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Variations on a theme: diverse *N*-acyl homoserine lactone-mediated quorum sensing mechanisms in Gram-negative bacteria

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ABSTRACT

Many Gram-negative bacteria employ a mechanism of cell–cell communication known as quorum sensing (QS). The role of QS is to enable the cells in a culture to coordinate their gene expression profile with changes in the population cell density. The best characterized mechanisms of QS employ N*-acylated homoserine lactones (AHLs) as signalling molecules. These AHLs are made by enzymes known as LuxI homologs, and accumulate in the culture supernatant at a rate proportional to the increase in cell density. Once the AHL concentration exceeds a certain threshold value, these ligands bind to intracellular receptors known as LuxR homologs. The latter are transcriptional regulators, whose activity alters upon binding the AHL ligand, thereby eliciting a change in gene transcription. Over the last five years, it has become increasingly obvious that this is a rather simplistic view of AHL-dependent QS, and that in fact, there is considerable diversity in the way in which LuxI-R homologs operate. The aim of the current review is to describe these variations on the basic theme, and to show how functional genomics is revolutionizing our understanding of QS-controlled regulons.*

Keywords: N*-acyl homoserine lactones, biofilms, cell-cell communication, LuxI, LuxR, quorum sensing, signal transduction*

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Introduction: scope of the current review

Over the last decade, it has become increasingly clear that many microbes, previously long viewed as being archetypal single-celled organisms, can coordinate their population behaviour in a process now known as ''quorum sensing'' (QS). Cell–cell communication plays a central role in mediating this cooperative behaviour and the last few years have witnessed an explosion in research in the area, primarily due to the involvement of QS in the control of virulence factor production and the formation of antibiotic-insensitive biofilms by clinically-important pathogens. QS, and the phenotypic traits it controls in different organisms, has been extensively reviewed in the past and there are many excellent reports describing this (see refs. 1–4 for some recent reviews). However, in this review, we aim to emphasize the mechanistic *diversity* of QS. For reasons of space limitation and clarity, we will focus mainly on *N*-acyl-homoserine lactone (AHL)-mediated QS in Gram-negative bacteria, but we will also touch on some other signalling systems that impinge on this. QS in Gram-positive organisms has been comprehensively reviewed recently[5,6]. The current review is not intended to be exhaustive or fully comprehensive; we cannot possibly hope to do justice to the full range of QS systems now being investigated. Instead, we will use just a few well-characterized examples to illustrate how our preconceptions about QS have changed in recent years, and in particular, emphasize just how prevalent variations on the basic theme of AHL-mediated QS have become.

Cell–cell signalling in *Vibrio fischeri*: the paradigm for QS

The earliest work on QS as we know it today was done in the 1970s in the lab of Hastings[7,8], although the term ''quorum sensing'' was not coined until much later[9]. Hastings and colleagues were investigating the origins of light production by the Gram-negative

bioluminescent marine bacterium, *Vibrio fischeri*. This organism is perhaps best known for its ability to form a symbiotic relationship with marine fauna, including several sepiolid squids and monocentrid fishes, although it is also found in the gut of some marine animals, and is an occasional pathogen of certain invertebrates. *V. fischeri* can also exist in the free-living state in sea water, where it lives off suspended and dissolved organic matter, although it rarely achieves a cell density greater than *ca* 10^4 cells per ml in this milieu. In the case of at least one host organism – the Hawaiian bobtailed squid, *Euprymna scalopes* – much has been garnered about the mechanism of colonization. Newly hatched squid become colonized by free-living *V. fischeri* within an hour of hatching. The presence of free-living bacteria stimulates mucus secretion by the host, and within hours, the bacteria form dense aggregates of cells within this matrix. These cell aggregates migrate through pores into the crypt structures of the *Euprymna* light organ. Once there, nurtured by a ready source of host-supplied amino acids, the *V. fischeri* multiply, eventually reaching maximal post-inoculation levels (*ca* 10^{10} bacteria per ml) after just 24 h[10]. By this time, the bacteria in the light organ bioluminesce strongly, and the squid may exploit this feature in a nocturnal counter-illumination strategy designed to evade predator (the role of bioluminescence in *V. fischeri* has recently been discussed[11]). In the morning, the light organ is periodically vented, releasing *ca* 90% of its contents back into the seawater. The remaining 10% goes on to seed the next cycle of bioillumination.

Hastings *et al*. found that the *lux* genes required for light production by *V. fischeri* exhibit a pronounced growth phase-dependency in their expression. During the early log-phase of growth, the bacteria exhibited very little *lux* gene expression, but as the cultures enter the stationary phase of growth, bioluminescence markedly increases (by a factor of $>10^4$). Crucially, and differentiating this expression profile from that of other growth-phase-dependent phenotypes, these workers noted that *lux* gene expression could be advanced simply by adding spent stationary phase culture supernatant to log-phase cells. Moreover, the diffusible signal factor responsible for this advancement was species-specific; addition of stationary-phase culture supernatants from *V. fischeri* to that of a related bioluminescent bacterium, *Vibrio harveyi* (or *vice versa*) did not restore light production, so the signalling molecule(s) involved were denoted ''autoinducers''. That is, the bacteria were concertedly responding *en masse* to a population cell density-dependent signal, rather than a growth phase-

dependent signal. We now know that the autoinducers are *N*-acylated homoserine lactone (AHL) derivatives; *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) in the case of *V. fischeri*, and *N*-(3-hydroxybutanoyl)-L-homoserine lactone (hydroxyl-BHL) in the case of *V. harveyi*[12,13].

The *V. fischeri lux* genes were subsequently cloned in *E. coli*[14], where it was found that the bioluminescence functions are encoded in two linked, but divergently transcribed genetic units, separated by about 150 bp of intergenic DNA. One of these units encodes a 250 residue-long transcriptional activator protein, LuxR. This protein is thought to be the receptor for OHHL, and although nominally globular and cytoplasmic, is normally found associated with the cell membrane. The distal transcriptional unit encodes an operonic cluster, *luxICDABEG*. The *luxCDABEG* structural genes encode the proteins required for production of light *per se*, although in *E. coli*, *luxG*, which encodes an FMN reductase, appears to be dispensable. However, the first gene in the operon, *luxI*, encodes a protein required for biosynthesis of OHHL, and can support the synthesis of this molecule when expressed in heterologous hosts. Subsequent work has shown that LuxI utilizes *S*-adenosylmethionine and 3-oxohexanoyl ACP (probably drained off the main pool of fatty acid biosynthetic intermediates[15]) to synthesize OHHL. Centered 42.5 bp upstream from the transcriptional start site of *luxICDABEG* is a small (*ca* 20 bp) inverted repeat sequence called the *lux* box, which has been shown to bind activated (*i.e.*, OHHL-bound) LuxR, initiating transcription of the adjacent *luxICDABEG* cluster[16,17]. At the same time, LuxR-binding to this operator region also seems to alter expression of *luxR* itself (autoregulation). Proteins homologous to the LuxI/LuxR signal generator/receptor pair have been identified in a variety of other organisms, and are frequently arranged in a divergently-transcribed bicistronic pair like that described above. Such LuxIR pairs are not always genetically linked to the genes they control – this is the case for *V. harveyi*.

The current working model of QS in *V. fischeri* is that LuxI constitutively produces OHHL, which passively diffuses across the membrane and accumulates in the culture supernatant at a rate proportional to the increase in population cell density. At the same time, LuxR is expressed and accumulates intracellularly, binding OHHL as it does so. Ligand binding is non-cooperative and reversible. Once the bulk AHL concentration exceeds a critical threshold value (defined by the K_d (0.1 μM) of OHHL for LuxR, and by the expression level of the latter), LuxR undergoes a

conformational change, which, in turn, leads to a change in the multimeric state of the protein. The multimerized protein then binds to the operator region (*lux* box) upstream of *luxICDABEG* and stimulates binding of RNA polymerase to the intervening promoter region[17]. Detailed analysis of LuxR and its orthologs has been frustrated by the intransigence of these proteins to biochemical manipulation – most LuxR-type proteins form insoluble inclusion bodies during over-expression, and do not readily refold upon solubilization/renaturation. However, molecular-genetic[18–21] and more recently, limited biochemical[17] analysis of LuxR has revealed that it is comprised of two domains; an N-terminal OHHL-binding domain, and a C-terminal DNA-binding domain containing a helix-turn-helix motif (residues 190–210). Within each domain, mutational analyses have revealed a more subtle distribution of labour across the molecule, with different sub-functionalities being associated with defined portions of the LuxR sequence. For example, mutation of residues 10–20 affects LuxR autoregulation, but not *lux* box binding. The next 136 residues are involved in OHHL binding, since deletion of these residues results in OHHL-independent *luxICDABEG* expression. Residues 79–127 appear to play a role in ligand-binding, while residues 116–161 are involved in multimerization. Finally, at the extreme C-terminal end of the molecule, the last 20 residues (230–250) appear to be required for the transcriptional activation of *luxICDABEG*, but not for *luxR* autoregulation. These various sequence determinants are likely to be generally functionally conserved across most LuxR-type proteins.

A second class of signal generators

In 1995, a second autoinducer molecule was identified in *V. fischeri*; *N*-octanoyl-(L)-homoserine lactone, or OHL[22]. Intriguingly, the synthesis of this molecule was found to be catalyzed by a 45.6 kDa protein, AinS, which has no similarity to LuxI, but does show partial homology to the LuxM protein from *V. harveyii*. AinS employs the same basic substrates for OHL synthesis (SAM and octanoyl-ACP) as LuxI, although it can also accept CoA derivatives in lieu of charged ACPs[23]. This finding suggests that QS signalling systems have evolved at least twice during evolutionary history, and that the different systems can be mixed-and-matched. The current model for *V. fischeri* QS posits that AinS-derived OHL is produced at relatively low cell densities (*ca* $< 10^8$ cells per ml) and is required for the initial stages of light organ colonization[10,24]

(Figure 1A,B). At this time, the bacteria do not need to luminesce, so *luxICDABEG* expression is not activated. Then, *via* a mechanism involving a non-LuxR-type membrane-associated AHL receptor protein, AinR, and a LuxU-dependent phosphorelay system, OHL inactivates LuxO, which normally acts to repress the expression of *litR*[10,25]. The LitR protein is a positive transcriptional activator of *luxR*, and is also required for the expression of early factors necessary for the successful (and species-specific) colonization of the light organ. At higher cell densities, the induction of *luxR* expression by LitR activates the expression of *luxICDABEG* and the other late-colonization genes. OHL also participates directly in this process since, in the absence of an activating AHL ligand, the expressed LuxR will not stimulate *luxI* expression on its own. Therefore, even though it binds to LuxR with much lower affinity than OHHL, the small amount of bound OHL is both necessary and sufficient to initiate the positive feedback loop that rapidly ramps up *luxICDABEG* expression (Figure 1C). Thus, QS in *V. fischeri* is a finely-tuned, sequential process that ensures the proper timing of gene expression during colonization.

