Universidade do Minho
Escola de Engenharia

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Characterization of the molecular interactions between *Staphylococcus epidermidis* biofilm infections and the host immune system

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PhD in Biomedical Engineering

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DECLARAÇÃO

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Characterization of the molecular interactions between *Staphylococcus epidermidis* biofilm infections and the host immune system

ABSTRACT

Staphylococcus epidermidis ranks first among the causative agents of nosocomial infections associated with indwelling medical devices. This association is due to the microorganism's ability to colonize the surface of these devices and form biofilms. The biofilm lifecycle is divided into initial adhesion, accumulation and maturation, and biofilm disassembly. The major clinical complications of biofilm formation is their high resistance to antimicrobials and to the host immune system, resulting in the development of chronic infections. To uncover the mechanisms by which biofilms evade the host immune system and cause chronic infections, a transcriptomic analysis of *S. epidermidis* biofilms exposed to human blood was performed. Our results revealed extensive changes in the transcriptome, suggesting that a quick adaptation to the new environment was made. Genes involved in amino acids biosynthesis and iron utilization were strongly affected, indicating that these mechanisms are important factors in *S. epidermidis* biofilm survival in human blood. The biofilm disassembly stage has been associated with the development of acute infections, however, despite its importance in the clinical setting, it is the less studied of the biofilm lifecycle stages. Hence, to comprehend the interactions between biofilm disassembly and the host immune system, biofilm-released cells were characterized with reference to several virulence parameters. Our results revealed that S*. epidermidis* biofilm-released cells are unique in their phenotype and virulence potential, sharing some features with planktonic cells, but simultaneously displaying features similar to biofilm cells. The phenotypic differences were also manifested as differences in the *S. epidermidis* transcriptome in response to immune cells. Thus, targeting the particular properties of biofilm-released cells could be important to prevent the serious acute infections associated with biofilm dissemination. As a preventive measure, the ability of a monoclonal antibody raised against PNAG to inhibit *S. epidermidis* biofilm accumulation was tested. Interestingly, it was observed that depending on the strain, the antibody present variable effect resulting, in some cases, in the enhancement of biofilm accumulation *in vitro*. In conclusion, the work described throughout this thesis has given an important contribution to the knowledge of biofilms-related infections, what will open new opportunities to effectively prevent the pathologic events associated with these serious and prevalent infections.

A caracterização das interações moleculares entre as infeções por biofilme de *Staphylococcus epidermidis* e o sistema imunitário do hospedeiro.

RESUMO

A espécie *Staphylococcus epidermidis* é atualmente considerada uma das principais causas do desenvolvimento de infeções nosocomiais, com particular associação a pacientes com dispositivos médicos invasivos. Esta associação é devida à capacidade desta bactéria aderir e formar biofilmes na superfície desses dispositivos. A formação do biofilme é classicamente dividida em adesão inicial, acumulação e maturação e, finalmente, a libertação de células do biofilme para o meio envolvente, num processo designado por dispersão. As grandes implicações clinicas da formação de biofilmes são a sua elevada tolerância aos agentes antimicrobianos e à resposta do sistema imunitário do hospedeiro, o que leva ao desenvolvimento de infeções crónicas. De forma a desvendar quais os mecanismos usados pela bactéria para escapar à resposta do sistema imunitário e causar infeções crónicas, foi caracterizado o transcriptoma de biofilmes de *S. epidermidis* cultivados na presença de sangue humano. Os resultados revelaram que a presença de sangue humano estimula uma extensa e rápida remodelação do transcriptome da bactéria, provavelmente para promover a adaptação ao novo e complexo ambiente envolvente. Os genes envolvidos na síntese de aminoácidos e na utilização de ferro sofreram as alterações mais pronunciadas, sugerindo que estes dois mecanismos são importantes fatores na sobrevivência da bactéria no sangue humano. Outra das etapas com grandes implicações clinicas é a dispersão de células do biofilme para o meio envolvente, uma vez que está diretamente associada ao desenvolvimento de infeções agudas importantes no hospedeiro. No entanto, esta é a etapa do ciclo de vida dos biofilmes de *S. epidermidis* menos bem compreendida. Assim, de forma a explorar as interações que ocorrem entre o sistema imunitário do hospedeiro e as células libertadas dos biofilmes de *S. epidermidis,* estas foram caracterizadas em diversos e importantes parâmetros envolvidos na sua virulência. Os nossos resultados revelaram que as células libertadas pelos biofilmes apresentam um fenótipo único, exibindo características particulares das células planctónicas, bem como das células derivadas do biofilme resultando também num estímulo particular do sistema imunitário do hospedeiro. Assim, estes resultados demonstram que um estudo mais aprofundado destas células poderá revelar novas oportunidades no desenvolvimento de estratégias preventivas contra as consequências patológicas associados à dispersão de células do biofilme. Numa perspetiva mais preventiva, a capacidade de um anticorpo monoclonal, específico para a PNAG, de inibir a acumulação dos biofilmes foi testada num sistema *in vitro*. Curiosamente, dependendo da estirpe testada, o anticorpo apresentou

eficácia variável resultando, em alguns casos particulares, no aumento da capacidade da estirpe de formar biofilme.

O trabalho descrito nesta tese contribuiu, significativamente, para aumentar o conhecimento sobre as infeções-associadas à formação de biofilmes e a interação destes com o sistema imunitário do hospedeiro. Este conhecimento irá, seguramente, abrir novas oportunidades para prevenir os eventos patológicos associados com estas infeções, por vezes severas, altamente prevalentes na sociedade.

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ABBREVIATION LIST

Agr	Acessory gene regulator
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
Aps	Antimicrobial peptide sensing system
AtlE	Autolysin E
B	Biofilm cells
Brc	Biofilm-released cells
BSA	Bovine serum albumin
CoNS	Coagulase-negative staphylococci
CFU	Colony forming units
CSLM	Confocal scanning laser microscopy
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DAPI	4',6-diamidino-2-phenylindole
RNA	Ribonuclecleic acid
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FL	Filter
FW	Forward
GO	Gene ontology
Ica	Intercellular cluster adhesion
Ig	Immunoglobulin
IL	Interleukin
i.v.	Intravenous
mAbs	Monoclonal antibodies
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NHS	Normal human serum
NRS	Normal rabbit serum
OD	Optical density
P	Planktonic cells

pAbs	Polyclonal antibodies
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline supplemented with 0.05 % Tween20
PGA	Poly-γ- DL-glutamic acid
PI	Propidium iodide
PNAG	Poly-N-acetylgçucosamine
PSM	Phenol soluble modulins
qPCR	Quantitative Polymerase Chain Reaction
RFU	Relative fluorescence units
RNA-seq	RNA sequencing
RPKM	Reads *per* kilobase per million
Rpm	Rotations *per* minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RV	Reverse
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TT	Tetanus toxoid

SCIENTIFIC OUTPUTS

PAPERS IN PEER REVIEWED JOURNALS:

1. **França A**, Vilanova M, Cerca N, Pier G. Monoclonal antibody raised against PNAG present variable effect in *S. epidermidis* biofilm accumulation *in vitro*. International Journal of Biological Sciences. 2013, 9(5): 518-20.
2. **França A**, Freitas AI, Henriques AF, Cerca N. Optimizing a qPCR Gene Expression Quantification Assay for *S. epidermidis* Biofilms: a Comparison between commercial kits and a customized protocol. PloS One. 2012, 7(5):e37480.
3. **França A**, Melo LDR, Cerca N. Comparison of RNA extraction methods from biofilm samples of *Staphylococcus epidermidis.* BMC Research Notes. 2011, 4(1): 572.

ORAL PRESENTATIONS:

1. **França A**, Pérez-Cabezas B, Carvalhais V, Freitas AI, Pier G, Vilanova M, Cerca N . A first new look into the interaction of *Staphylococcus epidermidis* biofilm-released cells with the host immune system in BioMicroWorld 2013 V International Conference on Environmental, Industrial and Applied Microbiology, Madrid, Spain. October 2nd-4th, 2013.
2. **França A**, Freitas AI, Carvalhais V, Cywes-Bentley C, Maira-Litrán T, Vilanova M, Pier G, Cerca N. *Staphylococcus epidermidis* biofilm dispersal cells: an intermediary phenotype? in Biofilms 5, Paris, France. December 10-12th, 2012.

POSTERS:

1. **França A**, Carvalhais V, Maira-LitránT, Vilanova M, Cerca N, Pier G. Whole transcriptome analysis of *Staphylococcus epidermidis* biofilms upon contact with human blood, in Eurobiofilms2013: Third European Congress on Microbial Biofilms, Ghent, Belgium. September 9-12th, 2013.
2. **França A**, Carvalhais V, Vilanova M, Pier G, Cerca N. *Staphylococcus epidermidis* biofilm-detached cells: differential gene expression and antibiotic resistance, in 6th ASM Conference on Biofilms, Miami, Florida, USA, September 28th-October 4th, 2012.

3. **França A**, Vilanova M, Cerca N. High variability of gene expression in *S. epidermidis* biofilm population, in BioMicroWorld2011: IV International Conference on Environmental, Industrial and Applied Microbiology, Torremolinos, Spain. September 14-16th, 2011.
4. **França A**, Bento J, Pier GB, Vilanova M, Cerca N. Comparison of RNA extraction methods from biofilm samples of *Staphylococcus epidermidis* and other bacterial pathogens, in FEMS2011, Geneva, Switzerland. June 26th -30th, 2011.

CHAPTER 1.

General Introduction

I. Biofilms and human disease

Traditionally bacteria have been regarded as individual organisms growing in homogenous planktonic, free-floating cultures [1,2]. In fact, for many centuries humans have suffered from acute bacterial infections caused by planktonic cells of specialized pathogens, which mounted life-threatening attacks [3]. However, in the last two decades it has been recognized that bacteria live preferentially in communities attached to a surface and surrounded by an extra-polymeric matrix mainly composed of polysaccharides, proteins, lipids and nucleic acids called biofilms [3-7]. Indeed, it is estimated that 99.9% of the bacterial communities occur in biofilms rather than in planktonic phenotypes [8], indicating that biofilm formation is an integral component of the prokaryotic lifecycle [9].

In the clinical setting, biofilms are responsible for 65% of the of the infections treated in the developed world [10,11], being implicated in a variety of human diseases such as dental disease, endocarditis, urinary tract infections, cystic fibrosis [12], and in several infections due to indwelling medical devices [13-15]. Biofilm formation on medical devices is characterized by the development of chronic and recalcitrant infections, which are less aggressive than acute infections, but persist for months or even years [10]. The chronicity of these infections are due to their complex 3-dimensional structures as well as their phenotypic heterogeneity [16-18], which promotes high resistance to antibiotic therapy [19,20] and to an attack from the host immune system effectors [21,22]. Hence, infections associated with biofilm formation on indwelling medical devices are hard to treat, often necessitating the removal of the infected device, increasing morbidity and ultimately contributing to increased patient mortality [23].

Staphylococcus epidermidis biofilms and its clinical implications

The genus Staphylococcus consists of non motile, non spore-forming, spherical and Gram-positive cocci, with sizes between 0.5-1.5 µm in diameter. They are facultative anaerobes that grow by aerobic respiration or by fermentation, and tolerate high concentrations of salt [24]. There are approximately 40 different species within the genus Staphylococcus [25]. These species are commonly classified based on their ability to produce the enzyme coagulase. *S. aureus* is the most clinically significant among the coagulase-positive staphylococci, while *S. epidermidis* is the most clinically important species of all the coagulase-negative staphylococci (CoNS), representing up to 90% of the infections caused by CoNS [26,27].

CoNS typically reside on healthy skin and mucous membranes of humans and animals, rarely causing disease, and are most frequently encountered as contaminants of microbiological cultures [28]. However, in the last two decades, they have been increasingly recognized as a leading cause of several clinically relevant infections [29,30], with a particular association with the use of indwelling medical devices [31-33] (see Table 1.1 for more information).

Table 1.1. *S. epidermidis* infections. Table adapted from [29].

Infection/place of infection	% of CoNS
Infective endocarditis	11-17%
Surgical site infections	13.7%
Native valve Infective endocarditis	25%
Cardiac implantable electronic devices	27%
Bacteremia and intravascular catheter infections	30%
Central venous catheters associated bloodstream infections	32.1%
Central line associated bloodstream infections	34.1%
Cebrospinal fluid shunts	37%
Hip and knee replacement	36-77%

This association is related to the strong ability of *S. epidermidis* to colonize the surface of indwelling medical devices and form biofilms [3], as well as to their ubiquity on healthy human skin [34] that greatly increases the risk of infection of the devices that penetrate skin [23,28]. Even though *S. epidermidis* biofilm infections rarely develop into life-threatening diseases, their high frequency and recalcitrant nature have important consequences on the patient's quality of life, and are a significant burden to the public health system since the treatment of these infections often involves the physical removal of the infected devices [23].

To be pathogenic, *S. epidermidis* elaborates several factors that contribute to its virulence, turning it into a successful pathogen in the context of biomaterial-associated infections [29]. The known virulence factors involved in *S. epidermis* pathogenesis are presented in the Table 1.2.

Table 1.2. Virulence factors presented by *S. epidermidis*. Table adapted from [23,35-37]. PNAG-poly-N-acetylglucosamine; PGA-polyglutamic acid; AMP-antimicrobial peptides; CP-complement proteins.

Virulence factor	Gene/Operon	Function
Initial adhesion		
Autolysin adhesion	*aae*	Fibrinogen, fibronectin and vitronectin
Autolysin	*atlE*	Binding to polystyrene and vitronectin
Serine aspartate binding protein	*sdrF*	Binding to collagen
Fibrinogen binding protein (fbe)	*sdrG*	Binding to fibrinogen
Elastin binding protein	*ebp*	Binding to elastin
Extracellular matrix binding protein	*embp*	Fibronectin binding
Teichoic acids (cell wall)	*Multiple genes*	Binds to fibronectin
Biofilm accumulation		
PNAG	*icaADBC*	Bacterial cell-cell interaction
Biofilm-associated protein	*bhp*	Bacterial cell-cell interaction
Accumulation-associated protein	*aap*	Bacterial aggregation after proteolysis
Extracellular matrix binding protein	*embp*	Bacterial aggregation
Immune evasion		
PNAG	*icaADBC*	Protects from AMPs, phagocytes, IgG and CP
PGA	*capABCD*	Protects from AMPs and phagocytes
Extracellular matrix binding protein	*embp*	Protects from phagocytes
Resistance to AMPs		
SepA protease	*sepA*	Involved in AMPs degradation
AMP sensing system	*apsRS*	Senses AMPS and resistance mechanisms
Toxins		
Phenol soluble modulin	*psmδ*	Pro-inflammatory cytolysin
Exoenzymes		
Lipases GehC, GehD	*gehC, gehD*	Persistence on human skin
Serine protease	*sspA*	Degradation of fibrinogen, CP; Tissue damage
Cysteine protease	*sspB*	Tissue damage

S. epidermidis biofilm lifecycle

Due to its clear importance in human health and disease, the *S. epidermidis* biofilm lifecycle has been studied for years, and it is now described as a 3 stage process (Figure 1.1): I) initial adhesion, II) accumulation and maturation, and finally, III) biofilm disassembly [11,23,38].

I. Initial adhesion

The mechanisms and molecules involved in this step are directly dependent on the properties of the bacterial surface. In the case of abiotic surfaces, initial adhesion is mainly mediated by the physicochemical properties of both the bacteria and solid surface, as well as by the non-specific van der Waals, Lewis acid-base and electrostatic forces [39,40]. Nevertheless, there are some surface bacterial molecules that can play an important role at this stage due to their hydrophobic characteristics, such as

the autolysins AtlE [41] and Aae [42], accumulation associated protein (Aap) [43-46] and teichoic acids [47]. Conversely, in the case of biotic surfaces or abiotic surfaces coated with host matrix proteins, the initial adhesion is governed via specific, receptor-mediated interactions [48]. *S. epidermidis* expresses a large variety of surface-anchored proteins, collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [49]. These surface molecules bind specifically to host matrix proteins, allowing the attachment of the bacteria to the surface, and posterior biofilm formation. Since indwelling medical devices are readily coated with host matrix plasma proteins, these MSCRAMMs are fundamental for *S. epidermidis* biofilm formation on the surface of indwelling medical devices [41] and thus, *S. epidermidis* virulence.

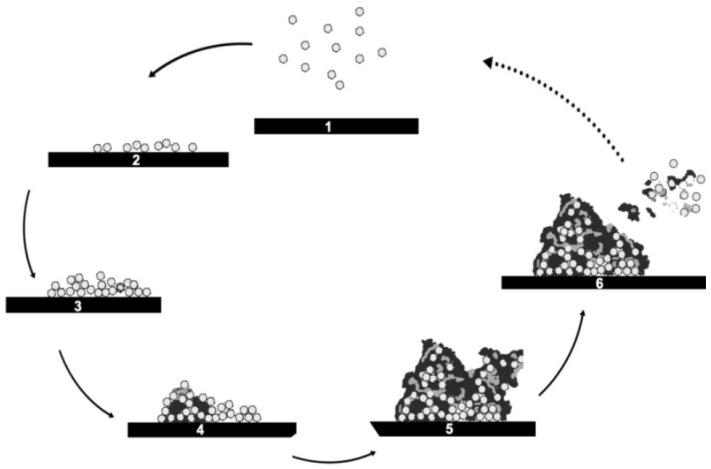

Figure 1.1. Schematic model of *S. epidermidis* biofilm lifecycle stages. Planktonic bacteria (1) will first adhere to a surface (2) followed by growth in clusters (3). With bacterial growth adhered to the surface, the matrix starts to be formed (4). Over time the biofilm becomes thicker and the matrix more prominent (5). Bacteria will then detach from the biofilm and will colonize other surfaces (6). Adapted from [50].

II. Maturation

After adhesion, bacteria start to divide and accumulate as multilayered cells in a process dependent on the synthesis of several extracellular and adhesive molecules, such as polysaccharides, proteins, lipids and nucleic acids, that will provide the framework into which bacterial cells are encased [4,51,52]. The production of the extracellular matrix is considered the hallmark of biofilm formation [3]. In this stage, the complex and typical tri-dimensionality of biofilms is achieved through a strong equilibrium between

both adhesive and disruptive forces that will allow the creation of channels for nutrients and waste circulation [53,54]. In order to form a cohesive structure, growing cells start to produce adhesive molecules that promote cell-cell inter-adhesion [23,38]. Unquestionably, poly-N-acetylglucosamine (PNAG), an exopolysaccharide, is the most important adhesive molecule for *S. epidermidis* biofilm formation [55], and for this reason it is also known as polysaccharide intercellular adhesin (PIA) [56]. This polysaccharide is synthesized by proteins encoded in the *icaADBC* operon, where IcaA and IcaD proteins produce a chain from activated N-acetylglucosamine monomers followed by its elongation and exportation to the cell surface, mediated by the IcaC protein [57]. Finally, N-acetylglucosamine monomers are partially de-acetylated by the cell-surface enzyme IcaB [58,59]. The acetylation process removes some of the N-acetyl groups from the polymer which confers the cationic character, that is essential to the attachment of the polymer to the bacterial surface [59]. Nevertheless, PNAG is not the only molecule involved in biofilm formation as several clinical isolates that lack the *ica* operon are able to form biofilms [60-62]. Recent studies have shown the involvement of surface proteins such as biofilm associated protein (Bap) [43,63,64], Aap [44] and extracellular matrix biofilm protein (Embp) [35] in biofilm formation. Additionally, teichoic acids, a characteristic component of the surface of Gram-positive bacteria [65], as well as DNA resultant from lysed bacteria [66], have also been recognized as important adhesive molecules in *S. epidermidis* biofilms maturation due to their negative charge that allows the interaction with other surface molecules. Despite the adhesive molecules involved in this stage, disruptive forces are also needed in order to create viable biofilms which require the formation of channels through which nutrients can penetrate into deeper biofilm layers [67]. These factors can, ultimately, lead to the dispersion of cells in to the involving environment regulating, therefore, the thickness and the expansion of the biofilm [53].

III. Disassembly

Biofilm disassembly, or also termed dispersion, is the last stage of the biofilm lifecycle. Even though the benefits provided to bacteria through the biofilm mode of growth are evident, under some particular conditions, the biofilm lifestyle may no longer be advantageous, and therefore, in such cases, it is essential for some cells to leave the biofilm and assume the planktonic lifestyle [68]. Biofilm disassembly is believed to be a combination of complex, multi-factorial and highly regulated processes, that can be triggered by several external and/ or by bacterial-derived signals [69] (see Table 1.3). Nutrient availability, oxygen depletion, low levels of nitric oxide, changes in temperature, high or low levels of

iron, accumulation of wastes, the appearance of antimicrobial compounds or other threats are categorized as environmental cues [69]. On the other hand, acyl homoserine lactones, cell-cell autoinducing peptides (*agr* quorum sensing system), intracellular second messenger cyclic di-GMP, diffusible fatty acids and D-amino acids are signals produced by the bacteria themselves [54]. However, biofilm disassembly may also occur due to shear forces, abrasion or predator grazing [54], which are considered passive mechanisms, know as detachment. Under favorable conditions biofilms seem to continually release small amount of cells, however, it is thought that after large periods of growth they may undergo major disassembly events [70].

Table 1.3. **Biofilm maturation and disassembly determinants in Staphylococci**. Table adapted from [38,68].

Effectors	Mechanism/ Function	Species
PSMs		
PSMα-type	Detergent like structure; disruption of the non covalent	*S. aureus*
PSMβ-type	Detergent like structure; disruption of the non covalent	*S. aureus* and *S. epidermidis*
δ – toxin	Detergent like structure; disruption of the non covalent	*S. aureus*
Proteases		
Dispersin B	Degradation of the polysaccharides matrix	*S. epidermidis*
Lysostaphin	Degradation of the polysaccharides matrix	*S. aureus* and *S. epidermidis*
Protease K	Protease K, trypsin, Esp, V8	*S. aureus* and *S. epidermidis*
Nucleases		
DNase I	Degradation of extracellular DNA	*S. aureus* and *S. epidermidis*
Nuc1	Digestion of DNA-based biofilm matrix	*S. aureus*
Nuc2	Digestion of DNA-based biofilm matrix	*S. aureus*
Regulators		
CidA	Control of autolytic activity, eDNA release	*S. aureus*
LrgAB	Inhibitor of CidA-mediated lysis	*S. aureus* and *S. epidermidis*
SarA	Control of proteases	*S. aureus* and *S. epidermidis*
Agr	Control of PSMs and proteases	*S. aureus* and *S. epidermidis*
Others		
pH	Reactivation/ inactivation of *agr* regulatory system	*S. aureus* and *S. epidermidis*

Despite all the signals that can trigger biofilm dispersion, bacterial cells within the biofilm are enclosed in the matrix that needs to be dissolved by effector molecules to allow the release of the cells into the surrounding environment [71]. These effector molecules include surfactants such as phenol soluble modulins (PSM) [71], polysaccharide degrading enzymes [72,73], proteases [71,74,75], nucleases [76,77] and also bacteriophages [4,78] (for more detail see Table 1.3). The release of the cells from the biofilm into the involving environment has been implicated in the development of several serious infections such as bacteremia [79], embolic events of endocarditis [80] and pneumonia [81] contributing, therefore, to the pathogenicity of *S. epidermidis* biofilms infections.

II. *S. epidermidis* biofilms interaction with the host immune system

The infections caused by bacteria upon entering the human body can be divided into acute and chronic. This classification usually reflects differences in the lifestyle of the bacteria that are causing the infection [82]. Frequently, acute infections involve planktonic bacteria, which cause severe clinical symptoms but that can, generally, be prevented or treated efficiently with the use of vaccines, antibiotics and infection control measures [83,84]. On the other hand, chronic infections are typically developed in the presence of bacterial biofilms [82,83] and are characterized by slow progression and low grade symptoms [83]. These infections are very difficult, if not impossible to treat with current therapies, because bacteria within biofilms have shown incredible tolerance to antibiotics [85] and to the potent host innate and adaptive immune effectors [86-88]. Although the nature of the antibiotic resistance have been explored for years, much less is known about the mechanisms employed by *S. epidermidis* biofilms to evade the host immune system.

