Future research trends in the major chemical language of bacteria

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Microbiology was revolutionized in the 1990's by the discovery that many different bacterial species coordinate their behavior when they form a group. In fact, bacteria are now considered multicellular organisms capable of communicating and changing behavior in relation to their cell-density; since 1994 this has been called quorum sensing. This group behavior ensures survival and propagation of the community in many natural environments. Bacterial intercellular communication is mediated by different chemical signals that are synthesized by bacteria which are then either secreted or diffused in the external environment. Bacteria are then able to detect the type and concentration of the signal resulting in regulation of gene expression and, consequently, a synchronized response by the community. The predominant signalling molecules produced by Gram-negative bacteria are *N*-acyl derivatives of homoserine lactone (AHLs) which have been shown to be produced by over seventy bacterial species. In this essay we discuss the importance of quorum sensing via AHLs and highlight current and future trends in this important field of research. [DOI: 10.2976/1.3065673]

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The discovery of chemical communication among bacteria revolutionized the thinking that bacteria exist in isolation as single-celled organisms. It has become evident in the last fifteen years that bacteria have the potential to establish highly complex and often multi-species communities. In fact, most bacteria monitor their population density via the production and detection of small signaling compounds (also called autoinducers) in a process which has been termed “quorum sensing” (QS) (Fuqua *et al.*, 1994). At low cell density bacteria produce low levels of signaling compounds which are released from the cells. As the bacterial population increases, for bacteria in enclosed niches, the freely diffusible chemical signal accumulates and bacterial density reaches a critical level (quorum). Bacteria now detect and respond to the signal through the regulation of their gene expression. This results in coordinated

and synchronized community behavior. QS signaling molecules are implicated in the regulation of an array of important bacterial phenotypes that benefit the community including cell division, production of virulence factors, motility, sporulation, nodulation, plasmid transfer, antibiotic production, bioluminescence, and biofilm formation (Bassler, 2002; Waters and Bassler, 2005; Whitehead *et al.*, 2001). Recently, it has also been suggested that bacteria produce and respond to signaling molecules in order to learn the diffusion rates in the surrounding environment. The term “diffusion sensing” has, therefore, been introduced in order to highlight this possibility (Hense *et al.*, 2007; Redfield, 2002). Many bacterial signaling molecules have been isolated and their structures are quite diverse (Fig. 1); it is very likely that many more will be found in the near future (Ryan and Dow, 2008; Waters and Bassler,

Figure 1. Examples of the chemical diversity of bacterial quorum sensing signaling molecules. On the left panel are the structures of the *N*-acyl homoserine lactones (AHLs) produced by Gram-negative bacteria. At the top of the panel shown is 3-oxo-HSL, in the middle 3-OH-HSL, and at the bottom un-substituted AHL. The R in all three situations varies from C1 to C15. The AI-2, autoinducer-2, is a furanoyl borate ester form and is produced by both Gram-negative and Gram-positive bacteria. PQS, Pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone is produced by some Gram-bacterial species. DSF, diffusible signal factor, is methyl dodecanoic acid and is produced by several Gram-bacterial species.

2005). Gram-positive bacteria most commonly use modified peptides as signal molecules whereas Gram-negative bacteria synthesize primarily *N*-acyl-homoserine lactones (AHLs) (Fig. 1). Both Gram-positive and Gram-negative bacteria produce AI-2 family molecules which are derived from the precursor 4,5-dihydroxy-2,3-pentanedione (DPD) produced by the LuxS synthase (Ryan and Dow, 2008).

Currently, AHL mediated QS is regarded as the paradigm for bacterial intercellular communication with over seventy examples in literature. Hence, we intend to focus on QS via AHLs which is predominant in Gram-negative bacterial species. In this perspective essay, we would like to present the reader with future research directions in bacterial chemical communication which is dictated by current research trends in this field.

FEATURES OF AN ARCHETYPAL AHL QUORUM SENSING SYSTEM

The first example of bacterial “talking” in Gram-negative bacteria was the demonstration in 1970, that the cooperative regulation of bioluminescence in the marine bacterium *Vibrio fischeri* occurred through the production and detection of diffusible signal compounds (Nealson *et al.*, 1970). *V. fischeri* colonizes the light organ of the Hawaiian bobtail squid (*Euprymna scolopes*) and, in this environment, the bacteria manages to grow very efficiently activating bioluminescence in response to high cell density. This light provided by the bacteria allows the squid to mask its shadow and thus

avoid predation; in exchange, the bacteria grow in a safe and nutrient rich niche reaching cell densities which cannot be achieved in seawater. Subsequently, in 1981 Eberhard *et al.* identified the signalling compound as an acylated homoserine lactone; more precisely, an *N*-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981). Two years later the genetic loci responsible for the production and regulation of bioluminescence were identified and characterized (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984). The signal 3-oxo-C6-HSL is synthesized via the LuxI synthase from S-adenosyl methionine and 3-oxo-hexanoyl-acyl-carrier protein. The AHL then moves freely across the bacterial membranes. Upon reaching a certain concentration at high cell densities (10^{10} cells mL⁻¹), it interacts with the modular sensor/receptor LuxR protein inside the cell forming an active complex. The LuxR-AHL complex has increased affinity for the palindromic *lux* box *cis*-element localized in the promoter region of the *lux* operon (*luxICDABEG*) which encodes enzymes required for bioluminescence. The *luxR* gene is adjacently and divergently transcribed from the *lux* operon. The *luxI* gene being part of the operon is under a positive induction feedback loop forming a regulatory circuit (Stevens and Greenberg, 1997).