In 2002, the genome sequence of one strain of *V. fischeri* (ATCC 700601, isolated in the late 1980's from a *Euprymna* light organ) was completed, and has yielded a wealth of information about this model symbiont and its relationship with host organisms[26]. In particular, the genome sequence opened up the possibility of using microarray analysis to define globally the QS regulon(s) controlled by the *ain* and *lux* QS systems in *V. fischeri*[10,27], and to screen the genome for genes preceded by potential *lux* box operator sequences. In addition to cataloging which genes are under the control of QS, these analyses have, by-and-large, confirmed the postulated link between the *ain* and *lux* systems, and further, have revealed hitherto unknown QS-controlled regulators that likely hardwire QS into other regulatory circuits such as the *rpoS* regulon. These and other findings are challenging the rather elementary view from the pre-genomic era, that QS systems are simple modular control units which simply serve to tie in gene expression to population cell density changes. A good example of this is seen in the ongoing work to characterize the interface between QS and virulence in various pathogenic organisms.

QS can be repressive

Members of the *Erwinia* (*Pectobacterium*) genus are phytopathogens active against a range of plants. One of these, *Erwinia*

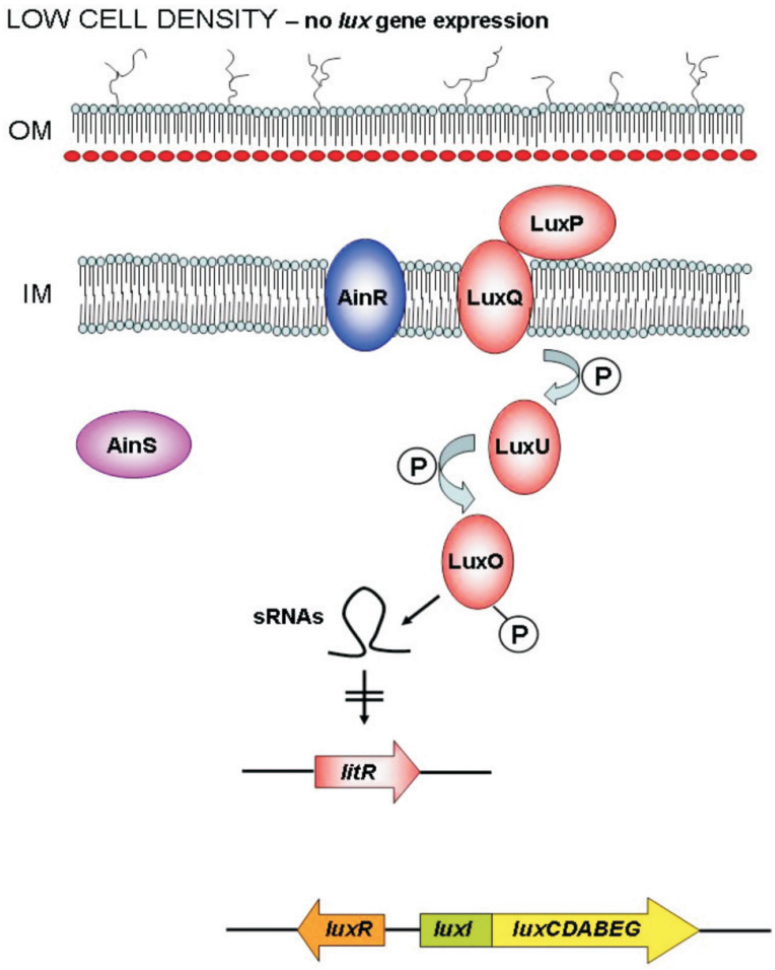

Fig. 1. *Control of bioluminescence in* Vibrio fischeri. (**A**) *In conditions of low cell density, a phospho-relay system involving LuxQ and LuxO (a signal integrator) maintain LuxO in a phosphorylated state. LuxO is a transcriptional activator which, in the phosphorylated state, increases the expression of several small RNA species. These, in turn, bind to the litR transcript and target it for degradation. In the absence of LitR, luxR is not expressed.*

carotovora subsp. *atroseptica* (Eca), is responsible for causing blackrot in potato plants and soft-rot in stored tubers[28], and consequently, is of considerable economical importance. Because of this, the genome sequence of Eca was recently determined[29]. Eca is phytopathogenic because it produces a welter of secreted virulence factors capable of macerating and digesting plant cell walls, including cellulases (Cels), pectin methylesterases, pectate

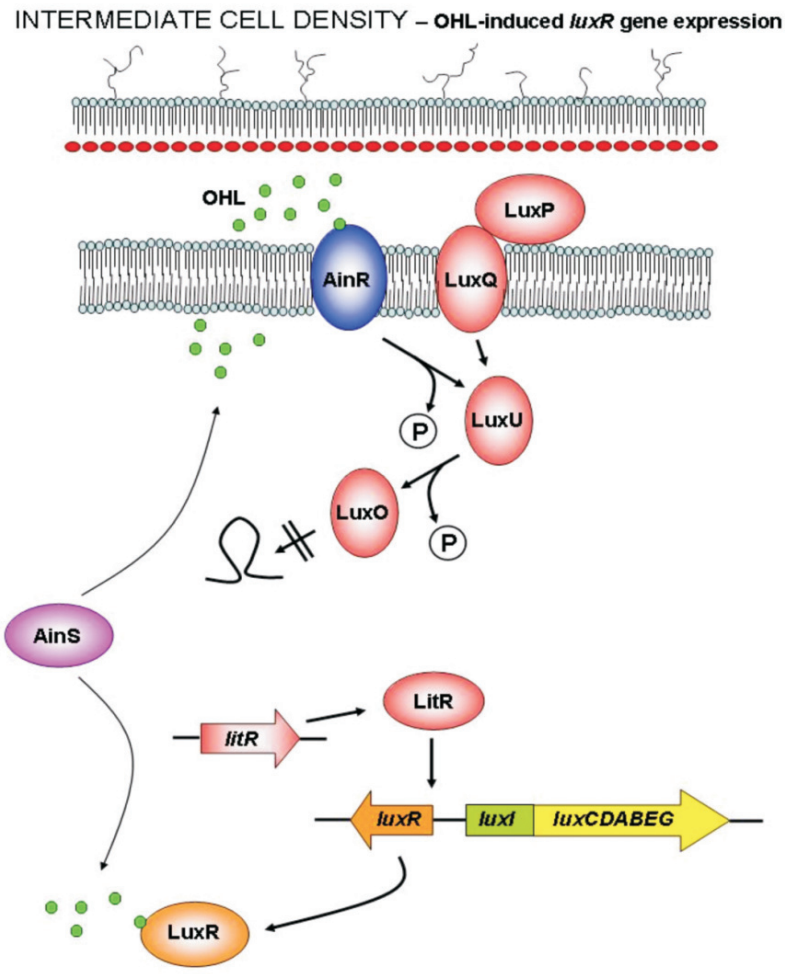

Fig. 1. *Control of bioluminescence in* Vibrio fischeri. (**B**) *At intermediate cell densities (*ca *10^8 cells/ml), AinS synthesizes OHL, which accumulates in the culture medium. The OHL is sensed by a membrane-associated receptor, AinR, causing it to switch its biochemical activity from being a kinase to being a phosphatase. This decreases the phosphorylation level of LuxU (and consequently, LuxO) leading to an increase in litR expression. LitR activates luxR expression. The LuxR is able to bind OHL (albeit with low affinity), thereby activating it. This allows the LuxR-OHL complex to start stimulating the expression of the lux genes, including luxI.*

lyases (Pels), pectin lyases, polygalacturonases, and proteases (Prts). As in many other Gram-negative pathogens, the production of many of these virulence factors is under QS control[30,31], making this a good target for the development of novel anti-bacterial

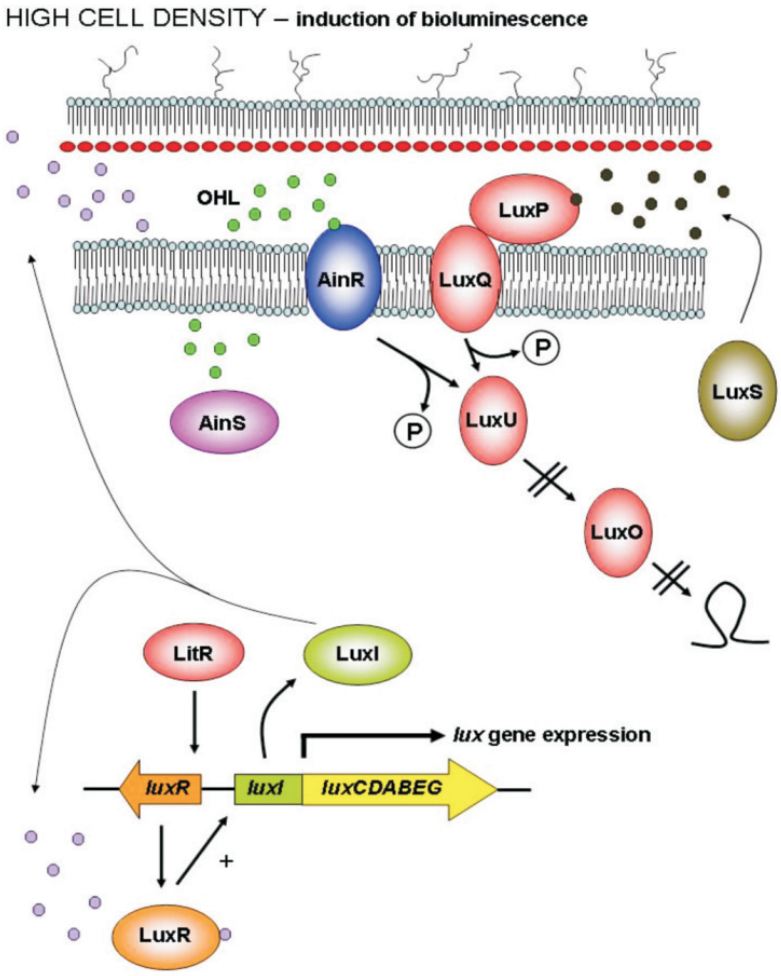

Fig. 1. *Control of bioluminescence in* Vibrio fischeri. (**C**) *At high cell densities, the LuxI protein generates OHHL, which binds to LuxR with high affinity. The LuxR-OHHL complex positively auto-regulates luxI expression, thereby rapidly ramping up the expression of the lux gene cluster, giving rise to bioluminescence.*

