

# REGULATORY CIRCUITS AND COMMUNICATION IN GRAM-NEGATIVE BACTERIA

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It is increasingly apparent that, in nature, bacteria function less as individuals and more as coherent groups that are able to inhabit multiple ecological niches. The increased awareness of the role of cell–cell communication in the ecology of Gram-negative bacteria is matched by an understanding of both the physiology and the molecular biology that underlie this process. In particular, the regulatory circuits and the structure of one of the important regulatory proteins have recently been described. Here, we review the current understanding of quorum-sensing circuits in bacteria, and the role of the regulatory LuxR-type proteins in particular.

## AUTOINDUCER

A small molecule that allows intercellular chemical communication by bacteria. These molecules are responsible for an auto-induction (positive feedback) regulatory mechanism.

## PHEROMONE

A small molecule that is involved in chemical communication. Autoinducers are a specific class of pheromone.

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Quorum sensing (QS) is the process of bacterial cell–cell communication that involves the production and detection of diffusible signalling molecules called AUTOINDUCERS, which have been referred to as bacterial PHEROMONES. It allows populations of bacteria to collectively control gene expression and synchronize group behaviour, and this synchronization is generally timed to coincide with attaining a high population density. Discovered more than ten years ago in the bacterium *Vibrio fischeri* (also known as *Photobacterium fischeri*) (BOX 1), QS research has progressed rapidly, mainly through studies at the molecular level. QS is now regarded as a general system among Gram-negative bacteria and numerous examples of QS have been described in several different bacterial species (TABLE 1) (for a selection of excellent recent reviews, see REFS 1–6).

Bacterial intercellular communication is based on the detection of diffusible signal molecules. Bacteria use a wide variety of signalling molecules, signal-detection systems and signal-transduction mechanisms to convert the information contained in the signal into changes in gene regulation. In Gram-negative bacteria, the signalling molecules are often ACYLATED HOMOSERINE LACTONES (AHLs). In Gram-positive bacteria, signalling molecules are often peptides (BOX 2). Autoinducers enable specific intraspecies communication, and although interspecies communication does occur, it is

less well understood. Recently however, a new autoinducer known as AI-2 has been proposed to function as a universal signal for interspecies communication (reviewed in REF 7).

The *V. fischeri* LuxR/LuxI system was the first bacterial cell–cell communication system to be characterized and soon became a paradigm. In Gram-negative bacteria, two important proteins are involved in the regulation of QS<sup>8</sup> — an R protein, which is a transcriptional regulator that is homologous to LuxR of *V. fischeri*, and an I protein, which is homologous to the autoinducer synthase LuxI of *V. fischeri* and is the enzyme that synthesizes the signalling molecule, which is usually an AHL.

Recognition of the ligand AHL by the LuxR transcriptional regulatory protein is extremely specific. Each LuxR transcriptional regulator activates transcription in response to a specific autoinducer signal. For example, Schaefer and co-workers showed that the LuxR transcriptional regulator from *V. fischeri* is only able to fully activate transcription of its target genes when the cognate autoinducer 3O-C6-HSL (3O-C6-homoserine lactone) is supplied in the culture medium<sup>9</sup>. This explains why specific autoinducer molecules facilitate intraspecies, but not interspecies, communication. The specificity of the interaction between the R protein and its cognate AHL is essential

Box 1 | The LuxIR system of *Vibrio fischeri*

The concept of quorum sensing (QS) originated with studies in *Vibrio fischeri* (formerly known as *Photobacterium fischeri*), which has two lifestyle modes: first, it grows in the sea to a low population density and does not luminesce; second, it forms symbiotic associations with fish and squid species such as *Euprymna scolopes*<sup>94</sup> and luminesces. In the figure, an adult Hawaiian bobtail squid is shown, which is ~2 cm long. There is a light organ close to the ink sac in the mantle cavity of the animal. The light organ contains ~10<sup>11</sup> *V. fischeri* cells per ml. The squid is nocturnal and light is emitted downwards through the mantle cavity. By matching the light intensity to the moon and starlight above, the squid becomes invisible to predators below. In symbiotic associations, newly hatched juvenile squids acquire their symbionts from the surrounding seawater. Colonization is a specific process and *V. fischeri* is the sole organism that colonizes the squid light organ. After entering the crypts — histological structures inside the light organ that are delimited by epithelial cells — of a nascent light organ in a juvenile squid, the bacteria undergo morphological and physiological changes in response to colonization of the host tissue. Bacteria proliferate and synthesize autoinducer, which can activate expression of the bacterial genes that produce luminescence. The autoinducer has to reach a threshold concentration before the luminescence genes are activated. Inside the crypts, densely packed bacteria are surrounded by a matrix fluid that contains millimolar concentrations of amino acids in the form of peptides. Each morning more than 95% of the bacterial culture is expelled from the crypts, and by the evening the remaining symbionts repopulate the organ through division<sup>95</sup>. So, there is a daily cycle of symbiont proliferation, expulsion and regrowth that is supported by nutrients that are supplied by the surrounding host tissues. First discovered in the 1980s, this phenomenon soon became a model for studies of the regulatory molecular mechanisms that are involved and also for studies of the symbiotic processes of *V. fischeri*<sup>61,96,97</sup>. Image kindly provided by Edward G. Ruby (University of Wisconsin, Madison, USA).

for bacteria to distinguish the AHLs produced by their own species from the AHLs that are produced by other species. However, this specificity is not absolute, and some non-cognate AHLs can elicit partial responses through interaction with LuxR-type transcriptional regulators.

A simple model for QS signalling is shown in FIG. 1. At low cell densities AHLs are synthesized at a basal level by the low concentrations of the constitutively transcribed and translated I protein. Newly synthesized AHLs diffuse out from the cell and form a concentration

gradient. Synthesis of the AHL, and accumulation in the local environment, continues during bacterial growth. The local environment is often a confined space or a niche in which restricted diffusion of molecules occurs<sup>10</sup>. When the population density, and therefore the AHL concentration, reaches a threshold value, sufficient AHL signal has been synthesized to allow the AHL ligand to interact with its cognate R protein. Binding of the AHL ligand activates the R protein transcriptional regulator, which, in turn, binds to specific DNA sequences in the promoter region of target genes and activates their transcription through DNA binding. In most systems that have been characterized, the *I* gene is a target of the R protein, and is rapidly transcribed once the threshold concentration of AHL is reached, which in turn results in production of increased amounts of AHL so that an autoinduction loop is set up. Therefore, the AHL is often referred to as an autoinducer. The amplification of the AHL signal ensures that all the target genes of the R protein are regulated in response to the AHL signal<sup>11</sup>. Consequently, intracellular responses are synchronized and perhaps even whole communities of cells are synchronized with population density<sup>11</sup>. The population-density-dependent regulatory circuits ensure that maximal production of specific proteins occurs during the late-logarithmic and stationary phases of growth when the population density is high. The physiological processes that are controlled by QS are diverse, but are often related to virulence in pathogenic organisms (TABLE 1).

