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Chemical Signaling among Bacteria and Its Inhibition

Review

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Generations of chemists and biologists have conducted research on natural products and other metabolites produced by bacteria and other microorganisms. This has led to an explosion in knowledge concerning the mechanism by which such natural products are made, ultimately allowing custom redesign of many of these molecules for increased potency and selectivity as therapeutic drugs [1]. Along the way, scientists have begun to appreciate that the bacterial world is teeming with life on a scale hardly conceivable, with constant communication within the bacterial world and with outside neighbors, such as plants and mammals. Only in recent years have some of the signaling molecules that comprise these elaborate forms of communication been characterized in any sort of chemical detail, which has in turn peaked interest in the intricate biology of this micro-world and its interactions with the macro-world.

Intraspecies Communication

Bacteria have evolved elaborate means to communicate with each other, both within and between species. Intraspecies communication is far and away the best characterized, simply due to the ease of working with pure cultures of bacteria. From this work, it has been shown that signaling pheromones in gram-positive bacteria are generally peptides, while the vast majority of such pheromones in gram-negative bacteria are small molecules, such as N-acyl homoserine lactones. These signaling pheromones accumulate with increasing cell density, triggering signaling events when a "quorum" is reached; hence the name "quorum sensing" (QS) to describe this phenomenon [2].

The general paradigm is that peptides in gram-positive bacteria signal through receptor-histidine kinases (RHKs) embedded in the membrane [3], while small molecules can diffuse across the cytoplasmic membrane in gram-negative bacteria to bind to regulatory proteins within the cell to trigger transcriptional changes (Figure 1). However, there are already exceptions to this paradigm, as will be discussed in this review, and it is also most likely the case that many peptides and small molecules exist and signal through membrane-bound or cytoplasmic receptors in all types of bacteria and as of yet remain undiscovered. This is supported by genomic data indicating the presence of putative signaling peptides and transporters in gram-negative bacteria [4] and the characterization of small signaling molecules, known as γ-butyrolactones, that appear to function in a cell density-dependent manner to elicit antibiotic production in the gram-positive genus *Streptomyces* [5]. Further study of signaling mechanisms in *Streptomyces* is of particular importance given the fact that strains in this genus produce thousands of bioactive natural products, many of which are important in medicine and agriculture. The complete genome sequences of *Streptomyces coelicolor* and *Streptomyces avermitilis* were recently published, which should greatly aid further efforts to characterize signaling in these bacteria [6, 7].

Intraspecies Communication in Gram-Positive Bacteria: The Agr System of Staphylococcus aureus

There are at least 17 putative two-component signaling systems in the genome of the gram-positive bacterial pathogen *Staphylococcus aureus*, all of which play some role in cell-cell or cell-environment communication [8]. Many functions in *S. aureus*, including virulence, are controlled by at least one of these two-component systems, known as the accessory gene regulator (*agr*) operon (reviewed in [9, 10]). As *S. aureus* cells grow, a small ($<$10 amino acid) extracellular peptide, known as the autoinducing peptide (AIP), is secreted and accumulates. This AIP is derived from processing of the propeptide, AgrD, by the putative processing enzyme, AgrB. Upon reaching a threshold concentration in the tens of nanomolar range, the AIP binds to and triggers activation of the receptor-histidine kinase, AgrC. This activation results in increased transcription of the unique regulator, RNAIII, ultimately leading to increased secretion of virulence and other accessory factors and downregulation of various surface proteins. This signaling process is but one example of density-dependent or quorum-sensing systems widespread in bacteria (Table 1 and Figure 2) [11, 12].

The sequence of the AIPs is highly variable, resulting in at least four specificity groups of strains within *S. aureus* and many more ($>$25) in other staphylococci [13, 14]. A group is defined as the collection of strains that produce the same AIP. The *agrB*, *D*, and *C* regions vary in concert to maintain the specificity of AIP processing and function, and this specificity results in four different receptors for the AIPs in *S. aureus*, designated AgrC-I, -II, -III, and -IV, reflecting the group that expresses them. Remarkably, there is extensive cross-communication at the level of ligand-mediated signaling, as most AIPs activate their cognate receptor while inhibiting activation of nonnative receptors [15]. This inhibition is a form of bacterial interference that does not result in growth inhibition but rather in the block of accessory gene functions, presumably resulting in an advantage for the strain producing the most abundant and/or most potent AIP.

The *S. aureus* AIPs are 7–9 residues in length, depending on the group, and contain a thiolactone ring structure in which the α-carboxyl group at the C terminus is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid from the C terminus of the peptide (Figure 2) [16]. Note, the AIP from *S. intermedius* has recently been shown to contain a lactone ring rather than the more usual thiolactone constraint [17]. A combi-

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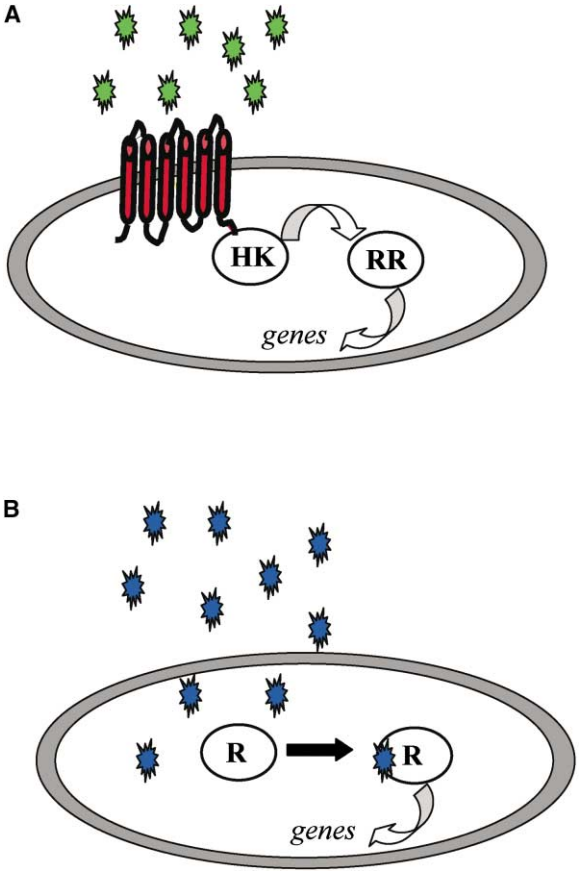

Figure 1. Schematic of Chemical Signaling in Bacteria

(A) Peptide signaling through receptor-histidine kinases (RHKs) in gram-positive bacteria. The extracellular signaling molecules, shown as stars, bind to the sensor domain of the RHK, triggering activation via phosphorylation or dephosphorylation of the HK domain. A classic phosphorelay to or from the response regulator (RR) ensues, which controls gene expression at the level of transcription. The sensor domain of RHKs contains a variable number of transmembrane helices, with 6–8 TM helices as the standard for peptide binding.

