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Quorum sensing inhibition in *Pseudomonas aeruginosa* biofilms: new insights through network mining

Martín Pérez-Pérez[a], Paula Jorge[b], Gael Pérez Rodríguez[a], Maria Olívia Pereira[b] and Anália Lourenço[a,c]

[a]ESEI – Escuela Superior de Ingeniería Informática, Universidad de Vigo, Ourense, Spain; [b]CEB – Centre of Biological Engineering, LIBRO - Laboratory of Research in Biofilms Rosário Oliveira, University of Minho, Braga, Portugal; [c]CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal

ABSTRACT

Quorum sensing plays a pivotal role in *Pseudomonas aeruginosa*'s virulence. This paper reviews experimental results on antimicrobial strategies based on quorum sensing inhibition and discusses current targets in the regulatory network that determines *P. aeruginosa* biofilm formation and virulence. A bioinformatics framework combining literature mining with information from biomedical ontologies and curated databases was used to create a knowledge network of potential anti-quorum sensing agents for *P. aeruginosa*. A total of 110 scientific articles, corresponding to 1,004 annotations, were so far included in the network and are analysed in this work. Information on the most studied agents, QS targets and methods is detailed. This knowledge network offers a unique view of existing strategies for quorum sensing inhibition and their main regulatory targets and may be used to readily access otherwise scattered information and to help generate new testable hypotheses. This knowledge network is publicly available at http://pcquorum.org/.

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Introduction

Quorum sensing (QS) is a communication mechanism that regulates gene expression in response to fluctuations in cell-population density (Waters & Bassler 2005). In QS, bacteria and fungi produce signal molecules, termed auto-inducers (AI), that increase in concentration as a function of cell density (Dixon & Hall 2015). Alteration in gene expression occurs when the concentration of an AI reaches a minimal threshold (Hawver et al. 2016). Usually, AI regulate genes encoding virulence factors, such as genes involved in biofilm formation and enhanced motility, but they can also coordinate interactions between microorganisms (intra- and inter-species) and between the microorganism and the host (Grandclément et al. 2016; Knecht et al. 2016).

Given the important role of QS on microorganism communication and virulence, agents with anti-QS activity – known as quorum quenching (QQ) agents – hold promising potential as antimicrobials (Chan et al. 2015). The antimicrobial capacity of QQ relies more on the reduction of virulence rather than the killing of the targeted bacteria. This type of approach is believed to not only diminish the development of antibiotic resistance, but also to improve the treatment of recalcitrant MDR infections (Hirakawa & Tomita 2013; Reuter et al. 2016).

While it is challenging to gather information about QQ agents, because it is scattered across the growing volume of scientific literature, the development of computational workflows to retrieve and integrate such information has the potential to uncover interesting links and may lead to new insights into QS-centric therapeutics. In particular, text mining and network mining approaches can support systematic literature processing and information integration from various sources, creating a comprehensive knowledge map. Indeed, network approaches have already been applied to the study of virulence and antibiotic resistance in *P. aeruginosa* (Hwang et al. 2016); however, anti-QS information has yet to be covered.

In previous work, the authors developed bioinformatics frameworks for the general retrieval of antimicrobial textual evidences (Kolchinsky et al. 2013, 2015) and for the reconstruction of antimicrobial interaction networks (Jorge et al. 2014, 2016). In the current paper, the objective was to extend this framework to extract additional information types and apply it to the retrieval and curation of research articles about antimicrobial strategies against *P. aeruginosa* QS.

CONTACT Anália Lourenço analia@uvigo.es

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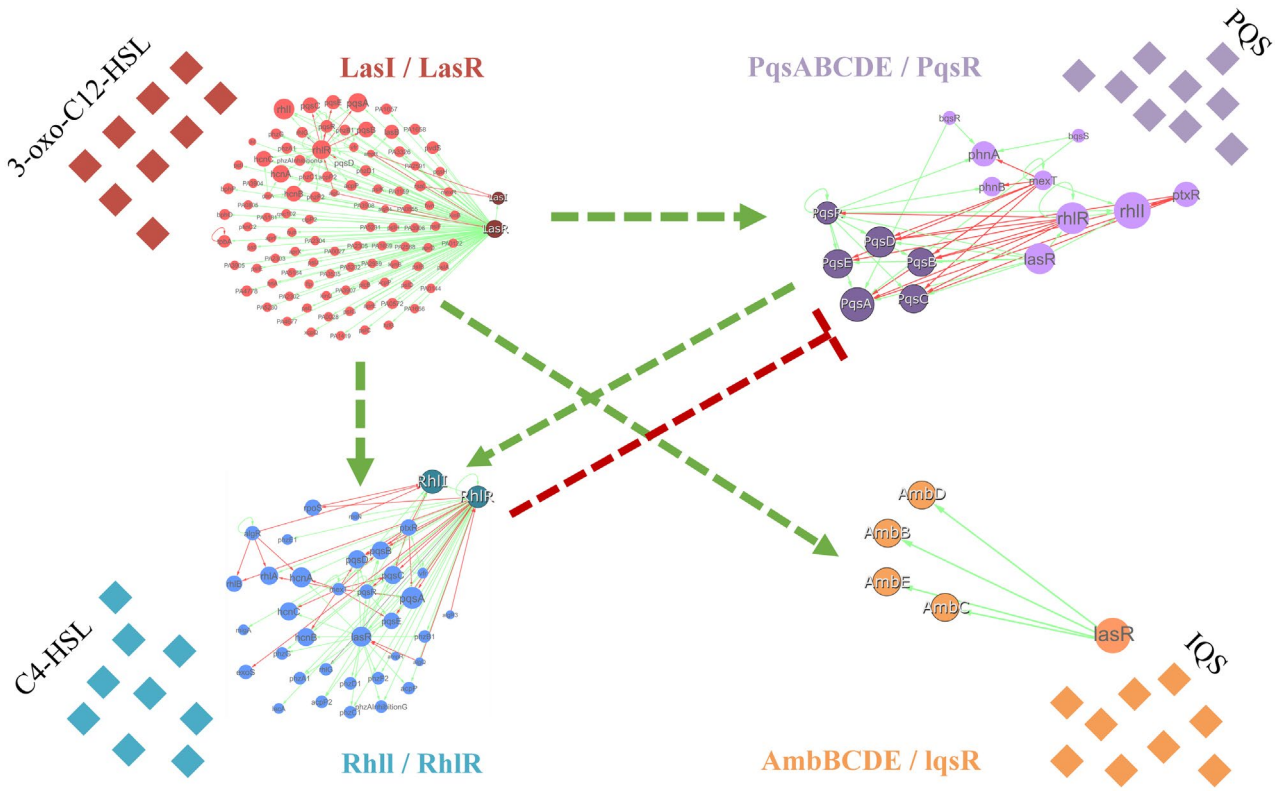

Figure 1. QS systems and their respective AI in *P. aeruginosa*. The regulatory networks for each QS system show key genes and their immediate neighbours, ie their regulators or nodes under their regulation.
Note: Green arrows depict stimulation and red arrows depict inhibition.

This was carried out with the aim of constructing a free and public knowledge network of potential anti-QS agents that will help researchers to readily access otherwise scattered information and to help generate new testable hypotheses.

