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Bacterial signaling as an antimicrobial target

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Abstract

Antibiotics profoundly reduced worldwide mortality. However, the emergence of resistance to the growth inhibiting effects of these drugs occurred. New approaches to treat infectious disease that reduce the likelihood for resistance are needed. In bacterial pathogens, complex signaling networks regulate virulence. Anti-virulence therapies aim to disrupt these networks to attenuate virulence without affecting growth. Quorum-sensing, a cell-to-cell communication system, represents an attractive anti-virulence target because it often activates virulence. The challenge is to identify druggable targets that inhibit virulence, while also minimizing the likelihood of mutations promoting resistance. Moreover, given the ubiquity of quorum-sensing systems in commensals, any potential effects of anti-virulence therapies on microbiome function should also be considered. Here we highlight the efficacy and drawbacks of anti-virulence approaches.

Introduction

Over the past century, antibiotics have served as the mainstay for treating bacterial infections. Antibiotics are small molecules that inhibit bacterial growth and, therefore, exert strong selective pressures on bacterial populations, which favor the emergence of antibiotic resistant (AR) clones. Indeed, AR isolates continue to emerge with each new class of antibiotics [1]. Approximately 2.8 million infections by AR pathogens occur annually in the United States, resulting in at least 35 000 deaths [2]. These alarming trends highlight the pressing need for alternative strategies to treat bacterial infections that also reduce the likelihood of resistance.

Anti-virulence therapies represent an alternative approach for treating bacterial infections. In contrast to antibiotics, anti-virulence agents do not directly inhibit bacterial growth [3], which is hypothesized to reduce the selective pressures exerted on pathogen populations. Instead, anti-virulence agents are designed to disrupt intercellular signaling networks essential for host colonization and induction of disease [4]. The challenge is to identify targets within these complex networks that inhibit virulence, while also minimizing the

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emergence of mutations or compensatory functions that enable pathogen circumvention of the anti-virulence therapy. Secondly, given the ubiquity of cross-species signaling within bacterial communities, another challenge is to inhibit pathogen virulence while avoiding detrimental effects to microbiome function. Herein, we review the implementation of anti-virulence strategies that target quorum sensing in pre-clinical models of infection, with a particular focus on *Staphylococcus aureus* and *Escherichia coli*, frequent members of human microbiomes that can also become dangerous opportunistic AR pathogens. The implementation of such strategies with *Pseudomonas aeruginosa*, another clinically important AR opportunistic pathogen, has been recently reviewed elsewhere [5–8].

Bacterial intercellular signaling through quorum sensing (QS)

Bacteria employ numerous strategies to sense their local environment and integrate this spatial information to regulate their growth, physiology, and behaviors. QS is a form of cell-to-cell communication that enables bacterial populations to coordinate their behaviors in an environmental and cell density-dependent manner [4,9]. The primary components of QS systems include: (1) biosynthetic machinery that generates the signal (autoinducer); and (2) cognate sensory machinery that recognizes and responds to the signal (Figure 1). QS systems represent attractive anti-virulence targets because they often activate virulence-associated functions essential for establishing infection [4]. Moreover, QS can mediate the switch from commensal to opportunistic pathogen and serve as bacterial sensors for host signals that enable appropriate deployment of pathogen virulence programs [10,11]. Thus, much effort has been dedicated toward discovering small molecules that inhibit QS systems [3,5,12,13] (Figure 1).

Targeting QS systems in *S. aureus*

S. aureus is a skin and/or nasal commensal in approximately 30% of the human population that, with inappropriate access to deeper tissues, can become an opportunistic pathogen responsible for potentially lethal infections [14–16,17•]. In *S. aureus*, the transition from commensal to pathogen is mediated by the accessory gene regulation (*agr*) locus, which encodes a QS system that activates numerous virulence factors and represses cell surface proteins that contribute to asymptomatic colonization of the nasal cavity and skin. Noticeably, the Agr system cross talks with other regulatory systems, such as SarA/SarR. SarA activates expression of the *agr* locus, and transcription of *sarA* is induced by SarA itself, while it is repressed by SarR [18,19] (Figure 2). The importance of *agr* in *S. aureus* infections is well established in various rodent models [20–23]. The *agr* locus encodes the machinery that synthesizes and secretes the QS signal autoinducing peptide (AIP), its receptor AgrC, its cognate response regulator (RR) AgrA, the RNAIII regulatory RNA and δ-toxin. AIP is encoded by the *agrD* gene. After translation, the AgrD propeptide is processed into AIP by AgrB [19,24] (Figure 2). AgrA and RNAIII together regulate about 200 genes in the *S. aureus* genome, including virulence-associated functions such as toxins, phenol soluble modulins and proteases [19,25]. Thus, the *agr* locus represents an attractive therapeutic target for treating *S. aureus* infections and preventing loss of commensalism in *S. aureus* carriers.

