Controlling infection by tuning in and turning down the volume of bacterial small-talk

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As the prevalence of bacterial resistance to multiple antibiotics increases it is becoming progressively more difficult to treat infections and, in many cases, the available therapeutic options are severely limited. Hence, there is a growing urgency to the search for novel targets and the development of new antimicrobials. To infect a host and cause disease bacteria produce an array of virulence determinants that contribute to pathogenesis. It is now known that many different Gram-positive and Gram-negative pathogens communicate via the production and sensing of small, diffusible signal molecules, to coordinate virulence determinant production. As a consequence, this event, now termed quorum sensing, represents a novel therapeutic target offering the opportunity to attenuate virulence, and thus control infection, by blocking cell-to-cell communication.

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The introduction of antibiotics in the 1930s heralded a new era in the treatment of bacterial infections, which resulted in a marked decrease in the number of deaths from bacterial infections. However, by the 1950s the emergence of resistance to clinically useful antibiotics was already apparent. More recently, it has become increasingly difficult to treat many infections since therapeutic options are severely limited, and in some cases non-existent, due to the emergence and spread of resistant bacteria. Multiple-resistant organisms, for example the meticillin-resistant *Staphylococcus aureus*, (MRSA), are an increasing problem in hospital environments, despite the instigation of strict control guidelines.[1] In the UK, the Public Health Laboratory Service has reported that, as a proportion of all *S aureus* causing septicaemia, MRSA has risen from 2% in 1990 to greater than 40% in 2000.[2] Of particular concern is the recent report of the first clinical isolate of *S aureus* to be classified as completely resistant to vancomycin,[3] which is one of the last lines of defence in the treatment of many bacterial infections. Such rapid emergence of resistance epitomises the problems associated with antibiotic development. Millions of dollars and pounds, and years of development, are devoted to antibiotics that almost immediately start to become less therapeutically useful when they finally reach the marketplace. However, as the urgency for novel antibacterials continues to grow, pharmaceutical companies are now focusing much attention and resources in this area with the common aim of finding novel bacterial targets and new ways to control and eradicate bacterial infections. In this context, the development of novel broad-spectrum antimicrobials that can target bacteria quickly and safely without succumbing to bacterial resistance continues to represent the ideal goal. Historically, most pushes for drug development have been aimed at developing agents that prevent bacterial reproduction, either bactericidal or bacteriostatic. This type of approach has been highly successful but transient in nature because the population of bacteria it is either forced to adapt—ie, become resistant—to ensure survival, or be eradicated. The inhibition of bacterial growth in vivo during the infection process is not the only approach to eradicate an invading pathogen. The ability to inhibit interactions between the pathogen and the host by attenuating virulence of the former, but not viability itself, represents an alternative and promising therapeutic approach.

To cause an infection a pathogen must gain entry into the host and then withstand host defence mechanisms to become established and cause the signs and symptoms of disease by damaging host tissues. To achieve this aim, bacteria synthesise a repertoire of products, or virulence determinants, which can be either cell-associated or extracellular, that contribute to their ability to cause disease and are a measure of a pathogen's virulence. Although the production of virulence determinants is required in vivo for the pathogen to successfully infect a host and cause disease, they are generally not strictly essential for the viability of organism, and thus represent a particularly attractive therapeutic target. Importantly, since such virulence determinants are unique to bacteria, and absent from the host, they also provide a degree of selectivity which is a prerequisite for any antimicrobial therapy. Furthermore, virulence determinants are tightly regulated by the bacterial pathogen to ensure they are deployed only at the appropriate stage of infection. For example, when an infection becomes systemic the expression of exotoxins and host-avoidance factors are normally upregulated with the concomitant down-regulation of factors required for adherence to, and colonisation of, host tissues. Consequently, regulators that control the expression of genes that encode for such virulence determinants are also a potential target

Quorum sensing: bacterial cell-to-cell communication

For many pathogens the outcome of the interaction between host and bacterium is greatly influenced by bacteria

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population size. For an invading pathogen, the ability of a single bacterial cell to communicate with its neighbours makes sense. It would be advantageous for the survival of a pathogen to instigate a unified attack on the host by ensuring that the bacterial cell population has reached a "critical mass" before individual cells commit to the production of its arsenal of virulence determinants and, thus, make certain that the host has insufficient time to mount an effective defence against the invading bacterial population. Such a strategy depends on the ability of an individual bacterial cell to sense information from other bacterial cells and, in response, to coordinate the expression of virulence genes when a sufficient population size has been reached. This cell-to-cell communication relies on the production and sensing of small signal molecules also termed "pheromones". Historically, these signal molecules have also been referred to as "autoinducers", implying a positive feedback or autoregulatory effect. However, since this is not always the case the generic use of this term can be misleading. Since the level of signal molecule is a reflection of the population size, the term "quorum sensing" has been adopted to describe the occurrence whereby the accumulation of signal molecules enables an individual bacterium to perceive when a minimum population unit (or quorum), is reached.[4,5] Transduction of the signal depends on the activation of a response regulator protein, which ultimately brings about the coordinated changes in gene expression needed to mount a specific population response.

In recent years there has been an explosive increase in quorum sensing research. The potential application for the design of novel antibacterial agents is only now being appreciated. It is now known that bacteria employ a whole range of chemical "languages" and several distinct families of signalling molecules have been characterised.[4,5] Furthermore, individual bacterial species can produce multiple distinct signals and many organisms use complicated regulatory hierarchies that control signal production, detection, and response.[6,7]

The chemical "languages" of Gram-negative bacteria

In Gram-negative bacteria, although several signal molecule families have been identified, the most intensively studied and understood are those that belong to the *N*-