strategies[32]. In line with this, QS mutants of Eca show reduced virulence factor production and reduced virulence *in planta*. Only one QS signal generator, ExpI, has been identified experimentally in Eca, and analysis of the genome sequence suggests that this is the only AHL synthase present[29,30,31]. Like LuxI, ExpI produces OHHL (along with smaller quantities of the non-3-oxo derivative, HHL), although there is some strain-to-strain variation in the nature of the AHL produced (summarized in reference 33), and

at least two broad classes of AHL-producing *Erwiniae* have been identified[34]. For the purposes of clarity, we will restrict our discussion here to QS in the sequenced strain of Eca (SCRI1043); the mechanistic details of QS in other *Erwinia* strains varies considerably, and has been reviewed recently[33]. The *expI* gene is part of a convergently transcribed bicistronic unit, the other component of which is a *luxR* homolog, *expR* (Figure 2). The 3' ends of each gene overlap slightly, indicating that they are evolutionarily tightly linked. However, the precise role of ExpR in virulence was questioned for many years because knockouts of the corresponding gene had very little impact on any aspect of virulence in many strains of *Erwinia*[35,36]. In contrast, inactivation of *expI* essentially abrogated virulence[36]. This issue was resolved by the finding (from genome interrogation[29]) that Eca contains an additional LuxR homolog, ECA1561, now denoted VirR[36]. As with *expR*, inactivation of *virR* had very little effect on virulence factor production. However, when introduced into an *expI* mutant (*expI* and *virR* are unlinked), the *virR* mutation fully restored exoenzyme production, even though OHHL production was abolished. This suggested – contrary to expectation – that VirR normally represses exoenzyme production (presumably through binding to some *lux*-type box at the 5' end of target genes, or perhaps by controlling the level of some intermediate regulator such as *rsmA*[37,38]). A working model is that at low cell densities, VirR binds to operator sequences upstream from its target genes (*e.g. celV*, *pelC*, *pehA*, *nip* and *svx*) and prevents their expression, but as the cell density rises, ExpI-derived OHHL complexes with VirR and relieves this repression (Figure 2C). VirR has also been implicated in controlling the expression of *rsmA*, another global regulator of exoenzyme production[36,37,38], and it is possible that many of the phenotypic effects of *virR* mutants are mediated *via* disruption of RsmA-mediated signalling (Figure 2A and B). The role of ExpR in this, if any, is not yet clear, although ExpR has been shown to exert some control over *rsmA* expression in certain strains of *Erwinia*[37]. Also, recent studies using purified ExpR have shown that the protein is certainly capable of binding OHHL, albeit with relatively low affinity ($K_d = 7.5\,\mu M$)[39]. This alone was good "smoking gun" evidence that another LuxR homolog was involved, since virulence factor production can be induced at much lower OHHL concentrations ($< 1\,\mu M$). This notwithstanding, the discovery of VirR is a good example of how genomics can assist in elucidating the components of a QS system. Equally, these findings also highlight the value of searching for QS-bypass mutants (*i.e.*,

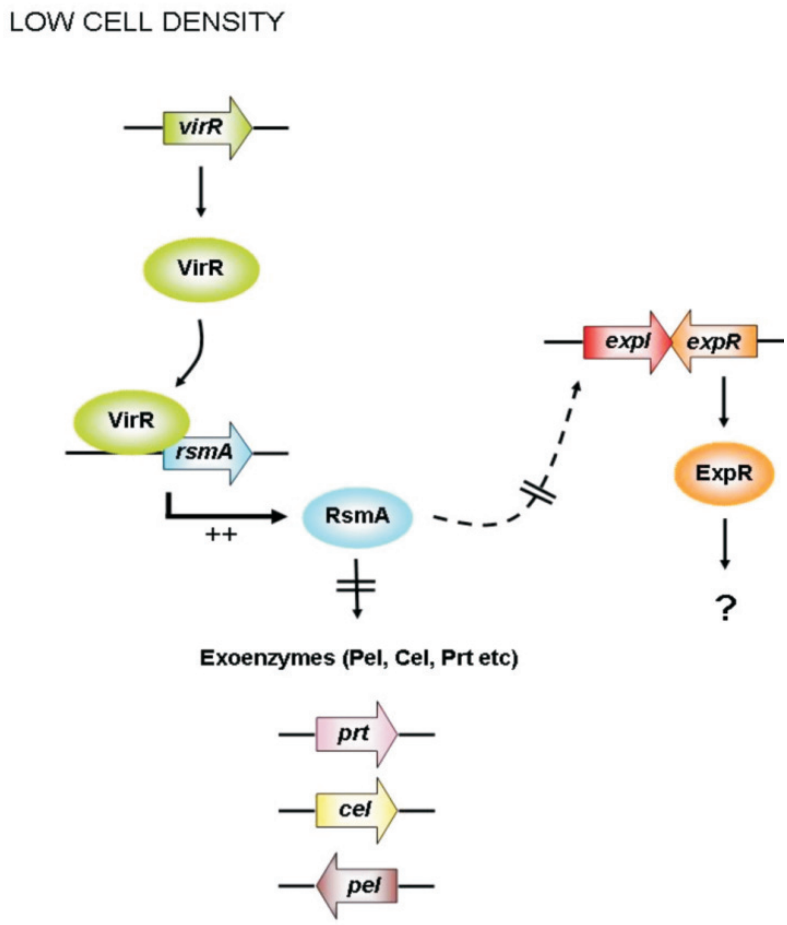

Fig. 2. *Quorum sensing control of exoenzyme production in the plant pathogen,* Erwinia carotovora. (**A**) *At low cell density, VirR activates the expression of RsmA. This protein binds to the exoenzyme transcripts and targets them for degradation. RsmA is also thought to inhibit the expression of expI.*

those mutants that restore AHL-dependent phenotypes in *luxI* homolog mutants).

Eca is not the only phytopathogen to exhibit QS-dependent negative regulation; indeed, the first such example was described in *Pantoea stewartii* subsp. *stewartii* (Pss), the causative agent of Stewart's wilt in corn crops[40]. The disease is caused by accumulation of an exopolysaccharide, stewartan, which is thought to block xylem vessels in the plant, leading to loss of turgor pressure and subsequently, wilt. Stewartan is a complex polymer composed of heptameric oligosaccharide repeat units containing glucose, galac-

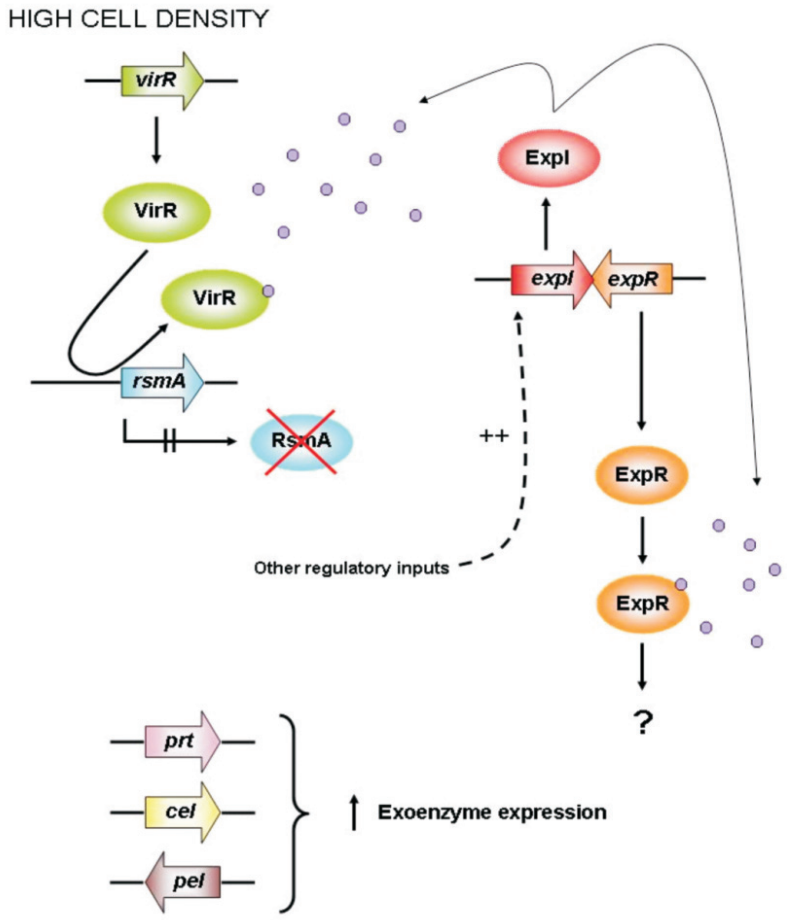

Fig. 2. *Quorum sensing control of exoenzyme production in the plant pathogen,* Erwinia carotovora. (**B**) *At higher cell densities, other regulatory inputs overcome the inhibitory effect of RsmA on expI expression, leading to the synthesis of OHHL. The AHL binds to VirR, causing it to dissociate from the RsmA promoter, thereby reducing RsmA levels and de-repressing exoenzyme synthesis. The precise role of ExpR, which is also capable of binding OHHL, is not yet known.*

tose and glucuronic acid, and is synthesized by a large 14-gene cluster (encoding the *cps* genes). Expression of the *cps* genes is controlled partly by QS; *esaI* and *esaR* are convergently transcribed ORFs encoding the AHL synthase/receptor pair in Pss, and partly by a multicomponent phosphorelay signal transduction system comprised of RcsA, RcsB, RcsC and YoiN[41,42]. Deletion of *esaI* results in abolition of OHHL synthesis by the organism, and loss of

