QUORUM SIGNALING AND SYMBIOSIS IN THE MARINE LUMINOUS BACTERIUM *VIBRIO FISCHERI*

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Recent studies of the biology of the marine bacterium *Vibrio fischeri* have focused primarily on two related characteristics of this species: its signature capacity to produce bioluminescence and its ability to symbiotically colonize the light-emitting organs of certain species of squids and fishes (65, 77). Not surprisingly, these environment-specific activities have been shown to be under the control of a series of cellular signal and response systems that include two-component phosphorelays (59, 101, 105), chemical receptors (6, 94), and quorum sensing (30, 96).

Acyl-homoserine lactone (AHL)-based quorum sensing was first described in *V. fischeri* (64) in the early 1970s and has subsequently been found to be present in at least 70 species of other gram-negative bacteria, including a number of important pathogens (39, 99). In an idealized and simplified model of the quorum-sensing process (sometimes called "autoinduction"), cells continuously emit species-specific AHLs and sense the presence and abundance of related bacteria by the accumulation of these autoinducers in their environment. As a result, the quorum-induced cells are able to differentially regulate genes whose products convey a selective advantage only at a high bacterial density (30). AHL signaling was initially identified because it regulated the expression of the *lux* gene clusters in *V. fischeri* and *Vibrio harveyi* (64) and encoded bacterial luciferase and other proteins required for bioluminescence (20). Later it was recognized that quorum sensing controls additional gene products in these and other microbial species (see references 8, 42, and 56). This signal-response system is both biologically and genetically flexible and has evolved in different bacteria to regulate a variety of different genes, even within a single genus (60). Genetic investigations have revealed that several species encode two or more AHL systems, often with (i) one controlling the expression of the other, and/or (ii) each operating independently but providing parallel inputs into a circuit that appears to function like a coincidence detector (30, 62). The study of quorum sensing over the last 3 decades has been responsible for a fundamental change in the way we think about bacterial behavior (39), yet the single most

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intriguing characteristic of this form of communication may be that it is deeply embedded in the way bacteria have evolved specific relationships, either beneficial or pathogenic, with eukaryotic hosts.

Recognition of the ubiquity and importance of bacterial symbioses in animals and plants has led to an increasing interest in how bacterium-bacterium and bacterium-host signaling maintains specificity and modulates functionality in beneficial associations. The list of microbial symbioses in which AHL quorum sensing has been identified continues to grow (33, 54, 87, 96, 97, 102) and has led to the development of a number of natural experimental systems for studying the mechanisms of this cell-cell signaling process and its biological consequences under conditions that exist in the real world (95).

In this chapter we review the role of quorum sensing in *V. fischeri*, focusing on recent developments in our understanding of the genetics and physiology of cell-cell signaling by populations of these bacteria, both in culture and in their light-organ symbioses. Particular emphasis is placed on outlining the regulatory factors and pathways by which quorum sensing coordinates the biological activities of this bioluminescent microbe.

MJ1 AND ES114: THE DIFFERENCE A STRAIN MAKES

The last 15 years have brought an easily overlooked shift in the major strains being used to study quorum sensing in *V. fischeri*, and important differences between these isolates bear emphasizing. Quorum sensing was discovered because it was used by several marine bacteria to control the induction of the *luxICDABEG* operon, which is responsible for generating bioluminescence, an easily measured phenotype. Earlier work typically utilized very bright isolates of *V. fischeri* that formed visibly luminescent colonies on plates and intensely light-emitting broth cultures. This is, after all, the phenotype that drew interest to *V. fischeri* and quorum sensing in the first place, and it is the image of this bacterium that most readers will have in their mind's eye. The seminal paper describing autoinduction in luminous bacteria used a strain designated MAV (63). When MAV was lost, *V. fischeri* MJ1 became the workhorse strain in the biochemical and molecular dissection of luminescence regulation, starting with the isolation and chemical identification of the first AHL signal (22), and continuing into the cloning, sequencing, and functional characterization of the MJ1 *lux* region (24, 25). Although strain ATCC 7744 has also been used (19), sequence differences between the genes of the ATCC 7744 and MJ1 *lux* operons are minimal (18), and while these strains behave similarly with respect to luminescence and autoinduction, ATCC 7744 often forms distinct colonies less readily. Thus, MJ1 became a useful type strain, and most of our fundamental knowledge of the mechanisms underlying quorum sensing in *V. fischeri* was discovered in MJ1 or inferred from experiments using the MJ1 *lux* genes cloned in *Escherichia coli*.

Development of an Environmentally Relevant Model System of Quorum Signaling

Unfortunately, MJ1 and other bright strains posed an experimental limitation: they could not be studied under the natural conditions in which *V. fischeri* produces light. Specifically, MJ1 was isolated from the light-emitting organ of the Japanese pinecone fish, *Monocentris japonica*, where *V. fischeri* is highly bioluminescent (79); however, these animals have yet to be induced to reproduce in the laboratory, making them an intractable model to manipulate and study the initiation of symbiosis. As interest shifted to the implications of quorum sensing in the environment, particularly during growth in a host, the inability to reconstitute a natural symbiosis with MJ1 became problematic. For example, although addition of autoinducers was discovered to stimulate transcription of non-*lux* genes in MJ1 (8), there was no way to determine which, if any, of these genes were important for this strain's interactions with *M. japonica*.

A breakthrough in *V. fischeri* research came when this bacterium was described as the light-

organ symbiont of the Hawaiian bobtailed squid, *Euprymna scolopes* (100). This invertebrate had previously been reared in the laboratory (1), and when its juveniles hatched, they were aposymbiotic (i.e., free of symbionts) and acquired *V. fischeri* from the surrounding seawater (100). Early work showed that *V. fischeri* strains native to *E. scolopes* were especially well adapted to this host and that nonnative strains such as MJ1 did not colonize juvenile squid well; thus, MJ1 was not an appropriate strain for studying the symbiosis between *V. fischeri* and *E. scolopes* (77). Instead, *V. fischeri* ES114, which was isolated from an *E. scolopes* light organ (3), was chosen for the subsequent array of ongoing symbiotic studies.

