

## *Vibrio fischeri* Uses Two Quorum-Sensing Systems for the Regulation of Early and Late Colonization Factors

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*Vibrio fischeri* possesses two quorum-sensing systems, *ain* and *lux*, using acyl homoserine lactones as signaling molecules. We have demonstrated previously that the *ain* system activates luminescence gene expression at lower cell densities than those required for *lux* system activation and that both systems are essential for persistent colonization of the squid host, *Euprymna scolopes*. Here, we asked whether the relative contributions of the two systems are also important at different colonization stages. Inactivation of *ain*, but not *lux*, quorum-sensing genes delayed initiation of the symbiotic relationship. In addition, our data suggest that *lux* quorum sensing is not fully active in the early stages of colonization, implying that this system is not required until later in the symbiosis. The *V. fischeri luxI* mutant does not express detectable light levels in symbiosis yet initiates colonization as well as the wild type, suggesting that *ain* quorum sensing regulates colonization factors other than luminescence. We used a recently developed *V. fischeri* microarray to identify genes that are controlled by *ain* quorum sensing and could be responsible for the initiation defect. We found 30 differentially regulated genes, including the repression of a number of motility genes. Consistent with these data, *ain* quorum-sensing mutants displayed an altered motility behavior in vitro. Taken together, these data suggest that the sequential activation of these two quorum-sensing systems with increasing cell density allows the specific regulation of early colonization factors (e.g., motility) by *ain* quorum sensing, whereas late colonization factors (e.g., luminescence) are preferentially regulated by *lux* quorum sensing.

The association between the luminescent marine bacterium *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*, is a model system for the study of mutualistic bacterium-host interactions that has been successfully used to investigate the processes underlying the initiation, accommodation, and persistence of symbioses (33, 34). The association is initiated every generation, as newly hatched juvenile squids collect their bacterial symbionts from the surrounding seawater (30). Within the first hour after hatching, bacterial cells present in the ambient seawater trigger the secretion of mucus from the superficial epithelium of the squid light organ. *V. fischeri* cells attach to this mucus, forming dense aggregates. Between 3 and 6 h after hatching, the bacterial aggregates begin to move towards pores that lead into the interior of the light organ. After entering the pores, bacterial cells travel through ciliated ducts into the deep crypt regions of the light organ. Interestingly, this latter process is *V. fischeri* specific and requires flagellum-driven motility (30). Once inside the light organ, *V. fischeri* cells grow on nutrients supplied by the squid host and reach maximal colonization levels at approximately 24 h post-inoculation (15, 35). Several genetic factors required for the different stages of this cooperative association have been identified; for example, (i) motility is essential for the initiation of the symbiosis (14, 26–28), (ii) the ability to acquire iron is important for the symbionts to maintain a normal level of colonization (16), (iii) defense against oxidative stress is nec-

essary to efficiently colonize (44), (iv) luminescence expression is required for persistent colonization (41), and (v) the regulation of colonization gene expression is important at each of these stages (22, 28, 41, 45, 46).

The coordinated expression of colonization factors can be controlled by a process termed quorum sensing, which relies on the secretion of small signal molecules that accumulate in the surrounding environment. Once the bacterial population reaches a threshold cell density, the concentration of the quorum-sensing signal is sufficient to induce gene expression either directly by interacting with a transcriptional regulator or indirectly by activation of a signaling cascade. Thus, colonization factors are expressed only when they are beneficial to the bacterial cell (e.g., when they are associated with the host), avoiding the execution of costly processes in nonpermissive environments (11, 47). In gram-negative bacteria the most commonly used signals are either (i) acylated homoserine lactones (acyl-HSLs) synthesized by a LuxI or LuxM type of enzyme or (ii) autoinducer-2 (AI-2), a furanosyl borate diester synthesized by the LuxS enzyme (5, 47). While acyl-HSLs are generally species specific due to differences in the acyl side chain, the LuxS signal appears to be essentially ubiquitous; i.e., many gram-negative and gram-positive species produce and respond to this signal molecule (2).

Quorum sensing based on the *lux* system was first described in *V. fischeri* as the principal process regulating luminescence expression (7). The expression of the *lux* genes, *luxICDABEG*, in *V. fischeri* is regulated in a cell density-dependent fashion through the LuxI-directed synthesis of *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) and its subsequent binding to the transcriptional activator protein LuxR (8, 25). The LuxR-acyl-HSL complex interacts with the *lux* promoter and

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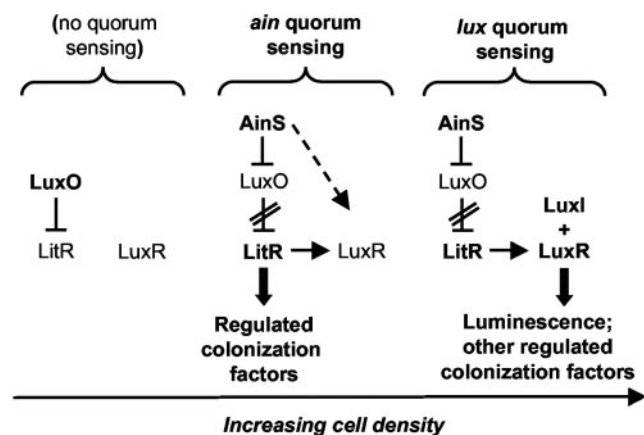


FIG. 1. Model illustrating gene regulation by the two major *V. fischeri* quorum-sensing systems, *ain* and *lux*. See the introduction for explanation.

induces the transcription of the *luxICDABEG* locus (12). The *V. fischeri lux* system is required for both luminescence expression in vivo and persistence of the bacterial symbiont in the squid light organ (41). *V. fischeri* possesses a second quorum-sensing system based on AinS, an enzyme belonging to the LuxM type of acyl-HSL synthases, which synthesizes an *N*-octanoyl homoserine lactone signal (C8-HSL) (13). We previously proposed a model of luminescence gene regulation by the two quorum-sensing systems *ain* and *lux* (22) (Fig. 1). At low cell densities, luminescence gene regulation is repressed through LuxO (22), a negative modulator of the transcriptional regulator LitR (29), (Fig. 1). At the intermediate cell densities achieved in laboratory culture, the AinS-synthesized signal has two effects, i.e., induction of luminescence gene expression through direct interaction with LuxR and inactivation of LuxO

(22); the second effect results in increased transcription of *litR* (29) (Fig. 1). LitR positively regulates transcription of *V. fischeri luxR* (9), thereby connecting the two quorum-sensing systems *ain* and *lux* and allowing the sequential induction of luminescence gene expression with increasing cell density (22) (Fig. 1). Inactivation of the *V. fischeri ain* system results in a luminescence defect and affects colonization competence (22). The *V. fischeri LuxS* signal AI-2 was shown to be involved in the regulation of luminescence expression and colonization competence as well; however, compared to that of *ain* and *lux* quorum sensing, the impact of AI-2 was quantitatively small (21).

