

Pseudomonas aeruginosa and *Staphylococcus aureus* communication in biofilm infections: insights through network and database construction

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ABSTRACT

The polymicrobial nature of most infections is often characterized by complex biofilm communities, where pathogen interactions promote infection progression and severity. Quorum-sensing, the major regulator of virulence and inter-species communication, is a promising target for new anti-infective strategies. This study aimed at collecting and analysing experimental information on the molecular basis of *Pseudomonas aeruginosa* and *Staphylococcus aureus* interactions in biofilms. Data were systematically annotated from relevant full-text papers optimally retrieved from PubMed, reconstructed as networks and integrated with specialized databases to identify promising antimicrobial targets. Network analysis revealed key entities regulating *P. aeruginosa*/*S. aureus* interactions, for instance the PqsABCDE/PqsR quorum-sensing system, which affects *S. aureus* growth and biofilm formation. By identifying the most reported *P. aeruginosa* virulence factors affecting *S. aureus*, for example, HQNO and siderophores, a list of experimentally validated agents affecting those factors, ranging from synthetic drugs to natural plant extracts, was constructed. The complex experimental data on *P. aeruginosa*/*S. aureus* interactions were for the first time systematically organized and made publically available in the new Inter-Species CrossTalk Database (www.ceb.uminho.pt/ISCTD).

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Introduction



Despite important advances in its research, biofilms remain a critical concern for many biomedical applications, being responsible for approximately 80% of human bacterial infections, such as those associated with the implant of medical devices and chronic infections, for example, lung infections, wounds, among others (Fey 2010; Jorge et al. 2012).

Within the biofilm context, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are frequently coexisting pathogens, responsible for co-colonization and co-infection in predisposed patients (Hoffman et al. 2006). The success of these infections is greatly due to their individual pathogenicity emanating from their metabolic versatility (Frimmersdorf et al. 2010), intrinsic and acquired antibiotic resistance (Schmidtchen et al. 2001), biofilm formation (Costerton 2001), and production of multiple virulence factors (Archer et al. 2011; Balasubramanian et al. 2013). Quorum sensing (QS) plays a major role in regulating all these individual traits, but it also controls inter-species communication,

phenomenon that often leads to the progression and exacerbation of infection, usually ending in a worse disease prognosis (Castillo-Juárez et al. 2015; Jennings et al. 2017; Sobin et al. 2017).

P. aeruginosa and *S. aureus* pathogenicity

P. aeruginosa is a well-known Gram-negative opportunistic human pathogen capable of causing both acute and chronic infections, being highly related to nosocomial infections (Sharma et al. 2014). The lungs are one of the most relevant niches for *P. aeruginosa* colonization, making it one of the most common pathogens isolated from respiratory infections, such as cystic fibrosis (CF) lung infection (Folkesson et al. 2012; Rodrigo-Troyano and Sibila 2017). Moreover, its great adaptability, phenotypic and genomic plasticity, ubiquity, and opportunistic sense (Shen et al. 2006; Silby et al. 2011; Brown et al. 2012) enables *P. aeruginosa* association with other types of infection, such as burns, wounds (Mulcahy et al. 2014; Turner et al. 2014), and those associated with biomaterials (Bredenstein et al. 2011).

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To aggravate this scenario, multidrug-resistant (MDR) *P. aeruginosa* strains are emerging with increasing frequency, rendering ineffective many of the existing antibiotic treatments (Chopra et al. 2008; Boucher et al. 2009). In fact, the World Health Organization (WHO) has recently identified carbapenem-resistant *P. aeruginosa* as a critical threat for which there is an urgent need for new therapies (WHO 2017a). *P. aeruginosa* displays intrinsic resistance to different types of antibiotics, for example, aminoglycosides, quinolones, and β -lactams, due to resistance mechanisms such as low outer membrane permeability, multi-drug efflux systems, and inactivating enzymes (e.g. β -lactamase) (Pang et al. 2019). *P. aeruginosa* can also acquire resistance genes from other microorganisms by horizontal gene transfer, while also being able to experience adaptive resistance, as is the case of biofilm formation (Jorge et al. 2019).

The long persistence of *P. aeruginosa* infections seems to be related with complex mechanisms of adaptation in which virulence factors are expressed according to the infection stage. Several factors have been accounted for the pathogenic potential of this bacterium, with many playing a role in its biofilm formation and dispersal. Such factors include those related with motility (e.g. flagella, pili) (Kazmierczak et al. 2015), enzymes (e.g. proteases) (Lee and Zhang 2015), siderophores (pyoverdine and pyochelin) (Reinhart and Oglesby-Sherrouse 2016), surfactants (e.g. rhamnolipids) (Solano et al. 2014), toxins (e.g. exotoxin A, pyocyanin) (Michalska and Wolf 2015; Hall et al. 2016), and the type-3 secretion system (T3SS) (Hauser 2009).

S. aureus is a Gram-positive human commensal bacteria frequently found in the mucosal surfaces of the nose and respiratory tract, and on the skin (Wendlandt et al. 2013; Lister and Horswill 2014). Consequently, it is easily transmitted by direct contact, making most of the population prone to infection. In the United States of America, a large part of healthy individuals are colonised with *S. aureus* (30–50%), within which 1% are colonized with methicillin-resistant *S. aureus* (MRSA) (Bhattacharya et al. 2015). Because of this, *S. aureus* is very often associated with nosocomial infections, since it is commonly transmitted by colonised healthcare staff through direct contact or invasive medical procedures (Bhattacharya et al. 2015). Its ability to evolve and adapt to multiple settings has led MRSA to rapid disperse over the globe, being considered a high threat according to the WHO (WHO 2017b; Jorge et al. 2019). Additionally, MRSA has been developing resistance to virtually all antibiotics classes that are used to treat it, either by making use of its intrinsic resistance factors or by acquiring more through mutations or horizontal

gene transfer from other microorganisms (Watkins et al. 2019).

Biofilms of *S. aureus* are related to many serious acute and chronic infections whose treatment can be complicated since many of its clinical isolates are either MRSA or MDR (Archer et al. 2011; Nair et al. 2014). Furthermore, it has been reported that the presence of *S. aureus* in heterogeneous biofilms increases the rate of plasmid horizontal transfer, which increases the antibiotic resistance of the biofilm (Venkatesan et al. 2015). The ability of *S. aureus* to survive host immune defences and cause a diverse range of diseases has been attributed to the expression of a broad set of virulence factors (Kong et al. 2016). For example, cell wall-anchored proteins (e.g. clumping factors, fibronectin proteins, protein A, and collagen adhesin) enable tissue attachment and evasion of the host immune system, allowing biofilm formation (Foster et al. 2014). Extracellular toxins (e.g. haemolysin, leukotoxin, exfoliative toxin, enterotoxin, and toxic-shock syndrome toxin-1) and enzymes (e.g. coagulase, proteases, and staphylokinase) are secreted to help in tissue penetration and host invasion (Kong et al. 2016). Surface-associated factors are down-regulated and surfactants are expressed in later stages leading to biofilm dispersion and infection spreading (Lister and Horswill 2014). The phenotype of small colony variants (SCV) in *S. aureus* has also been linked to infection persistence, as they seem to be able to establish intracellular infection and have a reduced metabolic state, thus lowering antibiotic efficacy (Garcia et al. 2013; Proctor et al. 2014). SCV also stimulate a reduced immune response and express increased adhesins and reduced toxins (Tuchscherr et al. 2010).

***P. aeruginosa* and *S. aureus* quorum sensing regulation**

QS is a communication mechanism that regulates gene expression in response to fluctuations in cell-population density (Waters and Bassler 2005). In QS, bacteria produce signal molecules, termed auto-inducers (AI), whose concentration increases as a function of cell density (Dixon and Hall 2015). Alterations in gene expression occur when the concentration of an AI reaches a minimal threshold (Hawver et al. 2016). Usually, AI regulate genes encoding virulence factors, such as those 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 (Knecht et al. 2016; Grandclément et al. 2016).