(B) Small molecule signaling through intracellular receptors in gram-negative bacteria. An intracellular receptor protein, labeled R, is stabilized upon binding the diffusible or actively transported signaling molecules (shown as stars). This receptor protein then binds to DNA and modulates gene expression.

nation of chemical synthesis, genetics, and structural and biological analysis has been used to study the structure-activity relationships within the AIPs and the RHK, AgrC [16, 18–24]. This integrated approach has revealed some of the structural features important for the activation and inhibition activities of the AIPs (summarized in Figure 3) and has paved the way to the rational design of global inhibitors of *S. aureus* virulence (see below). A particularly remarkable finding relates to the effects of changing the thiolactone linkage within the 16-atom membered macrocycle of the AIP. Lactam analogs of AIP-I and AIP-II are potent cross-group inhibitors, but activate receptors within their group only at very high concentrations [21, 23]. NMR analysis of the AIP-II lactam analog revealed dramatic differences in the backbone chemical shifts of residues within the ring (to roughly the same extent as linearizing the peptide), whereas the chemical shifts of the tail residues were essentially unaffected [24]. This points to the structural independence of the exocyclic (i.e., tail region) and endocyclic (i.e., within the macrocyle) regions of the molecule. Perhaps more importantly, these studies strongly suggest that the molecular recognition mechanisms underlying the competitive receptor-agonist and receptor-antagonist interactions are different; modification of the thiolactone moiety dramatically affects the structure of the macrocycle, yet this perturbation results only in loss of agonist activity.

Based on the above studies, we now have a basic understanding of the mechanisms underlying agonism and antagonism of AgrC by native AIPs. However, our understanding of how AIP binding leads to presumed AgrC autophosphorylation is still in its infancy. The biosynthetic mechanism by which the AgrD propeptide is converted into the mature AIP is equally poorly understood. There is good evidence that the integral membrane protein, AgrB, is responsible for the posttranslational modification of AgrD and possibly the secretion of mature AIP [25, 26]. For processing to occur, the propeptide must be cleaved internally in two locations, along with cyclization to form the thioester linkage. It is tempting to speculate that the cleavage of the C-terminal portion of the AIP from within the propeptide could occur through the formation of a acyl-enzyme intermediate, which would then be primed for nucleophilic attack by the sulfhydryl of the cysteine in the AIP, thus causing cyclization via thioester formation. However, the mecha-

Table 1. Some Bacterial Processes Controlled by Quorum Sensing

Biological Processes	Bacterial Species	Signaling Molecules	Inhibitors
virulence/competence	*Streptococcus pneumoniae*	CSP, BlpC*	RHK inhibitors
virulence	*Enterococcus faecalis*	GBAP, $CylL_s''$	
virulence	*Staphylococcus aureus*	AIP	TrAIP-II and other AIP analogs
competence	*Bacillus subtilis*	ComX	
bacteriocin production	*Lactococcus lactis*	Nisin	
bioluminescence	*Vibrio harveyi*	HSLs, AI-2	
virulence	*Vibrio cholerae*	CAI-1, AI-2	
biofilms/virulence	*Pseudomonas aeruginosa*	HSLs	Furanones, modified HSLs
conjugation	*Agrobacterium*	HSLs	
plant infection	*Bradyrhizobium japonicum*	Bradyoxetin	

Gram-negative bacteria are in the last five rows.

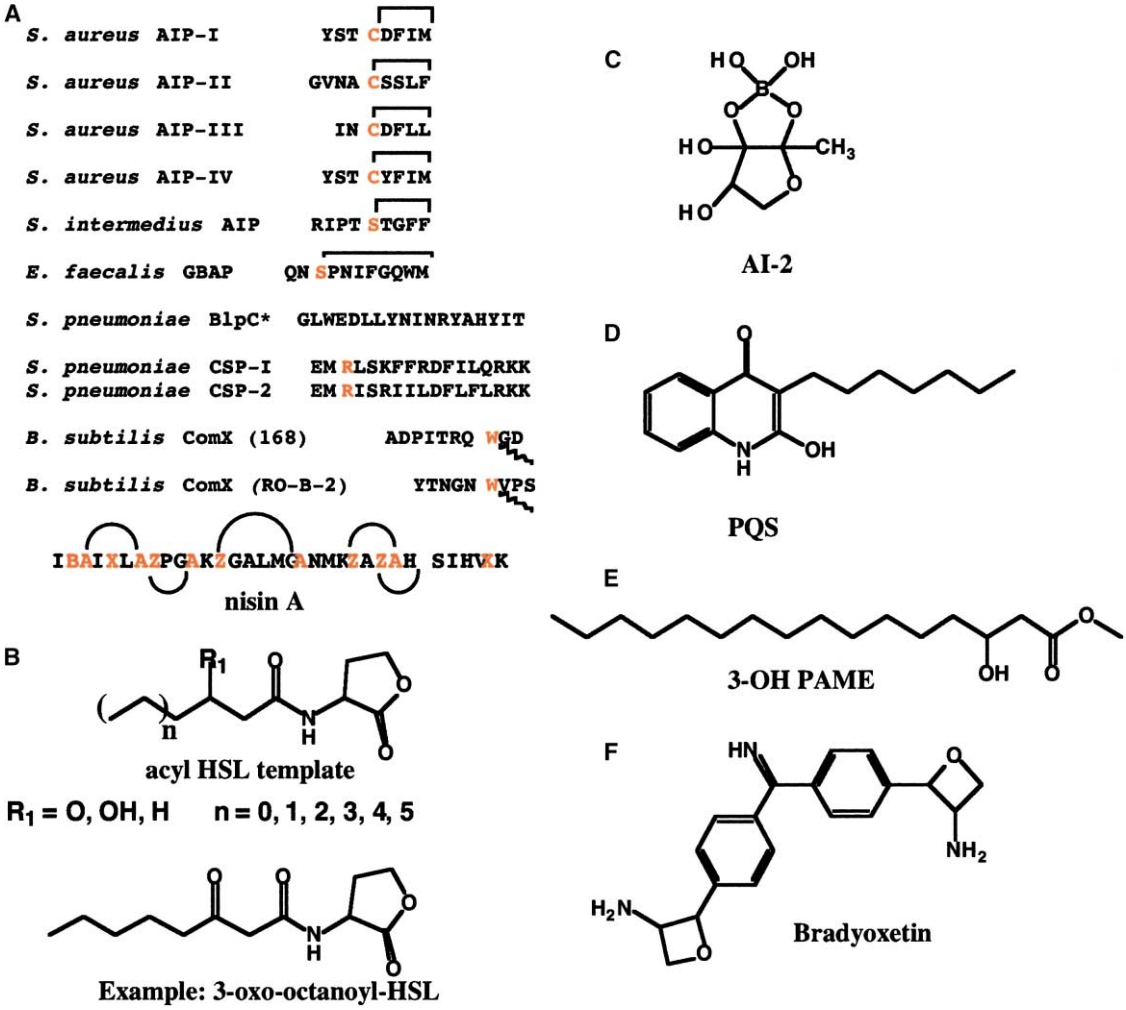

Figure 2. Chemical Composition of Bacterial Signaling Molecules.

(A) Signaling peptides in gram-positive bacteria. Conserved residues that are posttranslationally modified and/or are critically important for agonist activity are marked in red. The connectivities for cyclization in the AIPs are shown with semicircles or lines. For nisin A, the lanthionine bridges are indicated by semicircles. B, dehydrobutyric acid (Dhb); X, dehydroalanine (Dha); Z, aminobutyric acid (Abu). The lipid modifications, which are different from each other in composition (see main text), on the tryptophan of *B. subtilis* AIPs are marked with a squiggly line.

(B) Acyl-HSLs in gram-negative bacteria. A generic structure depicting some of the possible HSLs is shown, although this is by no means comprehensive, and all of the possible combinations have not yet been isolated. An example from *Agrobacterium tumefaciens* is shown for clarity. Furthermore, some HSLs contain an unsaturated double bond in their acyl chain, and the acyl chains of virtually all HSLs have an even number of carbons regardless of chain length as a necessity of their metabolic synthesis.