We asked here whether the stepwise activation of the *ain* and *lux* quorum-sensing systems with increasing cell density is functional in the symbiosis. We found that (i) *ain*, but not *lux*, quorum sensing is required for efficient initiation of colonization; (ii) the *lux* quorum-sensing system is not maximally induced until the later stages of colonization; and (iii) *ain* quorum sensing differentially regulates several factors, such as flagellar motility, with potential importance for colonization initiation.

MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. *V. fischeri* strains were grown at 28°C either in a seawater-based nutrient medium (SWT) (3) or in Luria-Bertani salt medium (LBS) (14). *E. coli* strains were grown at 37°C in Luria-Bertani medium (36). Media were solidified with 1.5% (wt/vol) agar as needed. Antibiotics were added to the media at the following concentrations when appropriate: chloramphenicol (Cam), 2.5 µg/ml for *V. fischeri* and 20 µg/ml for *Escherichia coli*; erythromycin, 5 µg/ml for *V. fischeri* and 150 µg/ml for *E. coli*; and kanamycin (Kan), 100 µg/ml for *V. fischeri* and *E. coli*. 3-Oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) was obtained from Sigma-Aldrich Corp. (St. Louis, MO); octanoyl-L-homoserine lactone (C8-HSL) was obtained from Aurora Biosciences (Coralville, IA). Other medium reagents were purchased from Difco Laboratories (Sparks, MD) and Sigma-Aldrich.

**Colonization assays.** Colonization levels were determined as the number of CFU per squid light organ present at 12 h postinoculation (34). For each

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
<i>E. coli</i> strains		
CC118λpir	Cloning strain	18
DH5α	Cloning strain	17
<i>V. fischeri</i> strains		
Wild-type ES114	Isolate from an <i>E. scolopes</i> light organ	3
<i>ainS</i> mutant CL21	<i>ainS</i> gene partially deleted and replaced by a chloramphenicol resistance ( <i>cat</i> ) marker	22
<i>litR</i> mutant PMF8	<i>litR</i> gene inactivated by insertion of a kanamycin resistance marker ( <i>kanR</i> )	9
<i>luxI</i> mutant VCW2G7	<i>luxI</i> gene inactivated by a frameshift mutation	22
<i>luxO</i> mutant CL42	<i>luxO</i> gene inactivated by insertion of a kanamycin resistance marker ( <i>kanR</i> )	22
<i>luxR</i> mutant CL53	<i>luxR</i> gene inactivated by insertion of a erythromycin resistance marker ( <i>ermR</i> )	21
<i>luxS</i> mutant CL39	<i>luxS</i> gene inactivated by insertion of a kanamycin resistance marker ( <i>kanR</i> )	21
<i>ainS luxI</i> mutant CL24	Double mutant carrying mutations as described above	22
<i>ainS luxO</i> mutant CL64	Double mutant carrying mutations as described above	22
<i>ainS luxS</i> mutant CL41	Double mutant carrying mutations as described above	21
LuxO(D47E) mutant CL59	LuxO protein carrying a T-to-G point mutation resulting in an aspartate-to-glutamate substitution at position 47	This study
Plasmids		
pCL145	pEV579 carrying a 1.8-kb <i>V. fischeri</i> ES114 DNA fragment with the <i>luxO</i> gene	22
pCL150	pCL145 with LuxO mutated by an aspartate-to-glutamate substitution at position 47	This study
pDM104-6	pVO8 carrying a <i>flaA::lacZ</i> transcriptional fusion	48
pEV579	Allelic exchange vector	40
pKV112	pVO8 carrying the <i>gfp</i> gene under P <sub>lac</sub> control	K. Visick
pKV31	pVO8 carrying a 1-kb Sall-PvuII fragment with the intact <i>luxR</i> gene	K. Visick
pVO8	<i>V. fischeri</i> cloning vector, <i>camR</i> and <i>ermR</i>	43

experiment, 12 to 20 newly hatched squids were placed into 50 ml of filter-sterilized seawater containing about 3,000 CFU of the indicated strain per ml. Another group of animals was placed into filter-sterilized seawater without added bacteria. After incubation for 12 h in the dark to prevent the usual expulsion of bacteria at sunrise (15, 19), the animals were homogenized, and the homogenate was diluted and spread onto SWT agar. The colony number was counted after overnight incubation, and the mean number of CFU per squid was calculated.

To determine the relative competitive index of the mutant strains, 10 to 15 newly hatched squids were placed into 50 ml of filter-sterilized seawater containing approximately 4,000 CFU of each of two competing strains and were incubated for 12 h. An aliquot of the inoculated seawater was spread onto LBS agar to determine the number and exact ratio of the two strains in the inoculum. At 48 h postinoculation, squids were homogenized and a dilution of the homogenate spread onto LBS agar. Approximately 100 CFU from the inoculum, as well as from each of the homogenates, was patched onto both antibiotic-containing and antibiotic-free LBS agar to determine the ratio of *V. fischeri* wild-type (Cam and Kan sensitive) to *luxO* mutant (Kan resistant) or *ainS* mutant (Cam resistant) cells. The relative competitive index was calculated as the ratio of the two strains in the symbiosis divided by the ratio in the inoculum.

To observe aggregation behavior, newly hatched squids were incubated with an inoculum of 500,000 CFU per ml of either wild-type *V. fischeri* or the *ainS* mutant, each carrying pKV112, a green fluorescent protein expression plasmid (Table 1). Every 30 min, two squids per treatment were dissected and the formation of aggregates was observed using fluorescence microscopy as previously described (30).

**Mutant construction.** Genetic techniques (DNA extraction, PCR, ligation, conjugation, and DNA sequencing) were carried out as previously described (22).

The *LuxO*(D47E) mutant was constructed using the QuikChange site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene Inc., La Jolla, CA). Plasmid pCL145 (Table 1), carrying the intact *luxO* gene in the allelic exchange vector pEV579 (Table 1), served as a template to create the site-directed mutation. The asparagine GAT codon in position 47 of the *LuxO* protein was changed into a glutamine GAG codon by introducing a T→G point mutation. The resulting plasmid, pCL150, was transferred into the *V. fischeri luxO* mutant CL42 by triparental mating, and double recombinants that had lost kanamycin resistance were selected as previously described (39), generating the *V. fischeri LuxO*(D47E) mutant strain CL59. The introduction of the point mutation into the genome was confirmed by sequence analysis of the mutant's *luxO* gene. Luminescence and growth characteristics were determined as previously described (22).

