

**Name of journal:** *World Journal of Clinical Cases*

**ESPS Manuscript NO:** 14531

**Columns:** REVIEW

**Role of quorum sensing in bacterial infections**

Castillo-Juárez I *et al.* QS and infections

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**Author contributions:** All authors designed the review and wrote the paper.

**Supported by** JSPS grant Challenging Exploratory Research, No. 25660062; SEP-CONACyT grant, No. 152794 (García-Contreras R); Fideicomiso COLPOS 167304 and Programa Cátedras-CONACyT 2112 (Castillo-Juárez); the Miguel Servet Program (C.H.U.A Coruña and ISCIII) (Tomás M); the CONACyT grant number 441393/269132 (Mandujano-Tinoco EA); and the Biotechnology Endowed Chair at the Pennsylvania State University.

**Conflict-of-interest:** The authors declare no conflict of interest.

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**Received:** October 9, 2014

**Peer-review started:** October 13, 2014

**First decision:** November 14, 2014

**Revised:** November 30, 2014

**Accepted:** April 16, 2015

**Article in press:**

**Published online:**

## **Abstract**

Quorum sensing (QS) is cell communication that is widely used by bacterial pathogens to coordinate the expression of several collective traits, including the production of multiple virulence factors, biofilm formation, and swarming motility once a population threshold is reached. Several lines of evidence indicate that QS enhances virulence of bacterial pathogens in animal models as well as in human infections; however, its relative importance for bacterial pathogenesis is still incomplete. In this review, we discuss the present evidence from *in vitro* and *in vivo* experiments in animal models, as well as from clinical studies, that link QS systems with human infections. We focus on two major QS bacterial models, the opportunistic Gram negative bacteria *Pseudomonas aeruginosa* and the Gram positive *Staphylococcus aureus*, which are also two of the main agents responsible of nosocomial and wound infections. In addition, QS communication systems in other bacterial, eukaryotic pathogens, and even immune and cancer cells are also

reviewed, and finally, the new approaches proposed to combat bacterial infections by the attenuation of their QS communication systems and virulence are also discussed.

**Key words:** Quorum sensing; Virulence; Infections; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; Animal models

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**Core tip:** In this manuscript we discuss the basics aspects of quorum sensing (QS) and its relationship with human infections, focusing in two major QS bacterial models, the opportunistic Gram negative bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Castillo-Juárez I, Maeda T, Mandujano-Tinoco EA, Tomás M, Pérez-Eretza B, García-Contreras SJ, Wood TK, García-Contreras R. Role of quorum sensing in bacterial infections. *World J Clin Cases* 2015; In press

## INTRODUCTION

Several important bacterial pathogens, like *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Vibrio cholerae*, utilize quorum sensing (QS) cell communication to coordinate the expression of multiple virulence factors and associated behaviors such as swarming and biofilm formation, once a population size threshold is reached. QS systems consist of an enzyme that catalyzes the synthesis of the signal (such as acyl-homoserine lactones or cyclic peptides) and a receptor that binds the signal and reprograms the expression of several genes, including those encoding the enzyme that produces the signal, creating a positive feedback loop. In bacterial pathogens, most of the QS controlled genes codify

several different virulence factors, such as proteases, toxins, and adhesins<sup>[1]</sup>. The expression of QS controlled phenotypes is energetically costly to the cells and only provides an advantage if it is expressed when cells reach high densities<sup>[2,3]</sup>; hence, in the context of bacterial infections, the expression of QS regulated virulence factors is delayed until a sufficient bacterial load is achieved and once this threshold is reached, bacteria coordinate their attack against the host, which maximizes the probability of establishing the infections and disseminating them, hence increasing the pathogen fitness. In fact, QS along with subversion of the immune system are the main factors that determine the bacterial infectious doses. Hence those bacterial pathogens that need small doses to infect, generally lack QS systems but are very effective at inactivating the immune response by killing professional phagocytes. In contrast, those bacterial pathogens that need high infectious doses rely in QS for the coordination of the expression of virulence<sup>[4]</sup>. In this review, the current knowledge about QS control of virulence factors in two main model bacterial pathogens, *P. aeruginosa* and *S. aureus* (which are also responsible for nosocomial and wound infections), will be discussed along with the relationship of their QS systems, its virulence in animal infection models, and the data available from human infections. Furthermore, the role of QS in other important infections and the role of QS in immune and cancer cells are discussed. Finally, proposed novel approaches of blocking QS/virulence as an alternative in fighting recalcitrant bacterial infections are also reviewed.

## **QS-CONTROLS OF THE EXPRESSION OF VIRULENCE FACTORS *IN VITRO***

### ***P. aeruginosa***

*P. aeruginosa* possesses at least three functional QS circuits; two of them are mediated by N-acyl homoserine lactones (HSL) signals and the other mediated by quinolones (Figure 1). The HSL-QS systems were first described and they were named after the virulence factors that were first identified under their control; hence, the Las system was discovered as a positive regulator for elastase

production through the expression of the structural elastase gene *lasB*<sup>[5]</sup>. This system (by LasI HSL- acyl- synthase) produces the 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), that binds its receptor LasR which then dimerizes and binds promoters that contain *las* boxes, turning on the expression of several genes, including *lasI*, which then in a positive feedback loop increases the production of 3-oxo-C12-HSL, the other HSL mediated QS system was named Rhl since it controls the expression of the biosurfactant rhamnolipids<sup>[6]</sup>. This system (RhlI) produces N-butyryl-L-homoserine lactone that is sensed by RhlR and also shows positive autoregulation<sup>[7]</sup>. The third QS system is mediated by different kinds of signals, alkyl quinolones, specifically 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal or PQS) which is synthesized from anthranilate by the products of *pqsABCDEH* genes and sensed by PqsR (MvfR)<sup>[8,9]</sup>. The three systems are interconnected and function in a hierarchical way<sup>[10]</sup>; the Las system is the first to become activated, and it in turn it stimulates the Rhl and PQS systems<sup>[11,12]</sup>, while PQS activates Rhl<sup>[13]</sup> and Rhl inhibits PQS<sup>[11,14]</sup>. Moreover, 3-oxo-C12-HSL, the Las signal, is able to bind functional RhlR dimers, promoting their dissociation and inactivation<sup>[15]</sup>. In addition to control *lasB* elastase, the Las system also controls the expression of *lasA* elastase, exotoxin A (PA1148), and alkaline protease (PA1246)<sup>[16]</sup>, and the Rhl also controls the expression of the phenazine pyocyanin a pigment able to cause oxidative damage to the eucaryotic host, promoting the production of reactive oxygen species (ROS) and depleting the host antioxidant defense mechanisms<sup>[17]</sup>, while the PQS system increases the expression of *lasB* elastase and pyocyanin<sup>[9]</sup>. In fact, the regulation of virulence factors by these 3 QS systems is complex and often overlaps<sup>[18]</sup>; for example, RhlR is apparently enough to compensate the absence of LasR at least in stationary phase cells in which it promotes the production of exoproteases, pyocyanin, PQS, and the 3-oxo-C12-HSL<sup>[18,19]</sup>. To add even more complexity, recently the role of environmental signals, such as the availability of iron and phosphate in influencing QS systems has been beginning to be explored<sup>[20]</sup>. In addition, other ions such as calcium strongly

influence the production of QS modulated virulence factors such as pyocyanin, and proteases<sup>[21]</sup> and in fact there is solid evidence that indicates that the chemical composition of the sputum in cystic fibrosis patients promotes the use of the PQS system for communication, preferentially over the HSL systems<sup>[22]</sup>. Moreover the presence of metabolites like 2,3-butanediol(end product of bacterial fermentation from species that coexist with *P. aeruginosa* in the lung of cystic fibrosis patients) enhance the production of QS controlled virulence factors (phenazines and exotoxin) and improve biofilm formation *via* the Las QS system<sup>[23]</sup>; hence, the expression of QS-virulence factors *in vivo* is likely influenced by several variables, related with the state of the host as well as the presence or absence of other bacterial species. Indeed, the simultaneous utilization of several QS systems in bacteria, may serve different purposes like identifying community composition<sup>[24]</sup> or distinguish phases in population development<sup>[25]</sup>, and a recent study shows that the concomitant utilization of Las and Rhl systems allows *P. aeruginosa* to simultaneously assess their population density and the presence of nutrients by combinatorial communication. Therefore, the secretion of QS controlled factors is subjected to “AND-gate” like responses to multiple signal inputs, allowing effective expression of secreted factors in high-density and low mass-transfer environments<sup>[26]</sup>. Another important role of QS systems in regulating bacterial physiology is that they are implicated in the tolerance against stress<sup>[27-29]</sup> that allow them to maximize their chances to effectively contend and survive the immune system attack<sup>[30]</sup>, which may be a major determinant for the establishment and progression of *P. aeruginosa* and other pathogens infections.

### ***S. aureus***

*S. aureus* produces several virulence factors and many of them are regulated by QS. In Gram positive bacteria, regulation by QS is generally mediated by autoinducing cyclic peptides. Specifically for *S. aureus*, QS controls the expression of virulence factors such as hemolysins, leukocidins, cell surface adhesins,

exoenzymes, and biofilm formation via the Agr system, which relies on the autoinducing peptide (AIP) (Figure 1E). AIP is encoded by *agrD* and consists of 7–9 amino acids, and has a 5-membered thiolactone ring<sup>[31-33]</sup>; this peptide is secreted by the membrane protein AgrB and activated by the AgrC sensor kinase<sup>[1]</sup>. The Agr system regulates the expression of several genes by the production of two regulatory RNAs, RNAII and RNAIII<sup>[34]</sup>, which are produced from promoters P2 and P3 respectively<sup>[34,35]</sup>. Transcription from the *agr* operon (*agrA*, *agrB*, *agrC* and *agrD*) is regulated by a phosphorylated AgrA homodimer from P2<sup>[36]</sup>, while RNAIII is produced by AgrA from P3. RNAIII, which is the effector of the system, upregulates *a*-haemolysin, and increases the production of proteases, toxins, and the synthesis of capsule, while it represses protein A (which allows *S. aureus* to evade opsonization), and the expression of surface adhesions<sup>[1,31,34,35,37,38]</sup>. Such modulation of the expression of several virulence factors by the Agr system allows *S. aureus* to express a different repertoire of those determinants according to the kind of disease and the environmental conditions including the host status. Noteworthy is that *in vitro* the appearance of clones with diminished QS had been observed; these clones are apparently social cheaters which exploit cooperative individuals without contributing with the production of virulence factors. The presence of cheaters during infections may be very relevant for disease progression, since in controlled experiments, the ratio between cheaters and cooperating individuals strongly affects the mortality rate and extent of infection; i.e., the severity of the infections are inversely proportional to the percentage of cheaters in the population<sup>[39]</sup>. Among the QS-controlled virulence factors in *S. aureus*, RNAIII is very important since it regulates biofilm formation, and resistance to antibiotics as well as the establishment of chronic infections is intimately related to the biofilm formation abilities of pathogens<sup>[40]</sup>. However, the *in vivo* biofilms in which *S. aureus* exists can be very complex environments, due to the presence of several other bacterial species and their multiple interactions with each other and with the host, hence *in vitro* models for studying *S. aureus* virulence



may have the disadvantage of not revealing the real expression of virulence factors. This hypothesis is supported by significant differences in the expression of several virulence factors in *S. aureus* grown in calf serum compared with those grown in defined CDM medium, since in serum the expression of hemolysins, enterotoxins, proteases, iron acquisition factors, and of RNA III is significantly higher than in standard growth medium, and such differences are partially due the low iron concentration in serum<sup>[41,42]</sup>.

