Review

Applications of small molecule activators and inhibitors of quorum sensing in Gram-negative bacteria

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Quorum sensing is a form of intercellular communication used by many species of bacteria that facilitates concerted interactions between the cells comprising a population. The phenotypes regulated by quorum sensing are extremely diverse, with many having a significant impact upon healthcare, agriculture, and the environment. Consequently there has been significant interest in developing methods to manipulate this signalling process and recent years have witnessed significant theoretical and practical developments. A wide range of small molecule modulators of quorum sensing systems has been discovered, providing an expansive chemical toolbox for the study and modulation of this signalling mechanism. In this review, a selection of recent case studies which illustrate the value of both activators and inhibitors of quorum sensing in Gram-negative bacteria are discussed.

Basics of quorum sensing

Quorum sensing is mediated by small diffusible signalling molecules termed autoinducers. These are synthesised intracellularly by bacterial cells throughout their growth and are continually released into the surrounding milieu; consequently, autoinducers typically accumulate in proportion to the cell density of the bacterial population [1]. Once the extracellular concentration of autoinducer reaches a certain threshold level (at which point the population is considered to be 'quorate'), a signal transduction cascade is triggered leading to population-wide changes in gene expression and the initiation of 'co-operative' behaviours that benefit the community as a whole [1–7]. Between different bacterial species there is variation in one or more aspects of this signalling process, that is, the exact nature of the chemical signals, receptors, mechanisms of signal transduction, and phenotypic consequences [1,8]. Nevertheless, with regard to intraspecies communication, most Gram-negative bacteria use quorum sensing systems which utilise one of two distinct types of small molecule autoinducer [1]. *N*-acylated-L-homoserine lactones (AHLs) are the most common class of autoinducer used by Gram-negative bacteria. They are produced by LuxI-type synthase enzymes and bind to cytoplasmic LuxR-type receptors to exert a regulatory output [9]. Cyclic peptides are the major class of autoinducer in Gram-positive bacteria. These are recognised by either membrane-associated histidine kinases or cytoplasmic receptors [9]. Recently, a family of molecules generically termed autoinducer-2 (AI-2) has been discovered. It has been suggested that AI-2 is a non-species specific autoinducer, capable of mediating intra- and interspecies communication among Gram-negative and Gram-positive bacteria (Figure 1) [1,10–12].

The dependency of quorum sensing upon a 'language' of autoinducers provides the chemical opportunity to manipulate this signalling process at a molecular level using non-native compounds [1,13,14]. Indeed, recent years have witnessed significant efforts directed towards the discovery of molecules capable of interfering with various components of the quorum sensing communication circuit (Box 1) [1].

In this review we describe some quorum sensing-regulated behaviours that have significant impacts upon human healthcare, agriculture, and the environment. The discussion is primarily focused upon the phenotypes of Gram-negative bacteria regulated by AHL-based signalling, as this represents the most thoroughly studied and best-understood class of quorum sensing systems [1] (for a discussion of intercellular signalling in Gram-positive bacteria the reader is directed towards some recent specialised reviews [9,15]). The potential real-world value of small molecule modulators of the relevant quorum sensing systems is highlighted, with a particular emphasis upon compounds which are thought to act via interaction with LuxR-type receptor proteins (Box 2 and Box 3). Where possible, examples of such agents are provided, with a focus upon most recent developments. Overall, these case studies provide a clear and timely illustration of the wide-ranging importance of quorum sensing and the significant promise offered by small molecule activators or inhibitors of this form of intercellular communication.

Quorum sensing and human healthcare

Numerous species of clinically relevant pathogenic bacteria use quorum sensing systems to regulate processes associated with virulence [16,17]. This allows the bacterial cells to multiply without displaying overt virulent behaviour until a certain threshold population density is reached [17]. Consequently, a coordinated attack on the

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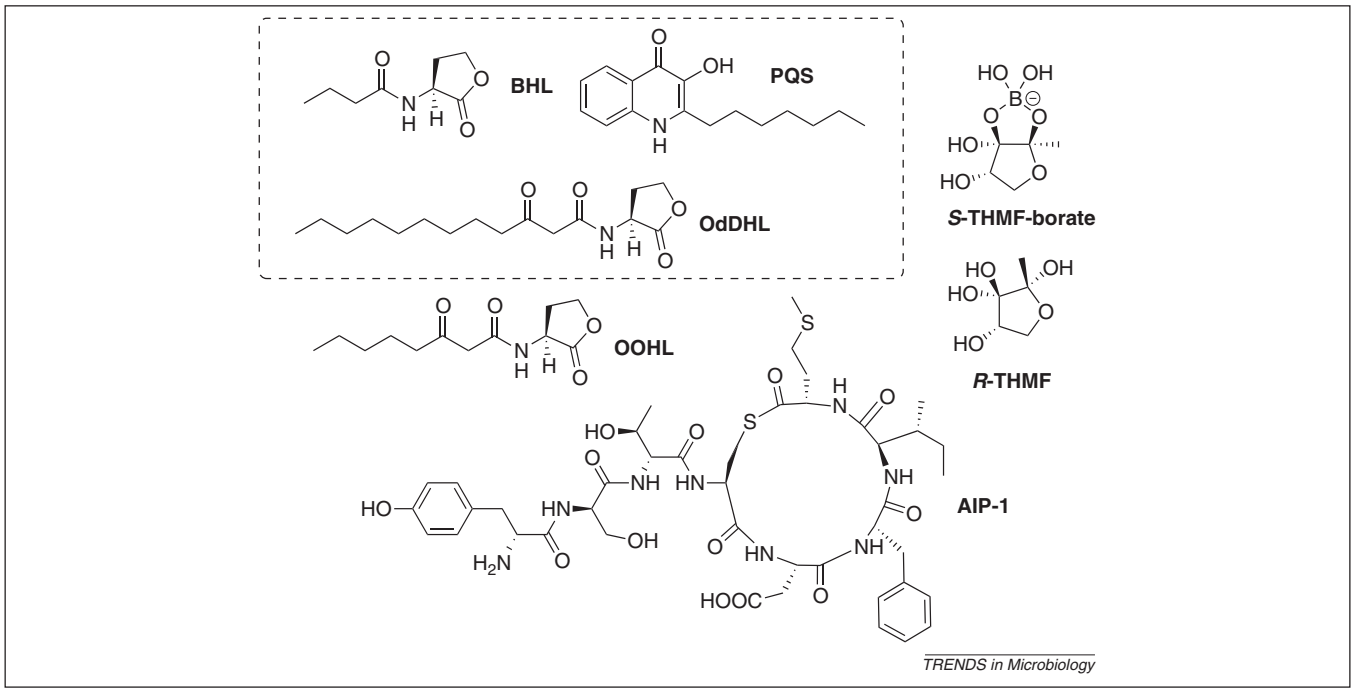