(C–F) (C), AI-2 has been shown to trigger bioluminescene and virulence in *V. harveyi* and *V. cholerae*, respectively; (D), PQS (*Pseudomonas* quinolone signal), 2-heptyl-3-hydroxyl-4-quinolone; (E), 3-OH PAME (3-hydroxypalmitic acid methyl ester); (F), bradyoxetin.

nistic details of this fascinating biotransformation remain to be elucidated, including how the respective enzymes faithfully process staphylococcal AIPs that vary in length from 7–9 amino acids, where this length difference is entirely determined by the varying N-terminal cleavage sites within the corresponding AIP propeptides.

Given the detailed understanding that has emerged concerning AIP-induced signaling in *S. aureus*, along with the naturally occurring cross-inhibition that has been characterized, it is only logical that efforts would be undertaken to develop inhibitors of this signaling, with an eye toward the development of novel antiinfectives. Substantial progress has been made toward this goal, which will be discussed later in this review in a separate section focusing on inhibitors of quorum sensing in general (and see Table 1 for a list of such inhibitors).

Virulence Control in Enterococcus faecalis

There are at least nine putative two-component systems found in the genome of *Enterococcus faecalis*, some of which represent potential therapeutic targets [27]. Analogous to the *agr* system in *S. aureus*, there exists one similar autoregulated two-component system in the bacterial pathogen *E. faecalis* known as the *E. faecalis* regulator (*fsr*) [28]. This locus includes a receptor-histidine kinase, FsrC, a response regulator, FsrA, and a putative AgrB-like processing enzyme, FsrB. It has been

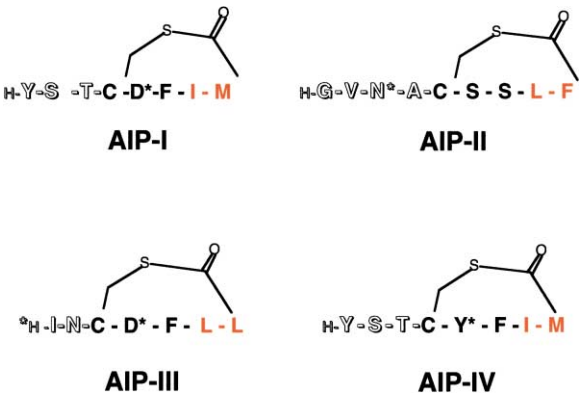

Figure 3. Composition and Key Determinants of the *S. aureus* AIPs

Standard single-letter codes for amino acids are indicated. The sulfur atom of the cysteine and the carbonyl contributed from the C-terminal amino acid are shown in a thioester linkage, which closes the macrocycle. Exocyclic (tail) residues are represented by outlined and shaded text. Residues that are critical for receptor activation are marked with an asterisk. The N terminus of AIP-III is marked with an asterisk to reflect the fact that additional amino acids on the N terminus abolish receptor activation. The two C-terminal amino acids, highlighted in red, are conserved in terms of hydrophobicity in all staphylococcal AIPs characterized to date.

shown that all three genes in the *fsr* operon are important for the production of virulence factors, such as gelatinase and a serine protease, and that mutation of these genes results in attenuated virulence in a mouse peritonitis model [29] and a relatively new *C. elegans* killing model [30]. In contrast with the *agr* system, where the AIP is processed from a dedicated propeptide AgrD, the *E. faecalis* AIP (also referred to as GBAP) is likely derived from the C terminus of the putative processing enzyme, FsrB [31]. However, there is ~19% sequence identity between FsrB and *S. aureus* AgrB-I-IV, and the propeptides in both systems are cleaved internally to release AIPs with new N and C termini. Furthermore, both AIPs contain a cyclic structure formed from the condensation of the α-carboxyl group of the peptide with a nucleophilic side chain situated on an amino acid located N-terminal to this in the AIP (Figure 2). It is likely that this cyclization is mediated by their respective processing enzymes, AgrB and FsrB. For the one characterized *E. faecalis* AIP, the nucleophile corresponds to the hydroxyl group on a serine residue nine amino acids away from the AIP C terminus, thus forming a lactone peptide. The use of lactone peptides for bacterial cell-cell communication is further supported by the recent discovery of a *S. intermedius* lactone AIP [17].

To date, no inhibitors of *E. faecalis* AIP-induced signaling have been reported. However, further structure-activity relationship studies of the *E. faecalis* AIP will most likely reveal key residues that are important for receptor activation but do not affect receptor binding. Such AIP analogs would constitute competitive antagonists, much like what has been developed in the *S. aureus agr* system (see below), and thus might have therapeutic utility.

Competence in Streptococcus pneumoniae and Bacillus subtilis

Some of the first studies hinting at the existence of hormone-like signaling in bacteria related to the control of competence in *Streptococcus pneumoniae* [32]. Many years later, the signaling molecule that controlled competence development was characterized as an unmodified heptadecapeptide (competence stimulating peptide, CSP-1) (see Figure 2), and the receptor, ComD, was subsequently identified (reviewed in [33]). A later study identified a new set of *Streptococcus pneumoniae* strains making a different heptadecapeptide, CSP-2, differing from CSP-1 at eight residues [34]. Further work demonstrated that many different pherotypes of CSP exist within many different streptococcal species, including *S. gordonii*, *S. oralis*, *S.mitis*, and *S. mutans* [35, 36]. Most recently, it has been shown that CSP signaling via ComD stimulates not only competence within most pneumococcal cells but also coordinated DNA release by donor cells within the same population, thus highlighting the beautiful elegance of quorum-sensing-mediated genetic exchange [37]. Furthermore, it has been shown that DNA transformation in *S. mutans* is not only dependent on CSP signaling but also increases dramatically within its natural biofilm habitat of dental plaque [36]. Lastly, there is now accumulating evidence that competence induction plays a vital role in the virulence of *S. pneumoniae* [38] and that two-component signaling in general (with at least 13 systems characterized in *S. pneumoniae*) plays a significant role in pathogenesis [39].

Natural genetic competence in *Bacillus subtilis* is also controlled by quorum sensing. The signaling peptide ComX is detected by the two-component system ComP-ComA, which then triggers the expression of many genes required for competence development [40]. There are in fact at least 29 kinase-regulator pairs in the *B. subtilis* genome, and all of these have been studied to various extents by DNA microarray analysis [41]. In the case of ComP-ComA, novel genes were identified that are controlled by this system and will require further study, just as in the *S. aureus* agr system, where similar studies yielded many new targets of regulation [42]. Recently, the structural composition of ComX, a decapeptide with a previously unknown modification, has been deduced [43]. This study demonstrated that Com X is isoprenylated (Figure 2) and that this isoprenoid modification varies in different strains (sometimes being a farnesyl, geranyl, or as yet uncharacterized modifications), thus giving rise to at least four groups in *B. subtilis*, some of which may cross-inhibit other groups. These groups vary not only in ComX but also in the sensor domain portion of ComP [44, 45], reminiscent of the *S. aureus* agr system. The isoprenoid modification of ComX is consistent with data showing that the processing enzyme ComQ contains an isoprenoid binding domain [46]. A remarkable finding that opened the door to ComX characterization was that expression of ComQ and ComX in *E. coli* was sufficient for pheromone production and secretion from the cells [45]; such results have thus far not been attained when attempting to obtain other processed and secreted gram-positive signaling peptides in *E. coli*. It is unknown whether the lipid-modified ComX simply diffuses or is actively transported out of *E. coli* cells. Lastly, the development of sporulation and competence is further modulated by another peptide, an unmodified pentapeptide (sequence = ERGMT),

CSF, that does not interact with a receptor at the cell surface but rather is actively transported into the cell by the oligopeptide permease, Opp, where it interacts with intracellular receptors to regulate transcription [47].

