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Novel approaches to bacterial infection therapy by interfering with bacteria-to-bacteria signaling

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Summary

The growing challenge of antimicrobial resistance and the paucity of novel antibiotics underscore the importance of developing novel therapeutics. Interference with bacterial cell-to-cell signaling constitutes a novel drug target. Quorum sensing (QS) is a cell-to-cell signaling mechanism that refers to the ability of bacteria to respond to chemical hormone-like molecules called autoinducers. QS is responsible for controlling a plethora of virulence genes in several bacterial pathogens. Antagonists to autoinducers will intercept bacterial intercellular communication hindering their ability to act in a coordinate manner to express virulence traits. Moreover, because QS is not directly involved in essential processes, such as growth of the bacteria, one can reason that inhibition of QS will not yield a selective pressure for development of resistance.

Keywords

quorum sensing; epinephrine; bacterial pathogenesis; enterohemorrhagic *E. coli* (EHEC)

Quorum sensing

Quorum sensing (QS) is a bacterial cell-to-cell signaling mechanism through which bacteria assess the density of their population. Such bacteria secrete hormone-like compounds, usually referred to as autoinducers. When these autoinducers reach a threshold concentration, they interact with transcriptional regulators to drive bacterial gene expression. Quorum sensing was first described in the regulation of bioluminescence in *Vibrio* species [1,2]. The luciferase operon in *Vibrio fischeri* is regulated by two proteins, LuxI, which is responsible for the synthesis of the acyl-homoserine-lactone (AHL) autoinducer, and LuxR, which is activated by this autoinducer to increase transcription of the luciferase operon [1,3]. Since this initial description, QS was then shown to be a widespread mechanism of gene regulation in bacteria. Three QS systems have been implicated in bacterial pathogenesis: 1) the LuxR/I–type systems, primarily used by Gram-negatives, in which the signaling molecule is an acylhomoserine lactone (AHL); 2) the peptide signaling systems used primarily by Gram-positives; and 3) the autoinducer-3 (AI-3)/epinephrine/norepinephrine signaling system that constitutes an inter-species signaling system in bacteria, and also has an intrinsic role in inter-kingdom communication with the host.

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Inhibitors of the LuxR/I QS systems

Subsequent to its initial description in *Vibrio fischeri*, homologues of LuxR and LuxI have been identified in other bacteria. These LuxR/I systems regulate the transcription of several genes involved in a variety of phenotypes including the production of antibiotics in *Erwinia*, motility in *Yersinia pseudotuberculosis*, pathogenesis and biofilm formation in *Pseudomonas aeruginosa*, etc...[4-6]. The LuxI-type proteins are the AHL synthases. AHLs have a conserved homoserine lactone ring connected through an amide bond to a variable acyl-chain. Acyl chains vary among 4 to 18 carbons and the third position may or not be modified (carbonyl group, hydroxyl or fully reduced). Different acyl-chains ensure that different AHLs will be recognized by different LuxR-type proteins. The substrates for AHL synthesis are S-adenosyl-methionine (SAM) (yields the homoserine lactone ring) and the acyl chains carried by acyl-carrier-proteins (ACP) from the lipid metabolism [7-9]. The LuxR-type proteins are transcription factors, which upon binding to the AHL signal, regulate transcription of their target genes. It has been shown that AHL binding to these proteins is essential for their stabilization; otherwise, in the absence of signal, they are targeted to degradation [10-12•]. The LuxR-type proteins recognize a specific AHL. Due to this feature, this signaling system has been primarily associated with intra-species signaling.

Pseudomonas aeruginosa uses QS to activate several genes involved in colonization and persistence within the host [4]. *P. aeruginosa* is an opportunistic pathogen of immunocompromised individuals, including those with burns, HIV, or cystic fibrosis [4]. The morbidity and mortality associated with cystic fibrosis is due to the chronic colonization of the pulmonary airways by *P. aeruginosa* biofilms. QS controls production of an array of virulence factors (elastase, exotoxin A, piocyanin, etc...) and biofilm development in this organism. Disruption of the QS system diminishes *P. aeruginosa* virulence in plants and animals and inhibits biofilm formation [13-15]. The QS system of *P. aeruginosa* is very complex and hierarchical. *P. aeruginosa* produces two AHLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-AHL) and *N*-butanoyl-L-homoserine lactone (C4-AHL) [16,17]. These AHLs bind to and activate LasR and RhlR transcription factors, respectively [4]. LasR complexed with 3OC12-AHL activates the transcription of *rhlR* and *rhlI* (RhlI is the synthase for C4-AHL). Therefore, LasR is at the very top of the *P. aeruginosa* QS signaling cascade [4].