### **QS-CONTROLS OF THE EXPRESSION OF VIRULENCE FACTORS *IN VIVO* *PSEUDOMONAS AERUGINOSA* QS AND VIRULENCE IN ANIMAL MODELS**

A number of animal models including the nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*) and mouse (*Mus musculus*), have been used to identify and define the role of virulence determinants in the pathogenesis of *P. aeruginosa*<sup>[43,44]</sup>. The attenuation of its QS is achieved by two basic strategies: (1) the utilization of mutant strains with QS genes disrupted; and (2) the quenching of QS by treatments that interfere with it; these methods have shown that QS systems as well as QS-independent virulence determinants are required for *P. aeruginosa* infections in animals.

The main animal model that was used to discover the relationship between QS and virulence of *P. aeruginosa* is the nematode *C. elegans*. In 1999 Tan and coworkers first described conditions to test the role of QS in virulence using this model, showing that the reference strain PA14 kills the nematode either after days (slow killing) or quickly after a few hours (fast killing)<sup>[45]</sup>. Their evidence indicate that fast and slow killing occur by distinct mechanisms; the slow killing involves an infection-like process and correlates with accumulation of PA14 within worm intestines, while the fast killing is mediated by the production of phenazines (regulated by QS); that increase active oxygen species<sup>[45,46]</sup>. A third mode by which *P. aeruginosa* can kill *C. elegans* is lethal paralysis; this mechanism is mediated by QS since Darby and coworkers, using QS-less mutant strains of *P.*

*aeruginosa*, found that the lethal effect is associated with a rapid neuromuscular paralysis, caused by the action of diffusible unidentified factors whose production requires the *las* and *rhl* genes, since the infection with a *lasR* mutant and with a *rhlR* reduces the paralysis (by 28%-100% and 100% respectively)<sup>[47]</sup>. A potential target of these diffusible factors is the EGL-9 worm protein, which is expressed in the neuronal muscle tissues<sup>[47]</sup>. In a recent study, a reduction of 83% in the death of the nematodes by the double mutant (*PA14rhlRlasR*) was reported; however, the analysis of individual mutants, revealed that only the *rhlR* mutant reduced death 69%, implying that the RhlR system is crucial for infection under their experimental conditions<sup>[48]</sup>. In addition to lethal paralysis and slow and fast killing, a fourth kind of *C. elegans* death induced by *P. aeruginosa* is the “red death”, characterized by the formation of red precipitate (PQS+Fe<sup>3+</sup> complex) within the intestine of the nematodes. This mode of death is mediated by the quinolone dependant QS system Mvfr-PQS in coordination with the PhoB phosphate sensor and the pyoverdine iron acquisition system<sup>[49]</sup>. The role of QS in *P. aeruginosa* infectivity and virulence in *C. elegans* is also evidenced by the effect of QS inhibitors, since a synthetic analog of HSL, meta-bromo-thiolactone (Figure 2A) that partially inhibits *in vitro* the LasR and RhlR systems also reduces the death of worms infected with PA14, to 60% at 24 h. Interestingly, the *in vivo* action of the quencher in the worm model occurs mainly through the RhlR system<sup>[48]</sup>. Moreover, phenylacetic acid (Figure 2B), which is a byproduct of the degradation of antibiotics such as penicillin G and cephalosporin G by G acylase<sup>[50]</sup>, increases the survival of PAO1 infected nematodes by 53%, while untreated worms die within 72 h. This protective activity is perhaps a consequence of interfering with the LasR and RhlR systems, due to the structural similarity of phenylacetic acid with salicylic acid, a quorum quencher<sup>[51]</sup>. Another compound, 2,5-piperazinedione (Figure 2C), increases the survival of worms by 66%, compared to untreated ones, and it was shown by molecular docking that it interacts with an amino acid residue (E145) in LasR, which is required for correct binding of the natural HSL

ligand<sup>[52]</sup>. Similarly curcumin (Figure 2D), a secondary metabolite from *Curcuma longa*, increases the survival of worms by 28%; this compound decreases the expression of genes involved in biofilm formation and attenuates HSL production in PAO1. Thus, it was suggested that it may act as a quorum quencher, delaying the synthesis of HSL molecules or by impairing autoinducers perception<sup>[53]</sup>. Moreover, various enzymes that degrade natural autoinducers are able to decrease the pathogenicity of *P. aeruginosa*; for example, adding the purified acylase PvdQ to *C. elegans* infected with *P. aeruginosa* PAO1, strongly reduces their pathogenicity and increases the nematodes life span<sup>[54]</sup>. Although the utilization of *C. elegans* as a model for studying *P. aeruginosa* infections has been very fruitful, recently it was proposed to use the fruit fly (*D. melanogaster*) as an animal model for the study of the *P. aeruginosa* pathogenesis, since the fly has a higher similarity to human<sup>[55-57]</sup>. The importance of both *P. aeruginosa* HSL QS pathways for infection was also demonstrated in *D. melanogaster* using the feeding assay, in which the bacteria are ingested and a local infection type is established in the intestine. In this assay the PA103 (*lasR*), PDO100 (*rhII*), PDO111 (*rhIR*), PAOR1 (*lasR*) and PAOJP2(*lasI/rhII*) mutant strains were avirulent with respect to wild-type PAO1 whose infected flies were killed at 14 d post-infection. Similarly, using the nicking assay (needle pricking), in which an injury is produced in the dorsum of the flies and *P. aeruginosa* is added to the wound, all mutant strains showed a lower death rate than wild-type, including the PDO100 mutant (*rhII*) with 50% survival of the flies compared to 90% death for the PAO1 wild-type 35 d post-infection<sup>[57]</sup>. However, in contrast to the work with *C. elegans*, to date the effect of quorum quenching in *P. aeruginosa* virulence in the fly was not yet evaluated. With regard of these two infection models, Clatworthy and colleagues pointed out that a drawback to study *P. aeruginosa* infections using invertebrate hosts are the differences between their immune response and the one of vertebrates. For example, *C. elegans* and *D. melanogaster* do not have an adaptive immunity, or complex multilineage immune cells, such as those present in vertebrates<sup>[58]</sup>. Thus it is important to analyze the

participation of QS in the pathogenesis of *P. aeruginosa* in vertebrate animal models, like zebrafish and mice. Specifically for zebrafish, the microinjection of PA14 QS-mutant strains (*lasR* and *mvfR*) during two different stages of fish development [28 and 50 h post-fertilization (hpf)], revealed that the participation of these two transcriptional activators during the infections is different and is influenced by the maturity of the immune system at different stages of the embryo development, since for the *lasR* mutant, only a 40% decrease in the death of the embryos at 50 hpf (a developmental stage when both macrophages and neutrophils are present) was recorded, whereas the *mvfR* mutant showed a moderate effect by decreasing death by 20% to 28 hpf (an stage in which only macrophages are present), but a higher effect of 60% decrease at 50 hpf<sup>[58]</sup>.

For murine models, different protocols have been used to determine the participation of QS in *P. aeruginosa* pathogenicity<sup>[43,44]</sup>. The thermal induced injury model is frequently used and consists of producing a burn of second or third degree on the dorsal side of the mouse using water at 90°C and subsequent inoculation of *P. aeruginosa*. Several experiments using this model have linked QS and virulence; for example, a PAO1-R1 ( $\square$ *lasR*) mutant has a diminished ability to spread systemically, as well as lower dispersion through the lesion at early stages<sup>[59,60]</sup>. Also, mice infected with PA14 *pqsA* show a 75% survival rate in contrast to 10% survival with wild-type PA14<sup>[61]</sup>. Similarly, virulence is reduced in PAO1 *lasR*, *lasI*, and *rhII* mutants, with the greatest effect seen for the double mutant *lasI-rhII* that decreased the mortality of animals by ~88%, significantly reduced the number of c.f.u in the lesion, liver and spleen, and delayed the spread of the bacteria from the lesion<sup>[59]</sup>. In agreement, similar results were found using the pneumonia model in neonatal mice, in which *lasR* mutants showed reduced virulence and are unable to replicate efficiently in the lung tissue; as a consequence, less damage occurs and the bacterial infection does not spread<sup>[62]</sup>. A third kind of experimental infection, the foreign-body infection model, consists in introducing a fragment of *P. aeruginosa* infected silicone into the peritoneal cavity

of mice. This model was successfully used to determine the participation of QS in biofilm formation<sup>[43]</sup>. In this system, the mutant strain *lasR-rhlR* disappears from the silicone fragments during the first 7 d of infection, in contrast with wild-type PAO1 cells which remain in the silicon implant for at least 14 and up to 21 d. Critically, the establishment of the PAO1 infection in the implants depends in the mouse strain that is used, since for Balb/c, bacterial counts in the implants decayed constantly from day one and several of the implants were completely cleared after 21 d, while for the NMRI strain, bacterial counts initially decreased (day 1 to 4), then remained constant and finally increased at levels similar to the initials at day 15<sup>[63]</sup>.

Regarding studies testing the quorum quenching effect on virulence, by using the foreign body model, it was found that the intraperitoneal addition of furanone C-30 greatly increased the bacterial clearance rate (Figure 2E)<sup>[63]</sup>. In agreement a similar effect was observed with the lung infection model in mice in which a related compound, C-furanone 56 (Figure 2F) accelerated the bacterial clearance from the lungs, reducing the severity of the damage and significantly increasing mice survival<sup>[64]</sup>. Another quorum quencher, the synthetic molecule ajoene (ajoene 4,5,9,-trithiadodeca-1,6,11-triene-9-oxide)(Figure 2G), is able to attenuate the production of various *P. aeruginosa* QS-controlled virulence factors *in vitro*, while in the pulmonary infection model in mice infected with PAO1, its prophylactic administration from two days before the infection and during its course, reduced the bacterial c.f.u. in the lungs 500-fold relative to the non-treated mice<sup>[65]</sup>. Moreover, in the burn mice model, the intravenous administration of three related quorum quenchers, the anthranilic acid analogues: 2-amino-6-chlorobenzoic acid, 2-amino-6-fluorobenzoic acid, and 2-amino-4-chlorobenzoic acid (Figure 2H-J), that inhibit the biosynthesis of quinolone signals and disrupt the MvfR-dependant gene expression, restrict the systemic spread of the PA14 strain and decreases the animals death by 30% to 50%<sup>[61]</sup>.