QS in P. aeruginosa *biofilms*

P. aeruginosa is a well-known and broadly studied opportunistic pathogen, responsible for several nosocomial infections, usually associated with the formation of biofilms, and often resistant to conventional antibiotics treatments (Mulcahy et al. 2014; Rybtke et al. 2015).

QS is dependent on systems of proteins that synthesise/recognise AI. To date, four main QS systems have been identified in *P. aeruginosa* (Figure 1), namely the LasI/LasR and the RhlI/RhlR systems (Pesci et al. 1997), the PqsABCDE/PqsR system (Dubern & Diggle 2008), and the AmbBCDE/IqsR system (Lee et al. 2013). Each system has its own AI: 3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL), N-butanoyl homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal - PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (integrated quorum sensing signal - IQS), respectively (Lee & Zhang 2015).

The QS systems regulate each other (Figure 1), in a hierarchical manner, with the LasI/LasR system regulating all other three systems, the RhlI/RhlR and the PqsABCDE/PqsR systems regulating each other, and the AmbBCDE/IqsR system regulating the PqsABCDE/PqsR system. These systems also regulate the expression of various genes related with motility, biofilm formation, immune evasion, iron scavenging and antibiotic resistance (Jakobsen et al. 2013).

The expression of virulence factors in *P. aeruginosa* is much diversified and, as shown in Figure 2, several have been shown to play a role in the process of biofilm development (Sauer et al. 2002). The PqsABCDE/PqsR system influences the production of extracellular DNA (eDNA), a matrix component important for the formation of stable and mature biofilms (Whitchurch et al. 2002; Allesen-Holm et al. 2006), and lectins, such as LecA and LecB, which influences biofilm formation and enhances colonisation and infection establishment (Adam et al. 1997; Lee & Zhang 2015). The RhlI/RhlR system controls the expression of rhamnolipids, which are important for late stage biofilm formation, tolerance against the host's immune cells and biofilm dispersion (Davey et al. 2003; Lequette & Greenberg 2005; Jensen et al. 2007). This system also

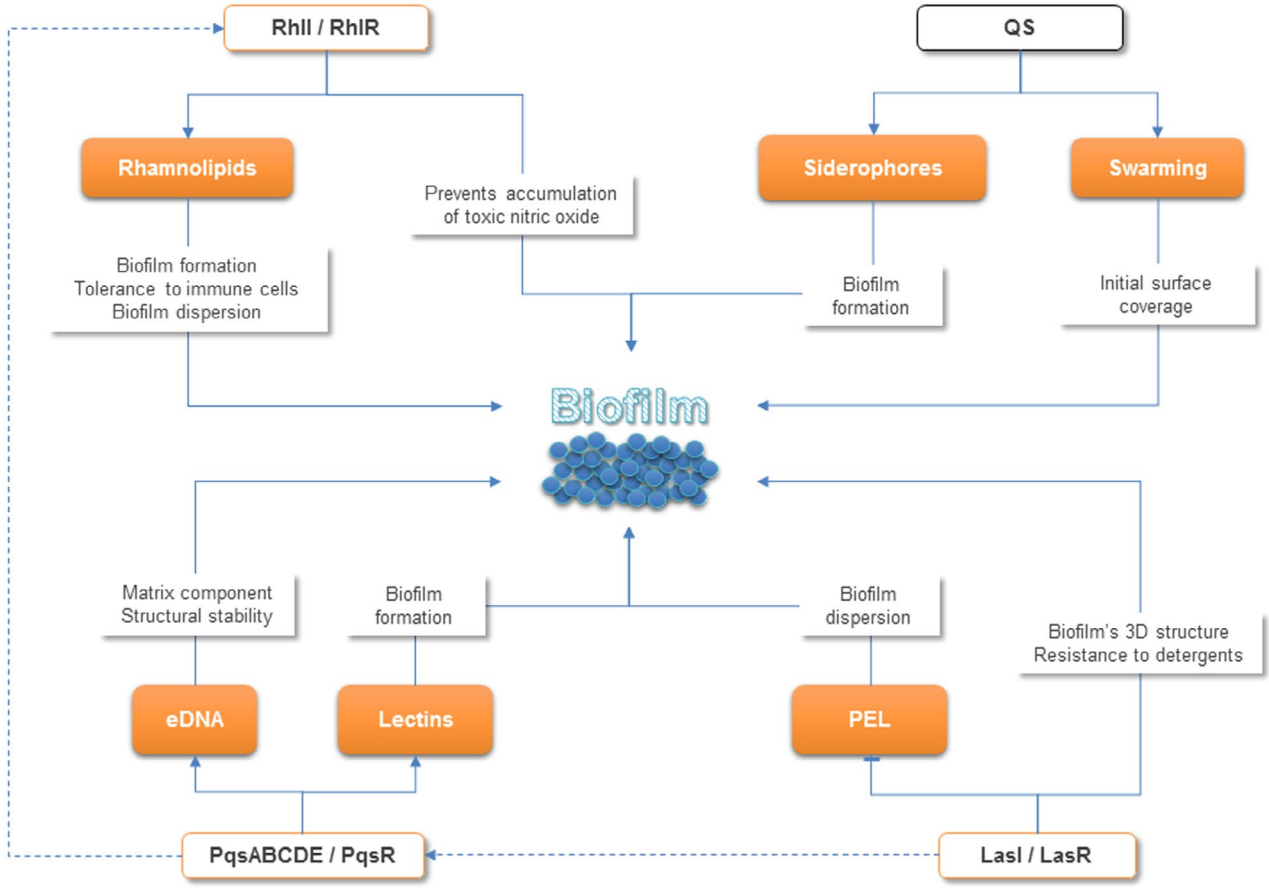

Figure 2. Overview of biofilm QS regulation.
Note: Arrows indicate stimulatory effect, bar-headed line indicates inhibitory effect and dashed arrows indicate inter QS system regulation.

prevents the accumulation of toxic nitric oxide in *P. aeruginosa* biofilms (Ciofu et al. 2015).

The LasI/LasR system diminishes the production of PEL, an exopolysaccharide and major component of the biofilm matrix, inducing biofilm dispersion (Ueda & Wood 2009), and also intervenes in the formation of mushroom-like biofilms and the resistance to detergents, like sodium dodecyl sulphate (SDS) (Davies et al. 1998).

In a more general way, QS influences the production of siderophores (such as pyoverdine and pyochelin) that are involved in biofilm formation (Banin et al. 2005); swarming motility, which has been linked to early stages of biofilm formation (Shrout et al. 2006); and it has also been linked to antibiotic tolerance found in *P. aeruginosa* biofilms but not in planktonic cells (Ciofu et al. 2015). These specific and important roles that QS plays in biofilms of *P. aeruginosa* make it a valuable target for the treatment of biofilm related infections.

Materials and methods

This section describes the steps followed to curate and obtain information on *P. aeruginosa* QQ and outlines how to properly visualise the data in the reconstructed network.