**Microarray comparison.** Wild-type *V. fischeri* and the *ainS*, *luxO*, and *LuxO*(D47E) mutants were grown in SWT medium to an optical density (OD) at 600 nm of 2.5, a point at which *V. fischeri* cells still grow exponentially and, based on luminescence expression data (22), *ain* quorum sensing is maximally induced. RNA extractions were performed as previously described (37). cDNA synthesis was carried out using 10 to 12 µg of purified RNA, 15 µg random decamer primers (IDT, Coralville, IA), a deoxynucleoside triphosphate mixture (Amersham Biosciences, Piscataway, NJ) containing a 3:2 ratio of aminoallyl-dUTP (Sigma-Aldrich) to dTTP, and 500 units of Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). RNA was removed by alkaline hydrolysis, and cDNA was purified and concentrated using Microcon-30 filters (Millipore Corp., Billerica, MA). Labeling of cDNA was carried out using mono-reactive Cy3 and Cy5 dyes according to the manufacturer's protocol (Amersham Biosciences), and labeled cDNA was desalted with a PCR purification kit (QIAGEN Inc., Valencia, CA). Nucleotide and label dye concentrations were determined by measuring absorption at 260 nm (nucleotide), 550 nm (Cy3), and 650 nm (Cy5). Microarray glass slides spotted three times with PCR products representing approximately 95% of all *V. fischeri* open reading frames (Integrated Genomics Inc., Chicago, IL) were incubated for 45 min at 42°C in prehybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] [Sigma-Aldrich], 0.001% sodium dodecyl sulfate [SDS], 0.5% bovine serum albumin). The two differentially labeled samples were mixed at a Cy3: Cy5 dye ratio of approximately 1:1 and concentrated to a final volume of 10 µl using Microcon-30 columns (Millipore Corp.). To each sample, 3.5 µl of 1-mg/ml salmon sperm DNA, 3.5 µl of 1-mg/ml yeast tRNA, and 18 µl PerfectHyb Plus hybridization buffer (Sigma Chemical Co.) were added, and the mixture was denatured at 99°C for 1.5 min. Each sample was added to the microarray glass slides and hybridized for 14 to 18 h at 42°C. The slides were washed at approximately 24°C for 5 min each in (i) 1× SSC, 0.001% SDS; (ii) 0.1× SSC, 0.001% SDS; (iii) 0.001% SDS; and (iv) water. After drying, fluorescence intensities were determined using a 428 Array Scanner (Affymetrix Inc., Santa Clara, CA). For each

TABLE 2. Colonization levels of quorum-sensing mutants at 12 h postinoculation

Strain	% of wild-type colonization level <sup>a</sup>
Wild type.....	100 (17)
<i>ainS</i> .....	45 (14)
<i>luxI</i> .....	115 (19)
<i>ainS luxI</i> .....	79 (15)
<i>luxS</i> .....	95 (30)
<i>ainS luxS</i> .....	48 (7)
<i>luxO</i> .....	37 (12)
<i>ainS luxO</i> .....	36 (12)
<i>litR</i> .....	51 (12)
<i>luxR</i> .....	119 (17)

<sup>a</sup> Colonization levels are the means from two experiments. In each experiment 12 animals were inoculated with one of the strains. The values are normalized to the levels reached in a wild-type colonization (averaging about 20,000 CFU/light organ), with standard errors indicated in parentheses.

hybridization spot, background intensities were subtracted from spot intensities. The data were then log transformed and normalized by equalizing 25% and 75% tiles. Ratios of the normalized Cy3 and Cy5 dye intensities were determined for each spot and averaged for each microarray. For statistical analysis, a two-sided, paired Student *t* test was performed.

**Transcriptional activity of *flaA*.** Plasmid pDM104-6 (Table 1) was introduced into *V. fischeri* wild-type and *ainS* mutant strains by triparental mating as previously described (39). Both strains were cultured in SWT plus erythromycin, and *flaA* transcriptional activity was estimated by assaying beta-galactosidase activity in six samples from two independent cultures at different time points during growth, using a standard method (36).

**Motility assays.** Motility and chemotaxis behaviors of *V. fischeri* were determined by growing wild-type and mutant strains in the appropriate liquid medium to an optical density of approximately 0.4. Three microliters of the culture was spotted on the surface of a fresh 0.25% agar medium plate, and the relative diameters of the colony and the expanding rings of migrating cells were determined after 6 to 24 h of incubation at 25°C. Assays were carried out on (i) SWT medium or (ii) defined medium (10 mM NH<sub>4</sub>Cl, 0.33 mM K<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 50 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, 10 mM KCl, 0.01 mM FeSO<sub>4</sub>, and 50 mM Tris-HCl [pH 7.4]) containing either 0.3% (wt/vol) Casamino Acids (CAA) or 0.5 g/liter *N*-acetylglucosamine (NAG) as a carbon source.

## RESULTS

**Colonization initiation by *V. fischeri* quorum-sensing mutants.** To determine whether any of the *V. fischeri* quorum-sensing systems is important for the initial stages of light-organ colonization, we determined the number of CFU in the organ 12 h after inoculation with either wild-type *V. fischeri* or the *luxI*, *ainS*, or *luxS* mutant (Table 2). At this early point in colonization, the number of CFU in squid colonized with the *luxI* mutant was indistinguishable from wild-type levels (Table 2). The fact that *luxI* mutant-colonized animals do not produce detectable luminescence (i.e., <1% of the wild-type level) (22) suggests that even a severe defect in light production does not affect colonization efficiency during the initial stages of colonization. In contrast, the *ainS* mutant, while expressing low (only 10% of wild-type) luminescence levels (22), was able to colonize the light organ only to <50% of the wild-type number of bacterial cells. Thus, although light emission was affected by a mutation in *ainS*, this gene probably regulates additional factors beyond luminescence that are important for colonization initiation. Squid colonized by the *luxS* mutant displayed colonization levels indistinguishable from those of wild-type-colonized animals at 12 h postinoculation (Table 2), indicating that *luxS* is not required for normal initiation of colonization.



TABLE 3. Comparison of the *V. fischeri ainS* and *luxO* deletion mutants with the constitutively active LuxO(D47E) mutant

Phenotype	Value for <i>V. fischeri</i> strain				
	Wild type	LuxO(D47E)	<i>ainS</i>	<i>litR</i>	<i>luxO</i>
Doubling time <sup>a</sup>	48 (7)	46 (4)	47 (6)	51 (6)	45 (6)
Growth yield <sup>a</sup>	7.8 (0.7)	7.4 (0.1)	6.1 (0.3)	5.9 (0.1)	7.8 (0.5)
Luminescence <sup>b</sup>	3.2 (0.4)	0.6 (0.1)	BD	0.1 (0.0)	3.5 (0.8)
Luminescence with C8-HSL <sup>b</sup>	4.3 (0.9)	0.7 (0.1)	3.3 (0.3)	0.2 (0.1)	7.8 (2.0)
Luminescence with 3-oxo-C6-HSL <sup>b</sup>	842 (70)	724 (87)	2.9 (0.6)	659 (168)	1036 (73)
Colonization level 12 h postinoculation <sup>c</sup>	100 (17) <sup>d</sup>	52 (10)	45 (14) <sup>d</sup>	51 (12) <sup>d</sup>	37 (12) <sup>d</sup>
Colonization level 72 h postinoculation <sup>c</sup>	100 (11)	97 (7)	29 (15) <sup>e</sup>	ND	95 (12) <sup>e</sup>

<sup>a</sup> Doubling time (minutes) and growth yield (optical density at 600 nm) were determined in SWT medium; the values are averages from three independent experiments, with the standard deviation indicated in parentheses.

