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Exploiting Quorum Sensing To Confuse Bacterial Pathogens

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SUMMARY

Cell-cell communication, or quorum sensing, is a widespread phenomenon in bacteria that is used to coordinate gene expression among local populations. Its use by bacterial pathogens to regulate genes that promote invasion, defense, and spread has been particularly well documented. With the ongoing emergence of antibiotic-resistant pathogens, there is a current need for development of alternative therapeutic strategies. An antivirulence approach by which quorum sensing is impeded has caught on as a viable means to manipulate bacterial processes, especially pathogenic traits that are harmful to human and animal health and agricultural productivity. The identification and development of chemical compounds and enzymes that facilitate quorum-sensing inhibition (QSI) by targeting signaling molecules, signal biogen-

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esis, or signal detection are reviewed here. Overall, the evidence suggests that QSI therapy may be efficacious against some, but not necessarily all, bacterial pathogens, and several failures and ongoing concerns that may steer future studies in productive directions are discussed. Nevertheless, various QSI successes have rightfully perpetuated excitement surrounding new potential therapies, and this review highlights promising QSI leads in disrupting pathogenesis in both plants and animals.

INTRODUCTION

Current Obstacles in the Treatment of Bacterial Pathogens

We find ourselves facing a significant moment in modern health care where many antibiotics have lost their effectiveness in treating life-threatening and debilitating diseases. Meanwhile, as the world's population continues to increase rapidly, agricultural markets are tasked with meeting worldwide nutritional needs. The expanding global distribution of crops has placed an added incentive on finding new ways to increase production and enhance disease resistance of plants and to extend the shelf lives of plant-derived products. Unfortunately, bacterial pathogens have outpaced our abilities to manage them.

There is a critical need to discover new antimicrobial compounds and to identify new methods for disease prevention and treatment. Drugs recently developed to thwart emerging antibiotic resistances, such as resistance to vancomycin, linezolid, and the latest beta-lactams, have themselves already lost effectiveness against some bacterial strains (1–3). Even more discouraging, development of new drug leads has slowed dramatically over the past 10 years, and newer drugs that have been successfully developed are strictly reserved to treat only the most serious infections, so as not to repeat overusage mistakes of the past (4). It is therefore more important than ever to develop therapies that will provide sustainable, long-term effectiveness against bacterial pathogens. Since current therapies rely on antibiotic treatments that result in death of invading bacteria and their clearance from the body, they place a strong selective pressure (arguably the strongest possible) on bacteria to develop resistance mechanisms. Generating new therapies that minimize pressures selecting for resistance would, in theory, be possible by avoiding growth-inhibitory effects. Newer strategies have sought to target components of bacteria that are responsible for pathogenesis rather than targeting components that are essential for growth and, as such, have garnered the name "antivirulence" or "antipathogenesis" therapies (for a review, see reference 5). Antitoxin therapies and some vaccines fit into this design for new treatments, and these strategies will undoubtedly continue to lead to new effective products. This review, however, focuses on a similar strategy which aims to interfere with the coordinated regulation of virulence factor production, rather than the virulence factors themselves, and summarizes the development and current status of strategies that target bacterial communication known as quorum sensing.

Quorum Sensing and "Antivirulence" Therapies

Blocking communication of one's adversaries serves as an effective tactic to disrupt cooperative actions among individuals or groups. The knowledge gained over the last 40 years that bacteria commonly benefit from social interactions and intercellular signaling presents an opportunity to interfere with their ability to coordinate efforts to invade their hosts, whether human, animal, or plant. In fact, it is now realized that communication interference naturally exists in the microbial world, and it stands to reason that this ploy to gain an advantage over competitors was originally invented by bacteria.

Cell-to-cell communication in bacteria (quorum sensing [QS]) relies on small, secreted signaling molecules, much like hormones in higher organisms, to initiate coordinated responses across a population. Discussed in the next section are common paradigms for several well-studied systems. In many cases, the responses elicited by QS signals are ones that contribute directly to pathogenesis through the synchronized production of virulence determinants, such as toxins, proteases, and other immune-evasive factors. Additionally, QS can contribute to behaviors that enable bacteria to resist antimicrobial compounds or drugs, such as biofilm development. If these efforts to coordinate were blocked, it is theorized that bacteria would lose their ability to mount an organized assault on the host's defense or immune system or would be less able to form organized community structures that promote pathogenesis, such as biofilms. For some bacteria, working together as a group provides a means to build a defense or to surmount a barrier that individual bacterial cells find impossible to achieve. Blocking interactions between bacteria would effectively force bacteria to live as individuals fending for themselves.

One key advantage proposed in targeting quorum sensing is based on the premise that a treatment that does not suppress growth of a cell will not exert a selective pressure to develop resistance to that treatment. Quorum sensing is not an essential process, and QS mutants in general have not displayed growth defects. Granted, interfering with the regulation of virulence factor production will likely reduce fitness for survival in certain situations, but if maintaining a delicate control over quorum-sensing-regulated genes is critical to the cell (and the exquisite layers of complexity found in many quorum-sensing pathways bolster this assumption), then developing resistance mechanisms against quorum-inhibiting therapies may be a difficult proposition for bacteria, which could help promote long-term efficacy of anti-QS therapies.

QUORUM-SENSING SYSTEM ARCHITCTURES

All quorum-sensing systems utilize small, secreted signaling molecules known as autoinducers (AIs). From an historical perspective, the most commonly studied autoinducers belong to one of the following three categories: acylated homoserine lactones (AHLs), used by Gram-negative bacteria (also sometimes referred to as autoinducer-1 [AI-1]); peptide signals, used by Gram-positive bacteria; and autoinducer-2 (AI-2), used by both Gram-negative and Gram-positive bacteria. There are also other quorum-sensing signals that go beyond these classes, including *Pseudomonas* quinolone signal (PQS), diffusible signal factor (DSF), and autoinducer-3 (AI-3), and new molecules will undoubtedly be discovered as the study of quorum sensing expands to species of bacteria yet to be investigated. Although this review focuses on quorum-sensing inhibition (QSI) (also referred to as "quorum quenching") as a therapeutic approach, understanding the potential mechanisms of quorum quenching first requires knowledge of the basic quorum-sensing systems that are utilized by bacterial pathogens. Therefore, we first offer brief summaries of quorum-sensing systems utilizing various autoinducer molecules. We also offer a more focused summary of the quorum-sensing system architecture of *Pseudomonas aeruginosa*, as this

TABLE 1 Quorum-sensing systems utilized by selected bacteria and associated phenotypes

Organism	Signal	Synthase(s)	Receptor(s)	Selected phenotypes	Reference(s)
P. aeruginosa	C4HSL	RhlI	RhlR	Exoenzymes, virulence, biofilm formation, motility, iron acquisition, pyocyanin	6, 7
	3OC12HSL	LasI	LasR, QscR		
	HHQ, PQS	PqsA to -D, PqsH	PqsR		
P. syringae	3OC6HSL	AhlI	AhlR	EPS, plant colonization	8
E. carotovora	3OC6HSL	CarI	CarR, ExpR, VirR	Carbapenem, exoenzymes, virulence	9
P. stewartii	3OC6-HSL	EsaI	EsaR	Adhesion, EPS, plant colonization	8
B. glumae	C8HSL	TofI	TofR	Motility, toxoflavin, lipase, virulence	10
A. tumefaciens	3OC8HSL	TraI	TraR	Ti plasmid conjugation, virulence	8
C. violaceum	C6HSL	CviI	CviR	Exoenzymes, antibiotics, violacein	11
S. liquefaciens	C4HSL	SwrI	SwrR	Swarming motility, biofilm formation	12
V. harveyi	3OHC4HSL	LuxM	LuxN	Bioluminescence, siderophores, protease and EPS production, virulence	13, 14
	AI-2	LuxS	LuxP		
	CAI-1	CqsA	CqsS		
V. cholerae	AI-2	LuxS	LuxP	Virulence, biofilm formation, EPS	13, 14
	CAI-1	CqsA	CqsS		
V. fischeri	3OC6HSL	LuxI	LuxR	Bioluminescence, host colonization, motility	13
	C8HSL	AinS	AinR		
	AI-2	LuxS	LuxP		
E. coli/*S.* Typhimurium	3OC8HSL[a]	NA[d]	SdiA	Motility, acid resistance	15
	AI-2	LuxS	LsrB	*lsr* operon expression (AI-2 uptake)	16
	AI-3	Unknown	QseC	Virulence, motility, biofilm formation	17
S. aureus	AIP	*agrD*[b]	AgrC	Virulence, exotoxins, biofilm dispersal	18
E. faecalis	GBAP	*fsrD*[b]	FsrC	Gelatinase, protease	19
	cCF10	*ccfA*[b]	PrgX	Adhesion, conjugation	20
S. pneumoniae	CSP	*comC*[b]	ComD	Competence, virulence, autolysis	21, 22, 23
B. thuringiensis	PapR	*papR*[b]	PlcR	Exoenzymes	20
	NprX	*nprRB*[b,c]	NprR	Toxins, sporulation, necrotrophism	24, 25
X. campestris	DSF	RpfB, RpfF	RpfC	Virulence, biofilm dispersal, EPS	26

[a] SdiA can also bind C4HSL, C6HSL, 3OC6HSL, and 3OC12HSL but does so with less affinity than 3OC8HSL.
[b] The peptide signal is genetically encoded by the indicated gene.
[c] The gene encoding NprX-containing propeptide is also referred to in the literature as *nprX*.
[d] NA, not applicable.

species has been used extensively for studies on quorum-sensing inhibition for reasons discussed below. QS system components and associated phenotypes of various bacterial species discussed in this review are outlined in Table 1, and illustrations of several of these systems are provided in Fig. 1. For the sake of space, this QS systems overview is broad in nature, so for more detailed discussions we refer the reader to other recently published reviews and the relevant primary literature cited below.

AHL-Based Quorum Sensing

N-Acyl homoserine lactones (AHLs) are the molecules most commonly used by Gram-negative bacteria as quorum-sensing autoinducers. These molecules are comprised of an invariant homoserine lactone (HSL) ring attached to an acyl chain that can vary in length between 4 and 18 carbon atoms. In addition to acyl chain length, AHLs can also differ in the saturation state of the acyl chain and the oxidation state at position 3 (Fig. 2). Generally speaking, AHLs are biosynthesized by members of the LuxI family of AHL synthases using the substrates *S*-adenosylmethionine (SAM) and an acylated acyl carrier protein (acyl-ACP) (27). Exceptions to this are LuxM and AinS of *Vibrio harveyi* and *Vibrio fischeri*, respectively, which catalyze the same reaction but do not share homology with LuxI-type proteins (28). In general, each AHL synthase predominantly makes a single type of AHL, although some synthases also produce additional AHLs in smaller amounts. Following synthesis, AHLs generally diffuse freely across the cell envelope and accumulate in the local environment. Alternatively, there is evidence that several AHLs may be actively transported across the cell membrane in certain bacterial strains (29–31). Once a critical concentration threshold is achieved, interaction between the AHL and a LuxR-type receptor protein in the cytoplasm of the cell becomes favorable. LuxR family members are transcriptional regulators whose DNA-binding activities change upon ligand interaction, resulting in modulation of target gene regulation in response to AHL accumulation. A straightforward example of LuxI/R signaling is in *Chromobacterium violaceum*, which utilizes CviI, CviR, and the AHL C6HSL to regulate violacein production (Fig. 1A) (11). Alternatively, some AHLs are detected by membrane-bound sensor kinases, such as LuxN of *V. harveyi*, that initiate phosphorelay signaling cascades following ligand binding (32). In either case, each AHL receptor protein demonstrates some degree of AHL binding specificity based on the length, saturation, and oxidation of the AHL acyl chain. Accordingly, each bacterial species carries a cognate synthase/receptor pair that produces and responds to a specific AHL molecule. Exceptions to this are species that utilize multiple synthase/receptor pairs that generate and respond to distinct AHL molecules, as is the case for *P. aeruginosa* (Fig. 1B) (7), as well as species that carry so-called "solo" LuxR-type receptors that have no cognate LuxI-type synthase, such as QscR of *P. aeruginosa* and SidA of *Escherichia coli* (33).

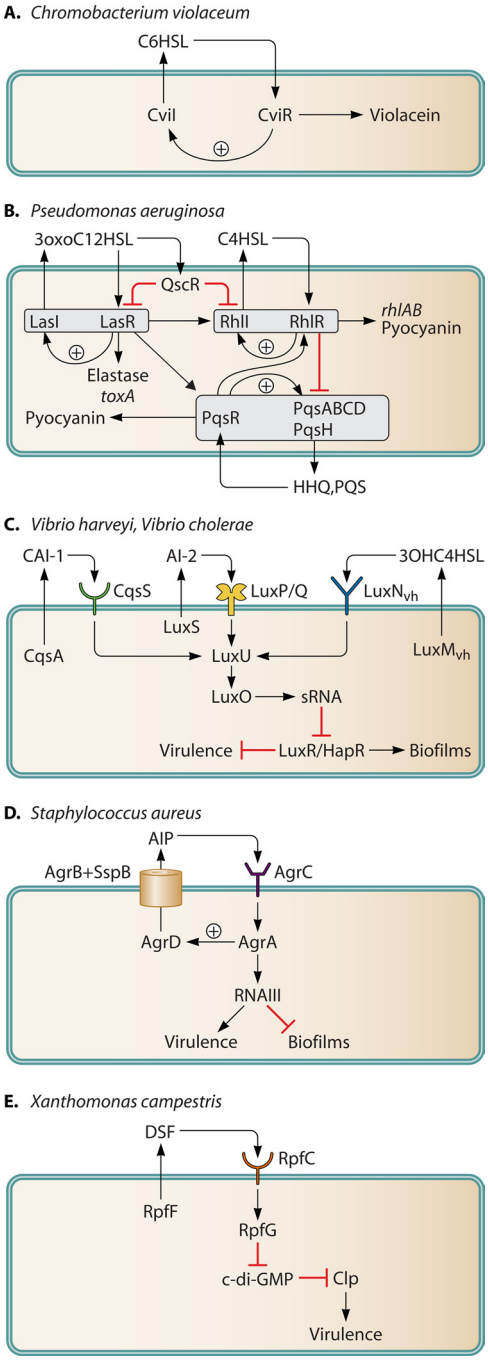


FIG 1 Simplified schemes of five quorum-sensing networks. Primary synthases, receptors, and other regulatory proteins are arranged around each cell to illustrate regulatory pathways. Arrows imply information flow, whereas T-bars indicate negative regulation. Arrows labeled "+" indicate documented positive feedback. LuxN and LuxM are unique to *Vibrio harveyi*, as indicated with the subscript 'vh' in panel C.

Peptide-Based Quorum Sensing

Gram-positive bacteria do not harbor LuxI or LuxR homologues and instead utilize modified oligopeptides as autoinducer molecules. These are genetically encoded and are generated ribosomally within the cell. Specialized transporters are responsible for active transport of these peptides out of the cell due to their inability to permeate the cell membrane. At points between translation, export, and detection, peptides are subject to various modification events, including processing and/or cyclization. Detection of these peptide signals can occur either at the surface of the cell or intracellularly. Many peptide autoinducers known to date are detected by a membrane-bound sensor kinase, which switches its kinase/phosphatase activity in response to interaction with peptide, which alters the phosphorylation state of the cognate response regulator and ultimately results in activation or repression of QS target genes. Systems utilizing extracellular detection include the *agr* system of *Staphylococcus aureus* and the *fsr* system of *Enterococcus faecalis*, which both control virulence factor production. The *agr* system of *S. aureus* centers around cyclic autoinducing peptides (AIPs) belonging to four distinct groups (Fig. 2) that interact with cognate AgrC sensor kinases of the same group to regulate exotoxin production and biofilm dispersal (18). The *fsr* system utilizes a different cyclic peptide, GBAP (gelatinase biosynthesis-activating pheromone) (Fig. 2), which is detected by the sensor kinase FsrC and induces production of gelatinase (19). Other bacteria utilize linear peptide autoinducers that are detected extracellularly, including the competence-inducing QS system of *Streptococcus pneumoniae* which is mediated by the competence-stimulating peptide (CSP) (Fig. 2) (22). Alternatively, some linear-peptide-based QS systems actively transport the autoinducers back into the cell where the peptide signal can interact directly with a cognate regulator to alter target gene expression. Such is the case for the PrgX system of *E. facaelis* (20) and the PlcR and NprR systems of *Bacillus thuringiensis* (20, 24, 25) (Fig. 2). As no investigation of quorum quenching against intracellular peptide-regulated systems has been published to date, these systems will not be discussed further in this review.

AI-2-Based Quorum Sensing

AI-2 is generated from the precursor *S*-adenosylhomocysteine (SAH) by the sequential enzymatic activities of 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) (also known as Pfs) and the metalloenzyme LuxS (34). The molecule resulting from these reactions is 4,5-dihydroxy-2,3-pentanedione (DPD), which is unstable in aqueous solution and undergoes spontaneous rearrangement into multiple interconvertible cyclic furanone compounds that, as a group, are termed AI-2. AI-2 is thought to freely diffuse out of both Gram-negative and Gram-positive bacteria and, as is the case for all other QS signals, accumulates in the extracellular milieu. Many species produce and respond to AI-2, and AI-2 receptors have been identified in *V. harveyi* and *Salmonella enterica* serovar Typhimurium. *V. harveyi* detects a boric acid-complexed form of AI-2 via the LuxP/LuxQ receptor/sensor kinase complex (Fig. 2) (35, 36). In contrast, the *S.* Typhimurium transporter LsrB interacts with a nonborated form of AI-2, leading to its internalization, phosphorylation, and interaction with the cytoplasmic transcriptional regulator LsrR (Fig. 2) (37). Thus, different bacterial species can detect different forms of AI-2, and detection can take place either extra- or intracellularly, depending on the bacterium.

Quorum-Sensing Systems Utilizing Other Autoinducers

As mentioned above, in addition to the three main categories of autoinducers, there are also other autoinducer molecules that do not fit within these classes. 2-Heptyl-3-hydroxy-4(1*H*)-quinolone (*Pseudomonas* quinolone signal [PQS]) and its precursor 2-hep-

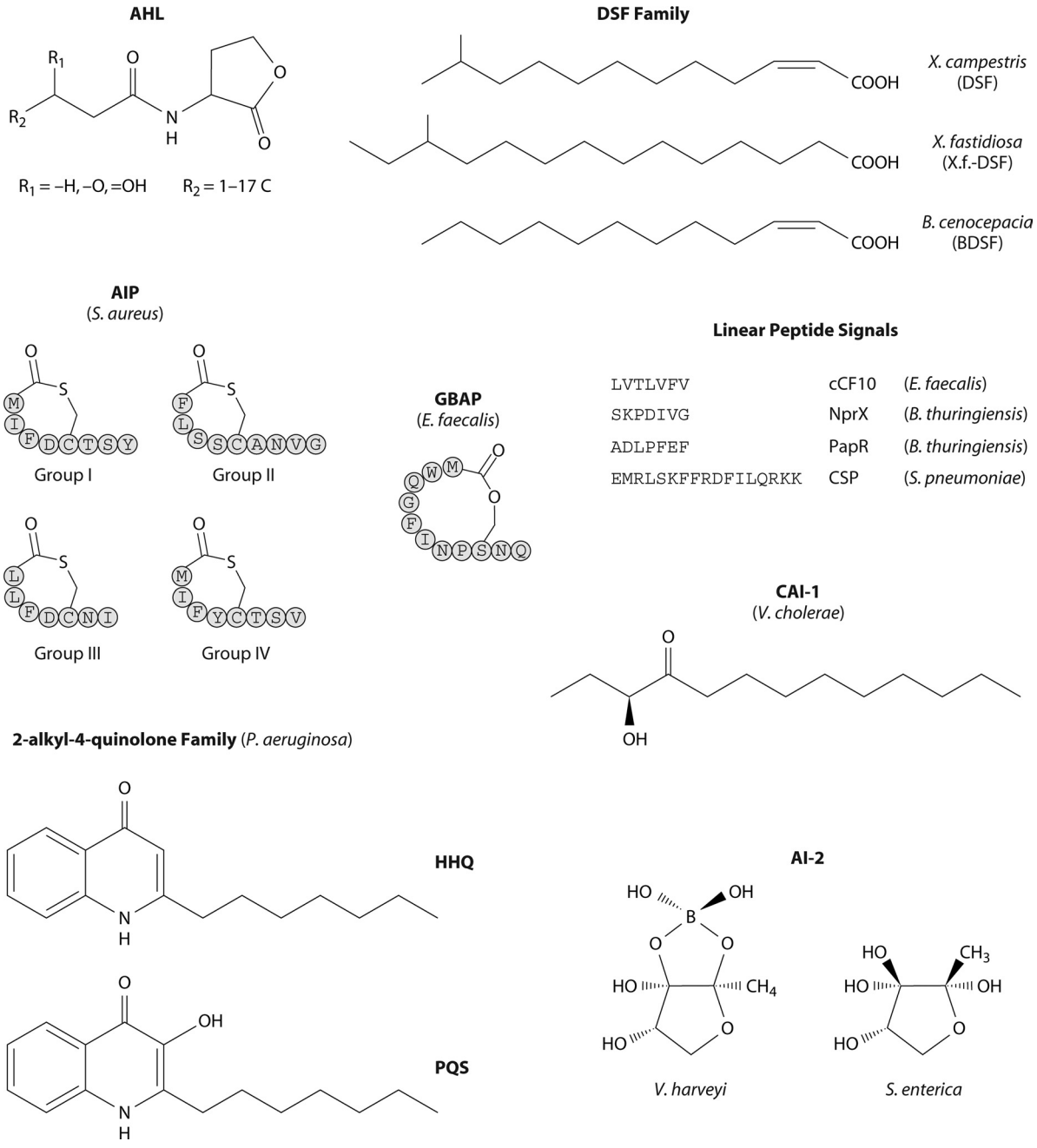


FIG 2 Structures of selected QS signal molecules.

tyl-4(1*H*)-hydroxyquinoline (HHQ) (Fig. 2) are the biosynthetic products of the *pqsABCDE* operon, which mediates the condensation of anthranilate and a β-keto-fatty acid. Both PQS and HHQ act as QS autoinducers via their interaction with the transcriptional regulator PqsR, which results in modulation of target gene expression (Fig. 1B). The only structural difference between the two molecules, an additional hydroxyl group added to the 3′ carbon atom of HHQ by the enzyme PqsH, appears to be important for the ability of PQS to function in iron acquisition in addition to its role in quorum sensing. Although only *P. aeruginosa* produces PQS, other species of *Pseudomonas* as well as *Burkholderia* spp. utilize HHQ as a quorum-sensing signal (6, 7).