Immune system: an overview

The function of the immune system is to protect the host against microbial infections. The development of an infectious disease involves complex interactions between the microbe and the host. The key events during infection development include: 1) entry of the microbe, 2) invasion and colonization of the host tissues, and 3) tissue injury [89]. In order to control the key events of infection, the host immune system present two types of immune responses: innate and adaptive. The major difference between these 2 types of responses is that the adaptive immune response is highly specific for the pathogen and improves with each encounter with the same antigen, while innate immunity consists of mechanism that exist before infection, and do not improve even after a repeated encounter with the same antigen [90] (see Table 1.4 for more detail). Therefore, the defense against microbes is mediated by the early reactions of the innate immune system and the later responses of adaptive immunity.

Innate immunity

The principal components of the innate immunity are the physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces, phagocytic cells (neutrophils and macrophages), natural killer cells, blood proteins, including members of the complement system and other mediators of inflammation, such as cytokines [90]. Each of these cells and proteins play a different

role in the response to microbes. In brief, neutrophils, mononuclear phagocytes and natural killer cells attack microbes that have breached the epithelial barriers and entered into tissues or the circulation. Macrophages and natural killer cells, secrete cytokines, which in turn activate phagocytes and stimulate the cellular reaction of the innate immunity, the inflammation. If microbes enter the circulation, they are attacked by various plasma proteins, the complement system being the major circulating protein cascade of the innate immunity [92].

Table 1.4. Principal distinctive features between innate and adaptive immunity together with their specific components. Table adapted from [91]. NK-natural killer; AMPs-antimicrobial peptides.

	Innate	**Adaptive**
Features		
Specificity	Pathogen-associated molecular patters	For antigens of microbes
Diversity	Limited	Very large
Memory	None	Yes
Response time	Immediately	Days
Components		
Physical /chemical barriers	Skin, mucosal epithelia AMPs	Lymphocytes and antibodies at epithelial surfaces
Blood proteins	Complement	Antibodies
Cells	Macrophages, neutrophils, NK cells	Lymphocytes B and T

Adaptive immunity

Innate and adaptive immunity are in constant interaction where the cells and factors of each component work cooperatively. The innate immune reactions against microbes stimulates adaptive immune responses and influences the nature of the adaptive responses. On the other hand, adaptive immune responses use many of the innate effectors to eliminate microbes [91]. Adaptive immune responses are divided into 2 types: humoral and cell-mediated immunity, which are facilitated by different components of the immune system and function to eliminate different types of microbes [91] Humoral immunity is mediated by antibodies produced by B lymphocytes activated by specific antigen exposure and maturation into plasma cells. Antibodies bind proteins and polysaccharides from the bacterial cell wall and also bacterial toxins [90]. Antibodies also function as molecular tags since they mark antigens for destruction; bacteria covered with antibodies are readily ingested by phagocytes or destroyed by complement proteins present in the blood [91]. However, antibodies are not effective against pathogens that are present inside the cells, hence in these particular cases cell-mediated immunity takes place, which is mainly governed by T lymphocytes [90].

S. epidermidis immune evasion

Contrary to what was previously thought, biofilms can actually engage the host immune system as well as their planktonic counterparts do [83]. However, the efficiency of the immune response in eliminating infecting microbial cells is impaired in biofilms [3]. One of the first hypothesis to explain the impairment of the immune effectors in biofilms was the physical barrier posed by the matrix [93]. However it was shown that leukocytes were able not only to penetrate the biofilm structure but also phagocytose the bacteria within the biofilms [22,94]. Therefore, several studies were performed in order to unravel the mechanisms and/or molecules involved in biofilm evasion. Besides its clear importance in biofilm accumulation, PNAG is also involved in *S. epidermidis* biofilm immune evasion [95-97]. Due to its positive charge [57], PNAG protects biofilm cells against the action of the cationic AMPs through electrostatic repulsions [58,98]. Interestingly, it also protects against anionic AMP by sequestering these molecules in a similar way that alginate protects against tobramycin in *Pseudomonas aeruginosa* [99]. In addition, it was shown that PNAG-producing *S. epidermidis* strains are more resistant to phagocytosis by neutrophils [58,97] and macrophages [100], than isogenic *ica*-negative mutants, probably due to the ability of PNAG to decrease the deposition of both antibodies and components of the complement system proteins onto the bacterial surface [96]. In addition, PNAG purified from *S. aureus* was shown to induce the production of antibodies to PNAG [101-103], which were able to confer protection [104-106] and to diffuse efficiently into deeper layers of *S. epidermidis* biofilms [86].

Another protective exopolymer involved in immune evasion is the extracellular anionic polymer poly-γ-DL-glutamic acid (PGA). This molecule that was first described in *Bacillus anthracis* [107] and is encoded by the *cap* locus that was found to be more expressed when cells are in the biofilm phenotype [108]. Similar to PNAG, PGA has an important role in the protection of *S. epidermidis* against the innate host defense. It was shown that PGA decreased the efficacy of phagocytic process as well as AMPs action, contributing therefore, for *S. epidermidis* biofilms pathogenicity [109]. In addition to these expolymers, an antimicrobial peptide sensing system (aps) was found in *S. epidermidis* which is crucial for *S. epidermidis* immune evasion [110]. The aps system senses the presence of AMPs produced by the host and up-regulates AMP-defensive mechanisms such as d-alanylation of teichoic acids [111,112] and the lysinylation of phospholipids by the MprF enzyme [113], which will decrease the anionic charge of the bacterial surface and prevent the binding of the cationic AMPs [110]. Furthermore, the VraF and VraG proteins possibly function as an exporter that removes AMPs from the bacterial cytoplasmic membrane [114]. In addition to the mechanisms controlled by the aps system, the production of proteases such as SepA also play an important role in degradation of the AMPs that reside inside neutrophils that are used

to kill bacteria upon phagocytosis [115]. Thus, SepA is important for *S. epidermidis* immune evasion [87]. Recently it was shown that *S. epidermidis* produces the PSMδ that displays potent cytolytic activity against neutrophils being, therefore, one more mechanism to subvert the host immune system [87].

The role of the specific, adaptive immune response to *S. epidermidis* infection is less well understood and the knowledge very scarce. The fact that our immune system has difficulties clearing long lasting *S. epidermidis* infections, despite the production of antibodies against *S. epidermidis* proteins, indicates that the adaptive host defense system might not be efficient against *S. epidermidis* [23]. However more studies are needed to better understand the nature of *S. epidermidis* resilience to the potent host immune responses.

III. AIMS AND OBJECTIVES

Due to the debilitating nature and economic impact of *S. epidermidis* biofilm infections, preventing or treating these infections is of paramount significance in modern medicine. Hence, it is essential that we understand the mechanisms whereby *S. epidermidis* evades the host immune system. Therefore, this thesis has as its primary objectives:

I. The study of the interaction between *S. epidermidis* biofilms and human blood-circulating immune cells and soluble factors;
II. The characterization of the phenotypic traits and interaction of the *S. epidermidis* biofilm-released cells with the host immune system using *in vitro* and *in vivo* models.

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CHAPTER 2.

Optimizing a qPCR gene expression quantification assay for *S. epidermidis* biofilms

ABSTRACT

Biofilm gene expression analysis by quantitative PCR has been increasingly used to understand the role of biofilm formation in the pathogenesis of *S. epidermidis* infections. However, depending on the RNA extraction procedure, cDNA synthesis kit and qPCR master mixes used, gene expression quantification can be suboptimal. Due to biofilm composition, in particular the presence of the extracellular matrix, some RNA extraction kits are not suitable for *S. epidermidis* biofilms. In this chapter, we describe a custom RNA extraction assay followed by the evaluation of gene expression using different commercial reverse transcriptase kits and qPCR master mixes. Our custom RNA extraction assay was able to produce good quality RNA with reproducible gene expression quantification, reducing the time and the costs associated. We also tested the effect of reducing cDNA and qPCR reaction volumes and, in most of the cases tested, no significant differences were found. Finally, we titered SYBR Green I concentrations in standard PCR master mixes and compared the normalized expression of the genes *icaA, bhp, aap*, *psm*β and *agrB* using 4 distinct biofilm forming *S. epidermidis* strains to the results obtained with commercially available kits. The overall results demonstrated that despite some statistically differences detected, the customized qPCR protocol resulted in the same gene expression trend presented by the high standards commercially available kits used.

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BACKGROUND

The study of gene expression is of one of the most important tools used to unravel the biological processes occurring in an organism under a particular condition [1]. Gene expression has proved to be a useful tool to the validation, for instance, of the transcriptional measurements associated with the shift of *S. epidermidis* to the mode of infection [2-4]. Advances in molecular biology and bioinstrumentation have led to the development of several new techniques with a range of sensitivities, throughputs and quantitative capabilities [1] such quantitative PCR (qPCR), microarrays and RNA sequencing analysis, that allows to analyze gene expression. Although qPCR is used for the analysis of a restricted number of genes, is still widely used due to the easiness, versatility and availability of the systems used for such analysis. Additionally, due to its high sensitivity, qPCR is the technique of choice for microarrays or RNA sequencing results validation [5,6]. Nonetheless, the success of the existing or any emergent RNA-based analysis relies on the quality of the messenger (m) RNA, since its purity and integrity can impact the accuracy of subsequent analytic techniques [7,8]. Currently, there are several commercially available kits for RNA extraction, however, most of these kits were not tested in biofilm cultures, and depending on the principle and properties applied by each kit, the quantification of mRNA transcripts can be impaired [9,10]. Additionally, it has been shown that different cDNA synthesis kits and gene expression quantification detection systems for qPCR can also be a source of variation that can largely impair gene expression quantification [11]. Hence, in this chapter, we describe the comparison of different commercially available kits and, simultaneously, the development of a customized protocol for gene expression quantification for qPCR, using *S. epidermidis* biofilm as samples. The custom protocol was optimized to maximize reliability of results, reduce time, and minimize the costs involved.

MATERIALS AND METHODS

Bacterial strains and growth conditions

For this study the following *S. epidermidis* biofilm-forming strains were used: 9142, LE7, IE186 and M129 [12]. Biofilms were formed as previously optimized [13]. In brief, a single colony of each *S. epidermidis* strains used was inoculated into Tryptic Soy Broth (TSB) (Oxoid, Cambridge, UK) and incubated at 37 °C and 120 rpm overnight. Afterwards, 1:100 dilution of the overnight culture was

performed in 1 mL of fresh TSB supplemented with 1% (w/v) of glucose (Fisher Scientific, MA, USA) to induce biofilm formation, and placed into 24-well plate (Orange Scientific, Braine-l'Alleud, Belgium). The plates were then incubated at 37^{o}C and 120 rpm for 24 ± 2 hours. Prior to any analysis, biofilm supernatants were removed, biofilms washed once and suspended in 1 mL of 0.9% NaCl. Planktonic bacteria were grown in 2 mL TSB in 15 mL tubes at 37 °C and 120 rpm for 18 ± 1 hours.

RNA extraction with commercially available kits

For RNA extraction we have selected two commercially available kits, previously tested by our research group [9], which applies distinct principles: 1) Fast RNA® Pro Blue (MP Biomedicals, CA, USA), that uses mechanical and chemical lyses along with organic purification, and 2) PureLink™ RNA Mini Kit (Invitrogen, CA, USA), which employs enzymatic lyses together with silica-membrane based purification. The procedures were performed as recommended by the manufacturers with a particular improvement: the enzymatic lyses was performed using 15 mg/mL of lysozyme (Sigma, MO, USA) for 60 min at 37 ^{o}C. This optimization increased the yield of total RNA for the double.

Customized RNA extraction protocol

The custom protocol described here was devised based on the efficacy of the mechanical and chemical lyses together (glass beads combined with phenol), and the easiness and quickness of the silica-membrane purification (ISOLATE RNA Mini Kit columns system). In brief, bacterial pellets were suspended in 100 µL RNase-free water and transferred into a 2 mL safe lock tube containing 0.4 g of acid-washed 150-212 mm silica beads (Sigma), 400 µL lyses buffer R (provided by the kit) and 500 µL 90% phenol solution (AppliChem, Darmstadt, Germany). This mixture was vortexed for 20 seconds. Thereafter, bacterial cell lyses was performed using a FastPrep® cell disruptor (BIO 101, Thermo Electron Corporation, Thermo Scientific, MA, USA) at a 6.5 meter/second during 35 seconds. The samples were then cooled on ice for 5 minutes and the lyses cycle repeated twice. Afterwards, samples were centrifuged at 16000 *g* for 5 minutes, and supernatants transferred into a new tube and mixed with equal volume of 100% ethanol (Fisher Scientific). From here all steps were done at room temperature except where otherwise noticed. The samples (including any remaining precipitate) were transferred into the ISOLATE RNA Mini kit column system (Bioline, London, UK) and centrifuged at 12000 *g* for 15 seconds. The flow-through was discarded and each column washed

with 700 µL of wash buffer I and centrifuged at 12000 *g* for 15 seconds. The flow-through was discarded and the column inserted into the same collection tube. Thereafter, 500 µL of wash buffer II was added to each column and centrifuged at 12000 *g* for 15 seconds. The flow-through was discarded and the columns reinsert into a new collection tube for a new centrifugation at 12000 *g* for 2 minutes in order to remove any trace of ethanol. The collection tube was discarded and each column was inserted into a recovery tube. Finally, RNA was eluted by adding 45 µL of RNase-free water to the center of the membrane, incubated for 1 minute and centrifuged for 1 minute at 12000 *g*.

DNase treatment

In order to degrade any possible genomic DNA co-purified with total RNA, the samples were treated with DNase I kit (Fermentas, Vilnius, Lithuania). Briefly, 2 µL of DNase I and 5 µL of reaction buffer were added to the RNA samples and incubated at 37 ^{o}C for 30 minutes. Subsequently, 5 µL of 25 mM EDTA was added, and the DNase I enzyme inactivated by heating the samples at 65 ^{o}C during 10 minutes.

RNA quality determination

The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop 1000^{TM} (Thermo Scientific). The absorbance ratio $A_{260}/_{A280}$ was used as an indicator of protein contamination, and A_{260}/A_{230} as an indicator of polysaccharide, phenol, and/or chaotropic salts contamination [14]. The integrity of the total RNA was assessed by visualization of the 23S/16S rRNA banding pattern. Electrophoresis was carried out at 80 Volts for 60 minutes using a 1.5% agarose gel. The gel was stained with ethidium bromide (Fisher Scientific) and visualized using a GelDoc2000 (Bio-Rad, Hercules, CA, USA). RNA was stored at -80 ^{o}C until further use.

Complementary DNA synthesis

Complementary DNA (cDNA) synthesis was performed using 4 different available commercial kits: 1) Super Script® VILO^{TM} (Invitrogen), 2) RevertAaid^{TM} First Strand cDNA Synthesis kit (Fermentas), 3) iScript^{TM} cDNA synthesis (Bio-Rad) and 4) qScript^{TM} cDNA Synthesis (Quanta BioSciences, MD, USA),

following the manufacturer's instructions. The same amount of total RNA (500 ng/20 µL) was reverse transcribed using two reaction volumes: 20 µL, as described by the manufacturer, or 10 µL. Genomic DNA carry-over was determined by performing a control that lacks the reverse transcriptase enzyme (no-RT control). All RNA extracted was absent of significant genomic DNA, as determined by an average cycle threshold difference of 18.5 ± 3.5, equivalent to a maximum quantification error of 0.0003%.

Gene expression quantification by qPCR

Oligonucleotide primers for the detection of 16S rRNA, *icaA*, *aap, bhp, agrB and psm*β were designed using the Primer3 software [15] having, respectively, either *S. epidermidis* RP62A (PubMed accession number NC_002976.3) or ATCC12228 (PubMed accession number NC_004461.1) genome as template (Table 2.1).

Table 2.1. Oligonucleotide primer sequences used for qPCR analysis. FW-forward; RV-reverse; bp-base pair

Target gene		Oligonucleotide primers sequence (5' to 3')	TM (°C)	Amplicon size (bp)
16S	FW	GGGCTACACACGTGCTACAA	59.79	176
	RV	GTACAAGACCCGGGAACGTA	59.85	
icaA	FW	TGCACTCAATGAGGGAATCA	60.20	134
	RV	TAACTGCGCCTAATTTTGGATT	59.99	
aap	FW	GCACCAGCTGTTGTTGTACC	59.22	190
	RV	GCATGCCTGCTGATAGTTCA	59.98	
bhp	FW	TGGACTCGTAGCTTCGTCCT	60.01	213
	RV	TCTGCAGATACCCAGACAACC	60.13	
agrB	FW	AATTCGTTTAGGGATGCAGGT	59.85	142
	RV	ACCGTGTGCATGTCTCCTAAT	59.49	
***psm*β**	FW	AGCAGAAGCTATTGCAAATACAG	57.96	105
	RV	CCTAATACGCTAACGCCACTTT	59.72	

qPCR analysis was performed using 4 different commercial qPCR mixes: 1) mi-real-time EvaGreen® Master (Metabion, Martinsried, Germany), 2) Maxima® SYBR Green Master Mix (Fermentas), 3) iQ™ SYBR® Green Supermix (Bio-Rad) and 4) PerfeCTa® SYBR® Green SuperMix (Quanta BioSciences), and also by using 3 standard PCR kits based on Taq polymerase, that were mixed with concentrated SYBR Green I (Invitrogen) for transcripts detection: 1) DyNAzyme™ II PCR Master Mix (Finnzymes, Vantaa, Finland), 2) MyTaq PCR mix (Bioline, London, UK) or 3) EzWay Direct Taq PCR MasterMix (Koma

Biotech, Seoul, South Korea). Different concentrations of SYBR Green I, ranging from 3.2x to 0.1x, were used. Two qPCR reaction volumes were also tested: 20 and 10 µL. The 20 µL reactions contained 2 µL diluted cDNA or no-RT control, 10 pmol of each primer, 6 µL nuclease free H_2O, and 10 µL of the respective 2x master mix. The 10 µL reactions contained half the respective volumes. Primer efficiencies were determined by the dilution method as well as performing a temperature gradient reaction from 50 to 65^{o}C [9]. At 60^{o}C, both set of primers had the best and more similar efficiencies values. qPCR run was performed on a CFX^{TM} 96 (Bio-Rad) with the following cycle parameter: 95^{o}C for 30 seconds, 39 cycles of 95^{o}C for 5 seconds, 60^{o}C for 15 seconds and 68^{o}C for 15 seconds. qPCR products were analyzed by melting curves for unspecific products or primer dimer formation. Relative fold increase of specific mRNA transcripts in biofilms comparing with planktonic cultures, was calculated using $2^{\Delta Ct}$ method, a variation of the Livak method, where 2 stands for the 100% reaction efficiency (the reaction efficiency was determined experimentally and thus 100% efficiency was replace by the real efficiency) and ΔCt = Ct (housekeeping gene)-Ct (target gene). The data analysis was based on at least 3 independent experiments.

Statistical analysis

All the assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and Tukey's multiple comparisons test, and also the paired sample t-test, using SPSS. Student's t-test was applied to all experimental data for rejection of some experimental values. All tests were performed with a confidence level of 95%.

RESULTS

RNA extraction and quality assessment

Two commercially available RNA extraction kits with distinct principles were selected: FastRNA® Pro Blue, which uses mechanical and chemical lyses together with organic extraction, and PureLink^{TM} RNA Mini Kit, which uses enzymatic lyses and silica-based membrane extraction. We then combined the best features of both kits, namely the high yield resulting from the glass beads- and phenol-based lyses and the fast isolation protocol provided by the columns system [9]. For the custom extraction, we tested 4 different column-based isolation kits. As illustrated by the results in Table 2.2, the

PureLink™ kit yielded very low concentration of RNA. However, when PureLink™ column system was combined with the custom lyses, we were able to recover 26-fold more RNA, obtaining very similar values as that obtained when using the Fast RNA® Pro Blue kit.

Table 2.2. Comparison of RNA yield and purity obtained by the different RNA extraction procedures used. Twenty-four hours biofilms were disrupted and RNA extraction performed using commercially available kits or an optimized custom procedure. The values represent the mean plus or minus the standard deviation of 3 independent experiments. Statistical differences between custom and commercial kits were analyzed with paired t-test. ***p*<0.01

RNA extraction kit	RNA yield (ng/µL)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio
FastRNA®	499±74	2.2±0.0	2.1±0.1**
PureLink™	17±3**	2.0±0.1	1.4±0.2**
Custom RNA w/ PureLink™	453±49	2.0±0.1	1.4±0.6**
Custom RNA w/ FavorPrep™	226±31**	1.8±0.1**	1.8±0.2**
Custom RNA w/Direct-zol™	182±5**	2.1±0.1	2.2±0,2**
Custom RNA w/ISOLATE RNA mini kit	422±84	1.9±0.1	1.6±0.1**

While all RNA extraction procedures resulted in acceptable low levels of protein contamination (A_{260}/A_{280}>1.8), some of the kits presented an A_{260}/A_{230} below 1.8. Total RNA integrity was assessed by visualization of the 23S/16S rRNA banding pattern. As can be seen in the Figure 2.1, the RNA extracted using the different procedures was intact since no smear was detected. No integrity information was assessed for the RNA extracted with PureLink™ RNA Mini Kit, as the low yield was below the limit of detection of our image system.

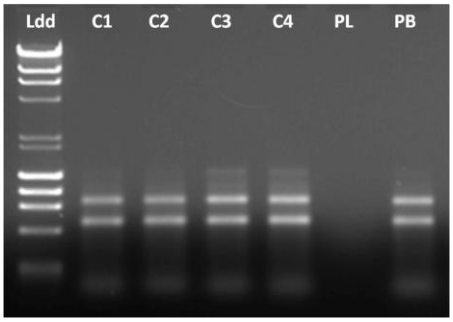

Figure 2.1. RNA integrity determined by visualization in ethidium bromide stained agarose gel. Ldd-DNA ladder (23 Kbp), C1-Custom with PureLink™ Mini Kit; C2-Custom with FavorPrep™ Blood/cultured cell total RNA; C3-Custom with Direct-zol™ RNA MiniPrep; C4-Custom with ISOLATE RNA Mini kit; PL-PureLink™; PB-FastRNA® Pro Blue.

cDNA kits and qPCR master mixes influence in gene expression quantification

In qPCR a common and important optimization step is the determination of the optimal cDNA dilution that should be used in order to obtain reproducible and meaningful results. Undiluted cDNA can interfere with the PCR reaction and thus, several cDNA dilutions were tested by determining the *icaA* gene fold increase in biofilms samples (Figure 2.2).