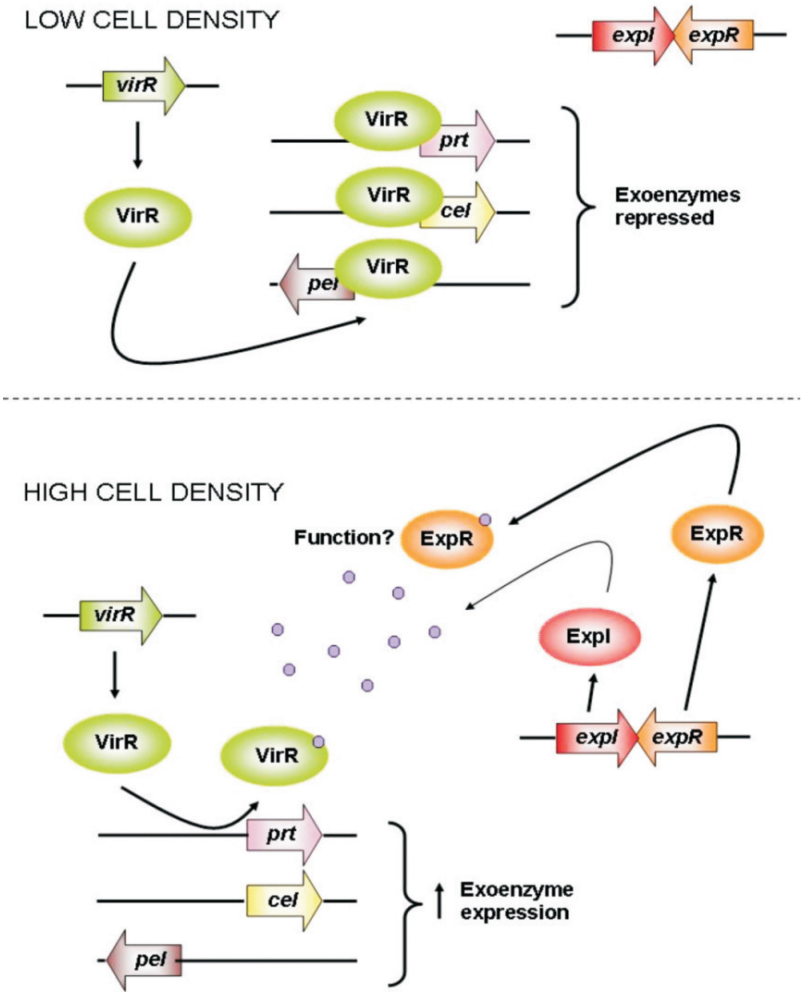

Fig. 2. *Quorum sensing control of exoenzyme production in the plant pathogen,* Erwinia carotovora. (**C**) *An alternative hypothesis for VirR action is that at low cell densities, this protein binds directly to the target gene (exoenzyme) promoters and represses expression from these. At higher cell densities, OHHL made by ExpI binds to VirR, causing it to dissociate from the target promoters, thereby allowing exoenzyme expression.*

virulence. However, deletion of *esaR* or deletion of *esaR* and *esaI* together results in a hypermucoid phenotype in which stewartan is overexpressed. Subsequent studies have shown that EsaR directly represses the transcription of *rcsA*. The product of this gene, RcsA, along with RcsB, is required for the activation of *cps* transcription. In the absence of bound EsaR, RcsA probably positively auto-

regulates itself, but in the repressed state, the concentration of any RcsA that is made by leaky expression is probably kept in check by Lon-mediated turnover, keeping the system tightly controlled[41]. EsaR has also been shown to have an autoregulatory role, providing further proof for its QS inhibitory activity[42]. EsaR is one of the few LuxR homologs for which there is good biochemical data, and experiments have shown that EsaR dimerizes and binds, independent of OHHL, to an operator sequence which spans the -10 region of a σ^{70} promoter consensus sequence upstream of *esaR*. This binding is relieved in the presence of OHHL, indicating that, like VirR in *Erwinia*, EsaR primarily operates as a repressor[42].

QS-repressive activity has been identified in other systems too (to date, all of them in the *enterobacteriaciae*). One of the best characterized of these is in the opportunistic pathogen, *Serratia marcescens*, which infects a range of mammalian, insect and plant hosts. Many strains of this organism produce copious quantities of virulence factors, including secreted secondary metabolites and exoenzymes (such as hemolytic proteases, lipases, nucleases, and chitinases), and these phenotypes are often found to be under QS control[43,44]. In addition, in strain ATCC39006, production of the vivid red pigment, prodigiosin, by a 15-gene biosynthetic cluster (*pigA-O*) is under QS control[45] (Figure 3). ATCC39006 contains a convergently-transcribed LuxI-R pair denoted *smaI* and *smaR*. Inactivation of *smaI* leads to loss of pigmentation, while inactivation of *smaR* has no effect on this phenotype. However, pigment production in a *smaI–smaR* double mutant is restored, indicating that SmaR normally represses pigment production at low cell densities, and that this repression is relieved upon binding the AHL ligand[45]. In line with this, purified SmaR was shown to bind to a DNA fragment encompassing the promoter region upstream of the *carA* gene (which is known to be under the control of QS in this strain), and this binding was essentially abolished in the presence of AHL[46]. Intriguingly, although β-galactosidase expression from a *pigA*::*lacZ* transcriptional fusion was reduced in a *smaI* mutant, and restored by addition of exogenous AHL, the exogenous signalling molecule did not act to advance expression of the *pig* cluster[45]. Clearly, more is being sensed here than just the population cell density, and the precise timing of pigment production is influenced by other factors. Recent work has indicated that pigment production is also subject to regulation by multiple environmental cues, mediated by a diverse set of regulatory proteins including a GacAS-like two-component system, an

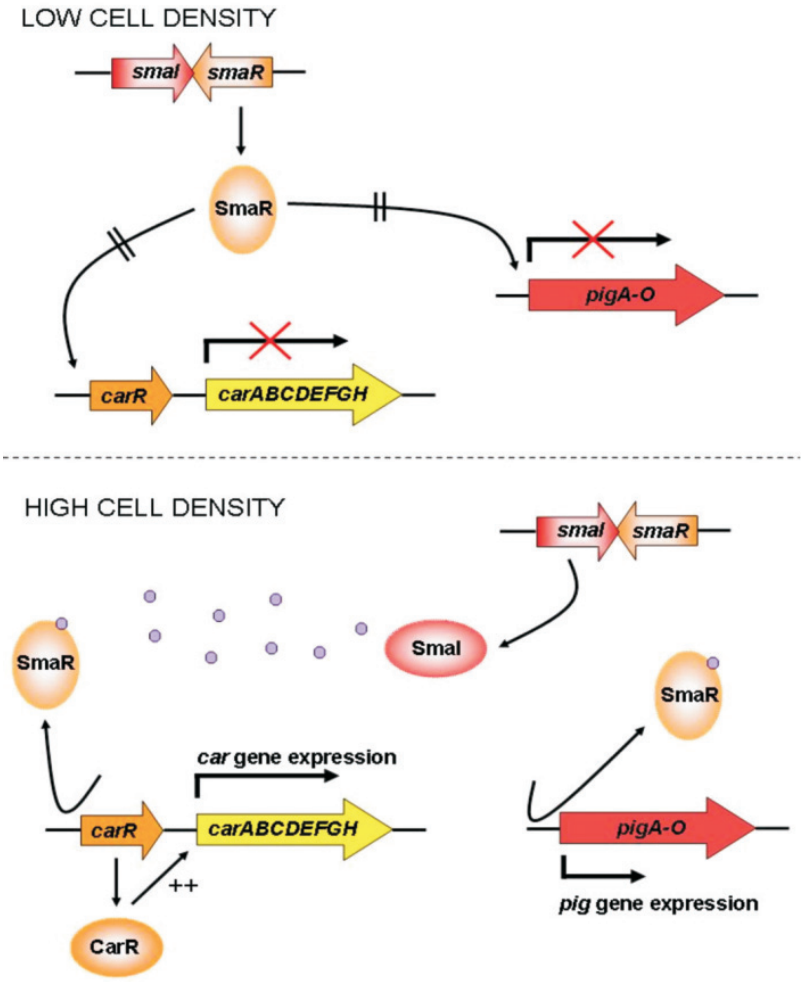

Fig. 3. *Quorum sensing in* Serratia sp*: orphan regulators and surrogate phenotypes. At low cell densities, the SmaR protein represses expression of the pigA-O cluster and of the carR gene. At higher cell densities, BHL produced by the SmaI protein accumulates in the culture and binds to SmaR. This causes the latter to dissociate from the pig promoter, allowing production of the prodigiosin biosynthetic genes. At the same time the BHL-independent CarR protein binds to the promoter region in front of the carA-H gene cluster, activating expression of the carbapenem biosynthetic genes. Additional, multiple regulatory inputs also impinge on expression of the car and pig clusters.*

adenylate cyclase, and several pleiotropic regulators of regulators (such as Rap and PigP)[46].

Unlike *E. carotovora* and *V. fischeri*, the SmaI protein encoded by *Serratia* sp. ATCC39006 primarily produces butyryl-(L)-homo-

serine lactone (BHL)[47], rather than OHHL, and this is reflected by the altered ligand specificity of SmaR[45,46]. Recent work aimed at engineering an altered specificity in LuxR[48] has shed light on how this altered specificity might have arisen. Presumably, *smaI* must have picked up mutations that influenced the ligand it makes (presumably by changing the size/nature of the acyl-ACP binding site). In turn, the *smaR* gene must have undergone a parallel selection to ensure that it responded appropriately to the new ligand. This could have arisen by horizontal transfer of a new *luxR* homolog with the correct ligand specificity, but given the very tight linkage between *smaI* and *smaR* – the genes overlap and are convergently transcribed – it is far more likely that the existing SmaR underwent a contemporaneous change of ligand specificity itself. [This does not mean that this change in specificity originally occurred in a *Serratia* genetic background – indeed, in ATCC39006, the *smaIR* locus is flanked by remnants of insertion sequences, suggesting that this locus was mobile as a unit in the distant past[47,49].] The recent work with LuxR has provided an explanation as to how this might have occurred. The most likely first step will have been a relaxation in the specificity of SmaR to enable it to recognize and respond to a range of AHLs. Acquisition of such "promiscuous" functionality[50] is a feature of many evolutionary intermediates during "respecialization"; it is rare for a single mutation to confer a new function and at the same time, completely abolish an older one. However, the broader specificity of the intermediate state would almost certainly incur a fitness cost, in which case, there would be a strong selective pressure to accrue mutations that minimize the deleterious nature of these, and in doing so, increase the specificity of the protein. Collins *et al.*[48] recently exploited this dual selection route to engineer a LuxR protein with specificity for decanoyl-(L)-homoserine lactone (DHL) instead of OHHL.