The signaling molecule *cis*-11-methyl-2-dodecenoic acid (diffusible signal factor [DSF]) was originally identified in *Xanthomonas campestris* pv. campestris but has since been determined to belong to a family of QS signals utilized by a variety of bacterial species, including *Burkholderia cenocepacia* and *Xylella fastidiosa* (Fig. 2). Production of DSF or *B. cenocepacia* DSF (BDSF) requires RpfB of *X. campestris* or its homologue Bcam0581 of *B. cenocepacia*, respectively (26, 38), and it was recently shown that BDSF is generated from the acyl carrier protein (ACP) thioester of 3-hydroxydecanoic acid by sequential Bcam0581-catalyzed dehydration and thioester cleavage reactions (38). DSF family signal detection appears to differ between species, although both currently

known mechanisms share the common feature of altering intracellular levels of cyclic di-GMP (c-di-GMP) (26, 39). Upon accumulation of DSF in the extracellular milieu of *X. campestris*, it interacts with the sensor kinase RpfC to induce a phosphorelay cascade that activates the response regulator RpfG. Activated RpfG functions to degrade c-di-GMP, and the subsequent decrease in the intracellular concentration of c-di-GMP results in activation of Clp regulators, which directly and/or indirectly regulate target gene expression (Fig. 1E). DSF signaling in *X. fastidiosa* appears to require the same genetic components; however, the precise system architecture appears to differ from that of *X. campestris* and needs further characterization (26). In *B. cenocepacia*, BDSF is detected by the receptor protein RpfR, which contains a PAS domain, a GGDEF domain, and an EAL domain. The phosphodiesterase activity of RpfR conferred by the EAL domain is stimulated upon interaction of RpfR with BDSF, thereby directly linking BDSF signal perception with reduction in c-di-GMP levels (39).

The autoinducer AI-3 is an aromatic signaling molecule produced by bacteria of the human intestinal microflora as well as certain enteric pathogens. The molecular structure of AI-3 is unknown, as is the gene responsible for AI-3 production. AI-3 detection by the enteric pathogens *Escherichia coli* and *Salmonella* Typhimurium occurs via the sensor kinase QseC, which then phosphorylates the response regulator QseB to activate transcription of target genes. The same QseC/B two-component system is also used for the bacterial detection of epinephrine and norepinephrine produced by the host, and thus it is hypothesized that the structure of AI-3 may resemble that of the two hormones (16, 17).

Other QS systems found in various *Vibrio* spp. as well as in *Legionella pneumophila* utilize α-hydroxyketones (AHKs) as signaling molecules. The *Vibrio cholerae* and *V. harveyi* genomes contain the *cqs* gene cluster, which is responsible for the production and detection of the AHK signal 3-hydroxytridecan-4-one (cholera autoinducer-1 [CAI-1]) (Fig. 2). The synthase responsible for CAI-1 production is the enzyme CqsA. CqsA uses the substrates (*S*)-2-aminobutyrate and decanoyl coenzyme A (decanoyl-CoA) to generate amino-CAI-1, which is then converted to CAI-1 in a CqsA-independent reaction. Subsequent interaction of CAI-1 with the sensor kinase CqsS results in a phosphorelay cascade involving LuxU and LuxO, two proteins which are also involved in AI-2 signaling in both species (as well as AHL signaling in *V. harveyi*). Thus, *V. cholerae* and *V. harveyi* simultaneously integrate CAI-1, AHL, and AI-2 signaling to regulate virulence-related processes via use of common downstream regulatory proteins (Fig. 1C) (40).

Quorum Sensing in the Model Pathogen *P. aeruginosa*

The human pathogen *P. aeruginosa* has been used extensively for studies on quorum-sensing inhibition for multiple reasons. First, the quorum-sensing network of *P. aeruginosa* is one of the best characterized quorum-sensing systems to date. Second, quorum sensing regulates the expression of numerous virulence-related products and has been shown to be important for *P. aeruginosa* pathogenesis in various model infection systems. Third, this pathogen poses a large burden on the medical community due to its extensive resistance to antibiotics and the current lack of effective treatment options (7), and thus discovery of quorum-quenching inhibitors amenable to therapeutic applications would be of high value. Quorum sensing in *P. aeruginosa* is a complex network comprised of at least three distinct signal/receptor pairs that function in a hierarchical fashion (Fig. 1B). *P. aeruginosa* has two LuxIR homologue pairs, LasIR and RhlIR, which produce and detect the AHL signaling molecules 3OC12HSL and C4HSL, respectively. The LasR-3OC12HSL complex induces expression of LasI as well as expression of RhlR, thereby positively regulating both AHL-based QS systems. The RhlR-C4HSL complex subsequently mediates its own autoinduction but has no direct effect on the LasR system. LasR and RhlR additionally positively and negatively regulate, respectively, expression of genes involved in the third quorum-sensing loop, which is based on the autoinducer PQS. PQS autoinduces its own production while concomitantly enhancing RhlR expression, thereby self-limiting its expression via an extended negative-feedback loop. PQS has no direct regulatory activity on the Las QS system. Each QS system also regulates various virulence factor-encoding genes, some of which are coregulated by the other QS systems. Additionally, there is also a solo LurR-type protein, QscR, that binds 3OC12HSL and subsequently inhibits both the *las* and *rhl* QS systems by multiple mechanisms (6, 7).

Categories of QSI Strategies

Although each quorum-sensing circuit utilized by a given bacterium is unique in its own right, all QS systems share a mechanism comprised of signal production, signal accumulation, and signal detection. All QS inhibitors that have been mechanistically characterized to date have been found to target at least one of these three steps. Therefore, we have organized this review by categorizing known quorum-sensing inhibitors according to the following activities: quorum-sensing signal degradation and inactivation, inhibition of QS signal biosynthesis, and inhibition of signal detection.

QS SIGNAL DEGRADATION AND INACTIVATION

One of the most extensively studied QSI strategies to date is degradation and modification of the quorum-sensing signals themselves. Most enzymes identified thus far target AHL molecules, although enzymatic inactivation of DSF, PQS, and AI-2 has also been reported. Due to the generally broad substrate specificity of proteases, enzymatic degradation of peptide-based QS signals has not garnered much consideration. A list of characterized quorum-quenching enzymes is presented in Table 2, and these are discussed in more detail below. It should be noted that Table 2 is not fully comprehensive, as many studies have identified putative autoinducer-inactivating bacterial species or genes but have not yet characterized their activity biochemically.

Enzymatic Degradation and Inactivation of AHLs

There are three known classes of enzymes which target AHL signals: lactonases, acylases, and oxidoreductases. AHL lactonases are metalloproteins (with the exception of AiiM) that hydrolyze the ester bond of the homoserine lactone ring to yield the corresponding acyl homoserine molecule (Fig. 3) (57, 85). Hydrolysis of this ester bond can also occur spontaneously at alkaline pH, and acidic conditions can restore the bond and AHL signaling activity following biological or abiotic hydrolysis (86). Lactonases generally have very broad AHL substrate specificity, likely due to the fact that the targeted homoserine lactone ring is conserved among all AHLs and the variable acyl chain makes only nonspecific interac-

TABLE 2 Characterized quorum-quenching enzymes

Group	Protein	Source	AHL specificity and preference, protein family, or enzyme class[a]	Localization, cofactor, or target signal[b]	Reference(s)
Acylases	PvdQ	*Pseudomonas aeruginosa*	≥7 carbons	Cell associated	41, 42, 43, 44
	QuiP	*Pseudomonas aeruginosa*	≥7 carbons	Cell associated	45
	AiiD	*Ralstonia* sp. strain XJ12B	3OC8-, C4-, and 3OC12-HSLs[c]	Cell associated	46
	Aac	*Ralstonia solanacearum* GMI1000	≥7 carbons	Cell associated	47
	AhlM	*Streptomyces* sp. strain M664	≥6 carbons, unsubstituted	Secreted	48
	AiiC	*Anabaena* sp. strain PCC 7120	4–14 carbons	Cell associated	49
	Aac	*Shewanella* sp. strain MIB015	≥8 carbons, unsubstituted	ND	50
	HacA	*Pseudomonas syringae* B728a	≥8 carbons; unsubstituted	Secreted	51
	HacB	*Pseudomonas syringae* B728a	≥6 carbons	Cell associated	51
	HacB	*Pseudomonas aeruginosa*	≥6 carbons	Cell associated	52
	ND	*Variovorax paradoxus*	4–14 carbons	ND	53
	ND	*Comamonas* sp. strain D1	4–16 carbons	ND	54
	ND	*Rhodococcus erythropolis*	6–14 carbons, 3-oxo-AHLs	ND	55
Lactonases	AiiA	*Bacillus* sp.	Metallo-β-lactamase superfamily	Zn^{2+}	56–57, 58, 59, 60, 61
	AiiB	*Agrobacterium tumefaciens* strain 58	Metallo-β-lactamase superfamily	Zn^{2+}	62, 63
	AttM	*Agrobacterium tumefaciens* strain 58	Metallo-β-lactamase superfamily	Zn^{2+}	63, 64, 65
	AhlD	*Arthrobacter* sp. strain IBN110	Metallo-β-lactamase superfamily	Zn^{2+}	66
	AhlK	*Klebsiella pneumoniae* KCTC2241	Metallo-β-lactamase superfamily	Zn^{2+}	66
	QsdR1	*Rhizobium* sp. strain NGR234	Metallo-β-lactamase superfamily	Zn^{2+}	67
	QlcA	Soil metagenome	Metallo-β-lactamase superfamily	Zn^{2+}	68
	AidC	*Chryseobacterium* sp. strain StRB126	Metallo-β-lactamase superfamily	Zn^{2+}	69
	QsdA	*Rhodococcus erythropolis*	Phosphotriesterase family	Zn^{2+}	70
	AiiM	*Microbacterium testaceum* StLB037	α/β-Hydrolase family	None	71
	AidH	*Ochrobactrum anthropi*	α/β-Hydrolase family	Mn^{2+}	72
	QsdH	*Pseudoalteromonas byunsanensis* strain 1A01261	GDSL hydrolase family	None	73
	BpiB01	Soil metagenome	Hypothetical protein	Zn^{2+}	74
	BpiB04	Soil metagenome	Glycosyl hydrolase family	Zn^{2+}	74
	BpiB07	Soil metagenome	Dienelactone hydrolase family	Zn^{2+}	74
	DhlR	*Rhizobium* sp. strain NGR234	Dienelactone hydrolase family	ND	67
	BpiB05	Soil metagenome	Hypothetical protein	Ca^{2+}	75
	PON1-3	Mammals (humans)	PON family	Ca^{2+}	76, 77, 78
Other enzymes	CYP102A1	*Bacillus megaterium*	Oxidoreductase	AHLs	79–80
	ND	*Rhodococcus erythropolis*	Oxidoreductase	3-Oxo-AHLs	55
	BpiB09	Soil metagenome	Oxidoreductase	AHLs	81
	CarAB	*Paenibacillus*, *Staphylococcus*, *Bacillus*, *Microbacterium*, and *Pseudomonas* spp.	Carbamoyl phosphate synthase	DSF	82
	Hod	*Arthrobacter nitroguajacolicus*	Dioxygenase	PQS	83
	LsrK	*E. coli*	Kinase	AI-2	84

[a] The AHL specificity and preference, protein family, and enzyme class are given for acylases, lactonases, and other enzymes, respectively. For the acylases, numbers refer to the preferred acyl chain length, and the preference for 3-oxo-substituted versus unsubstituted AHLs (if observed) is indicated.
[b] The localization, cofactor, and target signal are given for acylases, lactonases, and other enzymes, respectively. ND, not determined.
[c] Other AHLs were not tested.

tions within the active-site cavity of the enzyme (58). In contrast, AHL acylases hydrolyze the acyl-amide bond between the acyl tail and latone ring of AHLs in a nonreversible manner, resulting in the release of a fatty acid chain and a homoserine lactone moiety (Fig. 3) (46, 53). Unlike lactonases, acylases can exhibit substrate specificity based on the acyl chain length of the AHL and substitution on the third position of the chain (Table 2) due to a constrained binding pocket in the enzyme that must adapt structurally upon interaction with its ligand (41). The enzymes belonging to the third class, the oxidoreductases, do not degrade the AHL but rather modify it to an inactive form by oxidizing or reducing the acyl side chain (55), and these are the least abundant and least studied of the AHL-targeting enzymes identified to date.

Lactonases. The first report of biological inactivation of AHL signaling molecules was published in 2000 following the screening of more than 500 bacterial field isolates and lab strains. An AHL-inactivating isolate was characterized to be a *Bacillus* sp., and genetic studies allowed for the identification of the gene responsible for the inactivation activity, which was designated *aiiA* (autoinducer inactivation gene) (57). Although the *aiiA* gene did not share significant homology with any known sequences, it did encode an HXHXD sequence motif common to metallo-β-lactamases. Purified AiiA protein inactivated multiple AHLs *in vitro* (57), and biochemical analysis confirmed that AiiA functioned as a lactonase (85). Importantly, when cloned into the plant pathogen *Erwinia carotovora*, the *aiiA* gene reduced AHL release from the pathogen, decreased QS-controlled enzyme activities, and reduced soft rot disease symptoms on potato, eggplant, Chinese

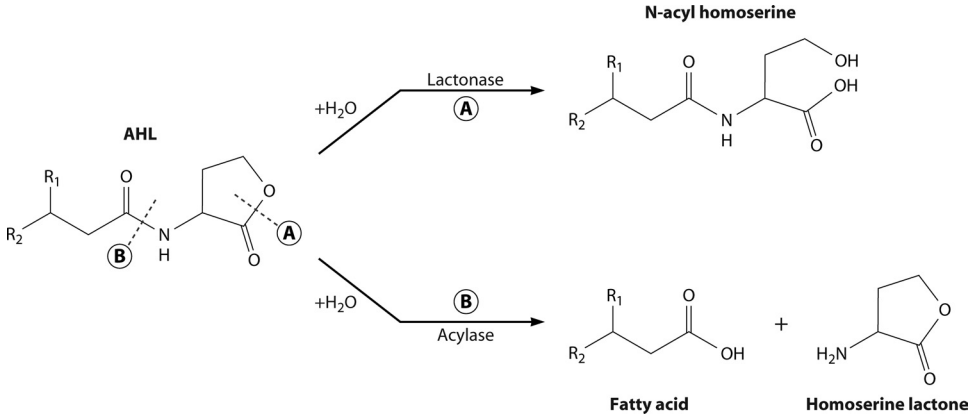


FIG 3 Mechanistic actions of AHL lactonase and AHL acylase enzymes. Dashed lines in the AHL schematic indicate the site of bond cleavage by lactonase (A) or acylase (B).

cabbage, carrot, and celery plants (57). Additionally, transgenic potato and tobacco plants carrying the *aiiA* gene showed enhanced resistance to *E. carotovora* infection (Fig. 4) (85), demonstrating for the first time the potential of quorum quenching in disease prevention. Following these initial reports, *aiiA* genes were identified in various other *Bacillus* species (56, 60, 87–89), and heterologous expression of various *aiiA* alleles in numerous pathogenic bacteria, including *P. aeruginosa* and *Burkholderia thailandensis*, reduced AHL accumulation and altered QS-dependent behaviors (60, 90). Additional studies have since provided further evidence of the utility of enzymatic quorum quenching for disease prevention and treatment in a variety of plant models using both transgenic plant species (91, 92) and wild-type (WT) and engineered AiiA-expressing bacteria (88, 89, 93–96). Importantly, the level of *aiiA* expression appears to be a crucial determinant in the efficiency of AHL degradation and subsequent success in disease prevention. Various subspecies of *Bacillus thuringiensis* exhibited diverse AHL-degrading activities, with the variation attributed to differential expression of the *aiiA* gene in each subspecies rather than different enzymatic activities. Furthermore, the biocontrol activity of an engineered AiiA-expressing *E. coli* strain was greater than that of the *B. thuringiensis* strain from which the *aiiA* gene was derived, perhaps due to low-level *aiiA* expression in the parent strain (88).

The second AHL lactonase identified, AttM, was discovered in the plant pathogen *Agrobacterium tumefaciens* following a transposon mutagenesis screen for aberrant AHL production. AttM and was only 24.8% identical at the DNA level to *aiiA* but shared the conserved HXDH motif (65). Initial studies described AttM as a lactonase induced in stationary phase that was responsible for termination of AHL-induced plasmid conjugation (65), but this may be a strain-specific pheonomenon (64). Additional studies have since indicated a specific role for AttM within the plant gall tumors characteristic of *A. tumefaciens*-mediated disease (63). AttM appears to contribute to bacterial fitness within the plant tumor (63) and may also function in interkingdom signaling between the plant and the bacterium. Salicylic acid and the nonprotein amino acid GABA, both naturally found in plants, have been shown to induce *attM* expression (97–99), and overexpression of either of these compounds in plants increases the plant's resistance to *A. tumefaciens* infection (97, 98). Additionally, proline, which is a competitive antagonist of GABA import into *Agrobacterium*, accumulates in plant gall tumors, where it may function to limit GABA-induced AHL degradation (100). Overall, the bacterial response to various plant signals, which includes differential expression of AttM, has been postulated to help modulate quorum sensing of *Agrobacterium* in such a way as to limit the unwanted elicitation of the plant immune response (100). Other

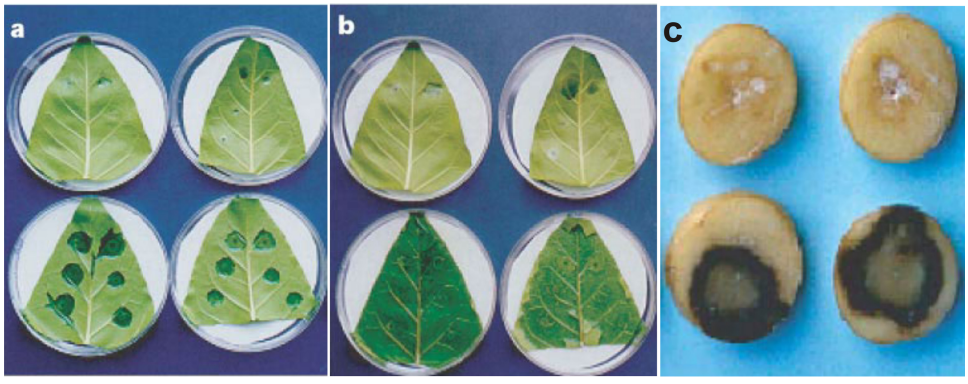


FIG 4 Lactonase AiiA expression in plants inhibits virulence effects of *Erwinia carotovora*. *E. carotovora* was inoculated onto leaves of tobacco (a and b) or potato tuber slices (c), transformed with an *aiiA* expression vector (top row) or untransformed (bottom row). The extent of tissue maceration was measured at 20 h (a), 40 h (b), and 48 h (c). (Adapted from reference 85 with permission of MacMillan Publishers Ltd.)

