features were genotypically accessed, namely adhesins and mucin-binding proteins, which establish the ability to colonize and to provide antagonistic effects against pathogens. Again, PCR using orthologous primers suggested evolutionary relatedness. In this work we will present the above mentioned biochemical parameters, and will discuss the relationship and the potential of different methods to detect probiotic features.

Reference

282

Improvement of fructooligosaccharides yield and productivity by solid-state cultivation of *Aspergillus japonicus* on corn cobs

Mussatto, Solange Inês; Rodrigues, Ligia Raquel; Teixeira, José Antonio

IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Portugal

E-mail: solange@deb.uminho.pt

Keywords: fructooligosaccharides, solid-state fermentation, *Aspergillus japonicus*

Abstract

Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety, which are widely used as an ingredient in functional foods. Conventionally, FOS is mainly produced on industrial scale from the disaccharide sucrose by microbial enzymes having transfructosylating activity, but the FOS production yields by this process are normally low (55–60%) [1]. In a previous work it was observed that *Aspergillus japonicus* ATCC 20236 immobilized in corn cobs has great potential for industrial application in FOS production, because high amounts of cells adhered to this material and produced FOS with high yield (66%) and productivity (6.61 g/l.h) [2]. Solid-state fermentation systems, which consist in a fermentation process allowing the growth of microorganisms on moist solid materials in the absence of free-flowing water [3], have been few explored for FOS production. Therefore, aiming to improve the previously obtained results, the present work evaluated the FOS production by *Aspergillus japonicus* cultivated on corn cobs under solid-state cultivation conditions.

A medium containing (% w/v): sucrose 20, yeast extract 2.75, $NaNO_3$ 0.2, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.05, and KCl 0.05, was prepared for the substrate (corn cobs) moistening. For the reactions, 3.5 grams of the autoclaved substrate were mixed with the required volume of medium to give 70% moisture content, and inoculated with a spore suspension to obtain $2 \cdot 10^6$ spores/gram dry substrate. The spore suspension was prepared by scrap down the spores of *Aspergillus japonicus* ATCC 20236 from PDA plates with a sterilized solution of 0.1% (w/v) Tween 80, and counted in a Neubauer chamber. Experiments were carried out in Petri plates statically incubated at 28ºC for 48 h.

FOS production in absence of free-flowing water (present work) was higher and faster than in submerged fermentation. By solid-state cultivation, total FOS concentration of 172.1 g/l was obtained after only 16 h of fermentation, corresponding to a yield of 87% and productivity of 10.76 g/l.h. Such values are 32% and 63% higher than those attained in submerged fermentation with immobilized cells. These results are of great relevance and contribute for the development of a process able to maximize FOS production at industrial level.

Acknowledgements: This work was supported by grant SFRH/BPD/38212/2007 from FCT.

[1] Yun, J.W. Enzyme Microb Technol 19 (1996) 107–117.
[2] Mussatto, S.I. Aguilar, C.N., Rodrigues, L.R., Teixeira, J.A. J. Mol. Catal. B: Enzymatic 59 (2009) 76–81.
[3] Pandey, A., Soccol, C.R., Mitchell, D. Process Biochem. 2000, 35, 1153–1169.

Reference

284

Kinetic and morphometric evaluation of fucoidan-degrading fungal strains

Rodríguez-Jasso, Rosa María (1); Mussatto, Solange I. (1); Pastrana, Lorenzo (2); Aguilar, Cristobal Noé (3); Teixeira, José A. (1)

1: Institute for Biotechnology and Bioengineering, University of Minho, Portugal;
2: Department of Biochemistry, Genetics and Immunology. University of Vigo, Spain;
3: Food Research Department, University of Coahuila, Mexico

E-mail: rosy_rodriguez@deb.uminho.pt

Keywords: Fucoidan, Fungal strains, Growth rate, Hyphal morphometry

Abstract

Fucoidan is a sulfated fucose hetero-polysaccharide found in brown algae. This compound has a wide variety of biological activities including anticoagulant, antithrombotic, antitumoral and antiviral (Alexeeva et al. 2002; Ellouali et al. 1993; McClure et al. 1992). Specific enzymes able to degrade fucoidan matrix are important tools to establish structural characteristics and biological functions of this polysaccharide. Such enzymes, called fucoidanases, have been only isolated from marine organisms (Sakai et al. 2004; Giordano et al. 2006). Reports of fungal microorganisms with enzymatic activity over this sulfated-polysaccharide are scarce.

Mycelial growth and morphology of filamentous fungi can be mathematically described by kinetic models, through the estimation of specific growth rate of molds on plates containing target polysaccharide as sole carbon source, using image processing techniques (Loera and Viniegra 1998). In this sense, the aim of this work was to identify fungal strains able to growth over fucoidan media as sources of active fucoidanases, by quantification of kinetic and morphology features, to establish the influence of media composition on growth patterns.

Aspergillus niger PSH, *Penicillium purpurogenum* GH2 and *Mucor sp.* 3P were the screened strains. Different culture media with and without mineral salts were tested for microbial growth. Fucoidan of *Laminaria japonica* and urea were used as carbon and nitrogen source. Radial growth rate (U_r) was kinetically monitored measuring colony diameters. Hyphal length (L_{av}) and diameter (D_h) were quantified by image analyses measurements.

All the evaluated strains were able to growth on different fucoidan-urea media, and their plate invasion capacity and radial growth rate were directly proportional to measured morphometric parameters. The three fungi strains synthesize acting metabolites toward fucoidan matrix, and are important tools for the synthesis of sulfated fucan-degrading enzymes. These results are, until now, the first report of enzymes able to growth and degrade fucoidan obtained by terrestrial fungus.

Alexeeva YV, Ivanova EP, Bakunina IY, Zvaygintseva TN, Mikhailov VV (2002). Lett Appl Microbiol 35:343-346
Ellouali M, Boisson-Vidal C, Durand P, Jozefonvicz J (1993) Anticancer Res 13:2011-2019
Giordano A, Andreotti G, Tramice A, Trincone A (2006) Biotechnol J 1:511-530
Loera O, Viniegra G (1998). Biotechnol Tech 12:801-804
McClure MO, Moore JP, Blanc DF, Scotting P, Cook GM, Keynes RJ, Weber JN, Davies D, Weiss RA (1992). AIDS Res Human Retroviruses 8:19-26
Urvantseva AM, Bakunina IY, Nedashkovskaya OI, Kim SB, Zvyagintseva TN (2006). Appl Biochem Microbiol 42:484-491
Sakai T, Kawai T, Kato I (2004) Mar Biotechnol 6:335-346

Reference

291

Screening of yeasts and industrial medium optimization for bioethanol production

Pereira, Francisco; Guimarães, Pedro M. R.; Teixeira, José A.; Domingues, Lucília

Universidade do Minho, Portugal

E-mail: franciscopereira@deb.uminho.pt

Keywords: Bioethanol, fermentation, yeast, medium oprimization, factorial design,

Abstract

Ethanol is an important industrial chemical with emerging potential as a biofuel to replace vanishing fossil fuels. Our aim is to screen ethanol-tolerant yeast (*Saccharomyces cerevisiae*) strains and develop fermentation media (based on industrial substrates/by-products) that enable to attain high fermentation rates and high ethanol titres at the end of the fermentation process, therefore minimizing distillation costs (which are considered a major constraint in industrial bioethanol production).

We have studied fermentation kinetics of different *S. cerevisiae* strains in a medium containing 300 – 350 g/L glucose with 100 g/L of corn steep liquor (CSL) as the sole nutrient source, using Erlenmeyer flasks fitted with glycerol-locks. Under this fermentation conditions, CEN.PK 113-7D (laboratory strain), CA11 and CA1162 (both isolated from "cachaça" production in Brazil), were able to produce 15.9 ± 0.4% (v/v), 16.4 ± 0.1% (v/v) and 17.1 ± 0.1% (v/v) ethanol, respectively. However, a medium with such high CSL concentration (100 g/L) could compromise the economical viability of industrial fermentation processes. Thus, using factorial design approaches we intend to partially replace CSL with other cheap nutrient sources to optimize the ethanol productivity and reduce the medium costs. For this optimization process we are using a basic medium consisting of 300 g/Lglucose syrup and 15 g/L CSL. A screening of nine supplements including nitrogen sources and trace elements was initially performed with strain CEN.PK 113-7D. CSL, Urea, $MgSO_4$ and $CuSO_4$ were identified as the most significant supplements to yield a higher ethanol titre. After the optimization process, it was observed that the ethanol titres obtained from 325 g/L initial glucose has increased from 15.9 ± 0.4% (v/v) (supplementation with 100 g/L CSL) to 17.3 ± 0.2% (v/v) when supplemented with the critical concentrations of CSL, $MgSO_4$, Urea and $CuSO_4$provided by the model (significance level of 95%). A screening of vitamins, unsaturated fatty acids and ergosterol was conducted with the optimized medium and the results will be discussed.

Furthermore, different strains isolated from "cachaça" and bioethanol production facilities in Brazil, which demonstrated high ethanol productivity when grown in the original medium (100 g/L CSL), are being tested with the optimized medium for bioethanol production.

Reference

295

Detection of *Yersinia enterocolitica* in raw pork by conventional culture methods and PCR based methods

Duarte, Sara (1); Torres, Elsa (1); Campos Cunha, Isabel (2); Saraiva, Margarida (2); Domingues, Lucília (1)

1: IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Portugal;
2: Laboratório de Microbiologia, Departamento de Alimentação e Nutrição, Centro de Saúde Pública Dr. Gonçalves Ferreira, Instituto Nacional de Saúde Dr. Ricardo Jorge, Portugal

E-mail: luciliad@deb.uminho.pt

Keywords: *Yersinia enterocolitica*; detection; polymerase chain reaction; foodborne pathogen; pork

Abstract

Yersinia enterocolitica is an emerging pathogenic microorganism associated with food. Its ingestion, through contaminated food, may cause different kinds of intestinal disorders. Since there is not much information about the presence of *Y. enterocolitica* concerning the consumption of food in Portugal and the conventional methodology is not very effective, this study proceeded, by implementing the PCR methodology, in order to detect the pathogenic microorganism in pork meat.

One hundred samples of raw minced meat were acquired in supermarkets and butcher's shops in the Greater Oporto and Braga area, with the purpose of determining the occurrence of *Y. enterocolitica*.

The detection limit of the conventional method (ISO 10273: 2003) was determined to be 10^5 CFU/g using CIN culture medium and 10^4 CFU/g using a pre-treatment step with KOH, which highlights the difficulties in detecting *Yersinia* using this methodology. A molecular PCR-based method was implemented, using BDC followed by cellular alkaline lysis to extract the samples' DNA (BDC-PCR-based method). The primers used in this study were the 16S rRNA gene, which allowed the detection of the genera *Yersinia,* and the *yst* gene to detect the pathogenic strains of the microorganism. The detection limit was studied in both sets of primers. The values obtained were 10^2 CFU/g for the 16S rRNA gene and 10^3 CFU/g for the *yst* gene for a pre-enrichment time of 24 h. Nevertheless, we have implemented a combined culture and PCR method for detection of *Yersinia* that besides ensuring the viability of the cells detected, showed to be more sensitive than the BDC-PCR-based method. All the samples were analysed by the BDC-PCR-based method and 25 by the three methodologies. The different methodologies will be discussed and the incidence of *Y. enterocolitica* in raw minced meat pork evaluated.

The results of this study show that the molecular methodology and the combined methodology that were here adopted are more sensitive than the common methodology. Therefore, it can become an important tool in food sample control, since it allows quicker results in a smallest time span. It is also more reliable and easier to work with when compared to other conventional methods.

Reference

299

SYBR Green I Real-time polymerase chain reaction as a tool to detect poultry's meat adulteration with pork's meat

Amaral, Joana S. (1,2); Mafra, Isabel (1,3); Soares, Sónia (1,2); Oliveira, M. Beatriz P.P. (1)

1: REQUIMTE, Serviço de Bromatologia, Faculdade de Farmácia, Universidade do Porto;
2: Instituto Politécnico de Bragança, Portugal;
3: CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa

E-mail: jamaral@ipb.pt

Keywords: Real-time PCR; species identification; adulteration; meat products; pork meat

Abstract

Nowadays, meat species adulteration in ground and comminuted products is being considered as a widespread problem in retail markets [1]. This problem encompasses many issues, such as adulteration by substitution with lower value meats, the presence of undeclared species and the fraudulent substitution of meat by lower price vegetable proteins. Another issue to be considered is related to religious practices since pork's meat consumption is sometimes forbidden. Several techniques are currently used for meat species identification in complex mixtures, including different protein-based methods such as HPLC, ELISA and electrophoretic techniques. Nevertheless, these methods can be significantly less sensitive and difficulties can arise in the case of thermally processed foods. Due to the higher stability of DNA molecules compared to proteins, and to its ubiquity in every type of cell, they are currently preferred as target compounds for meat species identification. Moreover, the analysis of DNA coupled with polymerase chain reaction (PCR) presents a fast, sensitive and highly specific alternative to protein-based methods [2].