Surrogate phenotypes and orphan regulators

In both *Serratia* sp. ATCC39006 and in *Erwinia carotovora* subsp. *carotovora* (Ecc) strain ATTn10, the QS system controls not only virulence factor production, but also the biosynthesis of a beta-lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid (car). This antibiotic is thought to be made in order to reduce competition for the nutritional windfall created at the site of an infection. The genes required for carbapenem production are encoded by an 8 gene cluster denoted *carA-H*[51]. The first 5 of these genes, *carA-E*,

are required for carbapenem biosynthesis, while *carF* and *carG* encode an apparently novel resistance mechanism necessary to prevent the cell from killing itself upon induction of carbapenem production. No function has yet been assigned to *carH*. The *car* cluster is preceded by 150 bp intergenic region containing a σ^{70}-like promoter and a range of inverted/direct repeats that are candidate binding sites for regulatory factors. Within the cluster, a weak constitutive promoter exists between *carE* and *carF*, probably maintaining a constant low-level stream of pre-formed resistance proteins. However, a much stronger, QS-dependent promoter is located at the 5' end of the cluster. Interestingly, in both *Serratia* ATCC39006 and in Ecc ATTn10, the *carA-H* genes are located adjacent to a divergently transcribed *luxR* homolog, denoted *carR*[52] (Figure 3). In the case of Ecc, CarR is a potent OHHL-dependent activator of carbapenem production, and has been shown to bind directly to the *carR-carA* intergenic region[53]. Indeed, CarR was the first LuxR homolog to be purified and biochemically characterized in any great detail[53]. The purified protein bound OHHL with a K_d of 1.8 μM, which is within the range of OHHL concentrations (1–3 μM) normally produced by this organism when grown in LB. Ligand binding was monitored by exploiting the ability of AHLs to alter the fluorescence of a highly conserved Trp residue in CarR, and calculations showed that 1 OHHL molecule was bound per monomeric unit of the protein. Recent biophysical data have confirmed this (MW and DRS, data not shown). The cognate LuxI homolog which generates the OHHL to which CarR responds is unlinked to *carR*, and corresponds to the *expI* gene in Eca (although in Ecc it is known, for historical reasons, as *carI*). The CarR protein therefore appeared to be an orphan LuxR homolog, and since CarR is not known to control any other phenotypes in Ecc, it looks as if the *carR/carA-H* locus has been introduced into Ecc from elsewhere (by horizontal gene transfer) and has come under the control of the endogenous QS system. Carbapenem production in Ecc is therefore a "surrogate" QS phenotype. Whether the cluster arrived in an OHHL-responsive state, or whether CarR evolved to become responsive to endogenous OHHL, is not clear. However, although not all strains of Ecc have the potential to produce carbapenem, a number of them carry "cryptic" carbapenem clusters which can be activated to produce the antibiotic when functional ATTn10 *carR* is expressed *in trans*[54]. Most of these cryptic strains have apparently lost their ability to make carbapenem due to the presence of defects in the endogenous *carR* genes or in the *carR* promoter region.

Whatever the reason for the persistence of this evolutionary wreckage, it shows that it is relatively straightforward to acquire new surrogate QS-controlled functions – a feature that we will return to presently.

As in Ecc, carabapenem production in *Serratia* ATCC39006 is also controlled by CarR. However, early studies indicated that the *Serratia* CarR operates in a ligand-independent manner. The *Serratia* CarR protein contains a Trp→Cys substitution in its ligand binding site. This eliminates the Trp residue most likely responsible for causing AHL-dependent quenching of intrinsic Trp fluorescence in Ecc CarR[39,53]. Indeed, in a subsequent X-ray crystallographic analysis of the tertiary complex formed between TraR (a LuxR homolog from *Agrobacterium tumefaciens* – see below), its cognate AHL (3-oxo-octanoyl-(L)-homoserine lactone, or OOHL), and a target DNA sequence, Vannini *et al*.[55] and Zhang *et al*.[56] independently showed that this highly conserved Trp residue plays a central role in binding the homoserine lactone moiety of AHLs[53]. However, recent mutational analysis of LuxR has challenged the generality of this conclusion, suggesting instead that the Trp side chain may make contacts with the 3-oxo moiety in ligand-bound LuxR[57]. Unexpectedly, in *Serratia*, expression of this AHL-independent CarR protein results in AHL-*dependent* carbapenem production[45]. That is, in either a *carR* mutant, or in a *smaI* mutant, transcription of the *carA-H* cluster is abolished. Slater and colleagues[45] have now elegantly elucidated much of the molecular basis for this, and have proposed a plausible model for how *carR* expression might be controlled by SmaR. In essence, at low cell densities, they propose that SmaR binds to the *carR* promoter region, repressing *carR* transcription. However, at higher cell densities, BHL accumulates in the culture and binds to SmaR, causing it to dissociate from the *carR* promoter, thereby relieving *carR* repression and allowing synthesis of the antibiotic.

One simple, but very illuminating recent study has shown just how easy it is to acquire surrogate QS-dependent phenotypes through phage-mediated horizontal gene transfer[58]. In pigmented *Serratia marcescens*, the *pig* gene cluster is always located between *cueR* and *copA*, whereas in non-pigmented strains, *cueR* and *copA* are contiguous, implying that at some ancestral stage, the *pig* cluster inserted itself between these genes. *Sma* 274 is a pigmented environmental *Serratia* isolate which does not carry out AHL-dependent QS, while *Sma* 12 is a non-pigmented clinical isolate which contains an active QS system, but no *pig* cluster. Coulthurst *et al*. used a generalized transducing phage, Φ3M, to introduce the

pig cluster from *Sma* 274 into *Sma* 12. Remarkably, they found that not only was the *pig* cluster expressed in *Sma* 12, but it also came under the control of the endogenous QS system. Conversely, when these workers introduced the *smaR* gene from *Sma* 12 into the non-QS strain *Sma* 274, they found that *pig* gene expression could be made dependent on exposure to exogenous BHL. The authors speculate that SmaR-mediated repression of some pleiotropic regulator present in all *Serratias* might be responsible for this apparently facile imposition of QS control. Whatever the mechanism involved, this little study illustrates just how easy it is to introduce QS, and therefore population cell density-dependent control of a phenotypic trait. It also suggests that QS is not always strictly necessary for control, raising the question of just how widespread the phenomenon of QS really is.

LuxIR orthologs have now been identified in a range of α, β and γ *Proteobacteria*, where they have been shown to be responsible for controlling a diversity of phenotypic traits. However, relatively few *Proteobacteria* (just 4% of genera from all orders at the last count[59]) are known to exploit AHLs for QS, and most of these are limited to just 3 orders; the *enterobacteriaciae*, *pseudomonaciae*, and the *rhizobiales*. This is remarkable if we accept that the last common ancestor of all five Proteobacterial classes probably carried a LuxIR-type QS system[60], suggesting that over the course of time, this particular signalling mechanism has been largely dumped. Intriguingly, those species that have kept AHL-mediated QS systems are mostly pathogens, indicating that QS is a good way of controlling virulence factor production.

Extrachromosomal QS systems

So far, all of the QS systems described above have been encoded on the bacterial chromosome. However, in some cases, such as *Agrobacterium tumefaciens* and in certain *Rhizobial* species, QS system(s) are plasmid encoded. *A. tumefaciens* is a more insidious phytopathogen than the *Erwiniaciae*; it transfers oncogenic T-DNA from tumor-inducing (Ti)-plasmids directly into the nucleus of host (dicotyledonous) plant cells, where it stimulates overproduction of plant growth hormones and consequent rapid neoplastic tissue proliferation to generate a crown gall tumor[61,62]. Some 21 *vir* genes, encoded in six operonic units (denoted *virA-G*) are located on the Ti plasmid and are required for T-DNA processing and transfer. The expression of these genes is induced by the presence of plant-derived signals, including pH, monosaccharides and certain

phenolic compounds (reviewed in ref. 62). In addition, other genes on the Ti plasmid direct the host cell to produce a novel class of signalling compound called opines (derived from sugars and amino acids). The two main classes of opines that concern us here are the nopalines (*e.g.* agrocinopines) and octopines, and the synthesis of these is directed by subtly-different types of Ti-plasmid. These opines serve not only as a nutrient source for the bacteria; they are also plant-derived signals which stimulate Ti plasmid transfer to nearby plasmid-less, avirulent bacteria. These arise because *A. tumefaciens* that have lost their Ti plasmids have a strong growth advantage and so soon out-compete organisms that have retained their plasmids; as such, they are evolutionary "cheats" which piggy-back on the nutritional windfall created by their plasmid-bearing partners[63]. However, this process is reversed in late stage gall formation by opine-stimulated conjugal Ti plasmid transfer between bacterial donors and recipients. Octopines and agrocinopines bind to opine-responsive transcription factors (OccR and AccR, respectively), thereby relieving repression of the respective *occ* and *acc/arc* operons (Figure 4). These operons mostly contain genes involved in the uptake and catabolism of opines, but in addition, they also contain a *luxR* homolog called *traR*. Therefore, synthesis of the AHL receptor protein is under the control of a pathogen-directed host-derived signal molecule (the opine), ensuring that conjugal transfer can only occur in the environment of the plant tumor. It is of interest to note that the simple insertion of *traR* into the *arc* or *occ* operons introduces an element of cell-density control into this system, highlighting the importance of gene context.

The target genes of TraR are encompassed in two divergently transcribed *tra* operons, and in the *trb* and *rep* operons. Each of these operonic units is preceded by an 18 bp operator region known as a *tra* box, analogous to the *lux* box described earlier[64–67]. The *tra* and *trb* operons encode functions required for conjugal transfer, while the *rep* operon encodes the plasmid replication machinery. The first gene within the *trb* operon (in both nopaline and octopine Ti plasmids) is a *luxI* homolog, *traI*[66,67]. The encoded TraI protein makes OOHL, which is the cognate AHL recognized by TraR. In the apo-state, TraR is a membrane-associated monomer[64]. However, the protein is unstable in the absence of the ligand and is rapidly degraded by endogenous proteases[65]. This is prevented in the presence of OOHL[64,65]. It is thought that as the nascent TraR protein is being extruded from the ribosome, it folds around the AHL molecule, forming an essentially irreversible, but stable active

