JOURNAL OF BACTERIOLOGY, June 2004, p. 3873–3881 Vol. 186, No. 12
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.12.3873–3881.2004

Vibrio fischeri LuxS and AinS: Comparative Study of Two Signal Synthases

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Received 25 February 2004/Accepted 29 February 2004

Vibrio fischeri **possesses two acyl-homoserine lactone quorum-sensing systems, *ain* and *lux*, both of which are involved in the regulation of luminescence gene expression and are required for persistent colonization of the squid host, *Euprymna scolopes*. We have previously demonstrated that the *ain* system induces luminescence at cell densities that precede *lux* system activation. Our data suggested that the *ain* system both relieves repression and initially induces the *lux* system, thereby achieving sequential induction of gene expression by these two systems. Analysis of the *V. fischeri* genome revealed the presence of a putative third system based on the enzyme LuxS, which catalyzes the synthesis of the *Vibrio harveyi* autoinducer 2 (AI-2). In this study, we investigated the impact of *V. fischeri* LuxS on luminescence and colonization competence in comparison to that of the *ain* system. Similar to the *ain* system, inactivation of the AI-2 system decreased light production in culture, but not in the squid host. However, while an *ainS* mutant produces no detectable light in culture, a *luxS* mutant expressed approximately 70% of wild-type luminescence levels. A mutation in *luxS* alone did not compromise symbiotic competence of *V. fischeri*; however, levels of colonization of an *ainS luxS* double mutant were reduced to 50% of the already diminished level of *ainS* mutant colonization, suggesting that these two systems regulate colonization gene expression synergistically through a common pathway. Introduction of a *luxO* mutation into the *luxS* and *ainS luxS* background could relieve both luminescence and colonization defects, consistent with a model in which LuxS, like AinS, regulates gene expression through LuxO. Furthermore, while *luxS* transcription appeared to be constitutive and the AI-2 signal concentration did not change dramatically, our data suggest that *ainS* transcription is autoregulated, resulting in an over 2,000-fold increase in signal concentration as culture density increased. Taken together, these data indicate that *V. fischeri* LuxS affects both luminescence regulation and colonization competence; however, its quantitative contribution is small when compared to that of the AinS signal.**

Vibrio fischeri, the bacterial light organ symbiont of the Hawaiian squid *Euprymna scolopes*, utilizes at least two quorum-sensing systems whose relative importance is dependent on cell density: (i) the well-known *lux* system, consisting of LuxI, which synthesizes *N*-(3-oxo-hexanoyl) homoserine lactone (3-oxo-C6-HSL), and the transcriptional regulator LuxR (14); and (ii) the *ain* system, which includes the second *V. fischeri* acyl-HSL synthase AinS, generating *N*-octanoyl HSL (C8-HSL) (16, 23, 24). We have previously demonstrated that these two systems are important at different cell densities: i.e., the *ain* system is the predominant inducer of luminescence in culture (24), whereas the *lux* system is necessary for luminescence expression at the higher cell densities present in the squid light organ (44). This sequential induction of luminescence gene expression is accomplished by a hierarchically regulated cascade in which the activated *ain* system exerts two effects: (i) relief of a negative regulation of gene expression of the *lux* system and (ii) low-level induction of the *lux* system through binding of the AinS signal to LuxR, which results in initial *lux* expression (24). The relief of gene repression is carried out through inactivation of the transcriptional regulator protein LuxO. LuxO inactivation results in an increased transcription of *litR* (32), which encodes a positive regulator of *luxR* transcription (10), thereby linking the *ain* and *lux* quorum-sensing systems in *V. fischeri* (24). Mutation of either the *ain* or the *lux* system results in a light organ colonization defect of *V. fischeri*, demonstrating the importance of quorum-sensing-dependent luminescence and possibly other colonization gene regulation in the *Vibrio*-squid symbiosis (24).

The recently sequenced genome of *V. fischeri* (http://www.ergo-light.com/ERGO/) revealed the presence of a putative third system, consisting of LuxS, the enzyme synthesizing a molecule named autoinducer 2 (AI-2), a furanosyl borate diester (7), and the putative AI-2 receptor proteins LuxP and LuxQ. LuxS was originally discovered in *Vibrio harveyi* as part of the quorum-sensing circuit that regulates luminescence gene expression (43). This signal synthase is hypothesized to serve in interspecies communication, as it can be found in many gram-negative and gram-positive bacterial species (29, 50). Luminescence gene expression in *V. harveyi* is coordinately controlled by both the LuxS-derived AI-2 and *N*-(3-hydroxybutanoyl) HSL (3-OH-C4-HSL), synthesized by the AinS homolog LuxM (33). Both signals interact with cognate hybrid two-component sensor-kinase proteins, LuxPQ and LuxN, respectively (2, 13), which transduce information through a phosphorelay cascade involving a protein designated LuxU (12), leading to inactivation of the transcriptional regulator LuxO (3, 11). Inactivation of LuxO results in increased transcription of *V. harveyi luxR* (a *V. fischeri litR* homolog), which positively regulates luminescence gene expression (32). Besides our own study of *V. fischeri* (24), other recent studies have demonstrated the involvement