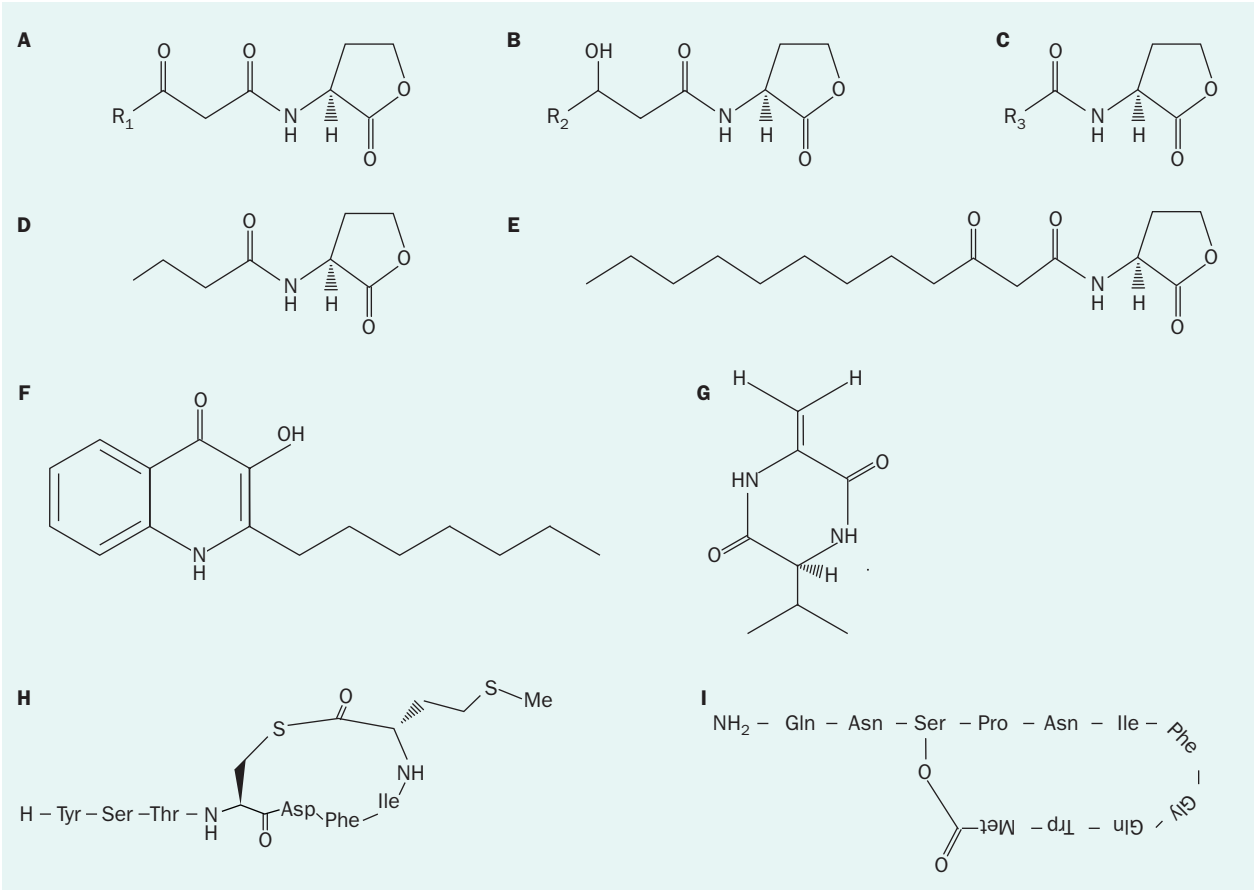

Figure 1. Structures of quorum sensing signal molecules. Generic structures for the N-acylhomoserine lactone family of signal molecules, produced by Gram-negative bacteria, with either (A) -oxo, (B) –hydroxy, or (C) no substituent at the 3 carbon position of the acyl side chain are shown. R=remaining number of carbons within the acyl side chain. For the P aeruginosa *AHLs shown, N-butanoylhomoserine lactone (C4-HSL) (D) and N-(3-oxododecanoyl)homoserine lactone (3-oxo-C12-HSL) (E), R_3 is $CH_3(CH_2)_2$ and R_1 is $CH_3(CH_2)_8$. The "pseudomonas quinolone signal molecule" (PQS), 2-heptyl-3-hydroxy-4-quinolone (F), and the diketopiperazine (DKP) cyclo(ΔAla-L-Val) (G) are also produced by* P aeruginosa*. The quorum sensing peptides produced by the Gram-positive bacteria* S aureus *(Group I) (H) and* E faecalis *(the gelatinase biosynthesis-activating pheromone, GABP) (I) are also shown.*

Examples of AHL-mediated quorum sensing regulation in human pathogens.

Bacterium	LuxR/I homologues	Major AHL	Phenotypes
Pseudomonas aeruginosa	LasR, LasI	3-oxo-C12-HSL	Exoenzymes, Xcp secretion apparatus,
	RhlR, RhlI	C4-HSL	biofilm maturation, RhlR
			Exoenzymes, cyanide, RpoS, lectins,
			pyocyanin, rhamnolipid
Burkholderia cepacia	CepR, CepI	C8-HSL	Protease, siderophore
Chromobacterium violaceum	CviR, CviI	C6-HSL	Antibiotics, violacein pigment, exoenzymes, cyanide
Yersinia pestis	YpeR, YpeI	Unknown	Unknown
Yersinia enterocolitica	YenR, YenI	C6-HSL	Unknown
Yersinia pseudotuberculosis	YpsR, YpsI	3-oxo-C6-HSL	Bacterial motility and clumping
	YtbR, YtbI	C8-HSL	Unknown
Aeromonas hydrophila	AhyR, AhyI	C4-HSL	Extracellular protease, biofilm formation
Brucella melitensis	Unknown	C12-HSL	Inhibition of *virR/virS*

acylhomoserine lactone (AHL) family (figure 1A–E). AHL-mediated communication was for many years believed to be limited to a few related marine bacteria in which it regulates bioluminescence. However, it is now evident that diverse Gram-negative bacteria produce AHLs, with examples of species belonging to the α, β, and γ, but not the δ or ϵ, subclasses of the proteobacteria. Importantly, a number of opportunistic human pathogens, including *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Yersinia pseudotuberculosis*, and *Serratia marcescens*, produce AHLs that are used to regulate diverse physiological processes such as the production of virulence determinants, which are capable of contributing to virulence by causing tissue damage (table).[4,5] Obligate human pathogens such as *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* do not seem to make AHLs[8] and no Gram-positive AHL-producers have yet been identified. Of note, AHL production has not been seen in *Escherichia coli* nor *Salmonella typhimurium*, although the latter has been reported to respond to exogenous AHLs suggesting that they can at least tune in to this signalling language.[9]