The significance of the studies in *V. fischeri* took time to be widely recognized and, in the last fifteen years, several LuxI-LuxR type AHL QS systems controlling many different phenotypes in a wide range of Gram-negative Proteobacteria have been isolated and studied (Fuqua and Greenberg, 2002; Fuqua *et al.*, 2001; Von Bodman *et al.*, 2003; Waters and Bassler, 2005; Whitehead *et al.*, 2001). In fact, Gram-negative bacteria predominantly communicate via AHL whose synthesis is dependent on a LuxI-family protein; a cognate LuxR-family protein then recognizes the AHL and the complex then affects transcription of target genes. This type of QS system is exclusively found in Gram-negative bacteria and it is a rather simple process with the mechanism of action of the LuxI/R pairs being very much conserved. A model depicting the common components of an AHL QS circuit is shown in Fig. 2.

The LuxI-family protein synthesizes a specific AHL by direct amide bond formation between the acyl side chain of a particular acyl-acyl carrier protein (acyl-ACP; from the fatty acid biosynthesis machinery) and the amino group of S-adenosylmethionine (SAM). Many different LuxI-family proteins that direct synthesis of AHLs with different acyl chain lengths (from 4 to 18 carbons), as well as variation in the oxidation state of C3 (which is either part of methylene group or carries an oxo- or hydroxyl group) have been identified from different species. However, not all AHL synthases belong to the LuxI family proteins; AHLs are also synthesized by LuxM/AinS type of proteins. In contrast to LuxI-family proteins, AinS of *V. fischeri* catalyses the synthesis of C8-HSL from SAM and octonoyl-ACP or octonoyl coenzyme A conjugates. LuxM of *V. harveyi* shares sequence

Figure 2. Schematic representation of a typical *N*-acyl homoserine lactone (AHL) dependent quorum sensing (QS) system in Gram-negative bacteria. The LuxI-type proteins are the main class of enzymes capable of synthesizing AHLs and they use the cellular metabolites *S*-adenosyl-methionine and acetylated acyl carrier proteins to form AHLs. At high population density, the AHL signal accumulates and, at quorum concentrations, it interacts directly with the LuxR-type protein. This induces a conformational change (usually allowing homo-dimerization) resulting in the alteration of the affinity for specific DNA sequences (known as *lux*-boxes) at target gene promoters changing gene expression.

similarity with the C-terminal part of AinS and directs synthesis of 3-OH-C4-HSL (Lupp and Ruby, 2004). Short chain AHLs (<C8) are believed to be able to freely diffuse across the cell envelope; whereas AHLs with longer acyl side chains may depend on transport systems (Kohler *et al.*, 2001). Several LuxI family proteins have been studied at the biochemical and structural level revealing their precise mode of action (Pappas *et al.*, 2004). The LuxR-family proteins are transcription factors which specifically bind the cognate AHL which, in most cases, results in the formation of homodimers. LuxR-AHL complexes can then bind at specific promoter sequences called *lux*-boxes of QS-regulated genes affecting their expression. The amino-terminal regions of LuxR-family proteins are involved in AHL binding, whereas the C-terminal domains are implicated in DNA-binding; numerous biochemical and structural studies have elucidated their mechanism of action (Pappas *et al.*, 2004).

EXAMPLES OF THE BEST CHARACTERIZED LuxI/R-TYPE QUORUM SENSING SYSTEMS

Among the most extensively studied systems are the ones present in *Pseudomonas aeruginosa* (Juhas *et al.*, 2005; Venturi, 2006). In fact, *P. aeruginosa* has two AHL-dependent QS systems, the LasI/R and the RhII/R system. LasI directs the synthesis of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) which interacts with LasR, whereas RhII synthesizes *N*-(butanoyl)-L-homoserine lactone (C4-HSL) which is the cognate AHL for RhIR. Many

studies have been carried out in *P. aeruginosa* as it is an important opportunistic pathogen in immunocompromised people. It is especially frequent in cystic fibrosis patients where it causes chronic colonizations of pulmonary airways (www.pseudomonas.com). The two AHL systems are intimately connected, the LasI/R system regulating *rhII/R* forming a cascade and together are involved in the regulation of many virulence factors including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, superoxide dismutases, and biofilm formation (Smith and Iglewski, 2003). In addition, QS mutants are considerably attenuated in several models including mouse, plant, and nematode indicating a major role in the pathogenicity of *P. aeruginosa* (Smith and Iglewski, 2003). The QS regulon has also been deciphered revealing the AHL QS in *P. aeruginosa* to be a complex global regulatory system affecting the expression (either in a positive or negative way as well as the timing) of approximately 300 genes (Hentzer *et al.*, 2003b; Schuster *et al.*, 2003; Wagner *et al.*, 2003).