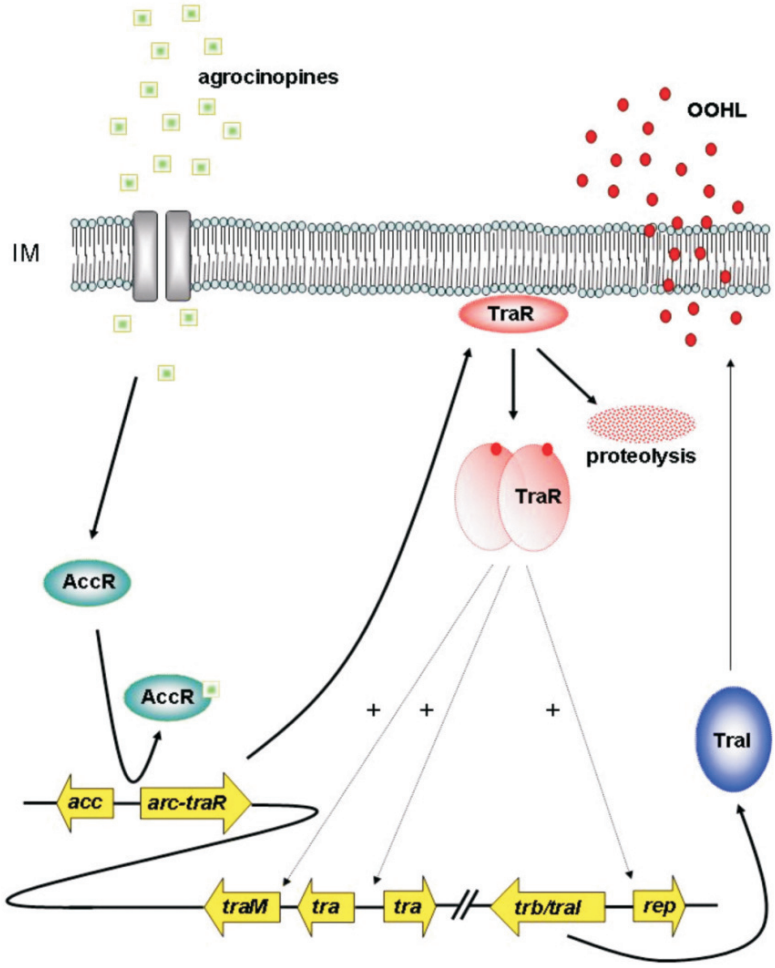

Fig. 4. *Quorum sensing control of plasmid transfer functions in* Agrobacterium. tumefaciens. *For simplicity, only nopaline signalling is illustrated, but the same principles also apply to opine-mediated signalling (see text for details). Plant (host) cells containing T-DNA produce agrocinopine signals which are taken up by the Agrobacteria. Once inside the cells, the nopaline binds to the AccR receptor protein, causing it to dissociate from the promoter controlling expression of the arc-traR operon on the Ti-plasmid. This permits expression of TraR, which, in the monomeric form is membrane-associated and rapidly turned over by endogenous proteolytic degradation processes. Low-level constitutive expression of traI, the gene encoding the OOHL synthase, eventually generates sufficient AHL to allow binding to the TraR. This causes the latter to dimerize and stabilize, and the resulting transcriptional activator stimulates expression of the tra/rep functions required for conjugative plasmid transfer/replication. TraR also stimulates expression of a negative regulator, TraM (see text for details).*

complex[68]. Not all LuxR homologs exhibit this type of behaviour – others show straightforward reversible binding to the AHL molecules[39,42,53]. Once bound to OOHL, the protein undergoes a conformational change and dimerizes, permitting the HTH motif to bind the major groove of the *tra* box, thereby stimulating transcription of the downstream genes[55,56]. Given that TraR controls TraI expression, these proteins positively cooperate to rapidly ramp up expression of the genes required for conjugal Ti plasmid transfer, and ensure that conjugation becomes dependent on the population cell density of the *donor* cells. Quite how the cell density of plasmid-less *recipients* is measured – if this is at all important for effective conjugal transfer – is not clear, since these cells do not produce OOHL and therefore should be effectively ''invisible'' to any quorum count. The whole system is driven by the fact that *traR* and *traI* both exhibit a low level of constitutive expression; in the complete absence of OOHL, TraR would not be able to function as a transcriptional activator. In the pre-quorate period, active TraR levels are kept in check by TraM. This small anti-activator protein, whose expression is under the control of TraR, binds to the C-terminal HTH-domain in the latter, effectively inactivating it until (it is thought) active TraR levels outtitrate TraM[70]. TraR activity has also been shown to be antagonized under certain circumstances by TrlR; this LuxR homolog contains a frameshift at residue 182, but encodes a functional, OOHL-binding N-terminus that forms inactive heterodimers with TraR[71,72]. No other *luxIR* homologues have been identified in the chromosome of the nopaline *A. tumefaciens* ''genome'' strain, C58.

In 2000, Zhang and colleagues announced that they had identified an enzyme in a *Bacillus* sp., denoted AiiA (for ''autoinducer inactivation'') that was capable of hydrolyzing the homoserine lactone ring moiety in AHLs[73,74]. Recent biochemical and structural analyses[75,76,77] of AiiA have established that this enzyme is a zinc metallo-enzyme, although some workers contest this[78]. Expression of AiiA in transgenic plants protected them from attack by *Erwinia carotovora*[74]. This exciting finding proved that such ''quorum quenching'' enzymes hold great biotechnological potential, and confirm QS as an excellent target for antimicrobial intervention. Two years later, the same team showed that an orthologous enzyme, denoted AttM, was able to degrade OOHL in *A. tumefaciens*[79]. AttM forms part of an operon comprised of *attK*, *attL* and *attM*. Expression of this operon is under the control of the IclR-like regulator, AttJ, and the same workers subsequently demonstrated that the *relA*-mediated stringent response also

imposes on this system[80]. However, recent findings[81,82] have brought this area of research full-circle by showing that γ-amino butyric acid (GABA), a plant-derived metabolite produced during wounding, is able to induce AttJ-dependent transcription of the *attKLM* cluster, thereby preventing Ti-plasmid conjugal transfer. Quite what advantage this confers upon the bacterium is not clear. AHL-degrading systems have now been identified in a diverse variety of bacteria[83,84,85], and not all of them are lactonases[86,87,88]. One protein that holds particular intrigue is PvdQ from *Pseudomonas aeruginosa*, which acts as a potent quorum quencher *via* its AHL acylase activity[89,90].

Although the *A. tumefaciens* QS system may seem convoluted, it is actually relatively straightforward compared to the systems involved in controlling phenotypes including conjugal plasmid transfer in some *Rhizobiales* [reviewed recently in ref. 91]. For example, in *R. leguminosarum*, the *cinI-cinR* locus has been shown to be a master regulator for at least three subordinate AHL-dependent QS systems (*raiI–raiR*, *rhi–rhiR*, and *traI–triR*), making this one of the most complex QS organisms currently known, and a world away from the simplistic LuxI–LuxR paradigm of just a decade ago.

QS in *Pseudomonas aeruginosa* – time for a conceptual re-think?

QS in *Pseudomonas aeruginosa* represents the ultimate *pot pouri* of signalling systems discussed so far; it is hierarchical, hardwired to other regulons, exploits a variety of signalling molecules, and involves an orphan LuxR homolog (QscR) that controls its own, discrete set of surrogate phenotypes. *P. aeruginosa* has become a paradigm for the study of long-term, chronic bacterial infections, although it is also associated with certain acute infections. *P. aeruginosa* has been associated with some 10% of all nosocomial (hospital-acquired) infections, and of the Gram-negative organisms, seems to be the worst offender in this regard[92]. As succinctly pointed out by Todar in his concise little *curriculum vitae* for the organism[93], *P. aeruginosa* is the epitome of an opportunistic pathogen; it rarely causes infections in healthy hosts, but in the immune-compromised, there is hardly any tissue that it will not attack. This is particularly true for cystic fibrosis (CF) patients, who, by virtue of their defective CFTR pump, accumulate large amounts of viscous mucus in the lung tissue, which is readily colonized by *P. aeruginosa* and members of the *B. cepacia*

complex . Nowadays, most CF patients succumb to the effects of these long-term, chronic infections, which are a leading cause of mortality and morbitity in this population. QS plays a central role in *P. aeruginosa* pathogenicity, and as a consequence, perhaps more is known about AHL-signalling in this organism than any other. AHLs have been readily isolated from the sputum of CF patients[94], and QS mutants display reduced virulence in model systems[95,96]. Interestingly, although the presence of a functional QS system seems to be pre-requisite for effective colonization, it is not required for persistence in the host, since many *Pseudmonas* isolates from CF sputum appear to have at least partially lost their ability to do QS.

Several excellent reviews on *P. aeruginosa* QS have been presented recently, and the reader is referred to these for a detailed description of the system and the various phenotypic traits under its control[2,92,97]. Briefly, the QS system in *P. aeruginosa* is hierarchical, and consists of two AHL-dependent signalling systems, both *luxI–luxR* homologs (Figure 5). At the top of the regulatory hierarchy is LasI, which makes 3-oxo-dodecanoyl-(L)-homoserine lactone (OdDHL), and the cognate receptor for this ligand, LasR. On binding OdDHL, LasR multimerizes and becomes active as a transcriptional regulator. Until recently, genes under the control of the *las* system were thought to be preceded by operator-like palindromic *las* boxes about 20 bp in length. However, new data[98] indicates that many *las* boxes lack dyad symmetry, and display notable variation in their LasR-binding properties, particularly with respect to cooperative associations. The heterogenous nature of *las* operator sequences may indicate that they are responsive to more subtle variations in LasR concentration/activity than previously thought. In addition, there is good evidence that LasR can also repress the expression of certain target genes (Wagner *et al*. identified 222 genes that are QS-repressed[99]).

Subordinate to the *las* system, and controlled by it, is the RhlI–RhlR signalling pair (*rhl* sub-system), which is involved in the generation and reception of BHL. The *rhl* signalling system was probably acquired by *P. aeruginosa* after the *las* system[49]. In contrast to LasR, RhlR requires its cognate ligand for activity as a transcriptional activator, but not for dimerization[100]. Unlike the genes controlled by the *las* system, RhlR promoters absolutely require dyad symmetry in the corresponding *rhl* box operator sequences. Many *rhl*-regulated genes also accept a regulatory input from the *las* system, but it is interesting to note that most of the virulence-related genes in the *P. aeruginosa* QS regulon are