Figure 1. Selected natural quorum sensing autoinducers. BHL, OdDHL and OOHL are examples of the *N*-acylated-L-homoserine lactone (AHL) class of autoinducers. BHL, OdDHL and PQS are native quorum sensing molecules in the Gram-negative bacterium *Pseudomonas aeruginosa*. OOHL is a signalling molecule in the Gram-negative bacterium *Agrobacterium tumefaciens*. AIP-1 is an example of the cyclic peptide class of autoinducers employed by Gram-positive bacteria (in this case, *Staphylococcus aureus*). (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate) and (2*R*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) are known AI-2 signalling compounds, which can interconvert with each other via a complex equilibrium process. *S*-THMF-borate is commonly referred to as AI-2 in the literature. Abbreviations: BHL, *N*-butanoyl-L-homoserine lactone; OdDHL, *N*-(3-oxododecanoyl)-L-homoserine lactone; OOHL, *N*-3-oxooctanoyl-L-homoserine lactone; PQS, *Pseudomonas* quinolone signal; AIP-1. autoinducing peptide 1.

host (or on a competing microorganism) is only made when the bacterial population is high, which increases the likelihood that any defences will be successfully overwhelmed, thereby enhancing the survival prospects of the bacteria [18,19].

Given the link between quorum sensing and virulence, it is unsurprising that disruption of this signalling process has emerged as an attractive new therapeutic strategy for the treatment of a variety of human infections caused by bacteria [1,17,20–22]. The inhibition of quorum sensing is commonly referred to as 'quorum quenching'. The expression was originally coined to describe the disruption of Gram-negative quorum sensing resulting from the enzymatic hydrolysis of AHL autoinducers [23]. However, over the course of the past decade this definition has evolved; the phrase quorum quenching is now commonly used in a more general sense to refer to any inhibition of quorum sensing (i.e., inhibition resulting from both enzymatic and

Box 1. Small molecule intervention of AHL quorum sensing systems: potential targets

There are three core components of all AHL-based quorum sensing systems: (i) the LuxI-type synthase (which generates the signalling molecule); (ii) the AHL signalling molecule itself; and (iii) the LuxR-type receptor protein (which binds to the signalling molecule). In principle, each of these components represents a possible target for external intervention using small molecules [1,13].

Interference with LuxI-type synthase activity is a conceptually straightforward method for quorum sensing modulation as this will affect the level of the signalling molecule present. Surprisingly, however, there are relatively few reports describing the use of synthetic small molecules to target such proteins [13]. Most studies on the chemomodulation of LuxI-type synthases have focused upon the use of analogues of natural molecules involved in the AHL biosynthesis process [1]. Crystal structures for several LuxI-type proteins have been reported recently; such information is expected to prove useful in the rational design of new synthetic modulators of these synthases [1,13].

Modulation (typically degradation) of the AHL signalling molecules themselves would be expected to interfere with normal communication pathways. Several prokaryotic and eukaryotic species are believed to degrade AHL signals in order to inhibit the quorum sensing by invading or cohabiting bacteria [13,87]. However, it is difficult to envisage how small molecules can be used in a direct manner to promote the degradation of autoinducers [1].

The majority of work on the small molecule modulation of AHL-based quorum sensing has focused upon the identification of compounds that can interact with LuxR-type receptor proteins [1,13]. In this context, the terms agonist and antagonist have often been used in the literature to describe molecules that act at the receptor level. However, it has been noted that there are pitfalls associated with attributing a definite type of biological effect (i.e., always acting as an agonist or always acting as an antagonist) to any given small molecule quorum sensing modulator: clear distinctions between antagonist and agonist activity often cannot be made, with many agents able to both slightly activate and slightly inhibit a quorum sensing system depending upon their concentration [1,13]. As such, it has been argued that it is more valuable to consider activity against LuxR-type receptors as a continuum from activation to inhibition and group molecules together accordingly (i.e., activators and inhibitors) [1,13]. However, this terminology is not yet widely adopted; therefore, in this review we use the terms agonist/activator and antagonist/inhibitor as specified in the relevant primary literature [1,13].

Box 2. Chemomodulation of LuxR-type receptors: what compounds to screen?