## QS IN *PSEUDOMONAS AERUGINOSA* HUMAN INFECTIONS

The importance of the QS systems of *P. aeruginosa* in human infections is highlighted by their presence in most clinical strains that were isolated during the moment of the infection. This was demonstrated in 2004 by Schaber *et al*<sup>[66]</sup>, by screening 200 isolates from patients with urinary tract, lower respiratory tract, and wound infections. Of those isolates, 97.5%(195 isolates)had robust functional HSL-QS communication systems and hence were able to produce elastase (codified by the genes *lasB* and *lasA*, which expression is QS dependent through LasR) and high levels of both HSL autoinducers while only 5 isolates failed to satisfy those criteria; however, 2 isolates were identified as being the same bacteria, but isolated at two different times from the same patient, and for one isolate there was no clinical data available to support that it was implicated in an infective process<sup>[66]</sup>. Hence only ~ 1% of the isolates that were implicated in infections appeared to be QS deficient. Critically, one of these isolates had no *lasR* and *rhlR* functional genes. In addition the authors demonstrated that those isolates deficient in HSL-QS systems produced high levels of non-QS controlled virulence factors, such as the ExoS and ExoT proteins that are components of the type three secretion system. Hence, perhaps this was an adaptive response that potentially could compensate for the decrease in virulence caused by QS deficiency., Nevertheless, the production of those proteins in the QS proficient isolates was not evaluated, and the virulence of the isolates was not tested using infection models. Other studies have reported similar results;, for example, the characterization of 442 *P. aeruginosa* isolates colonizing the respiratory tract of 13 intubated patients identified 9 genotypically different strains and of these, 6 strains produced both HSL-autoinducers and the virulence factors: elastase, exoproteases, rhamnolipid, hydrogen cyanide, and pyocyanin *in vitro*, and two of them had mutations in both *lasR* and *rhlR* genes, while the third had a mutant *lasR* gene<sup>[67]</sup>. Another study performed with 100 isolates from patients with respiratory infections that were collected from sputum, tracheal aspirate, and bronchoalveolar lavage identified 11 HSL-QS deficient

isolates, six of them with absent QS genes (one isolate negative for *rhlR*, two isolates negative for *rhlI* and *rhlR*, and three isolates were negative for *rhlI*, *rhlR*, *lasI*, and *lasR*). Interestingly, this study found a negative correlation between the expression of QS controlled virulence factors and antibiotic resistance<sup>[68]</sup>. Furthermore, the analysis of 82 *P. aeruginosa* clinical strains isolated from urinary tract infections identified 6 isolates deficient in the production of both HSL autoinducers, biofilm, rhamnolipids, and elastase, correlating with the absence of the *lasR* gene in one isolate and the absence of *lasI*, *lasR*, *rhlR* in another isolate, while the other 4 isolates harbored point mutations that probably inactivated their *lasI*, *lasR*, *rhlR*, and *rhlI* genes <sup>[69]</sup>.

Taken together, these independent studies indicate that about 90% of *P. aeruginosa* isolates that cause infection generally preserve active HSL-QS systems, although clearly a small percentage of the isolates have those systems impaired by mutations or loss of the important QS regulatory genes., nevertheless, in all these studies, the third QS system of *P. aeruginosa*, the quinolone dependent system, was not evaluated; hence, it is not reliable to conclude that these isolates were indeed 100% QS deficient. In addition, the existence of cell communication systems not yet described in this organism cannot be ruled out, and indeed in the reference strain PAO1, cell communication by fatty acids was recently discovered (DSF-like fatty acids, cis-2-decenoic acid) (Figure 1D)<sup>[70]</sup>. Another possibility that may explain the isolation of QS deficient strains from infections is the presence of multiple *P. aeruginosa* strains in the infection site and that the QS deficient isolates coexist with QS proficient strains; this was demonstrated recently in 8 patients with cystic fibrosis (CF), in which a complex mixture of QS-proficient and deficient isolates were found. Interestingly, among all the patients, the deficiency of the isolates in individual QS regulated phenotypes (LasA and LasB elastase, rhamnolipids, growth in adenosine, and HSL signals) ranged from 0 to ~90% and no single patient with 100% QS deficient isolates was found.

Such high diversity in isolates from the same patient likely is the result of a complex and multifactorial selective process, perhaps including social components like the advantages accrued by QS-deficient clones that use the resources made by the QS positive strains (siderophores, proteases, *etc.*), without contributing to the generation of the public goods; these bacteria are termed social cheaters<sup>[71]</sup>. Regarding the importance of QS for infections, these results indicate that at the population level, QS may be essential for CF infections; however, more studies increasing the number of CF patients and including other kinds of infections are necessary to better understand the importance of *P. aeruginosa* QS in the infective process. In addition, the elucidation of factors that shape the mosaic-like composition of isolates in patients or in animal models need to be determined in order to design better anti-QS therapies since the current ones are focused on laboratory strains with QS-proficient systems rather than clinical strains recently isolated from infections<sup>[72,73]</sup>. Although such factors are still unknown, some variables like: the severity and progression of the infection, the nutritional, health, and immunological status of the patients, the exposure of the susceptible individuals to only one, a few, or several strains and the bacterial loads during the infections could be involved. In this sense, animal models would be useful to evaluate the role of these and other valuables in the colonization diversity in the patients, for example experiments comparing the colonization of well feed animals and animals with a deficient nutrition, immune competent animals and immunosuppressed ones, or healthy animals compared to animals harboring important disorders such as the alpha-1-antitrypsin deficiency that promotes major pulmonary inflammation, degradation of lung tissue, and eventually manifestations of pulmonary emphysema, *etc.* using several bacterial strains (QS proficient and QS deficient) alone or in combination could be very valuable to determine the factors involved in the *in vivo* bacterial ecology in infections.

In addition, although *P. aeruginosa* virulence is multifactorial<sup>[74]</sup>, the individual importance of QS controlled virulence factors in different kinds of infections is a



current research area, and the role of molecules from the HSL autoinducers themselves, to extracellular factors like rhamnolipids, elastase, pyocyanin, *etc.* have been established. For example several independent studies have shown that the main *P. aeruginosa* autoinducer N-(3-oxododecanoyl)-HSL is readily detected in sputum samples collected from patients with cystic fibrosis<sup>[75-77]</sup>, which correlates with a QS dependent gene expression during the infections<sup>[78-80]</sup>. However, besides its role as a signal, the autoinducer is also able to inhibit lymphocyte proliferation as well as secretion of tumor necrosis alpha by macrophages and interferon gamma by T-cells<sup>[27]</sup>. Moreover QS controlled secreted factors such as alkaline protease can interfere with the classical and the lectin pathway-mediated complement activation *via* cleavage of C2, blocking phagocytosis and killing of *P. aeruginosa* by neutrophils<sup>[81]</sup>. Also elastase, by cleaving the pulmonary surfactant protein-A, can contribute to phagocytosis evasion<sup>[82]</sup>. Furthermore, rhamnolipids are able to disrupt calcium-regulated pathways and protein kinase C activation, preventing the induction of human beta-defensin-2 in keratinocytes<sup>[83]</sup>. Remarkably, the production of rhamnolipids in mechanically ventilated patients is associated with the development of life-threatening ventilator-associated pneumonia (VAP), while elastase production and QS independent production of the cytotoxins ExoU and ExoS are not<sup>[84]</sup>. Another QS controlled virulence factor, the polysaccharide alginate, protects *P. aeruginosa* biofilm cells from IFN-gamma-mediated macrophage killing<sup>[85]</sup>. Surprisingly, the importance of pyocyanin, a blue redox-active compound which is one of the main *P. aeruginosa* virulence factors studied *in vitro* and one of the more notorious in infections (present in large quantities in sputum from patients with cystic fibrosis infected by *P. aeruginosa*) during clinical infection is still underexplored<sup>[86]</sup>.

### **STAPHYLOCOCCUS AUREUS QS AND VIRULENCE IN ANIMAL MODELS**

The participation of SarA and Agr *S.aureus* QS systems in pathogenicity has been evaluated using numerous animal models, in which the bacteria induce diseases

such as osteomyelitis, septic arthritis, endocarditis, endophthalmitis and soft tissue abscesses. By using the mutant strains *agr* and *sarA*, as well as QS inhibition, the participation of these systems in the infectivity of the bacterium and the damage of tissues has been proved. Intravenous inoculation of the bacteria in mice induces the development of septic arthritis. In this model, *agr* mutants showed a reduced ability to induce the pathology since it is produced in only ~10% of the animals while the wild-type strain produces it in ~60% of the inoculated mice. Furthermore, in mice infected with the mutant strain, the arthritis severity is less, and only a few developed erosive arthropathy in contrast to those infected with wild-type<sup>[87]</sup>. Similarly in the endophthalmitis-rabbit model, which is established by the intraocular injection of the bacteria, *agr* mutants produced a smaller loss of neuroretinal function during the first 3 d of the infection, with respect to the wild-type strain. In addition, those infected with the mutant strain had normal eye histology, whereas those infected with the wild-type strain showed focal retinal destruction and mild vitritis<sup>[88]</sup>. In a subsequent study employing the same animal model, no significant differences in the rabbits eyes infected with the mutant strain (*sarA*) and with the wild-type were found; however, the simultaneous deletion of genes *agr* and *sarA* resulted in a near to complete attenuation of virulence<sup>[89]</sup>. Moreover, employing the model of endocarditis in rabbits, which consists in introducing a catheter into the ventricle and subsequently colonizing it by intravenous administration of the bacteria, it was observed that single mutations (*sarA* and *agr*) diminish the bacterial ability to induce the pathology, while a *agr/sarA* double mutant was incapable of inducing endocarditis in 100% of the animals inoculated with  $10^3$  or  $10^4$  c.f.u.<sup>[90]</sup>. Another infection model in mammals is the murine brain abscess model in which lesions are produced by embedding bacteria in agarose beads that are later inoculated in the cranial cavity. In this model, the *agr/sarA* double mutant, but not the single mutants, had reduced virulence, lower proliferation in the brain and poorly developed abscesses that were drastically smaller than those produced by the wild-type strain. Furthermore,

the double mutation attenuates the expression of pro-inflammatory cytokines and chemokines<sup>[91]</sup>. Similarly invertebrate models such as the nematode *C. elegans*, which is killed by feeding on *S. aureus*, showed similar outcomes, since mutating *sarA* or *agr* increased the survival of the worms with respect to the wild-type strain<sup>[92]</sup>.

Regarding the effect of quorum quenching in *S. aureus* animal infections, several inhibitory peptides have been evaluated; for example, in the murine subcutaneous abscess model, the administration of the synthetic autoinducer analog of AIP-II peptide (Figure 2K) in a single dose was able to decrease the formation of abscesses, and although AIP-II prevents expression of the *S. aureus agr* QS regulon for only a short time period, this transient inhibition is sufficient to achieve significant effects<sup>[93]</sup>. Other QS inhibitory peptides such as the RNAIII-inhibiting peptide (RIP) and its analogues (Figure 2 K and 2L), that inhibit the phosphorylation of a target protein called "target of RNAIII-activating protein" (TRAP), leading to the suppression of virulence factor production *in vitro*<sup>[94,95]</sup>, are also effective *in vivo*. For example, in the vascular-graft rat model, the administration of RIP (Figure 2L), both locally and systemically, is able to completely inhibit the formation of biofilms in graft and in polymethylmethacrylate beads infected with methicillin-susceptible and resistant *S. aureus*. Similarly, in the mouse sepsis model, administering RIP significantly reduced the bacterial load and mice mortality; this effect is potentiated by co-administration of antibiotics like cefazolin, imipenem, or vancomycin<sup>[96]</sup>. In addition, using the graft rat model, a RIP treatment increases its effectiveness in combination with antibiotics rifampin and temporin, and the complete elimination of infection is achieved by combining it with temporin A<sup>[97]</sup>. The same phenomenon has been documented with a derivative of RIP, termed FS3, which contains a substitution of alanine in the second position, since FS3 in combination with daptomycin has higher efficiency than single compounds, in the rat model of vascular graft staphylococcal infection<sup>[98]</sup>. Also in this model, a similar effect was

obtained, by combining tigecycline and the RIP analogue called FS8 which contains a terminal alanine (Figure 2M)<sup>[99]</sup>. Taken together these extensive studies demonstrate the participation of *S. aureus* QS systems in its pathogenicity and indicate that QS inhibition in combination with antibiotics is a promising new strategy that may be effective to treat the infections produced by this important pathogen.

