Roles of Bacterial Regulators in the Symbiosis between *Vibrio fischeri* and *Euprymna scolopes*

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1 Introduction

In a symbiosis, two or more evolutionarily distinct organisms communicate with one another in order to co-exist and co-adapt in their shared environment. The mutualistic symbiosis between the bioluminescent marine bacterium *Vibrio fischeri* and the Hawaiian squid *Euprymna scolopes* provides a model system that allows scientists to examine the mechanisms by which this communication occurs (McFall-Ngai and Ruby 1991). The squid, although *V. fischeri*-free (aposymbiotic) at hatching, rapidly acquires this bacterium and promotes its growth in a special symbiotic organ called the light organ (LO). In exchange for nutrients and a niche safe from competing bacteria, *V. fischeri* provides the bioluminescence used by *E. scolopes* to camouflage itself from predators.

In this chapter, we will give an overview of the early events in establishing the symbiosis and describe associated developmental changes triggered in each organism by the interaction. We will then discuss bacterial regulators and, where known, the traits they control that are necessary for a productive interaction between *V. fischeri* and *E. scolopes*. Finally, we will conclude by highlighting important directions for future investigation.

2 Early Events in the *Euprymna scolopes – Vibrio fischeri* Symbiosis

2.1 *Vibrio fischeri* strains are specifically recruited from the seawater

V. fischeri comprises less than 0.1% of the total bacterial population in the seawater inhabited by the squid (Lee and Ruby 1992), yet this organism alone

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is found in the light organ association (Boettcher and Ruby 1990). Furthermore, inoculation in the laboratory with bacteria closely related to *V. fischeri*, including *V. harveyi* and *V. parahaemolyticus*, fails to result in colonization (McFall-Ngai and Ruby 1991; Nyholm et al. 2000). In addition to this species-specific selection, strain-specific enrichment also occurs. Both visibly luminescent and non-visibly luminescent strains of *V. fischeri* co-exist in the seawater, but only the latter strains colonize the squid LO in nature (Lee and Ruby 1994b). This strict limitation on the species and strains of bacteria capable of colonizing the LO suggests that a specific exchange of signals must occur between the squid and the bacteria early during colonization.

Within hours of hatching, *E. scolopes* recruits *V. fischeri* from the surrounding seawater. The presence of bacteria or the bacterial cell wall component peptidoglycan in the seawater causes the squid to secrete mucus (Nyholm et al. 2002), allowing *V. fischeri* cells to aggregate near pores leading into the LO (Fig. 1). Other bacteria such as *V. parahaemolyticus* also exhibit the ability to aggregate in squid mucus, suggesting that *E. scolopes* does not distinguish between *V. fischeri* and other Gram negative bacteria at this stage (Nyholm et al. 2000). However, when both *V. parahaemolyticus* and *V. fischeri* are present, the latter organism becomes the dominant species in the aggregate (Nyholm and McFall-Ngai 2003), indicating that *V. fischeri* may participate in establishing specificity at this stage.