Staphylococci exhibit subtle strain-level differences in the chemical structures of their AIPs [26]. *S. aureus* secretes one of four AIP types that activate their cognate AgrC receptor and inhibit other AgrC alleles [23]. AIPs range from seven to nine aminoacids, and their types stems from variations in the *agrD* gene. Structural differences in AIP, combined with differences in the N-terminal region of AgrC, which binds this ligand, dictate the outcomes of activation or deactivation of this signaling cascade [19]. Identifying or synthesizing competing analogs to AIPs that inhibit AgrC has been an anti-virulence strategy to treat *S. aureus* infections [23]. Interspecies QS crosstalk also occurs between *S. aureus* and staphylococcal skin commensals. For example, *Staphylococcus epidermidis's* AIP is able to inhibit the Agr system of most *S. aureus*, except for one subgroup (subgroup 4), which in turns inhibits the Agr response of *S. epidermidis.* The AIP produced by *Staphylococcus caprae* was able to inhibit the Agr system of all classes of *S. aureus* [27,28,29•,30,31••] (Figure 2). Thus, candidate anti-virulence agents should ideally inhibit all four AIP types in *S. aureus*, while also avoiding harmful effects to the skin microbiome. Another strategy to inhibit *agr* activation is to target AgrA, which is more conserved among *S. aureus* strains. For example, savirin is a QS inhibitor that binds residues uniquely present in the DNA-binding domain of *S. aureus* AgrA and thus exhibits minimal effects on *agr* activation in the skin commensal *S. epidermidis*. Through inhibition of AgrA, savirin also inhibits transcription of RNAIII, which is activated by AgrA [25]. In acute models of skin infection, savirin treatment attenuates pathology as well as local and systemic burdens of *S. aureus* [25]. Importantly, in contrast to repeated antibiotic treatment, continuous *in vivo* passaging of *S. aureus* together with savarin coadministration did not produce savarin-resistant isolates [25]. Another strategy developed to inhibit AgrA and RNAIII was the development of peptide conjugated nucleic acids (PLNAs) that are modified antisense RNAs conjugated to a cell-penetrating peptide [32]. Interestingly, during acute lung infection, nitric oxide (NO) produced by the innate immune system also targets AgrA through a similar mechanism. NO inactivates AgrA through the *S*-nitrosylation of residues that also interfere with DNA binding and *agr* activation, thus resulting in attenuated disease. However, one has to be cautious of this approach given that NO production may engender changes in multiple protein targets and have off target pleiotropic effects [71••]. These findings, together with other studies [32–34], highlight the potential of targeting QS systems as an effective strategy for treating acute infections caused by *S. aureus*.

Commensal microorganisms also produce signaling molecules that interfere with pathogen QS systems to compete for limited nutrients and space. For example, some staphylococcal skin commensals produce AIPs that inhibit the AgrC receptor in *S. aureus* [27,28,29•, 30,31••,56,57] (Figure 2). Similarly, nasal commensals such as *Corynebacterium* spp. inhibit virulence-associated functions in *S. aureus* by blocking QS activation through an undefined mechanism [58,59•]. This antagonistic relationship can be potentially exploited as another antivirulence strategy to treat infections. Indeed, simultaneous administration of live commensal staphylococci or *Corynebacterium* attenuates *S. aureus agr* activation, abscess colonization and skin lesion formation [29•,31••,58]. Similarly, therapeutic administration of purified AIP from *S. caprae* reduces pathogen burdens and accelerates resolution of skin pathology [29•,31••]. Together, these studies demonstrate the potential for developing probiotic treatments that augment the inhibitory effects of commensals on pathogen QS

systems. More broadly, studying commensal-pathogen interactions within naturally occurring microbial communities could enable further discovery of antagonistic relationships that can be repurposed for treating infection and maintaining microbiome homeostasis.