<sup>b</sup> Specific luminescence values ( $10^{-2}$  quanta  $s^{-1}$  cell $^{-1}$ ) are essentially constant for cultures with an optical density (at 600 nm) between 1.0 and 5.0. The means from several samples from each culture are given, and standard errors of the mean are indicated in parentheses. Cultures were grown in SWT medium or in SWT medium supplemented with 120 nM of either 3-oxo-C6 or C8-HSL. Presented are results from a representative experiment, which was repeated with the same outcome. BD, below detection (i.e.,  $<2 \times 10^{-4}$  quanta  $s^{-1}$  cell $^{-1}$ ).

<sup>c</sup> Colonization levels are the means from two experiments that included 12 animals each, normalized to the percentage of the wild-type level (standard errors are indicated in parentheses). ND, not determined.

<sup>d</sup> These data are also presented in Table 2 and are included here for comparison.

<sup>e</sup> From reference 22.

A secondary mutation in either *luxI* or *luxS* did not enhance the colonization defect of the *ainS* mutant (Table 2), suggesting the absence of a synergistic effect of these mutations in colonization initiation.

One of the early events in colonization initiation is the formation of bacterial aggregates outside the squid light organ (30). Because a defect in this activity could explain the observed initiation phenotype, we examined aggregation behavior of the *ainS* mutant in comparison to wild-type *V. fischeri*. However, we could not detect any difference between the two strains, either in the timing of aggregate formation or in the size of the bacterial aggregate formed (data not shown). These results suggest that the *ainS* mutant is not defective in attaching to the squid mucus and that the colonization process(es) regulated by *ainS* becomes important after bacterial aggregation.

**Regulation cascade of the initiation defect.** In our previous study (22) we demonstrated that the *ainS* mutant displays a persistence defect; i.e., the number of CFU is similar to that of the wild type at 24 h after colonization (the earliest time point examined) but becomes significantly lower by 48 and 72 h. This persistence defect could be reversed by introducing a *luxO* mutation into the *ainS* mutant background, suggesting that AinS regulates gene expression through the inactivation of LuxO (22). LuxO, in turn, down-regulates transcription of *litR*, a gene encoding a positive regulator of *luxR* (9, 29). To investigate whether the colonization factors underlying the initiation defect of the *ainS* mutant are regulated through this AinS-LuxO-LitR pathway (Fig. 1), we compared light-organ colonization levels at 12 h postinoculation with either the *V. fischeri* wild-type, *luxO*, *ainS-luxO*, *litR*, or *luxR* strain (Table 2).

Previous work (22) has shown that a *luxO* mutation does not interfere with the ability of *V. fischeri* to persist in the squid light organ after 24 h of colonization. However, the results presented here demonstrate that *luxO*, like *ainS* and *litR* but not *luxR*, is required for a normal level of colonization by 12 h (Table 2). Furthermore, the *luxO* mutant displayed a competition defect that was comparable to that of the *ainS* mutant. Specifically, when juvenile squids were colonized with a 1:1 mixed inoculum of either *luxO* mutant and wild-type cells or

*ainS* mutant and wild-type cells, the average relative competitive index of both mutants was 0.4 (data not shown). Thus, mutations in both positive (AinS and LitR) and negative (LuxO) regulators in the pathway (Fig. 1) resulted in a similar defect in the level of colonization at 12 h (Table 2), which might also lead to a competitive defect. One interpretation of this surprising outcome is that, rather than a simple on-off switch, the AinS-LuxO-LitR pathway serves to maintain the colonization factor(s) it regulates at a proper level of expression. Because inactivation of *luxR* had no effect on the initiation of symbiosis (Table 2), the *lux* system is apparently not important for early stages of colonization, and thus the initiation defect of the *ainS* mutant does not operate through LuxR (Fig. 1).

**Growth, luminescence, and colonization phenotypes of the *V. fischeri* LuxO(D47E) mutant.** While inactivation of *V. fischeri ainS* results in both initiation and persistence defects, a mutation in *litR*, operating downstream of *ainS*, affected only colonization initiation (Table 2) (9, 22), suggesting that the effect of *ainS* on colonization initiation may be distinct from that on colonization persistence. We therefore hypothesized that the AinS-LuxO-LitR cascade might directly activate genes important for colonization initiation, while the absence of *ainS*-dependent induction of *lux* quorum sensing results in a defect in colonization persistence (Fig. 1).

To investigate this possibility further, we constructed a site-directed LuxO(D47E) mutant encoding an aspartate-47-to-glutamate substitution that has been shown to mimic the phosphorylated, and thus active (i.e., repressive), state of the *Vibrio harveyi* LuxO protein (10). According to the model of quorum sensing (Fig. 1), we predicted that this mutation would simulate the effects of an *ainS* mutation on the LuxO-LitR regulatory cascade but would allow the induction of *lux* quorum sensing (22). When we compared the growth, luminescence, and colonization phenotypes of the LuxO(D47E) mutant with those of the *ainS*, *litR*, and *luxO* mutants (Table 3), we found that their behavior was consistent with our hypothesis. Specifically, the growth yield of the LuxO(D47E) mutant in culture was reduced, while its doubling time was indistinguishable from that of the wild-type strain. However, the growth yield defect

of the LuxO(D47E) mutant was smaller than those of the *ainS* and *litR* mutants, suggesting that LuxO(D47E) might not repress downstream genes to the same extent as the native phosphorylated LuxO protein. As expected, the growth defect could not be eliminated by exogenous addition of C8-HSL (data not shown), because the mutated LuxO(D47E) protein only mimics the phosphorylated LuxO state and cannot be dephosphorylated (and thus deactivated) by *ain* signaling. Luminescence of the LuxO(D47E) and *litR* mutants in culture was decreased compared to wild-type levels but was not eliminated (Table 3) (9). This result is consistent with the model of regulation (Fig. 1) in which AinS has two effects on luminescence regulation: the inactivation of LuxO repression and the activation of the *lux* operon through a C8-HSL–LuxR interaction (22). Thus, inactivation of AinS is expected to result in a more severe luminescence defect than a constitutively active LuxO protein or a mutation in *litR*. The luminescence levels of the LuxO(D47E) and *litR* mutants did not change in response to exogenous C8-HSL addition, implying that both mutant strains synthesize sufficient amounts of C8-HSL to induce luminescence through C8-HSL–LuxR interaction. When 3-oxo-C6-HSL was provided, luminescence reached nearly wild-type levels, indicative of proper *lux* quorum-sensing induction. As previously shown, the *ainS* mutant displayed essentially opposite effects, i.e., an increase in luminescence to wild-type levels when C8-HSL was added but a reduced response to 3-oxo-C6-HSL (22) (Table 3). This effect may be due to the absence of C8-HSL–LuxR interaction, which is necessary for proper *lux* quorum-sensing induction (22).