Four main QS systems have been identified in *P. aeruginosa* (Figure 1), namely the LasI/LasR and the RhII/RhIR systems (Pesci et al. 1997), the PqsABCDE/PqsR system (Dubern and Diggle 2008), and the AmbBCDE/lqsR system (Lee et al. 2013). Each system has a corresponding 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 and Zhang 2015). The QS systems regulate the expression of various genes related with motility, biofilm formation, immune evasion, iron scavenging, and antibiotic resistance (Jakobsen et al. 2013), as well as each other, in a hierarchical manner (Figure 1).

The virulence factors expressed by *P. aeruginosa* are diversified and several of them play a role in the process of biofilm development (Pérez-Pérez et al. 2017). For example, the PqsABCDE/PqsR system is responsible for extracellular DNA (eDNA) and lectin production, which are related to biofilm formation and structural stability (Allesen-Holm et al. 2006; Lee and Zhang 2015). In addition, the RhII/RhIR system controls the expression of rhamnolipids that influence the late stage of biofilm formation and its dispersal (Davey et al. 2003; Lequette and Greenberg 2005). The latter phenomenon is also controlled by the LasI/LasR system by downregulation the expression of PEL, a major biofilm matrix component (Ueda and Wood 2009). QS also influences swarming motility, which has been linked to early stages of biofilm formation (Shrout et al. 2006) and is also related to the antibiotic tolerance found in *P. aeruginosa* biofilms but not in planktonic cells (Ciofu et al. 2015; Jorge et al. 2019).

As regards to QS in *S. aureus*, this bacterium deploys a wide collection of virulence factors in order to establish and sustain infection that are primarily coordinated by a complex global regulatory QS system known as accessory gene regulator (Agr). This QS system encodes a signalling circuit that produces and senses the extracellular AI auto-inducing peptide (AIP) and the intracellular effector RNAIII (Figure 1) (Recsei et al. 1986; Le and Otto 2015), being responsible for inducing the expression of toxins, which include haemolysins, phenol-soluble modulins (PSM), toxic shock syndrome toxin (TSST), enterotoxins, and Panton-Valentine leukocidin (PVL), among others (Otto 2014; Cheung et al. 2014; Fisher et al. 2018). Apart from toxins, the secretion of several *S. aureus* enzymes, such as proteases, staphylokinase (SAK), and lipases, has been related to the Agr system (Le and Otto 2015; Pietrocola et al. 2017). Biofilm formation has also been

strongly associated with Agr function, with downregulation leading to excessive biofilm thickness and lack of structuring, and upregulation inducing biofilm dispersal (Vuong et al. 2000, 2004; Cheung et al. 2011), indicating that the absence of Agr functionality may be advantageous for the success of persistent *S. aureus* infections (Goerke et al. 2000; Otto 2014). Also, the emergence of SCV, strongly associated with chronic and recurrent infections and lack of ability to produce cytolytic toxins, is due to reduced Agr activity and disrupted electron transport chain (Tuchscherer et al. 2011; Pader et al. 2014).

A second QS system, closely related to Agr, is the TRAP/RAP system. The RNAIII-activating peptide (RAP) is an AI that causes phosphorylation of its target protein (TRAP). TRAP is responsible for inducing the production of adhesion proteins, stimulating biofilm formation, and also activates the Agr system. The TRAP/RAP system is mainly active during the early/mid exponential growth phase and it is followed by the Agr system, which is mainly active during the mid/late exponential growth phase (Ciulla et al. 2019). In addition to these two QS systems, other regulatory systems, namely the staphylococcal accessory regulator (SarA), the *S. aureus* exoprotein (Sae) operon, and the staphylococcal alternative sigma factor B (SigB), regulate the production of virulence factors and intervene in *S. aureus* biofilm formation (Zielinska et al. 2012; Le and Otto 2015; Liu et al. 2016).

***P. aeruginosa* and *S. aureus* in polymicrobial biofilm infections**

P. aeruginosa and *S. aureus* co-occur in many biofilm-related infections and the relation between them is known to be competitive in nature (Nair et al. 2014). *P. aeruginosa* can inhibit the growth and even kill *S. aureus* when present in close proximity due to the production of several toxins, such as pyocyanin, hydrogen cyanide, and alkyl-hydroxyquinoline N-oxides, which can block the electron transport chain of *S. aureus*. Accordingly, these toxins also mediate the spatial segregation between the two species (Nair et al. 2014; Stacy et al. 2016). Despite this competition, *S. aureus* usually persists in *P. aeruginosa* infections, which might be related to the restriction of cell migration in *P. aeruginosa* highly viscous biofilms (Alves et al. 2018). Also, in biofilms where segregation between the two species is not complete, non-resistant *S. aureus* can benefit from being surrounded by resistant *P. aeruginosa* cells (Nair et al. 2014; Stacy et al. 2016). Lastly, *S. aureus* can employ mechanisms to counteract the effect of *P. aeruginosa* toxins, such as the formation