Small molecule modulators of quorum sensing are generally discovered through the biological evaluation of: (i) 'unbiased' libraries of synthetic or natural derivatives, or (ii) synthetic compounds resulting from a semirational design process whereby the structure of a known autoinducer is used as a template for the design and synthesis of structurally novel agents [1,33]. The latter is arguably the most commonly employed strategy for the discovery of small molecules that target LuxR-type receptors. Indeed, non-native AHLs represent the most extensively studied class of synthetic quorum sensing modulators reported to date. 'Random' chemical modifications around the native AHL scaffold can be made to yield novel compounds; the structure–activity (SAR) data obtained from the screening of such derivatives may facilitate further, more considered molecular changes to improve upon the biological properties of the molecule (e.g., efficacy) [1]. In addition, the X-ray crystal structures of some natural AHL ligands bound to their cognate LuxR-type receptors are available and these have been used to guide the design of synthetic AHL ligands [1,18,88]. Computational pharmacophore modelling has also proven valuable in this context, providing an understanding of the mechanism of action of such agents in terms of the fundamental bonding interactions involved [1]. There are, however, drawbacks associated with the use of AHL-based molecules. For example most LuxR-type receptors show a high level of specificity and affinity for their cognate autoinducers; consequently, the rational design and optimisation of AHL-based modulators is challenging as only slight deviations from the parent AHL structure are tolerated without large drops in binding affinity [1,78]. The majority of abiotic AHL-type compounds, including antagonists, are presumed to act in a competitive manner (due to their structural similarities with natural autoinducers), targeting the site on the LuxR-type receptor protein that is normally occupied by the natural ligand [1]. Therefore, they would only really be useful if they had a greater affinity for the LuxR-type receptor than the cognate natural AHL. However, this is difficult to achieve given the aforementioned specificity issues. In addition, the homoserine lactone moiety of AHLs is known to be readily degraded by mammalian lactonases, which could limit the efficacy of any AHL-based pharmaceutical agent [1,89]. Consequently, the identification of new classes of small molecule modulators of LuxR-type proteins, which are structurally distinct from AHLs, has attracted considerable interest in recent years [1]. Currently, such agents are generally discovered by the screening of 'unbiased' molecular collections (i.e., molecules not necessarily predicted *a priori* to affect quorum sensing). There is a relative dearth of reports detailing the X-ray crystal structures of LuxR-type receptors with non-native ligands, which has hindered the *de novo* rational design of abiotic ligands that are distinct from the general AHL structure [1,80]. The mode of action of non-AHL based modulators (in terms of which quorum sensing system is affected or which component of the system in targeted) is often difficult to delineate precisely; indeed, in many cases such information is not specified.

non-enzymatic methods, such as the application of small molecules). In principle, quorum quenching, as with any antibiotic intervention, would allow the host immune system a better chance of clearing the infection before the bacteria cause too much tissue damage [24,25]. Given the emergence and increasing prevalence of multidrug resistance in pathogenic bacteria, the development of novel therapies for the treatment of bacterial infections, such as those based on quorum quenching, would be of huge clinical significance [17]. It has been argued that one of the most appealing aspects of the quorum quenching approach is that although quorum sensing systems are often used to regulate virulence, they are not essential for bacterial survival. Thus, selective disruption of quorum sensing should attenuate pathogenicity without imposing the level of selective pressure associated with antibacterial treatments [1,13,20,26].

Box 3. Screening for LuxR modulators: biological considerations

Small molecules that affect LuxR receptor activity are typically discovered through culture-based screening assays that employ bacterial reporter strains (so-called biosensor strains) where the expression of an easily assayable phenotypic output is under the control of a LuxR-regulated promoter [13,33]. Such strains lack functional AHL synthases. Therefore, transcription of the reporter gene is, in principle, entirely dependent upon the addition of the exogenous AHL (or a functional equivalent) [13]. Agonism screening trials are performed without the addition of the natural AHL; instead, the non-native compounds being analysed are added at various concentrations, with LuxR agonists able to activate the transcription of the reporter gene [13]. In antagonism screening trials, the cognate autoinducer is added at a fixed concentration (typically just enough to stimulate expression of the reporter gene); antagonists are identified by their ability to compete with the native autoinducer and reduce the reporter read-out [33]. Such biosensor-based assays represent idealised cases. Even though they provide a very useful means of rapidly identifying potential quorum sensing modulators, their relevance to quorum sensing under natural conditions is not always apparent [63]. For example, the antagonism assay system does not accurately mimic the situation in wild-type cells, where the endogenous AHL is continually produced and can therefore more effectively outcompete any added antagonist. As a consequence, antagonists that appear extremely potent when identified using reporter systems often fail to elicit the anticipated response when tested in wild-type cells [33].

In general, there are a relatively limited number of examples describing the biological evaluation, under natural conditions, of small molecule quorum sensing modulators identified through culture-based assays [63]. Such studies are significant as they provide proof-of-concept for the alteration of a bacterial behaviour (e.g., pathogenicity to a host organism) through the modulation of a quorum sensing system in a real-world context [63]. There remains a definite need for further developments in this area.

The largest body of work in the area of small molecule-mediated quorum quenching of human pathogens pertains to the discovery of inhibitors of the relevant signalling receptors [1]. As a representative example, the regulation of virulence in *Pseudomonas aeruginosa* is discussed in more detail.

P. aeruginosa

P. aeruginosa is a clinically important opportunistic human pathogen often associated with multidrug resistant infections in immunocompromised patients [27–31]. *P. aeruginosa* infections are difficult to eradicate due to a combination of high levels of intrinsic antibiotic resistance and the predilection of *P. aeruginosa* to form antibiotic-resistant biofilms (Box 4) [16].