Targeting QS systems in Enterobacteriaceae

E. coli encompasses genetically and functionally diverse species that range from benign commensal to professional pathogen. Commensal *E. coli* are usually present in the gut microbiota [14]. Like *S. aureus*, endogenous *E. coli* can become opportunistic pathogens with inappropriate access to extra-intestinal niches and cause diseases such as meningitis, sepsis and urinary tract infections [35–37]. The outgrowth of commensal *E. coli* within the gut has also been linked to chronic, immune-mediated diseases such as inflammatory bowel disease (IBD) [38]. In contrast, pathogenic *E. coli* reside in environmental reservoirs such as ruminants and cause human diarrheal diseases upon ingestion of contaminated food and water sources [39,92].

The QseC signaling cascade is a highly conserved QS response system in Gamma-Proteobacteria that consists of the histidine sensor kinase (HK) QseC and its cognate RR QseB [40] (Figure 3). QseC senses the bacterial signal autoinducer-3 (AI-3), which encompass a family of pirazynone molecules that are derived from products from threonine dehydrogenase (Tdh) combined with abortive tRNA synthase reactions. The AI-3 family is synthesized by a variety of bacterial species. Importantly the most active molecule in this AI-3 family is a new pyrazinonetype of metabolite, with very potent activity [94]. QseC and a second HK QseE also sense the host neurotransmitters epinephrine and norepinephrine [41,42], thus enabling *E. coli* to coordinate population-level behaviors in direct response to the host. QseC activates QseB, thus stimulating flagella biosynthesis [40]. The kinase activity of QseC is promiscuous and also activates the non-cognate RRs, KdpE and QseF, which substantially expands the QseC regulon [43] (Figure 3).

Genetic studies have demonstrated that QseC is an important activator of virulence in pathogenic *E. coli* and in the closely related murine pathogen *Citrobacter rodentium* [44–46,90]. In enterohemorrhagic and enteropathogenic *E. coli*, QseC regulates the locus of enterocyte effacement (LEE), a pathogenicity island that is essential for causing disease [44,45,90]. QseC activates the LEE through KdpE and QseF-regulated sRNAs [43,47] (Figure 3). Despite employing divergent mechanisms for causing disease, the QseC regulon also modulates virulence-associated functions in other *E. coli* pathovars such as UPEC and in pathogens such as *Francisella tularensis* and *Salmonella enterica* [48–52,90,91]. Indeed, the integration of QseC into the intracellular signaling cascades that modulate virulence is wired differently between bacterial strains [49,53–55,91]. Importantly, the QseC signaling cascade is exploited by many Gram-negative bacterial pathogens to promote virulence. QseC activates expression of a multi and varied array of virulence genes in these pathogens, such as expression of type three secretion systems (T3SS), LPS modification enzymes that promote resistance to stress and antimicrobial peptides, flagella and motility genes, promotion of biofilm formation, expression of multiple iron uptake systems, pili/fimbriae and adhesins, and toxins [49,51,53–55,91].

Consequently, pharmacological inhibition of QseC with LED209 is effective in attenuating pathogen virulence and disease in diverse models of acute infection [46,49,51,90], thus demonstrating the potential efficacy of QseC as an anti-virulence target in numerous pathogens. LED209 was identified through a screen of a small molecule chemical library. Extensive structure activity relationship (SAR) studies revealed that LED209 is a potent pro-drug that is highly selective for QseC. Its warhead allosterically modifies lysines that are only present in QseC, impairing its function, and preventing the activation of the virulence program of several Gram-negative pathogens both *in vitro* and during murine infection. LED209 does not interfere with pathogen growth, possibly leading to a milder evolutionary pressure toward drug resistance. LED209 has desirable pharmokinetics and does not present toxicity *in vitro* and in rodents [51,90].