AHL-dependent QS has been intensively studied also in the plant pathogen *Agrobacterium tumefaciens* (White and Winans, 2007). *A. tumefaciens* possesses the ability to transform plant cells by directly transferring DNA fragments (called T-DNA) to their genomes. This causes the infected cells to proliferate developing tumors (also called crown galls) and to produce novel compounds called opines which are sources of nutrients for the colonizing bacteria. Nearly all the genes necessary for tumorigenesis are harbored in a large

plasmid called the Ti (tumour-inducing); this plasmid also harbors all the functions to self-conjugate into other bacterial cells. The *A. tumefaciens* Ti plasmids carry the AHL system called TraI/R. TraI synthesizes *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL) and, at high cell densities, the TraR/3-oxo-C8-HSL complex activates transcription of several target genes on the Ti plasmid, most importantly the ones responsible for conjugation. However, the expression of the *traR* regulator gene requires the presence of opines and the AHL system is functional only in the tumor environment. This means that plasmid conjugation actively takes place in crown gall tumors allowing consolidation of *A. tumefaciens* colonization and simultaneously keeping away other plant-associated bacteria. Experiments on the timing and regulation of the TraI/R system as well as extensive biochemical/structural studies have prompted important concepts and working models of AHL QS systems (White and Winans, 2007).

Other important contributions to understanding AHL QS systems include (i) studies on the regulation of antibiotic production and virulence factors in plant pathogenic soft rot

Erwinia species (Barnard *et al.*, 2007) and (ii) studies on the regulation of virulence in the plant pathogen *Pantoea stewartii*, a vascular pathogen which causes wilt and leaf blight in sweet corn and maize. Interestingly, the AHL EsaI/R system of *P. stewartii* is the paradigm system where the LuxR-family members act as repressors. More precisely, EsaR represses transcription in the absence of the cognate AHL produced by EsaI (i.e., 3-oxo-C6-HSL) and, at high cell densities upon binding the AHL, the EsaR/3-oxo-C6-HSL complex can no longer bind to DNA, thereby de-repressing target genes (Barnard *et al.*, 2007; Von Bodman *et al.*, 2003). A list of some of the most intensively investigated QS systems are listed in Table I.

ARCHITECTURAL DIVERSITY OF MULTIPLE AHL QUORUM SENSING SYSTEM CIRCUITS

An important fact that has come to light as a result of studies on QS in Gram-negative bacteria is that many of them possess multiple QS systems that interact extensively among themselves. *P. aeruginosa* provides an excellent example of both hierarchical and complex natures of QS systems

Table I. Examples of some of the best characterized *N*-acyl homoserine lactone based quorum sensing systems.

Organism	QS system	Major signals	Function(s) regulated by QS system	Reference
<i>Agrobacterium tumefaciens</i>	TraI/R	3-oxo-C8-HSL	Virulence plasmid copy number and conjugal transfer	(Hwang <i>et al.</i> , 1994; White and Winans 2007)
<i>Agrobacterium vitis</i>	AvsI/R	C14:1-HSL 3-oxo-C16:1-HSL	Virulence	(Hao and Burr, 2006)
<i>Burkholderia cenocepacia</i>	CepI/R CciI/R	C6-HSL, C8-HSL	Virulence functions and biofilm formation	(Malott <i>et al.</i> , 2005; Sokol <i>et al.</i> , 2003)
<i>Erwinia carotovora</i>	CarI/R (ExpI/R)	3-oxo-C6-HSL	Carbapenem antibiotics and exoenzymes	(Bainton <i>et al.</i> , 1992; Barnard <i>et al.</i> , 2007; McGowan <i>et al.</i> , 1995)
<i>Pantoea stewartii</i>	EsaI/R	3-oxo-C6-HSL	Exopolysaccharide	(Beck von Bodman and Farrand, 1995; Von Bodman <i>et al.</i> , 2003)
<i>Pseudomonas aeruginosa</i>	LasI/R, RhII/R	3-oxo-C12-HSL C4-HSL	Virulence factors Rhamnolipid synthesis and virulence	(Ochsner <i>et al.</i> , 1994; Passador <i>et al.</i> , 1993)
<i>Pseudomonas aureofaciens</i>	PhzI/R CsaI/R	3-OH-C6-HSL	Phenazine biosynthesis Biosynthesis of cell surface components	(Pierson, 3rd, <i>et al.</i> , 1994; Zhang and Pierson, 2001; Khan <i>et al.</i> , 2005)
<i>Rhizobium leguminosarum</i> <i>bv. viciae</i>	CinI/R RhlI/R RaiI/R TraI/R	7-cis-C14-HSL C6-HSL C7-HSL C8-HSL 3-oxo-C8-HSL 3-OH-C8-HSL	Functions required for symbiosis	(Lithgow <i>et al.</i> , 2000; Rodelas <i>et al.</i> , 1999; Wilkinson <i>et al.</i> , 2002; Wisniewski-Dye <i>et al.</i> , 2002)
<i>Serratia liquefaciens</i>	SwrI/R	C4-HSL	Swarming motility	(Eberl <i>et al.</i> , 1996)
<i>Vibrio fischeri</i>	LuxI/R AinS/R	3-oxo-C6-HSL C8-HSL	Bioluminescence Bioluminescence	(Engebrecht and Silverman, 1984; Gilson <i>et al.</i> , 1995)
<i>Yersinia pseudotuberculosis</i>	YpsI/R YtbI/R	C6-HSL 3-oxo-C6-HSL C8-HSL	Motility and clumping	(Atkinson <i>et al.</i> , 1999)