*V. fischeri* has a second acyl-HSL synthase, AinS, which is not homologous to LuxI and which synthesizes the C8-HSL autoinducer<sup>12</sup> (see BOX 2 for details of the system in *V. harveyi*). The QS circuits — including the responses to both 3O-C6-HSL, which is synthesized by LuxI, and C8-HSL, which is synthesized by AinS — regulate a group of genes that comprise a QS regulon<sup>13</sup>, and these QS-regulated genes include the bioluminescence genes and possibly other colonization factors of the squid symbiont host *Euprymna scolopes*<sup>14</sup>.

### QS circuits — themes and variations

Since the characterization of the *V. fischeri* LuxR/LuxI bioluminescence system (R/I system), many QS circuits have been described in different species of Gram-negative bacteria<sup>1</sup>. Only a few of these systems have been studied at the molecular level. In this review, selected examples will be used to illustrate the complexity that underlies quorum sensing in specific bacterial models (TABLE 1). It has become clear that there are several differences among QS systems. On the basis of the *V. fischeri* system, it was assumed that only one R/I system would regulate QS target genes in each bacterial species. Indeed, for many of the bacteria that have been studied, only one QS system has been identified. Some bacteria however, have been found to contain two or more QS systems. The most complex system described so far is that of *Rhizobium leguminosarum* biovar *viciae* (TABLE 1), in which there are five R proteins, four I proteins and a cocktail of six different acyl-HSL molecules that are used for signalling<sup>15</sup>.

#### ACYLATED HOMOSERINE LACTONES

A type of autoinducer that is used by Gram-negative bacteria and which is composed of an acyl chain attached to a homoserine lactone.

**Anti-activators.** The first example of added complexity to the basic QS paradigm was the discovery of anti-activator proteins, such as those that are found in *Agrobacterium tumefaciens*, the plant pathogen that induces crown gall tumours in host plants. Anti-activators modulate QS systems by interacting with the R proteins. The infectivity of *A. tumefaciens* is mediated by the transfer of oncogenic DNA fragments from a tumour-inducing (Ti) plasmid into the nuclei of the plant cell. Ti plasmids also mediate their own transfer among agrobacteria through a process that is partially controlled by QS. The protein **TraI** (an I protein) synthesizes 3O-C8-HSL, which binds to and activates **TraR** (an R protein). TraR activates the target genes *tra* and *trb*, which in turn stimulate the transfer of Ti plasmids. This system is regulated by two anti-activator proteins, **TraM** and **TrhR** (also known as TraS), which can bind to the TraR protein and inhibit DNA binding by TraR, thereby inhibiting transcriptional regulation by TraR. The physiological role of TraM might be to repress conjugal transfer of the Ti plasmid at low cell densities<sup>16</sup>. Interestingly, expression of the *traM* gene is positively regulated by activated TraR, showing that the anti-activators have a clear role in QS circuits in the cell<sup>17,18</sup>.

**Multiple QS systems.** The second example of increased complexity is the presence of multiple QS systems in the same bacterial species. These different systems are linked through intricate regulatory connections. In *Pseudomonas aeruginosa*, two complete QS systems, comprising the **LasR** (R protein), **LasI** (I protein) plus 3O-C12-HSL and the **RhlR** R protein, **RhlI** I protein plus C4-HSL systems, have been identified, as well as a third regulatory protein (QscR) that belongs to the LuxR family and is thought to be an anti-activator. Unlike the R proteins, there is little sequence homology between different anti-activator

proteins; however, anti-activators do have common functions. A large number of genes, including numerous virulence factors, are regulated by QS in *P. aeruginosa*. Each system modulates the expression of a regulon that comprises an overlapping set of genes. However, the Las and Rhl systems are not independent and form a regulatory cascade in which LasR in combination with its cognate ligand 3O-C12-HSL activates the expression of *rhlR* and *rhlI*<sup>19,20</sup>. The anti-activator protein QscR seems to have an important role in the interactions between the Las and Rhl QS systems<sup>21,22</sup> (see below).

In *R. leguminosarum* biovar *viciae*, the QS network is extremely complex and incompletely understood. This bacterium has four separate QS systems (TABLE 1), which communicate through six different acyl-HSLs. In this system, the chromosomally encoded CinIR proteins seem to be at the top of a regulatory hierarchy<sup>23,24</sup> that regulates the other plasmid-encoded QS systems.

**Multiple signalling molecules.** In some bacteria, including *P. aeruginosa* and *V. fischeri*, multiple AHL signalling molecules can be sensed by the QS systems. In *P. aeruginosa*, the AHL synthase RhlI can synthesize multiple, chemically distinct autoinducer molecules in different proportions<sup>25</sup>. In general, only the effects of the most abundant AHL species have been characterized for any one species. The reason for the synthesis of multiple AHLs is unclear, but one hypothesis is that the relative concentrations of the different AHLs could vary during the bacterial growth phase so that growth conditions could fine-tune QS regulatory systems. In *V. fischeri* different AHLs are synthesized by different proteins, including LuxI and AinS. LuxI synthesizes a 3O-C6-HSL that regulates the transcription of the *lux* operon in *V. fischeri* whilst a C8-HSL<sup>12,26</sup> molecule is synthesized by AinS.

Table 1 | **Examples of I/R QS systems**

Organism	I/R genes	AHLs synthesized	QS-regulated phenotypes	References
<i>Vibrio fischeri</i>	<i>luxR, luxI</i> <i>ainS</i>	3O-C6-HSL C8-HSL	Bioluminescence Colonization factors	13,14,102
<i>Pantoea stewartii</i>	<i>esaR, esaI</i>	3O-C6-HSL	Exopolysaccharide production	50
<i>Agrobacterium tumefaciens</i>	<i>traR, traI</i> , <i>trIR (traS)</i>	3O-C8-HSL	Virulence plasmid copy number and conjugal transfer	72
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	<i>carR, carI</i> ( <i>expR, expI</i> )*	3O-C6-HSL	Production of carbapenem antibiotic and exoenzymes	118,119
<i>Pseudomonas aeruginosa</i>	<i>lasR, lasI</i> <i>rhlR, rhlI</i> <i>qscR</i>	3O-C12-HSL C4-HSL	Virulence, biofilm formation, other cellular functions	49,53,93
<i>Rhizobium leguminosarum</i> biovar <i>viciae</i>	<i>rhlR, rhlI</i>	C6-HSL C7-HSL C8-HSL	Nodulation efficiency	15,120
	<i>raiR, rail</i>	C6-HSL C7-HSL C8-HSL	Function unknown	15,24
	<i>bisR, traR, traI</i> <i>cinR, cinI</i>	3OH-C8-HSL 3OH-C14:1-HSL	Plasmid transfer Growth inhibition	12 23

\**expIR* and *carIR* loci are highly homologous and were isolated from two different strains of *Erwinia carotovora* subsp. *carotovora* but do not represent two sets of IR loci in the same genus.

PAS AND GAF DOMAINS

PAS and GAF are two structural motifs found in small molecule binding proteins involved in signal transduction. The PAS motif is smaller and more common than the GAF domain, and is more closely linked to small-molecule binding.

HTH

(helix–turn–helix). A structural motif that is common in DNA-binding proteins; typically the second helix fits into the DNA major groove.

