SIGNAL PROCESSING IN
BACTERIAL QUORUM SENSING

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To my parents
Abstract

Cell-to-cell communication in bacteria is a process known as quorum sensing (QS) that relies on the production, detection, and response to the extracellular accumulation of signaling molecules called autoinducers (AIs). The signal processing circuit in the model quorum-sensing bacterium *Vibrio harveyi* is exquisitely designed which involves integration of the information from multiple autoinducers and complicated feedback regulations of the signaling components. Applying quantitative methods and physical reasoning, we investigated how cells integrate and interpret the information contained within multiple autoinducers. Using single-cell fluorescence microscopy, we quantified the signaling responses to and analyzed the integration of multiple autoinducers in the *V. harveyi* cells. Our results revealed that signals from two distinct autoinducers, AI-1 and AI-2, are combined strictly additively in a shared phosphorelay pathway, with each autoinducer contributing very nearly equally to the total response. We found a coherent response across the population with little cell-to-cell variation, indicating that the entire population of cells can reliably distinguish several distinct conditions of external autoinducer concentration. We speculate that the use of multiple autoinducers allows a growing population of cells to synchronize gene expression during a series of distinct developmental stages. We also identified and characterized two negative feedback loops that act to facilitate precise quorum-sensing signaling. The quorum-sensing central response regulator LuxO autorepresses its own transcription and the Qrr small regulatory RNAs (sRNAs) posttranscriptionally repress *luxO* by base-pairing with the *luxO* mRNA.
transcript. We discovered that the two cooperative negative feedback loops determine the point at which *V. harveyi* has reached a quorum and control the range of autoinducers over which the quorum-sensing responses occur. Our findings suggest that sRNA-mediated feedback regulation is a common design feature that permits fine-tuning of gene regulation and maintenance of homeostasis.
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Chapter 1 Introduction to bacterial quorum sensing

1.1 Cell-to-cell communication in bacteria

Cell-to-cell communication is a complex signaling process that is fundamental to the proper functioning and normal development of all living cells [1]. Errors in communication can be fatal, and are implicated in many human diseases. The capability of cells to correctly communicate with one another had been considered to exist only in multicellular organisms. The exchange of chemical signals between cells was assumed to be a trait highly characteristic of eukaryotes. Bacteria, as a simple unicellular organism, were believed to be seclusive, in the sense that communication among individual bacterial cells never happened.

The notion that bacteria live in isolated lifestyle was changed about three decades ago by the discovery of two luminous marine bacterial species, *Vibrio fischeri* and *Vibrio harveyi* [2,3]. Both species were observed to emit light only at high cell-population density in response to the accumulation of secreted signaling molecules. Study of these two luminescent bacterial species proved that cell-to-cell communication using chemical molecules also exist in the primitive bacteria [4,5,6]. Recent explosion of advances in the field of cell-to-cell communication in bacteria has now shown that many or most bacteria
communicate using secreted chemical molecules to coordinate behaviors of the whole group.

This prevalent bacterial cell-to-cell communication process is referred to as quorum sensing (QS) [7,8,9]. In quorum sensing, bacteria produce, release, and detect chemical signaling molecules, called autoinducers (AIs), which accumulate in a cell-density-dependent manner. The accumulation of a stimulatory concentration of extracellular autoinducers can only occur when a sufficient number of cells, i.e. a “quorum”, are present. Thus, autoinducer concentration serves as a proxy for cell number and the process is proposed to be a mechanism for census taking (Figure 1.1-1). Quorum sensing controls diverse behaviors of bacteria. Many of these behaviors are unproductive when undertaken by an individual bacterium but become effective by the simultaneous action of a group of cells. For example, quorum sensing regulates bioluminescence, virulence factor expression, biofilm formation, sporulation, antibiotic production, conjugation, and fruiting body development [8].

Furthermore, we now know that a vast assortment of different types of chemical signaling molecules are employed, and individual species of bacteria usually use more than one type of chemical signal to communicate. Various quorum sensing networks in different bacterial species often involve complex signaling pathways and hierarchical regulatory circuits, which have evolved to properly integrate and accurately process the sensory information. Quorum sensing is not limited to intra-species. Common autoinducers are produced by many different bacterial species to enable inter-species talk.
Figure 1.1-1 Autoinducer concentration serves as a proxy of population cell density in bacterial quorum sensing. At low cell density (LCD), the endogenously produced autoinducers (blue triangle and red circles) are secreted outside of cell and quickly diffuse away. At high cell density (HCD), autoinducers are accumulated and can be detected by other bacteria in the neighborhood. Black circles inside individual bacterial cells indicate chromosomes. Blue and red regions represent genes encoding enzymes for autoinducer production.
Bacteria even communicate with their eukaryotic host, referred as inter-kingdom communication [10,11]. The ability for bacteria to correctly coordinate and precisely integrate all the information obtained from the complicated communications with various groups of living organisms in their natural habitats is critical for their survival and development [12].

1.2 Quorum sensing in gram-negative bacteria

Understanding of bacterial quorum sensing was founded on the studies of the density-dependent production of bioluminescence in the marine symbiotic bacterium Vibrio fischeri [3], which lives in the light organ of the Hawaiian squid Euprymna scolopes [13]. Inside this organ, V. fischeri cells grow to high cell density and genes encoding enzymes for bioluminescence are induced. The squid utilize the light provided by these bacteria for counter-illumination to mask its shadow and avoid predation [14]. The bacteria also benefit since the light organ is rich in nutrients and allows better growth than in seawater.

The light production in V. fischeri is controlled by two regulatory proteins named LuxI and LuxR. LuxI is the autoinducer synthase that catalyzes synthesis of the acyl-homoserine lactone (AHL) autoinducer. LuxR is the cytoplasmic autoinducer receptor and transcriptional activator. LuxR can become active only when bound to autoinducer and thus promote transcription of the operon luxCDABE encoding luciferase and other
enzymes responsible for light production [4, 5]. The AHL autoinducers freely diffuse in and out of the cell and concentration of these signaling molecules increase with increasing cell density [15]. When the autoinducer reaches a critical, threshold concentration, it binds to LuxR and activates it to function as a transcriptional regulator and control expression of quorum-sensing target genes [16]. The LuxR-AHL complex also induces expression of itself – the luxR gene – and the autoinducer synthase luxI. This regulatory configuration creates a positive feedback loop that floods the environment with both signals and receptors and causes the entire population to switch into “quorum-sensing mode” and committee to coordinated behaviors, such as to produce light (Figure 1.2-1).

The quorum sensing circuit in V. fischeri is considered as the paradigm for quorum sensing in most gram-negative bacteria. This simple signal-response mechanism is employed by a large number of other gram-negative bacteria for the control of various cell-density-dependent functions [17, 18]. LuxI-type synthases in different bacteria species produce AHL autoinducers only differ by a particular fatty acyl chain. A diverse set of fatty acyl side chains of varying length, backbone saturation, and side-chain substitutions are incorporated into the AHL signals. These differences are crucial for signaling specificity [19]. Structural analysis of LuxR suggests that the receptors also possess specific acyl-binding pockets that allow each LuxR to be bound and activated only by its cognate AHL autoinducer [20, 21]. In mixed-species environment where multiple AHL autoinducers are present, each species can distinguish, measure, and respond only to the buildup of its own signal. Therefore, the LuxI/LuxR systems are used
Figure 1.2-1 The canonical gram-negative LuxI/LuxR-type quorum-sensing system. Autoinducers are produced by LuxI and can freely diffuse in and out of the cell. With increased cell density, a critical concentration of autoinducers is reached and AI-LuxR complex is formed to activate transcription of the quorum-sensing dependent genes, including luciferase for light production, as well as luxI/luxR to create a positive feedback loop for a quick switch into the “quorum-sensing mode”. AI: autoinducer.
predominantly for intra-species communication due to extreme specificity between the LuxR receptors and their cognate AHL autoinducers.

Additional complexity exists in many of the LuxI/LuxR systems. Actually, bacteria rarely rely exclusively on one LuxI/LuxR quorum-sensing system. Rather, they use one or more LuxI/LuxR-type systems, usually in conjunction with other types of quorum-sensing circuits. Many examples exist in which the backbone of the quorum sensing mechanism is a LuxI/LuxR-type signal-response circuit, upon which further levels of regulation have been layered [22,23].

1.3 Quorum sensing in gram-positive bacteria

Gram-positive bacteria also employ quorum sensing to regulate a variety of processes in response to cell density. However, in contrast to Gram-negative bacteria, which use AHL autoinducers and the LuxI/LuxR-type signaling systems, Gram-positive bacteria communicate using modified oligopeptides as signals and two-component-type membrane-bound sensor histidine kinases as receptors (Figure 1.3-1). In general, the autoinducing peptide is secreted outside of the cell through a dedicated ATP-binding cassette (ABC) transporter and increase in concentration as a function of cell density. In most cases, concomitant with signal release is signal cleavage and modification [24]. The peptide autoinducers are detected by membrane-bound two-component sensor kinases. Similar to the mechanisms by which gram-negative bacteria use the LuxI/LuxR quorum-sensing systems, each gram-positive bacterium uses a unique peptide signal different
Figure 1.3-1 A canonical peptide-mediated two-component model for quorum sensing in Gram-positive bacteria [12]. Autoinducer peptide genes encode peptide signal precursors, which are further modified and secreted through a dedicated ABC transporter. When extracellular concentration of the matured peptide autoinducers (blue octagon with tail) reaches a critical stimulatory level, the peptide ligands bind to the membrane-bound histidine kinase of a two-component signaling system. The sensor kinase autophosphorylates on a conserved histidine residue (H), and subsequently, pass on the phosphoryl group (PO$_4$ or P) to a cognate response regulator on a conserved aspartate residue (D). The phosphorylated response regulator activates the transcription of target genes.
from all other bacteria and the cognate receptors are exquisitely sensitive to that particular oligopeptide. Thus, as in LuxI/LuxR systems, peptide quorum-sensing circuits in Gram-positive bacteria are also used to confer intra-species communication.

Binding of the peptide ligand to the kinase receptor initiates signal transduction via a phosphorylation cascade [25,26]. A series of phosphoryl transfer events culminate in the phosphorylation of a cognate response regulator protein. Phosphorylation of the response regulator activates it and allows it to bind DNA and regulate transcription of the quorum sensing-controlled target genes (Figure 1.3-1).

Many gram-positive bacteria communicate using multiple peptides in combination with other types of quorum-sensing signals. Signaling of quorum-sensing is often coupled with signaling of other environmental cues and complex gene regulations. A variety of processes in Gram-positive bacteria are regulated by quorum sensing. Among these are competence for DNA uptake and sporulation in Bacillus subtilis [27], competence in Streptococcus pneumonia [28], virulence in Staphylococcus aureus [29], and conjugation in Enterococcus faecalis [30].

1.4 The quorum sensing circuit in Vibrio harveyi

Typically, Gram-negative bacteria use acyl-homoserine lactones and Gram-positive bacteria use peptides as autoinducers. To our knowledge, these two kinds of molecules most often promote intra-species cell-cell communication because a particular acyl-
homoserine lactone or particular peptide can only be detected by the bacterial species that produces it [10]. In addition, a non-species-specific autoinducer called AI-2, which is a family of inter-converting molecules all derived from the same precursor 4,5-dihydroxy 2,3-pentanedione, is produced and detected by a large variety of both Gram-negative and Gram-positive bacteria [30,32]. Interestingly, many bacterial species use more than a single autoinducer molecule for quorum sensing. For example, Gram-negative bacteria (e.g. *Rhizobium*) can use multiple homoserine lactones and likewise, Gram-positive bacteria (e.g. *Bacillus*) can use several peptides for communication [10,12,35]. These bacteria have evolved sophisticated quorum-sensing circuits to detect and integrate the information contained in multiple autoinducers.

The first observation that bacteria could communicate with multiple quorum-sensing signals was in the Gram-negative, bioluminescent marine bacterium *Vibrio harveyi* [33,34]. *V. harveyi* produces and detects three autoinducers: AI-1 (3-hydroxybutanoyl homoserine lactone), CAI-1 ((S)-3-hydroxytridecan-4-one), and AI-2 ((2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuranborate) [30,36,37]. AI-1 is only produced by *V. harveyi*, CAI-1 is produced by *V. harveyi* as well as other Vibrios, and AI-2, as discussed, is produced by many bacterial species. Thus AI-1, CAI-1, and AI-2 could provide information about the numbers of *V. harveyi*, Vibrios, and total bacteria in the vicinity, respectively. The three autoinducers are detected extracellularly by their cognate transmembrane receptors: LuxN, CqsS, and LuxPQ, respectively [38]. Information from the autoinducer-sensing pathways is transduced through shared components LuxU and LuxO [39,40,41], and five small regulatory RNAs (sRNAs) [42,43] to the master quorum-sensing regulator LuxR [44] (Figure 1.4-1). LuxR
Figure 1.4-1 The quorum-sensing circuit of wild-type *Vibrio harveyi*. The wild-type quorum-sensing circuit consists of three parallel signaling pathways with three different autoinducers – AI-1, CAI-1, and AI-2. Their synthases are LuxM, CqsA, LuxS, and their transmembrane receptors are LuxN, CqsS, LuxPQ, respectively. In the absence of autoinducers (i.e. at low cell density), the receptors act predominantly as kinases and pass phosphate (designated as “–P”) to LuxU and thence to LuxO. Phosphorylated-LuxO activates transcription of genes encoding five small regulatory RNAs (sRNAs). These sRNAs inhibit the translation of LuxR. In the presence of autoinducers (i.e. at high cell density), the receptors switch to a predominantly phosphatase-active state which reverses the direction of phosphoryl transfer through the circuit, so that LuxO is dephosphorylated and becomes inactive. Therefore, the genes encoding the five sRNAs are not transcribed, luxR mRNA is translated, and LuxR protein is made.
activates and represses genes including those required for bioluminescence, siderophore production, type III secretion, and metalloprotease production [10,12,45,45,46].

1.5 Overview

Quorum sensing is a fundamental, yet very complex signaling process existing in most bacterial species. Quorum sensing allows the unicellular bacteria to communicate within their own species, with other bacterial species in the environment, and also with their host eukaryotes. For bacteria, information obtained from all sorts of communications as well as from environmental conditions must be integrated in a proper and precise way to optimize the control of coordinated behaviors and make best strategies for survival. Therefore, bacteria must have employed exquisite design in the quorum-sensing circuit in order to implement the complicated computations on signaling information. To investigate the mechanisms and principles underlying signal processing in bacterial quorum sensing, we focused on the model quorum-sensing organism *V. harveyi* and applied quantitative methods and physical reasoning to unveil questions such as integration of multiple autoinducers (Chapter 2), feedback regulations on the signaling components (Chapter 3), and other complexity in the quorum-sensing circuit (Chapter 4).
Chapter 2 Quantifying signal integration in *V. harveyi* quorum sensing

2.1 Introduction

It remains a mystery how and why bacteria integrate multiple autoinducer signals and what additional information multiple autoinducers reveal about the cells’ environment that one autoinducer cannot reveal [48]. Furthermore, while in principle, quorum sensing enables bacteria to act in synchrony, the behavior of the entire population is ultimately dictated by events inside single cells. Recent single-cell studies of gene expression in bacteria have revealed that noise is inevitable even for isogenic cells in essentially homogeneous environments and that noise can result in heterogeneous phenotypes within a population [49-55]. Likewise, in quorum sensing, noise could make individual cells behave differently from one another even if they receive identical autoinducer inputs. To understand quorum-sensing signal integration and, ultimately, the evolution of cooperative behaviors at the population level, it is imperative to understand how cells behave individually. Specifically, do cells respond in unison or do they maintain population diversity? Bulk measurements – which focus on the population’s response – generally mask the behavior of individual cells and thus lose information about cell-to-cell variation. To fully understand the molecular mechanism underlying quorum sensing as well as the general principles underlying bacterial communication and cooperation, we must study this process at the single-cell level.
The quantitative single-cell fluorescence-microscopy studies of *V. harveyi* quorum sensing have allowed us to define the mechanism of quorum-sensing autoinducer signal integration. Our studies revealed highly uniform behavior in individual cells, suggesting that the *V. harveyi* quorum-sensing circuit is designed to tightly synchronize the population response to autoinducers. This network operates in stark contrast to other regulatory circuits (e.g., such as that underpinning sporulation in *Bacillus subtilis*), which appear designed to generate diversity among the members of the population [56-59]. We also discovered that information from the different autoinducers is integrated in a strictly additive way, with an unexpected balance between the signaling strengths of the different autoinducers, allowing the population as a whole to distinguish multiple states of autoinducer concentration. These results have important implications for the developmental cycle of *V. harveyi* and possibly for other bacteria that use multiple autoinducers.