Knowledge integration

The information needed to reconstruct the QQ network for *P. aeruginosa* was obtained from the PubMed database using the E-utilities tool (Kans 2016). This tool facilitates the access to large volumes of information contained in PubMed and it is highly customisable, so it is possible to filter the retrieved documents. In this case, the following considerations were applied: (1) only documents in English; (2) discard revision documents; (3) focus the query on *P. aeruginosa*. Next, a more specific filtering was applied in order to obtain only documents with relevant information about anti-QS agents in *P. aeruginosa*. A classification model was used to automatically prioritise documents (Abi-Haidar et al. 2008; Lourenço et al. 2010), which were further confirmed by human experts. Taking all these considerations in mind, reconstruction was based on 188 documents from a total of 364 documents retrieved from PubMed.

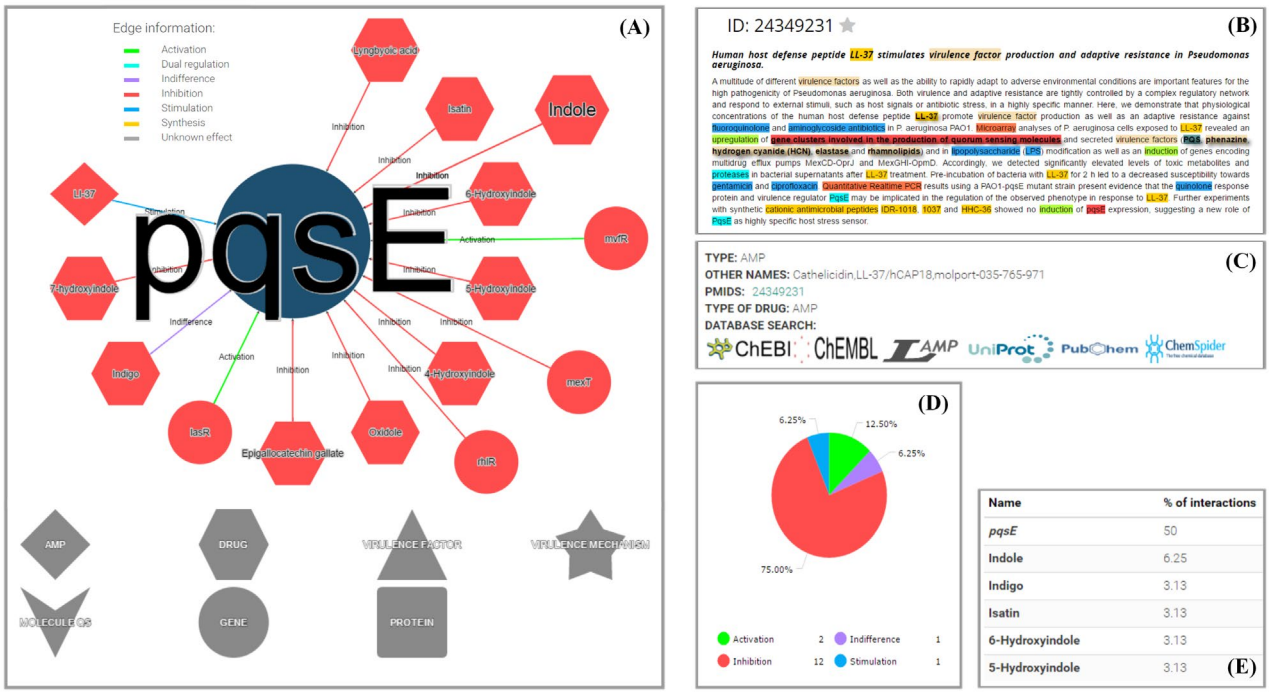

Figure 3. (A) Exemplification of sub-network visualisation by selecting the node *pqsE*; (B) visualisation of expert annotations in Markyt tool for the abstract of the document referring the interaction of LL-37 with pqsE; (C) detailed information about the AMP node LL-37; (D) information about the number of edges in the sub-network; (E) information about the % of interactions in the selected sub-network.

After relevance assessment, human experts revised term annotations, ie the annotation of textual mentions of interest (drug, antimicrobial peptides (AMP), protein, gene and organism), in the title and abstract of the documents. Automatic entity recognition was supported by a set of tools as follows: LINNAEUS was used to tag organisms (Gerner et al. 2010), ABNER was used to recognise genes and proteins (Settles 2005), and an in-house automatic tagger with the help of a custom dictionary was used to tag drugs and AMP. The drug lexicon was downloaded from DrugBank (Law et al. 2014), CHEBI (Hastings et al. 2013), PubChem (Wang et al. 2014), CHEMBL (Bento et al. 2014), the protein lexicon from Uniprot (Consortium 2015), and the peptide lexicon was downloaded from CAMP (Waghu et al. 2014) and LAMP (Zhao et al. 2013). The main aim of expert manual curation is to ensure the correct and comprehensive annotation of important entities and to ensure the annotation of the interactions between QS entities and agent entities. Inhibitory and non-inhibitory interactions (stimulation, indifference) were duly annotated in order to achieve a more comprehensive picture of the effects of the tested agents. This curation was performed with the help of the Markyt curation tool (Pérez-Pérez et al. 2014).

The *P. aeruginosa* genome database (Winsor et al. 2016) and recent studies on its regulatory systems, namely the works of Balasubramanian et al. (2013) and Lee and Zhang (2015), were used to obtain detailed information on the QS systems and the regulatory cues leading or involving the gene/protein targets present in the QQ network.

Knowledge visualisation

The knowledge network is publicly accessible at http://pcquorum.org. This web application allows users to perform network searches and navigate through agent-QS interactions in various ways. For example, the user may search for interactions based on one or two specific nodes (eg look into the sub-network of *pqsr*E, as illustrated in Figure 3A), or for all interactions of a specific type (eg stimulation). The user could also look into particular QQ sub-networks by inspecting the annotation heat map, which provides visual indication of the number of combinations supporting the existing antimicrobial agents and QS-related targets. All data (interaction type, organism, strain, mode of growth, method, and PubMed reference) are easily accessible either by direct network visualisation (node and edge names), by clicking in network elements (ie nodes and edges) or by checking the 'agent interactions' tab below the network. Combination details include known synonyms of the names of antimicrobial agents, genes, proteins and other virulence or QS-related components, and links to main biological databases like CHEBI (Hastings et al. 2013), PubChem (Wang et al. 2014), CHEMBL (Bento et al. 2014) and the protein catalogue of Uniprot (Consortium 2015) (Figure 3C). Finally, users

