Interfering with Bacterial Quorum Sensing

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ABSTRACT: Quorum sensing (QS) describes the exchange of chemical signals in bacterial populations to adjust the bacterial phenotypes according to the density of bacterial cells. This serves to express phenotypes that are advantageous for the group and ensure bacterial survival. To do so, bacterial cells synthesize autoinducer (AI) molecules, release them to the environment, and take them up. Thereby, the AI concentration reflects the cell density. When the AI concentration exceeds a critical threshold in the cells, the AI may activate the expression of virulence-associated genes or of luminescent proteins. It has been argued that targeting the QS system puts less selective pressure on these pathogens and should avoid the development of resistant bacteria. Therefore, the molecular components of QS systems have been suggested as promising targets for developing new anti-infective compounds. Here, we review the QS systems of selected gram-negative and gram-positive bacteria, namely, Vibrio fischeri, Pseudomonas aeruginosa, and Staphylococcus aureus, and discuss various antivirulence strategies based on blocking different components of the QS machinery.

KEYWORDS: Vibrio fischeri, Pseudomonas aeruginosa, Staphylococcus aureus, quorum sensing inhibitors, autoinducer, virulence

Introduction

Quorum sensing (QS) is a signaling mechanism that is quite common in bacteria and involves the exchange of small chemicals between bacteria. It was first identified in the marine bacterium Vibrio fischeri.1–3 QS describes the ability of an organism to adapt the activity of its gene expression machinery to the population density in the nearby environment. This allows bacteria to act as a community, and thus express phenotypes that are beneficial for the group. Single bacteria release internally synthesized chemicals (autoinducers, AIs) either by actively transporting them across the bacterial cell membrane or by letting them passively diffuse through the membrane. In this manner, the external AI concentration automatically reflects the cell population density. When a certain cell population density, that is, AI density, is reached, the gene expression program of bacterial cells is altered and the transcription of certain genes is switched on or off. Thus, in adapting their behaviors to various environments, bacteria can regulate genes that are advantageous for their survival. Such cell-to-cell communication is important, for example, to organize light-emitting reactions (bioluminescence), to form biofilms, to produce antibiotics, to express virulence factors, or for the transfer of genetic material (conjugation and transformation).4,5

The mechanistic details of QS are different between gram-negative and gram-positive bacteria. The main difference is that the AI molecules themselves differ between gram-negative and gram-positive bacterial species. Gram-negative bacteria utilize N-acyl N-homoserine lactones (AHLs), which are homoserine lactone (HSL) rings with an additional fatty acid side chain.6,7 The fatty acid chains differ in length, and their residue is dependent on the bacterial species. P. aeruginosa also uses alkyl quinolines.7 In contrast, gram-positive bacteria utilize secreted peptides as signal molecules.8 Different AIs of three well-studied bacterial systems are illustrated in Table 1. In general, AI molecules produced by gram-negative bacteria diffuse passively in and out of cells, whereas AIs synthesized by gram-positive bacteria are actively transported.8 Nevertheless, it was found that in several gram-negative bacterial families such as Enterobacteriaceae or Pasteurellaceae, AI-2 can be actively transported as well.9 Moreover, it was shown that AHLs can also be actively transported through the cellular membrane.10,11

The inhibition of QS mechanisms has been discussed as an attractive way of combating bacterial infections because it is thought to exert a reduced pressure to select resistant bacterial strains from the population. The field has been reviewed at regularly intervals. For example, the recent review by Scutera et al.12 emphasized the selection of targets and the different classes of chemicals developed against them. QS inhibitors were also reviewed in detail in the book Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight.12 Chapters 4
and 5 of that book provide detailed discussions of various classes of natural QS inhibitors, such as bacterial AHLases that inactivate AHLs (lactonases, acylases, or oxidoreductases) as well as synthetic QS inhibitors. Here, we take a more biological route than those authors and put a strong focus on the gene-regulatory machineries related to QS.

**QS in Bacteria**

Various genera, such as *Aliivibrio*, *Escherichia*, *Pseudomonas*, and *Staphylococcus*, utilize QS for cell-to-cell communication enabling them to adapt their gene expression levels to phenotypes that are advantageous for the group. In the following section, we describe the well-understood QS systems of the model system *V. fischeri* and of the two pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Table 2 gives an overview of the genes necessary for cell-to-cell communication in these species.

**QS in V. fischeri.** The marine luminous bacterium *V. (Aliivibrio) fischeri* forms a symbiotic relationship with various eukaryotic hosts, whereby *V. fischeri* benefits from nutrient supply while the host takes advantage of the luminescence reaction carried out by this bacterium. Light emission is thereby used in different ways, for example, to produce counterillumination that prevents detection by natural enemies (camouflage), to support hunting, to provide protection against predators, or to help in alluring mates. For instance, the fish *Monocentris japonicus* exploits this light reaction to impress and lure a mating partner. On the other hand, the light organ of bobtail squid *Euprymna scolopes* accommodates *V. fischeri* to exploit its light emission at night so that its contrast against the bright moonlight is minimized.