### 2.2 Results

#### 2.2.1 Construction of *V. harveyi* sensor mutant strains

To investigate the mechanism underlying how *V. harveyi* integrates the information contained in its multiple autoinducers, we engineered strains that allowed us to examine each quorum-sensing signaling pathway in isolation as well as strains that allowed us to analyze the signaling properties of the combined pathways. We focused only on
integration of signals from autoinducers AI-1 and AI-2 through the LuxN and LuxPQ pathways, respectively. We did not study CAI-1 signaling through CqsA. Our rationale is as follows: first, under our laboratory conditions, the CAI-1 pathway is the weakest of the three signaling pathways, and thus AI-1 and AI-2 are the major inputs influencing quorum-sensing-controlled gene expression; second, we wanted to analyze the simplest possible case of integration of two signals. For this set of experiments, we constructed *V. harveyi* strains carrying only the LuxN, only the LuxPQ pathway, or both pathways. In each case, the *V. harveyi* strains lacked the CqsS pathway. To enable quantitative measurements of signaling through the individual and combined pathways, all the strains were engineered to contain a transcriptional fusion of *gfp* fused to a quorum-sensing responsive promoter. Additionally, all of our strains constitutively produced red fluorescent protein (mCherry) which we used as an internal standard [60] (Figure 2.2-1).

The strains used are as follows: the LuxN⁺ strain carries wild type *luxN* on the chromosome and is deleted for *cqsS* and *luxPQ*. The strain is also deleted for *luxM*, encoding the AI-1 synthase LuxM, and is therefore exclusively responsive to exogenously added AI-1. Similarly, the LuxPQ⁺ strain is deleted for *luxN* and *cqsS* as well as *luxS* encoding the AI-2 synthase. This strain is only responsive to exogenous AI-2. The combined LuxN⁺ LuxPQ⁺ strain lacks *cqsS*, *luxM*, and *luxS* and is responsive to exogenously supplied AI-1 and AI-2. In each strain, *gfp* is fused to the *qrr4* promoter which is one of the genes encoding the quorum-sensing sRNAs that are activated by LuxO-P (Figure 2.2-1). mCherry is driven by the constitutive promoter Ptac inserted at an intergenic region of the chromosome. Because mCherry is expressed constitutively, it reports on the cell’s overall protein level, including variations due to cell size and phase
Figure 2.2-1 Quorum-sensing circuit in the engineered *Vibrio harveyi* sensor mutants. In the LuxN<sup>+</sup> sensor mutant (top), the genes encoding *cqsS*, *luxPQ*, and the gene encoding the AI-1 synthase *luxM* are deleted. As a result, this mutant only responds to exogenously added AI-1. The LuxPQ<sup>+</sup> sensor mutant (middle) responds exclusively to exogenous AI-2, and the LuxN<sup>+</sup> LuxPQ<sup>+</sup> sensor mutant (bottom) responds to exogenous AI-1 and AI-2. We quantify the responses using a *qrr4-gfp* transcriptional reporter fusion that is activated by LuxO-P. As an internal standard for fluorescence, the gene encoding mCherry is fused to a constitutive promoter *Ptac* and integrated at an intergenic region of the chromosome.
of the cell cycle. Normalizing the reporter GFP intensity by the internal standard mCherry intensity therefore provides an accurate measure of quorum-sensing receptor activity, and eliminates errors caused by uneven illumination or inaccurate segmentation of cells during image processing. The engineered *V. harveyi* strains were grown to steady state (Figure 2.2-2) in broth medium containing particular autoinducer concentrations. Cells were transferred to glass slides on a microscope, and phase-contrast and fluorescence snapshots were taken. Microscopy images were processed automatically by a custom computer program to obtain fluorescence intensities of individual cells.

2.2.2 Responses of individual autoinducer-detection pathways

Each autoinducer-detection pathway contributes uniquely to the overall *V. harveyi* integrated quorum-sensing response. Thus, to understand how cells communicate, understanding the signaling properties of the individual quorum-sensing pathways is imperative. Toward this end, we measured dose responses of individual cells of the LuxN*+* mutant responding to AI-1. LuxN*+* mutant cells were grown in series-diluted concentrations of exogenous AI-1 and the distributions of P_{Qrr4}-GFP intensities of individual cells at each AI-1 concentration were obtained (Figure 2.2-3). A gradual increase in the mean P_{Qrr4}-GFP intensity distribution occurred with decreasing AI-1 concentration, reflecting increasing kinase activity of LuxN, and, consequently, increasing LuxO-P concentration. While we observed heterogeneity in P_{Qrr4}-GFP expression over the population, the distribution of P_{Qrr4}-GFP intensities remained single-
Figure 2.2-2 The engineered *V. harveyi* strains were grown to steady state. Sensor mutants LuxN\(^+\) (blue squares), LuxPQ\(^+\) (red circles), and LuxN\(^+\) LuxPQ\(^+\) (black triangles) were grown in AB medium at 30°C. At time zero, cell cultures were diluted into fresh AB medium to OD\(_{600}\)=10\(^{-7}\)~10\(^{-6}\). After 12 hours growth, cell samples were extracted for snapshots under the microscope. For each data point, 100 cells were measured, and the means (symbols) and standard deviations (error bars) of normalized GFP are plotted. Apparently, under the specified conditions, cells are in steady state in P\(_{Qr4}\)-GFP expression between 12 and 14 hrs.
Figure 2.2-3 Single-cell microscopy images and GFP fluorescence distributions of LuxN\(^+\) cells of \textit{V. harveyi}. (A)-(D) Snapshots of cells growing exponentially at different AI-1 concentrations (indicated above images) and the corresponding GFP fluorescence distributions of single cells. The mean GFP fluorescence intensity of each cell is normalized by the cell’s mean mCherry fluorescence intensity. Each distribution is obtained from 100 cells. A. U. denotes arbitrary units.
Figure 2.2-4 Cell-to-cell variation in $P_{Qrr4}$-GFP expression is smaller after normalizing by mCherry intensity. (A) Cell-to-cell variation, represented by relative noise, i.e. the standard deviation (SD) of the population divided by the mean, versus mean GFP intensity for LuxN$^+$ (blue crosses), LuxPQ$^+$ (red pluses), and LuxN$^+$ LuxPQ$^+$ (black dots) cells at different autoinducer concentrations. (B) Cell-to-cell variation for the same cell samples as in (A), but with the GFP intensity of each cell normalized by the same cell’s mCherry intensity. Cell-to-cell variation (relative noise) is smaller after normalization.
peaked with moderate variance around the population average at all AI-1 concentrations (cell-to-cell variation was somewhat smaller after normalizing by mCherry intensity, see Figure 2.2-4). This result suggests that all the *V. harveyi* cells respond identically to AI-1, which promotes well-coordinated cellular behavior across the population. The shift in the mean $P_{Qrr4}$-GFP intensity between zero and saturating AI-1 is obviously larger than the standard deviation within the population at any AI-1 concentration, suggesting that cell-to-cell variation, or noise, in quorum sensing is low enough to allow the cells to reliably mount distinct responses to low and high AI-1 concentrations.

We performed similar individual-cell dose-response experiments on the *V. harveyi* LuxPQ$^+$ mutant strain to determine the signaling properties of the AI-2 pathway. For comparison, in Figure 2.2-5 we show dose-response curves for both the LuxN$^+$ and LuxPQ$^+$ mutant strains to AI-1 and AI-2, respectively. Means and standard deviations over a population of cells are reported for each strain. Similar to the results shown in Figure 2.2-4, at all autoinducer concentrations, the normalized $P_{Qrr4}$-GFP-intensity distributions are single-peaked, with standard deviation over the mean always smaller than 0.4. For each data point, the population sample consists of 100 individual cells, thus the standard error of the mean is one tenth of the standard deviation of the population. Each dose-response curve can be described by a simple Hill function $\alpha_{AI} + \beta_{AI} / (1 + [AI] / K_{AI})$ with Hill coefficient equal to 1. The inhibition constants for AI-1 and AI-2 are $K_{AI-1} = (6.9 \pm 0.5)$ nM and $K_{AI-2} = (6.4 \pm 0.5)$ nM, respectively. Note that a 1 nM concentration is approximately 1 molecule of autoinducer in the volume of a single *V. harveyi* cell, indicating an extremely sensitive response of *V. harveyi* cells to
Figure 2.2-5 Dose responses of LuxN$^+$ cells to AI-1 (blue) and LuxPQ$^+$ cells to AI-2 (red). Each average and standard deviation (error bar) of normalized GFP was obtained from microscopy images of 100 cells. Curves were fitted using $\alpha_{AI} + \beta_{AI}/(1+[AI]/K_{AI})$ with $\alpha_{AI-1} = 0.07$, $\beta_{AI-1} = 2.9$, $K_{AI-1} = 6.9$ nM and $\alpha_{AI-2} = 0.09$, $\beta_{AI-2} = 1.9$, $K_{AI-2} = 6.4$ nM. A.U. denotes arbitrary units.
autoinducers. The LuxN\textsuperscript{+} strain has approximately 50\% higher P\textsubscript{Qrr4}-GFP levels than the LuxPQ\textsuperscript{+} strain at low autoinducer concentrations where LuxO-P and P\textsubscript{Qrr4}-GFP are maximal. However, the two strains have similar residual levels of P\textsubscript{Qrr4}-GFP, which remain measurable above background at saturating autoinducer concentrations.

2.2.3 Response of combined autoinducer-sensing pathways

The above experiments allowed us to determine the signaling response of the LuxN pathway to AI-1 and that of the LuxPQ pathway to AI-2 when each pathway is present alone. We likewise wondered how the cells respond to AI-1 and AI-2 when the two pathways are present together. To examine this, we performed experiments analogous to those above with the \textit{V. harveyi} LuxN\textsuperscript{+} LuxPQ\textsuperscript{+} strain in the presence of combinations of AI-1 and AI-2. Surprisingly, we found that although the amplitudes of the autoinducer responses are different when the two quorum-sensing pathways are present individually (Figure 2.2-5), the amplitudes of the AI-1 and AI-2 responses are nearly identical when the two pathways are present simultaneously (Figure 2.2-6). In particular, the dose-response curves for AI-1 and AI-2 almost overlap (red and blue curves) in both the case when one autoinducer is present alone and in the case when a saturating amount of the other autoinducer is also present. Critically, the overlap of these curves depends on the extremely similar amplitudes of the responses as well as the similar inhibition constants for AI-1 and AI-2 as observed in Figure 2.2-5. The very
similar amplitudes of the two autoinducer dose-response curves demonstrate that each autoinducer-sensing pathway contributes approximately half of the total response.

Figure 2.2-6 clearly shows that when both pathways are present (e.g. in the LuxN<sup>+</sup> LuxPQ<sup>+</sup> strain), each autoinducer alone is only capable of partial inhibition of P<sub>Qrr4</sub>-GFP expression. When AI-1 and AI-2 concentrations are increased together, with similar concentrations of each autoinducer present, the resulting dose-response curve of P<sub>Qrr4</sub>-GFP expression covers the entire dynamic range (yellow-green curve). The P<sub>Qrr4</sub>-GFP distribution is always single-peaked and noise in GFP expression is always moderate, with the standard deviation over the mean no more than 40%. Again, we take this to mean that despite the existence of noise in the quorum-sensing pathway, individual cells are able to discriminate several distinct states. For example, the P<sub>Qrr4</sub>-GFP distributions do not substantively overlap for these three cases: when both AI-1 and AI-2 are below 1nM, both around 10nM, and both above 100nM. Thus, it appears that individual <i>V. harveyi</i> cells can accurately determine the level of external autoinducers. This result suggests that, in principle, <i>V. harveyi</i> cells can not only detect low and high cell-density states with low and high autoinducer concentrations, but also some intermediate cell-density states represented by intermediate autoinducer concentrations.

To obtain a more comprehensive view of the autoinducer response of the LuxN<sup>+</sup> LuxPQ<sup>+</sup> strain, we explored a grid of possible combinations of AI-1 and AI-2 concentrations. In this way, the complete dose-response surface was obtained (Figure 2.2-7). This surface, displaying average P<sub>Qrr4</sub>-GFP production, is almost mirror-symmetric with respect to the equal-AI-1-and-AI-2 diagonal, i.e. P<sub>Qrr4</sub>-GFP expression is
Figure 2.2-6 Dose responses of LuxN\textsuperscript{+} LuxPQ\textsuperscript{+} cells to either AI-1 (blue) or AI-2 (red) while the other autoinducer is either absent (open squares and circles) or present at a saturating concentration (solid squares and circles). Data in yellow-green represent the response to approximately equal amounts of AI-1 and AI-2 (x-axis values indicate total autoinducer concentrations).
Figure 2.2-7 Dose-response surface of LuxN$^+$ LuxPQ$^+$ cells to various combinations of AI-1 and AI-2. Each vertex of the grid is the averaged normalized GFP fluorescence intensity obtained from a population of 100 cells exposed to the specified AI-1 and AI-2 concentrations. The dose-response curves in Figure 2.2-6 correspond to cuts through this surface.
almost invariant with respect to exchange of AI-1 and AI-2 concentrations. Notably, there are at least three distinct states of the output $P_{Qrr4}$-GFP level: high (both AI-1 and AI-2 concentrations low, indicated by the red area in Figure 2.2-7), intermediate (one autoinducer concentration low and the other high, indicated by the two green areas) and low (both AI-1 and AI-2 concentrations high, indicated by the blue area). This surface confirms that more than two quorum-sensing states can be deciphered by the cells. However, interestingly, under these conditions, high AI-1, low AI-2 is apparently not distinguished from low AI-1, high AI-2.