Chemically, AHLs consist of a homoserine lactone ring attached, via an amide bond, to an acyl side chain of varying length (figure 1A–E). So far, naturally occurring AHLs with 4–14 carbons in length have been identified, which may be saturated or unsaturated and with or without a hydroxy-substituent, oxo-substituent, or no substituent on the carbon at the 3 position of the *N*-linked acyl chain.[4]

Although AHLs are common to several Gram-negative bacteria, many Gram-negative bacteria also use signalling molecules unrelated to AHLs. In addition to AHLs *P aeruginosa* also produces the "pseudomonas quinolone signal" molecule (PQS), (figure 1F) which structurally resembles the quinolone antibiotics.[10] This molecule has been shown to modulate AHL-mediated quorum sensing in *P aeruginosa*. In addition, several Gram-negative pathogens including *P aeruginosa* produce cyclic dipeptides (diketopiperazines, DKPs), such as cyclo(Δala-L-val) (figure 1G).[11] Although the physiological function of DKPs remains unclear, they are capable of cross-activating, or antagonising, AHL-regulated processes. In other bacteria that are not human pathogens several other distinct signal molecules have been discovered[4] and, hence, it would be a reasonable to assume that many more diverse bacterial signalling molecules have yet to be discovered

Gram-positive quorum sensing molecules

Gram-positive bacteria are known to quorum-sense, although the nature of the signal molecules differs from those in Gram-negative bacteria. By contrast, Gram-positive bacteria use peptides as quorum sensing signalling molecules (figure 1H, I). Peptide-based adaptation is now known to be used by several pathogenic bacteria to regulate physiological processes. For example, both *Bacillus subtilis* and *Streptococcus pneumoniae* use peptides as quorum sensing molecules to regulate development of bacterial competence, as well as sporulation in the latter. Moreover, in *Enterococcus faecalis* and staphylococci, peptide-mediated quorum sensing is involved in virulence gene regulation.[12,13]

Is there a "universal" signalling language in bacteria?

For novel therapies, a common or universal chemical language that spans the Gram-positive/Gram-negative boundary and has widespread potential, would be a particularly attractive target. While the AHLs and modified peptides described above are confined to a reasonably narrow range of bacteria, recent evidence has suggested the existence of a universal quorum sensing language. A family of molecules, termed AI-2, common to many Gram-negative and Gram-positive pathogens including *E coli*, *Helicobacter pylori*, *Neisseria meningitides*, *Porphyromonas gingivalis*, *Proteus mirabilis*, *S typhimurium*, *E faecalis*, *Streptococcus pyogenes*, and *S aureus* has recently been described.[14,15] However, there is no direct evidence for the involvement of AI-2 in the regulation of pathogenic traits. Furthermore, whether AI-2 has a true role in quorum sensing signalling in general has recently been questioned, with suggestions that in most bacteria AI-2 is simply a metabolic side product, which casts doubts on its suitability as a target in the context of quorum sensing inhibition.[14,16]

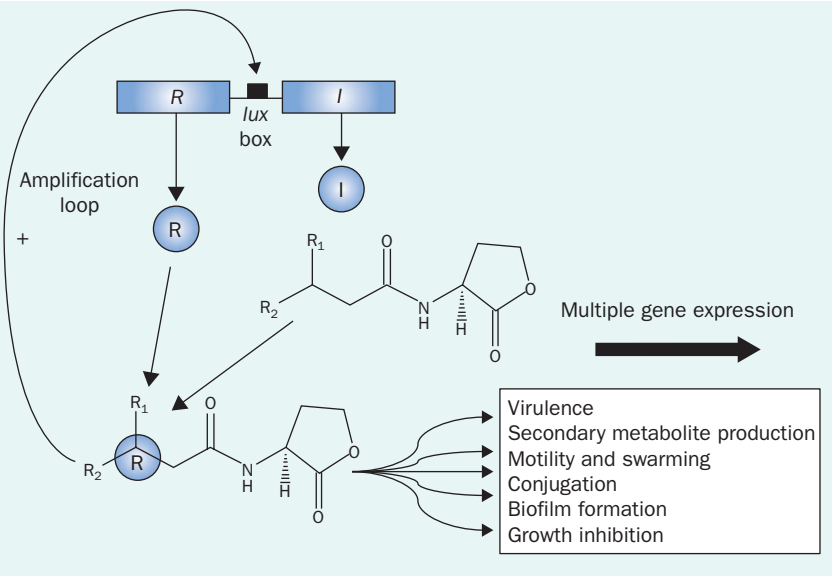

Figure 2. The LuxRI/AHL-dependent quorum sensing circuitry. The "R" and "I" genes are homologues of the Vibrio fischeri luxR *and* luxI *genes in which the R protein is the response regulator and the I protein is the AHL synthase. Arrangement of the* R/I *genes varies between bacteria, being convergent, divergent, in tandem, or unlinked. After synthesis AHLs either diffuse (short chain) or are pumped out (long chain) of the bacterial cell into the surrounding environment before being taken up into neighbouring bacterial cells. AHLs bind to and activate R proteins and the R protein/AHL complex then activates or represses the expression of multiple targets genes by binding to a conserved DNA sequence, termed a lux box or a lux-box-like sequence, just upstream of the target gene(s). In many (but not all) cases, the R protein/AHL complex also activates the* I *gene creating an amplification loop resulting in a rapid increase in* I *gene expression and AHL production.*

AHL-dependent quorum sensing: its role in virulence and the infection process

AHL-dependent quorum sensing systems have now been described in a range of bacterial species and, typically, the two central players belong to the LuxI and LuxR protein families, which function as AHL synthases and transcriptional regulators, respectively.[4] AHLs, synthesised via the LuxI protein either diffuse (for AHLs with a short acyl side chain), or are pumped out of (for AHLs with a long acyl side chain) the bacterial cell, across the cell envelope, and accumulate in the extracellular milieu. Once a sufficient concentration of AHL has been reached, the AHL binds to and activates the transcriptional regulator LuxR protein to form a complex that is responsible for activating or repressing the target genes.[4] In some cases, the gene that encodes for the LuxI AHL synthase (*luxI*) is itself activated by the LuxR/AHL complex resulting in a positive autoinduction circuit in which the AHL signal molecule also controls its own synthesis (figure 2). Some bacteria, for example the opportunistic human pathogens *P aeruginosa* and *Y pseudotuberculosis*, possess multiple LuxR/I type quorum sensing circuits which form a regulatory hierarchy in the control of gene expression.[6,7] Importantly, other classes of AHL synthases distinct from the LuxI family have also been identified. An additional family (LuxM) in *Vibrio* species and a putative novel class of AHL synthases (HdtS) in *Pseudomonas fluorescens* have been identified.[17,18]