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
V. fischeri		
ES114	Wild-type isolate from *E. scolopes* light organ	5
ainS mutant CL21	*ainS* gene partially deleted and replaced by chloramphenicol resistance (*cat*) marker	24
litR mutant PMF8	*litR* gene inactivated by insertion of kanamycin resistance marker (*kanR*)	10
luxI mutant VCW2G7	*luxI* gene inactivated by a frameshift mutation	24
luxO mutant CL42	*luxO* gene inactivated by insertion of kanamycin resistance marker (*kanR*)	24
luxR mutant CL53	*luxR* gene inactivated by insertion of erythromycin resistance marker (*ermR*)	This study
luxS mutant CL39	*luxS* gene inactivated by insertion of kanamycin resistance marker (*kanR*)	This study
ainS luxS mutant CL41	Double mutant carrying mutations as described above for CL21 and CL39	This study
luxS luxO mutant CL90	Double mutant carrying mutations as described above for CL39 and CL42	This study
ainS luxS luxO mutant CL91	Triple mutant carrying mutations as described above for CL21, CL39 and CL42	This study
V. harveyi		
BB120	Wild type	1
BB152	AI-2 producer	1
BB170	AI-2 sensor	1
BB886	AI-2 sensor mutant	1
Plasmids		
pCL112	2.1-kb HaeIII fragment carrying *ainS* gene in pVO8	24
pCL115	2.7-kb *V. fischeri* ES114 DNA with *luxS* gene cloned into pEVS79	This study
pCL122	pCL115 with random Tn*Kan* insertion 70 bp downstream of the *luxS* transcriptional start site	This study
pCL125	1.1-kb *V. fischeri* ES114 DNA with *luxS* gene cloned into pVO8	This study
pCL145	1.8-kb *V. fischeri* ES114 DNA with *luxO* gene cloned into pEVS79	24
pCL149	Allelic exchange vector pEVS79 carrying partially deleted *luxR* gene replaced by erythromycin resistance (*erm*) marker	This study
pCL152	pCL112 with *lacZ* gene from pKV124 cloned into EcoRV site approximately 200 bp downstream of translational start site	This study
pCL154	pCL125 with the *lacZ* gene from pKV124 cloned into the EcoRV site approximately 200 bp downstream of translational start site	This study
pCL155	pCL145 with *luxO* gene inactivated by insertion of *ermR*-marker into NsiI site	This study
pEVS79	Allelic exchange vector, *camR*	41
pEVS94	Source of the *ermR* cassette	41
pKV30	Cloning vector carrying partially deleted *luxR* gene replaced by erythromycin resistance (*erm*) marker	44
pKV124	Source of *lacZ* gene	47
pMU106	Allelic exchange vector carrying partially deleted *ainS* gene replaced by chloramphenicol resistance (*cat*) marker	24
pVO8	*V. fischeri* cloning vector, *ermR*	45

of homologous proteins of this regulatory cascade in colonization factor expression in *Vibrio anguillarum* (8, 31), *Vibrio cholerae* (30, 52), and *Vibrio vulnificus* (22, 26, 39), and both *Vibrio alginolyticus* and *Vibrio parahaemolyticus* have been reported to possess AI-2 activity (1). While the complete regulatory system remains to be determined for most of these species, the accumulating data are suggesting a common mechanism in the genus *Vibrio* (27, 32).

These homologies within the genus *Vibrio*, as well as the fact that the *ain* system is apparently conserved between *V. fischeri* and *V. harveyi*, led us to speculate that an AI-2 system might also operate in *V. fischeri*. Besides the potential impact of AI-2 on the expression of luminescence, we were curious to learn whether this signal is also important in light organ colonization by *V. fischeri*, as AI-2 has been implicated in colonization factor regulation in a variety of bacterial species (50). Furthermore, the *V. fischeri ainS* mutant is defective in colonizing its squid host, suggesting that *ainS* regulates the expression of genes important in symbiosis (24). If the AI-2 system functions in *V. fischeri* as it does in *V. harveyi* (33), those factors might also be coordinately regulated by *luxS* and *ainS*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. Medium components were purchased from Difco Laboratories, Inc. (Sparks, Md.), and Sigma Chemical Co. (St. Louis, Mo.). Strains of *V. fischeri* were grown at 28°C in either a seawater-based nutrient medium (SWT) containing Bacto-Tryptone (Difco), yeast extract, and glycerol (5) or Luria-Bertani salt (LBS) medium (17). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium (37). Media were solidified with 1.5% (wt/vol) agar as needed. Antibiotics were added to the media at the following concentrations when appropriate: chloramphenicol at 2.5 μg/ml for *V. fischeri* and 20 μg/ml for *E. coli*, kanamycin at 100 μg/ml for *V. fischeri* and *E. coli*, and erythromycin at 5 μg/ml for *V. fischeri* and 150 μg/ml for *E. coli*. 3-Oxo-C6-HSL was obtained from Sigma Chemical Co., and C8-HSL was obtained from Aurora Biosciences (Coralville, Iowa).

Genetic techniques. Genomic and plasmid DNAs were extracted by using the Qiagen DNeasy and Qiaprep Miniprep systems (Qiagen, Inc., Valencia, Calif.), respectively. PCR was performed according to standard protocols (37) with AmpliTaq DNA polymerase (Perkin-Elmer, Inc., Branchburg, N.Y.). For plas-

mid constructions, restriction enzymes and DNA ligase were obtained from New England Biolabs, Inc. (Beverly, Mass.), and used according to the manufacturer's protocol. Transfer of plasmids into *E. coli* host strains was accomplished by standard techniques (37). Triparental conjugation was used to transfer plasmids into *V. fischeri* strains (40). Sequencing was carried out on a Perkin-Elmer ABI Prism automated sequencer at the University of Hawaii Biotechnology/Molecular Biology Instrumentation and Training Facility.

Construction of *V. fischeri* mutants. To generate the *luxS* and the *ainS-luxS* mutant strains, a 2.7-kb fragment carrying the *luxS* gene was PCR amplified from *V. fischeri* strain ES114 based on the genomic sequence provided by Integrated Genomics, Inc. (Chicago, Ill.), at http://www.ergo-light.com/ERGO/. The fragment was cloned into the mobilizable vector pEVS79 (Table 1), generating pCL115. Plasmid pCL115 was randomly mutagenized with the EZ::TN <Kan-2> insertion kit (Epicentre, Madison, Wis.). One clone, designated pCL122, was identified that carried the <Kan-2> cassette insertion 70 bp downstream of the translational start site of the *luxS* gene. Plasmid pCL122 was transferred into both the wild-type (ES114) and the *ainS* mutant (CL21) strains of *V. fischeri* by triparental mating. Single and double recombinants were selected as previously described (40), generating the *V. fischeri luxS* and the *ainS-luxS* mutant strains (CL39 and CL41, respectively). Introduction of the *luxS* mutation into the genome of *V. fischeri* was confirmed by PCR analysis. The *luxS*-complementing plasmid pCL125 was generated by subcloning a 1.1-kb BamHI-EcoRI fragment carrying the intact *luxS* gene from pCL115 into the *V. fischeri* cloning vector pVO8 (Table 1).