Finally, while the colonization levels of the LuxO(D47E), *litR*, and *ainS* mutants were comparable at 12 h postinoculation, only the *ainS* mutant displayed a persistence defect at 72 h (Table 3). Thus, the *ainS* mutant is compromised in both the initiation and persistence of symbiosis; however, the underlying causes of these two colonization defects appear to be distinct.

**Onset of *lux* quorum sensing.** Having established that *lux* quorum sensing does not affect the ability of *V. fischeri* to initiate colonization, we asked whether this system is fully active during the early stages of colonization. Because induction of the *lux* system is required for light expression in the host (i.e., *luxI* mutant-colonized animals are dark [22]), *lux* activity can be easily monitored by measuring symbiotic light production. In culture, where the *lux* system is inactive, the exogenous addition of saturating concentrations of the LuxI-synthesized 3-oxo-C6-HSL stimulates light expression more than 1,000-fold (22). We used a similar approach to determine the level of *lux* activity during the initiation of symbiosis by comparing luminescence expression by wild-type-colonized juvenile squid exposed to seawater containing (i) no addition, (ii) 120 nM 3-oxo-C6-HSL, or (iii) 1,200 nM 3-oxo-C6-HSL during the first 24 h of colonization (Fig. 2A). To minimize any possible effects of 3-oxo-C6-HSL on colonization phenotypes other than luminescence expression, juvenile squids were incubated with wild-type *V. fischeri* cells for 6 h before being transferred into seawater containing 3-oxo-C6-HSL. Almost immediately after exposure to 3-oxo-C6-HSL, and 3 h earlier than the no-addition control, squid became detectably luminous (Fig. 2A). This positive effect of 3-oxo-C6-HSL on luminescence expression continued until approximately 12 h postinoculation, after

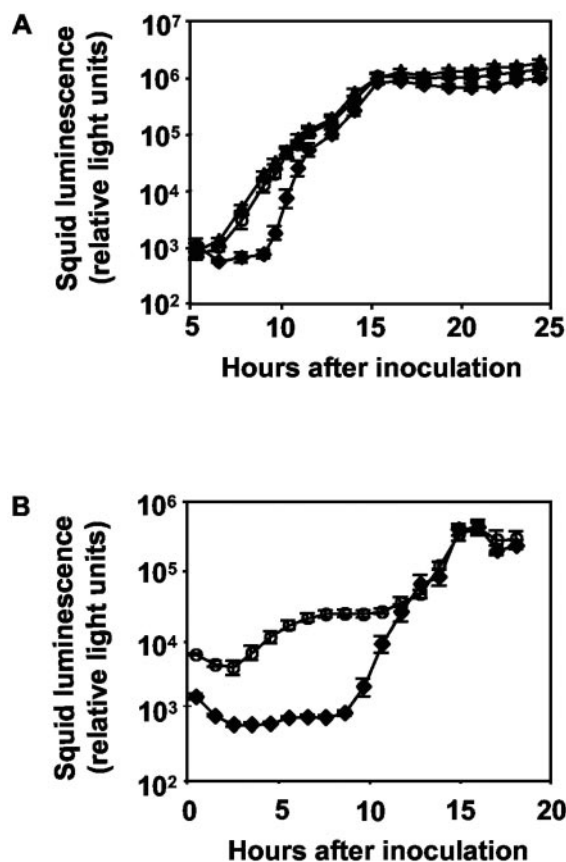


FIG. 2. Symbiotic *lux* quorum-sensing activity during initial stages of colonization. Presented are the mean luminescence levels of 24 animals for each treatment, with standard errors. A. Effect of 3-oxo-C6-HSL addition on luminescence of *V. fischeri*-colonized juvenile squid. Animals were inoculated with *V. fischeri* wild-type cells for 6 h and then transferred to seawater only (diamonds) or seawater containing either 120 nM (circles) or 1,200 nM (triangles) 3-oxo-C6-HSL. B. Effect of *luxR* gene overexpression on luminescence of *V. fischeri*-colonized juvenile squid. Animals were inoculated with *V. fischeri* wild-type cells carrying either pKV31 (circles) or pVO8 (diamonds), a vector control.

which luminescence levels were indistinguishable between exposed and control animals (Fig. 2A). In contrast, exposure of wild-type-colonized squid to 1,200 nM of the AinS-synthesized signal C8-HSL had no significant effect on the development of luminescence expression (data not shown), suggesting that this signal is produced in saturating concentrations by 6 h postinoculation. Because acyl-HSLs are susceptible to hydrolysis, it was a concern that the absence of any positive effect on luminescence at time points beyond 12 h was due to degradation of the compound. However, because luminescence expression by animals exposed to 10 times the saturating concentration (1,200 nM 3-oxo-C6-HSL) was indistinguishable from that by animals exposed to 120 nM 3-oxo-C6-HSL (Fig. 2A), it seemed unlikely that degradation of 3-oxo-C6-HSL affected the outcome of this experiment.

Finally, we tested our hypothesis that LuxR, a target of LitR induction (Fig. 1), is limiting during the early stages of colonization. When juvenile squids were colonized with wild-type *V. fischeri* overexpressing the *luxR* gene in *trans*, luminescence