P. aeruginosa is arguably the most-studied and best-understood bacterium using quorum sensing to regulate pathogenic processes [32]. At least three different quorum sensing pathways are used. Two of these systems employ AHLs as the signalling molecules; specifically, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) with cognate receptor LasR and *N*-butanoyl-L-homoserine lactone (BHL) with the cognate receptor RhlR. These AHL-based systems are interlinked with a third signalling system

Box 4. Bacterial biofilms

A bacterial biofilm is a sessile community of bacterial cells attached to a surface and embedded within a self-produced matrix of polysaccharide material [90]. Bacterial biofilms are ubiquitous in nature, being formed by nearly all known bacterial species [91]. The formation of a biofilm confers several advantages on the constituent cells [81]. Biofilms provide a mechanically stable and protective environment resulting in a higher tolerance against a wide range of environmental insults such as UV exposure, extremes of pH, and exposure to antibiotics and antimicrobial agents [91].

Bacterial biofilm formation has a significant impact upon many human endeavours. For example, mixed species biofilms are useful in an industrial context for the bioremediation of human and manufacturing waste [92]. However, the presence of certain mixed species biofilms on industrial metal surfaces may result in corrosion [92]. Moreover, biofilm formation on food contact surfaces can lead to product contamination during food processing, which can result in foodborne illness and reduced product shelf-life [92]. Bacterial biofilms are of particular importance from a therapeutic perspective. Within a clinical context, several aspects of pathogenesis are directly related to the development of biofilms [92]. There are numerous types of surfaces within clinical settings (including wounds, teeth, and medical instruments) that can support biofilm development [92]. Bacteria growing as a biofilm have increased resistance to host immune defences, antibiotic treatments, antiseptics, and other cleaning agents relative to their planktonic counterparts [90]. Consequently, their eradication from patients using conventional chemotherapeutics, or from contaminated surgical equipment using cleaning products is extremely difficult [32,90]. Thus, novel methods to inhibit the growth of biofilms or accelerate the breakdown of existing biofilms would be extremely useful in a variety of fields [93,94]. In this context, the inhibition of bacterial quorum sensing has emerged as an attractive strategy. Quorum sensing has been shown to regulate biofilm formation in several clinically important bacterial species; consequently, inhibitors of this signalling process represent promising potential antibiofilm agents [94]. In a significant recent study, the therapeutic value of combining traditional antibiotics with quorum sensing inhibitors was examined [94]. Molecules known to inhibit AHL-based quorum sensing were found to increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo* [94]. Recently, a range of 2-aminobenzimidazole derivatives were discovered which had quorum sensing modulatory activity in *P. aeruginosa* reporter strains and which were also capable of strongly inhibiting the growth of, and dispersing, *P. aeruginosa* biofilms [93]. It is worth noting, however, that the precise role of quorum sensing in biofilm formation remains a topic of some debate in the literature. For example, it is known that the impact of quorum sensing upon biofilm formation in *P. aeruginosa* is dependent upon the growth conditions used (indeed, quorum sensing-negative mutants were reported to be still able to form biofilms when grown in certain nutritional environments) [95].

employing a chemically distinct autoinducer termed *Pseudomonas* quinolone signal (PQS), forming an intricate hierarchical quorum sensing network (see Figure 1 for structures) [1,29,33,34]. *P. aeruginosa* is known to produce a range of other quinolone-type molecules which may also play a role in quorum sensing in this organism [35].

Quorum sensing plays a key role in the pathogenesis of *P. aeruginosa* [16,25–27,32] regulating the timing and production of multiple virulence factors [36,37] and biofilm formation (in many growth conditions) [16]. The Las system is considered to stand at the apex of the hierarchy [1,33]. Consequently, the LasR receptor is usually the main target for inhibitor development [1,14,33].

Many synthetic LasR antagonists share the same general structural framework as the natural autoinducer, OdDHL (e.g., compounds **1–4**, Figure 2) [1,13,33]. Synthetic antagonists based around non-AHL frameworks are also known but are less common [1]. Synthetic furanones are an important example, their structures based around those of naturally occurring furanones produced by the marine algae *Delisea pulchra* [38] (note that these natural compounds are not able to inhibit quorum sensing in *P. aeruginosa*) [1,22]. For example, *in vitro* studies demonstrated that compound **5** (Figure 2) was capable of disrupting AHL-based quorum sensing in *P. aeruginosa* [1,20,39]. Compound **5** and a related analogue **6** (Figure 2)