However, given that QseC homologues are also expressed by gut commensals, including endogenous *E. coli*, studies investigating the effects of QseC inhibition on the human microbiome and on commensal-to-pathogen transitions are important next steps to assess the safety and specificity of this anti-virulence target in pre-clinical models of infection.

Targeting QS systems in chronic infection and disease

While anti-virulence strategies can be effective in models of acute infection, the implementation of such therapies to treat chronic infections may be challenging because of selective pressures continuously exerted by the host [60,61]. Whole genome sequencing has revealed that isolates recovered from chronic disease patients accumulate mutations that fundamentally alter the function of the pathogen [7,16,62–64]. Moreover, in chronic infections that are likely caused by commensal-turned-opportunists, infection-associated isolates harbor mutations that functionally distinguishes the pathogen from its putative commensal ancestor [16,17•,63].

Chronic infections—Chronic *S. aureus* infections are associated with loss-of-function *agr* mutations in a subset of patients, which correlates with poorer prognosis and increased mortality [16,62,63,65,66]. The AgrC receptor in chronic infection isolates frequently acquire mutations that increase the AgrC activation threshold or completely inactivates the receptor [17•,62,67••,68•]. These *agr* dysfunctional isolates generally exhibit decreased virulence potential *in vitro* and in animal infection models [17•,62,67••,68•]. However, one study reported that an *agr* dysfunctional bloodstream isolate retained its virulence in an intravenous infection model, which corresponded with the upregulation of agr-regulated virulence factors [17•]. In contrast, *agr* inactivation in a closely related *S. aureus* nasal strain from the same patient attenuated its virulence during bloodstream infection [17•]. Moreover, within a population of *agr* dysfunctional isolates, a subset of cells revert back to an *agr*+ phenotype through phase variation [69••]. Together, these studies suggest that chronic infection isolates can acquire mutations or functions that bypass the need for *agr*-dependent activation of its virulence programs, an outcome that could also arise with chronic administration of anti-*agr* agents.

Another common characteristic of *agr* dysfunctional mutants is the production of robust biofilms, a functional characteristic that can be recapitulated in isogenic *agr* mutants [62,

67••,70]. *Agr* dysfunctional mutants exhibit increased fitness in biofilm infection models compared to *agr*+ strains, which corresponds with the formation of impenetrable *in vivo* biofilms that protect the pathogen from leukocyte-mediated killing [67••]. Indeed, *agr* dysfunctional mutants are detected as early as one week following *S. aureus* biofilm infection, which is consistent with strong host pressures that select for *agr* dysfunction to avoid immune-mediated clearance [67••]. In contrast, *agr*+ isolates exhibit greater fitness and are more virulent during acute skin infections [67••]. Similarly, QS inactivating mutations have also been reported in *P. aeruginosa* clinical isolates associated with chronic disease [61,72–74]. However, there is a debate on whether these QS mutants in biofilms may be eventually selected out of the population. Taken together, these findings suggest that anti-virulence treatments are likely to be challenging in chronic infections because of the microevolutionary processes that result in loss-of-function mutations in pathogen QS systems. We still need to better understand the arms race between QS proficient and deficient strains within these environments. Another consideration is whether a multi-pronged approach targeting signaling cascades at several levels would work better than a specific target.

Chronic inflammatory diseases—Chronic inflammatory diseases are often associated with compositional changes to the human microbiome that contribute to disease pathogenesis — a state known as dysbiosis. Atopic dermatitis (AD), a chronic skin condition, is associated with dysbiosis of the skin microbiome, which is often characterized by the expansion of commensal *S. aureus* and a decrease in *S. epidermidis* [75,76]. The severity of AD corresponds with an elevated *S. aureus* to *S. epidermidis* ratio [77]. In a murine model of skin infection, co-administration of the skin commensal *Staphylococcus hominis*, or its purified AIP, with *S. aureus* infection attenuates *agr* activity, inflammation and lesion formation, which corresponds to decreased activation of host proteases that damage the epidermis to cause disease [31••]. Interestingly, the efficacy of *S. hominis* treatment was lost when co-administered with *S. aureus* at a 1:10 ratio, which is representative of the dysbiosis observed in AD patients [31••]. Thus, this study demonstrates the potential of utilizing commensal QS systems to treat chronic skin diseases and restore homeostasis within the skin microbiome.