*V. fischeri* uses the well-understood QS system, as shown in Figure 1, to control and regulate the bioluminescence reaction. The signaling system requires two regulatory proteins, encoded by the genes *luxI* and *luxR*, to carry out central functions. *luxI* is organized in the *luxICDABE* operon that also harbors the genes needed for the luminescence reaction itself. The two luciferase subunits, needed for the luminescence reaction, are expressed by *luxAB*, while the proteins expressed from

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**Table 1.** Signal molecules (autoinducers) of three bacterial species (*V. fischeri*, *S. aureus*, and *P. aeruginosa*). Autoinducers that are used in different QS systems belong to different signal molecule classes. The structures were drawn with ChemBioDraw Ultra 14.0 and Inkscape.

| BACTERIAL SPECIES | SIGNAL MOLECULE CLASS | SIGNAL MOLECULE | CHEMICAL STRUCTURE | QS SYSTEM |
|-------------------|-----------------------|-----------------|--------------------|-----------|
| *V. fischeri* (gram-) | Acetyl homoserine lactones (AHLs) | LuxI | ![LuxI structure](image) | Lux |
| *P. aeruginosa* (gram-) | Alkyl Quinolones (AQ) | N-(3-oxododecanoyl)-l-homoserine lactone (OdDHL) | ![OdDHL structure](image) | Las |
| | | N-butyl-l-homoserine lactone (BHL) | ![BHL structure](image) | Rhl |
| | Autoinducing peptides AIPs | 2-heptyl-4-hydroxyquinoline (HHQ) | ![HHQ structure](image) | Pqs |
| | | 2-heptyl-3-hydroxy-4(1H)-quinolone/Pseudomonas quinolone signal (PQS) | ![PQS structure](image) | |

*S. aureus* (gram+) | Autoinducing peptides AIPs | AIP-I | ![AIP-I structure](image) | Agr |
| | | AIP-II | ![AIP-II structure](image) | |
| | | AIP-III | ![AIP-III structure](image) | |
| | | AIP-IV | ![AIP-IV structure](image) | |
### Table 2. Quorum-sensing genes of V. fischeri, P. aeruginosa, and S. aureus. Gene and protein information based on NCBI, Uniprot, and explanation from the text.

| V. fischeri | P. aeruginosa | S. aureus |
|-------------|---------------|-----------|
| **GENE NAME** | **PROTEIN NAME** | **PROTEIN FUNCTION** | **GENE NAME** | **PROTEIN NAME** | **PROTEIN FUNCTION** | **GENE NAME** | **PROTEIN NAME** | **PROTEIN FUNCTION** |
| luxI | Acyl-homoserine-lactone synthase | Synthesizes autoinducer OHL (N-(3-oxohexanoyl)-L-homoserine lactone). Autoinducer binds to LuxR to regulate bioluminescence. | lasI | Acyl-homoserine-lactone synthase | Synthesizes autoinducer OdDHL (N-(3-oxodecanoyl) homoserine lactone). Autoinducer binds to LasR to regulate elastase expression. | agrB | Accessory gene regulator protein B | Converts AgrD into the autoinducing peptide (AIP). AIP binds to AgrC to activate AgrA that regulates virulence factor expression. |
| rhlI | Acyl-homoserine-lactone synthase | Synthesizes autoinducers BHL (N-butanoyl-L-homoserine lactone) and HHL (N-hexanoyl-L-homoserine lactone). Autoinducer BHL binds to RhlR to regulate elastase expression. | | | | |
| pqsA-E | PqsA-E | Produces autoinducers HQQ and PQS (2-heptyl-3-hydroxy-4(1h)-quinolone) | | | | |
| pqsH | | | | | | |
| **Regulator activation** | | | | | | |
| luxR | Transcriptional activator protein LuxR | Activates transcription of bioluminescence operon. | lasR | Transcriptional activator protein LasR | Binds OdDHL. Activates transcription of elastase structural gene (LasB). | agrA | Accessory gene regulator protein A | Regulates expression of various virulence factors. |
| rhlR | Regulatory protein RhlR | Binds BHL and HHL. Activates transcription of rhlAB and of elastase structural gene (lasB). | | | | |
| pqsR | Transcriptional regulator PqsR | Binds PQS. Activates expression of pqs operon, of HQQ and of several virulence factors. | | | | |
| mvfR | | | | | | |
The QS system of *P. aeruginosa* is shown in Figure 2. In contrast to *V. fischeri* that uses only one QS circuit, *P. aeruginosa* exhibits the three QS circuits named *Las*, *Rhl*, and *Pqs* that are interconnected with each other. *Las* and *Rhl* are in fact homologous systems. In *V. fischeri*, the QS circuits are hierarchically regulated. The *Las* system activates both the *Rhl* and *Pqs* systems, while *Rhl* can suppress *Pqs* and *Pqs* activates *Rhl*. Although details of this activating mechanism still need to be deciphered, the involvement of protein PqsE in *Pqs* signaling, rather than *Pseudomonas* quinolone signal (PQS) biosynthesis, has been suggested.33