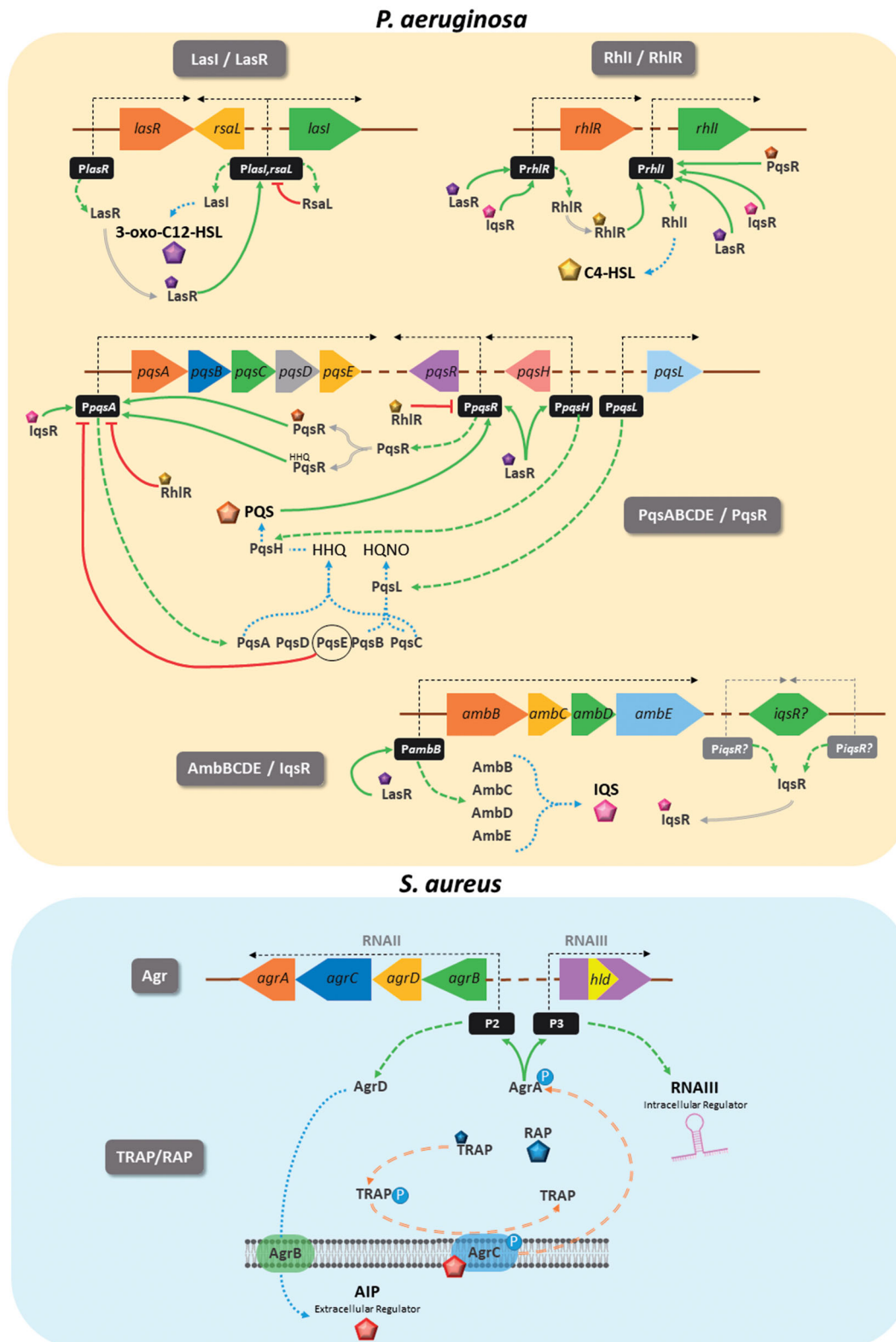


Figure 1. QS regulation in *P. aeruginosa* and *S. aureus*. (A) Hierarchical regulation of the four QS systems in *P. aeruginosa*. At the top of the hierarchy is the LasI/LasR system, which, when activated by 3-oxo-C12-HSL, upregulates itself (positive feedback loop) and the other three QS systems. The RhlI/RhIR system also upregulates itself and downregulates the PqsABCDE/PqsR system. The latter also has a positive feedback loop and upregulates the RhlI/RhIR system. It is noteworthy that this system is responsible for the synthesis of 2-alkyl-4-quinolones, namely PQS, HHQ, and HQNO, which are known to influence *P. aeruginosa*/*S. aureus* interactions. Finally, the more recently described AmbBCDE/IqsR system upregulates the RhlI/RhIR system. (B) *S. aureus* Agr and TRAP/RAP regulatory systems. The *agr* operon consists of two transcriptional units, RNAII and RNAIII, driven by the promoters

of SCV (Nair et al. 2014). All these factors increase the antimicrobial resistance of the polymicrobial biofilm and enhance the severity of the related infection.

Despite new insights into the complexity and impact of multi-species infections on human health, the impact of polymicrobial interactions on infectious diseases remains challenging to examine in laboratory context (Lyczak et al. 2002; Rogers et al. 2010; Price et al. 2013; Filkins et al. 2015). The gap is centred on the dearth of appropriate *in vitro* models that accurately reproduce the host environments, particularly the availability of required nutrients and the impact of host immune factors.

This manuscript discusses various aspects of the social behaviour of *P. aeruginosa* and *S. aureus* interactions in infectious diseases, according to experimental findings reported in scientific literature. The main goals are to map the mechanisms underlying those interactions, through the collection and curation of scientific textual evidences, in order to gain insight into the implications that *P. aeruginosa* and *S. aureus* interactions have on the progression and outcome of polymicrobial infections, and analyse this information to pinpoint critical communication channels to be explored for antimicrobial therapy. Therefore, the main contributions of this work are the reconstruction of a knowledge network of up-to-date experimental results on this subject, the deposition of the gathered data in a newly constructed and publicly accessible database (www.ceb.uminho.pt/ISCTD), and the identification of promising therapeutic targets for *P. aeruginosa*/*S. aureus* co-infections and their respective reported inhibitors.

Materials and methods

This section describes the steps followed to obtain and curate the information on *P. aeruginosa* and *S. aureus* communication. Moreover, it presents the bioinformatics tools available to explore the gathered data.

Information retrieval and annotation

The information needed to reconstruct the *P. aeruginosa*/*S. aureus* communication network was retrieved from PubMed (Sayers et al. 2019), with emphasis put on the

compilation of experimentally validated interactions between the two species. PubMed queries were optimised in terms of number of hits (too low: relevant papers were missed; too high: too many irrelevant papers and impracticable annotation effort) and scope, which was narrowed to experiments mentioning the names of the two species and common terminology denoting simultaneous culturing (e.g. co-cultivation, co-infection, co-culture, polymicrobial, multispecies, mixed-biofilm, etc.). This ensured that PubMed results encompassed almost all the body of work in the field. Subsequently, the relevance of each retrieved document was assessed and relevant information (e.g. textual mentions to organisms, strains, mode of growth, interaction outcome, diseases, and experimental methods) was annotated from the full-text documents. The entity and interaction categories used in expert annotation are described in Tables 1 and 2, respectively.

Information representation and integration

The network analysis software Cytoscape (Shannon et al. 2003) was used to represent the gathered data in network format. All the curated data was made publicly available in the newly constructed Inter Species CrossTalk Database (ISCTD), the first database solely focussed on microorganism communication, at www.ceb.uminho.pt/ISCTD. All data were transformed into JSON format and the online database constructed using Visual Studio software and HTML, CSS, JavaScript, and jQuery programming languages. Textual evidences were integrated with pertinent regulatory data in the literature for *P. aeruginosa*. Data on anti-QS agents experimentally validated for *P. aeruginosa* was retrieved from the PCQuorum database (Pérez-Pérez et al. 2017) and integrated with the new gathered data.

Results and discussion

Database and knowledge network overview

The gathered data stemmed from an annotation effort that resulted in the reconstruction of a network comprehending a total of 670 interactions between

P2 and P3, respectively. RNAII is a four gene operon, *agr* BDCA, encoding AgrB responsible for processing and exporting AgrD, the AIP precursor. AIP is an exclusively extracellular AI produced in the mid exponential growth phase, whose threshold levels cause AgrC to autophosphorylate, leading to the phosphorylation of AgrA. AgrA activates RNAIII expression, an intracellular effector responsible for increasing the secretion of *S. aureus* toxins and enzymes. In turn, RAP (RNAIII-activating peptide) is another *S. aureus* AI that, in the initial exponential growth phase, induces the phosphorylation of TRAP, its target protein and master regulator of *S. aureus* pathogenesis, activating it. This causes the passage from the planktonic to the biofilm mode of growth and the activation of the Agr system. The two QS systems are therefore connected and are phase-dependent, with AIP indirectly downregulating TRAP phosphorylation. Solid arrows: upregulation of gene expression; dashed arrows: gene expression; dotted arrows: synthesis process; lines with flat ends: downregulation of gene expression; double solid arrows: receptor agonism; double dashed arrows: activation process.

P. aeruginosa and *S. aureus* described in 61 different scientific publications (dating from 1993 until October 2019). The annotated information on *P. aeruginosa*/*S. aureus* interactions was made publicly accessible at www.ceb.uminho.pt/ISCTD, in which users can perform searches and navigate through the annotated data. As

illustrated in Figure 2, database users are allowed to do more or less refined searches depending on their specific interests. Specifically, users may search for interactions based on one or two specific entity categories (source and target) or considering a specific interaction direction (*P. aeruginosa* > *S. aureus* or *S. aureus* > *P. aeruginosa*) (Figure 2(A)). The respective data table is generated, showing an organized view of all interaction details, such as organism strains, mode of growth, experimental methods, observations made by the experts, and PubMed references. Users can then narrow down their search by querying for specific terms within the generated table (Figure 2(B)), for example “biofilm”.

Figure 3 depicts a representation of this curated knowledge network, illustrating the complexity and the wide array of entities so far reported as involved in this inter-species interaction. This complexity is also

Table 1. Annotated entity categories.

Entity Categories	Examples
Cell*	<i>P. aeruginosa</i> , <i>S. aureus</i>
Gene	<i>pvl</i> , <i>pvdE</i> , <i>pchA</i>
QS Molecule or AI	2-heptyl-4-hydroxy quinoline N-oxide (HQNO), Pseudomonas Quinolone Signal (PQS)
Virulence Mechanism	Biofilm, SCV
Virulence Factor	Siderophores, DesB
Other	Non-virulent proteins (e.g. TatC), metabolites (e.g. acetate), cell components (e.g. peptidoglycan), among others ...