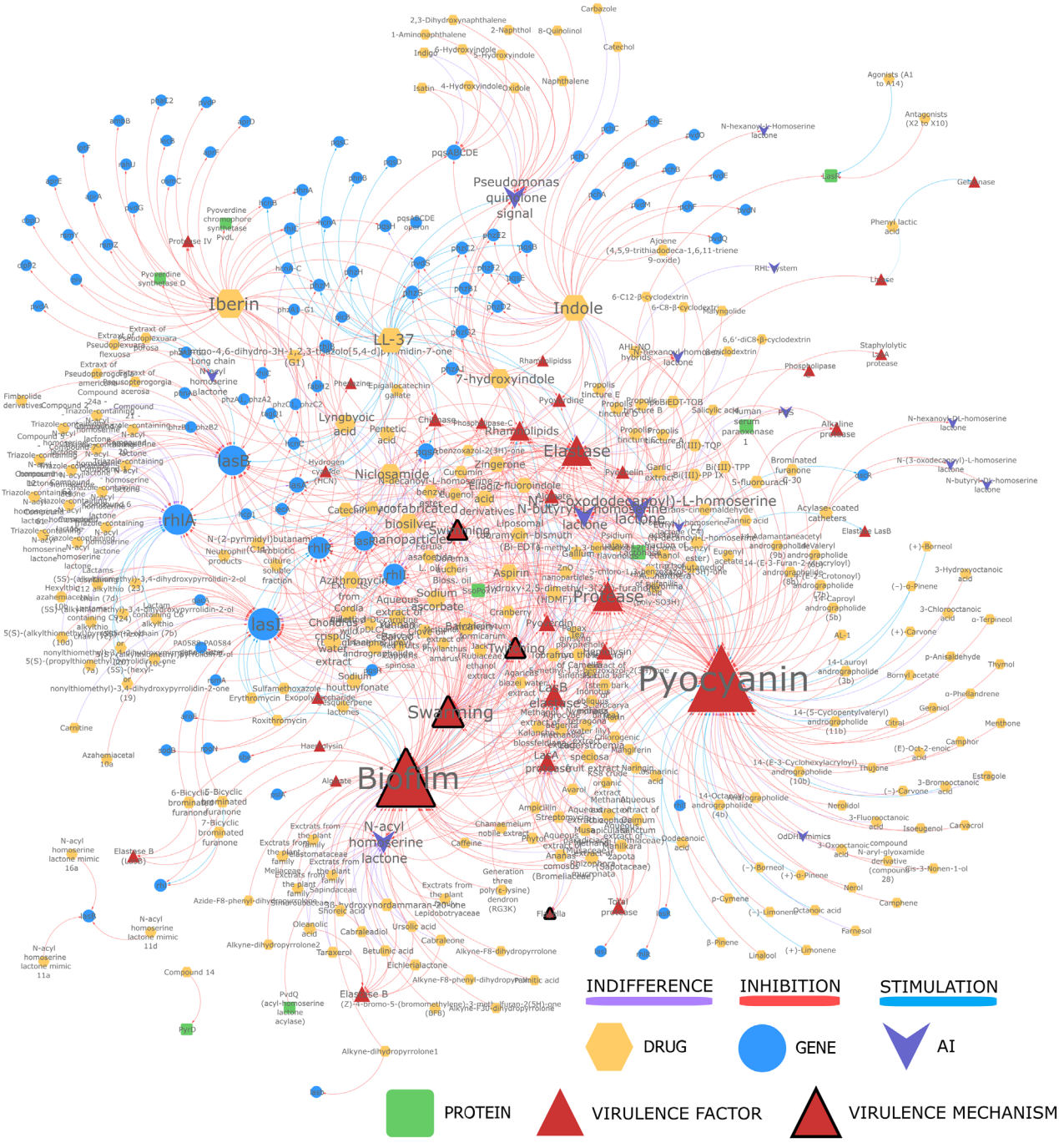

Figure 4. Overview of the agent-QS network reconstructed for *P. aeruginosa*. Nodes and edges are coloured based on the corresponding category whereas node size indicates the level of connectivity.

can read the abstracts of the documents supporting the annotated combination in the Markyt platform (Figure 3B) and inspect various statistics about the visualised network, such as the distribution of combination effects or the most represented nodes (Figure 3D and E).

In terms of technology, HTML5 (http://www.w3.org/TR/html5/) and the Cystoscape web plugin (Lopes et al. 2010) support the interactive and customised visualisation and analysis of the knowledge network. The back-end of the application relies on PHP programming language (version 5.5) and the MySQL database engine (version 5.5).

Results and discussion

Characterisation of the P. aeruginosa *agent–QS knowledge network*

Presently, the constructed network contains 1,004 annotated agent–QS interactions supported by 110 scientific

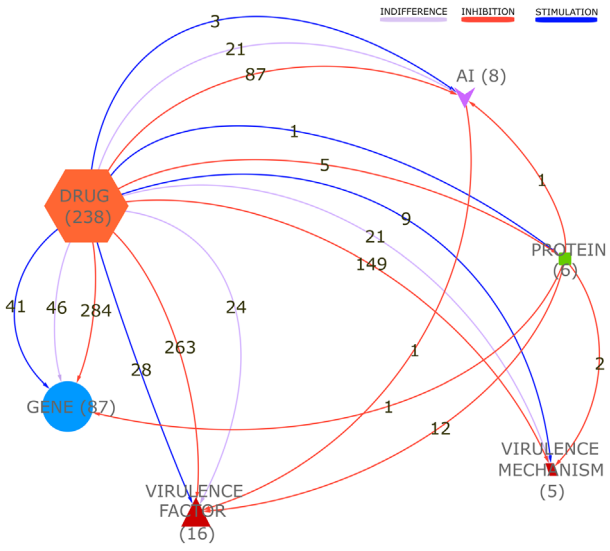

Figure 5. Simplified representation of the network. The value at the edges indicates the number of interactions between the two connected nodes. The value at the nodes indicates the number of entities falling in that category (eg the network includes 238 unique drugs).

For a bird's eye view, network contents can be summarised in terms of agents and targets, and main interaction types (Figure 5). It was noticeable that some of the studies encompassed high-throughput analyses of agent effect over QS genes, which resulted in a high number of curated genes. Although there is knowledge of only four AI in *P. aeruginosa*, the network describes eight 'variants' of these AI, because some studies tested HSL of different sizes (C6- and C8-HSL), other studies mentioned HSL without specifying the size, and some others tested different HSL enantiomers (D-HSL and L-HSL). Moreover, and as expected, the majority of studies focused on reporting inhibitory interactions.

Looking into node connectivity, the most frequently used agents and QS targets are shown in Table 1. Azithromycin (PubChem CID: 447043) is an antibiotic and was the most commonly used agent throughout the 110 annotated documents. This antibiotic was shown to inhibit important QS targets such as *lasI* and *rhlA* (Köhler et al. 2010; Swatton et al. 2016). Other agents, namely tannic acid (PubChem CID: 16129778), iberin (PubChem CID: 10455), 14-alpha-lipoyl andrographolide (PubChem CID: 5318517) and indole (PubChem CID: 798) were the second most frequently used agents. The first three are natural plant products and the latter is a natural bacterially produced compound, and all showed inhibitory effects against important QS virulence factors such as pyocyanin (Lee et al. 2009; Tashiro et al. 2010; Zeng et al. 2011; Naik & Mahajan 2013), pyoverdine (Lee et al. 2009; Tan et al. 2014), rhamnolipids (Lee et al. 2009; Jakobsen et al. 2012) and biofilm (Lee et al. 2009; Zeng et al. 2011; Tan et al. 2014). In the retrieved documents, the most studied QS genes belonged to the LasI/LasR and RhlI/RhlR systems. At the top of the curated QS genes was *lasI*, which codes for the AI 3-oxo-C12-HSL that is recognised by LasR. Given the regulatory ascendance of this system over the other three QS systems, it is understandable that so many QQ approaches studied *lasI* as a QS target.