TABLE 4. Transcriptome analysis of *V. fischeri* quorum-sensing mutants

Category and ORF <sup>b</sup>	Annotation (putative function)	Ratio <sup>a</sup>	
		Wild type/ <i>ainS</i> <sup>c</sup>	<i>luxO</i> /LuxO(D47E) <sup>d</sup>
<b>Motility</b>			
VF0777	Methyl-accepting chemotaxis protein	-3.0 (0.6)	-2.9 (6.5)
VF1836	Flagellar biosynthesis protein, FlhF	-2.3 (0.9)	-3.3 (0.7)
VF1846 <sup>e</sup>	Flagellar protein, FlhL	-1.8 (6)	-3.1 (0.3)
VF1853 <sup>e</sup>	Flagellar hook-basal body protein, FlhE	-1.9 (0.4)	-4.2 (0.9)
VF1862	Flagellin, FlaE	-5.0 (0.4)	-15.5 (0.2)
VF1863	Flagellin, FlaD	-4.0 (0.3)	-8.3 (0.7)
VF1864 <sup>e</sup>	Flagellin, FlaC	-1.7 (18)	-4.2 (0.1)
VF1865 <sup>e</sup>	Flagellin, FlaB	-1.6 (12)	-3.0 (6.4)
VF1866 <sup>e</sup>	Flagellin, FlaA	-1.8 (7)	-7.2 (4.1)
VF1874 <sup>e</sup>	Flagellar hook protein, FlgE	-1.4 (28)	-3.8 (0.9)
VF1875	Basal-body rod modification protein, FlgD	-5.6 (0.2)	-5.1 (0.1)
VF1876	Flagellar basal-body rod protein, FlgC	-11.8 (0.1)	-11.1 (0.1)
VF1877	Flagellar basal-body rod protein, FlgB	-3.3 (0.3)	-8.6 (0.7)
VF1881	Negative regulator of flagellin synthesis, FlgM	-3.7 (0.7)	-7.2 (32.3)
VF2079	Flagellin, FlaF	-7.1 (0.1)	-8.0 (0.1)
<b>Transcriptional regulators</b>			
VF1573	DNA-binding protein	-3.1 (0.1)	-2.7 (2.5)
VF2177	Transcriptional regulator, LitR	10.3 (4.2)	5.9 (<0.1)
VFA1014	Hypothetical protein (sensory transduction)	2.5 (10.6)	2.2 (17.5)
VFA1015	Sigma factor, RpoS like	5.5 (0.1)	2.3 (2.9)
VFA1017	Possible response regulator	7.0 (4.0)	4.9 (0.3)
<b>Metabolism</b>			
VF0008	Cystine-binding protein transporter	-3.8 (<0.1)	-2.5 (25)
VF0316	Drug/metabolite exporter	-3.2 (3.8)	-5.2 (5.5)
VF2247	Dihydroneopterin aldolase (folate biosynthesis)	-2.5 (19)	-3.1 (2.0)
VFA0452	Formiminoglutamase (histidine metabolism)	2.3 (0.8)	2.6 (0.5)
VFA0806	Glucose-1-phosphate adenylyltransferase (polysaccharide biosynthesis)	3.9 (0.4)	3.1 (1.6)
VFA0995	High-affinity ferrous iron permease	-5.6 (0.3)	-2.3 (0.6)
<b>EPS biosynthesis</b>			
VF0171	Hypothetical protein	4.2 (0.4)	2.4 (17.5)
VF0172	<i>O</i> -Acetyltransferase	3.6 (0.1)	3.8 (0.5)
<b>Hypothetical proteins</b>			
VF1355	Hypothetical protein	2.0 (3.5)	2.8 (1.1)
VF2255	Hypothetical protein	2.3 (0.3)	3.2 (0.2)

<sup>a</sup> The *V. fischeri* microarray includes 95% of the ORFs present in the genome, arrayed in three replicates. Negative values represent repression by *ain* quorum sensing. Indicated in parentheses are the results of a two-tailed, paired Student t-test, expressed as percent probability that the data sets are identical.

<sup>b</sup> ORF designations are those found at <http://www.ergo-light.com/ERGO/>.

<sup>c</sup> Average ratios of six values for each ORF (two microarrays, with three replicates on each array).

<sup>d</sup> Average ratios of three values for each ORF (one microarray, with three replicates on each array).

<sup>e</sup> Regulation of the ORFs was selected with less stringent criteria (see Results).

levels were higher than those of animals colonized with *V. fischeri* carrying the vector control in *trans* (Fig. 2B). Again, the differences in the levels of light expression were observed only during the initiation of colonization. These data suggest that, because of the limited expression of LuxR, the *lux* system is not maximally induced until approximately 12 h postinoculation, coincident with the time at which the squid light-organ crypts become densely colonized with *V. fischeri* cells (30).

**Microarray comparison of wild-type *V. fischeri* and quorum-sensing mutants.** In an effort to identify factors regulated by *V. fischeri ain* quorum sensing that could be responsible for the observed colonization defects, we performed three independent microarray experiments using culture-grown cells. Two microarray experiments compared the transcriptomes of the *ainS* mutant and the wild type. To ensure that the regulation was *ain* quorum-sensing specific and not a result of gene regulation by the C8-HSL-LuxR interaction (Fig. 1), a third experiment compared the transcriptomes of the LuxO(D47E)

and *luxO* mutants. We used the two selection criteria of (i) greater than twofold differential regulation in each of the three experiments and (ii) a *P* value of <0.05 to identify genes that were differentially regulated by *ain* quorum sensing (Table 4).

The most striking finding of the microarray study was that genes encoding several flagellins and flagellar basal-body proteins, appeared to be negatively regulated by the *V. fischeri ain* quorum-sensing system (Table 4). In addition, one of the 31 putative methyl-accepting chemotaxis protein genes (<http://www.ergo-light.com/ERGO/>) appeared to be differentially repressed by *ainS*, suggesting a possible role of quorum sensing in the regulation of a behavioral response to a specific environmental condition (Table 4). Finally, the gene for the *V. fischeri* FlgM homolog, a putative sigma-28 antagonist, appeared to be down-regulated as well, indicating that the repression of sigma-28-dependent motility genes by *ain* quorum sensing is unlikely to function through FlgM (Table 4). The majority of the *V. fischeri* motility genes are located in a distinct chromosomal re-

gion (VF1826 to VF1881 [http://www.ergo-light.com/ERGO/]). When we lowered the selection criteria somewhat to include genes that were on average greater than twofold differentially regulated (yet still with a *P* value of <5%), we found an additional six motility genes that were down-regulated by *ain* quorum sensing (Table 4).

We confirmed the microarray data for one of these genes (*flaA*) by comparing the beta-galactosidase activity levels of a *flaA::lacZ* fusion carried in *trans* by both wild-type *V. fischeri* and the *ainS* mutant. The beta-galactosidase activity was on average 3.3 times higher in the *ainS* mutant than in wild-type *V. fischeri* (data not shown), supporting a role of *ainS* in the expression of this important flagellin (26).

The genes for several regulatory proteins also appeared differentially regulated by *ain* quorum sensing (Table 4), suggesting that the *V. fischeri* quorum-sensing regulon is connected to other regulatory circuits. For instance, the previously proposed positive regulation of *V. fischeri litR* by *AinS* (22) was confirmed (Table 4). While the *V. fischeri ainS* mutant does not express detectable luminescence levels in culture and the specific luciferase activity is only 10% of that of the wild type (22), we could not detect differential luminescence (*luxCDABE*) or regulatory (*luxR*) gene expression by *ain* quorum sensing. We believe that this result may reflect how luciferase specific activity increases dramatically around an OD of approximately 1.0 and stays constant thereafter (22). Thus, at the OD of 2.5 at which the sample was taken for microarray analysis, positive regulation of *lux* transcription by *ain* quorum sensing may have already ceased.