### **QS IN STAPHYLOCOCCUS AUREUS HUMAN INFECTIONS**

Although it is not always pathogenic to humans, the Gram (+) bacterium *S. aureus*, is frequently found in the human respiratory tract and on the skin and it is considered as a transient member of the human microbial flora<sup>[32]</sup>, it is able to cause several kind of infections with a plethora of clinical manifestations. There are risk factors that complicate the infection caused by *S. aureus*, including the presence of prosthetic material and immunosuppression<sup>[100]</sup>, and it is considered one of the three main causes of nosocomial bacterial infections. Among the many kinds of *S. aureus* infections, skin ones are very common; for instance, in children it is the main cause of impetigo, a superficial skin infection that according to its clinical manifestations is divided into non-bullous and bullous impetigo, the non-bullous is the most common form, the lesions begin as papules that progress to vesicles with erythema on its periphery. These become pustules that later form adherent crusts with a golden appearance and can be accompanied by regional lymphadenitis, although systemic symptoms are usually absent. Bullous impetigo is seen in young children in which the vesicles enlarge to form flaccid blisters with clear yellow liquid, which later becomes darker and turbid, leaving a thin brown crust<sup>[3,4]</sup>. Other common infections produced by *S. aureus* are hair follicle infection or folliculitis, furunculosis, and cellulitis. In addition to skin infections, *S. aureus* also causes respiratory tract infections such as nosocomial and septic pneumonia, septic pulmonary emboli and post viral empyema. It also infects the apocrine glands, causes musculoskeletal infections, produces bacteremia and its

complications like sepsis, septic shock, and infective endocarditis and is able to produce toxic shock syndrome and food poisoning. Therefore, *S. aureus* is a major health concern worldwide.

Although the role of QS in regulating the expression of several virulence determinants including toxin production, and biofilm formation have been extensively studied *in vitro* and in animal models, its importance in actual human infections is yet under studied; nevertheless, it is known that as in the case of *P. aeruginosa* the great majority of *S. aureus* clinical isolates implicated in human infections possess active QS systems (*agr*<sup>+</sup>). Although some *agr*<sup>-</sup> strains are also commonly found in *S. aureus* infections<sup>[101-103]</sup>, the presence of both kind of strains during infection indicates that *agr*<sup>+</sup> and *agr*<sup>-</sup> variants may have a cooperative interaction<sup>[103]</sup> and also raises the possibility that social interactions like QS cheating may exist during the infections<sup>[39]</sup>. In addition, the link of QS and biofilm formation in *S. aureus* strongly suggests that this QS is important for the development and establishment of its chronic infections<sup>[32]</sup>; however, further work in this area is needed to define the importance and the specifics of QS in regulating *S. aureus* virulence in human infections.

## QS ANTIVIRULENCE DRUGS

### *P. aeruginosa*

To date, there are a large number of quorum quenching (QQ) compounds reported. In general, the three types of QQ compounds are degraders of AHL autoinducers, synthase inhibitors, and receptor inhibitors. Here the QQ compounds used against *P. aeruginosa* are sorted into seven categories.

### *Halogenated compounds*

One of the best characterized QQ compounds is the synthetic brominated furanone 4-bromo-5-(bromomethylene)-2(5H)-furanone known as C-30 (Figure 2E)<sup>[104]</sup>. This compound was synthetically modified from the natural brominated

furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone of the algae *Delisea pulchra*. Another furanone compound is 5-(bromomethylene)-2(5H)-furanone, also called furanone C-56 (Figure 2F) which is a derivative of the secondary metabolites produced by the algae<sup>[105]</sup>. Interestingly, although C-30 is effective for the attenuation of several QS-dependent virulence factors *in vitro* and in animal models, resistance against this compound has been found both in laboratory PA14 derived mutants and in clinical isolates; to date, the only resistance described mechanism is the active efflux of this compound by the MexAB-OmpR pump but the existence of other mechanisms cannot be ruled out<sup>[72,106,107]</sup>. Furanone C-56 affects the processes of biofilm formation and dispersal although it does not influence initial attachment to abiotic substrata. In addition, indole (Figure 3A) produced from L-tryptophan by a variety of bacteria and 7-hydroxy indole (7HI) (Figure 3B), an oxidized compound of indole created by bacterial oxygenases, are extracellular signals that attenuate the production of biofilm and virulence factors in *P. aeruginosa*<sup>[108]</sup>. In addition, among 31 natural and synthetic indole analogs, 7-fluoroindole (7FI) (Figure 3C) was identified to be a QQ compound capable of reducing the production of virulence factors such as 2-heptyl-3-hydroxy-4(1H)-quinolone, pyocyanin, rhamnolipid, pyoverdine, and pyochelin<sup>[109]</sup>. 7FI shows higher inhibition toward biofilm formation than indole or 7HI. As another fluorine compound which shows the QQ ability, 5-fluorouracil (5FU) (Figure 3D), an anticancer uracil analog, also is a potent inhibitor of *P. aeruginosa* virulence<sup>[110]</sup>. Since 5-FU is basically used for clinical purposes as a chemotherapeutic approach in patients with cancer, it holds promise as a QQ compound for clinical use. However, clinical strains resistant against this compound have been identified<sup>[72,107]</sup>.

Based on the previous report that chlorolactone (CL) is an inhibitor to the QQ receptor<sup>[111]</sup>, three other synthetic CL analogs were tested for QQ effects. As a result, meta-bromo-thiolactone (mBTL) (Figure 2A) was the most effective QQ compound since pyocyanin production and biofilm formation were inhibited in

the presence of mBTL, in addition, mBTL moderately protected *C. elegans* and human lung epithelial cells from killing by *P. aeruginosa*<sup>[48]</sup>. Other halogenated QQ compounds include the derivatives of anthranilic acid which is the primary precursor of 4-hydroxy-2-alkylquinolines (Figure 2H-J)<sup>[61]</sup>. The halogenated anthranilic acid analogs inhibit quinoline biosynthesis and the expression of QS-related genes. Beyond that, halogenated maleimide analogs also are QQ compounds; in particular, bromo- and iodo-substituted maleimides decrease bacterial attachment and biofilm formation whereas chloro-N-methyl-maleimide has bacteriocidal action rather a QQ effect<sup>[112]</sup>. In addition to this, 5-chloro-1,3-benzoxazol-2(3H)-one<sup>[113]</sup> also called chlorzoxazone<sup>[114]</sup> are QQ compounds that contain a halogen group (Figure 3G).

### *Lactonases and acylases*

Degrading enzymes such as lactonases and acylases are another class of QQ compounds. Their effect on QQ is due to the degradation of AHL-based autoinducers. To date, some unique lactonases have been characterized; for example, the halotolerant lactonases derived from *Bacillus* spp<sup>[115]</sup> and a thermally-stable lactonase from *Bacillus weihenstephanensis* P65<sup>[116]</sup>, which may be useful for future applications. Lactonase itself or in combination with ciprofloxacin prevented systemic spread of the bacteria in murine burn wounds infected with *P. aeruginosa*, while for the combination mice mortality was completely abolished and skin regeneration was promoted<sup>[117]</sup>. In addition, immobilized esterases and acylases embeded on medical plastic materials inhibit biofilm formation<sup>[118]</sup>. Another unique approach using a lactonase is to utilize an engineered *Lactobacillus plantarum* strain expressing the lactonase AiiA from *Bacillus thuringiensis* 4A3<sup>[119]</sup>. Extracellular virulence factors such as pyocyanin, protease, elastase, and rhamnolipids of multi-drug resistant clinical isolates of *P. aeruginosa* were inhibited and the attachment to uroepithelial cells was reduced by co-culturing *P. aeruginosa* with the engineered strain. The original trial was

performed using a *P. aeruginosa* strain capable of expressing a lactonase derived from *Microbacterium testaceum*, which led to reduced production of virulence factors and attenuated cytotoxicity against human lung epithelial cells<sup>[120]</sup>. A new trial using genetic engineering has been recently reported; this is by an engineered T7 bacteriophage expressing a lactonase with activity for a broad-range of bacterial hosts<sup>[121]</sup>. The engineered T7 bacteriophage was able to inhibit the biofilm formation of a consortium of *P. aeruginosa* and *Escherichia coli*.

### **QQ compounds found by several screening approaches**

For searching for novel QQ compounds, structure-based computational screens and high-throughput screens have been conducted. An ultra-high-throughput, cell-based assay to screen a library of approximately 200000 compounds was used to find an inhibitor which can decrease the gene expression regulated by the Las system<sup>[122]</sup>. As a result, PD12 (Figure 3J), a tetrazole with a 12-carbon alkyl tail and V-06-018, a phenyl ring with a 12-carbon alkyl tail (Figure 3K), which have both similarity with the structure of 3OC12-HSL, were identified as QQ compounds. In addition, a compound having QQ ability was also found among a series of 1,3-benzoxazol-2(3H)-one derivatives<sup>[113]</sup>; thereby, 1,3-benzoxazol-2(3H)-one (Figure 3F), 5-chloro-1,3-benzoxazol-2(3H)-one (Figure 3G), 6-methyl-1,3-benzoxazol-2(3H)-one (Figure 3I), and 5-methyl-1,3-benzoxazol-2(3H)-one (Figure 3H) have QQ ability. As another approach for clinical application of QQ compounds, the thousands of drugs clinically used in the treatment of different diseases were screened to find drugs with QQ properties which can be applicable to humans. By the screening, it was found that an anthelmintic drug, niclosamide (Figure 3L) strongly inhibits the QS response by *P. aeruginosa*<sup>[123]</sup>, although the active compound was demonstrated to be 5-FU which was already described as a QQ agent<sup>[107, 110]</sup>. Moreover, since antibiotics are also robust compounds for clinical use, inhibition of QS by antibiotics was surveyed. As a result, it was found that low concentrations of azithromycin, ceftazidime, and ciprofloxacin inhibit QS in *P.*



*aeruginosa*<sup>[124]</sup>. In addition, QS in a *P. aeruginosa* environmental isolate was inhibited at sub-inhibitory concentrations of tobramycin<sup>[125]</sup> although other studies demonstrated that a low concentration of tobramycin induces biofilm formation<sup>[126]</sup>. Screening using a computational approach and molecular docking analysis has also been useful for evaluating the binding capacity of QQ compounds to receptor proteins; thereby, new potential QQ compounds were identified. Pharmacophore modeling and *in silico* screening to find an antagonist for QS in *P. aeruginosa* indicated that a compound with tetravalent lead has QQ ability<sup>[127]</sup>. Another two compounds thimerosal (Figure 3M) and phenyl mercuric nitrate (Figure 3N) were selected as QQ compounds based on their similarity to the Pb-QQ compound. Also, the automated docking program by which the docking capability of a ligand to a receptor can be analyzed identified 5 potential new QQ compounds; among the candidates, baicalein (Figure 3O) has the strongest QQ ability as it inhibits biofilm formation of *P. aeruginosa* and the QQ effect by baicalein increases synergistically in the presence of ampicillin<sup>[128]</sup>. Also, another 5 compounds were identified to be QQ by using a structure-based virtual screening approach targeting the QS receptor LasR; of the 5 compounds, the most promising was 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one also called G1 (Figure 3P)<sup>[129]</sup>.

