HHS Public Access

Author manuscript

Expert Rev Anti Infect Ther. Author manuscript; available in PMC 2024 April 21.

Published in final edited form as:

Expert Rev Anti Infect Ther. 2020 June ; 18(6): 499–510. doi:10.1080/14787210.2020.1750951.

Alternative approaches to treat bacterial infections: targeting quorum-sensing

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Abstract

Introduction—The emergence of multi- and pan-drug-resistant bacteria represents a global crisis that calls for the development of alternative anti-infective strategies. These comprise anti-virulence approaches, which target pathogenicity without exerting a bacteriostatic or bactericidal effect and are claimed to reduce the development of resistance. Because in many pathogens, quorum-sensing (QS) systems control the expression of virulence factors, interference with QS, or quorum-quenching, is often proposed as a strategy with a broad anti-virulence effect.

Areas covered—We discuss the role and regulatory targets of QS control in selected Gram-positive and Gram-negative bacteria, focusing on those with clinical importance and QS control of virulence. We present the components of QS systems that form possible targets for the development of anti-virulence drugs and discuss recent research on quorum-quenching approaches to control bacterial infection.

Expert opinion—While there has been extensive research on QS systems and quorum-quenching approaches, there is a paucity of in-vivo research using adequate animal models to substantiate applicability. In-vivo research on QS blockers needs to be intensified and optimized to use clinically relevant setups, in order to underscore that such drugs can be used effectively to overcome problems associated with the treatment of severe infections by antibiotic-resistant pathogens.

Keywords

Quorum-sensing; anti-virulence; quorum-quenching; *Staphylococcus aureus* ; *Pseudomonas aeruginosa* ; biofilm

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1. Introduction

Antimicrobial resistance (AMR) has been recognized as an enormous threat to global public health. There are now a series of multidrug-resistant (MDR) and pan-drug resistant (PDR) bacteria for which there is virtually no cure. In 2019, the World Health Organization reported that AMR causes at least 700,000 deaths per year worldwide and it is predicted that the annual death toll will reach 10 million by 2050 if no action is taken. Around 2.4 million people could die in high-income countries between 2015 and 2050 without a sustained effort to contain AMR [1]. With the last novel class of antibiotic drugs discovered in the 1980s, there has been a paucity of new therapeutic approaches over the past quarter century to respond adequately to the widespread development of antibiotic resistance. Therefore, clinicians and scientists are searching for new ways to combat antimicrobial-resistant bacteria, which in particular includes the development of therapies targeting bacterial virulence mechanisms and virulence regulators, including those controlling cell-cell communication [2].

Quorum-sensing (QS) is a way of cell-cell communication that describes the ability of bacteria to sense the bacterial cell density and respond with gene expression changes to adapt to the changing environmental conditions that arise as a consequence of the increased number of bacteria and decreased availability of nutrients [3]. This type of regulation occurs in both pathogenic and non-pathogenic species, but QS systems of pathogens have been in the focus of investigation because they frequently control virulence determinants [4,5]. The biological purpose of QS regulation of virulence factors is believed to be due to the fact that in the beginning of an infection the production of QS-controlled pro-inflammatory toxins would alert immune defenses, while during progressed infection such immune evasion factors become increasingly necessary for bacterial survival. Furthermore, bacteria that have established an infection need degradative exoenzymes to acquire nutrient sources; and thus, such enzymes are also often QS-controlled [6,7].

Both Gram-positive and Gram-negative bacteria use QS for communication, but the composition of the systems differs considerably. One main difference is the chemical nature of the secreted molecules used to signal the status of cell density. These have frequently been called “autoinducers” (AIs), but also, in analogy to signals of higher organisms, pheromones. Gram-positive systems mainly use secreted autoinducing peptides (AIPs), while Gram-negative systems mainly use N-acylated homoserine lactones (AHLs). Furthermore, Gram-negative signals are sensed by intracellular molecules, while Gram-positive signals commonly bind to the extracellular part of two-component systems that transfer the stimulation by protein phosphorylation inside the cell [3,8-10]. Moreover, there is wide variety even within Gram-negative and especially Gram-positive bacteria in terms of the specific QS systems that different species employ. There are exceptions of more widespread systems, such as the AI2 LuxS system, but whether this system truly has a QS role in all bacteria is debated [11,12]. Thus, with possibly rare exceptions, QS-targeting strategies differ significantly depending on the targeted pathogen or pathogen group.

2. Quorum sensing in Gram-positive bacteria

Quorum-sensing systems in Gram-positive bacteria usually communicate via two-component systems (TCS), which consist of a membrane-located histidine kinase signal receptor paired with a cytoplasmic response regulator (Fig. 1). Upon binding of the extracellular QS signal (the AIP), the histidine kinase initiates a phosphorylation cascade that activates the response regulator, which is a DNA-binding transcription factor. This transcriptional activation directly or indirectly influences the expression of QS-controlled target genes [10].