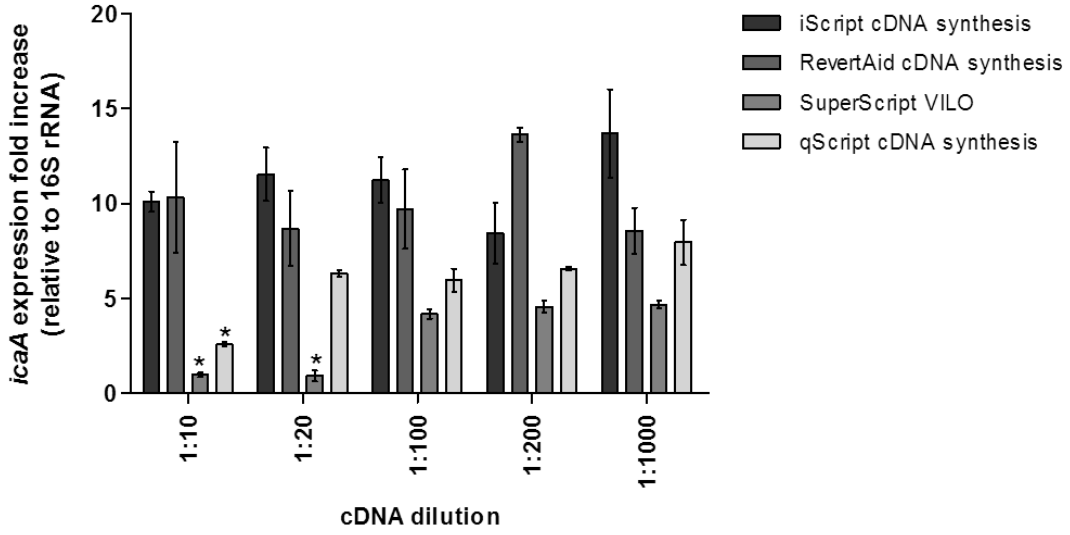

Figure 2.2. Effect of cDNA dilution in *icaA* gene expression quantification. cDNA synthesized from biofilms and planktonic cultures of *S. epidermidis* strain 9142 was diluted from 1:10 to 1:1000 fold and *icaA* transcripts quantified by qPCR. cDNA replicates were synthesized using the same RNA sample but independent cDNA synthesis reactions. The values represent the mean plus or minus standard error of the mean of 3 independent experiments. Statistical differences were analyzed with one-way ANOVA and Tukey's multiple comparison test. *p<0.05.

The lowest dilution common to all the 4 tested kits that shown reliable results, as assessed by no significant variation between the tested cDNA concentrations, was the 1:100 dilution. Therefore, for all the further studies cDNA was diluted 100-fold. As different RNA extraction kits resulted in variable RNA quality, we also sought to determine whether the cDNA synthesis and qPCR kits would have similar variability. Therefore, several cDNA kits and qPCR master mixes commercially available were tested. Using the cDNA synthesized by different kits, *icaA* gene expression was quantified using different master mixes. Interestingly, as illustrated in Figure 2.3, significant differences were found in *icaA* quantification when varying the cDNA kit (p<0.05, ANOVA), but not when varying the qPCR master mix (p>0.05, ANOVA) demonstrating that, besides RNA extraction procedure, the selection of the cDNA synthesis kit will also impact gene expression quantification. Despite the consistent *icaA* gene expression determination with different qPCR kits, the PCR efficiency varied somewhat between 84 ± 4% for iQ™ SYBR® Green, 84 ± 7% for Maxima ® SYBR Green 78 ± 5% for PerfeCTa® SYBR® Green and 87 ± 6% for mi-real time EvaGreen® master mixes. The efficiency of PerfeCTa® SYBR® Green was significantly different from the efficiency of mi-real-time EvaGreen® Master mix (p<0.05, ANOVA), however no important consequences on gene expression quantification were observed.

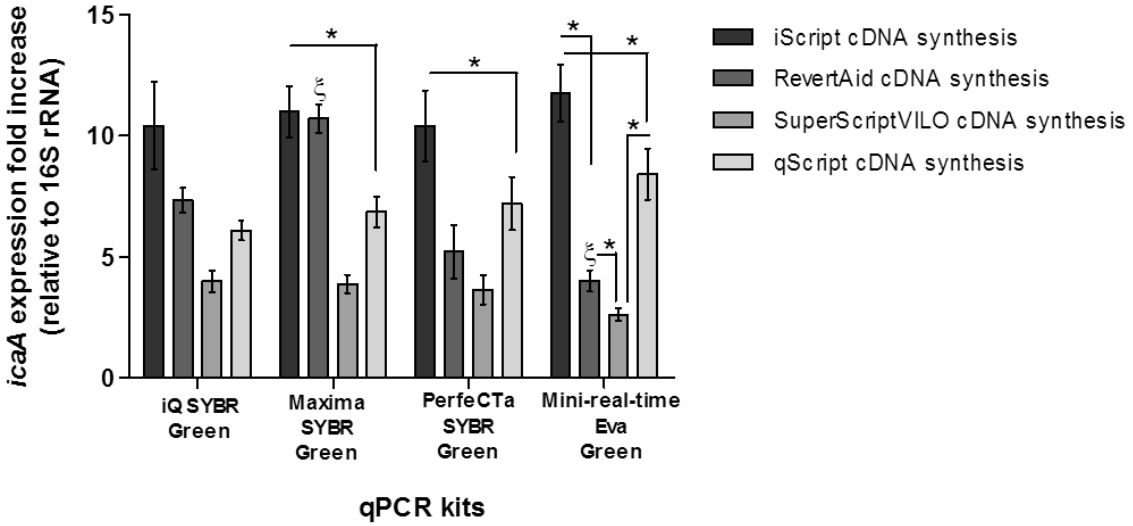

Figure 2.3. The impact of different cDNA and qPCR commercial kits in *icaA* gene expression quantification. cDNA (1:100) from biofilms and planktonic cultures of *S. epidermidis* strain 9142 was synthesized using different kits. The impact of different qPCR master mixes in *icaA* quantification was also tested. The values represent the mean plus or minus standard error of the mean of 3 independent experiments. Statistical differences between cDNA kits (*) or qPCR master mixes (ξ) were analyzed with ANOVA and Tukey's multiple comparison test. */ξ p<0.05.

Reduction of the reverse transcriptase and qPCR volume reaction are among the possible ways to reduce costs associated with gene expression analysis. To determine if a lower volume of reaction could still provide consistent and reproducible results, reverse transcriptase reactions were performed using either 10 or 20 µL of volume, and quantified with 20 µL volume reaction of Maxima® SYBR Green Master Mix. Simultaneously, cDNA obtained from a 20 µL reaction with RevertAid™ First strand cDNA synthesis kit was quantified using either 10 or 20 µL of qPCR reaction volume. As shown in Figure 2.4, the variation of qPCR volume did not affect the quantification of *icaA* gene expression (p>0.05, paired sample t-test). The same was not true for the reverse transcriptase reactions, since significant variation was found, particularly, in the cDNA obtained using SuperScript® VILO™ cDNA synthesis kit (Figure 2.5).

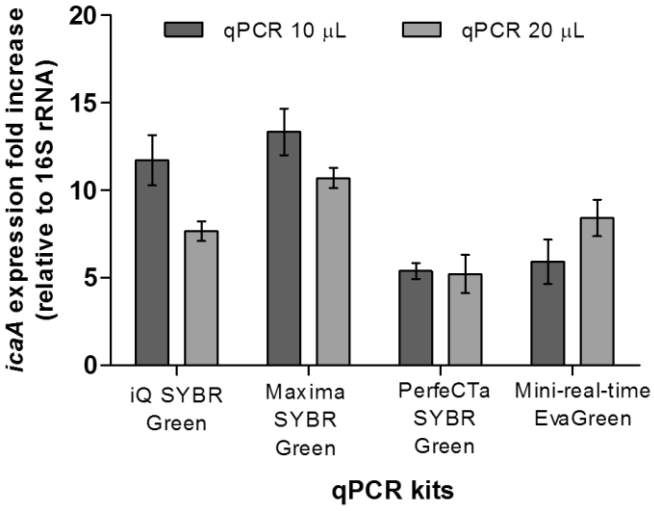

Figure 2.4. Variation in *icaA* gene expression quantification using different qPCR reaction volumes. cDNA from biofilms and planktonic cultures synthesized using RevertAid™ First Strand cDNA synthesis kit (20 µL reaction) was used for *icaA* transcripts quantification by different qPCR master mixes and using different reaction volumes. The values represent the mean plus or minus standard error of the mean of 3 independent experiments. Statistical differences between 10 µL and 20 µL reactions were analyzed with paired t-test.

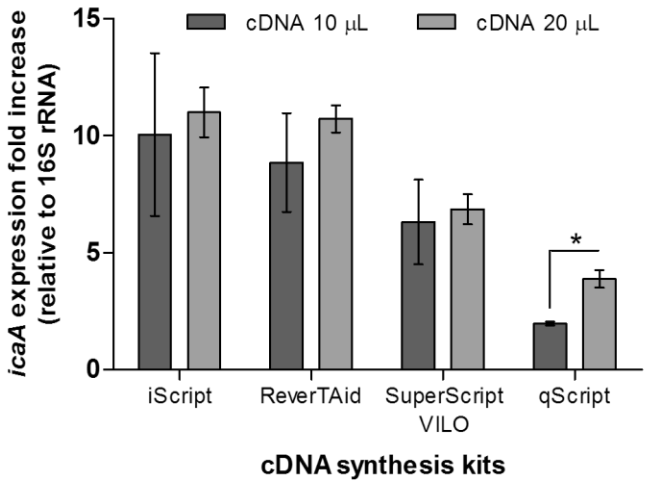

Figure 2.5. Variation in *icaA* gene expression quantification using different cDNA. cDNA from biofilms and planktonic cultures synthesized using 20 µL or 10 µL reaction volumes, was used for *icaA* transcripts quantification. The transcripts were detected using Maxima® SYBR Green Master Mix. The values represent the mean plus or minus standard error of the mean of 3 independent experiments. Statistical differences between 10 µL and 20 µL reactions were analyzed with paired t-test. *p<0.05.

Optimization and validation of a custom qPCR reaction for *S. epidermidis* biofilm gene expression

Another way to reduce costs associated with gene expression analysis by qPCR is to prepare a custom SYBR Green qPCR mix. This can be achieved by using a common PCR mix (or the individual components of the mix, namely Taq polymerase + dNTPs + buffers) and adding the fluorescent dye. This approach requires several optimization steps, since SYBR Green I concentration can interfere with the PCR reaction [16,17]. Using a 10000× solution of SYBR Green I (Invitrogen, NY, USA) different PCR mixes were titrated, ranging in final concentrations of SYBR Green I from 4× to 0.5×. As expected, SYBR Green I concentration strongly influenced the relative fluorescence units (RFU) detected in each reaction (Figure 2.6).

Generally, the higher the concentration, the higher the RFU detected. However, in the custom mixes based on DyNAzyme™ II PCR Master Mix and MyTaq™ PCR, SYBR Green I concentrated 4 × resulted in no RFU detection. To determine if this absence of RFU was result of any signal interference with the fluorescence detector or a PCR reaction inhibition, the products of the qPCR were run on a 1.5% agarose gel (Figure 2.7).

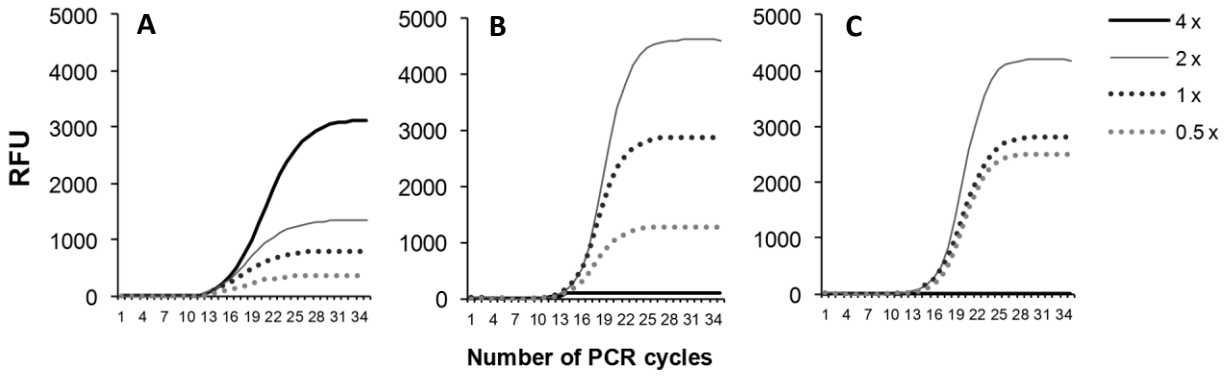

Figure 2.6. SYBR Green I dilution influence in qPCR assay using different Taq polymerase PCR kits. The tested SYBR Green I concentrations ranged from 0.5 to 4× using the following commercially available PCR kits: Ezyway Direct PCR Mix (A), MyTaq PCR mix (B) and DyNAzyme™ II PCR Master Mix (C). The data presented are representative of two independent experiments.

It was observed that the absence of RFU in the qPCR mix with 4× SYBR Green I was the result of an inhibition of the PCR reaction. To validate the custom qPCR mix, we selected the DyNAzyme™ II PCR Master Mix supplemented with 1x SYBR Green I, and compared the outcome of gene expression to that obtained using Maxima ® SYBR Green Master Mix. We selected a set of genes that are known to be involved in biofilm formation and accumulation, namely *bhp, icaA*, and *aap* [18,19], and also some genes involved in biofilm modulation, such as *agr* and *psm*β [2].

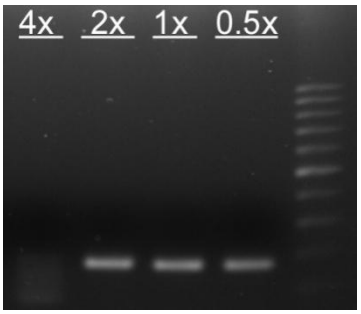

Figure 2.7. Effect of SYBR Green I concentration in the inhibition of the qPCR. The qPCR was performed using the DyNAzyme™ II PCR Master Mix.

RNA was extracted from biofilm and planktonic cultures from four distinct *S. epidermidis* strains, that were previously characterized in terms of biofilm formation [12]. The cDNA used for the validation of the custom qPCR mix was synthesized with RevertAid™ First Strand cDNA synthesis kit in a 20 µL reaction volume. No significant differences were found in the PCR efficiency when using either custom or commercial mixes (88 ± 7% for the custom assay). Additionally, the results obtained with

the custom qPCR were consistent with the results obtained with the commercial Maxima® SYBR Green Master Mix, being either up- or down-regulated genes detected in similar quantities (Table 2.3). Nevertheless, statistically significant differences were found in 5 of the 20 comparisons (16, if excluded the genes that were not detected) (p<0.05, paired-samples t-test).

Table 2.3. Comparison of gene expression quantification using a custom qPCR mix and Maxima® SYBR Green Master Mix. cDNA from biofilms and planktonic cultures was synthesized using a 20 µL reaction iScript™ cDNA synthesis kit, and quantified in a 10 µL qPCR reaction using Maxima® SYBR Green Master Mix or custom made master mix. Values represent the fold difference between biofilm and planktonic population plus or minus standard deviation of 3 independent experiments. Values above 1 indicate up-regulation in biofilm, and below 1 indicates down-regulation. Statistical differences between custom and Maxima® SYBR Green Master Mix reactions were analyzed by paired-sample t-test. * p<0.05. ND-not detected.

		***S. epidermidis* strains**			
		9142	**IE186**	**M129**	**LE7**
icaA	*Custom*	8.31±3.39*	41.12±17.50	45.63±21.14	6.40±2.42
	Maxima®	12.87±2.51*	56.89±22.11	71.53±59.28	5.50±3.33
bhp	*Custom*	5.43±0.82*	2.02±1.31	ND	ND
	Maxima®	7.02±1.11*	2.33±2.00	ND	ND
aap	*Custom*	1.62±0.20*	2.80±2.34	5.81±1.04*	3.47±0.74*
	Maxima®	2.14±0.32*	2.10±0.77	8.49±2.12*	5.57±0.61*
psmβ	*Custom*	1.46±0.64	0.35±0.20	0.19±0.14	0.48±0.34
	Maxima®	1.13±0.39	0.35±0.07	0.21±0.11	0.31±0.11
agrB	*Custom*	1.31±0.26	0.42±0.24	0.99±0.94	0.60±0.36
	Maxima®	1.06±0.47	0.68±0.26	0.77±0.09	0.77±0.09

DISCUSSION

The aim of this study was to optimize the gene transcript quantification of *S. epidermidis* biofilm samples using qPCR. This optimization included minimization of costs, and the maximization of reproducibility and sensitivity. To achieve that we assessed the three key steps of qPCR gene transcript analysis, namely RNA extraction, cDNA synthesis, and finally, the qPCR reaction. RNA extraction, as a first step, is often considered the most important step, since poor quality RNA will unquestionably influence the reliability and reproducibility of the downstream applications [7].

Common indicators of RNA extraction success include the concentration, purity and integrity of RNA [20]. These indicators are influenced by both the sample's nature and the principle of the RNA extraction kit used [21-23].

Complex samples, such as *S. epidermidis* biofilms, are notoriously difficult to disrupt and the high content of proteins and polysaccharides in the biofilm matrix can interfere with downstream analysis, as we have shown previously [9]. In that study, since FastRNA® Pro Blue was the only kit using mechanical lyses and had the highest performance, we tried to optimize the RNA extraction with the other kits tested (PureZOL™ from Bio-Rad and PureLink™ from Invitrogen) by performing the mechanical lyses step of the FastRNA® Pro Blue kit, and using the lyses buffers included with the respective kits. However, this modification did not significantly increase the RNA yield [9], suggesting that the high efficiency of FastRNA® Pro Blue was not only due to the mechanical lyses, but also due to the chemical composition of the buffer. We have reported similar results for other bacterial species that form biofilms, such as *Listeria monocytogenes* [10].

Analyzing FastRNA® Pro Blue buffer composition, we devised the custom procedure described here, wherein 90% phenol solution was added to the buffer of each silica-based membrane commercial kits in a proportion of 1:1. This approach significantly increased the RNA yield, with no detectable reduction of RNA purity and integrity. To see if other commercial silica-based column kits could also be used with this approach, three other kits were successfully tested. Of note, the FavorPrep™ kit was originally optimized to RNA extraction from human cells, but was easily adapted for bacterial cultures (Table 2.2). The custom protocols did not surpass the FastRNA® Pro Blue kit in terms of RNA quality or yield, however, we also evaluated the time necessary to perform the protocol and the cost associated with each one. In comparison with FastRNA® Pro Blue kit, we could achieve a 68% cost reduction, *per* reaction, when using our custom RNA protocol (Table 2.4 and Table 2.5). Furthermore, the overall experiment took us only 40 minutes to process 6 samples, versus nearly 4 hours with the FastRNA® Pro Blue.

Table 2.4. Analysis of the percentage of cost reduction when using the custom RNA extraction and qPCR instead of the commercially available kits. The price *per* reaction already includes all the extra-reagents needed. The values and comparison presented are relative to the price of largest kit available on the market. NA-not applicable.

		Kit	Price/ reaction (€)	% of cost reduction
RNA extraction	Commercial	FastRNA® Problue	7.15	NA
	Custom	Based on PureLink™	5	30%
		Based on ISOLATE™	4.2	41%
		Based on Direct-Zol™	4	44%
		Based on FavorPrep™	2.3	68%
qPCR assay	Commercial	Maxima ® SYBR Green Master Mix	0.48	NA
	Custom	DyNAzyme™ II PCR Master Mix+SYBR Green I	0.13	73%

Table 2.5. Kits and reagents used for the RNA extraction. All the prices listed were obtained by quote during January 2012. * ethanol is used on variable volume. An overestimated 1 mL volume was used for the purpose of price calculations.

Kit (Manufacturer)	Number extractions *per* kit	Prices (€) *per* reaction
FastRNA® Pro Blue (MPBiomedicals)	50	7.14
PureLink™ RNA Mini Kit (Invitrogen)	10-50	9.70-4.46
ISOLATE RNA Mini Kit (Bioline)	10-250	5.30-3.68
Direct-zol™ RNA MiniPrep (Zymo Research)	50-200	4.19-3.41
FavorPrep Blood/Cultured Cell Total RNA (Favorgen)	50-300	2.50-1.80
Ethanol 100% (Fisher)	2500*	0.006
Chloroform (Fisher)	3333-8333	0.002-0.001
Phenol (AppliChem)	277-1387	0.08-0.06
Glass beads, acid-washed, 150-212 µm (Sigma)	25-1250	1.53-0.33
RNAse & DNAse free tubes with screwcap (BioPlastics)	500	0.12

Without questioning the importance of RNA extraction step, a previous study regarding the optimization of cDNA synthesis using commercially available kits, revealed a high variability in the results obtained by some of the kits tested, indicating that the reverse transcriptase reaction is also crucial to obtain reliable measurement of mRNA transcripts [11]. Our results have also confirmed those observations [11], since a high variability was found in the quantification of cDNA obtained with different synthesis kits. Curiously, no significant variation was found in the reverse transcriptase kits when quantified by the iQ™ SYBR® Green Supermix (p>0.05, ANOVA) (Figure 2.2). The presence

of PCR inhibitors in the cDNA was tested by serial dilution of the samples. Using the $2^{\Delta Ct}$ method, a variation of the Livak method [24], if no PCR inhibitors are present in the cDNA, the relative fold increase of a specific gene should remain constant as cDNA is diluted (assuming a reasonable dilution range) [20]. While this was true for some cDNA synthesis kits, there were others that clearly contained PCR inhibitors. Using 100-fold cDNA dilution, we found that regardless the qPCR master mix used, no significant variation in gene expression quantification was detected, even when using different cDNA sources.

In the Sieber *et al.* study, besides the reverse transcriptase variability, they also reported, although lower, some variability associated with the qPCR kit used [11]. Actually, the majority of the qPCR master mixes tested here, including the custom qPCR mix, presented similar efficiencies (85±6% average) with the exception of the PerfeCTa® SYBR Green SuperMix (78 ± 5%) (p<0.05, ANOVA). While many qPCR kits recommend the use of 50 µL reactions, we previously reduced the volume to 25 µL and were able to properly detect gene expression both in *E. coli* [25] and *S. aureus* [26]. The reduction of reaction volume is appealing as it reduces the costs associated with an experiment. However, smaller volumes can introduce more pipetting errors and may reduce the limit of detection. To address this issue, reverse transcriptase and qPCR reactions were performed in either 10 or 20 µL volumes.

Interestingly, no significant differences were found between 10 or 20 µL qPCR reactions in any of the tested kits (Figure 2.4). On the other hand, with the cDNA synthesis kits tested, the variation was higher in 10 µL reverse transcriptase reactions, as noted by the higher standard deviation presented (Figure 2.5). The reduction of either cDNA or qPCR volume reaction from 20 to 10 µL, will unquestionably allow the reduction of some of the costs associated with gene expression analysis. Nevertheless, regarding the cDNA synthesis, we observed, in some particular cases, significant variability associated with reduced volume reactions. This higher variability would require an increase in the number of technical replicates in order to obtain reliable and meaningful results. Therefore, in our judgment, the reduction of the reverse transcriptase volume reactions might not be advantageous and ultimately, might not reduce overall costs (Table 2.6).

Table 2.6. cDNA synthesis kits used and price *per* reaction. All the prices listed were obtained by quote during January 2012.

Kit (manufacturer)	20 µL reactions *per* kit	Prices (€) *per* reaction
SuperScript® VILO™ synthesis (Invitrogen)	50-250	10.08-8.88
RevertAid™ First Strand cDNA synthesis (Fermentas)	20-100	4.25-2.80
iScript™ cDNA synthesis (Bio-Rad)	25-100	6.48-4.97
qScript™ cDNA synthesis (Quanta BioSciences)	25-500	4.52-3.04

Contrary to the reverse transcriptase reaction, a reduction in the qPCR volume reaction was not associated with changes in the outcome of the experimental assay. Therefore, the use of 10 µL volume reaction instead of the 25 or 50 µL recommended by the manufacturer's will allow to perform between 2.5 to 5 more reactions with the same cost (Table 2.7). A further approach to reduce experimental costs is to add SYBR Green I to a PCR mix, as such mixes are often considerably cheaper than qPCR mixes (Table 2.7).

Table 2.7. qPCR kits and reagents used and prices *per* reaction. All the prices listed were obtained by quote during January 2012 * kit to which SYBR Green I was added.