### 2.2.4 The two autoinducer inputs are integrated additively

For a signal-integration circuit such as the quorum-sensing circuit in *V. harveyi* that involves multi-step bidirectional biochemical reactions, one might expect the two signals to be integrated in a complicated nonlinear manner. Surprisingly, however, we found quite the opposite. That is, AI-1 and AI-2 signal integration is simply additive. The dose-response surface of the LuxN$^+$ LuxPQ$^+$ strain can be accurately described by the additive function

\[
[GFP] = \gamma_0 + \frac{\gamma_{AI-1}}{1+[AI-1]/K_{AI-1}} + \frac{\gamma_{AI-2}}{1+[AI-2]/K_{AI-2}},
\]

where the $\gamma$s and $K$s are fitting parameters. The inhibition constants have the same values as in the individual pathways: $K_{AI-1} = 6.9$ nM and $K_{AI-2} = 6.4$ nM (Figure 2.2-5 and Figure 2.2-6). As shown in Figure 2.2-8, the average $P_{Qrr4}$-GFP expression values
Figure 2.2-8 Autoinducers are integrated additively in the LuxN\textsuperscript{+} LuxPQ\textsuperscript{+} Strain. The response of LuxN\textsuperscript{+} LuxPQ\textsuperscript{+} cells to combined AI-1 and AI-2 shown in Figure 2.2-7 can be well described by a simple additive model 

\[ \gamma_0 + \frac{\gamma_{\text{AI-1}}}{1 + [\text{AI-1}]/K_{\text{AI-1}}} + \frac{\gamma_{\text{AI-2}}}{1 + [\text{AI-2}]/K_{\text{AI-2}}} \]

with \( \gamma_0 = 0.16 \), \( \gamma_{\text{AI-1}} = 1.53 \), \( \gamma_{\text{AI-2}} = 1.49 \), \( K_{\text{AI-1}} = 6.9 \text{ nM} \), \( K_{\text{AI-2}} = 6.4 \text{ nM} \). The red line has a slope equal to 1.
obtained from Equation (1) agree with the measured ones over the entire dose-response surface. The two non-cooperative Hill functions correspond to the individual responses of the LuxN and the LuxPQ pathways, respectively. Therefore, we conclude that LuxN and LuxPQ make independent, additive contributions to GFP levels presumably via additive contributions to LuxO-P.

2.2.5 The two autoinducer pathways contribute differently to noise

Although the two autoinducer signals are combined additively with approximately equal weights in their input to the circuit, we find that the two pathways contribute differently to the noise in P_Qrr4-GFP expression. As shown in Figure 2.2-9A, the LuxPQ+ strain (with no LuxN receptor) has significantly larger relative noise, i.e. larger cell-to-cell variation, than does the LuxN+ strain (with no LuxPQ receptor) for the same mean P_Qrr4-GFP level. Apparently, signaling through the LuxPQ receptor introduces more noise to the circuit than does signaling through the LuxN receptor. This difference is confirmed by the distinct noise levels observed for the LuxN+ LuxPQ+ strain treated with either saturating AI-1 or saturating AI-2 (Figure 2.2-9B). In the LuxN+ LuxPQ+ strain, the mean P_Qrr4-GFP levels are nearly identical under these two conditions, but the relative noise is almost a factor of two larger when only LuxPQ contributes kinase activity (AI-1 saturating) than when only LuxN contributes kinase activity (AI-2 saturating). Indeed, as shown in Figure 2.2-9B, noise in the LuxN+ LuxPQ+ strain is at its absolute maximum when only LuxPQ contributes kinase activity.
Figure 2.2-9 The two autoinducer sensing pathways contribute differently to GFP expression noise. (A) Relative noise, i.e. the standard deviation (SD) of the population divided by the mean, versus mean normalized GFP fluorescence intensity for LuxN$^+$ cells at different AI-1 concentrations (blue) and for LuxPQ$^+$ cells at different AI-2 concentrations (red). (B) Relative noise for LuxN$^+$ LuxPQ$^+$ cells as a function of AI-1 and AI-2 concentrations.
2.2.6 The kinase activities of LuxN and LuxPQ are regulated by autoinducers

Our observation that the LuxN and LuxPQ pathways contribute independently and additively to Pqrr4-GFP expression implies that the kinase activities of LuxN and LuxPQ must be regulated by the autoinducers. We draw this conclusion from the following simple model for the signaling pathway leading to Pqrr4-GFP expression: we assume that LuxN and LuxPQ are the dominant kinases and phosphatases for LuxU, that phosphotransfer between LuxU and LuxO is reversible, and that Pqrr4-GFP expression is a linear function of LuxO-P concentration [O-P]. The final assumption follows from the observed additivity of Pqrr4-GFP expression with respect to AI-1 and AI-2, which is difficult to understand unless [O-P] is in the linear regime of the qrr4 promoter driving gfp, i.e. the maximal [O-P] is far below the level required to half saturate the promoter activity. The kinetic equations describing this model are

\[
\begin{align*}
\frac{d[U-P]}{dt} &= \left( K_N + K_{PQ} \right) [U] - \left( P_N + P_{PQ} \right) [U-P] - k_+ [U-P] [O] + k_- [U] [O-P] \\
\frac{d[O-P]}{dt} &= k_+ [U-P] [O] - k_- [U] [O-P],
\end{align*}
\]

(2)

where [U-P] is the LuxU-P concentration, and \( K_N, K_{PQ}, P_N, \) and \( P_{PQ} \) are the total cellular kinase and phosphatase activities of LuxN and LuxPQ, respectively. At steady state, the time derivatives in Equation (2) can be set to zero, yielding

\[
[O-P] = \frac{K_N + K_{PQ}}{K_N + K_{PQ} + k_- / k_+ \left( P_N + P_{PQ} \right)} [O]_{tot},
\]

(3)
where \([O]_{\text{tot}}\) is the total concentration of LuxO. To explain the observed broad range of additivity of \(P_{\text{Qrr4-GFP}}\) expression with respect to the autoinducers, Equation (3) must be separable into two terms, one of which depends only on AI-1 and the other of which depends only on AI-2. This is possible if the autoinducers regulate the receptor kinase activities \(K_N\) and \(K_{PQ}\), but not if the autoinducers regulate only the receptor phosphatase activities \(P_N\) and \(P_{PQ}\), since the latter appear only in the denominator of Equation (3). Indeed, for additivity to be achieved, the denominator of Equation (3) must be approximately constant, which implies one of two scenarios: (1) only the kinase activities of LuxN and LuxPQ are regulated by autoinducers while phosphatase activities are not, and the kinase and phosphatase activities satisfy \(K_N + K_{PQ} \ll k_+/(k_+ \cdot (P_N + P_{PQ})\) implying that LuxO-P levels are far from saturation, i.e. \([O-P] \ll [O]_{\text{tot}}\); (2) the kinase and phosphatase activities are both regulated, but their sum is independent of autoinducer concentration such that \(K_N + K_{PQ} + k_-/k_+ \cdot (P_N + P_{PQ})\) remains constant. Unlike the first scenario, the second scenario requires fine-tuning of reaction rates and therefore seems less likely. While the signaling pathways leading to LuxO-P are likely to include some processes not considered in our simple model (e.g. intrinsic dephosphorylation of LuxU-P and LuxO-P), our qualitative conclusions – in particular that the kinase activities of LuxN and LuxPQ must be autoinducer regulated – are robust to such quantitative corrections.

Since the amplitudes of the responses to AI-1 and AI-2 are almost identical in the LuxN\(^+\) LuxPQ\(^+\) strain (Figure 2.2-6), the maximum total kinase activities of the two
receptors LuxN and LuxPQ must be nearly the same, i.e. $K_N \approx K_{PQ}$. However, for the strains expressing only a single receptor type, the peak $P_{Qrr4}$-GFP expression is 50% higher for LuxN+ than for LuxPQ+ strain (Figure 2.2-5). This apparent discrepancy can be readily accounted for if the total phosphatase activity of LuxPQ is higher than that of LuxN, i.e. $P_{PQ} > P_N$ (including possible differences in receptor concentration).

2.3 Discussion

Living cells monitor their environment using a variety of signal-transduction systems, ranging from simple two-component systems in prokaryotes to highly complex signal-transduction networks in mammalian cells. Since environmental cues are always numerous, the ability to integrate multiple signals is indispensable if cells are to behave appropriately. However, the mechanisms and logic by which cells integrate environmental signals remain by and large poorly understood. Here, we have quantitatively analyzed the integration of multiple autoinducer signals by the model quorum-sensing bacterium *Vibrio harveyi*, using single-cell fluorescence microscopy. Our studies reveal a unified response across the population, with moderate cell-to-cell variation. We find that signals from two distinct autoinducers, AI-1 and AI-2, are combined strictly additively in a single phosphorelay pathway, with each autoinducer contributing very nearly equally to the total response. Moreover, the cell-to-cell variation
in response is small enough that the entire population of cells can reliably distinguish at least three distinct conditions of external autoinducer concentration.

We used GFP under the control of the chromosomal sRNA Qrr4 promoter as a reporter of the activity of the quorum-sensing signaling pathway (Figure 2.2-2). In all our strains, the GFP distribution was always single peaked at all autoinducer concentrations, with cell-to-cell standard deviation no more than 40% of the mean, suggesting that populations of *V. harveyi* cells respond coherently to autoinducer signals. By contrast, genes in some other bacterial systems are known to have bimodal (two-peaked) expression distributions. In many cases, bimodal gene expression is also hysteretic, i.e. cells remain for a long time in one state of expression, which constitutes a form of cellular “memory”. For instance, bimodal distributions in gene expression enable sporulation and competence in *Bacillus subtilis* [56-59], stringent response in mycobacteria [61], and induction of the *lac* operon in *E. coli* [62,63]. In all these cases, bimodality and hysteresis are believed to provide advantages to the organism by enabling phenotypic diversity within isogenic populations. In general, hysteresis in gene expression requires some form of positive feedback. The lack of bimodality in our engineered strains of *V. harveyi* is expected since there is no positive-feedback loop in the circuit controlling Qrr sRNA expression in these cells. Since our engineered strains lack both the downstream transcription factor LuxR and the autoinducer synthases, there exists the possibility that the sRNAs or LuxR could feed back positively to the synthases and produce a bistable circuit in wild-type cells. In quorum sensing, bistability has only been reported for a rewired LuxIR circuit in *V. fischeri* [64]. In this case, the positive feedback and the resulting bistability and hysteresis occur at the population level and
divide the entire population into two separate subpopulations, each with a unique phenotype. Our consistent observation of a narrowly peaked distribution of quorum-sensing responses strongly suggests that \textit{V. harveyi} cells respond in unison to the presence of autoinducer signals. For quorum-sensing cells, in contrast to bacteria undergoing competence, sporulation, or the stringent response, operating as a coherent population appears to be more important than maintaining phenotypic diversity.

Given that the autoinducer signals are combined in one pathway in \textit{V. harveyi}, why should the signals be combined additively, as we observe for AI-1 and AI-2? Simple alternatives would be for saturating autoinducer levels to be combined in “logic gates” such as AND, in which both autoinducer signals would be required for a full response, or OR, in which either signal would be sufficient for a full response. However, these logic gates have only two possible output states, on or off. In contrast, the addition of the two autoinducer signals allows for more than two output states of the signaling pathway, and therefore, potentially allows for more than two expression states of quorum-sensing regulated genes. Indeed, we discovered three distinct levels of signaling strength, represented by the heights of the plateaus in Figure 2.2-7. Moreover, the standard deviation of \textit{P}_{Qrr4}-GFP expression across the population of cells was sufficiently small (\textit{cf.} Figure 2.2-9B) that the entire population can apparently distinguish the three distinct plateau heights. This means that, in principle, every cell in the population can distinguish three external autoinducer conditions: both autoinducers low, both autoinducers high, and a third condition in which one autoinducer is high and the other is low. The reliability with which cells can distinguish among these three conditions is increased by the equal spacing of the plateau heights as shown in Figure 2.2-7. Given a uniformly-distributed
input of autoinducer concentration and the observed level of noise (cell-to-cell variation in \( P_{Qrr4} \)-GFP expression), a significantly unequal spacing of the plateau heights would lead to overlapping distributions of \( P_{Qrr4} \)-GFP expression for the two more closely spaced plateaus. The implication is that noise might then cause some cells to misinterpret external conditions, and regulate quorum-sensing genes inappropriately. The need for all cells to reliably distinguish among multiple autoinducer conditions may therefore explain not only the additivity of the quorum-sensing pathway, but also why the contributions of the AI-1 sensor LuxN and the AI-2 sensor LuxPQ to the total kinase activity are so nearly equal – equal kinase activities mean equally spaced plateau heights, which in turn mean that individual cells are less likely to confuse one autoinducer condition with another.

The existence of multiple quorum-sensing output states potentially underpins diverse patterns of quorum-sensing regulated gene expression. For example, in previous studies the quorum sensing circuit of \( V. harveyi \) was found to act as an autoinducer “coincidence detector” (i.e. requiring both AI-1 and AI-2) for full induction of bioluminescence [38,110]. Thus, in the present context, the three distinguishable levels of signaling output (indicated by \( Qrr4 \) promoter activity) appear to be collapsed by downstream signal processing events to two levels of bioluminescence. More generally, the target genes of quorum sensing could be tuned to different signaling output levels so that only particular classes of genes are switched on/off at early, middle, or late stages of community development. Alternatively, some genes could have graded expression between these different developmental stages. The requirement for multiple distinct output states might also explain our observation of a graded, rather than switch-like, response of the \( Qrr4 \) promoter. Specifically, our dose-response data are well described by
a non-cooperative, $n = 1$ Hill function response to both autoinducers. Cooperativity would have resulted in an $n > 1$ Hill function and therefore a more switch-like response of $P_{Qrr4}$-GFP to autoinducers. During the signaling process, cooperativity could in principle have arisen from binding of autoinducers to receptors, transfer of phosphate among the protein components in the phosphorelay, and/or binding of phosphorylated LuxO to DNA. Our results suggest that in fact all of these steps are non-cooperative, despite the fact that the receptors are likely dimers [41] and that LuxO may function as a tetramer or octamer [Tu KC unpublished]. Indeed, a graded non-cooperative response of Qrr expression to autoinducers is essential for the existence of multiple distinguishable quorum-sensing states, as a switch-like response of the Qrr expression would have allowed for only two states.

Based on a simple kinetic model for signaling (Equation (2)), we have argued that the kinase activities of LuxN and LuxPQ are regulated by autoinducers, whereas for most two-component receptors, it is still an open question whether the kinase or phosphatase or both activities are regulated by input stimuli. Previously, LuxN receptors have been successfully modeled as switching between two states: the ON (kinase dominant) and OFF (phosphatase dominant) states [66,67]. Each receptor has intrinsic kinase and phosphatase rates depending only on the state in which the receptor exists. Extending this model to LuxPQ, the total cellular kinase activities $K_N$ and $K_{PQ}$ consist of a major contribution from those receptors in the ON state with little or no contribution from those in the OFF state. From the constraints set by additivity, we conclude that the phosphatase activities $P_N$ and $P_{PQ}$ are unregulated, i.e. receptors have the same phosphatase rates in
both the ON and OFF states. Note that autoinducer concentrations only affect the thermal balance between ON and OFF states, and therefore the kinase and phosphatase activities are regulated only via the biasing of receptors between states (of course, the total kinase and phosphatase activities also depend on receptor concentrations). The low levels of $\text{P}_{\text{Qrr4}}$-GFP expression with saturating AI-1 in the LuxN$^+$ strain, saturating AI-2 in the LuxPQ$^+$ strain, and saturating AI-1 plus AI-2 in the LuxN$^+$ LuxPQ$^+$ strain indicate that kinase rates in the OFF states are much smaller than those in the ON states for both LuxN and LuxPQ. By decreasing the fraction of receptors in the ON state, autoinducers reduce the total kinase activity of the quorum-sensing receptors in \textit{V. harveyi}. (See Appendix A for more details.)