TWO-COMPONENT SIGNAL-TRANSDUCTION SYSTEM

A bacterial signalling system that is composed of a sensor kinase protein, usually localized in the membrane, and a cytoplasmic response regulator protein. A specific signal is detected by the sensor kinase, allowing autophosphorylation of this protein. The phosphate is then transferred from the sensor kinase to the response regulator. The phosphorylated regulator protein is activated and modulates target gene transcription.

Recently, a new QS molecule, 2-heptyl-3-hydroxyl-4-quinolone, known as PQS, was identified as a signalling molecule that has a role in the QS hierarchy of *P. aeruginosa*. Although PQS is chemically distinct from the other AHLs that form the basis of communication in the *P. aeruginosa* QS circuits, it is an integral part of the QS hierarchy. PQS, in common with the AHLs, is a diffusible signalling molecule that accumulates in the local environment and can modulate gene expression<sup>27</sup>.

In addition to sharing similar QS mechanisms, many Gram-negative bacteria have symbiotic or pathogenic relationships with eukaryotic hosts. It is not surprising that symbiosis, pathogenesis and QS are intertwined in a complex regulatory network.

**QS regulators: structure and biochemistry**

Recently, crystal structures of a dimeric TraR protein in complex with its cognate AHL (3O-C8-HSL) and a DNA-promoter fragment containing the TraR-binding site (known as the *tra* box) were solved<sup>28,29</sup>. These **TraR structures** (FIG. 2) have considerably advanced the understanding of signalling in QS systems and have highlighted several fascinating questions about the functions of R proteins.

*Structural features of the activator TraR.* TraR is composed of two structurally and functionally distinct protein domains that are separated by a linker region

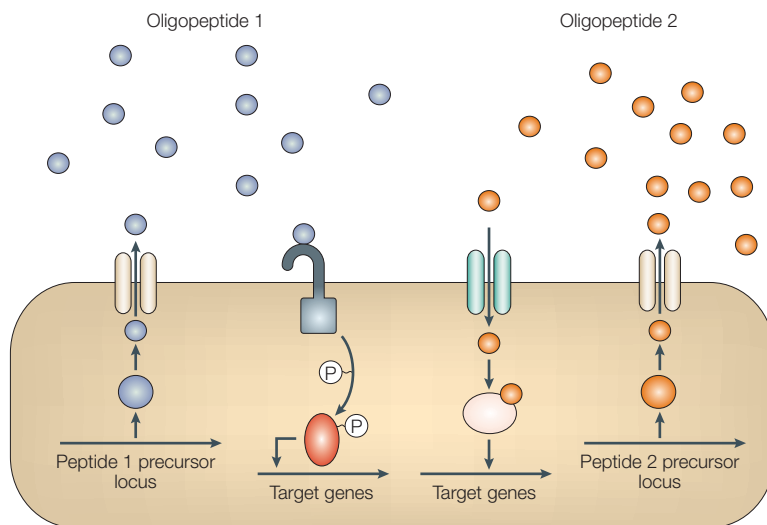
(residues 163–174). The amino-terminal portion of the protein is involved in ligand recognition and binding, whereas the carboxy-terminal domain is responsible for DNA-sequence recognition and binding. Interestingly, the crystal structures showed that the two domains can independently form symmetrical homodimers, but the overall structure of the dimer that is formed from two full-length TraR proteins is asymmetric.

The structure of the N-terminal ligand-binding domain (residues 1–162) is a helix–sheet–helix sandwich. In this sandwich, the ligand is completely buried between the concave surface of the five-stranded β-sheet and the superposed helices, whereas the three helices underneath the β-sheet are involved in dimerization of this domain. The position of the ligand that is buried in the hydrophobic interior of the domain indicates that AHL binding and release must be associated with large-scale modifications of the protein structure. Structurally, the N-terminal domain resembles a PAS or GAF domain. PAS AND GAF DOMAINS are two distinct structural motifs that are found in many signalling proteins.

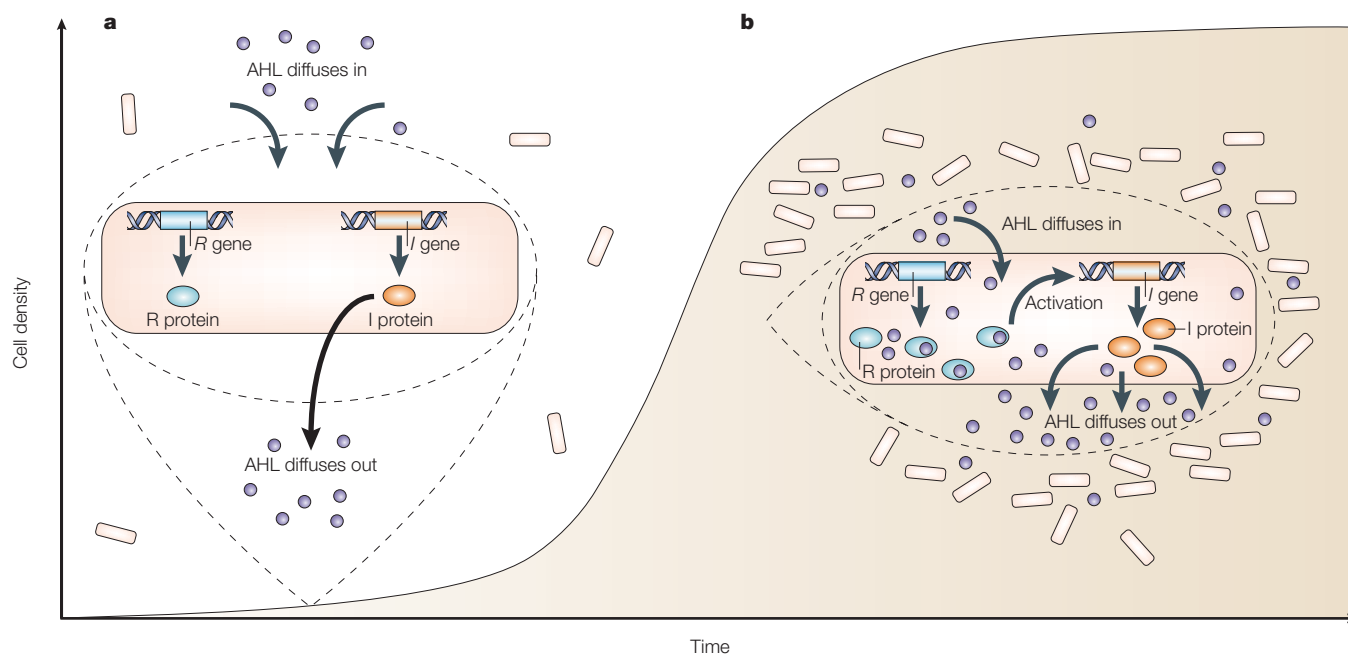
The C-terminal domain (residues 175–234) has a four-helix bundle containing a DNA-binding helix–turn–helix (HTH) motif. The C-terminal domain is dimeric and the dyad symmetry axis corresponds to the dyad axis of the cognate DNA. This structure is particularly well adapted for recognition of the *tra* box, an inverted