Kit (Manufacturer)	20 µL reactions *per* kit	Prices (€) *per* reaction
mi-real-time EvaGreen® Master (Metabion)	250-1250	0.44-0.35
Maxima ® SYBR Green Master Mix (Fermentas)	250-5000	0.81-0.48
iQ™ SYBR® Green Supermix (Bio-Rad)	250-5000	0.77-0.59
PerfeCTa® SYBR® Green SuperMix (Quanta BioSciences)	250-5000	0.70-0.58
DyNAzyme™ II PCR Master Mix (Finnzymes)*	500-2500	0.12-0.11
MyTaq™ Mix (Bioline)*	500-2500	0.21-0.18
EzWay Direct PCR Master Mix (Koma Biotech)*	100	1.40
SYBR green I nucleic acid gel stain (Invitrogen)	12500-25000	0.03-0.02

A fundamental step to be taken in consideration is an initial titration of the SYBR Green I, as a concentration can diminish the sensitivity and limit of detection and a higher concentration can inhibit the PCR reaction, as shown in our results. According to Figure 2.6, a titration of 0.5x SYBR Green I in DyNAzyme™ II PCR Master Mix, would be sufficient to detect the PCR products. However, as qPCR's done using Maxima ® SYBR Green Master Mix would yield RFU levels of around 4000-5000,

we decided to use the 1× SYBR Green I concentration (since no PCR inhibition was detected) in order to obtain similar RFU levels.

To validate our protocol, RNA from 4 different *S. epidermidis* strains grown in planktonic and biofilms from was extracted and analyzed as described. Several known genes involved in *S. epidermidis* biofilm formation, accumulation and modulation were selected as a control since their function and expression levels have been widely studied [27-33]. Both commercial and custom master mix detected the expected gene transcript levels in *S. epidermidis* biofilms when compared with planktonic cultures, validating our custom qPCR master mix (Table 2.3). Despite the statistically significant differences (p<0.05, paired-samples t-test) found between the commercial and the custom qPCR master mixes in the expression of *S. epidermidis* 9142 *icaA*, *bhp* and *aap genes*, or in *S. epidermidis* M129 and LE7 *aap gene*, these differences were small (below 1.8 fold), with no biological significance. Furthermore, both increases and decreases in transcript levels were detected in both experimental setups. Since the overall priming efficiency of the custom qPCR was similar to the commercial kits, we hypothesized that those small differences could be the result of variations in each SYBR Green I titration of the standard PCR mix, as we have detected some batch to batch variations in RFU and PCR efficiencies. As the initial cost of SYBR Green I is high, it can be used in other applications, such as agarose gel DNA/RNA staining. Once we thaw the aliquots, we kept them at 4^{o}C, protected from light. We did not address the effect of storing SYBR Green I at 4^{o}C, although the manufacturer indicates that short-term storage is possible. For future reference, smaller aliquots should be prepared, so that freshly thawed SYBR Green I could be used.

The qPCR custom master mix developed in this study not only produced comparable results to those obtained using commercially available master mixes, it also, allowed considerable reduction in the cost associated with gene expression quantification, around 70% (Table 2.4).

CONCLUSIONS

Currently, qPCR is considered the gold standard technique to study transcript levels of a specific set of genes under specific treatment or stress conditions, and to validate the results obtained in genome-wide analysis such as DNA microarrays and RNA sequencing. Therefore, qPCR is a technique in high demand that has to assure high reliability, sensitivity and reproducibility. Herein, we describe

a custom procedure for RNA extraction and qPCR analysis that present the same high standards as the commercially available and reduces the high costs normally associated with gene expression quantification.

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CHAPTER 3.

S. epidermidis biofilm transcriptome alterations upon interaction with whole human blood

ABSTRACT

Pathogens that cause systemic infections typically present special features that help them to escape and colonize host's organs. Obviously, the ability to withstand the high bactericidal activity of host's blood is essential for biofilm maintenance, and thus essential for pathogenesis. Therefore, the mechanisms used by *S. epidermidis* biofilms to overcome and escape the high antimicrobial activity of human blood need to be identified and characterized. Hence, we aimed to elucidate *S. epidermidis* biofilms transcriptome dynamics when in contact with human blood. Gene ontology enrichment analysis showed that the up-regulated genes included those involved in biosynthesis and metabolism of amino acids, small molecules, carboxylic acid, ketones and glutamine. One of the striking changes observed was the increase in the expression of genes involved in iron recognition and uptake, suggesting that iron utilization may constitute one of the most important mechanisms used by *S. epidermidis* biofilms to survive in human blood.

BACKGROUND

The most important virulence factor of *S. epidermidis* is its ability to tenaciously adhere to the surfaces of indwelling medical devices that penetrate the skin and form adhesive biofilms. Frequently, *S. epidermidis* develops biofilms on the surface of peripheral or central intravenous catheters, accounting, at least, for 22% of the cases of bloodstream infections detected in patients in intensive care units in the USA [1]. Pathogens that cause systemic infections typically present special features that help them to escape and colonize host's organs [2]. Obviously, the ability to withstand the high bactericidal activity of host's blood is essential for biofilm maintenance, and thus indispensible in pathogenesis. Therefore, the mechanisms used by *S. epidermidis* to overcome and escape the high antimicrobial activity of human blood need to be identified and characterized. Due to the development of high-throughput nucleic acid identification and sequencing techniques, such as microarrays and RNA sequencing (RNA-seq), the analysis on how pathogenic microorganisms react during culture in human blood has been completed for important pathogens such as *Candida albicans* [2], *Staphylococcus aureus* [3], group A *Streptococcus* [4], *Streptococcus agalactiae* [5] and *Enterococcus faecalis* [6]. These data provided valuable information for understanding dissemination and virulence of these pathogens in the bloodstream. However, despite their importance in human health and disease, no such information has been reported regarding the transcriptome of biofilm cells for these pathogens or for *S. epidermidis*. The identification of *S. epidermidis* genes differentially expressed upon contact with human blood will be of crucial importance in understanding the strategies used by this bacterium to evade the host immune response and cause systemic infections. Since adaptive gene expression will determine whether bacteria successfully persist and disseminate in the host, in this chapter we describe the characterization of the transcriptome of *S. epidermidis* biofilms upon contact with human blood.

MATERIALS AND METHODS

Bacteria and growth conditions

For this study, *S. epidermidis* RP62A was used. One single colony was inoculated into 2 mL of TSB and incubated overnight at 37^{o}C and 700 rpm (VorTemp^{TM} 1550, Labnet International, USA). The overnight suspension was diluted 1:600 into fresh TSB supplemented with 1% glucose (v/v) (TSBG), distributed (1mL) into 24-well tissue culture plates (Costar®Corning) and incubated at 37^{o}C, 100 rpm for 24 hours.

Blood collection and blood fractionation

Peripheral blood was collected from healthy adult volunteers by venipuncture into BD Vacutainer® sodium heparin tubes (Becton Dickinson, NJ, USA). All donors gave written informed consent to have blood taken. Blood was collected under the 1999-P-001173/48 protocol approved by the Partner's Health Care System Institutional Review Board (Boston, MA, USA).

***S. epidermidis* biofilms co-incubation with whole human blood**

Twenty-four hour old *S. epidermidis* biofilms (grown as described above) were washed once with fresh TSB. Afterwards, 1 mL of whole blood or TSB was added to the wells containing the biofilms and allowed to incubate for 2 or 4 hours at 37^{o}C in 5% CO_2 with slight agitation. After the incubation period, blood and TSB were gently removed and the biofilms washed twice with phosphate buffered saline (PSB) with 0.05% Tween20 (PBST) (Boston BioProducts, MA, USA), supplemented with a protease inhibitor cocktail tablet (Roche, Basel, Switzerland). The biofilms were immediately suspended in 1 mL of the RNA protect™ bacterial reagent (QIAGEN, Hilden, Germany) diluted 2:1 in PBS. Each condition was carried out in triplicate, and then the biofilms pooled together; 1.5 mL of this suspension was used for RNA extraction. This experiment was performed 6 independent times with different healthy blood donors. For cDNA library construction, 3 of the independent experiments were mixed together in order to decrease donor-dependent variation and 2 sets of independent samples were created.

Total RNA Extraction

RNA was extracted using RNeasy mini kit (QIAGEN) following the manufacturer's instructions with some alterations (complete description in Chapter 2). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

Removal of genomic DNA

One cycle of TURBO DNA-free enzyme (Ambion, NY, USA) was performed following manufacturer's recommendations. To improve genomic DNA removal and remove salts introduced by the DNase treatment, RNA was treated with a mixture of 1:5 Acid-phenol:chloroform (Ambion) (pH 4.5). Briefly, the RNA volume was adjust to 200 µL with nuclease-free water and transferred to phase lock heavy gel

tubes. Afterward, 200 µL of 1:5 Acid-phenol:chloroform was added, the tube inverted several times to mix, incubated for 3 minutes at room temperature (RT) and centrifuged for 2 minutes at 16000 *g* and 4°C.The RNA fraction was then recovered by precipitation using 0.1 volume of 3 M sodium acetate (Ambion), 5 µg glycogen (Ambion) and ice-cold 100 % ethanol (Fisher Scientific) and incubated for 1 hour at 80°C. RNA was centrifuged at 16000 *g* for 30 minutes and 4°C, and washed twice with ice-cold 70 % ethanol (Fisher Scientific). The pellets were then centrifuged briefly and residual supernatant collected carefully. Finally RNA pellet was allowed to air-dry for 5 minutes at RT and dissolved in 30 µL of Tris-EDTA buffer (TE) (Ambion).

Removal of eukaryotic RNA

In order to remove any contaminating eukaryotic RNA derived from mammalian cells present in the blood, the samples were treated with MICROB*Enrich*™ kit (Ambion) following the manufacturer's instructions. Briefly, 50 µg of RNA was prepared to hybridize with the magnetic beads to the polyadenylated 3' ends of eukaryotic mRNA. The magnetic beads bound to the 18S and 28S rRNA and polyadenylated mRNA were pulled to the side of the tube with a magnet. The enriched bacterial RNA (in the supernatant) was transferred into a new tube and the RNA integrity assessed using a 2100 Bioanalyzer (Agilent).

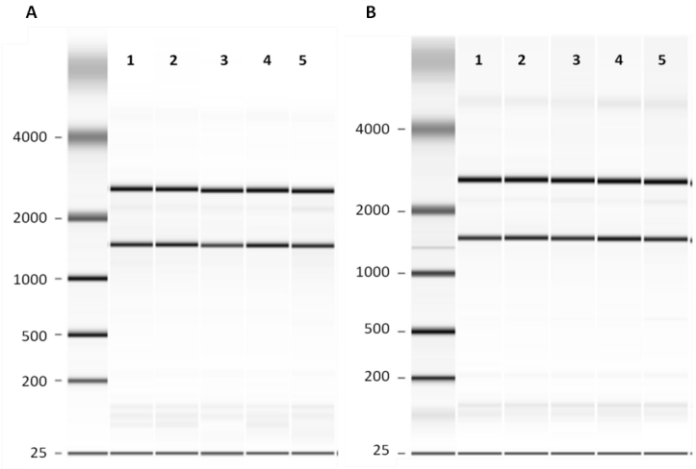

Figure 3.1. RNA integrity profile of the samples used for the first (A) and second (B) RNA-seq runs. (1) RNA extracted from biofilms before the addition of blood, **(2)** 2 hours of incubation with TSB, **(3)** 2 hours of incubation with whole human blood, **(4)** 4 hours of incubation with TSB and **(5)** 4 hours of incubation with whole human blood. The RNA 6000 ladder was used and contains six RNA fragments ranging in size from 0.2 to 6 Kilobase.

Prokaryotic rRNA depletion

Prokaryotic mRNA was enrich by depleting the ribosomal RNA using Ribo-Zero™ rRNA removal kit for Gram-positive bacteria (Epicentre, WI, USA), following the manufacturer's instructions. mRNA was recovered by precipitation as indicated by the manufacturer. The pellets were then dissolved in 18 µL of Elute, Prime and Fragment buffer (Illumina, San Diego, USA) a part of Illumina TruSeq verison 2 kit.

Libraries preparation

Library preparation for RNA-seq was performed using the Illumina TruSeq™ RNA kit, version 2 (Illumina) following the manufacturer's instructions. Briefly, 400 ng of mRNA was chemically fragmented and immediately first and second strand complementary DNA (cDNA) prepared. Later, the overhangs were converted into blunt ends and a single *A*-nucleotide was added to the 3'ends allowing the ligation of different bar codes. At the end, a selective enrichment of the double stranded-cDNA fragments that had adapter molecules on both ends was performed by PCR. Library construction was validated through a high quality control: 1) Agilent 2200 TapStation System (High Sensitivity D1K Screen Tape) to determine the size of the products, which should be around 260-300 base pairs, and 2) quantitative PCR to determine functionality (hybridization to flow cell) and the exact concentration of the libraries to create optimum cluster densities across every lane to obtain the highest quality data on Illumina sequencing platforms.

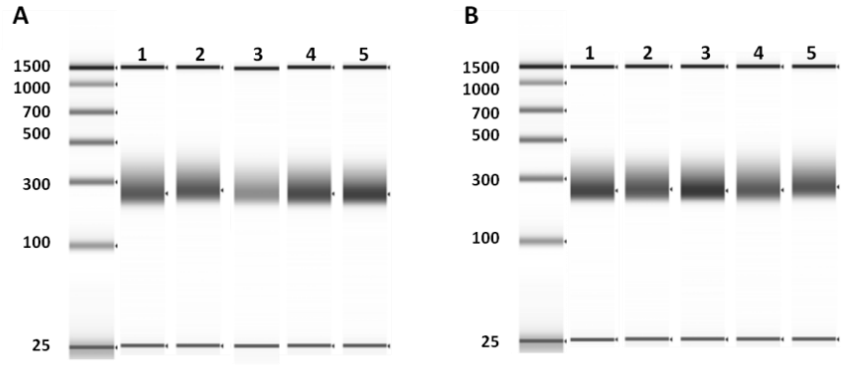

Figure 3.2. Sizes of the libraries created for RNA-seq run 1 (A) and 2 (B). Libraries prepared with RNA extracted from biofilms at time 0 **(1)**, 2 hours post incubation with TSB **(2)** or whole human blood **(3)** and, 4 hours post incubation with TSB **(4)** or whole human blood **(5)**. The DNA ladder contains five DNA fragments ranging in size from 0.2 to 1 kilobase pairs.

RNA-seq data analysis

The reads obtained were aligned to the genome of *S. epidermidis* RP62A (RefSeq accession number NC_002976.3), and the total reads/gene were normalized using reads *per* kilobase per million (RPKM), that account for both library size and gene length effects within-sample comparisons, as described by Mortazavi and his collaborators [7]. Differential gene expression between biofilms incubated with TSB or human blood, and biofilms at the start of the culture was then calculated. In order to determine statistically significant differences in gene expression between the tested conditions, Baggerly's statistical [8] test with false discovery rate (FDR) and *p* values correction was applied [9]. All these steps were performed using CLC Genomics Workbench version 5.1 (MA, USA).

Genes that were not detected (RPKM=0) and with fold changes with *p* values greater than 0.05 were discarded. Using Venn diagrams [10] the genes that were expressed in both biological replicates were identified. A list of the fold change values obtained in each run, for each gene, was created and the values were averaged. Pearson correlation was performed in order to assess the agreement between biological replicates. For further analyses, only genes with fold changes above two were selected for inclusion.

Finally, in order to simplify the global analyses and to identify the principal biological processes enhanced upon contact with human blood, gene ontology (GO) enrichment of the up- and down-regulated genes was accomplished using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 9.05 [11]. Only gene-sets passing significance thresholds (FDR, *p* value<0.05) were selected for display. The visualization of the interactions between genes was performed using Cytoscape version 2.8.3 [12]. Protein localization within the bacterial cell was predicted using PSORTb version 3.0.2 [13].

In order to confirm the data obtained by RNA-seq, a few genes of interest were selected (Table 3.1) and qPCR performed as described in Chapter 2.

Table 3.1. Oligonucleotide primer sequences used for RNA-seq confirmation by qPCR.

Target gene		primers sequence (5' to 3')	TM (°C)	Amplicon size (bp)
16S	FW	GGGCTACACACGTGCTACAA	59.79	176
	RV	GTACAAGACCCGGGAACGTA	59.85	
aap	FW	GCACCAGCTGTTGTTGTACC	59.22	190
	RV	GCATGCCTGCTGATAGTTCA	59.98	
bhp	FW	TGGACTCGTAGCTTCGTCCT	60.01	213
	RV	TCTGCAGATACCCAGACAACC	60.13	
icaA	FW	TGCACTCAATGAGGGAATCA	60.20	134
	RV	TAACTGCGCCTAATTTTGGATT	59.99	
lrgB	FW	ATATCGCAAGCGCGAAGTAT	59.87	165
	RV	ATTGCTGTCGTTGCAGCTT	59.61	

Assessment of biofilms viability upon exposure to human blood by flow cytometry

Biofilm viability was determined by flow cytometry as described elsewhere [11,12], with minor alterations. In brief, biofilms were washed twice, suspended in 1 mL of PBS, sonicated for 10 seconds at 18 Watt (Branson model W 185 D ultrasonic cell disrupter; Heat Systems-Ultrasonics, Plainview, New York, USA), and vortexed at maximum speed. Finally, 180 µL of a solution with SYBR green I (1:5000 dilution, Invitrogen, California, USA), 1 µg/mL of propidium iodide (PI) (Sigma, MO, USA), 10 µL of quantification beads, and 10 µL of 1:100 diluted bacteria were mixed together by vortexing and the cells counted in a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., CA, USA). SYBR green I fluorescence was detected on the FL1 channel while PI fluorescence was detected on the FL3 channel. For all detected parameters, amplification was carried out using logarithmic scales. The concentration of bacteria was determined by acquiring the counts for a specific number of microspheres. Statistical differences between groups were determined using unpaired t-test and *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Confirmation of RNA-seq results

In order to confirm the results obtained with RNA-seq analysis, 4 genes of interest were selected and their expression determined by qPCR using the same biological model. As can be seen in Table 3.2,

although qPCR was able to detect higher amount of mRNA transcripts [3,4,6], the trend observed was the same as in RNA-seq. Thus, these results validated and confirmed the results obtained using RNA-seq analysis.

Table 3.2. Verification of the RNA-seq data by qPCR. Results are expressed as the mean of relative fold expression obtained in 3 independent experiments plus or minus standard error of the mean from bacteria cultured in blood for 2 or 4 hours comparatively to the time zero. ND-not detected; NA-not applicable.

	T2h blood		T4h blood	
Gene	**RNA-seq**	**qPCR**	**RNA-seq**	**qPCR**
aap	2.76 ± 1.95	8.97 ± 3.65	1.77 ± 0.459	11.64 ± 1.99
bhp	2.36 ± 1.67	8.49 ± 3.92	ND	NA
icaA	31.16 ± 22.03	89.64 ± 73,93	21 ±2.73	223.98 ± 44.88
lrgB	113.08 ± 79.96	10.25 ± 4.67	78.7 ± 65.6	25.86 ± 7.68

S. epidermidis biofilms survival in human blood

Human blood is a complex mixture of immune circulating cells and soluble factors that are very active against invading pathogens. However, pathogens that promote systemic infections have developed mechanisms that enable them to circumvent the high microbicidal properties of the host's blood [14]. In order to evaluate the stress induced by human blood on the ability of cells within *S. epidermidis* biofilms to survive, biofilm viability was evaluated by flow cytometry. Despite some lost of viability, we have determined a survival percentage of 59% ± 11.4% and 54% ± 5.6%, respectively, upon 2 and 4 hours incubation with whole human blood (Figure 3.3). These results clearly show the ability of *S. epidermidis* biofilms to survive in such harsh environment, and the importance of studying the mechanisms employed by the bacterium to survive, which ultimately will allow dissemination into host's organs and cause infection.

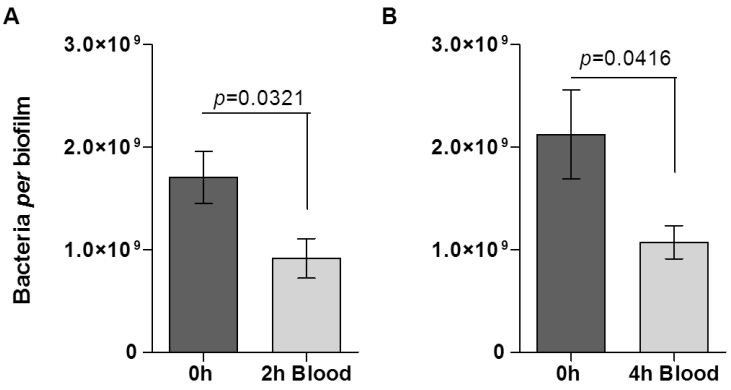

Figure 3.3. Total number of viable cells *per* biofilm upon incubation with human blood for 2 (A) and 4 (B) hours. The total number of viable cells was assessed by flow cytometry using SYBR green I/ PI staining. The bars represent the average plus or minus the standard error of the mean of 4 to 6 independent experiments. Statistical difference between groups was determined using unpaired t-test.

The *ex vivo* model and global transcriptomics analysis

To gain enhanced understanding of the mechanisms used by *S. epidermidis* to survive and evade the anti-bacterial activities of human blood, global changes in its transcriptome upon contact with blood were assessed. Obviously, the most relevant data would be provided by analyzing bacterial gene expression during the progress of biofilm-related infections in patients. However, this kind of study is extremely difficult, if not impossible, to conduct. A model that completely mimics the *in vivo* environment with the inclusion of all the variables which are likely to influence bacterial responses and the course of infection is inaccessible [4]. Nevertheless, the incubation of microorganisms with human blood samples seemed to be an attractive model for mimicking *in vivo* environment, and has been increasingly used in the last years [2-6,14-16]. Thus, *S. epidermidis* biofilms were incubated with heparinized human blood for 2 or 4 hours. Sodium heparin was chosen as anticoagulant over EDTA or sodium citrate due to its action on anti-thrombin III rather than ions that may be essential for bacterial growth [17]. Furthermore it was shown that group B meningococci incubated with blood samples collected with sodium citrate were killed faster than in heparinized blood. On the other hand, another study has reported that *Candida albicans* gene expression is influenced by the presence of heparin in blood samples. Nevertheless, only a very small group of genes (10 out of 2002) was affected [14]. Importantly, when working with human samples, there is a significant source of variability that is due to differences among individual. It is known that factors such as age [18], gender [19], exposure factors such as smoking, diet and medication [20], the proportion of the different cell populations comprising

the blood [21], and even the time of day at which the sample was taken are responsible for inter-individual sample variation [22]. In order to reduce possible donor-associated gene expression variability, blood from 6 different donors was used for this experiment. At the end, total RNA resultant from 3 of these 6 experiments were pooled together creating two set of samples that were processed independently. Nevertheless, besides the attempt to decrease donor-dependent variability, differences between biological duplicates were observed (Figure 3.4), resulting in Pearson correlation coefficients of 0.7 and 0.8, respectively, 2 and 4 hours after incubation with human blood.

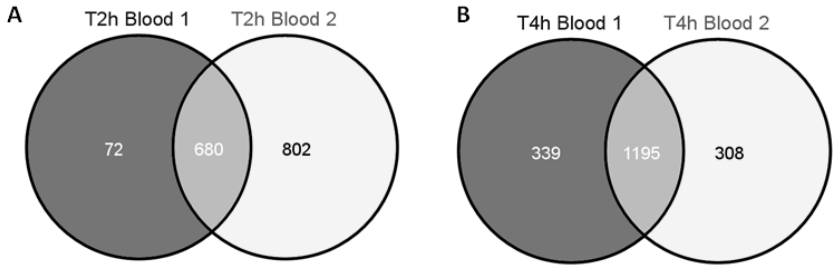

Figure 3.4. Venn diagrams showing the number of common transcripts (overlapping circles) and unique transcripts (non-overlapping circles) between the 2 biological replicates upon 2 (A) or 4 (B) hours of incubation with whole human blood. All further analysis was performed using the 680 and 1195 genes that are common to both independent experiments in order to study phenomena that are present in all donors.