While the *Las* and *Rhl* systems use AHLs as AIs, the *Pqs* system uses 2-alkyl-4-quinolones (AQs), most predominant, 2-heptyl-4-hydroxyquinoline (HHQ), and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), as signaling molecules. The AIs, N-(3-oxo-dodecanoyl)-1-homoserine lactone (OdDHL) and N-butyryl-1-homoserine lactone (BHL), are synthesized by LasI and RhII, respectively. The Pqs signaling molecule is produced by PqsH and PqsA-E that are organized in one operon. When a minimal concentration threshold is reached, AIs bind to their respective transcriptional regulators LasR–OdDHL, RhlR–BHL, and PqsR–PQS/HHQ. The AI protein complexes regulate multiple virulence genes and regulate also the expression of each other. LasR–OdDHL formation. This allows distracting the host defense systems and provokes chronic infections. Examples of virulence factors are LasA, LasB, and Exotoxin A (ToxA). The elastases LasA and LasB were shown to have an impact on cell wall flexibility and in consequence hinder the healing process. Exotoxin A is a transferase that is associated with cellular death. The blue pigment pyocyanin is a redox-active virulence factor that affects multiple cellular functions, for instance, cellular respiration and electron transport. *P. aeruginosa* also produces hydrogen cyanide, which is a potent inhibitor of cellular respiration and associated with compromised lung function in patients.

The QS in *P. aeruginosa* is a gram-negative bacterium that causes chronic lung infections in patients suffering from cystic fibrosis based on biofilm formation. In total, 8.5% of all infections acquired in the hospital are due to the pathogen *P. aeruginosa*. This pathogenic phenotype is especially critical in patients who are coinfected with HIV. Selective pressure exerted by anti-infective treatments positively selects multidrug-resistant *P. aeruginosa* strains. In addition, this effect challenges the treatment of this pathogen. Resistance is acquired either by incorporating plasmid-encoded resistance genes or by spontaneous resistance mutations. *P. aeruginosa*, for instance, may overexpress several multidrug-resistant efflux pumps that confer drug resistance to this pathogen.

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activates the Rhl and Pqs circuit by binding to the promoter regions of rhlR and pqsR. Moreover, LasR–OdDHL activates lasI resulting in a positive feedback loop. LasR–OdDHL also activates pqsH that is needed to synthesize the signaling molecule PQS from HHQ. In contrast, RhlR–BHL represses the expression of the PqsA-E operon, whereas PqsR–PQS activates the expression of PqsA-E. The Pqs and Rhl systems also interact via PqsE. Moreover, PqsE was recently found to function as thioesterase and is involved in the synthesis of the signaling molecule HHQ that is the precursor of PQS. Wade et al investigated transcriptional start sites and showed that the binding of PqsR to the promoter region of pqsA can increase the PQS signal, while pqsR in turn is activated by LasR and repressed by RhlR.

QS in S. aureus. S. aureus is a gram-positive bacterium responsible for infections of the skin and soft tissue, bacteremia, endocarditis, sepsis, and toxic shock syndrome. As for P. aeruginosa, treating S. aureus is complicated due to the evolvement of multidrug-resistant S. aureus strains, known as methicillin-resistant S. aureus (MRSA). Strains that are not resistant to antibiotics are termed methicillin-susceptible S. aureus; one can distinguish between the hospital-acquired MRSA and community-acquired (CA-MRSA) forms. In general, methicillin-resistant pathogens are a huge burden that one needs to overcome, especially in the healthcare sector.

The various infections that are caused by S. aureus are facilitated by several (intrinsic) virulence factors. Virulence factors comprise a large spectrum of various enzymes and exotoxins that enable the evasion of the immune system and tissue adhesion or cause damages to the host cell. Proteases, lipases, and nucleases, for example, take part in the tissue invasion, whereas leukocidins enable the evasion of the immune response. Sepsis, on the other hand, is associated with enterotoxin release such as the toxic shock syndrome toxin. Further virulence factors that are secreted by S. aureus are the alpha, beta, gamma, and delta hemolysins (the order corresponds to their sequence of discovery). α-Hemolysin, for example, triggers the destruction of membrane structures and can cause pneumonia. Surface proteins such as the microbial surface components recognizing adhesive matrix molecules exhibit different functions – all are important for S. aureus survival – comprising the adherence to the host tissues or immune system evasion. These proteins also enable the formation of bacterial biofilm. Thus, virulence factors are a crucial part in the pathogenesis of bacterial infections.

The expression of different virulence factors depends on external influences and is regulated by the cell-density-dependent QS accessory gene regulator (agr) system of S. aureus, shown in Figure 3. The agr locus consists of the five genes agrA, agrB, agrC, agrD, and hld. As suggested by the names, agrA to agrD are organized in one operon. Thereby, the agr operon and hld are controlled by different promoters, termed P2 and P3, respectively. Each of those proteins takes over a different function in the QS system: AgrB, a transmembrane protein, as well as SpsB, a type I signal peptidase, converts the AgrD pro-peptide into the autoinducing peptide (AIP) that is used as cellular signaling molecule. While AgrB removes the charged AgrD carboxy tail, a type I signal peptidase, termed SpsB, is responsible for the removal of the amphipathic N-terminus.

In contrast to gram-negative bacteria, short peptides rather than HSLs are used as signaling molecules in S. aureus. The length of these AIPs varies between 7 and 9 amino acids, whereby S. aureus encodes four different allelic AIP variants (AIP-I to AIP-IV). At the C-terminus, five residues form a
thiolactone ring. Each secreted AIP binds specifically to the respective AgrC histidine kinase.