wherein activated LasR regulates the expression of *rhIR* which leads to the induction or repression of specific sets of genes. In addition, several global regulators have also been reported to influence the QS circuitry of *P. aeruginosa* (Venturi, 2006). Another example of a highly complex QS network is the one found in *Rhizobium leguminosarum* bv. *viciae* which has been reported to have at least four complete QS systems, namely, CinI/R, TraI/R, Rail/R, and RhlI/R. Here, the CinI/R system appears to be at the highest level of the regulatory pathway as mutants in this system exhibit decreased levels of all of the short chain AHLs that are produced by the other three QS systems (Gonzalez and Marketon, 2003). Additionally, different QS signals may be integrated into the same signal transduction pathway to modulate cellular functions. This may be seen in *V. harveyi*, where there are three different systems, namely, 3-hydroxy-C4-HSL dependent LuxM/N system (QS-1), AI-2 dependent LuxS/PQ system (QS-2), and the CAI-1 dependent CqsA/S system. The AHL dependent LuxM/N system is different from the LuxM/N system in several ways. First of all, the LuxM synthase responsible for 3-OH-C4-HSL is not similar to the LuxI-family protein, although both carry out similar function. Additionally, the AHL signal is not detected at first by the LuxR-family protein but by a membrane-bound two-component receptor protein, LuxN. Other autoinducer molecules like the AI-2 and CAI-1 are also detected at cell surface by such proteins which alternate between kinase and phosphatase activities, depending on the presence of the signal. At high population density, the signals are sensed by different sensors, LuxN, LuxQ, via the periplasmic LuxP and CqsS, respectively, but all signaling pathways converge by regulating the phosphorylation status of another protein, LuxO. Dephosphorylated LuxO is inactive and cannot initiate LuxR mRNA degradation, which is then available for expression of genes required for bioluminescence and other functions (Milton, 2006). These examples merely draw attention to the diversity of QS networks in different bacteria, and studies aimed at deciphering the details of their functioning as well as their relation to other regulatory pathways would be very important in the future.

AHLs AND BACTERIAL INTERSPECIES SIGNALING

Signal specificity in a LuxI/R-type QS system is provided by the LuxR-family protein being able to bind a specific AHL which is synthesized by the cognate LuxI-family protein. It is, therefore, possible that bacterial species with similar/compatible LuxI/R systems colonizing or sharing the same niche have the potential for interspecies signaling via their AHLs. This has been demonstrated in mixed biofilms of *Burkholderia cepacia* and *P. aeruginosa*. These two bacterial species are phylogenetically distant but phenotypically close to being able to colonize similar habitats (for example, the lungs of cystic fibrosis patients and the rhizosphere of plant roots) and both possess AHL QS systems (Venturi *et al.*,

2004). It has been demonstrated that *B. cenocepacia* can detect and respond to AHLs produced by *P. aeruginosa* when both bacterial species are grown in a mixed biofilm (Riedel *et al.*, 2001). This interspecies communication was also proven at the single cell level using GFP-based AHL responsive systems showing that *B. cenocepacia* responded to AHLs synthesized by *P. aeruginosa* (Riedel *et al.*, 2001). Since bacteria in nature are mostly found in polymicrobial communities, the possible role of AHLs in interspecies signaling remains largely unexplored. Interestingly, it appears that the “AHL-calling distance” in plant roots can reach rather long distances (e.g., 37 μ M) in proportion to bacterial size. This opens the question whether AHL communication can also take place over long distances between individual bacteria (Gantner *et al.*, 2006). A topic for future research will most probably focus on the role of AHLs in multispecies community structure and cooperation, especially in the biofilm mode of growth.

There have been a few reports of bacterial species which do not produce AHLs but, nevertheless, possess an unpaired LuxR-family protein which is able to respond to them. For example, *Escherichia coli* and *Salmonella enterica* serovar Typhimurium do not produce AHLs but possess the QS LuxR-family protein called SdiA, which is able to bind and respond to AHLs, and, consequently, affect target gene expression (Ahmer, 2004). The biological significance of a bacterial species being able to respond to exogenous AHLs is currently not known: it has been proposed that it might be a way for bacteria to detect bacterial density in the environment (Ryan and Dow, 2008). Analyzing the genome sequences of many Gram-negative bacteria, it has been found that the presence of unpaired QS LuxR-family genes/proteins is actually rather common (this is discussed in more detail below) (Case *et al.*, 2008). It is, therefore, important to establish the probable role that these genes/proteins play in interspecies communication and polymicrobial communities.