In the present work, the development of a real-time PCR technique for pork's meat detection in complex matrices is reported. To achieve this objective, DNA was extracted from reference binary meat mixtures containing known percentages of pork's meat. The real-time PCR approach was based on the specific amplification targeting the 18S rRNA mitochondrial gene for pork species detection and targeting a eukaryotic DNA fragment as a reference gene for quantification. The amplification products were monitored by using the fluorescent dye SYBR Green I associated with melting curve analysis to verify the specificity of obtained fragments. Under our experimental conditions, pork and eukaryotic detection systems produced fragments with 149 bp and 140 bp, and with melting temperatures of 83.5ºC and 87.5º, respectively. Calibration curves were obtained with the cycle threshold (Ct) values by using the DDCt method. The detection and quantification of pork's meat was achieved in the range of 0.1% to 25%, with a high correlation coefficient (R^2=0.9943) and a PCR efficiency of 88.7%. The developed methodology was successfully validated using blind samples and applied to the quantitative evaluation of pork's meat in different poultry processed meat products, including sausages, hamburgers and nuggets.

[1] Asensio, L., González, I., García, T., Martín, R. (2008). Food Control, 19, 1–8.
[2] Mafra, I., Ferreira, I.M.P.L.V.O., Oliveira, M.B.P.P. (2008). Eur. Food Res. Technol. 227:649-665.

Reference

305

The combined effect of high pressure and temperature on V_{max} and K_M of the enzyme horseradish peroxidase

Gomes, Isabel R.; Pinto, Andreia A.; Santos, Mickael da C.; Salvador, Ângelo C.; de Oliveira, Marise F.; Martins, Cátia F.; Marques, Clara F.; Medde, Rosa; Saraiva, Jorge A.

Universidade de Aveiro, Portugal

E-mail: jorgesaraiva@ua.pt

Keywords: Keywords: peroxidase activity, high pressure, temperature, activation volume, activation energy, Michaelis-Menten constant

Abstract

Firstly used in the fields of chemistry and physics, the use of the so called high (hydrostatic) pressure (HP) is now an established method for cold pasteurization of foods. In what concerns enzymes, HP has the potential to change enzymes activity and selectivity. [1] The effect of HP on an enzymatic reaction is governed by the activation volume (Va) of the reaction: if Va is positive (negative), the reaction rate is decreased (increased) by pressure, while if it is zero there is no effect.

Due to its ubiquitous distribution in nature, high thermal resistance, and catalytic activity over several substrates, peroxidase (EC 1.11.1.7; donor:hydrogen-peroxidase oxidoreductase) is an enzyme used in industrial applications in several fields and with great potential for future applications. [2] Peroxidase also shows high resistance to HP (baric resistance). [3] Horseradish peroxidase is the most characterised and studied peroxidase and its main isoenzyme has been already cloned. [2]

In this work, the combined effect of HP (from atmospheric pressure to 500 MPa, ~5000 atm) and temperature (below and above room temperature) on the catalytic parameters (K_M and V_{max}) of horseradish peroxidase was studied. These type of works are on importance to help understand how living organisms thrive in deep sea waters, at high and low temperatures and under several hundreds atmospheres of pressure and the properties of its enzymes.

Although K_M changed over the combinations of HP and temperature studied, no particular trend was found. V_{max} showed more variation, with HP and temperature having antagonistic effects: i) above room temperature, the increase in activity caused by temperature was counteracted by HP; ii) below room temperature, the decrease in activity caused by temperature was counteracted by HP. V_{max} decreased for i) and increased for ii) several fold, compared to the value at atmospheric pressure (0.1 MPa). Generally, a different behaviour was found from atmospheric pressure up to 50-100 MPa and for higher pressures. The activation volume/activation (E_a) energy was calculated at the different temperatures/pressures studied, allowing to verify the effect of temperature on V_a and of HP on E_a and the pressure-temperature binomial for optimum activity.

1. Heremans K. and Smeller L., Biochim. Biophys. Acta-Protein Struct. Mol. Enzymol., 1998, 1386, 353-370.
2. Veitch, N. C., Phytochemistry, 2004, 65: 249–259.
3. Lemos, M. A., Oliveira, J. C., van Loey, A., Hendrickx, M. E., Food Biotechnol., 1999, 13:13-32

Reference

310

Restriction profiles of 26S rDNA as a molecular approach for wine related yeast identification

Duarte, Filomena Luz; Zanol, Geni Carmen; Baleiras-Couto, Margarida

L-INIA Dois Portos, INRB, Portugal

E-mail: evn.flduarte@mail.net4b.pt

Keywords: Wine yeasts, restriction profiles, ribosomal DNA

Abstract

Interactions between non-*Saccharomyces* wine yeasts and *Saccharomyces cerevisiae* during wine fermentation may interfere with the growth and/or change the fermentation behaviour of the starter yeast, thus ultimately influence wine quality. Moreover, the non-*Saccharomyces* yeasts can produce a diversity of enzymatic activities and fermentation metabolites of oenological importance. Therefore, the development of methods capable of rapid and efficient identification of most naturally occurring oenological yeasts is extremely important.

The sequence of the D1/D2 domain of the 26S ribosomal DNA reflects ascomycetous yeasts phylogenetic relationships to the species level and is widely used as a first approach for yeast identification in taxonomic studies. Thus we have chosen a region of the 26S rDNA, of around 1100 bp, comprising the D1/D2 domain, to be digested with restriction endonucleases (*Hinf*I, *Mse*I, *Apa*I, *Hae*III and *Cfo*I) in order to generate restriction profiles that differentiate wine related yeast species. With that purpose, in this work we have analysed 81 yeast strains (including 38 type strains) belonging to 54 species.

The restriction enzymes *Mse*I, *Hae*III and *Cfo*I revealed a high discrimination power at the species level. On the other hand, *Apa*I and *Hinf*I, were much less discriminant, though *Hinf*I presented high polymorphism. The profiles obtained with *Mse*I, *Hae*III and *Cfo*I enabled the clustering of the studied yeast strains, into 47 groups. From a total of 54 species covered in the study, there were only 3 groups that could not be resolved to the species level. One of the groups comprises 2 species of *Dekkera* (*D. anomala* and *D. bruxellensis*), another one 2 species of *Saccharomyces* (*S. cerevisiae* and *S. paradoxus*) and the last one, 5 other species, namely *S. bayanus*, *S. pastorianus*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii* and *Z. lentus*. The use of an additional enzyme, *Apa*I or *Hinf*I allowed the differentiation of the two *Zygosaccharomyces* spp. from the remaining species.

This methodology has enabled the identification of 17 yeast species among the isolates from a pilot scale red wine fermentation experiment, conducted with commercial additives; of 18 yeast species among the isolates from a wine filling plant, including the detection of dangerous spoilage yeast species; and of 16 yeast species among the isolates present in grapes from the portuguese variety Touriga Nacional.

Co-financed by the program POCI 2010 (FEDER/FCT, POCTI/AGR/56102/2004)

Reference

319

Synthesis of flavor esters by cutinase in miniemulsion and organic solvent media

P. C. de Barros, Dragana (1); P. Fonseca, Luís (1); K. Weiss, Clemens (2); Landfester, Katharina (2)

1: IBB-CEBQ, Instituto Superior Técnico,Lisbon, Portugal;
2: Max-Planck-Institute for Polymer Research, Mainz, Germany

E-mail: dragana@ist.utl.pt

Keywords: Flavor esters, cutinase, organic solvent system, miniemulsion

Abstract

Flavor esters, that belong to the group of alkyl esters, are very valuable compounds belong to an enormous group of flavor compounds that are very important components of natural aromas. They are engaged in fruit flavored products used in various food, beverage, cosmetic and pharmaceutical industries [1,2].

The main objective of this work was studying and testing the nature and influence of reaction media on the synthesis of flavor ester catalyzed by *Fusarium solani pisi* cutinase in two different media: first, organic media, in particular isooctane an organic solvent recognized as safe ingredient in food and beverage industrial processes, second in miniemulsion system [3,4] allowing to use water as media for ester synthesis.

Ester synthesis and cutinase selectivity for different chain length of acids and alcohols (ethyl and hexyl) were evaluated. In *iso*-octane, after 1 h of reaction, cutinase exhibits rates of esterification between 0.24 µmol × mg^{-1}× min^{-1} for ethyl oleate and 1.15 µmol × mg^{-1}×min^{-1} for ethyl butyrate, while in a miniemulsion system the rates were from 0.05 for ethyl heptanoate to 0.76 µmol × mg^{-1}× min^{-1} for ethyl decanoate. The reaction rate for the synthesis of hexyl esters in a miniemulsion system was from 0.19 for hexyl heptanoate to 1.07 µmol × mg^{-1}× min^{-1} for hexyl decanoate. High conversion yields of 95% at equilibrium after 8 h of reaction in *iso*-octane for pentanoic acid (C_5) with ethanol at equimolar concentration (0.1 M) was achieved. Additionally, this work showed that a significant and unexpected shift in cutinase selectivity occurred towards longer chain length carboxylic acids (C_8–C_{10}) in miniemulsion system as compared to organic solvent (iso-octane) and previous studies in reverse micellar systems. The possibility of working with higher concentration of substrates, without inhibitory effect on the enzyme, was another advantage of the miniemulsion system.

[1] S. Hari-Krishna, N.G. Karanth, Catalysis reviews, 2002, 44, 499-591.
[2] F. W. Welsh, R. E. William, K. H. Dawson, , J. Food Sci., 1990, 55, 1678-1682.
[3] K. Landfester , Macrom. Rapid Comm., 2001,22,896-936.
[4] K. Landfester, Annu. Rev. Mater. Res., 2006,36, 231-279.

Reference

326

Comparative analyze of the kefir fermentation process and microbiota, using milk and cheese whey as substrates

Magalhães, Karina Teixeira (1); Pereira, Maria Alcina (1); Dragone, Giuliano (1); Schwan, Rosane Freitas (2); Domingues, Lucília (1); Teixeira, José Antonio (1)

1: Universidade do Minho, Portugal;
2: Universidade Federal de Lavras, Brasil

E-mail: gt-magalhaes@uol.com.br

Keywords: Kefir, Fermentação, DGGE, Análise Química

Abstract

Kefir, a mixed culture that ferments lactose, is known for the production of a refreshing fermented beverage popular in Eastern-European countries by inoculating milk with kefir grains. Kefir grains are gelatinous white or cream-coloured, water insoluble, irregular granules with diameter ranging 0,3–3,5 cm. They are composed mostly of proteins and polysaccharides in which the complex microbiota is enclosed. The beverage consists of a microbial diversity that includes lactic acid bacteria, yeasts and their metabolites. The aim of this work was to compare the fermentation and the microbiota of kefir , using milk and cheese whey as substrates. The grains were added in the proportion of 5% in 250ml of each substrate. Assays were performed at 25 °C for 48h. The concentrations of lactose, ethanol, lactic acid and acetic acid were quantified by HPLC. To determinate the composition of microbiota in Kefir of fermentation, PCR-DGGE analysis was used.The fermentation of milk and cheese whey by kefir grains are observed in this study. It can be observed that the lactose concentration at the end of the milk fermentation was lower in comparison with that obtained at 48 h for cheese whey fermentation. Despite the higher lactose consumption during the fermentation of milk by kefir grains, the concentrations of ethanol, acetic acid and lactic acid did not show significant differences with those obtained during the cheese whey fermentation. No changes in the DGGE profiles in all fermentations were observed to fungal and bacterial communities. It was thus concluded that lactose from cheese whey is converted to products with higher yields than lactose from milk, in addition to showing the same group of microorganisms for both fermentation process.

Paraskevopoulou, A., Athanasiadis, I., Kanellaki, M., Bekatorou, A., Blekas, G., Kiosseoglou, V. Food Research International, (2003) 36, 431-438.

Beshkova, D. M., Simova, E. D, Frengova, G. I., Simov, Z. I., Dimitrov, Z. H. P., International Dairy Journal, (2003) 13, 529-535.