Box 2 | **Quorum-sensing circuits in Gram-negative and Gram-positive bacteria**

In Gram-negative bacteria, I proteins are enzymes that catalyse the synthesis of species-specific AHLs, which are detected by R proteins that are transcriptional regulators (FIG. 1). In Gram-positive bacteria, oligopeptides are the signalling molecules. A model for oligo-peptide-mediated quorum sensing in Gram-positive bacteria is shown in the figure. Oligopeptide signals are synthesized as precursor peptides that are processed, modified and exported using ABC export systems. The oligopeptides (shown as filled blue or orange circles) are synthesized in the form of precursors. After modification and processing, they are exported out of the cell into the environment by ABC transporters (shown as yellow channels) where the molecules accumulate as the cell density of the bacterial population increases. For some oligopeptides (blue circles) a cellular TWO-COMPONENT SIGNAL-TRANSDUCTION SYSTEM (shown in grey) detects the oligopeptide signal, which it transmits into the cytosol through phosphorylation-mediated signal transduction from the two-component sensor kinase to the two-component response regulator. The phosphorylated response-regulator protein is activated and regulates the transcription of target genes. For other oligopeptides (orange circles) the peptides are actively transported into the bacterial cell by a specific oligopeptide permease (shown in green). The peptide binds to a cognate regulatory protein (pink), which in turn regulates the transcription of target genes. Peptide signals can be detected either by two-component phosphorelay circuits, which are used to detect oligopeptide autoinducers like ComX that is involved in cell-density induction of competence in *Bacillus subtilis*, or can be peptides that are internalized by a specific oligopeptide permease, such as PhrC, another competence-stimulating factor (reviewed in REF. 98). A third QS mechanism has been described in *Vibrio harveyi*, which is another luminescent marine bacterial species. The *V. harveyi* system lacks the *luxIR* locus found in *V. fischeri*. Instead, two signals are synthesized: a typical AHL autoinducer (3OH-C4-HSL or AI-1) synthesized by a synthase that is homologous to AinS, which is known as LuxM, and a non-acyl-compound, which has recently been shown to be a furanosyl borate diester (AI-2)<sup>99</sup> that is synthesized by LuxS<sup>100</sup>. However, in *V. harveyi*, the perception mechanism is distinct because it does not involve an R protein (reviewed in REF. 101). Both signals are detected by two-component sensor kinase proteins — LuxN responds to AI-1 and LuxQ responds to AI-2. AI-2 has now been proposed to be a universal signal for interspecies communication because the presence of LuxS (and AI-2 production) is widespread among Gram-negative and Gram-positive bacteria<sup>101</sup>.







**Figure 1 | The quorum-sensing paradigm in Gram-negative bacteria.** The *R* and *I* genes are homologous to the *Vibrio fischeri luxR* and *luxI* genes. *luxR* homologues encode the transcriptional regulator (R protein) and *luxI* homologues encode the acyl homoserine lactone (AHL) synthase (I protein). **a** | At low bacterial cell densities AHL molecules are synthesized and accumulate. Depending on the length of the acyl chain, AHLs either diffuse or are pumped out of the cell into the local environment, where the molecules are available for diffusion into, or uptake by, bacterial cells. **b** | At high bacterial cell densities, a threshold concentration of AHL molecules has accumulated. The R protein forms a complex with its cognate AHL and this complex either activates or represses the transcription of target genes. As the *I* gene is usually positively regulated by the R protein in complex with the cognate AHL, rapid amplification of the AHL signal results, which facilitates the coordinate transcriptional regulation of multiple genes.

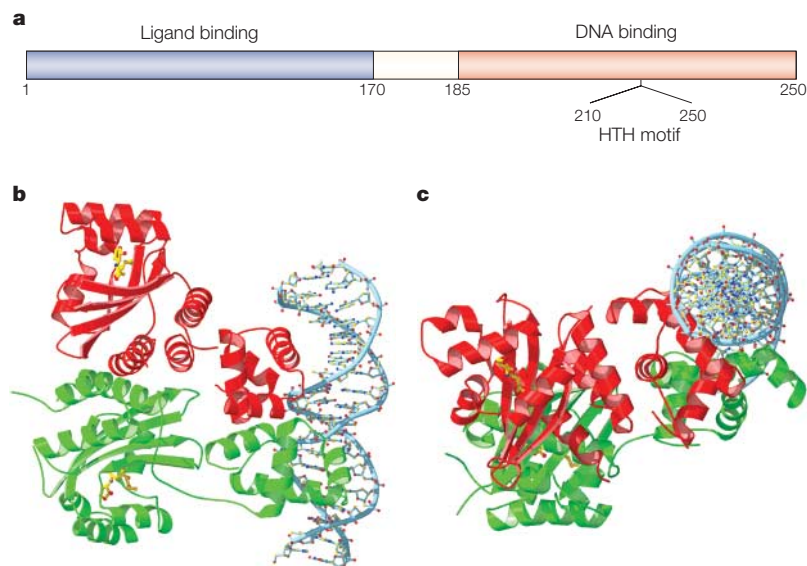
repeat recognition sequence, in which the interaction between the two monomers ensures a fixed length for the promoter sequence. The C-terminal domain is homologous to the small dimeric DNA-binding protein GerE from *Bacillus subtilis*, which is able to regulate gene expression by recruitment of the  $\sigma$ -subunit of RNA polymerase to the promoter of the regulated gene<sup>30</sup>. By contrast, the C-terminal domain of the *V. fischeri* LuxR protein interacts with the RNA polymerase  $\alpha$ -subunit to regulate transcription of the *lux* genes<sup>31</sup>.

The structures of TraR raise a number of important questions. How is gene transcription activated or repressed in response to ligand binding by the R protein? In organisms with multiple R proteins, are homodimers between R proteins formed or can heterodimeric R proteins function to regulate transcription? How did this class of regulators evolve?

The biochemical function of regulatory proteins such as TraR is to bind their cognate ligands and activate or repress transcription of target genes. It is clear that AHL-binding is associated with large conformational changes, which presumably expose the ligand-binding site that is completely buried in the hydrophobic core of the unliganded TraR protein. It can be hypothesized that the conformational changes that occur on ligand binding modify the DNA-binding capability of the TraR protein and/or its ability to interact with RNA polymerase. The nature of the ligand-binding site also offers an explanation for the extremely high affinity of

the R-protein family<sup>32</sup> — the  $K_d$  of TraR for its cognate AHL ligand is  $\sim 1$  nM<sup>33</sup> — which enables the bacteria to detect AHLs at concentrations close to one molecule per cell. The conformational changes that are hypothesized to occur after ligand binding are consistent with the altered protease sensitivity of TraR in complex with the AHL ligand<sup>34</sup>.

The reduced DNA-binding affinity of TraR in the absence of AHL<sup>33,35</sup> might indicate that in the unliganded state the N-terminal domain interferes with DNA binding. One simple model for dimer activation proposes that a ligand-induced conformational change exposes the DNA-binding site. Unfortunately, this model cannot be applied to all the R proteins because, in addition to their function as activators of transcription, some R proteins function as repressors. Examples include ExpR from *Erwinia chrysanthemi*<sup>36</sup> and RhIR from *P. aeruginosa*<sup>37</sup>. Furthermore, operator binding by the R protein does not always require ligand binding<sup>37</sup>. There is also evidence for modulation of the oligomerization state of the R proteins in some systems — such as in *P. aeruginosa* where QscR oligomerization is modulated by AHL binding<sup>22</sup>. The independence of the dimerization of the C-terminal and N-terminal domains and the variability of the length of the linker region<sup>29</sup> indicates that the geometry of the complexes that are formed between R proteins in the presence and absence of AHLs might be able to provide a structural basis for functional variations.