Given recent evidence that two-component signaling plays a significant role in pathogenesis in many bacterial pathogens, including *S. pneumoniae*, such signaling has become a target for inhibitor development both in academic circles and the pharmaceutical industry (see below).

Common Themes

It is worth noting that the vast majority, if not all, of the peptides (AIPs) involved in signaling in gram-positive bacteria have hydrophobic motifs, some of which have already been proven to be critically important for activity. This is true for the last two amino acids of the staphylococcal AIPs [16] and is also true for the vital isoprenoid modifications on the *B. subtilis* AIPs [43]. It is probable that the mechanism of binding for all of these AIPs to the receptor-histidine kinases (RHKs), which are polytopic integral membrane proteins, involves a strong hydrophobic interaction followed by receptor activation mediated by other interactions, perhaps of a hydrophilic and/or electrostatic nature. Such a hypothesis can be confirmed through further structure-activity relationship studies of the remaining AIPs along with detailed characterization of the ligand binding contacts within the RHKs.

Quorum Sensing and Bacteriocin Production

The production in a cell-density-dependent manner of antimicrobial compounds (bacteriocins) by lactic acid bacteria is well established and extensive (for reviews see [48, 49]). As one example, nisin is the prototype of a large class of bacteriocins, the lantibiotics [50], which are heavily posttranslationally modified peptides characterized by the presence of dehydrated amino acids and, typically, (β-methyl)lanthionine bridges (Figure 2). Genetic analysis has revealed that nisin production is stimulated in a cell-density-dependent fashion by nisin itself. However, the antibacterial activity and the signaling activity of nisin are distinct, with dependence on different amino acid residues, although ring formation in the peptide is required for both activities [51].

Another example of quorum-sensing-based control of bacteriocin production is illustrated by the regulation of cytolysin production in *E. faecalis* [52]. Genetic evidence suggests that the cytolysin subunits are related to the lantibiotics. However, unlike lantibiotics, cytolysin is lytic for eukaryotic as well as prokaryotic cells, and it consists of two structural subunits. The synthesis of cytolysin is quite elaborate, involving posttranslational modification, proteolytic cleavage, secretion, and an additional step of extracellular proteolytic degradation of two subunits to produce the mature product. One of the precursor subunits, $CylL_s''$, induces transcription of the structural genes for both cytolysin subunits. The amount of induction depends on the amount of $CylL_s''$ added, suggesting that cell-density-dependent accumulation of one subunit signals for the production of both subunits. The precise structure and chemical composition of the signaling molecule $CylL_s''$ is unknown but appears to have at least one lanthionine bridge as well as other not yet fully characterized modifications [53]. Not only is the signaling molecule itself rather unique, but so too is the signal-sensing apparatus. Signaling does not proceed through a classic two-component histidine-kinase/response regulator pair but rather involves a transmembrane protein of unknown function, CylR1, and an apparent helix-turn-helix DNA binding protein, CylR2. In the absence of $CylL_s''$, these two proteins appear to act together to repress gene transcription. Further work will be required to elucidate the exact mechanism of cell-density-dependent cytolysin induction by $CylL_s''$.

In the majority of instances, the signaling molecules that switch on bacteriocin production are distinct from the bacteriocins themselves, e.g., bacteriocin production in *S. pneumoniae* has recently been shown to be controlled by a processed and secreted linear peptide (BlpC*, also called SpiP) that signals through a classic two-component signaling cascade (Figure 2) [54, 55]. There are at least three BlpC* peptides in different strains that have been identified, with corresponding amino acid changes in the respective receptor-histidine kinases.

Although the mechanism by which bacteriocins are induced via quorum sensing is fascinating, it is perhaps the bacteriocins themselves that are of even more widespread interest due to their potential utility in the food and pharmaceutical industries. In fact, nisin is already used as a food additive in over 50 countries, including the EU and the USA. Other bacteriocins of potential medical interest are gallidermin, active against the causative agent of acne, *Propionibacterium acnes*, and mersacidin, shown to be active in vivo against methicillin-resistant *S. aureus*. The scope of bacteriocin research is truly enormous and lies outside the scope of this review; the reader is instead referred to comprehensive reviews of the field ([50, 56] and references therein).

Intraspecies Communication in Gram-Negative Bacteria: Acyl-HSL-Based Signaling

Many gram-negative bacteria use acylhomoserine lactones (acyl-HSLs) as intercellular signals in density-dependent gene regulation (reviewed in [57, 58]). The first acyl-HSL, *N*-(3-oxohexanyoyl)-L-homoserine lactone, was identified in the marine luminescent bacterium *Vibrio fischeri* in 1981 [59]. Since that time, numerous bacteria, including *Pseudomonas aeruginosa, Agrobacterium tumefaciens, Rhizobium leguminosarum*, and *Rhodobacter sphaeroides*, have been shown to produce a wide range of acyl-HSLs, all differing in the length of the acyl moiety and in the degree of oxidation at the C3 position (Figure 2). Acyl-HSLs are known to signal through a protein known as LuxR (or its homologs) and are produced by an enzyme known as LuxI (or its homologs).

LuxR contains two domains: the N-terminal region contains conserved residues known to be required for acyl-HSL binding, and the C-terminal region of the protein contains a predicted helix-turn-helix motif that has been implicated in DNA binding. It has been surmised that density-dependent accumulation of acyl-HSLs from basal LuxI-mediated production leads to increased binding of acyl HSLs to the N-terminal domain of already formed LuxR, thus relieving an autoinhibited conformation of the protein (reviewed in [58, 60]). However, recent structural studies on a LuxR homolog, TraR, from *Agro-*

bacterium tumefaciens have shown that the pheromone, at least for TraR, is deeply embedded in a hydrophobic cavity with virtually no solvent contact [61, 62]. Indeed, there is evidence that TraR is stabilized toward cellular proteolysis by binding to the pheromone [63, 64], suggesting that the pheromone might indirectly affect gene transcription by stabilizing functional TraR dimers. It remains to be seen whether or not this mechanism of pheromone-induced protein stabilization holds true for other LuxR homologs, especially given the fact that it appears that some LuxR-related proteins bind DNA in the absence of acyl-HSLs [65].

Acyl-HSLs are produced by the LuxI family of synthases from the substrates acylated acyl carrier protein (acyl-ACP) and S-adenosyl-L-methionine (SAM) (reviewed in [58]). The enzymology of acyl-HSL synthesis has been investigated extensively, culminating most recently with the crystal structure of the LuxI homolog, EsaI [66]. This study revealed structural similarities between EsaI and *N*-acetyltransferases, including a common phosphopantetheine binding fold as the catalytic core. The structure provides support for a sequential ordered reaction [67] in which the acyl chain of the acyl-ACP, which is presented as a thioester of the ACP phosphopantetheine prosthetic group, is attacked by the nucleophilic amine of SAM. This is followed by lactonization, which occurs by intramolecular nucleophilic attack on the γ carbon of SAM by its carboxylate oxygen to produce the homoserine lactone product (Figure 4). Furthermore, as acyl-HSLs produced by different bacterial species vary both in the length of the acyl chain as well as in the degree of oxidation at the C3 position, the structure suggests that such differences can be accommodated by coordinated sequence differences in and near the binding pocket, much like what is seen in HSL binding by LuxR homologs [61, 62]. Lastly, there are other groups of HSL biosynthetic enzymes that appear to have no significant homology to the LuxI enzyme, although they appear to catalyze HSL synthesis from the same substrates, at least for the LuxM type of enzymes [68, 69].