One of the most intensively studied pathogens, with respect to quorum sensing, is *P aeruginosa*. This ubiquitous opportunistic human pathogen can cause infections of the blood, skin, eye, gut, respiratory, and genitourinary tract systems in patients immunocompromised by surgery, cytotoxic drugs, or burn wounds. In particular, it is a problem in cystic fibrosis where it efficiently colonises host lung tissue causing chronic pulmonary damage.[19] The virulence of *P aeruginosa* depends on an armoury of virulence determinants, whose production is subjected to AHL-mediated quorum sensing regulation, to varying degrees (figure 3). These include tissue degrading exoproteases (elastase [*lasB*], the LasA protease [*lasA*], and alkaline protease [*aprA*]), haemolytic rhamnolipids, cytotoxic lectins, the free-radical-generating pigment pyocyanin, hydrogen cyanide, the siderophore pyoverdin, catalase, superoxide dismutase, and exotoxin A (*toxA*), as well as the secretion apparatus (*xcp*) needed for the export of many of these extracellular products.[4,20,21] Importantly, under certain experimental conditions, quorum sensing has been shown to play a part in the late stages of biofilm formation in *P aeruginosa*, which may have significant implications for the treatment of biofilm-associated infections.[22]

P aeruginosa has two AHL-dependent quorum sensing systems—namely the *las* and *rhl* systems comprising the LuxRI homologues LasRI and RhlRI, respectively. Whereas LasI directs the synthesis of *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL) (figure 1E), RhlI is responsible for generating *N*-butanoylhomoserine lactone (C4-HSL) (figure 1D).[23,24] Importantly, the two AHL-dependent quorum sensing systems are not independent of each other, but form a regulatory hierarchy in which the Las system regulates the Rhl system. Furthermore, the genes that are regulated by Las and Rhl systems are not mutually exclusive and considerable regulatory overlap exists. In this regard, it has been estimated that 1–4% of all genes in *P aeruginosa* may be controlled to some degree by quorum sensing.[25] More recently, a third LuxR homologue termed QscR has been identified, which further increases the complexity of the regulatory network in *P aeruginosa*. QscR has been shown to regulate the expression of the genes that encode for the LasI and the RhlI AHL synthases.[26]

Considerable evidence has amassed to confirm the role of quorum sensing in the regulation of virulence determinant production in vitro. Moreover, there is also evidence to show that quorum sensing does occur in vivo and is important during the infection process. The contribution of quorum sensing to pathogenesis has been most extensively studied in *P aeruginosa* using various experimental animal models. In a neonatal mouse model of acute pneumonia, a *lasR*-negative mutant of *P aeruginosa* showed significantly less virulence than the parent strain.[27] Furthermore, using the same model a *lasI/rhlI* double mutant was almost avirulent.[28] Similar