**Figure 2 | Structures of R proteins.** **a** | The domain structure of a typical R protein is shown, with an amino-terminal ligand-binding domain (blue) and a carboxy-terminal DNA-binding domain (orange) that incorporates a helix–turn–helix motif and a region that is involved in activation. **b,c** | Two views of the TraR dimer bound to the *tra* box operator DNA are shown. The two monomers are coloured red and green to show the asymmetric dimer that the R protein forms on binding to the acylated homoserine lactone (AHL; yellow). The view in **(b)** is shown along the crystallographic *a*-axis and view in **(c)** is shown along the DNA axis. Reproduced with permission from REF. 29 © (2002) EMBO J. Nature Publishing Group.

**Modulating R-protein function.** Accumulating evidence indicates that heterologous interactions between different proteins could modulate R-protein function. TraM, which is an anti-activator of the *A. tumefaciens* TraR protein, binds to TraR close to the linker region and inhibits TraR-mediated transcriptional activation<sup>38</sup>. The three different R proteins RhIR, QscR and LasR in *P. aeruginosa* can form heterodimers *in vivo* after expression in the heterologous host *Escherichia coli*<sup>22</sup>. If heterologous dimers can also form in *P. aeruginosa*, this would add a further layer of complexity to the interactions between QS systems and could provide a structural basis for the recognition of the multiple, diverse operator regions that are found upstream of R-protein target genes, might not involve sites that have dyad symmetry and could involve recognition sites of varying size. It is unclear whether the formation of heterodimers can change the function (transcriptional activation or repression) of the R protein in response to binding of either of the cognate AHL molecules, or indeed whether either cognate AHL binds to R proteins that have formed heterodimers. This situation could prove yet more complex because a non-cognate AHL can change the structure of RhIR homodimers<sup>39</sup>, which might complicate communication networks but could also be more pertinent to intraspecies rather than interspecies communication.

The R proteins have a modular structure that consists of a DNA-binding domain that is typical of many transcriptional regulatory proteins and a separate ligand-binding domain. The ligand-binding domain contains motifs with structural homology to GAF

or PAS domains. This is of interest because these domains are ubiquitous and are often found in proteins that function in signal transduction or which bind small molecules. GAF and PAS domains are common in signalling and sensor proteins, but are usually confined to proteins that have enzymatic domains, such as bacterial histidine kinase proteins, including the nitrogen regulation protein NtrB of *Azospirillum brasilense*<sup>40</sup>, rather than proteins with DNA-binding domains. R proteins seem to represent a minimal signal-transduction pathway in which the sensor domain and the regulator domain are fused. This arrangement minimizes the overhead of the system, but lacks amplification. It is unclear if this minimalist signal-transduction system represents an example of ancestral simplicity or has resulted from a reduction in complexity during evolution.

### Physiological functions of R proteins

Transcriptional activators and repressors often interact with DNA through a HTH motif<sup>41</sup>. An important clue to the function of a regulator is the position of the DNA-binding site relative to the transcription start site in the target gene promoter because this determines the interaction of the regulatory protein with the RNA polymerase, and therefore its function. Transcription factors can function as activators or as repressors, depending on the site of interaction in the promoter of the regulated gene — for example, AraC can repress or activate the *araBAD* operon<sup>42</sup>.

**Transcriptional activators.** Many R proteins function as transcriptional activators<sup>43</sup>. However, although the R proteins have been shown to bind DNA, all the experiments have been carried out *in vitro* and DNA binding is dependent on the presence of the autoinducer. TraR, CarR, LuxR and LasR all bind to their DNA target promoters *in vitro*<sup>33,44–47</sup>. Palindromic ‘lux box’ DNA motifs have been identified in *V. fischeri* and are located in the promoters that are upstream of the R-protein-regulated genes and have been proposed to be binding sites for the R-protein–autoinducer complex<sup>48</sup>. In *P. aeruginosa*, the equivalent of the lux box is the las box — the consensus sequence of which was defined through alignment of eight promoters of LasR-regulated genes — but las boxes were only detected upstream of 7% of the QS-regulated genes identified in a global study<sup>49</sup>. This unexpectedly low proportion of genes that seem to represent *bona fide* targets for LasR indicates either that regulation is indirect, or that the bioinformatic method used was not sufficiently sensitive.

**Autoinducer-independent repression.** Some R proteins function as autoinducer-independent repressors. Although the ExpR proteins that have been studied in *Erwinia carotovora* subsp. *carotovora* and *E. chrysanthemi* have characteristics of repressors *in vitro*, their regulatory roles *in vivo* remain unclear. The EsaR protein of *Pantoea stewartii* (formerly known as *Erwinia stewartii*) is the best characterized of the repressor systems<sup>50,51</sup>. EsaR binds to target DNA in a ligand-free state and

represses its own transcription in the absence of the AHL that is synthesized by EsaI (3O-C6-HSL)<sup>51</sup>. The DNA-binding site of EsaR is located in the *esaR* promoter such that it sterically blocks transcriptional activation by RNA polymerase<sup>51</sup>. In this system, interactions with the AHL abrogate the transcriptional regulatory function of the R protein, in contrast with many other R proteins. However, it was recently shown that, although the AHL responses of both EsaR and ExpR are unlike most other R proteins, when bound *in vitro* in the appropriate manner to promoter DNA these proteins can also interact with RNA polymerase and function as transcriptional activators in the absence of AHLs<sup>52</sup>.

In engineered repressor systems in which a *lux* or a *tra* box was inserted between position 0 and the -35 site relative to the transcription start site in a promoter sequence, it was shown that LuxR and TraR could function as AHL-dependent repressors that bind to these artificial promoters<sup>35,46</sup>. This indicates that AHL-dependent repression might also occur in natural systems. Recently, numerous QS-repressed genes were identified using microarray studies in *P. aeruginosa*<sup>49,53</sup>. Only a small proportion of these genes had a *las* box upstream of the transcription start site, which might indicate that indirect control by QS mediates a global negative effect on gene expression. Further work is required to clarify whether repression by LasR or RhlR proteins is physiologically relevant.

**Layering control: anti-activators.** In *A. tumefaciens*, the two anti-activators TraM and TrlR modulate TraR-dependent transcriptional activation through protein-protein interaction. Both of these proteins function through the formation of inactive complexes with TraR. The TraM protein prevents TraR from activating target-regulated genes under non-inducing (no AHL present) conditions<sup>17</sup>. TraM forms inactive heteromultimers with TraR through interactions with the TraR C-terminal domain, although TraM shares no obvious sequence similarity with TraR<sup>38,54</sup>. Yeast two-hybrid studies and far-western analyses of TraM binding to TraR have indicated that monomeric TraM associates with a region in the C-terminal domain of dimeric TraR<sup>54</sup>. By contrast, TrlR is a truncated R protein in which the N-terminal 181 amino acid domain of TrlR is homologous to the N-terminal domain of TraR — 88% identical at the amino acid level<sup>55,56</sup>. Comparison of the nucleic acid sequences shows that there is a frameshift mutation after residue 181, which renders the sequence of the C-terminal domain of TrlR different to that of TraR. TrlR-mediated inhibition of TraR function requires the formation of TraR–TrlR heterodimers. These heterodimers are unable to bind to *tra* boxes with high affinity<sup>57</sup>. Inhibition occurs as there is no DNA-binding domain and the inhibitory effects of TrlR can be removed by inserting a DNA-binding domain into this protein<sup>56</sup>.