Chronic gastrointestinal diseases such as IBD are also associated with a dysbiotic gut microbiota. In Crohn's disease, dysbiosis is often characterized by the expansion of commensal Proteobacteria such as *E. coli*, which includes increased mucosal colonization of a functionally distinct subset known as adherent-invasive *E. coli* (AIEC) [78,79]. AIEC strains utilize type 1 pili to colonize the intestinal epithelium, which enables aberrant stimulation of mucosal inflammatory responses mediated by AIEC expression of flagellin [80–83,93]. Chemical inhibition (using LED209) of QseC, which stimulates flagella and type 1 pili biosynthesis, attenuates colitis induction by AIEC and inhibits Enterobacteriaceae outgrowth in several experimental models of IBD [40,84•]. Thus, targeting the QseC system in AIEC strains may help alleviate overstimulation of the mucosal immune system while also restoring homeostasis within the gut microbiota of IBD patients. However, it remains unknown whether QS components such as QseC in commensal *E. coli* are vulnerable to the same selective pressures as *S. aureus* or *P. aeruginosa* during chronic disease. A recent study

reported the emergence of point mutations in *qseC* in the aminoacid residues S8R and I283L in *Klebsiella pneumoniae* that contributed to the development of polymixin resistance following repeated antibiotic exposure. However, the only mutations that inactivate QseC function, which are the direct targets of LED209 are in K256 and K427 [85,86]. Taken together, futures studies are clearly needed to assess the microevolution of QS systems in human commensals and opportunistic pathogens and to define the implications of these genetic events on bacterial function, host response and treating disease.

Concluding remarks and future considerations

Over the past decade, numerous studies have demonstrated the promising potential of anti-virulence agents in preclinical models of bacterial infections. However, the efficacy of targeting QS systems seems to be dependent on the type of bacterial infection (acute versus chronic) and the site of infection. Chronic disease can lead to the emergence of QS dysfunctional mutants and functionally heterogeneous pathogen populations that can render the administration of QS inhibitors challenging. Moreover, given the ubiquity of QS systems in commensals and the potential for crosstalk within the microbiota and between commensal and pathogen [87–89], further studies investigating the contribution of QS in modulating microbiome function and commensal-pathogen interactions are needed in order to evaluate the safety and long-term efficacy of these agents in preclinical models of human disease.