One interesting regulatory locus contains the genes VFA1013 to VFA1017, some of which were positively regulated by *ain* quorum sensing (Table 4). The proteins encoded by both VFA1014/VFA1013 and VFA1016/VFA1017 have regions homologous to protein kinases and response regulator pairs, respectively (http://www.ergo-light.com/ERGO/). Located between these putative two-component regulatory systems is another gene (VFA1015) that was positively regulated by *ain* quorum sensing (Table 4). This gene is most closely related to the stationary-phase sigma factor gene *V. fischeri rpoS* (VF2067) (http://www.ergo-light.com/ERGO/). The functions of the genes in this regulatory locus remain to be determined; nevertheless, the *ainS* regulation of VFA1015, together with the growth yield defect of the *ainS* mutant (Table 3), led us to the hypothesis that the genes in the VFA1013-to-VFA1017 locus may encode important sensory and response systems used by *V. fischeri* to switch from the planktonic to the symbiotic lifestyle.

**Motility phenotype of *V. fischeri* quorum-sensing mutants.** Because the microarray results indicated that flagellin and flagellar basal body genes are regulated by the *ain* system, we assessed the motility behaviors of several of the quorum-sensing mutants. As we reported previously (22), the swimming behaviors of the *ainS* and the *luxI* mutants on rich SWT medium containing either 0.4% or 0.7% agar were indistinguishable from that of the wild type. However, at a lower agar concentration (0.25%) on both SWT and a minimal salts medium supplemented with either CAA or NAG, differences in motility behavior between wild-type and mutant strains became apparent (Table 5).

In general the effects of quorum-sensing gene mutations on

TABLE 5. Relative migration of *V. fischeri* quorum-sensing mutants in soft agar

Strain	Migration distance in <sup>a</sup> :		
	SWT	CAA	NAG
Wild type	1	1	1
<i>ainS</i>	1.4	1.6	2.4
<i>luxO</i>	0.9	BD <sup>b</sup>	BD
<i>ainS luxO</i>	0.9	BD	BD
<i>luxS</i>	1.1	1	1
<i>ainS luxS</i>	1.4	1.6	2.4
<i>litR</i>	1.4	1.6	2.2
<i>luxI</i>	1	1	1
<i>luxR</i>	1	1	1

<sup>a</sup> Migration distances in 0.25% agar medium relative to those of wild-type *V. fischeri*. The data are from a representative experiment, which was repeated with the same outcome. Measurements are accurate to within approximately 10%. Migration diameters were determined in a rich SWT medium or in a minimal medium with either CAA or NAG as the sole carbon source.

<sup>b</sup> BD, below detection (no migration observed).

motility were more pronounced in the defined media (Table 5), suggesting that nutrient availability affects *V. fischeri* motility by an unknown mechanism. While the *ainS* mutant displayed a hypermotile phenotype in all media, the *luxO* mutant produced slightly smaller migration ring diameters in SWT, and no migration could be observed in minimal medium containing either CAA or NAG (Table 5). The migration patterns of the *ainS-luxO* mutant in each medium were indistinguishable from those of the *luxO* mutant, suggesting that *luxO* has a dominant effect on the motility phenotype. The *luxS* mutant migrated slightly faster than the wild type in SWT, but the migration was not affected in minimal medium (Table 5). Furthermore, the introduction of the *luxS* mutation into the *ainS* mutant did not noticeably enhance the hypermotile phenotype of the *ainS* mutant (Table 5), suggesting the absence of a synergistic effect. The *litR* mutant displayed essentially the same phenotype as the *ainS* mutant (Table 5), consistent with the gene regulation model (Fig. 1) in which *litR* functions downstream of *ainS* (22). Neither mutation of *luxI* nor mutation of *luxR* resulted in an altered motility behavior under the conditions tested (Table 5). All of the strains grew with identical doubling times in each of the media, and only the hypermotile *ainS*, *ainS luxS*, and *litR* strains (22) (Table 3) displayed a growth yield defect (data not shown), indicating that the observed phenotypes are not due to a metabolic defect.

## DISCUSSION

In this study we investigated the impact of the *V. fischeri* quorum-sensing systems *ain* and *lux* on the early stages of colonization. Our data demonstrate that (i) *ain* (but not *lux*) quorum sensing is required for the normal initiation of colonization, (ii) the *lux* system is not fully active during initiation of colonization, and (iii) the *ain* system regulates not only the *lux* system but also several putative early colonization genes, including those controlling motility. Taken together, these data demonstrate for the first time the differential activation of two bacterial quorum-sensing systems at distinct stages of a beneficial host tissue colonization.

**A model of colonization factor regulation by *V. fischeri* quorum sensing.** Many gram-negative bacteria possess multiple



quorum-sensing systems (47), a phenomenon that has led to an examination of how these systems may function together. Our results show that in the case of *V. fischeri* two systems are arranged to allow the induction or repression of colonization factors important during specific temporal phases of the symbiotic relationship. The fact that the *ain* system is functional and essential at early stages of colonization, whereas the *lux* system is neither fully induced nor required at this time (Fig. 2), suggests that the *ain* system is operative at a lower threshold cell density than the *lux* system and provides evidence for a stepwise pattern in their control during the onset of symbiosis. The sequential nature of quorum-sensing induction is further supported by our previous finding that in symbiotic strains of *V. fischeri* the effect of the *ain* system on luminescence is apparent at the relatively moderate bacterial concentrations occurring in culture ( $10^8$  to  $10^9$  cells ml<sup>-1</sup>), whereas the *lux* system is the predominant inducer of luminescence expression at the very high cell densities ( $>10^{10}$  cells ml<sup>-1</sup>) achieved only within the squid light organ (22).

We proposed previously a model by which luminescence is regulated by *ain* and *lux* (22). The results of this present study expand this model to include the regulation of non-*lux* genes at specific phases in the colonization of *E. scolopes* by *V. fischeri*. At the very low cell densities found in seawater ( $<10^4$  cells/ml), the absence of *ain* quorum sensing allows repression of colonization gene expression through the transcriptional regulator protein LuxO (Fig. 1). When *V. fischeri* initiates colonization of its host by aggregating and then migrating through the squid light-organ ducts (30), the cell density increases to the threshold cell density of *ain* quorum sensing. This threshold level is approximately  $10^8$  cells/ml, based on both *ain*-dependent luminescence induction and C8-HSL signal production in culture (21, 22), and correlates with the cell densities occurring during the initiation process (35). At this stage the AinS signal, C8-HSL, inactivates LuxO, allowing the appropriate level of expression of early colonization genes through LitR (Fig. 1). This model is consistent with two observations: the *ainS*, LuxO(D47E), and *litR* mutants are similarly defective in colonization initiation, and a double *ainS luxO* mutant displays an initiation defect comparable to those of the single *ainS* and *luxO* mutants (Table 2), suggesting that their effects are modulated through a common pathway. While our evidence supports the notion that both luminescence genes (22, 29) and motility genes (Tables 4 and 5) are regulated through the AinS-LuxO-LitR pathway (Fig. 1), it remains possible that LuxO regulates some colonization factors independently of LitR.

