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Quorum-Sensing Signal-Response Systems in Gram-Negative Bacteria

Kai Papenfort[1,#] and **Bonnie Bassler**[2,3,#]

[1]Department of Biology I, Ludwig-Maximilians-University Munich, Martinsried, Germany

[2]Department of Molecular Biology, Princeton University, USA

[3]Howard Hughes Medical Institute, Chevy Chase, MD 20815

Abstract / Preface

Bacteria use quorum sensing to orchestrate gene expression programmes that underlie collective behaviours. Quorum sensing relies on the production, release, detection and group-level response to extracellular signalling molecules, which are called autoinducers. Recent work has discovered new autoinducers in Gram-negative bacteria, shown how these molecules are recognized by cognate receptors, revealed new regulatory components that are embedded in canonical signalling circuits and identified novel regulatory network designs. In this Review we examine how, together, these features of quorum sensing signal–response systems combine to control collective behaviours in Gram-negative bacteria and we discuss the implications for host–microbial associations and antibacterial therapy.

Introduction

Quorum sensing is a cell-to-cell communication process that enables bacteria to collectively modify behaviour in response to changes in the cell density and species composition of the surrounding microbial community. Quorum sensing involves the production, release and group-wide detection of extracellular signalling molecules, which are called autoinducers. Autoinducers accumulate in the environment as bacterial population density increases. Bacteria monitor changes in the concentration of autoinducers to track changes in their cell numbers and to collectively alter global patterns of gene expression. Processes that are controlled by quorum sensing, such as bioluminescence, the secretion of virulence factors, the production of public goods and the formation of biofilms, are unproductive and costly when undertaken by a single bacterial cell, but become effective when undertaken by the group[1].

Both Gram-positive and Gram-negative bacteria use quorum sensing. Gram-positive systems typically use secreted oligopeptides and two-component systems, which consist of membrane-bound sensor kinase receptors and cytoplasmic transcription factors that direct alterations in gene expression. The biological roles of quorum sensing in Gram-positive

[#]*Corresponding authors:* Bonnie Bassler, bbassler@princeton.edu, Phone: +1-609-258-2857, Department of Molecular Biology, Princeton University, Lewis Thomas Laboratory, Washington Road, 08544 Princeton, NJ, USA, Kai Papenfort, kai.papenfort@lmu.de, Phone: +49-89-2180-74502, Department of Biology I, LMU, Munich, Biocenter, Groβhaderner Str.2-4, 82152 Martinsried, Germany.

bacteria have been extensively reviewed elsewhere[2–4]. In this Review, we focus on quorum sensing in Gram-negative bacteria and highlight unusual signalling molecules, novel regulatory components and heterogeneity in quorum sensing responses.

Four common features are found in nearly all known Gram-negative quorum sensing systems[5]. First, the autoinducers in such systems are acyl-homoserine lactones (AHLs) or other molecules that are synthesized from *S*-adenosylmethionine (SAM), and they are able to diffuse freely through the bacterial membrane. Second, autoinducers are bound by specific receptors that reside either in the inner membrane or in the cytoplasm. Third, quorum sensing typically alters dozens to hundreds of genes that underpin various biological processes. Fourth, in a process called autoinduction, autoinducer-driven activation of quorum sensing stimulates the increased synthesis of the autoinducer, which establishes a feed-forward loop that is proposed to promote synchronous gene expression in the population.

Gram-negative bacteria often use several autoinducers, and new studies are revealing the molecular determinants that provide the receptors extraordinary specificity in distinguishing between closely related molecules. Quorum sensing information is often integrated by small RNAs (sRNAs)[6] that control target gene expression and that also function in feedback loops. Quorum sensing network architectures promote signalling fidelity, temporal control and flexible input–output dynamics. Important questions regarding quorum sensing are: how do bacterial cells prioritize one autoinducer over another? How do network features enable optimal performance? And what are the requirements that enable quorum sensing systems to tune their input–output relations to changing stimuli?

Quorum sensing underpins collective behaviours that often involve expensive public goods[7]. Placing such assets under collective control avoids exploitation of those goods. Nonetheless, recent evidence suggests that stochastic processes are also relevant; for example, phenotypic heterogeneity that stems from pathways that are controlled by quorum sensing may enable bet-hedging and division of labour among constituent members of a bacterial population[8]. How individual heterogeneity can be embedded in processes that are synchronously executed at the population level is being intensively investigated[9]. Quorum sensing heterogeneity could also be crucial for neighbouring cells that are not close relatives — for example, in the microbiota of the host[10]. Autoinducers and other molecules that are produced by both prokaryotic and eukaryotic organisms could be used for one-way, two-way or multi-way communication. Appropriate interpretation of the information that is contained in such chemical blends at the individual and population levels could be crucial for the survival of individual cells and for protection of the host and its established microbiota from bacterial, fungal or viral invaders. Indeed, in eukaryotic hosts, autoinducers provide probiotic functions, alter the composition of the microbiota, affect the expression of virulence genes and encourage pathogens to disperse from biofilms[11].

This Review focuses on new Gram-negative bacterial autoinducers, receptors, design principles that control regulatory network architectures and the coordinated responses that quorum sensing controls. We discuss newly discovered functions that are mediated by quorum sensing, highlight their relevance for collective bacterial behaviours, the possibility

of heterogeneity in quorum sensing responses, and we emphasize roles in host–bacterial interactions.

Autoinducers, receptors, and specificity

Bacteria that live in heterogeneous populations presumably encounter complex mixtures of autoinducers that are produced by themselves, their clonal siblings, close relatives, and their non-kin neighbours, which could be fierce competitors[7,12,13]. Thus, bacteria face the challenge of extracting information from mixtures of related and unrelated molecules. This issue is compounded by the fact that bacteria often rely on producing and detecting several autoinducers. How bacteria correctly interpret blends of molecules that are produced by themselves and by other species in the vicinity, and how they elicit appropriate and coordinated changes in gene expression in response to these blends are important questions.

Autoinducers

In Gram-negative bacteria, AHLs are the most common class of autoinducers. They have a core *N*-acylated homoserine-lactone ring and a 4–18 carbon acyl chain that can contain modifications[14] (FIG. 1a). Hundreds of bacterial species contain LuxI-type synthases that produce these AHLs[15]. The length of the acyl chain can affect stability, which may have consequences for signalling dynamics[16].

LuxI enzymes produce AHLs by deriving the lactone moiety from SAM, and, in most cases, the particular acyl chain is obtained from intermediates of fatty acid biosynthesis. One remarkable exception is the plant-associated photosynthetic bacterium *Rhodopseudomonas palustris* in which the LuxI-type enzyme, 4-coumaroyl-homoserine lactone synthase (RpaI), produces *p*-coumaroyl-homoserine lactone (HSL), for which the acyl group comes from the host metabolite *p*-coumarate[17] (FIG. 1a). Using a plant-derived compound enables *R. palustris* to connect its quorum sensing response to bacterial population density and the availability of plant consumables. Other plant-associated bacteria synthesize unusual HSL autoinducers. *Bradyrhizobium japanicum* and *Aeromonas* spp. produce isovaleryl-HSL18, whereas *Bradyrhizobium* BTAi produces cinnamoyl-HSL[19] (FIG. 1a), but all of these species use bacterial substrates.

Two other plant-associated bacteria, *Ralstonia solanacearum* and *Xanthomonas campestris*, produce atypical autoinducers. Depending on the strain, the PhcB protein of *R. solanacearum* synthesizes one of two related autoinducers, 3-hydroxypalmitic-acid-methyl-ester (3-OH PAME)[20] and (*R*)-methyl-3-hydroxymyristate ((*R*)-3-OH MAME; FIG. 1b)[21]. These autoinducers control virulence and the formation of biofilms[22]. *X. campestris* also uses *cis*-11-methyl-2-dodecenoic acid, which is known as diffusible signal factor (DSF; FIG. 1c), to modulate transitions between its planktonic and biofilm-associated lifestyles[23]. Structural homologues of DSF have been discovered recently, including in human pathogens, such as *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*[24]. All DSF-type molecules are synthesized by RpfF proteins[25] (FIG. 1c). Interestingly, one organism can generate several DSF signals, all of which are synthesized by a single RpfF protein[26].