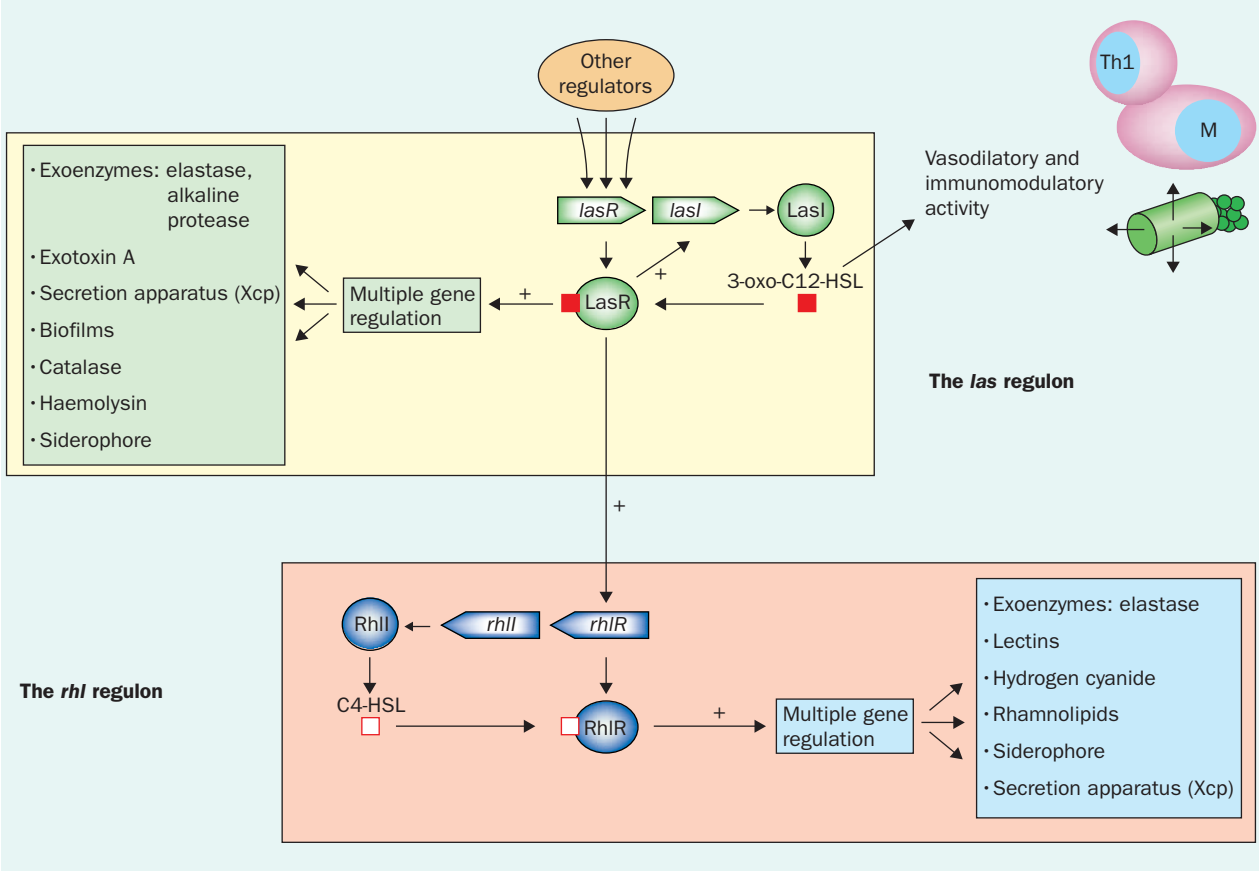

Figure 3. Hierarchical organisation of quorum sensing systems in P aeruginosa*. A simple schematic of the genes encoding for the Las and Rhl quorum sensing systems in* P aeruginosa*. LasR activates the expression of* lasI *to generate 3-oxo-C12-HSL. The LasR/3-oxo-C12-HSL complex positively drives the expression of multiple target genes. The RhlR protein drives the expression of* rhlI*, and hence C4-HSL, and the RhlR/C4-HSL complex is responsible for controlling multiple target genes. LasR/3-oxo-C12-HSL also positively regulates the expression of* rhlI*. Of particular note is the overlap, with respect to the regulation of target genes, between the two systems.*

effects were reported in a burned mouse model of infection, where strains deficient in *lasR*, *lasI*, or *rhlI* were less virulent than the parent strain.[29] By contrast, no reduction in virulence was reported with a *lasR* defective strain in a mouse model of corneal infection.[30] However, together with results obtained for a *lasR* mutant in other model systems,[31] these data suggest that for *P aeruginosa*, AHL-dependent quorum sensing is a general feature of pathogenesis in a range of hosts.

In addition to mutant studies, confirmation that AHLs are produced by *P aeruginosa* in vivo during infection has recently been obtained, using a combination of both direct and indirect methods. Sputa and lung tissue biopsies from cystic fibrosis patients infected with *P aeruginosa* and *B cepacia* have been assayed directly for the presence of AHLs with AHL biosensors.[32–34] Positive results have been obtained for sputa from patients infected with either *P aeruginosa* or *B cepacia* and the presence of AHLs was confirmed by rigorous chemical analyses.[32] Wu et al[35] have also shown the production of AHLs in mouse lung tissue after experimental infections with *P aeruginosa*. Additional evidence in support of AHL production in vivo was reported by Stickler et al[36] during analyses of sections of indwelling urethral catheters that had become colonised by bacterial biofilms while in use. Sections from four of nine of these catheters tested positive for AHLs while unused catheters were negative, which suggests that AHLs were produced in situ in the urinary tract.

Since it is evident that AHLs are produced in vivo, the question now arises as to whether these compounds exert any effects on the host itself that may also contribute to the success of an invading pathogen. In this context, recent work suggests that the *P aeruginosa* AHL signal molecule produced by LasI, 3-oxo-C12-HSL (figure 1), may function as a virulence determinant in its own right since this quorum sensing signal molecule has been shown to have proinflammatory, immune modulatory, and vasorelaxant properties[37–39] 3-oxo-C12-HSL modulated both T-cell and B-cell function and, for example, blocked lipopolysaccharide-stimulated production of tumour necrosis factor α (TNFα) by peritoneal macrophages.[37] Furthermore, 3-oxo-C12-HSL mediated switching of the T-helper-cell response from the antibacterial Th1 response (characterised by interleukin 12 and interferon γ production) to a Th2 response. Since T-cell responses constitute an important component of the host immune defense against *P aeruginosa*, such signal-molecule-mediated suppression of T-cell activity is likely to be advantageous to this pathogen. In addition, 3-oxo-C12-HSL also exerts a pharmacological effect on the cardiovascular system suggesting that host

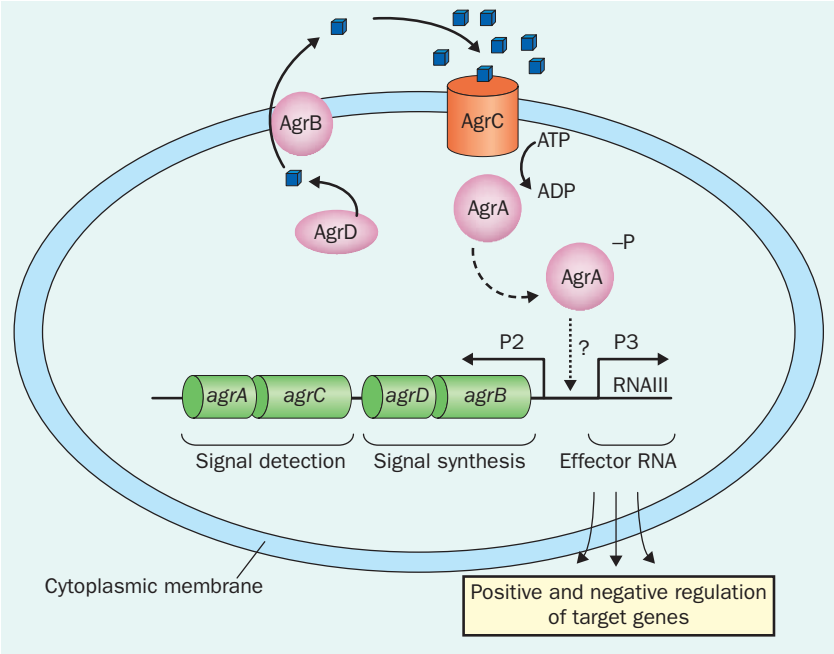

Figure 4. Quorum sensing in S aureus. *In the Gram-positive human pathogen* S aureus, *the quorum sensing molecule is generally a small modified peptide and signal transduction involves a two-component system comprising a sensor protein and a response regulator protein. The* agr *locus consists of two divergent transcriptional units. As shown, the leftward operon is expressed from the P2 promoter and consists of* agrABCD, *which encode the proteins that are responsible for generating and sensing the peptide signal molecule; the rightward operon, transcribed from the P3 promoter, encodes* δ*-haemolysin and RNAIII. The quorum-sensing peptide is derived from the gene product of* agrD *by the action of the AgrB protein, which is also responsible for the export of the peptide out of the cell. As the bacterial population increases, the peptide accumulates in the extracellular environment. Once a critical concentration is reached, the peptide interacts with the AgrC membrane associated sensor protein (a histidine protein kinase) causing it to autophosphorylate. The phosphate is then transferred to the response regulator and in its phosphorylated state AgrA then activates expression of RNAIII, the effector molecule, which mediates the changes in expression of multiple target genes resulting in the* agr *response. Since the genes responsible for peptide production (*agrBD*) are also activated, this results in the production of more peptide signal and the generation of an autoinduction feedback loop.*

cardiovascular function may be modulated, or influenced, by bacterial quorum sensing molecules. In isolated porcine coronary arteries, 3-oxo-C12-HSL caused a concentration-dependent relaxation effect on thromboxane mimetic-induced contractions;[38] of note, it also induced marked bradychardia when administered to live conscious rats.[40] Consequently, this signal molecule may not only have a role in regulating the genes that encode for virulence determinants in *P aeruginosa*, but also in modifying host responses to maximise its own chances of survival and proliferation by capitalising on the increased supply of nutrients via the bloodstream, in combination with a down-regulation of host defences.