In recent years, many investigators have begun to focus on quorum sensing in the opportunistic human pathogen *Pseudomonas aeruginosa* due to its role in a variety of human illnesses, including infections in immunocompromised patients suffering from AIDS, cystic fibrosis (CF), severe burn wounds, or other ailments (reviewed in [58, 70] and references therein). *P. aeruginosa* produces and secretes multiple extracellular virulence factors, including proteases, hemolysins, exotoxin A, exoenzyme S, and pyocyanin, all of which can cause extensive tissue damage in humans and other mammals. *P. aeruginosa* produces at least two quorum-sensing acyl-HSLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL), which signal through the LuxR homologs LasR and RhlR, respectively. Signaling through these quorum-sensing circuits potentially coordinates the expression of hundreds of genes during *P. aeruginosa* growth, as deduced from transcriptome analysis [71, 72]. Abundant evidence indicates that mutation of these quorum-sensing circuits results in virulence attenuation in burn, respiratory infection, and other animal models of human disease. Similarly, the role of quorum sensing in *P. aeruginosa* infection of CF patients is also well established, including in the regulation of biofilm formation [73]. It is worth noting that there are other potential acyl-HSLs in *P. aeruginosa* [74], although it is not known what the functions of these putative molecules might be. Given the serious nature of bacterial infections, including those caused by gram-negative bacteria and particularly *P. aeruginosa*, the acyl-HSL based quorum-sensing circuitry has become an important target for drug discovery efforts, some of which will be discussed later.

Other Signaling Molecules

In gram-negative bacteria, there is already evidence that other signaling molecules exist beyond just acyl-HSLs that could be involved in intraspecies communication, including peptides [4] and cyclic dipeptides [75, 76]. Another known molecule is the *Pseudomonas* quinolone signal (PQS), namely 2-heptyl-3-hydroxy-4-quinolone, now shown to be involved in the quorum-sensing pathways of *P. aeruginosa* [77–79] and known to be upregulated during lung infections in CF patients [80, 81]. In fact, initial efforts at inhibiting this pathway have shown that one precursor to PQS, anthranilate, can be converted into an inhibitor of PQS production by modifying it to methyl anthranilate, thereby reducing production of at least one virulence factor, elastase, in vitro [82]. A molecule named bradyoxetin has been shown to be involved in quorum sensing in the symbiotic bacterium *Bradyrhizobium japonicum* [83]. Chemical analysis of this cell density factor (CDF) showed that it is distinctly different from acyl-HSLs, with a proposed structure containing oxetane rings, namely 2-{4-[[4-(3-aminooxetan-2-yl)phenyl](imino)methyl]phenyl}oxetan-3-ylamine [83] (Figure 2). Another example of a different signaling molecule is 3-OH PAME (3-hydroxypalmitic acid methyl ester), shown to be involved in virulence regulation in *Ralstonia (Pseudomonas) solanacearum*, which is a soil-borne phytopathogen that causes a wilting disease of many important crops [84, 85]. It is likely that an enormous number of other unknown compounds and signaling cascades are just waiting to be discovered, which will then open the door to further antiinfective drug discovery efforts aimed at the inhibition of these pathways.

Interspecies Communication/Warfare

AI-2: A Common Language among Bacteria?

The quorum-sensing circuitry in the bioluminescent bacterium *Vibrio harveyi* contains an intriguing blend of gram-positive and gram-negative aspects. *V. harveyi* makes a typical gram-negative acyl-HSL autoinducer, AI-1 [*N*-(4-hydroxybutyl)-L-homoserine lactone], but this autoinducer signals through a typical two-component system, much like in gram-positive bacteria. Furthermore, an additional molecule, AI-2, is made that does not resemble any other known signaling molecule and yet also signals through a two-component system, with both pathways ultimately leading to bioluminescene in this organism. For many years, the structural identity of AI-2 remained elusive, but recently the crystal structure of AI-2 in complex with its required signaling partner, LuxP, was determined [86], revealing that AI-2 is a novel furanosyl borate diester (Figures 2 and 4). The presence

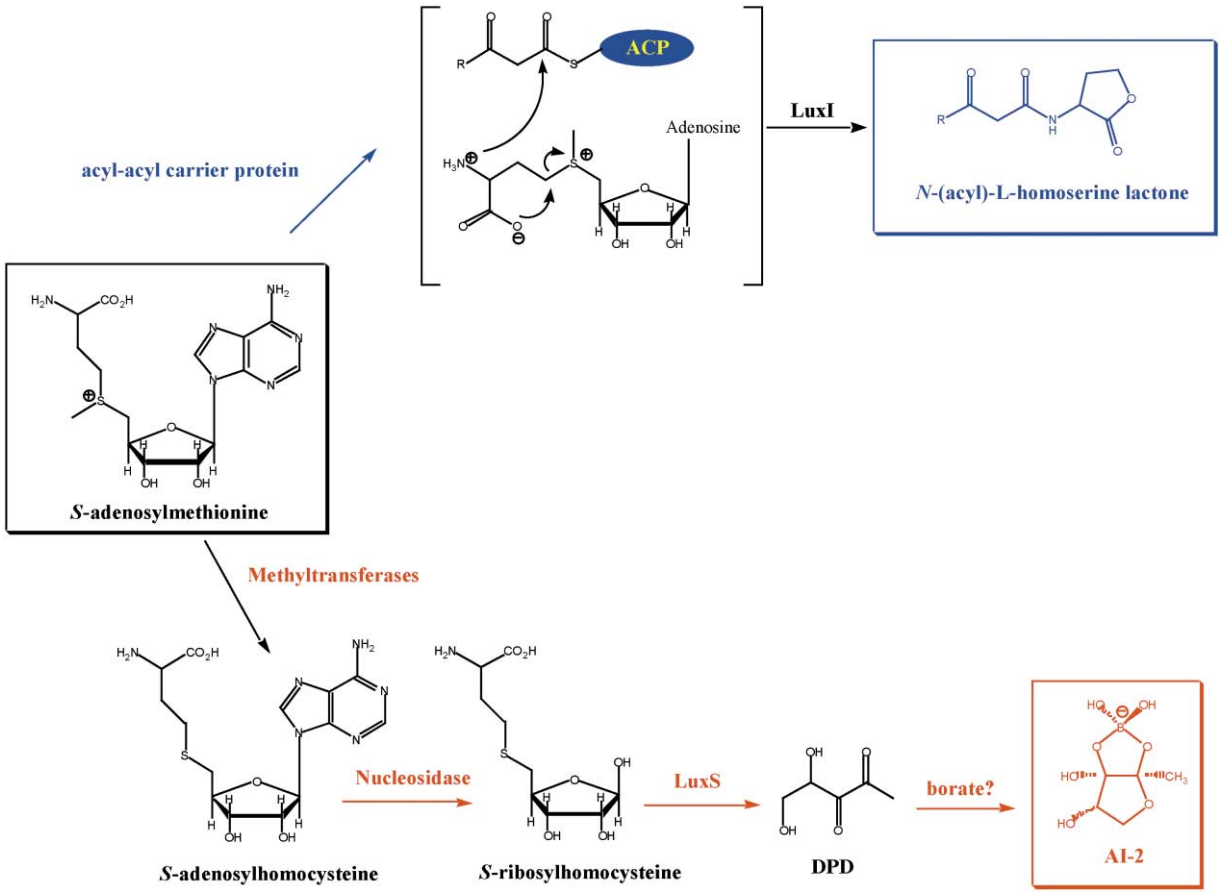

Figure 4. Biosynthesis of *N*-(Acyl)-L-Homoserine Lactones and AI-2, a Furanosyl Borate Diester

Both signaling molecules are derived from *S*-adenosylmethionine. The synthase enzymes and cosubstrates involved in the ASL and AI-2 pathways are indicated in blue and red, respectively. The mechanistic details of these transformations are still poorly understood, although structures of LuxI and LuxS enzymes have recently been determined (see main text). DPD, 4,5-dihydroxy-2,3-pentadione.

of a boron atom in AI-2 was a surprise and is quite intriguing because very little is known concerning the role of boron in biological systems, although it is known to be essential in many plants. As shown in Figure 4, it is thought that boron is added as the final step in the biosynthesis of AI-2, using 4,5-dihydroxy 2,3-pentanedione (DPD) as the precursor, which is itself derived from the cleavage of S-ribosylhomocysteine (SRH) by the enzyme LuxS (reviewed in [87]). The mechanism of this unusual enzymatic transformation remains to be elucidated.