Many bacteria produce and detect several autoinducers. The bioluminescent marine bacterium *Vibrio harveyi* was the first bacterium that was discovered to use several autoinducers and it remains the model for understanding how bacteria process chemical blends. *V. harveyi* uses three autoinducers for intra-species, intra-genera and inter-species communication[27] to regulate approximately 600 target genes[28]. *V. harveyi* produces a canonical AHL, 3OH-C4-HSL (HAI-1; FIG. 1a), using the LuxM synthase[29,30]. Surprisingly, LuxM is not a LuxI homologue, but it carries out analogous reactions using SAM and fatty-acid intermediates as substrates[31]. As far as is known, only *V. harveyi* and its closest relative *Vibrio parahaemolyticus* produce HAI-1, which suggests that this autoinducer is used for intra-species communication[5].

V. harveyi also uses (*Z*)-3-aminoundec-2-en-4-one as an autoinducer (FIG. 1d). The related molecule, (*S*)-3-hydroxytriecan-4-one, was discovered first as an autoinducer in *Vibrio cholerae*[32] (FIG. 1d). Collectively, these molecules are called cholera autoinducer 1 (CAI-1). In *V. cholerae*, the CAI-1 autoinducer synthase (CqsA) acts on SAM and decanoyl-CoA to produce amino-CAI-1, which is immediately converted, possibly spontaneously, into CAI-1. Both amino-CAI-1 and CAI-1 are biologically active; however, CAI-1 predominates in cell-free culture fluids[33–35]. Amino-CAI-1 is more stable than CAI-1 (REF. 33), which raises the possibility that CAI-1 promotes a rapid response to fluctuations of autoinducer. CqsA enzymes exist in all *Vibrio* spp. and they can produce various CAI-1 moieties that have different acyl chain lengths and modifications. *Vibrio* spp. respond to each other's CAI-1s with different affinities than to their own CAI-1s, which suggests that CAI-1 is used for intra-*Vibrio* communication. Curiously, other than *Vibrio* spp., c*qsA* homologues exist in the distantly related bacteria *Legionella pneumophila* and *Janthinobacterium* sp. HH01 (REFS 36,37). In *L. pneumophila*, the corresponding autoinducer, 3-hydroxypentadecane-4-one (LAI-1), regulates DNA uptake and host cell interaction, which implicates LAI-1 in inter-kingdom communication[38].

The final autoinducer in *V. harveyi* is autoinducer 2 (AI-2), which consists of a set of interconverting autoinducer molecules that are all derived from 4,5-dihydroxy-2,3-pentanedione (DPD; FIG. 1e)[39]. LuxS, the DPD synthase, is present in more than 500 bacterial species, making AI-2 the most common bacterial autoinducer identified to date[40]. DPD is highly reactive and it spontaneously cyclizes into various furanone moieties. Specific bacterial species detect different forms of DPD as their active AI-2 signals. For example, in *V. harveyi*, AI-2 contains boron[41], whereas, in *Escherichia coli* and *Salmonella* spp., the AI-2 signal is a non-borated cyclized DPD moiety[42] (FIG. 1e). As the different DPDs rapidly interconvert, AI-2 provides a means for inter-species communication[13,43]. Certain bacteria, such as *P. aeruginosa*, do not encode LuxS and thus do not make AI-2. Nonetheless, they can detect AI-2 produced by other bacterial species, and AI-2 alters their gene expression programmes[44].

P. aeruginosa uses two canonical AHL autoinducers (FIG. 1a) as well as non-AHL autoinducers for quorum sensing. Specifically, cyclic dipeptides (2,5-diketopiperazines; DKPs) are generated by tRNA-dependent cyclodipeptide synthases[45] and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) is produced by proteins that are encoded by the non-ribosomal peptide synthase gene cluster *ambBCDE*[46] (FIG. 1f). In addition, a

quinolone (2-heptyl-3-hydroxy-4-quinolone, known as PQS; FIG. 1g) is used as an autoinducer[47]. PQS is produced by proteins that are encoded by the *pqsABCDH* genes, and, together with the two AHLs, controls the formation of biofilms and the production of virulence factors[48]. Quinolones are widely known for their antibiotic and anticancer activities[49], which demonstrates the multi-functionality of particular autoinducers.

Autoinducer multi-functionality has also been reported for *Photorhabdus* species. *Photorhabdus asymbiotica* is an insect and human pathogen that produces dialkylresorcinols[50] (DARs; FIG. 1h), whereas *Photorhabdus luminescens*, in which virulence is limited to nematodes, synthesizes photopyrone autoinducers[51] (PPYs; FIG. 1i). In addition to quorum sensing, PPYs function as insect toxins, whereas DARs have antibiotic activity[50]. Eight different PPYs (PPYA, PPYB, PPYC, PPYD, PPYE, PPYF, PPYG and PPYH) are produced by the PpyS synthase and the DarB ketosynthase produces 2,5-dialkylcyclohexane-1,3-diones (CHDs) from fatty-acid-derived precursors, which can be further oxidized into DARs by the DarA aromatase[52].

Receptors and specificity

Commonly, Gram-negative bacteria use LuxR-type receptors, which are cytoplasmic transcription factors that detect AHLs produced by partner LuxI-type synthases. LuxR proteins contain two functional domains: an amino-terminal ligand-binding domain and a carboxy-terminal DNA-binding domain[53]. In the absence of the cognate autoinducer, most LuxR-type receptors fail to fold and are degraded. By contrast, LuxR proteins that are bound to an autoinducer are stable, dimerize and bind to DNA[54,55]. LuxR–autoinducer complexes associate with short DNA sequences termed '*lux* boxes' upstream of target genes[56–58]. Interestingly, EsaR, the LuxR-type protein from *Pantoea stewartii*, functions as a transcriptional repressor in the absence of its cognate autoinducer and releases DNA following autoinducer binding[59,60].

Structures of four full-length LuxR-type receptors have been solved: TraR (FIG. 2a) from *Agrobacterium tumefaciens*[55,61] and *Rhizobium* sp. NGR234 (REF. 62), QscR (FIG. 2b) from *P. aeruginosa*[63] and CviR (FIG. 2c) from *Chromobacterium violaceum*[64]. Structures of the ligand-binding domains of LasR65 from *P. aeruginosa* and SdiA66 from *E. coli* have also been solved. In all cases, LuxR-type receptors form homodimers. The N-terminal regions of LuxR-type receptors resemble GAF and PAS domains, which are well-known mediators of signal transduction processes[67]. The C-terminal regions have DNA-binding helix–turn–helix domains, which are characteristic of many bacterial transcription factors[68]. Polar residues in the N termini, including three highly conserved tryptophan residues, contact the HSL moiety of the autoinducer, which defines the binding orientation. Residues that provide hydrophobic and van der Waals interactions to acyl chain moieties are less conserved. The acyl chains can occupy the binding pocket in different configurations: short AHLs are extended and point toward the solvent, whereas long chains are bent and face the interior of the pocket[69]. Seemingly, LuxR proteins use a combination of amino-acid variation and flexibility in the binding pocket to achieve AHL binding specificity.

Approximately 76% of annotated LuxR proteins belong to the so-called LuxR-solo class of transcription factors[70], that is, they have no accompanying LuxI synthases. This suggests

that many more autoinducers could exist that are produced by non-LuxI synthases or that are supplied by other bacteria and modulate the activity of these receptors. QscR from *P. aeruginosa* is probably the best-characterized LuxR-solo receptor. QscR has relaxed ligand-binding specificity compared with the two non-solo LuxR receptors, LasR and RhlR. Indeed, QscR activates target gene expression at nanomolar concentrations of C8-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL, 3-oxo-C12-HSL and C14-HSL71 (FIG. 1a). Thus, QscR may be used by *P. aeruginosa* to detect autoinducers that are produced by cohabitating species, such as *Burkholderia cepacia*[72].

The second major class of Gram-negative quorum sensing receptors are the two-component membrane-bound histidine kinases that signal to cytoplasmic transcription factors through phosphorylation. The best-studied examples come from *V. harveyi* and *V. cholerae*[5]. HAI-1, CAI-1 and AI-2 are detected by LuxN[73], CqsS[32] and LuxQ[74], respectively (FIG. 1a,d,e). LuxN is specific to *V. harveyi*, whereas the other two receptors (CqsS and LuxQ) are conserved in *V. cholerae*. The detection of AI-2 also requires the periplasmic protein LuxP[75]. LuxN and CqsS are predicted to contain nine and six transmembrane spanning helices, respectively, which prevent the use of structure-based methods to define autoinducer detection and specificity determinants. Rather, receptor mutagenesis coupled with permutation of the AHL and CAI-1 ligands revealed the LuxN and CqsS binding pockets and uncovered the 'gatekeeper' amino acids that are crucial for distinguishing between autoinducers[12,76]. Both receptors show strict specificity for their cognate ligands. Indeed, LuxN is not activated by any AHL variant and longer AHLs function as potent antagonists[12], which suggests that *V. harveyi* detects non-cognate autoinducers that are produced by competitors and, in response, turns off quorum sensing to avoid the exploitation of its public goods. With respect to CqsS, CAI-1 derivatives that have altered acyl chains fail to activate CqsS, whereas enlargement of the head group converts the autoinducer into an antagonist[76].