Regulation of the kinase activities of LuxN and LuxPQ appears to be necessary to achieve three equally spaced levels of LuxO-P (Equation (3)). The requirement for kinase regulation in \textit{V. harveyi} quorum sensing therefore appears to stem from the need to combine multiple input signals into more than two distinguishable output levels of LuxO-P. One prediction from this analysis is that the sensor CqsS, which was not present in our strains, is likely to also have its kinase activity regulated by its autoinducer CAI-1. Moreover, CqsS is likely to contribute additively to total kinase activity and with a strength comparable to that of LuxN and LuxPQ, resulting in four maximally distinguishable levels of kinase activity and therefore four distinguishable autoinducer conditions.

The similarity of the responses to AI-1 and AI-2 is striking, not only in the amplitudes but also in the inhibition constants. We speculate that \textit{V. harveyi} usually
encounters similar amounts of AI-1 and AI-2, and the responses of receptors have been optimized to match the natural dynamic range of autoinducer concentrations. It has been demonstrated that single mutations in the receptors LuxN and LuxPQ can result in dramatic changes in their inhibition constants [41,67], so the similar values for AI-1 and AI-2 may represent an evolved optimum.

We also quantified the noise in $P_{Qrr4}$-GFP expression in our three reporter strains. Noise is an inherent feature of signal transduction and gene expression both in prokaryotes and eukaryotes. Due to the low copy number of cellular components and the stochastic nature of biochemical reactions, fluctuations are inevitable. Large fluctuations might be deleterious for processes requiring precise control but beneficial for those providing phenotypic diversity. In quorum sensing, bacterial cells detect population cell density to coordinate their behavior on a community-wide scale. Low noise in quorum-sensing signal transduction might therefore benefit the population of cells by allowing all cells to behave correctly and in unison at each stage of community development. Indeed, we observed low noise in $P_{Qrr4}$-GFP expression in all our strains. At all autoinducer concentrations the standard deviation over the mean was less than or close to 0.4 (Figure 2.2-9). In other systems, the dominant source of cell-to-cell variation in gene expression has been attributed to extrinsic noise, e.g. differences among cells in concentrations of general purpose cellular components such as RNA polymerases and ribosomes [49]. In the quorum-sensing circuit we have studied, the noise we have observed is also likely due to extrinsic factors rather than to biochemical noise in phosphotransfer or transcription and translation of $P_{Qrr4}$-GFP. The most likely source of the noise we observed is fluctuations in concentrations of the pathway components such as the receptors LuxN and
LuxPQ and the response regulator LuxO. The noisier response in LuxPQ pathway is very likely caused by variations in the copy number of the LuxPQ receptors, which suggests that there could be some additional regulation of receptor expression in the quorum-sensing circuit.

2.4 Materials and methods

2.4.1 Bacterial strains, media and DNA manipulations

All *V. harveyi* strains used in this study were derived from the wild-type strain BB120 [68] and grown aerobically at 30°C in Autoinducer Bioassay (AB) broth. *E. coli* S17-1λpir was used for general DNA manipulation and grown with aeration at 37°C in LB broth. The relevant strains and plasmids are listed in Table 1 and 2.

DNA manipulation was performed using standard procedures [69]. Phusion DNA polymerase was used for PCR reactions. dNTPs, restriction enzymes, and T4 DNA ligase were obtained from New England Biolabs. DNA purification kits were provided by Qiagen. *E. coli* was transformed by electroporation using a Bio-Rad Micro Pulser. Plasmids were introduced into *V. harveyi* by conjugation [33] and exconjugants were selected using the antibiotic resistances carried on the plasmids together with polymyxin B.
2.4.2 Fluorescent protein reporter and sensor mutant construction

A *cat*-resistance cassette from pKD3 [70] was cloned into vector pCMW1 [48] downstream of *gfp* at the BamH1 site, making pTL3. The GFP-Cm\(^r\) fragment from this construct was subsequently amplified by PCR and recombined using the \( \lambda \) red technique [70] into a cosmid to replace the wild-type *qrr4* gene, producing pTL20. Lastly, \( P_{Qrr4} \)-GFP-Cm\(^r\) was introduced onto the chromosome to replace *qrr4* by allelic recombination. Ptac-mCherry was amplified from the vector pEVS143-mCherry containing an IPTG inducible mCherry gene and cloned into pKD13 [70] at the NheI site, resulting in pTL82. The cosmid, pTL83, was constructed using the \( \lambda \) red technique by recombining the Ptac-mCherry-Kan\(^r\) fragment into the intergenic region downstream of the entire *lux* operon. Final insertion of Ptac-mCherry-Kan\(^r\) onto the *V. harveyi* chromosome was accomplished by allelic recombination.

To construct the various *V. harveyi* sensor mutants, pKM780 carrying \( \Delta luxS::Cmr \), pJMHH291 carrying \( \Delta luxN::Cmr \), pDL500 carrying \( \Delta luxPQ::Cmr \), pJMHH244 carrying \( \Delta cqsS::Cmr \), and pKM705 carrying \( \Delta luxR::Kan \) were used to sequentially delete the corresponding wild-type genes by allelic recombination. Following each gene deletion, the plasmid pTL18 containing an IPTG inducible FLP recombinase, derived from pEVS143 and pCP20 [70] (Appendix B), was introduced into the *V. harveyi* strain to eliminate the antibiotic resistance marker on the chromosome.
2.4.3 Single-cell fluorescence microscopy

For dose-response experiments, *V. harveyi* strains LuxN+ (TL87), LuxPQ+ (TL88), and LuxN+ LuxPQ+ (TL89) were grown in AB medium for 8~12 hrs. Growth was monitored by measuring optical density at 600 nm. Cultures were diluted to OD$_{600}$ = 10$^{-6}$ ~ 10$^{-7}$ and exogenous autoinducers were added at the specified concentrations. Following growth to steady state (13~14 hrs; OD$_{600}$ = 0.005 ~ 0.05), cells were concentrated by centrifugation and maintained on ice until measurements were made. 1 µl of cell culture was spread on a glass slide and covered with a 1% AB agarose pad as well as a coverslip.

Phase-contrast and fluorescent images were taken at room temperature using a Nikon TE-2000U inverted microscope. Custom Basic code was written to control the microscope. Images were acquired using a X100 oil objective and a cooled CCD camera (-65°C, Andor iXon). Segmentation of individual cells was performed on phase-contrast images (). Background and cellular auto-fluorescence values were subtracted from the green and red channels, respectively. Total fluorescence intensity of each cell was obtained by summing all pixels and fractions of pixels in the segmented cell region. Normalized GFP values for each cell were calculated by normalizing total green to total red fluorescence intensity.

2.5 Acknowledgement

Chapter 2 is an extension of the published work [71].
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<tr>
<th>Strain</th>
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### Table 2 Plasmids used in Chapter 2

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Chapter 3 Negative feedback loops precisely control \textit{V. harveyi} quorum-sensing response

3.1 Introduction

Complicated feedback regulations are incorporated into the quorum-sensing circuit in \textit{V. harveyi} to promote accurate signaling and facilitate precise population-wide change of behaviors in response to autoinducer concentrations. Here we identify and characterize two negative feedback loops that act in the \textit{V. harveyi} quorum-sensing circuit (Figure 3.1-1). Both feedback loops involve the central quorum-sensing response regulator LuxO. We find that LuxO negatively autoregulates its own transcription irrespective of its phosphorylation state and the Qrr sRNAs posttranscriptionally control LuxO production by pairing with and repressing the \textit{luxO} mRNA transcript [74]. Thus, both feedback loops reduce LuxO production which, in turn, reduces \textit{qrr} expression. Disruption of these negative feedback loops results in increased phosphorylated LuxO (LuxO~P) and, as a consequence, an alteration in the timing and levels of the expression of quorum-sensing-regulated genes. We propose that the two feedback loops exist to precisely control LuxO protein levels in order to tightly regulate Qrr production. This arrangement primes the Qrr sRNAs to respond accurately to changes in AI levels and, therefore, permits a faithful quorum-sensing response and properly aligned QS-regulated
Figure 3.1-1 Two negative feedback loops involve the central quorum-sensing response regulator LuxO. LuxO negatively autoregulates its own transcription irrespective of its phosphorylation state. And the Qrr sRNAs posttranscriptionally control LuxO production by pairing with and repressing the luxO mRNA transcript. These two feedback regulations are highlighted in red and are the main focus of this chapter. There are feedback loops involving the master quorum-sensing regulator LuxR too: LuxR binds its promoter and inhibit its own transcription [75]; LuxR also enhances transcription of the Qrr sRNAs, which actively pair with luxR mRNA and degrade the transcript [44,75].
gene expression with AI concentration. We suggest that sRNA-mediated feedback regulation could be a common signaling network design feature that permits fine-tuning of gene regulation and maintenance of homeostasis.

3.2 Results

3.2.1 Disruption of the feedback loops alters LuxO~P levels

We have identified two negative feedback loops that function to repress LuxO production. First, LuxO negatively regulates its own transcription independent of its phosphorylation state and, second, the Qrr sRNAs posttranscriptionally repress LuxO translation by binding to the luxO 5’ UTR [74]. More importantly, we were able to make mutations in the promoter region of luxO to disrupt each feedback loop individually and also in combination: the mutation in the LuxO-binding site, LuxO Loop− (Figure 3.2-1A and Figure 3.2-1C green box), abolishes LuxO autorepression; the single point mutation near the luxO translational start site, sRNA Loop− (Figure 3.2-1B and Figure 3.2-1C blue box), abolishes base-pairing between the Qrr sRNAs and luxO; and finally, the combination of the LuxO Loop− and sRNA Loop− mutations abolishes both LuxO regulatory feedback loops (Figure 3.2-1 purple box). We call this final construct Double Loop−. Our next goal was to introduce these mutations into the V. harveyi chromosome and study the consequences of misregulation of LuxO production on QS-dependent gene expression.
Figure 3.2-1 Disruption of feedback loops and various loop mutants. (A) Disruption of the LuxO autorepression loop. Sequences of the two luxO promoters are shown: In the WT sequence, the black box indicates the LuxO-binding site; In the LuxO Loop- sequence, the line represents the mutations (highlighted in bold) in the LuxO-binding site that eliminate LuxO autorepression. (B) Predicted Qrr4-luxO duplex formation and complementary base-pairing regions are indicated by black dots. RBS: ribosome-binding site. AUG: LuxO start codon. Disruption of the Qrr4-luxO duplex, also known as sRNA Loop-, was achieved by engineering a U-to-A point mutation in the luxO promoter, as indicated. (C) Color coded wild-type strain and various loop mutants.
Because LuxO protein levels increase in the absence of each LuxO feedback loop, we first wondered what effect this has on LuxO activity. The \textit{qrr} genes are activated by LuxO--P at low cell density (LCD), so we can use \textit{qrr} expression as a direct readout of LuxO--P activity in \textit{V. harveyi}. We used a \textit{V. harveyi} strain that is locked in the low-cell-density (LCD) state, specifically, the autoinducer synthases for AI-1 and AI-2 and the sensor kinase for CAI-1 are deleted (\textit{\textDelta luxM, \textDelta luxS, \textDelta cqsS}) [71]. This locked LCD strain makes high levels of LuxO--P because the two intact AI sensors are locked in kinase mode and because the CqsS sensor is deleted, the CAI-1 system provides no input into the quorum-sensing circuit. We engineered the feedback loop mutations individually and in combination into this locked LCD strain, transformed a \textit{qrr4-gfp} transcriptional fusion reporter into the mutants, and quantified fluorescence. Figure 3.2-2A shows that \textit{qrr4-gfp} expression is high in the wildtype LCD strain (red bar). In the LuxO Loop© strain, \textit{qrr4-gfp} expression is increased compared to wildtype (green bar), and a similar increase in \textit{qrr4-gfp} expression occurs in the sRNA Loop© strain (blue bar). These results suggest that, in the absence of either the LuxO or sRNA negative feedback loop, total LuxO--P increases at LCD. Moreover, in the Double Loop© strain, there is a much greater increase in \textit{qrr4-gfp} expression (purple bar), indicating that the two feedback loops cooperate to control LuxO--P levels.

To examine the role of an individual Qrr sRNA in feedback regulation, we engineered a \textit{V. harveyi} strain deleted for four of the five \textit{qrr} genes, leaving only the \textit{qrr4} gene remaining in the chromosome (we denote this strain \textit{qrr4^+}). We chose to leave Qrr4 intact because \textit{qrr4} is the most highly expressed \textit{qrr} gene at LCD, and thus we suspected it might have the most important role in feedback regulation. \textit{qrr4-gfp} expression is
Figure 3.2-2 Disruption of the negative feedback loops increases LuxO−P. (A) A qrr4-gfp transcriptional fusion was transformed into a *V. harveyi* ΔluxM, ΔluxS, ΔcqsS strain, denoted WT (red bar). Into this strain, the following mutations were engineered: LuxO Loop− (green bar), sRNA Loop− (blue bar), and Double Loop− (purple bar). (B) The qrr4-gfp fusion was transformed into the qrr4+ *V. harveyi* strains: qrr4+ (red bar), LuxO Loop− (green bar), sRNA Loop− (blue bar), and Double Loop− (purple bar). For (A) and (B), fluorescence production was measured in overnight cultures grown in triplicate and error bars denote the standard deviation of the mean.
significantly higher in the qrr4+ strain than in wildtype V. harveyi (compare red bars in A and B in Figure 3.2-2). This result is expected because elimination of four of the five Qrrs reduces sRNA-mediated repression of both luxO and luxR mRNAs, and the resulting higher levels of LuxO–P and LuxR lead to increased activity of the qrr4 promoter (Figure 3.2-1) [77]. The green bar in Figure 3.2-2B shows that qrr4-gfp expression is further increased in the LuxO Loop– strain. However, there is no increase in qrr4-gfp expression in the sRNA Loop– strain (Figure 3.2-2B, compare red bar to blue bar). Disruption of the sRNA loop does, however, cause a sizable increase in qrr4-gfp level in the qrr4+ strain already lacking the LuxO feedback loop (Figure 3.2-2B, Double Loop–, purple bar). We interpret these results to mean that qrr4 levels are not sufficient for repression of LuxO in the qrr4+ strain possessing an intact LuxO feedback loop (Figure 3.2-2B, compare red bar and purple bar). However, in the context of the LuxO Loop– background, Qrr4 levels are sufficiently high to repress LuxO translation via the sRNA negative feedback loop, and hence elimination of this loop leads to an increase in LuxO and a concomitant increase in qrr4 expression. One possible explanation for the apparent threshold of qrr4 expression required to repress luxO is that Qrr4 may have a higher binding affinity for luxR than luxO mRNA, and thus, luxR is the primary target and luxO is the secondary target of regulation by Qrr4. In this scenario, Qrr4 only represses luxO, and, in turn, reduces its own expression after luxR has been repressed and only if there remain excess Qrr4 sRNAs to do so. The different affinities of the sRNAs for their target mRNAs could allow limited sRNAs to prioritize the regulation of different target mRNAs.
3.2.2 The feedback loops affect expression of quorum-sensing regulated genes in response to autoinducer concentrations