It is well established that AI-2 plays an important signaling role in *Vibrio harveyi*, and it has been recently demonstrated that AI-2 and an uncharacterized molecule, CAI-1, also signal to control virulence in the related bacterial pathogen *Vibrio cholerae* [88, 89]. Furthermore, there is ample evidence that the biosynthetic enzyme LuxS is abundant in bacteria, with LuxS being conserved in 35 of the 89 currently available complete bacterial genomes (National Centre for Biotechnology Information [NCBI]) [87]. This has led to the suggestion that the same or similar AI-2 molecule is synthesized and signals in many different bacterial pathogens, thus serving as a form of interspecies communication for the purpose of all-inclusive bacterial quorum ensing [90].

There is accumulating evidence that AI-2 is made in many different bacterial species and that disruption of the *luxS* gene results in dramatic transcriptional changes throughout these bacteria (reviewed extensively in [87]). However, to date the signaling apparatus for AI-2 signal reception, including LuxP-homologs (other than ribose binding proteins) and the homologous two-component systems, has only been found in *V. harveyi*, *V. cholerae*, and *S. typhimurium*. Furthermore, it has been recently suggested that AI-2 (or its precursor, DPD; Figure 4) could represent a toxic byproduct of an essential metabolic process in the cell, namely the active methyl cycle [91, 92], and that this toxic metabolite could be excreted from the cell at early points of growth to be internalized later for degradation in a controlled manner. This internalization could be proceeding through a recently characterized uptake system in *S. typhimurium* [93] .

Until further studies have elucidated the signaling role of AI-2 in other bacterial species, its function as an interspecies communication signal will remain a matter of debate. If AI-2's universal role in bacterial cell-cell communication is confirmed, then this signaling pathway will become an important target for general quorum-sensing inhibition in bacteria. Along these lines, the structures of various LuxS homologs have been eluci-

dated by several groups, thereby opening the door to possible structure-based drug design of LuxS inhibitors [94–96].

Prokaryotic-Prokaryotic and Prokaryotic-Eukaryotic Communication and/or Warfare

Given the fact that intraspecies communication is so widespread in nature, it is not unexpected to discover that bacteria might compete with each other at the level of signaling and quorum sensing. We described above how *S. aureus agr* groups compete with each other at the level of ligand binding to the RHK, AgrC. It is also quite possible that an acyl-HSL produced by one bacterial species may inhibit the activity of an acyl-HSL produced by another species, especially given the fact that extending the acyl side chain from six carbons to ten or more carbons can convert agonists into antagonists in some cases (see below for further details). In addition, bacteria have evolved other, recently characterized mechanisms to compete with each other at the level of quorum sensing, all of which thus far revolve around signal modification and/or degradation.

A lactonase enzyme has been characterized, present in 2%–3% of ~800 soil bacteria tested, that is able to open the HSL ring of acyl-HSLs, thereby virtually abolishing their activity [97]. This enzyme was found in all isolates of *Bacillus thuringiensis* and in some, but not all, other isolates of bacilli. As this gram-positive bacterial species does not produce acyl-HSLs, this suggests that a type of bacterial warfare is most likely occurring at the level of signaling. Other groups have recently characterized acyl-HSL-acylases in soil bacteria or bacteria from mixed biofilms that cleave the acyl side chain off the HSL ring [98, 99]. In this regard, it is worth mentioning that some studies are finding that cross-communication with acyl-HSL signaling occurs between the bacterial pathogens *Pseudomonas aeruginosa* and *Burkholderia cepacia* in the context of mixed biofilms [100, 101], thus making it all the more likely that cooperation and competition exist in such communities in the bacterial world. This is further supported by evidence suggesting that *Salmonella typhimurium* responds to heterologous HSLs via the LuxR homolog SdiA [102].

One of the first characterized examples of eukaryotic-prokaryotic quorum-sensing warfare came from studies on the Australian macroalga *Delisea pulchra* [103]. These studies showed that certain furanone-based secondary metabolites produced in this seaweed are capable of interfering with acyl-HSL-mediated quorum sensing in *P. aeruginosa* and thus bacterial biofilm formation. The use of these algal-derived furanone compounds for QS inhibition is an active area of research (described below) not only in the area of bacterial pathogenesis but also for the prevention of fouling of ships and nets in marine waters. Collectively, this discovery and others described below have led to the hypothesis that the widespread occurrence of acyl-HSL-mediated quorum sensing has resulted in the evolution by higher organisms of specific means to either interfere with, escape from, or exploit these signaling pathways to their advantage (reviewed in [104]).

Evidence is mounting that higher organisms such as plants respond to and exploit bacterial communication. At least one eukaryotic host, the model legume *Medicago truncatula*, detects nanomolar to micromolar concentrations of different acyl-HSLs produced by both symbiotic (*Sinorhizobium meliloti*) and pathogenic (*P. aeruginosa*) bacteria, resulting in significant changes in the accumulation of over 150 plant proteins [105]. Furthermore, exposure to acyl-HSLs causes the secretion of QS-mimicking signals of unknown structure [105] which are produced by this and other plant species [106]. It has also been shown that the motile zoospores of the green seaweed *Enteromorpha* detect and respond to acyl-HSLs produced by bacteria in order to find and attach to bacterial cells in marine biofilms [107]. The mechanism by which the zoospores detect and respond to acyl-HSLs is unknown, and it is also uncertain if other marine species, such as *D. pulchra* (noted above), have similar ways of detecting acyl-HSLs. It is possible that some marine species not only can detect and respond to acyl-HSLs by attachment to bacterial cells (such as with *Enteromorpha*), but that, once attached, begin secreting QS inhibitory substances (such as with *D. pulchra*) to compete with their bacterial neighbors.

While prokaryotic-prokaryotic and eukaryotic-prokaryotic communication and/or warfare already exist in the environment, it is in mankind's nature to try to improve on Mother Nature. In this regard, plant biologists have realized the enormous potential of quorum sensing for plant communication with, and perhaps protection from, bacterial pathogens. In one instance, plants genetically modified to produce acyl-HSLs communicated with their bacterial pathogens, which actually restored the pathogenicity of a relatively large inoculum of an acyl-HSL-negative mutant of *Erwinia carotovora* [108]. In another ingenious example of plant genetic engineering, the recently characterized acyl-HSL lactonase was inserted into tobacco and potato plants, thus conferring resistance to *Erwinia carotovora* infection [109].