2.1 Quorum sensing in *Staphylococcus aureus*

One of the best-studied Gram-positive QS systems is the Agr (accessory gene regulator) system in *S. aureus*. The *S. aureus* Agr system consists of four genes, which are encoded by the RNAII region of the *agr* locus: *agrB*, *agrD*, *agrC*, and *agrA*. RNAII transcription is driven by the P2 promoter, and transcription of RNAIII, which is encoded adjacent to RNAII but in opposite direction, is driven by the P3 promoter [13]. The gene product of *agrD* is the AIP precursor [14]. While the exact reactions involved in producing mature AIP are still not fully understood, it is known that the *agrB* gene encodes a transmembrane endopeptidase, which post-translationally modifies AgrD by cleaving the C terminus and introducing the thiolactone structure that is characteristic for almost all staphylococcal AIPs [14], with rare exceptions that have an (oxo-) lactone ring [15]. AgrB is then believed to export the modified AgrD across the plasma membrane [16,17], where it is further proteolytically processed at the N-terminus possibly involving signal peptidase I (SpsB) [18] and a membrane-located protease, MroQ [19]. Extracellular AIP is recognized by AgrC, a histidine kinase transmembrane receptor. Once AIP binds to AgrC, the kinase autophosphorylates on a histidine residue [20]. AgrC then phosphorylates the response regulator, AgrA, on an aspartate residue. This activates AgrA and enables it to bind to both the P2 and P3 promoters, upregulating transcription and inducing a positive feedback loop [21].

Targets of the Agr system are regulated predominantly via RNAIII, a small regulatory RNA that exerts many of its effects via blocking translation of Rot (repressor of toxins) [22,23]. As a result, translation of enterotoxins, alpha-toxin, leukocidins, degradative exoenzymes, and further targets is upregulated, whereas some surface proteins such as protein A are down-regulated [6]. In addition to the P2 and P3 promoters, AgrA can bind to the promoter regions of phenol-soluble modulin (PSM) *psmα* and *psmβ* operons [24], exerting exceptionally strict and direct control of these virulence determinants. The PSMs are amphipathic, surfactant-like peptides with multiple functions, including lytic activity on leukocytes, erythrocytes, and other cell types, biofilm structuring and dispersal, and further activities believed to be important during the commensal state of staphylococci [25]. The PSM family also includes δ-toxin, which is encoded within RNAIII [22].

The biological role of *S. aureus* Agr QS during infection is believed to consist in delaying production of toxins and degradative exoenzymes until a point in infection where there is sufficient bacterial cell density to withstand immune attacks that earlier would be stimulated by toxin production in a way that would be counterproductive for bacteria survival. Down-

regulation by QS of surface proteins has been believed to enable colonization of tissues at earlier stages of infection, but this sort of regulation is not consistent for all matrix-binding surface proteins and probably is more complex [6,26]. More recently, evidence has been achieved that points to a role of Agr in asymptomatic colonization of the skin and the intestine in the commensal state, possibly explaining why the Agr system is present in less pathogenic and non-pathogenic staphylococci as well [27,28].

2.2 Quorum sensing in other *Staphylococcus* species

Staphylococcus species other than *S. aureus* generally also have Agr systems, which are homologous to that found in *S. aureus* [29-32]. More detailed research on the tasks of non-*S. aureus* Agr systems has been limited so far mostly to *S. epidermidis*. *S. epidermidis* is a skin commensal that is associated with biofilm formation on indwelling medical devices and associated bacteremia [33]. The *S. epidermidis* Agr system downregulates genes involved in cell division, translation, coenzyme transport, and metabolism, while upregulating genes involved in fermentation [34]. Additionally, similar to *S. aureus*, *S. epidermidis* upregulates virulence factors, which in *S. epidermidis* mainly include PSM peptides and extracellular degradative enzymes [34,35]. The regulation of PSMs is as strict as in *S. aureus*, resulting in a significant impact of *S. epidermidis* Agr on pro-inflammatory capacity [35] and other PSM-mediated phenotypes such as biofilm structuring and expansion control [36,37]. The role of QS in modulating pathogenesis in other staphylococcal species has not been examined yet.

2.3 Quorum sensing in *Enterococcus faecalis*

E. faecalis resides in the human gastrointestinal tract as a member of the endogenous microbiota. Clinical isolates of *E. faecalis* show frequent resistance to vancomycin, representing a severe threat to public health systems [38]. *E. faecalis* has a QS system for cell density-dependent regulation of virulence called Fsr (fecal streptococci regulator) that is a homolog of the staphylococcal Agr system with its components FsrA, FsrC, FrsB, and FsrD believed to exert the corresponding tasks [7]. The Fsr system positively regulates the transcription of two important virulence factors, the metalloproteases gelatinase (encoded by *gelE*) and serine protease (encoded by *sprE*) [7,39]. The AIP of the Fsr system is called GBAP (gelatinase biosynthesis-activating pheromone), an 11-amino acid peptide containing a lactone structure [40].

2.4 Quorum sensing in *Clostridioides* species

Many pathogenic *Clostridioides* species produce aggressive toxins involved in disease progression [41,42]. *C. botulinum* and *C. perfringens* have an Agr-like system that regulates toxin production and sporulation [43,44]. The *C. botulinum* genome contains two distinct *agr* loci, *agr*-1 and *agr*-2, which both contain homologues of the *agrBD* genes from *S. aureus*, termed "*agrBD1*" and "*agrBD2*". The *agrD1* and *agrD2* genes code for very similar AIP peptide precursors; however, the putative ring structure of *C. botulinum* AIP1 differs from that of AIP2. A strain of *C. botulinum* containing a mutant version of *agrD*1 showed a significant reduction in spore formation compared to the wild type and the *agrD*2 mutant. Conversely, the *agrD2* mutant showed lower toxin production than the wild type and the *agrD1* mutant. This suggests that each AIP plays a specific role in gene regulation

[43]. Similar to *C. botulinum*, *C. perfringens* regulates sporulation via Agr. *C. perfringens* contains only one *agrBD* locus, which is involved in the production of *C. perfringens* enterotoxins (CPE), perfringolysin O (PFO), alpha-toxin (CPA) and beta2 toxin (CPB2) [44]. In certain strains of *C. difficile*, the most frequent cause of nosocomial antibiotic-associated diarrhea [45], the *agr* locus can affect intestinal colonization and other virulence factors [46]. *C. difficile* carries a complete *agrBDCA* operon. Interestingly, the hypervirulent strain *C. difficile* 027 has an additional chromosomal copy of *agrBD* [46]. *C. difficile agr* has an effect on the production of the flagellar regulon and the toxin TcdA [46,47]. However, the detailed molecular mechanisms underlying this regulation remain unclear.