AHL lactonases belonging to the metallo-β-lactamase superfamilly have also been discovered in bacteria of the genera *Agrobacterium* (AiiB), *Arthrobacterium* (AhlD), *Klebsiella* (AhlK) (62, 66), and *Chryseobacterium* (AidC) (69).

Various AHL lactonases not belonging to the AiiA clade have also been identified. Metagenomic analyses faciliated the discovery of QlcA, BipB01, BipB04, BipB05, and BipB07 (68, 74, 75), and screens for AHL-degrading bacteria led to the identification of QsdA of *Rhodococcus erythropolis* strain W2 (70), AiiM of *Microbacterium testaceum* (71), AidH of *Ochrobactrium* sp. strain T63 (72), and QsdH of *Pseudoalteromonas byunsanensis* strain 1A01261 (73). Despite their differences in DNA sequence and metal ion dependence (Table 2), all of these enzymes were confirmed to function as lactonases following biochemical analysis of reaction products. Additionally, *in vitro* and *in vivo* studies have confirmed the overall value of these lactonases in quorum quenching and disease prevention against *E. carotovora* (62, 68, 71, 72), akin to what was originally shown with AiiA.

In addition to the bacterial enzymes described above, a set of mammalian enzymes, paraoxonases 1, 2, and 3 (PON1 to -3), has also been found to have AHL lactonase activity. Initial observations that human airway epithelia and mammalian sera were able to inactivate AHLs (101, 102) and the enzymatic similarities between the suspected serum lactonase and known mammalian paraoxonases (102, 103) led researchers to examine the effect of PON expression on AHL degradation. CHO cells expressing any of the murine PON genes were able to degrade 3OC12HSL at levels comparable to those in cells expressing the known AHL lactonase gene *aiiA* (102), and treatment of serum with PON inhibitors reduced 3OC12HSL degradation (103). Evolutionary relationship analysis of PONs and studies of PON1 reactivity with more than 50 different substrates demonstrated that PON1 can hydrolyze lactones, esters, and phosphotriesters using the same active site, but its primary activity appears to be as a lactonase (76, 77). Interestingly, in addition to differential expression at various sites within the host (PON1 and PON3 are expressed in the liver and sera, whereas PON2 is a cell-associated enzyme expressed in various tissues [103]), the different PON enzymes appear to have unique enzymatic activities. All three purified PONs had AHL lactonase activity *in vitro*, but each enzyme was determined to have a distinct specificity against a range of substrates, with PON1 and PON3 acting on the widest range of substrates but PON2 being the most active against AHLs (76, 103–105). *In vitro* results have supported a role for PON2 in AHL degradation by murine tracheal epithelial cells (104), and other experiments have demonstrated that both serum PON1 and purified PON1 reduce *P. aeruiginosa* biofilm formation *in vitro* (103). However, PON1 knockout (KO) mice were found to be more resistant to *P. aeruginosa* infection by WT bacteria, whereas a *lasI* mutant of *P. aeruginosa* unable to make 3OC12HSL was equally virulent against both WT and PON1 KO mice (103). Based on gene expression studies, the authors suggest that increased *pon2* and/or *pon3* expression in the PON1-KO mice may compensate for the lack of PON1, which could result in enhanced 3OC12HSL degradation due to the higher AHL-inactivation activity of PON2 compared to PON1 (103), although this has yet to be confirmed experimentally.

Acylases. AHL acylase-like activity was first detected in a soil isolate of the betaproteobacterium *Variovorax paradoxus* that was able to degrade and utilize multiple AHLs as a source of both energy and nitrogen (53). This enzymatic activity was not fully defined at the time but was predicted to be the result of an acylase due to HSL accumulation in the culture medium (53). Following this initial report, acylase activity was demonstrated in another betaproteobacterium, a *Ralstonia* isolate, and the responsible AHL inactivation gene was named *aiiD* (46). The AiiD protein was most similar to aculeacin A acylase (AAC) from *Actinoplanes utahensis* but also shared similarity with other acylases responsible for hydrolysis of penicillin and cephalosporin (46). Interestingly, homologues of AiiD were also found in various other *Ralstonia* and *Pseudomonas* spp. (46), and these bacteria were later confirmed to also possess AHL acylase activity (42, 44, 45, 47, 51, 52). Biochemical analysis of the fate of 3OC10HSL incubated with purified AiiD protein confirmed that AiiD functions as an AHL acylase, releasing HSL and 3-oxodecanoic acid as the major degradation products (46). It was also demonstrated that AiiD does not degrade penicillin G or ampicillin and that porcine kidney aclyase and penicillin acylase are unable to degrade AHLs, indicating that certain acylase enzymes with unique specificity for AHL molecules exist (46). It was hypothesized that AHL acylases could potentially modulate bacterial behavior by interfering with quorum sensing (53), parallel to what had been demonstrated with AHL lactonases, and Lin et al. confirmed this by demonstrating that expression of AiiD in *P. aeruginosa* PAO1 decreased the accumulation of 3OC12HSL and C4HSL, consequently altering virulence factor production and swarming motility (46). Furthermore, expression of AiiD in *P. aeruginosa* attenuated its virulence in a nematode model of infection (46), substantiating the notion that AHL-degrading enzymes may have utility in modulating bacterial behavior and potentially pathogenesis.

Since the identification of AiiD, a few other AHL acylases with distinct substrate specificities have been identified and characterized to various degrees (Table 2). The first gammaproteobacteria reported to have AHL aclyase activity were the soil *Pseudomonas* isolate PAI-A and *P. aerguinosa* PAO1, both of which were shown to be able to utilize AHLs with acyl chains of at least 8 carbons for growth, although with differing kinetics (42). Sequence analysis revealed that the *P. aeruginosa* PAO1 genome encodes four homologues predicted to belong to the Ntn hydrolase superfamily, and thus far three of these (PvdQ, QuiP, and HacB) have been functionally characterized (43). PvdQ (PA2385) is an AHL aclyase with specificity for long-chain AHLs (42, 44), and crystallization studies revealed that AHL hydrolysis proceeds by a mechanism typical of the Ntn hydrolase superfamily (41). The observation that a *pvdQ* mutant of PAO1 retained its ability to grow on long-chain AHLs suggested that other AHL-degrading enzymes were also employed by *P. aeruginosa* (42), and additional studies confirmed that two other Ntn hydrolase homologues, QuiP (PA1032) and HacB (PA0305), also function as AHL acylases with substrate specificity similar to that of PvdQ (45, 52). Importantly, although PvdQ, QuiP, and HacB were all capable of modulating the accumulation of the endogenous *P. aeruginosa* signal 3OC12HSL (44, 45, 52), their abilities to modulate *P. aeruginosa* behavior and virulence *in vivo* were distinct. Whereas overproduction of PvdQ in *P. aeruginosa* or exogenous addition of purified PvdQ protein attenuated *Pseudomonas* virulence in both fast and slow killing assays in the nematode *Caenorhabditis elegans* (106), overproduction of HacB showed only limited effects on pathogenesis in nematodes, with differences in virulence being detectable only after 5 days of infection (52). These disparate effects help to underscore

the importance of *in vivo* studies of all AHL-targeting enzymes even when their substrate specificities are comparable.

Unfortunately, studies of various other AHL acylases have not done much to increase our understanding of the potential of these enzymes as novel therapeutic agents *in vivo*. The first identified extracellular AHL acylase (AhlM) was found in the Gram-positive bacterium *Streptomyces* sp. strain M664 and had broader substrate specificity than previously identified acylases, being active against both medium- and long-chain AHLs, as well as penicillin G (48). Although purified AhlM protein decreased 3OC12HSL accumulation and virulence factor production when added to cultures of *P. aeruginosa* (48), no *in vivo* pathogenesis studies have been published. In later studies, the AHL acylases AiiC and Aac were identified from the cyanobaterium *Anabaena* sp. strain PCC 7120 and a *Shewanella* sp. isolated from the fish gut, respectively (49, 50). Applied studies with Aac have been limited to transformation of *aac* into the fish pathogen *Vibrio anguillarum*, where it was demonstrated to decrease AHL accumulation and interrupt biofilm formation *in vitro* (50); no studies of AiiC inhibition of pathogenesis have been published. Most recently, *Pseudomonas syringae* was shown to harbor two AHL acylases (HacA and HacB) with different localizations and substrate specificities (51), and an *aac* gene with 83% identity to *aiiD* was identified in *Ralstonia solanacearum* (47). The antivirulence utility of these enzymes has been examined only cursorily *in vitro* (47, 51). Additional bacterial species reported to contain yet-to-be-identified genes/proteins responsible for amidolytic hydrolysis of AHLs include *Comamonas* sp. strain D1 (54) and *Rhodococcus erythropolis* (55).

Oxidoreductases. Compared to the abundance of data regarding AHL degradation by lactonases and acylases, there have been very few reports of inactivation of AHLs via oxidation or reduction of the acyl side chain. This type of AHL inactivation was first discovered in *Rhodococcus erythropolis*, a bacterium able to utilize a range of AHLs as both a carbon and nitrogen source (55). High-pressure liquid chromatography (HPLC) analysis of 3OC14HSL following incubation with *R. erythropolis* revealed a new peak with a retention time equivalent to that of 3OHC14HSL, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) results confirmed the presence of this reduced form of the AHL (55). Nonstereospecific reduction of most, but not all, other AHLs tested was observed, and all AHLs tested were eventually degraded by the cells, indicating the presence of another enzymatic activity against AHLs in addition to oxidoreduction (55). The gene responsible for this activity has yet to be identified. Two years later, the *Bacillus megaterium* enzyme CYP102A1, a P450 monooxygenase, was found to oxidize AHLs with both natural and nonnatural stereochemistries, as well as the various products of lactonase or acylase hydrolysis (79). A follow-up paper examining the same enzyme found that hydroxylated C12HSL retained a significant amount of its signaling activity, and *B. megaterium* AHL inactivation rates were only half those of analogous AiiA-expressing *Bacillus* spp. (80), indicating that AHL oxidation by CYP102A1 will likely not be the most effective approach for quorum quenching. A third oxidoreductase, the NADH-dependent enzyme BpiB09, was identified by metagenomic analysis as able to inactivate 3OC12HSL (81). Interestingly, the authors proposed that in addition to 3OC12HSL, BpiB09 may also act on intermediates of the fatty acid cycle, which would indirectly inhibit AHL biosynthesis by lowering available 3-oxo-acyl-ACP in the cell, thereby hindering AHL signaling in two ways. Heterologous expression of *bpiB09* in *P. aeruginosa* reduced AHL accumulation, consequently inhibiting swarming and swimming motilities, pyocyanin production, and biofilm formation *in vitro* (81). Furthermore, *bpiB09*-expressing *P. aeruginosa* was less pathogenic against *C. elegans* than WT bacteria (81), providing the first evidence that oxidoreductases can function to reduce virulence by inhibiting quorum sensing *in vivo*.

Unique quorum-quenching capacity of *Rhizobium* sp. strain NGR234. To date, most bacteria known to be capable of AHL inactivation have been found to contain only one or two enzymes responsible for this activity. However, there are exceptions, including *P. aeruginosa*, which employs multiple AHL acylases (PvdQ, QuiP, HacB, and PA3922) (42, 44, 45, 52). One unique exception is the bacterium *Rhizobium* sp. strain NGR234, which appears to carry at least five distinct AHL-degrading loci as determined by sequence- and function-based screening (67). All five loci were shown to inactivate 3OC8HSL and alter *P. aeruginosa* motility, biofilm formation, and pyocyanin production *in vitro*. Following further genetic analysis, authors identified the following five genes as having AHL inactivation activity: *dhlR*, *qsdR1*, *qsdR2*, *aldR*, and *hydR-hitR*. Biochemical studies demonstrated that both DlhR and QsdR1 were AHL lactonases, and both enzymes interfered with QS-regulated processes when heterologously expressed in *A. tumefaciens*. Additionally, overexpression of either *dhlR* or *qsdR1* in NGR234 resulted in aberrant colonization of cowpea roots, indicating that these genes may play an important role in bacterial fitness within the host rhizosphere by degrading either bacterially derived AHLs or yet-to-be-determined plant molecules. The mechanisms of action of the other three putative AHL-degrading enzymes of this strain have yet to be elucidated, although data suggest that the *qsdR2* product also functions as a lactonase (67).

Enzymatic Inactivation of Non-AHL QS Signals

Although most investigations have focused on inactivation of AHLs, several reports have described enzymatic inactivation of other QS autoinducers. Members of the DSF family of molecules (Fig. 2) are used in quorum sensing by both *Xylella fastidiosa*, the causative agent of Pierce's disease of grapes and other plants, and several *Xanthomonas* species, which cause black rot in cabbage and other cruciferous crops. Importantly, DSF signaling directly affects the virulence of these two pathogens (26). Newman et al. screened native bacteria associated with plants infected with DSF-producing pathogens for inhibition of DSF signaling as assessed by a reporter strain of *X. campestris* (82). This screen revealed 8 bacterial strains from multiple genera, including *Bacillus*, *Staphylococcus*, and *Pseudomonas*, with DSF inactivation activity. Transposon mutagenesis of *Pseudomonas* sp. strain G led to the identification of *carA* and *carB* as the genes responsible for this activity (82). CarA and CarB are the subunits of the heterodimeric complex responsible for the synthesis of carbamoylphosphate, a required precursor for arginine and pyrimidine biosynthesis (107). Surprisingly, although the percentage of environmental isolates initially identified as being able to degrade DSF was low, long incubation times (24 h or more) of laboratory strains with DSF revealed that almost all tested strains had low-level DSF-degrading activity (82). The lab strain *E. coli* DH10B was one such strain, and mutational analyses confirmed that *carAB* were responsible for DSF inactivation by this species as well. Thus, it appears that CarAB function to inactivate DSF in multiple bacterial species,

although the rate at which this inactivation occurs is strain specific (82). Biochemical analyses of the reaction products of CarAB-mediated DSF inactivation revealed the presence of at least two distinct modified forms of DSF with greatly diminished signaling capacity (82); however, the mechanism of DSF modification and the chemical nature of these altered forms have not been elucidated. Importantly, it was shown that DSF-degrading strains are capable of disease control *in vivo*. Coinoculation of bacteria exhibiting high levels of DSF degradation activity with *X. campestris* or *X. fastidiosa* into plant leaves resulted in decreased the severity of black rot and Pierce's disease, respectively, and this attenuation was *carB* dependent (82).

Recognition of the structural similarity between the *P. aeruginosa* QS signal PQS (Fig. 2) and 3-hydroxy-2-methyl-4(1*H*)-quinolone (MPQS), an intermediate of the quinaldine utilization pathway in *Arthrobacter nitroguajacolicus*, by the labs of Williams and Fetzner led to the postulation that the deoxygenase responsible for cleavage of MPQS, Hod, would also recognize PQS as a substrate (83). Experiments confirmed this hypothesis to be correct; Hod-mediated degradation of PQS produced *N*-octanoylanthranilic acid and carbon monoxide, presumably resulting from 2,4-dioxygenolytic ring cleavage (83). Experiments using purified Hod enzyme demonstrated enzyme-mediated inhibition of PQS signaling in cultures of *P. aeruginosa*, but Hod progressively lost activity during incubation in spent culture supernatants due to proteolytic degradation by secreted bacterial proteases. Using a lettuce leaf model of *P. aeruginosa* infection, Pustelny et al. were also able to demonstrate that loss of PQS, by either deletion of *pqsA* or coinjection of Hod enzyme, resulted in attenuation of virulence *in planta* (83).

In another study, LsrK from *E. coli* was examined for its ability to exploit AI-2 signaling. LsrK is the cytoplasmic enzyme responsible for phosphorylation of AI-2 following import into the cell (108). Roy et al. found that by providing LsrK exogenously to *E. coli* or *S.* Typhimurium cultures, they could inhibit QS activation by blocking AI-2 import (the negative charge of phospho-AI-2 prevents its transport via the Lsr transporter) (84). Interestingly, AI-2 responses of *V. harveyi* cultures were also inhibited by LsrK-treated AI-2, despite the fact that AI-2 signaling proceeds through extracellular detection via a membrane-bound sensor kinase in this species. Additionally, exogenously added LsrK blocked quorum sensing under coculture conditions (84), suggesting that LsrK could be useful for quenching both intraspecies and interspecies quorum sensing.

Nonenzymatic Inactivation and Sequestration of QS Signals Using Antibodies

In addition to enzymatic degradation and inactivation of QS signals as discussed above, nonenzymatic means of signal inactivation and sequestration, centering mainly on antibodies, have also been recently investigated. The development of monoclonal antibodies (MAbs) against AHLs was first achieved using lactam-containing haptens whose linkers mimicked naturally occurring AHL acyl chains of various lengths and oxidation states (109). The highest-affinity MAbs were specific for 3OC12HSL, and investigators were able to demonstrate that one of these MAbs, RS2-1G9, inhibited QS signaling and QS-regulated pyocyanin production in *P. aeruginosa in vitro*, suggesting therapeutic potential of antibody-mediated QSI (109). Shortly thereafter, Miyairi and colleagues demonstrated that active immunization with bovine serum albumin (BSA)-conjugated 3OC12HSL increased survival of mice following intranasal challenge with *P. aeruginosa*, despite having no effect on bacterial burdens in the lung (110). Experiments confirmed that immunized mice had lower levels of free 3OC12HSL in both serum and lung homogenates than nonimmunized mice, and sera from immunized mice were able to protect macrophages from the cytotoxic effects of 3OC12HSL, suggesting that antibody targeting the AHL was responsible for protection following immunization (110). The MAb RS2-1G9 was also tested for its ability to protect murine macrophages from cytotoxicity effects of 3OC12HSL, and it was demonstrated that RS2-1G9 protected macrophages from 3OC12HSL-induced apoptosis in a concentration-dependent manner (111). As such, antibody sequestration of AHLs may be therapeutically beneficial by two routes, both by inhibiting activation of QS cascades and subsequent production of bacterial virulence factors and also by preventing host cell cytotoxicity induced by the AHL molecules themselves.

Catalytic antibodies designed to hydrolyze target AHLs have also been developed. Following immunization with a hapten containing a squaric monoester motif, Marin et al. identified the MAb XYD-11G2 as a catalytic antibody capable of hydrolyzing 3OC12HSL with moderate kinetics and inhibiting *P. aeruginosa* pyocyanin production *in vitro* (112). Later, Kapadnis et al. designed sulfones resembling transition state analogues in AHL hydrolysis reactions with the specific intention of generating AHL-targeting catalytic antibodies with higher levels of activity than previously achieved (113). Transition state analogues are chemical compounds that are designed to mimic the native substrate during the catalytic transition state within the enzyme. Such analogues are predicted to bind to the enzyme with greater affinity than the native substrate, making transition state analogues attractive candidates for enzyme inhibitors (114). These sulfones were not biologically active as QS agonists or antagonists but were able to competitively inhibit AiiA-dependent AHL hydrolysis, suggesting that the sulfones were accurate transition state analogues (113). Unfortunately, no additional data regarding the effectiveness of using these sulfones as haptens for the generation of AHL-specific catalytic antibodies have been published.

Importantly, antibody-based quorum quenching has also been developed for Gram-positive peptide signals. Park et al. designed hapten AP4-5 to resemble the natural QS signal AIP-4 utilized by *S. aureus* (115). Following conjugation of AP4-5 with carrier proteins and immunization of BALB/c mice, one of 20 prepared MAbs, AP4-24H11, was found to have strong binding affinity and high specificity against AIP-4 compared with AIP-1,-2, or -3. *In vitro* evaluations showed that AP4-24H11 was able to alter the production of various *S. aureus* exoproteins, including known virulence factors, in an AIP-4-dependent manner and was most active against *agr* group IV strains, although some cross-reactivity against strains of other *agr* group types was evident (115). Also, biofilm formation, which is known to be negatively regulated by QS in *S. aureus*, was increased in the presence of AP4-24H11, consistent with effective QS inhibition. Encouragingly, AP4-24H11 was also shown to limit *S. aureus* pathogenicity *in vivo*. Coinjection of *S. aureus* with AP4-24H11 prevented the formation of lesions in a murine subcutaneous infection model, and passive immunization with AP4-24H11 prior to lethal challenge with *S. aureus* protected all mice from death (115). Thus, immunopharmacotherapy appears to be a viable approach for quorum-

quenching-mediated reduction of virulence in both Gram-positive and -negative bacterial pathogens *in vivo*.

INHIBITION OF QS SIGNAL BIOSYNTHESIS

An alternative approach for limiting signal accumulation is inhibition of signal synthesis. Although reports of signal synthesis inhibition are far less numerous than those of signal degradation and inactivation, evidence indicates that inhibition of signal production is feasible and effective both *in vitro* and *in vivo* for several types of autoinducers.