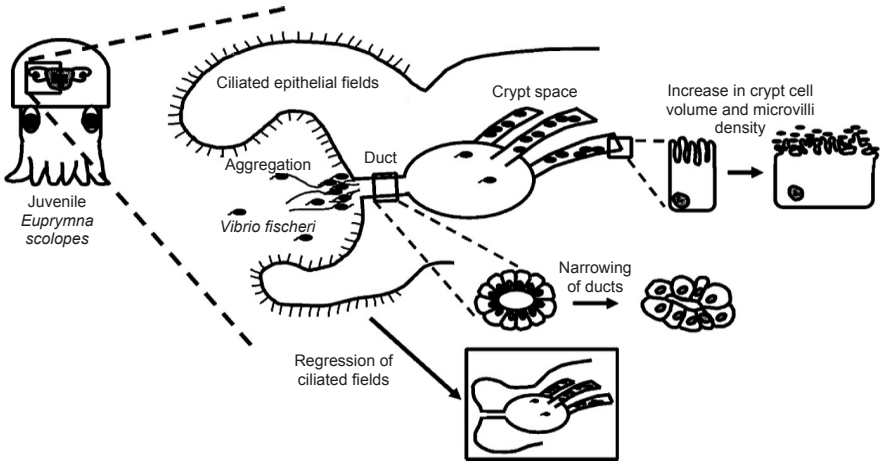

Fig. 1. Cartoon depicting the structure of and developmental changes in the juvenile squid LO during colonization. The position of the LO in a juvenile squid is shown on the *left*, while an enlarged cross section is shown on the *right*. The juvenile LO contains three pores on each side (six total), only one of which is depicted at the opening of the duct. *Arrows* indicate developmental events that occur within the first 4 days after exposure to *V. fischeri*. *Dashed lines* indicate an enlargement of the boxed area. *V. fischeri* cells are shown as *black ovals* aggregated in the mucus (depicted as *wavy lines*) outside the pore and in the crypt spaces (without flagella). This depiction of the light organ is based on Visick and McFall-Ngai (2000) and references described therein.

2.2 *Vibrio fischeri* cells navigate physical and chemical barriers to colonize *Euprymna scolopes*

From the aggregates, the *V. fischeri* cells migrate through LO pores, reaching ducts that ultimately lead into crypts, the sites of colonization (Fig. 1). In the ducts, the bacteria must move through mucus against an outward current generated by ciliated cells lining the passageway (McFall-Ngai and Ruby 1998). As a further barrier to colonization, the ducts contain high levels of nitric oxide, an anti-microbial agent that may function as a layer of defense against invasion by non-specific bacteria (Davidson et al. 2004). In the crypts, *V. fischeri* cells may encounter macrophage-like cells, a potential immune surveillance system (Nyholm and McFall-Ngai 1998). In addition, the bacteria may be exposed to toxic oxygen radicals such as hypohalous acid, produced by a halide peroxidase enzyme secreted by epithelial cells within the crypts (Weis et al. 1996; Small and McFall-Ngai 1999). Despite this plethora of potential host defenses, *V. fischeri* cells can enter the LO and grow to high cell density, approximately 10^{11} cells/cm^3 (Visick and McFall-Ngai 2000). Thus, *V. fischeri* must possess mechanisms by which it can evade host defenses and thrive in the LO environment.

Growth to high cell density does not represent the endpoint of the symbiosis. Rather, the symbiosis is dynamic. Each morning the squid expels between 90 and 95% of the bacterial population from its LO (Lee and Ruby 1994a). During the day, the *V. fischeri* cells retained in the squid divide to repopulate the LO. Therefore, persistent colonization actually consists of cycles of expulsion and re-growth, requiring the symbiotic bacteria to adapt to changing environments within the LO.

2.3 Both Organisms Undergo Developmental Changes in Response to the Symbiosis

The interaction between *E. scolopes* and *V. fischeri* induces a number of developmental and morphological changes in each organism (Fig. 1). Ciliated epithelial cells, present on a field that projects outward from the LO, likely function to facilitate recruitment of *V. fischeri* by drawing the bacteria-laden seawater into the mucus matrix near the LO pores. Once the symbiont has successfully migrated into the LO, apoptosis and subsequently regression of these ciliated fields results in their loss over the course of four days (Montgomery and McFall-Ngai 1994; Foster and McFall-Ngai 1998). The consequence, presumably, is a reduction in any further recruitment of additional symbiotic bacteria.

A bacterial signal that triggers some of the developmental changes in the epithelial fields is the bacterial cell wall component lipopolysaccharide (LPS). Purified LPS is sufficient to induce apoptosis in the fields (Foster et al. 2000). Most likely, the highly conserved lipid A portion of LPS is responsible, as LPS purified from many species of Gram negative bacteria can induce apoptosis. Possibly, an LPS detection pathway similar to the Toll-like receptor pathway found in many organisms (Gerard 1998) recognizes bacterial LPS and triggers apoptosis.

The LPS signal, however, is not sufficient to trigger regression of the epithelial fields; this suggests that more than one signal is required for this developmental change (Foster et al. 2000). *V. fischeri* strains that do not enter the LO fail to induce regression, suggesting signaling occurs between the bacteria and squid cells in the LO (Doino and McFall-Ngai 1995). Although regression requires a bacterial signal, the program continues regardless of the presence of bacteria: removing *V. fischeri* with antibiotic treatment after 12 h does not stop or reverse regression (Doino and McFall-Ngai 1995).