There is also accumulating evidence that communication exists between bacteria and animals. For example, acyl-HSLs have been shown to have immunomodulatory activities in mammalian cells both in vitro and in vivo [110–113]. Furthermore, there is one study that has shown a possible cross-communication between the epinephrine hormone signaling system and enterohemorrhagic *E. coli* O157:H7, possibly exploited by the bacterium to sense that it is within the gut and to activate genes essential for intestinal colonization [114]. The activation of quorum sensing by host signals seems counterintuitive, at least from the perspective of the bacteria, as it should be advantageous for the bacteria to remain in control of the relative amount of activating signal in parallel with the quantity of bacteria present. Otherwise, early activation of quorum sensing might provoke a host response when the bacteria are badly outnumbered. Further studies will need to address the relevance and importance of such cross-communication between the eukaryotic host and bacteria.

Chemical Probes and/or Inhibitors of Bacterial Communication

Inhibitors of virulence that do not have detrimental effects on bacterial growth can be useful chemical probes

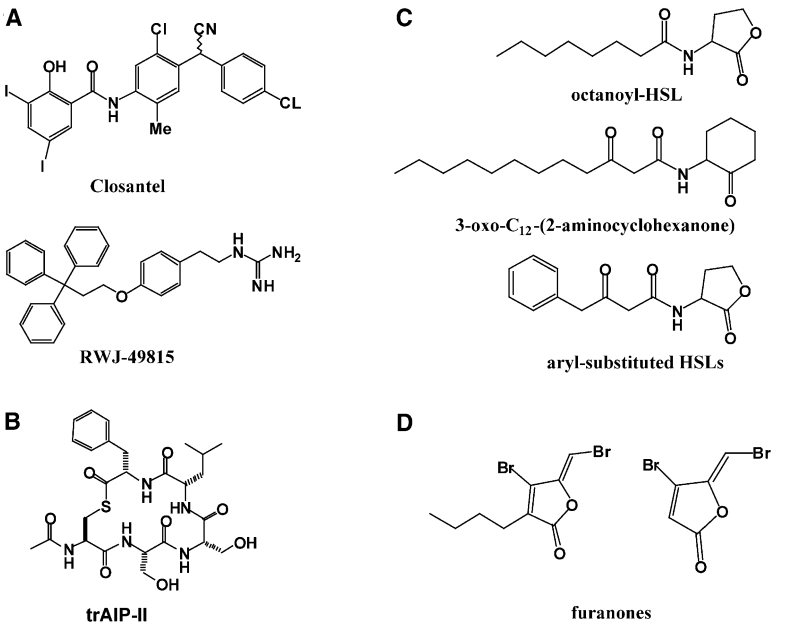

Figure 5. Inhibitors of Bacterial Communication

(A) Histidine kinase inhibitors: two examples of compounds identified from in vitro screens are shown.

(B) Global inhibitors of virulence in *S. aureus*: TrAIP-II (a truncated derivative of the AIP-II thiolactone peptide) containing amino acid sequence Ac-*cyclo*[CSSLF].

(C) Specific inhibitory acyl-HSL analogs: 3-oxo-C12-(2-aminocyclohexanone), aryl-substituted acyl-HSLs, and *N*-(octanoyl)-HSL (see main text for further details).

(D) Furanones as acyl-HSL QS inhibitors.

for studies on bacterial virulence and for possible drug development. While many studies have focused on screening for compounds that target traditional pathways or particular virulence determinants, such as adhesion or type III secretion [115] (reviewed in [116]), other groups have begun to focus on identifying inhibitors of regulatory and/or quorum-sensing-based pathways. We will focus on the latter efforts.

Histidine Kinase Inhibitors

The widespread prevalence of two-component signaling in bacterial pathogens, some of which are considered essential for growth, has prompted substantial interest within the pharmaceutical and academic sectors in the development of broad-spectrum two-component inhibitors. For example, of the 13 two-component systems found in the genome of *S. pneumoniae*, eight kinase-regulator pairs are required for virulence in a murine respiratory tract model, thus highlighting the central role of these systems in bacterial pathogenesis [39]. Initial efforts have focused on screening assays based on the in vitro autophosphorylation reactions of sensor kinases and subsequent phosphotransfer to their cognate response regulators. Other efforts have focused on bacterial cell-based assays with downstream reporter genes used to monitor two-component signaling activity in the presence of tested compounds. From such studies, a number of compounds have emerged [117], only some of which have been thoroughly characterized with respect to their mechanism of action in bacteria. Indeed, some studies have demonstrated that many HK inhibitors identified using high-throughput in vitro screens act nonspecifically in cells either by disrupting membrane integrity [118] or causing protein aggregation, in the case of Closantel and RWJ-49815 (Figure 5) [119]. However, the recent crystal structure of the nucleotide binding domain of a sensor kinase (CheA) from *Thermotoga maritima* in complex with ADP and various analogs of ATP bodes well for future structure-based design of inhibitors specifically targeting the autokinase domain of sensor kinases and avoiding the homologous domains of host ATPases [120]. For comprehensive reviews of the growing field of two-component inhibition, see [117] and [121].

Rationally Designed Antagonists of Receptor-Ligand Interactions: The S. aureus AIPs

Recent evidence from the *agr* system *in S. aureus* suggests that specific and extracellular antagonism of two-component signaling at the level of receptor-ligand binding could represent a generalizable approach to virulence inhibition [23]. This is perhaps possible in *S. aureus*, because *agr* mutants are greatly attenuated for virulence in several animal models of infection (reviewed in [10]), suggesting that blockade of *agr* signaling in vivo might have therapeutic utility. Toward this end, the availability of naturally occurring peptide-based *agr* antagonists (see above) has opened the door to peptidomimetic and/or small molecule drug discovery efforts, much akin to what has been done so successfully in the pharmaceutical industry for the inhibition of G protein-coupled receptors and other hormonally based signaling pathways [122]. In fact, the *agr* system is one of the few bacterial systems described thus far where interference with quorum sensing by the addition of an exogenous substance, i.e., a cross-inhibitory AIP, has been shown to attenuate virulence in an animal model of infection [16]. In this study, a subcutaneous abscess mouse model of *S. aureus* infection was used to demonstrate that coinjection of *agr* group I *S. aureus* with the antagonist, AIP-II, led to greatly attenuated abscess formation. Other researchers have suggested that another molecule, known as RIP, can inhibit staphylococcal biofilm formation and infections in vivo ([123] and references therein), although the validity of some of these experiments has been called into question [124].

Structure-activity studies on the AIPs [16, 20] have provided important insights that have allowed the rational design of AIP analogs that are global inhibitors of *S. aureus* virulence (one of these is shown in Figure 5).

This is best illustrated through our work on AIP-II, where we found that residues in the tail of the molecule are critical for activation of the cognate AgrC-II receptor. Based on this finding, we reasoned that removal of the tail would afford a peptide that could still bind the receptor but no longer activate the signaling response. Accordingly, we demonstrated that the truncated version of AIP-II (trAIP-II) was an inhibitor of all four *S. aureus* groups as well as some other *Staphylococci* species tested [20]. Importantly, cyclic peptides such as trAIP-II are excellent starting points for peptidomimetic-type strategies designed to improve the bioavailability or potency of the initial compounds.