To generate the *luxS luxO* and the *ainS luxS luxO* mutants, the *luxO* gene carried on pCL145 (Table 1) was mutagenized by inserting a 1.2-kb erythromycin resistance (*ermR*) gene from pEVS94 (Table 1) into the NsiI site located approximately 300 bp downstream of the *luxO* gene start site. The resulting plasmid, pCL155, was transferred into *V. fischeri luxS* by triparental mating, and single and double recombinants were selected as previously described (40), generating the *V. fischeri luxS luxO* mutant CL90. Similarly, plasmid pMU106, carrying a partially deleted *ainS* gene replaced by a chloramphenicol resistance marker, was introduced into the *luxS luxO* mutant to create the *ainS luxS luxO* mutant strain CL91. Using a previously described bioassay (38), we saw no evidence that strain CL91 produces C8-HSL activity, confirming that the *ainS* mutation was introduced into the genome.

The *luxR* mutant was constructed by subcloning a 4.1-kb HaeIII fragment from pKV30 into the mobilizable vector pEVS79 (Table 1). This fragment carries the *luxR* gene from *V. fischeri* ES114, which has been inactivated by partial deletion and insertion of an erythromycin resistance cassette (44). The resulting plasmid, pCL149, was transferred into the chromosome of *V. fischeri* ES114 by triparental mating. Single and double recombinants were selected as previously described (40), generating the *V. fischeri luxR* mutant strain CL53. The introduction of the mutation into the *V. fischeri* genome was confirmed by PCR analysis. Furthermore, luminescence characteristics of strain CL53 were determined and found to be similar to the luminescence characteristics of a previously described *V. fischeri luxR* mutant strain (44). Specifically, luminescence in culture was decreased to approximately 10 to 20% of wild-type levels. Similarly, whereas wild-type luminescence was induced several 100-fold upon exogenous addition of 120 nM 3-oxo-C6-HSL, as expected, this addition produced no induction of the *luxR* mutant.

Luminescence in culture. To determine their luminescence characteristics, *V. fischeri* wild-type and mutant strains were inoculated to an optical density at 600 nm (OD_{600}) of about 0.05 into 10 ml of SWT containing either (i) no addition, (ii) 120 nM C8-HSL, or (iii) 120 nM 3-oxo-C6-HSL. Cultures were kept shaking at 28°C, samples were taken at various times during growth, and luminescence and OD were measured. Maximum specific luminescence (luminescence/OD) values were averaged for measurements made between ODs of 1.0 and 5.0. Growth rate and growth yield were also determined during these experiments by measuring OD as a function of time. Experiments were repeated at least once.

Colonization assays. Three colonization phenotypes of *V. fischeri* wild-type and mutant strains were assessed.

(i) Symbiotic bioluminescence. The progress of early colonization events was monitored as described previously (35). Briefly, newly hatched squids were placed into vials with 4 ml of filter-sterilized seawater (FSSW) containing an inoculum of approximately 1,000 CFU of the indicated strain per ml. Twenty-four individual animals were infected per treatment group; 6 animals served as uninoculated controls and were placed into FSSW without added bacteria. Animal bioluminescence, an indirect measure of the degree of squid colonization, was monitored periodically over 24 h using a modified Packard Tri-Carb 2100TR scintillation counter (Packard Instruments Inc., Meriden, Conn.) as a photometer.

(ii) Colonization level in the squid light organ. The number of CFU per squid was determined at 24 and 48 h postinoculation following a previously described method (34). Newly hatched squid were placed into 50 ml of FSSW containing about 1,000 CFU of the indicated strain per ml, incubated for 12 h, and then transferred into FSSW. Some animals were placed into FSSW without added bacteria. At subsequent times, 15 animals per treatment group and 2 uninoculated 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.

(iii) Competitive phenotype. The ability of bacterial symbionts to compete during host colonization under conditions of coinoculation was tested for the *V. fischeri* wild-type and *luxS* mutant strains, using a previously described approach (46) with the following modifications. About 15 newly hatched squid were placed into 50 ml of FSSW containing approximately 1,000 CFU of each of the competing strains per ml and were incubated for 12 h. An aliquot of the inoculated seawater was spread onto LBS agar to determine the total 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 and each of the homogenates was patched onto antibiotic-containing or antibiotic-free LBS agar to determine the ratio of *V. fischeri* wild-type (Kan^s) to *luxS* mutant (Kan^r) cells.

LuxS and AinS signal activities of *V. fischeri* cultures. A modification of a previously described assay (1) was used to determine the ability of *V. fischeri* to produce LuxS signal activity. Briefly, *V. fischeri* strains were grown to the indicated OD in SWT. The medium was separated from the cells by centrifugation and subsequent filtration through a 0.2-μm-pore-size filter. Overnight cultures of *V. harveyi* strains BB170 (AI-2 sensor) and BB886 (AI-2 sensor mutant) (Table 1), grown in autoinducer bioassay (AB) medium (18), were diluted 1:5,000 into fresh AB medium. Cell-free culture supernatants of *V. fischeri* strains grown in SWT were added to these bioassay strains to a final concentration of 10% (vol/vol). The luminescence of 1-ml aliquots of the *V. harveyi* bioassay cultures was periodically measured with a TD20/20 luminometer (Turner Design, Sunnyvale, Calif.). SWT served as a negative control, and cell-free AB broths of an overnight culture of either the *V. harveyi* wild type (BB120) or the acyl-HSL-defective *V. harveyi* BB152 strain were used as the AI-2-producing positive controls. Results are reported as a percentage of the induction level produced by the positive control.

The concentration of C8-HSL produced by *V. fischeri* strains grown in SWT was determined with the same cultures assayed above. Supernatants of cultures at different ODs were extracted with acidified ethyl acetate and concentrated by evaporation. The amount of C8-HSL present in the concentrated samples was determined by using a previously described β-galactosidase-linked bioassay (38).

LuxS signal and AinS signal were extracted from *V. fischeri* cultures in three independent experiments. From each extraction, both the induction of luminescence expression of the AI-2 reporter strain and the induction of β-galactosidase expression in the C8-HSL reporter strain were determined in triplicate. It should be noted that while the concentration of C8-HSL can be determined directly by using synthetic C8-HSL standard, determination of AI-2 activity is indirect and assumes a linear relationship between signal concentration and induction of luminescence in the *V. harveyi* reporter strain.