*Entity and entity category were termed “cell” when the effector or target was unspecified.

Table 2. Annotated interaction categories.

Interaction Category	Description
Upregulation	Increase regarding the expression of a gene/protein.
Downregulation	Decrease regarding the expression of a gene/protein.
Stimulation	Increase in the appearance/amount of virulence mechanisms/factors (or other molecules) or bacterial growth/viability.
Inhibition	Decrease in the appearance/amount of virulence mechanisms/factors (or other molecules) or bacterial growth/viability.
Null effect	No alteration in the expression of a gene/protein or in the appearance/amount of virulence mechanisms/factors (or other molecules) or bacterial growth/viability.
Protection	Protective effect of one bacteria onto another.
Synergism	Aggravation of the co-infection's severity/resilience in comparison with the single-species case.

INTER-SPECIES CROSSTALK DATABASE

(A) **STEP 1 Choose Interaction Direction** **STEP 2 Choose Source Entity Category** **STEP 3 Choose Target Entity Category**

(B) **Type keywords below to further narrow down your search!**

Search table (such as: type of interaction (eg. downregulation), disease (eg. cystic fibrosis), etc...)

Year	PMID	Source	Source Entity	Source Type	Interaction	Target	Target Entity	Target Type	Method	Disease	Strain	Observations
2005	16030221	<i>P. aeruginosa</i>	hcnB	Gene	Inhibition	<i>S. aureus</i>	Cell	Cell	CFU; RT-PCR	Cystic Fibrosis	<i>P. aeruginosa</i> UCBPP-PA14; <i>S. aureus</i> MN8	<i>P. aeruginosa</i> produces factors that mediate <i>S. aureus</i> lysis, many of which were induced (pqsA, phzE2, hcnB genes) during CF sputum growth. Co-culture revealed that within 5 h, <i>S. aureus</i> numbers level off and then decrease.
2005	16030221	<i>P. aeruginosa</i>	phzE2	Gene	Inhibition	<i>S. aureus</i>	Cell	Cell	CFU; RT-	Cystic	<i>P. aeruginosa</i> UCBPP-PA14;	<i>P. aeruginosa</i> produces factors that mediate <i>S. aureus</i> lysis, many of which were induced (pqsA, phzE2, hcnB genes)

Figure 2. Schematisation of the search flow available in the ISCTD. Users go through (A) a selection flow in which they can specify the interaction direction and the source and target entity categories. When hitting “Search”, the database automatically outputs (B) a table containing all annotated data for that search. Users can then search keywords within the table in the search panel. In this figure, the exemplified table shows the effect of *P. aeruginosa* genes on *S. aureus*.

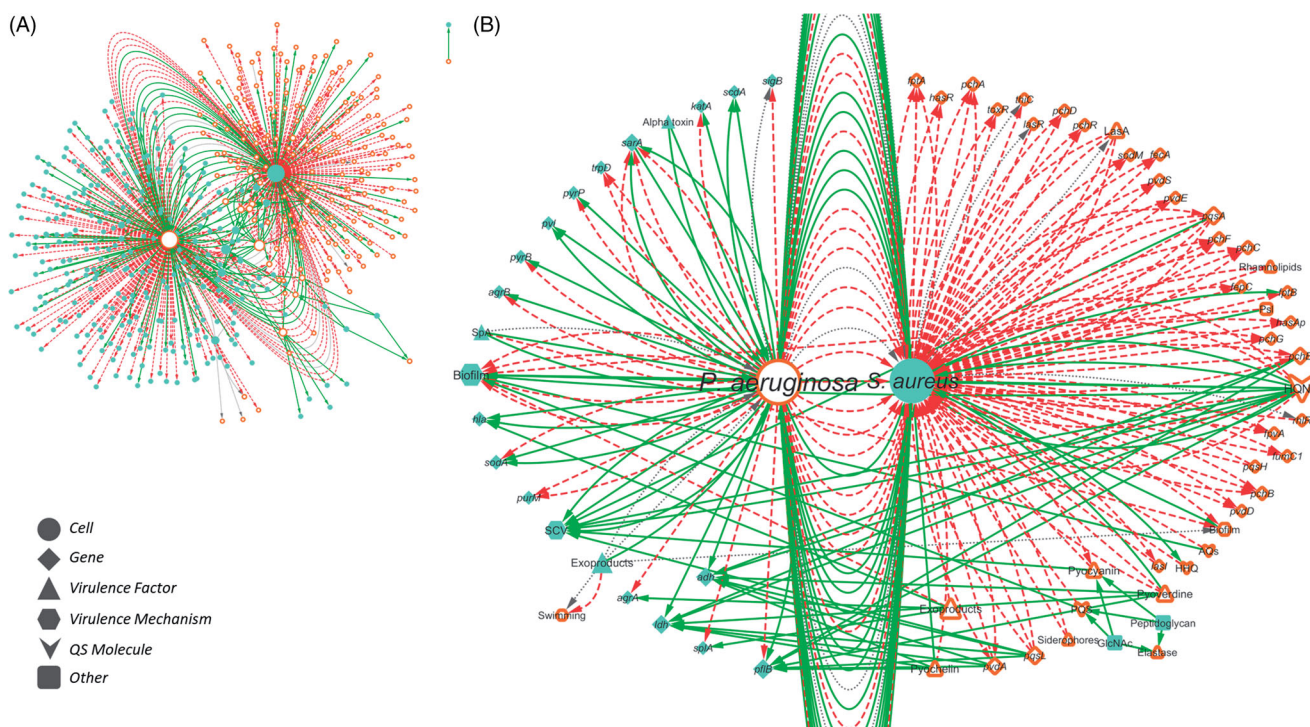


Figure 3. *P. aeruginosa/S. aureus* interaction network. (A) Overview of all annotated interactions reported in the scientific literature; (B) Most reported interactions (annotated more than once). Solid arrows: positive interactions (e.g. upregulation, stimulation); dashed arrows: negative interactions (e.g. downregulation, inhibition); dotted arrows: null effect. *P. aeruginosa* and *S. aureus* nodes are depicted in unfilled and filled shapes, respectively; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

demonstrated when taking into consideration the different types of interactions, both positive (e.g. stimulation, upregulation) and negative (e.g. inhibition, downregulation) that are established between both bacteria.

All the annotated data is summarised in Table 3 according to interaction directionality, i.e., *P. aeruginosa* > *S. aureus* or *S. aureus* > *P. aeruginosa* interactions, and mode of growth. Interestingly, the majority of the annotated interactions have *P. aeruginosa* as the source microorganism (64%), which is corroborated by Figure 3(A) in which it is clear that the *S. aureus* node has a greater number of inward edges (interactions) than the *P. aeruginosa* node. In terms of the mode of growth used in all the analysed studies, no major differences were seen between biofilm and planktonic growth, with 46% of studies performed on biofilms and about 44% on planktonic bacteria. *In vivo* was the least studied setting, representing only 11% of the studies, and was mainly related to clinical studies of human samples rather than *in vivo* laboratorial testing.

Looking into studies concerning a disease scenario, the majority were CF related (Table 3). CF is a genetic disease associated with the dysfunction of a transmembrane chlorine channel that causes secretion of a

viscous mucus layer on the respiratory epithelium that facilitates microbial colonisation, with lung disease being the major cause of reduced life expectancy and death in these patients (Scoffone et al. 2019). Although *S. aureus* is typically one of the primary microorganisms isolated in CF lungs during early childhood, it is quickly followed by *P. aeruginosa*, which progressively increases its prevalence as patients grow older (Razvi et al. 2009; Price et al. 2013). When reaching 18 years of age, the most problematic microorganism for these patients is *P. aeruginosa*, with 80% being colonised with this bacterium (Høiby 2011), which becomes the most common pathogen isolated from CF sputum at this stage (Folkesson et al. 2012). The problematic of CF lung infection is a great example of the interplay between the two species and may explain why the majority of the studies focus on disclosing the mechanisms through which *P. aeruginosa* and *S. aureus* interact in the CF lung.