We have previously demonstrated that *ain* quorum sensing is required for appropriate expression of the *lux* quorum-sensing system (22). Thus, inactivation of *ainS* produces two effects in vivo: an initiation defect resulting from the loss of regulation of early colonization factors (Table 2) and a persistence defect caused by the absence of appropriate *lux* quorum-sensing induction (22). As predicted, both the LuxO(D47E) and the *litR* mutants display an initiation defect (Table 2) but not a persistence defect (Table 3) (9).

Finally, once *V. fischeri* has densely colonized ( $>10^{10}$  cells/ml) the deeper crypt regions of the squid light organ, and after initial induction by the *ain* system, *lux* quorum sensing induces expression of late colonization genes, such as those encoding luminescence (Fig. 1). A previous study demonstrated that

mutations in either *luxI*, *luxR*, or *luxA* (*luxA* encodes one of the subunits of luciferase), produced similar defects in colonization persistence (41), suggesting that luminescence might be the only colonization factor regulated by the *lux* quorum-sensing system. However, a separate study of the fish light-organ symbiont *V. fischeri* MJ1 showed that *lux* quorum sensing regulated the expression of five non-*lux* proteins (4). Inactivation of one of the corresponding genes, *qsrP*, in the squid light-organ symbiont *V. fischeri* ESR1 resulted in a competitive defect, suggesting that at least this *lux* quorum-sensing-regulated protein is a colonization factor.

**Quorum sensing affects *V. fischeri* motility.** When planktonic *V. fischeri* cells colonize juvenile *E. scolopes*, both the animal host and the bacterial symbiont undergo dramatic developmental changes (24). Two of the most apparent phenotypes of symbiotic *V. fischeri* cells are luminescence induction and an apparent loss of flagella (35). The ability to express luminescence during colonization is principally regulated by *lux* quorum sensing (42). Based on the data presented here (Tables 4 and 5), we hypothesize that, while flagellated planktonic *V. fischeri* initiate the colonization process, as they reach the deeper crypt tissues where proliferation occurs, flagellar elaboration is repressed as a consequence of *ainS* signaling.

There are several lines of evidence that the altered (either decreased or increased) motility behavior of mutants in the *ain* quorum-sensing pathway is the cause of the colonization initiation defect (Fig. 1; Table 2). Proper motility behavior has been shown previously to be essential for colonization initiation; i.e., both nonmotile and hypermotile mutants are significantly impaired in establishing a light-organ colonization (14, 27). However, at later stages of colonization the majority of bacterial cells have lost their flagella, implying that proper swimming motility is not required for maintenance of the symbiosis (35). The results presented here are consistent with this paradigm: both the hypermotile *ainS* and *litR* mutants and the motility-deficient *luxO* mutants (Table 5) display a delay in colonization initiation (Table 2). The presence of this delay in mutants predicted to have either higher (*luxO*) or lower (*ainS* or *litR*) expression of LitR suggests that targets of this regulator, like motility genes, must be carefully modulated.

Whole-genome transcription analysis showed that *ain* quorum sensing regulates both flagellar basal body and flagellin genes (Table 4). Motility gene regulation follows a hierarchical regulation cascade allowing the assembly of the flagellar apparatus in a sequential manner. The different classes of motility genes include early genes encoding regulatory proteins that control the entire motility regulon, middle genes encoding structural components of the hook-basal body and the type III secretion apparatus, and late genes encoding the flagellar filament, chemotaxis, and motor force generator proteins (1). The motility regulatory cascade in *V. fischeri* has not been completely elucidated; however, the analysis of regulatory proteins and the promoter regions of motility genes shows similarities to the regulatory cascade of *Vibrio cholerae* (26, 28, 31, 48). For example, inactivation of the early sigma-54-dependent master regulator (*frA*), as well as sigma-54 (*rpoN*) itself, abolishes motility, suggesting that, as in *V. cholerae*, *frA* is the master regulator of the *V. fischeri* motility regulon (28, 31, 48). Because *ain* quorum sensing regulates both sigma-54-dependent middle genes (i.e., *flgBCDEM*, *flhF*, *fliEL*, and *flaA*) (28)



and sigma-28-dependent late genes (i.e., *flaBCDEF*) (26), it is not clear at which regulatory level quorum sensing affects motility gene regulation. Interestingly, while *V. cholerae* quorum sensing also negatively regulates motility, only chemotaxis genes were shown to be differentially regulated (49), suggesting that the regulatory level at which quorum sensing affects the motility regulon is different in these two *Vibrio* species.

***ain* quorum-sensing regulon.** While our findings are consistent with the hypothesis that the altered motility behavior of the *ain* quorum-sensing mutants is, by itself, sufficient to explain their inability to efficiently initiate colonization, it remains possible that AinS and LuxO also regulate other, perhaps disparate, initiation factors. Our transcriptome analysis revealed several additional genes that are regulated by *ain* quorum sensing (Table 4) and might play a role in colonization initiation. For instance, *V. fischeri* may adapt to growth conditions in the light-organ crypts by using *ain* quorum sensing to regulate factors involved in nutrient uptake or metabolism (Table 4). Furthermore, the differential regulation of these genes provides a possible explanation for the growth yield defect exhibited by the *ain* quorum-sensing mutants in vitro (22) (Table 3).

Another result of *ain* quorum sensing appears to be the induction of genes in a putative exopolysaccharide (EPS) locus (Table 4). This locus contains many genes homologous to the *Escherichia coli* *wzy* locus, a system involved in O-antigen biosynthesis (VF0151 to VF0201 [http://www.ergo-light.com/ERGO/]). The consequences of EPS production in bacterium-host associations are diverse, but they can include enhanced adherence to tissue surfaces and/or protection against the challenges of complement-mediated phagocytosis and killing by the innate immune system (32). While the role(s) of EPS in this association remains to be determined, both of these challenges are faced by symbiotic *V. fischeri* (42). Mutations in homologs of LuxO or LitR in other *Vibrio* species affect colony morphology and biofilm formation, which in some cases correlates to changes in EPS production (6, 20, 23, 38, 49). Accordingly, the *V. fischeri* *litR* mutant was reported to display a more translucent colony morphology and to produce more biofilm than the wild type (9; P. Fidopiastis, personal communication). Not surprisingly, we found that the *V. fischeri* *ainS* mutant displayed similar phenotypes (data not shown); however, it remains to be determined whether these phenotypes are related to LPS and/or EPS production.

Our analysis indicates that *ain* quorum sensing has an effect on the transcription of a number of genes, including some unique ones (VF1355 and VF2255) with unknown functions (Table 4). While the functions of others can be inferred, it is not yet clear whether their regulation is direct or is an indirect effect of a multistep signaling pathway involving other transcriptional factors. In any case, the discovery of these regulated genes not only has given us insight into the way in which *ainS* signaling controls both early and late stages of symbiosis but also has provided candidate genes that may encode novel colonization determinants.

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