Our next question was whether alteration in LuxO~P levels affects the ability of *V. harveyi* to properly control downstream quorum-sensing gene expression in response to autoinducers. To examine this, we measured the *V. harveyi* bioluminescence as an output in response to AI-1 and AI-2 in our mutants (Figure 3.2-3). Because the strains lack the AI-1 and AI-2 synthases, this strategy allowed us to precisely control the autoinducer inputs and measure the corresponding quorum-sensing outputs. The EC$_{50}$ values for the wild-type (WT), LuxO Loop$^-$, sRNA Loop$^-$, and Double Loop$^-$ strains are 25 nM, 61 nM, 64 nM, and 192 nM respectively (Figure 3.2-3A). Thus, in the absence of either the LuxO or sRNA feedback loop, *V. harveyi* becomes two to three times less sensitive to AIs, whereas in the absence of both feedback loops *V. harveyi* becomes eight times less sensitive to AIs (Note, a two-fold change in the EC$_{50}$ values is at the margin of significance). We conclude that the two feedback loops cooperate to significantly increase *V. harveyi*'s sensitivity to AIs. Importantly, Figure 3.2-3A shows that disruption of the feedback loops does not substantially alter the slopes of the dose-response curves (Hill coefficient values for WT, LuxO Loop$^-$, sRNA Loop$^-$, and Double Loop$^-$ strains are 2.4, 2.8, 2.7, and 2.2, respectively), suggesting that these two feedback loops do not impinge on the downstream amplitude of the quorum-sensing response, but rather, adjust the autoinducer concentration at which *V. harveyi* detects that it has reached a quorum and, therefore, the two feedback loops control the timing of quorum-sensing gene expression.
Figure 3.2-3 The negative feedback loops make *V. harveyi* more sensitive to autoinducers. Dose-response curves using bioluminescence as readout for the quorum-sensing-activated luciferase operon (A) in wild-type (WT) *V. harveyi* and various loop mutants and (B) in the *qrr4* strains with various loop deletions. Data were fit with variable-slope sigmoidal dose-response curves \[ \log(\text{lux}) = \text{bot} + \frac{\text{top} - \text{bot}}{1 + \left(\frac{\text{EC}_{50}}{[\text{AI}]}\right)^{\text{HillCoeff}}} \] to determine the concentration of half-maximal response (EC_{50}) and the slope of dose-response curve (Hill Coefficient) for each strain. RLU: relative light units in counts min^{-1} ml^{-1}/OD_{600}. HAI-1: *harveyi* AI-1.
Revealed from the dose-response curves of bioluminescence expression, the EC\textsubscript{50} values in the \textit{qrr4}\textsuperscript{+} strain, the LuxO Loop\textsuperscript{−} strain, the sRNA Loop\textsuperscript{−} strain, and the Double Loop\textsuperscript{−} strain are 13 nM, 34 nM, 15 nM, and 139 nM, respectively (Figure 3.2-3B), demonstrating that in a strain containing only \textit{qrr4}, elimination of the two feedback loops renders \textit{V. harveyi} ten-fold less sensitive to autoinducers. Similar to the results observed for cells containing all five sRNAs, the slopes of the dose-response curves are not substantially altered (Hill coefficient values for the \textit{qrr4}\textsuperscript{+}, LuxO Loop\textsuperscript{−}, sRNA Loop\textsuperscript{−}, and Double Loop\textsuperscript{−} strains are 1.8, 1.7, 1.6, and 1.6, respectively). We note that the EC\textsubscript{50} value for bioluminescence in the \textit{qrr4}\textsuperscript{+} strain (13 nM) is two-fold less than that of the wildtype strain containing all five \textit{qrr} genes (25 nM). This difference suggests that \textit{qrr4} alone cannot fully repress \textit{luxR} translation, and due to increased LuxR protein levels in the \textit{qrr4}\textsuperscript{+} strain relative to wildtype, it takes fewer autoinducers to increase LuxR levels to above the threshold required for LuxR to activate the \textit{lux} operon.

### 3.2.3 Determination of LuxR protein levels at single-cell resolution

The behavior of the QS population is ultimately dictated by the behavior of individual cells. Up to this point, we have focused on the regulation of quorum-sensing target genes at the population level. However, our goal is to relate the individual cell behavior to that of the population. Toward this end, we explored how individual cells respond to autoinducer inputs and determined their cell-to-cell variation with and without the LuxO regulatory feedback loops. We focused on quantifying LuxR levels because
LuxR is the master regulator of quorum-sensing target genes, and LuxR ultimately dictates the pattern of quorum-sensing gene expression. We constructed a full-length LuxR-mCherry protein fusion and introduced it onto the *V. harveyi* chromosome at the native *luxR* locus in the wild type and the feedback-loop mutants. Subsequently, we measured LuxR-mCherry in individual cells under different autoinducer input conditions. The individual cell dose-response profiles of LuxR in the various feedback-loop mutants mirror the patterns we observed at the population level with bioluminescence (Figure 3.2-4A). The calculated EC$_{50}$ values for the wild-type, LuxO Loop$^-$, sRNA Loop$^-$, and Double Loop$^-$ strains are 36 nM, 57 nM, 62 nM, and 271 nM with Hill coefficients 1.3, 1.1, 1.2, and 1.0, respectively (Figure 3.2-4A). Thus, with regard to LuxR levels in individual cells, in the absence of either the LuxO or sRNA feedback loop, *V. harveyi* becomes roughly two times less sensitive to autoinducers, whereas in the absence of both feedback loops, *V. harveyi* becomes eight times less sensitive to autoinducers. The Hill coefficients of the LuxR dose-response curves are approximately 1, supporting our previous results that LuxR protein levels increase in a graded manner in response to increasing AI concentrations [48]. The present results show that there is roughly a fivefold difference between LuxR protein levels under minimal and saturating autoinducer levels (Figure 3.2-4A), which is the range of LuxR that determines the temporal order of expression of quorum-sensing-repressed and QS-activated target genes.

We also measured LuxR protein levels in response to autoinducers in single cells containing only *qrr4*. The calculated EC$_{50}$ values for the *qrr4* strain, the LuxO Loop$^-$ strain, the sRNA Loop$^-$ strain, and the Double Loop$^-$ strain are 20 nM, 43 nM, 16 nM, and 46 nM with Hill coefficients 1.1, 1.5, 1.7, and 1.5, respectively (Figure 3.2-4B). As in
Figure 3.2-4 LuxR single-cell dose-response curves and relative noise. (A) Individual-cell dose-response curves were obtained using the same *V. harveyi* strains as in Figure 3.2-3A but containing a LuxR-mCherry protein fusion integrated in the native *luxR* locus: WT (red squares), LuxO Loop (green triangles), sRNA Loop (blue circles), and Double Loop (purple diamonds). A.U.: arbitrary units. HAI-1: *harveyi* AI-1. (B) LuxR dose-response curves in *qrr*4+ background. The *V. harveyi* strains from Figure 3.2-3B, but containing a LuxR-mCherry protein fusion integrated in the native *luxR* locus, were analyzed: *qrr*4+ (open red squares), LuxO Loop (open green triangles), sRNA Loop (open blue circles), and Double Loop (open purple diamonds). Data were fit and coefficients were obtained as in Figure 3.2-3. Each symbol in (A) and (B) represent the average mCherry value obtained from microscopy images of 100 cells. (C) The relative noise, *i.e.* the standard deviation (SD) of the population divided by the mean, versus the mean LuxR-mCherry fluorescence intensity for wild-type *V. harveyi* with various feedback loop mutations (closed symbols) and for the *qrr*4+ strain with various feedback loop mutations (open symbols).

In the case of bioluminescence, these results show that in a strain containing *qrr*4 only, disruption of the sRNA feedback loop alone has no significant effect on the LuxR dose-response curve. We note that the basal level of LuxR protein in the various *qrr*4+ strains at low autoinducer concentration is approximately two-fold higher than that in the corresponding strains containing all five *qrr* genes. This finding confirms our expectation based on Figure 3.2-2B, that *qrr*4 alone cannot fully repress *luxR* translation to wild-type levels. Due to this increased LuxR level in the *qrr*4+ strain at LCD, there is only a three-fold difference between LuxR protein concentrations under minimal and saturating autoinducer levels, giving *V. harveyi* a reduced window of quorum-sensing.
target gene expression. Thus, the changes in lux expression in Figure 3.2-3 can be directly attributed to corresponding changes in LuxR protein levels, suggesting that autoinducer-directed alterations in LuxR concentrations are faithfully transduced into alterations in downstream quorum-sensing target gene expression.

Our measurements of LuxR protein at single-cell resolution enabled us to quantify the cell-to-cell variation in LuxR in the presence of increasing AI concentrations. Figure 3.2-4C shows the relative noise in LuxR protein in the eight different V. harveyi mutants. At low autoinducer concentrations, the standard deviation over the mean is approximately 0.4. Thus, when minimal LuxR is present (LCD), the system is, not surprisingly, somewhat noisy. However, at high autoinducer concentrations, the standard deviation over the mean is approximately 0.2, indicating that there is little LuxR variation between single cells at HCD. Surprisingly, there is no significant difference in the relative noise observed in cells containing or lacking the various feedback loops or in cells that contain multiple versus one Qrr sRNA (Figure 3.2-4C).

### 3.2.4 The feedback loops do not affect the kinetics of the HCD to LCD transition

Figure 3.2-3 indicates that the presence of the feedback loops makes wild-type V. harveyi more sensitive to autoinducers. Previous experiments have discovered a negative feedback loop in the V. harveyi QS circuit in which LuxR activates the expression of the qrr2, qrr3, and qrr4 genes, leading to a rapid transition out of HCD mode into LCD
mode [76]. To determine whether the feedback loops involving LuxO and the Qrr sRNAs likewise play a role in controlling the dynamics of the HCD to LCD transition, we grew the same *V. harveyi* strains as in Figure 3.2-3A in saturating AI concentrations to simulate HCD mode, washed away the autoinducers with fresh medium to simulate an immediate transition to LCD mode, and subsequently measured cellular Qrr sRNA levels (Figure 3.2-5A). Qrr levels increase to maximal values within 10 minutes after the removal of autoinducers in all four *V. harveyi* strains. There do not appear to be significant differences in the rates at which Qrr sRNAs increase or the maximum values reached in the feedback loop mutants, suggesting that the feedback loops do not play a significant role in the kinetics of this transition.

### 3.2.5 Qrr sRNA induction eliminates luxR, but not luxO, mRNAs

It has been previously demonstrated that the Qrrs base-pair with luxR mRNA and destabilize it to prevent further translation [42]. To determine whether repression is identical for luxO mRNA, we focused on the cellular mRNA levels of luxR and luxO during the HCD to LCD transition shown in Figure 3.2-5A. Consistent with previous observations, luxR mRNA levels decline from the maximal amount present at HCD to a low basal amount within 3 minutes of the transition into LCD mode (Figure 3.2-5B). The rapid decrease in luxR mRNA levels matches exactly the fast increase of Qrr sRNA levels (Figure 3.2-5A), confirming that luxR mRNA is indeed completely degraded as a consequence of Qrr sRNA repression. Surprisingly, the pattern for luxO mRNA is
different (Figure 3.2-5C): there is a small drop in cellular luxO mRNA levels in all mutant strains during the first 3 minutes after the HCD to LCD transition. However, 30 minutes after the transition, luxO mRNAs remain at levels either the same or only slightly lower than the original HCD levels before the transition. Figure 3.2-5C also shows that the relative amounts of luxO mRNA in the wild-type and mutant strains are consistent with the relative LuxO protein levels inferred from qrr4-gfp expression (Figure 3.2-2A). The initial slight drop in luxO mRNA levels following the HCD to LCD transition cannot be attributed to sRNA repression, since the sRNA Loop and the Double Loop strains display the same pattern. Moreover, the subsequent maintenance of luxO mRNA levels cannot be attributed to limited availability of Qrr sRNAs, since the Qrr sRNAs remain at abundant levels even after all luxR mRNAs are degraded (Figure 3.2-5AB). Taken together, these results suggest that the Qrr sRNAs do not have the dramatic on/off effect on luxO mRNA as they have on luxR mRNA. Nevertheless, the Qrr sRNAs clearly do repress LuxO protein production via direct base-pairing with luxO mRNA (Figure 3.2-1B). One possibility is that Qrr sRNAs repress luxO mRNA by sequestration. A Northern blot showed that deleting all Qrrs does not change luxO mRNA half-life, and overexpressing Qrr4 extends luxO mRNA half-life [74]. In contrast, Qrr sRNAs decrease luxR mRNA half-life by 15-fold. Furthermore, deleting or overexpressing the Qrrs barely changes cellular luxO mRNA levels, but, at the same time, causes dramatic changes in cellular luxR mRNA levels. These results support the idea that the Qrrs sequester luxO mRNA and block its translation. However, sequestration alone cannot explain the differences in luxO mRNA levels seen in the strains with and without the sRNA loop (Figure 3.2-5C).
Figure 3.2-5 Cellular sRNA and mRNA levels during the HCD to LCD transition. (A) The feedback loops do not affect dynamics of Qrr sRNAs during the transition from HCD to LCD. The identical *V. harveyi* strains shown in Figure 3.2-3A were grown in duplicate in the presence of saturating AI-1 and AI-2: WT (red squares), LuxO Loop− (green triangles), sRNA Loop− (blue circles), and Double Loop− (purple diamonds). Autoinducers were washed away with fresh medium and sRNA levels were quantified using a QuantiGene Plex Reagent System (Panomics). Error bars are standard deviations of samples in duplicate. A.U.: arbitrary units. (B) *luxR* mRNA levels in the cells from panel A decline rapidly during the HCD to LCD transition for all four *V. harveyi* strains. (C) *luxO* mRNA levels show different patterns compared to *luxR* mRNA in these strains during the HCD to LCD transition.

3.3 Discussion

Coordination of quorum-sensing processes often involves multi-step signal transduction pathways that control the expression of master transcription factors, which in turn, regulate genes responsible for group behaviors. Quorum-sensing networks must be designed to precisely translate external autoinducer concentrations into proper patterns of internal quorum-sensing target gene expression. The molecular components making up the *V. harveyi* quorum-sensing circuit have been defined and characterized, allowing us to undertake an analysis of the regulatory features of the network that optimize the quorum-sensing output response. We identified two cooperative negative regulatory feedback loops involving LuxO and the Qrr sRNAs. Specifically, LuxO negatively regulates its own transcription by virtue of a LuxO-binding site in the *luxO* promoter. Negative regulation does not depend on the phosphorylation state of LuxO, suggesting
that LuxO competition with RNAP for DNA-binding at this LuxO/-35 site sets the level of *luxO* transcription. LuxO is also subject to posttranscriptional feedback regulation by the Qrr sRNAs; thus *luxO* mRNA constitutes a new target of the Qrr sRNAs in *V. harveyi*. The two feedback loops function to make *V. harveyi* more sensitive to changes in external autoinducer concentrations and thereby set the threshold population density at which cells reach a quorum.