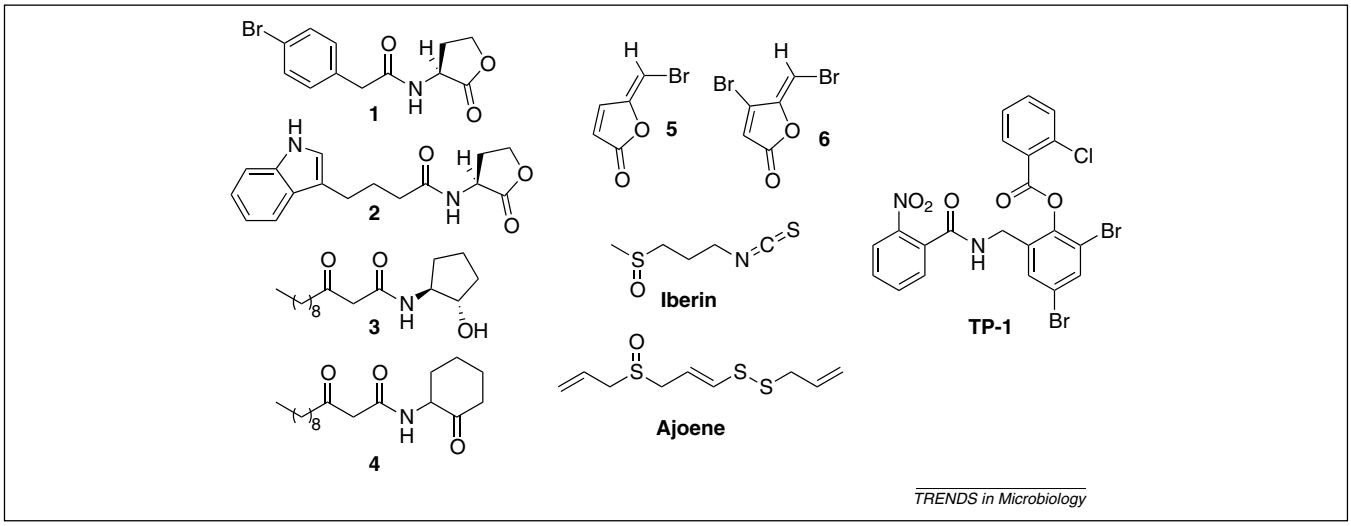

Figure 2. Some examples of small molecules capable of modulating quorum sensing in *Pseudomonas aeruginosa*. Compounds **1** [83], **2**[83], **3**, and **4** [84,85] are synthetic structural mimics of the native *P. aeruginosa* autoinducers *N*-butanoyl-L-homoserine lactone (BHL) and *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL). Compounds **1–4** are known to be LasR antagonists. Compounds **5** [39] and **6** [21,40] are synthetic furanone derivatives with quorum sensing inhibitory activity. Iberin (from horseradish) [41] and ajoene (from garlic) [42] are examples of naturally occurring substances capable of inhibiting quorum sensing. Iberin is thought to act in a competitive-type manner at the RhlR (and possibly LasR) receptor. It has been reported that in mouse models of pulmonary infection a significant clearance of infecting *P. aeruginosa* was detected in ajoene-treated mice [42]. TP-1 is a synthetic superagonist of the LasR quorum sensing system. TP-1 is thought to act directly through the LasR receptor in a highly selective manner [1,80,86]. Note that the original structure proposed for TP-1 in 2006 [86] was subsequently revised to that shown [80].

were also examined for their effects on *P. aeruginosa* lung infections in mice [21,40]. Both compounds accelerated bacterial clearance by the host [21,40] and reduced the severity of lung pathology [40]. In addition, treatment with either **5** or **6** significantly increased the survival times of mice with lethal *P. aeruginosa* infections [40]. Critically, the effects of these furanones were shown to be mediated through the quorum sensing systems. These studies are extremely significant; they clearly demonstrate that the virulence of *P. aeruginosa* can be attenuated by the inhibition of quorum sensing using small molecules and therefore provide a proof-of-concept for the treatment of Gram-negative bacterial infections in general by small molecule-mediated quorum quenching [1]. The ability of *D. pulchra* furanones and synthetic analogues to disrupt quorum sensing in a variety of Gram-negative bacterial strains is well documented. However, the precise mode of action of such agents remains elusive [1].

Many naturally occurring substances are also known to antagonise quorum sensing in *P. aeruginosa* [1] (e.g., iberin from horseradish [41] and ajoene from garlic [42], Figure 2). However, the precise structure of the bioactive component(s) of such substances is not always available; even when structures can be obtained, determination of the mode of action is difficult as there is generally little structural correlation with any other quorum sensing modulator whose molecular target is known [1].

PQS-based quorum sensing has also been implicated as playing a key role in the virulence of *P. aeruginosa* [43–45]. The attenuation of PQS signalling has received considerable interest as a potential antibacterial strategy in recent years and there is significant scope for further exciting developments to be made in this area [29,46]. Recent studies have identified a third LuxR-type protein in *P. aeruginosa*, termed QscR (quorum sensing control repressor) that is not linked to an AHL synthase. QscR is involved in AHL-based quorum sensing in this organism, including the regulation of virulence, and has been cited as a target for the modulation of quorum sensing controlled genes [1,47].

In summary, small molecule-mediated quorum quenching is considered by many to be an attractive strategy for the treatment of bacterial infections. However, although this approach has shown promise in *in vitro* and mouse infection models, human applications still remain a long way off. A major concern is compound toxicity, which is related to off-target effects. For example, furanones are not widely considered specific (and thus viable) quorum sensing inhibitors; indeed, furanone 6 has been shown to be toxic to rainbow trout at high concentrations which underlines the need for the development of novel, less toxic small molecule inhibitors of quorum sensing and a better understanding of the modes(s) of action of such agents [48]. In addition, the therapeutic use of AHL-based antagonists is complicated by the fact that some AHLs possess immunomodulatory activities and affect certain cells and tissues in the body [49]. Furthermore, many current small molecule inhibitors of quorum sensing would be expected to have low aqueous solubility, limiting their potential to be used as a drug. Recent results also challenge the prevailing concept that small molecule inhibition of quorum sensing should cause relatively little Darwinian selection pressure for bacterial resistance [50]. Indeed, it appears that bacteria can more readily obtain resistance to antivirulence compounds, such as quorum quenching small molecules, than was originally envisaged. This clearly has important repercussions for any future clinical utilisation of such agents [50].

Potential value of superagonists

Given the role that quorum sensing plays in the initiation of virulence processes, the use of quorum sensing antagonists to attenuate bacterial pathogenicity is a conceptually straightforward strategy. Less obvious perhaps is the opposite approach, employing agonists [1,51]. The basic premise behind this strategy is that if quorum sensing systems regulating virulence factor production could be artificially activated at lower population cell densities, this would stimulate the host immune system at a point when fewer bacterial cells are present and therefore increase the likelihood of the infection being successfully cleared [1,51]. This strategy is dependent on the identification of non-native agonistic compounds that display heightened activities relative to the native autoinducers, so-called superagonists [1,51]. However, there is some controversy surrounding the therapeutic potential of superagonists [1]. For example, there is evidence that the timing of the onset of quorum sensing-controlled gene induction in *P. aeruginosa* is regulated by other factors in addition to the concentration of the autoinducer [21,52]. Nonetheless, the identification of quorum sensing superagonists in human pathogenic bacteria is still an active area of research. For example, TP-1 (Figure 2) was recently identified as a superagonist of LasR-based quorum sensing in *P. aeruginosa*. Overall the field of superagonism of quorum sensing is very much in its infancy, especially relative to the more well-studied area of antagonism. There are currently only a handful of known, characterised superagonists of LuxR-based signalling and there is a definite dearth of studies examining the *in vivo* effects of these compounds [51].