The other two highly studied genes were *rhlA*, which belongs to the RhlI/RhlR system and codes for rhamnolipids, and *lasB*, which belongs to the LasI/LasR system and codes for LasB elastase. Rhamnolipids are virulence factors that allow *P. aeruginosa* to evade the host's immune system and contribute to biofilm development and dispersion (Davey et al. 2003; Lequette & Greenberg

Table 1. The top three curated QQ drugs and QS targets.

Drug[1]	Gene[2]	AI[2]	Virulence factor[2]	Virulence mechanism[2]
Azithromycin (1.6%)	*lasI* (11%)	3-oxo-C12-HSL (25%)	Pyocyanin (39%)	Biofilm (53%)
Tannic acid, iberin, 14-alpha-lipoyl andrographolide, indole (0.8%)	*rhlA* (10%)	PQS (22%)	Elastase LasB (23%)	Swarming (23%)
Naphthalene (0.4%)	*lasB* (10%)	C4-HSL (21%)	Protease LasA (20%)	Twitching (12%)

Note: [1]% relative to the total number of different drugs per document; [2]% relative to the total number of annotated QS targets.

Table 2. The top three experimental methods used in the analysis of each type of curated QS target.

Gene	AI	Virulence factor	Virulence mechanism
RT-qPCR (25%)	β-galactosidase assay (19%)	Quantification by absorbance (32%)	Crystal violet staining (38%)
GFP production (23%)	*C. violaceum* bioassay (14%)	Elastin Congo red assay (20%)	Motility assay (33%)
β-galactosidase assay (19%)	TLC (9.5%)	Protease assay (6.1%)	SEM (7.5%)

Abbreviations: RT-qPCR, quantitative reverse-transcription real-time polymerase chain reaction; GFP, green fluorescent protein; TLC, thin-layer chromatography; SEM, scanning electron microscopy.
Note: % relative to the total number of annotated QS targets.

articles, dated from 2009 till present (Figure 4). Agents are mainly categorised into antibiotics, antifungals, AMP, disinfectants, and natural and synthetic products. QS targets are divided into genes (eg *lasR*, *rhlI*), proteins (eg LasR, synthetases), AI (eg 3-oxo-C12-HSL, PQS), virulence factors (eg pyocyanin, rhamnolipids) and virulence mechanisms (eg biofilm, swarming). The majority of the effects annotated were of inhibition (81%), but also indifference (11%) and stimulation (8.2%).

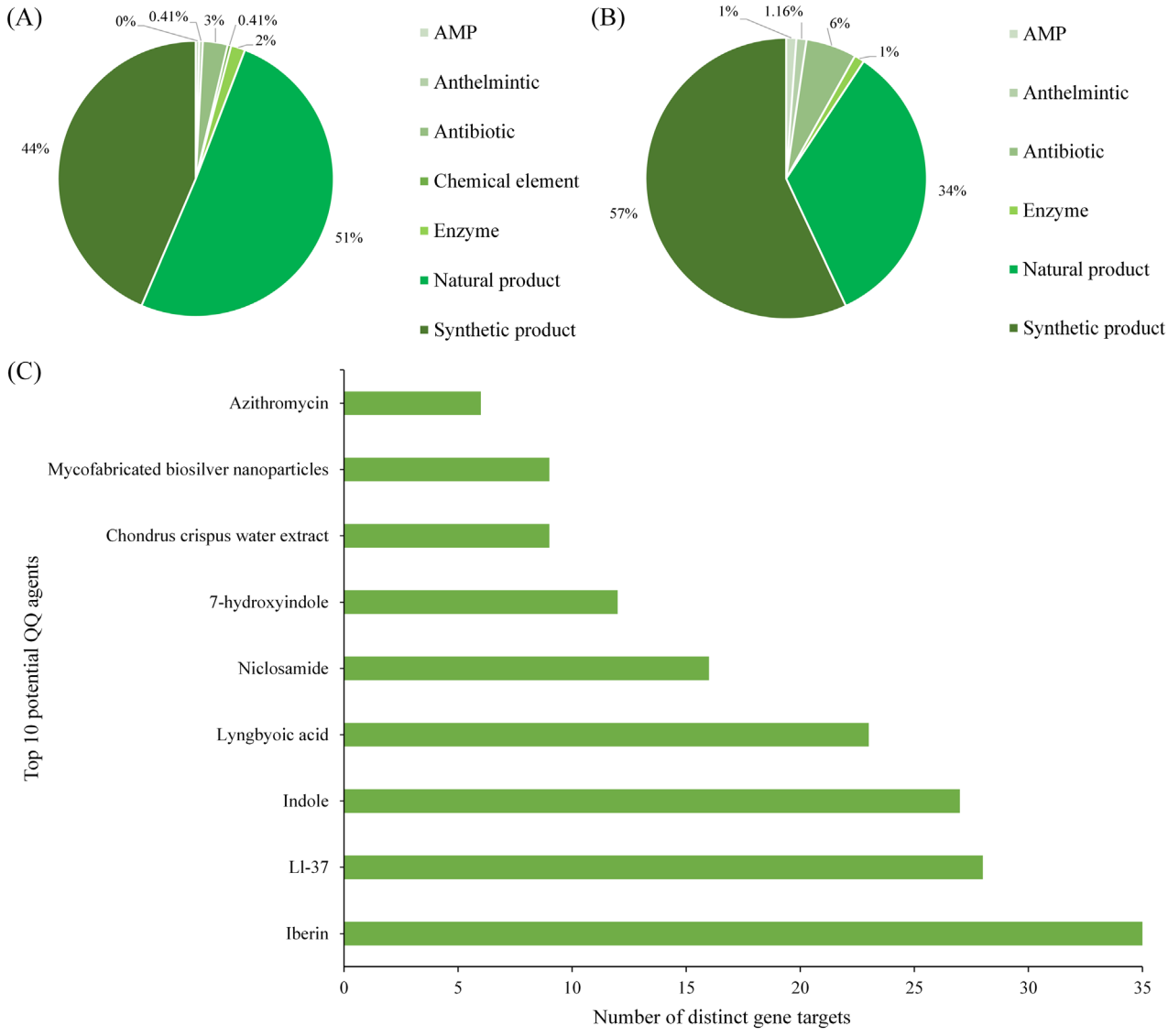

Figure 6. Distribution of potential QQ agents per type for all curated targets (A) and gene targets (B); top 10 potential QQ agents for gene targets (C). % relative to the total number of different agents per document.

2005; Jensen et al. 2007). The virulence factor LasB elastase allows the bacteria to degrade elastin, collagen, and other host proteins and to acquire iron from them (Wolz et al. 1994; Yanagihara et al. 2003). Interestingly, the LasB elastase, the protease LasA and pyocyanin were the most studied virulence factors. LasA allows *P. aeruginosa* to disrupt the host's epithelial barrier facilitating colonisation, to evade the immune system and to lyse *Staphylococcus* cells (Kessler et al. 1993; Park et al. 2000). Similarly, pyocyanin helps in the establishment of infection by facilitating host colonisation and immune evasion (Lau et al. 2004).