Transcriptional activity of *luxS* and *ainS*. Two plasmids were generated to estimate the transcriptional activity of the *luxS* and *ainS* genes in *V. fischeri*. The *lacZ* gene was isolated from plasmid pKV124 (Table 1) by BamHI restriction digestion and was blunt ended with Klenow fragment. To obtain the transcriptional fusions *ainS*::*lacZ* and *luxS*::*lacZ*, the *lacZ* gene was ligated either (i) into the EcoRV site located approximately 200 bp downstream of the translational start site of the *ainS* gene carried on pCL112 (Table 1), creating pCL152, or (ii) into the EcoRV site located approximately 200 bp downstream of the translational start site of the *luxS* gene carried on pCL125 (Table 1), creating pCL154. Each of these low-copy-number plasmids was introduced into *V. fischeri* wild-type and mutant strains by triparental mating.

V. fischeri strains carrying in *trans* either pCL152 or pCL154 were cultured in either SWT plus erythromycin or SWT plus chloramphenicol, and the relative transcriptional activity of *luxS* and *ainS* was assayed in terms of the β-galactosidase activity of triplicate cultures at different times during growth with a standard *o*-nitrophenyl-β-D-thiogalactopyranoside (ONPG) method (37). Because these are in *trans* fusions, their expression levels are used only to estimate the level of native transcriptional activity.

RESULTS

LuxS signal activity in *V. fischeri*. To investigate whether *V. fischeri* produces a LuxS-dependent signal activity, we tested the ability of culture supernatants of the wild type and the *luxS*

TABLE 2. LuxS activity of *V. fischeri*[a]

Strain[b]	% LuxS activity in *V. harveyi* reporter strain:	
	BB170 (AI-2 sensor)	BB886 (AI-2 sensor mutant)
V. harveyi		
Wild-type BB120	147 (14)	100 (37)
AI-1 mutant BB152	100 (18)	1 (12)
V. fischeri		
Wild type (vector)	49 (3)	0 (1)
luxS (vector)	0 (3)	1 (2)
Wild type (pCL125)	44 (8)	2 (2)
luxS (pCL125)	41 (16)	2 (2)

[a] The *V. harveyi* reporter strains BB170 (AI-2 sensor) and BB886 (AI-2 sensor mutant) were incubated in medium containing 10% (vol/vol) culture supernatants of either the *V. fischeri* wild type or the *luxS* mutant carrying in *trans* either a vector control (pVO8) or a functional copy of the *luxS* gene on pCL125 (Table 1). Shown is the normalized response of a representative experiment. Standard errors are indicated in parentheses.

[b] The addition of culture supernatants from either wild-type *V. harveyi* BB120 (which produces both *V. harveyi* AI-1, an acyl-HSL, and AI-2) or *V. harveyi* BB152 (which produces only AI-2) served as positive controls.

TABLE 3. Luminescence of *V. fischeri luxS* mutants[a]

Strain type	Luminescence values (10^{-2} quanta s^{-1} $cell^{-1}$) with addition of[b]:		
	None	C8-HSL	3-Oxo-C6-HSL
Wild type	5.7 (0.1)	8.2 (1.1)	17,000 (3,900)
luxS	4.2 (0.2)	5.1 (0.4)	22,000 (3,600)
ainS	BD[c]	4.3 (0.2)	5 (1)
ainS luxS	BD	2.6 (0.1)	4 (1)
luxO	6.8 (0.8)	12.2 (2.9)	24,000 (4,000)
luxS luxO	8.3 (1.1)	17.2 (4.0)	37,000 (1,500)

[a] Specific luminescence values are the means of cultures with an OD_{600} of between 1.0 and 5.0; standard errors of the means are indicated in parentheses. The results shown are from a representative experiment.

[b] Cultures were grown in SWT either without additions or with the addition of either 120 nM C8-HSL or 120 nM 3-oxo-C6-HSL.

[c] BD, below detection (<0.02 quanta s^{-1} $cell^{-1}$).

mutant to induce luminescence in the *V. harveyi* reporter strains BB170 (AI-2 sensor) and BB886 (AI-2 sensor mutant). Cell supernatants harvested from mid-log-phase cultures of the *V. fischeri* wild type, but not of the *luxS* mutant, induced luminescence in the AI-2 sensor strain to approximately 50% of the level of culture supernatants from either wild-type *V. harveyi* BB120 or the acyl-HSL mutant *V. harveyi* BB152 (Table 2). Complementation of the *V. fischeri luxS* mutation by providing a functional *luxS* gene in *trans* on pCL125 restored the ability to induce luminescence (Table 2). These results establish that *V. fischeri* possesses an activity capable of inducing *V. harveyi* luminescence and that this activity is dependent on a functional *luxS* gene. Neither the *V. fischeri* wild type nor the *luxS* mutant induced luminescence in the AI-2 sensor mutant, whereas medium conditioned by wild-type *V. harveyi* BB120 did (Table 2), demonstrating that the *V. fischeri* LuxS activity is dependent on the presence of the AI-2 receptor protein. Taken together, these data indicate that *V. fischeri* cells produce an activity in culture that is indistinguishable from that of *V. harveyi* AI-2.

Luminescence of *V. fischeri luxS* mutants in culture. Having shown that *V. fischeri* possesses LuxS signal activity, we investigated whether this signal contributes to light production in *V. fischeri* as it does in *V. harveyi*. The luminescence levels of the *V. fischeri* wild type, as well as the *luxS*, *ainS*, and *ainS luxS* mutants, were determined in culture either with or without the addition of C8-HSL or 3-oxo-C6-HSL (Table 3). Because *V. fischeri* strain ES114 produces only very low levels of 3-oxo-C6-HSL activity in culture (5), exogenous supplementation with this compound triggers a dramatic response in light production (24, 44). In contrast, C8-HSL has been previously demonstrated to affect luminescence gene expression of *V. fischeri* in culture by two means: (i) relief of *lux* repression through inactivation of LuxO and (ii) direct induction of the *lux* system, apparently through low-affinity binding to and activation of LuxR (24). Thus, a mutation in *ainS* results in a dark phenotype due to repression and absence of induction of *lux*. Exogenous addition of C8-HSL to wild-type *V. fischeri* stimulates light production in a LuxR-dependent manner, although to a lesser extent than 3-oxo-C6-HSL addition (24, 44).