### ***Other AHL antagonists***

Some of QQ compounds described above are antagonists of AHL molecules; hence their QS inhibition effect is triggered by interrupting the binding (interaction) between AHL molecules and receptors. To date, there are a large number of AHL antagonists; for example, patulin (Figure 3Q)<sup>[130]</sup>, salicylic acid (Figure 3R)<sup>[114]</sup>, 3-oxo-C12-(2-aminophenol) (Figure 3S)<sup>[131]</sup>, and nifuroxazide (Figure 3T)<sup>[114]</sup> as well as C-30 (Figure 2E)<sup>[104]</sup>. In addition, 4-nitropyridine-N-oxide (Figure 3U) is a QQ compound<sup>[132]</sup>, which also reduces bacterial adhesion to silica-coated surfaces<sup>[133]</sup>. Other QQ compounds are pyrimidine (Figure 3V)<sup>[134]</sup>, N-decanoyl-L-homoserine benzyl ester (C2) (Figure 3W)<sup>[135]</sup>, 2,5-piperazinedione

(Figure 2C)<sup>[52]</sup>, and phenylacetic acid (Figure 2B)<sup>[51]</sup>. The bacterial sensitivities to several antibiotics (tobramycin, gentamycin, cefepime, and meropenem) in the presence of C2 were higher than those without C2<sup>[135]</sup>. This may be due to the synergistic interactions between C2 and the antibiotics. In addition, QQ by the cyclic dipeptide 2,5-piperazinedione (Figure 2C) might be due to interference with the binding of the natural ligand 3-oxo-C12-HSL to its receptor protein based on the molecular docking analysis<sup>[52]</sup>. Phenylacetic acid (Figure 2B), which is similar to salicylic acid, has been reported to be a QQ compound<sup>[51]</sup>.

### *Inhibitors with different QS targets*

There are some reports on inhibitors with QS targets different than AHLs and receptors. A new class of antivirulence compounds was reported by Shouldice *et al*<sup>[136]</sup>; the QQ compounds interact with the bacterial periplasmic protein DsbA, which is essential for the folding and function of exported virulence factors. Another target of QQ compounds is mono-ADP-ribosyltransferase which functions as a bacterial toxin<sup>[137]</sup>. Some newly-identified QQ compounds were found by using a virtual screen of commercially available compounds combined with a directed poly(ADP-ribose) polymerase; thereby, V23 (Figure 3X), V30 (Figure 3Y), and P1 (Figure 3Z) compounds as well as NAP (Figure 3A1)<sup>[137]</sup> and PJ97A (Figure 3B1)<sup>[138]</sup> were identified as inhibitors of toxin production<sup>[137]</sup>. Other antagonists are a series of compounds targeting PqsR, the receptor of the *pqs* system<sup>[139]</sup>. Among the analogs of 2-heptyl-4-hydroxyquinoline (HHQ) synthesized, three HHQ analog with 6-CN (Figure 3C1), 6-CF<sub>3</sub> (Figure 3D1), or 6-NO<sub>2</sub> (Figure 3E1) along with n-C<sub>7</sub>H<sub>15</sub> are the best competitors<sup>[139]</sup>, which are promising starting compounds for further drug design.

### *Cell extracts and secretion products from isolated microorganisms*

Based on the concept that microbial interaction (inhibition, repression, acceleration, and dependence) is a complex phenomenon due the large numbers of microbes, a

new approach to isolate unique microorganisms with QQ ability and to utilize cell extracts and secretion products has been recently reported. Among the 46 marine bacterial isolates, 11 extracts from *Bacillus*, *Marinobacter*, *Halobacillus*, *Staphylococcus*, or *Ferrimonas* species showed antibiofilm activity against *P. aeruginosa*<sup>[140]</sup>. The partially-purified antibiofilm compound from S6-15 (similarity with *Bacillus pumilus*) is stable up to 60 °C and under neutral and alkaline conditions. In addition, its QQ ability was inactivated by the treatment by enzymes such as proteinase K, trypsin, and lysozyme<sup>[140]</sup>. Also, bacteria able to utilize AHL molecules as a sole source of carbon and nitrogen have been isolated and characterized as AHL-degrading bacteria<sup>[141]</sup>. Among 41 isolates which retained QQ activity after heat treatment, some of the isolates showed impaired QS inhibition after the treatment by proteinase K whereas the other isolates remained active. In addition, actinomycetes with QQ activity were also isolated from marine sponge. In this study, methanol extracts of 12 actinomycetes had an inhibitory effect on the production of QS-mediated virulence factors<sup>[142]</sup>; in particular, of the three strains which showed very good anti-QS activity, the most promising strain is NIO 10068 (*Streptomyces* sp.) that secretes cinnamic acid and/or linear Pro-Gly dipeptide which may be QQ compounds. Further bacteria capable of having QQ ability were also isolated from healthy coral species<sup>[143]</sup>; of 120 bacterial isolates, up to 24% of the isolates showed anti-QS activity. In particular, a *Favia* sp. coral isolate inhibits the biofilm formation of *P. aeruginosa* by secreting a low-molecular mass compound which is not inactivated by heat and proteinase K<sup>[143]</sup>. Also, a cell-free lysate of endophytic bacteria isolated from *Pterocarpus santalinus* Linn. also showed QQ activity<sup>[144]</sup>. *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 isolated as the endophytic bacteria exhibit potent AHL degrading ability by inhibiting about 80% violacein production in a biosensor strain. QQ activity by the cell lysate was effective against biofilm formation rather than to planktonic cells, and the QQ activity was due to the presence of AHL lactonase in cell-free lysate<sup>[144]</sup>.

Moreover, a small cyclopropane-containing fatty acid, lyngbyoic acid (Figure

3F1), a major metabolite produced by the marine cyanobacterium, *Lyngbya cf. majuscula* has been identified to be a QQ compound capable of strongly inhibiting Las-QS system<sup>[145]</sup>. In addition, the biosurfactant, lunasan produced by *Candida sphaerica* UCP 0995 is also a QQ compound<sup>[146]</sup>. Recently, it was discovered that a conditioned high density lipoprotein is also a QQ compound capable of reducing the virulence of *P. aeruginosa* by influencing *las*-and *rhl*-QS systems as well as biofilm formation<sup>[147]</sup>. Furthermore, ultra-small solid lipid nanoparticles for the pulmonary delivery, which are prepared by using various pharmaceutical lipids, are fabricated to deliver QQ compounds to a target site without any penetrable cellular barrier<sup>[148]</sup>. In this study, plain small solid lipid nanoparticles exhibited anti-virulence properties themselves.

#### *QS inhibitors from food and plant sources*

Since biocompatibility of QQ compounds to higher organisms is one of the important requirements for clinical use, there are a lot of trials to find QS inhibitors from food and plants. The anti-QS activity of aqueous extracts from edible plants and fruits, like pineapple, plantain, and sapodilla, was evaluated; most of these extracts showed QQ activity without inhibiting bacterial growth in *P. aeruginosa*<sup>[149]</sup>. Also, analogs from a natural bicyclic diterpenoid lactone, andrographolide which is the main phytoconstituent from *Andrographis paniculata* Nees (herb), were screened to evaluate QQ activity<sup>[150]</sup>. An andrographolide-based compound, 14-(5-cyclopentylvaleryl) andrographolide (compound 11b) (Figure 3G1) had the best QQ activity among all the new compounds. In addition, some QQ compounds were found from traditional Chinese medicine by using a molecular docking analysis and QS assays<sup>[151]</sup>. As a result, emodin (Figure 3H1) had a certain antibiofilm activity as well as the ability to increase the activity of ampicillin against *P. aeruginosa*.

Furthermore, five sesquiterpene lactones of the goyazensolide (Figure 3I1) and isogoyazensolide-type (Figure 3J1) isolated from the Argentine herb *Centratherum*

*punctatum*<sup>[152]</sup>, iberin (Figure 3K1) from horseradish<sup>[153]</sup>, allicin (Figure 3L1) from garlic<sup>[154]</sup>, and phenolic components ([6]-gingerol (Figure 3M1), [6]-shogaol (Figure 3N1), zingerone (Figure 3O1)) from ginger<sup>[155]</sup> and anacardic acids<sup>[156]</sup> are QQ compounds, which might be suitable for further development of antivirulence and antibacterial agents.

### *Staphylococcus aureus*

Since QS regulates the expression of multiple *S. aureus* virulence determinants, and since the frequency of drug resistant clinical strains causing infections is rising (like methicillin-resistant *S. aureus* “MRSA”), several compounds aiming to disrupt these regulatory interactions have been identified; among them, perhaps the best characterized is the QS inhibitor RNAPIII-inhibiting peptide (RIP) (Figure 2L), an endogenous *S. aureus* peptide that is able to decrease the damage of *S. aureus* in several animal models as discussed before<sup>[94,157]</sup>. At the molecular level, the production of several toxins is activated in a cell density manner by the RNAPIII-activating protein (RAP) and by the autoinducing peptide (AIP), and is inhibited by RNAPIII-inhibiting peptide (RIP) and by inhibitory AIPs; RAP participation in the pathogenesis consists in inducing the phosphorylation of a 21-kDa protein (known as target of RAP or TRAP). While RIP inhibits its phosphorylation, the phosphorylation of TRAP is essential to create the autoinducing loop since it leads to the activation of RNAPIII synthesis<sup>[94]</sup>. In addition to decreasing the damage of *S. aureus* during infection, RIP treatment also is able to prevent its adhesion to human kidney cells and its biofilm formation on dialysis catheters<sup>[158]</sup>. Other effective peptides analogues to RIP are FS3 and FS8 (Figure 2M) discussed previously<sup>[98,99]</sup>. Moreover, recently four AIP non-functional peptide analogues were identified; these peptides have an ample spectrum since they can repress many AgrC receptors (type I–IV) and have a very high affinity. For example, treatment with the peptides block hemolysis (at picomolar concentrations) and attenuate the production of toxic shock syndrome toxin-1 by 80% at nanomolar concentrations;

hence, these compounds are the most potent synthetic inhibitors of QS in *S. aureus* to date<sup>[159]</sup>.