AHL-mediated quorum sensing as a therapeutic target for novel antimicrobials

As widespread resistance to traditional antibacterial agents continues to pose a major threat, the demand for novel therapeutic approaches to the treatment of infectious diseases is increasing. The ability to switch off virulence gene expression exogenously, and thus attenuate virulence, may therefore offer a novel strategy for the treatment or prevention of infection. In pathogens such as *P aeruginosa* that use AHLs to regulate virulence, the most obvious strategies for disrupting AHL-dependent quorum sensing are either to deplete concentrations of the signal molecules—by destruction of the AHL signal molecule or by inhibiting it synthesis—or alternatively to block transmission of the signal by antagonising the formation of the LuxR/AHL complex.

The stability of AHLs is pH-dependent and even at a physiological pH the integrity of the molecule is unstable.[41] However, at more alkaline pHs AHLs are highly susceptible to lactonolysis (opening of the lactone ring). Yates et al[41] have shown that when either *Y pseudotuberculosis* or *P aeruginosa* are grown in complex nutrient media, AHLs accumulate during the exponential growth phase of the culture but then are largely inactivated during the stationary phase. This is primarily as a consequence of the pH-dependent opening of the lactone ring, such that in the more alkaline pHs achieved in the stationary phase nutrient media, the ring-open form accumulates. Resistance to ring opening, caused by an increase in pH, increases with the length of the acyl side chain.[41] Since the ring opened form cannot activate LuxR homologues, this perhaps explains why homoserine lactone itself, which lacks an acyl side chain, does not function as a signal molecule, because it is rapidly inactivated at pH measurements below the physiological level. Furthermore, such chemical instability of AHLs lends itself to exploitation in the development of novel therapies by reducing or abolishing virulence-factor production by depleting AHL concentrations. In this context, AHL-degrading enzymes and their corresponding genes (*aiiA* genes)—which are capable of degrading AHLs produced by *P aeruginosa* and thus reduce the production of AHL-regulated virulence determinants—have recently been isolated from *Bacillus* species.[42] However, AiiA-type enzymes seem to inactivate AHLs by lactonolysis[43] and since this is a pH-dependent, reversible chemical reaction, one drawback to their use is the possibility that inactivated ring-opened forms could revert to the active ring closed version. However, while such enzymes could conceivably be used topically—eg, on pseudomonas burn wound infections—due to problems associated with the delivery of large proteinaceous therapeutic agents to the site of infection, they are unlikely to be useful for systemic administration.

More promising for systemic applications is the potential

to interfere with the transmission of the signal by using antagonists that compete with the cognate AHL for binding to the LuxR transcriptional regulator proteins, thereby attenuating the pathogen by switching off virulence gene expression. LuxR-type proteins are DNA-binding proteins and when AHLs bind to and activate LuxR, the resultant complex regulates gene expression by binding to a regulatory DNA region— the *lux* box—which is located just upstream of the target gene. Data from several studies has shown that the C-terminal region of LuxR proteins is needed for DNA-binding while the N-terminal contains the AHL-binding domain.[4,5]

Structure activity relationship studies have identified the key structural features of the AHL molecule needed for activity. For a given LuxR homologue, analogues of the natural AHL ligand may function as agonists or antagonists and, in this regard, the ability of various analogues to inhibit the action of the cognate AHL has been shown.[44–47] For example, in the opportunistic human pathogen *A hydrophila*, regulation of exoprotease production by its cognate AHL, C4-HSL, is inhibited by AHLs with acyl side chains of ten, 12, or 14 carbons in vitro.[48]

Although these studies have been crucial to further our understanding of the antagonism of LuxR-mediated regulation, so far the large-scale synthesis and screening for AHL antagonists has not been reported. However, Givskov et al[49] have recently shown that other natural products can antagonise AHL-mediated quorum sensing. Halogenated furanones, which are structurally related to AHLs, are produced by the macroalga *Delisea pulchra* in which they represent a natural biological example of quorum-sensing blockade by acting to discourage bacterial colonisation of this alga. Indeed, such furanones have also been shown to inhibit AHL-regulated processes, including biofilm formation, in a number of bacteria.[50] Although it was originally thought that these compounds acted by competing with the natural AHL ligand for a common binding site on the LuxR protein, recent evidence suggests that the reported inhibition of AHL-mediated gene expression is a consequence of accelerated degradation of the LuxR protein in the presence of the furanones resulting in reduced concentrations of LuxR protein within the cell.[51] This effectively interferes with the reception and response to AHLs. It is likely that these halogenated furanones will be toxic to higher organisms, nevertheless their ability to control *P aeruginosa* infections in appropriate animal models is of considerable scientific interest and may offer important structural leads for the design of antagonists for inhibiting AHL-dependent quorum sensing.