Given its intrinsic role in *P. aeruginosa* pathogenesis, several approaches have been developed to interfere with AHL QS signaling. One approach was to chemically synthesize compounds modeled on the natural AHL signals [18,19]. A second approach was to characterize natural products that can mimic AHLs and block LuxR-type of receptors. One such approach is the use of halogenated furanones synthesized by the marine macroalga *Delisea pulchra*, which act as competitive inhibitors of the bacterium's AHL-based QS system [20]. Furthermore, exposure of plants to AHLs induced the secretion of compounds that mimic QS signals and thus have the potential to disrupt QS in associated bacteria [21]. A third approach was to utilize enzymes, such as lactonases, which can degrade AHLs. The soil bacterium *Bacillus* produces a lactonase enzyme that hydrolyzes the lactone ring of AHLs. This lactonase enzyme probably interferes with AHL-signaling by other bacterial species with which *Bacillus* competes in nature [22•]. An example of the utilization of this enzyme to interfere with QS signaling was the engineering of transgenic plants expressing the *Bacillus* lactonase. These plants were resistant to QS-dependent bacterial infection [22•]. Recently, Muh et al. [23•] screened a large library of synthetic molecules to identify AHL QS inhibitors. This screen identified two inhibitors that resembled the AHL 3OC12-AHL, blocking its binding to LasR. The first compound is a tetrazole with a 12-carbon alkyl tail, and the second has a phenyl ring with a 12-carbon alkyl tail. A third thiophenyl compound, which is structurally unrelated to 3OC12-AHL, was subsequently shown to also specifically inhibit the binding of this AHL to LasR [24].

Inhibitors of peptide QS signaling systems

QS in Gram positive organisms relies on auto-induction by small peptides, which interact with two-component systems ultimately regulating gene transcription. These small peptides are derived from oligopeptides that are cleaved and modified before being secreted by the bacterium. These peptides are recognized by sensor kinases that initiate a phosphorylation cascade within the bacterial cell to regulate gene expression.

Staphylococcus aureus is one of the most common commensal Gram positive organisms in humans, however it can lead to: pneumonia, endocarditis, osteomyelitis, wound infections, and other complications. The QS system that this bacterium utilizes is one of the most studied systems in Gram positive organisms. This QS system is termed the accessory gene regulator (Agr) system and regulates virulence gene expression in staphylococci. At low cell density, the bacteria express proteins required for attachment and colonization and as the cell density becomes higher, this expression profile switches to express proteins involved in toxin and protease secretion [25].

The *S. aureus* autoinducing peptide (AIP) is encoded by the *agrD* gene. AgrB, then adds a thiolactone ring to this peptide and transports the AIP out of the cell. The AgrC sensor kinase is the AIP receptor, and upon autophosphorylating in response to this signal, it then transfers this phosphate to its cognate response regulator ArgA. Phosphorylated ArgA activates transcription of the *agr* operon for autoregulation and in addition, activates transcription of the RNAPIII regulatory RNA, which in turn represses expression of cell adhesion factors and induces expression of secreted factors [25].

The specificity of the Agr system is such that, not only will non-cognate AIP and receptors not specifically recognize each other, each AIP also represses the other AIPs by competitive binding to non-cognate receptors. Thus far, there are four known specificity groups, which have been characterized by sequence variation and each group seems to result in a different disease. For example, *S. aureus* group III seems to correlate with menstrual toxic shock syndrome, type III with necrotizing pneumonia, and type Is and type IIs have been associated with vancomycin resistance. Thus, in co-infection models, one group will out-compete the other leading to only one disease progression. This might have allowed evolution of *S. aureus* into suited environments [25]. The interference AIPs with non-cognate AgrC kinases prevent these kinases from binding to their cognate AIPs, and inhibits their signaling cascades. This phenomenon has been exploited as a means to create chimeric AgrC proteins with broadened specificity, tightening specificity, and receptors that lack activation specificity [26]. This inhibitory effect has therapeutic potential, and further understanding of the AIP-AgrC interaction will enable the design of more potent inhibitors.