Beyond the QS inhibitory peptides, several other interesting molecules able to block the expression of *S. aureus* virulence factors have been discovered, among them, the small molecule biaryl compounds in which the aromatic rings either are either fused or separated by a short linker. This result is particularly interesting, since they are able to inhibit the production of the alpha-hemolysin and the modulin- $\alpha$  toxin in a dose-dependent manner without inhibiting bacterial growth, since they are effective against methicillin-resistant *S. aureus*, and since one of the effective compounds is diflunisal (Figure 3P1), an FDA-approved nonsteroidal anti-inflammatory drug<sup>[160]</sup>. Diflunisal has the clear advantage that it could be easily repurposed for treating *S. aureus* infections or could be used to coat catheters and other medical devices just as was recently done for 5-FU(Figure 3D)<sup>[161]</sup>, which has QS inhibitory activity against *P. aeruginosa*<sup>[110]</sup>, *E. coli*<sup>[162]</sup> and perhaps several other pathogens. In addition, some natural products with QS inhibition activity against *S. aureus* like 2,5-di-O-galloyl-dhamamelose (hamamelitannin) (Figure 3Q1), a non-peptide analog of RIP found in the bark of the plant *Hamamelis virginiana* had been identified. This compound is effective *in vitro* to inhibit virulence without affecting growth, and *in vivo* in a rat graft model, preventing device-associated infections<sup>[163]</sup>. Moreover, recently the screening of 83 essential oils led to the identification that black pepper, cananga, and myrrh oils and their common constituent cis-nerolidol (Figure 3R1) strongly attenuate *S. aureus* biofilm formation, its hemolytic activity, and protect *C. elegans* against its infection. Although their mechanism is not fully understood yet, transcriptional analyses showed that at least black pepper oil treatment inhibited the expression of the  $\alpha$ -toxin gene (*hla*), nuclease genes, and QS regulatory genes<sup>[164]</sup>; similar effects can be observed with treatments with trans-stilbene (Figure 3S1)and resveratrol (Figure 3T1)<sup>[165]</sup>.

## QS SYSTEMS ARE PRESENT IN SEVERAL OTHER IMPORTANT BACTERIAL PATHOGENS

In addition to *P. aeruginosa* and *S. aureus*, several other bacterial pathogens utilize QS systems to control the expression of multiple virulence factors during infection. Among the more relevant for human health are *Vibrio* spp, *Acinetobacter* spp, *Burkholderia cepacea*, and enteric bacteria like *Escherichia* spp. and *Salmonella typhimurium*. The following section is an overview of their known QS systems and their relationship with virulence.

### *Vibrio* spp.

*Vibrio* is a genus of facultative anaerobic Gram-negative bacteria possessing a curved rod shape (comma shape) typically found in saltwater. Several species are pathogenic to animals including humans and are responsible for food borne infections that are usually associated with eating contaminated food or water. In addition, they also cause wound infections and septicemia. The first QS system was described in the bioluminescent marine bacterium *Vibrio fischeri*, considered the paradigm for QS found in most Gram-negative bacteria. *Vibrio fischeri* colonizes the light-emitting organs of the squid *Euprymna scolopes*, in which it multiplies and reaches a high population density and induces the expression of luminescence genes. This gene expression occurs in a coordinated fashion<sup>[166]</sup>. The squid uses the light conferred by the bacteria to hide its own shadow in shallow waters and thus avoid predators<sup>[167]</sup>. To date several QS systems have been described in *Vibrio* spp.

In *Vibrio harveyi*, the following three QS systems are known: (1) LuxM (synthase), LuxN (receptor) and 3OHC4HSL (signal); (2) LuxS (synthase), LuxP (receptor) and AI-2 (signal); and (3) CqsA (synthase), CqsS (receptor) and CAI-1 (signal).

These systems are associated with bioluminescence, siderophores, protease and extracellular polysaccharide (EPS) production, and other virulence factors<sup>[168-170]</sup>.

In *Vibrio cholerae*, two QS systems have been described: (1) LuxS (synthase), LuxP (receptor) and AI-2 (signal); (2) CqsA (synthase), CqsS (receptor) and CAI-1 (signal).

These systems have been associated with biofilm formation, extracellular polysaccharide (EPS) production, and other virulence factors<sup>[169]</sup>.

Finally, in *Vibrio fischeri* three QS systems are known: (1) LuxI (synthase), LuxR (receptor) and 3OC6HSL (signal); (2) AinS (synthase), AinR (receptor) and C8HSL (signal); and (3) LuxS (synthase), LuxP (receptor) and AI-2 (signal).

These systems are associated with bioluminescence, host colonization, and motility<sup>[168,170]</sup>. Other QS systems found in various *Vibrio* spp. and in *Legionella pneumophila* utilize hydroxyketones (AHKs) as signalling molecules<sup>[170]</sup>.

### ***Acinetobacter* spp.**

*Acinetobacter* is a genus of aerobic, non-motile Gram-negative bacteria that are widely distributed in nature, commonly occurring in soil. Among them, some species like *A. baumannii* are frequently isolated in nosocomial infections, especially in intensive care units, since they attack debilitated and immunocompromised patients; in addition they have a high tolerance against antibiotics and an inherent ability to acquire antibiotic resistance genes, being therefore a serious emergent health problem. Their QS systems consist of homologues of the LuxR and LuxI proteins of *Vibrio fischeri* known as AbaR (receptor) and AbaI (synthase) and play a role in biofilm formation and motility in *Acinetobacter* spp<sup>[171]</sup> and in *Acinetobacter baumannii*<sup>[172]</sup>. This QS system is an important virulence factor responsible for the outstanding antibiotic resistance and survival properties in the latter species<sup>[173]</sup>. However, the role of QS systems in the regulation of other virulence factors implicated in the development of infection has not yet been established<sup>[174]</sup>.

Synthesis of N-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C12-HSL) is catalyzed by AbaI from *Acinetobacter* strain M2 (initially characterized as



*Acinetobacter baumannii*, although genomic sequencing studies have distinguished this strain as *Acinetobacter nosocomiales*<sup>[174,175]</sup>. The completed genome sequence of *A. baumannii* strain ATCC 17978 indicates that autoinducer synthase AbaI (gene A1S\_110) and acyltransferases may be the sole participants in the synthesis of AHL signals of variable chain length by the organism<sup>[176]</sup>. Many strains of *Acinetobacter* (63%) produce more than one AHL. However, none of the AHL signals can be specifically assigned to a particular species of the genus<sup>[177]</sup>. *Acinetobacter* quorum signals are not homogeneously distributed, and therefore distinction between virulent and non-virulent strains on the basis of QS signals is difficult. Communication between bacteria with respect to cell density is integral to the maturation of *Acinetobacter* spp. Biofilm<sup>[176,178]</sup>. Mutation of *abal*, which produces the acyl-homoserine lactone molecule, resulted in a 30%-40% reduction in biofilm production relative to that of the isogenic parental strain<sup>[173]</sup>. Exogenous addition of purified *Acinetobacter* acyl homoserine lactone restored biofilm maturation in the *abal* mutant<sup>[176]</sup>.

### ***Burkholderia cepacea***

The *Burkholderia cepacia* complex is a group of Gram-negative bacteria composed of at least 18 different species; they are important human pathogens which produce pneumonia in immunocompromised individuals that are affected by lung diseases such as cystic fibrosis. All *Burkholderia cepacea* complex members encode at least one QS system that consists of homologues of the LuxR and LuxI proteins of *Vibrio fischeri* [CepI(synthase), CepR (receptor), and AHLs C8-HSL and C6-HSL(signal)]. AHL production in the *Burkholderia cepacea* complex is strain-dependent with respect to both the quantity and type of AHL molecules<sup>[179,180]</sup>. Another QS system in the *Burkholderia cepacea* complex is the CciIR system [CciI(synthase), CciR (receptor), and/AHLs C8-HSL and C6-HSL(signal)]<sup>[181]</sup>. Phenotypic assays and global transcript and protein analysis with *cepIR* and *cciIR* mutant strains have shown that AHL-mediated QS controls various functions, including swarming

motility, biofilm formation and the production of virulence factors, such as proteases (e.g., the metalloproteases ZmpA and ZmpB), siderophores, toxins and antifungal agents<sup>[179]</sup>.

In 2008, Boon *et al*<sup>[182]</sup> reported the identification of a novel fatty acid signal molecule that is produced by several *B. cenocepacia* strains. The structure of the molecule synthesized by *B. cenocepacia* J2315 was identified as *cis*-2-dodecenoic acid, referred to as BDSF (*Burkholderia* diffusible signal factor). BDSF is structurally related to DSF (diffusible signal factor, *cis*-11-methyl- 2-dodecenoic acid), which was first isolated from supernatants of *Xanthomonas campestris* pv *campestris*. The BDSF-regulated QS system is involved in the control of several functions. Mutation of *rpfFBc* resulted in decreased motility, reduced adherence to porcine mucin, diminished exopolysaccharide (EPS) production and lowered protease activity. In addition, the BDSF mutant strains were found to be more susceptible to antimicrobial agents, and their ability to form biofilms was shown to be strongly reduced<sup>[179]</sup>.

### ***Escherichia spp./Salmonella typhimurium***

*E. coli* and *S. typhimurium* are related enteric Gram-negative, facultative anaerobic bacteria. Although most *E. coli* strains are commensal for warm-blooded organisms, such as mammals, some serotypes cause serious food poisoning and other kinds of infections like urinary tract infections and neonatal meningitis, while *S. typhimurium* and other *Salmonella* pathogenic serovars are responsible for Salmonellosis, an infection that causes diarrhea, fever, vomiting, and abdominal cramps. Although usually the illness resolves after four to seven days without medical treatment, several million people are infected by this bacterium each year. In *E. coli* and *S. typhimurium*, three QS systems have been described: (1) Unknown (synthase), SdiA (receptor) and 3OC8HSL (signal). This system has been associated with motility and acid resistance<sup>[183]</sup>; (2) LuxS (synthase), LsrB (receptor) and AI-2 (signal). Lsr operon expression (AI-2 uptake)<sup>[184]</sup>; and (3) Unknown (synthase),

QseC (receptor) and AI-3 (signal). This system has been implicated in virulence, motility and biofilm formation<sup>[185]</sup>.