The crystal structures of LuxP in complex with the periplasmic domain of LuxQ were determined[74,75]. In the absence of AI-2, the two LuxPQ complexes form a symmetric heterotetramer, which, following AI-2 binding, undergoes a substantial conformational change. Protomer rotation in the periplasmic region breaks the symmetry of the LuxPQ–LuxPQ tetramer, which prevents the phosphorylation of the cytoplasmic domains (see below). In LuxP from *V. harveyi*, two positively charged arginine residues that are located in the binding pocket stabilize the boron-complexed, negatively charged AI-2 moiety and facilitate hydrogen bonding of the ligand to five additional amino acids. Interestingly, AI-2 binding also promotes clustering of LuxPQ–LuxPQ tetramers, which can influence AI-2 sensitivity and response dynamics[75].

Several proteobacteria have an alternative AI-2 detection system. The *lsrACDBFGE* (*lsr* stands for LuxS regulated) operon encodes an ATP-binding cassette transporter (ABC transporter) that internalizes AI-2. The operon also encodes enzymes that are responsible for the degradation of AI-2 (REF.40). The operon is regulated by the LsrR repressor, which binds to a processed AI-2 product in the cytoplasm. In this case, LsrB is the equivalent of LuxP and is located in the periplasm (FIG. 1e). LsrB binds to a boron-free cyclized AI-2 moiety. Three crystal structures of LsrB complexed with AI-2 show that, despite sequence

and structural variation, six highly conserved amino acids drive the interaction of LsrB with AI-2 (REF. 77). Given the low percentage (~11%) of sequence identity between LuxP-like and LsrB-like receptors, it is possible that additional, yet undiscovered, AI-2 receptors exist.

Quorum sensing network architectures

To accurately execute quorum sensing behaviours, bacteria must detect, interpret and integrate extracellular chemical information and convert that information into changes in gene expression. How bacteria achieve these feats is especially interesting when several autoinducers are used and in mixed species consortia[78]. Moreover, the information can be corrupted by internal noise (such as fluctuations in transcript or protein numbers), external changes (temperature, pH, osmolarity, and so on), or if competing bacteria contribute or consume autoinducers, and all of these features require compensation. Systems biology approaches have uncovered common network design principles that occur in quorum sensing systems that are able to overcome these issues[79]. Below, to illustrate these principles, we discuss the two most common canonical network architectures using *Pseudomonas* spp. and *Vibrio* spp. as examples.

Pseudomonas spp. quorum sensing

Pseudomonas spp., specifically *P. aeruginosa*, use a dense network of quorum sensing receptors and regulators (FIG. 3). The major *P. aeruginosa* receptors are LuxR-type receptors that, following autoinducer binding in the cytoplasm, function as DNA-binding transcriptional activators[80]. There are currently four well-known quorum sensing pathways in *P. aeruginosa*: two LuxR and LuxI-type systems called LasR and LasI and RhlR and RhlI, the PqsR-controlled quinolone system and the IQS system that functions under phosphate-limiting conditions[46].

The systems are organized in a hierarchy with LasR at the top of the cascade (FIG. 3). LasR, in complex with 3-oxo-C12-HSL (FIG. 1a), activates a large regulon of downstream genes that includes the *lasI* synthase gene, which leads to autoinduction[81]. The LasR–autoinducer complex also activates the expression of *rhlR* and *rhlI*, which encode the second quorum sensing pathway[48], and the *pqsR* and *pqsABCDH* genes, which encode the PQS system[82]. RhlR operates similarly to LasR, and when bound to C4-HSL (FIG. 1a), activates its own regulon that includes *rhlI* and thereby establishes the second autoinduction feed-forward loop[83,84]. The PqsR–PQS complex feeds back to activate *rhlRI*85, which connects the three signalling modules. In addition, RhlR inhibits the expression of *pqsR* and *pqsABCD*, and this loop is suggested to ensure the correct ratio of 3-oxo-C12-HSL to C4-HSL, which, in turn, dictates the activation of PQS[86]. A recent survey of regulators that affect quorum sensing in *P. aeruginosa* listed 13 transcription factors of which 10 repressed and 3 activated Rhl-directed and/or Las-directed functions[48]. This high degree of interconnectivity highlights how several intracellular and extracellular cues are integrated to modulate the quorum sensing output. Presumably, fine-tuning the response through several layers of regulation enables robust cell–cell communication under diverse conditions.

Interestingly, RhlR is a key quorum sensing component in *P. aeruginosa* that controls the expression of virulence genes[80]. As *rhlI* can be activated by either LasR or PqsR, together

with RhlR bound to C4-HSL, at least one other autoinducer is required for pathogenicity. In wild-type *P. aeruginosa*, the additional required autoinducer is usually supplied by the Las system. However, isolates of *P. aeruginosa* from patients with cystic fibrosis frequently have mutations in *lasR*[87]. In this case, the phosphate starvation protein PhoB can override the necessity for LasR through the activation of IQS production. In turn, IQS activates the expression of the *pqs* genes (FIG. 3), which produces the additional required autoinducer through activation of *rhl* expression[46]. This alternative by-pass mechanism makes virulence gene expression in *P. aeruginosa* immune to mutations in LasR, which could be particularly relevant during chronic infection[48].

Vibrio spp. quorum sensing

V. harveyi and *V. cholerae* provide the second example of a canonical quorum sensing circuit; in this example, the system relies on membrane-bound receptors. Although the advantages and disadvantages of cytoplasmic DNA-binding transcription factors versus membrane-bound receptors are not fully understood, one issue is clear: both types of system must avoid responding to endogenously produced autoinducers before achieving ‘a quorum’. Rapid degradation of LuxR-type proteins in the absence of autoinducer prevents the premature activation of quorum sensing in *Pseudomonas*-type systems[88], whereas localization of the receptors to the membrane in *Vibrio*-type systems decouples the cytosolic production of autoinducers from detection in the periplasm[5].

V. harveyi and *V. cholerae* use CqsS and LuxPQ as quorum sensing receptors, which interact with CAI-1 and AI-2, respectively. In addition, *V. harveyi* uses a third HAI-1 binding receptor, LuxN. In both species, the signalling relays are arranged in parallel (FIG. 4). In the absence of autoinducers, LuxN, LuxPQ and CqsS are kinases that autophosphorylate and shuttle phosphate to LuxU, which passes the phosphate to the response regulator LuxO[89]. Phosphorylated LuxO functions together with σ^{54} (REF. 90) to activate the transcription of genes that encode four (*V. cholerae*) or five (*V. harveyi*) homologous sRNAs, known as the quorum regulatory sRNAs (Qrr sRNAs)[91]. The Qrr sRNAs are Hfq-dependent sRNAs that regulate gene expression by base-pairing with target mRNAs and altering translation[6,92]. The Qrr sRNAs activate or repress the translation of 20 mRNAs[93]. Most importantly, they activate translation of the mRNA that encodes the low cell density master regulator, AphA, and they repress translation of the mRNAs that encode the high cell density master regulators, LuxR in *V. harveyi* and HapR in *V. cholerae*[91] (FIG. 4).

At high cell density, autoinducer binding inhibits autophosphorylation, which enables the phosphatase activities of the receptors to dominate[94]. Dephosphorylated LuxO is inactive, which terminates expression of the *qrr* genes. In the absence of the Qrr sRNAs, *luxR* or *hapR* is dere-pressed and *aphA* is not activated. Under this condition, LuxR or HapR is produced and it activates genes that underpin collective quorum sensing behaviours (FIG. 4).

In addition, the Qrr sRNAs repress *luxMN*, which encode an autoinducer synthase and receptor pair[95] (FIG. 5), and repress translation of *luxO*[96]. The Qrr sRNAs repress *luxR* or *hapR* through catalytic degradation of the mRNA, repress *luxMN* by coupled degradation of the mRNA, repress the translation of *luxO* by sequestering *luxO* mRNA, and they activate *aphA* by revealing the ribosome binding site[97]. Although catalytic degradation of the *luxR*

or *hapR* mRNA by the Qrr sRNAs does not alter the Qrr pool, coupled degradation and sequestration remove Qrr sRNAs from the system[97]. These regulatory mechanisms are crucial for the maintenance of appropriate Qrr pools and overall quorum sensing dynamics (FIG. 5).