In order to study *S. epidermidis* transcriptome changes independent of donor variability, only genes that were differentially transcribed in both samples (Figure 3.5) were considered for further analysis. Additionally, in order to distinguish between alterations caused by the unique environment of human blood and non-specific alterations required for growth in fresh medium, biofilms were also incubated with TSB, a medium regularly used in laboratory studies for staphylococcus growth. The identification of the genes uniquely expressed in blood will be important both for understanding the infection process and also to uncover the specific mechanisms employed by the bacterium for immune evasion. As can be seen in the Figure 3.5, within 680 genes only 139 genes were uniquely expressed in biofilms after 2 hours of exposure to whole human blood. Within these 139 genes, 41 (29.49%) were up-regulated and 34 (24.46%) down-regulated, while 63 genes (45.3%) presented very small changes in their transcription (around 2-fold change). Four hours after exposure, among the 1195 genes differentially expressed, only 445 genes were uniquely expressed in the presence of human blood, whereas 94 (21.12%) were up-

regulated and 97 (21.79%) down-regulated, while 254 (57.08%) presented very small changes in their expression profile (around 2-fold change). Hence, for further analysis only the genes that were uniquely expressed in the presence of human blood were considered for inclusion.

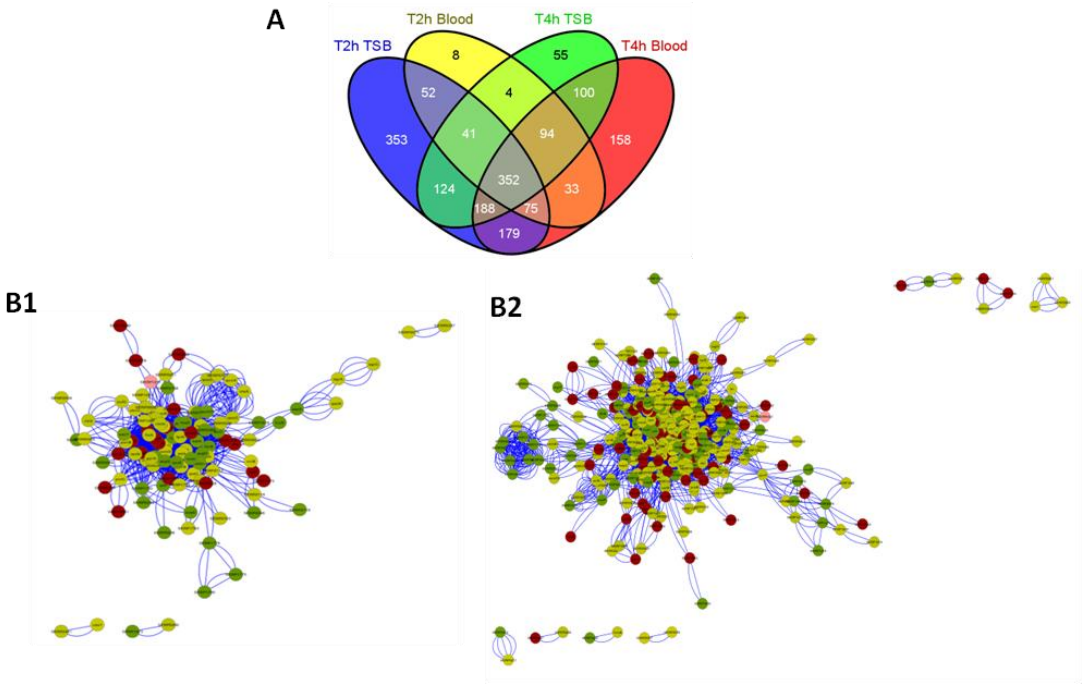

Figure 3.5. (A) Venn diagram showing the number of common transcripts (overlapping circles) and unique transcripts (non-overlapping circles) in all the conditions tested; (B) Network of the interactions of all the genes uniquely expressed within biofilms exposed for 2 (B1) or 4 hours (B2) to human blood. Nodes represent genes that are connected with edges representing pair-wise interactions. Colors indicate up-regulated genes (dark green), down-regulated genes (red) and genes that presented with small variations in their expression (around 2-fold change) (light green).

S. epidermidis biofilms major transcriptomic changes upon contact with human blood

Upon contact with human blood, *S. epidermidis* biofilm transcriptomes undergo dramatic changes in response to the stress created by the presence of human blood components. To better understand the significance of the changes it is essential to narrow down the analysis of such complex data and to highlight the most important biological processes occurring. Hence GO enrichment analysis was performed using STRING. As can be seen in the Figure 3.6, the biological processes that were found enhanced within the up-regulated genes 2 hours after incubation with human blood are linked to amino

acid, small molecules and carboxylic acid biosynthesis. After 4 hours of exposure, it was also found that genes involved in the biosynthesis of organic acids, cellular ketone and glutamine, as well as oxoacid and carboxylic acid metabolism were also enhanced. No enhancement in biological processes within down-regulated genes 2 or 4 hours after incubation with human blood was found. Interestingly, the up-regulation of amino acid biosynthesis and metabolism was also observed in *S. aureus* both during blood [3] and lung infection [23], as well as in other microorganisms such as group A Streptococcus [4] and *Candida albicans* [14] when incubated with human blood.

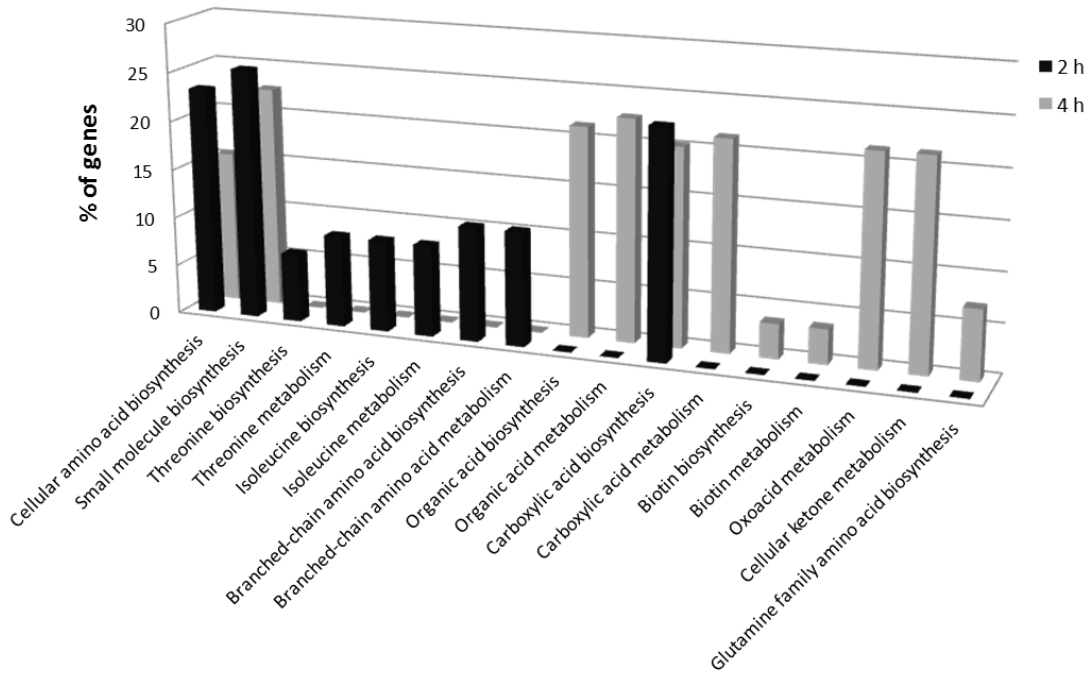

Figure 3.6. Percentage of up-regulated genes within the different biological functional classes upon 2 or 4 hours of exposure to human blood. GO enrichment was performed using STRING. Only set-genes with *p* values less than 0.05 (with FDR) were selected for display.

Biofilm formation and virulence determinants

One of our primary interests was to assess the changes that occurred in the expression of genes involved in the biofilm lifecycle and immune evasion. A closer look into several of those genes (Table 3.2) revealed that those involved in the biofilm lifecycle as well as in immune evasion were up-regulated in both blood and TSB cultures. However, in the first 2 hours after exposure to human blood, genes that promote

biofilm formation genes such as *aap, bhp, icaA* were expressed at lower levels while the negative regulator *saeR* was found expressed at higher levels in cells within biofilms under humans blood stress. This decrease in biofilm formation is probably related to an efficient energetic strategy, whereby the energy applied for other functions not related to bacterial survival in human blood is redirected.

Table 3.3. Expression of genes involved in biofilm formation and immune evasion during exposure to human blood for 2 and 4 hours. ND-not-detected; IgG-immunoglobulin G; CP-complement proteins.

Gene	Fold change (2h)		Fold change (4h)		Function
	TSB	Blood	TSB	Blood	
aap	3.4	2.7	ND	1.7	Bacterial aggregation after proteolysis
atlE	6	3.6	1.6	2.4	Binding to polystyrene and vitronectin
bhp	5.5	2.3	1.7	ND	Bacterial cell-cell interaction
capB	6.7	ND	2.8	2.4	Protects from AMPs and phagocytes
gehD	7	3.1	2.3	5.4	Binding to collagen; Persistence on skin
gehC	ND	3.1	3.6	7.8	Persistence on skin
icaA	67	31	15.07	21.0	Protects from AMPs, phagocytes, IgG and CP; Bacterial cell-cell interaction
saeR	16	101	10.6	105	Two component signaling system
sepA	3.05	ND	ND	ND	Involved in AMPs degradation
sdrG	6.7	11	2.08	4.3	Binding to fibrinogen
sdrF	1.8	ND	ND	ND	Binding to collagen
sspA	1.07	1.7	ND	1.4	*Serine protease*
sspB	ND	ND	ND	ND	Tissue damage

As we observed previously by GO enhancement, biological processes such as amino and carboxylic acid biosynthesis and metabolism were enhanced rather than genes involved in virulence or biofilm formation. Nevertheless, 4 hours after exposure to human blood, a slight increase in the expression of several of these genes seemed to indicate that bacteria started adapting to the environment created by the human blood components. Thus, the expression of genes involved in biofilm maintenance and virulence started to increase, reaching or even surpassing the expression observed within biofilms incubated with TSB. Interestingly, in the case of the response regulator *saeR* this was not observed. Despite the involvement of *saeR* in biofilm formation through the repression of proteases and nucleases that are involved in biofilm dispersion [24], it was also shown that *saeR* is responsive to several phagocytosis-related stimuli including pH, oxidative stress and the presence of defensins, factors essential for immune evasion [25-27]. Furthermore, in agreement with our findings, it was found that *saeR* is also up-regulated in *S. aureus* upon incubation with human blood [3]. Additionally, it was found that *saeR* expression increases in the presence of hemoglobin [28].

Iron uptake and sequestration

Genes associated with iron uptake, recognition and sequestration were found up-regulated 4 hours after incubation with human blood (Table 3.4). Interestingly, some genes were also shown to be up-regulated in TSB, but only in the first 2 hours of incubation. Iron is an essential cofactor in basic metabolic pathways to both pathogenic microorganisms and their hosts. During the years of co-evolution, the shared requirement for both the pathogen and the host to acquire and use iron has shaped the pathogen–host relationship [29,30]. For bacteria, iron is essential in many biological processes such as nucleotide biosynthesis, aerobic respiration, tricarboxylic acid cycle and oxidative defense systems that protect bacteria from the reactive oxygen species produced by the host [30,31]. Thus, not surprisingly, almost all bacteria require iron to grow and establish infection in their hosts [29]. Hence, one of the first lines of defense against bacterial infection is sequestering iron to prevent bacterial outgrowth in a process termed nutritional immunity [32].

Table 3.4. Genes involved in iron uptake and metabolism that were found differentially expressed 2 and 4 hours after incubation with human blood. The localization each of the protein was determined using PSORTb version 3.0.2. ND-not-detected; a-this receptor may be located on the cell wall, extracellular or in the cytoplasmic membrane.

Gene	Fold change (2h)	Fold change (4h)	Function	Localization
SERP0400	16.6	19.4	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0401	15.7	18.0	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0402	11.8	10.0	Iron compound ABC transporter, ATP-binding protein	Cytoplasmic membrane
SERP0949	8.0	8.0	Transferrin receptor	Unkown[a]
SERP0403	30.9	7.3	Transferrin receptor	Cytoplasmic membrane
SERP1953	ND	4.16	*HssR*, two-component regulatory system HssRS	Cytosplasmic
SERP1775	5.5	3.81	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP1776	4.5	3.05	Iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0306	ND	2.75	Iron compound ABC transporter, ATP-binding protein	Cytoplasmic membrane
SERP1951	ND	-2.70	*HrtA*, ABC transporter complex HrtAB involved in hemin import	Cytoplasmic membrane

In humans, iron is primarily found sequestered within ferritin (iron storage protein) or transferrin (iron transporter), or complexed within the porphyrin ring of the heme cofactor of hemoglobin and myoglobin (oxygen carrying and storage proteins) being, thus, inaccessible to bacteria [33]. This way, the host environment contains very low levels of free iron (10^{-18}M) [23,32,34]. Nonetheless, pathogenic bacteria have evolved several mechanisms that are able to sequester iron present in the host [34]. One of the mechanisms described for *S. aureus* is the use of the two component Heme-*S*ensor *S*ystem (HssRS). This system responds to heme, hemin, hemoglobin or blood and activates the expression of the heme-regulated transporter (HrtAB) efflux pump, an ABC-type transporter involved in the alleviation of hemin toxicity and thus, playing a pivotal role in the intracellular heme homestasis [35,36]. Orthologs of *hssRS* and *hrtAB* are also found in *S. epidermidis* as well as other Gram-positive bacteria, suggesting a conserved mechanism by which these pathogens can acquire iron and modulate virulence [35,37,38]. Although 2 hours after incubation with blood both *hssRS* and *hrtAB* were not expressed, after 4 hours, as can be observed in the Table 3.4, the expression of the DNA-binding response regulator *hssR* is increased indicating that *hssS* was activated and therefore the bacterial cells are sensing the presence of heme in the cytoplasm. However, *hrtA* was found down-regulated. Not being mobilized, heme is excreted via HrtAB complex to avoid heme-related toxicity the down-regulation of *hrtA* suggests that all the heme in the cytoplasm was used for other cellular functions [35].

Besides this two component sensing system there are also important transferrin receptors that recognize specifically transferrin from the human blood and use the iron that is bound for cellular functions [39,40]. These results clearly indicate that iron uptake may constitute an important mechanism of survival in human blood. The requirement for iron ensures that the systems involved in iron uptake are located at the surface during infection. Thus, the inactivation of these receptors and systems have been proposed as interesting candidate vaccines for several pathogens including *Neisseria meningitides* [41], the *Hemophilus ducreyi* [42], *S. aureus* [43], and *E. coli* [44].

CONCLUSIONS

Characterization of the bacterial transcriptome during host-pathogen interactions is a fundamental step to understand the infectious processes caused by human pathogens. In this chapter, we have described, for the first time, *S. epidermidis* biofilms transcriptome upon exposure to human blood using an *ex vivo*

model of infection. A careful analysis revealed that *S. epidermidis* biofilms undergo a rapid adaptive response that enable it to survive and persist in host's blood over the time course of the infection. Moreover, these results indicate that *S. epidermidis* survival in human blood is primarily related to the ability to synthesize several essential molecules that are not available in this setting, rather than the expression of classic virulence factors, implying a protective rather than an aggressive strategy of survival. In addition, iron recognition and uptake seems to be the one of the most important mechanisms that *S. epidermidis* biofilms use to respond to the presence of human blood. This issue needs to be further investigated in order to determine if this mechanism may be used to identify potential vaccine candidates based on their in vivo expression as has already been proposed for other pathogenic bacteria.

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SUPPLEMENTARY DATA

Table S 3.1. List of top 10 up- and down-regulated genes uniquely expressed in biofilms upon 2 hours of contact with whole human blood. The localization each of the protein was determined using PSORTb. a-this protein may be located on cytoplasm or cytoplasmic membrane.

Gene	Fold change	Product	Localization
		Up-regulated	
SERP2326	228.7	acetoin dehydrogenase, E1 component, alpha subunit	Cytoplasmic
ppdk	30.4	pyruvate phosphate dikinase	Cytoplasmic
leuA	19.3	2-isopropylmalate synthase	Cytoplasmic
ilvC	16.7	ketol-acid reductoisomerase	Cytoplasmic
SERP0479	14.6	truncated IS1272 transposase	Unknown
hisD	13.4	histidinol dehydrogenase	Cytoplasmic
hom	11.8	homoserine dehydrogenase	Unknown
SERP1780	11.0	transporter, putative	Cytoplasmic membrane
SERP2260	11.0	PTS system, fructose-specific IIABC components	Cytoplasmic membrane
		Down-regulated	
SERP0079	-6	hypothetical protein	Cytoplasmic
SERP0244	-5.6	oxidoreducatese, Aldo/Keto reductase family	Cytoplasmic
mqo-3	-5.5	malate:quinone oxidoreductase	Cell wall [a]
SERP1868	-5.3	Transporter, putative	Cytoplasmic membrane
SERP0080	-4	cobalamin synthesis protein	Cytoplasmic
moaB	-3.4	molybdenum cofactor biosynthesis protein B	Cytoplasmic
SERP0294	-2.9	hypothetical protein	Cytoplasmic membrane
scdA	-2.9	cell wall biosynthesis	Cytoplasmic
SERP1664	-2.8	hypothetical protein	Cytoplasmic
SERP2173	-2.7	hypothetical protein	cytoplasmic

Table S 3.2. List of top 10 up- and down-regulated genes uniquely expressed in biofilms upon 4 hours in contact with whole human blood. The localization each of the protein represented was determined using PSORTb.

Gene	Fold change	Product	Localization
Up-regulated			
leuD	31.9	isopropylmalate isomerase small subunit	Unknown
SERP1395	27.7	amino acid ABC transporter substrate-binding protein	Cytoplasmic membrane
SERP1864	26.3	bioY family protein	Cytoplasmic membrane
SERP0400	19.4	iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP0401	18.0	iron compound ABC transporter, permease protein	Cytoplasmic membrane
SERP1703	18.0	single-stranded DNA-binding protein family	Cytoplasmic
ilvA	17.7	threonine dehydratase	Cytoplasmic
SERP2141	17.3	regulatory protein, putative	Cytoplasmic membrane
argC	11.1	N-acetyl-gamma-glutamyl-phosphate reductase	Unknown
SERP0402	10.0	iron compound ABC transporter, ATP-binding protein	Cytoplasmic membrane
Down-regulated			
arcB-1	-120.0	ornithine carbamoyltransferase	Cytoplasmic
arcA	-75	arginine deiminase	Cytoplasmic
arcD	-60.4	arginine/ornithine antiporter	Cytoplasmic membrane
SERP0958	-16.8	phosphate ABC transporter, permease protein	Cytoplasmic membrane
cysS	-12.4	cysteinyl-tRNA synthetase	Cytoplasmic
SERP0171	-10.7	hypothetical protein	Cytoplasmic
SERP2005	-10.6	amino acid ABC transporter, amino acid-binding protein	Unknown
SERP2425	-8.7	hypothetical protein	Cytoplasmic membrane
SERP1655	-7.9	hypothetical protein	Cytoplasmic membrane
SERP0094	-7.9	cysteine synthase/cystathionine beta-synthase family protein	Unknown

CHAPTER 4.

S. epidermidis biofilm-released cells: phenotype characterization and a first look into its interaction with the host immune system

ABSTRACT

S. epidermidis biofilm disassembly has been associated with the development of serious biofilm-associated infections. However, little is known about the phenotype and the interaction of biofilm-released cells with the host immune system. In this chapter we describe the characterization of biofilm-released cells in several important parameters such as antibiotic susceptibility, total protein and gene expression profiles, the ability to adhere to abiotic surfaces and their susceptibility to human immune effectors using *in vitro* and *in vivo* models. The results showed that biofilm-released cells present a particular phenotype. These cells display, simultaneously, features of planktonic cells, such as expression of *psm*β and *icaA*, or the ability to colonize host organs in the first hours of infection, as well as biofilm features, such as high antibiotic tolerance, and lower ability to stimulate the production of IL-6. Moreover, *S. epidermidis* biofilms produced a unique protein that is not detected in the other phenotypes. Hence, this study shows, for the first time, that *S. epidermidis* biofilm-released cells present an intermediary phenotype, that should be take into consideration in the pathogenesis of biofilm-related infections.

BACKGROUND

Biofilm disassembly, the release of bacterial cells within the biofilm into the involving environment, has been associated with the development of severe infections such as bacteremia [1], embolic events of endocarditis [2] and pneumonia [3]. However, despite its clear importance in the clinical setting, little is known about the particular features of the biofilm-released cells and their interaction with the host immune system. Similarly to the other phases of the biofilm lifecycle, biofilm disassembly is believed to be a combination of complex, multi-factorial, and highly regulated processes that can be triggered by several external and/ or by bacterial-derived signals [4-6]. In the beginning of the biofilm formation, planktonic attached bacteria undergo several physiological and genetic expression modifications that will lead to the biofilm phenotype [7]. Reasonably, it was initially hypothesized that after disassembly, biofilm-released cells would revert to the initial planktonic phenotype [8]. However, it has been recently shown that cells released from *Pseudomonas aeruginosa* [9] and *Streptococcus mutans* [10] biofilms present particular and distinctive features from the ones presented by their planktonic and biofilm counterparts. An in-depth understanding of the particular properties of biofilm-released cells and its interaction with the host immune system is needed to help to prevent the pathologic events associated with biofilm cells dissemination to more distant sites. Therefore, in this chapter, we describe *S. epidermidis* biofilm-released cells ability to adhere to abiotic surfaces, tolerance to antibiotics, total protein profile, expression of genes with particular interest in biofilm formation, maturation, and disassembly, and tolerance to opsonophagocytic killing. In addition, the ability of these cells to colonize systemic organs and persist within the host was also evaluated.

MATERIALS AND METHODS

Bacteria and growth conditions

For this study, the biofilm forming strain *S. epidermidis* 9142 was used [11]. One single colony grown in Tryptic Soy Agar plates (TSA, Liofilchem, Roseto degli Abruzzi, Italy) was inoculated into 2 mL of Tryptic Soy Broth (TSB, Liofilchem) and incubated overnight at 37^{o}C and 120 rpm. The overnight-grown cells were diluted in TSB in order to obtain a suspension with an optical density (OD) at 640 nm of 0.250 (±0.5) which correspond, approximately, to 1.5×10^{8} Colony Forming Units (CFU)/mL. Biofilms were formed by inoculating 15 µL of this suspension into 1 mL of TSB supplemented with 0.4% (v/v) glucose (TSBG), and

incubated in a 24-well plate (Orange Scientific, Braine-l'Alleud, Belgium) at 37°C and 120 rpm. Twenty-four hours later, spent medium was removed and the biofilms were washed twice with phosphate buffered saline (PBS, Boston Bioproducts, MA, USA). One mL of fresh TSBG was then carefully added to allow additional 24 hours growth in the same conditions. Planktonic cultures were prepared by adding 150 µL of a bacterial suspension with OD_{640nm} of 0.250 (±0.5) into 10 mL TSBG, and incubated for 24 hours at 37°C and 120 rpm.

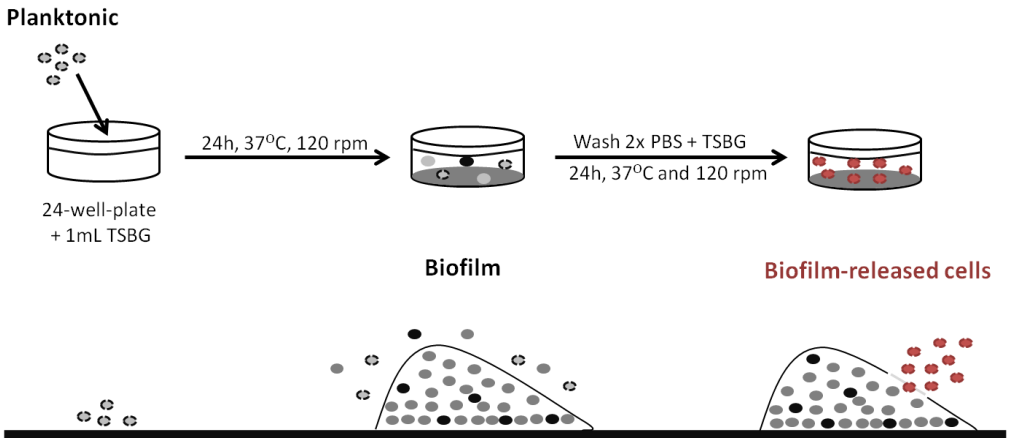

Figure 4.1. Schematic fed batch model used to collect *S. epidermidis* biofilm-released cells.