The 3-oxo-C12-HSL, PQS and C4-HSL were the top three annotated AI. IQS was only recently described as the fourth AI of *P. aeruginosa* QS, which explains why it was not tested in any of the curated documents. In terms of virulence mechanisms, studies focused on biofilms and cell motility mechanisms, namely swarming, twitching and swimming (data not shown).

The methodologies supporting anti-QS study varied in nature (Table 2). When genes were the target, the majority of the works used methods that are able to analyse a set of specific genes simultaneously, either by using DNA/RNA amplification with PCR or by coupling genes with detectable proteins, such as green fluorescent protein (GFP) or β-galactosidase. Although offering a comprehensive look of agent effects over QS and helping in the identification of multi-target agents, high-throughput methods, such as microarrays, were only occasionally used (9.3%). AI were typically detected by bioassays that use reporter strains unable to produce AI and that harbour reporter genes. These genes typically included the *lacZ* gene, eg in *E. coli* pKDT17, which is detected by the β-galactosidase assay,

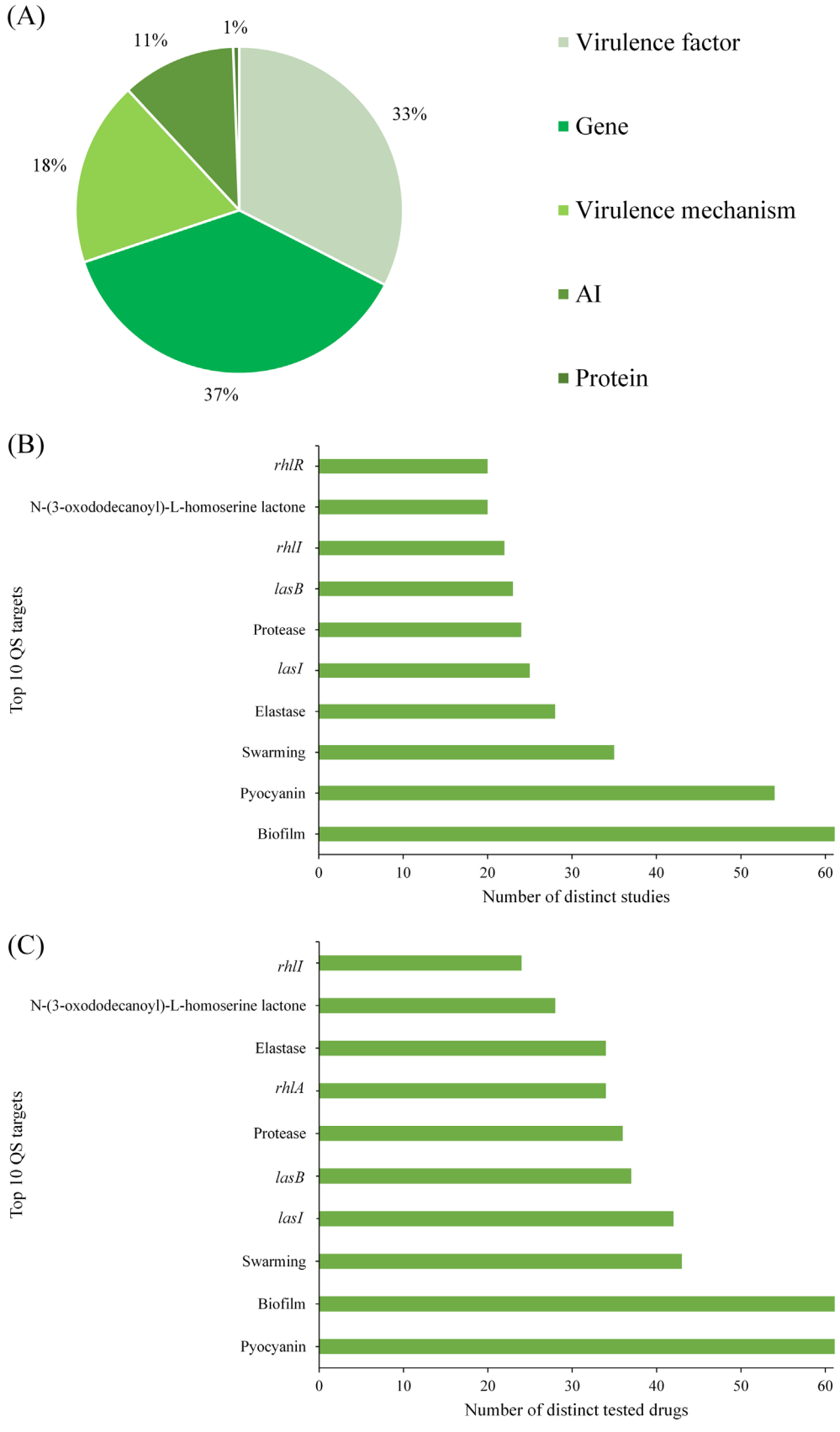

Figure 7. (A) Distribution of type of QS target; (B) number of studies supporting the top 10 QS targets; (C) number of potential QQ agents for the top 10 QS targets.