## **QS SYSTEMS BEYOND BACTERIA**

QS systems have been extensively studied in bacteria and are of great interest for understanding the development of clinically-significant infections, but whether eukaryotes have cell signaling systems similar to bacterial QS mechanisms is a question that has recently drawn the attention of research worldwide. In this section, we will discuss some examples of eukaryotic microorganisms and human cells that use QS for the development of certain biological functions. The first report of a QS system in eukaryotes was carried out more than 40 years ago, when it was observed that dense cultures of the fungi *Candida albicans* show a reduced tendency towards the morphological transition from yeast to hypha, which is considered a key virulence factor for this opportunistic fungal pathogen<sup>[186]</sup>. To date, several compounds have been identified as responsible for this phenomenon, such as 2-phenylethanol, tryptophol, farnesol, farnesoic acid, and tyrosol<sup>[187]</sup>; these QS molecules are secreted by *C. albicans* and when they accumulate over a threshold level, they trigger changes in: (1) fungal dimorphisms<sup>[186]</sup>; (2) biofilm formation<sup>[188]</sup>; and (3) expression of virulence genes<sup>[189]</sup>. In addition, in other dimorphic fungi (*Mucor rouxii*, *Histoplasma capsulatum*, *Ceratocystis ulmi*), the “inoculum size effect” is usually observed; however, QS autoinducers in these organisms have not been identified<sup>[187]</sup>. In 1997, it was discovered that in the protozoan parasite of humans *Trypanosoma cruzi*, the differentiation of replicating and slender forms to non-dividing and stumpy ones is also a density-dependent (quorum) response that limits the population size<sup>[190]</sup>. This phenomenon is mediated by the soluble factor SIF (stumpy induction factor) that is released by trypanosomes, and a recent study revealed that the QS signaling in *T. cruzi* shares components with the quiescence pathways of mammalian stem cells, providing novel therapeutic targets *via* QS interference<sup>[191]</sup>. Like those parasitic species

described thus far, in 2006 it was demonstrated that the budding yeast *Saccharomyces cerevisiae* endure morphological transition from the yeast form to a filamentous form in response to both cell density and the nutritional state of the environment. This induction is mediated by the phenylethanol and tryptophol auto signaling molecules, that regulate the transcription of a set of ~ 150 genes and which include FLO11, an essential gene for filamentous growth as well as several others genes that may play a role in the transition from the exponential to the stationary growth phase<sup>[192,193]</sup>. However, not only parasitic infections or traits present in unicellular eukaryotes are controlled by QS, since surprisingly in our body the number of cells of the immune system is maintained throughout a similar mechanism, where IL-2 is produced and secreted by activated CD4+T cells and sensed with high affinity (IL-2R $\alpha$ ) by a population of CD4+Treg, which in turn can regulate the number of total CD4+T population<sup>[194]</sup> by competition for the IL-2 factor<sup>[195]</sup>. Failure of QS due to the absence of IL-2 or by defects on the sensor IL-2R $\alpha$  leads to lymphoid hyperplasia and autoimmune diseases<sup>[196]</sup>. Furthermore, in 2009 Hickson and coworkers proposed that cancer cells may use QS mechanisms to operate as communities and regulate different multicellular functions as the metastatic process resembles bacterial biofilm formation and dispersion. This idea emerged based on several lines of evidence that suggested a close relationship between high cancer cell densities and high metastatic ability<sup>[197]</sup>. This relationship can be possibly explained by the fact that the cells secrete paracrine factors or autoinducers that increase their metastatic efficiency; these observations have been made since the 90's<sup>[198]</sup> and recently by combining mathematical modeling with experimental evidence, the presence of QS systems in cancer was confirmed<sup>[199]</sup>. Such interesting findings are now opening new areas of study, including the development of future clinical applications (a summary of all the QS reviewed is provided in Table 1).

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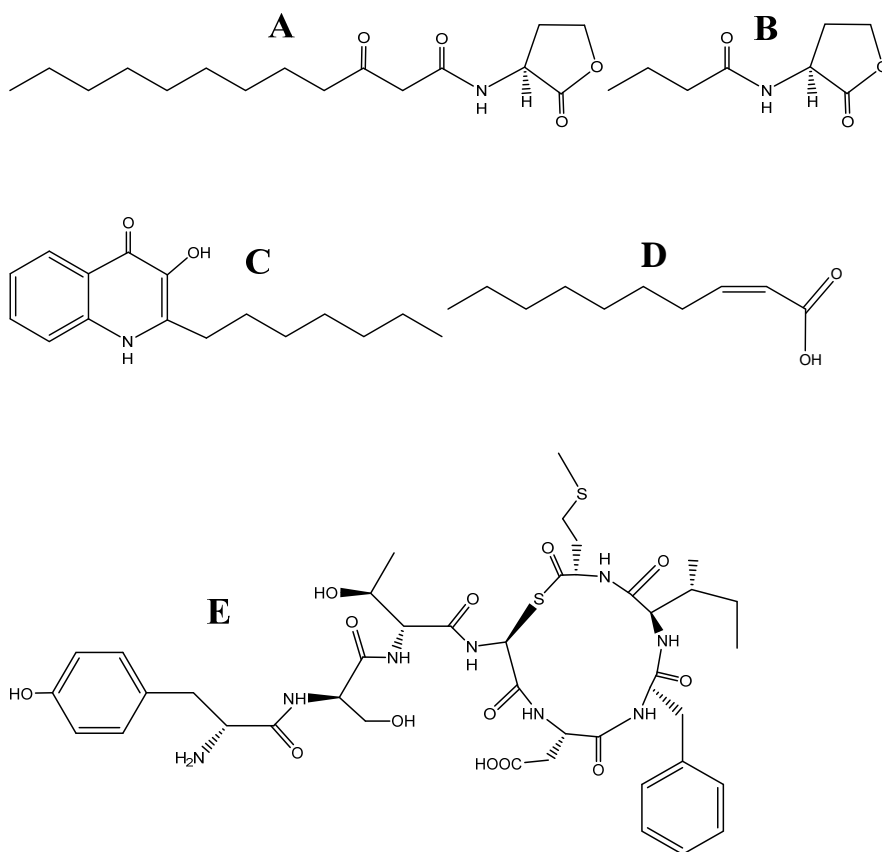
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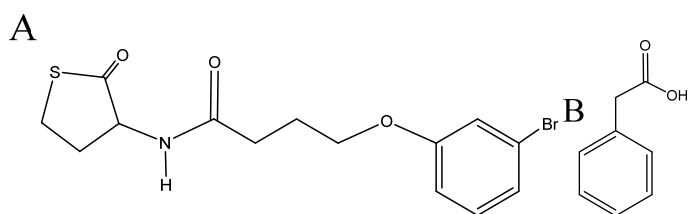
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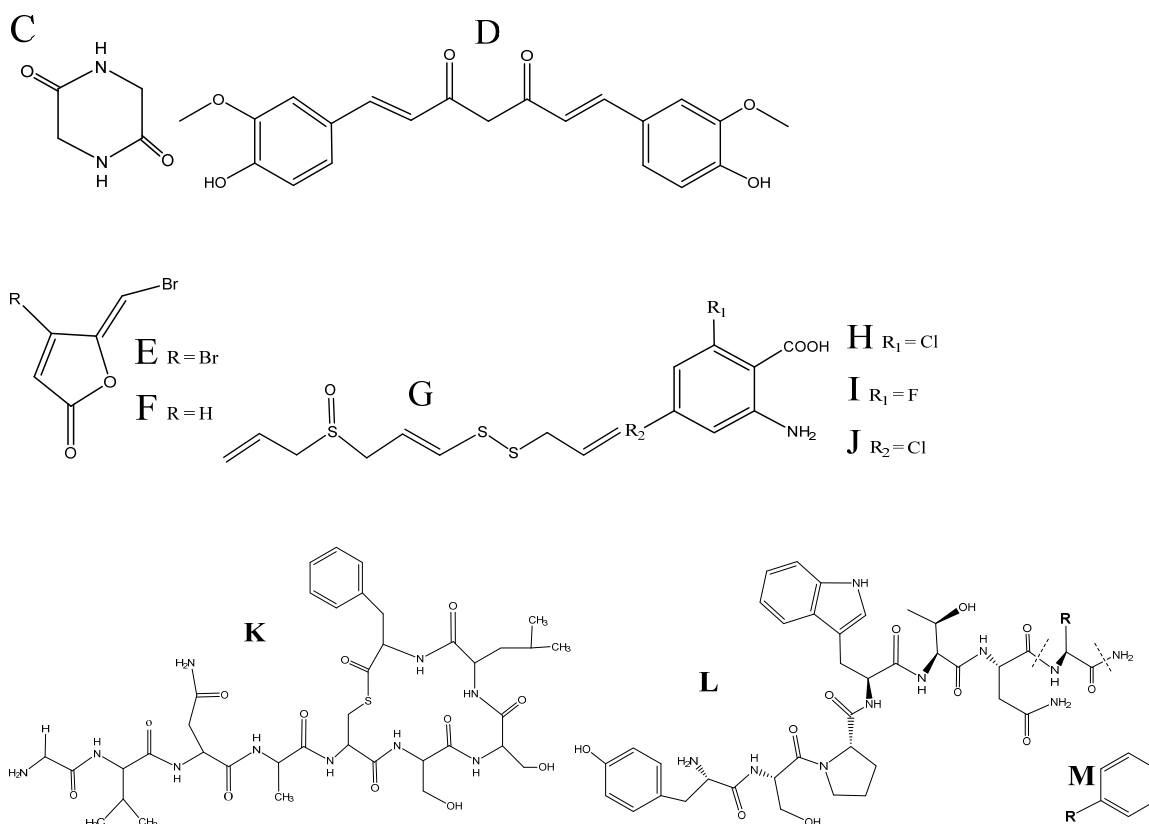
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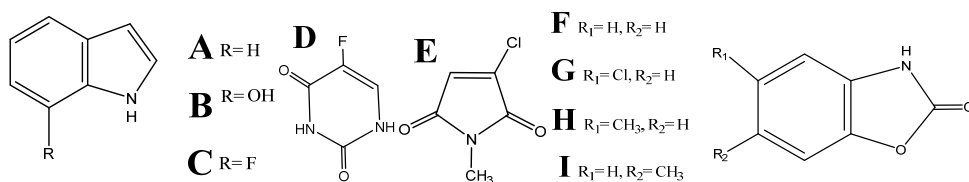
**Figure 1 Structures of representative QS signal molecules of *P. aeruginosa*. A: 3-oxo-C12-homoserine lactone; B: N-butyryl-L-homoserine lactone; C: 2-heptyl-3-hydroxy-4-quinolone; D: DSF-like fatty acids, *cis*-2-decenoic acid) and *S. aureus* (E: AIP group I).**

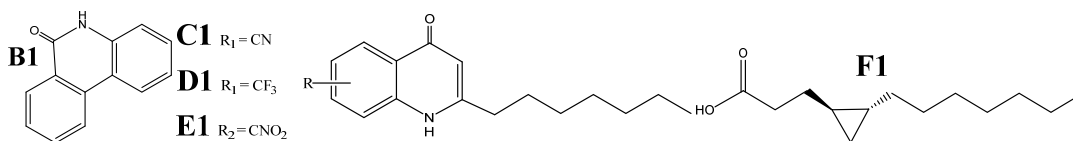
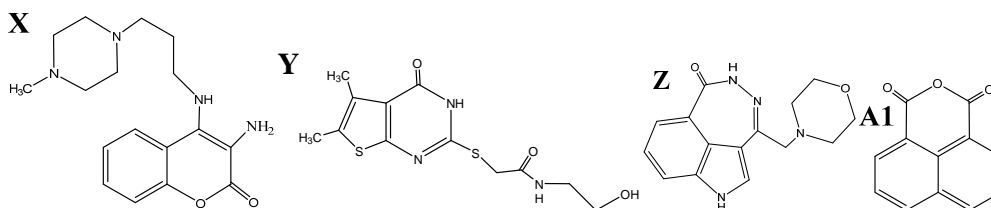
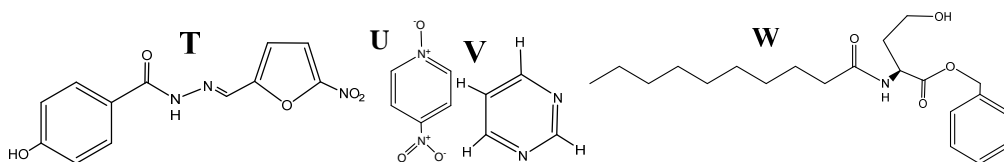
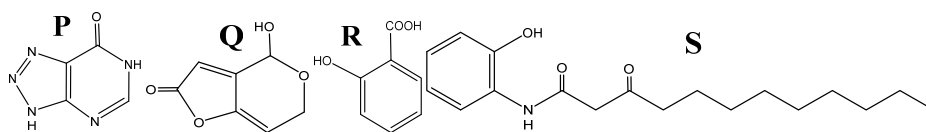
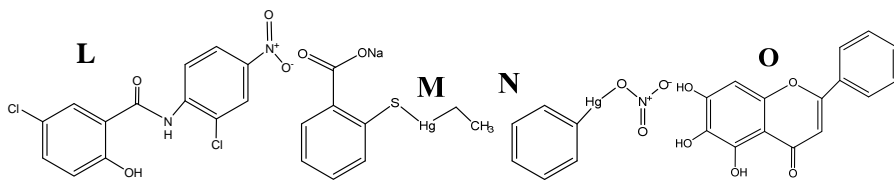
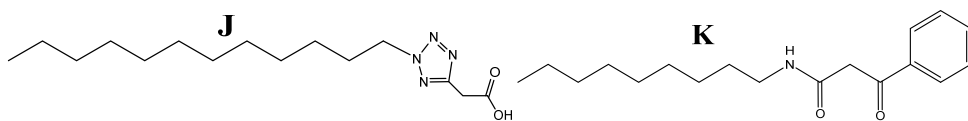