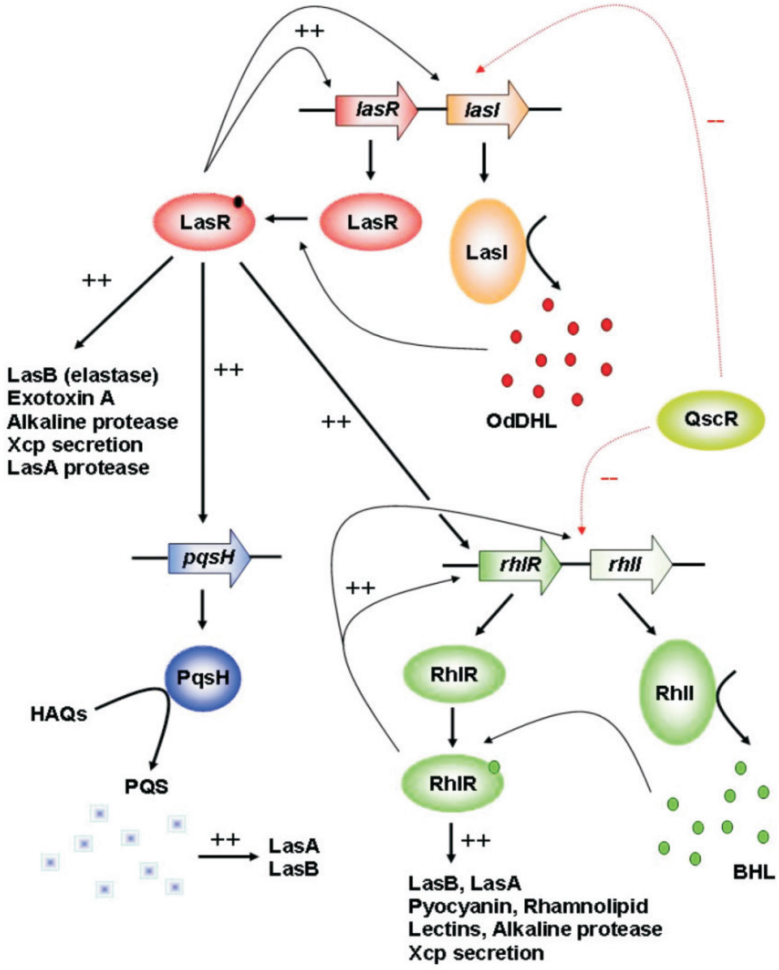

Fig. 5. *Hierarchical control of virulence factor production in* Pseudomonas aeruginosa. *At low cell densities, the QscR protein is thought to repress expression of lasI and rhlI, although other regulators such as MvaT, RsaL, RelA, RsmA, GacA, Vfr and RpoS (not shown) also affect the expression of one or both of these genes. However, as the cell density increases, this repression is relieved and the OdDHL made by the LasI protein binds to LasR. The active LasR-OdDHL complex further stimulates lasI expression, setting up a positive feedback loop that rapidly up-regulates expression of the genes under the control of the las system, including the signalling pathway responsible for PQS synthesis and elements of the rhl signalling system. Like the las system, the RhlI-RhlR system also positively auto-regulates itself, and controls the production of most of the known virulence factors associated with* Pseudomonas aeruginosa *infections. The rhl signalling system appears to be hardwired into the central regulatory circuitry of the cell controlling transition from exponential phase growth to stationary phase growth (via RelA and RpoS), and to the PQS signalling pathway (not shown).*

primarily *rhl*-controlled[97]. On the basis of this, Schuster and Greenberg[97] have proposed that acquisition of the *rhl* system was tied in with the adoption of a more pathogenic lifestyle by this organism. It is interesting to note that BHL and OdDHL are very different in size and degree of acyl-chain substitution, ensuring that these signalling systems do not cross-talk to any appreciable extent through inappropriate ligand-binding to the ''wrong'' LuxR homolog. Together, the LasI–LasR and RhlI–RhlR signalling systems control the expression of a large number of phenotypic traits including secretion of extracellular virulence factors such as exotoxins, proteolytic haemolysins, phospholipases and secondary metabolites (Figure 5), and crucially, also biofilm formation[101,102]. In the last few years, a third signalling factor, the *Pseudomonas* quinolone signal (PQS) has become implicated in linking the *las* and *rhl* systems, and probably controls its own subset of phenotypic traits[103–106]. A parallel PQS signalling system has recently been reported in *Burkholderia* sp[107]. The synthesis of PQS is under the positive control of the *las* system, but is negatively controlled by the *rhl* system, indicating that the ratio between OdDHL and BHL is critical for its production. PQS is derived from 4-hydroxy-2-alkylquinolones made by the *pqsABCDE* and *phnAB* operons, whose expression is controlled by PqsR. The product of these steps, 4-hydroxy-2-heptylquinolone, is then converted to PQS by PqsH, whose expression is controlled by LasR. Like OdDHL, PQS is a hydrophobic molecule that is relatively insoluble in aqueous solutions. However, recently, Mashburn and Whiteley[108] reported that PQS (but not the AHLs) can be shuttled between individual cells in small vesicles budded off from the cell surface. This vesicle budding was itself stimulated by PQS production, since it was absent in a mutant (*pqsH*) unable to make PQS. It seems likely that the insertion of PQS itself into the outer membrane can stimulate vesicle formation and budding.

Plasmodium aeruginosa may be unique in terms of the number of independent microarray studies that have been done in order to characterize the genes under QS control. In three microarray studies[99,109,110], 866 genes were identified (in total) as being QS-induced. This is a conservative figure since one of these studies (that of Hentzer *et al.*[109]) employed a more stringent cut-off (5-fold) than the others. However, although the individual studies identified between 3 and 7% of the genome as being QS-modulated, the overlap between them was small, with only 97 genes being found in common. This common set of genes may represent a ''core'' QS regulon which is expressed in most growth conditions. Moreover,

the study of Wagner *et al*. identified >200 genes as being repressed, whereas Schuster *et al*. only found 38 QS-repressed genes. This may reflect differences in either the growth conditions employed, or differences in the way in which a gene was defined as being QS-regulated. Also, the very large numbers of genes identified in these microarray studies as being QS-regulated may be misleading; transcriptomic analysis alone cannot discriminate between cause and effect, and a large number of the modulated genes are likely to be only indirectly controlled by QS. Indeed, just 7% of the QS-controlled genes identified by Wagner *et al*. were preceded by *las* or *rhl* boxes[99]. Although this can be partially accounted for by the presence of multi-gene operons, each associated with just a single *las*/*rhl* box, it is noteworthy that *ca* 9% of all QS-controlled genes in that study were classified as transcriptional regulators or two-component systems of some kind. Therefore, many of the genes identified as being supposedly ''QS-regulated'' are likely to be controlled indirectly by other pleiotropic regulators[97]. Even relatively minor changes in growth conditions may therefore bring about some variation in the apparent QS regulon[99], reinforcing the remarkable adaptability of this organism to environmental challenges[111]. It seems that in *P. aeruginosa*, QS has become so deeply embedded into the existing regulatory networks that it has become hardwired to the organism's ''CPU'' machinery.

Another key feature to come out of the microarray analyses relates to the timing of signal molecule production; not all QS-controlled genes were induced at the same point in the growth curve[110]. That is, the ''traditional'' view of QS in which gene expression is triggered once a defined AHL concentration is exceeded does not hold here, and there seems to be a continuum of both signal specificities and timing of gene expression in this organism[110]. Furthermore, QS mutants grown in the presence of saturating concentrations of AHLs showed similar gene expression timing to the wild-type strain, indicating that the trigger for QS gene activation was not the signal molecule concentration *per se*. This is reminiscent of events required for progress through the eukaryotic cell cycle – checkpoint steps, which ensure that inappropriate gene expression is suppressed until the cell is ready to commit to *e.g*. virulence factor production. To complicate the issue further, it seems that certain complex media contain a *P. aeruginosa*-metabolizable substance which inhibits AHL-dependent gene expression, which may partially explain the (very large) differences between the apparent QS regulons defined for different media[112].

P. aeruginosa also contains an "orphan" LuxR homolog, denoted QscR. Mutants in *qscR* are hypervirulent, suggesting that this protein operationally functions as a repressor[113]. QscR forms heterodimers with LasR and RhlR, and there is good evidence to suggest that the protein binds OdDHL[114]. This situation is reminiscent of the TraR-TrlR interaction observed in *A. tumefaciens*, where TrlR effectively "titrates" TraR and thereby suppresses any phenotypes under its control. However, unlike TrlR, QscR contains a functional DNA-binding domain, indicating that it is capable of controlling the expression of target genes itself. Indeed, studies with purified QscR have shown that it can bind operator regions with dyad symmetry (*ca* 20 bp in length) associated with the promoters of certain genes (*e.g.* PA1897, adjacent to *qscR* itself), and that this binding is enhanced by OdDHL[115]. Therefore, in addition to functioning as a repressor, QscR can also activate gene expression. Clearly, unlike the *rhl* sub-system, which is dependent on the *las* sub-system at a transcriptional level, the QscR regulon is subordinate to the *las* system because of its dependency on the OdDHL ligand produced by LasI. Depending on the promoter, binding affinities vary from 0.3 →4 nM, and may or may not exhibit weak cooperativity[115]. QscR-dependent promoters exhibit similarities to LasR-dependent promoters, although the two are clearly distinct. However, QscR appears to have a relaxed signal specificity compared to LasR[115], and may even bind ODHL (3-oxo-decanoyl-(L)-homoserine lactone) with higher affinity than OdDHL, suggesting that this protein may also respond to signals produced by other organisms. A recent study defined the QscR regulon, by using microarray analysis to compare the transcriptome of a *qscR* mutant with that of the wild-type[116]. Some 424 genes showed altered expression – most of them appearing to be repressed by QscR, although, consistent with the *in vitro* biochemical characterization of the protein, the expression of 76 genes appeared to be activated. As might be expected, the QscR regulon substantially overlaps those of LasR and RhlR, although within this, a subset of genes also appear to be exclusively regulated by QscR itself. Quite how QscR fits in to the overall QS system is not yet clear, although it probably functions to tightly repress QS-controlled virulence factor production during the early (pre-quorate) stages of growth.

Perhaps the most important QS-controlled phenotype in *P. aeruginosa* is biofilm formation. Although the precise role of biofilms in *P. aeruginosa* pathogenicity has not yet been established, the more-or-less constant inflammatory response stimulated

by the presence of bacterial cells in the lung tissue plays a major role in the progressive lung degeneration seen in many CF patients. Viewed close-up, *P. aeruginosa* biofilms grown in flow cells exhibit a three-dimensional architecture composed of mushroom-like towers interspersed by water-filled channels[117,118], although there is considerable variation in biofilm morphology from strain-to-strain and in different growth conditions[118]. The cells that comprise the biofilm are embedded within a secreted exopolysccharide matrix consisting largely of alginate, sometimes augmented by protein and nucleic acids, which apparently acts to glue the whole structure together[117,119]. The release of genomic DNA from *Pseudomonas* biofilms is dependent on the AHL and PQS signalling systems[119], and may play a role in adaptive evolution.

P. aeruginosa biofilms go through a well-defined series of developmental stages before achieving their final structural configuration[120]. Briefly, planktonic founder cells first establish the colony by adhering to the solid substratum. The micro-assemblage then proliferates and subsequently differentiates, in a multi-step process known as "maturation". This is often followed by the dispersal of new planktonic seed cells from discrete pockets on the mushroom-like structures. These maturation steps often involve additional sculpting, which in one case, has been shown to be mediated by a prophage-dependent remodeling process[121]. Indeed, it is interesting to speculate why biofilms have not evolved further into "true" (*i.e.*, genetically pre-determined, distinct) multicellular entities. Presumably the answer lies in the fact that once formed, a multicellular organism cannot "dissociate" into its component parts and seed new habitats, whereas biofilm cells can. That is, phenotypic plasticity seems to be favored over phenotypic determination. Mutants affected in QS, particularly the *las* component of the QS system, form flat, undifferentiated biofilms[101]. However, one recent report has challenged the role of the *las* system in this and has posited that the subordinate *rhl* system makes a greater contribution to biofilm formation[102]. Whatever the case, drug-like QS blockers would almost certainly reduce biofilm formation *in vivo*, and therefore increase the fraction of the bacterial population that are susceptible to conventional antibiotic intervention. Promising work done by the Givskov/Hoiby groups in Denmark has identified a number of furanone-like small molecules that apparently block QS in *P. aeruginosa* and indeed, attenuate virulence in mouse models *in vivo* and reduce biofilm formation *in vitro*[122,123,124]. Several other groups are also working on the

development of small molecule QS blockers, and this has been reviewed recently in reference (125).