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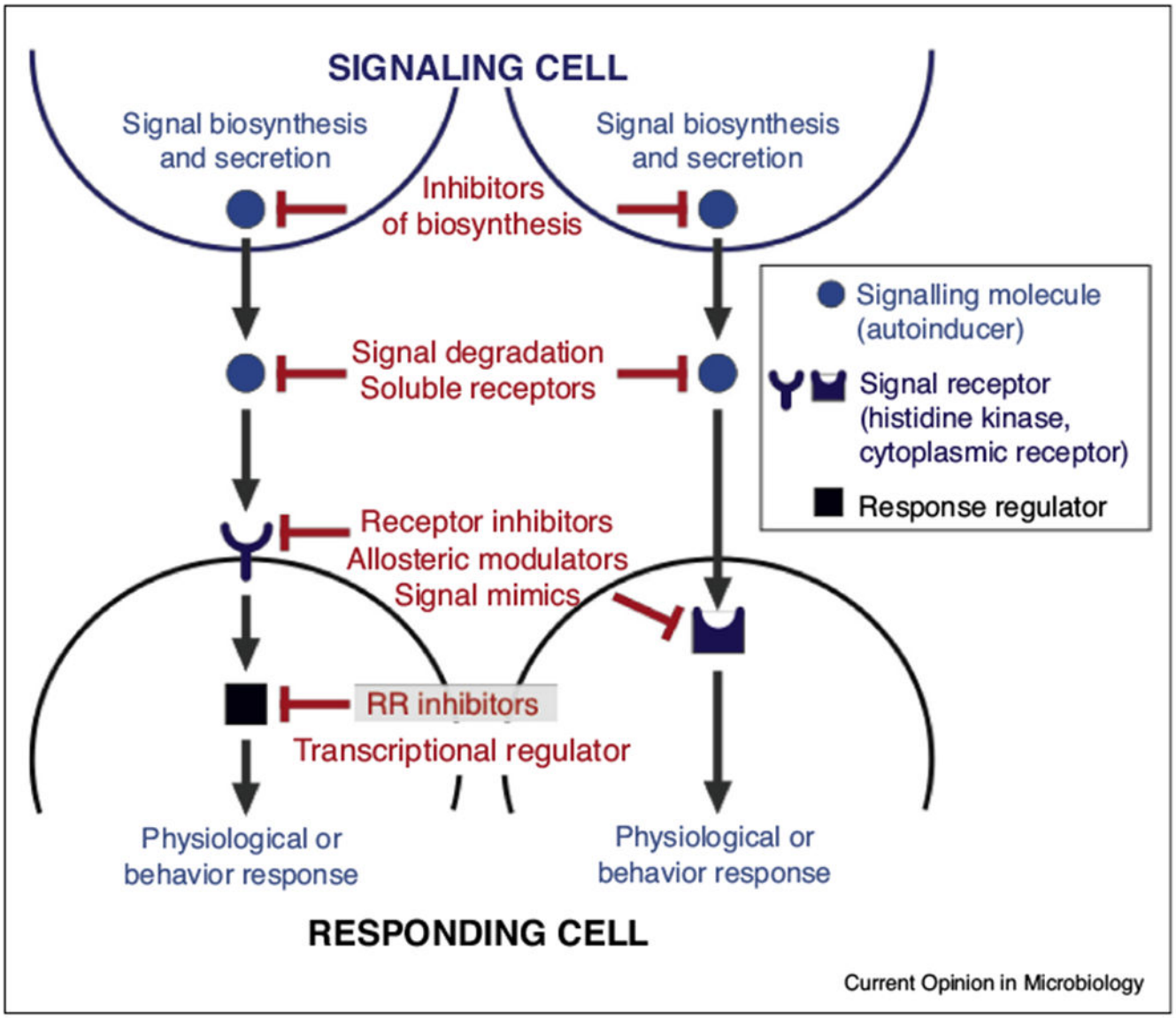

Figure 1.

Basic structure of quorum sensing systems in bacteria. Quorum sensing (QS) is a form of cell-to-cell communication that enables bacterial populations to coordinate their behaviors in an environmental and cell density-dependent manner. The primary components of QS systems include the biosynthetic machinery that generates the QS signal and its cognate sensory machinery that recognizes and responds to the signal. The latter is usually a cytoplasmic receptor or histidine sensor kinase (HK), which upon its activation phosphorylates its cognate response regulators (RR). However, other transcription factors can also be inhibited. The QS cytoplasmic receptors and RRs then modulate bacterial behaviors and functions through their functions as transcription factors.