By contrast, the *P. aeruginosa* QscR protein, which has 32% sequence identity with RhlR and 29% sequence identity with LasR, is thought to be an anti-activator protein but has both of the R-protein regulatory domains — an AHL-binding domain and a DNA-binding domain. FLUORESCENCE ANISOTROPY measurements and

*in vivo* crosslinking studies have shown that QscR can form different complexes depending on the experimental conditions; multimers form in the absence of AHLs, lower-order oligomers form when QscR is complexed either with C4-HSL or 3O-C12-HSL and heterodimers form when QscR is mixed with LasR or RhlR in the absence of AHLs<sup>22,39</sup>. Taken together, these data are consistent with the hypothesis that, at the start of exponential growth when the concentrations of AHLs are low, QscR forms inactive heterodimers with LasR and/or RhlR, thereby inhibiting expression of some QS-regulated genes. However, at the onset of stationary phase when the AHL concentration has increased, the equilibrium would be displaced towards the formation of LasR homodimers bound to 3O-C12-HSL and/or RhlR homodimers bound to C4-HSL, leading to the regulated expression of target genes.

What is the physiological significance of these QscR complexes? It is important to consider that the formation of a complex is dependent on the relative amounts of regulatory proteins that are present at the same time in the cell and on the relative concentrations of the different AHLs. One study has shown that in *R. leguminosarum* different AHLs are synthesized with a complex temporal pattern and there are important differences between the results of research on isolated systems and results obtained with these integrated systems<sup>58</sup>. Additionally, spatio-temporal regulation of *lasI* transcription has been observed during biofilm maturation in *P. aeruginosa*<sup>59</sup>.

Therefore, studies of the activities of the R proteins and of the production of the AHLs under different growth conditions would be helpful to delineate the molecular mechanisms of integration of QS networks in bacteria.

### Integrating QS with other control circuits

How are the synthesis and activities of the R proteins integrated into other cellular regulatory systems? How is the timing of QS established during growth and colonization of bacterial communities? Recently, research has begun into the integration of QS control into bacterial global regulatory networks. Such integration links QS regulation to other aspects of cell physiology and expands the range of signals — environmental and/or physiological — that influence target gene expression. A complex scheme of regulatory processes that integrate with QS-regulated genes is emerging from convergent studies in different laboratories; for example, the regulatory circuitry in the QS systems of *P. aeruginosa* that is shown in FIG. 3.

**AHL concentration.** The canonical LuxI/LuxR model from *V. fischeri* hypothesized that when a threshold AHL concentration was reached, the R protein activates the transcription of its target genes. QS regulation, and the perception of cell density, were thought to result from variations in the AHL concentration inside and outside cells. The trigger for the induction of the QS-regulated genes was signal accumulation. Indeed, there is considerable evidence for the regulation of AHLs at the level of

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A measure of the polarization of fluorescent light that can be used to measure the speed of rotation of a pigment molecule or protein.

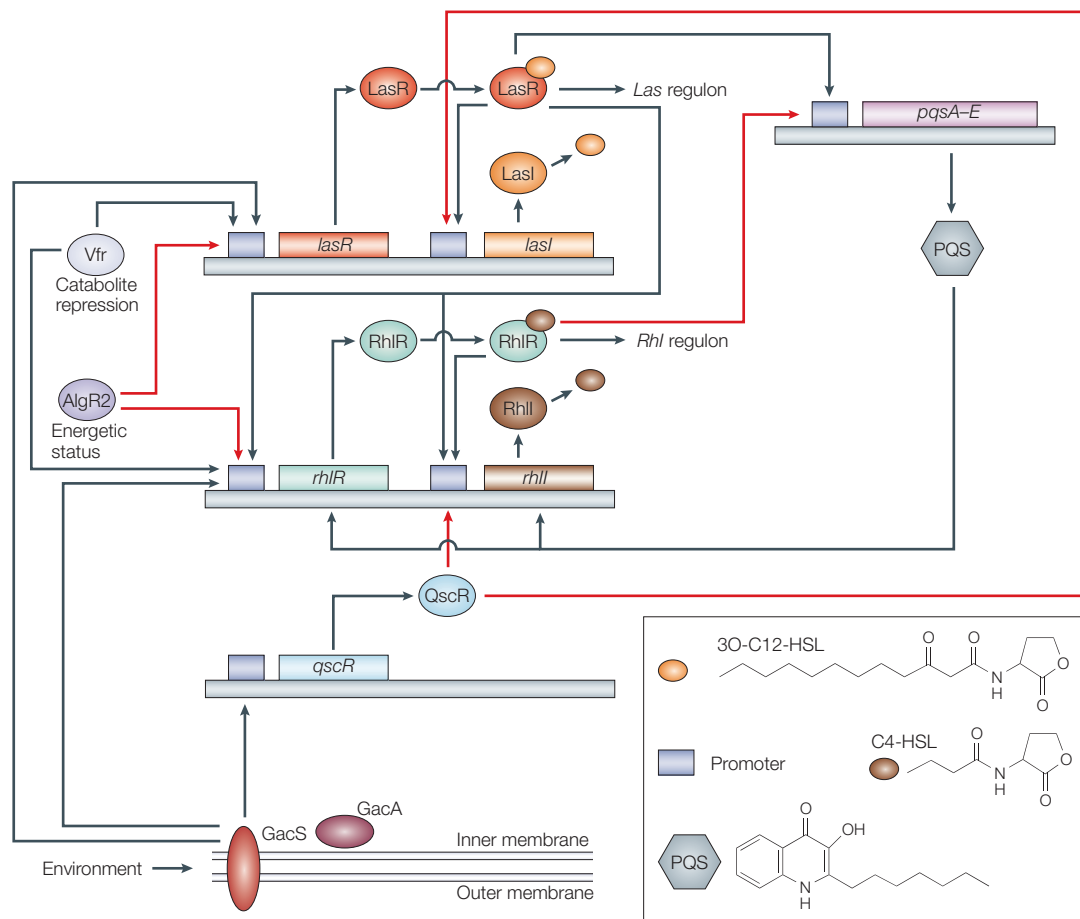


Figure 3 | **The Las, Rhl and Qsc quorum-sensing systems in *Pseudomonas aeruginosa*: hierarchies and integration into cellular control circuits.** For each circuit in the cell the interactions between the different QS systems are indicated by arrows. Black arrows indicate positive regulation and red arrows indicate negative regulation. Signals from the environment, the intracellular metabolic status of the cell and other regulators, such as RpoS, RsmA and MvaT, also interface with this cellular circuitry.