After AIP synthesis, the signaling molecule is transported out of the cell by AgrB. AIP then binds to the extracellular part of agrC that is also an integral membrane protein. As mentioned earlier, AgrC functions as a histidine kinase that in turn autophosphorylates the response regulator AgrA. This autophosphorylation is established by an AIP-induced change in the AgrC conformation that enables a connection between the sensor and kinase domains. AgrA in turn upregulates the expression of the hld and agr operons by binding to the intergenic DNA between promoters P2 and P3. The hld gene encodes the RNAIII effector molecule that posttranscriptionally regulates several virulence factors (eg, α-hemolysin). In consequence, the agr system regulates the expression of virulence factors but, in addition to other global regulators, it also regulates its own expression. In total, RNAIII and AgrA regulate the transcription of ∼200 genes that comprise virulence factors.

Interspecies and interkingdom communication. Bacteria normally coexist with other bacterial species in multispecies communities inside the host (eg, in the gastrointestinal tract or the oral cavity). Interestingly, both gram-negative and gram-positive bacteria are able to cross talk by recognizing and processing autoinducing signals of other species.

For example, biofilms in the lungs of patients suffering from cystic fibrosis often consist of P. aeruginosa and Burkholderia cepacia bacterial strains. Applying green fluorescent protein tags, Riedel et al showed that these two bacterial species, which both use AHLs as signaling molecules, were able to cross talk in a murine infection model. In this case, B. cepacia could recognize AHL signals synthesized by P. aeruginosa but not vice versa (unidirectional signaling).

It was also shown that pathogenic bacteria can interact with eukaryotic host cells, and vice versa, by utilizing each other’s autoinducing signals. Enterohemorrhagic Escherichia coli (EHEC) is responsible for bloody diarrhea and also uses QS for cellular communication. It was shown that E. coli can communicate with the signaling molecules of the host cell. An EHEC knock-out mutant, which was not able to synthesize AI, was found to respond to signaling molecules produced by the host that could trigger the expression of several virulence genes. On the other hand, it is also possible that signaling molecules synthesized by the host can inhibit bacterial QS systems. Chun et al found that human airway epithelia can suppress the cell-to-cell communication of P. aeruginosa. They proposed that this is a defense mechanism of the mammalian airway system against pathogens.

The AI-2 QS system. Beside AHL autoinducer molecules, another AI termed AI-2 was first discovered in the bioluminescent marine bacterium Vibrio harveyi. AI-2 has been proposed to be present in several QS systems of gram-negative and gram-positive bacteria, while its precursor molecule 4,5-dihydroxy-2,3-pentanedione (DPD) was found in >70 bacterial species. It was shown that AI-2 enables interspecies communication (various examples are given in the study by Lowery et al.

AI-2 is synthesized by AI synthase LuxS via the precursor product DPD. The luxS gene was first discovered in E. coli, Salmonella typhimurium, and V. harveyi and was shown to be associated with the expression of bioluminescence and virulence factors as well as biofilm formation. Two other proteins, LuxP and LuxQ, serve as the AI-2 sensor. The structure of AI-2, in complex with V. harveyi sensor protein...
protein LuxP, was revealed via X-ray crystallography.\textsuperscript{63} LuxP is a periplasmic protein that, when bound to the AI, interacts with the LuxQ sensor protein.\textsuperscript{63} LuxQ is a two-component protein that contains both a sensor kinase domain and a response regulator domain.\textsuperscript{64} Depending on the cell density (low or high), either the kinase or regulator domain is activated, which leads to a switch between repression and production of light, respectively. At a low cell density, LuxQ autophosphorylates and passes the phosphate to the phosphotransferase LuxU, which then transfers it to the response regulator LuxO.\textsuperscript{64} LuxO is activated by this phosphorylation and represses luxCDABE, which encodes luciferase, by destabilizing, with the help of other factors, the mRNA that encodes the transcriptional activator LuxR.\textsuperscript{65,66} In consequence, no light is produced. At a high cell density, the AI-2 and LuxQ interact. LuxO then acts as phosphatase, leading to a reversion of the signaling process: the phosphate is transferred back from LuxO to LuxU, which leads to an inactivation of LuxO.\textsuperscript{64} Since LuxO is inactive, LuxR is translated, binds to the luxCDABE promoter, and thus activates the transcription of luxCDABE, leading to light production.\textsuperscript{67} In addition to the AI-2 QS system of \textit{V. harveyi}, two other systems exist, which are described elsewhere.\textsuperscript{47}

**Interferring with QS to Develop New Antivirulence Therapies**

A number of studies have succeeded in exploiting the bacterial QS system as target for treatment of bacterial infections. Targeting the QS system is believed to be advantageous over conventional therapeutic strategies, because only the communication mechanism between the bacteria is disrupted without killing the individual cells. Hence, this strategy should generate a lower selective pressure and reduce the rate at which antibiotic resistance develops during the treatment.\textsuperscript{5,6,8} Since bacteria use the QS system also to regulate the expression of virulence factors and biofilm formation, inhibiting the signaling system should, in principle, favor the viability of less virulent strains and prevent or minimize the establishment of pathogenic biofilms.\textsuperscript{5} Since experimental conditions may strongly affect experimental outcome, we are lacking a clear, consistent characterization how agr interference affects biofilm formation.\textsuperscript{46}