P. aeruginosa-derived AHLs also have pathogenicity-related effects on the host immune system. For example, OdDHL has been shown to stimulate T-cells to produce interferon-γ. Furthermore, and in one of the most exciting developments in the field in recent years, the *P. aeruginosa* outer membrane protein, OprF, was found to be a receptor for this cytokine[126,127]. Interferon-γ was found to up-regulate a number of QS phenotypes, and this up-regulation was abolished in *rhlI* and *rhlR* mutants. This indicated that the binding of IFN-γ to OprF leads, by mechanisms unknown, to an up-regulation of *rhl*-dependent phenotypes (including virulence factor production). In this way, a cycle of communication between the pathogen and the host immune cells is set up, rapidly ramping up the production of virulence factors. Other immune-modulatory effects of OdDHL have also been reported (reviewed recently in (128).

Biofilms and the possible origins of QS

QS has now been shown to control biofilm formation in many microbes, and the Centers for Disease Control (CDC) has recently estimated that around 80% of chronic bacterial diseases involve these structures[129]. This notwithstanding, remarkably, there is still controversy over precisely what biofilms are. Are they distinct growth states, or are they simply extreme versions of an otherwise continuum of growth phenotypes? What is their relationship to say, colonies on an agar plate? The latter do not need QS to form, although certain QS mutants of *P. aeruginosa* often display small-colony morphologies (MW, unpublished observations). Also, the question of whether biofilms secrete the same spectrum of virulence factors as planktonic cells has yet to be addressed. Presumably, the answers to these fascinating problems will be forthcoming in the future. However, rather more is known about how biofilm cells differ from planktonic cells, mainly as a result of several detailed transcriptomic analyses. For example, in the case of *P. aeruginosa*, Whitley *et al.*[130] found that the expression of just 78 genes (out of a total complement of *ca* 5600 ORFs) was altered between exponential-phase planktonic and mature biofilm cells. This result suggested that sessile biofilm cells resemble actively-growing exponential-phase cells in most respects, although this remains to be proven. As these authors acknowledge though, biofilms are heterogenous communities that are likely to exhibit different gene

expression profiles depending on where in the biofilm the sample is derived. In one detailed study of this issue, it was found that *lasI* expression diminishes in biofilms over time, while *rhlI* expression remains essentially unaffected. Moreover, both genes are expressed to a greater level at the base of the biofilm, adjacent to the substratum[131]. In contrast, global *proteomic* analyses of biofilm and planktonic cell cultures present a different story; about half of the *P. aeruginosa* soluble proteome is altered in mature biofilms relative to their planktonic counterparts[120]. This lack of congruence between transcriptomic and proteomic studies is not uncommon. However, although it is accepted that most regulation occurs at the transcriptional level in prokaryotes, the functional molecules in the cells are proteins and metabolites, and there are arguments for both technologies being complimentary to one another. Interestingly, there are very few reports of global metabolomic analysis being applied to the study of biofilms and QS, although studies along these lines are well-underway in the author's laboratories. These data indicate that QS mutants exhibit well-defined metabolomes, and we can readily discriminate between say, wild-type cells on the one hand, and *lasI* mutants, *rhlI* mutants or *pqsR* mutants on the other, on the basis either of their secreted secondary metabolome, or their primary metabolome (MW & PWD, unpublished observations). These observations reinforce the notion that QS has global effects and that it substantially impinges on the central metabolic pathways of the cell.

Biofilms need not be composed of a single species of organism. Indeed, mixed biofilms are probably the norm (especially in environmental isolates), and it is becoming increasingly clear that as many as 99% of all species of bacteria are probably unculturable when grown alone[132]. This raises an interesting issue with regards to the definition of an ecological ''niche'', since a niche can no longer be considered to be the habitat occupied by just one species. Mixed biofilms of ''unculturables'' potentially permit sharing of the metabolic burden associated with colonization of the niche, allowing greater adaptive flexibility. That is, metabolic products produced by one cell can potentially diffuse across to a different cell where they are metabolized further, and *vice versa*. Cell–cell communication, both through direct physical contact and *via* diffusible chemical signals, must play a key role in the development of this cooperative behaviour since, at the very least, the players necessarily need to coordinate their replication; if one of the partners in this intercellular metabolic relay dies, so will the other, providing a strong selection pressure for the evolution of

intercellular signals. That is, the primordial intercellular signal(s) may have arisen from say, a shared metabolite within a mixed biofilm. A good example of this type of signal is seen with the *P. aeruginosa* siderophore, pyoverdine, which can function as both an iron scavenger and a signal molecule[133]. Winzer *et al.*[134] have provided a good working definition for a cell–cell signalling molecule, arguing that 4 simple conditions must be met before a molecule can be regarded as a true signalling agent. Perhaps most importantly, they point out that the cellular response, post-reception of the signal, must extend beyond the physiological change(s) required to metabolize or detoxify the compound. Furthermore, for a signal molecule to be classed as a *quorum sensing* signalling agent, its production needs to be coupled to population growth in the culture. These are crucial points, especially given the recent controversy over "autoinducer-2".

Bacterial esperanto? The controversy over AI-2

As outlined in an earlier section, the control of bioluminescence in *V. harveyi* is under the control of AHL-dependent QS. However, this description is rather overly simplistic since it ignores the contribution of autoinducer-2 ("AI-2"), which regulates bioluminescence in conjunction with *N*-3-hydroxybutanoyl-(L)-homoserine lactone. AI-2 is made by the LuxS enzyme, which has since been identified in numerous species of bacteria[135,136], including pathogens such as *V. cholerae*, where it has been implicated in the control of virulence factor production[137]. The function of LuxS is to hydrolyze *S*-ribosylhomocysteine, generating homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD)[138,139]. As such, it plays an important role in the activated methyl cycle of these cells, since *S*-ribosylhomocysteine is obtained from demethylation of *S*-adenosylmethionine. Not all bacteria exploit this pathway, and some, such as *P. aeruginosa*, use an *S*-adenosylhomocysteine hydrolase to circumvent this step, cleaving SAH to generate homocysteine and adenosine. The role played by DPD and LuxS in central metabolism has frustrated the characterization of AI-2 as a signal molecule, since *luxS* mutants would be expected to be pleiotropically affected in metabolism. [This said, in normal laboratory culture conditions, *luxS* mutants rarely display profound growth defects, although in their natural environment, the selection pressure is likely to be dramatically different.] One suggestion has been that while AHLs serve as *intra*-species QS molecules, AI-2 could serve as an *inter*-species signalling agent,

perhaps functioning to inform those cells with appropriate receptors for this molecule about how crowded the neighborhood might be, or telling the organism when it has entered a particular environmental niche (*e.g.* the gut). For example, there is good experimental evidence to suggest that *P. aeruginosa* responds to the AI-2 made by other host microflora[140]. Another possibility, likely to hold for organisms such as the *Vibrios*, is that AI-2 acts as part of a "coincidence" sensor, allowing multiple signalling inputs to be simultaneously assessed[136]. However, at least one study, involving *Serratia marcescens*[141], showed that there is considerable strain-to-strain variation in the phenotypes of *luxS* mutants, even within a given "species", indicating that we are only just scratching the surface of the potential biology involved.

DPD is only a precursor for AI-2, and needs to undergo non-enzymatic rearrangement to generate the active signalling compound(s). However, this inter-conversion can generate several different molecular species (all of them furanones), depending on the conditions prevailing at the time, so the term AI-2 should be construed as referring to a collection of related compounds[142]. Reinforcing this, two different DPD-derived compounds have been identified in co-crystals of AI-2 receptors. In the structure of the *V. harveyi* LuxP/AI-2 complex, AI-2 appeared to be a furanosyl borate diester[138]. However, in the structure of the *Salmonella typhimurium* LsrB protein complexed with AI-2[143], the signalling molecule appeared to be (*2R,4S*)-2-methyl-2,3,3,4-tetrahydroxyterahydrofuran ("*R*-THMF"). This molecule does not contain boron, even though, during its preparation for crystallization, there would have been ample opportunity for borate to become complexed with the ligand. It seems then, that AI-2 is a heterogenous collection of molecular species, although these are all dependent upon the presence of a functional LuxS protein. Whether subtle alterations to the ligand turn it from being a metabolic intermediate into a signalling molecule remain to be seen. In theory, DPD is a good precursor for a QS molecule, since its synthesis, being tightly linked to a central metabolic flux, ought to be proportional to the rate of increase in the cell density. The signal transduction pathways mediated by AI-2 in *S. typhimurium* and *V. harveyi* have been reviewed recently[142]. These systems have so far fallen into two categories. One, exemplified by signalling in the *Vibrios*, involves the perception of extracellular AI-2 and the subsequent activation of a signal transduction cascade. The other system, characterized in *E. coli* and several other enterics, involves the uptake of AI-2 into the cell and its subsequent metabolism. Likewise, *P. aeruginosa*,

which does not encode *luxS*, is also capable of taking up and metabolizing exogenous AI-2[140]. It is tempting to speculate that these catabolic systems evolved, at least in part, as a means of scavenging AI-2 made by other organisms. In relation to this, several bacterial species have been shown to not only degrade, but also to utilize the AHL signalling molecules produced by other microbes as a sole C-source (*e.g.* see ref. 89). This suggests that the metabolism of signal molecules generally may be a widespread phenomenon.

Concluding remarks

The last 5 years or so have seen a veritable explosion in our knowledge and understanding of QS in Gram-negative bacteria. Much of this progress has been driven by the ready availability of technological platforms that enable facile transcriptional and proteomic profiling. The challenge now will be to try and put together the various (currently, rather disparate) studies and generate useful, holistic models of QS in various organism(s). Furthermore, by its very nature, many functional genomic analyses are ''hypothesis-generating'' rather than ''hypothesis-testing'' (although this is not always the case), and it is becoming clear that a good deal of molecular analysis lies ahead before we will truly understand the systems concerned. The aim of this review is to highlight the *differences* between QS systems, rather than their similarities. If the last few years have taught us anything, it is that this diversity is substantial, and much remains to be learned about this fascinating signalling paradigm.

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