Reference

333

Effect of several antimicrobial agents upon the survival of *L. monocytogenes* strains

Barbosa, Vanessa Raquel; Pedrosa, António José; Paula, Teixeira; Joana, Silva

Escola Superior de Biotecnologia, Centro de Biotecnologia e Química Fina, Universidade Católica Portuguesa, Rua Dr. António Bernardino Almeida, P-4200-072 Porto, Portugal

E-mail: raquel_gb@live.com.pt

Keywords: *Listeria monocytogenes*, biofilm, disinfectants, persistence

Abstract

Listeria monocytogenes is a widely distributed pathogenic bacterium, responsible for outbreaks of listeriosis in humans and animals. This microorganism has the ability to form biofilms on different types of food-processingsurfaces, potentially leading to food product contamination.
In order to eliminate *L. monocytogenes* from the processing surfaces, several sanitizing agents are commercially available. However, the mishandling of these agents may lead to the regular exposure of the contaminants to sub-lethal conditions in hard to reach places and consequently to the emergence of persistent strains that are difficult to eradicate. The properties of *L. monocytogenes* that differentiate persistent from non-persistent strains are yet to identify. Nevertheless, it has been suggested that the persistence may be related to the ability of some strains to form biofilms and survive sanitizing treatments.
The aim of this study was to elucidate the possible relationship between the ability to resist the antimicrobial agents and the production biofilm with the persistence associated to some strains of *L. monocytogenes.* Minimum inhibitory concentrations (MIC) of three commercially available detergents (Betelene-F3, bleach and Topax 66) were determined against two strains of *L. monocytogenes* isolated from a cheese industry, one considered persistent and the other non-persistent. The antimicrobial efficacy of the same agents against cells in suspension and biofilm formation was also evaluated.
Both strains showed similar MIC values to the three detergents tested. Concerning the inactivation of cellular suspensions, there was a significant difference between both strains, when using Betelene-F3 and bleach, being the persistent strain more affected by these detergents than the non-persistent strain. No significant difference was observed concerning biofilm formation between the two strains. Thus the results suggest that the persistence of *L. monocytogenes,* in food industry, can not be, solely, justified by its resistance to detergents and biofilm production.

Aarnisalo, K., Lundén, J, Korkeala, H. & Wirtanen, G. LWT, 40, 1041-1048 (2006).
Lundén, J., Autio, T., Markkula, A., Hellström, S., and Korkeala, H. International Journal of Food Microbiology, 82, 265–272 (2003).

Reference

335

Antimicrobial activity of ginja cherries (*Prunus cerasus* l.)

Demiray, Simge; Piccirillo, Clara; Franco, Albina Ribeiro; Castro, Paula Lima; Pintado, Maria Manuela

Universidade Católica Portuguesa, Portugal

E-mail: sdemiray@esb.ucp.pt

Keywords: Ginja cherry, polyphenols, antimicrobial activity

Abstract

Ginja cherry ***(****Prunus cerasus L.)* is a kind of cherry native to Portugal, widely used to make the traditional liqueur Ginjinha. Sour cherry may acquire new interest due to its high content of antioxidant compounds and it can be considered as "functional food" [1]. Although many cherries contain macro and micronutrients, their diverse biological properties relate to their high levels and wide diversity of phenolic-type phytochemicals. Polyphenolic compounds are believed to be responsible for their wide range of observed biological properties including antioxidant, antiallergenic, anti-inflammatory, anti-viral, anti-microbial, anti-proliferative, anti-tumorigenic, anti-anxiety, and anti-carcinogenic [2].

Previous work [3, 4] showed that extracts obtained from Ginja cherries and the liqueur- Ginjinha are both rich in polyphenolic compounds. Identification of phenolic compounds supported the high antioxidant activity of the samples. In a previous study, it has been demonstrated that some phenolic compounds possess antimicrobial properties owing to their interaction with microbial cell wall [5]. In this study we report on the antimicrobial properties of Ginja cherries. Antimicrobial tests were performed on the cherry pulp as well as on their extracts; Gram-positive, Gram-negative bacteria and yeasts strains were used. A correlation was established between the antimicrobial activity upon the target microorganism and the polyphenolic content.

[1] Blando, F.; Gerardi, C.; Nicoletti, I. J. Biomed. Biotechnol. 2004, 5, 253-258.
[2] Middleton, E. Adv. Exp. Med. Biol. 1998, 439, 175-182.
[3] S. Demiray, C.L. Rodrigues, M. Politi, F.X. Malcata, M.E. Pintado, P.M.L. Castro, CHISA 2008, Prague, Czech Republic.

Reference

341

Fungal growth on coffee husks and spent ground under solid-state cultivation conditions

Machado, Ercília M. S.; Rodriguez-Jasso, Rosa M.; Mussatto, Solange I.; Teixeira, José A.

Universidade do Minho, Portugal

E-mail: lyamachado86@gmail.com

Keywords: Coffee Husks, Spent Ground, Solid-state cultivation, fungal growth

Abstract

The processing of coffee generates significant amounts of agricultural wastes. Coffee husks, comprised of dry outer skin, pulp and parchment, are probably the major residues from the handling and processing of coffee (1). Coffee spent ground is the main coffee industry residue obtained during the processing of raw coffee powder to prepare “instant coffee”. Coffee husks and spent ground are generated in more than two millions tons yearly (2), and the major problem encountered by the industries is the disposal of these residues, since they contain some amount of caffeine, polyphenols and tannins, which makes them toxic in nature (3).

Filamentous fungi are microorganisms able to growth over complex substrates behind minimal conditions, and play an important role in the generation of natural compounds with high commercial interest. Therefore, the aim of the present work was to evaluate the ability of some fungal strains to growth on coffee husks (basically the parchment skin - the hull that surrounds the coffee bean), and spent grounds, as an alternative to add value to these toxic residues.
Strains from the genus *Aspergillus*, *Penicillium*, *Mucor* and *Neurospora* were used. Microbial growth was carried out in Petri plates containing 30% of coffee husks or spent ground and 70% of Czapek-Dox saline media, pH 5.0. The plates were inoculated with a suspension containing $5´10^6$ spores/g dry residue, and maintained at 28ºC for 5 days. The spore suspension was prepared by scrap down the spores from PDA plates with a sterilized solution of 0.2% Tween 80, and counted in a Neubauer chamber. Cultivations were done in duplicate to each fungal in each different substrate. Radial growth rate (Ur, mm/h) was monitored kinetically measuring colony diameters every 12h.
All the evaluated fungal strains showed mycelium presence over both residues. For almost all the strains, the invasion capacity was higher in coffee spent ground than in coffee husks. Highest growth rates were obtained with *Neurospora crassa*, with values of 0.99 and 0.76 mm/h for spent ground and husks, respectively. It was thus concluded that coffee husks and spent grounds can be successfully used as substrate for fungal strains growth. Among the evaluated strains *Neurospora crassa* gave the best results and could be thus evaluated in solid-state fermentation processes for the obtainment of compounds with commercial interest from these two agro-industrial residues.

(1) Saenger M., Hartge E.-U., Werther J., Ogada T., Siagi Z. (2001) Renew. Energy 23: 103–121.
(2) Fan L., Pandey A., Mohan R., Soccol C. R. (2000) Acta Biotechnol. 20:41-52.
(3) Pandey A., Soccol C.R., Nigam P., Brand D., Mohan R., Roussos S. (2000) Biochem. Eng. J. 6:153-162.

Reference

352

Antimicrobial and antioxidant activity of different extracts of *Mentha cervina* from Portugal

Rocha, Joana (1); Carvalho, Susana; Politi, Matteo; Castro, Paula; Pintado, Manuela

Escola Superior de Biotecnologia-UCP, Portugal

E-mail: joanarocha31@hotmail.com

Keywords: antimicrobial activity, antioxidant capacity, Menta cervina, DNA protection, phenolic compounds

Abstract

The main objective of this work was to analyze the antimicrobial and antioxidant activity of *Mentha cervina* extracts in order to assess its potential for the development of functional ingredients that may be applied in the food or cosmetic industries. Antimicrobial activity of essential oil of the plant was evaluated by disc-diffusion assays; the tinctures (ethanol/water mixtures) and infusions were assessed by well-diffusion assay using as target microorganisms Gram-negative bacteria, *E. coli* and *Salmonella* spp., Gram-positive bacteria, *Staphylococcus aureus*, *Listeria innocua* and *Bacillus cereus*, and yeasts, *Candida albicans* and *Yarrowia lipolytica*. Tinctures and infusions did not show any inhibition activity whereas the essential oil inhibited all the tested microorganisms. The extract showed the highest activity towards *Candida albicans* and *Yarrowia lipolytica* and the lowest upon *Listeria innocua* and *S. aureus*. The antioxidant capacity of tinctures, infusions and essential oils was determined by the $ABTS^{\cdot+}$ method and by the DNA assay — where protection of DNA from degradation was determined, and total phenol content was assessed by the Folin-Ciocalteu method. The ascorbic acid equivalent (g L^{-1}) values ranged from 0,571 ± 0,017 for 65% tincture, down to 0,044 ± 0,005 for plant infusion. The gallic acid equivalent (g L^{-1}) values range for 0,724 ± 0,025 for 65% tincture, down to 0,011 ± 0,002 for infusion plant. The extracts of tinctures showed effective protection for 100%, 65%, 40%, 20% and 0% of etanol/water mixtures at 40 mg/ml and absence of protection for essential oil at 40 mg/ml. No extract revealed pro-oxidant activity. The microbiological results were related with phenolic compounds present in the extracts.

Reference

356

Microencapsulation of probiotic bacteria in alginate-soy protein mixtures

Cardoso, Sónia (1); Cardoso, Celsa (1); Andrade, José Carlos (1,2)

1: Instituto Superior de Ciências da Saúde, Gandra, Portugal;
2: CICS - Centro de Investigação em Ciências da Saúde. Gandra, Portugal

E-mail: jose.andrade@iscsn.cespu.pt

Keywords: Probiotics, Microencapsulation, alginate, soy protein, Lactobacillus casei

Abstract

Due to their health benefits, probiotic bacteria have been increasingly included in food products, especially in yoghurt and fermented milks during the past two decades. In order for these microorganisms to exert positive health effects, they have to reach the large intestine of the host, alive and in sufficient number. Therefore, conditions that increase the survival and viability of probiotic bacterial strains during the whole lifespan of the food and through the gastrointestinal transit are needed. The main objective of this work was to study the microencapsulation of the probiotic *Lactobacillus casei* in mixtures of alginate and soy protein by the emulsification/ internal gelation method in order to improve its viability and functionality. The results obtained have showed that it is possible to make microparticules of alginate and soy protein by the emulsification/ internal gelation method with a mean size lower than 60 µm. However, particle size varies depending on the conditions used: speed of agitation, alginate/soy protein ratio and the amounts of calcium and acid added. The microencapsulation of *L. casei* didn't improved the viability and functionality to conditions such as high (60°C e 75°C) and low (-20°C) temperature when compared to free cells. However, the survival of encapsulated cells in simulated gastric juice at pH values of 1,5 and 2,0 was significantly ($p < 0.001$) better than that of non-encapsulated.

Reference

364

Microbiological quality of ready-to-eat edible crab (*Cancer pagurus*)

Marques, Pedro (1); Pedro, Sónia (2); Marques, António (2); Pomba, Constança (1)

1: FMV/UTL, Portugal;
2: INRB, I.P./ IPIMAR, Portugal

E-mail: dr_pmarques@hotmail.com

Keywords: edible crab, food safety, shelf-life

Abstract

The shelf life of refrigerated cooked whole edible crab was determined by microbiological and sensorial analysis. Shelf life, defined as the time taken for bacterial counts in the flesh to reach 5 log10 cfu g^{-1}, depending primarily on the storage temperature of cooked raw material (refrigerated vs. frozen). Bacterial counts indicated that product prepared from refrigerated material had a shelf-life of 7–10 days, while those prepared from frozen material had a shelf life of at least 12 days. The microbiological quality of ready-to-eat edible crab (Cancer pagurus) placed in the market was also assed. High total viable counts were detected in some market samples.

Reference

371

Evaluation of antimicrobial activity of lactic acid bacteria, previously isolated from traditional Portuguese cheeses

Pereira, Cláudia Inês; Arez, Eliane; Mendes, Marta; Gomes, Ana; Malcata, F. Xavier

CBQF/Escola Superior de Biotecnologia - Universidade Católica Portuguesa, Portugal

E-mail: cipereira@mail.esb.ucp.pt

Keywords: Inhibition, antimicrobial compounds, pathogens, spoilage microorganisms, traditional Portuguese cheeses

Abstract

Traditional Portuguese cheeses have for long been recognized for their unique organoleptic characteristics. They are manufactured from raw milk, and their composition depends chiefly on their indigenous microflora – which is mainly composed by lactic acid bacteria (LAB), together with (potential) pathogen and spoilage bacteria which are contributed by the inner and outer udder, as well as by the farmer's hands and milking equipment.

Adventitious LAB behave as spontaneous starters in Portuguese traditional cheesemaking (e.g. *Serra da Estrela* cheese), thus contributing to acidify the curdled matrix – that aids in draining whey, and influences the nature and extent of enzyme-mediated changes during ripening. Besides their contribution to flavour and texture development in cheese –in addition to indigenous milk enzymes and residual rennet, they also eventually prevent cheese spoilage and pathogen transmission to humans, owing to several antimicrobial compounds produced thereby – e.g. organic acids, hydrogen peroxide and bacteriocins.