Concerning the most reported effector (source) entities, most annotated interactions reported effects of bacteria as a whole (annotated as “cell”) (Table 3), meaning that no molecular entity was identified/tested. Most annotated interactions, except those annotated in *in vivo* studies, have genes as the top target category

Table 3. Top 3 curated information for *P. aeruginosa*/*S. aureus* interactions based on the source organisms and mode of growth.

Source organism	No. of interactions	Mode of growth	No. of interactions	Source entity ^a	Target entity category ^a	Interaction category ^a	Disease ^b	Experimental method ^b
<i>P. aeruginosa</i>	432 (64%)	Biofilm	308 (71%)	Cell (83%) HQNO (2.9%) Pyochelin, Pyoverdine (1.6%)	Gene (79%) Cell (25%) Virulence Mechanism (5.2%)	Downregulation (56%) Upregulation (23%) Inhibition (11%)	Cystic fibrosis (54%) Wounds (21%) Chronic suppurative otitis media, Dental infections (3.6%)	CFU (37%) RT-PCR (12%) Crystal violet assay (7.7%)
				Cell (60%) Exoproducts (7.8%) HQNO (6.9%)	Gene (55%) Cell (38%) Virulence Factor (4.3%)	Inhibition (34%) Upregulation (30%) Downregulation (25%)	Cystic fibrosis (58%) Chronic lung infection, Wounds, Peritoneal infection (4.2%)	CFU (20%) RT-PCR (13%) Spectrophotometry (8.7%)
				Cell (100%)	Cell (88%) Virulence Mechanism (13%)	Synergism (75%) Inhibition, Stimulation (13%)	Cystic fibrosis (100%)	Lung function evaluation, Bacterial cultivability (38%) Bacterial lawn growth, Colony morphology analysis (13%)
<i>S. aureus</i>	238 (36%)	Biofilm	39 (16%)	Cell (82%) SpA (10%) Exoproducts (7.8%)	Gene (67%) Cell (26%) Virulence Mechanism (5.2%)	Upregulation (33%) Downregulation (28%) Null Effect (15%)	Cystic fibrosis (45%) Wounds (36%)	CFU (23%) RT-PCR, Crystal violet assay (14%) RNAseq (9.1%)
				Cell (91%) Exoproducts (3.1%) GlcNAc, Peptidoglycan (1.6%)	Gene (85%) Cell (5.2%) Virulence Factor (4.2%)	Downregulation (68%) Upregulation (15%) Stimulation (7.8%)	Cystic fibrosis (57%) VAP, Wounds, Peritoneal infection (7.1%)	CFU, RT-PCR (13%) Motility assay in agar, Flow cytometry, Spectrophotometry (6.7%)
				Cell (100%)	Cell (100%)	Synergism (100%)	Cystic fibrosis (100%)	Lung function evaluation, Bacterial cultivability (50%)

^a% relative to the total number of annotated interactions for the respective mode of growth. ^b% relative to the sum of the number of different diseases or experimental methods per document for the respective source organism and mode of growth. ^cClinical studies. HQNO: 2-heptyl-4-hydroxy quinoline N-oxide; GlcNAc: N-Acetylglucosamine; SpA: staphylococcal protein A; GlnAC: N-Acetylglucosamine; VAP: ventilator associated pneumonia; CFU: colony-forming units; RT-PCR: reverse transcriptase-polymerase chain reaction; RNAseq: RNA sequencing.

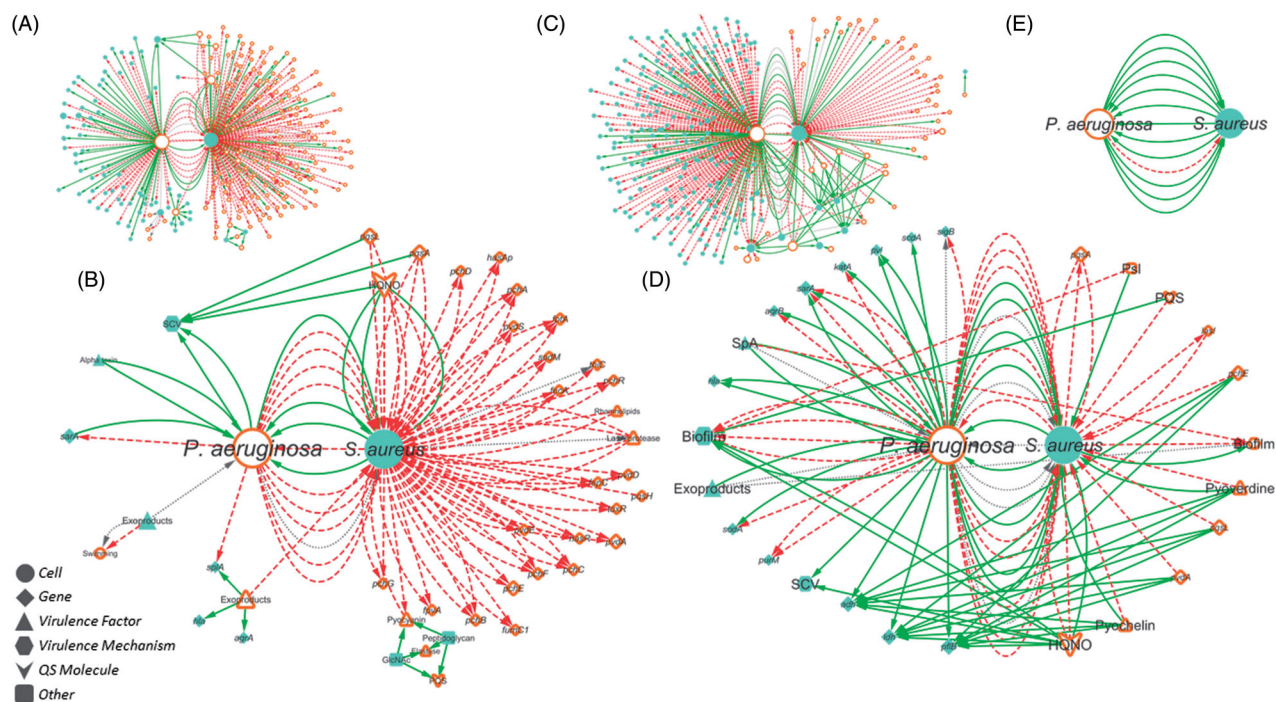


Figure 4. *P. aeruginosa/S. aureus* interaction networks in planktonic, biofilm, and *in vivo* settings. (A) All annotated interactions in the planktonic mode of growth; (B) Most reported interactions (annotated more than once) in the planktonic mode of growth; (C) All annotated interactions in the biofilm mode of growth; (D) Most reported interactions (annotated more than once) in the biofilm mode of growth; (E) All annotated interactions *in vivo*. Solid arrows: positive interactions (e.g. upregulation, stimulation); dashed arrows: negative interactions (e.g. downregulation, inhibition); dotted arrows: null effect. *P. aeruginosa* and *S. aureus* nodes are depicted in unfilled and filled shapes, respectively; node and node label sizes are directly proportional to the number of related (outward and inward) edges (interactions).