Inhibitors of Acyl-HSL Signaling

Since the discovery of acyl-HSLs, many investigators have performed structure-activity relationship analyses of them, with an initial focus on the acyl chain [125–129]. These studies collectively demonstrated that (1) the length of the acyl chain can be altered somewhat with minimal effect on activity; (2) dramatic changes in acyl side chain length and/or changes at the 3 position eliminate activity; and (3) extended acyl chain geometry is required, as constrained analogs are inactive. One such study [128] showed that the autoinducer of TraR in *Agrobacterium tumefaciens*, *N*-(3-oxooctanoyl)-L-homoserine lactone, can be converted into an antagonist of similar potency by simply replacing the carbonyl at the 3 position with a methylene to form *N*-(octanoyl)-L-homoserine lactone (Figure 5). This led to the conclusion that the 3-oxo group is unnecessary for TraR binding but that it must play an important role in TraR activation.

A more recent study [130] involved the synthesis of 22 novel acyl-HSLs bearing various substituents at the C4 position of the acyl chain of either *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) or C6-HSL. These analogs were then assayed systematically for activation or inhibition of bioluminescence signaling in *V. fischeri*. Dose-response curves for activation and inhibition in the presence of the natural agonist 3-oxo-C6-HSL were generated, allowing comparisons between compounds to be made. This analysis revealed an interesting difference between alkyl- versus aryl-substituted analogs, in which most alkyl analogs retained some potency as agonists, while most aryl-substituted analogs were converted into antagonists (Figure 5). The authors surmise that a specific inhibitory interaction occurs between the substituted aryl group and some residue(s) in the acyl-HSL binding pocket of the LuxR protein, which cannot occur with the alkyl substituents.

A number of structure-activity studies have also been performed on the macrocyclic part of acyl-HSLs. Reminiscent of the situation with the *S. aureus* AIPs, the nature of the heteroatom in the ring appears to be important for biological activity. For example, the thiolactone analog of the *P. aeruginosa* autoinducer *N*-(3-oxo-dodecanoyl)-L-homoserine lactone retained activity, while the corresponding lactam analog had markedly reduced activity [127]. Several studies have also explored the effects of adding substituents onto, or altering the size of, the 5-membered HSL ring [131, 132]. For example, Nielsen et al. focused on synthesizing molecules with substituents in the 3 and 4 positions of the HSL ring of 3-oxo-C8-HSL, inspired in part by the resulting structural similarity with furanones (see below). They devised a new synthetic strategy to incorporate hydroxyl groups into these positions, which could then serve as handles to introduce more bulky substitutions via acylation, carbamoylation, or alkylation [131]. All molecules were tested for activity in a LuxR-based QS reporter system. The 3-hydroxy *cis* or *trans* analogs were only very weak activators at high concentrations and were in fact also inhibitors at high concentrations, leading one to speculate that they are acting as either partial agonists or in some nonspecific manner. On the other hand, the 4-hydroxy *cis* or *trans* analogs were activators, with the *cis* analog being as potent as the control acyl-HSL agonist, and the *trans* analog losing one order of magnitude in potency. Substitution with carbamate vastly decreased activity in all compounds tested. The authors conclude that further substitution at these positions should yield compounds with dramatically increased potency [131].

Suga et al. synthesized a library of 96 acyl-HSL analogs in which the macrocycle was systematically altered [132, 133]. These compounds were tested against two LuxR-type proteins, LasR and RhlR, both from *P. aeruginosa*. These studies revealed surprising differences in the tolerance of these two related receptors to changes in the HSL ring. For example, cyclohexanone analogs of the respective acyl-HSL's were inactive in LasR activation but active against RhlR, thus leading the authors to conclude that not all LuxR-type proteins recognize the HSL moiety in the same way. This could be viewed as surprising given that the two residues (Asp70 and Trp57) in TraR involved in polar interactions with the lactone moiety of acyl-HSLs are strictly conserved in LuxR-type proteins [61, 62], thus suggesting that the binding of the lactone moiety of N-acyl HSLs to LuxR-type proteins should be very similar in the homoserine lactone cavity. However, the overall sequence identity between LasR and TraR is only $\sim$17%, and the results of Suga et al. suggest that subtle differences in the binding cavity between the two proteins may result in dramatic effects on activation by certain analogs. Lastly, the authors show that some of the tested analogs, e.g., 3-oxo-C12-(2-aminocyclohexanone) (see Figure 5), are antagonists of quorum sensing in vitro, including against biofilm formation [132]. Parenthetically, it is assumed by this study and others that these antagonists function by competitive antagonism; however, recent structural studies on TraR suggest that acyl-HSL's are buried deeply in the protein and function by stabilizing an unstable protein (see above) [61, 62]. It is difficult to envision with a model of competitive antagonism how these antagonists gain access to this deep pocket to displace the acyl-HSL. An alternative mechanism is that these antagonists bind deeply within newly synthesized LuxR-type proteins, thus preventing incorporation of acyl-HSLs and thereby keeping the proteins in an unstable state. Future efforts in the field should focus on clarifying the mechanism of antagonism by these compounds and also on showing whether these or other compounds have efficacy in vivo against bacterial infections.

Furanone compounds are also being tested for inhibition of quorum sensing (see above), with evidence accumulating of in vitro efficacy [134, 135] and, most notably,

in vivo efficacy in a mouse pulmonary infection model with *P. aeruginosa* infection [136] (see Figure 5 for representative compounds). It had been assumed that furanones act by competing with acyl-HSL's for binding to LuxR-type proteins. However, the mechanism of this inhibition as well as the inhibition observed with some inhibitory acyl-HSL analogs has recently received renewed attention in light of studies suggesting that acyl-HSLs function by stabilizing unstable LuxR-type proteins [64]. One group has reanalyzed some of their earlier data [137] and has now demonstrated that, rather than displacing the AHL signal from LuxR, the furanones function by increasing LuxR turnover [138]. The authors suggest that halogenated furanones interact with LuxR, producing conformational changes that result in rapid proteolytic degradation of the complex; however, this complex was not detected even when proteolysis was halted in the cell to trap it out. Therefore, the precise structural mechanism by which furanones stimulate LuxR turnover remains somewhat unclear, especially given the fact that furanone compounds lacking the acyl side chain appear to be more active than those containing it (Figure 5) [138].

Conclusions

It is apparent that scientists have barely skimmed the surface in terms of understanding communication in the bacterial world. For example, given the complexity of two-component signaling in gram-positive bacteria, with at least 17, 9, 13, and 29 putative histidine kinase-response regulator pairs in *S. aureus, E. faecalis, S. pneumoniae*, and *B. subtilis*, respectively, along with untold numbers in many other gram-positive and gram-negative bacteria, it is obvious that currently characterized RHK ligands are vastly under-represented. As discussed above, there are many other signaling systems being discovered in gram-negative bacteria, with plenty more to be discovered. Furthermore, many of these new molecules will signal through novel uncharacterized proteins, as has already been suggested with the case of cytolysin induction in *E. faecalis* [52].

The past decade has seen the emergence of a new way of thinking about bacteria. Rather than existing as individual cells in the environment (and in biologists' culture flasks, for that matter!), bacteria grow in communities (notably biofilms) dominated by diversity, hence requiring forms of intra- and interspecies communication. The scientific community has only just begun to understand some of the languages of a few members of the bacterial world. Given that the vast majority of bacteria from the soil and deep oceans are not even culturable, it will remain a major challenge to understand the bacterial world literally all around us. Efforts are ongoing to mine the genomes of the bacterial world for unusual and interesting natural products [139, 140], which have yielded and will continue to yield new avenues of therapy against human infectious and other diseases. It is not yet proven that understanding bacterial communication will directly lead to new therapies against bacterial infections [116]; however, studying the modes of chemical communication that exist in the bacterial world will surely further our understanding of the extraordinary diversity that surrounds us.

Acknowledgments

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