Another developmental event in the squid may also function to reduce LO accessibility. Within 12 h after symbiotic colonization, a two- to three-fold increase in actin levels occurs within the apical surface of epithelial cells lining the LO ducts (Kimbell and McFall-Ngai 2004). This increase in actin is correlated with a narrowing of the ducts, which decrease in size two-fold (Fig. 1). The narrowing of the ducts, along with the loss of the ciliated fields on the LO surface, likely limits entry into the LO. However, the LO remains at least somewhat open to the environment, as marked bacteria introduced into the seawater can subsequently be isolated from the adult LO (Lee and Ruby 1994b). Because *V. fischeri* remains the only bacterial resident, mechanisms must remain in place to prevent other species from infecting the LO.

Changes also occur in crypt epithelial cells immediately adjacent to the colonizing *V. fischeri* bacteria. Within 72 h of symbiotic colonization, these cells increase in volume as they develop from columnar to cuboidal cells (Fig. 1) (Montgomery and McFall-Ngai 1994). Concurrently, the microvilli on their surfaces increase in density and complexity (Lamarcq and McFall-Ngai 1998). These alterations likely increase the surface area available for interactions with the symbiotic bacteria. These structural changes require the persistent presence of bacteria (Doino 1998; Lamarcq and McFall-Ngai 1998), suggesting that a continual signal exchange occurs between the squid and bacteria throughout the symbiosis.

V. fischeri cells also undergo developmental changes upon colonization of the LO. Planktonic *V. fischeri* are flagellated and motile, traits that are essential for the bacteria to enter the LO (Graf et al. 1994; Millikan and Ruby 2003). Within 24 h of colonization, however, most of the bacteria lose their flagella and become non-motile. The cells re-grow flagella and regain motility shortly after expulsion from the LO (Ruby and Asato 1993). The bacteria also decrease in size in the LO and, after attaining high cell density, induce light

production (Ruby and Asato 1993) to a level 100-fold higher than in culture (Boettcher and Ruby 1990; Stabb et al. 2004). This luminescence is essential for persistent infection (Visick et al. 2000). Thus, in addition to signaling *E. scolopes* to induce developmental changes during the onset of symbiosis, *V. fischeri* also recognizes signals within the LO environment and adapts accordingly.

3 Regulatory Systems Employed by *Vibrio fischeri* to Promote the Symbiosis

3.1 Two-Component Signal Transduction Systems

Many bacteria, including *V. fischeri*, recognize and respond to their environments using two-component regulatory systems (Fig. 2A, reviewed in Stock et al. 2000). These systems are composed of a sensor histidine kinase