Recent research has demonstrated that interspecies interactions between bacterial community members often take place with non-AHL signaling molecules. For example, the signaling molecule AI-2 (a furanosyl borate diester) (Fig. 1) is produced by a wide range of Gram-positive and Gram-negative bacteria and thus, it is regarded as a universal interspecies signal (Federle and Bassler, 2003; Ryan and Dow, 2008; Schauder *et al.*, 2001). However, the ability of bacteria to respond to AI-2 would depend on the presence of a complete AI-2 signaling pathway made up of homologs of LuxPQ-receptor of *Vibrio harveyi* and Lsr ABC-transporter of *Salmonella enterica* serovar Typhimurium. In the absence of AI-2 signal receptors, the metabolic role of LuxS would involve generation of S-adenosyl-L-methionine (SAM) and the recycling of methionine; the AI-2 molecule precursor is usually produced as a by-product of this reaction. It appears that these AI-2 signaling components are restricted to bacte-

ria belonging to *Vibrio* sp. and a few animal pathogens, indicating that LuxS at large would have a metabolic role in other bacteria (Rezzonico and Duffy, 2008). Scientists are now also beginning to address the prospect of interspecies and interkingdom signaling using various signaling molecules among distantly related organisms which colonize similar niches (Cugini *et al.*, 2007; Ryan *et al.*, 2008). This signaling can lead to cooperation as well as competition/inhibition. As bacteria often form complex multispecies consortia in nature, a future challenge will be to decipher how the polymicrobial community establishes and maintains itself.

AHL RESPONSE BY UNPAIRED QUORUM SENSING LuxR-FAMILY PROTEINS

Research in the past few years has led to the identification of QS signal receptors or LuxR homologs that occur without a cognate signal generator or LuxI homolog nearby or anywhere else in the genome. They have been identified in both bacteria that do not produce AHL type signaling molecules as well as in those that possess one or more complete QS systems. The first unpaired LuxR homolog to be reported was SdiA, initially identified in *E. coli* as the factor that promotes cell division and later on found also in *S. typhimurium* (Ahmer *et al.*, 1998; Wang *et al.*, 1991). Both these bacteria are not reported to produce AHLs. As mentioned before, SdiA has been proposed to function in interspecies signaling and this is due to the observations that it binds to more than one type of AHL molecule and regulates target genes in an AHL dependent manner (Michael *et al.*, 2001; Smith and Ahmer, 2003; Yao *et al.*, 2006). SdiA also functions in mediating the effects of indole, a stationary phase signaling molecule of *E. coli* that may also serve as an interspecies signal as its levels can be changed by other bacteria in a mixed community (Lee *et al.*, 2006). It seems that SdiA can respond to at least two different kinds of signaling molecules, AHLs (foreign) and indole (endogenous), and modulate cellular functions appropriately. Future studies need to be done on these aspects in a mixed bacterial community to uncover similar signaling pathways.

QscR is another well studied LuxR orphan regulator of *P. aeruginosa* which has two complete QS systems; LasI/R and RhII/R (as explained above). QscR responds to 3-oxo-C12-HSL concentration and its main function is to prevent premature expression of quorum signals and virulence factors like pyocyanin, HCN, elastase, etc. (Chugani *et al.*, 2001; Lee *et al.*, 2006; Lequette *et al.*, 2006). QscR therefore interferes with the expression of the endogenous QS system(s) and influences intraspecies signaling. Additionally, in response to 3-oxo-C12-HSL, QscR also regulates a set of target genes distinct from those regulated by the two endogenous QS systems (Lequette *et al.*, 2006). However, QscR has also been found to respond better to 3-oxo-C10-HSL and regulate target gene expression, thus showing a response to a

broader range of AHLs (Lee *et al.*, 2006). *P. aeruginosa* produces this AHL via LasI; however, it cannot be excluded that QscR may also function in detection of signals from other species.

Another orphan LuxR homolog, BisR (bacteriostasis induction sensor) of *Rhizobium leguminosarum* bv. *viciae* strain A34, is a regulator of growth inhibition of this strain in the presence of 3OH-C14:1-HSL, produced by the CinI/R QS system, and controls the frequency of pRL1J1 plasmid transfer (Wilkinson *et al.*, 2002). BisR, located on the pRL1J1 plasmid, represses the expression of *cinI* and thereby maintains minimal amounts of 3OH-C14:1-HSL in donor cells. This prevents BisR and 3OH-C14:1-HSL mediated activation of *traR* and other plasmid transfer functions. Upon sensing a critical concentration of 3OH-C14:1-HSL produced by an optimum number of recipient cells (where chromosomal *cinI* is not under BisR mediated repression due to absence of plasmid), BisR induces the plasmid transfer functions (Danino *et al.*, 2003). BisR functioning therefore seems to be closely connected to the QS system of these bacteria.

Other LuxR proteins that have been reported in bacteria containing complete quorum sensing systems include ExpR of *Sinorhizobium meliloti* as well as AvhR and AviR in *Agrobacterium vitis* (Hao *et al.*, 2005; Pellock *et al.*, 2002; Zheng *et al.*, 2003). *Brucella melitensis*, which does not contain a *luxI* homolog but, however, appears to produce AHLs, contains two unpaired LuxR proteins. These are called VjbR and BlxR and have been functionally characterized (Delrue *et al.*, 2005; Rambow-Larsen *et al.*, 2008).