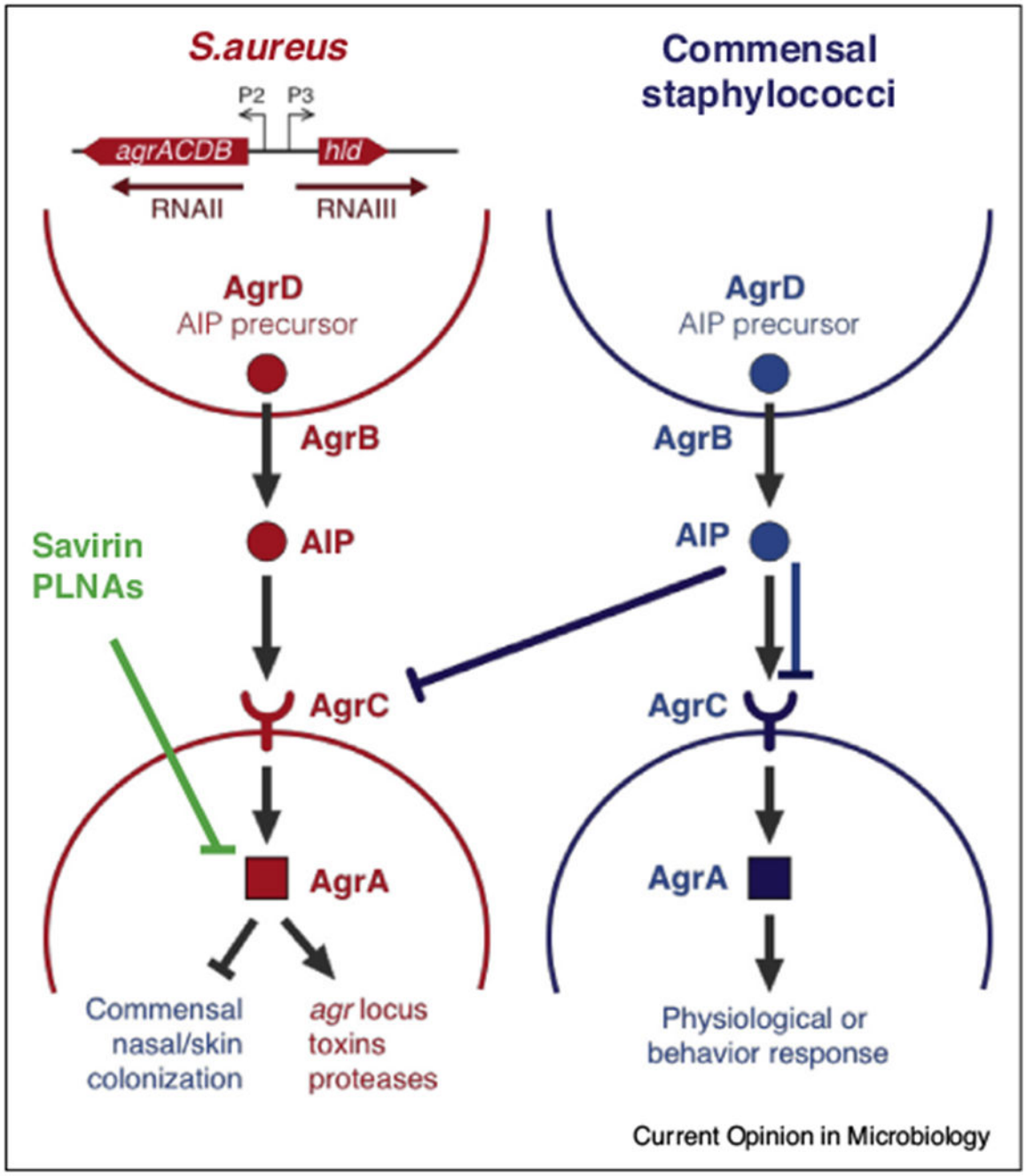

Figure 2.

The *agr* quorum sensing system in *S. aureus.* The *agr* locus consists of two divergently encoded operons, *agrACDB* and RNAIII, which are activated by the transcription factor AgrA. The *agrACDB* operon encodes AgrD, which is the precursor for the QS signal autoinducing peptide (AIP); AgrB, which is the machinery that processes and secretes AIP; and the AgrAC two-component system comprises the AIP receptor AgrC and its cognate response regulator AgrA. The RNAIII operon consists of the regulatory RNA, RNAIII, and the δ-toxin encoded by *hld.* AgrA and RNAIII together activate virulence-associated

functions such as toxin production, secretion of phenol soluble modulins (PSMs) and protease activity and inhibit the expression of cell surface proteins associated with commensalism. Coagulase negative staphylococcal commensals produce AIPs that inhibit the AgrC receptor in *S. aureus*, thus attenuating its virulence, they may also inhibit AgrCs from other commensals. Additionally Savirin and PNLAs also inhibit Agr signaling.