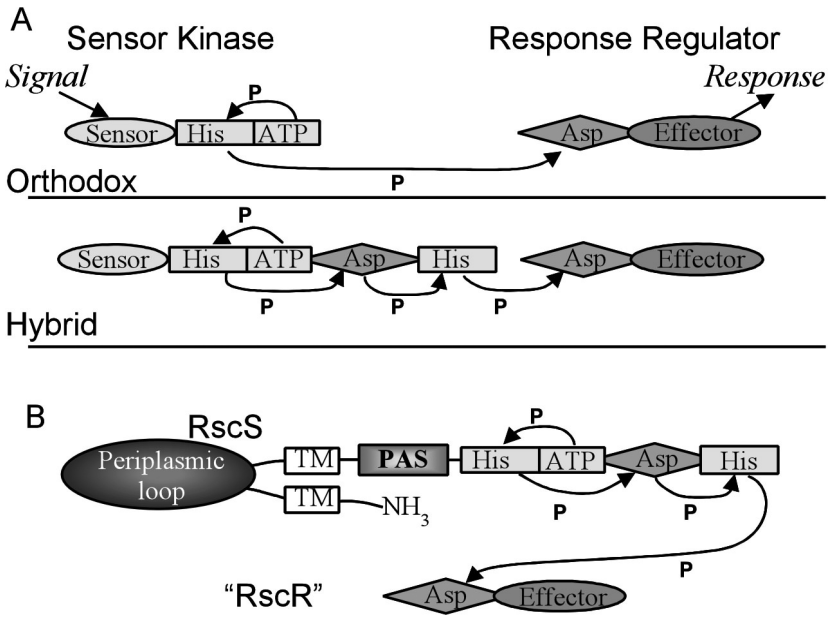

Fig. 2. Two-component regulatory systems. A. The phospho-relay in orthodox (*top*) and hybrid (*bottom*) two-component systems. Upon detection of signal, phosphate generated from a bound ATP is passed from conserved His to Asp residues until finally being transferred to an Asp in the response regulator, resulting in a response, either altered transcription or protein function. B. RscS is a hybrid sensor kinase. The sensor domain of RscS is composed of two transmembrane helices (*TM*), a large periplasmic loop and a PAS domain

protein that recognizes and transmits an environmental signal (through autophosphorylation on a His residue) to a second protein, the response regulator, which (when phosphorylated on a conserved Asp residue) carries out a response. Most frequently, the response consists of a change in gene expression; alternatively, changes in protein activity can result.

Given the changes in environmental conditions that *V. fischeri* cells experience as they travel from seawater into the LO, it is not surprising that colonization by *V. fischeri* requires two-component regulators. At least two such regulators are required for efficient initiation of symbiotic colonization: the sensor kinase RscS (Visick and Skoufos 2001) and the response regulator, GacA (Whistler and Ruby 2003). A transcriptional regulator, FlrA, which exhibits limited similarity to response regulators and is required for initiation (Millikan and Ruby 2003) will also be discussed here.

rscS. Mutations in *rscS* severely reduce the ability of *V. fischeri* to initiate symbiotic colonization: most animals remain uncolonized following exposure to *rscS* mutants, although other animals become colonized after a delay of several hours (Visick and Skoufos 2001). These results suggest that mutants are blocked at an early stage of colonization, but that they can occasionally by-pass this block and ultimately achieve what appears to be normal colonization. In culture, *rscS* mutants do not exhibit defects in growth, motility, or the timing and level of bioluminescence induction, traits known to be important for colonization (Visick and Skoufos 2001). Thus, to date no clues to *rscS* function have been garnered by phenotypes observed in culture.

The sequence of *rscS* suggests that it encodes a hybrid sensor kinase similar to ArcB and BvgS (Fig. 2B) (Visick and Skoufos 2001). These proteins contain, in addition to the conserved His residue that serves as the site of autophosphorylation, two additional domains with conserved residues (Asp and His) predicted to be sequentially phosphorylated and that may serve as sites of additional regulation (Fig. 2B). Upon receipt of a colonization signal, RscS is predicted to autophosphorylate and transfer the phosphate to an as-yet-unidentified response regulator, termed RscR, which may regulate genes or activities essential for symbiosis.

What serves as the colonization signal, and how is it detected by RscS? Clearly, many possibilities exist, and include in addition to bacterially produced molecules and seawater signals, components of the LO mucus, cell surface signals and nutrients. Determining the portion of RscS responsible for detecting the colonization signal will advance our understanding of symbiotic signal exchange. In many cases, the amino terminal periplasmic portion of sensor kinase proteins receives the environmental signal (Stock et al. 2000). For example, *Salmonella* PhoQ detects Mg^{2+} in the environment through its periplasmic domain; binding of Mg^{2+} to this domain results in a conformational

change and inactivation of the response regulator PhoP (Vescovi et al. 1997). RscS is predicted to possess a periplasmic domain of ~200 residues (Visick and Skoufos 2001); the large size of this region suggests it may play a role in RscS function, possibly signal detection.

In addition to a potential periplasmic signaling domain, RscS contains a second input domain, known as PAS. In other proteins, PAS detects signals such as small ligands, or changes in light levels, oxygen concentration or redox potential (Taylor and Zhulin 1999). Whether the PAS domain contributes to signal detection by RscS during colonization remains unknown. However, the transition from seawater to the nutrient-rich LO could plausibly affect the energy status of the *V. fischeri* cells thereby altering their redox potential or oxygen concentration, which could be sensed by the PAS domain. Thus, investigations of the PAS and periplasmic domains of RscS will be fruitful for exploring bacteria-host interactions. Perhaps each domain detects a distinct condition, allowing RscS to integrate multiple signals from the squid environment to regulate the initiation of colonization.