Inhibitors of the AI-3/ epinephrine/ norepinephrine signaling system

The bacterial QS chemical signal AI-3 is produced and utilized by several bacterial species, and is known to be responsible for the activation of enterohemorrhagic *E. coli* (EHEC) O157:H7 virulence genes. This signaling system, however, has far reaching implications because it is also used for inter-kingdom signaling between prokaryotic and eukaryotic cells. Namely, AI-3 cross-signals with the eukaryotic hormones epinephrine and/or norepinephrine in an agonistic fashion [27,28]. AI-3 is an aromatic signaling molecule produced by the normal human gastrointestinal microbial flora whose structure is not yet defined [27•]. Both epinephrine and norepinephrine are present in the gastrointestinal tract. Norepinephrine is synthesized within the adrenergic neurons present in the enteric nervous system (ENS) [29]. Epinephrine is synthesized in the central nervous system (CNS) and in the adrenal medulla, it acts in a systemic manner after being released into the bloodstream, thereby reaching the intestine [30]. Both hormones modulate intestinal smooth muscle contraction, submucosal

blood flow, and chloride and potassium secretion in the intestine [31]. Epinephrine and norepinephrine are recognized by adrenergic receptors in mammalian cells, and Freddolino et al. [32] reported that the ligand-binding sites for epinephrine and NE in a human adrenergic receptor are similar. Extensive evidence indicates that both epinephrine and NE are recognized by the same receptors and play important biological roles in the human GI tract.

EHEC is responsible for major outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS) throughout the world. Annually in the United States, EHEC is responsible for an estimated 73,000 illnesses, 1,800 to 3,600 hospitalizations and from 61 to 541 deaths with combined annual economic costs of up to \$405 million [33]. During the recent outbreak in the United States of EHEC from contaminated raw spinach, 199 people were infected, 102 were hospitalized, 31 suffered from HUS, and 3 patients died. In Argentina, Chile and Uruguay, EHEC is responsible for 40% of the cases of bloody diarrhea. In the U.K., EHEC incidence has increased over the years to 2.7/100,000. In an outbreak in 1996 in Sakai, Japan, there were over 7,500 cases [34] (CDC webpage).

EHEC has a very low infectious dose (as low as 50 cfu), which is one of the major contributing factors to EHEC outbreaks. EHEC colonizes the large intestine where it causes attaching and effacing (AE) lesions. The AE lesion is characterized by the destruction of the microvilli, and the rearrangement of the cytoskeleton to form a pedestal-like structure that cups the bacterium. The genes involved in the formation of the AE lesion are encoded within a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE). The LEE region contains five major operons: *LEE1*, *LEE2*, *LEE3*, *tir/LEE5* and *LEE4*, which encode a type III secretion system (TTSS) [35], an adhesin (intimin) [36] and its receptor (Tir) [37], and effector proteins, which together with Tir are translocated into the epithelial cell through the bacterial TTSS [37]. The mortality associated with EHEC infections stems from the production and release of a potent Shiga toxin by these bacteria. Shiga toxin induces cell death in endothelial cells, primarily in the urinary tract, causing HUS [38]. The genes encoding Shiga toxin are located within the late genes of a λ -like bacteriophage, and are transcribed when the phage enters its lytic cycle [39-41]. Disturbances in bacterial envelope, DNA replication, or protein synthesis (which are targets of conventional antibiotics) trigger an SOS response in the bacterial cells that signals the bacteriophage to enter the lytic cycle. The phage replicates, produces Shiga toxin and lyses the bacteria releasing Shiga toxin. Consequently, treatment of EHEC infections with conventional antimicrobials may enhance EHEC-mediated disease [42,43].

The AI-3/epinephrine/NE inter-kingdom signaling cascade activates expression of virulence genes in EHEC [27,44-46••]. AI-3 and epinephrine/NE are agonistic signals, and response to both signals can be blocked by adrenergic antagonists [27••,45••,47]. These signals are sensed by sensor kinases in the membrane of EHEC that relay this information to a complex regulatory cascade that activates the flagella regulon (facilitating EHECs mobility through the intestinal mucus layer within the intestinal lumen), the LEE (necessary for intestinal colonization through the formation AE lesions), and Shiga toxin expression. The quorum sensing *E. coli* regulator C (QseC) is one of these sensor kinases. QseC specifically senses AI-3/ epinephrine/ norepinephrine to augment its phosphorylation state and directly binds to norepinephrine. Thus, QseC is a bacterial adrenergic receptor functional analog. QseC's recognition of these signals can be blocked with the α -adrenergic antagonist phentolamine. QseC is essential for virulence in EHEC, given that a *qseC* mutant is attenuated for virulence in a rabbit animal model [45••]. Upon sensing AI-3/epinephrine/NE, QseC phosphorylates the QseB response regulator, which activates expression of several transcription factors and virulence genes, initiating a complex regulatory cascade in EHEC to coordinately express virulence traits [45••].