The probability that a particular receptor is in the kinase or phosphatase state is dictated by the difference in free energy between the two configurations[73]. This molecular architecture is analogous to chemotaxis receptors in *E. coli* and suggests the general relevance of two-state, free-energy models for bacterial sensor kinases[98].

Importantly, because all quorum sensing receptors in *Vibrio* spp. have both kinase and phosphatase activity, and transfer phosphate to and from the same phosphorelay protein, LuxU, quorum sensing can never be fully turned on or fully turned off unless all the autoinducers are present or absent, respectively.

Quorum sensing dynamics in *Vibrio* spp. are further modulated by the above-mentioned feedback loops as well as other regulatory feedbacks that tune the information that flows through the network. There are six known feedback loops (FIG. 5): First, LuxO autorepresses its own transcription[99]. Second, the Qrr sRNAs sequester the *luxO* mRNA, which represses *luxO* translation[96,97]. Both of these loops limit the production of LuxO at low cell densities, which sets the lower limit below which the Qrr sRNAs, and thus quorum sensing, cannot be further repressed[97,100]. Third, LuxR or HapR activates the expression of the *qrr* genes[101], and the Qrr sRNAs feedback to destabilize the *luxR* or *hapR* mRNA[91]. This double loop makes LuxR-driven or HapR-driven quorum sensing transitions faster[102]. Fourth, LuxR or HapR represses its own transcription, which avoids the runaway production of LuxR or HapR at high cell densities, which places a limit on the possible quorum sensing output[103,104]. Fifth, AphA and LuxR or HapR reciprocally repress one another's transcription, which ensures maximal production of AphA at low cell density and maximal production of LuxR or HapR at high cell density[103]. Sixth, at low cell density, the Qrr sRNAs facilitate the degradation of *luxMN* mRNA, which decreases the synthesis and detection of HAI-1. This loop de-emphasizes the HAI-1 signal at low cell density and enhances HAI-1 sensitivity at high cell density[95]. Presumably, at low cell density, numbers of non-kin cells are crucial to track but at high cell density, monitoring and cooperating with kin cells are key. Indeed, theoretical work suggests that in mixed-species communities, the broad signal AI-2 is more informative during the early stages of biofilm formation, whereas species-specific autoinducers dominate in single-species communities or during later stages of biofilm formation[105,106]. Together, all of these feedback loops guarantee optimal dynamics, fidelity and smooth transitions between quorum sensing states.

Quorum sensing functions

Traditionally, quorum sensing was defined as cell–cell communication among bacteria that results in changes in transcription factor activity, and thus, changes in gene expression. Quorum sensing-directed behaviours were defined as those that require all of the bacteria in the population to act in unison to make the behaviours successful[107,108]. New research broadens these definitions by showing inter-kingdom communication[109], responses by

intracellular small-molecule chemical signals[110], and heterogeneity in gene expression that is controlled by quorum sensing[8].

Quorum sensing has long been known to control the production of virulence factors and the formation of biofilms[3]. Similarly, biofilms and virulence are known to rely on intracellular second-messenger signalling molecules, including cyclic dimeric guanosine monophosphate (c-di-GMP) and cyclic adenosine monophosphate (cAMP)[111]. This overlap is exemplified in *B. cenocepacia*: the DSF-family autoinducer *cis*-2-dodecenoic acid (BDSF) binds to RpfR, which is a protein that contains GGDEF and EAL domains. The binding of BDSF to RpfR causes a decrease in the intracellular concentration of c-di-GMP, which affects swarming motility, the formation of biofilms and virulence[112]. There are other examples of quorum sensing connections to c-di-GMP and cAMP in *Vibrio* spp., pseudomonads and other Gram-negative pathogens[113]. Linking quorum sensing to nucleotide-based second messengers enables the conversion of complex information encoded in autoinducer blends into a single, general intracellular signalling molecule.

Quorum sensing behaviours are often studied in isolation, that is, in well-mixed, shaken cultures and/or in the absence of cooperating or competing microorganisms. However, non-uniform growth conditions and/or mixed communities influence the functions of quorum sensing[114]. For example, fluid flow, especially in complex geometries, influences the temporal and regional activation of quorum sensing-controlled biofilm formation genes among individual members of *V. cholerae* communities, which leads to complex patterns of colonization[115,116]. In the oral cavity, which is non-uniform and subject to flow, AI-2-based communication is required for the formation of multispecies biofilms and the development of dental plaque[117]. In other biofilm communities, quorum sensing promotes competition, at least among non-kin. For example, in *V. cholerae*, quorum sensing activates type VI secretion, causing lysis of neighbouring non-kin cells[118–120], which promotes the scavenging of DNA from lysed cells and horizontal gene transfer[121].

In the gut, AI-2 signalling was recently reported to promote the expansion of Firmicutes over that of Bacteroidetes after antibiotic treatment, which suggests that quorum sensing at least partially shapes the composition of the microbiota[122] (FIG. 6). Interestingly, a much greater proportion of species in the Firmicutes than in the Bacteroidetes encode functional AI-2 signalling systems. Furthermore, AI-2 produced by the gut commensal bacterium *Blautia obeum* (formerly known as *Ruminococcus obeum*) restricts the virulence of *V. cholerae*, which is relevant during recovery from cholera[123]. Interestingly, the *V. cholerae* receptor that is relevant for AI-2 sensing under these conditions is the LuxR-solo transcription factor, VqmA, rather than LuxPQ[124,125].

Modulation of the gut microbiome and/or its activities can also result from inter-kingdom autoinducer signalling[109]. For example, exposure of mammalian epithelial cells to AI-2 induces the production of the inflammatory cytokine interleukin-8 (REF. 126). AI-2 produced by *P. aeruginosa* causes apoptosis in some mammalian cell types[127]. Conversely, enteric bacteria detect the hormones adrenaline and noradrenaline produced by the host using the sensor kinases QseC and QseE, respectively[128]. Most recently, mammalian epithelial cells were found to release an AI-2 mimic in response to bacteria or to the

disruption of tight junctions. The AI-2 mimic is detected by the bacterial AI-2 receptor, LuxP or LsrB, and it activates quorum sensing-driven gene expression in the bacteria[129] (FIG. 6). Exploiting AI-2 as a general communication signal, as opposed to other species-specific autoinducers, could be a strategy that enables the host to maximally communicate with and drive global changes in gene expression in mixed populations such as those that exist in the gut. Similarly, diverse plants and algae produce autoinducer mimics that influence quorum sensing-controlled behaviours in their bacterial colonizers, although, in many cases, the significance of these interactions is unclear[130].

Finally, not all quorum sensing-pathways promote the synchronization of gene expression among all group members (FIG. 6). This phenotypic heterogeneity is considered an important bet-hedging strategy[131]. Quorum sensing-driven heterogeneity has been extensively studied in *V. harveyi* and can be attributed to the phosphorylation status of LuxO (FIG. 4), which has consequences for the formation of biofilms[132,133]. Nonconformist cells have also been reported in other systems; however, in most cases, the molecular mechanisms that underlie heterogeneity are not defined[8]. Recent work in *Pseudomonas putida* suggests that the production of autoinducers can be heterogonous in immature biofilms and that autoinducers can trigger self-induction of quorum sensing functions in individual cells[9], which indicates that the biological function of a quorum sensing signal can vary depending on the growth conditions.

Conclusions

Chemical communication among bacteria through quorum sensing is a central feature of bacterial life that enables bacteria to take a census of the population and discern who their neighbours are, whether they are kin or non-kin, and/or friend or foe. Quorum sensing enables bacteria to orchestrate collective behaviours. In this Review, we have summarized how quorum sensing systems function using a similar set of operating principles, which are embedded in the physical and chemical properties of the autoinducers, the corresponding receptors and their downstream regulators. Quorum sensing is crucial for many bacterial processes, and not surprisingly, synthetic modulators of quorum sensing are being actively pursued to alter bacterial behaviour on demand (BOX 1). It is possible that the principles that underlie bacterial quorum sensing networks are also crucial for collective behaviours in higher organisms. For example, social insects, such as honeybees and ants, use quorum sensing to determine nesting sites[134,135]. Another tantalizing example is that animal hair follicles can only regenerate in concert with nearby follicles, and this collective process follows a quorum sensing-like logic[136]. This and other new research raise the exciting, but now plausible possibility that quorum sensing is not restricted to microorganisms, but rather, is a general mechanism that functions throughout the tree of life.