(Table 3). However, this does not translate to a high number of studies analysing gene modulation, which comprise only 11% of the total analysed, but rather reflects the great amount of results that more in-depth molecular methodologies, such as microarrays and RNA sequencing, output, each translating into an annotated interaction. The concomitant analysis of both bacteria grown as a dual-species biofilm demands the adaptation and optimisation of methodologies typically designed for single-species analysis. This setback can be what is currently impairing a greater use of in-depth methods for the analysis of *P. aeruginosa* and *S. aureus* interactions at the molecular level.

***P. aeruginosa* and *S. aureus* biofilm network**

As stated, biofilms are known to be the mode of growth of up to 80% of human bacterial infections (Fey 2010). Thus, although planktonic testing is practical and informative, studies entailing biofilms do a better job at mimicking a real-life infection scenario. The biofilm, planktonic, and *in vivo* *P. aeruginosa/S. aureus* interaction networks are represented in Figure 4. Biofilms represent the mode of growth of 52% of the total annotated interactions, which is still far from ideal.

Interestingly, when looking into *P. aeruginosa* > *S. aureus* interactions, most of them were reported in biofilm scenarios (71%), with only 27% using planktonic growth (Table 3). Regarding *S. aureus* > *P. aeruginosa* interactions, planktonic was the most used mode of growth, namely in over 80% of the annotated interactions. This is also observable in Figure 4, where there is a clear contrast between the number of edges, i.e., interactions, targeting each bacteria when comparing the biofilm (Figure 4(C)) and the planktonic (Figure 4(A)) networks. This observation illustrates a gap in studies concerned with the effect of *S. aureus* on *P. aeruginosa*, revealing an urgent need for more biofilm testing in order to originate a levelled understanding of the bi-directional interplay of both species within mixed consortia.

The most annotated effector entities, apart from “cell”, for *P. aeruginosa* > *S. aureus* biofilm interactions were HQNO, pyochelin, and pyoverdine (Table 3), as best represented in Figure 4(D). HQNO is a major compound produced by the *pqsABCDE* operon of *P. aeruginosa* (Figure 1) and is described as anti-staphylococcal molecule acting through the inhibition of the oxidative respiration (Williams and Cámara 2009). In turn, pyoverdine and pyochelin are the two major *P.*

aeruginosa siderophores, that is, iron-chelating molecules, produced in iron-limited conditions (Brandel et al. 2012) that are able to inhibit *S. aureus* biofilm formation. In fact, it has been shown that HQNO and the two siderophores are both required for efficient killing of *S. aureus* by *P. aeruginosa* (Filkins et al. 2015).

In turn, in *S. aureus* > *P. aeruginosa* interactions, the most annotated source entities, apart from “cell”, were exoproducts and the staphylococcal protein A (Spa) (Table 3), as best shown in Figure 4(D). Spa is a secreted factor that not only mediates interaction with host cells but has also been shown to bind cell surface structures of *P. aeruginosa*, inhibiting two persistence-associated behaviours of this bacterium: biofilm formation and uptake by host immune cells (Armbruster et al. 2016). The testing of exoproducts was actually generally used in all scenarios and interaction directions, except for *in vivo* settings. Both HQNO and staphylococcal protein A are examples of exoproducts, but the general term here annotated was stemmed from studies where a specific molecule was not tested but rather the cell-free supernatant of the bacterial culture. This was an expected feature of these studies given that interspecies communication and QS are mainly based on the production and excretion of molecules to the extracellular media that are sensed by other cells.

As stated, gene was the most annotated target type in all scenarios except “*in vivo*”. For *S. aureus* > *P. aeruginosa* interactions, most annotated target genes in planktonic settings related to the iron binding and transport. Iron is a vital nutrient for bacterial growth but relatively scarce in most infection sites, acting as a limiting factor (Nguyen and Oglesby-Sherrouse 2015). *P. aeruginosa* secretes exoproducts that negatively impact *S. aureus* growth and survival causing cell death and subsequent iron release, which can be further utilized by *P. aeruginosa* for its own benefit (Mashburn et al. 2005). The presence of *S. aureus* during co-culture seems to be related with a decreased transcription of *P. aeruginosa* iron regulated genes as 51% of annotated target genes pertained to this category, with 96% of them being downregulated, indicating that the presence of *S. aureus* increases usable iron for *P. aeruginosa*. In biofilm settings, QS-related genes, such as *rhIR*, *pqsH*, and *lasI*, were one of the top annotated targets (23%). Looking at the types of established interactions, it is not surprising that *P. aeruginosa* QS genes were downregulated or unchanged in biofilms where *S. aureus* was present. *P. aeruginosa* produces multiple virulence factors that contribute to the removal of *S. aureus* from dual-species biofilms, with the LasI/LasR, PqsABCDE/PqsR, and RhIR/RhIR QS systems being the major regulators of these

factors (Lee and Zhang 2015). In fact, when *P. aeruginosa* mutants lacking one or both major QS genes (*lasI*, *rhII*, and *lasI/rhII*) were co-cultured in dual-species biofilms with *S. aureus*, a less effective removal of *S. aureus* was observed, suggesting that *S. aureus* thrives when *P. aeruginosa* QS is inactivated (Woods et al. 2018).

As to *P. aeruginosa* > *S. aureus* interactions in planktonic settings, most annotated target gene pertained to functions related to molecule biosynthesis (35%), mostly of purines and pyrimidines, key components of DNA/RNA synthesis and whose production was upregulated. This could point to an early state of nitrogen starvation in co-culture (Tognon et al. 2019). In biofilm settings, most annotated targets were related with ion and molecule transmembrane transport (17%) and biosynthesis (13%), mostly downregulated (77% and 93%, respectively), which may contribute to a state of metabolic quiescence, decrease in biofilm formation (Miller et al. 2017), and, possibly, SCV induction.

Discerning *P. aeruginosa* > *S. aureus* communication in biofilms

In clinical settings, biofilm infections caused conjointly by *P. aeruginosa* and *S. aureus* are more virulent than those caused by each independent species, and are frequently associated with increased disease severity and chronicity (DeLeon et al. 2014). Nevertheless, the ecological relationship established between *P. aeruginosa* and *S. aureus*, in such niche, is competitive rather than cooperative. The effect of *P. aeruginosa* in *S. aureus* appears to be preferably studied and will be used here to showcase the complexity and diversity of established interactions.

As demonstrated in Figure 5, a complex and intricate network of regulators dictates the expression of pathogenicity factors in *P. aeruginosa*, which play an important role in its social interplay with *S. aureus* within the dual-species biofilms. *P. aeruginosa* secretes several extracellular substances, such as hydroxy-2-alkylquinolines (PQS and HQNO) and hydrogen cyanide, which hinder the proliferation of *S. aureus* biofilms (Palmer et al. 2005; Biswas et al. 2009; Filkins et al. 2015). Both PQS and HQNO are products of the PqsABCDE/PqsR QS system, whose action accounted for 18 of the biofilm annotated interactions in over 5 different documents, including stimulation of *S. aureus* biofilm formation and *S. aureus* growth inhibition (Figure 5). In fact, out of the existing four, this QS system was the most reported as acting, directly or indirectly, upon *S. aureus*, followed by the RhII/RhIR system. PQS is a signalling molecule that positively regulates a subset of QS dependent genes involved in siderophore-mediated iron uptake and iron chelating activity in *P. aeruginosa*