Although MJ1 continues to be used productively for biochemical studies of *lux*-encoded proteins, ES114 has become the wild-type strain of choice for most researchers investigating quorum sensing and/or symbiosis. For this reason, ES114 was the first *V. fischeri* strain to have its genome sequenced (80), and as a result, commercially produced microarrays are based on the gene content of ES114 and most mutant analyses are now done in ES114. Furthermore, many of the recent advances in understanding quorum sensing in *V. fischeri*, as well as all of the symbiotic studies described below, have used ES114 as the wild-type and/or parent strain. For these reasons, the following sections of this chapter focus primarily on *V. fischeri* ES114; however, before doing so, it is relevant to summarize certain important similarities and differences between strains ES114 and MJ1.

A Comparison of Quorum Signaling between *V. fischeri* Strains

The most obvious difference between MJ1 and ES114 is that the latter is not visibly luminescent in culture. While the specific luminescence (i.e., light emission per cell) of ES114 increases 2 to 3 orders of magnitude when it colonizes the *E. scolopes* light organ (3), the luminescence of cultured ES114 cells, even at very high cell density, typically can be detected only using a luminometer. Interestingly, even in the symbiosis, where cells are packed densely and luminescence is fully induced, ES114 cells produce only 1/10 the luminescence of cultured MJ1 cells. This puzzling property of poor light output (i.e., cell-specific luminescence level) in culture is conserved among virtually all *V. fischeri* isolates from *E. scolopes* (49, 50), and the reason for the prevalence of this trait among the symbionts of this host, but not others (27, 68), is not well understood. Importantly, it is not due to a large-scale genetic difference: the orientation and function of the *lux* genes, which are described below and underpin the quorum-sensing and bioluminescence functions of *V. fischeri*, are similar in ES114 and MJ1 (38). Other components of the *V. fischeri* quorum-sensing system are also found in both strains.

The relatively dim luminescence of ES114 in culture has been attributed to a low production of the AHL autoinducer *N*-3-oxo-hexanoyl-homoserine lactone (3-O-C_6-HSL) (3), and a convincing argument can be made for this assertion. For example, in culture ES114 makes only 0.01% of the 3-O-C_6-HSL that MJ1 does (Table 1). Moreover, even when the *lux* operon of ES114 is fully induced with exogenous 3-O-C_6-HSL, de novo production of this autoinducer is far lower than what is observed in MJ1 (38). On the other hand, a low level of 3-O-C_6-HSL production is a somewhat unsatisfying and circular explanation for low luminescence, considering that the 3-O-C_6-HSL autoinducer synthase gene, *luxI*, is cotranscribed with the *luxCDABEG* genes that encode the enzymes responsible for bioluminescence. Thus, one might reasonably assert that low 3-O-C_6-HSL production does not cause low luminescence in ES114 so much as both phenomena are the result of some other mechanism that attenuates expression of the *luxICDABEG* operon (at least in culture). Interestingly, 3-O-C_6-HSL levels are considerably higher in the light organ of colonized *E. scolopes* than they are in cultures of ES114 grown outside the host (4), and engineered overexpression of ES114 *luxI* and the other *lux* genes within cells of ES114 leads to a high level of luminescence (38). These results indicate that strain ES114 has the metabolic means

TABLE 1 Output of HSL autoinducers by *V. fischeri* strains ES114 and MJ1

Culture optical density	3-oxo-C_6-HSL (nM)		C_8-HSL (nM)	
	ES114	MJ1	ES114	MJ1
0.5	0.01	2	35	11
1.5	0.1	2,100	1,100	180
2.8	0.2	7,400	ND	1,700

to produce higher levels of both luminescence and 3-O-C_6-HSL than it normally does and suggest that an important regulatory difference between MJ1 and ES114—located outside the *lux* operon—exerts an effect on this locus.

One candidate regulator known to affect bioluminescence in *V. fischeri* is *ainS* (32), which produces a second autoinducer, *N*-octanoyl-homoserine lactone (C_8-HSL), as discussed in greater detail below. Production of C_8-HSL initiates stimulation of the *lux* operon at moderate cell densities; if ES114 had relatively low expression of AinS and low C_8-HSL output, this deficiency could potentially explain why ES114 is so much dimmer than MJ1. However, not only is C_8-HSL production unimpaired in ES114, it is even greater than the output of this autoinducer by MJ1 (Table 1).

Most likely, then, the large difference in the levels of 3-O-C_6-HSL production and luminescence seen between cultures of ES114 and MJ1 is due to external regulatory influences on the autoinducer synthase genes, and such regulation may be multifactorial. In this regard, it is worth noting that bioluminescence is affected by environmental conditions very differently in these two strains. One example of this difference is how the expression of luminescence in ES114 and MJ1 varies with culture aeration (Fig. 1). In the experiment presented, aliquots from each culture were vigorously shaken (so that the cell's luciferase was fully oxygenated) immediately before their specific light emission was measured in a luminometer. With ES114, the more highly aerated the culture, the greater the maximal expression of luminescence (Fig. 1A); in contrast, while differences in aeration affected the culture density (A_{595}) at which MJ1 cells induced luminescence, they did not affect maximal luminescence output (Fig. 1B). Similarly, two other notable and documented differences between *lux* regulation in ES114 and MJ1 are that glucose (29, 94) and iron (41) repress luminescence in strain MJ1, but these physiological factors do not affect strain ES114 in culture (3).