2.5 Quorum sensing in *Listeria monocytogenes*

Listeria monocytogenes is a food-borne pathogen that can form biofilms on abiotic surfaces such as stainless steel and plastic [48]. *L. monocytogenes* has a complete *agrBDCA* operon and uses a thiolactone-containing pentapeptide AIP [49,50]. *L. monocytogenes* Agr appears to be necessary for the development of biofilms on abiotic surfaces [51] and the production of cell-wall associated and other virulence factors [52,53]. It has been shown to affect virulence in a systemic mouse infection model [50].

2.6 Quorum sensing in *Streptococcus* spp.

One of the earliest descriptions of bacterial cell-to-cell communications arose from a study in *Streptococcus pneumoniae*, where activated bacteria conferred their competence to inactivated bacteria via a competence factor in culture supernatants [54,55]. The QS signal responsible for competence induction is a heptadecapeptide, named CSP (competence-stimulating peptide) [54], which derives from a precursor (ComC) by cleavage and transport into the medium by an ATP-binding cassette (ABC) transporter, ComAB [56]. CSP acts through a receptor (ComD) and a response regulator (ComE) to activate the *comAB* and *comCDE* operons [57], establishing a positive feedback loop ensuring an abrupt rise in CSP levels, making all cells in a culture competent simultaneously. In addition to competence, ComDE QS in *S. pneumoniae* and group B streptococci such as *S. mutans* regulates biofilm formation [58,59]. In *S. pneumoniae*, ComDE is up-regulated in and has a positive impact on chronic (lung) in contrast to acute infection (bacteremia), where it is down-regulated [58].

Recently, regulator gene of glucosyltransferase (Rgg) and small hydrophobic peptide (SHP) have been discovered as a new QS system in streptococci [60]. The Rgg/SHP complex is involved in regulating virulence phenotypes, including biofilm formation [61] and surface polysaccharide production [62]. In addition, there are homologs of the Rgg QS system, such as RopB, Rgg2, Rgg3, and ComR [61,63]. SpeB, which is regulated by RopB [64], is a virulence factor that plays a prominent role in *S. pyogenes* pathogenesis [65,66]. It is a cysteine protease that cleaves host molecules such as antimicrobial peptides and activates host matrix metalloproteinases (MMPs). MMP dysregulation can lead to intensified host tissue damage and loss of barriers to protect against microbial dissemination [67].

The streptococcal invasion locus *sil* system is comprised of the *silAB, silEDC*$_R$ and *silC* genes. SilA-SilB represent a response regulator and a histidine kinase; SilD and SilE are putative ABC transporters; and SilC$_R$ is the autoinducer peptide [68,69]. SilC may act as

a transcriptional regulator of virulence factors [70]. $SilC_R$ affects the gene expression of ScpC, a protease that can degrade host CXC chemokines which are important in neutrophil recruitment to the infection site [71].

3. Quorum-sensing in Gram-negative bacteria

Quorum sensing in many Gram-negative bacterial species is dependent on membrane-diffusible N-acylhomoserine lactone (AHL)-type autoinducers, which directly bind to the cognate transcription factors without signal transduction by a membrane-located receptor (Fig. 1). The LuxI/R type is the most common QS pathway in Gram-negative bacteria [72]. LuxI or its homologs are responsible for the production of AHLs, which upon reaching a threshold level activate LuxR, leading to virulence gene expression. While the general chemical nature of the AHLs is conserved, their specific structure and that of the involved QS system can vary greatly from one bacterial species to another.

3.1 Quorum sensing in *Vibrio* species

Many Gram-negative QS types were first described in the marine bacteria *V. fischeri* or *V. harveyi*. The QS effect that controls luminescence in *V. fischeri* has been known since 1972 [73] and the structure of the autoinducing AHL substance, N-3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL), was described in 1981 [74]. Genetic analysis later identified the LuxI-LuxR system to control the induction of luminescence within growing cultures [75] and LuxI to synthesize the AHL autoinducer [76], which binds and activates the transcription factor LuxR when it reaches a threshold level [77]. However, it has been shown that the induction of luminescence can also be indirectly controlled by AinS-AinR [78] and LuxS-LuxP/Q [11,79], two additional QS systems in *V. fischeri*. AinS synthesizes N-octanoyl-homoserine lactone (C8-HSL), which is detected by the histidine kinase AinR, while LuxS synthesizes boron-containing autoinducer 2 (AI-2), which binds to the periplasmic protein LuxP [80]. AHL systems are used by many other Gram-negative bacteria, including by pathogens to control virulence determinants, while AI-2 has been named a “universal” QS system due to its occurrence in Gram-negative and Gram-positive bacteria [81]. However, AI-2 is the byproduct of a metabolic reaction in the activated methyl cycle and given the absence of a known import or signal transduction system for AI-2 in Gram-positive bacteria, it remains unclear whether it has a QS rather than merely metabolic function in those organisms [12].

The pathogenic species *V. cholerae* is best known for causing the severe diarrheal disease, cholera. *V. cholerae* produces two critical virulence factors, cholera enterotoxin (CT) and intestinal colonization factor (toxin-coregulated pilus, TCP), which are regulated by the ToxR/S TCS [82]. QS additionally regulates virulence and virulence determinant expression in *V. cholerae* via the LuxO-P cascade, sRNAs called Qrr1-4, and a DNA-binding repressor of virulence gene expression called HapR, a homolog of LuxR [83,84].