Inhibition of AHL Production

AHL signals are produced by acyl-HSL synthases belonging to the LuxI or AinS family and are formed from the substrates SAM and acyl-ACP (27, 28). As such, inhibition of AHL production could theoretically be achieved through suppression of SAM biosynthesis, interference with generation of acyl-ACPs, or inactivation of the synthase enzyme. As of yet, there are only limited reports of inhibition of AHL production by these means. It should also be mentioned that inhibitors of MTAN have also been investigated as potential quorum-quenching compounds, but given that MTAN is directly involved in synthesis of both AHLs and AI-2, inhibitors of this enzyme are discussed separately below.

FabI (NADH-dependent enoyl-ACP reductase) is the bacterial enzyme responsible for catalyzing the final step in the biosynthesis of acyl-ACP, thereby providing the acyl chains for AHL synthesis. The family of short-chain alcohol dehydrogenases to which FabI belongs is known to be inhibited by antibacterial diazobroines and triclosan. Hoang and Schweizer confirmed that FabI in *P. aeruginosa* is one of multiple enoyl-ACP reductases harbored by the bacterium and that FabI is likely involved in generation of butyryl-ACP for use in the synthesis of C4HSL. Importantly, triclosan was shown to be an inhibitor of FabI that could successfully suppress the production of C4HSL in an *in vitro* synthesis system, and *fabI* mutant cultures accumulated only 50% of the AHLs found in WT cultures, corroborating the role of FabI in AHL synthesis (116). Unfortunately, *P. aeruginosa* is resistant to triclosan due to the activity of a multidrug efflux pump (117), so alternative FabI inhibitors will need to be developed before the *in vivo* efficacy of this approach can be evaluated.

In the Gram-negative pathogenic bacterium *Burkholderia glumae*, the causative agent of rice grain rot, the QS signal C8HSL is produced by the LuxI homologue TofI, and QS is involved in the production of the key virulence factor toxoflavin. A screen of 55 "hits" from a previous quorum-sensing screen in *P. aeruginosa* (118) yielded the identification of two strong inhibitors of QS in *B. glumae* (119). Although both compounds inhibited C8HSL production, they were found to do so in different ways; whereas both E9C-3oxoC6 and J8-C8 (Table 3) suppressed toxoflavin production in WT cells, only E9C-3oxo-C6 had an effect in a *tofI* mutant strain, suggesting that the two compounds functioned by different mechanisms and that the molecular target of J8-C8 was the TofI enzyme (119). Direct inhibition of TofI by J8-C8 was subsequently confirmed *in vitro*. In contrast, E9C-3oxoC6 was found to competitively inhibit C8HSL binding to the AHL receptor TofR. Structural studies of TofI in its apo form and in complex with J8-C8 revealed that the inhibitor binds in a pocket with high specificity for the octanoyl chain, suggesting that J8-C8 is a competitive inhibitor of the acyl-ACP carrier utilized for C8HSL synthesis, and thus it has been postulated that modulation of acyl chain length in J8-C8 mimics may be a means by which to rationally design enzyme-specific LuxI family inhibitors (119). Separately, holo-ACP, the SAM analogues *S*-adenosylhomocysteine, *S*-adenosylcysteine, and sinefungin, the reaction product methylthioadenosine (MTA), and the possible reaction intermediate butyryl-SAM were found to inhibit C4HSL production by RhlI *in vitro* (27), demonstrating that LuxI-type synthase activity could also potentially be inhibited by end products, reaction intermediates, and/or substrate analogues.

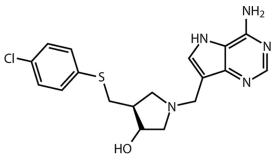
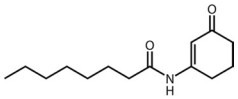
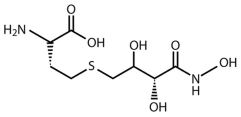
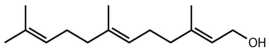
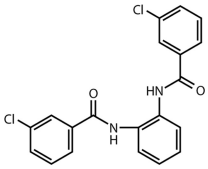
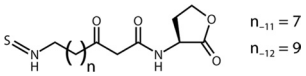
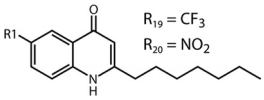
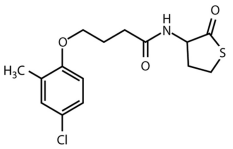
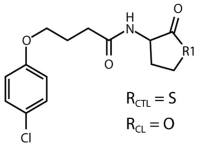
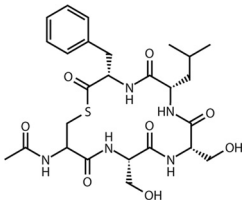
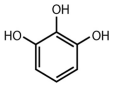
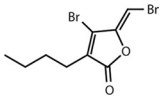
Inhibition of AI-2 Production

AI-2 is generated from the precursor SAH by two enzymes that function in succession. The nucleosidase MTAN (also known as Pfs) catalyzes the removal of adenine from SAH to produce *S*-ribosyl-L-homocysteine (SRH), and SRH is subsequently converted to homocysteine and DPD by the metalloenzyme LuxS (34). This conversion proceeds through sequential aldose-ketose and ketose-ketose isomerization steps and a final β-elimination step that removes homocysteine from a 3-keto intermediate to yield DPD. DPD is unstable in aqueous solution and undergoes spontaneous rearrangement into multiple interconvertible cyclic furanone compounds that as a group are termed AI-2. Although different bacteria sense different forms of AI-2, all AI-2 (DPD) is synthesized by LuxS, and thus the LuxS enzyme makes an attractive target for generation of inhibitors that may function to prevent quorum sensing in multiple bacterial species. Additionally, the LuxS enzyme is absent from mammalian species, lowering the potential for unwanted off-target effects of inhibitors in host cells. MTAN inhibitors have also been investigated for potential as quorum quenchers but are discussed separately below due to the role of MTAN in both AI-2 and AHL biosynthesis.

The most common method for identification of LuxS inhibitors has been rational design of substrate analogues that are unable to undergo either the isomerization or β-elimination steps catalyzed by LuxS. Alfaro et al. published the first report of LuxS inhibition by two substrate analogues, *S*-anhydroribosyl-L-homocysteine and *S*-homoribosyl-L-cysteine. These two analogues were determined to be competitive inhibitors that prevented the first and last steps of the catalytic mechanism, respectively; however, their inhibition activities were weak (142). Shortly thereafter, a series of publications reported LuxS inhibition by a variety of other substrate and intermediate analogues. The most potent LuxS inhibitors were identified when replacement of the unstable enediolate moiety of a predicted enediolate intermediate with a stable hydroxamate group yielded two stereoisomeric inhibitors, compound 10 (Table 3) and compound 11, having K_i values in the submicromolar range (123). Cocrystallization studies showed that both inhibitors were able to bind the homocysteine-binding pocket of LuxS and interact with the active-site metal ion. Furthermore, structural comparisons between compounds 10 and 11 and other, less active compounds suggested that both the amino acid moiety and binding to the metal center are crucial for high-affinity binding and inhibition of LuxS (123).

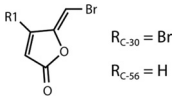
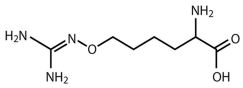
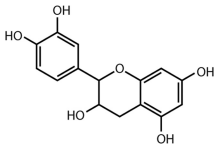
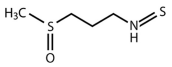
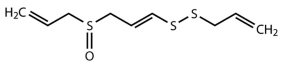
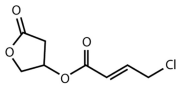
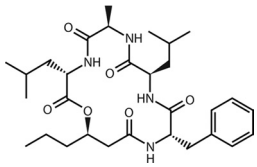
Other substrate analogue inhibitors have since been identified but have only moderate activity against LuxS. These include the SHR analogue 3,5,6-trideoxy 6-fluorohex-5-enofuranose (compound 44), which lacks the C3 hydroxyl group required for conversion to DPD (143), various SHR analogues modified at the ribose C3 position (144), and multiple SHR analogues in which the furanose ring oxygen is replaced by a nitrogen atom (145). Of

TABLE 3 Structures of selected QSI compounds and their targets

Category	Inhibitor	Structure	Target(s)	Reference(s)
Synthase inhibitors				
Synthetic	*p*ClPhT–DADMe–ImmA		MTAN	120–121, 122
	JA-C8		TofI (LuxI family, *B. glumae*)	119
	Compound 10		LuxS	123
Natural source	Farnesol		PqsA	124
Receptor inhibitors				
Synthetic	TP-5		LasR	125
	Itc-11, -12		LasR (covalent)	126
	Compounds 19 and 20		PqsR	127
	4606-4237		LuxN, CviR	128–129
	CTL, CL		CviR	128
	trAIP-II		AgrC	130
Natural sources	Pyrogallol		AI-2 analog	131
	Furanone C-2		LuxR family (and possibly LuxS)	132

(Continued on following page)

TABLE 3 (Continued)

Category	Inhibitor	Structure	Target(s)	Reference(s)
	Furanone C-30, -56		LuxR family	133, 134
	Canavanine		LuxR family	135
	Catachin		LuxR family	136
	Iberin		LasR, RhlR	137
	Ajoene		LuxR family	138, 139
	Honaucin A		LuxR family	140
	Solonomide A, B		AgrC	141

note, these studies revealed that whereas some compounds acted as simple, competitive inhibitors, others exhibited time-dependent inhibitory activity (144, 145). This time-dependent inhibition is likely the consequence of low binding affinity of the compound for LuxS; with time, the compound binds to LuxS and some type of structural conversion of the compound within the active site of LuxS results in a tighter enzyme-substrate complex and enhanced inhibition (144, 145).

As an alternative approach to rational analogue design, phage display has also been utilized to identify novel peptide inhibitors of LuxS. Purified LuxS was used to screen a phage display library, and five LuxS-binding peptide sequences were identified. However, only one of these peptides, TNRHNPHHLHHV, was found to inhibit LuxS activity, and inhibition was only 25% (146). There has also been a single report of inhibition by covalent modification of LuxS. Bromonated furanones have long been recognized as inhibitors of AHL-mediated quorum sensing in Gram-negative bacteria (see "Furanones" below), and Zang et al. identified multiple brominated furanones that inhibited LuxS to various degrees (147). One such furanone, designated C-2 (Table 3), was further characterized mechanistically and was found to covalently modify LuxS, resulting in a single LuxS adduct detectable by mass spectrometry. Matrix-assisted laser desorption ionization (MALDI)-MS results were consistent with the loss of one bromide atom from C-2 following modification of LuxS (147). The mechanisms of the remaining furanone inhibitors were not elucidated.

An obvious limitation in the current understanding of LuxS-based quorum quenching is the lack of data regarding the efficacy of inhibiting AI-2 production in whole cells. All of the papers described above screened compounds for inhibition of purified LuxS *in vitro* and thus do not address the ability of such compounds to reach the bacterial cytoplasm where the LuxS enzyme resides. Given the modest potency of most of the inhibitors identified to date, it may be efficacious to develop more potent inhibitors prior to inhibition studies in whole cells, but such testing will be a crucial step in determining if quorum quenching of AI-2 signaling via inhibition of LuxS *in vivo* is feasible.

Inhibition of MTANs

As mentioned above, MTAN is directly involved in the biosynthesis of both AHL and AI-2 autoinducers. In AI-2 bysynthesis, MTAN catalyzes the hydrolytic depurination of SAH to generate SRH, the substrate of LuxS and direct precursor of DPD (AI-2) (34). In AHL biosynthesis, MTAN is responsible for depurinating MTA, a by-product of AHL synthesis and various other cellular metabolic pathways that is known to inhibit AHL synthase activity (27). Of note, in addition to disruption of AHL and/or AI-2 biosynthesis, MTAN inhibition could also interfere with important metabolic pathways, including polyamine biosynthesis and methionine salvage, in which SAH or MTA is a product inhibitor (148), potentially resulting in aberrant growth and increased pressure to develop resistance.

Studies utilizing kinetic isotope effects (KIEs) and crystal structures of MTANs in complex with known transition state analogues have allowed for transition state modeling of MTANs from *E. coli*, *S. pneumoniae*, *Neisseria meningitidis*, *S. aureus*, and *V. cholerae* and the subsequent design of analogue inhibitors (120–122, 149–153). Thus far, it appears that most MTANs share a fully dissociated S_N1 transition state characterized by a ribooxacarbenium ion (121, 122, 149, 152), with the exception of MTANs from

N. meningitides and *Helicobacter pylori*, which have an early S_N1 transition state (151, 154). Two general classes of MTAN inhibitors tested to date are immucillin A (ImmA) derivatives, which mimic the early dissociative transition states, and DADMe-ImmA derivatives, which mimic late dissociative transition states (120, 150). Interestingly, despite the high homology between the active sites of MTAN from *E. coli* and *S. pneumoniae* and their similar transition state structures, the inhibition constants for the two enzymes of various analogues differed widely, presumably due to the different catalytic efficiencies of the enzymes (120, 122). The MTAN of *E. coli* is more catalytically efficient than that of *S. pneumoniae* with either MTA or SAH as a substrate, resulting in greater inhibition of *E. coli* MTAN than of *S. pneumoniae* MTAN by any of the inhibitors tested (122). For example, the most potent inhibitor of *S. pneumoniae* MTAN was *p*-chlorophenylthio-DADMe-ImmA (*p*ClPhT-DADMe-ImmA) (Table 3), which had a K_i^* of 360 pM (122), where K_i^* is the final equilibrium dissociation constant calculated for a second linear reaction following slow-onset tight binding. The same analogue had a K_i^* of 47 fM for the *E. coli* MTAN (120). Impressively, this binding affinity is 91 million times greater than the affinity of *E. coli* MTAN for the native substrate SAH, placing 5′-thio-substituted DADMe-immucullins among the most potent competitive transition state analogue inhibitors identified to date (120). The same general relationship between catalytic efficiency and inhibitor potency was also observed with MTAN of *V. cholerae*, which has a catalytic efficiency between those of MTANs from *E. coli* and *S. pneumoniae* and also had inhibitor affinities intermediate to those observed for the other two enzymes (149).

The majority of the transition state analogue inhibitor characterization has been performed using purified MTANs *in vitro*. However, there has been some preliminary work done to assess the effects of MTAN inhibitors on quorum sensing and biofilm formation in *V. cholerae* and *E. coli*. MTAN has traditionally been considered an antibiotic lead due to its central role in various metabolic pathways (148), and indeed some MTAN inhibitors have been shown to have antimicrobial activity (155). However, growth of *V. cholerae* or *E. coli* in the presence of ImmA and DADMe-ImmA derivatives was unchanged compared to that of untreated cultures at concentrations sufficient to inhibit QS signal production (149, 156). Additionally, inhibition of AHL production in *V. cholerae* and of AI-2 production in both *V. cholerae* and *E. coli* was sustained for extended growth cycles without observable effects on growth (149), suggesting that some MTAN inhibitors do not place a burden on cellular processes that would select for rapid development of resistance. Importantly, MTAN inhibitors were also demonstrated to inhibit biofilm formation by both strains to differing degrees (149), indicating that MTAN inhibitors can effectively cross into cells to inhibit quorum-sensing-regulated virulence-related phenotypes. It should be noted that although transition state analogues are more potent *in vitro* inhibitors of *E. coli* MTAN than *V. cholerae* MTAN, inhibition of *E. coli* AI-2 production was less than that for *V. cholerae* (149), suggesting that differences in cellular permeability may influence the efficacy of MTAN inhibitors against different bacterial pathogens.

Inhibition of PQS Production

PQS and its precursor HHQ result from a condensation reaction between the precursor anthranilate and a β-keto-fatty acid (157). The first report of inhibition of PQS production was published in 2001, when, with the knowledge that anthranilate analogues can act as inhibitors of anthranilate synthesis in *E. coli*, Calfee et al. examined the ability of the analogue methyl anthranilate to modulate PQS production in *P. aeruginosa* (157). Exposure of *P. aeruginosa* to methyl anthranilate resulted in a decrease in PQS accumulation in a concentration-dependent manner without affecting bacterial growth. Congruent with inhibition of PQS signaling, activity of the PQS-controlled virulence factor elastase was also suppressed by methyl anthranilate. Years later, halogenated anthranilate analogues were demonstrated to inhibit PQS biosynthesis and subsequent PQS-dependent gene regulation (158). Excitingly, these compounds were shown to have therapeutic benefits *in vivo*, where they increased survival and limited systemic dissemination of *P. aeruginosa* in a thermal injury mouse model (158). These effects were concluded to be due to QSI, as HHQ and PQS production at the infection site was reduced in the presence of the compounds. Interestingly, the halogenated anthrnilate analogues were also shown to increase the osmosensitivity of a range of bacterial pathogens in a QS-independent manner (158), suggesting that these compounds may have broader-range therapeutic potential than originally anticipated. The identification of PqsA as the carboxylic acid-CoA ligase responsible for activation of anthranilate has now provided an additional means by which to screen for inhibitors of PQS production (159). Initial experiments have shown that PQS synthesis inhibitors can be either substrates or nonsubstrates of PqsA, and thus it appears that either competitive inhibition of anthranilate binding or inhibition of downstream enzymes may be a successful approach for PQS quorum quenching (159).

PQS production was also found to be suppressed by the sesquiterpene farnesol. Farnesol (Table 3) is a signaling molecule produced by *Candida albicans*, an opportunistic fungal pathogen often found in mixed infections with *P. aeruginosa* in clinical settings. Farnesol-mediated inhibition of PQS production and PQS-controlled pyocyanin production was concentration dependent and was due to decreased expression of the *pqs* operon, which the authors propose to result from the farnseol-mediated formation of nonproductive interactions of the transcriptional regulator PqsR with the *pqsA* promoter (124). In addition, it was shown that naturally occurring levels of farnesol produced by *C. albicans* were sufficient to suppress PQS production during coculture, suggesting a physiologically relevant role for farnesol in modulation of PQS signaling. Interestingly, other compounds containing the long-chain isoprenoid backbone were also able to repress PQS production, although to differing degrees, potentially providing a novel scaffold for the design of PQS synthesis inhibitors (124).

Inhibition of Peptide Autoinducer Production

Studies investigating inhibitors of peptide autoinducer production are very limited, likely because the enzymes responsible for their synthesis, such as ribosomes and peptidases, are commonly essential for the growth and survival of the bacterial cells (160). Therefore, inhibitors of these enzymes would be expected to have bactericidal activity in addition to quorum-quenching activity, which would theoretically increase the pressure on bacteria to develop resistance. Despite this, several studies investigating inhibitors of peptide signal production in *S. auerus* and other Gram-positive bacteria have been published, although these studies were geared toward mechanistic understanding of signal synthesis

rather than signal production inhibition as a potential antivirulence approach.

S. aureus AIPs are generated from the precursor ArgD following two proteolytic cleavage events that remove the leader and tail segments of the propeptide. The protein AgrB removes the C-terminal tail of the propeptide (161, 162), while removal of the N-terminal leader segment is catalyzed by the type I signal peptidase SpsB (Fig. 1D) (163). With the intention of probing the activities of SpsB in whole cells, Kavanaugh and colleagues designed a linear peptide with a proline at the +1 position that they predicted would inhibit cleavage based on known requirements for SpsB processing. Along with this peptide, which they called P+1, they also generated a fluorescein derivative of the same peptide, which they called P+1f. P+1f was resistant to cleavage by SpsB *in vitro*, and P+1 moderately inhibited SpsB-mediated cleavage of the peptide Pep1, a mimic of the AgrD N terminus and substrate for SpsB, both *in vitro* and in whole-cell assays. Furthermore, addition of the P+1 inhibitor to cultures of *S. aureus* strain AH462 inhibited quorum sensing in a concentration-dependent manner and at levels that did not affect bacterial growth (163). Unfortunately, the P+1 inhibitor was not especially stable in bacterial cultures, likely due to the presence of various bacterial proteases to which P+1 is sensitive. To decrease the rate of degradation of the inhibitor, the alanine residues of P+1 were replaced, generating peptide NIF. NIF was as effective at inhibiting SpsB activity as and better at inhibiting quorum sensing than the P+1 inhibitor. Additionally, NIF was able to inhibit AIP biosynthesis in whole cells, whereas no reduction in AIP levels was detected in the presence of P+1 (163). Unfortunately, and in agreement with other studies examining the effect of signal peptidase I inhibition (160), the growth-inhibitory MIC for NIF was much lower than that for P+1 (163), indicating that SpsB may be a good target for novel antibacterial drugs with the additional benefit that AIP-mediated quorum sensing may also be inhibited.