Advances in defining the role of LuxI and its homologues in AHL production has highlighted AHL synthesis as a conserved target for the inhibition of AHL-dependent quorum sensing. AHLs are synthesised from precursors derived from aminoacid and fatty acid metabolism, with the LuxI proteins being the predominant family of AHL synthases,[4] although at least two other unrelated synthases (LuxM family and HdtS)[17,18] have been identified. LuxI proteins are not particularly closely related and it is not possible to predict the nature of the AHL produced by a given LuxI protein. However, in vitro studies using recombinant proteins have shown that LuxI-type proteins catalyse the formation of AHLs from the appropriately charged acyl carrier protein (acyl-ACP), as the major acyl chain donor, and *S*-adenosyl methionine (SAM), which provides the homoserine lactone moiety.[52–54] The main conundrum has always been how a particular LuxI-type protein distinguishes and selects an acyl side chain of the correct length from a pool of potential precursors of varying length. Although protein sequence analyses in conjunction with mutagenesis studies have identified residues that are essential for function,[4,5] neither has contributed greatly to furthering our understanding of the mechanism of AHL biosynthesis or the role of specific LuxI homologue aminoacid residues involved in catalysis or acyl side chain selection. However, the publication of the first crystal structure of a LuxI protein homologue[55] has provided new insights into the function of these AHL synthases and will aid in the design of novel inhibitors. Possible strategies include the development of analogues that compete with the natural precursors for the active site of LuxI, or in some way interfere with the enzymatic process itself. Up to now, intermediates in the AHL synthetic pathway have been reported to inhibit AHL synthesis, as have analogues of the AHL precursor SAM.[56] Furthermore, AHL synthesis may also be blocked by targeting the key stages in fatty acid metabolism which supply the acyl side chains. It seems that the LuxM family of AHL synthases share many common features in the AHL synthetic pathway, opening up the potential for the design of inhibitors that block AHL synthesis in both enzyme families.[57]

Quorum sensing in Gram-positive bacteria: its role in virulence and potential as a novel therapeutic target

In Gram-positive bacteria, the quorum sensing signal molecules are generally small modified peptides instead of AHLs, and transduction of this signal usually involves a two component system phosphorylation cascade that ultimately triggers the required change in gene expression. Indeed, opportunistic human pathogens such as *S aureus*, *E faecalis*, *S pneumoniae*, and *B subtilis* regulate a variety of cellular processes via these peptide-mediated quorum sensing systems.[12,13]

S aureus is, for example, a primary pathogen causing both pyogenic and toxin-mediated infections in human beings and animals, which due to multiantibiotic resistance poses a major health threat, particularly in hospital environments.[2,58] The less toxigenic *Staphylococcus epidermidis* and other coagulase-negative staphylococci are, however, associated with chronic infections especially those associated with indwelling medical devices (see page 677).[59] Both *S aureus* and *S epidermidis* produce several cell wall components and exoproteins, required for virulence, which are controlled via quorum sensing through the accessory gene regulatory locus (*agr*) (figure 4).[6,12,59] In *S aureus*, a range of cell surface proteins (eg, protein A, coagulase, fibronectin-binding proteins) and exo-proteins (eg, proteases, haemolysins, toxic shock syndrome toxin 1 [TSST-1], and enterotoxin B) are regulated by *agr* in a

cell-density-dependent fashion.[12] Hence, as *S aureus* reaches the stationary phase of growth, *agr* represses the expression of genes coding for cell surface proteins involved in colonisation while activating the expression of genes coding for exotoxins and tissue-degrading exoenzymes. Moreover, several experimental infection studies have underlined the key role of the *agr* locus in the pathogenicity of *S aureus*.[12,60,61]

In staphylococcal species, the quorum sensing peptide (figure 1H) is produced by the processing of AgrD by AgrB, which is also responsible for the export of the signal molecule (figure 4). The peptide interacts with the AgrAC two component system, in which AgrC is the membrane-bound sensor kinase and AgrA is the cytoplasmic response regulator. The system is activated through the binding of a quorum sensing peptide to AgrC leading to a phosphorylation cascade. Signal transduction ultimately results in the production of the effector molecule, an RNA species termed RNAIII, which is responsible for triggering the *agr* response.[12]

S aureus strains can be subdivided into four groups (I–IV), in which the AgrD-derived peptide pheromone from strains of one group can cross-activate the *agr* response, and thus virulence, in other strains of that group but inhibit the *agr* response of members of other groups.[62] For instance, the addition of a group III peptide thiolactone to the culture medium of a group I strain virtually abolishes production of TSST-1, enterotoxin C3, and lipase.[63] Furthermore, co-inoculation of a virulent group 1 strain with the group II peptide resulted in a reduction in virulence of the group I strain in a mouse subcutaneous abscess infection model.[60] For *S epidermidis*[64,65] and *Staphylococcus lugdunensis*[62] only one peptide pheromone group has so far been identified. Although the various peptide groups differ in their aminoacid sequence, they all possess a cyclic thiolactone moiety (figure 1H).[60,63,64] Detailed structure activity studies of group I and group II *S aureus* peptide thiolactones respectively, identified analogues that either exhibited reduced activity as activators, were converted to inhibitors, or possessed no activity as either activators or inhibitors of self.[60,63] Furthermore, while the *S epidermidis* peptide thiolactone inhibits the *agr* response in *S aureus* groups I, II, and III but not group IV, only the group IV peptide was able to inhibit the *S epidermidis agr* response.[66]

It seems that staphylococcal species themselves have evolved an innate mechanism for warding off competitors and, in conjunction with the information summarised above, suggests that the peptide thiolactones represent an excellent starting point for the design of universal inhibitors of staphylococcal quorum sensing. However, of crucial importance when considering the design of novel antagonists that inhibit the *agr* response is that an inhibitor of one *agr* group may be a potent activator of virulence in another. As such, although a particular peptide analogue may prove useful in attenuating the virulence of invading *S aureus* strains, due consideration must be given to the potential of the analogue to act as an activator in the context of *S epidermidis* and other coagulase-negative staphylococci. One final and important consideration with respect to the inhibition of *agr* system is that the *agr*-dependent downregulation of cell-surface adhesions is likely to be lessened. This could potentially facilitate tissue colonisation and enhance biofilm formation,[67] which could have important implications for the development of chronic infections. In particular, in the context of medical-device-associated infections the formation of biofilms, recalcitrant to antibiotic therapy, are considered to make an important contribution to pathogenesis, especially of chronic infections..