Light production by the *luxS* mutant was decreased to between 60 and 80% of the wild-type level (Table 3). Addition of C8-HSL to the *luxS* mutant increased light production, but not to wild-type levels, indicating that C8-HSL cannot fully complement the AI-2 deficiency. In contrast, when the LuxI-derived 3-oxo-C6-HSL was supplied exogenously, the luminescence levels of the *luxS* mutant and the wild type were both increased dramatically to levels that were approximately the same (Table 3), suggesting that the response of *V. fischeri* to 3-oxo-C6-HSL is not dependent on a functional AI-2 system. Both the *ainS* and *ainS luxS* mutants were not detectably luminous in culture (Table 3). The addition of C8-HSL could restore light production of the *ainS* and the *ainS luxS* mutants to nearly wild-type and *luxS* mutant levels, respectively. Similarly, the luminescence levels of *luxS* and *ainS luxS* were indistinguishable from those of their parent strains (wild type and *ainS*, respectively) when 3-oxo-C6-HSL was supplied exogenously. Because complementation of both the *luxS* and *ainS* mutations with functional genes in *trans* could restore luminescence to wild-type levels (data not shown), these mutations are unlikely to exert a downstream effect.

In *V. harveyi*, AI-2 activates a phosphorylation cascade resulting in the inactivation of LuxO, a negative regulator of luminescence (3, 11). To determine whether this pathway is conserved in *V. fischeri*, we compared the luminescence of a *luxS luxO* double mutant to that of a *luxO* mutant and found that the levels of light production by these two strains were similar, either with or without the addition of acyl-HSLs (Table 3). These data are consistent with a model in which *V. fischeri* LuxS produces a signal that regulates luminescence through inactivation of LuxO, as has been reported for the AinS signal, C8-HSL (24).

Symbiotic luminescence and colonization competence of *V. fischeri luxS* mutants. To determine whether symbiotic competence of *V. fischeri* is compromised by a mutation in *luxS*, we monitored the luminescence of juvenile animals colonized by the *V. fischeri* wild type or the *luxS*, *ainS*, or *ainS luxS* mutants during the first 24 h of colonization (Fig. 1). As previously shown (24), *ainS* mutant-colonized animals expressed between 10 and 20% of the luminescence level of those colonized by the wild type. In contrast, the luminescence of *luxS* mutant-colo-

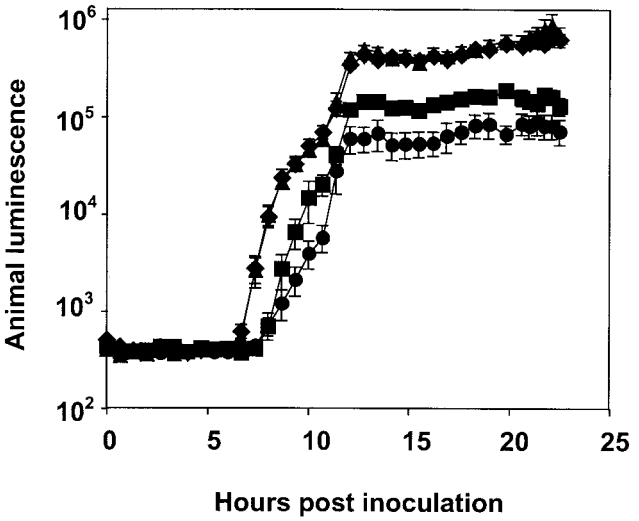

FIG. 1. Luminescence of *luxS* mutants in the symbiotic light organ. Animal luminescence was monitored during the initial stages of *E. scolopes* colonization by *V. fischeri* wild-type (solid diamonds), *ainS* mutant (solid squares), *luxS* mutant (solid triangles), and *ainS luxS* mutant (solid circles) strains. For each time point, mean values of 24 animals were calculated and standard errors of the mean are indicated. The experiment was repeated with the same outcome.

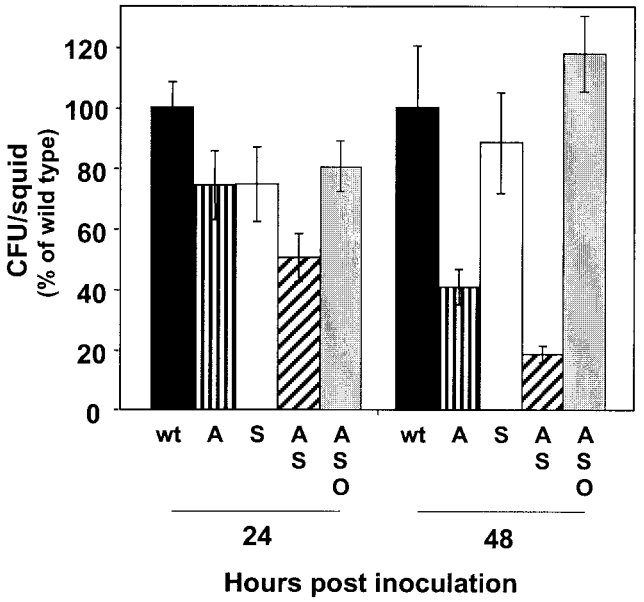

FIG. 2. Colonization competence of quorum-sensing mutants. Colonization levels of the *ainS* mutant (A), *luxS* mutant (S), *ainS luxS* mutant (A S), and *ainS-luxS-luxO* (A S O) mutant relative to *V. fischeri* wild type (wt) were measured at 24 and 48 h postinoculation. Each bar represents the mean value of 15 animals with the associated standard errors. The experiment was conducted twice with the same outcome.

nized animals was indistinguishable from that of wild-type-colonized animals (Fig. 1). However, the luminescence levels of animals colonized by the *ainS luxS* mutant were consistently 50% lower than those of animals colonized by the *ainS* single mutant, suggesting that either their specific luminescence or their colonization levels were decreased (Fig. 1).