More recently, a screen of fungal secondary metabolites for inhibition of QS in *E. faecalis* revealed that ambuic acid can inhibit the biosynthesis of cyclic peptides in multiple Gram-positive species (164). The *E. faecalis fsr* QS system involves the cyclic peptide GBAP, and GBAP biosynthesis is believed to proceed by a mechanism similar to that of the AIP molecules. Although the precise target of ambuic acid remains unknown, the reduction in gelatinase production in *E. faecalis* cultures by ambuic acid could be counteracted by the addition of exogenous GBAP, indicating that GBAP biosynthesis was inhibited, and further evidence suggests that this occurs posttranslationally (164). Although ambuic acid was also able to inhibit peptide signal production in *S. aureus* and *Listeria innocua*, the level and sustainability of inhibition in all species were low (164), indicating that more potent inhibitors will need to be developed prior to testing antivirulence efficacy *in vivo*.

INHIBITION OF SIGNAL DETECTION

Synthetic Signal Analogues and Receptor Antagonists

One of the most intuitive means by which quorum-sensing inhibitors have been developed is the generation of analogues of native signals and known inhibitors. It has been hypothesized that alterations in native signals can be engineered to maintain the signal-receptor interaction while disrupting downstream signaling by generating nonproductive signal-receptor complexes that competitively block binding by the native signal. Likewise, it may be possible to modify structures of known inhibitor molecules in such ways to enhance their inhibitory activities or alter their target specificities. High-throughput and computer-aided screens of small molecules have also expanded the list of known agonists and antagonists of various QS systems and have increased the number of structural scaffolds on which novel inhibitory compounds can be based.

Inhibitors of AHL receptors. Multiple groups have examined the interactions of various LuxR-type receptor proteins, most commonly LuxR, TraR, and LasR, with both natural and nonnatural AHL molecules and have evaluated their subsequent activities. In addition to variations in the length, saturation, and oxidation states of natural AHLs, a plethora of nonnatural AHL analogue substitutions have been synthesized and tested to date, including but not limited to thiolactones (165, 166), lactams (167), triazolyldihydrofuranones (168), and urea analogues (169). Analogues have been found that exhibit a range of different activities, including no activity, pure agonism or pure antagonism, partial agonism, and synergistic agonism (167, 170–172). The different AHL analogues tested to date and their specific activities against the various receptor proteins are too numerous to report here in detail, so for a more comprehensive and specific discussion of these molecules we refer the reader to other recently published reviews (173, 174). There are some general findings of these investigations, however, that we have chosen to discuss below, as well as several specific examples of AHL receptor antagonists that show promise for the future development of analogue-based QSI strategies.

The elucidation of the crystal structures of TraR (175, 176), SdiA (177), LasR (178, 179), and more recently QscR (180) has provided pivotal information regarding the key interactions between LuxR-type receptors and both native and nonnative ligands. Unfortunately, because the precise binding requirements distinguishing agonists from antagonists remain unclear and appear to differ between individual receptor proteins, the crystal structures of these proteins have not been especially helpful in the rational design of AHL analogues or non-AHL-like molecules with specific agonist or antagonist activity. However, molecular docking programs have enhanced the ability to predict compounds that would be capable of binding in the ligand-binding pockets of the solved crystal structures or that have two-dimensional (2D) structures similar to those of known agonists or antagonists and thereby would be likely to modulate receptor activity. Such modeling has been utilized by multiple groups and has successfully led to the identification of novel AHL-like and non-AHL-like agonists and antagonists (125, 181–184). Crystal structures have also helped in the development of reactive QS probes that target LasR. The binding pocket of LasR contains a nucleophilic cysteine residue that allows for covalent modification by reactive probes. Two such isothiocyanate-based probes, Itc-11 and Itc-12, (Table 3), were shown to covalently modify the ligand-binding domain of LasR, presumably at Cys79, and isothiocyanate-based probes were also shown to inhibit QS in WT *P. aeruginosa in vitro* (126). As an alternative approach, high-throughput non-structure-based screening methods have been employed to identify AHL receptor antagonists with diverse molecular structures (129, 185–188). Such high-throughput screening has also led to the identification of two classes of molecules able to suppress *V. cholerae* virulence factor expression through modulation of either the noncanonical AHL receptor LuxN or the NtrC-like response regulator LuxO (189). Additionally, structure-based derivatiza-

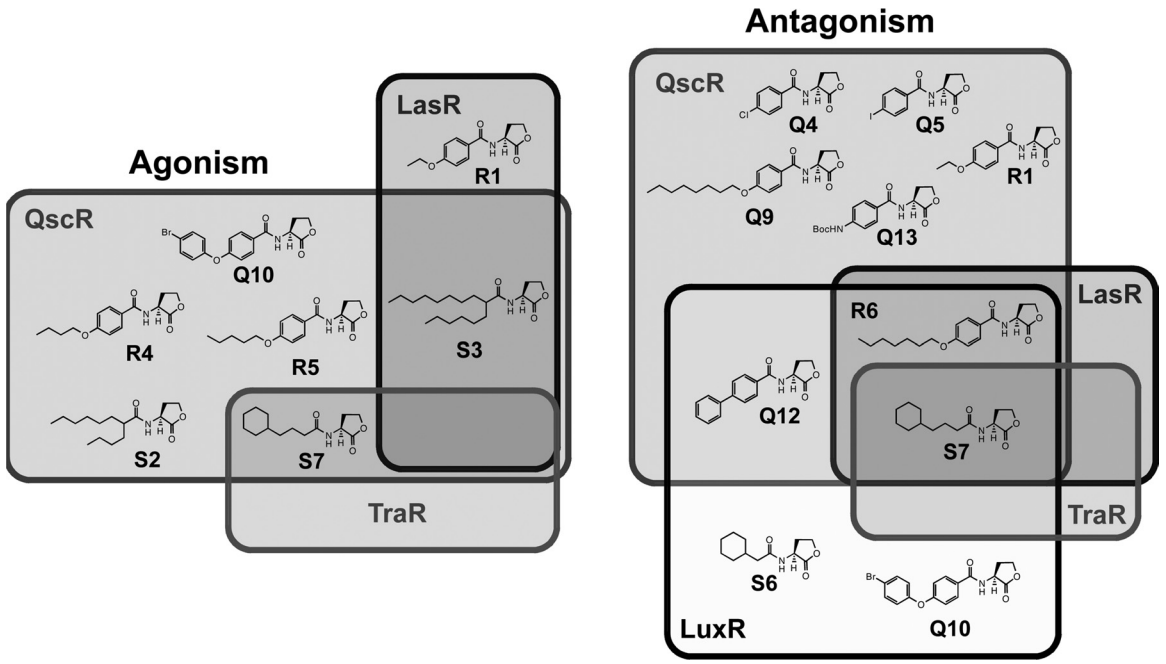


FIG 5 Venn diagrams showing "degenerate" AHL analogues tested for activity on LasR, TraR, LuxR, and QscR. Overlapping regions indicate ligands with notable activity in two or more receptors. (Reprinted from reference 198. Copyright 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.)

tion of previously identified antagonists has led to the discovery of multiple non-AHL pharmacophores with activity against LuxR-type proteins, including disubstituted imidazolium salts (183, 190) and *N*-acyl cyclopentylamides (191, 192).

Overall, structure-activity relationship (SAR) and molecular modeling studies have indicated that both agonistic and antagonistic interactions require sufficient favorable hydrogen bonding, hydrophobic, and van der Waals interactions between the ligand and ligand-binding site of the receptor to surmount any steric hindrance imposed by the presence of nonnative ligand substituents (125, 171, 193). This is in agreement with studies demonstrating that even minor changes in the length or substituents of the acyl tail, which contributes to the binding affinity between the receptor and the ligand (11, 166, 194–196), can have large effects on its activity (11, 170, 197). Alteration of the chirality of the lactone ring or substitutions of part or all of the lactone head group can also alter the activity of a given molecule for a given AHL receptor (11, 118, 165–167, 196, 197), likely because of important conserved intermolecular hydrogen bonds between the AHL lactone head group and the ligand-binding pockets of AHL receptors (175–180). In addition to promoting ligand-receptor interaction, hydrogen bonding may also help to determine the type of activity exhibited by a given compound. For example, the triphenyl signal mimic TP-1 acts as an agonist of LasR and is predicted to form hydrogen bonds with the conserved residues Trp60 and Asp73 in the ligand-binding pocket of the receptor. In contrast, the TP-1-derivative TP-5 (Table 3) is predicted to make only the Asp73 hydrogen bond and instead functions as an antagonist of LasR (125). However, this antagonistic activity could also be the result of predicted steric interference between the third aromatic ring of TP-5 and the ligand-binding pocket, which could induce an aberrant conformation of the receptor (179), and additional structural data will need to be acquired before the precise mechanism of TP-5 antagonism is established.

Interestingly, different AHL receptor proteins can engage in different binding interactions with a given ligand despite the fact that the ligand-binding sites of the various LuxR-type proteins on average share 75% homology (167, 171, 198–200). This has been confirmed by crystal structures demonstrating that a given AHL can occupy slightly different conformations within the ligand-binding sites of different LuxR-type proteins and that conserved binding-site residues can serve distinct functionalities for ligand binding within these different receptors (178). These differences assuredly contribute to species-specific activation of a receptor by its cognate ligand and will likely facilitate the development of small-molecule modulators designed to have more restricted or more global target specificity. In agreement with this conclusion, individual inhibitors that either have highly specific activity for a single LuxR-type protein or antagonize multiple AHL receptors have been identified. A large portion of this work has come from the Blackwell lab, which has worked to identify and refine "degenerate" analogues of AHLs that target members across the LuxR family of receptors. Systematic evaluation of four focused libraries of nonnative AHLs for activity against the receptors TraR, LasR, and LuxR yielded sets of ligands that modulated either one, two, or all three of the receptor proteins (171). A later study expanded upon this initial finding by screening various ligands against the solo LuxR-type receptor QscR in addition to TraR, LasR, and LuxR, and this resulted in the identification of multiple compounds with activity against one, two, or three receptors, as well as the ligand S7, which was capable of antagonizing all four receptors (Fig. 5) (198). Other studies from the Blackwell lab have demonstrated the applicability of various compounds against the pathogenic actions of *E. carotovora* on *Solanum tuberosum* (potato) and of *P. syringae* on *Phaseolus vulgaris* (green bean), and several compounds showed promising results modulating quorum sensing in both bacteria, though the timing and dosage of treatments, especially in the potato model, were critical (199, 201).

In another example of cross-inhibition, a high-throughput chemical screen of approximately 35,000 compounds led to the identification of 15 small molecules that inhibited the LuxN/AI-1 QS circuit in *V. harveyi* (129). Despite the fact that these molecules were initially identified in a screen for inhibition of the membrane-bound sensor kinase LuxN, one of these molecules, 4606-4237 (Table 3), which carries a phenoxyl group at the terminus of the acyl chain and has a homocysteine thiolactone group in place of the homoserine lactone moiety, was later demonstrated to act as an antagonist of the cytoplasmic receptor CviR of *C. violaceum* (128). Thus, despite the differences in sequence and/or signal transduction mechanisms by AHL receptor proteins, the similarities of their ligand-binding pockets can be sufficient to allow for cross-inhibition by the same inhibitor compound.

Interestingly, the mechanism by which analogues antagonize LuxR-type receptor activity also appears to be variable depending on the ligand and the receptor. Swem et al. generated and tested various analogues of the previously identified CviR antagonist 4606-4237, leading to the identification of C10HSL, CTL, and CL as additional CviR antagonists (Table 3) (128). Studies using purified CviR protein complexed with these antagonists indicated that three of the antagonists function by preventing receptor association with target DNA, while C10HSL functions instead by preventing a productive interaction with RNA polymerase while having limited impact on DNA binding by CviR (128). More recently, structure-function studies and cocrystallization were used to further elucidate the mechanism by which these analogues inhibit CviR activity. The crystal structure of CviR in complex with CL suggests that CL binds in the native ligand-binding pocket and induces the CviR dimer to assume a crossed-domain closed conformation that prevents binding of DNA (202). This closed conformation involves the repositioning of the key residue Met89. Importantly, the relative inhibition potencies of the various antagonists were found to directly correlate with the progressive repositioning of the Met89 side chain, a result that helps to explain how small changes in ligand structure can induce large changes in receptor activity (202). For other AHL receptors, including TraR and LasR, the native ligands are postulated to trigger a protein folding switch that is required for proper folding of the receptor, which enhances protein stability (175–179). In these cases, antagonists may function by promoting the adoption of nonnative conformations which could both prevent productive DNA-protein interactions and promote receptor degradation. In all likelihood, there are multiple effective mechanisms of antagonism against AHL receptors depending on the native role of ligand binding in receptor physiology and the specific interactions between a given antagonist and a receptor.

Unfortunately, the majority of the reports regarding AHL receptor ligands published to date have focused on chemical, structural, and *in vitro* biological characterization of the various agonists and antagonists rather than on their effective disruption of virulence-related phenotypes or pathogenicity *in vivo*. However, there are some data addressing these issues. Various compounds have been demonstrated to inhibit *in vitro* behaviors, including but not limited to *P. aeruginosa* biofilm formation (118, 126, 168, 200, 203), *Serratia marcascens* swarming and biofilm formation (192), and carbapenem production in *Serratia* spp. (186). Furthermore, studies with the CviR antagonist CL have shown promise in *in vivo* infection models, as administration of CL at a concentration of 20 μM was able to increase the life span of *C. elegans* following infection with WT *C. violaceum* from 2 days to 7 days (128). Given that a *cviI* mutant deficient for QS is able to kill *C. elegans* in an average of 12 days, the 5-day increase in survival afforded by CL treatment is substantial (128). Obviously, the testing of other AHL receptor antagonists *in vivo* will be a crucial step in evaluating their therapeutic potential, especially for AHL-like molecules containing a lactone ring moiety that may be subject to the same degradation or inactivation as native AHLs in the host.

AI-2 analogues. With the concept of molecular mimicry in hand and the goal of identifying novel antagonists of AI-2 signaling, Wang and colleagues published a series of papers in which they screened a large number of compounds that they believed would have the potential to complex with the *V. harveyi* receptor LuxP (131, 204, 205). They chose to focus on compounds with molecular structures significantly different from those of the previously examined nonaromatic cyclic polyols which generally exhibited agonist activity (206–208). The first two screens involved diol-containing compounds, which have the ability to complex with boric acid, and native boronic acid compounds (131, 205), all chosen due to their similarities to the 2,3-borate diester form of AI-2 recognized by LuxP (Fig. 2) (35, 36). The third screen tested approximately 1.7 million compounds from commercial compound databases by *in silico* analysis against the LuxP-AI-2 holoform crystal structure (204). These three screens yielded a total of 12 AI-2 QS inhibitors with 50% inhibitory concentrations (IC_{50}s) in the micromolar range (single-digit micromolar range for the 10 compounds identified in the first two screens) in whole-cell assays with *V. harveyi*, without significant effects on bacterial growth. The best inhibitor identified from these screens was pyrogallol (Table 3), with an IC_{50} of 2 μM. More recently, there have been multiple reports of the generation and screening of C1- and C2-alkyl DPD derivatives. C1-alkyl derivatives have included linear, branched, cyclic, and aromatic DPD analogues (209–213), whereas the C2 derivatives tested have thus far been limited to linear substitutions of various alkyl chain lengths (214). None of these C1- or C2-alkyl analogues were more potent inhibitors than the previously identified pyrogallol, but some did have AI-2-modulating activity. During the preparation of this review, a study utilizing structure-based virtual screening identified seven novel compounds that inhibit AI-2 recognition by *V. harveyi* LuxPQ with IC_{50}s in the micromolar range and with little to no bacterial cytotoxicity (215), increasing the number of molecular scaffolds now known to antagonize AI-2 QS.

Results from these investigations have revealed two types of QS-modulating activity that are both analogue and target system specific. First, as anticipated, numerous DPD derivatives were found to be AI-2 QS antagonists in one or multiple bacterial species (209–213). Second, some of the analogues were found to be synergistic agonists despite having no agonist activity on their own (209–211, 213). For both types of QS-modulating activity, species specificity was apparent. For example, propyl-DPD and other analogues with longer alkyl chain substitutions were all antagonists of AI-2 QS in *E. coli*, but only butyl-DPD had antagonistic activity in *S.* Typhimurium (212). Likewise, cyclic DPD analogues generally inhibited AI-2 QS in *E. coli*, whereas they either had no activity or functioned as synergistic agonists in *S.* Typhimurium (209). Additionally, the activity of a given analogue in a given strain may be context dependent, as multiple C1-alkyl DPD derivatives were found to function as either antagonists or synergistic agonists in *V. harveyi* under different assay conditions (210–213).

Overall, these studies have several important implications for developing AI-2 QSI strategies. Biochemical and biological analyses indicate that phosphorylation of AI-2 analogues is necessary but not sufficient for antagonism in *E. coli* and *S.* Typhimurium (212) and that both the shape and flexibility of the analogue are likely important for the generation of productive interactions between the analogue and AI-2 receptor (209). Also, given the species specificity exhibited by many of the analogues tested to date, disruption of AI-2 signaling in multispecies environments will likely require multiple AI-2 analogues used in combination that can inhibit AI-2 QS in various species simultaneously (209, 212) or in combination with inhibitors of other QS pathways that contribute to virulence alongside the AI-2 pathway. Finally, although AI-2 derivatives do not appear to be cytotoxic toward mammalian cells (211), work will need to be done to assess the stability and efficacy of AI-2 analogues *in vivo*. One concern for *in vivo* efficacy is the general instability of AI-2 molecules. However, Guo and colleagues recently demonstrated that a *bis*-ester-protected "prodrug" form of isobutyl-DPD effectively inhibited AI-2-based quorum sensing in *E. coli* comparably to the unprotected inhibitor (216). Furthermore, the *bis*-ester-protected isobutyl-DPD was a selective antagonist for *E. coli* despite the fact that the unprotected analogue antagonized AI-2-mediated quorum sensing in both *E. coli* and *S.* Typhimurium (216). The reason for this selectivity was not elucidated, but it was reasoned to be the result of differential diffusion of the prodrug into the cells and/or activation of the prodrug following permeation. In either event, ester analogues of AI-2 derivatives may prove helpful in alleviating instability issues of these types of molecules while simultaneously increasing target selectivity (216).

Peptide analogues. Quorum sensing in *S. aureus* is a unique case wherein natural variants of the QS signals function as QS inhibitors against AgrC receptors of strains belonging to different *agr* groups. There are at least four different *agr* groups within *S. aureus* (Fig. 2), and many other *agr* groups exist in other staphylococci. The AgrC receptor of each group responds to its own AIP molecule by activating the *agr* regulon, but this activation is inhibited by heterologous AIPs of other groups (217). The exceptions to this are the AIPs of of groups I and IV, which differ by only a single endocyclic residue; AIP-I strongly activates its cognate AgrC receptor while weakly activating AgrC of group IV strains, while AIP-IV strongly activates the *agr* response in both groups I and IV (218). The molecular determinants for AIP specificity have been shown to reside entirely in the interaction between the AIP signal and the AgrC receptor (130). Both cognate activation and heterologous inhibition activities require the ring structure of AIP, as linear AIPs have no detectable activity in any *agr* group (219, 220), but the requirement of the thioester linkage has been debated (218–220). The tail of the AIP molecule is required only for activation, as mutation of the tail amino acid residues or removal of the tail did not alter inhibition of the *agr* response in heterologous groups despite eliminating self-activation activity (130, 219). Numerous SAR studies have mapped amino acid residues critical for AIP activity, and overall the results demonstrate that critical amino acids differ between AIP groups (218–220). For example, in AIP-I the endocyclic aspartic acid residue adjacent to the cysteine residue is critical, as replacement of this amino acid with alanine converts AIP-I to a potent inhibitor of AgrC of groups I and IV (218, 219). In contrast, mutation of the exocyclic residue asparagine of AIP-II to alanine converted the peptide to a self-antagonist, while mutation of the endocyclic residues did not alter its activity (221). Geisinger et al. recently examined inhibition of constitutively active AgrC mutants by a range of AIP analogues and found that heterologous AIPs can inhibit AgrC activity by distinct mechanisms. Some heterologous AIPs were competitive inhibitors of the cognate AIP, while others were found to act as inverse agonists, inducing a nonactive AgrC conformation even in the absence of activating ligand (222). This may help to explain why residues critical for activity differ between AIP groups. Systematic mutation of native AIP molecules has generated analogues with various changes in activity, including reduced activation activity, enhanced activation activity, loss of all activity, conversion of self-activation to self-inhibition activity, and a unique case of conversion of cross-inhibition to cross-activation activity (218–221). Combining multiple mutations known to provide antagonist activity, for example, truncating the tail of a self-inhibitory AIP-I mutant, has also been shown to have potential in the generation of stronger inhibitors that function against all *agr* groups (218).