Various classes of chemical compounds as well as different targets have been proposed that interfere with different parts of the QS cascade. All QS systems share a general pattern or signaling cascade: an AI is synthesized, then reaches a certain concentration threshold and binds to a transcriptional regulator that subsequently activates or represses certain genes. This opens up four potential strategies for blocking QS.\textsuperscript{5} First, one may suppress the synthesis of the AI; second, target the AI (either by decomposing the AI in an enzymatic reaction or by deactivated it using antibodies); third, the antagonism of the regulator; and fourth hinder the regulator protein from binding to DNA. Possibilities to inhibit AI-2 synthesis are described in a recent book.\textsuperscript{12}

In the following section, we describe different approaches that are followed to disrupt the bacterial QS systems of \textit{V. fischeri} and the pathogens \textit{S. aureus} and \textit{P. aeruginosa}.

**Targeting \textit{V. fischeri}**. The QS system of \textit{V. fischeri} was targeted in several studies. Schaefer et al investigated synthetic HSL analogs in terms of their binding affinity to LuxR and their ability to reduce the luminescence reaction.\textsuperscript{69} They identified several LuxR binders that induced a luminescence reaction and also identified competitive HSL compounds, which were not capable of activating the luminescence reaction and could thus be applied to inhibit QS-dependent gene expression.

Both Piletska et al.\textsuperscript{70} and Cavaleiro et al.\textsuperscript{71} studied the ability of polymers to attenuate QS in \textit{V. fischeri}. These synthetic polymers (eg, itaconic acid based) were able to sequester the autoinducing signal and are thus termed signal molecule-sequestering polymers (SSPs).\textsuperscript{70} SSPs showed affinities to the HSL signaling molecule and prevented the \textit{V. fischeri} bioluminescence reaction by absorbing the AI.\textsuperscript{70}

One advantage of these polymers, in comparison with other anti-infectives, is the decrease of harmful side effects.\textsuperscript{70} When applied to mammalian cells, these polymers were also shown to be non-cytotoxic.\textsuperscript{71} Moreover, polymeric structures were favorably used in various applications, especially as surface-coating structures of medical devices (for instance, catheter or prosthetics) to, for example, hinder biofilm formation of various pathogens.\textsuperscript{70,71}

The authors of this study suggested that it should be possible to transfer the findings of these studies to pathogenic bacteria in order to develop SSP-based QS inhibitors in other bacterial systems.\textsuperscript{70}

**Targeting \textit{S. aureus}**. \textit{Attacking the AI}. Park et al applied an immunopharmacotherapeutic approach and investigated monoclonal antibodies (mAbs) in terms of their ability to neutralize the AI peptide AIP-IV via sequestration.\textsuperscript{72} Thereby, an AIP-IV hapten was synthesized to provoke an anti-AIP-IV immune reaction in mice. Out of 20 produced anti-AIP-IV, one antibody (AP4-24H11) with high binding affinity was highly specific toward AIP-IV. Moreover, applying AP4-24H11 to different \textit{S. aureus} strains resulted in a decreased α-hemolysin production. This antibody was also successfully applied to an infected murine model showing abscess formation.\textsuperscript{72} These results highlight that the removal of an autoinducing signal peptide from a bacterial system results in the inhibition of QS-dependent gene expression, without tampering bacterial genetic information. Kaufmann et al provided an example of QS interference with monoclonal antibodies and reported an AHL-specific mAb that was able to inhibit the \textit{P. aeruginosa} QS cascade.\textsuperscript{73} For more information on antibody-based approaches, readers are referred to the study by Scutera et al.\textsuperscript{5}

\textit{Preventing AI–regulator interactions}. Mansson et al investigated the potential of marine bacteria to decrease the pathogenicity of \textit{S. aureus} by attacking its \textit{agr} QS system.\textsuperscript{74} They
showed that the investigated marine photobacterium produces two AI antagonists named solonamide A and B (Fig. 4A) that were able to inhibit QS in a highly virulent CA-MRSA strain. Note that *S. aureus* strains are grouped based on which AIP and AgrC are present. This leads to four (I–IV) different *S. aureus* groups each causing different disease(s), for example, toxic shock syndrome (group III). Tal-Gan et al identified peptides (synthesized AIP analogs; Fig. 4B) that were able to inhibit AgrC receptors in all four strains.76

Murray et al synthesized several small-molecule inhibitors that interact with the cytoplasmic membrane and appear to affect the AIP–AgrC interaction as allosteric noncompetitive inhibitors.77 The most potent inhibitor (Fig. 4C) was tested in a mouse model that was infected with *S. aureus*. The experiments showed that the inhibitory effect toward the *agr* system could decrease nasal colonization in mouse.

**Inhibiting regulator binding to DNA.** Since the *S. aureus* *agr* system was shown to be involved in skin and soft tissue infections,77 Sully et al.78 aimed at identifying a small-molecule inhibitor that disrupts the *S. aureus* signaling cascade but omits suppressing that of commensal *Staphylococcus epidermidis*. The reason behind this was that *S. epidermidis* is an important gram-positive bacterium involved in host defense mechanisms against skin pathogens and is thus important for human skin flora.79 To ensure specificity toward *S. aureus*, they investigated the structural differences between the components of the *agr* systems of *S. aureus* and *S. epidermidis*. Since the AgrC residues, which are crucial for *agr* functionality, were found to be conserved between *S. aureus* and *S. epidermidis*, AgrA was selected as target protein. The authors applied high-throughput screening to 24,087 compounds and discovered inhibitors of the *agr* signaling cascade that suppress the upregulation of virulence factors. The inhibitor was named savirin short for signaling cascade that suppress the upregulation of virulence.