Prior to any of the analysis described below, biofilm-released cells were collected by careful aspiration of the biofilm culture supernatants; biofilms were washed twice with PBS, and then suspended in 1 mL of TSB (for antibiotic assays) or PBS (for the other studies) by scraping the cells from the plastic surface. Afterwards, planktonic, biofilm and biofilm-released cells were sonicated for 15 seconds at 7 Watt (VC600, Sonics, CT, USA) in order to dissociate cell clusters and create a homogenous suspension. The viability of the suspended cells was not reduced by this methodology, as determined before [12].

Total proteins extraction

Total proteins extraction of the different *S. epidermidis* cell preparations was performed by using lysostaphin digestion as described elsewhere [13], with some modifications. In brief, each population was grown and processed as described above. Afterwards, the OD_{640nm} of each population was adjusted to 1, which corresponds to approximately 1×10^9 CFU/mL, and 2 mL of each suspension harvested by

centrifugation for 10 minutes at 16000 *g* and 4°C. Pellets were washed twice with PBS, and finally suspended in 200 µL of PBS supplemented with 300 µg/mL of lysostaphin (Ambicin® L, AMBI, Inc., NY, USA) and a tablet of protease inhibitor cocktail (Roche, IN, USA). This suspension was incubated for 90 minutes at 37°C with rocking. After the lysostaphin digestion, the suspensions were centrifuged for 15 minutes at 8000 *g* and 4°C to remove protoplasts. The supernatants were then transferred into a new tube and treated with Turbo™ DNase (Ambion, CA, USA) for 30 minutes at 37°C. Finally, proteins were recovered with acetone precipitation, adding 4 volumes of ice-cold acetone to the suspension, and followed by an overnight incubation at -20°C. Proteins were recovered by 30 minutes centrifugation at 16000 *g* and suspended in 100 µL of PBS. Total proteins were quantified using Nanodrop™1000 (Thermo Scientific, MA, USA) and the concentration between samples normalized to 30 µg, in NuPAGE® LDS (Novex®, CA, USA) sample buffer and NuPAGE® Sample Reducing Agent (Novex®). The samples were boiled for 5 minutes and then loaded into NuPAGE® Novex 4-12% Bis-Tris Gel (Novex®) that was immersed in MEPs buffer (Novex®). Electrophoresis was carried out at 120 Volts for 60 minutes. The gel was stained with Bio-Rad silver stain (Bio-Rad, CA, USA) following the manufacturers' instructions.

RNA extraction

Total RNA extraction was performed by using FastRNA® Pro Blue (MP Biomedicals, CA, USA) kit with small modifications, as described before [14]. In brief, the different *S. epidermidis* populations were harvested by centrifugation for 10 minutes at 16000 *g* and 4°C. Bacterial pellets were then suspended in 850 µL of RNApro™ Solution and transferred into lysing matrix B tubes (supplied by the kit). The lyses was then carried out using the Fast Prep™ cell disruptor FP120 (Thermo Scientific) at 6.5 meter/second for 35 seconds. This cycle was repeated 3× with intervals of 5 minutes on ice. The tubes were then centrifuged, supernatants transferred into a new tube, mixed with 300 µL of chloroform (Sigma, MO, USA), incubated at room temperature (RT) for 5 minute and finally, centrifuged for 5 minutes at 4°C and 16000 *g*. This step was repeated twice in order to obtain higher purity. The aqueous phase was then carefully transferred into a new tube, mixed with cold 100% ethanol and incubated at -20°C for at least 30 minutes. RNA was recovered by 30 minutes centrifugation at 16000 *g* and 4°C. RNA pellets were washed twice with 75% ethanol and suspended in 50 µL of DEPC-treated water. In order to remove any co-purified genomic DNA, one step of DNase treatment (Fermentas, Ontario, Canada) was performed following the manufacturer's instructions. RNA quantity (ng/µL) and purity (A_{260}/A_{280} and A_{260}/A_{230}) were assessed using Nanodrop™ 1000 (Thermo Scientific) spectrophotometer. RNA integrity was determined

by electrophoresis loading 1-2 µg of RNA sample into a 1.5% agarose gel and run at 80 Volts for 60 minutes in 1 × Tris Acetate-EDTA buffer. The bands were reveled with ethidium bromide (Fisher Scientific, PA, USA) and images were taken using the Gel Doc 2000 (Bio-Rad).

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was used to evaluate the presence of PNAG on the surface of the three *S. epidermidis* populations, as elsewhere described [15,16]. Briefly, cells were washed twice with PBS and a 10 µL aliquot of each cell suspension was air-dried onto a glass slide. Bacteria were fixed to the slide with methanol for 1 min at RT. At this point *S. epidermidis* cells were incubated for 2 hours at RT with antibodies anti-PNAG, human monoclonal antibody F598 conjugated to Alexa Fluor 488 at 5.2 µg/mL, in PBS with 0.5% bovine serum albumin (BSA) and 10% NRS. As controls we included *S. epidermidis* samples labeled with a human alginate-specific monoclonal antibody F429 conjugated to Alexa Fluor 488 at 5.2 µg/mL. After incubation, samples were washed 3× with PBS and further incubated for 1 and 2 hours, at RT with, respectively, a secondary donkey anti-goat IgG conjugated to Alexa Fluor 568 (Invitrogen, NY USA) diluted 1:250 in PBS 0.5% BSA, and the nucleic acid stain Syto 83 (5mM) (Invitrogen) at a final concentration of 5 µM. Samples were washed twice with PBS, and mounted with Mowiol mounting media and a glass coverslip. Slides were observed with a Zeiss LSM 5 Pascal confocal inverted microscope (Oberkochen, Germany) equipped with an Argon 488 nm laser, a HeNe1 543 nm laser, and a HeNe2 633 nm laser. Samples were viewed with a Plan Apochromat 63×/1.4 oil objective and data analyzed with Zeiss LSM Imaging software.

Quantitative PCR

Quantitative PCR (qPCR) analysis was performed as described in Chapter 2 with minor alterations. In brief, total RNA was reverse transcribed into complementary (c) DNA using the iScript^{TM} cDNA sysnthesis kit (Bio-Rad). The primers used for qPCR experiments were designed using Primer3 software [17] having *S. epidermidis* RP62A complete genome (PubMed accession number NC_002976.3) or ATCC 12228 (for *psm*β, PubMed accession number NC_004461.1) as a template (Table 4.1). The run was performed using CFX96^{TM} thermal cycler (Bio-Rad) with the following cycling parameters: 10 minutes at 95^{o}C followed by 40 repeats of 5 seconds at 95^{o}C, 10 seconds at 60^{o}C and 20 seconds at 72^{o}C, using 5 µL of iQ^{TM} SYBR

Green Supermix (Bio-Rad), 0.5 µL of each forward and reverse primers at 10 µM, 2 µL of ultrapure water, and finally, 2 µL of 1:100 diluted cDNA. The data analysis was based on 3 independent experiments.

Table 4.1. Oligonucleotide sequence of the primers used in this study. Bp-base pair.

Target gene		Primers sequence (5' to 3')	TM (^{o}C)	Amplicon size (bp)
16S	FW	GGGCTACACACGTGCTACAA	59.79	176
	RV	GTACAAGACCCGGGAACGTA	59.85	
icaA	FW	TGCACTCAATGAGGGAATCA	60.20	134
	RV	TAACTGCGCCTAATTTTGGATT	59.99	
atlE	FW	GTAGATGTTGTGCCCCAAGG	60.38	180
	RV	TGGAAGAGGAACAGTTTGGAC	59.17	
***psm*β**	FW	AGCAGAAGCTATTGCAAATACAG	57.96	105
	RV	CCTAATACGCTAACGCCACTTT	59.72	

Antibiotic susceptibility

The antibiotics and respective concentrations used in this study were: 40 mg/L of the cell wall synthesis inhibitor vancomycin (Sigma), 16 mg/L of the protein inhibitor tetracycline (Research Products International Corp., IL, USA), and 10 mg/L of the DNA-dependent RNA polymerase enzyme inhibitor rifampin (Fisher Scientific). The antibiotic concentration used was the peak serum concentration determined for each antibiotic [17]. Briefly, the different *S. epidermidis* populations were collected and processed as described above. A dilution was made in TSB in order to obtain an OD_{640nm} of 0.4 that corresponds to 3×10^8 Colony Forming Units (CFU)/mL, and then diluted 10× in order to obtain approximately 3×10^7 CFU/mL. Afterwards, these suspensions were diluted 1:2 in fresh TSB containing each antibiotic and allowed to grow up to 6 hours at 37^{o}C and 120 rpm. A control was obtained by diluting the suspension in fresh TSB without adding antibiotic. One mL of each sample was collected after 2 and 6 hours of incubation, centrifuged for 10 minutes at 16000 *g* and 4^{o}C, the pellet washed twice, and finally suspended into 1 mL of PSB 0.05% Tween20 (PSBT) (Boston BioProducts). These suspensions were sonicated for 15 seconds at 7 Watt and then vortexed at maximum speed for 10 seconds. The number of viable cells upon antibiotic exposure were quantified by performing 10-fold serial dilutions in PBST and

plated onto Trypticase™ Soy Agar 5% sheep blood (TSAsb) plates (Becton Dickinson, NJ, USA). Plates were incubated for 48 hours at 37 °C. This experiment was repeated at least 5 times.

Normal human serum collection

Fresh human blood was collected from healthy adult volunteers under the 1999-P-001173/48 (BWH Legacy #: 84-01009) protocol approved by the Partner's Health Care System Institutional Review Board (Boston, MA, USA). All donors gave written informed consent to have blood taken. Blood was then transferred to BD Vacutainer® tubes (Becton Dickinson) and centrifuged at 13000 *g* and 4°C for 30 minutes. Normal human serum (NHS) was then collected and stored at -80°C until further use.

Initial adhesion quantification

The initial adhesion ability of the biofilm-released cells comparatively to their planktonic and biofilm counterparts was determined using abiotic surfaces and assessed over the time, as described before [18]. Sterile acrylic (2 × 2 cm) and silicone (2 × 2 cm) surfaces were placed onto 6-well tissue culture plate (Orange Scientific), and covered with 4 mL of each bacterial suspension at 1×10^7 CFU/mL. In the case of glass surfaces (Ø1cm), these were placed into 24-well tissue culture plate and covered with 0.3 mL of each bacterial suspension at the same concentration. In order to determine the effect of surface coating by host matrix proteins in the adhesion of the biofilm-released cells, glass surfaces were incubated with NHS. In brief, sterile glass surfaces were covered with 0.1 mL of 10% NHS diluted in PBS, and incubated for 15 minutes at RT [19]. The surfaces were then washed twice with PBS to remove unbound proteins. Coated or uncoated surfaces were incubated for 10, 30 or 60 minutes at 37°C and 120 rpm. After incubation, the surfaces were washed twice in PBS to remove non-adhered cells and stained, for 10 minutes, with 2.5 µg/mL of 4,6-diamino-2-phenylindole (DAPI). The adhered cells were observed under an Olympus BX51 epifluorescent microscope equipped with a CCD color camera DP71 (OLYMPUS,PA, USA) which acquires images with 1360 × 1024 pixel resolution at a magnification of 200×. For each surface, at least 10 TIFF images were taken randomly over the entire surface. The enumeration of the adhered cells *per* cm^2 of surface was determined using the image analysis automated enumeration software (SigmaScan Pro 5.0, Systat Software, Sigma). In these conditions, 18420 ± 1575 pixels were equivalent to 0.0025 cm^2 [20]. The experiment was repeated at least twice and with 2 technical replicates.

Opsonophagocytic killing assay

Opsonophagocytic killing assays were performed as described elsewhere [21], with some minor alterations. The Human promyelocytic leukemia cell line HL-60 (American Type Culture Collection, VA, USA) was differentiated into neutrophils in the presence of 0.8% of dimethylformamide (Sigma) for 5 to 6 days at 37^{o}C and 5% CO_2. Using trypan blue staining (Sigma), to differentiate dead from live cells, the final HL-60 count was adjusted to 1 × 10^8 cells/mL. Bacterial suspensions of each *S. epidermidis* populations were adjusted with TSB to 3 × 10^8 CFU/mL and serial diluted in order to obtain 1 × 10^6 CFU/mL. NHS was used at concentration of 10%. All the dilutions were performed in *Roswell Park Memorial Institute* (RPMI) (Gibco, NY, USA) supplemented with 1% bovine serum albumin (American BioAnalytical, MA, USA) and 10 mM of HEPES (Gibco) (designed as complete RPMI). In Brief, the assay mixture contained 100 µL of HL-60 (at a concentration of 1x10^8 cells/mL), 100 µL of each bacterial suspension (at a concentration of 1×10^6 CFU/mL), 100 µL of a 10% NHS and 100 µL of complete RPMI. Tubes with bacteria only, bacteria plus HL-60, and bacteria plus 10% NHS were used as controls and for validation of the assay. The reaction mixture was incubated on a rotor rack at 37°C for 90 min. After incubation, the tubes were sonicated for 10 seconds at 7 Watt followed by 10 seconds vortex at maximum speed. The suspensions were serial diluted in PSBT and plated onto BBL™ Trypticase™ Soy Agar with 5% Sheep Blood (TSA II) (Becton Dickinson). The percentage of the surviving bacteria was calculated by determining the ratio of the CFU/mL surviving in the test tubes and the control tube with bacteria only. This experiment was performed three independent times with technical triplicates.

Mice

Female BALB/c mice were purchased from Charles River (Barcelona, Spain) and kept under specific-pathogen-free conditions at the Animal Facility of the Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), the 86/609/EEC directive, and Portuguese rules (DL 129/92).

Challenge infections

The bacterial inocula of the different *S. epidermidis* populations were adjusted to 5 × 10^8 cells/mL in a flow cytometer using counting beads and SYBR Green I /propidium iodide staining to differentiate

between live and dead bacteria, as described previously [12]. Female BALB/c with 8-10 weeks were injected intravenously (i.v.) in the lateral tail vein with 1×10^8 of planktonic, biofilm or biofilm-released cells in 0.2 mL PBS. Control mice were injected with 0.2 mL of PBS. Each challenge was then confirmed by plating the inoculum in Trypticase™ Soy Agar plates (TSA, Becton Dickinson).

Bacterial dissemination assessment

Two, 6 and 14 hours post-infection, liver and spleen were aseptically removed, homogenized mechanically in 3 mL of PBS, and quantitatively cultured on TSA plates (Bencton Dickinson). Plates were then incubated at 37°C for approximately 20 hours.

Cytokine/ chemokine quantification

Interleukin (IL)-6 (eBioscience, CA, USA) and the chemokines KC (CXCL1) and MCP-1 (CCL2) (R&D DuoSet®, MN, USA) were quantified following the manufacturer's instructions.

Statistical analysis

Statistical significance between groups was evaluated by either one-way ANOVA with Tukey's multiple-comparisons test or two-way ANOVA with Bonferroni post test. Percentage values were previously transformed to arcsin and then analyzed by the appropriated statistical analysis test. All tests were performed with the GraphPad Prism version 5 (GraphPad Software, CA, USA). Differences between groups were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

The total protein profile of biofilm-released cells resembles biofilm phenotype

The evaluation of the total protein profile is one quick method to assess genotypic and phenotypic differences between bacterial populations. By using this approach, we could observe that *S. epidermidis* biofilm-released cells present more similarities to biofilm than to their planktonic cell counterparts (Figure 4.2). The more evident differences/similarities were detected in protein bands with apparent

molecular mass between 14 and 3 kDa. Besides the clear qualitative differences, these small protein bands are present in higher amounts in biofilm-released than in planktonic or even biofilm cell extracts. Moreover, biofilm-released cells present a unique protein band with approximately 14 kDa. In order to correlate this unique protein with possible higher or lower virulence potential, a bioinformatics analysis using ExPASy, TagIdentTool [22], based in the molecular weight of the protein was performed.

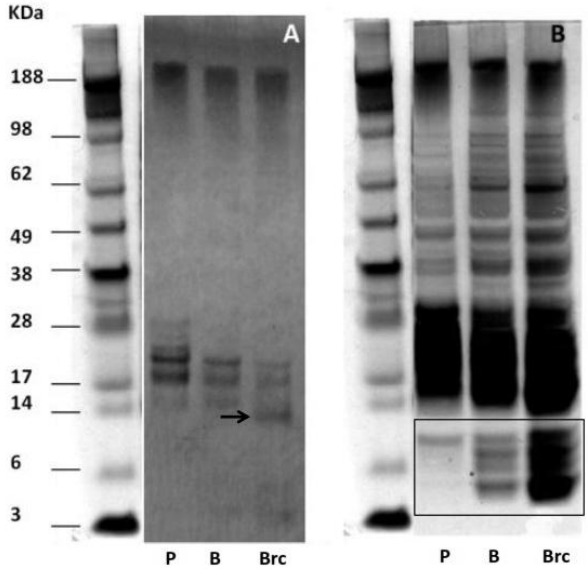

Figure 4.2. Total protein profile of *S. epidermidis* populations. Protein band migration profile of total protein cell extracts obtained from planktonic (P), biofilm (B) or biofilm-released cells (Brc) using lysostaphin digestion. Samples were loaded (30 µg protein/lane) into 4-12% Bis-Tris gel that was stained using Bio-Rad silver stain. **(A)** 5 minutes, **(B)** 15 minutes of developing process. The row indicates the unique protein presented in the biofilm-released cells and the square the group of proteins with clear qualitative and quantitative differences between the populations.

This analysis suggested that this protein belongs to a group of ribosomal proteins involved in translation or rRNA and tRNA processing, toxin-antitoxin module, holin-like protein, glycine cleave system H, Initiation-control protein YabA, protein ArsC or VraC, ribosome-binding factor A. The protein may also belong to a group of uncharacterized proteins. However, due to the high number of possible functions, the isolation and identification of this protein should be done in the future, as it may be a possible target of anti-microbial strategies to be used against the infections developed by disassembled cells.

The expression of biofilm-related genes presented by biofilm-released cells is shared with their planktonic and biofilm counterparts

To determine the involvement of *S. epidermidis* biofilm lifecycle-associated genes in the phenotype of the biofilm-released cells and evasion from the immune system, we have quantified the expression of the autolysin *atlE,* that mediates the adhesion to biotic and abiotic surfaces, as well as cell lyses and consequent release of DNA that acts as adhesive molecule [23,24]; *icaA*, that encodes one of the enzymes involved in PNAG synthesis, which is a key molecule in biofilm accumulation [25] and immune evasion [21,26,27]; *psm*β that is involved in biofilm disassembly [1,6] and, finally, *rsbU,* a positive regulator of biofilm formation [28,29]. As shown in Figure 4.3, significant differences in genetic expression were found between the populations.

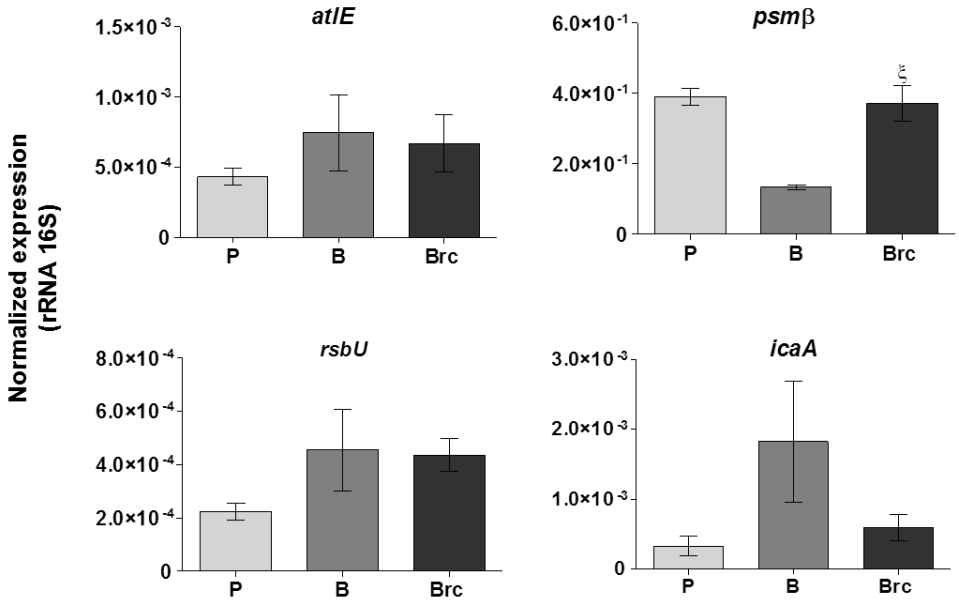

Figure 4.3. Expression of genes involved in *S. epidermidis* biofilm lifecycle. Normalized expression values were calculated using 16 rRNA ribosomal subunit by applying the $2^{\Delta Ct}$ mathematical model. The bars represent mean plus or minus standard error of the mean of 3 independent experiments. Statistical differences between groups were analyzed with one-way ANOVA and Tukey's multiple comparisons test. $^{\xi}p<0.05$ when comparing biofilm-released with biofilm phenotype.

When analyzing biofilm-released cells gene expression profile, we observed, in the case of the *psm*β, that these cells produce as much modulin as their planktonic counterparts, both twice as more as biofilm-derived cells (Figure 4.3). In accordance with our results, Wang and his collaborators (2011) have shown

that cells in the effluent of biofilm cultures expressed higher levels of *psm*β suggesting that *psm*β expression leads to biofilm cluster disassembly during biofilm development. This indicates that in our study bacteria were released from the biofilm through an active mechanism of disassembly [1]. Using a foreign body infection mice model, it was shown that *psm*β play an essential role in biofilm virulence, since biofilm formed by *S. epidermidis psm*β isogenic mutant on the surface of catheters, presented less ability to disseminate into the host organs [1]. In the case of *atlE* and *rsbU* expression, biofilm-released cells clearly resemble the biofilm cells, while in the case if *icaA* gene expression, biofilm-released cells display the same trend as planktonic cells, even though no statistically significant differences were found.