In terms of quorum-quenching utility, multiple papers have reported encouraging results for the use of AIP analogues as novel therapeutics. Lyon et al. described an AIP-II derivative lacking the exocyclic tail portion of the molecule, designated trAIP-II (Table 3), which functioned as an inhibitor in all four *agr* groups, including in cognate group II strains, making this molecule the first known AIP-based global inhibitor of *S. aureus agr* activation (130). Furthermore, addition of trAIP-II to *S. aureus* cultures reduced production of δ-toxin, a known virulence factor (130), indicating a reduction in virulence potential. Separately, 14 macrocyclic peptide-peptoid hybrid molecules lacking thioester linkages were designed as AIP-1 analogues, and one was found to alter *S. aureus* biofilm formation *in vitro*, a known AIP-modulated behavior, although the mechanism of action by the molecule was not investigated (223). The most persuasive evidence that AIP analogue inhibitors can be potential antivirulence compounds comes from experiments done in mice. Subcutaneous coadministration of synthetic AIP-II along with a group I *S. aureus* strain was shown to significantly reduce abscess formation in mice compared to administration of the group I strain alone (219). Wright et al. (224) used *in vivo* imaging to demonstrate that *agr* expression is activated quickly following injection of bacteria into mice but is then downregulated during a "metbolic eclipse period" of 36 to 48 h, after which the *agr* regulon is reactivated. Administration of an antagonistic AIP both weakened and delayed the original activation of *agr* by an average of 3 h and prevented later reactivation of the locus. Furthermore, although *agr* expression was not prevented completely, the delay and dampening of *agr* expression prevented abscess formation from occurring in mice (Fig. 6) (224), suggesting that therapeutic manipulation of the timing of QS activation via administration of quorum-quenching compounds may be sufficient to reduce virulence of at least some bacterial species.

The regulatory pathway controlling competence development in *Streptococcus pneumoniae* was among the very first quorum-sensing systems identified for any bacterium (225), but only in recent years has a link been made between this peptide-signaling system and *S. pneumoniae* pathogensis (21, 23). *S. pneumoniae* competence is induced by the linear, 17-amino-acid competence-stimulating peptide (CSP), which is recognized at the cell surface by the histidine kinase ComD (22). In studies probing critical

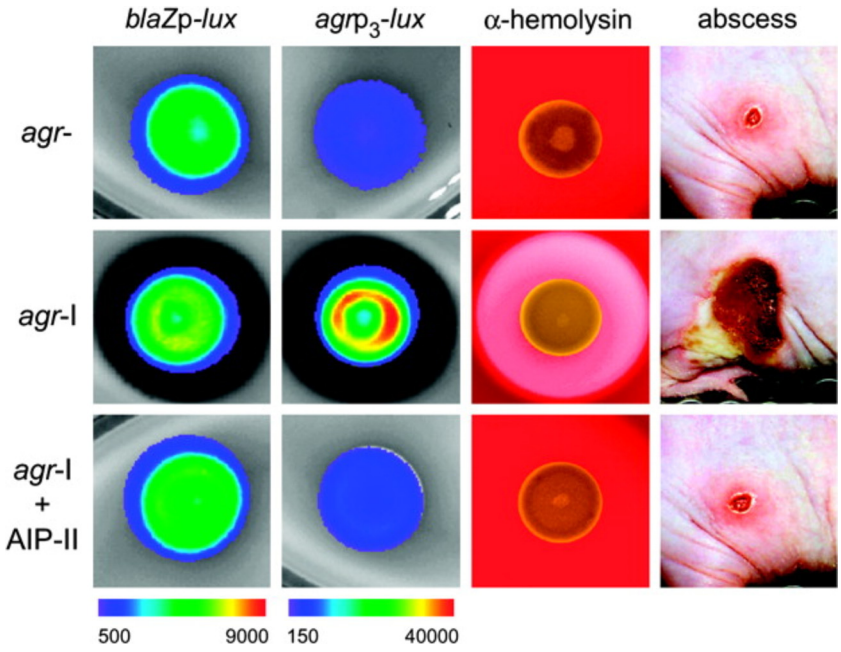


FIG 6 Inhibition of *Staphylococcus aureus agr* quorum sensing. Activation of the *agr* system can be visualized with an *agr*P_3-*luxABCDE* reporter (column 2) and is required for hemolysin activity on blood agar plates (column 3) and lesion formation in a subcutaneous infection model in mice (column 4) (compare each to the first row, where *agr* is mutated). The effect of AIP-II on the agr-I strain phenocopies the *agr* mutant, demonstrating inhibition of *agr* signaling. (Reprinted from reference 224. Copyright 2005 National Academy of Sciences, U.S.A.)

residues of CSP, alanine substitution of the first amino acid generated a peptide (CSP-E1A) that could block the ability of the native CSP to bind and stimulate ComD signaling (226). During *in vitro* studies, adding CSP-E1A to cells significantly decreased virulence factor expression. More impressively, coinjection of the inhibitor peptide with *S. pneumoniae* in a mouse lung infection model led to improved mouse survival compared to a no-treatment control or to coinjection of *S. pneumoniae* with a noninhibitory peptide analogue. Additionally, coinjection of the inhibitor blocked the bacteria's ability to transform into an antibiotic-resistant strain within the mouse when DNA encoding antibiotic resistance was supplied simultaneously (226). These results indicate a potential to block acquisition of new genes by bacteria, provided that an inhibitor is supplied at a time when extracellular DNA is available. Certainly further testing will be needed to verify these results, and studies designed to measure peptide stability under *in vivo* conditions will be needed to address concerns underlying the use of linear peptides as potential quorum-quenching therapies.

Small-molecule inhibitors of other QS receptors. Following optimization of a lead compound derived from an anti-QseC small-molecule screen in enterohemorrhagic *E. coli* (EHEC), Rasko et al. generated the QseC antagonist LED209 (188). The compound was shown to inhibit virulence factor gene expression *in vitro* in response to either AI-3 or the host adrenergic signaling molecule epinephrine and abolished lesion formation in cell culture despite having no effect on EHEC growth. Although LED209 was unable to significantly affect EHEC colonization of infant rabbit intestines regardless of the timing of inhibitor administration, oral administration of LED209 was able to attenuate virulence of *S.* Typhimurium in mice following intraperitoneal (i.p.) injection. Additionally, a single oral dose of LED209 afforded protection to mice that were preexposed to *Francisella tularensis*, another pathogen in which AI-3-based QS contributes to virulence (188). Unfortunately, although LED209 did not inhibit EHEC growth (188), a recent study found that deletion of *qseC* results in large metabolic changes in both *F. tularensis* and EHEC that consequently affect virulence factor production (227). Thus, QseC inhibition is likely both an antimicrobial and an anti-QS strategy.

Only within the last year have PqsR antagonists been identified. Initial SAR studies of PQS in 2010 led to the identification of molecular determinants important for agonism of PqsR. These included the alkyl chain, which exhibited a direct relationship with the potency of activation, and electron density of the aromatic ring. Unfortunately, although various PQS derivatives exhibited agonist and synergistic agonist activity, no antagonism was detected (228). In 2012, however, Lu et al. generated and tested a set of HHQ analogues with various side chains and benzene ring substituents and identified the first reported PqsR antagonists (127). Although none of the alkyl side chain analogues showed antagonist activity, the introduction of strong electron-withdrawing groups, such as nitrile, trifluoromethyl, or nitro, at position 6 of the benzene ring resulted in potent antagonistic activity. The most potent inhibitors identified were compounds 19 and 20 (Table 3), which had trifluoromethyl and nitro functional groups, respectively, and IC_{50}s of near 50 nM. Further experiments indicated that both compounds were competitive inhibitors of PQS, and direct binding of an antagonist to PqsR was confirmed by surface plasmon resonance (SPR) biosensor experiments (127). Altering the alkyl chains of the antagonists resulted in decreased activity, confirming the importance of an alkyl chain at least 6 carbons in length for both agonism and antagonism of PqsR. Antagonist analogues had no observable antibacterial effect, but compound 19 was able to inhibit pyocyanin production by 74% in *P. aeruginosa* PA14 (at a concentration of 3 μM). Surpris-

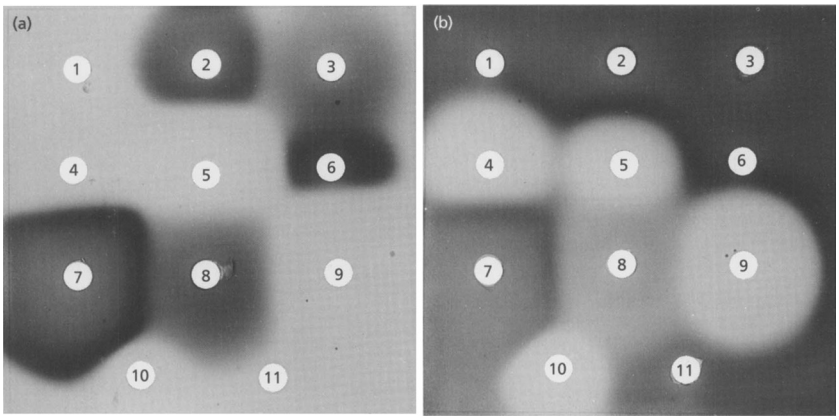


FIG 7 Induction (a) and inhibition (b) of violacein synthesis in *Chromobacterium violaceum* CV026 by synthetic AHLs. (a) CV026 seeded in agar; (b) CV026 plus 1 μM C6HSL. Various AHL compounds, numbered 1 to 11, were added to wells cut into the agar. Violacein production (dark halos around wells in panel a) indicates agonistic activity, and white halos (b) indicate violacein inhibition and hence AHL antagonism. (Reprinted from reference 11 with permission of the Society for General Microbiology.)

ingly, however, elastase production and rhamnolipid production were not inhibited even at higher concentrations of the compound, but no explanation for these results was provided (127). In a follow-up report by the same group, rational design strategy and SPR biosensor analysis were used to identify additional PqsR binders (229). The κ-opioid receptor agonist (±)-*trans*-U50488, which was previously shown to activate quinolone signaling in *P. aeruginosa* (230), was used as a starting point for fragment-based design of novel inhibitors. This ultimately led to the identification of the hydroxamic acid-derived antagonist compound 11, which inhibited pyocyanin production by *P. aeruginosa in vitro* with an IC_{50} of 23.6 μM (229). Although the potency of compound 11 was less than that of HHQ analogues 19 and 20, compound 11 has a lower molecular weight and is therefore more amenable to further optimization for use *in vivo*. This second report also helped define structural features of PqsR ligands that determine agonism versus antagonism (229), making rational design of potent PqsR antagonists more feasible moving forward.

Natural-Product QS Inhibitors

Natural sources of small-molecule inhibitors provide an unremitting source of bioactive compounds. In recent years, more than 60% of new molecular entities introduced as agents to fight cancer have come from natural sources (231). The success that natural sources have in producing active compounds often stems from the fact that nature has the ability to produce highly complex molecules. These characteristics, such as having multiple chiral centers and complex bonding structures, make such compounds difficult to design and synthesize in the lab. With the widespread availability of bacterial reporter strains that provide robust autoinducer responses with easily measurable products, such as luciferase, pigments, and enzymes, the ability to test samples of naturally occurring compounds is limited only by the ability to gather and acquire sizeable amounts of starting material.

The most frequently utilized reporter system that provides a quick assessment of AHL-related inhibitory activity has been that of *C. violacium*. The hallmark characteristic of this bacterium is its ability to produce the water-insoluble purple pigment violacein, for which production is positively regulated by AHLs. A *C. violaceum* mutant, CV026, contains a transposon insersion in *cviI*, a *luxI* gene homologue, rendering the strain unable to produce its native AHL, C6HSL (11). However, this strain can detect exogenously provided AHLs with acyl chain lengths of eight carbons or fewer when growing on agar plates (coculturing of microbes alongside *C. violaceum* is also commonly employed) (Fig. 7a). When CV026 is provided with short-chain acyl homoserine lactones (or other QS agonists), violacein is generated, providing a clear visual indicator of functional signaling. To determine whether an unknown compound has antagonistic activity, typically a subsaturating concentration of AHL (e.g., 1 μM C6HSL) is provided to cells to induce violacein production; if production of the pigment is disrupted by the tested sample, thereby resulting in a clear zone on a field of blue pigment, one would conclude that it contains inhibitory activity (Fig. 7b) (11).

Several other reporter systems have been designed to improve efficiency and sensitivity in screening for inhibitors. A fine example is the quorum-sensing inhibitor selector (QSIS) vectors developed by the Givskov group (139). These vectors were designed to select for, or screen activity of, inhibitors of AHL-mediated signaling. They utilize the *V. fischeri* LuxR or *P. aeruginosa* LasR and RhlR proteins as targets, whose activities can be assessed upon regulation of toxic gene products (phospholipase A or levansucrase) or reporter gene products (β-galactosidase or green fluorescent protein). A clear demonstration of this system can be found in studies that tested 100 extracts from 50 species of *Penicillium*, finding that one-third of species produce QSI compounds, including penicillic acid and patulin (232). Staphylococcal AIP reporters that make use of genetic reporters, such as β-galactosidase fused to central virulence genes of the *agr* QS system, have also been developed, which have allowed, for example, the identification of trihydroxy methyl xanthones from fungal sources as inhibitors of AIP signaling (233).

Cell-cell communication among bacteria undoubtedly promotes beneficial interactions among cells, but it has been considered likely that signal interference plays a natural part in interactions between microbial species and between microbes and higher organisms. Screening for inhibitory characteristics using the approaches outlined above has yielded numerous leads from diverse

sources. Small molecules produced by bacteria, fungi, plants, and small animals are highlighted here.

Furanones. Brominated furanones were among the first recognized small-molecule inhibitors of quorum sensing. Though concerns with toxicity remain a blockade for their commercial or therapeutic use, furanones have served as helpful molecular probes in understanding signaling and the consequences of its inhibition.

From 1974 to 1981, the Roche Research Institute of Marine Pharmacology collected samples of marine specimens for purposes of identifying new natural sources of antimicrobial compounds. Several algal species, including those of *Delisea*, were found to have broad-spectrum antimicrobial activities targeting both Gram-negative and Gram-positive bacteria (234). In subsequent studies, compound isolation focused on *Delisea pulchra* (132, 235, 236) due not only to its ability to produce interesting secondary metabolite compounds but also to its profound capability to stave off colonization by common microbial epiphytes. Active compounds produced by *Delisea* were repeatedly found to be halogenated (234), and several new brominated and chlorinated furanones were isolated (132). Insightful comparisons of structural similarities between furanones (five-membered lactones) and acylated homoserine lactones led to the hypothesis that the algal metabolites may bind AHL receptors. Tests of this hypothesis were first conducted on *Serratia liquifaciens*, due to its recognized reliance on AHLs to initiate swarming motility (133). Applying brominated furanones to agar led to a dose-dependent inhibition of swarming while not affecting general swimming motility, a QS-independent behavior (133). Negative swarming effects could be overcome by increasing concentrations of AHL, suggesting a competitive interaction between AHL and furanone for the LuxR-type receptor protein.

Diverse sets of furanone compounds have been generated by chemical synthesis (237, 238), yielding inhibitors tested most frequently and rigorously on *P. aeruginosa* quorum sensing and on the *V. fischeri* LuxR protein heterologously expressed in *E. coli*, where compounds C-30 and C-56 (Table 3) were the most effective inhibitors (134, 237, 239–243). More recently, additional synthetic schemes have led to new varieties of furanones, but their inhibitory properties remain relatively untested (244, 245).

Despite new compound development, toxicity remains a concern that may prevent widespread application for therapeutic uses. Evidence for these concerns has come from two aquaculture trials, one using rainbow trout and the other with rotifer *Brachionus plicatilis*, where furanones were added directly to the aquatic tanks. At the higher concentrations tested (1 μM C-30 in trout experiments and ~24 μM C-2 in rotifer experiments), the compounds resulted in abrupt fish death (in <4 h) and severely reduced growth rates of rotifers (242, 246). In another study, toxicity to human fibroblasts was found for C-56 at concentrations of >10 μM (245), yet levels of up to 20 μM C-30 have not been toxic on HeLa human cancer cells (242), and in *in vivo* mouse studies, where up to 17 μg/g body weight (BW) C-56 or up to 2 μg/g BW C-30 was used, no obvious health effects on mice were seen. Certainly, further toxicity studies will be required before use can be expanded for broader applications.

QS inhibition by halogenated furanones has been documented for various plant and animal pathogens. Bioluminescence in *Vibrio harveyi* is inhibited greater than 1,000-fold at micromolar concentrations of furanone (247). Correspondingly, survival of brine shrimp after challenges with pathogenic *V. harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* improved with low-micromolar treatments of synthetic furanones (248). Similar results could be seen in treating *Brachionus* rotifer aquaculture infected with *V. harveyi* (246), and treatment of *V. anguillarum* infections with furanone in rainbow trout delayed mortality by several days (242). The ability of the plant pathogen *Erwinia carotovora* to produce carbapenem antibiotics was found to be disrupted when grown in the presence of furanone, and the compound slightly decreased protease and cellulase activities (249).

Furanone inhibition of *P. aeruginosa* signaling has focused on disrupting virulence gene expression. Exoprotease, pyoverdin, and chitinase activities were reduced, and biofilms were easily penetrated and disrupted by synthetic furanones (240). *In vitro*, polymorphonuclear leukocytes added to *P. aeruginosa* biofilms responded with an oxidative burst only toward bacteria pretreated with C-30 (250). Disruption of QS by furanones has translated to improved animal survival when challenged with lethal *P. aeruginosa* inoculations. In one study, C-56 furanone fed to mice orally over a three-day trial period increased survival rates nearly 4-fold over those for untreated animals when challenged with a lethal dose of *P. aeruginosa* (243). In separate studies, bacterial loads in lungs were three orders of magnitude lower when C-30 furanone treatment was provided subcutaneously every 8 h over 7 days following bacterial challenge (240). Impressively, even if bacterial infection was allowed to establish for 2 days before treatment, *P. aeruginosa lasB* expression could be suppressed for up to 6 h following a single administration of C-30 intravenously (240).

The AI-2 pathway has also been proposed to be affected by furanones in both Gram-negative and Gram-positive bacteria. For instance, chemotaxis and flagellar biosynthesis genes of *E. coli* that were reportedly induced by exogenous AI-2 were inhibited when the furanone C-2 was added to cultures (251). It remains unclear, though, how *E. coli*'s chemotaxis and flagellar responses to AI-2 are propagated, since the Lsr system, the only known AI-2 sensory pathway in enteric bacteria, has not been directly linked to these chemosensory systems. AI-2 signaling in Gram-positive bacteria is even less clear, since no signaling pathways have been described, yet staphylococcal biofilms were inhibited from growing on materials used in medical devices when furanone compounds were integrated into them (252). Likewise, synthetic furanones and furanone analogues incorporating thiophenone moieties decreased staphylococcal biofilm formation mediated by AI-2 (245, 253). However, these reports are in conflict with findings that *luxS* mutants of *Staphylococcus epidermidis* display increased biofilm formation (254) and that furanones supplied at sub-MICs actually enhance biofilm development (255). It will be difficult to resolve these apparently contradictory studies without further investigations; however, in agreement among them were findings that furanone concentrations of above 10 μM were cytotoxic, and since the strongest biological activities were seen near these concentrations, perhaps their effects involve nonspecific stress responses in addition to AI-2 responses. Similar effects may account for findings in *Bacillus anthracis* as well. For this bacterium, mutations in *luxS* lead to growth suppression (256), and this served as the explanation for why inhibiting *luxS* or the AI-2 pathway with addition of 5 μg/ml furanone attenuated growth and 40 μM led to a complete lost of viability (257). Thus, whether effects seen in these Gram-positive bacteria are a result of AI-2 signal inhibition, disruption

of LuxS-mediated metabolism involving the reactive methyl cycle, or off-site toxic effects of furanones remains unknown.

The large underlying question surrounding furanone-based inhibition that remains unanswered is how these compounds inhibit signaling. The presumed mechanism, and basis for testing, came initially from structural similarities between furanones and AHL signaling molecules. They were proposed to function as competitive inhibitors of autoinducer receptors (133). However, demonstrating direct interaction between furanones and receptor proteins has been difficult. Further complicating matters is that both AHL and AI-2 signaling pathways are purportedly disrupted by the compounds, indicating that these molecules can inhibit two different types of receptor families. Evidence of direct binding to either type of receptor comes from genetic and biochemical studies indicating that furanones bind at or near signal-binding pockets but interact with them in a significantly differently way than agonistic molecules. Illustrating this idea, LuxR mutant proteins that interfere with C8HSL binding impacted sensitivity to furanone treatments only slightly, indicating different binding modes for the two types of ligands (258). However, these modest inhibitory effects were reversed with further C8HSL addition, supporting the notion of a competitive interaction for the binding site. Separately, furanones have been observed to enhance cellular turnover of LuxR proteins (237, 258), counteracting the stabilizing effects provided by AHLs (259). Taking the data together, furanones appear not to inhibit LuxR activation by a "classic" competition mechanism, but rather they appear to engage LuxR at sites that are somewhat different from those used in AHL interactions and thus cause protein instability. Less is clear on how furanones interfere with AI-2-based signaling, though covalent modification of LuxS by C-2 has been implicated (147) (see "Inhibition of AI-2 Production" above) and may explain findings of reduced AI-2 production by this compound in some species (251, 253). To date, no studies have investigated if AI-2-binding proteins are directly affected by furanones.