3.2 Quorum sensing in *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous Gram-negative bacterium that naturally occurs in soil and water and an opportunistic pathogen that primarily infects immunocompromised individuals [85].

The LasI/R QS system, a homolog of the *V. fischeri* LuxI/R system is the first QS system of *P. aeruginosa* that has been described [4]. However, *P. aeruginosa* has later been found to express several other QS systems, including RhlI/R [86], the PqsR-controlled quinolone system [87], and the IQS system [88], which are arranged in a regulatory cascade with the LasI/R system on top. The *lasI* gene encodes the enzyme producing N-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C_{12}-HSL) [89], which binds and activates the cognate response regulator LasR [90,91], resulting in the regulation of target gene expression [91-93]. The LasR–AHL complex then stimulates expression of *rhlR* and *rhlI* [94,95], and the *pqsR* and *pqsABCDH* genes of the quinolone system [96]. RhlI synthesizes N-(butanoyl)-homoserine lactone (C_4-HSL), which interacts with the cognate RhlR [97]. In addition, RhlR inhibits the expression of *pqsR* and *pqsABCDH*, and this loop is suggested to ensure the correct ratio of 3-oxo-C_{12}-HSL to C_4-HSL, which, in turn, dictates the activation of quinolone (2-heptyl-3-hydroxy-4-quinolone, known as PQS) [98]. Lastly, the IQS system with the signal molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde is induced under phosphate starvation. The regulation of that system is not well understood [99].

The role of QS in *P. aeruginosa* virulence has been investigated using a number of animal models of indwelling device-associated and wound infection [100,101]. Most work has been performed on the LasI/R and RhlI/R systems, which control factors and processes that are fundamental to the pathogenicity of *P. aeruginosa* [102], including elastase, alkaline protease, exotoxin A, rhamnolipds, and pyocyanin, and biofilm formation [103-105]. *P. aeruginosa* is also known as a predominant factor exacerbating lung disease in cystic fibrosis (CF) patients [106]; and while animal models for CF are difficult [107], active levels of HSLs have been detected in sputum from CF patients, suggesting that QS systems are active and may control virulence factor expression in the lungs of patients with CF [108].

3.3 Quorum sensing in *Acinetobacter baumannii*

A. baumannii is a major cause of hospital-acquired infections associated with multi-drug resistance and high mortality rates [109]. The *A. baumanii* AbaI/R QS system is a homolog of the *V. fischeri* LuxI/R system and uses the signal N-(3-hydroxydodecanoil)-L-homoserine lactone (3-OH-C12-HSL). It regulates biofilm formation [110], surface motility [111], and production of the reactive oxygen species resistance enzymes, superoxide dismutase (SOD) and catalase [112]. In a *Galleria* infection model, an *abaI* mutant caused less mortality than the wild-type strain, indicating a role of the AbaI/R system in virulence [113].

3.4 Quorum sensing in *Enterobacter* spp.

Enterobacter is a Gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacterium belonging to the family *Enterobacteriaceae*. It can be found in the soil, water, food and the gastrointestinal tracts of animals and humans. The *Enterobacter* genus comprises 22 species; however, *E. aerogenes* and *E. cloacae* are the two most prominent pathogens in the genus, with serious implications in respiratory and urinary tract infections [114]. *Enterobacter* is one of the ESKAPE pathogens, deemed serious threats to public health due to AMR. In contrast to AMR, virulence and QS mechanisms of *Enterobacter* species remain largely unclear. It has only been shown that some *Enterobacter* species from humans can produce long-chain AHLs [115].

4. Targeting quorum-sensing by anti-virulence strategies

The co-occurrence of increasing rates of resistance to current antibiotics and the slowing in the development of novel antibiotics remain major problems for the treatment of infectious diseases. Anti-virulence drugs have been in the focus of the search for alternatives to antibiotics [2]. Frequently alleged advantages of anti-virulence drugs are related to the fact that they specifically target the expression of virulence determinants without affecting bacterial growth. These advantages comprise first and foremost a lack of impact on the existing natural microbiota and a reduction of resistance development. While antibiotics can virtually eradicate the microbiota or lead to severe dysbiosis, anti-virulence drugs are believed not to affect non-pathogenic bacteria, maintaining the ability of the microbiota to exert natural "colonization resistance" toward the pathogen. This is of particular advantage for patients at high risk to develop infections with, for example, *C. difficile* that occur due to intestinal dysbiosis as a result of antibiotic use [116]. Additionally, it is often argued that the development of resistance to anti-virulence drugs is decreased because the bacteria do not face a direct selective pressure as they do with antibiotics [117]. Many pathogens express a series of virulence factors that are often collectively controlled by global regulators, in particular by QS systems. As targeting such regulators offers the advantage of suppressing a series of virulence factors at a time, QS systems have been in the focus of anti-virulence research. Potential targets in a QS circuit, all of which are essential for QS activity, comprise QS signaling molecules, their biosynthesis, and QS signal transduction via receptor/signal transduction systems. Suppressing QS activity by such means is commonly called "quorum-quenching" (QQ). Many drugs with alleged QQ effects have been described in the literature, and for some animal infection studies have been used to analyze in-vivo efficacy. Here, we attempt to discuss principles rather than give an exhaustive list of the many reported drugs and related studies and focus on studies that include in-vivo analysis where available.

4.1 Targeting QS signaling molecules

One way by which the QS circuit can be interrupted is by enzymatic destruction of the QS signal with so-called QQ enzymes. QQ enzymes have been extensively studied in Gram-negative bacteria, focusing on AHL-degrading enzymes, of which there are two main groups, AHL-lactonases and AHL-acylases (Fig. 2).