The negative feedback loop is a common network-design motif in signal transduction pathways and gene regulatory networks. Negative feedbacks are generally believed to play three major roles: reducing “noise”, i.e. temporal fluctuation or cell-to-cell variation [78,79]; decreasing response rise times [80,81,82]; and producing graded responses [83]. All three roles have been demonstrated for negatively auto-regulated transcription factors. First, high concentration of a transcription factor will result in repression, while low concentration will result in increased production, leading to a narrower distribution of protein levels than for proteins that are not auto-regulated. This feature reduces expression noise of the transcription factor. Second, a transcription factor subject to negative feedback with a stronger promoter than a non-self-regulated one, can nonetheless reach the identical steady-state level. The negatively regulated transcription factor will increase rapidly until it reaches the threshold level required for repressing its own expression. This feature results in a shorter gene-expression rise time [81]. Third, a particular amount of inducer will result in a lower level of a negatively auto-regulated transcription factor than a non-regulated one due to auto-repression of expression. This feature decreases the slope of the dose-response curve and generates a graded response.
We determined whether the two negative feedback loops under study play any of these three canonical roles. In order to measure noise in the quorum-sensing response, we used single-cell fluorescence microscopy to quantify LuxR levels of *V. harveyi* in individual cells at various autoinducer concentrations. The relative noise, namely the standard deviation over the mean, is only 20% at high autoinducer concentrations (Figure 3.2-4C). Thus, *V. harveyi* cells act in unison at high cell density. However, surprisingly, there is no discernable difference in relative noise in the various feedback loop mutants, whether in the presence of a single or multiple sRNAs. A possible explanation for this observation is that the two central transcriptional regulators in the quorum-sensing circuit – LuxO and LuxR – are both subject to transcriptional and posttranscriptional negative feedback regulation. The feedback loops on LuxO may primarily contribute to noise reduction in LuxO expression levels, while the feedback loops on LuxR filter out noise originating from various sources, including noise in LuxO levels, to stabilize the LuxR expression level. In any event, we find that the two feedback loops on LuxO do not noticeably contribute to the low relative noise in LuxR expression.

To test whether the translational repression of LuxO by Qrr sRNAs decreases rise time relative to steady-state levels of the Qrrs during the HCD to LCD transition, we measured Qrr sRNA levels in both wild-type cells and in the engineered strains with the feedback loops disabled (Figure 3.2-5A). Our results show that the transition happens rapidly – Qrr levels reach maxima within 10 minutes – in all strains. There is no discernible difference either in the rise times or the plateau levels of Qrrs in the presence or absence of the feedback loops. Therefore, the LuxO-Qrr feedback loop does not function to allow a fast transition out of HCD mode.
We also considered whether the feedback loops involving LuxO and Qrr sRNAs might function to generate a graded response. However, as shown in Figure 3.2-4A, the slopes of the LuxR dose-response curves are not substantially different for wild-type and the various loop mutants. All the Hill coefficients are ~1 from the fitting curves, suggesting a non-cooperative graded quorum-sensing response irrespective of whether or not the negative feedbacks were present. With only a single Qrr present, the Hill coefficients for the loop mutants are modestly larger than when both loops are present (Figure 3.2-4B, 1.5-1.7 vs. 1.1), but the difference is within our experimental error. Thus, we conclude that the negative feedback loops do not make the quorum-sensing response to autoinducers more graded.

In summary, the two negative feedback loops reported here do not appear to play any of the usual roles of negative feedback loops in gene-regulatory networks. However, the feedback loops clearly do function to tune the sensitivity of the quorum-sensing response to autoinducer concentrations, and in so doing, establish the point at which the population of *V. harveyi* reaches a quorum, and thus set the timing of downstream quorum-sensing target gene expression. It is puzzling why this fine-tuning of the quorum-sensing threshold is achieved via negative feedbacks rather than, e.g., by adjusting parameters of reactions in the signaling and gene regulatory circuit.

If these two negative feedback loops were not wired into the quorum-sensing circuit, we speculate that there would be a loss of homeostatic control due to unchecked LuxO and LuxR levels. Specifically, we suggest that the two negative feedback loops have evolved to couple the natural dynamic range of autoinducer concentrations with the
desired dynamic range of downstream quorum-sensing-regulated gene expression. Likewise, negative feedback regulation increases signaling fidelity in the mating pheromone pathway in yeast [84]. In particular, the specific architecture of the LuxO-Qrr negative feedback loop prevents over-accumulation of Qrrs, and thus prevents the targets of Qrrs from “bottoming out”, i.e. declining to extremely low levels. Intuitively, the obligate activation of Qrr sRNAs by LuxO~P/LuxR places a cap on Qrr expression: if Qrr levels start to become too high, luxO/luxR mRNA is decreased due to the excess of Qrrs, and as a consequence, qrr expression declines due to decreased levels of LuxO~P/LuxR. Therefore, all targets of the Qrrs are protected from “bottoming out” (approaching zero) by the feedback loops in which the Qrrs negatively regulate their own activators, LuxO~P and LuxR. Consider the following simplified kinetic equation for a target mRNA (e.g. luxR or luxO):

$$\frac{d[mRNA]}{dt} = \gamma - \mu \cdot [mRNA] \cdot [sRNA] - \delta \cdot [mRNA],$$  

(4)

where $\gamma$ is a constant transcription rate of the mRNA, $\mu$ is the coefficient for the rate of mutual degradation (or sequestration) of sRNA and mRNA, and $\delta$ is the rate of ordinary degradation/dilution of mRNA. At steady state, the time derivative of the mRNA concentration must be zero, and so the cellular mRNA level is:

$$[mRNA] = \frac{\gamma}{\mu \cdot [sRNA] + \delta}$$  

(5)

If the sRNA level becomes very high, the denominator in Equation (5) will be extremely large and result in a “bottoming out” of the mRNA. It is obvious that by setting an upper limit on Qrr sRNA levels, mRNA levels are thereby prevented from approaching zero.
Physiologically, it is likely to be important to maintain a basal level of translation of the mRNAs of key quorum-sensing players – including LuxO and LuxR – otherwise these proteins will be diluted out during cell growth and division. Thus, the multiple negative feedback loops function to keep LuxO, the Qrrs, and LuxR levels in check, and therefore aid in maintaining the expression of both Qrrs and QS-regulated target genes within restricted windows. We suggest that sRNA-based regulation of mRNAs of the essential quorum-sensing components is indispensible for proper functioning of quorum-sensing signaling.

Our results suggest that there are roles negative feedback loops can play in signaling and gene regulatory networks beyond those already recognized. While the LuxR-Qrr feedback loop accelerates the HCD to LCD transition [76], the topologically identical LuxO-Qrr feedback loop appears to play a different role – namely fine-tuning the threshold where the bacterial population reaches a quorum while controlling the dynamic range of expression of quorum-sensing target gene expression. We suggest that bacterial sRNA posttranscriptional negative regulatory feedback loops, and potentially, eukaryotic miRNA negative feedback loops may prove to be a common network design motif. They provide features such as noise reduction, rise time shortening, grading of responses, as well as new ones including the dynamic range control proposed here in the context of the *V. harveyi* quorum-sensing circuit.
3.4 Materials and methods

3.4.1 Fluorescence analysis and bioluminescence assays

All gfp expression analyses were performed on a Becton Dickinson FACS Aria cell sorter, and data were analyzed using FACS Diva software as described previously [76]. For dose-response curve experiments, cultures were grown for 14 h in LM medium and subsequently diluted 1:1000 in fresh medium. In a 96-well microtiter plate, 90 µl of culture was added to 5 µl of 100 µM AI-1 and 5 µl of 100 µM AI-2, and serial dilutions were made to final concentrations of 10 pM total of both AI-1 and AI-2. The plates were incubated for 6 h and then each sample was transferred to a 5 mL round-bottom tube (BD Biosciences) and fluorescence was measured. For dose response curves measuring bioluminescence, an identical procedure was used except that cultures were grown in AB medium in quadruplicate. Bioluminescence and OD$_{600}$ were measured using a Perkin Elmer EnVision plate reader.

3.4.2 Single-cell fluorescence microscopy

For measurements of LuxR-mCherry in individual cells, wild-type, $qrr4^+$, and the corresponding feedback loop mutant $V$. harveyi strains (KT841 KT843 KT845 KT847 KT849 KT851 KT853 KT855) were grown in AB medium for 16 hrs. Growth was
monitored by measuring optical density at 600 nm. Cultures were diluted to OD$_{600}$ = 0.0005 and exogenous autoinducers were added at the specified concentrations. After growing for 6 hrs, cells were concentrated by centrifugation and maintained on ice until measurements were made. 1 µl of cell culture was spread on a glass slide and covered with a 1% AB agarose pad as well as a coverslip. Phase-contrast and fluorescent images were taken at room temperature using a Nikon TE-2000U inverted microscope. Custom Basic code was written to control the microscope. Images were acquired using a 100x oil objective and a cooled CCD camera (-65°C, Andor iXon). Segmentation of individual cells was performed on phase-contrast images. Background and cellular auto-fluorescence values were subtracted from the red channel. Total fluorescence intensity of each cell was obtained by summing all pixels and fractions of pixels in the segmented cell region. Cell volume was estimated by considering each cell as a cylinder plus two semi-spheres. LuxR-mCherry concentration in each cell was calculated as the total mCherry fluorescence intensity over the volume of that cell.

3.4.3 Quantification of cellular RNA levels

*V. harveyi* cell cultures grown in LM medium for 14 hrs were diluted to OD$_{600}$ = 0.005 in 5ml AB medium in duplicate with 0.5 µM AI-1 and 0.5 µM AI-2 added to the cultures. After 6 hrs growth at 30°C, 1 ml cell culture of each strain was centrifuged and the pelleted cells were transferred into 2ml fresh AB medium and put back into a shaking incubator at 30°C. At specified time points, 20µl cell cultures were taken out and mixed
with 180ul RNAlater® (Ambion) solution to preserve cellular RNA. Quantification of RNA levels was later performed using QantiGene Plex 2.0 Reagent System (Panomics) that quantifies RNA directly from crude cell lysates using complimentary oligonucleotides attached to beads without any RNA purification, reverse transcription, or target amplification. The assay was performed according to the manufacturer’s recommendations.

3.5 Acknowledgement

Chapter 3 is part of the submitted work [74].
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Chapter 4 Complexity in the processing of *V. harveyi* quorum-sensing signals

4.1 Signaling strength of the two autoinducer-sensing pathways is modulated

We have previously shown that information from the two autoinducers – AI-1 and AI-2 – is integrated in an additive way, with an unexpected balance between the signaling strengths of the different autoinducer-sensing pathways (Figure 2.2-6). This equal weighting of the two signal detection pathways is suggested to minimize interference between pathways and allow bacteria to learn maximal amount of information of individual signals [85]. Specifically, in *V. harveyi* quorum-sensing circuit, multiple autoinducer-sensing pathways are converged in a phosphorelay through LuxU and LuxO (Figure 1.4-1). If signaling response in one pathway is much stronger than others, the converged output of all pathways will be dominated by the strongest input. In particular, Qrr4-GFP or LuxR-mCherry level will only be responsive to the preponderant autoinducer and mask the subtle changes caused by other weaker autoinducer-sensing pathways. Therefore, in order to properly integrate multiple quorum-sensing signals, the signaling strength of these autoinducer-sensing pathways must be modulated to optimize obtained information.
Interestingly, the relative signaling strength of the AI-1-sensing/LuxN and the AI-2-sensing/LuxPQ pathways is different under various circumstances. In 2.2.3, we have shown that in the LuxN$^+$ LuxPQ$^+$ strain (TL89), Qrr4-GFP expression was almost identical in response to either AI-1 or AI-2. However, when we applied bioluminescence as the quorum-sensing output and similarly measured the dose-response curves for individual pathways or combined pathways, a distinction in the response to AI-1 and the response to AI-2 was observed (Figure 4.1-1). As clearly shown in Figure 4.1-1B, responses of the LuxN$^+$ LuxPQ$^+$ strain (TL27) to AI-1 and AI-2 are not identical as in Figure 2.2-6. The difference in AI-1 AI-2 dose responses suggest the two signaling pathways are not of equal strength any more.

Since bioluminescence is produced by genes in the lux operon activated by the master quorum-sensing regulator LuxR, we would like to know whether LuxR shows different responses to AI-1 and AI-2. Toward this end, we further examined the dose responses of sensor mutant strains that carry LuxR-mCherry on the chromosome as the quorum-sensing output (TL110, TL111, and TL112). Similar to the previous sensor mutant strains using P$_{Qrr4}$-GFP as reporter and Ptac-mCherry as internal standard (Chapter 2), these LuxR-mCherry strains also carry a constitutively expressed internal standard – a GFP driven by the Ptac promoter placed at the same intergenic region on chromosome as the Ptac-mCherry fusion was placed in the previous strains. Similar single-cell dose-response experiments were performed on these LuxR-mCherry reporter strains and the results are shown in Figure 4.1-2. We notice that the dose-response curves resemble the ones observed in the bioluminescence reporter strains (Figure 4.1-1). The
Figure 4.1-1 **Dose-response curves of *V. harveyi* sensor mutants using bioluminescence as quorum-sensing output.** (A) Dose responses to AI-1 (blue squares) of the LuxN+ strain (TL25) and to AI-2 (red circles) of the LuxPQ+ strain (TL26). (B) Dose responses to AI-1 or AI-2 with saturating or zero amount of the other autoinducers of the LuxN+ LuxPQ+ strain (TL27). Error bars indicate standard deviations obtained from triplicate.
Figure 4.1-2 Autoinducer dose responses using LuxR-mCherry as quorum-sensing output. (A) Dose responses of LuxN$^+$ cells (TL110) to AI-1 (blue) and LuxPQ$^+$ cells (TL111) to AI-2 (red). Each average and standard deviation (error bar) of normalized LuxR-mCherry was obtained from microscopy images of 100 cells. Normalization was done by dividing individual cells’ mCherry intensity over their own GFP intensity. Curves were fitted using $\alpha_{\text{AI}} + \beta_{\text{AI}}/(1+[\text{AI}]/K_{\text{AI}})$ with $K_{\text{AI-1}} = 5.6$ nM and $K_{\text{AI-2}} = 1.6$ nM. A.U. denotes arbitrary units. (B) Dose responses of LuxN$^+$ LuxPQ$^+$ cells (TL112) to either AI-1 (blue) or AI-2 (red) while the other autoinducer is either absent (open squares and circles) or present at a saturating concentration (solid squares and circles). Curves were fitted using $\alpha_{\text{AI}} + \beta_{\text{AI}}/(1+[\text{AI}]/K_{\text{AI}})$ with $K_{\text{AI-1,saturating AI-2}} = 4.2$ nM, $K_{\text{AI-2,saturating AI-1}} = 0.34$ nM for dose-response curves with saturating amount of the other autoinducer and $K_{\text{AI-1, no AI-2}} = 13$ nM, $K_{\text{AI-2, no AI-1}} = 1.3$ nM for dose-response curves with zero amount of the other autoinducer.