Quorum sensing and the environment: biofouling

Biofouling is the process by which all unprotected artificial and natural substrata in marine environments become colonised by micro- and macroorganisms [53–55]. Biofouling is a serious problem for marine industries and navies; micro- and macrofoulers can promote metal corrosion, reduce the efficiency of heat exchangers and increase the drag of ships [53,54,56]. Biofouling is typically controlled through the use of substances that are toxic to biofouling organisms [53]. However, some target organisms are known to be resistant and many of these chemicals are also toxic to other organisms and pollute the aquatic environment [53]. Thus, there is a need for the development of new antifoulant strategies, particularly those which are more environmentally friendly [53].

The disruption of bacterial quorum sensing has recently emerged as an attractive alternative approach for the inhibition of biofouling [53–55]. The formation of microbial biofilms on water-exposed surfaces can play an important role in the settlement of the larvae of macrofouling species [53,54]. The larvae of many marine invertebrates

preferentially settle upon bacterial biofilms [55] and, for many bacterial species, quorum sensing is involved in regulating the formation and maturation of biofilms (Box 4) [55]. Thus, interference with bacterial quorum sensing may lead to a disruption of associated biofilms which, in turn, could result in a reduction of macrofouling of submerged surfaces [55]. This area has attracted significant interest in recent years, with a particular focus upon Gram-negative bacteria due to their predominate presence in the marine environment [53–55]. Encouragingly, there have been studies (although under non-natural conditions) that provide proof-of-concept for the reduction of marine biofouling by the inhibition of quorum sensing using the small molecule kojic acid [54] and various furanone-based compounds [53]. Conceivably, active small molecules of this sort could be incorporated into surface coatings that could be applied to the surface of interest to inhibit the normal biofouling process (e.g., chemically enhanced paint for the hulls of ships) [56,57]. Overall, it is hoped that further studies into the role of bacterial quorum sensing in biofouling will lead to the development of novel and less toxic methods for its control.

Quorum sensing and agriculture

Global demand for food and agricultural crops is increasing at a rapid pace, propelled by continuing increases in global population and the accelerating use of grain for biofuel production [58,59]. With regard to land-based crops, there are two broad options available for satisfying this demand: (i) increasing the land area used for crop production or (ii) increasing the productivity on existing farmland [58]. Bringing large amounts of new land into production is not attractive from an environmental perspective [58]. Thus, there is a continuing interest in the development of methods to increase crop productivity [58]. In addition, more extensive use of non-land-based sources of food represents an attractive complement to traditional agricultural crops. In this regard, the ability to modulate quorum sensing could prove to be useful. Various species of plant pathogens and pathogens of aquatic-based foodstuffs, which are a cause of tremendous losses in food production, use quorum sensing systems to regulate processes associated with their virulence [32]. Thus, quorum quenching through the use of small molecules may prove valuable for the treatment of such diseases. Furthermore, there are some examples of symbiotic plant bacteria that use quorum sensing systems to regulate phenotypes of potential benefit to the host [32]. In principle, therefore, stimulation of these systems using small molecules could be desirable in an agricultural context [32]. Some representative case studies illustrating the potential agricultural value of activators and inhibitors of quorum sensing are discussed in more detail.

Quorum sensing in plant pathogenic bacteria

Examples of plant pathogens that use quorum sensing systems to regulate processes associated with their virulence include *Erwinia carotovora* (recently renamed *Pectobacterium carotovora*, which causes soft rot in a variety of plants), [60] and *Pantoea stewartii* (causes leaf blight in sweet corn and maize crops) [61]. A particularly well-studied case involves the bacterium *Agrobacterium tumefaciens*, which is capable of causing crown gall tumours in plants [61]. *A. tumefaciens* employs an AHL-based quorum sensing system that uses *N*-3-oxooctanoyl-L-homoserine lactone (OOHL, Figure 1) as the autoinducer and TraR as the LuxR-type receptor [1,61]. TraR-based quorum sensing is thought to contribute towards the aggressiveness of *A. tumefaciens* plant infections [61,62]. Several small molecule inhibitors of this signalling pathway have been developed [1]. However, most of these studies employed culture-based reporter gene assays and it is unclear if this antagonism would translate into useful biological activity (i.e., suppression of tumour formation) under native conditions on plant hosts. A similar situation is seen in the case of *P. carotovora* [1] [63]. In a significant recent study, various AHL-based modulators (which had been identified in culture-based reporter assays) of quorum sensing in *P. carotovora* and an additional plant pathogen *Pseudomonas syringae* B278A were examined for their ability to modulate quorum sensing regulated virulence in infection assays using the native plant hosts (potato and green bean, respectively) [63]. The compounds largely retained their activity profiles when introduced into the plant host; however, it was observed that inhibition of virulence in these wild-type infections was related to the timing of compound dosing.

In addition to inhibiting quorum sensing, artificial stimulation using non-native small molecules has been identified as an alternative method for the treatment of plant pathogenic bacteria (as discussed for human pathogens, *vide supra*) [32,64]. For example, it has been observed that disease in tobacco plants caused by *P. carotovora* can be reduced by the application of the pathogens own AHL [64,65]. However, the use of non-native small molecules in this context has not been examined to any great extent.