AHL-lactonase, a member of the metallo-β-lactamase superfamily, was first described in *Bacillus* sp. isolate 240B1 [118]. It cleaves the homoserine lactone ring present in AHLs in a reversible manner by hydrolysis, which renders the QS molecule incapable of binding to the target transcriptional regulator and attenuates the effectiveness of the signal molecule. Genes encoding the AHL-lactone-degrading enzyme are widespread in many bacteria, including *Bacillus* spp., *Agrobacterium tumefaciens*, *Rhodococcus* spp., *Streptomyces* spp., and *Arthrobacter* spp. [119]. Several studies have shown that AHL lactonases can degrade AHL signals in a series of pathogenic Gram-negative bacteria, including *P. aeruginosa* and *A. baumannii*, impacting virulence and virulence-associated phenotypes [120-123],

The AHL-acylase, a member of the Ntn-hydrolase superfamily that was first described in the *Variovorax paradoxus* strain VAI-C, irreversibly hydrolyzes the amide linkage between the acyl chain and homoserine moiety of AHL molecules. This process releases homoserine

lactone and the corresponding fatty acid, which do not exhibit any residual signaling activity. Expression of AiiD, an AHL-acylase from *Ralstonia* strain XJ12B, in *P. aeruginosa* led to decrease in its ability to swarm and produce elastase and toxin [124]. The AHL-acylase AhlM from *Streptomyces* sp. strain M664 decreases the production of virulence factors, including elastase, total protease, and LasA in *P. aeruginosa* by reducing the accumulation of AHLs [125]. AhlM is also a penicillin acylase; and penicillin acylases and AHL acylases can often hydrolyze both β-lactams and AHLs [126]. AHL acylases have been found and characterized in many bacteria, such as *Ralstonia erythropolis* W2, *Comamonas* spp. strain D1, *Shewanella* spp. strain MIB015, and *Streptomyces* spp. [119].

Several bacteria express QQ enzymes that target their own QS signals. One example is *A. baumannii*, which encodes several AHL lactonases in addition to an AHL-degrading enzyme, AidA, with an α/β-hydrolase activity [127,128]. Specific QQ enzymes that act by hydrolysis of Gram-positive signals are not known. However, the peptide character of Gram-positive QQ signals makes them susceptible to degradation by non-specific proteases.

4.2 Targeting QS signaling molecule synthesis

Inhibition of QS signal synthesis can be achieved by drugs interfering with the respective biosynthetic enzymes. For example, ambuic acid, a fungal secondary metabolite, binds to and inhibits the enzymatic activity of AgrB homologs, as shown for *S. aureus*, *E. faecalis*, and *L. innocua*, leading to reduced AIP production [129] (Fig. 3). Sinefungin and the S-adenosyl-L-methionine (SAM) analogs, S-adenosylhomocysteine and S-adenosylcysteine, can decrease AHL synthesis by inhibiting RhlI activity in *P. aeruginosa* [130] (Fig. 2).

4.3 Targeting the QS receptor and signal transduction

Most QS inhibitors target the interaction of the signal with its receptor by an either competitive or non-competitive mode, or – in Gram-positive bacteria – inhibit the signal transduction process mediated by the TCS systems by interfering with the activity of the response regulator. From a pharmacological point of view, the membrane location of the receptor in Gram-positive bacteria offers the advantage of being able to use more hydrophilic drugs that would not be able to penetrate through the membrane to reach the cytoplasmic signal receptors in Gram-negative bacteria.

The first QQ molecules to be described were furanone-based inhibitors of AHL QS, namely halogenated furanones from the marine algae, *Delisea pulchra* [131], and synthetic compounds with the same activity were investigated extensively for their QQ activity ever since [132]. Originally believed to work in a competitive fashion, it was later found that these compounds increase turnover by LuxR [133]. Halogenated furanones were shown to inhibit virulence factor expression, increase biofilm susceptibility to antibiotics, and increase clearance of *P. aeruginosa* in an acute model of pulmonary infection [134] and in a device-associated model when given together with an antibiotic [135]. While furanones have been proposed to inhibit a series of QS activities in a broad array of pathogens [132], in-vivo evidence underscoring their usefulness to control bacterial infection is often lacking. Many other compounds, including flavonoid derivatives [136]and N-decanoyl-L-homoserine benzyl ester [137], a further structural analog of *P. aeruginosa* AHL, have also been shown

to inhibit *P. aeruginosa* QS and reduce virulence gene expression. Moreover, AHL analogs have been developed that effectively inhibited QS in other Gram-negative bacteria such as *V. fischeri* and *Agrobacterium tumefaciens* [138]. However, similar to a plethora of additional compounds reported to inhibit QS in *P. aeruginosa* and other Gram-negative bacteria in vitro, these have not been investigated for efficacy in animal infection models. Finally, there are many more studies on QQ compounds and their in-vitro and in-vivo efficacy to treat infections by *P. aeruginosa* and other Gram-negative bacteria; but many of these studies failed to provide clear evidence of an exclusively QQ rather than general antibacterial effect.