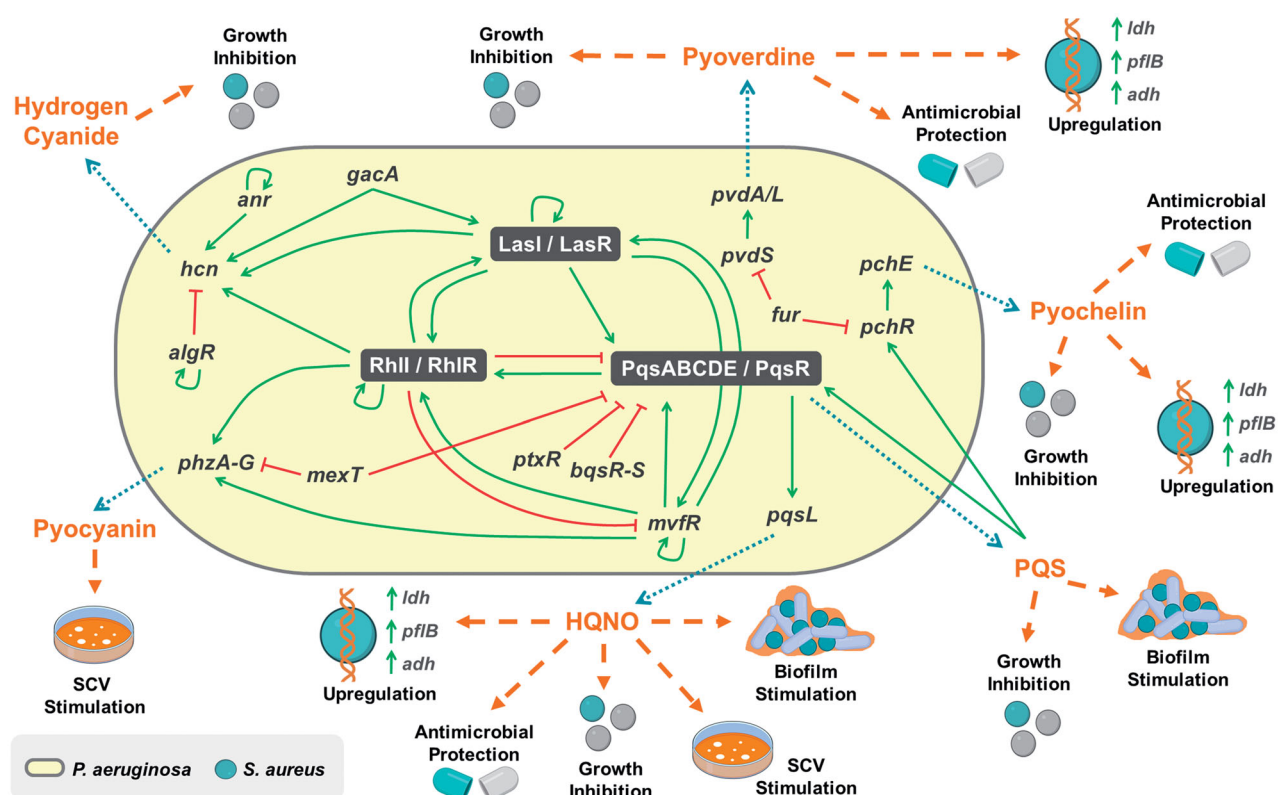


Figure 5. Schematic representation of the effect of *P. aeruginosa* on *S. aureus* in dual-species biofilms. Annotated interconnections between the molecules produced by *P. aeruginosa*, their effect on *S. aureus*, and the main regulatory network involved in *P. aeruginosa* > *S. aureus* interactions is depicted. Solid arrows: upregulation of gene expression; lines with flat ends: downregulation of gene expression; dotted arrows: synthesis process; dashed arrows: effect on *S. aureus*.

(Bredenbruch et al. 2006). Specifically, PQS can prompt the expression of genes involved both in the regulation (*pvdS*) and biosynthesis of pyoverdine (e.g. *pvdA* and *pvdE*) and pyochelin (*pchE*) (Diggle et al. 2007). Despite the reported inhibitory effect of pyoverdine and pyochelin on *S. aureus* (Figure 5), both siderophores were found to be involved in its protection from antibiotic treatment in a biofilm model of CF infection (Orazi and O'Toole 2017).

In fact, the growth of *S. aureus* is not completely inhibited by *P. aeruginosa*. *S. aureus* has defence mechanisms that help it outcompete *P. aeruginosa* in the same infection, thus coexisting as a persister (Nair et al. 2014). For instance, *P. aeruginosa* excretes HQNO, which activates the alternative sigma factor B in *S. aureus* and alters the expression of several virulence factors, including those that regulate adherence, invasiveness, and persistence within host cells, and facilitates the emergence of SCV (Mitchell et al. 2010). The SCV phenotype can survive in proximity with *P. aeruginosa*, which allows *S. aureus* persistence. The stimulation of biofilm formation by PQS and HQNO also contributes to this persistence, as biofilm-enclosed cells become more protected from external stresses (Fugère et al. 2014).

Although the exact regulatory mechanisms behind *P. aeruginosa*/*S. aureus* communication in biofilms are not

fully clear, the reconstructed knowledge-networks highlight the complexity and multi-tiered regulatory processes by which *P. aeruginosa* controls expression of virulence factors that critically affect *S. aureus*, impacting the pathogenicity of the biofilm-related infection. Specifically, the networks revealed that *P. aeruginosa* QS signalling plays an important role in this dual-species interplay, since three QS systems, namely the systems LasI/LasR, RhII/RhIR and PqsABCDE/PqsR, positively regulate the expression of specific *P. aeruginosa* virulence factors affecting *S. aureus*.

Besides QS, the integrated regulatory network of *P. aeruginosa* shows that other genes can also play key roles and control multiple pathways that trigger inter-species interactions. For example, PtxR is a transcriptional regulator that reduces the expression of the *pqsABCDE* operon (Figure 5), which negatively affects the production of pyocyanin (Carty et al. 2006). Stressful environmental conditions may also be controlling the differential expression of the regulatory cascade. For instance, *mvfR* is involved in regulating critical physiological processes, being known to control the transcription of iron-related genes, under low-iron concentrations (Ochsner et al. 2002), and upregulating the transcription of the three QS systems and pyocyanin

synthesis (Figure 5). Once intracellular iron levels are high, uptake systems and their regulators, including *mvfR*, are repressed by the *fur* transcriptional regulator (Troxell and Hassan 2013). This negative feedback loop also turns down the production of all other systems under *mvfR* control (e.g. pyocyanin and HQNO synthesis) (Maura et al. 2016). Under low oxygen or anoxia, *anr* controls hydrogen cyanide production and other cellular responses, including biofilm formation associated with chronic infections (Hammond et al. 2015).

Targeting *P. aeruginosa*/*S. aureus* biofilm communication

Antivirulence agents are presented as alternative therapeutics that can circumvent antibiotic resistance by targeting virulence factors rather than bacterial growth pathways (i.e. the target of traditional antibiotics) (Totsika 2016). This strategy is quite advantageous considering the large number of putative virulent targets (Allen et al. 2014). Since QS is the main virulence regulator in bacteria, inhibition of QS mechanisms using quorum quenching (QQ) agents appears to be a promising strategy to modulate the virulence of bacterial pathogens (Chan et al. 2015). Moreover, as the target may constitute an extracellular factor, the emergence and spread of resistance could be less likely (Fetzner 2015). Nevertheless, the selection of the target is of critical importance for the effectiveness of the antimicrobial strategy. In line with this, it is essential to understand the full dynamics of action of the targeted virulence factor as well as the dynamics of production (Dickey et al. 2017). The expression of certain virulence factors could be subjected to the control of several regulatory mechanisms other than the targeted QS system (Arya and Princy 2016).

Figure 6 summarizes the antivirulence agents that have so far shown inhibitory effect against two major virulence factors produced by *P. aeruginosa* and annotated as affecting *S. aureus* in biofilm settings, HQNO and siderophores (pyoverdine and pyochelin). A search was made in <http://pcquorum.org/> for inhibitors targeting not only the molecules of interest but also their coding genes. A total of 25 and 19 reported agents were encountered inhibiting HQNO and siderophores, respectively, with four of them being active against both pyoverdine and pyochelin (Figure 6).