Quorum sensing in plant symbiotic bacteria

Pseudomonas aureofaciens (now commonly referred to as *P. chlororaphis*) strain 30–84 is a symbiotic bacteria which uses an AHL-based quorum sensing circuit to regulate the production of phenazine antibiotics that can protect wheat from a disease caused by the fungus *Gaeumannomyces gramnis* var. *tritici* [32,66,67]. Thus, activation of *P. chlororaphis* quorum sensing could promote an antifungal environment for the wheat host [32]. Quorum sensing has also been shown to play an important role in both the establishment and regulation of the symbiotic interactions between nitrogen-fixing bacteria and the plant hosts in a variety of species of legume-nodulating rhizobia [68,69]. Stimulation of quorum sensing in these bacteria could enhance nitrogen fixation, thus decreasing the amount of fertiliser that needs to be added to the crop hosts, which has both financial and environmental benefits. However, despite the potential agricultural benefits associated with small molecule modulation of quorum sensing systems, this field, in general, has received comparatively little attention.

Quorum sensing in aquaculture

The inhibition of quorum sensing has also been identified as a strategy to combat bacterial infections in aquaculture (the cultivation of aquatic organisms such as molluscs, fish,

and shrimp) [64]. For example, the aquatic pathogens *Aeromonas hydrophila* and *Aeromonas salmonicida* use AHL-based quorum sensing to regulate processes associated with virulence [64]. The most intensively studied organism in this context is the marine bacterium *Vibrio harveyi*. This organism, and closely related species, are among the most important pathogens in the intensive farming of a range of creatures, especially shrimp [70]. The virulence of *V. harveyi* towards different host organisms has been shown to be dependent upon quorum sensing *in vivo* [70]. Various halogenated furanones are known to disrupt AHL- as well as AI-2-mediated signalling in Gram-negative bacteria [1] (*vide supra*) and it has been demonstrated that the addition of the natural furanone (5*Z*)-4-bromo-5-(bromomethykene)-3-butyl-2(5*H*)-furanone to culture water increased the survival of brine shrimp larvae challenged to different pathogenic isolates including *V. harveyi* [70,71]. However, the therapeutic index of the furanone is probably too low to be used in practice (indeed, due to the relatively high toxicity of the compound towards the organism it is possible that the quorum quenching effect is secondary) [70]. Numerous other natural and synthetic small molecule inhibitors of quorum sensing in *V. harveyi* are known [1]. However, in the majority of cases such compounds were not discovered by observing their effects upon virulence, but rather their ability to modulate bioluminescence, another quorum sensing-regulated phenotype. As such, the ability of such quorum sensing inhibitors to affect *V. harveyi* pathogenicity is unclear. Overall, data pertaining to the impact of quorum sensing upon virulence in aquatic pathogens are lacking and the use of small molecules to inhibit quorum sensing is a largely unexplored strategy for the treatment of bacterial infections in aquaculture [64]. However, it is expected that this area will receive considerable attention in the coming years; aquaculture is one of the fastest-growing food-producing industries and current methods for the treatment of aquatic diseases (antibiotics and disinfectants) have only had limited success in the treatment of aquatic disease [64].

Enzymatic degradation of quorum sensing autoinducers

In addition to the use of small molecules, alternative strategies for inhibiting quorum sensing in Gram-negative bacteria have been explored [72]. The most intensely studied (and arguably the most significant) of these is the enzymatic degradation of the AHL autoinducers. Several quorum quenching enzymes have been identified in a range of organisms, which can hydrolyse either the amide or lactone moieties of AHLs (acylases and lactonases, respectively) to produce products that are no longer active signalling agents [56]. In principle, such enzymes could find applications in a number of the diverse range of fields affected by quorum sensing [56,72]. For example, there is proof-of-concept that the expression of quorum quenching enzymes in edible crops could be used to combat diseases caused by certain pathogenic bacteria (in which virulence is regulated by quorum sensing) [72,73]. Arguably, this method is more cost-effective than spreading anti-quorum sensing small molecules on crop surfaces. In a therapeutic context, there is proof-of-concept demonstrating the reduction of virulence of *P. aeruginosa* in an animal infection model through the application of an AHL acylase [74]. It has therefore been proposed that the external addition of purified quorum quenching enzymes may represent a novel general antibacterial therapy [74,75]. These examples highlight the potential value of quorum quenching enzymes in areas in which the inhibition of quorum sensing is desirable. However, there are several issues associated with the application of quorum quenching enzymes in real-world settings. For example, how selective is a given enzyme of this sort? Will the enzyme hydrolyse other non-AHL molecules and what would be the effects of such processes [72]? Are there any AHL-regulated microbial functions that are beneficial to the host that may be inhibited by enzyme activity (e.g., the production of phenazine antibiotics by *P. chlororaphis* strain 30–84) [72]? In terms of the expression of quorum quenching enzymes in edible crops and therapeutic applications it is also important that the products of AHL degradation are not toxic to humans [72]. In addition, as a consequence of their bacterial origin, AHL-degrading enzymes may show poor pharmacokinetic potential due to rapid proteolytic clearance and immune surveillance in the host. Furthermore, it has been noted that the widespread expression of an AHL degrading enzyme in plants may create selective pressure for the evolution of bacterial strains that are capable of inhibiting the activity of the enzyme or even bypassing their dependence on AHLs for the expression of virulence determinants [72]. Recently, the 3D structures of some lactonases have been revealed. These have provided further understanding of the molecular mechanisms underlying the action of such quorum quenching enzymes, which may stimulate further advancements in this field (e.g., the design of more effective catalysts) [76,77].