As staphylococci and several other clinically important Gram-positive bacteria have the Agr QS system, Agr inhibitors have been in the focus of QQ drug development for Gram-positive bacteria [139]. Almost all investigated Agr-inhibiting drugs target the AgrC receptor or the AgrA response regulator, most of which work as competitive inhibitors of AIP binding to AgrC (Fig. 3). The latter include derivatives of AIP, a strategy based in part on the finding that naturally occurring AIPs from different staphylococcal species or Agr subgroups within a species cross-inhibit [140,141]. For example, an early study showed that a specific AIP derivative globally inhibits all Agr specificity groups in *S. aureus* [10]. More recent research followed up on those earlier findings by using cross-inhibiting AIPs to decrease *S. aureus* pathogenicity during skin infection [142]. However, these compounds contain a thiolactone, a relatively unstable structure, which severely impacts systemic in-vivo applicability. Other cyclodepsipeptides as analogs of AIPs have been tested as well, including some isolated from actinomycetes [143] or ngercheumicins and solonamide B produced by photobacteria [144,145]. These have not been tested in infection models. Lastly, fengycin lipopeptides from *Bacillus* species have recently been shown to inhibit *S. aureus* Agr via competitive interaction at AgrC. Application of *Bacillus* spores, which germinate in the gastro-intestinal tract, were demonstrated in that study to eliminate *S. aureus* intestinal colonization in a QQ probiotic fashion [27].

Another class of Agr inhibitors target AgrA. The best studied is savirin, whose in-vivo efficacy was investigated in a series of animal infection models with the community-associated high Agr-activity MRSA strain LAC. In an air pouch model, it led to a 1 – 2 log reduction of bacterial burden in the kidneys. In a dermonecrosis model, wound formation was almost completely abolished when savirin was injected at the time of infection. This reflects results with a more recently reported AgrA inhibitor, apicidin, which was also shown to reduce dermonecrosis when co-injected with LAC [146]. However, when savarin was injected 1 or 2 days after infection, better mimicking a scenario of clinical relevance, abscess formation was only barely reduced, but there was a significant yet minor decrease in CFUs in the abscess and spleen [147]. Furthermore, several biaryl hydroxyketone compounds identified in a screen for AgrA interaction were reported to inhibit Agr QS activity and increase healing of LAC-infected wounds [148]. One compound, named F19, has also been shown more recently to reduce mortality in a systemic infection model [149].

QS signaling transduction has also been targeted in streptococci. Inhibition of the CSP receptors ComD1 and ComD2 by dominant negative competence-stimulating peptide (dnCSP) attenuated acute *S. pneumoniae* lung infection and increased survival rate of mice

[150]. In *S. mutans*, interference with the ComA receptor by synthetic 1,3-di-m-tolylurea (DMTU) led to decreased dental biofilm plaque formation in a rat model of caries [151].

5. Expert opinion

QS blockers have been widely promoted as promising alternatives to antibiotics, and we presented in this review principles and specific examples that have been described as potential QQ therapeutic strategies. However, after there has been strong initial enthusiasm in the field, a more in-depth look at what has been accomplished leaves us with the realization that there are manifold challenges; and not much evidence has been provided yet in thorough in-vivo and pharmacological studies to substantiate therapeutic promise of such drugs. The related issues will be discussed in the following.

First, especially for QQ of infections caused by Gram-negative bacteria, there is a paucity of in-vivo animal work to underscore the claims originating from the plethora of in-vitro examinations on QQ compounds. This is particularly noteworthy for the treatment of *P. aeruginosa*-associated CF infection, the initial focus of QS research, for which animal evidence of QS function and promise of QQ drugs is largely missing. The situation is somewhat better for non-CF *P. aeruginosa* infections and in the Gram-positive infection field, where several animal models have been performed, for example analyzing *S. aureus* infection. However, even in this case the infection models that have used been mostly comprise models of infection types for which treatment is not complicated, such as skin infections, while for the more serious infection types that would require alternative drugs, such as staphylococcal sepsis or lung infection, there is also almost no research on QQ intervention.

Biofilm-associated infections deserve particular reference regarding the role of QS and the applicability of QQ strategies. There is still frequent reference to QS positively controlling biofilm formation and QQ being a general strategy to interfere with biofilm infections, which is based mostly on an early study reporting increased depth of *P. aeruginosa lasI*-deficient in-vitro biofilms [152]. However, there is now strong and increasing evidence that these early findings cannot be generalized. For example, in staphylococci, the most frequent cause of biofilm infections on indwelling medical devices, QS-deficient biofilms grow thicker and more compact, as shown both in vitro and in vivo, and inhibiting QS leads to increased biofilm formation [36,153,154]. The common theme that appears to underlie QS control of biofilm formation is that QS systems often control biofilm structuring factors, whose absence can have different effects on overall biofilm formation [155]. Furthermore, QS cheaters are known to arise in biofilm-associated infections caused by *P. aeruginosa* and staphylococci [154,156,157], whose in-vivo advantage for survival has recently been explained in *S. aureus* infection by increased resistance of the resulting enhanced biofilms to innate host defenses [158]. Altogether, QQ for the control of biofilm-associated infection cannot generally be regarded as a promising strategy and has to be investigated thoroughly in every infection type, as it may be counterproductive.

Further frequent problems in QQ research include a lack of ascertaining QS-blocking specificity and missing exclusion of mere growth versus solely virulence effects. If growth

effects are not rigorously ruled out, the effects of alleged QQ drugs, particularly in vivo, may not represent more than common antibacterial effects, negating the specific claimed advantages of a QQ approach. Another frequently encountered problem in the experimental in-vivo approach consists in the fact that many researchers pre-incubate the QQ drug with the inoculum or inject the drug together with the inoculum. Such experiments are not genuine in-vivo investigations, because the inhibiting interaction may have occurred pre-injection. This procedure is particularly problematic if there is, as is often the case, only a narrow concentration window between only QQ and bactericidal effects, and appropriate concentrations below the MIC are calculated for the final concentration in the animal, but not the inoculum. Moreover, this sort of drug application hardly adequately represents a clinical scenario. Interestingly, when a thorough analysis by direct comparison was made, such as in the *S. aureus* savirin study, the more clinically representative delayed application of the drug resulted in dramatically decreased efficacy [147].