Other natural product modulators of QS. (i) Plant-derived compounds. Plants have served as excellent systems in which to study microbe-microbe and interkingdom interactions. Plants produce compounds that serve as both autoinducer agonists and antagonists (260, 261), and dozens of plant molecules, whose production profiles are found to change with the developmental stage of the plant, have been isolated and cataloged for their potential to interfere with bacterial signaling (262). For example, exudates from alfalfa seeds (*Medicago* spp.) have revealed at least one active compound, canavanine, an arginine analogue (135), that inhibited AHL signaling in *C. violaceum* and could inhibit production of extracellular polysaccharide II (EPSII, a necessary component for plant cell invasion in the symbiont *Sinorhizobium meliloti* (135). The mechanism by which canavanine inhibits QS is unknown, but this demonstrates that plants can manipulate bacterial cohabitants, even those with which they have symbiotic relationships.

Plants are also privy to the information shared among bacteria, and evidence shows that they can adjust gene expression accordingly and, in response, jam bacterial signaling. Roots of the model host plant *Medicago truncatula* (legume), used to study symbiotic relationships with *Sinorhizobium meliloti* and pathogenic relationships with *P. aeruginosa*, were treated with nanomolar concentrations of AHLs, and subsequent changes in the plant proteome were measured (263). Cellular levels of at least 154 proteins were found to significantly change in the presence of AHLs, with concentrations of some increasing and those of others decreasing. Astonishingly, the response to AHL treatments included secretions by the root tissues that contained compounds having QS agonist properties that were able to induce a LasR reporter, as well as QS-inhibiting properties that were able to block an AI-2-based reporter (263). Though studies have yet to identify the compounds produced by the plant roots, the work offers a glimpse of the complex interplay between plants and microbes, whether their relationships are symbiotic or pathogenic.

Plant-based food sources and traditional medicines that have been customarily associated with healthy diets and well-being have received special attention as testable sources of QSI compounds. Extracts of fruits (e.g., blackberries, cranberries, vanilla, and citrus) and herbs (e.g., rosemary and turmeric) and extracts and oils of other plant materials (garlic, clove, and cinnamon) and medicinal plants (e.g., betel nut and notoginsing) have all displayed QSI activity to some degree, typically found via inhibition of violacein production in the *C. violaceum* bioassay (137, 138, 264–275). In very few cases, however, have individual compounds been isolated or identified from these kinds of complex sources. Separation of complex material into homogenous parts is necessary to understand the true activity of individual compounds, which thus would improve the likeliness of understanding mechanisms of inhibition. For instance, curcumin, a phenolic compound, was tested for quorum-quenching activity independently from its source, turmeric, itself a traditional antimicrobial agent. Subinhibitory concentrations of curcumin were found to affect quorum sensing in *P. aeruginosa*, decreasing biofilm formation and virulence factor production (276). Effects of downregulated virulence factors *in vitro* were translated to decreases in pathogenicity of *P. aeruginosa* on plant tissues (using *Arabidopsis thaliana* as a model) and in an invertebrate animal model (*C. elegans* infection); however, a molecular explanation for how curcumin affects *P. aeruginosa* quorum sensing remains to be seen. Separately, in studies of 50 medicinal plants from South Florida, six plants representing five different families were found to have QSI activity against *C. violaceum* and *A. tumefaciens* and could inhibit production of elastase, pyoverdin, and biofilms of *P. aeruginosa* (277, 278). Several flavenoid-like compounds were found to be produced by one of these species, a member of the *Combretum* family, and the molecule catachin (a flavan-3-ol) was one compound isolated that accounted for these activities (136).

The popular herbal medicine goldenseal (*Hydrastis canadensis*) is known in traditional medicine for its anti-inflammatory and antimicrobial properties and is found to produce an abundance of alkaloid compounds with a potential for bioactivity (279). A recent investigation found that extracts of goldenseal were able to inhibit the Agr signaling pathway of *S. aureus*, as determined by luciferase reporters that indicate quorum-sensing activity, and were shown to reduce alpha-toxin production (280). Application of the extracts to cultures of methicillin-resistant *S. aureus* (MRSA) grown with keratinocytes reduced keratinocyte toxicity incurred by the bacteria. Interestingly, the extracts were active against three of the four *agr* groups, indicating the active component has a generalized mechanism of inhibition against the signaling pathway (280). In addition to the benefits that a broad Agr inhibitor would provide as a therapeutic, the compound(s), once identified, will serve as a valuable molecular probe to help understand signal transduction of the Agr pathway.

Recently, two studies have used activity-based purifications to elucidate active QSI compounds from garlic and horseradish. From horseradish, the isothiocyanate iberin (Table 3) was identified and shown by transcriptome profiling to substantially inhibit expression of LasR- and RhlR-regulated genes in *P. aeruginosa* (137). From garlic extracts, an allyl sulfide called ajoene (Table 3) was isolated using the activity monitored by the QSIS reporter system (138, 139). Interestingly, ajoene specifically affected a narrow set of QS-regulated genes in *P. aeruginosa*, including those involved in rhamnolipid production, and thus prevented *P. aeruginosa* biofilms from killing polymorphonuclear leukocytes in flow chambers, an effect attributable to rhamnolipid production (138, 139). Additionally, ajoene, as well as the furanone C-30, was shown to synergize with the antibiotic tobramycin in killing of *P. aeruginosa* biofilms and to improve clearance of *P. aeruginosa* from lungs in a mouse model of pulmonary infection (138, 139).

Another such food additive that has been investigated is the flavoring agent cinnamaldehyde. Studies have demonstrated its ability to disrupt QS signaling in *Vibrio* (281, 282) and *Burkholderia* (283) species, and it or its structural analogues were effective at protecting *Artemia* shrimp (281) and *C. elegans* (284) from *V. harveyi*, *V. anguilarum*, and *V. vulnificus* infections while not inhibiting growth of the bacteria. The proposed mechanism of action for cinnamaldehyde is disruption of protein-DNA interactions of the QS-responsive master regulatory protein, LuxR (not to be confused with LuxR-type AHL receptors), with targeted promoter sequences. SAR studies found that cinnamaldehyde analogues disrupted, modestly, LuxR's interaction with the LuxR consensus binding sequence (281); however, mechanisms explaining these effects have not been clear, and the knowledge that cinnamaldehyde is able to covalently modify cysteine residues of various proteins (285) leaves open the possibility that other targets are at play. The decreased toxicity of food and herbal extracts compared with other better-studied but cytotoxic QS inhibitors offers a practical rationale to mine natural dietary substances for novel QSI compounds.

Bacterial plant epiphytes also influence plant pathogens by interfering with QS. Perhaps not surprising is the finding that various epiphytes produce molecules that can be both agonistic and antagonistic on pathogens. Work in the Lindow lab has found examples of complex relationships among epiphytes and the pathogen *P. syringae* on plant leaves. Several species of *Erwinia*, *Pantoea*, and *Pseudomonas* were found to be capable of producing high levels of AHL (at levels up to 18 times higher than those produced by *P. syringae*) (286). Since swarming motility is a key aspect for invasion and spread for *P. syringae* but is negatively regulated by QS in this organism, high levels of epiphyte-produced AHLs on the leaf surface were found to reduce the frequency of brown spot disease on bean plants caused by *P. syringae* (286). Alternatively, several other plant epiphytes were found to produce secreted factors capable of QS inhibition. Eleven percent of 123 plant epiphyte strains were able to interfere with *P. syringae* QS-mediated regulation, as determined by changes seen in AhlR (a LuxR family protein)-dependent regulation (287). Surprisingly, inhibition by epiphytes was tightly correlated with their ability to limit amounts of iron available to the pseudomonads by producing higher-affinity siderophores. Because *P. syringae* forgoes QS under iron-limiting conditions, the sequestration of iron by epiphytes represents a novel mechanism of QS inhibition (287, 288). Separately, another study found that iron limitation was the cue for *P. aeruginosa* to produce a factor that inhibited *Agrobacterium tumefaciens* from forming biofilms. Unexpectedly, production of this new substance appears to be neither regulated by classic iron regulators nor controlled by the *las*, *rhl*, or *pqs* quorum-regulated system. Iron sequestration by *P. aeruginosa* did not account for the inhibitory effects on *A. tumefaciens*; instead, the produced factor appears to stimulate biofilm dispersal (289).

(ii) Compounds from aquatic sources. Aquatic ecosystems continue to reveal diverse microorganism taxonomies and to yield enormous diversity in secondary metabolite products. Numerous reports are emerging that provide empirical data demonstrating QSI activity from various marine sources that include sponges (290), microalgae (291), bryozoa (292), and alga- and coral-associated bacteria (293, 294). Low-micromolar concentrations of phenethylamide compounds from the marine bacterium *Halobacillus salinus* were found to inhibit *V. harveyi* luminescence and *C. violaceum* pigment production without inhibiting growth (295). Secondary metabolites isolated and purified from sponges (290) and Gram-negative cyanobacteria (296) also exemplify successes where low-micromolar concentrations of purified compounds could inhibit QS reporters. Recently, for example, low-molecular-weight compounds isolated from the marine cyanobacterium *Leptolyngbya* and named honaucins A to C (for Honaunau Bay, HI, the location of the reef where the bacteria were isolated) (Table 3) exhibited potent, low-micromolar QSI activity in both a *V. harveyi* bioluminescence assay and an *E. coli* AHL reporter assay (140). Halogenated derivatives of these compounds, however, showed impressive submicromolar activity. Intriguingly, these compounds were also found to inhibit inflammation responses in activated macrophages, reminiscent of the inhibitory activity that 3OC12HSL exhibits on the NF-κB pathway (297), which helps to emphasize that bioactive small molecules are capable of targeting several seemingly unrelated pathways. In another study, two novel cyclic depsipeptides were isolated from a marine *Photobacterium* species and termed solonomides A and B (Table 3); they were identified by screening inhibitory effects against the *Staphylococcus aureus agr* signaling pathway (141). The four amino acid ring structures, linked via 3-hydroxyoctanoic or 3-hydroxyhexanoic fatty acids, closely resemble the structures of the Agr AIP peptides and would seemingly compete with AIPs in binding to AgrC.

(iii) Peptide-associated inhibitors. Microbial diversity also flourishes in soils, and actinomycete species, such as *Streptomyces*, are famously important sources of antimicrobial compounds. In a screen for compounds that inhibited *Enterococcus faecalis* production of gelatinase and a secreted serine protease, which are regulated by the *fsr* quorum-sensing system (298), a *Streptomyces* soil isolate extract containing the tricyclic polypeptide siamycin blocked gelatinase production at sub-growth-inhibitory concentrations (299). In *E. faecalis* the autoinducer GBAP triggers the two-component signal transduction system FsrC/FsrA, and data indicate that siamycin most likely inhibits signaling through this pathway. Together these studies may enhance an understanding of how cyclic peptides of some Gram-positive bacteria are recognized by their respective membrane-spanning sensor kinase proteins, which have been exceedingly difficult receptor proteins to study, especially from a structural biology standpoint.

Other microbially produced compounds that inhibit *Staphylococcus agr* signaling have been identified from the human commensal bacterium *Lactobacillus reuterii*, a vaginal isolate thought beneficial in maintaining a healthy vaginal environment (300).

Cyclic dipetides (or diketopiperizines [DKPs]) account for this observed inhibitory activity, and cyclo[L-Tyr-(L or D)-Pro] and cyclo(L-Phe-L-Pro) were identified to inhibit *agr* activities of all four *agr* groups, including expression of the toxic shock syndrome toxin (TSST). Cyclic dipeptides are produced by numerous other bacterial species and are capable of interfering with (both promoting and inhibiting) AHL signaling pathways. Gram-positive (301) and Gram-negative (302, 303) species produce DKPs and appear to be capable of targeting LuxR proteins in engineered biosensors, but they were also found to affect *S. liquifaciens* swarming (303). The oral pathogen *Streptococcus mutans* utilizes multiple peptide-based quorum-sensing systems that promote bacteriocin production, competence, and biofilm development. Mixed cultures of *S. mutans* with other oral bacteria, whether commensal species such as *Streptococcus salivarius* or pathogens such as *Porphyromonas gingivalis* and *Treponema denticola*, present situations where the competing species inhibit *S. mutans*' ability to regulate QS-controlled behaviors and do so via an extracellular factor (304, 305). Though mechanisms for this inhibition have not been discerned, the inhibitory activity can be abolished by boiling active supernatants (305) or by supplementing cultures with excessive *S. mutans* CSP (304). Clearly, niches where large amounts of microbial interaction and competition exist may provide excellent examples of natural QS antagonism.

ANTIBIOTICS AS QS INHIBITORS

Although this review focuses on studies that investigated the mechanisms and feasibility of inhibiting quorum-sensing signaling, which have emerged largely as efforts motivated to find alternative methods to control harmful bacterial behaviors without a reliance on antimicrobial therapies, there have been several recent studies indicating a link between antibiotic treatment and quorum-sensing effectiveness. There is growing awareness that many antibiotics affect multiple modes of bacterial physiology by means that are beyond direct inhibition of currently understood primary targets of each antibiotic class (306), and evidence suggests that QS is one alternative target of antibiotics in some species. Therefore, it is worth discussing interactions between antibiotics and bacterial cell-cell communication.

In the 1980s it was recognized that treatment of diffuse panbronchiolitis (a biofilm-associated disease of the lung) with macrolides, such as erythromycin, was beneficial in long-term disease prognosis and survival (307, 308). Similar findings were reported for cystic fibrosis patients infected with *P. aeruginosa* (309–311), where improved lung function in children was seen following six to 15 months of azithromycin (AZM) treatment. This was an unexpected finding, considering the high levels of resistance that *P. aeruginosa* shows toward these drugs, and general anti-inflammatory effects associated with macrolides were suggested to account for the positive results (312). However, *in vitro* testing with subinhibitory concentrations of macrolides indicated an additional effect of their application on reduction of *P. aeruginosa* virulence factor expression, including decreased production of exotoxin A, proteases, elastase, DNase, leukocidin, and phospholipase C (313–315). Positive outcomes from sub-MIC AZM treatments against *P. aeruginosa* in a murine pneumonia model provided further laboratory evidence that macrolides could affect the physiology of the bacteria without inhibiting their growth *in vivo* (316).

In characterizing the outcomes of subinhibitory drug treatments on *P. aeruginosa*, Sofer et al. were first to demonstrate that erythromycin treatment reduced AHL production (317); these findings were further elaborated by Tateda et al. (318), who quantified the reductions of C4HSL and 3OC12HSL to be greater than 70 and 90%, respectively, in PAO1 treated with 2 μg/ml AZM. These reductions coincided with reduced Las- and Rhl-dependent gene transcription and have been corroborated by microarray and proteomic studies (319, 320), which found, in addition to QS gene misregulation, a reduction in levels of oxidative stress and motility-related genes but an increase in type III secretion (TTS)-related genes. The diminished response to oxidative stress conditions may account for the reduced cell viability in late stationary phase that was reported to occur with increasing concentrations of macrolides (318, 319). Likewise, the finding that TTS was induced with exposure to AZM may explain the paradoxical finding that pretreatment of bacterial cultures with macrolides prior to intranasal inoculation in mice led to an enhanced lethal effect (321). It is plausible that inducing the TTS system before infection primed the bacteria to enter a virulent state, essentially providing a head start in combating the host's immune response.

Further animal studies have reaffirmed findings that AZM improves *P. aeruginosa* infections. In a cystic fibrosis mouse model (utilizing $Cftr^{-/-}$ mice [322]), treatment with AZM reduced the bacterial load in lungs of mice, and gene expression of the QS-regulated *lasB* gene was downregulated *in vivo* (323). AZM treatment also downregulated production and polymerization of alginate, which, combined with decreasing QS responses, was a major contribution to enhanced sensitivity to H_2O_2 and complement and loss of viability in the stationary phase of growth. The benefits of AZM treatments have also been tested in experimental urinary tract infections (UTIs) in mice. Inhibition of motility and biofilm formation by AZM likely contributed significantly to clearance of bacteria from murine renal tissues after 5 days (324).

Although azithromycin has shown the highest efficacy in inhibiting QS, other classes of antibiotics have also been shown to have similar QSI activities, raising the question as to how these compounds inhibit production of autoinducers. In a screen of 11 antimicrobial compounds, a cephalosporin (ceftazidime [CFT]) and a fluoroquinolone (ciprofloxacin [CPR]) were also able to inhibit AHL production in *P. aeruginosa* and showed regulatory effects very similar to that seen for AZM, as determined by transcriptional profiling after treatment with each antibiotic (320). As the structural compositions of AZM, CFT, and CPR are highly divergent, it appears unlikely that each directly blocks activity of AHL synthase proteins and instead seems more probable that a separate, unifying theme for inhibition is at work. It has been suggested that cellular responses to the drugs lead to decreased membrane permeability to block antibiotic penetration, and consequentially this also inhibits autoinducer transfer (320). Until this is tested directly, other mechanisms should be considered, including effects on general stress responses and resistance to reactive oxygen species.

Lastly, it is worth considering the combinatorial use of antibiotics with anti-QS strategies, since QSI could be effective at blocking bacterial signals that either enhance antimicrobial resistance (e.g., drug efflux) or promote physiological states that enhance persistence (e.g., biofilms). As such, QSI may render cells more susceptible to a variety of antimicrobial compounds. Two studies have recently assessed this possibility using QSI compounds found from natural sources together with tobramycin, a drug reported to inhibit biofilm development of *P. aeruginosa* QS mu-

tants (232, 250). In *P. aeruginosa* foreign-body infection models in mice, it was demonstrated that combinatorial treatments of tobramycin with either the furanone C-30, ajoene (from garlic), or horseradish extracts (with the major QSI component being iberin) all exhibited synergistic inhibitory activities toward viable bacterial cell counts when treatments were provided soon after implantation. In tests where treatments were initiated 11 days postimplant, they were not beneficial, indicating the need to intervene before bacteria enter a chronic infection state and/or before the host immune system walls off the infection. In separate studies, combinatorial application of the previously identified QSI compounds baicalin hydrate, hamamelitannin, and cinnamaldehyde with antibiotics was tested on *Burkholderia* spp., *S. aureus*, and *P. aeruginosa* to measure viable counts of bacteria in biofilms and in animal models of infection (283, 284). Synergistic effects were most pronounced in *Burkholderia cepacia* biofilms treated with baicalin hydrate combined with tobramycin, which resulted in an additional 2-log decrease in viable cell counts compared to tobramycin treatment alone. Enhanced bactericidal activity was also reported against some *P. aeruginosa* and *S. aureus* biofilms using combinatorial treatments (baicalin hydrate or cinnamaldehyde combined with tobramycin for *P. aeruginosa* and an AIP analogue, hamamelitannin, with clindamycin or vancomycin for *S. aureus*), though the enhancement of killing was less robust than that seen for *Burkholderia*. QSI compounds were also seen to improve infection outcomes and survival probabilities in invertebrate infection models and to decrease bacterial loads in mouse pulmonary tissues (283). These reports offer intriguing new data that may lead to new treatment options for difficult-to-eradicate bacterial infections.

QS AS A THERAPEUTIC TARGET

Quorum sensing has been touted as an ideal target for so-called "antivirulence" therapies for multiple reasons. These include the importance of QS for virulence in a variety of bacterial species, low selective pressure to develop resistance since QS is generally not required for growth, and the possibility of more species-specific effects of QSI drugs compared to traditionally antibiotics due to the species-specific nature of most QS systems. Unfortunately, although theoretically QSI compounds should be effective therapeutics for these reasons, there remain many concerns that the presumed benefits of QSI therapy compared to antibiotic treatment will not come to fruition. Although studies have demonstrated an important role for QS in the pathogenicity of numerous bacteria under laboratory conditions, the importance of QS in clinical settings and the ability of QS inhibitors to function *in vivo* remain debatable. Despite these concerns, there are examples of successful alleviation of pathogenicity using QSI compounds in various model systems, giving hope that quorum quenching will be an effective therapy against at least some bacterial pathogens.