To differentiate between these two possibilities, we compared the number of bacterial cells that were present in the light organ populations of animals colonized by either wild-type *V. fischeri* or the *luxS*, *ainS*, or *ainS luxS* mutants at 24 and 48 h (Fig. 2). While no significant difference in colonization ability was detected between the *luxS* mutant and wild-type *V. fischeri*, the *ainS luxS* double mutant colonized the juvenile squid to only 50 to 75% of the level reached by the *ainS* single mutant (Fig. 2), indicating a synergistic effect of these two mutations, similar to what has been shown for the effect of homologous systems on light emission by *V. harveyi* (33). Because squid luminescence levels were similarly reduced (Fig. 1), the levels of symbiotic light production per cell of the *ainS* and the *ainS luxS* mutants are likely to be the same, suggesting that colonization factors other than luminescence are cooperatively regulated by these signals. Complementation of the *ainS luxS* mutant with an intact *luxS* gene in *trans* restored luminescence and colonization to the levels of an *ainS* mutant, indicating that the combined effect is due to a mutation in *luxS* and not to an unrelated defect of this strain (data not shown). When squid were colonized with an *ainS luxS luxO* triple mutant, colonization levels were similar to wild type (Fig. 2). The latter result might be solely due to the reversal of the *ainS* phenotype by the *luxO* mutation, thus restoring the essentially wild-type phenotype of a *luxS* mutant. However, because the effect of a *luxS* mutation can only be detected in the absence of AinS, suggesting a synergistic effect, and AinS has been shown to operate through LuxO (24), it is possible that LuxS regulates colonization gene expression through LuxO as well.

To ensure that the observed limitation in colonization level was not a result of a general metabolic defect, we determined the growth characteristics of the *luxS* mutant strains. The *luxS* mutant exhibited both wild-type growth rate and yield in rich medium. In contrast, the *ainS luxS* mutant displayed a wild-type growth rate but was decreased in its final growth yield; however, this decrease was comparable to that previously reported for the *ainS* mutant (24). Thus, the absence of a functional *luxS* gene did not significantly impact the growth characteristics of *V. fischeri*.

Mixed-inoculum colonization experiments were performed to determine whether the *luxS* mutation would reduce the competitive competence of *V. fischeri*. We found no detectable defect in the competitive colonization ability of either the *luxS* mutant or the *ainS luxS* mutant relative to their parent strains (data not shown). Because the *ainS luxS* mutant displayed a colonization defect (Fig. 2) one might expect to also observe this defect in a mixed-inoculum experiment; however, because both LuxS and AinS synthesize small molecules that will diffuse into the surrounding environment (48), it is likely that the presence of wild-type cells can complement the deficiency of the *luxS* mutation.

LuxS and AinS signal synthesis during growth. To regulate gene expression in a cell density-dependent manner, quorum-sensing signals accumulate in the growing bacterial population until a threshold concentration is reached that is sufficient for gene induction (48). To determine whether the synthesis of either the *V. fischeri* LuxS signal or the AinS-derived C8-HSL signal, which both positively influence luminescence (Table 3), correlates with light production, the levels of both signal ac-

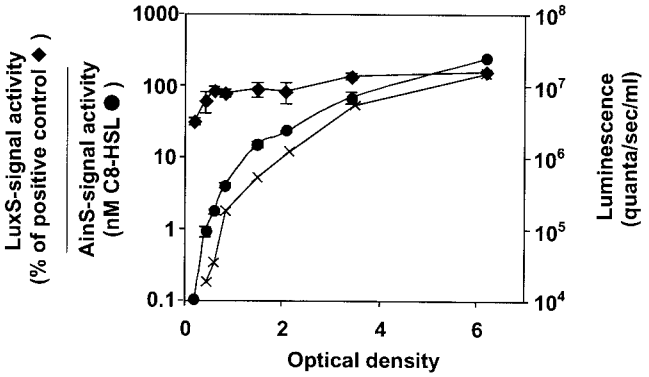

FIG. 3. Relationship between luminescence and quorum-sensing signal activity during growth of *V. fischeri* in culture. The relative level of LuxS signal activity (solid diamonds) in the culture supernatant is given as a percentage of the level produced by the positive control, an overnight *V. harveyi* BB152 culture (see Materials and Methods). The concentration of the AinS signal, C8-HSL (solid circles), in the *V. fischeri* culture was determined with synthetically produced C8-HSL as standard. Luminescence of the culture (crosses) is presented for comparison. Shown is a representative experiment; standard deviation bars are indicated.

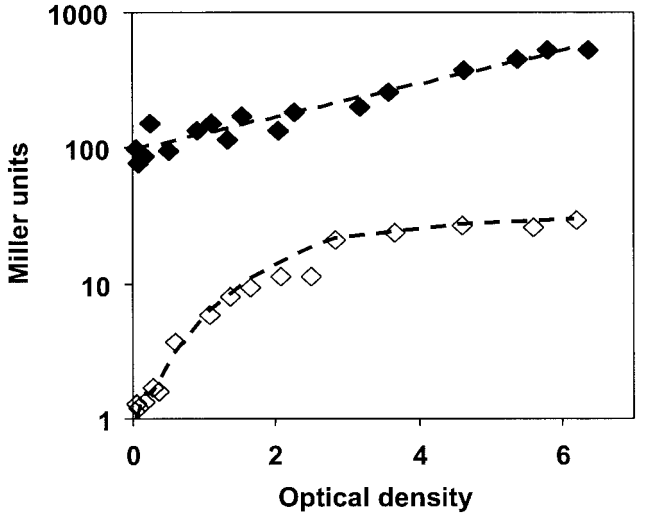

FIG. 4. Transcriptional activity of *luxS* and *ainS* in culture. β-Galactosidase activity of *V. fischeri* wild-type cells carrying either a *luxS*::*lacZ* fusion (solid diamonds) or an *ainS*::*lacZ* fusion (open diamonds) in *trans* on low-copy-number plasmids pCL152 and pCL154, respectively, was measured during growth. Shown are the cumulative data of three cultures; standard deviation bars were smaller than the symbols.

tivities were determined during growth (Fig. 3). While the concentration of the C8-HSL signal increased over 2,000-fold during the course of the experiment, mirroring the expression of luminescence in culture, AI-2 activity increased less than 4-fold (from 40 to 150% that of the positive control) (Fig. 3).

Transcriptional activity of *luxS* and *ainS*. The transcriptional autoregulation of a quorum-sensing signal synthase gene, as occurs in the *V. fischeri lux* system (15), provides a mechanism to rapidly induce gene expression once a threshold concentration of the signal is reached. To investigate whether the expression of either *ainS* or *luxS* is similarly autoregulated, we determined the relative transcriptional activities of these two genes throughout culture growth by using *lacZ* transcriptional fusions. The rate of *ainS* transcription changed significantly during growth, increasing exponentially until the culture reached an OD of approximately 2.0 (Fig. 4). This pattern coincided with the increase in both C8-HSL signal production and luminescence (Fig. 3). In contrast, the rate of increase in *luxS* gene transcription was much lower and did not change throughout the experiment (Fig. 4), correlating with the relatively constant production of the signal (Fig. 3) and consistent with a constitutive expression of this gene.