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Glossary

Two-component systems
A large group of signal-transduction circuits that typically consist of a membrane-bound histidine sensor kinase that detects a specific environmental stimulus and a cognate response regulator that mediates the cellular response, primarily through transcriptional regulation of target genes.

Small RNAs (sRNAs)
Bacterial small RNAs (sRNAs) are a heterogeneous group of post-transcriptional regulators that often act together with the chaperone Hfq.

Public goods
Common-pool resources that are frequently present in biological and social systems. Public goods are available to all members of the community, irrespective if a member contributed to their production or not. Therefore, public goods are prone to exploitation by non-producers.

Feed-forward loop
A common regulatory network motif in biological pathways. The feed-forward loop is composed of two input factors (usually transcriptional regulators), one of which regulates the other, such that both jointly regulate a downstream target genes.

Bet-hedging
A survival strategy that reduces the temporal variance in fitness at the expense of a reduced arithmetic mean fitness.

GAF and PAS domains
Domains that are often conserved in signaling proteins in which they function as ligand binding domains.

van der Waals interactions
Weak attractive or repulsive forces between molecules or atomic groups that donot result from covalent bonds or electrostatic interactions between ions or ionic groups.

ATP-binding casstte transporter (ABC transporter)
A member of a large superfamily of small molecule transport systems that are present in all phyla.

σ^{54}
An alternative sigma factor in bacteria that is encoded by the *rpoN* gene, which was originally identified as a regulator of genes that are involved in nitrogen metabolism.

Hfq
A globally acting RNA-binding protein that facilitates base pairing of bacterial small RNAs with their target mRNAs.

Cyclic dimeric guanosine monophosphate (c-di-GMP)
A second messenger molecule used in signal transduction in a wide variety of bacteria.

Cyclic adenosine monophosphate (cAMP)
A second messenger molecule important in many biological processes in organisms, ranging from bacteria to humans.

GGDEF domain and EAL domains
Protein domains that are ubiquitous in bacteria and function to synthesize and degrade the intracellular signalling molecule cyclic dimeric guanosine monophosphate (c-di-GMP), respectively.

Type VI secretion
Systems that are used by Gram-negative bacteria to inject effector proteins and virulence factors from across the interior of one bacterial cell into another cell called the prey.

Horizontal gene transfer
The exchange of genetic information between organisms in a manner other than by traditional reproduction. Horizontal gene transfer is key for acquisition of antibiotic resistance in bacteria and horizontal gene transfer also has an important role in evolution and generation of diversity.

Persister cells
Isogenic members of a bacterial population that have entered a non-growing or extremely slow-growing physiological state, which makes them tolerant to a wide range of antimicrobials.

Text Box 1: Synthetic Quorum-Sensing Modulators

Disabling bacterial quorum sensing with small molecules has been proposed as a strategy to prevent the formation of biofilms and pathogenicity. The quorum sensing circuits of *Pseudomonas aeruginosa* and *Vibrio cholerae* contain several possible targets.

In *P. aeruginosa*, LasR antagonists have been identified that are derivatives of the native acyl-homoserine lactone (AHL)[137–140]. Structurally unrelated compounds have also been developed and some LasR inhibitors are also RhlR inhibitors[141]. For example, meta-bromo-thiolactone (mBTL) partially represses LasR and RhlR, and blocks the production of virulence factors and the formation of biofilms[140]. Some small molecules can be antagonists of one receptor (for example, RhlR) and agonists of another receptor (for example, LasR)[142], which highlights the inherent complexity in successfully developing anti-quorum-sensing approaches. LasR and RhlR can have opposing regulatory roles for some targets (for example, LasR represses and RhlR activates certain targets and vice versa) and other regulatory pathways can have a role[142,143]. PqsR inhibitors have been synthesized based on the natural 2-heptyl-3-hydroxy-4-quinolone (PQS) ligand[135,136] and have recently been demonstrated to function as anti-virulence agents[144–146]. Of note, blocking the function of PqsR with a small molecule can also interfere with the formation of persister cells[147].

Blocking autoinducer synthases is another option; for example, the biosynthesis of PQS depends on anthranilate as an intermediate, and its production can be inhibited by the anthranilate analogue, methyl anthranilate[148]. A screen for inhibitors of the LasI and RhlI synthases identified salicylic acid, tannic acid and *trans*-cinnamaldehyde. Follow-up mechanistic analyses showed that tannic acid and *trans*-cinnamaldehyde inhibit RhlI[149].

In *V. cholerae*, quorum sensing represses the production of virulence factors and promotes biofilm dispersal. Thus, molecules that prematurely activate quorum sensing are being pursued for therapeutic development. The addition of synthetic cholera autoinducer-1 (CAI-1) represses the production of cholera toxin and the toxin co-regulated pilus[27,32]. Several small-molecule CqsS agonists have been identified that are specific for *Vibrio* spp.[150,151]. An alternative possibility is the inhibition of LuxO, which activates quorum sensing. A high-throughput screen led to the identification of a set of 6-thio-5-azauracil derivatives, such as AzaU, that are potent inhibitors of LuxO ATPase activity[152]. AzaU has broad-spectrum activity against pathogenic *Vibrio* spp. However, AzaU is specific for LuxO proteins and does not antagonize other NtrC homologues and thus, AzaU does not affect growth. Finally, a synthetic inhibitor of 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN; also known as Pfs), an enzyme that is involved in the synthesis of CAI-1 and autoinducer 2 (AI-2)[35,39], blocks quorum sensing in *V. cholerae*[153]. Although MTAN inhibitors block the production of autoinducers and the formation of biofilms, deletion of the gene that encodes MTAN does not prevent biofilm development, which indicates that MTAN inhibitors could operate by a pleiotropic mechanism[154].

Few clinical trials that involve these molecules have been conducted to date. One concern is that the inhibition of quorum sensing could increase the prevalence of virulent

genotypes[155]. Identification of the most effective, resistance-proof and reliable quorum sensing-modulators is a challenging task. Nonetheless, the promise of this innovative strategy for antimicrobial treatment in times of emerging multidrug-resistant bacterial pathogens has led to substantial interest and activity.