Despite the ecological competition involving LAB and unwanted microorganisms during cheesemaking, the exact mechanisms involved therein are not yet fully understood. Hence, the main objective of the present research effort was to hypothesize and test potential mechanisms for the antimicrobial behaviour of *Lactococcus lactis* LMG S 19870, *Lactobacillus brevis* LMG 6906 and *Lactobacillus plantarum* LMG S 19557, against 14 target microorganisms – including 7 beneficial strains and 7 unwanted microorganisms, using the agar well diffusion method. The organic acids released by such LAB exhibited a high antimicrobial activity against all target microorganisms, either in their exponential or stationary growth phases – especially those of *L. plantarum* and *L. brevis*. Furthermore, the nature of other antimicrobials was sought, the action of which was superimposed on that conveyed by said organic acids.

Reference

373

Genomic diversity of *Oenococcus oeni* from three viticulture regions of Portugal

Marques, Ana Paula (1); Chambel, Lélia (2); San Romão, Maria Vitória (1,3); Tenreiro, Rogério (2)

1: Instituto de Biologia Experimental e Tecnológica (IBET) and Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa;
2: Universidade de Lisboa, Faculdade de Ciências, Centro de Biodiversidade Genómica Integrada e Funcional (BioFIG);
3: Instituto Nacional de Investigação Agrária, ex-Estação Vitivinícola Nacional

E-mail: amarques@itqb.unl.pt

Keywords: Viticulture region, winery, *Oenococcus oeni*, M13-PCR fingerprinting, genomic diversity

Abstract

Oenococcus oeni is an alcohol-tolerant and acidophilic lactic acid bacterium thatplays an important role in the elaboration of wine, where it is often added as a starter culture to carry out the malolactic fermentation (MLF). Given the economic importance of MLF, the taxonomic structure of this species has been studied in detail.
In this study, 121 *Oenococcus oeni* isolates from wines of three viticulture regions of Portugal were genetically differentiated by M13-PCR fingerprinting. Twenty seven different genomic clusters represented by two or more isolates and 21 single-member clusters, at 85% similarity level, were recognized by a numerical analysis. Cluster analysis of the M13-PCR fingerprinting patterns revealed distinct region specificity of *O. oeni* strains and allowed to show the diversity of *O. oeni* isolates. Specific profiles tended to be restricted to *O. oeni* strains from a certain viticulture region. *O. oeni* isolates from the same wine were grouped in different clusters, which indicated the presence of different types of *O. oeni* strains.
The M13-PCR fingerprinting analysis could be a good methodology to study the *O. oeni* ecology of wine during malolactic fermentation and to evaluate the performance of new malolactic starter cultures and their dominance over the native microbiota.

Reference

377

Angiotensin-converting enzyme-inhibitory activity of extracts of hydrolysed k-casein glycomacropeptide: stability under simulated gastrointestinal digestion

Tavares, Tânia (1); Amorim, Manuela (1); Pintado, Manuela (1); Récio, Isidra (2); Malcata, F. Xavier (1)

1: CBQF/ Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal;
2: Instituto de Fermentaciones Industriales. CSIC, Madrid, Espanha

E-mail: mfamorim@mail.esb.ucp.pt

Keywords: Casein Glycomacropeptide, antihypertensive activity, gastrointestinal digestion, bioactive peptide, Cynara cardunculus

Abstract

Dietary proteins usually possess a wide range of nutritional, functional and biological properties; many of such biological properties have been attributed to physiologically active peptides, which are encrypted within the protein sequence.
k-Casein glycomacropeptide (CMP) – one of the main components of whey, is released in the first step of (enzymatic) cheese making – and has been claimed to act as mediator in important biological pathways.
Peptides released from CMP via enzymatic or microbial activity have indeed been proven to posses inhibitory activity against the angiotensin-converting enzyme (ACE). However, they will not act properly in the human body unless they can resist gastrointestinal digestion, as well as be absorbed and reach the cardiovascular system in active form. On the other hand, several studies have already demonstrated the important role of gastrointestinal digestion upon ACE-inhibitory peptide formation.
Therefore, the aim of this study was to evaluate the ACE-inhibitory activity of peptide extracts obtained via hydrolysis of CMP, using an extract of *Cynara cardunculus* as enzymatic vector. Moreover, such peptides were subjected to simulated gastrointestinal digestion, in order to assess their stability and the evolution of their ACE-inhibitory activity.
A 40 g/L solution of CMP, hydrolysed with 8.5 %(w/w) commercial crude extract of *C. cardunculus,* exhibitedACE-inhibitory activity corresponding to IC_{50} values of 296.0 mg/mL (total fraction), 63.0 mg/mL (<3000 Da fraction) and 717.0 mg/mL (>3000 Da fraction).
The identification of peptides proceeded by LC-MS, and permitted identification of the following peptides: MAIPPKKNDQD (k-CN f106-115), as potentially responsible for antihypertensive activity; and TVQVTSTAV (k-CN f161-169) and MAIPPKKNDQD (k-CN f106-115), as potentially responsible for antithrombotic activity, because both encompass sequences analogous to the bioactive fragment f400-411 of fibrinogen g-chain. A new peptide, KTEIPIN (k-CN f116-123), was also identified to relatively high concentrations, with a promising antihypertensive activity. Studies concerning the *in vitro* simulation of gastrointestinal digestion were conducted; the gastrointestinal stability of the total extract and of the <3000 Da peptide fraction, as well as of the plain CMP (used as control), following incorporation in water and fruit juice, was tested in said gastrointestinal model, but none of said fractions was significantly affected.

Reference

394

Towards the construction of a food-grade *Lactococcus lactis* strain for *in situ* production of mannitol in dairy products

Paixão, Laura; Gaspar, Paula; Isabel, Pacheco; Neves, Ana Rute; Santos, Helena

ITQB, Portugal

E-mail: lpaixao@itqb.unl.pt

Keywords: *Lactococcus lactis*, mannitol, food-grade, metabolic engineering

Abstract

Mannitol is a polyol with recognized health-promoting properties. Over a number of years, several metabolic engineering strategies have been attempted in our laboratory to improve mannitol production using the dairy GRAS bacterium *Lactococcus lactis.* The most promising results were attained by overproducing the enzymes directly involved in mannitol biosynthesis: the lactococcal mannitol-1-phosphate dehydrogenase and a mannitol-1-phosphate phosphatase (M1Pase) from the protozoan parasite *Eimeria tenella* (Gaspar *et. al*, 2004; Gaspar, 2008). In fact, during growth on glucose, M1Pase was the main bottleneck in mannitol production since the over-expression of the respective encoding gene resulted in an 8-fold increase in the mannitol yield.

Thus far, the only M1Pase amino acid sequences available (full sequence *Eimeria tenella* and internal fragments from *Caloglossa continua*) are from organisms that do not fulfill the GRAS status. Therefore, to construct a food-grade mannitol producer it is mandatory to identify a gene encoding M1Pase in organisms traditionally used in food.

Different approaches to identify M1Pase in *L. lactis* were unsuccessful, but we found a food-grade mannitol producer fungus with M1Pase activity. Hence, the purification of M1Pase activity was accomplished and the respective gene identified. The synthetic gene optimized for expression in *L. lactis* was cloned together with the lactococcal mannitol-1-phosphate dehydrogenase and the resulting construct introduced into the appropriate background. Results on the characterization of the resulting strain will be presented. This work represents an important step towards our final goal of obtaining a food-grade *L. lactis* high-level mannitol production.

P. Gaspar, A. R. Neves, A. Ramos, M. J. Gasson, C. Shearman and H. Santos. Appl. Environ. Microbiol. 70, 1466-1474 (2004).

P. Gaspar. Ph.D. thesis. Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (2008).

Reference

397

Studies of lignin and polysaccharides recovery from kraft liquor for biotechnological applications

Cabral, Diana (1,2); Ruzene, Denise S. (1,2,3); Silva, Daniel P. (1,2,3); Teixeira, José (2); Gonçalves, Adilson Roberto (1)

1: Escola de Engenharia de Lorena, Universidade de São Paulo, Brazil;
2: IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho;
3: ITP - Instituto de Tecnologia e Pesquisa, Tiradentes University

E-mail: adilson@debiq.eel.usp.br

Keywords: biofuels, polysaccharides recovery, kraft liquor, solvent extraction, lignin utilization

Abstract

According to the biorefinery concept, this study has the objective of evaluating alternatives for the valorization of all the kraft liquor fractions. This liquor consists mainly in lignin, cellulose and hemicellulose. Currently, cellulose pulp industries recover the cooking chemicals by burning and energy is introduced into the process. Sustainable development guidelines, regarding the costs and wastes reduction and biotechnology principles may present new solutions for the production of valuable products.

In this study, two methods for the polysaccharides extraction, in three different pH conditions, are presented. The only difference between these methods is the solvent applied: ethanol and 1,4-dioxane. All the samples were maintained 24 h at 298 K. After a filtration step, the solid fraction resultant from the ethanol treatment had a carbohydrate content of 40.51%, 44.64% and 49.53%, for pH values of 3, 4 and 6, respectively. The treatment with 1,4-dioxane, reached the following values: 21.17%, 18.41% and 29.73% for the same pH values. These results were obtained with HPLC analysis after polysaccharides hydrolysis. Three unknown peaks were detected that we considered to be sugar derivative compounds. Thus, the polysaccharides contents, for both treatments, might actually be superior.

Concerning to the product purity, the ethanol extraction revealed to be the less efficient. The lignin content in the solid fraction, ranged between 28% and 31%, with 1,4-dioxane extraction, and between 40% and 50%, with ethanol.

The lignin molecular weight was determined with GPC, after and before liquor pH lowering. Thereby, for pH 6, pH 4 and pH 3 the obtained MWs were: 2376 Da, 1477 Da and 3705 Da, respectively. The molecular weight increase may be due to the lignin repolymerization. These results suggest that the polysaccharides recovery and lignin molecular weight may be related. As the molecular weight increases or decreases, the polysaccharides percentage presents the same behavior, regardless of the product purity. The data obtained after FT-IR analysis suggested that there was no significant modifications on lignin structure.

Summarizing, these preliminary results indicate that there is a possibility of recovering the kraft liquor´s polysaccharide while the residual lignin can still be used for burning to energy recovery.

Financial Support: Erasmus Mundus - ISAC Program, FCT/Portugal, Millipore Brazil and CNPq-CAPES/Brazil

Reference

398

Microencapsulation of *Lactobacillus paracasei* LAFTI® L26 by extrusion in an alginate matrix

Sousa, Sérgio C. (1); Gomes, Ana M. (1); Pintado, Maria M. (1); Malcata, F. Xavier (1); Rodrigues, Dina (2); Rocha-Santos, Teresa (2); Silva, José P. (3); Lobo, José M. Sousa (3); Costa, Paulo (3); Amaral, Maria H. (3); Bahia, Maria F. (3); Freitas, Ana C

1: Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal;
2: ISEIT/ Viseu, Instituto Piaget, Estrada do Alto do Gaio, Galifonge, 3515-776 Lordosa, Viseu, Portugal;
3: Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4050-123 Porto, Portugal

E-mail: sdcruz@mail.esb.ucp.pt

Keywords: microencapsulation"extrusion"*Lactobacillus paracasei*

Abstract

Probiotic bacteria are currently used in the development of functional food products, yet sometimes face technological challenges when incorporated in food matrices with more aggressive environments – salt, acid or oxygen concentrations. Encapsulation is an efficient technique to overcome such difficulties since encapsulation microcapsules help in their protection from both the product intrinsic properties and the gastrointestinal tract. Among the many factors influencing encapsulation efficiency, capsule size is an important issue since it can affect the textural and sensorial properties of the food product to which they are added. In this research work the microencapsulation efficiency and stability throughout storage of calcium alginate capsules (produced by extrusion) of *Lactobacillus paracasei* LAFTI® L26 was studied. Initially, extrusion by coaxial flow was used for encapsulation. Storage in Ringer solution at 4 ºC in a 1:9 (g/mL) racio – and the effect of a protecting agent – lactose, were assessed (samples collected at 0, 3, 5, 7 and 14d). In order to reduce the capsules' size, extrusion by aerodynamically assisted flow was also tested and two different rupture solutions (sodium citrate 2 %(w/v) and phosphate buffer (0.5 M; pH 7)) were assessed. The capsules obtained via extrusion by coaxial flow presented dimensions superior to 200 µm. The incorporation method was shown to be effective. Suspension of the *L. paracasei* LAFTI® L26 alginate capsules in Ringer solution and storage at 4 ºC was shown to be a good preservation method and lactose did not present a protective effect. Such encapsulation increased survival of bacteria under storage at 4 ºC for two months (samples collected at 0, 3, 5, 7, 14, 21, 30 and 60d), reducing the decline of viable cell numbers when in comparison with free cells (3 log cycles versus 4 log cycles). The size of the capsules obtained using extrusion by aerodynamically assisted flow was smaller than 100 µm which allows the capsules to be incorporated in food products without a negative sensorial perception. The encapsulation method was also shown to be effective and no difference between rupture solutions was observed.