Given the dramatic differences between ES114 and MJ1, it is clear that in *V. fischeri* the processes of quorum sensing and *lux* regulation are strain dependent. Thus, when considering individual studies, readers should be careful to note which of these model strains is being used. The differences between gene regulation in

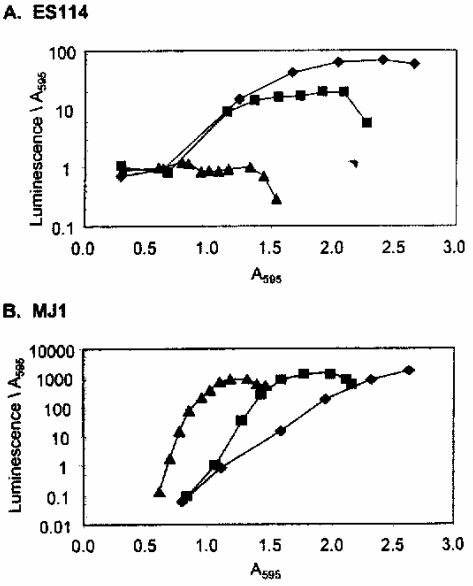

FIGURE 1 Specific luminescence (luminescence per A_{595}) of ES114 (A) or MJ1 (B) grown at 24°C in 250-ml flasks, shaken at 200 rpm, in 50 (diamonds), 100 (squares), or 200 (triangles) ml of SWTO, a rich nutrient medium (7, 66). Bacterial cell density was measured by absorbance of the culture at 595 nm (A_{595}).

ES114 and MJ1 raise interesting questions regarding the evolution of *V. fischeri* and provide the grist for potentially enlightening comparative studies; however, given our increased use of ES114 and the fact that researchers can utilize this strain in a reconstituted symbiosis, we focus the rest of our consideration on this strain.

A MECHANISTIC MODEL OF THE THREE QUORUM-SIGNALING SYSTEMS OF *V. FISCHERI*

The last 5 years have seen a considerable advance in our understanding of quorum sensing within the genus *Vibrio* and, specifically, *V. fischeri*. Figure 2 presents a summary of some of our current knowledge concerning the circuitry of signaling in *V. fischeri*. Three quorum-sensing signals have been described in *V. fischeri* ES114. As mentioned above, two of them are AHLs: (i) C_8-HSL, synthesized by AinS (32, 48), and (ii) 3-O-C_6-HSL, synthesized by LuxI (20, 22). The third signal is produced by *V. fischeri* LuxS, a homolog of the *V. harveyi* protein that synthesizes a furanosyl borate diester product called autoinducer-2 (AI-2) (9). These three signal synthases are all active in *V. fischeri* (55, 57), and their products accumulate in the environment around the cells. Together these signals comprise three systems that coordinately regulate specific genes in a cell-density-dependent manner (Fig. 2).

System 1: The 3-O-C_6-HSL Signal

The first level of quorum signaling recognized in *V. fischeri* was that controlled by the canonical LuxI-LuxR system. This system is embedded in the *lux* operon of both *V. fischeri* (24) and *Vibrio salmonicida* (67) and has been the subject of extensive physiological, biochemical, and genetic studies (20, 30). Briefly, the enzymes catalyzing the bacterial luminescence reaction are encoded by the last six genes of the *luxICDABEG* operon, which in *V. fischeri* is adjacent to, but divergently transcribed from, the *luxR* gene. The *luxA* and *luxB* genes encode, respectively, the α and β subunits of luciferase, an enzyme that catalyzes the conversion of a long-chain aldehyde, reduced flavin mononucleotide and oxygen to a long-chain fatty acid, flavin mononuleotide, H_2O, and light. The *luxC*, *luxD*, and *luxE* genes encode a fatty acid reductase complex that resynthesizes the aldehyde substrate, and *luxG* is involved in flavin mononucleotide biosynthesis. In *V. fischeri* the expression of these *lux* genes is regulated in a cell-density-dependent fashion through binding of the LuxI-synthesized AHL signal, 3-O-C_6-HSL, to the transcriptional activator protein LuxR, encoded by *luxR*. The LuxR-AHL complex then binds to the *luxICDABEG* promoter and induces the transcription of this operon (85). Thus, the transcription of *luxI* leads to the production of an AHL signal (autoinducer) that results in a further increase in its transcription (Fig. 2). Homologs of this LuxI-LuxR system are present in dozens of bacterial species, controlling a wide diversity of activities, including luminescence, motility, biofilm formation, pigmentation, antibiotic production, and virulence (16, 30, 60).

Systems 2 and 3: The C_8-HSL and AI-2 Signals

As first described in *V. harveyi* (99), parallel (or "hybrid") inputs are used by *V. fischeri* to sense the accumulation of both C_8-HSL and AI-2. The C_8-HSL synthesized by AinS presumably functions with its cognate receptor, termed AinR, activating it as a transcriptional regulator (32). The *ainR* gene is located immediately downstream of *ainS* (Fig. 2), and the two genes are transcriptionally linked (unpublished data). The arrangement of these genes is analogous to that of their homologs in *V. harveyi*, *luxM* and *luxN*. Biochemical and genetic studies of AHL signaling in *V. harveyi* have shown that, in the presence of an inducing concentration of the LuxM-synthesized AHL, the receptor, LuxN, participates in a phosphorelay cascade by stimulating the relative dephosphorylation of LuxU (Fig. 2).

The LuxS system of *V. fischeri* also appears homologous to that described in *V. harveyi*, although the roles and activities of several of the components have not been directly demonstrated in *V. fischeri*. As in other bacteria,