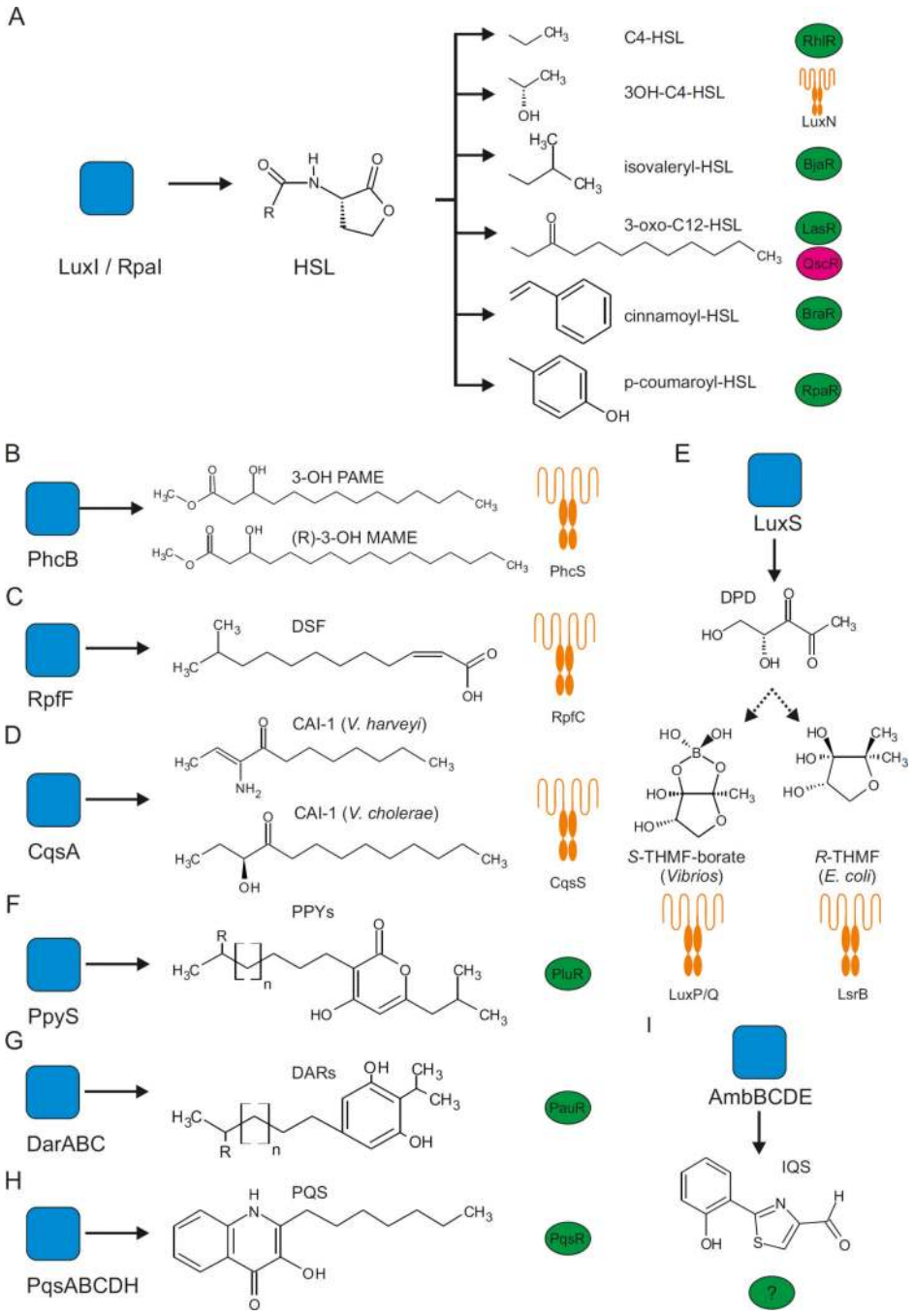

Figure 1. Quorum sensing synthases, autoinducers and receptors

This figure shows the structures of various autoinducers together with their corresponding synthases (blue) and receptors (transcription factors are shown as green and pink ovals and transmembrane receptors are shown as orange schematics). **a** | Homoserine lactone (HSL) autoinducers that are produced by different Gram-negative bacteria. **b** | 3-hydroxypalmitic-acid-methyl-ester (3-OH PAME) and (*R*)-methyl-3-hydroxymyristate ((*R*)-3-OH MAME) are produced and detected by *Ralstonia* spp. **c** | Diffusible signal factor (DSF) is used for quorum sensing in *Xanthomonas campestris*. **d** | The CAI-1 autoinducer synthase (CqsA)

and the CqsS receptor system produces and recognizes various cholera autoinducer 1 (CAI-1) molecules. The *Vibrio harveyi* and *Vibrio cholerae* CAI-1 molecules are shown. **e |** 4,5-dihydroxy-2,3-pentanedione (DPD) is synthesized by all LuxS enzymes and is thus the universal precursor to the widespread family of quorum sensing autoinducers that are collectively designated as autoinducer 2 (AI-2). In the presence of boron, AI-2 forms (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate), the active autoinducer in *Vibrio* spp. In the absence of boron, AI-2 exists as (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), the active autoinducer in enteric bacteria. The LuxPQ and LsrB receptor schematics shown are meant to designate that autoinducer recognition occurs in the periplasm, not the cytoplasm. LuxP and LsrB are homologues of ribose binding proteins. LuxP functions in conjunction with the two-component sensor kinase protein LuxQ and LsrB functions together with a membrane-spanning ATP binding cassette (ABC) transporter complex. **f |** 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) is produced by *Pseudomonas aeruginosa*. The IQS receptor is currently unknown. **g |** The 2-heptyl-3-hydroxy-4-quinolone (PQS) system is one of several quorum sensing systems in *P. aeruginosa*. **h |** *Photorhabdus asymbiotica* uses dialkylresorcinols (DARs) for cell–cell communication. **i |** PpyS of *Photorhabdus luminescens* produces several photopyrones, which are sensed by the PluR transcriptional regulator. *E. coli, Escherichia coli*; RpaI, 4-coumaroyl-homoserine lactone synthase.

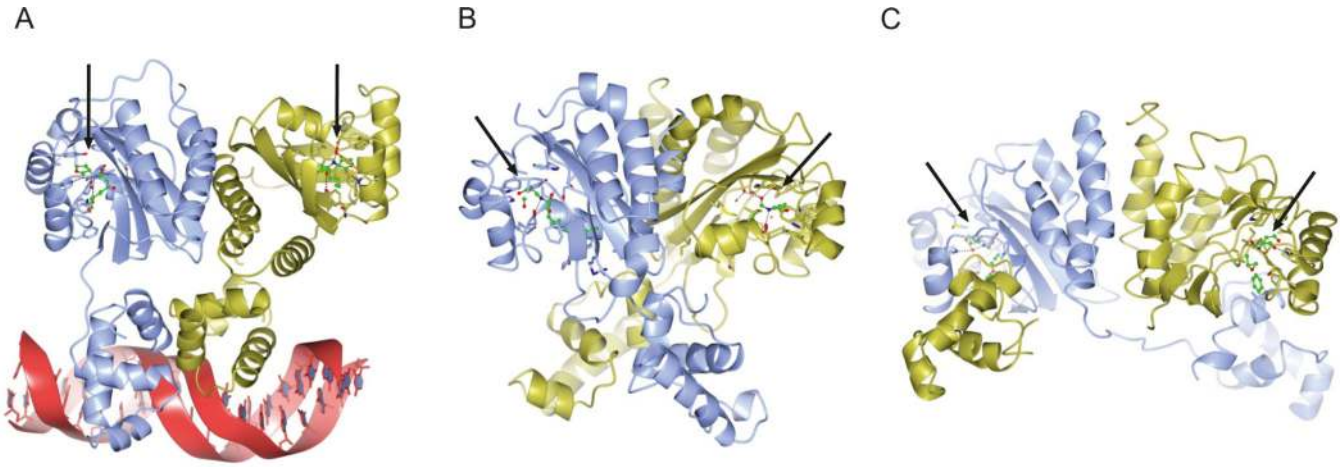

Figure 2. Structures of LuxR-type quorum sensing receptors

This figure shows the crystal structures of four LuxR-type receptors. **a** | TraR from *Agrobacterium tumefaciens* bound to autoinducer and DNA (Protein Data Bank (PDB) entry 1L3L). **b** | QscR from *Pseudomonas aeruginosa* bound to autoinducer (PDB entry 3SZT). **c** | CviR from *Chromobacterium violaceum* bound to an inhibitor called chlorolactone (PDB entry 3QP5). The arrows denote the positions of the ligands. The structures of the ligand-binding domains of all three proteins are similar; however, whereas TraR (panel **a**) adopts an asymmetric dimer, QscR (panel **b**) and CviR (panel **c**) form nearly symmetric cross-subunit architectures. The locations and conformations that are adopted by the DNA-binding domains differ substantially, enabling (panels **a** and **b**) or preventing (panel **c**) DNA binding and transcriptional activation of target genes.