Reference

413

Design and study of a thermo efficient closed-loop vertical photobioreactor for microalgae production

Matos de Sousa, Joao; Gouveia, Luísa

LNEG -Laboratório Nacional de Energia e Geologia, Portugal

E-mail: joao.sousa@ineti.pt

Keywords: photobioreactor microalgae energy environment CO2 mitigation

Abstract

With the variations of crude oil supply prices, depletion of resources and environmental problems related to green house gases, the design of energy efficient photobioreactors, for biofuel production from microalgae biomass, has recently aroused high interest due to its commercial potential.

A closed loop vertical tubular photobioreactor specially designed to operate under conditions of scarce flat land availability and irregular solar irradiance conditions, was used to study the potential of microlgaeproduction. The INETI's Lumiar Campus in the city of Lisbon, located on the western coast of Portugal (38º 42'N, 9º 11'W), was selected for the pilot study.

Culture agitation, air mixing and temperature control during the whole cultivation period are the main problems in actual photobioreactors leading to costly operation. To maintain an optimal temperature during day/night variations, during the day itself and seasonal variations, solar energy is expected to operate a cooling device. Irradiance will be controlled in order to avoid wavelengths that are harmful to microalgaegrowth while controlling the excessive temperature, using low-cost filtration material. In order to obtain a good mixing of air and liquid and at the same time reducing agitation cost, a venture system and a solar energy pump will be tested in the next months.

The admission and exhaust of CO2 will be monitored in order to control the rate conversion of microalgal biomass.

This PBR allows a good microalgae productivity with a very low energy cost , using only renewable energy sources and maximizing CO2 sequstration.

Oral Session:
S8 – Bioinformatics, Comparative Genomics and Evolution

Reference

424

Bacterial clone definition using microbial typing methods: Advantages and caveats

Carriço, João André

Instituto de Microbiologia, Faculdade de Medicina de Lisboa, Portugal

E-mail: jcarrico@fm.ul.pt

Keywords: Bacterial typing

Abstract

Bacterial typing methods allow microbiologists to discriminate isolates of the same bacterial species. Some of the first typing methods used, antimicrobial resistance profiles and serotyping, are examples of methods based on phenotypic characteristics. With the introduction of molecular biology, Restriction Fragment Length Polymorphism analysis (RFPL) and Pulsed-Field Gel Electrophoresis (PFGE) became standard methodologies in epidemiological studies, probing for markers at genomic level. Both techniques query the genome for the number and location of endonuclease recognition sequences, by generating gel-based band patterns. More recently, other pattern-based techniques, such Multi Locus Variable Number of Tandem Repeats (MLVA) have emerged. With the decreasing costs of sequencing technologies, and due to their superior reproducibility and portability, sequence-based typing methods such as Multi-Locus Sequence Typing (MLST) and other sequence-based species specific methods (such as *emm* typing), are becoming the mainstream for microbial identification at subspecies level.

Microbial typing is of extreme importance in several microbiology fields such as population genetic studies by allowing the inference of patterns of phylogenetic descent or in clinical microbiology by providing a way to track the dissemination of particularly pathogenic bacteria.

From the analysis of microbial typing results, researchers usually define groups of related strains that are dubbed “clones” or types. However, given the nature of some typing methods and the fact that the genome of most bacteria undergoes recombination processes, the term “clone” can be misleading.

In this presentation, we will demonstrate several applications of a methodological framework that can be used to shed some light on whether “clones” can be defined using a given microbial typing method or combination of methods. Specifically, the framework provides a quantitative approach to the congruence of typing methods. Analysis will be discussed for different species.

We will also highlight the importance of correctly choosing the data analysis methodology for the assignment of isolates to clusters or types of genetically related strains and some caveats of some commonly used methods.

To conclude, we will talk about future work and needs in the field of microbial typing, how the advent of high-throughput sequencing could potentially change the field and what bioinformatic challenges still lay ahead.

Reference

414

Systems Biology for the development of microbial cell factories

Rocha, Isabel (1); Lourenço, Anália (1); Rocha, Miguel (2); Ferreira, Eugénio C. (1)

1: IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal;
2: Department of Informatics / CCTC, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

E-mail: ecferreira@deb.uminho.pt

Keywords: Systems Biology, in silico metabolic engineering

Abstract

In recent years, with advances in Systems Biology, there has been an increasing trend towards using mathematical and computational tools for the in silico design of enhanced microbial strains, and it is expected that such strategies will become more efficient as more robust and reliable mathematical models describing the function of cell factories become available. The derivation of strategies for increasing the productivity of microbial cell factories by applying a Systems Biology perspective is being pursued at Bio-Process Systems Engineering group (BioPSEg).
The development of more reliable and standardized microbial models allows the improvement of model predictions and therefore makes possible the application of simulation and optimization algorithms for the design of rational metabolic engineering strategies. In that context, we have been focusing our research on generation of better mathematical models of microbial metabolism, applying Bioinformatics tools like Data Mining and Biological Text Mining.
Current projects include the reconstruction of the metabolic network of *Kluyveromyces lactis*, *Streptococcus pneumonia*, *Enterococcus* spp and improvement of existing models for *Escherichia coli* and *Helicobacter pylori*. For that aim, relevant data sources are identified and data integration strategies are defined for collecting and integrating available genomic and functional information for each organism. Model curation and validation are the subsequent steps in the iterative process of model building.
A new open-source, user-friendly, and modular tool, Optflux, is being introduced to support in silico metabolic engineering. It uses stoichiometric metabolic genome-scale models allowing the set of fluxes in the organism's metabolism to be determined, given a set of environmental constraints. The optimization tasks, i.e., the identification of metabolic engineering targets (the best set of gene modifications given an industrial goal), can be performed with Evolutionary Algorithms, Simulated Annealing and Local Search methods.

Reference

399

Evolutionary analysis of enzymes involved in the synthesis of di myo-inositol-phosphate

Gonçalves, Luís G (1); Borges, Nuno (1); Fernandes, Pedro (2); Dopazo, Hernán (3); Santos, Helena (1)

1: Instituto de Tecnologia Química e Biológica, Portugal;
2: Instituto Gulbenkian de Ciência, Portugal;
3: Centro de Investigación Príncipe Felipe, Spain

E-mail: lgafeira@itqb.unl.pt

Keywords: Hyperthermophiles, evolution, compatible solutes

Abstract

Over a number of years, our team directed considerable effort to characterize new compatible solutes used by hyperthermophiles, microorganisms adapted to thrive in environments with temperatures above 80ºC. Interestingly, the solutes found in these organisms clearly differ from those used by mesophiles. Generally, hyperthermophiles accumulate negatively charged compounds, while mesophiles usually accumulate neutral or zwitterionic solutes and the accumulation occurs not only in response to osmotic stress, but also to cope with heat stress. Indeed, many of these solutes have not been found in mesophiles.

Di-myo-inositol phosphate (DIP) is the most widespread solute in hyperthermophiles, being found in members of nearly all marine hyperthermophilic genera, both *Bacteria* and *Archaea*. The synthesis proceeds via 3 reactions: 1) Inositol-1P is activated with CTP into CDP-inositol, catalyzed by inositol-1P cytidylyltransferase (IPCT); 2) CDP-inositol is then condensed with another molecule of inositol-1P yielding di-*myo*-inositol phosphate phosphate (DIPP), catalyzed by DIPP synthase (DIPPS); and 3) DIPP is dephosphorylated into DIP by the action of a phosphatase. Recently, the genes and enzymes involved in this pathway were characterized (1). Homologs to the genes encoding IPCT and DIPPS were found in the genomes of all known DIP-accumulating organisms. In most cases, these two activities are fused in a single gene product, but separated genes were predicted in *Aeropyrum pernix*, *Hyperthermus butylicus,* and members of the order *Thermotogales*.

The present phylogenetic analysis used the 35 IPCT and DIPPS sequences available in public databases. The fit of the data to protein and DNA evolutionary models was done using ProTest and ModelTest programs. Phylogenetic trees based on protein sequences as well as on 16S rDNA sequences were constructed using maximum likelihood PhyML programs. The resulting phylogenetic trees indicated the organization of the IPCT and DIPPS proteins in three groups: one refers to the organisms in which IPCT and DIPPS are encoded by separated genes; a second one comprised the members of the *Aquificales* and the *Euryarchaeota*; the third group was less defined and comprised all the remaining sequences available. This study suggests that the genes encoding IPCT and DIPPS evolved from separated genes to a fused gene, encoding the two enzyme activities. Moreover, there is strong evidence for the occurrence of lateral gene transfer along the evolutionary process.

(1) M. V. Rodrigues, N. Borges, M. Henriques, P. Lamosa, R. Ventura, C. Fernandes, N. Empadinhas, C. Maycock, M. S. da Costa, H. Santos. 2007. J. Bacterol. 189:5405-12.

Reference

126

Global expression analysis of a *Staphylococcus aureus* mutant with an impaired cell wall

Sobral, Rita Gonçalves (1); Ludovice, Ana Madalena (1); Jones, Alison E. (2); Des Etages, Shelley G. (2); Dougherty, Thomas J. (3); Peitzsch, Robert M. (2); Gaasterland, Terry (4); De Lencastre, Hermínia (1, 5); Tomasz, Alexander (5)

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa, 2780 Oeiras, Portugal;
2: Genetic Technologies, Pfizer Global Research & Development, Groton, CT. 06340;
3: Antibacterial Biology, Pfizer Global Research and Development, Groton, CT;
4: Scripps Institute of Oceanography, La Jolla, Ca. 92093;
5: The Rockefeller University, 1230 York Avenue, New York, NY 10021

E-mail: sobral@itqb.unl.pt

Keywords: *Staphylococcus aureus*, microarrays, virulence, beta-lactam resistance.

Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) owes its high level resistance to *mecA* and also to the functioning of several native genes, one of which was identified as *murF*, coding for a cell wall biosynthetic protein. A *murF* insertion mutant (F9) was constructed in the background of MRSA strain COL; the dramatic decrease observed in the oxacillin MIC was accompanied by the emergence of highly frequent and stable resistant subpopulations (homostar F9H). The peptidoglycan changes observed included reduced cross-linking and accumulation of disaccharide tripeptide monomer and were much less significant for F9H strain (1).

The mutant and the homostar transcription profiles were determined by microarray analysis, using three biological replicates. Total RNA extracted from COL, F9 and F9H strains was hybridized against Affymetrix chips.

Overall, 196 genes were identified as being differently expressed in the three strains. Of these, 141 were up-regulated (100) or down-regulated (41) both in the mutant and in the homostar strain when comparing to COL. The expression level of the remaining genes was differently affected when comparing the mutant and the homostar profiles. This latter group of genes includes mainly determinants related to virulence and pathogenesis, as staphylococcal enterotoxin B, fibrinogen-binding proteins and hemolysins, all over-expressed in F9 mutant but showing unaltered expression in the homostar. Induction of virulence genes was also observed in a *murF* conditional mutant by a similar transcriptomic assay (2).

The majority of the genes with altered expression was common to F9 and F9H strains, suggesting that these specific transcriptional changes are the consequence of the impairment of *murF*. The differences observed in the mutant and in the homostar transcriptional profiles are probably related to the phenotypic traits which distinguish both strains, namely the oxacillin resistance level and the extent of peptidoglycan composition abnormalities. Surprisingly, although some of the identified genes are cell surface related, no gene of the cell wall biosynthetic pathway has an altered expression. Instead, a mutation in *murF* gene, responsible for a major cell wall defect, seems to trigger an overall aggressive/defensive reaction through the regulation of the transcription of virulence related determinants, suggesting a tight connection between two strain specific characteristics, the antibiotic resistance level and the virulence potential.

1. Sobral, R.G.et al. 2003. Microb. Drug Resist. 9: 231-241.
2. Sobral, R.G. et al. 2007. J. Bacteriol. 189: 2376-2391.

Reference

154

The regulatory network underlying the transcriptional up-regulation of the FLR1 gene in mancozeb stressed yeast cells: qualitative modeling and simulation

Monteiro, Pedro Tiago (2,3); Dias, Paulo Jorge (1); Ropers, Delphine (3); Oliveira, Arlindo Limede (2); Freitas, Ana Teresa (2); Sá-Correia, Isabel (1); Teixeira, Miguel Cacho (1)

1: IBB - Institute for Biotechnology and Bioengineering, CEBQ/IST, Portugal;
2: INESC-ID Lisboa / KDBIO, Portugal;
3: Institut National de Recherche en Informatique et en Automatique (INRIA), Unité de recherche Rhône-Alpes, France

E-mail: mnpct@ist.utl.pt

Keywords: regulatory network, computer modeling, fungicide resistance

Abstract

Multidrug resistance is often the result of the activation of drug efflux pumps able to catalyze the extrusion of the toxic compound to the outer medium, this activation being many times controlled at the transcriptional level. Although thousands of associations have been established between individual transcription factors and target genes in the model eukaryote *S. cerevisiae* [1,2], transcriptional regulation is expected in most cases to be the result of the interaction and cross-talk between networks of transcription factors. This is the case of the transcriptional activation of the *FLR1* gene in response to stress induced in yeast by the agricultural fungicide mancozeb. *FLR1* up-regulation depends on the integrated action of four transcription factors, which interplay to produce the observed transcriptional up-shift [3].
Using a qualitative model that is able to overcome the current lack of quantitative data on kinetic parameters and molecular concentrations, a model of the *FLR1* regulatory network was built and the response of *S. cerevisiae* to mancozeb stress in different genetic backgrounds was simulated. This allowed the identification of essential features of the transition from unstressed to fungicide stressed cells and to make new predictions on the dynamic behavior of the system following mancozeb exposure, some of which were validated experimentally. In particular, the role of Pdr3 in *FLR1* regulation was further scrutinized and the inter-dependent role of Yap1 and Yrr1 in the regulation of *PDR3* and *RPN4* was brought to light.