The studies carried out on unpaired LuxR family proteins point to their role in interspecies, intraspecies as well as interkingdom signaling, depending on the genomic context and the ecological niche of bacteria in which they occur. Clearly, these processes show a level of sophistication much more than just cell density dependent signaling that is characteristic of typical LuxR family proteins. Therefore, it is anticipated that future studies of orphan LuxR proteins or unpaired LuxR homologs will lead to a better understanding of these processes.

AHL EFFECT ON HUMAN AND PLANT CELLS

Recent studies have extended the function of AHLs from bacterial signaling molecules affecting gene expression in bacteria to being directly recognized by eukaryotic cells and affecting their behavior (Lowery *et al.*, 2008). It has been established that the 3-oxo-C12-HSL produced by *P. aeruginosa* impairs the ability of mammalian cells to respond to the invading bacterial pathogen via the innate immune system (Kravchenko *et al.*, 2008; Kravchenko *et al.*, 2006; Shiner *et al.*, 2006). One of the key steps in this response immediately after receptor-recognition of the pathogen is the activation of the nuclear transcription factor NF- κ B and consequential expression of genes encoding pro-inflammatory cytokines. The 3-oxo-C12-HSL of *P. aeruginosa* affects the

NF- κ B pathway regulation resulting in disruption of the immune response and promoting *P. aeruginosa* persistent infection, especially in cystic fibrosis patients. Current studies are now particularly focused on the identification of the mammalian receptor for 3-oxo-C12-HSL as this will provide important insight in host-pathogen interaction. A recent study hypothesized that the AHL of *P. aeruginosa* crosses cell membranes and possibly interacts with intracellular nuclear peroxisome proliferator-activated receptor (PPAR) which belongs to the nuclear hormone (NHR) superfamily (Jahoor *et al.*, 2008). These recent results extend the role of AHLs to interfering with the immune system in order to tailor the infection of the pathogen. It is expected that deciphering the mode of action of AHLs on mammalian cells is a field that will now grow dramatically.

Despite the fact that many important bacterial phytopathogens use AHL QS for their infection, less experimentation on the effects of AHLs on plant cells has been performed (Von Bodman *et al.*, 2003). A few years ago, the response to AHLs of the legume *Medicago truncatula* was studied using a proteomic approach (Mathesius *et al.*, 2003). *M. truncatula* is exposed to both beneficial and pathogenic bacteria which use AHL QS to interact with the plant. Plant roots were exposed to two different AHLs and proteomic analysis revealed that 154 proteins were upregulated, indicating a significant plant response to AHLs. Some of these proteins were found to have function in host defense, transcriptional regulation, stress, and plant hormone responses. Recent studies further highlighted that AHLs are perceived also by plants. It has been demonstrated that AHLs can be recognized by plants and, as a result, activate defenses during pathogen build up in the infection process. More precisely, experiments with *Serratia liquefaciens*, a strain that is capable of protecting plants against fungal pathogens, showed that AHL production in the rhizosphere of tomato plant roots was responsible for increasing systemic resistance against the fungal leaf pathogen *Alternaria alternata* (Schuhegger *et al.*, 2006). In another study, the response of the model plant *Arabidopsis thaliana* to different AHLs was investigated via microarray analysis (von Rad *et al.*, 2008). It was established that AHLs are taken up by *A. thaliana* and are distributed throughout the plant and that they cause distinct changes in transcription profile in roots and shoots. In this experimental set up, AHLs did not show activation of the plant defense and/or resistance genes; however, they affected levels of plant hormones resulting in lateral roots and root elongation. These first reports on plant responses to bacterial AHLs will most probably lead the way to further studies on how plants respond to AHLs produced by both pathogens and beneficial bacteria. The direct role of AHLs will most probably be very complex as plants and bacteria have co-evolved for a very long period of time.

PLANT INTERKINGDOM SIGNALING WITH BACTERIAL AHL QUORUM SENSING

One of the first examples that indicates eukaryotic interference with AHL QS was the observation that plants produce several chemical compounds that act as agonists or antagonists to bacterial AHLs, implicating interkingdom communication; these have been called AHL-mimics (Bauer and Mathesius, 2004). Several plant species secrete AHL mimics which can either stimulate or inhibit bacterial AHL QS systems (Bauer and Mathesius, 2004). Detection of AHL mimics have been reported in secretions of pea, rice, soyabean, tomato, crown vetch, *M. truncatula*, and from the unicellular algae *Chlamydomonas reinhardtii* (Degraassi *et al.*, 2007; Gao *et al.*, 2003; Teplitski *et al.*, 2004; Teplitski *et al.*, 2000). No evidence is currently available that these AHL mimics are interacting with QS systems *in vivo*; experiments in tomato with *A. tumefaciens*, in fact, have evidenced that these mimics cannot act as AHL agonists (Khan and Farrand, 2008). Consequently, the precise source, structure, and biological significance of these AHL mimics from plants is currently unknown. Very recently, two compounds, lumichrome and riboflavin, have been identified from *C. reinhardtii* which act as AHL mimics by activating the QS LasR protein of *P. aeruginosa* which normally recognizes 3-oxo-C12-HSL (Rajamani *et al.*, 2008). Since plants and algae commonly secrete these compounds, it cannot be excluded that these serve as signals which manipulate bacterial communication.