Biofilm-released cells, present lower amount of PNAG on the surface

PNAG is known to be involved in both biofilm accumulation [25] and immune evasion [21,26,27]. Therefore, higher or lower amounts of PNAG on the bacterial surface can affect virulence. When cells are released from the biofilm they are more exposed to host immune effectors. In this situation, the PNAG attached to the bacterial surface is crucial for protection against the action of antimicrobial peptides (AMPs) [30,31], and the deposition of antibodies and complement factors that will help the phagocytic process [26]. CLSM was thus performed to assess the presence or absence of PNAG on the surface of biofilm-released cells. Although CLSM analysis was used as a qualitative tool, it was able to show interesting differences between populations (Figure 4.4). Using the monoclonal antibody anti-PNAG, mAb F598, we were able to visualize that both biofilm-released (Figure 4.4 C1) and planktonic cells (Figure 4.4 A1) present only basal levels of PNAG. However, it is clear that biofilm cells present higher distribution of the PNAG on their surface (Figure 4.4 B1), which may suggest less susceptibility to phagocytosis and AMPs. As shown in the Figure 4.4 panel 4, corresponding to the control sample, no cross reactivity was observed when the different *S. epidermidis* populations were incubated with a mAb specific for the alginate capsule of *Pseudomonas aeruginosa.*

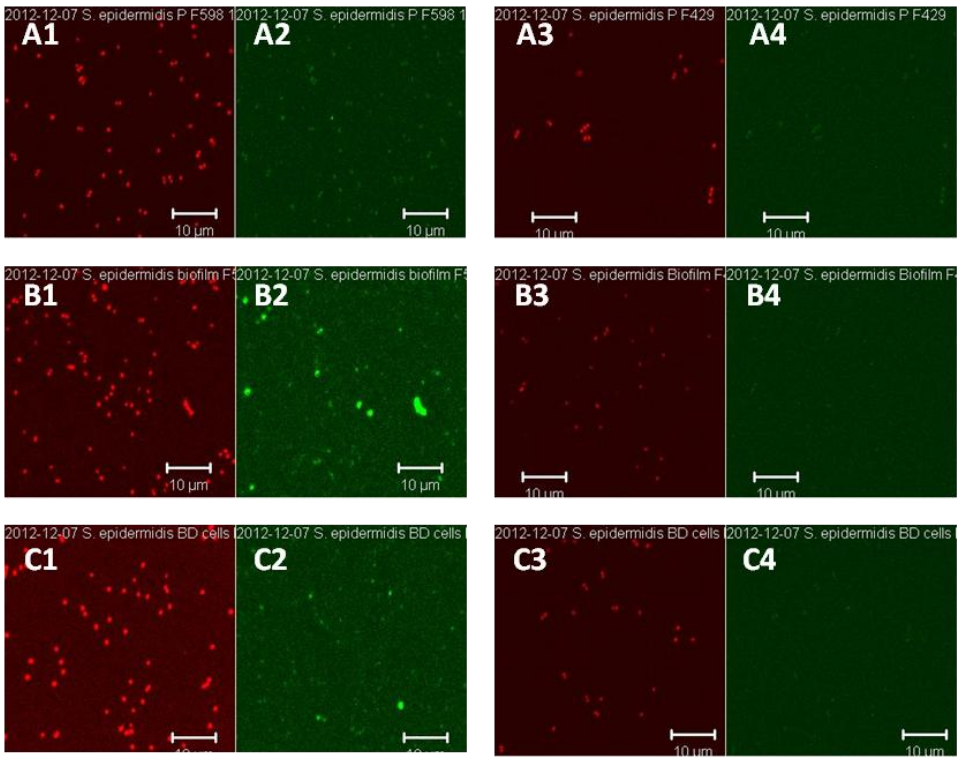

Figure 4.4. Confocal Laser Scanning Microscopy imaging of PNAG expression on the surface of planktonic (A), biofilm (B) and biofilm-released (C) cells. *S. epidermidis* cells were labeled with either mAb F598 to PNAG or mAb F429 to alginate, both conjugated to Alexa Fluor 488 (green). The panels 1 and 3 represent the binding of the nucleic acid stain SYTO 83 (red). The panel 2 represents the binding of the mAb F598 and the panel 4 the binding of the control mAb F429. Bar = 10 µm.

Biofilm-released cells show higher tolerance to tetracycline than planktonic growing cells

Considering the known differences in antibiotic susceptibility between planktonic and biofilm cultures, we have tested the antibiotic susceptibility of biofilm-released cells to peak serum concentrations of rifampin, tetracycline and vancomycin (Figure 4.5) [17]. The delta $\log_{10}$ CFUs/mL unit-reduction values presented 2 and 6 hours after exposure to antibiotics were calculated relatively to time zero. Interestingly, 2 and 6 hours after exposure to tetracycline, we observed that biofilm-released cells presented significant higher tolerance to tetracycline than their planktonic counterparts, showing only a 0.7 $\log_{10}$ CFU/mL unit-reduction versus the 2.2 $\log_{10}$ CFU/mL unit-reduction presented by planktonic cells. Six hours post-exposure, biofilm-released cells showed 1.7 $\log_{10}$ CFU/mL unit-reduction against the 2.5 $\log_{10}$ CFU/mL unit-reduction showed by planktonic cells.

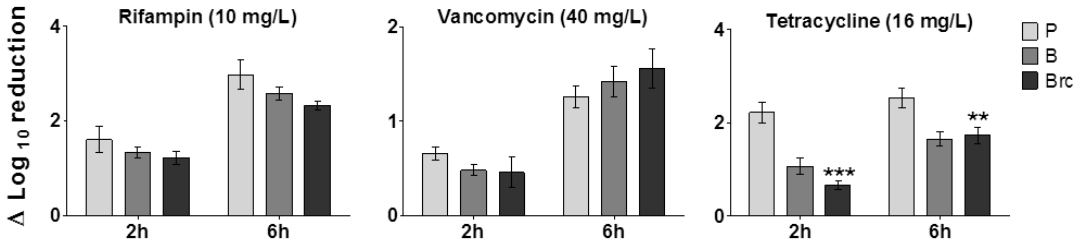

Figure 4.5. Base 10 logarithmic CFU/mL unit-reduction of *S. epidermidis* populations upon incubation with antibiotics. The 3 different populations were incubated for 2 and 6 hours with peak serum concentrations of rifampin, vancomycin and tetracycline. The columns represent the mean plus or minus standard error of the mean of 5 to 6 independent experiments. Statistical differences between groups were analyzed with two-way ANOVA and Bonferroni post test. ** $p<0.01$; ***$p<0.001$ when comparing biofilm-released with their planktonic counterparts. P-planktonic; B-biofilm; Brc-biofilm-released cells.

These results were different from what was shown previously for *Staphylococcus aureus* [32], where biofilm-released cells were as sensitive as planktonic cells to rifampin. Nevertheless, similar to our results, cell released from *Streptocccus mutans* biofilms showed to be more resistant to chlorhexidine than their planktonic counterparts [10]. The enhanced tolerance presented by the cells released from biofilms over their planktonic counterparts may have an important impact in the efficacy of both prophylactic and therapeutic approaches that do not account with this fact, leading to the decrease of its efficiency. Although this analysis have provided important insights in the tolerance of the biofilm-released cells to a representative group of antibiotics (cell wall, protein and RNA synthesis inhibitors), an array of different antibiotics should be tested in order to better understand the tolerance presented by these cells, and its consequence in the virulence of *S. epidermidis* biofilm-related infections.

Biofilm-released cells do not adhere better to abiotic surfaces than biofilm or planktonic cells

Taking into consideration the hypothesis that biofilm-released cells present higher potential to colonize other regions of the host, we assessed the initial adhesion ability of the 3 populations to different abiotic surfaces, uncoated (acrylic, glass and silicone) or coated (glass surfaces) with human serum proteins. The number of adhered cells *per* cm^2 of surface is presented in the Figure 4.6. Planktonic, biofilm and biofilm-released cells presented similar ability to adhere to any of the uncoated abiotic surfaces tested, independently of its hydrophobicity, a parameter known to affect initial adhesion [18].

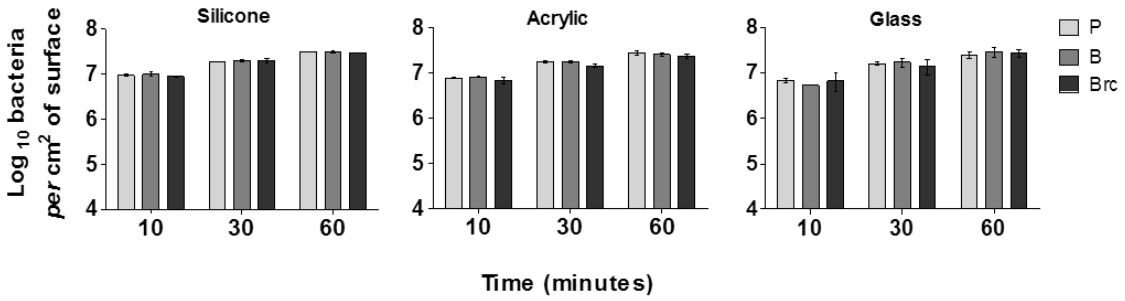

Figure 4.6. Number of base 10 logarithmic *S. epidermidis* cells adhered *per* cm^2 of silicone, acrylic and glass surfaces. The bars represent the average plus or minus the standard error of the mean of 3 independent experiments. Statistical differences between groups were analyzed with two-way ANOVA with Bonferroni post test. P-planktonic; B-biofilm; Brc-biofilm-released cells.

Additionally, in order to verify if a conditioning film composed of human serum proteins could provide an advantage for bacterial initial adhesion, glass surfaces were coated with 10% NHS and incubated with each bacterial suspension for 30 minutes. However, despite influencing the overall adhesion ability shown by all the populations, no significant differences were found between the 3 populations adherence to coated surface (Figure 4.7).

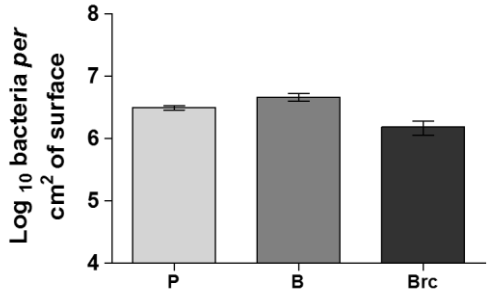

Figure 4.7. Number of base 10 logarithmic *S. epidermidis* cells adhered *per* cm^2 glass coated surface upon 30 minutes of incubation. The bars represent the mean plus and minus the standard error of the mean of 3 independent experiments. Statistical differences between groups were analyzed with one-way ANOVA with Tukey's multiple comparison test. P-planktonic; B-biofilm; Brc-biofilm-released cells.

Clearly all the populations present the same ability to adhere to both coated and uncoated surfaces, indicating no advantage of biofilm-released cells over the other phenotypes to adhere. Since initial adhesion studies are highly variable depending on the testing conditions, [33], we repeated the experiment with two more different initial inocula concentration (1×10^5 and 4×10^7), and two different

adhesion times (10 and 180 min). Again, no differences between the 3 populations were found (data not shown). However, we cannot rule out that different conditions could provide different results.

Biofilm-released cells are as sensitive to oposonophagocytic killing as planktonic cells

It was previously shown that biofilm cells are more resistant to oposonophagocytic killing than their planktonic counterparts [21]. Hence, we aimed to further assess possible differences in virulence potential of biofilm-released cells using an *in vitro* opsonophagocytic killing assay. The percentage of surviving CFU/mL of each population upon incubation with 10% NHS and the human promyelocytic leukemia cell line HL-60 was compared with CFU/mL obtained in tubes containing bacteria only. The other controls performed, bacteria plus 10% NHS or plus HL-60, showed no unspecific killing during the assay. As represented in Table 4.2, biofilm-released cells seem to be as sensitive as their planktonic counterparts to opsonophagocytic killing, when compared with biofilm cells, which presented, as expected, higher tolerance to the stress created by the presence of NHS and HL-60 cells. These results suggest that biofilm-released cells, as planktonic cells, may be less protected against opsonophagocytic killing than biofilms-derived cells, which is probably related to the lower contents of PNAG observed on the surface of these cells.

Table 4.2. Opsonophagocytic susceptibility of the different *S. epidermidis* populations in the presence of 10% NHS and the human cell line HL-60. The percentage of surviving bacteria presented is relative to the control (only bacteria after 90 minutes of incubation at 37^{o}C) plus and minus standard error of the mean. Statistical differences between groups were analyzed with two-way ANOVA with Bonferroni post test. $\xi\xi p<0.01$ when comparing biofilm-released with biofilm cells. P-planktonic; B-biofilm; Brc-biofilm-released cells.

	Percentage of surviving bacteria		
	P	**B**	**Brc**
Bacteria + 10% NHS	136.43 ± 31	124.20 ± 11	124.66 ± 17
Bacteria + HL-60	121.33 ± 14	112.50 ± 19	97.73 ± 13
Bacteria + HL-60 + 10% NHS	17.00 ± 1.6	26.00 ± 2.6	13.00 ± 0.56$^{\xi\xi}$

Biofilm-released cells stimulate a unique response from the host immune system

In order to explore the virulence of biofilm-released cells comparatively to their planktonic and biofilm counterparts, an intravenous mouse infection model was used. Bacterial dissemination into systemic

organs such as liver and spleen, and thus the ability to colonize and persist in the host was assessed 2, 6 and 14 hours after infection (Figure 4.8). The overall results showed that irrespectively to the phenotype, *S. epidermidis* populations are quickly cleared from the host as observed by the decrease in CFU levels recovered over the time-course of infection. Analyzing in more detail the bacterial load in both the liver and spleen, it can be observed that biofilm-released cells, despite the differences found, present a colonization profile very similar to planktonic cells. However, with the time-course of infection is it clear that these cells become different from both planktonic and biofilm counterparts, showing an intermediary colonization and persistence pattern.

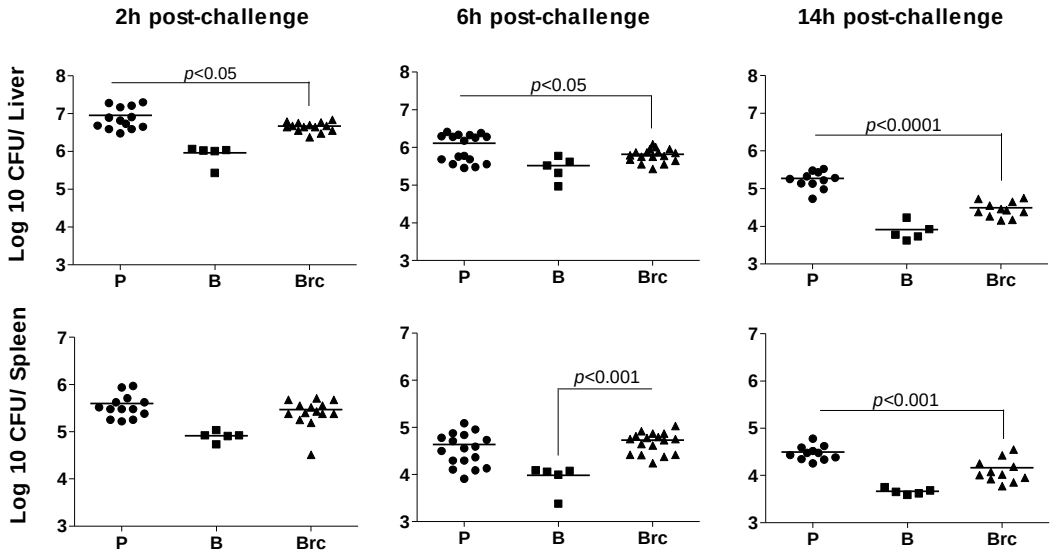

Figure 4.8. Number of base 10 logarithmic CFU recovered from both liver and spleen of BALB/c mice infected i.v. with 1 × 10^8 cells of *S. epidermis* planktonic, biofilm and biofilm-released cells, 2, 6 or 14 hours post-challenge. Each dot represent the value of an individual animal and the longitudinal line the average of 1 (biofilms) to 3 independent (planktonic and biofilm-released cells) experiments. Statistical differences between groups were analyzed with one-way ANOVA with Tukey's multiple comparison test. P-planktonic; B-biofilm; Brc-biofilm-released cells.

In addition to evaluate the colonization ability, we also aimed to assess if the different *S. epidermidis* populations could distinctly stimulate the host immune system, by quantifying the systemic signals of inflammation, and the activation and recruitment of macrophages and neutrophils, which are considered the first line of defense against bacterial infection. To achieve that, the concentration of IL-6 and the MCP-1 and KC chemokines was determined in the serum of infected mice (Figure 4.9).

As can be observed, the challenge by all the *S. epidermidis* populations stimulated a significant increase in the production of IL-6, KC and MCP-1 when compared with the control (non-infected group).

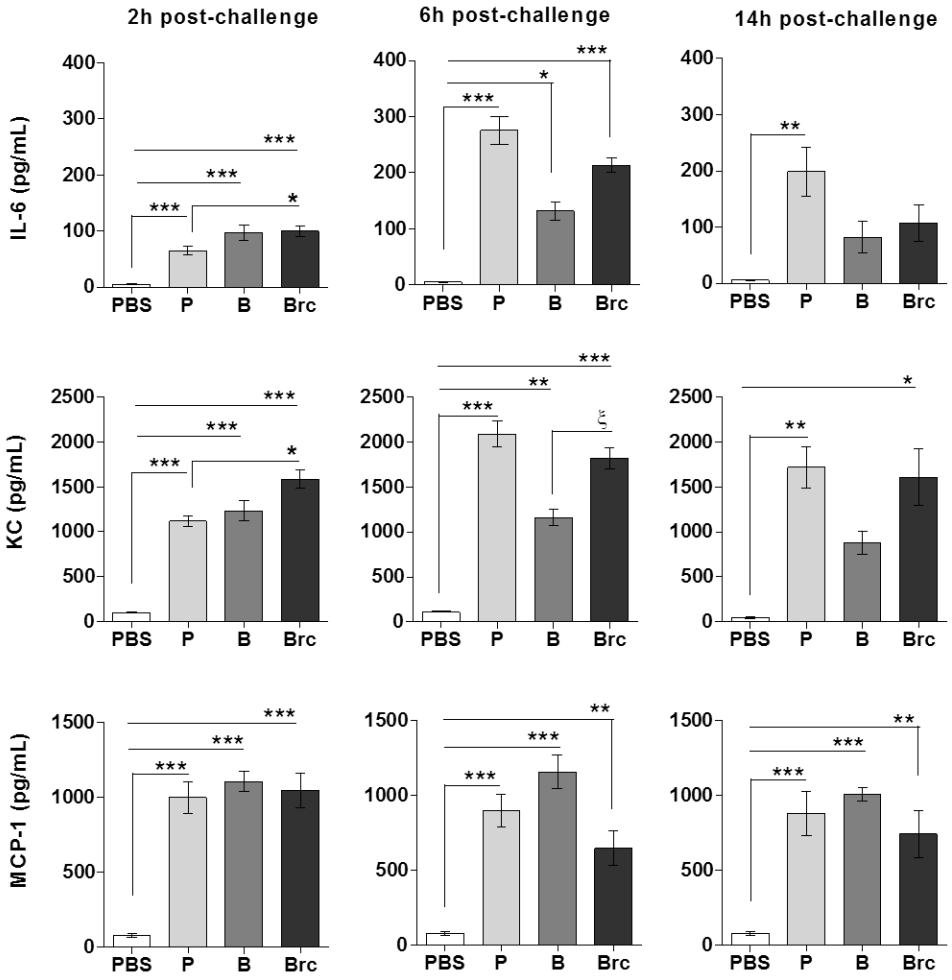

Figure 4.9. The level of the IL-6 (top) and the chemokines KC (middle) and MCP-1 (bottom) of BALB/c mice infected i.v. with 1 × 10^8 cells of *S. epidermis* planktonic, biofilm and biofilm-released cells, 2, 6 or 14 hours post-challenge. The bars represent the average plus or minus the standard error of the mean of 1 (biofilm-derived cells) to 3 independent experiments (planktonic and biofilm-released cells). Statistical differences between groups were analyzed with one-way ANOVA with Tukey's multiple comparison test. *p<0.05; **p<0.01; ***p<0.0001 when comparing all the *S. epidermidis* populations with the control PBS or when comparing biofilm-released cells with their planktonic counterparts. $^{\xi}p$<0.05 when comparing biofilm-released with biofilm phenotype. PBS-phosphate buffered saline; P-planktonic; B-biofilm; Brc-biofilm-released cells.

However, over the time-course of the infection some particularities associated with *S. epidermidis* biofilm-released cells were observed. Despite similar levels of IL-6 were induced by any of the

populations, a significantly higher IL-6 concentration was detected 2 hours after the i.v. challenge, in the sera of mice infected with biofilm-released cells comparatively to their planktonic counterparts. However, 6 and 14 hours post-infection, similar levels of IL-6 to those observed in their biofilm counterparts were detected, both lower than those detected in the sera of planktonic-infected mice. As shown in Figure 4.9, biofilm-released cells stimulated a higher production of KC than the planktonic population as detected 2 hours post-infection. However, 6 and 14 hours post challenge, mice infected with planktonic cells reached the same stimulation levels as mice infected with biofilm-released cells, both presenting higher levels of KC than biofilm-derived cells. Interestingly, no significant differences in the serum levels of the monocyte chemoattractant MCP-1 were observed between mice infected with either of the three *S. epidermidis* populations used. These results, altogether, show that biofilm-released cells present features distinct from the other known phenotypes. However, although these results provide interesting and important insights into the virulence of biofilm-released cells, a more detailed study is needed to better understand the persistence in the host organs, as well as the type of immune response elicited by biofilm-released cells and hence, its implications in *S. epidermidis* biofilm infection virulence.

CONCLUSIONS

The characterization of phenotypic features, as well as the characterization of the biofilm-released cells interaction with the host immune system, is of crucial importance to prevent the serious pathologic events associated with the biofilm cells dissemination. Therefore, by characterizing *S. epidermidis* biofilm-released cells virulence through several important parameters, we were able to advance the knowledge on this topic. Under our experimental conditions, *S. epidermidis* biofilm-released cells present different phenotypic features than the ones presented by either biofilm or planktonic cells, with an impact in their virulence potential. The most striking difference observed between the populations is the increased resistance to tetracycline shown by *S. epidermidis* biofilm-released cells. This resistance may have important consequences in the efficacy of prophylactic and therapeutic measures based in antibiotics that act in protein synthesis blockage, since the resistance observed seem to be related with the mechanism of action of the antibiotic. Although some authors claim that these cells will ultimately revert to the planktonic phenotype, more studies are needed to clarify that possibility. Furthermore, even if at longer time periods the disassembled cells would eventually reverted completely to the

planktonic phenotype, the result of this transient phenotype in virulence and bacterial survival should not be neglected.

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CHAPTER 5.

The use of anti-PNAG antibodies to inhibit *S. epidermidis* biofilms accumulation *in vitro*

ABSTRACT

Due to broad-spectrum antibiotics tolerance of bacteria within biofilms, the use of antibodies have been shown to be one promising alternative to target surface-attached molecules and inhibit biofilm formation. Because *S. epidermidis* biofilm accumulation is mainly mediated by PNAG, we have tested the ability of previously produced polyclonal or monoclonal antibodies anti-PNAG to inhibit *S. epidermidis* biofilm accumulation *in vitro,* using the standard crystal violet staining. Although the polyclonal antibodies 9GlucNH_2-TT and dPNAG-TT did reduce the biofilm accumulation *in vitro*, the control normal rabbit serum presented the same pattern of inhibition, indicating the presence of other factors in the serum able to inhibit biofilm accumulation in a PNAG-independent manner. In the case of the monoclonal antibody F598, the effect observed was clearly PNAG-dependent. However, depending on the *S. epidermidis* strain used, the monoclonal antibody F598 had differential effect, resulting in an inhibition or enhancement of the biofilm accumulation *in vitro*. Hence, this work have shown, that serum may present factors that effectively inhibit *S. epidermidis* biofilm accumulation and, on the other hand, that monoclonal antibodies should be tested in several strains in order to ensure that the pretended effect is transversal to several strains.

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BACKGROUND

Due to the ability of *S. epidermidis* to form biofilms on the surface of indwelling medical devices, these devices are a common source of serious biofilm-related infections [1]. Since *S. epidermidis* biofilms are highly tolerant to antibiotics [2] and to the host immune system effectors [3], the surgical removal of the infected devices is often required to resolve those infections [4] resulting in significant effects in a patient's quality of life, as well as an heavy burden to the public health system [5]. Hence, preventive approaches are clearly needed to overcome this challenge. Due to broad-spectrum antibiotics tolerance of bacteria within biofilms, among others, the use of antibodies have been shown to be one promising alternative to target surface-attached molecules and inhibit biofilm formation [6-8]. Since in most clinical *S. epidermidis* strains biofilm accumulation is mainly mediated by the polysaccharide poly-β-1,6-N-acetylglucosamine (PNAG) [9,10], it was hypothesized that the binding of this molecule by monoclonal or polyclonal antibodies anti-PNAG could impact biofilm accumulation. It was previously shown that human monoclonal antibodies (mAbs) and rabbit polyclonal antibodies (pAbs), specific for PNAG, were effective in killing *S. epidermidis* as well as other PNAG-producing bacteria in opsonophagocytic *in vitro* assays [3,11,12], and in protecting the murine host against these infections [13-15]. Nevertheless, the efficacy of these antibodies in inhibiting *S. epidermidis* biofilm formation *in vitro* has not previously been investigated. Hence, in this chapter, we describe the effectiveness of previously synthesized and characterized pAbs 9GlucNH_2-TT and dPNAG-TT, and mAb F598 to inhibit *S. epidermidis* biofilm accumulation *in vitro*.