*luxI* transcription, and fluctuations in AHL concentrations could be attributed in part to alterations in the expression of the *luxI* gene<sup>2</sup>. Moreover, a second level of control could be the lability of AHLs, allowing a cascade of differently timed responses to different molecules. For example, the *attM* gene of *A. tumefaciens* encodes an AHL lactonase, which is homologous to AiiA of *B. subtilis* (BOX 3), which inactivates the 3O-C8-HSL signal through hydrolysis of the HSL ring<sup>60</sup>. It is thought that *A. tumefaciens* adopts a signal-turnover system to enable exit from QS-dependent Ti plasmid conjugal transfer.

Nevertheless, this canonical scheme is too simple. First, most of the studies on the regulation of synthesis of the R proteins have been carried out using transcriptional gene fusions, which cannot provide information on post-transcriptional regulation. In fact, the apo-TraR protein (which is not complexed to AHL) is rapidly degraded by the Clp and Lon proteases in the absence of its cognate autoinducer<sup>34</sup>. Second, several diffusible SMALL-MOLECULE ANTAGONISTS have been identified that are produced by the same bacteria that produce AHLs. For example, cyclic dipeptides (diketopiperazines or DKPs) have been isolated from *P. aeruginosa* and other Gram-negative bacteria<sup>61</sup>. These diffusible antagonists could

modulate the activity of the R proteins by functioning as competitive inhibitors of AHLs<sup>62</sup>. Third, it is often difficult to prematurely induce the expression of QS-controlled genes in an exponentially growing culture by the addition of exogenous purified AHLs before the AHL would usually be produced in sufficient concentrations to activate the R protein. Although the transcription of some *P. aeruginosa* genes can be induced prematurely by adding AHLs<sup>63</sup>, in common with bioluminescence genes that can be induced prematurely in *V. fischeri*<sup>64</sup>, other QS-regulated genes cannot be activated prematurely<sup>63</sup>. Possible explanations are that the concentration of the R protein is limiting during exponential growth, or that additional uncharacterized regulatory proteins superimpose control on the expression of some QS-regulated genes.

**PQS.** The recently described QS molecule PQS is intricately linked to the other QS systems of *P. aeruginosa*. LasR regulates PQS production, which, in turn, is necessary for transcription of the *rhIR* and *rhII* genes, thereby creating a regulatory link between the *las* and *rhl* QS systems<sup>27,65</sup>. PQS is synthesized from anthranilate<sup>66</sup> by the products of the *pqs* operon

**SMALL-MOLECULE ANTAGONISTS**  
In this case, small molecules that are structurally related to AHLs that can compete with the autoinducer for binding to the regulatory R protein.



Box 3 | **Quorum sensing and microbial ecology**

The role of acyl homoserine lactone (AHL) signalling in intercellular interactions between bacteria and their biotic and abiotic environments is still poorly understood. As several different species seem to produce the same AHLs, these bacterial pheromones might be signals for communication between cells of different species, perhaps to coordinate community behaviour.

**Distribution of quorum sensing in bacteria**

The environmental distribution of AHL-mediated systems among bacteria has to be considered. It has been reported that production of AHLs is restricted to members of the Proteobacteria, and that within the Proteobacteria, only 21 bacterial genera (4% of the total number of bacterial genera) contain species that are known to produce AHLs<sup>102,103</sup>.

**Rhizosphere communities**

Steidle and co-workers showed that the indigenous bacterial communities that colonize the roots of tomato plants growing in non-sterile soil produce AHL molecules<sup>104</sup>. They also developed tools for *in situ* visualization of AHL-mediated communication between individual cells in the rhizosphere of tomato plants. One possibility is that, in such natural habitats, the different species communicate with one another to coordinate their behaviours.

**Biofilms and infection**

Many QS-controlled genes encode virulence factors that are important for bacteria–host interactions during infection. For example, QS mutants of *Pseudomonas aeruginosa* are attenuated in a mouse model<sup>105,106</sup>. QS regulation is also involved in biofilm development of *Serratia liquefaciens*<sup>107</sup> or of *P. aeruginosa*, in the lungs of cystic fibrosis (CF) patients<sup>59,108</sup>. Moreover, interspecies communication has been reported during infection; for example, in CF lungs that are colonized by both *P. aeruginosa* and *Burkholderia cepacia*. In addition to producing and responding to its own AHL, *B. cepacia* responds to AHLs that are produced by *P. aeruginosa*, which promotes the formation of a mixed-species biofilm<sup>109</sup>. Interactions between *P. aeruginosa* and avirulent oropharyngeal flora strains isolated from sputum samples of CF patients have also been investigated<sup>110</sup>. These studies indicated important contributions of the host microflora to *P. aeruginosa* infection by modulating gene expression through interspecies communication. Autoinducer-2 (AI-2) is the signalling molecule that is involved in this interspecies communication and was produced in sputum cultures by most isolates from CF sputum. However, the *P. aeruginosa* PAO1 genome does not contain *luxS*, which is necessary for production of AI-2 (REFS 100,110).

**Mixed communities**

Some eukaryotic hosts produce AHL analogues that could disrupt QS regulatory systems, probably for protection — for example, *Delisea pulchra* (red alga) produces halogenated furanones that are structurally similar to the AHL signals<sup>111,112</sup>. These halogenated furanones could inhibit AHL-mediated gene expression either by displacing the AHL signal from the R protein<sup>113</sup> or through accelerated R protein turnover<sup>114</sup>.

Some bacteria produce enzymes that interfere with AHL signalling presumably to gain a competitive advantage in specific ecological niches. An AHL lactonase (AiiA) from *Bacillus* species<sup>115</sup> inactivates AHLs by hydrolysis of the lactone bond. Expression of the *aiiA* gene in *Erwinia carotovora*, a plant pathogen, eliminates AHL production and decreases infectivity<sup>116</sup>. In a mixed biofilm, a *Ralstonia* strain (XJ12B) has been shown to produce another type of AHL hydrolase, AiiD, which has an AHL acylase activity (hydrolysis of the acyl side chain). Expression of the *aiiD* gene in *P. aeruginosa* quenched AHL-regulated behaviours such as swarming motility, virulence-factor production and paralysis of *Caenorhabditis elegans*<sup>117</sup>.

*pqsABCDEH*. The immediate precursor of the PQS signalling molecule is HHQ (4-hydroxy-2-heptyl-quinoline), which is itself released from, and taken up by, bacterial cells<sup>67</sup>. The expression of *pqsABCDE* is regulated by a LysR-like transcriptional regulator PqsR (designated MvfR in *P. aeruginosa* strain PA14; REF. 68). By contrast, *pqsH*, which is necessary for the conversion of HHQ to PQS, is positively regulated by LasR in complex with 3O-C12-HSL<sup>69</sup>. Production of PQS is not only induced by the *las* QS system but is also repressed by the *rhl* system, indicating that there is a balance between these QS systems<sup>70</sup>. PQS is produced maximally during the late stationary phase of growth<sup>65</sup>. Nevertheless, under certain growth conditions, PQS is detectable at the onset of stationary phase and is produced in the absence of LasR<sup>71</sup>. Under these conditions there is LasR-independent activation of the *rhl* QS system, which is accompanied by LasR-independent production of PQS. The molecular mechanism by which PQS controls gene expression is unknown.