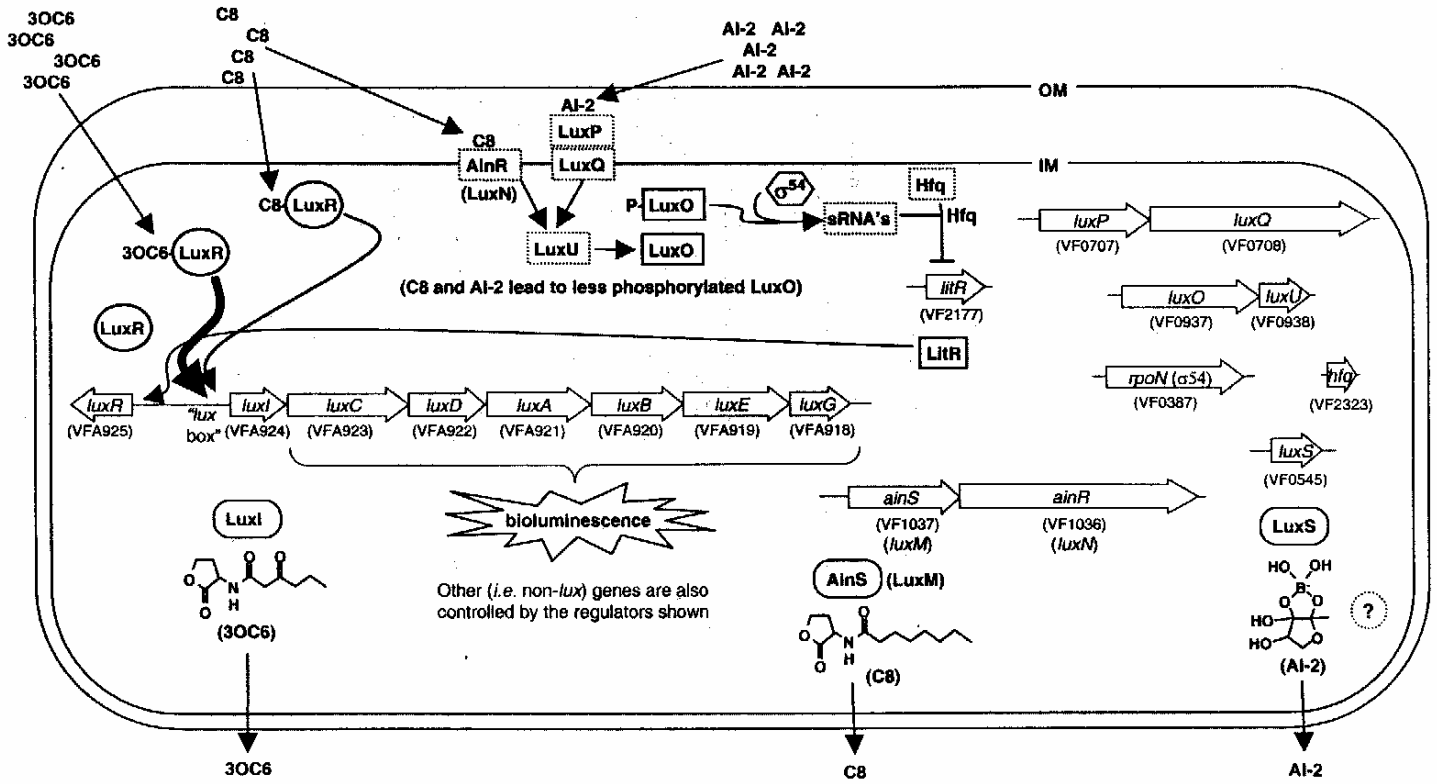

FIGURE 2 Model of quorum sensing in *V. fischeri* ES114. Each gene is indicated by a labeled open arrow with the open reading frame designation from the *V. fischeri* genome database provided underneath in parentheses (e.g., *luxI* is designated VFA924). The structures of three autoinducer molecules are presented underneath their respective synthases, and a "?" by AI-2 indicates that its structure is inferred but has not been identified in *V. fischeri*. Interactions between autoinducers, proteins, genes, and small RNAs (designated "sRNAs") are indicated, and dotted lines around sRNA or protein components indicate that these have been identified in the genome database but not functionally confirmed in ES114 experimentally.

the *V. fischeri* LuxS is responsible for AI-2 synthesis, and the integration of this system into quorum sensing is illustrated by the observation that a *luxS* mutant of *V. fischeri* is dimmer than wild type (55). LuxS can give rise to structurally distinct versions of AI-2 (58). To date, the specific structure of AI-2 produced by *V. fischeri* has not been determined; however, it seems likely that this species' bioactive AI-2 autoinducer is also a furanosyl borate diester. Also, by analogy to work done in *V. harveyi*, and on the basis of genomic analysis of *V. fischeri*, we predict that AI-2 is perceived by LuxQ via LuxP and that the resulting signal is transduced through the relative dephosphorylation of LuxU (Fig. 2).

Systems 2 and 3: Convergence on a Core Circuitry in *Vibrio* Quorum Sensing

As illustrated in Fig. 2, the *V. fischeri* C_8-HSL and AI-2 signaling systems converge at LuxU. In our current model, the presence of either autoinducer leads to the relative dephosphorylation of LuxU, which in turn leads to a more dephosphorylated, inactive condition for the sigma-54-dependent regulator LuxO (Fig. 2). As mentioned above with respect to transduction of the C_8-HSL signal, the genes encoding components of this pathway have been identified in the *V. fischeri* genome, but only the roles of LuxO, sigma-54, and LitR have been tested experimentally. Importantly, as predicted from the current model (Fig. 2), the influence of *luxS* on bioluminescence is dependent on *luxO* (55). Recently, another signaling input to LuxO has been discovered: the global regulator CsrA is implicated in quorum sensing by *Vibrio cholerae*, feeding information into LuxO that affects this protein's output (51). All sequenced *Vibrio* species, including *V. fischeri*, have homologs of CsrA, as well as small RNAs (sRNAs) that regulate it, encoded by several *csrB* genes (47). At present there is no direct evidence that *V. fischeri* quorum sensing is affected by the CsrA system; however, a mutation in GacA, which regulates CsrA in other species (89), suppresses induction of *V. fischeri* luminescence (101).