Similarly, understanding the recently recognised quorum sensing systems in the opportunistic human pathogen *E faecalis* could facilitate the development of novel anti-infectives. Although enterococci form part of the normal microflora of the human gastrointestinal tract, they are also among the major causes of nosocomial infections including wound infections, bacteraemia, endophthalmitis, and endocarditis. Furthermore, with the emergence of vancomycin-resistant enterococci attention has again been focused on the virulence of these organisms. Recently it has been shown that *E faecalis* uses peptide pheromone-based quorum sensing to regulate a number of virulence determinants.[68–71] The newly described *fsr* (*fsrABC*) system is homologous to the staphylococcal *agr* locus and is thought to function in a similar manner: peptide-mediated quorum sensing regulates two proteases and possibly additional factors that contribute to virulence. In this context, the Fsr system is required for virulence in both a mouse peritonitis[68] and a rabbit endophthalmitis[69] model, suggesting that it has an important role in both local and systemic infections. An equivalent of the peptide encoding *agrD* is absent from the *fsr* locus. Instead, the 3′ region of *fsrB* is homologous to *agrD*, and the 11 residue cyclic quorum-sensing peptide (figure 1I) is processed from the C-terminal end of FsrB.[70] It has been discovered that the production of a cytolysin, thought to contribute to the pathogenesis of enterococcal infections, is also regulated by quorum sensing (via a system distinct from *fsr*).[71] This discovery suggests that the development of inhibitors which block the quorum-sensing-regulated production of these virulence determinants is a viable approach in the struggle to control one of the primary causes of antibiotic-resistant hospital-acquired infections.

Does cross-talk between bacteria occur during infection?

Different Gram-negative bacteria are known to produce the same family of quorum sensing signalling molecules. Co-habitation of those organisms in the same environmental niche opens up the opportunity of interspecies cross-talk as a result of a commonality of language. In this context, two pathogens—namely *P aeruginosa* and *B cepacia*, which infect the lungs of patients with cystic fibrosis and are a major cause of mortality[17]—both possess AHL-based quorum sensing systems.[4,5] Evidence of cross talk between *P aeruginosa* and *B cepacia* in vitro was initially provided by McKenney et al,[72] who showed that the exogenous addition of spent culture supernatants from *P aeruginosa* increased production of quorum-sensing-regulated virulence determinants in *B cepacia*, a result that was attributed to the presence of AHLs in the added supernatant. Riedel et al,[73] working on mixed species biofilms, showed that *B cepacia* could perceive *P aeruginosa*-derived AHLs but not vice versa, suggesting the

Search strategy and selection criteria

English language articles for this review were identified by searches of the ISI Web of Science (http://wos.mimas.ac.uk/) and references from relevant articles; numerous articles were identified through searches of the extensive files of the authors. Search terms were "cell-to-cell communication", "quorum sensing", "*N*-acylhomoserine lactones", "peptide pheromones", "bacterial infection", "antibiotic resistance", and "virulence". Because of restrictions on the number of references allowed, key review articles have been cited as guidelines for further reading.

existence of unidirectional signalling between these two organisms. Confirmation that AHLs are also produced by both species in vivo by analyses of sputa from infected cystic fibrosis patients,[32] raised the possibility that interspecies cross-talk also occurs within the host.

In the context of cystic fibrosis lung microbiology, *B cepacia* and *P aeruginosa* are not the only pathogenic bacteria that infect the cystic fibrosis lung. Early in life, the lung of individuals with cystic fibrosis often become colonised by *S aureus* which, later in life, is usually replaced by *P aeruginosa*.[74] As to why the acquisition of *P aeruginosa* is apparently associated with the displacement of *S aureus* is the subject for much debate[74] and, at first, the involvement of cross talk between these two organisms may seem unlikely since they use distinct quorum sensing languages.[19,74] However, we have recently noted that the exogenous addition of AHLs produced by *P aeruginosa* to cultures of *S aureus* affected virulence-determinant production by *S aureus*.[75] More specifically, the production of haemolysins, TSST-1, protein A, and fibronectin binding proteins were reduced on addition of the *P aeruginosa* quorum sensing signal molecule 3-oxo-C12-HSL. Since many of these products are regulated by *agr* in *S aureus*, it suggests that *P aeruginosa* AHLs may interfere with the peptide-mediated quorum sensing regulation of gene expression in *S aureus*. Moreover, the transcription of RNAIII, which is the ultimate effector molecule of the *agr* response,[12] is also inhibited by 3-oxo-C12-HSL. Thus, the possibility arises that that 3-oxo-C12-HSL may have a role in the eradication of *S aureus* from the cystic fibrosis lung.

The pros and cons of quorum sensing inhibitor therapy

Several distinct chemical languages are used by bacterial pathogens and there is no doubt that many more signalling molecules have still to be discovered. Due to the lack of a truly universal bacterial language, however, any therapeutic agents capable of blocking quorum sensing are likely to have a much narrower spectrum of activity than conventional antibiotics. However, in theory, this should have the added benefit of causing less disturbance to the host's natural microflora. Unlike conventional antibiotics, quorum sensing inhibitors will not necessarily kill bacteria (or inhibit their growth) but, by attenuating virulence, will aid clearance of the pathogen by the host's own defence mechanisms. As a consequence, it is probable that the selective pressure for the emergence of resistance will be lessened. Furthermore, the non-bactericidal mode of action should help to minimise the release of bacterial endotoxins and, hence, septic shock during infection. However, since the very nature of their mode of action is reliant on host defences, it is unlikely that they will be suitable to treat immunocompromised patients and perhaps, in general, a prophylactic rather than a therapeutic use would be more appropriate. What is more, the most appropriate choice of antibiotic to treat an infection is normally guided by antibiotic sensitivity test results. Since the use of traditional in vitro sensitivity assays will not be applicable for quorum sensing inhibitors, the development of new diagnostic systems to establish their effectiveness will also be essential.

Concluding comments

The ability of pathogenic bacteria to modulate virulence gene expression as a consequence of changes in population density, enabling bacteria to act as a community rather than in isolation, is a far more complex event than initially anticipated. Quorum sensing systems can no longer be considered in isolation, as perhaps best exemplified by the hierarchical arrangement of multiple quorum sensing systems, which regulate multiple phenotypes, in *P aeruginosa*. We are only just beginning to elucidate and understand the intricacies of the systems involved, let alone begin to exploit them therapeutically, and much work has still to be done to fully understand the subtleties of quorum-sensing-mediated gene regulation. The major challenge that lies ahead is exploiting this knowledge to design quorum sensing inhibitors that attenuate virulence of an invading pathogen, and to assess their ability to treat infections.

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Conflicts of interest

We have no conflict of interest in relation to this review.

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Useful website

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