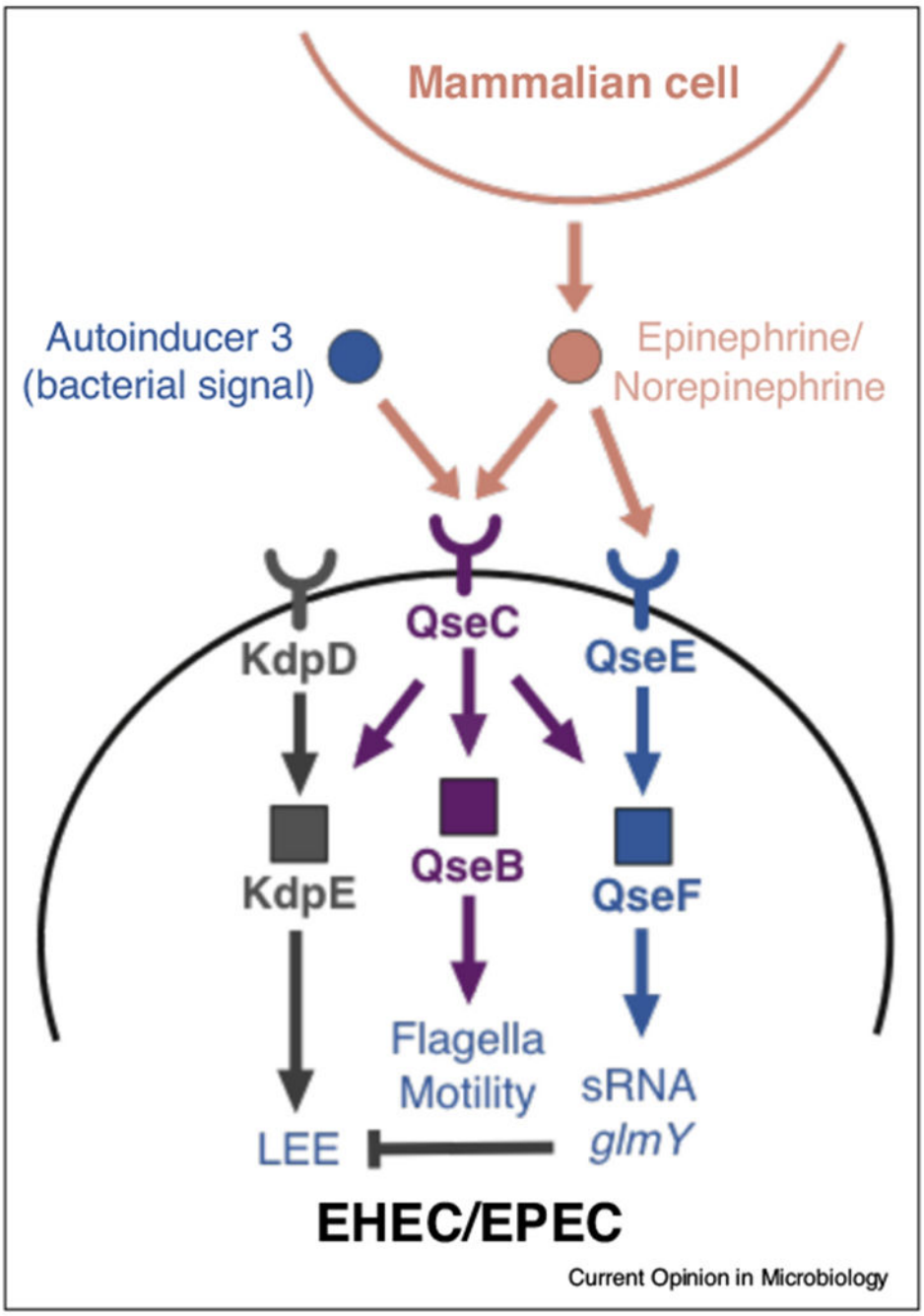

Figure 3.

The QseC quorum sensing system in pathogenic *Escherichia coli*. A major quorum sensing response system in *E. coli* consists of a two-component system comprises the histidine sensor kinase (HK) QseC and its cognate response regulator (RR) QseB. The QseC receptor is autophosphorylated upon engagement with the QS signal autoinducer-3 and then transfers the phosphate to QseB, which transcriptionally stimulates flagella biosynthesis. QseC, along with a second HK QseE, also sense the host neurotransmitters epinephrine and norepinephrine. Kinase activity in QseC is promiscuous and can activate two additional non-

cognate RRs, KdpE and QseF. In enterohemorrhagic and enteropathogenic *E. coli* (EHEC, EPEC), QseC also regulates the locus of enterocyte effacement (LEE), which is essential for causing intestinal disease. In EHEC and EPEC, QseC activates the LEE through KdpE, a positive regulator of the LEE, and inhibits the LEE through sRNAs that are modulated by QseF.