Although these antivirulence agents may be promising for attenuating *S. aureus* and *P. aeruginosa* resilience in co-infection scenarios, this antivirulence strategy has to face several challenges to become a feasible therapeutic option. One of the challenges in

disrupting QS networks is the fact that, in some cases, the interference with the target could promote virulence instead of attenuating it (García-Contreras et al. 2015). This point is even more relevant when considering polymicrobial infections. Because inter-species interactions could be mediated by QS-controlled factors, the interference with a QS system in a pathogen potentially could facilitate the pathogenicity of the co-infecting species (Radlinski et al. 2017). For example, the PqsABCDE/PqsR system influences the production of extracellular DNA (Whitchurch et al. 2002) and lectins, which influences biofilm formation and enhances colonisation and infection establishment in *P. aeruginosa* (Lee and Zhang 2015). In addition, this system positively regulates HQNO levels, involved in *P. aeruginosa*/*S. aureus* interactions (Figure 5). Depending on the environmental conditions found by these pathogens, different interactions were annotated, including stimulation of *S. aureus* SCV, antimicrobial protection, and inhibition of *S. aureus* growth. Therefore, the interference with the QS system could reduce the overall virulence, by inhibiting *P. aeruginosa* biofilm formation and possibly preventing the formation of *S. aureus* SCV. On the other hand, as HQNO inhibits *S. aureus* growth, the antivirulence therapies targeting this molecule could backfire and facilitate the spreading of *S. aureus*.

Consequently, the choice of antivirulence agents must pay attention to the multi-species community as a whole, that is, the impact on the remaining co-occurring pathogens must be considered as it can have a positive, hence undesired, effect on them. Moreover, to increase antimicrobial effectiveness in polymicrobial communities, empiric therapy would probably require the combination with other antimicrobial agents (including other antivirulence drugs or even antibiotics). Very few studies have tackled this approach in double-species biofilms of *P. aeruginosa* and *S. aureus*. The only example found in the literature using an adapted optimized query is the use of the QS inhibitor hamamelitanin targeting the RAP/TRAP QS system in *S. aureus*, which was combined with vancomycin in a gauze and successfully reduced biofilm formation of both species in an *in vitro* mixed biofilm model of chronic wound (Brackman et al. 2016). Although antivirulence approaches have substantially progressed, it appears that the search for antivirulence agents is still a challenging and unexplored area of investigation, with most of these antivirulence drugs tested *in vitro* and against single-species populations (Pérez-Pérez et al. 2017; Martínez et al. 2019).

Overall, this work was able to decipher the current knowledge on the complex interactions between *P.*

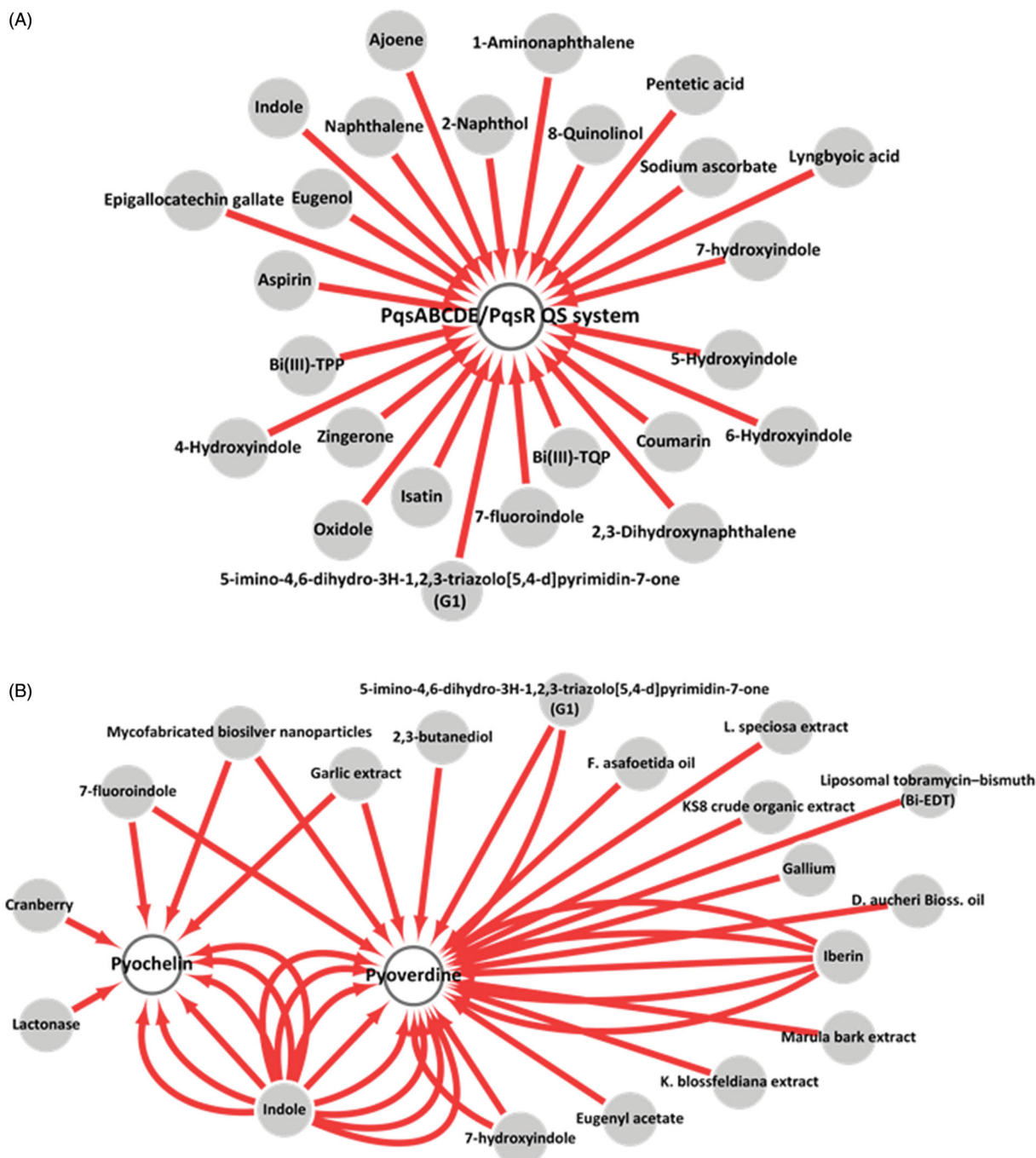


Figure 6. Networks of antivirulence agents targeting (A) the PqsABCDE/PqsR QS system and (B) the siderophores pyoverdine and pyochelin.

aeruginosa and *S. aureus* in the context of their competitiveness within the biofilm mode of growth, by implementing an innovative and systematic analysis of the scientific literature on this subject. The examination of the gathered information resulted in the clarification of the amount and types of studies being conducted, but more importantly, in the identification of the major and most studied molecular players in *P. aeruginosa*/*S. aureus* communication and/or interaction. More specifically, with *P. aeruginosa* revealed as key player,

pseudomonal factors affecting *S. aureus* were pointed out (e.g. HQNO and siderophores) and their respective antivirulence agents identified. Yet, we are still far away from fully understanding the dynamic and complex networks of interactions that occur in the natural infection environment. Although efforts are being made to deeply understand the interactions that those microorganisms experience during infection, as this work shows, the percentage of studies that have pointed out specific mechanisms of interaction remains relatively

small. The improvement of more powerful and efficient genetic tools and the design of feasible *in vitro* models that reflect the *in vivo* environment are pivotal for exploring unanswered questions about the ecology of *P. aeruginosa*/*S. aureus* biofilm infections. Expectantly, this work and its resulting database (www.ceb.uminho.pt/ISCTD.) will assist researchers aiming at diminishing the resilience of this bacterial consortium within biofilm related infections by downsizing time and work related costs.

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