MATERIALS AND METHODS

Bacteria and growth conditions

S. epidermidis RP62A, 1457, 1457-M10 and M184 were used in this work. One single colony of each strain was transferred from Trypticase™ Soy Agar 5% sheep blood (TSAsb) plates (Becton Dickinson, NJ, USA), not older than 2 days, into 2 mL of Tryptic Soy Broth (TSB) (Becton Dickinson), and incubated overnight at 37°C and 700 rpm (VorTemp™, Labnet International, NJ, USA). The overnight culture was then diluted 1:100 in fresh TSB for further experiments.

Polyclonal antibodies

The previous developed and synthesized pAbs raised against both naturally occurring and further de-acetylated PNAG (dPNAG) [16], and synthetic oligosaccharide of 9 monosaccharide units (9Glc-NH_2) [17], both conjugated with tetanus toxoid (TT) were used. Briefly, these antibodies were obtained by subcutaneous immunization of New Zealand White rabbits with 100 µg (dPNAG-TT) or 10-µg doses (9GlucNH_2-TT) of the polysaccharide emulsified in incomplete Freund's adjuvant. One week later the rabbits were injected intravenously, three times, with the antigen in saline solution, each injection spaced by 3 days. Rabbits were bled every 2 to 6 weeks, and serum was tested by ELISA using purified PNAG as the coating antigen [13]. Normal rabbit serum (NRS), previously tested for anti-PNAG immunoglobulin (Ig) contents, was used as a control.

Monoclonal antibodies

The previously developed and synthesized monoclonal antibody (mAb) IgG_1 F598 raised against PNAG [15] was used. In brief, B cells from a patient recovering from *Staphylococcus aureus* infection were transformed with Epstein-Barr virus and screened for their ability to bind either acetylated or de-acetylated PNAG. Ig variable region genes from hybridomas of interest were cloned into the IgG_1-TCAE6 vector and transfected into CHO cells for the production of fully human IgG_1 mAbs [15]. The mAb F429 raised against *Pseudomonas aeruginosa* alginate capsule was used as an isotype control and was developed and synthesized as described elsewhere [18].

Removal of endogenous immunoglobulin and inactivation of endogenous complement factors in polyclonal antibodies

Endogenous Ig present in both pAbs were removed by adsorption with a PNAG-negative strain, *S. epidermidis* 1457-M10 [19]. NRS was absorbed as well, however, using a PNAG-positive strain, *S. epidermidis* 1457, in order to remove both endogenous Ig and possible naturally occurring antibodies anti-PNAG. In brief, NRS and both pAbs were diluted 10 × in Minimum Essential Media (Gibco, NY, USA) supplemented with 1 % bovine serum albumin (Sigma, MO, USA) and incubated with bacterial suspension with an OD_{640nm}=1 for 30 minutes at 4°C with constant rocking. After incubation, bacteria were removed by 10 minutes centrifugation at 4°C and 16000 *g*. This procedure was repeated three times. Finally, in order to inactivate endogenous complement factors, both NRS and polyclonal

antibodies were incubated for 30 minutes at 56°C, and filtered through a Spin-X® Centrifuge tube with a 0.22 µm pore (Corning®Costar, NL, Mexico) to eliminate any remaining bacteria.

Biofilm inhibition assays using polyclonal antibodies

In order to test the ability of both dPNAG-TT and 9GlucNH_2-TT to inhibit biofilm accumulation, an overnight culture of *S. epidermidis* RP62A was diluted 1:100 in TSB, and 150 µL of this suspension distributed into 96-well tissue culture plates (Corning®Costar). Subsequently, absorbed and heat-inactivated pAbs or NRS were added to the appropriated wells, starting with 1:100 dilution and performing serial 2× fold dilutions until obtaining a 1:3200 dilution. The plates were then incubated statically at 37°C, for 1 hour, to allow antibody binding to the bacterium, and then placed at 250 rpm for 24 hours. The experiment was repeated twice and each pAb and NRS concentration evaluated in quadruplicates.

Biofilm inhibition assays using monoclonal antibody

In order to evaluate the ability of mAb F598 to inhibit *S. epidermidis* biofilm accumulation, overnight cultures of *S. epidermidis* RP62A, 1457, 1457-M10 and M184 were diluted and distributed as described for pAbs assays. Subsequently, 150 µL of the mAbs F598 or F429 previously diluted were added to the bacterial culture in order to obtain 333, 167, 67 and 6.7 nM of antibody in each appropriated wells. The plates were incubated, statically, at 37°C for 1 hour to allow antibody binding, and then incubated at 250 rpm, 37°C for 24 hour. The experiment was repeated twice and each mAb concentration evaluated in quadruplicates.

Biofilm quantification

Crystal violet staining was performed after 24 hours of co-culture of bacteria and either mAbs or pAbs in order to assess the *S. epidermidis* biofilm accumulation in the different conditions. The medium was removed and the biofilms were washed three times with PBS (Boston BioProducts). Microtiter plates (Costar®Corning) were then incubated at 37°C in an inverted position for approximately 2 hours to dry the biomass. Biofilms were then stained with 0.5% crystal violet (w/v) (Sigma) (dissolved in water) for 15 minutes at room temperature, washed 5 × with tap water, and then the plates were incubated in an

inverted position for a few seconds. Finally, crystal violet was dissolved with 200 µL of 33% acetic acid (v/v) (Fisher Scientific), and the absorbance was recorded at 595 nm in an ELISA microtiter reader (BioTek Instruments, VT, USA).

Statistical analysis

Statistical significance between groups was evaluated by either Kruskal-Wallis one-way ANOVA and Dunn's multiple comparisons test or Dunnett's multiple comparisons test, both with a 95% confidence level. Differences between groups were considered significant when $p<0.05$. Statistical analysis was carried out with GraphPad Prism version 5 (CA, USA).

RESULTS AND DISCUSSION

Polyclonal anti-PNAG antibodies inhibits *S. epidermidis* biofilm accumulation *in vitro* through a PNAG-independent mechanism

Because pAbs are produce by different B cells after encounter the antigen, they present different affinities, and therefore, different ability to recognize and bind to multiple epitopes. Having this into account, pAbs present a higher chance to more effectively bind to PNAG and thus inhibit cell-cell interaction and consequent biofilm accumulation. For this reason, we tested the ability of both dPNAG-TT and 9Gluc-NH_2-TT to impair *S. epidermidis* biofilm accumulation *in vitro.*

As show in the Figure 5.1, although biofilm accumulation was highly inhibited by both 9Gluc-NH_2-TT and dPNAG-TT, the NRS control did have similar effect on *S. epidermidis* RP62A biofilm accumulation. This may indicate the presence of endogenous anti-PNAG antibodies or other endogenous Ig in the serum and in the antiserum, respectively, that could be influencing biofilm accumulation.

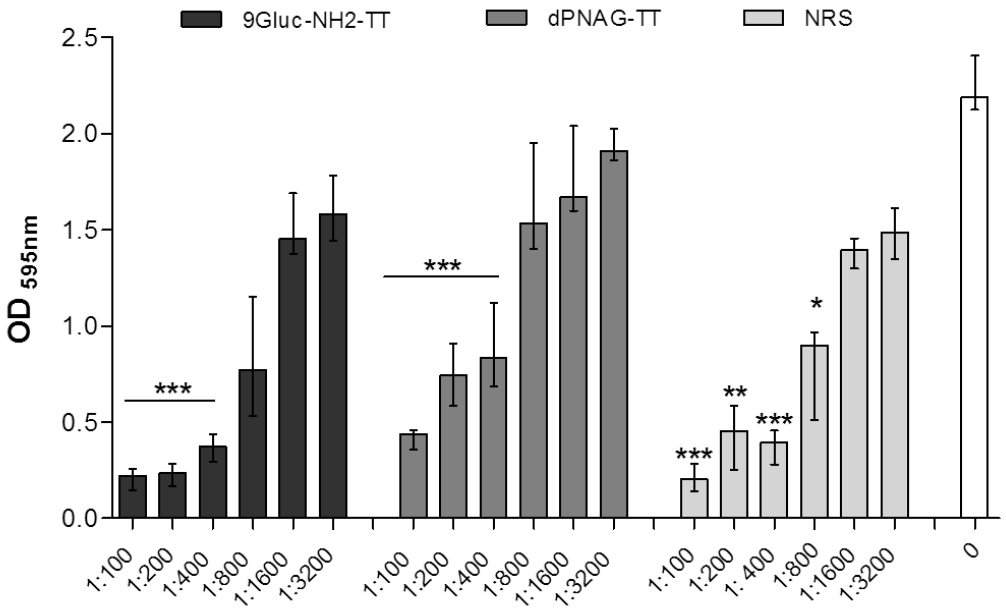

Figure 5.1. Effect of both 9Gluc-NH_2-TT and dPNAG-TT pAbs, and the control NRS on *S. epidermidis* biofilm accumulation *in vitro*. The bars represent the median with interquartil range of two independent experiments with quadruplicates for each concentration tested. Statistical significance was analyzed one-way ANOVA test and Dunn's multiple comparisons test with a 95% confidence level. * $p<0.05$, ** $p<0.01$; *** $p<0.001$ *vs.* 0 (TSB). NRS-normal rabbit serum; OD-optical density.

Thus, in order to remove these endogenous factors, both pAbs and NRS were absorbed with *S. epidermidis* PNAG-negative 1457-M10 or PNAG-positive strain 1457, respectively. Nevertheless, in both cases, no significant differences were found before and after the absorption showing that the mechanism by which the biofilm accumulation is being impaired is not PNAG-dependent (Figure 5.2).

Despite PNAG importance in biofilm formation and accumulation in *S. epidermidis*, it was shown that clinical isolates that do not produce PNAG can still form biofilms [20], indicating the involvement of other adhesive factors in biofilm accumulation. Indeed, in the last years, it has been shown the involvement of several proteins in *S. epidermidis* biofilm accumulation such as Aap [21], Bhp [14], Embp [22], SesC [7].

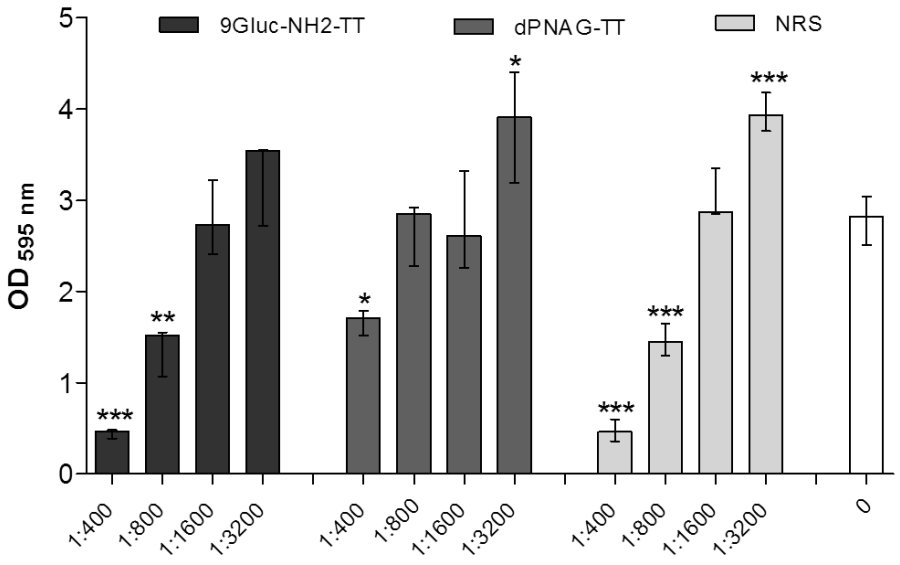

Figure 5.2. Effect of both 9Gluc-NH_2-TT and dPNAG-TT polyclonal antibodies as well as NRS in *S. epidermidis* biofilm accumulation *in vitro* after absorption. The bars represent the median with interquartil range of 3 technical replicates for each concentration tested. Statistical significance was analyzed by one-way ANOVA test and Dunnett's multiple comparisons test with a 95% confidence level. * p<0.05, ** p<0.01; *** p<0.001 *vs*. 0 (TSB). NRS-normal rabbit serum; OD-optical density.

Rabbit serum presents several proteases in its composition [23] which may be involved in the degradation of adhesive proteins and thus decrease *S. epidermidis* biofilm accumulation. On the other hand, because both pAbs and NRS were heat-inactivated, the possible involvement of complement proteins in biofilm accumulation impairment was discarded. Although we did not further characterize the involvement of possible proteases in the inhibition caused by the NRS, in *S. aureus*, it has been shown that normal human serum was able to inhibit biofilm formation [24,25], however, the factor that was mediating the inhibition was not proteinaceous [24]. Another hypothesis that could explain the biofilm formation inhibition in the presence of serum is its slightly alkaline pH, that can negatively influence biofilm formation [26]. Abraham and Jefferson [24] have shown that normal human serum, even buffered was still able to cause inhibition of *S. aureus* biofilm accumulation *in vitro*. Hence, further studies are needed to uncover the factors present in the serum that play a role in the inhibition of biofilm accumulation *in vitro* which later may help to understand biofilm formation *in vivo.*

Monoclonal antibody raised against PNAG has variable effects on *S. epidermidis* biofilm accumulation *in vitro*

Serum is complex milieu that harvest several molecules and factors of the host immune system. Hence, besides the possible endogenous Ig and natural occurring anti-PNAG antibodies, there are several protein and non-protein factors that may impact *S. epidermidis* biofilm accumulation, as we observed in the assays performed with NRS. Therefore, we have tested the ability of highly specific mAbs to impair *S. epidermidis* biofilm accumulation *in vitro*. Therefore, we have used an already characterized anti-PNAG mAb, IgG_1 F598, which presents high affinity for both acetylated and deacetylated forms of PNAG [15]. As an isotype negative control, the monoclonal antibody IgG_1 F429 produced against *P. aeruginosa* capsule was used [18].

As expected, the mAb F598 presented a doses-dependent effect in biofilm accumulation, while the mAb F429 control had no significant effect. Additionally, in the case of the PNAG-deficient, *ica*-mutant strain 1457-M10, no significant effect was found on the biofilm biomass as no PNAG is produced. Hence, these results suggest that the effect caused by the presence of the mAb is indeed PNAG-dependent. Interestingly, depending on the *S. epidermidis* strain used, the presence of mAb F598 had a differential effect on biofilm accumulation. In the case of the strain RP62A we observed a 42% reduction in biofilm biomass at the highest mAb concentration tested, while in the clinical strains 1457 and M184 the mAb F598 presented a doses-dependent increase of the biofilm accumulation of 300% and 333%, respectively. As observed in other studies that have used antibodies specific for *S. epidermidis* surface molecules, the observed enhancement of biofilm formation could be a result of increased PNAG expression caused by the early blockage of the polysaccharide [8]. On the other hand, monoclonal antibodies are highly specific and recognize only one particular epitope on the antigen, being, hence highly susceptible to small variations in the antigen. The specificity of mAb F598 for epitopes on PNAG that do not require the N-acetyl groups on the glucosamine monomers may have therefore contributed to the differential effects observed in biofilm accumulation. These would thus depend on the level of PNAG acetylation of individual strains, ultimately, controlled by the IcaB extracellular deacetylase [27], which was not addressed in this study. Therefore, mAbs directed to other epitopes of the PNAG might be better suited for inhibition of *in vitro* biofilm accumulation. A recent study reported that the blockage of *S. epidermidis* surface protein, Aap, by mAbs with specificity for different epitopes of the protein presented differential effect on biofilm accumulation *in vitro*: some of the mAbs decrease accumulation while others resulted in a biofilm accumulation enhancement [8].

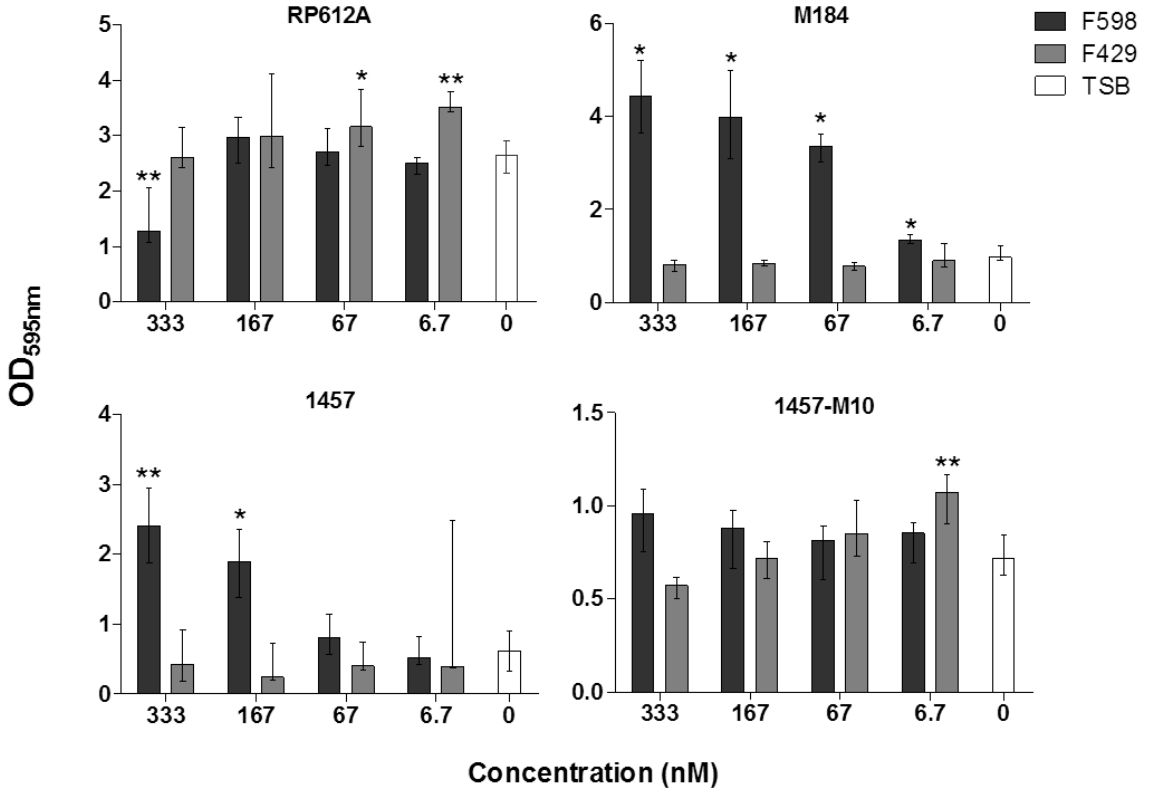

Figure 5.3. Effect of mAb F598 specific to PNAG on *S. epidermidis* biofilm accumulation *in vitro.* The bars represent the median with interquartil range of two independent experiments with quadruplicates for each concentration tested. Statistical significance was analyzed one-way ANOVA test and Dunn's multiple comparisons test with a 95% confidence level. *p<0.05, ** p<0.01 *vs.* 0 (TSB). NRS-normal rabbit serum; OD-optical density.

This result suggest a difference between the reported effect of mAb F598 against PNAG-producing bacteria in animal models [12,15], and its efficiency at inhibiting *in vitro* static biofilm accumulation among different *S. epidermidis* strains. While the stimulation of biofilm formation by *S. epidermidis* grown *in vitro* may raise questions regarding the usage of mAb F598 *in vivo,* the results do not necessarily exclude that mAb F598 could be effective *in vivo* against biofilm infections. Notably, many biofilms are formed under flow conditions and it is not clear to what extent shear stress from flow over *in vivo* biofilms contributes to biofilm formation, and whether under those conditions the effect of mAb F598 might be different.

CONCLUSIONS

Our findings have shown, in one hand, that normal rabbit serum present unknown factors that effectively inhibit *S. epidermidis* biofilm accumulation *in vitro*, which need to be studied in detail in order to better understand *S. epidermidis* biofilm formation *in vivo,* and thus the role of the host factors in the establishment of the biofilm. On the other hand, the study involving the monoclonal antibody further stress the necessity to use more than a few strains to test the effect of such antibodies since in particular cases it may increase biofilm formation resulting in a deleterious effect.

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CHAPTER 6.

Concluding remarks and Future perspectives

CONCLUDING REMARKS

Throughout this thesis the molecular interactions between *S. epidermidis* biofilms and the host immune system were addressed. Due to the high complexity of the host-pathogen interaction we focused our studies in key stages of the biofilm lifecycle, namely the interaction between mature biofilms or biofilm-released cells with the host immune system.

Our primary interest was to discover the genetic alterations achieved by *S. epidermidis* biofilms in the context of the host immune response. However, due to the known technical issues associated with RNA extraction from Gram-positive bacteria and biofilm samples, several RNA extraction kits were tested. It was concluded that **the most efficient RNA extraction kits for *S. epidermidis* biofilms were the ones with mechanical- and chemical-based lysis, yielding RNA with highest quality of all**. Interestingly, we also found that **different cDNA synthesis kits could strongly impact the outcome of gene expression analysis in *S. epidermidis* biofilms**. Furthermore, we have devised a custom made qPCR reaction that was able to achieve the high standards required for this kind of analysis, however reducing the final volumes of cDNA or qPCR reaction, which allowed us to save considerable amounts of money.

Having an efficient gene expression quantification workflow completely optimized, we then proceeded to the characterization of the transcriptomic alterations in *S. epidermidis* biofilms upon exposure to human blood. It was observed that ***S. epidermidis* biofilms were able to withstand the high bactericidal activity of human blood,** which was probably due to the extensive changes observed in its transcriptome. **One of the most important and striking observations was the great increase in the expression of iron uptake systems, which suggests this as an important mechanism for evasion and survival from the human blood-circulating immune effectors.**

We were also interested in studying the final stage of the biofilm lifecycle. Biofilm disassembly, and thus biofilm-released cells, has been associated with the development of several acute infections. Nevertheless, the virulence potential of these cells remained to be addressed. Hence, S. *epidermidis* biofilm-released cells were characterized with reference to several different potential virulence factors. Interestingly, despite earlier suggestions that biofilm-released cells would quickly revert to their planktonic phenotype, **it was observed that in the case of *S. epidermidis,* biofilm-released cells**

presented a particular phenotype displaying, simultaneously, features of both planktonic and biofilm cells. These differences had an impact in *S. epidermidis* virulence potential. **The most remarkable feature of the *S. epidermidis* biofilm-released cells was their high capability to withstand the action of tetracycline**. This resistance may have important consequences in the efficacy of prophylactic and therapeutic measures based in antibiotics that act in protein synthesis blockage. Nevertheless, this issue needs to be investigated in more detail.

Finally, we tested whether a monoclonal antibody raised against PNAG could be used as a therapeutic approach against *S. epidermidis* biofilm accumulation, since this has been considered one of the most promising strategies against biofilm formation by several microorganisms. Interestingly, we found out that due to the strain-to-strain variability, the tested antibody presented variable effects, sometimes resulting in an enhancement of biofilm accumulation.

FUTURE PERSPECTIVES

The work described in this thesis has provided valuable information on how *S. epidermidis* biofilms interact with the host immune system. Nevertheless it has also raised some important questions that need to be answered. Some of the suggestions that should be taken into consideration for future investigations are:

1) Transcriptomic analysis of *S. epidermidis* biofilm-released cells upon exposure to human blood;
2) The identification of the blood-circulating immune cells and soluble factors that induce transcriptomic changes in *S. epidermidis* biofilms and in its released cells;
3) Construction of mutants to identify the genes that are essential for survival and evasion of *S. epidermidis* biofilms and of its released cells.