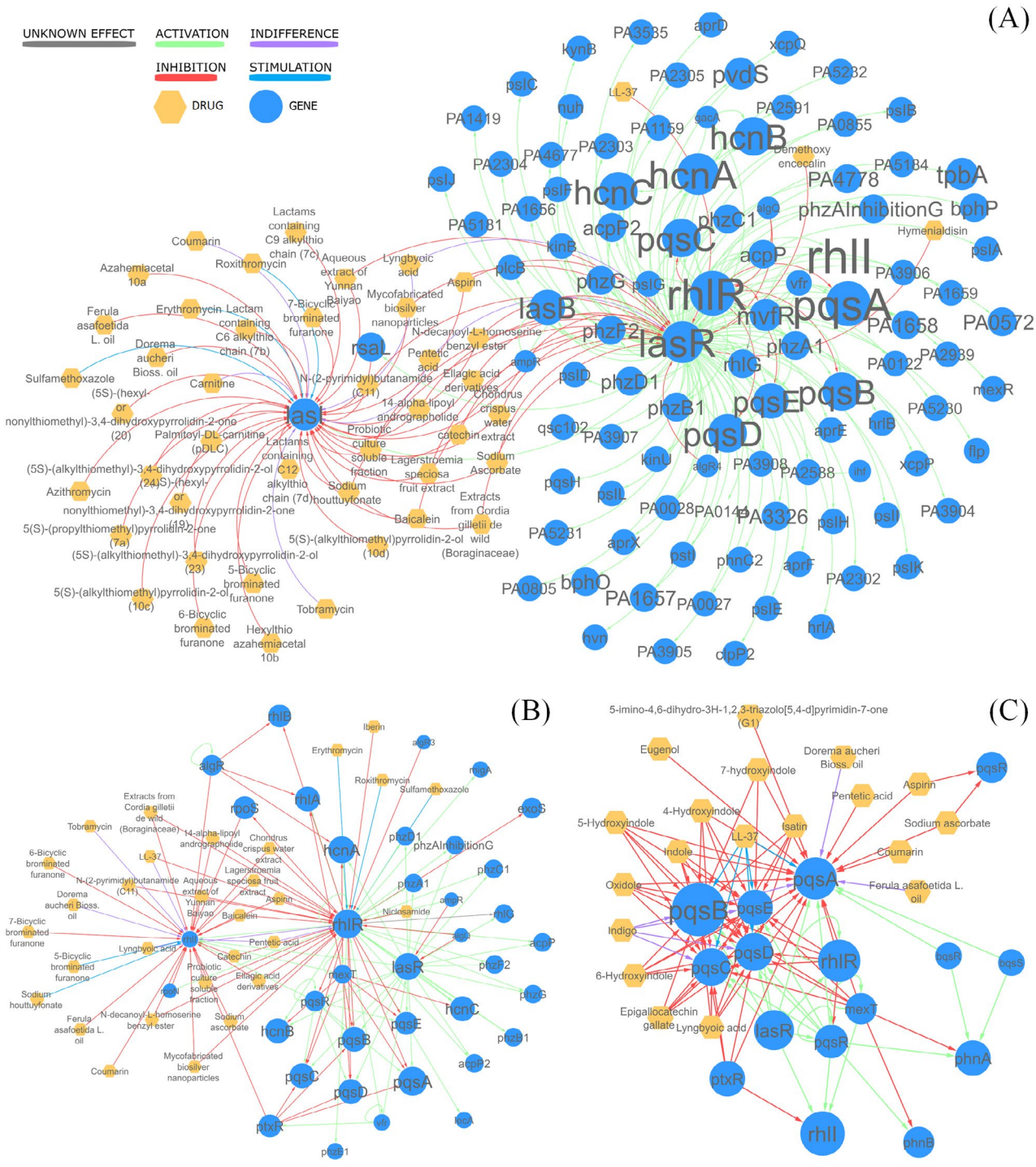

Figure 8. Representation of three different sub-networks. (A) Sub-network for the LasI/LasR QS system and its related nodes; (B) sub-network for the RhlI/RhlR QS system and its related nodes; (C) sub-network for the PqsABCDE/PqsR QS system and its related nodes.

and the violacein gene, in *Chromobacterium violaceum* CV026, which is detected by the production of the pigment violacein in the presence of added AI (McClean et al. 1997). Separation techniques, such as thin-layer chromatography (TLC), are also usually employed coupled with the bioassays in order to separate AI according to their size (Steindler & Venturi 2007). Virulence factors were tested as follows: pigments, such as pyocyanin, and siderophores, such as pyochelin and pyoverdine, were detected via spectrophotometric assays (Hoegy et al. 2014; Jayaseelan et al. 2014); LasB elastase was detected by the Elastin Congo red assay (Hall 1966) and LasA protease by protease assays, eg spectrophotometric (Ayora & Götz 1994) and staphylolytic (Kessler et al. 1993). Finally, the methods commonly

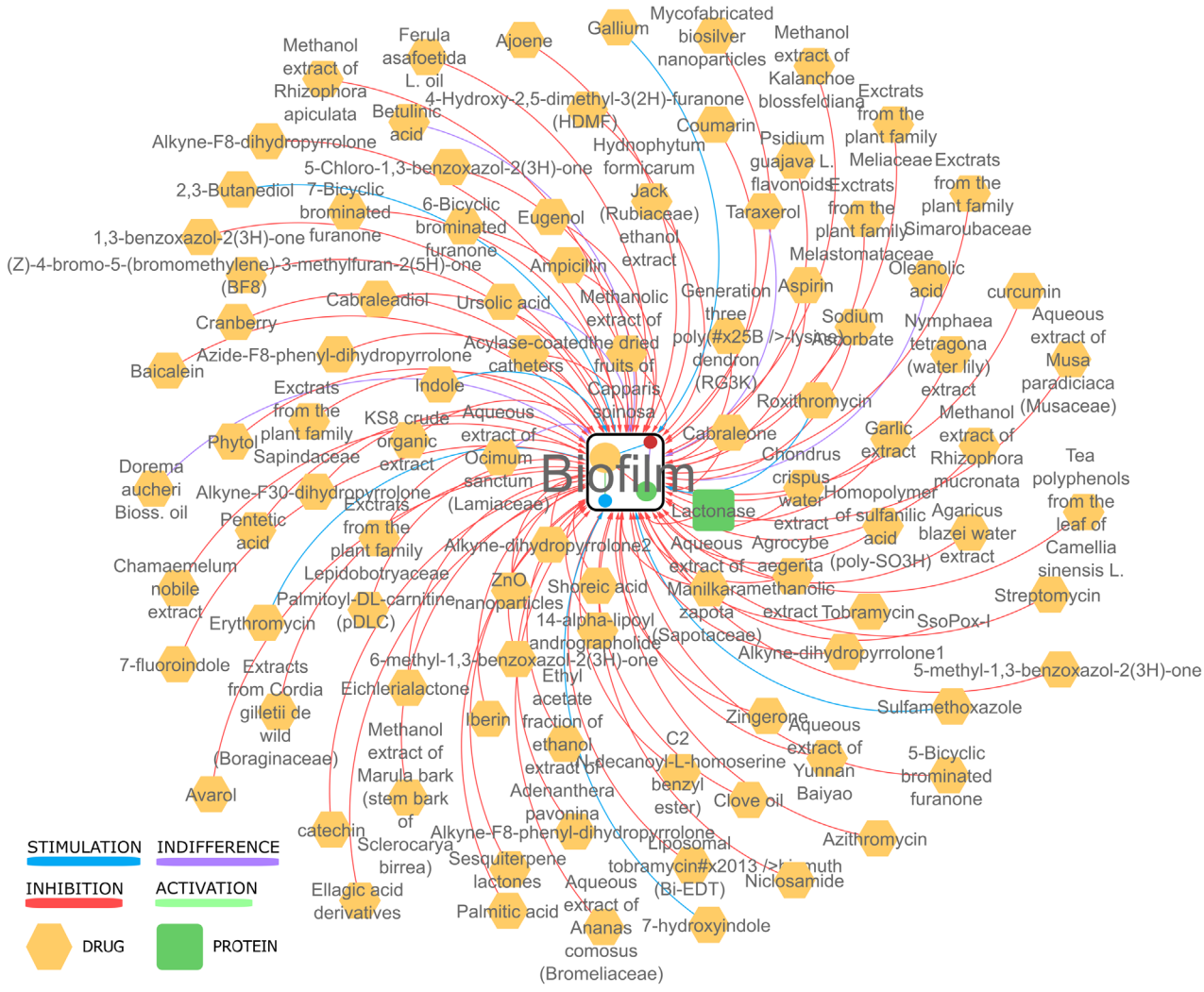

Figure 9. Agent-QS sub-network for the biofilm virulence mechanism.

used to analyse virulence mechanisms were crystal violet staining and scanning electron microscopy (SEM) for biofilm testing and motility assays for swarming, twitching and swimming testing.

As shown in Figure 6, most of the curated agents were natural (51%) or synthetic (44%) products. The same was true when analysing the agents tested on QS genes, for which 57% and 34% of the agents were synthetic or natural products, respectively. This shows that anti-QS studies are currently focusing on the discovery of new and natural QQ and in the use and design of synthetic products. Also interestingly, AMP (eg LL-37) and antibiotics (eg azithromycin) are part of the top 10 agents tested over a large numbers of QS genes, with indole (natural product) being the agent tested over more different QS genes.