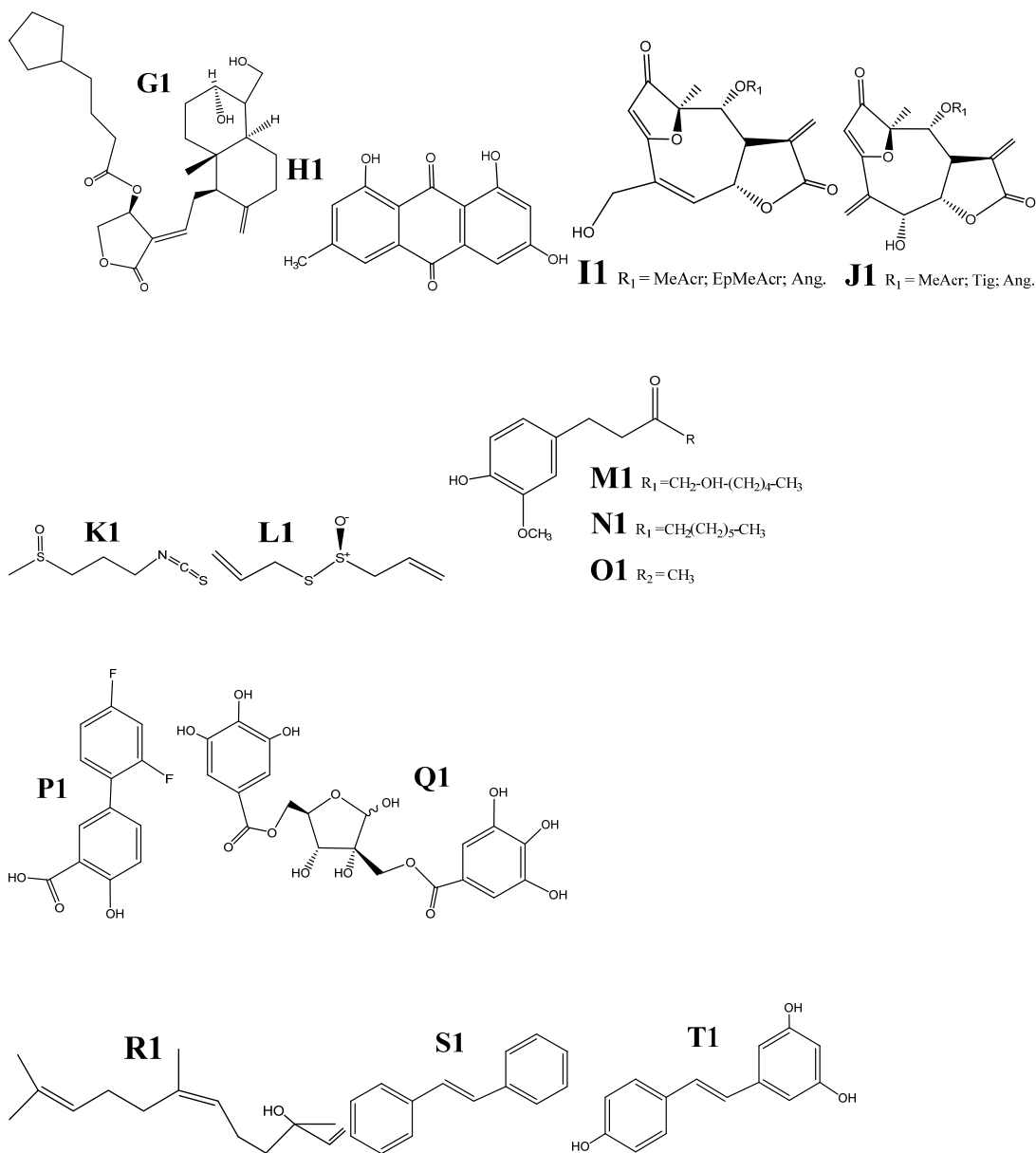




**Figure 2 Structures of QSI evaluated in animal models.** A: Meta-bromothirolactone; B: Phenylacetic acid; C: 2,5-piperazinedione; D: Cucurmin; E: Furanone C-30; F: Furanone C-56; G: Ajoene 4,5,9,-trithiadodeca-1,6,11-triene-9-oxide; H: 2-amino-6-chlorobenzoic acid; I: 2-amino-6-fluorobenzoic acid; J: 2-amino-4-chlorobenzoic acid; K: AIP-II; L: RIP (H-Tyr-Ser-Pro-Trp-Thr-Asn-Phe-NH<sub>2</sub>); M: FS8 (H-Tyr-Ser-Pro-Trp-Thr-Asn-Ala-NH<sub>2</sub>).







**Figure 3 Structures of representative quorum quenching molecules of *P. aeruginosa*.** A: Indole; B: 7-hydroxy indole; C: 7-fluoroindole; D: 5-fluorouracil; E: 2-chloro-N-methyl-maleimide; F: 1,3-benzoxazol-2(3H)-one; G: 5-cloro-1,3-benzoxazol-2(3H)-one (clorzoxazone); H: 5-methyl-1,3-benzoxazol-2(3H)-one; I: 6-methyl-1,3-benzoxazol-2(3H)-one; J: PD12; K: V-06-018; L: Niclosamide; M: Thimerosal; N: Phenylmercuric nitrate; O: Baicalein; P: 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-*d*]pyrimidin-7-one; Q: Patulin; R: Salicylic acid; S: 3-oxo-C12-(2-aminophenol); T: Nifuroxazide; U: 4-nitropyridine-N-oxide; V: Pyrimidine; W: N-

decanoyl-L-homoserine benzyl ester; X: V23; Y: V30; Z: P1; A1: NAP; B1: PJ97A; C1: 6-CN; D1: 6-CF<sub>3</sub>; E1: 6-NO<sub>2</sub>; F1: Lyngbyoic acid; G1: Andrographolide 14-(5-cyclopentylvaleryl); H1: Emodin; I1: Goyazensolide-type; J1: Isogoyazensolide-type; K1: Iberin; L1: Allicin; M1:[6]-gingerol; N1: [6]-shogaol; O1: Zingerone and *S. aureus*; P1: Diflunisal; Q1: Hamamelitannin; R1: Cis-nerolidol; S1: Trans-stilbene; T1: Resveratrol.

**Table 1 Quorum sensing systems and quorum sensing-virulence associated phenotypes in the reviewed organisms**

<b>Organism</b>	<b>QS systems</b>	<b>Regulated phenotypes</b>
<b>Gram (-) bacteria</b>		
<i>Pseudomonas aeruginosa</i>	1) LasI (S)-LasR (R)-HSL (s) 2) HSL RhlI-RhlR 3) Alkyl quinolones (PQS)	Expression of several virulence factors including: Pyocyanin, pyoverdine, elastase, alkaline protease, HCN, rhamnolipids and biofilm formation
<i>Vibrio harveyi</i>	1) LuxM (S)-LuxN (R)-3OHC4HSL (s) 2) LuxS (S)-LuxP (R)-AI-2 (s) 3) CqsA (S)-CqsS (R)-CAI-1 (s)	Expression of bioluminescence genes and several virulence factors including: Siderophores, protease, EPS production.
<i>Vibrio cholerae</i>	1) LuxS (S)-LuxP (R)-AI-2 (s) 2) CqsA (S)-CqsS (R)-CAI-1 (s)	Expression of several virulence factors including: Biofilm formation and EPS production.
<i>Vibrio fischeri</i>	1) LuxI (S)-LuxR (R)-3OC6HSL (s) 2) AinS (S)-AinR (R)-C8HSL (s) 3) LuxS (S)-LuxP (R)-AI-2 (s)	Expression of bioluminescence, host colonization and motility genes
<i>Acinetobacter spp</i>	1) Abal (S)-AbaR (R)-3OHC12HSL (s)	Expression of virulence factors including biofilm formation.
<i>Burkholderia cepacea</i>	1)Cep1 (S)-CepR (R)-	Expression of swarm motility

	C8HSL,C6HSL (s) 2)Ccil (S)-CciR (R)- C8HSL,C6HSL (s)	genes and several virulence factors including: proteases, siderophores, toxins, antifungal agents and biofilm formation.
<i>Escherichia coli</i> <i>Salmonella typhimurium</i>	1)Unknown (S)-SdiA (R)- 3OC8HSL (s) 2)LuxS (S)-LsrB (R)-AI-2 (s) 3) Unknown (S)-QseC (R)- AI-3 (s)	Expression of motility genes, acid resistance and virulence factors including biofilm formation
Gram (+) bacteria		
<i>Staphylococcus aureus</i>	1)AgrB (S)-AgrC (R)-AIP(s)	Expression of several virulence factors including: hemolysins, leukocidins, cell surface adhesins, exoenzymes, and biofilm formation
Fungi		
<i>Candida albicans</i>	2-phenylethanol, tryptophol, farnesol, farnesoic acid, and tyrosol as (s)	Fungal dimorphism, biofilm formation and expression of virulence genes.
<i>Mucor rouxii</i> , <i>Histoplasma capsulatum</i> , <i>Ceratocystis ulmi</i>	QS (s) unknown	Fungal dimorphism
<i>Saccharomyces cerevisiae</i>	phenylethanol and tryptophol as (s)	Transition from yeast to filamentous form
Protozoa		
<i>Trypanosoma cruzi</i>	SIF soluble factor as (s)	Differentiation of replicating to non-dividing forms
Mammal cells		

CD4+T cells	IL-2 (s) and IL-2R $\alpha$ (R)	Regulation of the CD4+T cells population
Cancer cells	Multiple (S) and (R) and paracrine factors as (s)	Regulation of the metastatic process

IL-2: Interleukin 2; SIF: Soluble inhibitory factor; QS: Quorum sensing.