Concerns

The first assumption underlying the hypothesis that QS is a valid target for antivirulence therapies is that quorum-sensing systems are actively utilized during infection and are important for virulence. It has become apparent, however, that this is not always the case. For example, *P. aeruginosa* QS systems have been demonstrated to be important for pathogenesis in multiple animal infection models (325–328), and AHLs and QS-regulated genes have been demonstrated to be expressed *in vivo* (329–331). However, analyses of clinical *P. aeruginosa* isolates have revealed that *lasR*, and less frequently *rhlR*, can be mutated during infection (332–336). In one study it was demonstrated that a single cystic fibrosis patient acquired at least four independent *lasR* mutants over an 8-year period (337), suggesting a strong selection pressure *in vivo* for abolishing LasR-mediated QS during chronic infections. Isolates in which this type of genetic adaptation has taken place would be unaffected by QSI therapies targeting LasR activity. It has also been shown that expression of virulence factors known to be LasR regulated can evolve to be QS independent when the organism is grown under conditions requiring QS (338). Interestingly, no suppressor mutants restoring virulence factor production arose in a *lasR rhlR* double mutant background, suggesting that by targeting both systems, it may be possible to limit such compensatory genetic adaptations (338). A different example of target receptor deficiency is the case of SdiA of *S.* Typhimurium. Although SdiA has been shown to respond to AHLs from *E. carotovora* during growth *in vitro* and therefore could theoretically be targeted by QSI compounds (339), SdiA was found to not be expressed during growth *in planta* (340). Thus, although laboratory experiments may indicate that a given compound can effectively inhibit quorum sensing in a given bacterium, it will be important to ensure that therapy is undertaken at times when the target molecule is present and functional QS contributes to pathogenesis.

The second assumption is that because QS is not essential for bacterial growth, inhibition of QS should not exert selective pressure on the bacteria to develop resistance. Unfortunately, although QS may not be required for growth under laboratory conditions, the growth benefits afforded by QS *in vivo* may not be fully appreciated. For example, although *P. aeruginosa* growth is not affected by *lasR* mutations under traditional laboratory conditions, LasR is known to regulate many genes involved in central metabolism (341, 342). Additionally, *lasR* mutants have been found to spontaneously emerge during growth on rich media and have been found to have a growth advantage on certain amino acids known to be present at high concentrations in CF patient airway secretions (337). This growth advantage is at least in part due to increased expression of the regulator CbrB, which regulates various catabolic pathways, suggesting that aberrant expression of other regulators resulting from the loss of LasR function may provide a growth benefit during growth *in vivo*. Such a growth advantage could explain the emergence of *lasR* mutant populations during *in vivo* infection (332–337).

P. aeruginosa also requires a functional LasR QS system in order to grow on adenosine (343), which has been postulated to be a physiologically relevant carbon source during growth *in vivo* (241), although it should be mentioned that the relatively high frequency of *lasR* mutant isolates recovered from chronic CF patients (332) contradicts this assumption unless adenosine utilization has become LasR independent in these strains. In any event, it seems likely that inhibition of LasR during growth on adenosine would result in selective pressure to develop resistance to the inhibitor in order to maximize growth. In agreement with this hypothesis, a transposon mutant screen of *P. aeruginosa* grown on adenosine in the presence of the QS inhibitor C-30 successfully yielded mutants with increased resistance to C-30 (241). The various mutants were found to carry transposon insertions in either *mexR* or *nalC*, both genes which encode repressors of the multidrug resistance efflux pump MexAB-OprM. Importantly, mutation of *mexR* had no effect on bacterial virulence but did abolish

the protective effects of C-30 treatment during infection in *C. elegans* (241). Interestingly, mutations in *mexR* and *nalC* have been found in clinical isolates of *P. aeruginosa* (344), and these isolates were resistant to C-30 (241), suggesting physiologically relevant pressures for mutations that could increase inhibitor resistance during growth *in vivo*. Of additional interest, MexAB-OprM was recently demonstrated to play a more ubiquitous role in quorum sensing in *P. aeruginosa*. It naturally functions to selectively export a variety of 3-oxo-AHLs with between 8 and 14 carbons out of the cell, ultimately changing the relative accessibility of different AHLs to LasR (345). This natural mechanism for signal selection may prove to be a broad obstacle for the use of AHL signal mimics as QSI compounds, as they may also be substrates for MexAB-OprM; however, the use of non-AHL-like inhibitors as well as quorum-quenching strategies not involving internalized inhibitor molecules may obviate this issue.

Central to debates regarding selective pressures resulting from QSI is the notion of "social conflict" arising between bacteria that produce and detect QS signals and respond accordingly (cooperators) and bacteria that no longer detect or respond to the QS signals (cheaters), and this phenomenon has been examined by multiple research groups (334, 346). The cheater group is referred to as such because although these bacteria no longer participate in QS, they can still benefit from any extracellular (shared) QS-dependent products generated by the cooperators without expending the energy or resources to make these products themselves. As such, it is reasoned that cheaters should have a growth benefit over cooperators due to a reduction in energy costs. This has been confirmed by *in vitro* experiments showing that the frequency of cheaters (receptor mutants) increased over time when cocultured with cooperators (WT bacteria) in media requiring extracellular QS-regulated products for growth (346, 347). The presence of cheaters in a mixed population would also be expected to incur a cost for the population as a whole, because as the cheater population grows, the relative amount of QS-regulated extracellular products produced per bacterium declines. This is precisely what was observed when WT *P. aeruginosa* (cooperators) was cocultured with *lasR rhlR* double mutant bacteria (cheaters) in QS-requiring media; overall, the growth of the culture was hampered compared to that of a pure WT culture (347). Similar results were observed with a single *lasR* mutant during coculture under conditions requiring QS for growth (348). However, private goods controlled by QS (for example, nucleoside hydrolase, which allows for growth of *P. aeruginosa* on adenosine) are found to limit the emergence of cheaters when resources attainable only by private goods are at sufficient levels in the environment (349). Under these circumstances, cheaters arise but are kept at very low levels. The presence of cheaters has been confirmed to exist *in vivo* when the population dynamics of *P. aeruginosa* were monitored during the first 20 days of infection in intubated patients. The proportion of patients carrying *lasR* mutants increased over time, but the WT population was never eliminated completely (334), presumably because the growth advantage of the mutant exists only in the presence of the cooperating strain and its QS-regulated products. Encouragingly, this study also found that ventilator-associated pneumonia occurred later in patients colonized with mixed populations of WT and QS mutant bacteria than in patients colonized with WT bacteria alone, suggesting that the presence of a cheater population may be sufficient to hamper overall virulence *in vivo* (334).

Relevant to quorum quenching, administration of QSI compounds would be expected to promote this type of social conflict when they are administered to mixed populations containing both QSI-sensitive and QSI-resistant bacteria. In the most recent publication on this subject, Mellbye and Schuster (347) used WT bacteria as functional mimics of QSI-resistant mutants (they continue to quorum sense and are thus cooperators) and QS receptor mutants as mimics of QSI-sensitive bacteria (cheaters). Given that WT bacteria (QSI-resistant mimics; cooperators) were outcompeted by the QS receptor mutant bacteria (QSI-sensitive mimics; cheaters) during *in vitro* coculture, their results suggest that social cheating would self-limit the enrichment of QSI-resistant strains (347), which agrees with *in vivo* results demonstrating that the frequency of *lasR* mutants increased over time in intubated patients (334). However, these *in vivo* results did not derive from QSI-treated patients, and *in vitro* coculture of only two strains engineered to represent QSI-resistant and QSI-sensitive populations may not accurately assess social conflict arising from QSI treatments. Multiple types of QS mutants have been shown to arise *in vivo*, including single receptor mutants and double receptor mutants with or without compensatory mutations allowing for QS-independent production of virulence factors. Additionally, mechanisms of QSI resistance could be diverse, including upregulation of efflux pumps or target modification that abolishes the inhibitory effect of a QSI compound. Bacteria could also evolve means to enzymatically inactivate QSI compounds either extra- or intracellularly, with the former allowing for the conferment of QSI resistance in *trans* across a population. Therefore, the effect of a given QSI compound on a mixed community of *P. aeruginosa* during infection would presumably depend on multiple factors: the distinct subpopulations of bacteria and their relative abundances within the bacterial community; the target specificity (i.e., only LasR or both LasR and RhlR), mechanism of action (i.e., signal degradation, receptor antagonism, etc.), and inhibitory efficiency of the QSI compound; the relative importance of both extracellular and intracellular QS-regulated factors for growth in a given *in vivo* environment; the presence of compensatory mutations permitting QSI-sensitive or QS-deficient strains to produce traditionally QS-regulated products; and the existence and mechanism(s) of QSI resistance.

Given that *lasR* mutations arise frequently during *in vivo* infection (332–336), it could also be reasoned that use of QSI compounds that target LasR may prevent the natural evolution of *lasR* mutant strains over time, given that the presence of the QSI compound would effectively make the bacteria *lasR* negative without genetic mutation. In agreement with this, azithromycin treatment of intubated patients colonized by *P. aeruginosa*, a treatment which inhibits both *las* and *rhl* QS systems, resulted in a decrease in the frequency of *lasR* mutants over time, whereas in the group treated with a placebo the frequency of *lasR* mutants increased. This would suggest that QSI therapy may select for virulent (WT) populations that could initiate late-onset infections once treatment was stopped (350). Thus, it is extremely difficult to gauge at this time if QSI against *P. aeruginosa* will be effective *in vivo*.

A third assumption underlying the belief that QS is a good therapeutic target is that the species specificity of most QS systems should reduce the issues of broad-spectrum activity and off-target effects traditionally associated with antibiotic treatment. Unfortunately, there are data indicating that this may not hold true. First, of all, certain methods of quorum quenching are inherently

nonspecific, especially for systems utilizing AHL signals. For example, AHL lactonases tend to degrade AHLs of all sizes and substitutions, which would result in the nonspecific quenching of all QS systems in the vicinity that utilize AHL signals. Second, despite the general species specificity of QS, certain receptor proteins, especially those for AHLs, have been found to have highly similar ligand-binding pockets. Given that these receptors often recognize the same or similar AHL molecules, it is not surprising that AHL signal analogues have been found with antagonistic activity against multiple bacterial species despite differences in the target AHL receptor proteins (171, 198). Another potential issue is that inhibition of QS in even a single strain of bacteria can result in unsolicited effects on other resident organisms. Morello et al. screened rhizosphere bacteria for the native ability to interrupt quorum sensing in the biocontrol bacterium *Pseudomonas aerofaciens* (351). QS in this bacterium controls both persistence in the rhizosphere and antibiotic production. Several isolates that could inhibit antibiotic production by *P. aerofaciens* were found, although the inhibitory molecules were not identified. Importantly, it was shown that the presence of the inhibitory isolates was sufficient to reduce *P. aerofaciens*-mediated inhibition of the fungal pathogen *Gaeumannomyces graminis* var. *tritici*, which causes take-all disease of wheat and barley (351). Although that study aimed to interrupt QS in a biocontrol agent, which would likely not be the goal of QSI therapies, it still highlights the unanticipated effects that quorum quenching can have on other interspecies and interkingdom interactions. This issue was more directly illustrated by Cirou et al. when they used biostimulation both to increase the native population of the AHL-degrading bacterium *Rhodococcus erythropolis* in the hydroponically grown potato rhizosphere and to facilitate rhizosphere colonization following introduction of the biocontrol isolate *R. erythropolis* strain R138 (352). Both treatment conditions resulted in an increase in the *R. erythropolis* population and a concomitant biasing of the bacterial community, including a decrease in the resident *Agrobacterium* population. Furthermore, artificial introduction of the biocontrol strain in combination with biostimulation treatment appeared to displace native AHL-degrading populations (352). Although overall the results demonstrate that beneficial bacteria can be selected for by using biostimulation approaches, they also indicate the potential impact that such selection could have on soil microbial communities and point out the need for more rigorous evaluation of global effects following QSI-based treatments.

Finally, even in the event that resistance to inhibitors does not develop and that inhibitors work selectively on targeted bacteria, it is possible that quorum quenching *in vivo* will not be effective for various other reasons. Rasch et al. investigated if QSI could be effective as a food preservation strategy but found that although some QSI compounds were successful in inhibiting QS-regulated protease activity *in vitro*, they had no effect on bean sprout spoilage by *Pectobacterium* strain A2JM (353). The reason for this ineffectiveness remains unknown. Separately, Park et al. hypothesized that since *B. glumae* requires AHL signaling for virulence in rice grain and seedlings, quorum quenching would be effective in limiting pathogenesis. Unfortunately, although an AiiA-transformed *B. glumae* strain had reduced AHL accumulation, there was no difference in toxoflavin production or pathogenicity (354). The authors postulated that this was due to residual C8HSL levels remaining even in the presence of AiiA, which could be sufficient to induce virulence factor production (354). Complete elimination of QS signals is not always required in order to achieve attenuation of virulence, however, as was seen in *S. aureus*, where even a delay in *agr* signaling was sufficient to reduce pathogenicity (224). Additionally, it is becoming increasingly apparent that there can be diversity in the QS regulon between different strains of the same species, as has recently been found in *P. aeruginosa* (355), in which case QSI may be more effective against certain strains of a pathogen and less effective against others. Thus, the efficacy of QSI will likely be species, and perhaps strain, specific due to the mechanistic intricacies of each individual system and the distinct role that QS plays during infection by different pathogens.

QSI Success Stories

Despite the concerns and reported difficulties in identifying successful QSI strategies, promising developments have been made toward treating some of the most important human pathogens of our time using QSI approaches. We conclude this review by highlighting some examples of these developments.

V. cholerae remains a perennial scourge to communities worldwide that lack reliable sanitary drinking water. *V. cholerae* utilizes a complex quorum-sensing pathway that integrates two separate signals to control virulence factor expression, biofilm formation, and protease secretion. Interestingly, *V. cholerae* QS is pivitol in switching from a virulent state at low cell densities to a dispersion state at high cell densitites, opposite to what is seen in many other pathogens. Virulence factors, such as cholera toxin, are inhibited upon application of autoinducers, whereas a protease is induced by them, leading to detachment from the intestinal epithelia (356–358). Thus, therapeutic treatment of cholera would call for quorum-sensing agonism while bacteria are at a low cell density to promote repression of virulence factor production and to facilitate premature exit from the intestine. Importantly, this treatment would need to be applied in the intestinal tract, where *V. cholerae* affects the host. In an innovative approach, Duan and March cloned and expressed *V. cholerae*'s CAI-1 synthase gene, *cqsA*, in a probiotic strain of *E. coli* Nissle. Exposure of *V. cholerae* to spent culture supernatants from the CqsA-expressing Nissle strain (Nissle-cqsA) or coculture with Nissle-cqsA led to decreases in cholera toxin production by the pathogen (359). Testing for effectiveness *in vivo*, Nissle-cqsA was prefed to mice that were subsequently challenged with *V. cholerae*. Astonishingly, Nissle-cqsA, but not the parent strain expressing the empty vector, dramatically enhanced survival of mice in a dose- and time-dependent manner (360). Furthermore, immunohistochemical staining for cholera toxin in treated mice showed significantly less toxin accumulation in intestinal epithelial cells than in those of untreated mice or mice fed the Nissle-vector strain (360). Thus, CAI-1 expressed by Nissle, presumably while colonizing the intestines, was effective in preventing severe *V. cholerae* infection. These results not only provide an encouraging new approach for treating cholera but also demonstrate the potential power of probiotic bacteria to serve as vectors for delivery of signaling molecules.

Antibiotic resistance has been most steadily emerging in the important human pathogen *S. aureus*, and a need to find new treatments to combat this organism is urgent. Therefore, it is worth reiterating in this section the promising results regarding QSI therapies against *S. arueus*. By coadministering inhibitory synthetic AIPs along with *S. aureus* bacteria, staphylococcal lesion formation in the skin of mice was reduced or prevented (Fig. 6)

(219, 224). Though a complete understanding of how the inhibitor peptides maintain a relatively long-lasting effect during the course of the infections is lacking, these experiments stand out as highly impressive among *in vivo* animal studies, especially given the strong phenotype seen in preventing lesion formation. Additionally, coinjection of *S. aureus* with the anti-AI-4 MAb AP4-24H11 or passive immunization with the MAb prior to lethal challenge with *S. aureus* fully protected mice from either skin lesion formation or death, respectively (115). Clearly, disrupting *agr* quorum sensing during the course of infection has beneficial results.

Further success stories can be taken from several field studies testing the utility of enzymatic quorum quenching in treatment of plant and aquaculture pathogens. Many transgenic plant species expressing AiiA have been shown to be more resistant to soft rot disease (85, 91, 92); however, the use of such plants in food production has been met with strong resistance from consumers, propelling the development of alternative quorum-quenching approaches. Bacterially mediated quorum quenching using AiiA was also demonstrated to be beneficial against plant pathogens, both when *aiiA* was expressed in the pathogenic bacterium itself and during coinoculation of the pathogen with bacteria naturally producing AiiA or engineered bacterial strains expressing heterologous AiiA (88, 89, 93–96). AiiA was also shown to have potential for use in aquaculture, as carp fed either recombinant AiiA protein or *Bacillus* expressing *aiiA* were more resistant to infection by the fish pathogen *Aeromonas hyrophila* (361, 362). Separately, AHL-degrading enrichment cultures (EC) of undefined microbial isolates naturally associated with marine organisms have been evaluated for use as probiotic cultures in aquaculture. EC5, isolated from the Pacific white shrimp *Penaeus vannamei*, was tested for the ability to protect the gnobiotic rotifer *Brachionus plicatilis* from *V. harveyi*-mediated growth retardation, a phenomenon known to require functional AHL and AI-2 QS systems (363). EC5 was able to enhance growth of *B. plicatilis* challenged with *V. harveyi* mutants lacking functional AI-2 signaling but not those lacking only AHL QS. This indicated that EC5 was capable of degrading 3OHC4HSL and alleviating AHL-mediated growth defects but was unable to inhibit AI-2, and it suggested that probiotic treatment combining AHL-degrading ECs with AI-2-inhibitory bacteria or compounds may be an effective way to protect *B. plicatilis* during aquaculture (363). More recently, Nhan et al. tested ECs from two types of sea bass for their abilities to improve the health of shrimp larvae exposed to waters contaminated with *V. harveyi* (364). Addition of ECs directly to the rearing water or through inoculation of larva food resulted in improved larval survival and larval quality compared to those of *V. harveyi*-challenged populations, confirming the utility of probiotic AHL-degrading bacteria in protecting against pathogenic bacteria. Interestingly, ECs also improved the health of larvae grown under normal hatchery conditions (not challenged with *V. harveyi*), indicating that AHL degradation may also protect shrimp larvae against harmful constituents of the native intestinal microflora (364). Thus, although the use of transgenic plants for food production may be limited due to consumer concerns, biocontrol methods using native WT strains of autoinducer-degrading bacteria may be more widely accepted and appear to be sufficiently effective against both plant and aquaculture pathogens.

Finally, we conclude with an example of a recently engineered system that utilizes quorum sensing in a way quite unlike the strategies we have recapitulated in this review; since its approach is unique, we feel compelled to mention it here. As we have described above, autoinducer signals are often unique to the species that produce them and serve in some ways as a distinctive "scent" that might be traced. Using synthetic biology approaches, Saeidi et al. generated an *E. coli* version of a bloodhound, capable of sniffing out and killing *Pseudomonas aeruginosa* (365). Expressed in *E. coli*, LasR enabled the bacterium to detect 3OC12HSL produced by *P. aeruginosa*. Additionaly, promoter sequences targeted by LasR when bound to 3OC12HSL were engineered upstream of genes encoding a pseudomonas-killing bactericidal compound, pyocin C5. When cultured together, the 3OC12HSL generated by *P. aeruginosa* triggered *E. coli* to produce and release pyocin C5. The engineered *E. coli* was thus able to kill the pseudomonads and was effective against *P. aeruginosa* growing both planktonically and in biofilms (365). Though these studies were conducted only under *in vitro* conditions, it will be interesting to watch as developments lead toward *in vivo* testing.

CONCLUDING REMARKS

In closing, since the initial discovery of quorum sensing more than 40 years ago, the mechanistic understanding of various QS systems and appreciation for the importance of QS in pathogenesis of many bacterial species have soared. As additional QS systems and signal molecules have been identified, so have the known natural incidences of quorum-sensing inhibition, by both signal-targeting enzymes and small-molecule inhibitors of signal synthases and receptors. The possibility of targeting quorum sensing as a means to alleviate or prevent infection has been met with strong optimism in the wake of the antibiotic resistance problem that currently impedes treatment of many bacterial pathogens. If numbers of intellectual property disclosures are an indicator of the interest in an idea, the growing number of patent applications in the past few years that focus on QSI approaches shows a bright future for this field (366). As is often the case with drug discovery, the large number of enzymes and compounds now known to have QSI activity far outnumbers those that have been tested for *in vivo* efficacy. Perhaps as the development of quorum-sensing inhibition expands from an academic environment, where mechanistic studies continue to identify new opportunities and proofs of concept, to an applied-science environment, where enterprise can drive innovation toward marketable products, treating infectious diseases by communication disruption will become a common, mainstream therapy.

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