Concluding remarks and future directions

A diverse range of bacterial behaviours, which have a significant impact upon a wide range of fields including healthcare, agriculture, and the environment, are regulated by quorum sensing systems [1]. Consequently, the ability to modulate quorum sensing systems using small molecules could have tremendous real-world implications. In addition to the significant potential applications of such compounds discussed in this review, a plethora of other diverse medical and industrial uses have been envisaged. For example, it has been proposed that small molecule quorum sensing inhibitors could be embedded into food packaging to keep the produce fresher for longer, in the plastic used to manufacture catheters to prevent infection and in toothpaste to help prevent bacterial infections leading to tooth decay (http://blog.ted.com/2009/04/08/the_secret_soci/#more). Unsurprisingly, therefore, this field has garnered significant interest in recent years; a range of potent activators and inhibitors of quorum sensing systems have been developed, providing an expansive set of chemical tools to study and manipulate this signalling process [1]. However, despite an extensive body of work in this area, real-life applications, in the main, remain a long way off. Significant advancements need to be made in both theoretical and practical aspects of this field [1].

The majority of known small molecule quorum sensing modulators have been discovered using culture-based assay systems and only evaluated under such conditions. The relevance of such compounds to native situations is unclear (Box 3). Thus, to facilitate the discovery of small molecule modulators of quorum sensing with useful real-world applications, there is a definite need for more studies which involve the testing of compounds in biologically relevant environments (e.g., animal infection model studies). Furthermore, in most cases, the specificity of quorum sensing modulators has not been evaluated. This is a significant issue, as there are potential off-target effects of such agents, which may be undesirable and difficult to predict *a priori* [14]. Standardisation of the assays used by different researchers to study small molecule modulation of quorum sensing pathways is also important [1,13,78]. Even in studies investigating the same receptor proteins, there is often variation in the assay conditions employed which can have a large effect upon the biological activity of a small molecule agent [1,13,78]. This means that a direct comparison of the levels of activities of small molecules obtained from different studies carried out by different researchers can be misleading and is not appropriate in many cases. In addition, many compounds (including antibiotics at sublethal concentrations [79]) will impinge upon virulence-associated phenotypic traits, yet this does not mean that these compounds interfere directly with quorum sensing. However, microarray technology may allow the judicious comparison of wild-type cells treated with or without a putative quorum sensing inhibitor (test compound) with a quorum sensing mutant to address the issue of specificity.

Another fundamental issue is what type of small molecules should researchers be screening for quorum sensory modulatory activity (Box 2)? The screening of libraries of random compounds (natural or synthetic) is always an option, but the testing of molecules which have been deliberately designed to interact with some component of the quorum sensing circuit may be more efficient in terms of hit rate. Overall, there is a definite need for more detailed fundamental studies into the molecular basis of small molecule modulation of quorum sensing to better understand how existing activators or inhibitors function in terms of the fundamental bonding interactions involved, which should facilitate the rational design of next-generation agents with improved properties [1]. In this context, the acquisition of X-ray crystal structures of LuxR-binding domains with non-native ligands would be particularly valuable and recent years have witnessed some notable achievements of this sort [1,80].

In addition, recent work suggests that disruption of quorum sensing can pose a selective pressure on bacteria [50,81,82]. It has therefore been argued that future research on quorum sensing inhibition should be preferentially directed towards strategies that include a lower risk of resistance development. In this sense, noncompetitive (or uncompetitive) inhibitors of quorum sensing may be more desirable than competitive inhibitors, as the effects of the latter can be easily titrated out by overexpression of quorum sensing core genes [81]. However, given the relatively low interspecies conservation of LuxR homologues [1], noncompetitive agents would almost certainly be rather narrow spectrum anti-infectives and may be prone to loss of efficacy *in vivo* due to mutation of their cognate binding sites. High affinity competitive inhibitors of quorum sensing offer the advantage of a more generic structural framework, yet so far, few analogues have proven effective at blocking quorum sensing in wild-type cells or in animal infection models. It is worth noting, however, that broad-spectrum quorum sensing inhibitors could potentially interfere with the commensal (and beneficial) microbial flora of the host, which may lead to undesirable consequences. A potential third generic class of LuxR-based quorum sensing blockers are the irreversible inhibitors that bind to and covalently block the active site of the LuxR homologue [78]. Again, however, advances here may be limited due to the nonconserved and variable nature of the AHL-binding site in different LuxR proteins.

In conclusion, despite significant progress in recent years the field of small molecule modulation of quorum sensing is, in many regards, still in its infancy. There is undoubtedly significant promise offered by small molecule activators or inhibitors of this form of intercellular communication; however, developments in both the practical and theoretical aspects of this field are needed before this potential can be exploited (Box 5). It is anticipated that the forthcoming years will witness considerable progress in this regard, with many significant, exciting and groundbreaking discoveries yet to be made.

Box 5. Outstanding questions

- What is the best way to identify useful small molecule modulators of quorum sensing? Are quorum sensing modulators discovered using culture-based assay systems relevant to native situations?
- How specific are current quorum sensing modulators? What are the off-target effects of such compounds?
- What type of small molecules should be screened for quorum sensory modulatory activity? What advancements need to be made to allow the *de novo* rational design of abiotic modulators that are structurally distinct from existing agents?
- What type of quorum sensing inhibitors should be made? *A priori,* high affinity noncompetitive inhibitors of LuxR-type proteins would be the most desirable compounds to make. However, will such agents actually be useful *in vivo*? Should more focus be placed upon the development of competitive inhibitors and irreversible inhibitors?

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