![Figure 4](image)

**Figure 4.** *S. aureus* quorum-sensing inhibitors. (A) Solonamide A and B76; (B) AIP D4A76; (C) most potent inhibitor found by Murray et al.77; (D) savirin78; (E) ω-hydroxyemodin.81
Inhibitors of autoinducer biosynthesis

PQS signalling

Anthrilate analogues

PqsD inhibitors

Inhibitors with unknown target

AHL signalling

Figure 5. *P. aeruginosa* QS inhibitors that inhibit autoinducer biosynthesis. (A) Calfee et al.82; (B) Lesic et al.84; (C) Coleman et al.83; (D) Storz et al.85; (E) Hinsberger et al.86; (F) Sahner et al.87; (G) Allegretta et al.88; (H) Weidel et al.89; (I) Zhou et al (eugenol)91; (J) Miller et al.92; (K) Chang et al.93 (salicylic acid); and (L) Chang et al.93 (trans-cinnamaldehyde).

PqsD (Fig. 5D) were developed by a ligand-based approach that also inhibited the biofilm formation in the target organism *P. aeruginosa*.85 Several structural scaffolds inhibiting PqsD have been discovered,86–90 see the review chapter by Maurer et al. in the book.12 and Figure 5E–H.

Zhou et al investigated the plant-based inhibitors and assessed their impact on the Las and Pqs systems for cell-to-cell communication.91 They found that eugenol (Fig. 5I) was able to suppress the expression of multiple virulence factors (e.g., elastase and pyocyanin). Moreover, biofilm formation was decreased. Miller et al identified a series of highly effective small molecules (Fig. 5J) that inhibited the production of pyocyanin by *P. aeruginosa*.92 Interestingly, the production of this virulence factor appeared to be affected through a novel pathway that is independent of LasR and RhlR.

Interference with the AHL production, considering RhlI and LasI systems, was investigated by Chang et al.93 They identified salicylic acid, tannic acid, and trans-cinnamaldehyde as strong inhibitors of AHL synthesis, while the last two were found to inhibit the RhlI–AHL production mechanism (Figs. 5K and L), respectively. To understand the underlying mechanism of AHL synthesis inhibition, they applied molecular docking to trans-cinnamaldehyde and the X-ray structure of LasI. While they concluded that the inhibitor is able to block the substrate-binding pocket needed for AHL production, a reduced AHL production was still observable. This suggests that there might exist another inhibition mechanism.

**Attacking AI.** A further antivirulence strategy is attacking the produced AI itself. AHL signaling molecules can, for example, be degraded by acylases94,95 or deactivated by lactonases.96 For more information, refer to Scutura et al.5

**Antagonism of the regulator.** The regulator PqsR, which is activated by a signaling molecule and regulates the expression of multiple virulence factors, has attracted attention as
a target, and antagonists of this receptor have been discovered starting from the natural ligand\textsuperscript{97,98} and by a fragment-based approach.\textsuperscript{99,100} These synthetic QS inhibitors are reviewed in detail in the chapter of Maurer et al.\textsuperscript{12} and are shown in Figure 6A–D. A first in vivo proof of concept of a PqsR antagonist as antivirulence agent was provided by Lu et al.\textsuperscript{101} The application of a HHQ-derived antagonist in the animal models Caenorhabditis elegans and Galleria mellonella led to a drastic reduction of the mortality rate caused by P. aeruginosa. Recently, Starkey et al identified a highly potent antagonist (Fig. 6E) in a high-throughput whole-cell screening which was also effective in mouse burn and lung infection models.\textsuperscript{102} They demonstrated the antagonists’ additional potential of preventing P. aeruginosa forming antibiotic-resistant persister cells.

Welsh et al investigated the effect of modulating the activity of the regulatory protein RhlR in wild-type P. aeruginosa.\textsuperscript{32} They monitored the production of the RhlR-regulated virulence factors pyocyanin and rhamnolipid and found that when AHL antagonists were applied, pyocyanin production was induced,

**Antagonists of the regulators**

**Antagonists of PqsR**

- **A**
- **B**
- **C**
- **D**
- **E**

**Antagonism of AHL regulators LasR or/and RhlR**

- **F**
- **G**

**AHL mimics**

- **H**
- **I**
- **J**

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**Figure 6.** P. aeruginosa QS inhibitors based on regulator antagonism. (A) Lu et al.\textsuperscript{97,101}; (B) Ilangoavan et al.\textsuperscript{98}; (C) Klein et al.\textsuperscript{99}; (D) Zender et al.\textsuperscript{100}; (E) Starkey et al.\textsuperscript{102}; (F) Wu et al.\textsuperscript{103} (C-30 furanone); (G) Musthafa et al.\textsuperscript{104} (2,5-piperazinedione); (H) McInnis and Blackwell\textsuperscript{105}; (I) Geske et al.\textsuperscript{106}; (J) Stacy et al.\textsuperscript{107}; and (K) O’Loughlin et al.\textsuperscript{109} (mBTL).
whereas the production of rhamnolipid was repressed. They also suggested that preventing the cross talk between the Rhl and Pps systems could suppress the development of virulence.