[1] Teixeira M.C., Monteiro P., Jain P., Tenreiro S., Fernandes A.R., Mira N.P., Alenquer M., Freitas A.T., Oliveira A.L., Sá-Correia I. 2006. The YEASTRACT database: a tool for the analysis of transcriptional regulatory associations in *Saccharomyces cerevisiae*. Nucleic Acids Res, 34: D446-D451.
[2] Monteiro P.T., Mendes N., Teixeira M.C., d'Orey S., Tenreiro S., Mira N.P., Pais H., Francisco A., Carvalho A., Lourenço A.R., Sá-Correia I., Oliveira A.L., Freitas A.T. 2008. YEASTRACT-DISCOVERER: new tools to improve the analysis of transcriptional regulatory associations in *Saccharomyces cerevisiae*. Nucleic Acids Res. Database Issue 36: D132-D136.
[3] Teixeira M.C., Dias P.J., Simões T., Sá-Correia I. 2008. Yeast adaptation to mancozeb involves the up-regulation of FLR1 under the coordinate control of Yap1, Rpn4, Pdr3 and Yrr1. Biochem Biophys Res Commun, 367: 249-255.

Reference

207

Evolutionary dynamics of *Chlamydia trachomatis* key antigen

Nunes, Alexandra; Borrego, Maria José; Florindo, Carlos; Gomes, João Paulo

National Institute of Health, Portugal

E-mail: alexandra.nunes@insa.min-saude.pt

Keywords: Chlamydia trachomatis, MOMP, evolution, antigen

Abstract

Chlamydia trachomatis is an obligate intracellular pathogen involving 18 serovars responsible for trachoma (A-C), genital infections (D-K) and lymphogranuloma venereum (L1-L3). Its major outer membrane protein (MOMP) is a well-known porin, adhesin, and the dominant antigen. Investigation of the *ompA* (that codes MOMP) variability on circulating *C. trachomatis* strains may contribute for understanding the impact of the host pressure on this key antigen. We aimed to study the *ompA* mutational trends in clinical specimens routinely collected in the Portuguese NIH over the last 7 years by analyzing the *ompA* evolutionary dynamics based on MOMP structure and on the size/position of antigenic regions.

From a total of 795 specimens that were successfully typed, 29.2% showed *ompA* nucleotide changes when compared with the respective prototype-strain; ~42% of them had never been described. Overall, 93.9% of the mutations that occurred in MOMP variable exposed domains yielded amino acid changes, while the reverse was seen for the rest of the protein ($P<10^{-4}$). More, amino acid alterations were 7.2-fold more frequent within B-cell epitopes ($P=0.012$), and some mutations were also found within or close to T-cell antigenic clusters. Some of these mutations are under positive selection, and are known to neutralize infectivity *in vitro*, which strongly suggests a mechanism of host immune evasion. Interestingly, the two most ecological succeeded genotypes, E and F, showed a mutation rate 60.3-fold lower than the other genotypes ($P<10^{-8}$), suggesting that the secret of their efficacy may reside on a better fitness for dealing with the host immune system rather than on specific virulence factors. Furthermore, the variability exhibited by some strains involved residues that are known to participate in the binding to the LPS as well as residues that play a critical role during the membrane mechanical movements, contributing to a more stable and flexible porin conformation, which suggests some plasticity to deal with environmental pressure.

Globally, these MOMP mutational trends yielded no mosaic structures or important phylogenetic changes, but instead point mutations on specific protein domains, which may enhance pathogen's infectivity, persistence, and transmission. We believe that investigation of the mutational trends of pathogen's key antigens may be essential to understand pathogen's feedback to host pressure, and consequently, to develop prophylactic or therapeutic measures.

Poster Session:
S8 – Bioinformatics, Comparative Genomics and Evolution

Reference

105

Highly penicillin-resistant multidrug-resistant *pneumococcus*-like strains colonizing children: genomic characteristics and implications for surveillance

Simões, Alexandra S. (1); Sá-Leão, Raquel (1, 2); Bootsma, Hester J. (3); Eleveld, Marc J. (3); Tavares, Débora A. (1); Carriço, João A. (4, 5); de Lencastre, Hermínia (1, 6); Hermans, Peter W.M. (3)

1: Laboratory of Molecular Genetics, Instituto de Tecnologia Quimica e Biologica (ITQB), Universidade Nova de Lisboa (UNL), Oeiras, Portugal;
2: Centro de Matemática e Aplicações Fundamentais (CMAF), Universidade de Lisboa, Lisboa, Portugal;
3: Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;
4: Instituto de Microbiologia, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Lisboa, Portugal;
5: Instituto de Engenharia de Sistemas e Computadores: Investigação e Desenvolvimento (INESC-ID);
6: The Rockefeller University, New York, NY, USA

E-mail: assimoes@itqb.unl.pt

Keywords: *Strepococcus pneumoniae*, Penicillin, Multidrug-Resistant, Multilocus Sequence Analysis (MLSA), Comparative Genomic Hybridization (CGH)

Abstract

While performing surveillance studies aimed to describe the impact of the pneumococcal conjugate vaccine in colonization, we observed an increase from 0.7% in 2003 to 5% in 2006 in the prevalence of penicillin resistance (MIC 2-6 mg/L) among isolates presumptively identified as pneumococci which could have serious clinical implications. Although 15 of the 22 penicillin-resistant isolates were optochin-resistant, they were bile soluble and thus considered to be bona fide pneumococci. This study aimed to clarify the nature of these isolates. By multilocus sequence analysis (MLSA) all isolates but one were “streptococcus of the mitis group”. A single isolate was identified as *S. pseudopneumoniae*. Of interest, an atypical *lytA* and the recently described mitilysin were widespread among the strains and five had pneumolysin. Furthermore, comparative genomic hybridization (CGH) indicated that a considerable part of the proposed pneumococcal core genome is conserved in these isolates, including several pneumococcal virulence genes. The results suggest that among pneumococci and closely related streptococci, universal unique phenotypic and genetic properties that could aid species identification are virtually impossible to obtain. When atypical strains are found during colonization studies, MLSA is an informative tool that can be used to complement routine tests. In our study, after correct identification of the penicillin-resistant isolates, we found that penicillin resistance levels among pneumococci remained stable from 2003 to 2006.

Reference

106

MAT genes and the assessment of biological species in *Diaporthe* and *Phomopsis*

Santos, Jorge M.; Correia, Viviana G.; Phillips, Alan J. L.

Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

E-mail: jmsantos@fct.unl.pt

Keywords: Diaporthe, Phomopsis, mating-types, ITS, EF1-α, phylogeny, taxonomy, biological species

Abstract

Sexual reproduction in ascomycete fungi is governed by a single *locus* called the mating-type (*MAT*) *locus*. The mating-type *loci* of *Diaporthe* and its *Phomopsis* anamorphs differ in a single gene: *MAT1-1-1* in mating-type *MAT1-1* and *MAT1-2-1* in mating-type *MAT1-2*. *MAT* genes, as master regulators of sexual reproduction, strongly influence sex, and hence play an important role in population genetics and evolution in fungi. Moreover, these genes are evolving rapidly throughout the eukaryotic kingdoms, apparently in conjunction with speciation. Two main problems are posed when studying phytopathogenic filamentous fungi: 1) the correct identification of the species involved in a certain disease and 2) the establishment of anamorph-teleomorph connections. In order to diagnose mating-types in *Diaporthe* and *Phomopsis* and evaluate their usefulness in teleomorph induction *in vitro* and biological species delimitation, we designed degenerate primers that differentially amplify part of the *MAT1-1-1* and *MAT1-2-1* genes. *MAT* phylogenies were generated and compared to ITS and EF1-α phylograms. Mating experiments were conducted to evaluate the existence of reproductive barriers between some isolates, and their anamorphic morphologies were compared. The primers proved to be useful not only in the mating-type diagnosis of isolates and selection of compatible mating pairs, surpassing classical methodological problems of teleomorph induction *in vitro*, but also in the assessment of biological species boundaries.

Reference

117

Do it yourself comparative genomics

Almeida, João Manuel Feio de

Universidade Nova de Lisboa, Portugal

E-mail: jmfa@fct.unl.pt

Keywords: bioinformatics,genomics,molecular evolution,data pipeline

Abstract

The amount of genome sequences available in public databases increases steadily. But the full exploitation of this bonanza of data is hampered by the limitations in sequence annotation. These limitations result from an imbalance between the rate of accumulation of new sequences, and the throughput of wet-bench researchers. The gap is usually filled by *in silico* analysis, mostly done through data pipeline software (*e.g.* EMBL Bank to TrEMBL). The results are more often than not stored in secondary databases after a most scant quality control assessment due to limitations in staff. This state of affairs results in the need to enforce a most strict set of parameters during the *in silico* analysis in order to avoid or limit the emergence of artifacts (*e.g.* annotation transfer from analogs).

Most genome centered databases (*e.g. e*!Ensembl) offer pre-computed comparative genomic results to speed the analysis required by the ordinary user. Those results are often available through rich graphic user interfaces that ease the burden of finding the intended data. So the ease of use is counterbalanced by conservative nature of released datasets.

Bi-directional BLAST is an accepted procedure for to homolog detection, but it must be supplemented with domain and syntheny information. As these analyses are computationally intensive there is still room for intermediate solutions, like complementing the analysis with global alignments of both the ORF and its product to remove false hits.

Software with this capabilities is not readily available, tend to need refactoring, and require the installation of the programming environment. To overcome these limitations a data pipeline was designed in Java to allow for automatic bi-directional NCBI BLAST of large sets of ORF sequences. The application has the ability to assign GO-slim terms for aggregate analysis on the results according to the three ontologies. When coding sequences are supplied the application allows for the refining of the hits through global alignment, based either on the nucleotide sequence or the conceptual translation. Further analysis is available such as molecular evolution rates obtained through PAML, and several related statistics (*e.g.* codon position conservation).

The application dump the results in a set text delimited files that can be fed to a spreadsheet or a RDBMS for subsequent analysis. This application enables the user to perform customized comparative genomic analysis in a regular personal computer.

[1] S. Altschul, T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman, Nucl. Acids Res., vol. 25, Sep. 1997, pp. 3389-3402.
[2] Z. Yang, Mol Biol Evol, vol. 24, Aug. 2007, pp. 1586-1591.
[3] P. Rice, I. Longden, and A. Bleasby, Trends in genetics, vol. 16, 2000, pp. 276-277.

Reference

120

SIVmnd-2 infection in captive-born mandrills (*Mandrillus sphinx*) housed at the Lisbon Zoo

Valente, Susana Soares (1); Venenno, Teresa (1, 2); Esteves, Aida (1, 2); Bernardino, Rui (3); Fernandes, Teresa Lobo (3); Parreira, Ricardo (1, 2); Piedade, João (1,2)

1: Unidade de Ensino e Investigação de Virologia, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa;
2: Unidade de Parasitologia e Microbiologia Médicas, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa;
3: Jardim Zoológico e de Aclimatação em Portugal, Lisboa

E-mail: svalente@ihmt.unl.pt

Keywords: Mandrillus sphinx, SIVmnd-2, pol/env genes, cytochrome b haplotype, phylogenetic analysis

Abstract

African non-human primates represent a large reservoir for simian lentiviruses and humans are, at present, potentially exposed to a large diversity of simian immunodeficiency viruses (SIV) through contact with wild and captive primates. Mandrills (*Mandrillus sphinx*) are large semiterrestrial primates from western central Africa, where Ogooué river (Gabon), functioning as a natural barrier, divides the species in two haplotype/phylogeographic groups, N (northern) and S (southern). Besides humans, these primates were the first to be reported to harbour two distinct lentiviruses, SIVmnd-1 and SIVmnd-2, and, interestingly, the distribution of the viral types follows the geographic separation, with type 1 being present in the south and type 2 in the north.
In 2006, immediately prior to being transferred to the Budapest Zoo, a 7-year-old mandrill housed at the Lisbon Zoo tested positive for serum HIV/SIV cross-reacting antibodies by standard enzyme immunoassays. Using the WHO algorithm for HIV screening, inspection of the remaining six individuals of the colony showed the presence of anti-SIV antibodies in five more animals. After PCR amplification of mitochondrial DNA, cloning and sequencing, phylogenetic analysis of a 424-bp-fragment from the cytochrome *b* gene revealed the genetic close relationship between the animals, as expected, and placed them in the haplotype N, suggesting ancestry in a maternal lineage originating from the northern natural range of the species (North Gabon, Equatorial Guinea, Southwest Cameroon). In addition, phylogenetic analysis of partial SIV gp41/*env* (460 bp) and integrase/*pol* (720 bp) sequences, amplified from proviral DNA, indicated that the animals were infected with SIVmnd type 2, with all the sequences grouping together in a tight monophyletic cluster, corroborating the association between SIVmnd-2 and haplotype N of the host. This analysis further revealed no evidence of recombination (at intra or intertype levels). Attempts to isolate this novel SIVmnd-2 strain *in vitro* are currently in progress.
Although it was one of the first primates identified as a natural host of SIV, the mandrill is still a less commonly studied model. Despite having a high prevalence of infection in the wild, mandrills represent a substantial part of the bushmeat trade. Moreover, SIVmnd replicates in human peripheral blood mononuclear cells and macrophages *in vitro*, thus assuming itself as a candidate for a new emerging lentiviral human pathogen.