The majority of the targeted QS entities on the curated documents were genes (37%) and virulence factors (33%), with the least targeted QS entity being the AI (Figure 7). It is noteworthy that, despite only representing 18% of the targets, the virulence mechanisms, specifically biofilms, were the most tested QS target over all the different curated studies, and the second most tested QS target over different types of agents. This means that biofilms mechanisms were consistently tested throughout the curated documents.

QS systems sub-network analysis

The knowledge network about QQ experimentally validated interactions were further explored in combination with regulatory (virulence-centred) data and addressed each of the *P. aeruginosa* QS systems. Figure 8A, B and C represents the integrated knowledge sub-networks for the three QS systems LasI/LasR, RhlI/RhlR and PqsABCDE/PqsR, respectively. A sub-network was not presented for the AmbBCDE/IqsR QS system because this system has been described relatively recently (Lee et al. in 2013) and, at the time of this reconstruction, no work described approaches targeting this system.

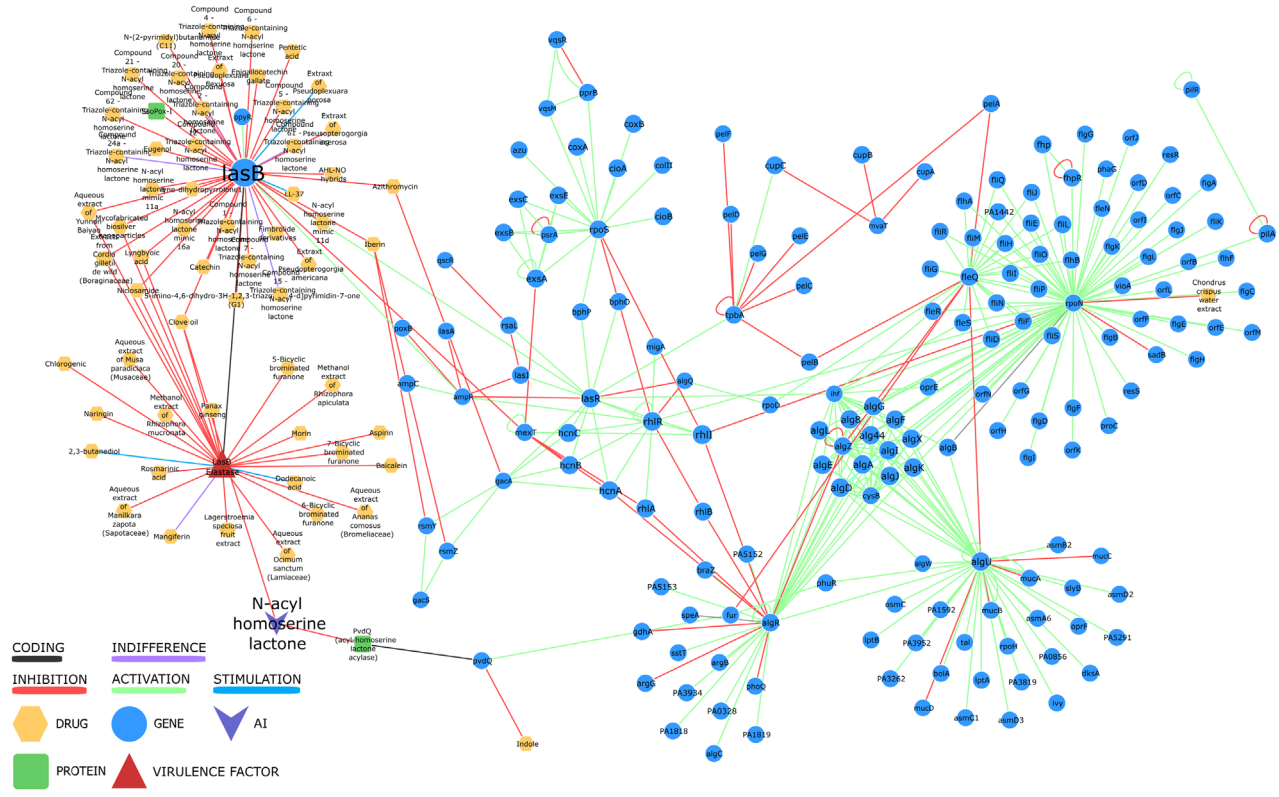

Figure 10. Interconnection between the agent-QS sub-network for the biofilm virulence mechanism and the regulatory network of *P. aeruginosa*.

Most studies focused on the LasI/LasR system. Figure 8A shows a total of 45 agents tested towards LasI/LasR, with 42 tested against *lasI* and 20 (17 of which common to *lasI*) against *lasR*, with 84% of them resulting in inhibitory effect. At the regulatory level, the high number of genes regulated by *lasR* (102) was noticeable, including genes participating in the other QS systems (eg *rhlI*, *rhlR*, *pqsABCDE* and *pqsR*). This regulatory intertwining makes the LasI/LasR system a valuable target for anti-QS therapies and explains why many studies test agents against this system.

A total of 30 agents were annotated in studies focused on the RhlI/RhlR system, more specifically 25 agents tested against *rhlI* and 22 agents tested (17 of which common to *rhlI*) against *rhlR*, with 70% of the reported tests resulting in inhibition (Figure 8B).

The PqsABCDE/PqsR QS system was the least studied of the three systems (Figure 8C). The majority of the curated agents (19) were tested against *pqsA*, 11 (all repeated) were tested against *pqsB* and *pqsE*, 10 (all repeated) against *pqsC* and *pqsD*, and two (both repeated) against *pqsR*. Moreover, 79% of the agents showed an inhibitory effect on the PqsABCDE/PqsR system.

Biofilm sub-network analysis

The network was further explored to uncover all the QQ interactions related to biofilms, a key virulence mechanism. Figure 9 shows all the 95 agent–biofilm interactions curated, from 62 documents. A total of 95 different agents were tested on biofilms and the majority of the reported interactions were inhibitions (86%). Figure 10 shows the integration of this sub-network with the corresponding regulatory sub-network (ie the regulatory paths related to biofilm formation). It serves both to illustrate the complexity underlying the regulation of biofilms and also to showcase all the agents present in our network that were tested on these genes. A total of 62 different agents are present in this sub-network, and 37 of these are not present in the biofilm sub-network (Figure 9), ie were not tested on biofilms in the curated documents. By acting upon biofilm related genes and virulence factors, these agents may be considered to have anti-biofilm potential.

Conclusions

The reconstructed knowledge network is being routinely updated and is freely available at http://pcquorum.org. It

currently describes potential anti-QS agents acting upon QS genes, AI, virulence factors and virulence mechanisms (eg biofilms), namely regulatory interactions and specific functional information. The website visualisation and analysis tools provide researchers with the means to explore the most promising anti-QS agents and their preferred targets, and eventually, to generate new research hypothesis. Namely, network search options allow the user to focus on different sub-networks, eg different agents or interactions, and inspect the regulatory profile of the targeted genes/proteins.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Paula Jorge http://orcid.org/0000-0001-7860-4179
Anália Lourenço http://orcid.org/0000-0001-8401-5362

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