In the *V. fischeri* core circuit, the C_8-HSL signal appears to have a considerably stronger influence on the expression of bioluminescence than does AI-2. For example, a *luxS* deletion mutant, which lacks AI-2 production, still achieves >70% of the level of bioluminescence produced by wild type (55). However, the importance of *luxS* is probably context dependent; i.e., AI-2 may have a significant effect on sensing and regulation of bioluminescence in certain environments. As one might predict (Fig. 2), the fold-decrease in luminescence associated with a *luxS* mutation is greater in an *ainS* mutant background (55), and conditions that favor AI-2 accumulation over that of C_8-HSL may change the extent of the regulatory contribution of each of these systems. As discussed below, environmentally responsive regulators can influence the rate of autoinducer signal production and accumulation, so it would not be surprising to find that environmental conditions influence the relative importance of the three quorum-signaling systems (Fig. 2).

In conjunction with the AI-2-activated circuit, the result of the parallel AHL-signaling pathway is to dephosphorylate the transcriptional regulator LuxO (52, 99). In *V. harveyi*, this inactivation reduces the concentration of several regulatory sRNAs whose presence inhibits the translation of the *V. harveyi* master regulator, LuxR (a homolog of *V. fischeri* LitR [Table 2]), resulting in an increase in expression of not only the *lux* operon but also a number of other recently discovered target genes (62). The components of this "core" phosphorelay system are encoded in the genomes of many if not all *Vibrio* species (61) (Table 2). While this core signaling circuitry is best described in the *V. harveyi* paradigm (99), it is already clear that there are both qualitative and quantitative differences among the phosphorelay cascades found in different species (15, 56, 106). For instance, in *V. fischeri*, LuxO may regulate the synthesis of as few as one sRNA (personal communication) (Fig. 2), rather than the four described for *V. harveyi* (51, 99). Similarly, while *Vibrio anguillarum* has all the typical core circuit components (Table 2), it regulates LuxO quite differently; e.g.,

TABLE 2 Presence of homologous quorum-sensing systems in some *Vibrio* species[a]

System	Ortholog in the following *Vibrio* species:					
	Vf	Vp	Vv	Vc	Vh	Va
System 1	**LuxI**	—	—	—	—	**VanI**
	LuxR	—	—	—	—	VanR
System 2	**AinS**	**LuxM**	—	—	**LuxM**	**VanM**
	AinR	LuxN	—	—	LuxN	VanN
System 3	**LuxS**	**LuxS**	**LuxS**	**LuxS**	**LuxS**	**VanS**
	LuxP	LuxP	LuxP	LuxP	LuxP	VanP
	LuxQ	LuxQ	LuxQ	LuxQ	LuxQ	VanQ
System 4	—	**CqsA**	—	**CqsA**	**CqsA**	ND
—	CqsS	—	CqsS	CqsS	ND	
Core circuit (for systems 2, 3, and/or 4)	LuxU	LuxU	LuxU	LuxU	LuxU	VanU
	LuxO	LuxO	LuxO	LuxO	LuxO	VanO
	LitR	OpaR	SmcR	HapR	LuxR[b]	VanT
	CsrA	CsrA	CsrA	CsrA	ND	ND

[a]Vf, *V. fischeri*; Vp, *V. parahaemolyticus*; Vv, *V. vulnificus*; Vh, *V. harveyi*; Va, *V. anguillarum*. Systems 1 to 4 are listed in the order in which they were discovered; the absence of a recognizable ortholog in the published genome is indicated by a dash (—). Core components constitute a signaling pathway shared by systems 2 and 3. Bold type indicates the signal synthases. ND, not determined (no genome sequence available).

[b]The *V. harveyi* LuxR is not a homolog of the *V. fischeri* LuxR.

phosphorelay signaling apparently can proceed independently of LuxU and is subject to feedback by the LitR homolog VanT (14, 17). Finally, there is evidence that, in addition to luminescence, the AinS/LuxO pathway regulates genes affecting general cellular functions; i.e., in contrast to a *luxI* mutant (92), the growth yield of an *ainS* mutant is only 75% of the level of wild-type cells (57). These differences may be mediated through the AinS/LuxO-dependent induction of a unique *V. fischeri* sigma factor (56) and have not been described in mutants of other *Vibrio* species.

System 4: a Parallel Input in Some Signaling Pathways

In the preceding sections we described the pathways by which two AHLs and the AI-2 signal work both in parallel and sequentially to regulate the induction of luminescence and other activities in *V. fischeri* (Fig. 2). In a subset of *Vibrio* species (Table 2), another signaling system feeds into the central parallel circuitry along with systems 2 and 3 (99). In *V. harveyi* the protein CqsS apparently synthesizes an as yet unidentified signal that interacts with CqsR, a membrane-bound sensor (43). Genetic studies indicate that the output of this sensor feeds into the core circuitry at the level of LuxU. CqsS signaling has also been described in *V. cholerae* (51), where it appears to play a more dominant role than in *V. harveyi* (53, 99). Examination of the genome of *V. fischeri* ES114 provides no evidence of a CqsS-CqsR system in this organism (Table 2).

Evidence for Sequential AHL Signaling in *V. fischeri*

In strain ES114, levels of C_8-HSL quickly become saturating in culture. While exogenous addition of this autoinducer can complement an *ainS* mutant's luminescence defect, it has no effect on either a wild-type or *luxI* mutant (57), an observation that is consistent with a sequential effect of the two AHLs (Fig. 2). Adding 3-O-C_6-HSL to an *ainS luxI* double mutant recovers luminescence only to the level characteristic of an *ainS* mutant, indicating that C_8-HSL signaling must be normal before cells can respond to even a high concentration of 3-O-C-HSL. Thus, the impact of C_8-HSL signaling (at least on ES114 *lux* gene expression) is evident at cell concentrations occurring in culture and continues to be important at the higher

densities more characteristic of the light organ environment. In contrast, the inducing effect of the LuxI signal is apparent only at the very high bacterial concentrations in the symbiosis. A similar mechanism for sequentially linking two AHL systems has been well documented in *Pseudomonas aeruginosa* (chapter 9), and results in a highly complex and interacting network of signaling (82).