Moreover, a large panel of antagonists of LasR have been identified, which are either structurally unrelated AHLs or derived from the native ligand (AHL mimics) and are comprehensively reviewed in the chapter of Maurer et al. A first non-AHL-based compound showing promising anti-QS effects is a synthetic analog of a marine natural product referred to as C-30 (Fig. 6F) that resulted in the inhibition of virulence factor production, biofilm formation, higher survival rate, and reduced pathogenicity in a C. elegans killing assay and in mice. Another in vivo study was conducted by Musthafa et al. They identified 2,5-piperazinedione (Fig. 6G) as a potent inhibitor suppressing P. aeruginosa QS and that reduced pyocyanin synthesis by 85%. The inhibitor led to increased survival rates in the C. elegans killing assay. Molecular docking suggested that 2,5-piperazinedione occupies the AHL-binding pocket of LasR.

A considerable number of AHL mimics have been reported (see the review by Maurer et al). Structural modifications imply the replacement of the hydrolyzable “head group” (eg, HSL moiety) or both. Selected examples are shown in Figure 6H–K. One example with a modified head group is the work reported by McInnis and Blackwell. They synthesized a focused library designed with the aim to enhance the stability of the hydrolyzable lactone ring. The biological evaluation resulted in the identification of antagonists largely selective for LasR, LuxR, and TraR using E. coli and P. aeruginosa reporter strains.

In a further work, this group reported on the identification of thiolactone analogs acting as LuxR-type receptor (LasR, LuxR, and TraR) antagonists using P. aeruginosa and E. coli (LasR) as well as V. fischeri (LuxR) and Agrobacterium tumefaciens (TraR) bacterial reporter strains. They were able to identify novel and highly active thiolactones to inhibit LuxR-type QS receptors.

By modifying the tail moiety, Geske et al evaluated ~90 compounds and showed that these LasR antagonists could strongly inhibit virulence factor production elastase B. Another AHL library, designed by Geske et al, resulted in the identification of several antagonistic library compounds active against LuxR-type receptors (LasR, LuxR, and TraR) using reporter gene assays (E. coli [LasR], V. fischeri [LuxR], and A. tumefaciens [TraR]). To identify QS LasR modulators, Stacy et al synthesized 72 triazole derivatives (AHL analogs) to block LasR activity. They found different QS antagonists that inhibited LasR activity using E. coli reporter gene assays (β-galactosidase).

O’Loughlin et al identified meta-bromo-thiolactone (mBTL) as a strong in vivo and in vitro inhibitor of LasR and RhlR QS. Besides the prevention of virulence factor production and biofilm formation by mBTL, this compound was additionally able to protect C. elegans and human lung epithelial cells against P. aeruginosa killing. On the basis of their previous work, Miller et al recently identified several novel antagonists that suppressed pyocyanin virulence factor synthesis. The underlying QS signaling pathway was independent of LasR and RhlR in this case.

Inhibiting regulator binding to DNA. Seet et al knocked out the newly identified P. aeruginosa anti-activator gene qslA and reported an increased expression of virulence factors. They reported that QslA interacts with LasR in such a way that LasR cannot bind to its target promoter. In consequence, QslA serves as QS inhibitor by suppressing the expression of rhlR. The binding mechanisms of QslA and LasR were further investigated by Fan et al who determined the crystal structure of QslA bound to the N-terminal ligand-binding domain of LasR. They reported that QslA blocks the LasR dimerization interface, which results in the disconnection of LasR from its target promoter.

In silico approaches. As in medicinal chemistry for the discovery of enzyme inhibitors, computer-based approaches have also been applied in the field of discovering anti-QS substances with the aim of saving experimental time and costs by preselecting promising candidates via virtual screening.

Preventing AI-regulator interactions. Annapoorni et al carried out virtual screening to find LasR and RhlR QS inhibitors in P. aeruginosa. Out of 1,920 compounds, docking identified five promising candidate binders for the LasR and RhlR receptors. They verified their potential to suppress the expression of virulence factors such as protease, elastase, and hemolysin, in vitro experiments.

Similarly, Tan et al screened a library of 3,040 natural derivatives to find new candidate QS inhibitors of the LasP receptor from P. aeruginosa. Based on the docking results of all these molecules, they were able to limit in vitro experimental procedures to only 22 promising candidate inhibitors. The best compound was experimentally investigated and reported to downregulate several virulence factors.

Finally, Yang et al screened the structural compound libraries, Supernatural and SuperDrug, that contain structures showing similarities to the so far identified inhibitors of the LasR receptor from P. aeruginosa. By applying virtual screening and molecular docking, they were able to identify three compounds that were able to suppress the QS signaling cascade.

Using a variant of Boolean network modeling, Schaadt et al presented an in silico multilevel modeling approach to study time-dependent properties of the Las, Rhl, and Pps signaling systems of P. aeruginosa. Their aim was to investigate the regulatory and metabolic interplay between QS inhibitors, receptor antagonism, signaling molecules, and expression of the virulence factors such as elastase, rhamnolipids, and pyocyanin. In the simulations, they found that signaling molecules HHQ and PQS are decreased when the expression of PqsBCD is suppressed by appropriate inhibitors. Using this,
network approach also enabled them to quantitatively predict the impact of Pps inhibitors and PqsR antagonists.