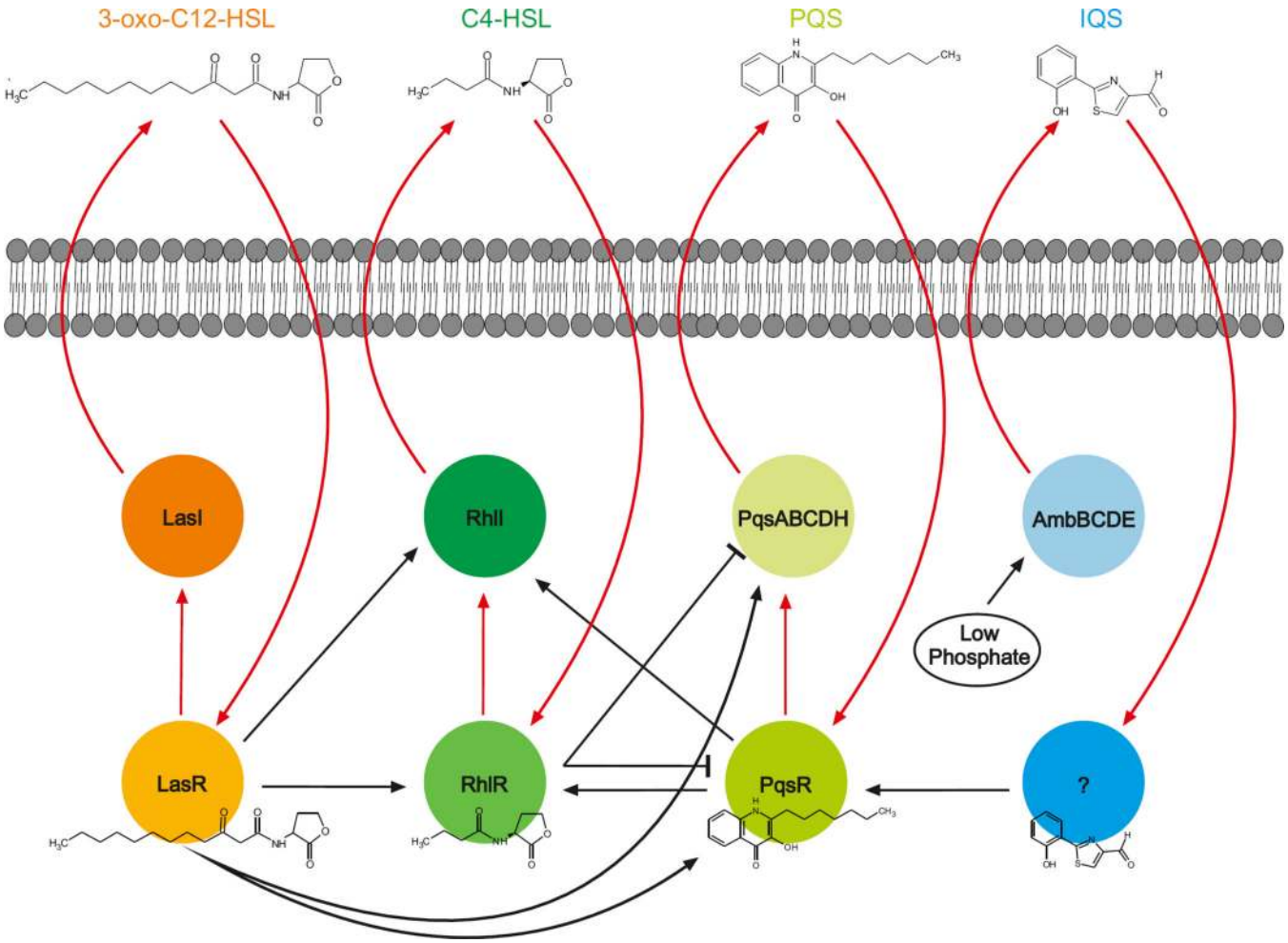

Figure 3. Quorum sensing circuits in *Pseudomonas aeruginosa*

The four autoinducer synthases, LasI, RhlI, PqsABCDH and AmbBCDE, produce the autoinducers, 3-oxo-C12-homoserine lactone (HSL), C4-HSL, 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), respectively. 3-oxo-C12-HSL, C4-HSL and PQS, are recognized by cytoplasmic transcription factors. The receptor for IQS is currently unknown. The production of the IQS signal is induced under phosphate starvation. The individual circuits are highly interconnected and involve autoinduction (red arrows).

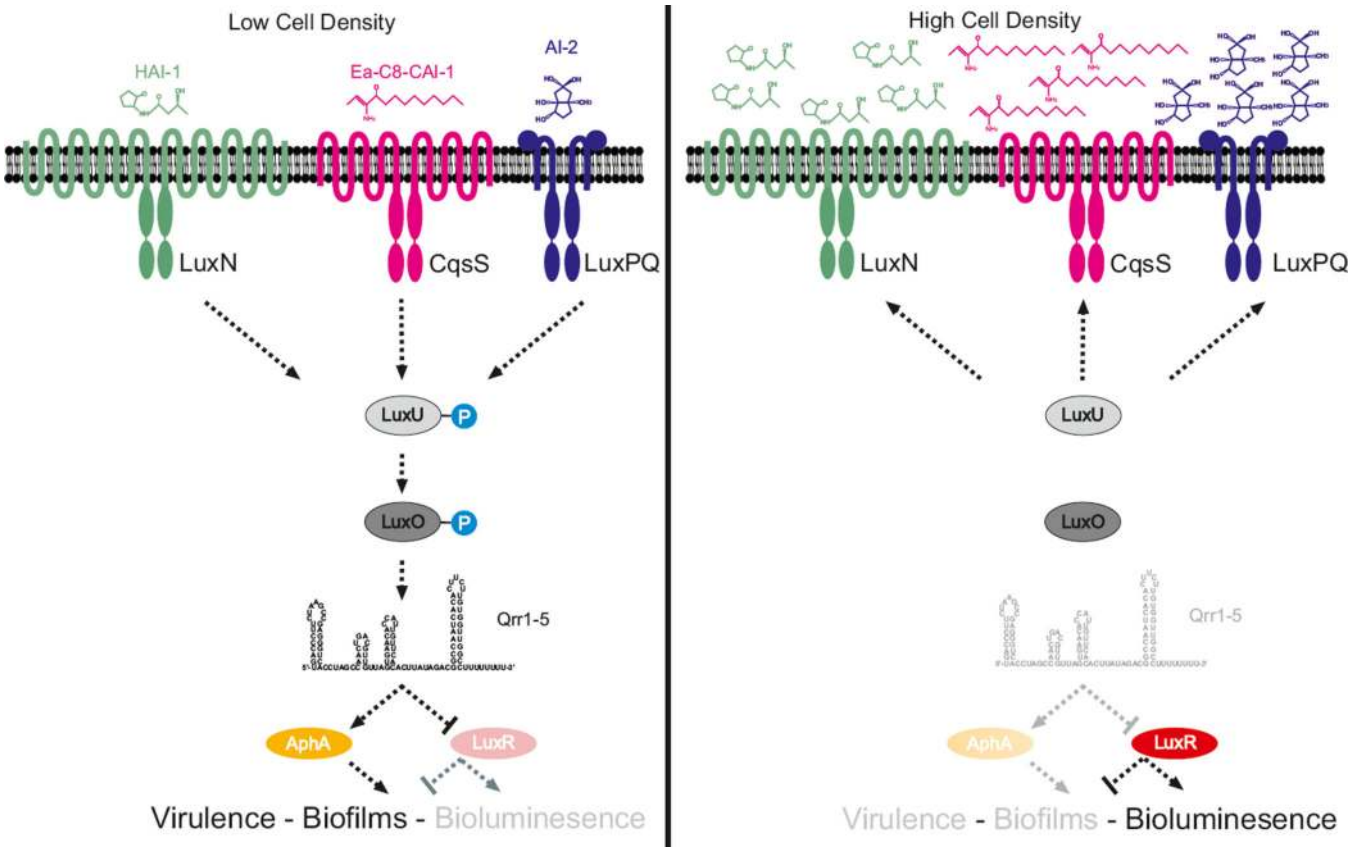

Figure 4. Quorum sensing circuits in *Vibrio harveyi*

Left panel: Signal transduction at low cell densities. During this stage, autoinducer levels are low and the LuxN, LuxPQ and CqsS receptors act as kinases. LuxO is phosphorylated and the quorum regulatory small RNAs (Qrr sRNAs) Qrr1, Qrr2, Qrr3, Qrr4 and Qrr5 (Qrr1–5) are transcribed. The Qrr sRNAs repress *luxR* and activate *aphA*. AphA controls genes that are involved in individual behaviours and activates genes that are required for virulence and the formation of biofilms (in *Vibrio cholerae*). Right panel: Signal transduction at high cell densities. During this stage, autoinducer levels are high and the LuxN, LuxPQ and CqsS receptors function as phosphatases. LuxO is dephosphorylated, the Qrr1–5 sRNAs are not transcribed; therefore, AphA is not produced, whereas LuxR is produced. LuxR controls genes that are required for group behaviours, including genes that are responsible for bioluminescence (in *Vibrio harveyi*). AI-2, autoinducer 2; Ea-C8-CAI-1, (*Z*)-3-aminoundec-2-en-4-one; HAI-1, 3OH-C4-homoserine lactone.