What is the identity of the cognate response regulator, RscR, and what genes or proteins are controlled by the RscS/R regulatory system? In many cases, the genes for sensor kinases and their cognate response regulators are linked on the chromosome, and in some cases, genes controlled by the regulators are also nearby. This is not the case for *rscS* and the gene encoding its response regulator. The advent of the *V. fischeri* genome sequencing project (http://ergo. integratedgenomics.com/Genomes/VFI), has made it possible to use bioinformatics to look for RscR. Using the sequences of known regulators, we have searched and identified about 40 response regulators (Hussa and Visick, unpubl. data). At least 14 appear unlinked to sensor kinase genes, and thus represent the best candidates for RscR. Current work is aimed at mutagenizing these candidates and asking whether any mutants exhibit *rscS*-like colonization defects. If *rscR* encodes a DNA binding protein, then newly available DNA microarrays will be used to explore the regulon controlled by RscS/R. Identification of the targets of RscS/R regulation may also suggest a role for this regulon in symbiosis initiation. Once a target(s) of these regulators is identified, experiments aimed at identifying the colonization signal can be formulated.

gacA. In a number of pathogenic bacteria, the two-component system GacS/A regulates expression of virulence and host association traits, such as production of exoenzymes in *Pseudomonas* spp. (Heeb and Haas 2001) and motility in *Salmonella* (Goodier and Ahmer 2001). *V. fischeri* GacA also plays a role in host association. Mutants defective for *gacA* exhibit severe defects in initiating colonization: only about 10% of animals become colonized and those animals that become colonized exhibit a nearly 100-fold reduction in the level of colonization (i.e., the number of bacteria residing in the LO) (Whistler and Ruby 2003). The role of GacA is likely to be quite complex. In culture, it is associated with a number of phenotypes known to be important for

symbiosis, including motility, nutrient acquisition, siderophore activity and luminescence (Whistler and Ruby 2003). The global control of disparate traits, all of which contribute to host-association, highlights the importance of such regulators in the evolution of symbiotic associations. As with RscS/R, neither the signal nor the gene/protein targets for GacA/S are known. Identification of targets of GacA regulation, possibly through DNA microarray experiments, will help elucidate the role of this regulator in symbiosis and potentially reveal previously unknown traits important for host-microbe interaction.

flrA. FlrA, a transcription regulator with limited sequence similarity to response regulators, functions as a master regulator of flagellar biosynthesis (Millikan and Ruby 2003). Given the absolute requirement for motility in symbiotic initiation, the requirement for FlrA seems straightforward as mutations lead to a lack of flagella. However, complemented *flrA* mutants showed restored motility but not normal colonization: initiation was delayed and the level of colonization at 48 h post-inoculation was reduced by 10-fold.

One explanation for the above result is that the timing and level of flagellar biosynthesis are critical for optimal initiation and colonization and these characteristics were not properly restored in the complemented strains. In support of this hypothesis, hyper-motile (hyper-flagellated) *V. fischeri* mutants also exhibit severe delays in initiating colonization and defects in the level of colonization 24 h post-inoculation (Millikan and Ruby 2002). Alternatively, an equally plausible explanation is that FlrA controls genes other than those involved in flagellar biosynthesis (Millikan and Ruby 2003) that are also required for colonization.

Several non-flagellar genes appear to be regulated by FlrA (Millikan and Ruby 2003). One gene that appears to be repressed by FlrA, *hvnC*, encodes a protein related to HvnA and HvnB, two secreted NAD^+ glycohydrolases found in *V. fischeri*. However, neither *hvnA* nor *hvnB* appears necessary for colonization (Stabb et al. 2001); therefore, the relevance of FlrA-mediated regulation of *hvnC* is unclear. A second putative FlrA-repressed gene is homologous to *V. cholerae kefB*. In *E.coli*, KefB is a potassium efflux protein that is important for protecting cells from toxic metabolites during growth on a poor carbon source (Ferguson et al. 2000). Possibly, the *V. fischeri* KefB homolog provides protection from a LO-specific toxin.