Inhibit regulator binding to DNA. Leonard et al determined the crystal structure of an AgrA LytTR domain in S. aureus that is necessary to bind DNA.119 They subsequently applied fragment virtual screening to a small library consisting of 500 compounds and found three inhibitors that disrupted binding of AgrA to DNA.

Outlook. Although several new discoveries in the field of bacterial QS and in the development of promising inhibitors have been reported recently, significant research gaps remain.

First of all, it is unclear whether all molecular components of QS systems and the respective regulators have been discovered up to date. For example, Miller et al recently identified novel antagonists of pyocyanin production in P. aeruginosa which appear to act through a pathway that is independent of the known regulators LasR and RhlR.92 Drees et al presented a differential equation and noise model for those network components in V. harveyi which are required to convert information about cell density into a corresponding concentration of AIs.120 They suggested that dynamic and noise measurements may be particularly helpful in identifying missing components and regulatory links. In a related work on V. harveyi, Plener et al assayed the activity of the QS cascade at population and at single cell levels.121 They found that the ratios of kinase to phosphatase activities of three hybrid sensor kinases were important for the signaling output as well as for the degree of noise in the system. The pools of phosphorylated LuxU/LuxO per cell determined the copy number of LuxR, generating a heterogeneous QS activation at the single cell level.

An important issue in antimicrobial drug development is the treatment of bacterial biofilms. Infections that are based on biofilms have a preference to be chronic as well as resistant to antibiotics.122 Thereby, QS might help bacteria to regulate group behavior in these densely packed bacterial biofilms.122 Here, a combination therapy of QS inhibitors and antibiotics could be beneficial: QSIs can enhance the susceptibility of bacterial biofilms to the treatment with antibiotics that resulted in increased in vitro (P. aeruginosa, Burkholderia cenocepacia, and S. aureus) killing and in vivo (C. elegans and G. mellonella) survival rates.123

Moreover, genes that are associated with virulence factor expression were detected to be often mutated during bacterial infection.124,125 For example, lasR of P. aeruginosa was found to be susceptible to mutations, resulting in the disruption of virulence factor production and/or biofilm formation.125 Thereby, lasR mutants were isolated from (chronic) cystic fibrosis patients suggesting that QS is actually downregulated in (biofilm based) chronic airway infections due to the selection against lasR wild-type strains and the establishment of lasR mutants that are not capable of QS.125 Another study reported on P. aeruginosa mucA mutants, also resulting in downregulation of AHL and PQS QS systems in cystic fibrosis lung infection.124 Thus, a treatment with QS inhibitors might not be effective as soon as a chronic (biofilm based) disease state is established.

Small molecule 2-amino acetophenone was found to, on the one hand, reduce acute pathogenic virulence in vivo, but, on the other hand, to promote mutations in virulence gene lasR resulting in the increased survival and persistence of bacterial cells during P. aeruginosa infections.126 Nevertheless, an aforementioned PqsR antagonist was able to prevent the formation of P. aeruginosa persistor cells.102 Moreover, the comparison of QS inhibitor azithromycin and placebos showed that the treatment with QSIIs in sub-MIC concentration to decrease virulence factor production resulted in the selection of more virulent wild-type strains since the fitness advantage of less virulent QS mutants is lost, if QS is blocked (https://clinicaltrials.gov, NCT00610623).127

In addition to “traditional” strategies to inhibit the QS signaling cascade, there exist also alternative strategies, such as inhibition via pheromone-guided antimicrobial peptides or the combination of QS inhibitors with antibiotics. These strategies are comprehensively reviewed in Chapter 6 of the book Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight.112

Despite the fact that multiple drugs have been tested in vitro and in vivo, very few clinical trials involving QS inhibitors have been conducted or initiated. Only three clinical trials, with verified status, are reported in the publicly available ClinicalTrials.gov database.121

Scutera et al speculated that the interest of pharmaceutical companies in the development of QS inhibitors is only moderate based on the imbalance between high costs for developing new drugs, while the market for these drugs seems to be restricted.5 They also suggested that the apparent advantage of avoiding drug resistance by targeting the signaling system may have the downside that strains with increased virulence could be selected.

Discovering the complex intricacies of QS systems and understanding the genetic, and possibly also epigenetic, mechanisms of bacterial adaptation under selective pressure are important research questions. For example, it is possible that when a certain signaling system of a certain species is targeted, other (pathogenic) bacterial species, the patient is infected with, may have an increased selective advantage. Moreover, bacteria may of course also become resistant to QS inhibitors. In the case of the agr system of S. aureus, for example, this may occur via the upregulation of efflux transporters.78 Fortunately, the recently discovered QS inhibitors reviewed in this article and elsewhere5 are nice tools for such mechanistic studies.

Author Contributions
KR wrote the first draft of the manuscript. All authors (KR, AS, VH) developed the structure and arguments for the paper,
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contributed to the writing of the manuscript, agree with manuscript results and conclusions, jointly made critical revisions and reviewed and approved the final manuscript.
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