Reference

159

New developments for evaluating the congruence between microbial typing methods

Carriço, João André (1); Severiano, Ana (1); Pinto, Francisco (1,2); Melo-Cristino, José (1)

1: Instituto de Microbiologia,Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal;
2: Centro de Química e Bioquímica, Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

E-mail: jcarrico@fm.ul.pt

Keywords: Microbial Typing Methods, Congruence of experiments, Data Analysis

Abstract

Microbial typing methods allow the differentiation for bacterial isolates of the same species, providing a fundamental tool for clinical microbiology, molecular epidemiology and population genetics studies. These methods allow for the identification of particularly virulent strains, to assess the transmission between different hosts and to study the population phylogeny and dynamics.
In these fields comparing typing methods classifications is a recurring task. Collections of bacterial and viral isolates are characterized by several microbial typing techniques, each isolate being classified into a given type or subtype.
Nevertheless, comparison of these classifications has relied on subjective and qualitative evaluation criteria. In order to quantitatively measure the congruence of typing methods results, we have successfully suggested the use of a set of known partition comparison coefficients: Adjusted Rand (AR) and Wallace (W) coefficients (Carriço, 2006).
The combined analysis of AR and W allows the evaluation of the strength of the agreement of two classifications and a finer understanding of how the results are related, allowing the supported choice of what typing methods should be used for a clone definition for a given species and how reliable that definition can be, based on the dataset used. A high value of AR is translated as a good agreement between the partitions formed by the two methods and W provides an indication of asymmetries in the directionality of concordance.
Recently, Pinto *et al* (Pinto, 2008) extended this framework, proposing the calculation of the expected W in the case of independence and the use of a confidence interval for W, adding statistical support for the comparison of these coefficients. These new measures reinforce the role of W in generating maps of types or subtypes equivalence between different typing methods, and facilitate the joint analyses of multiple typing methods.
In order to make them available for any field of study and to the microbial typing community in particular, we have implemented these new advances by updating the original user-friendly web-tool where researchers can analyze their data applying the proposed framework. It is freely available at www.comparingpartitions.info. Users can now calculate the proposed coefficients and respective confidence intervals. For added statistical support confidence, the user is also given the choice of calculating the confidence intervals by bootstrapping their data.

Carriço, J.A.; C. Silva-Costa; J. Melo-Cristino; F. R. Pinto; H. de Lencastre; J.S.Almeida; M.Ramirez, J Clin Microbiol. 2006 Jul;44(7):2524-32

Pinto FR, Melo-Cristino J, Ramirez M (2008). PLoS ONE 3(11): e3696.

Reference

238

Porphyromonas gingivalis proteases involved in periodontal disease

Correia, Maria José (1); Rosa, Nuno (1); Campos, Pedro (2); Filipe, Magda (2); Barros, Marlene (1)

1: Departamento de Ciências da Saúde, Universidade Católica Portuguesa;
2: Departamento de Biologia, Universidade de Aveiro

E-mail: mariacorrei@gmail.com

Keywords: P.gingivalis, proteases, PPI, periodontal disease

Abstract

Porphyromonas gingivalis is one of the major microbial agents involved in periodontal disease interacting with several constituents of the human host. *P.gingivalis* proteases are considered the major virulence factors involved in host-microbial interaction. The sequencing of the *P.gingivalis* genome has revealed that this bacterium is capable of producing several proteases included in the Aspartic, Cysteine, Metallo and Serine protease families as well as a number of proteases still unclassified in the existing families. Understanding of the role of *P.gingivalis* proteases in the host-bacterial interaction could lead the way to new strategies for periodontal disease management. This rationale has mainly been applied to the study of gingipains (3 different cysteine proteases produced by *P.gingivalis*), but could be extended to all the other known and predicted proteases.

Proteins and especially proteases exert their role in biological processes mainly through interactions with other proteins and the study of Protein Protein Interactions (PPIs) has know an exponential growth in the last decade. Several international databases have accumulated experimental and theoretical data on protein structure and function and powerful algorithms have been designed to perform the analysis of that data producing (PPI) databases. However, some of the interactions retrieved from the algorithm analysis are not biologically meaningful or even possible due to several "*in vivo*" constraints.

The aim of this work is to determine which of the PPIs predicted for *P.gingivalis* proteases using the String server are possible within the oral cavity and of those which are theoretically more meaningful for the host-bacterial interactions established during periodontal disease.

Information on the *P.gingivalis* proteases was obtained from UniprotKB (The UniProt Consortium, 2008) and Merops (Rawlings, *et al* , 2008) databases. Original data were curated to eliminate repeated entries or entries for isoforms of the same protease produced by different strains of *P. gingivalis*. The unique proteases identified were introduced in the STRING server (Jensen *et al*, 2009) where interactions were determined. The raw data obtained from the STRING server were manually curated.

The results of this analysis are important in establishing the best target molecules to be used in the modulation of periodontal diseases.

The UniProt Consortium, 2008 Nucleic Acids Res. 36:D190-D195(2008).

Rawlings, N.D., Morton, F.R., Kok, C.Y., Kong, J. & Barrett, A.J. (2008) Nucleic Acids Res 36, D320-D325

Lars J. Jensen, Michael Kuhn, Manuel Stark, Samuel Chaffron, Chris Creevey, Jean Muller, Tobias Doerks, Philippe Julien, Alexander Roth, Milan Simonovic, Peer Bork, and Christian von Mering. Nucl. Acids Res. 37: D412-D416.

Reference

314

The red yeast *Sporidiobolus salmonicolor* holds clues to a new intermediate sex system in fungi

Coelho, Marco A.; Sampaio, José Paulo; Gonçalves, Paula

Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

E-mail: madc@fct.unl.pt

Keywords: Evolution of sex, mating-types, Sporidiobolales, red yeasts

Abstract

In fungi, sexual identity is determined by specialized genomic regions called mating-type (*MAT*) loci which are comparable to sex chromosomes in animal and some plants. To date, only two mating systems were described in fungi: the bipolar system (if mating is dictated by two alternate sets of genes at the *MAT* locus) and the tetrapolar system (if two functionally distinct molecular recognition mechanisms, encoded by two unlinked *MAT* regions, constrain the selection of sexual partners). The tetrapolar system is found only in some basidiomycetes and can generate four different mating types per meiosis, potentially lowering the odds of inbreeding. The two *MAT* specific regions encode peptide pheromone/ pheromone receptors and homeodomain transcription factors and heterozygosity at both regions is a pre-requisite for sexual development. We found that *Sporidiobolus salmonicolor*, a basidiomycetous red yeast in the sub-phylum Pucciniomycotina, has a mating system unlike any previously described because it is intermediate between bipolar and tetrapolar. By studying all the available strains of *S. salmonicolor*, we show that: (i) mating behavior is bipolar; (ii) heterozygosity at both *MAT* regions is required for mating; and (iii) new mating types with no apparent fitness defect were generated by occasional disruptions of the genetic cohesion of the *MAT* locus. Moreover, the allele number and mode of evolution of the newly identified HD1/HD2 transcription factors resembles that of the tetrapolar systems.

We propose that the intermediate mating system is likely to be pervasive in some genera within the Pucciniomycotina and may be stable in species for which it provides the right balance between inbreeding and outcrossing.

Reference

320

Molecular modelling and phylogenetic analysis of the protein disulfide isomerase from *Besnoitia besnoiti*

Marcelino, Eduardo (1 2); Morais, Joana B. (1 2); Martins, Tiago M. (2); Cortes, Helder (3); Leitão, Alexandre (1); Novo, Carlos (2)

1: Instituto de Investigação Cientifica Tropical, CVZ, CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. Da Universidade Técnica 1300-477, Lisboa, Portugal;
2: Unidade de Tecnologias de Proteínas e Anticorpos Monoclonais (UTPAM), Instituto de Higiene e Medicina Tropical, Campus do Lumiar, Estrada do Paço do Lumiar, 22, Edifício F, r/c, 1649-38 Lisboa, Portugal;
3: Laboratório de Parasitologia, Núcleo da Mitra. ICAM, Universidade de Évora, Apartado 94, 7000-554 Évora, Portugal

E-mail: amedmarcelino@fmv.utl.pt

Keywords: Besnoitia besnoiti, protein disulfide isomerase, theoretical model, phylogeny

Abstract

Besnoitia besnoiti is an Apicomplexa parasite responsible for bovine besnoitiosis, a high prevalence disease in tropical and subtropical regions that apparently is re-emerging in Europe, with recent cases reported in Portugal, Spain, France and Germany. Despite the great economical losses associated with this disease, it has been underestimated and poorly studied, having neither an effective therapy nor a safe vaccine available. Protein disulfide isomerase (PDI) was demonstrated to be relevant in the host cell adhesion/invasion process and in the host immune response for the closely related *Neospora caninum* and *Toxoplasma gondii* and, for *B. besnoiti*, our preliminary results are in good agreement with those observations. In this work the gene sequence coding for the *B. besnoiti* PDI (BbPDI) was determined (GenBank™ accession number DQ490130) and a 3D theoretical model was built by comparative homology using Swiss-Model server covering the four domains **a**, **b**, **b'** and **a'**, and deposit in PMDB (http://mi.caspur.it/PMDB/) under de id PM0075875. BbPDI belongs to the Thioredoxin like Superfamily (cluster 00388) and is included in PDI_**a** family (cluster defined cd02961) and belongs to the PDI_**a**_PDI_**a´**_**c** subfamily (cd02995). According to the Conserved Domain Database (CDD) annotation, this subfamily includes endoplasmic reticulum (ER)-resident eukaryotic proteins involved in oxidative protein folding. They are oxidases, catalyzing the formation of disulfide bonds of newly synthesized polypeptides in the ER, and reductases, acting as isomerases to correct any non-native disulfide bond, having also chaperone activity to prevent protein aggregation and facilitate the folding of newly synthesized proteins. PDI's have the **abb'a'** domain structure (where **a** and **a'** are redox active TRX domains while **b** and **b'** are redox inactive TRX-like domains). PDI members also contain an acidic region (**c** domain) after the **a'** domain and show a wide substrate specificity. The phylogenetic analysis and the importance of PDI as a potential drug target for the treatment of besnoitiosis will be discussed.