A further significant indication that plants actually produce compounds which signal AHL QS systems is that recently two unpaired (lacking a cognate LuxI-family AHL synthase; see above) quorum LuxR-family homologs have been reported to respond to a plant compound. *Xanthomonas campestris* pv. *campestris* (Xcc; a crucifer pathogen) and *Xanthomonas oryzae* pv. *oryzae* (Xoo; a rice pathogen) are two plant pathogenic bacteria that do not produce AHLs but possess a LuxR-family protein, designated XccR and OryR, respectively, which respond to a plant compound (Ferluga *et al.*, 2007; Ferluga and Venturi, 2008; Zhang *et al.*, 2007). In Xcc, XccR was found to regulate the expression of a gene coding for proline imino peptidase, a virulence factor, by binding to the *lux* box in its promoter which was enhanced in the presence of plant extract. In Xoo, OryR has been demonstrated to regulate expression of 1, 4 beta cellobiosidase, a protein required for optimum virulence. Additionally, the OryR protein was shown to be solubilized by a component of macerated rice containing medium but not by any of the tested AHL molecules. This indicates that OryR mediated modulation of gene expression most probably occurs in response to the plant component but not in response to AHLs. It is most probable that unpaired LuxR homologs in these two bacteria participate in interkingdom signaling whereby they facilitate successful infection by detecting and binding to host signal molecules. Zhang *et al.* (2007) report the presence of unpaired LuxR homologs similar to XccR in other

plant-associated bacteria (both beneficial and pathogenic) underlining the importance of this type of signaling. Upcoming research on understanding the nature of plant component and its mode of action will help to eventually formulate effective intervention strategies. For example, by controlling the production of this compound, it might be possible to attenuate *Xanthomonas* plant infections.

An important very recent discovery demonstrated that a subgroup of LuxI/R family pairs in several plant-associated bacteria produce a new class of AHL signal molecules which requires a plant produced compound as a substrate for their biosynthesis (Schaefer *et al.*, 2008). It was observed that bacteria belonging to the *Rhodopseudomonas*, *Bradyrhizobium*, and *Silicibacter* genera use LuxI-family AHL synthases to produce coumaroyl-AHL using coumaric acid produced by the plant as substrate rather than fatty acids from cellular pools. The necessity of a plant produced compound as substrate integrates two different cues for these QS systems, high population density and availability of an exogenous substrate, thus confining the functioning of these QS systems to particular environments. Since coumaric acid is produced by plants, this QS system is most likely involved in establishing intimate interactions between plants and bacteria. It is most likely that more examples of QS systems requiring an exogenously provided substrate will be discovered in the near future.

AHL QUORUM SENSING AND SOCIAL EVOLUTION

Our improved understanding of chemical AHL-based communication among bacteria led scientists to begin to study its possible link with cooperation, social behavior, and evolution in a bacterial community. AHL QS allows a community to regulate the expression of genes mostly coding for energy demanding products (e.g., secreted enzymes, antibiotics, production of biofilm extracellular matrix) which are not significant if produced by a few cells, but instead would be valuable for cells existing in high population densities. This cooperation within a group of bacteria to produce an energy-demanding “public good” in fact provides benefit to all the bacteria present in the community (West *et al.*, 2006). If a community is composed of co-operators and the public good is advantageous, the population grows faster resulting in a successful colonization. If, on the other hand, the community is invaded by non-cooperative bacteria (cheaters) which do not produce the public good but can gain benefit and fitness from it, the community is at risk of growth and, possibly, survival. Two recent studies using *P. aeruginosa*, which is known to be able to colonize many different environments as well as being an important human opportunistic pathogen, showed that AHL QS is a social characteristic that is exploitable by cheaters (Diggle *et al.*, 2007; Sandoz *et al.*, 2007). These studies used co-cultures of wild-type and QS deficient *P. aeruginosa* strains grown in liquid minimal medium containing carbon sources which required the production of a

QS-regulated secreted protease to sustain growth. It was determined that, while cheaters were at a growth disadvantage as they did not produce the protease, they grew more rapidly than the wild-type and, when the proportion of cheaters increases beyond a critical point, the growth rate of the whole population declined. Diggle *et al.* (2007) went further to demonstrate that, if the interacting bacteria are close relatives, AHL QS is favored and exploitation by cheaters diminishes, meaning that QS is maintained via kin selection (Diggle *et al.*, 2007). The large body knowledge on AHL QS will continue to encourage social evolution studies in bacteria and it is not excluded that understanding behavioral patterns might lead to strategies for the control of pathogenic and/or beneficial bacterial communities.