The AI-3/epinephrine/NE signaling cascade is essential for EHEC pathogenesis. Of note this signaling cascade is present in several bacterial pathogens (e.g. *Shigella*, *Salmonella*, *Erwinia*

carotovora, *Pasteurella multocida*, *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae*, *Chromobacter violaceum*, *Coxiella burnetti*, *Yersinia*, *Francisella tularensis* and *Ralstonia solacearum*) [45,46], suggesting that this inter-kingdom cross-signaling is not restricted to EHEC. There are few if any good treatment options other than hydration with electrolytes and plasmapheresis for HUS. Antibiotics and antimotility agents are contraindicated for EHEC infections [48]. In fact, antibiotics affect or promote the expression and release of the potent Shiga toxins, thereby increasing the occurrence and severity of HUS and CNS involvement. The lack of efficient treatments for EHEC infections, due to the controversy surrounding the administration of conventional antibiotics, combined with the growing challenge of antimicrobial resistance, underscores the importance of understanding this signaling cascade to design and generate new classes of antimicrobials.

The cross signaling of AI-3 with epinephrine and norepinephrine, combined with the ability of adrenergic antagonists to block EHEC's recognition of all three signals (rendering it unable to induce its virulence genes), suggest an exciting potential alternative for treatment of EHEC infections by using adrenergic antagonists. Furthermore antagonists of this signaling cascade will not only be useful against EHEC, but possibly also against other human pathogens such as enteropathogenic *E. coli* (EPEC), *Salmonella*, *Shigella*, *Yersinia pestis*, and others all of which harbor this signaling cascade.

Conclusions

QS has been shown to be pivotal for bacteria to gauge the host environment, as well as competition from other bacterial cells within the host to initiate pathogenesis. Our understanding of the various QS signaling systems, and the connections amongst them, led to the development of potential novel therapeutic approaches to combat bacterial infections. QS is an emerging field and further development of the anti-QS potential therapeutics highlighted in this review, as well as others, will certainly contribute to the future of human health, and the fight against infectious diseases.

Expert commentary

One of the biggest challenges of medicine in this century is microbial antibiotic resistance. It was believed that modern medicine conquered infectious diseases through the discovery of antibiotics. However, very few novel antibiotics have been discovered in the past 30 years, and during this period, the rapid bacterial evolution, allowed them to evolve mechanisms of resistance to virtually all known antibiotics. Hence, diseases such as tuberculosis and whooping cough constitute again an important health problem, and have been labeled as re-emerging infectious diseases. Combined with multi-antibiotic resistance, another major problem are emerging infectious diseases such as bloody diarrhea caused by EHEC. Interference with QS signaling is an emerging field for development of novel therapeutics against bacterial infections. The advantage of this approach is that anti-QS drugs are unlikely to induce bacterial resistance. Unlike antibiotics that kill or hinder bacterial growth, QS antagonists will only confuse signaling. Hence, QS antagonists could be viewed as pathogenic blockers rather than antimicrobials. This concept is enticing in the view that these compounds, in theory, would not induce a selective pressure towards evolution of bacterial resistance.

Five-year review

Significant progress in the development of novel antimicrobial agents will require an increase in research and funding. In the next five years a better understanding of the intrinsic mechanisms of bacterial intercellular and bacteria-host signaling will provide the basis for the design of novel therapeutics. In addition, the compounds described in this review show promise, and further structural functional analysis, toxicity, and animal research will be

necessary to assess their drug potential. Interfering with bacterial chemical communication offers several advantages over conventional antibiotic targets. Conventional targets disrupt protein synthesis, DNA replication or bacterial cell wall synthesis, which compromise bacterial growth and survival, ensuing selective pressure towards evolution of bacterial resistance mechanisms. Quorum sensing antagonists may not interfere with such vital processes in bacteria, hence, it is anticipated that this strategy should not select for resistance mechanisms. It is now being appreciated that signaling in bacteria occurs through an array of chemical compounds, offering flexibility for development of several classes of antimicrobials.

Key issues

- Quorum sensing signaling is used by several important bacterial pathogens to modulate expression of their virulence armamentarium.
- Quorum sensing is also utilized for signaling between bacteria and host, playing an important role in bacterial recognition of the host environment.
- There is growing evidence that interference with quorum sensing signaling is a promising avenue towards development of novel therapeutics.
- It is anticipated that interference with bacteria cell-to-cell signaling will not engender a selective pressure for evolution of bacterial resistance mechanisms

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