Reference

384

The genome of a gut strain of *Bacillus subtilis*: molecular insights into a bi-phasic life cycle and the mechanisms of probiosis

Schyns, Ghislain (1); Pereira-Leal, José (2); Serra, Cláudia (3); Fickers, Patrick (4); Potot, Sébastien (1); Barbosa, Teresa (1); Chevreux, Bastien (1); de Saizieu, Antoine (1); Perkins, John B. (1); Wyss, Markus (1); Henriques, Adriano O. (3)

1: R&D, DSM Nutritional Products, Ltd., Kaiseraugst, Switzerland;
2: Instituto Gulbenkian de Ciência, Oeiras, Portugal;
3: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal;
4: Centre d'Ingénérie des Protéines, Université de Liège, Sart-Tilman, Belgium

E-mail: Ghislain_r.Schyns@dsm.com

Keywords: whole genome sequencing, probiogenomics, rates of evolution, gut microbiota

Abstract

Bacillus subtilis, a Gram-positive, rod-shaped, spore-forming bacterium, is both a model organism as well as an industry workhorse. The genome of the 168 strain, a derivative of the initial isolate known as the Marburg strain, was published in 1997, inspiring a wealth of studies on the most diverse aspects of the biology of this organism. It is now recognized, however, that several important traits have been attenuated or lost upon domestication of this strain. Here, we report the genome sequence of a gut-associated strain of *B. subtilis* hereinafter designated BSP1. The genome consists of a single chromosome of 3,985,000 bp specifying an estimated 3,693 protein-coding sequences and a plasmid of 62,231 bp, coding for 81 predicted proteins. BSP1 lacks most prophages and prophage-like elements found in the 168 strain. Several negative regulators of Spo0A a key regulatory protein in many post-exponential adaptive responses are also absent. Increased activity of Spo0A explains important biological properties of BSP1, including its potent anti-microbial activity or its ability to sporulate during growth, both of which traits that may be beneficial in the gut. BSP1 also carries at least 200 genes not found in *B. subtilis* 168, most of which were presumably acquired horizontally mainly from the closely related *B. licheniformis*. Some appear to control complex behaviors such as colony morphology and biofilm architecture, mucosal adhesion through a sortase-dependent collagen-binding protein, or hemicellulose utilization. While absent from the sequenced laboratory strain, some of these genes are signatures for probiotic bacteria. Overall, the BSP1 genome sequence supports the view that *B. subtilis* has a bi-phasic life style, cycling between the soil and the animal gastrointestinal tract. Importantly, several genes differ considerably between the gut strain and a standard laboratory strain and thus, while evidencing traits of life in the gut, the genome of strain BSP1 also provides insight into the adaptation of *B. subtilis* to life in the laboratory

Reference

400

Semantic tools for the management and integration of ecophysiological data from cultured multi-species biofilms

Bohn, Andreas; Antunes, Sofia

Instituto de Tecnologia Química e Biológica - Universidade Nova de Lisboa, Portugal

E-mail: abohn@itqb.unl.pt

Keywords: scientific workflow, semantic web, phototrophic biofilm, ecoinformatics

Abstract

The present work is based on experimental data from the concluded EU-project PHOBIA on phototrophic biofilms and their applications (http://www.photobiofilms.org). Experiments were performed in five different laboratories across Europe, all using the same freshwater and marine inocula of natural origin, as well as the same incubators and control-parameter protocols. The resulting data structure was typical for ecological datasets, showing a large number of variables and a small amount of repetitions.

Our goal was to develop a web-based scientific workflow consisting of a database connected to an analysis module for detecting significant correlations between the data pools and providing a predictive model for phototrophic biofilms. For this purpose, we implemented a recent database prototype (S3DB, http://www.s3db.org) relying on concepts from the semantic web, which has previously been applied successfully in multi-partner projects in biomedicine. Our modifications to S3DB significantly improved the velocity of uploading and querying the PHOBIA data, and enabled the storage of 1- and 2-dimensional data arrays, like biofilm growth curves and images. In addition, we created an interface for the interoperability of the database with analysis scripts written in the R Language for Statistical Computing.

In the present work, we demonstrate the current state of development of WebPHOBIA, and show how the semantic framework allows the management and description of the PHOBIA metadata with a formalism that is understandable also for non-computational scientists. We hypothesize that generalizations of the vocabulary used to describe the PHOBIA data could stimulate the development of a general framework for the description of microbial systems, supporting the integration, sharing and comparison of experimental data generated across the sub-disciplines of microbial biology.

We discuss how our approach aimed to deal with microbial population-level data could complement ongoing high-throughput molecular and cellular research, leading to the development of integrated multi-level models of the function of microbial systems under real-world conditions.

Reference

415

Integration of proteomic data for predicting dynamic behaviour in an *E. coli* central carbon network after genetic perturbations

Costa, Rafael S.; Machado, Daniel; Rocha, Isabel; Ferreira, Eugénio Campos

IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

E-mail: ecferreira@deb.uminho.pt

Keywords: *E. coli* metabolism, linlog kinetics, constraint-based approach, prediction, dynamic modelling, data integration

Abstract

One of the great challenges in the post-genomic era is to understand the dynamic behaviour of a living cell. For that purpose, quantitative models describing metabolic network dynamics are a powerful tool as “dry lab” platforms to simulate experiments before they are performed *in vivo*. Kinetic models and stoichiometric genome scale models of the microbial metabolism are usually the two large-scale modelling approaches most used. So far, few large scale kinetic models have been successfully constructed. The main reasons for this are not only the associated mathematical complexity, but also the large number of unknown kinetic parameters required in the rate equations to define the system. In contrast to kinetic models, the genome scale modelling approach bypasses these difficulties by using basically only stoichiometric information with certain physicochemical constraints to limit the space of a network without large fitted parameters sets. Although these constraint-based models are highly relevant to predict a feasible set of steady-state fluxes under a diverse range of genetic conditions, the steady-state assumption may oversimplify cellular behaviour and cannot offer information about time dependent changes. To overcome these problems, combining these two approaches appears a reasonable alternative to modelling large-scale metabolic networks.

In this work, we used a large-scale central carbon metabolic network of *E. coli* [1] to investigate whether including high throughput enzyme concentrations data into a model allows an improved prediction of the response to different single-knockouts perturbations. For this purpose, a model based on the flux balance analysis (FBA) approach and linlog kinetics was constructed. As a first validation, we applied it to predict steady-state changes in fluxes and metabolite concentrations, as well as dynamic responses to perturbations in the central *E. coli* metabolism. Then, the approach was evaluated by comparison with various sets of published *in vivo* measurements [2]. Our results indicate that integration of the quantitative enzyme levels into the kinetic models, in general, can be used to predict dynamic behavior changes.

[1] Chassagnole,C.; Noisommit-Rizzi,N.; Schmid,J.W.; Mauch,K.; Reuss,M. Biotechnology and Bioengineering 2002, 79: 53-73.

[2] Ishii, N. et al. Science 2007, 316: 593-597

Reference

416

The evolution of glycolysis in *Saccharomyces*: a possible link with growth temperature preferences

Valério, Elisabete; Almeida, João; Sampaio, José Paulo; Gonçalves, Paula

Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal

E-mail: evalerio@fct.unl.pt

Keywords: Saccharomyces, evolution rates, glycolysis, speciation

Abstract

The natural ecology of *Saccharomyces cerevisiae* and its close relatives is, in the post-genomic era, almost as obscure as it was 50 years ago. Several *Saccharomyces* species have been consistently isolated from oak bark and soil and were often found in sympatric association (i.e. two species in the same microenvironment) [1]. Sympatric pairs of species typically consisted of species with markedly different preferences with respect to growth temperature, such as *S. cerevisiae* (thermophilic) and *S. kudriavzevii* (cryophilic). We hypothesize that divergence in growth temperature preferences may have played a major role in the evolution of this group of yeasts, by allowing the species to share the same microenvironment while exploring differently the circadian and seasonal temperature regimes. We identified changes in evolution rate of glycolytic genes that may denote a selective pressure to adjust the performance of the glycolysis to the growth temperature range. Our results for two enzymes suggest that their temperature profile differs between thermophilic and cryophilic species, in line with the growth temperature preferences. Concurrent with the results obtained for the activities of individual enzymes, our results show that *S. kudriavzevii* has a better fermentative performance at low temperatures, while *S. cerevisiae* performs better above 30ºC. Taken together, our results show a species specific trend in the performance of the entire glycolytic pathway, suggesting that *S. kudriavzevii* specialized in ethanol production at lower temperatures, while *S. cerevisiae* seems to have evolved towards a better performance of the entire pathway at higher temperatures, through improved heat stability of glycolytic enzymes.

ACKNOWLEDGMENTS:
Fundação para a Ciência e a Tecnologia (PTDC/BIA-BDE/71734/2006)
Fundação para a Ciência e a Tecnologia (E. Valério is recipient of post-doc grant SFRH/BPD/46471/2008)

[1] J.P. Sampaio and P. Gonçalves. 2008. Applied Environmental Microbiology, 74 (7): 2144–2152

Reference

417

Multi-locus sequence typing, biogeography and ecology of the astaxanthin-producing yeast *Xanthophyllomyces dendrorhous*

Palma, Márcia C. David (1); Libkind, Diego (2); Sampaio, José Paulo (1)

1: Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516, Caparica, Portugal;
2: Laboratorio de Microbiologia Aplicada y Biotecnologia, Universidad Nacional del Comahue, Centro Regional Universitario Bariloche (CRUB)-Consejo Nacional de Investigaciones Científicas y Tecnológicas

E-mail: m.palma@fct.unl.pt

Keywords: Yeasts, *Xanthophyllomyces*, Biogeography, Sequencing

Abstract

The basidiomycetous yeast *Xanthophyllomyces dendrorhous* (asexual state: *Phaffia rhodozyma*) has biotechnological relevance due to the production of astaxanthin, a carotenoid pigment used as an additive in aquaculture feeds. This yeast was first isolated in the late 1960's by Herman Phaff [(1)] from nutrient-rich exudates of deciduous trees in Japan and Alaska. Subsequently many other strains have been isolated from tree exudates in the Northern Hemisphere and recently a remarkably new *Xanthophyllomyces* habitat was found in the Southern Hemisphere. In Patagonia (Argentina) *X. dendrorhous* was consistently isolated from the fruiting bodies of *Cyttaria hariotii*, an ascomycetous fungus that is a parasite of *Nothofagus* trees [(1)]. Sequences of the rRNA gene were used in the study of Patagonian and Northern Hemisphere isolates and the ITS-based phylogeny of *X. dendrorhous* was compared with that of its tree hosts (Betulaceae, Corneaceae, Fagaceae and Nothofagaceae) [(1)]. Although the results suggested a concordance between the two phylogenies and therefore an organization of *Xanthophyllomyces* populations according to their host type and not to geography, a more detailed analysis should aim at clarifying the global population structure of *X. dendrorhous*. In the present study we employed multi-locus sequence typing to analyze the genetic structure and biogeography of different lineages of *X. dendrorhous*. Variable regions of the genes *CRTI*, *CRTS* and *IDI* were sequenced and used for phylogenetic analysis. *X. dendrorhous* lineages were better resolved than in the previous ITS-based tree. One strain was heterozygous for two of the loci and the haplotypes had to be determined by molecular cloning. In general the *CRTI, CRTS* and *IDI* phylogenies were congruent with each other suggesting that clonality dominates the propagation of *X. dendrorhous* in spite of the fact that this yeast is capable of sexual reproduction [(3)]. Associations with host trees were observed for the isolatesfrom *Nothofagus* (Patagonia) and *Cornus* (Japan), which form two clearly distinct populations. However the isolates from *Betula* (Finland, Russia, Alaska and Japan) and *Fagus* (Japan) were poorly resolved. We will discuss the present organization of *X. dendrorhous* in three major clades in which the isolates from *Nothofagus* form a biogeographic unit whereas the isolates from *Cornus* and those from *Betula* + *Fagus* appear to escape this pattern.

This work was supported by an FCT grant (project PTDC/BIA-BDE/73566/2006).

1.Libkind D, Ruffini A, van Broock M, Alves L, Sampaio J. (2007) Appl Environ Microbiol 73:1120-1125.
2.Weber R, Becerra J, Silva M, Davoli P. (2008). Mycological Research II2: 861-867.
3.Golubev W. (1995). Yeast 11: 101-110.

Reference

427

Towards bioprocess control based on a reduced metabolic model of *Escherichia coli*

Soons, Zita I.T.A.; Rocha, Isabel; Ferreira, Eugénio Campos

IBB – Institute for Biotechnology and Bioengineering - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

E-mail: ecferreira@deb.uminho.pt

Keywords: Elementary mdes, controlled random search, *Escherichia coli*

Abstract

Most mathematical models used for optimization and control of biotechnological processes are relatively simple and the complex interactions between the extracellular environment and the thousands of intracellular enzymes and metabolites are generally ignored. The lack of this information in bioreactor monitoring and control can have a profound impact on biological systems. Nevertheless, the use of model-based methods in process monitoring and control is nowadays limited due to their complexity and the lack of appropriate methodologies. The challenge of the development of a model that predicts the dynamic response of cellular phenotypes to environmental conditions is not yet solved and will be addressed in the view of bioprocess control. First, stoichiometric models represent an infinite number of possible phenotypes; systems biology tools need to be applied such that the simulation matches the phenotypes in given conditions. Second, most tools in systems biology are designed for steady-state applications, whereas the aim of process control requires a dynamic approach. Third, as a consequence of the complexity of the models, the computational intensity is high.

One tool that has the potential to solve the above problems is Elementary Modes. EMs analysis identifies all minimal functional pathways possibilities inherent to a metabolic network. A consideration in the analysis of EMs for large metabolic networks is the problem of combinatorial explosion of possible routes across the networks. In many situations, more EMs exist than necessary to construct all admissible flux distributions. Therefore, some of them can be taken as a generator set of the whole admissible region. In this work, a controlled random search (CRS) algorithm combined with nonnegative least squares is developed to select a limited number of EMs matching the observed phenotype. The method minimizes the objective function in an iterative search. The objective function to be minimized consists of the weighted sum of squared errors and a penalty for inefficiency of each EM and for model size.

This case study considered the central carbon metabolism of *E. coli*. A single EM or a linear combination of EMs could be selected to match the experimental data. Using the CRS algorithm, the original model with 2706 EMs was reduced to a system of 3 modes for biomass growth, acetate production, and maintenance purposes that gives a good correlation with the measured data.