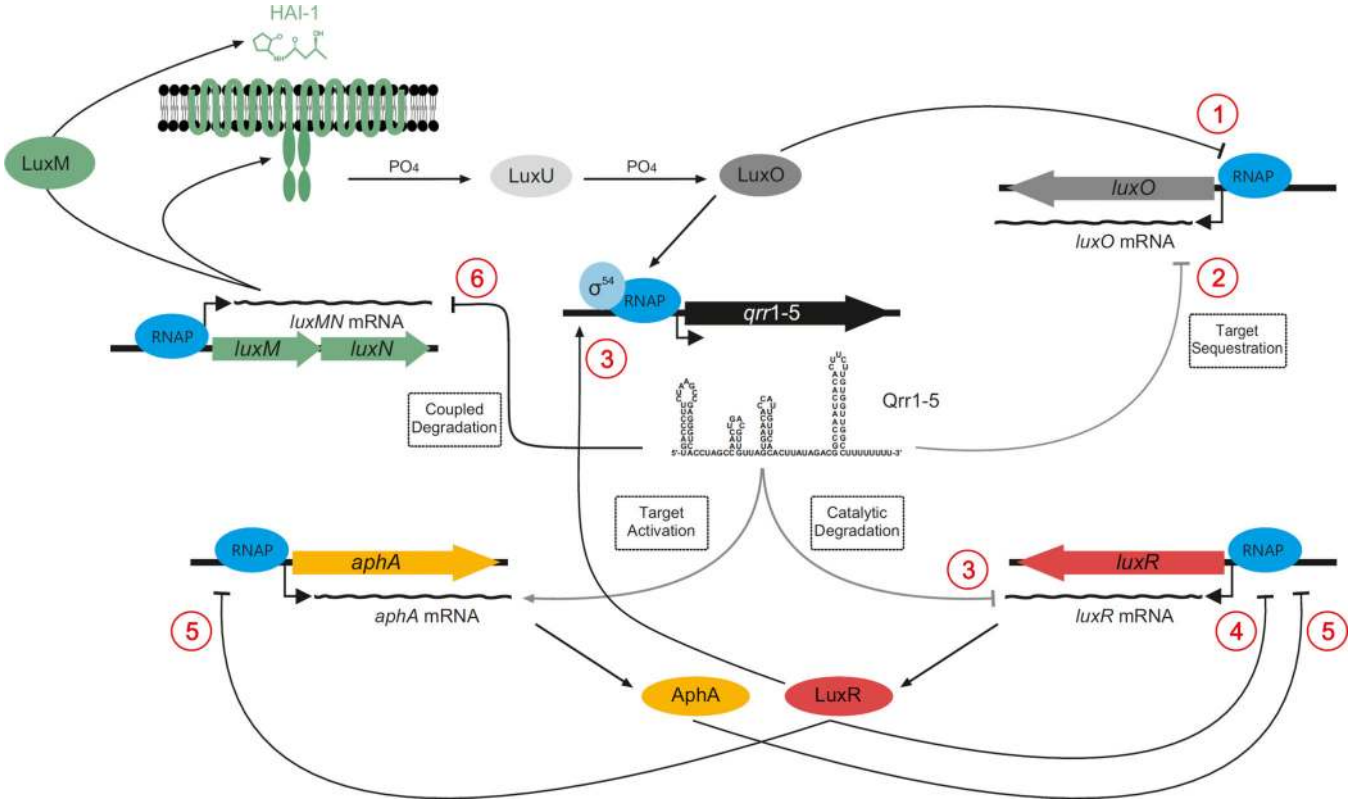

Figure 5. Feedback loops control *Vibrio harveyi* quorum sensing dynamics

Six different feedback loops are embedded in the *Vibrio harveyi* quorum sensing circuit. **a** | LuxO autorepresses its own transcription. **b** | The quorum regulatory small RNAs (Qrr sRNAs) inhibit *luxO* translation by mRNA target sequestration. **c** | LuxR activates *qrr* transcription. The Qrr sRNAs, in turn, inhibit the production of LuxR by catalytic degradation of the *luxR* mRNA. **d** | LuxR represses its own transcription. **e** | AphA and LuxR reciprocally repress each other's transcription. **f** | Base pairing of the Qrr sRNAs with the *luxMN* mRNA facilitates degradation of the RNA duplex (coupled degradation). The arrows denote activation. Inhibitory arrows denote repression. Grey arrows indicate post-transcriptional regulation. All of these feedback loops except the Qrr-to-*luxMN* loop also exist in *Vibrio cholerae*. In *V. cholerae*, LuxR is known as HapR. HAI-1, 3OH-C4-homoserine lactone; RNAP, RNA polymerase.

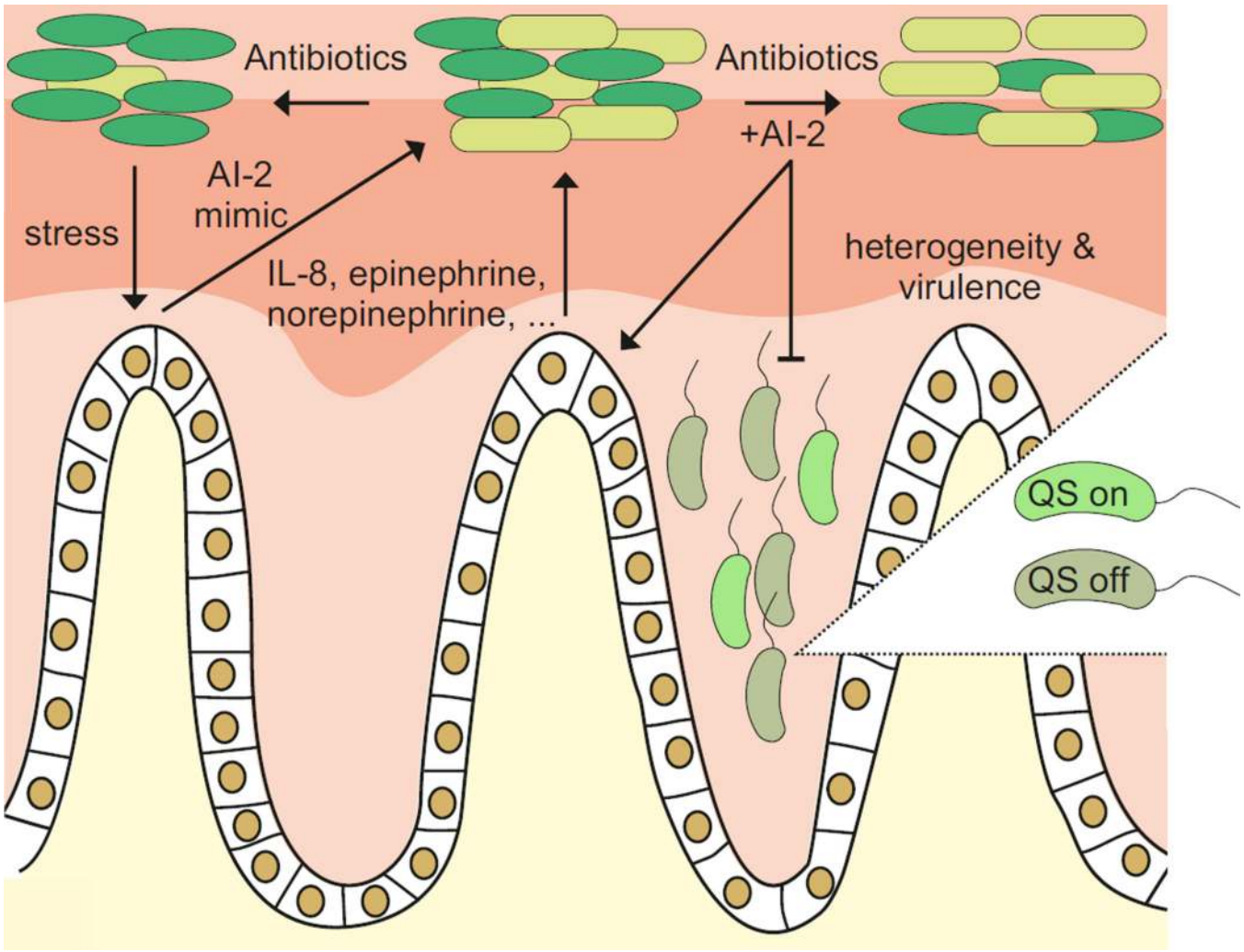

Figure 6. AI-2-mediated quorum sensing in the mammalian gut

Gut microorganisms communicate using autoinducer 2 (AI-2). Treatment with antibiotics can alter the composition of the microbiota, which can be ameliorated by modulating levels of AI-2. Eukaryotic cells produce cytokines, such as interleukin-8 (IL-8), in response to AI-2. Hormones (adrenaline and noradrenaline) and AI-2 mimics are produced by the host and can be detected by bacteria. Quorum sensing can alter phenotypic heterogeneity among isogenic members of a bacterial population, which affects virulence-related traits, such as biofilm formation.