**Environmental factors.** Environmental conditions can modulate expression of the QS regulators. The expression of the *traR* gene of *A. tumefaciens* is induced by nutrients called opines that are released from plant tumours<sup>72</sup>. Opines are imported into bacterial cells and metabolized. They bind to one of the bacterial regulatory proteins OccR or AccR, depending on the strain of *A. tumefaciens*. The activated regulatory protein regulates the initiation of transcription of *traR* through induction (OccR) or derepression (AccR)<sup>73</sup>. This control restricts the function of the R protein to a plant-associated (host) environment. So, although the QS network is classical, and similar to that of the *V. fischeri* model, an additional regulatory network, which is required for proper induction of conjugal plasmid transfer, is superimposed on the Tra system. In *P. aeruginosa*, the *lasR* and *rhlR* genes are positively controlled by the GacA two-component response regulator, which is a global regulator that is strictly required for the production of several virulence factors<sup>74</sup>. Moreover, the synthesis of a third *P. aeruginosa* R protein, QscR (which is thought to have a role as an anti-activator), is also

positively regulated by GacA<sup>22</sup>. Therefore, in response to unknown environmental signals, the expression of the three R genes can be modulated. In *P. aeruginosa*, the adenylate cyclase CyaB, its product cAMP and the cAMP-dependent transcriptional regulator Vfr constitute an important signalling network that acts as a master regulator of the expression of approximately 100 genes. Vfr, a functional homologue of the catabolite repressor protein CRP, responds to the metabolic and energetic status of the cell<sup>75,76</sup>. *lasR* gene expression is under Vfr control<sup>77</sup> and, more recently, *rhlR* expression has also been shown to be partly dependent on Vfr<sup>78</sup>.

**QS and growth-phase regulation.** Synthesis of the LasR and RhlR proteins is regulated by the growth phase and growth conditions<sup>49</sup>. The *rpoS* gene encodes the specific stationary phase sigma factor, RpoS. The connection between QS and RpoS is complex and the two regulatory systems have a variety of effects on each other. There were some controversies between several published data, but recent studies have confirmed that all the available data are consistent with QS stimulation of *rpoS* expression by about twofold<sup>19,80,53,49,79</sup>.

The regulatory protein AlgR2 (AlgQ), which was originally identified as a regulator of alginate biosynthesis<sup>81,82</sup>, seems to regulate several functions that are related to the metabolic and energetic status of the cell<sup>83</sup>, and also modulates transcription of *lasR* and *rhlR*<sup>84</sup>.

Recently, several loci have been identified that affect the timing of expression of QS-controlled genes in *P. aeruginosa*. Deletion of *rpoS*, *qscR* and *rsmA* led to premature expression of several QS-regulated genes including *rhlI* and *lasI*<sup>21,80,85</sup>. To identify other loci that affect the transcription of QS-regulated genes, a library of chromosomal transposon mutants was screened for altered expression of virulence factors<sup>86</sup>. Among the mutants that were isolated, one was located in the *mvaT* gene, a new member of the *P. aeruginosa* QS regulon. Mutations in *mvaT* led to increased expression of QS-regulated genes and premature expression of several QS-regulated genes after addition of exogenous AHLs. MvaT is homologous to a heterodimeric transcription factor in *Pseudomonas mevalonii*<sup>87</sup>, which regulates mevalonate catabolism. In *P. aeruginosa*, MvaT is a global regulatory protein involved in the growth-phase-dependent control of several QS-regulated genes<sup>86</sup>. MvaT also represses the expression of the three fimbrial *cup* gene clusters that are involved in biofilm formation<sup>88</sup>. MvaT proteins in *Pseudomonas* spp. were recently shown to be a new class of H-NS-like proteins<sup>89,90</sup>. H-NS proteins have a role in bacterial nucleoid organization and in the expression of genes involved in adaptation to environmental challenges<sup>89,91</sup>.

In conclusion, it seems that many different systems are involved in the growth-phase-dependent control of QS, and that a specific autoinducer concentration is required, but is not sufficient for, expression of some late QS-regulated genes. It seems likely that growth-phase-dependent control of QS-dependent genes is multifactorial and that there are additional layers of control that prevent late-response genes from being expressed early.

### Quorum sensing — a global analysis

In many bacteria, virulence factors are regulated by QS<sup>1</sup>, but how global is the impact of QS regulation on cellular physiology? Using microarrays to analyse transcriptional responses, a more comprehensive evaluation of *P. aeruginosa* QS regulation was recently made. Three different research groups have used microarrays to analyse the QS-regulated transcriptome of *P. aeruginosa*, enabling comparisons to be made between independent studies<sup>53,49,92,93</sup>. A large number of genes were shown to be QS-regulated, with 6–10% of the total genome affected; estimates of the number of QS-regulated genes included 315 activated genes and 38 repressed genes<sup>53</sup>, 394 activated genes and 222 repressed genes<sup>49</sup>, and 163 QS-regulated genes<sup>92</sup>. Ninety-seven induced genes were identified in all three studies. Some genes were transcriptionally activated or repressed in response to 3O-C12-HSL, some were transcriptionally activated or repressed in response to C4-HSL and a subset of genes responded differently to these two AHLs, indicating signal-specificity. Changes in media composition and oxygen concentration seemed to have significant effects on the microarray data produced. So, it is important to appreciate that under different experimental conditions, additional genes can be differently regulated by QS. However, some interesting and general observations can be made that add new insights into the links between QS regulation and cell physiology.

Many QS-regulated genes are involved in basic cellular processes such as DNA replication, RNA transcription and translation, cell division and amino acid synthesis. The diverse functions of QS-regulated genes increases the physiological impact of QS (BOX 3). An important finding of these global studies is that a significant number of genes seem to be repressed by QS. However, temporal patterns of gene expression during growth indicate that most genes are activated by QS during the transition from the logarithmic phase to the stationary phase of growth, when the cell density markedly increases. Nevertheless, a small number of loci showed the largest induction response early in growth when cell density is low. Finally, induction is not a permanent switch and transcription responses of some genes are transient and cease shortly after induction<sup>53</sup>. Finally, induction of most genes could not be prematurely induced by the addition of autoinducers. So, microarray analyses have confirmed and extended earlier observations that adding exogenous AHL prematurely has little effect on the timing of gene expression for a large number of genes<sup>53</sup>. It is important to determine if the physiological importance and diversity of QS can be extended to other bacterial species.

### Implications and future directions

The past ten years have seen the description of several QS systems in different bacteria. The mechanisms involved are much more complex than the simple models that were originally proposed. Solving the crystal structure of the TraR protein has advanced our understanding of the function of the R proteins, but TraR could be a specific case and we now eagerly anticipate molecular characterization of the other R proteins to enable several questions to be addressed. How

and when do they dimerize? How do they bind to their ligands? How do they interact with other proteins, such as the anti-activators that have been described for *A. tumefaciens* and *P. aeruginosa*? Are anti-activators common and can generalizations be made? R proteins can have positive and negative regulatory functions but whether they act as true repressors at the level of

transcription initiation remains to be proven. The integration of QS factors into the global regulatory networks of the cell is particularly important. There is clearly a need for systematic global and temporal methodologies for understanding QS, which could have implications for interruption of biofilm formation and the design of new antibacterial molecules.

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## Competing interests statement

The authors declare that they have no competing financial interests.

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