To determine whether proteins involved in the three *V. fischeri* quorum-sensing systems: *lux* (i.e., LuxR and LuxI), *ain* (i.e., AinS, LuxO, and LitR), and/or LuxS, regulate transcription of *luxS* and *ainS*, we measured the relative transcriptional activity of these two genes in a variety of quorum-sensing mutants. Because in several cases we were interested in the effect of the presence of the genes on their own transcription, the *lacZ* fusions had to be carried in *trans*. The inactivation of neither of these genes influenced *luxS* transcription (Table 4), providing further evidence for a constitutive transcription of this gene. In contrast, *ainS* gene transcription was decreased almost 10-fold in an *ainS* mutant, and transcription levels could be restored to wild-type levels either by supplying C8-HSL exogenously or by introducing a *luxO* mutation into the *ainS* mutant background (Table 4). These data are consistent with a model in which the *ainS* gene induces its own transcription through the synthesis of the AinS signal and this regulation involves inactivation of LuxO (Fig. 5). A similarly decreased level of *ainS* transcription was also observed in a *litR* mutant, but not in either a *luxR* or a *luxI* mutant, implying that the *ain* quorum-sensing system is autoregulated independently of the *lux* system. Mutations in neither *luxS* nor *luxO* changed transcription levels of *ainS* significantly (Table 4).

DISCUSSION

Previous studies have identified two acyl-HSL-based signaling systems in *V. fischeri*, designated *lux* and *ain* (14, 24). The

TABLE 4. Transcriptional activity of *V. fischeri luxS* and *ainS*

Strain type	β-Galactosidase activity (Miller units) from fusion to[a]:	
	luxS	*ainS*
Wild type	205 (46)	17.8 (4.4)
ainS	189 (15)	2.0 (0.1)
ainS (+ C8-HSL)[b]	ND[c]	17.2 (4.1)
luxS	204 (23)	16.0 (4.1)
ainS luxS	216 (35)	1.9 (0.1)
luxO	225 (46)	18.9 (3.9)
ainS luxO	ND	15.1 (6.0)
litR	181 (30)	2.7 (0.4)
luxR	167 (24)	17.4 (4.3)
luxI	206 (36)	17.4 (3.8)

[a] Shown is transcriptional activity of *luxS* and *ainS* in different genetic backgrounds. β-Galactosidase activity was measured during growth and averaged for several measurements taken at ODs of between 1 and 5. Shown is a representative experiment, with standard errors in parentheses.
[b] The *ainS* mutant was grown in the presence of 120 nM C8-HSL.
[c] ND, not determined.

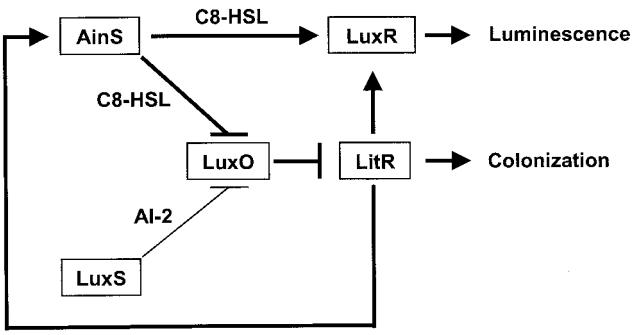

FIG. 5. Conceptual model for the regulation of luminescence and colonization genes by *V. fischeri* LuxS and AinS. Arrows indicate positive, inducing effects, and bars indicate negative, inhibitory ones. (See Discussion for explanation.)

activation of the *lux* system is dependent on *ain* induction, thereby providing a genetic mechanism to sequentially induce gene expression with increasing cell density (24). In this study, we investigated the impact of a putative third system, based on the enzyme LuxS, on luminescence and colonization ability in comparison to AinS. Our results suggests that (i) *V. fischeri* LuxS synthesizes a compound that is indistinguishable from *V. harveyi* AI-2; (ii) both *V. fischeri* AinS and LuxS signals exert their regulatory effects on gene expression through the same pathway, which is likely to operate through LuxO (Fig. 5); and (iii) *ainS* transcription and C8-HSL signal concentration are regulated by a positive feedback loop, whereas rates of *luxS* transcription and LuxS signal production appear to be relatively constant (Fig. 5).

Regulation of luminescence and colonization gene expression by LuxS. In *V. harveyi*, both the LuxM-derived 3-OH-C4-HSL and the LuxS-derived AI-2 regulate the expression of luminescence and other gene products through the inactivation of the transcriptional regulator LuxO (3, 11, 33). Similarly, our results demonstrate that the inactivation of the *V. fischeri luxS* gene causes a defect in luminescence expression in culture and that this defect could be relieved by the mutational inactivation of LuxO (Table 3). Furthermore, while a mutation in *luxS* did not affect colonization competence of *V. fischeri*, introduction of a *luxS* mutation into an *ainS* mutant background further decreased colonization levels (Fig. 2). These data, together with our previous finding that the signal synthesized by the LuxM homolog, AinS, operates through LuxO (24), suggest that these two systems regulate gene expression synergistically through the same downstream LuxO-mediated cascade (Fig. 5) that has been described in *V. harveyi* (3).

In contrast to *V. harveyi* (33), however, the effect of the *V. fischeri* LuxS-synthesized signal on luminescence and other colonization gene expression (Table 3) appears to be considerably smaller than that of the AinS signal (24). These findings are similar to the results of a recent study on the *V. cholerae* quorum-sensing circuit (30). *V. cholerae* possesses a regulatory cascade resembling that of *V. harveyi* (and now *V. fischeri*), with at least two signal inputs: the LuxS-derived AI-2 and a yet unidentified signal synthesized by an enzyme designated CqsA. While a mutation in *cqsA* abolished the ability of *V. cholerae* to express luminescence from *V. harveyi lux* genes carried in *trans*, a mutation in *luxS* did not have a significant effect. Similarly, the LuxO-regulated phenotype of biofilm formation by *V. cholerae* is under the control of CqsA, but the LuxS signal is largely dispensable (51). Taken together, these data suggest that although a regulatory circuit involving LuxO appears to be conserved in *Vibrio* species (32), the relative importance of each of the involved signals may vary widely among bacterial species.