Relative strength of each signaling pathway is the same in Figure 4.1-2 as in Figure 4.1-1, only with small difference in the absolute amplitude of dose responses to autoinducers, which is probably caused by the nonlinear amplification from the number of LuxR proteins to the amount of bioluminescence catalyzed by LuxR-activated luciferase. Nevertheless, the dose-response curves in Figure 4.1-2 confirmed that signaling strength of LuxN and LuxPQ pathways is no longer balanced to almost equal as observed in the previous $P_{\text{Qrr4}}$-GFP sensor mutant strains. Noticeably, the inhibition constants $K_{\text{AI-1}}$ and $K_{\text{AI-2}}$ for the two autoinducers also differ from each other, whereas the two constants are very similar in the $P_{\text{Qrr4}}$-GFP sensor mutants.
We wonder what factors modulate the signaling strength of the two autoinducer-sensing pathways. The major difference between the sensor mutants carrying P_{Qrr4}-GFP reporter and those carrying LuxR-mCherry reporter (or natural bioluminescence reporter) is the absence or presence of LuxR in the quorum-sensing circuit. In the P_{Qrr4}-GFP sensor mutant strains (TL87, TL88, and TL89), luxR gene was deleted to avoid extra feedback regulation on the P_{Qrr4}-GFP reporter. While in the LuxR-mCherry sensor mutants (TL110, TL111, and TL112), luxR is intact. It is very likely that LuxR protein or luxR mRNA plays some role in modulating the relative strength of the two signaling pathways. To further investigate this problem, we constructed P_{Qrr4}-GFP sensor mutant strains with luxR gene intact on the chromosome (TL116, TL117, and TL118) and performed the same dose-response experiments for individual autoinducer-sensing pathways and combined pathways. As shown in Figure 4.1-3B, responses to AI-1 or AI-2 are no longer identical as in Figure 2.2-6. The difference in relative signaling strength of AI-1 and AI-2 sensing pathways is consistent with that observed using LuxR-mCherry (Figure 4.1-2) and bioluminescence (Figure 4.1-1) as reporters. These results all confirmed that in the absence or presence of luxR gene, relative signaling strength of the two signaling pathways is different. As discussed in 2.3, signaling strength of individual autoinducer-sensing pathways depends on receptor number and the biochemical properties that determine the kinase and phosphatase activities of single receptor. Thus, the change in signaling strength suggests LuxR protein or luxR mRNA may play a role in regulating LuxN/ LuxPQ numbers or affect their kinase/phosphatase activities.
Figure 4.1-3 Autoinducer dose responses of *V. harveyi* sensor mutants using \( \text{P}_{\text{Qrr4}} \)-GFP reporter in the presence of *luxR* gene. (A) Dose responses of LuxN\(^+\) cells (TL116) to AI-1 (blue) and LuxPQ\(^+\) cells (TL117) to AI-2 (red). Each average and standard deviation (error bar) of normalized GFP was obtained from microscopy images.
of 100 cells. A.U. denotes arbitrary units. (B) Dose responses of LuxN$^+$ LuxPQ$^+$ cells (TL118) to either AI-1 (blue) or AI-2 (red) while the other autoinducer is either absent (open squares and circles) or present at a saturating concentration (solid squares and circles).

We now know LuxR protein or luxR mRNA modulate the total kinase/phosphatase activities of the two autoinducer-sensing pathways. But whether luxR affects only LuxN or LuxPQ pathway or both pathways is still a mystery. To answer this question, we conducted a side-by-side dose-response experiment of the LuxN$^+$ and LuxPQ$^+$ sensor mutants either with or without luxR gene on the chromosome using P$_{Qrr4}$-GFP as the reporter. As shown in Figure 4.1-4, AI-1 dose-responses of the strains possessing only LuxN pathway in the absence or presence of luxR gene are almost identical, whereas AI-2 dose-responses of the strains possessing only LuxPQ pathway in the absence or presence of luxR gene are quite different. These results indicate that luxR has a major influence in the kinase and/or phosphatase activities of the LuxPQ pathway, either by changing LuxPQ protein number or affecting the biochemical properties of LuxPQ. Using a bioinformatic method [75], we searched the promoter region of luxPQ and found on LuxR binding site. The involvement of luxR in modulating the signaling strength of LuxPQ pathway is probably indirect. Since luxR mRNA is one of the many repressed targets of the Qrr sRNAs, it is also likely that presence/absence of luxR mRNA titrates Qrr sRNAs amount and therefore affect the regulation of other mRNA targets, whose protein products could affect the signaling strength.
Figure 4.1-4 Side-by-side comparison of dose responses of sensor mutants with and without *luxR* gene. (A) AI-1 dose responses of LuxN⁺ LuxR⁻ cells (TL87, purple squares) and LuxN⁺ LuxR⁺ cells (TL116, blue circles). Each average and standard deviation (error bar) of normalized GFP was obtained from microscopy images of 100 cells. A.U. denotes arbitrary units. (B) AI-2 dose responses of LuxPQ⁺ LuxR⁻ cells (TL88, pink squares) and LuxPQ⁺ LuxR⁺ cells (TL117, blue circles).
More surprisingly, not only the master quorum-sensing regulator gene \textit{luxR} affects signaling strength of the autoinducer-sensing pathways, the autoinducer synthase genes are also found to modulate the relative strength of the two signaling pathways. In the LuxN\(^+\) and LuxPQ\(^+\) strains, only the corresponding autoinducer receptor LuxN or LuxPQ is remained while the other two receptors are knocked out. These two strains also lack the corresponding autoinducer synthases so that we can add autoinducers exogenously to precisely control the input into the quorum-sensing system. However, the other two synthases for the non-responsive autoinducers are still present in the cell. We further constructed the AI-1 sensing and AI-2 sensing strains, where all three genes for autoinducer synthases are deleted from the chromosome, and, similar to the LuxN\(^+\) and LuxPQ\(^+\) strains, only the corresponding autoinducer receptor LuxN or LuxPQ is kept in the AI-1 sensing or AI-2 sensing strains, respectively. Using LuxR-mCherry as the quorum-sensing reporter, we measured dose-response curves of the LuxN\(^+\) (TL110) and LuxPQ\(^+\) (TL111) strains, as well as the AI-1 sensing (TL150) and AI-2 sensing (TL151) strains (Figure 4.1-5). To our surprise, the two sets of dose-response curves revealed different relative signaling strength between the AI-1-sensing/LuxN and AI-2-sensing/LuxPQ pathways. It could be that the autoinducer synthases somehow indirectly involve in the regulation of total kinase/phosphatase activities of the two signaling pathways. Alternatively, the mRNAs of the synthases could be targets of the Qrr sRNAs, and as a result, presence or absence of the synthase genes could affect LuxN or LuxPQ receptor number through titration of sRNA and competition with other sRNA targets.
Figure 4.1-5 Autoinducer synthase genes affect relative signaling strength of the two signaling pathways. (A) Dose responses of the LuxN$^+$ cells (TL110) to AI-1 (blue square) and LuxPQ$^+$ cells (TL111) to AI-2 (red circle). Each average and standard deviation (error bar) of normalized LuxR-mCherry was obtained from microscopy images of 100 cells. A.U. denotes arbitrary units. (B) Dose responses of the AI-1 sensing cells (TL150) to AI-1 (blue squares) and AI-2 sensing cell (TL151) to AI-2 (red circles).
4.2 Signaling through multiple small RNAs in the quorum-sensing circuit

In the *V. harveyi* quorum-sensing circuit, information from multiple autoinducers is integrated into a central response regulator LuxO (Figure 1.4-1). Phosphorylated LuxO (LuxO-P) in turn activates five small regulatory RNAs. Eventually, all of these five small RNAs have the same repress target – *luxR* mRNA. The backbone of the *V. harveyi* quorum-sensing circuit possesses an interesting structure: multiple autoinducers converge to single LuxO, then diverge to multiple sRNAs, and converge again to single LuxR, finally diverge again to hundreds of genes activated or repressed by LuxR. The five sRNAs in *V. harveyi* are found to act additively to control quorum sensing by altering translation of the master transcriptional regulator, LuxR [43]. In a closely related species *V. cholerae*, there are four sRNAs sitting downstream of LuxO in a very similar quorum-sensing circuit. However, the four sRNAs *V. cholerae* act redundantly to control quorum-sensing gene expression by destabilizing the mRNA encoding the master quorum-sensing transcriptional regulator, HapR [42].

It remains a mystery why there are multiple sRNAs and what their roles are in quorum-sensing signaling besides simply being repressors of LuxR or HapR. One hypothesis is that LuxO-P activates different sRNAs at different threshold levels so that a particular set of sRNAs are turned on corresponding to each LuxO-P concentration. This setting presumably converts the continuous LuxO-P concentration to discrete sRNA levels and the resulting digitization of the quorum-sensing information is proposed to
reduce signaling noise. To test this hypothesis, we engineered sensor mutant strains that carry both $P_{Qrr4}$-GFP and $P_{Qrr2}$-mCherry reporters. By varying the input of autoinducer concentrations, the responsive LuxO-P levels are changing gradually from minimal to saturated amount. We would like to see whether $P_{Qrr4}$-GFP and $P_{Qrr2}$-mCherry would be turned on at different LuxO-P levels. Figure 4.2-1 showed that the dose-response curves of the LuxN$^+$ (TL105) cells to AI-1 and the LuxPQ$^+$ (TL106) cells to AI-2 have similar inhibition constants for $P_{Qrr4}$-GFP and $P_{Qrr2}$-mCherry (compare green and red curves in Figure 4.2-1). To activate the two different sRNAs – Qrr2 and Qrr4, the threshold of AI-1 concentration differs by less than 10-fold in the LuxN$^+$ strain and the threshold of AI-2 concentration is almost identical in the LuxPQ$^+$ strain. Therefore, at least for Qrr2 and Qrr4, the threshold of autoinducers (or LuxO-P) to turn them on is more or less the same.

In both *V. harveyi* and *V. cholerae*, the Qrr sRNAs repress translation of multiple target mRNAs, such as *luxO* and *luxR* [44,74,76,77]. The Qrr sRNAs in *V. cholerae* are also found to activate translation of a target mRNA [86]. It is likely that besides having common mRNA targets regulated by all Qrrs, each sRNA has its own mRNA targets that are only regulated by that specific Qrr. Primitive evidence showing the existence of Qrr-specific targets is that *V. harveyi* mutant cells possessing each one of the five sRNAs displayed different morphology (Figure 4.2-2). The “Qrr1 only” and “Qrr3 only” cells carry just Qrr1 or Qrr3 while have the other four Qrrs knocked out. These cells showed normal cell shape under microscope. However, the “Qrr2 only”, “Qrr4 only”, and “Qrr5 only” strains have a large portion of filamentous cells observed under microscope. Filamentation is not a consequence of lowered overall Qrr level since the LuxO$^-$ strain (no LuxO-P present and the Qrrs are not expressed) grow normally just as the “Qrr1 only”
Figure 4.2-1 Comparison of the activation threshold of autoinducers for Qrr2 and Qrr4 expressions. (A) Comparison of dose responses of the LuxN\textsuperscript{+} cells (TL105) to AI-1 using P_{Qrr4}-GFP (green squares) and P_{Qrr2}-mCherry (red squares) reporters. Green or red fluorescence intensity is normalized over the maximal value in each dose-response curve. Each average and standard deviation (error bar) of normalized P_{Qrr4}-GFP and P_{Qrr2}-mCherry was obtained from microscopy images of 100 cells. A.U. denotes arbitrary units. (B) Comparison of dose responses of the LuxPQ\textsuperscript{+} cells (TL106) to AI-2 using P_{Qrr4}-GFP (green squares) and P_{Qrr2}-mCherry (red squares) reporters.
Figure 4.2-2 The *V. harveyi* “Qrr only” strains showed different morphology.
and “Qrr3 only” strains. Therefore, the most likely explanation is that some genes associated with the filamentous phenotype must be regulated by some but not all of the sRNAs.

4.3 Temporal profile of autoinducer production and detection

An outstanding question is why *V. harveyi* and related species employ multiple autoinducer signals, but funnel all the information into a single pathway. We can envision two main possibilities (potentially in combination): the multiple autoinducers could reveal information about the community composition, e.g. which species are present and in what abundance, or the multiple autoinducers could reveal information about the stage of development of the community, e.g. the stage of growth of a biofilm. In support of the first possibility, the three autoinducers used by *V. harveyi* have distinct ranges of species specificity, intraspecies for AI-1, within Vibrios for CAI-1, and across many species for AI-2. Thus, different combinations of the three autoinducers could indicate different compositions of a bacterial community. In our experimental conditions, however, we found that cells could not distinguish very well between high AI-1, low AI-2 and high AI-2, low AI-1 (Figure 2.2-6 and Figure 2.2-7). This result argues for the second possibility, namely that different combinations of autoinducers represent different stages of community development. For example, if a growing *V. harveyi* community typically accumulates AI-2 before AI-1, then the signaling contour in Figure 2.2-7 would always
be traversed along the right edge, and cells could reliably interpret an intermediate signaling strength as a condition of high AI-2, low AI-1, since the opposite condition of high AI-1, low AI-2 would rarely if ever be encountered. In much of eukaryotic development, e.g. embryogenesis, the rate of development is fixed and driven by a clock [87], obviating the need for a signal representing the stage of development. However, without the support of a surrounding organism, the rate of development of a bacterial community depends on unpredictable environmental conditions such as nutrient availability, and therefore some means of determining stage of development is required so that cells in the community can behave appropriately. Recent models of biofilm growth suggest that communities may be mixed at early stages, but that at later stages competition for nutrients by overgrowth of neighboring cells can result in large domains of cells descended from a single progenitor, and therefore composed of a single species [88]. If so, generic signals such as AI-2 may be most informative at early stages of biofilm growth, while species specific signals such as AI-1 may be reserved for later stages.

To explore the order of accumulation of the *V. harveyi* autoinducers AI-1 and AI-2, we engineered autoinducer-sensing strains to report the temporal profile of autoinducer production of wild-type *V. harveyi* in a typical growing process. The AI-sensing strains have LuxR-mCherry as a readout for autoinducer detection and are only responsive to the specified autoinducers. When the AI-1 sensing strain (TL150) or AI-2 sensing strain (TL151) grow together with the wild-type strain in a mixed culture tube, the temporal AI-1 or AI-2 production profile of the wild-type cells can be probed by measuring LuxR-mCherry level inside the AI-sensing strains. The AI-sensing strains also have all three
autoinducer synthases deleted. So they would not contribute to any of the autoinducer concentrations in the environment and presumably not interfere with the normal growth or autoinducer production of the wild-type strain. The AI-1,2 sensing strain has two receptors and can be responsive to both AI-1 and AI-2. The specific AI-sensing strain is mixed with wild-type, both started from OD at $10^{-7}$. LuxR-mCherry intensity was measured by single-cell microscopy every hour and the corresponding OD of the mixed culture was recorded. Ratio of wild-type cells to AI-sensing cells at each time point was calculated by counting the fluorescent AI-sensing cells. To our surprise, we found the temporal profiles of LuxR-mCherry in all the three AI-sensing strains almost overlap and are not very distinctive with each other (Figure 4.3-1A). This suggests that the quorum-sensing responses to individual autoinducers, or to the combined two autoinducers, share similar temporal trait. Interestingly, signaling strength of the AI-1 sensing strain in response to AI-1 and the AI-2 sensing strain in response to AI-2 is very well balanced (Figure 4.3-1B). Combining information from Figure 4.3-1A and B, it is very likely that the two autoinducers AI-1 and AI-2 are produced to trigger equal amount of response in each AI-sensing pathway in a typical wild-type growing process.
Figure 4.3-1 Using AI-1 and AI-2 sensing strains to probe temporal profile of autoinducer production during the growth of wild-type *V. harveyi* cells. (A) AI-1 sensing (TL150, blue squares), AI-2 sensing (TL151, red circles), AI-1,2 sensing (TL123, green triangles) cells are mixed with wild-type cells, respectively. Each species started from OD = 10^{-7}. First time point was taken after 10 hours’ growth. WT (wild-type) OD was calculated using OD of the mixed culture and ratio of wild-type to AI-sensing cells. LuxR-mCherry intensity was an average of 100 cells from microscopy images and error bars represented standard errors. (B) Dose responses of AI-1 sensing (blue squares) and AI-2 sensing (red circles) strains.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB120</td>
<td>Wild-type</td>
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</tr>
<tr>
<td>TL25</td>
<td>ΔluxM ΔluxPQ ΔcqsS</td>
<td>71</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>TL87</td>
<td>ΔluxM ΔluxPQ ΔcqsS ΔluxR Δqrr4::gfp Ptac-mCherry-Kan</td>
<td>71</td>
</tr>
<tr>
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<td>This work</td>
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Quorum sensing is a cell-to-cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers. Quorum sensing enables groups of bacterial cells to coordinate their gene expression and behave collectively. Often, bacteria employ multiple autoinducers. In the model quorum-sensing bacterium *Vibrio harveyi*, three parallel autoinducer-sensing pathways converge into a shared response regulator in a phosphorelay signaling circuit. It is intriguing to consider why there are multiple autoinducers and how cells integrate and interpret the information contained within these multiple signals. To investigate this problem, we constructed a suite of mutant strains that have either a single autoinducer-sensing pathway or a combination of two of the three pathways. We also constructed several fluorescent protein reporters as outputs of different stages of the signaling system. Using single-cell fluorescence microscopy, we quantified the signaling responses in individual cells and analyzed the integration of multiple autoinducer signals in the quorum-sensing circuit. The results revealed that information from the two distinct autoinducers, AI-1 and AI-2, is combined strictly additively with each autoinducer contributing very nearly equally to the total response. An analysis using information theory shows that this equal weighting of information from both signals optimizes the total information obtained through quorum sensing [85]. Our results suggest that multiple autoinducers may be utilized by bacterial cells to indicate specific stages during the development of a microbial community. Bacteria might decipher characteristic
combinations and quantities of autoinducers as signatures of particular community conditions, and respond with appropriate programs of gene expression.