As discussed above, one alleged advantage of QQ drugs is a stated lack of resistance development. However, this hypothetical advantage is based on in-vitro findings; and it remains debatable if in an in-vitro situation, where the absence of virulence determinants is assumed to lead to increased elimination by host defenses, it would still be present – because it still would ultimately lead to a survival disadvantage. Furthermore, resistance development to the widely promoted QQ drug, brominated furanone C-30, has already been detected [159].

In conclusion, QQ is still seen as a promising alternative or additional therapeutic option, likely to be used in co-application with an antibiotic, especially for infections that are caused by antibiotic-resistant pathogens and those that have intrinsic antibiotic resistance, such as biofilm-associated infections. However, we have now learned that not all biofilm-associated infections react to QQ interference in a therapeutically beneficial way. On the other hand, the promise of QQ interference to treat serious or acute infections has not yet been sufficiently substantiated by animal infection models of systemic infection with appropriate technical setups and clear-cut demonstration of the QQ effect underlying therapeutic efficacy. While for biofilm infections, we will not be able to overcome the limitations that are set by the specific biological system, and for which QQ strategies may thus be limited to a restricted number of pathogens, we should optimize our efforts to find working QQ drugs for serious and life-threatening infections by antibiotic-resistant, dangerous pathogens such as *S. aureus*.

Financial and competing interests disclosure:

The authors report no conflicts of interest. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH) under project number 1 ZIA AI001080.

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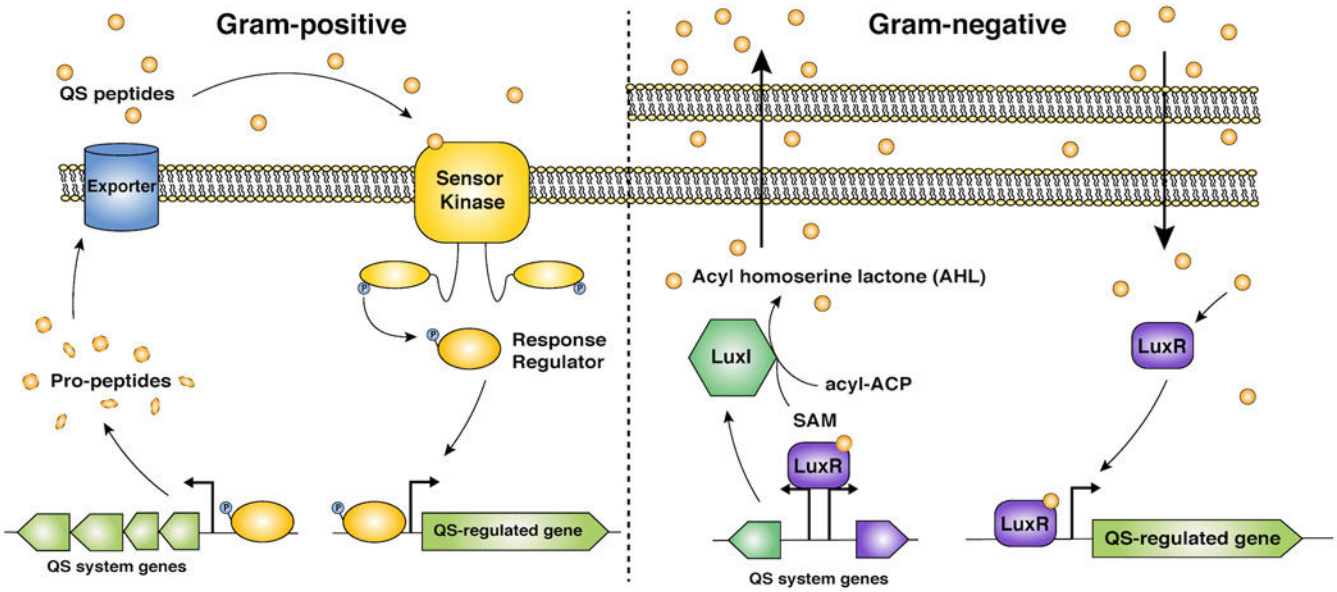

Fig. 1. Principles of QS in Gram-positive and Gram-negative bacteria.

In Gram-positive bacteria, usually QS genes encode pre-peptides that are exported and sometimes post-translationally modified. Upon reaching a cell density-determined threshold, they activate a membrane-located receptor (histidine kinase), which via a phosphorylation cascade activates a cytoplasmic DNA-binding protein (response regulator). The response regulator activates target genes directly or indirectly via further regulatory components such as regulatory RNAs and/or proteins, in addition to positively regulating the QS system genes, resulting in an autofeedback loop.

In Gram-negative bacteria, QS systems usually have membrane-diffusible signals that upon reaching a threshold directly activate DNA-binding proteins. Most Gram-negative bacteria use homologs of the LuxI/LuxR system and AHLs, but there are a variety of other systems.