Regulation of LuxS and AinS signal production. We determined the activity of the LuxS- and AinS-derived signals and estimated the transcriptional activity of the signal synthase genes, *luxS* and *ainS*, during growth in rich medium. Although AI-2 is produced in a growth phase-dependent manner in several bacterial species (6, 9, 21, 22, 42), the nutrient composition of the medium and other physiological factors appear to impact AI-2 production more than cell density (6, 19, 22, 43). We did not investigate the environmental conditions under which *V. fischeri* produces AI-2, but instead chose growth conditions that resulted in a >1,000-fold difference in luminescence output, a phenotype regulated by *luxS* (Table 3). Under these conditions, LuxS signal concentration and *luxS* transcription did not reflect the magnitude of change that occurred in bacterial luminescence (Figs. 3 and 4). Significantly, even at cell densities that preceded luminescence induction (i.e., OD of <0.5), both measures of LuxS activity were comparably high (Fig. 3 and 4), suggesting the absence of a characteristic signal dose response. As expected by the relatively constant transcription levels throughout growth (Fig. 4), *luxS* transcription apparently was not affected by mutation of any of the known *V. fischeri* quorum-sensing proteins (Table 4).

In contrast, the patterns of both C8-HSL concentration and *ainS* transcriptional activity correlated well with light emission; i.e., both were highest at cell densities at which luminescence is induced and reached saturation thereafter (Fig. 3 and 4). Because the changes in both the rate of C8-HSL signal production and the apparent transcriptional activity of *ainS* correlated, it is likely that *ainS* transcription rate directly determines signal concentration. The observation that *ainS* transcription was decreased considerably in the absence of C8-HSL (Table 4) suggests that the cell density-dependent increase in C8-HSL concentration is accomplished by an autoregulatory mechanism of the *ainS* gene. The inactivation of the transcriptional regulator LitR decreased the activity of the *ainS* transcriptional reporter as well, supporting the hypothesis that LitR positively regulates *ainS* gene transcription either directly or through another transcriptional protein (Fig. 5). The notion that C8-HSL activates a phosphorelay cascade resulting in the inactivation of LuxO, a negative regulator of *litR* transcription (24, 32), predicts that mutation of *luxO* should result in increased levels of *litR* and, therefore, increased levels of *ainS*. However, *ainS* reporter levels were indistinguishable between the wild type and the *luxO* mutant (Table 4). While this result might suggest an effect of *ainS* on *litR* that is independent of LuxO, a simpler explanation is that the positive effect of LuxO on *ainS* in this assay is below our detection levels. This idea is supported by the observation that *ainS* reporter levels were indistinguishable from those of the wild type when measured in an *ainS luxO* mutant background (Table 4). Mutations in neither *luxR* nor *luxI* affected *ainS* gene transcription, indicating that the regulation is independent of the *lux* quorum-sensing system (Fig. 5). Because our data suggest that LuxS and AinS operate through the same pathway, one would expect de-

creased *ainS* transcription levels in a *luxS* mutant as well. However, *ainS* transcription levels were indistinguishable in the *luxS* mutant and wild type (Table 4), providing additional evidence that the relative effect of the LuxS signal in this regulatory cascade is much lower than that of the AinS signal (Fig. 5).

LuxS signal synthase or a metabolic enzyme? Since the discovery of AI-2 activity in *V. harveyi*, the question of why a bacterium would utilize two inputs into the same regulatory cascade has led to much speculation. It was originally proposed that the acyl-HSL signal senses an individual species' cell density, whereas the nonspecific AI-2 senses the cumulative cell density of the bacterial community (1). The discovery that AI-2 signal production is dependent on the growth conditions rather than cell density per se has led to the alternative hypothesis that AI-2 reflects a change in environmental conditions through a change in the metabolic activity of the bacterial community (4, 43). These findings, together with the fact that LuxS has a role in central metabolism, led to the proposal that in most bacteria AI-2 is not a specific signal but a metabolic by-product of a common detoxifying pathway (49). On the other hand, the regulation by LuxS of niche-specific genes has been demonstrated for several pathogens (50), suggesting that this signal synthase has a specific role in host colonization. However, colonization competence of *luxS* mutants has been investigated in only a few bacterial species, and the observed defects were in many cases either small or not detectable (28), implying that other regulatory factors might be more important than the LuxS signal.

Our study does not definitively determine the role of LuxS in *V. fischeri*. The inactivation of *luxS* did not result in a growth defect either in culture (data not shown) or in symbiosis (Fig. 2), suggesting that the absence of LuxS activity does not significantly affect metabolic processes, at least under our experimental conditions. Instead, our data demonstrate that a *luxS* mutation has an effect on luminescence and that this effect can be relieved by a *luxO* mutation (Table 3 and Fig. 2), thereby providing evidence for a signal function exerting its effect through a transduction cascade common in *Vibrio* species. Although this putative signal alone did not appear to be important for the symbiotic lifestyle of *V. fischeri* cells in the environment of the squid light organ, inactivation of *luxS* had a significant effect on colonization competence in an *ainS* mutant background (Fig. 2). Thus, one might speculate that in environments in which the *ain* system is not active, the AI-2 system becomes an important player in the regulation of colonization factors. In addition to its monospecific light organ associations, *V. fischeri* is part of the multispecies bacterial community present in the enteric tracts of fishes and marine invertebrates (20, 25, 36). It is possible that the *V. fischeri* AI-2 system in these associations is important and/or that AI-2 produced by other bacteria might have an impact on colonization competence.

ACKNOWLEDGMENTS

B. L. Bassler kindly provided *V. harveyi* reporter strains. D. S. Millikan, P. M. Fidopiastis, and J. R. Graber provided helpful comments on the manuscript. Integrated Genomics Inc., Chicago, made *V. fischeri* ES114 genomic sequence information available.

This work was supported by the National Institutes of Health grant RR12294 to E. G. Ruby and M. J. McFall-Ngai; National Science Foundation grant IBN0211673 to M. J. McFall-Ngai and E. G. Ruby; and a W. M. Keck Foundation grant to E. P. Greenberg, E. G. Ruby, M. J. McFall-Ngai, and others.

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