Quorum-sensing bacteria also employ complex feedback regulations in the signaling circuit. In *V. harveyi*, we identified and characterized two negative feedback loops that act to facilitate precise transitions between low-cell-density (LCD) and high-cell-density (HCD) states. The quorum-sensing central response regulator LuxO autorepresses its own transcription and the Qrr small regulatory RNAs (sRNAs) posttranscriptionally repress *luxO* by base-pairing with the *luxO* mRNA transcript. Disruption of these negative feedback loops substantially increases the concentration of autoinducers required for cells to transit from LCD to HCD modes of quorum-sensing-regulated gene expression. We conclude that the two cooperative negative feedback loops determine the point at which *V. harveyi* has reached a quorum and control the range of autoinducers over which the transition occurs. Negative feedback regulation also constrains the range of quorum-sensing output – by preventing sRNA levels from becoming too high and preventing *luxO* mRNA levels from reaching zero. Our findings suggest that sRNA-mediated feedback regulation is a common design feature that permits fine-tuning of gene regulation and maintenance of homeostasis.

Quorum-sensing signaling involves complicated modulation in the signaling strength of multiple autoinducer-sensing pathways. We found that quorum-sensing components, such as the gene encoding master quorum-sensing regulator LuxR, as well as the autoinducer synthases, fine-tune the relative signaling strength of the AI-1 and AI-2 sensing pathways. We also found that the quorum-sensing response is coupled with
autoinducer production and perception in the specific niche where the bacteria live and a particular time stage which the microbial community develop into.

Although the simplest organisms, bacteria possess sophisticated signaling systems. They have the ability to accurately detect and properly respond to complicated environmental cues, to communicate with each other and act cooperatively like social animals, to carry out elaborate cellular differentiation and develop like multicellular organisms, and to quickly adapt to the constantly changing environment. Quorum sensing connects bacterial behaviors to an interacting and structured microbial community including both cooperativity and competition among individuals. Dealing with these intricate problems, quantitative methods and physical reasoning are often in demand. As the exploration of biology goes deeper and wider, qualitative experiments and descriptive methods will not be enough to understand the complexity of living systems. Quantitative experiments and systematic analysis are becoming more and more indispensible to obtain a comprehensive understanding of biological systems and to reveal the underlying principles in all aspects of biology. With an integration of physical sciences and biology, our knowledge about, not only quorum sensing or bacterial signaling, but the fundamental mechanisms and principles of the nature of all living things, will reach an unprecedented scope.
Appendix A  Constraints on Kinase and Phosphatase Rates Set by Additivity

Our discovery that the signals from the two autoinducers AI-1 and AI-2 are integrated in a strictly additive way in the \textit{V. harveyi} quorum-sensing circuit sets strong constraints on the parameters of the biochemical reactions within the circuit. Our analysis of these reactions is based on the two-state model for the quorum-sensing receptors [66,67]. Each of the receptors LuxN and LuxPQ are considered to have two distinct conformational states denoted as ON and OFF states. Associated with each state, there is an intrinsic kinase rate and an intrinsic phosphatase rate per receptor (the kinase rates are denoted $q_{ON}^{+}$ and $q_{OFF}^{+}$, and the phosphatase rates are $q_{ON}^{-}$ and $q_{OFF}^{-}$ for the ON and OFF states). For each type of receptor, LuxN or LuxPQ, the fraction of receptors in the ON state (which equals the probability of any given receptor being in the ON state) is

$$p_{ON} = \frac{1}{1 + e^{-F}} \quad , \quad \text{(A1)}$$

where

$$F = E_{ON} - E_{OFF} + \log \left( \frac{1 + [AI]/K_{d}^{OFF}}{1 + [AI]/K_{d}^{ON}} \right) \quad \text{(A2)}$$

is the free energy difference between the ON and OFF states (all energies are in units of the thermal energy $k_B T$). $E_{ON}, E_{OFF}$ are the free energies without autoinducer bound and $K_{d}^{ON}, K_{d}^{OFF}$ are the dissociation constants in the ON and OFF states. Note that for
\[ K_d^{\text{OFF}} << K_d^{\text{ON}} \text{ and } E_{\text{ON}} \leq E_{\text{OFF}}, \] the fraction of receptors in the ON state can be expressed as a simple non-cooperative Hill function of autoinducer concentration

\[ p_{\text{ON}} = \frac{\sigma}{1 + [\text{AI}]/K_{\text{Al}}}, \quad (A3) \]

where \( \sigma = 1/(1 + e^{E_{\text{OFF}} - E_{\text{ON}}}) \) and \( K_{\text{AI}} = \left(1 + e^{E_{\text{OFF}} - E_{\text{ON}}} \right) \cdot K_d^{\text{OFF}} \). This was found to be the case for LuxN in a previous study (67). Autoinducers regulate the fraction of receptors in each state but not the intrinsic kinase or phosphatase rates of the ON and OFF states. The total cellular kinase and phosphatase activities of the receptors can therefore be expressed as:

\[
\begin{align*}
K_N &= p_{\text{ON},N} \cdot [N]_{\text{tot}} \cdot q_{\text{ON,NN}}^+ + (1 - p_{\text{ON},N}) \cdot [N]_{\text{tot}} \cdot q_{\text{OFF,NN}}^+ \\
K_P &= p_{\text{ON},P} \cdot [PQ]_{\text{tot}} \cdot q_{\text{ON,PQ}}^+ + (1 - p_{\text{ON},P}) \cdot [PQ]_{\text{tot}} \cdot q_{\text{OFF,PQ}}^+ \\
K_{\text{NQ}} &= p_{\text{ON},\text{NQ}} \cdot [PQ]_{\text{tot}} \cdot q_{\text{ON,NQ}}^- + (1 - p_{\text{ON},\text{NQ}}) \cdot [PQ]_{\text{tot}} \cdot q_{\text{OFF,NQ}}^-
\end{align*}
\]

(A4)

The steady-state LuxO-P level is determined by these kinase and phosphatase activities of LuxN and LuxPQ according to Equation (3) in 2.2.6. Taking account Equations (A4), the denominator in Equation (3) can be rewritten explicitly as

\[ K_N + K_{\text{PQ}} + k_-/k_+ \cdot (P_N + P_{\text{PQ}}) = [N]_{\text{tot}} \left[ \beta_N + \left( \alpha_N - \beta_N \right) \cdot p_{\text{ON,N}} \right] + [PQ]_{\text{tot}} \left[ \beta_{\text{PQ}} + \left( \alpha_{\text{PQ}} - \beta_{\text{PQ}} \right) \cdot p_{\text{ON,PQ}} \right], \]

(A5)

where

\[
\begin{align*}
\alpha_N &= q_{\text{ON,NN}}^+ + k_-/k_+ \cdot q_{\text{OFF,NN}}^- \\
\beta_N &= q_{\text{OFF,NN}}^+ + k_-/k_+ \cdot q_{\text{OFF,NN}}^- \\
\alpha_{\text{PQ}} &= q_{\text{ON,PQ}}^+ + k_-/k_+ \cdot q_{\text{ON,PQ}}^- \\
\beta_{\text{PQ}} &= q_{\text{OFF,PQ}}^+ + k_-/k_+ \cdot q_{\text{OFF,PQ}}^-
\end{align*}
\]

(A6)

As discussed in 2.2.6, to achieve the observed additivity of AI-1 and AI-2 signal...
integration, this denominator, expressed in Equation (A5), must be approximately constant, independent of AI-1 and AI-2 concentrations. Therefore, the expression on the right hand side of Equation (A5) must be independent of $p_{ON,N}$ and $p_{ON,PQ}$, which requires $|\alpha_N - \beta_N| \ll \beta_N$ and $|\alpha_{PQ} - \beta_{PQ}| \ll \beta_{PQ}$, i.e.

$$\left[ q_{ON,N}^+ - q_{OFF,N}^- + k_- / k_+ \cdot (q_{ON,N}^- - q_{OFF,N}^-) \right] \ll q_{OFF,N}^- + k_- / k_+ \cdot q_{OFF,N}^-$$
$$\left[ q_{ON,PQ}^+ - q_{OFF,PQ}^- + k_- / k_+ \cdot (q_{ON,PQ}^- - q_{OFF,PQ}^-) \right] \ll q_{OFF,PQ}^- + k_- / k_+ \cdot q_{OFF,PQ}^-.$$  \hspace{1cm} (A7)

These conditions imply either one of two scenarios: the first one is

$$\begin{cases} q_{ON,N}^- \approx q_{OFF,N}^-, & q_{ON,N}^+ \ll k_- / k_+ \cdot q_{OFF,N}^- \\ q_{ON,PQ}^- \approx q_{OFF,PQ}^-, & q_{ON,PQ}^+ \ll k_- / k_+ \cdot q_{OFF,PQ}^- \end{cases}$$ \hspace{1cm} (A8)

and the second is

$$\begin{cases} q_{ON,N}^+ - q_{OFF,N}^- \approx k_- / k_+ \cdot (q_{OFF,N}^- - q_{ON,N}^-) \\ q_{ON,PQ}^+ - q_{OFF,PQ}^- \approx k_- / k_+ \cdot (q_{OFF,PQ}^- - q_{ON,PQ}^-) \end{cases}$$ \hspace{1cm} (A9)

The first scenario (A8) corresponds to the first one discussed in 2.2.6: the phosphatase rates in the ON and OFF states $q_{ON}^-$ and $q_{OFF}^-$ are approximately the same, which means the phosphatase activity $P_N + P_{PQ}$ is effectively unregulated by autoinducers; on the other hand, from the observed response to autoinducers, the kinase rate in the OFF state must be much smaller than that in the ON state, i.e. $q_{OFF}^+ \ll q_{ON}^+$, and the kinase activity $K_N + K_{PQ}$ must be much smaller than the effective phosphatase activity $k_- / k_+ \cdot (P_N + P_{PQ})$, resulting in a far-from-saturated LuxO-P level, i.e. $[O-P] \ll [O]_{tot}$.

The second scenario (A9) corresponds to the second one mentioned in the main text: both
kinase and phosphatase activities are regulated by autoinducers (with different kinase rates $q_{ON}^+$, $q_{OFF}^-$ and different phosphatase rates $q_{ON}^-$, $q_{OFF}^+$ in the two states), but the change in kinase activity due to autoinducer regulation, proportional to $q_{ON}^- - q_{OFF}^-$, just equals the negative change in the effective phosphatase activity, proportional to $k_-/k_+ \cdot \left(q_{OFF}^- - q_{ON}^+ \right)$. This condition requires fine-tuning of the reaction rates and we consider it less likely than the first scenario.
Appendix B  An efficient way to remove antibiotic resistance markers in \textit{V. harveyi}

Datsenko and Wanner developed a simple and efficient method to disrupt chromosomal genes in \textit{Escherichia coli} in which the phage \( \lambda \) Red recombinase is utilized to promote recombination between PCR product(s) synthesized with homologous primers and the targeted gene(s) [70]. Deletion of a particular gene generates a scar of antibiotic resistance gene in replacement of the original coding sequence. The antibiotic resistance genes are flanked by FRT (FLP recognition target) sites and can be further eliminated by using a helper plasmid pCP20 expressing the FLP recombinase, which acts on the directly repeated FRT sites flanking the resistance gene [89]. This system was adapted to the deletion of chromosomal genes in \textit{V. harveyi}. However, there are extra steps to be taken in order to utilize this method. First, a cosmid (pLAFR) containing the DNA sequence covering the targeted gene has to be transformed into the \textit{E. coli} strain used in the Datsenko and Wanner method to replace the gene’s coding sequence on the cosmid with an antibiotic resistance marker. The resulting cosmid is transformed into the parent \textit{V. harveyi} strain and the antibiotic resistance marker will be integrated into chromosome through homologous recombination. To get rid of the antibiotic resistance marker from chromosome, the \textit{E. coli} cells carrying the constructed cosmid have to be transformed with the plasmid pCP20 expressing the FLP recombinase to eliminate the antibiotic resistance marker on the cosmid. The resulting cosmid, which contains the DNA
sequence covering the targeted gene but with the coding sequence of that gene completely deleted, is then transformed back into the *V. harveyi* strain which has the targeted gene replaced by an antibiotic marker. With homologous recombination, some cells will have the marker replaced by the clean deletion. Although this method works, the efficiency is extremely low. A massive screen has to be taken to look for the antibiotic sensitive cells. Usually, the chance for such homologous recombination to happen is less than 1%.

I improved the way to eliminate antibiotic resistance marker on *V. harveyi* chromosome by directly introducing the FLP recombinase into the *V. harveyi* cells. Specifically, I constructed a plasmid pTL17 by inserting the coding sequence of FLP recombinase into plasmid pEVS143 at the AvrII and BamHI sites (Figure B-1). Plasmid pEVS143 can be replicated and maintained in *V. harveyi* and FLP recombinase expression can be induced in the *V. harveyi* cells by adding IPTG. By transforming pTL17 into the *V. harveyi* cells with antibiotic markers, the chance of finding an antibiotic sensitive colony is largely increased, from less than 1% to more than 50%. Also, this method skipped the steps to construct the cosmid with a clean deletion and saved several days in the process of cloning. Since the most commonly used antibiotic markers in *V. harveyi* cloning are chloramphenicol (Cm) and kanamycin (Kan), the pTL17 plasmid is further modified by inserting a coding sequence of TetR, the tetracycline (Tet) resistance protein, into the PvuI site inside the coding sequence of KanR, the kanamycin resistance protein, on pTL17 (Figure B-1). The resulting Tet resistant plasmid pTL18 can now be used to remove both Cm and Kan resistance markers.
Figure B-4.3-1 Construction of plasmids pTL17 and pTL18.
References