Are FlrA-repressed genes relevant to symbiotic colonization? FlrA-controlled flagella, which are required for initiation, become dispensable to colonized bacteria. Thus, a switch in flagella gene transcription may be coordinated with induction or repression of non-flagellar genes through FlrA. The regulation of FlrA itself may be at the level of transcription, analogous to cAMP-CRP mediated control of the master flagellar regulators *flhDC* in *E. coli* (Soutourina et al. 1999). In addition, the limited similarity of FlrA to response regulators suggests its activity could be regulated via phosphorylation by a sensor kinase. Future work will likely focus on

determining whether FlrA itself is transcriptionally controlled, whether over-expression of FlrA during colonization affects the level or timing of transcription of putative FlrA-controlled genes and whether such genes themselves promote (or interfere with) colonization.

3.2 Quorum-Sensing Regulatory Systems

First described in *V. fischeri*, quorum sensing is used by many bacteria to detect the presence of other bacteria in their surroundings (reviewed in Taga and Bassler 2003). This method of monitoring the environment involves the production of a small molecule known as an autoinducer (AI) by an autoinducer synthase. Secreted into the environment, AIs can be recognized in recipient cells either by a specific two-component sensor kinase, or more frequently in Gram-negative bacteria, by a DNA-binding protein in the LuxR family. In either case, the AI signal results in transcriptional control of target genes.

V. fischeri uses both the LuxR DNA binding protein and specific sensor kinases to detect at least three AI signals (Fig. 3). Both pathways contribute to the control of bioluminescence, a trait required for symbiosis. A mutant

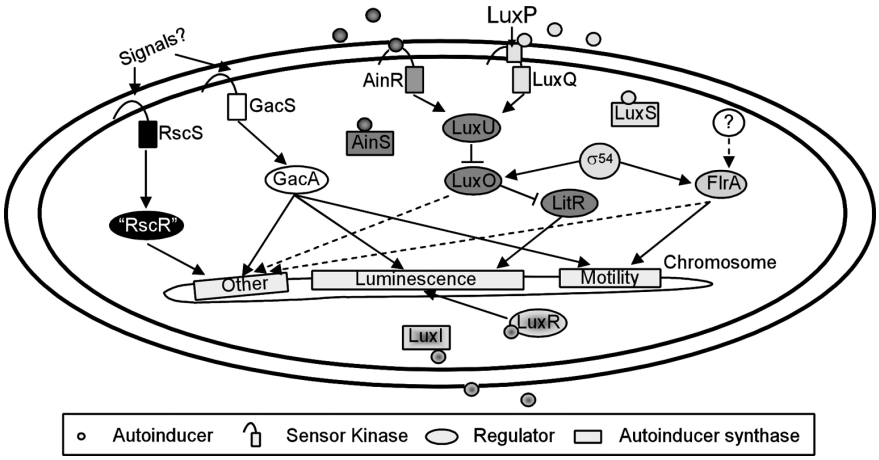

Fig. 3. Regulatory circuits required for symbiosis. *Dotted lines* represent hypothesized regulatory events. Activities required for symbiosis –"luminescence," "motility," and "other" – are represented as genes on the *V. fischeri* chromosome. Regulation of these activities may be through activation of transcription, as is the case for FlrA, through activation of transcription of a repressor, as is predicted to be the case for LuxO, or through modulating the activity of the protein product. The *V. fischeri* proteins AinS, AinR, and LitR are homologous to *V. harveyi* proteins LuxM, LuxN and LuxR, respectively

defective for *luxA*, one of two genes that encode bacterial luciferase, exhibits a three- to four-fold reduction in colonization level within 48 h post-inoculation (Visick et al. 2000). Encoded upstream of *luxA* are LuxR and LuxI, an AI synthase that produces the AI detected by LuxR. Mutations in either *luxR* or *luxI* result in a colonization defect similar to that of the *luxA* mutant, suggesting that these regulators are required for symbiosis due to their role in transcriptional control of the *lux* operon.