PRACTICAL APPLICATIONS IN THE CONTROL OF BACTERIAL AHL QUORUM SENSING

It is widely accepted that disruption or interference of AHL QS will diminish AHL-dependent expression of virulence determinants in bacteria; this could have practical applications especially due to the increasing emergence of antibiotic-resistant bacteria. It has now also become evident that bacteria in nature exist mainly as sessile, surface-associated communities which are called biofilms; their formation and maturation requires QS and biofilms allow a community of bacteria to survive hostile environments (Hentzer *et al.*, 2003a). Bacterial biofilms are significant in persistent and chronic infections allowing bacteria to withstand environmental stresses. Most importantly, they allow bacteria to endure the action of antibiotics and of the immune system. In recent years, several scientists have explored ways to control bacterial infections and pathogenicity via blocking or interference of the AHL QS response; this is most often referred to as quorum quenching or signal interference (Gonzalez and Keshavan, 2006; Hentzer *et al.*, 2003a,b; Zhang and Dong, 2004). Strategies to disrupt AHL QS include (i) depleting concentrations of the AHL signal molecule either by its degradation or inhibition of the synthesis or, alternatively (ii) by blocking the transmission of the AHL signal by interfering with the formation of the AHL/LuxR-family complex.

Intensive screening for small natural and synthetic compounds which inhibit QS resulted in the identification of several compounds which act by interfering with the binding of the AHL to the cognate Lux-family receptor, by decreasing its concentration via promotion of rapid protein turnover or also by decreasing its DNA binding activity (Defoirdt *et al.*, 2007; Dong *et al.*, 2007; Manefield *et al.*, 2002). One of the most potent inhibitors found is an halogenated furanone produced by the seaweed *Delisea pulchra* and related synthetic derivatives (Givskov *et al.*, 1996; Gonzalez and Keshavan, 2006; Hentzer *et al.*, 2003a). These compounds have been shown to be effective as anti-infective drugs. They block QS by targeting LuxR-family proteins in *P. aeruginosa* render-

ing the biofilms more susceptible to antibiotics as well as significantly decreasing the number and disease symptoms of *P. aeruginosa* in infected lung tissues (Hentzer *et al.*, 2003b). Unfortunately, these furanone compounds are toxic and, hence, they cannot be used by the pharmaceutical industry. Other natural compounds inhibiting QS have been found in garlic extracts and in members of the genus *Penicillium* (Gonzalez and Keshavan, 2006; Rasmussen *et al.*, 2005a; Rasmussen *et al.*, 2005b). Synthetic AHL analogs have and are currently being studied for their behavior in affecting QS systems; however, thus far they appear to be less effective than the natural compounds (Gonzalez and Keshavan, 2006; Olsen *et al.*, 2002; Reverchon *et al.*, 2002).

An alternative approach to interfering with QS is to quench AHL signals via the use of enzymes which have AHL-degrading activity. Two main classes of AHL-degrading enzymes have been found; an AHL lactonase activity initially found in *Bacillus* sp. and an AHL acylase first reported in *Variovorax paradoxus* that can also use AHLs as a unique source of carbon and energy (Dong *et al.*, 2007). Recently, a third class of enzymes has been reported; paraoxonase enzymes having AHL degrading activity have been first found in human epithelial cells and, later, also shown to be widely conserved in several other mammalian species (Dong *et al.*, 2007). These quorum quenching enzymes have been investigated for their possible role as antimicrobial agents against different pathogens with encouraging results. In fact, the expression of a lactonase in transgenic potato and tobacco plants conferred significant resistance to the plant bacterial pathogen *Erwinia carotovora* which uses AHL QS for activating expression of virulence genes (Dong *et al.*, 2001). Similarly, the action of AHL lactonases and acylases has been shown to be effective in the control of other bacterial pathogens; however, questions do remain about their limitations as, for example, affecting nontarget organisms (Dong *et al.*, 2007; Zhang and Dong, 2004).

The use of chemical inhibitors or enzyme quenchers of QS as a strategy to attenuate bacterial pathogenicity is rather attractive because it is not bactericidal and, therefore, does not create harsh selective pressure for the development of resistance mutants. Chemical attenuation is likely to be more suitable for treating human and animal infections, whereas the enzyme quenching approach could prove more appropriate for controlling bacterial plant virulence. This line of research is currently pursued by both academia and industry.

CONCLUSIONS

In this essay we focused on the future directions of AHL QS (as summarized in Fig. 3); the predominant language of Gram-negative bacteria. Research will now need to further understand how this and other chemical communication systems determine bacterial behavior relative to other bacterial species as well as when living in close association with eukaryotic hosts. Determining the role of LuxI/R systems in

Figure 3. Cartoon summary of the current and future research trends in the field of *N*-acyl homoserine lactone (AHL) dependent quorum sensing in bacteria.

interkingdom signaling will be a major challenge; for example, do many of these systems perceive signals produced by eukaryotes or how do AHLs influence the behavior of eukaryotic cells? Another aspect of chemical communication which remains largely unexplored is the role of signals in establishing and maintaining polymicrobial communities. Bacteria in the wild live, in most cases, in close association with many different bacterial species; it is most probable that extensive communication is taking place allowing recognition, maintenance, and growth of a bacterial community. Another aspect that is emerging is that many bacteria possess multiple QS systems which are then channeled into common response proteins extending the repertoire of signals that a bacteria can respond to. The discovery that bacteria communicate has revolutionized bacteriology and this essay highlights how this field of research is now moving onto the next phase which will also, hopefully, result in useful practical applications in the fight against bacterial diseases.

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