Multiple Roles of C_8-HSL in Quorum Sensing

The C_8-HSL autoinducer in *V. fischeri* functions through more than one mechanism (Fig. 2), although this signal apparently has other activities that cannot yet be integrated into the current model. Besides signaling through AinR and the "conserved core circuitry" described above, C_8-HSL can interact with LuxR directly. When 3-O-C_6-HSL is limiting, C_8-HSL can serve as a weak activator of LuxR (23, 81) (Fig. 2); however, when 3-O-C_6-HSL is abundant, C_8-HSL can actually inhibit 3-O-C_6-HSL signaling (48; unpublished data). This result can be interpreted as reflecting the differential capacity of these two AHLs to bind and activate LuxR. C_8-HSL signaling may work through another mechanism as well. While the *ainS* mutant of *V. fischeri* ES114 produces very little luminescence, the addition of 3-O-C_6-HSL does not induce luminescence to a wild-type level (57), suggesting that activation by C_8-HSL must precede or prepare the way for subsequent induction by 3-O-C_6-HSL.

From the current model (Fig. 2), one might predict that C_8-HSL accomplishes this preparatory activity by enhancing LitR levels and that this enhancement must be critical for LuxR accumulation and full induction by 3-O-C_6-HSL. However, it is difficult to reconcile such a model with all the available data: notably, the important phenotypic differences between *ainS* and *litR* mutants. For example, an *ainS* mutant is dark in culture, whereas a *litR* mutant is only delayed in its induction of luminescence (26, 57). Moreover, addition of 3-O-C_6-HSL stimulates luminescence in a *litR* mutant just as well as it does in wild type, while an *ainS* mutant remains more than a 100-fold dimmer (56). During the early stages of growth, this difference between the *ainS* and *litR* mutants may be due to C_8-HSL combining with LuxR to directly pre-induce the *lux* operon (including *luxI*), thereby "jump-starting" the powerful 3-O-C_6-HSL/LuxR transcriptional effects. However, late in growth, a *luxO* "on" mutation, which is unable to induce the repressing sRNAs, completely complements *ainS* (57). This result indicates that the *ainS* luminescence defect is expressed primarily through LuxO. The nature of this LitR-independent LuxO regulation remains unknown, but it may work through another, physiological, level of regulation of the *lux* operon (see below).

QUORUM SIGNALING IN A LIGHT-ORGAN SYMBIOSIS

The *V. fischeri–E. scolopes* Light-Organ Association

Even correcting for our disproportionate focus on pathogenic bacteria, almost all bacterial species recognized to use AHL quorum sensing are host associated (39). This trend suggests that for many microorganisms the biologically significant function of quorum signaling may be fully revealed only by studying the colonization of their natural host. The beneficial association between *V. fischeri* and its squid host is an example of just such a biologically relevant system. Symbiotic colonization of the light organ of *E. scolopes* proceeds through a series of well-described stages (72, 87) involving developmental adaptation and accommodation by both partners (96). Briefly, free-living *V. fischeri* cells present in the ambient seawater attach to a host-derived mucus matrix produced by epithelial appendages located immediately outside of pores that lie on the surface of the nascent light organ of a juvenile squid (69, 70). These pores lead to six epithelium-lined crypt spaces deep within the organ. The aggregated bacteria migrate along the mucus, through the pores and into the crypts, where they rapidly proliferate, colonizing the light organ with approximately 10^6 bacteria (72, 76). Once it reaches a critical cell density, the symbiont

population induces luminescence that is ultimately used by mature squid in their nocturnal behavior (44). At dawn every morning throughout its life, the squid expels 90 to 95% of the symbiont population, and the remaining cells repopulate the organ within a few hours, creating a fresh bacterial culture for the coming night (37, 71). The resulting cyclic daily rhythm plays an integral part in the biology of this dynamic association (5, 49).

The ability to colonize the squid light organ is remarkably specific: only *V. fischeri* and, to a much lesser extent, the very closely related *Vibrio. logei* (27) can successfully initiate an association. Studies of mutants of *V. fischeri* have shown that a number of bacterial products (e.g., capsule [96, 105]) and behaviors (e.g., motility and luminescence [36, 59, 92]) play important roles in the symbiont's ability to initiate and persist in the association. In all cases studied to date, these colonization factors are under signal-induced regulation involving two-component phosphorelays and/or quorum sensing (31, 96).

The symbiotic roles of the three *V. fischeri* quorum-sensing systems (Fig. 2) have been examined during the initiation and stabilization stages of the juvenile light-organ association. The two AHL quorum-sensing signaling systems of *V. fischeri* contribute to colonization of the light organ in distinct ways. Mutation of the *ainS* (but not the *luxI*) signal synthase delays initiation of the symbiosis, while mutation of *luxI* results in a much more severe luminescence defect in the symbiosis (57). As described below, loss of either of these AHL signals leads to an inability of *V. fischeri* to persist normally in the light organ beyond the first 24 h. In contrast, the role of LuxS signaling in colonization is minor and can only be observed as a small decrease in an *ainS luxS* double mutant background relative to the much larger *ainS* defect. While there is apparently little role for LuxS in the monospecific light-organ symbiosis, it remains possible that AI-2 signaling may serve *V. fischeri* in its other ecological niches (such as the enteric tracts of marine organisms [78]) where it resides in multispecies communities with other AI-2 signaling species (77).

The Importance of Cell-Cell Signaling in Symbiosis

At bacterial concentrations below 10^9 cells/ml (i.e., the maximum cell density normally achieved in broth cultures), C_8-HSL dominates the control of *lux* expression in *V. fischeri* ES114 (57). In contrast, in the light organ, where symbiont concentrations are well over 10^9 cells/ml, both signals are required for maximum luciferase synthesis and luminescence, although the ability to synthesize 3-O-C_6-HSL clearly plays the greatest role (57). Bioassays of 3-O-C_6-HSL in the adult light organ have indicated a concentration of approximately 100 nM, significantly above that necessary to fully induce luminescence in culture (4). Because a functional *luxI* gene is required for normal levels of light emission in the symbiosis, and mutants defective in luminescence fail to persist, 3-O-C_6 signaling is an important colonization factor (57, 92). Thus, although the LuxI signal has little effect on luminescence in culture, it is critical for symbiotic performance.