A second AI synthase, AinS, produces a distinct AI that also is required for symbiosis. The pathway through which the AinS-synthesized AI is detected and transmitted is predicted based on studies in the related bacterium, *V. harveyi* (reviewed in Taga and Bassler 2003). In *V. harveyi*, AIs signal through two hybrid sensor kinases, LuxN and LuxQ, using a phosphotransferase protein, LuxU, to ultimately affect the activity of a response regulator, LuxO. In the absence of AIs, LuxO negatively regulates *lux* genes by indirectly controlling transcription of a transcriptional activator (LitR in *V. fischeri* [Fidopiastis et al. 2002]). In *V. fischeri*, the AinS-produced AI likely is recognized by AinR, a sensor kinase with significant homology to *V. harveyi* LuxN (Gilson et al. 1995; Lupp et al. 2003) (Fig. 3).

A mutant defective for *ainS* exhibits a colonization level indistinguishable from that of *luxA*, *luxI* and *luxR* mutants (Lupp et al. 2003). However, whereas the *luxA*, *luxI*, and *luxR* mutants produce no symbiotic bioluminescence (at least 1000-fold decreased [Visick et al. 2000]), the *ainS* mutant produces approximately 10–20% of the wild-type bioluminescence. Thus, it seems probable that the role of *ainS* in colonization may be independent of its role in bioluminescence regulation. These phenotypes are difficult to separate, however: mutations in *luxO*, the response regulator through which the AI signals are transmitted, restore to wild-type levels both the slightly decreased symbiotic bioluminescence and the colonization defect of the *ainS* mutant (Lupp et al. 2003). Thus, an important direction will be to determine whether this pathway controls genes, other than *lux*, necessary for colonization.

V. fischeri encodes a third AI synthase, LuxS (Lupp and Ruby 2004). In *V. harveyi*, LuxS produces an AI that is detected by sensor kinase LuxQ through its interaction with the periplasmic protein LuxP (Taga and Bassler 2003). Because *V. fischeri* contains homologs for all of these genes (Lupp and Ruby 2004), it seems likely that this AI system functions similarly in the symbiotic organism (Fig. 3). A strain of *V. fischeri* in which *luxS* is mutated colonizes the LO as well as the wild-type strain; however the *luxS* mutation appreciably decreases the colonization efficiency of an *ainS* mutant, but not its per cell luminescence (Lupp and Ruby 2004). These data provide further support for a role of the AinS system in symbiosis distinct from that of luminescence. Further investigation of the three quorum sensing pathways likely will provide insight both into genes necessary for symbiotic colonization and, because the signals are known, signal transduction during symbiotic colonization.

4
Future Directions

The initiation of the symbiosis between *E. scolopes* and *V. fischeri* involves several regulatory systems, many of which affect traits known to be involved in colonization (Fig. 3). Many important questions remain. First, what is the relation of these regulatory circuits to one another? Both FlrA and the luminescence regulator LuxO modulate transcription by σ^{54}-containing RNA polymerase (Lilley and Bassler 2000; Millikan and Ruby 2003). Not surprisingly, a σ^{54} mutant is defective for colonization, motility and luminescence (Wolfe et al. 2004). Links between luminescence and motility also are found with the GacA mutant (Whistler and Ruby 2003) as well as with one class of hyper-motile mutants (Millikan and Ruby 2002), supporting coordinate regulation of motility and luminescence. Therefore, global regulation of the colonization response by *V. fischeri* may involve regulation of σ^{54} activity. Epistasis experiments could help to determine how these systems integrate to regulate colonization.

Second, what other traits, aside from luminescence and motility, are required for initiation of symbiosis? RscS is required for initiation, yet has no effect on motility or luminescence. GacA and FlrA both appear to regulate other functions as well. The major outer membrane protein OmpU is required for initiation of colonization (Aeckersberg et al. 2001) as is a recently identified gene cluster (Yip et al. 2005). These genes are possible targets of the systems described here. Identification of additional targets will be greatly aided by the *V. fischeri* genome sequence and available microarrays.

Finally, what signals are detected by the bacteria to regulate symbiosis? Aside from AIs produced by the quorum sensing systems, the signals received by the bacteria remain unknown. Predictions of the signals can be made based on our current knowledge of the environmental conditions in the LO. Furthermore, the recent sequencing of an *E. scolopes* expressed sequence tag library (http://trace.ensembl.org/) will facilitate identification of squid genes important for colonization and thus provide clues as to the conditions/signals the bacteria encounter in the LO. The answers to these questions will advance our understanding of the communication between and adaptation by *V. fischeri* and its host *E. scolopes*.

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