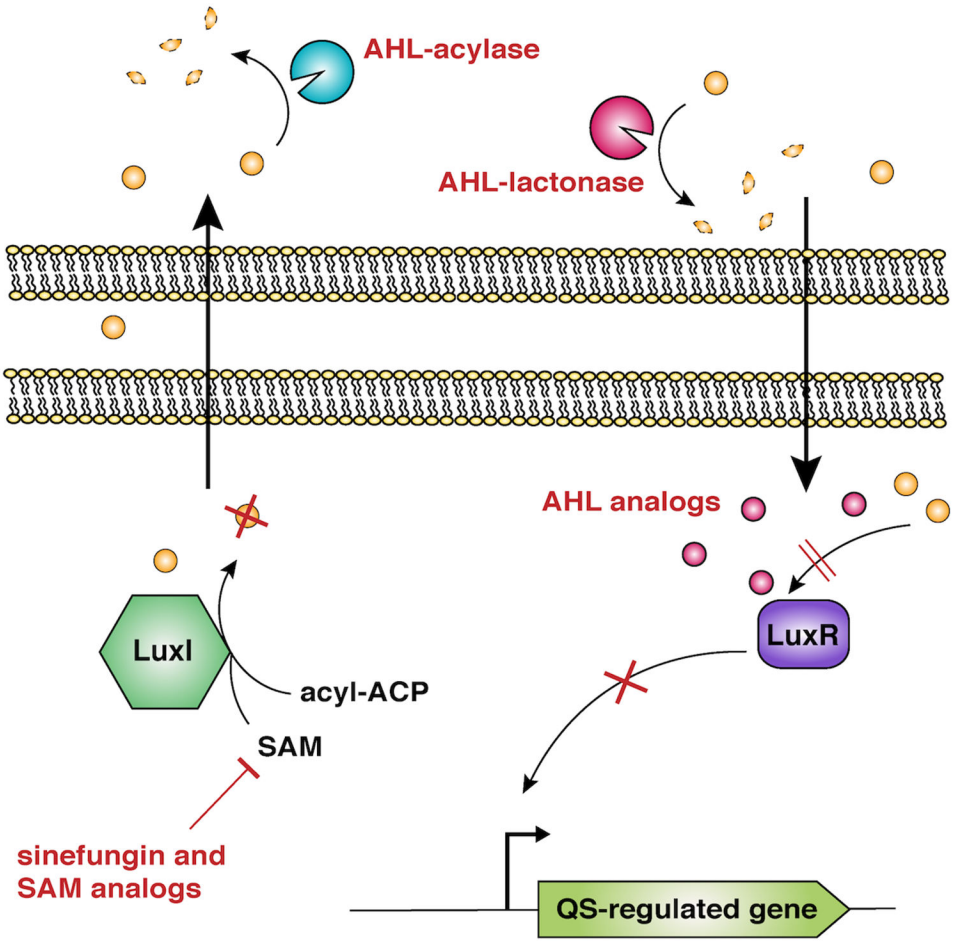

Fig. 2. QQ approaches in Gram-negative bacteria.

Many QQ approaches in Gram-negative bacteria are based on three mechanisms targeting LuxR/I homologs, (i) AHL-degrading enzymes (AHL acylase, AHL lactonase), (ii) AHL analogs that inhibit AHL binding to and activation of the DNA-binding LuxR homolog, or (iii) inhibitors of AHL biosynthesis.

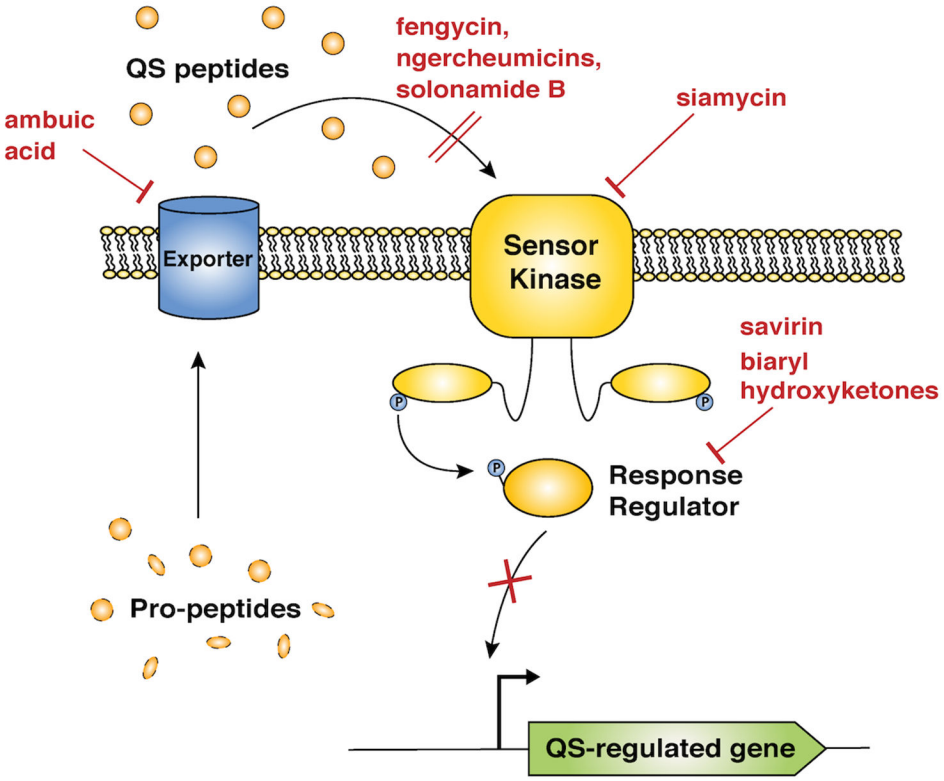

Fig. 3. QQ approaches in Gram-positive bacteria.

QQ approaches in Gram-positive bacteria comprise first and foremost (i) AIP analogs that inhibit AIP binding to the histidine kinase receptor, (ii) compounds that inhibit the DNA-binding response regulator, or (iii) biosynthesis of the AIP. Note several targets of Gram-positive QS systems can be reached by drugs from the extracellular space, allowing greater drug hydrophilicity.