While a *luxI* mutant initiates the symbiosis normally, an *ainS* mutant is delayed in colonization, suggesting that C_8-HSL signaling plays a role during the first few hours of the symbiotic interaction, as the bacteria form aggregates and enter the light organ (73). Thus, the sequential nature of the pathway of quorum signaling, proceeding from AinS to LuxI, is not only found in culture but is similarly evident in the natural process of colonization (57). *ainS* signaling is also required for normal persistence in the symbiosis (57); however, this requirement appears to extend beyond the role of C_8-HSL in inducing a normal level of luminescence. Specifically, an *ainS*$^+$ strain engineered to have a lower specific luminescence (i.e., less light emitted per cell) than the *ainS* mutant nevertheless persists normally (93). This and other observations suggest that some additional, nonluminescence activity that is regulated by *ainS* through the LuxO-dependent pathway contributes to symbiotic persistence in the light organ. It is not surprising, then, that other *ainS*-regulated cellular functions should exist, owing to the presence of a number of LuxO-regulated

phenotypes reported for different *Vibrio* species (52, 91, 107). Microarray analysis of the *ainS* regulon has presented a series of candidates required for symbiotic persistence, as well as for the pathway of their regulation downstream of LuxO (56).

Signaling between *V. fischeri* and Its Host

As described above, the initiation of light-organ colonization brings about a specific series of genetic and behavioral modifications in the bacterial symbionts, including several quorum-induced activities. Similarly, *V. fischeri* colonization of a juvenile squid triggers a program of biochemical and morphological development that transforms the nascent light organ into a mature symbiotic structure (72, 96). Recent studies have begun to reveal the signals that trigger host development and, to date, three bacterial signal compounds, all of them modified cell envelope components, have been identified. The first signal is the lipid A component of *V. fischeri* lipopolysaccharide, a potent morphogen inducing apoptosis of the mucus-secreting appendages that facilitate the bacterial attachment and colonization (28, 46). In addition, peptidoglycan (PGN) fragments shed by *V. fischeri* cells induce the secretion of mucus by the light organ (69), possibly by a signaling pathway using the host's PGN receptor proteins (34). Finally, a tetrapeptide fragment of PGN, first described as the tracheal cytotoxin of *Bordetella pertussis* (13) and the PGN-derived cytotoxin of *Neisseria gonorrhoeae* (11, 75), has been shown to induce trafficking of hemocytes into the light organ (45). Tracheal cytotoxin works synergistically with lipid A, inducing the full program of tissue regression as the organ matures (46). These three *V. fischeri*-derived signals, required for normal symbiotic development in the light-organ association, have previously been shown to function as virulence determinants in several pathogenic bacterial infections (12). Thus, the discovery of the remarkably different roles these compounds play in microbe-host interactions emphasizes the importance of context in the evolution of signal-response pathways. Currently, studies are under way to determine whether quorum signaling induces symbiotic *V. fischeri* cells to induce the release of lipid A and/or tracheal cytotoxin, either during the initiation of colonization or during the daily cycle of expulsion and regrowth that characterizes the persistent association.

While such indirect signaling of host development may result from *V. fischeri* quorum sensing, examples of a more direct action of bacterial AHLs on host tissue are appearing in pathogenic models (reviewed in reference 84), and this mode of signaling may be a factor in the light-organ symbiosis as well. Not only is 3-O-C_6-HSL present in the squid light-organ tissues at near-micromolar concentrations, but this AHL has also been shown to freely diffuse into those tissues (4). Current investigations by M. McFall-Ngai and her collaborators are aimed at determining whether this or other quorum-sensing signals of the *V. fischeri* symbionts are also sensed, and responded to, by the host, leading to changes in gene expression in the light-organ epithelium. In pathogenic associations, one response of animal tissue is the inactivation of bacteria-produced AHLs (10), possibly playing a role in the host's innate defenses. Such an activity in the light organ, while as yet unobserved, could modulate the response of both the host and the symbionts to bacteria-produced quorum-sensing signals.

POSITIVE FEEDBACK AND ENVIRONMENTAL CONTROL OF SIGNAL SYNTHESIS

In *V. fischeri*, as in many other bacteria, the rate of autoinducer accumulation is not constant but rather is tied to certain environmental conditions. Throughout this volume it is clear that environmentally responsive regulators often control the rate of autoinducer production, and also may modulate the rate of signal degradation by the autoinducer-producing bacterium (e.g., chapter 24). Moreover, several autoinducer systems control themselves in a positive feedback manner (40, 74, 83) and, as a consequence, regulatory inputs from environmental stimuli can be greatly amplified.

Both the *luxI* and *ainS* genes of *V. fischeri* are autostimulatory; i.e., an accumulation of 3-O-C_6-HSL and C_8-HSL results in an increased transcription of their synthase genes, *luxI* and *ainS*, respectively (55). The positive-feedback autoregulation of 3-O-C_6-HSL is readily illustrated in Fig. 2, which depicts how this signal combines with LuxR to stimulate expression of LuxI, the 3-O-C_6-HSL synthase. Multiple lines of evidence (55) suggest that C_8-HSL stimulates *ainS* transcription through the AinR, LuxU, and LuxO pathway, leading to an increased level of LitR (Fig. 2). Although AI-2 does not appear to affect transcription of *luxS* (55), this signal is predicted to increase LitR abundance and therefore AinS and C_8-HSL output. As described above, this output leads to positive-feedback regulation through core components common to the AI-2 signaling pathway. Thus, if environmentally responsive regulators modulate transcription of *luxI*, *ainS*, or possibly *luxS*, such control can be amplified greatly by the positive-feedback activity of the autoinducers.

In *V. fischeri*, environmental factors play a role in modulating autoinducer production, bioluminescence, and other coregulated functions, and autoinducer concentrations reflect both the ambient conditions and cell density, not simply the latter. That cell density is not the only determinant governing bioluminescence is illustrated by the observation that in colonies on agar medium and in the *E. scolopes* light organ, bacteria are packed to similarly high densities, yet only in the latter environment are they highly bioluminescent. Therefore, some environmental difference between the light organ and a nutrient-rich agar medium helps determine the level of *luxICDABEG* (and other gene) expression.

We recently discovered that the redox-responsive ArcA/ArcB two-component regulatory system, which is activated in response to more reduced conditions, mediates repression of the *luxI* promoter (J. L. Bose, C. S. Rosenberg, and E.V. Stabb, submitted for publication). The phosphorylated response regulator, ArcA-P, binds the *luxI* promoter proximal to the LuxR-binding site, and we propose that it functions as a repressor by interfering with LuxR-mediated activation. Interestingly, the ArcA/ArcB system does not repress luminescence during colonization of juvenile *E. scolopes*, and an *arcA* mutant achieves the same level of brightness in culture as wild-type cells do in the light organ (Bose et al., submitted). Thus, redox state appears to be a key environmental factor determining expression of *luxICDABEG*, and regulation by the ArcA/ArcB system could account for the differences in bioluminescence and autoinducer output observed in culture and in host tissues. Another intriguing and as yet untested implication of these findings is that one *V. fischeri* cell perceiving a more oxidized environment through its ArcA/ArcB system might transmit this information to neighboring cells in the form of an increased production of 3-O-C_6-HSL.

It seems likely that environmentally responsive regulators also modulate the C_8-HSL and AI-2 signals in *V. fischeri*, although data in this regard are scant. Interestingly, there is a large inverted repeat located near the 5′ end of the *ainR* gene of *V. fischeri* that is absent in its homolog (e.g., *luxN*) in other *Vibrio* species. The presence of this element could indicate a novel regulatory mechanism controlling the receptor for C_8-HSL, and because of the autostimulatory nature of this signal, such control might influence C_8-HSL accumulation as well. Similarly, environmental control of genes encoding several other intermediates in the C_8-HSL signaling pathway, such as sigma-54 (103) or LitR (26), could connect conditional regulators with C_8-HSL accumulation. There is also precedence for control of AI-2 accumulation by the modulation of expression of *luxS* or the related enzyme *pfs* (2, 98). This level of regulation might be active in *V. fischeri* as well, although *V. fischeri* apparently lacks the pathway for AI-2 destruction mediated by LsrF, LsrG, and LsrK in *E. coli* and *Salmonella* (90, 104). The possibility that the C_8-HSL or AI-2 signaling systems are modulated merits further investigation, as we may find that in certain environments these signals are even more important than is currently appreciated.

FUTURE DIRECTIONS

Recent advances in *V. fischeri* research, notably the completed genome sequence of strain ES114 (80) and the development of molecular genetics (21, 88), have enabled us to draw a fairly detailed model of how three autoinducer systems in this bacterium control the regulation of bioluminescence (Fig. 2); however, several aspects of this model await experimental confirmation. In particular, the details of the C_8-HSL and AI-2 pathways draw extensively from work performed on homologous components in *V. harveyi*, and genetic and biochemical tests of the roles for AinR, LuxP, LuxQ, Hfq, and sRNAs in *V. fischeri* await completion. Because these components are conserved in several *Vibrio* species (Table 2), we expect that they will function the same in *V. fischeri* as in these other systems. Nonetheless, careful examination of these components is likely to reveal differences unique to quorum sensing in *V. fischeri* and is necessary to build a complete understanding of quorum sensing in this model system. Such knowledge will be critical for elucidating exactly where and how environmentally responsive regulators feed into the quorum-sensing circuitry illustrated in Fig. 2.

Unraveling the complex series of interactions controlling autoinducer synthesis and bioluminescence in *V. fischeri* presents a challenge that is likely to grow exponentially as more regulatory components are uncovered. For example, environmentally responsive regulators may feed into this circuitry at any of a number of sites and in some cases may exert control over more than one component of the pathway. Importantly, as each new regulator is discovered, we are presented not only with the question of how it connects to the current model (Fig. 2), but we must also consider whether other regulators affect it and how. Such questions may best be addressed using mathematical models and integrated analysis to understand the dynamics of quorum-sensing regulatory networks (35, 86), allowing us to place quantitative values on the contributions to effector output of the individual pathways in the network (Fig. 2).

We expect that bioinformatic and computational approaches will help build and test such models and reveal new pathways in this regulatory web. In the past, components of the current model (Fig. 2) have been tested for their influence on bioluminescence, autoinducer synthesis, and/or transcription from the *luxI* or *luxR* promoters; in some cases, their dependence on other components of this regulatory pathway has been tested by mutational analyses. Such approaches will continue to be useful, but microarray analyses also now offer the opportunity to simultaneously and quantitatively assay regulatory effects on transcripts for multiple components of the pathway, potentially uncovering how a single regulator connects to the regulatory network at multiple nodes. Such analyses combined with computational approaches will prove a powerful way to model the many connections and their relative importance in this complex system.

The sequencing of the first *V. fischeri* genome has led to the construction and application of glass-slide and Affymetrix chip microarrays, opening up the opportunity to map out the transcriptional circuitry defining a quorum-sensing pathway in this bacterium (56). As a result, we can expect soon to see what other genes and functions are part of the AHL-induced regulon, as well as the ways the several quorum-sensing regulons in *V. fischeri* interact with each other and with other modulating circuitry, as described in *P. aeruginosa* (82). Recently a second *V. fischeri* isolate, strain MJ11 obtained from a *M. japonica* light organ, has had its genome sequenced, assembled, and annotated (unpublished data). The availability of the MJ11 sequence will open the door to exciting comparative studies of quorum-sensing networks in *V. fischeri*, with an eye to discovering the evolutionary flexibility of these signaling pathways as they adapt to the specific environments presented by different hosts.

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