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## QUORUM SENSING IN THE VIBRIO FISCHERI - EUPRYMNA SCOLOPES SYMBIOSIS

## A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

#### DOCTOR OF PHILOSOPHY

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#### ABSTRACT

Quorum sensing is a cell density-dependent bacterial gene regulatory mechanism used for the expression of colonization-related genes. The symbiotic relationship between the luminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* serves as a model system to study the molecular processes underlying bacterial colonization. This system is especially well-suited for the investigation of the impact of quorum sensing on colonization because (i) it is an easily accessible, natural, two-species colonization model, and (ii) quorum sensing regulates luminescence expression in *V. fischeri*, which allows the non-invasive detection of quorum-sensing activity both in culture and in symbiosis. While the impact of one of *V. fischeri*'s quorum-sensing systems, lux, on luminescence expression and symbiotic competence has been extensively studied, little was known about other putative systems.

The results of this study demonstrate that the *V. fischeri* ain system is essential for both maximal luminescence expression and symbiotic competence. The ain system predominantly induces luminescence expression at intermediate cell densities, which occur in culture, while the lux system is responsible for luminescence expression at the high cell densities found in symbiosis, suggesting the sequential induction of luminescence gene expression by these two systems. Furthermore, the ain quorumsensing system is important for the processes underlying colonization initiation, while the impact of the lux system is apparent only in later stages of the symbiosis, indicating distinct functions of these two systems during the colonization process. A global transcriptome analysis of quorum-sensing mutants revealed that ain quorum sensing

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represses motility gene expression, providing a likely explanation for the initiation defect. Although it has been known that many bacterial species possess multiple quorum-sensing systems, this is the first study demonstrating that two quorum-sensing systems are employed to specifically regulate functions important at distinct cell densities occurring during the colonization process.

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## CHAPTER 1

## GENERAL INTRODUCTION

#### BACKGROUND AND SIGNIFICANCE

# Quorum sensing is a bacterial gene regulatory mechanism utilized for the expression of colonization-related traits

Bacterial quorum sensing has been shown to be important for the transcriptional regulation of many genes that are essential in the establishment and the maintenance of bacteria-host associations. Quorum sensing constitutes a regulatory mechanism that allows bacteria the expression of colonization-related genes exclusively when they are at high cell densities; e.g., when they are associated with a host. A low constitutive secretion of quorum-sensing signals permits the bacterial cells to sense the density of surrounding cells by measuring the concentration of this signal and to induce the specific expression of genes once a certain threshold concentration of the quorum-sensing signal is achieved (Miller and Bassler, 2001).

Among Gram-negative bacteria, the most commonly used signals are (i) acylated homoserine lactones (acyl-HSLs), synthesized by two distinct classes of enzymes, homologs of *Vibrio fischeri* LuxI and *Vibrio harveyi* LuxM, and (ii) a LuxS-derived furanosyl borate diester, termed autoinducer-2 or AI-2 (Whitehead *et al.*, 2001). Whereas acyl-HSL signaling appears to be restricted to Gram-negative bacteria, signaling through AI-2 has been shown to be ubiquitous in both Gram-negative and Gram-positive species (Bassler *et al.*, 1997). Due to differences in the acyl-side chain, acyl-HSLs are specifically sensed by the bacterial species producing this signal (Fuqua *et al.*, 1996). In contrast, AI-2 is produced and recognized by many different bacterial species and was thus proposed to serve in inter-species communication (Xavier and Bassler, 2003). Quorum-sensing signal transduction is accomplished by two principal mechanisms, (i) the direct interaction of the signal with a transcriptional regulator or (ii) the activation of a signal transduction phosphorelay cascade through binding to a cognate receptor protein (Fuqua *et al.*, 2001; Bassler, 2002).

# The Vibrio fischeri – Euprymna scolopes symbiosis is an ideal model system to study the impact of quorum sensing on animal colonization

Quorum sensing employing acyl-homoserine lactones (acyl-HSL) as signaling molecules was originally discovered in V. fischeri as the mechanism underlying the regulation of bioluminescence in this species (Nealson et al., 1970; Eberhard, 1972; Nealson, 1977). Many more host-associated bacterial species have since been found to utilize quorum sensing for the cell density-dependent expression of colonization-related genes, including important pathogens such as Pseudomonas aeruginosa and Agrobacterium tumefaciens (Parsek and Greenberg, 2000). While most of our understanding of the molecular processes underlying quorum sensing relies on in vitro studies of the luminous bacterial species V. fischeri and V. harveyi, the investigation of the impact of quorum sensing on the colonization process requires adequate model systems. Although it is of great interest to understand the mechanisms resulting in human infectious disease, the available experimental systems are, for obvious reasons, non-natural infection models; i.e., cell cultures or mice. On the other hand, natural plant model systems, such as the plant crown gall tumor caused by A. tumefaciens, are readily accessible; however, the applicability of these models to human disease is limited because of the distinct pathologies caused by bacteria in plants and animals.

The symbiosis between the marine, luminescent bacterium V. fischeri and the Hawaiian bobtail squid E. scolopes represents an ideal model system to study the impact of quorum sensing on the colonization process of animals. This mutualistic association has been successfully used for the investigation of the processes underlying the initiation, accommodation and persistence of symbioses, and many of the discoveries obtained through these studies can be directly related to pathogenesis (Ruby, 1996; Ruby, 1999). V. fischeri is the sole symbiotic partner of E. scolopes; thus, the experimental system is a defined, natural two-species association. Because the bacterial partner is transferred

horizontally, it was possible to develop a standard colonization assay to investigate the early events of the symbiotic relationship between *V. fischeri* and *E. scolopes*. The symbiosis is exclusive but not essential under laboratory conditions; thus, each of the symbiotic partners can be maintained individually, allowing the comparison of the symbiotic vs. the aposymbiotic (squid) and free-living (bacteria) life-style (Ruby, 1996). Molecular techniques that allow the genetic manipulation of *V. fischeri* have been developed, permitting the construction of mutant strains, which can be assessed for their ability to colonize *E. scolopes* in comparison to their wild-type parent (Visick and Ruby, 1996; Visick and Ruby, 1997; Visick, 1998; Stabb and Ruby, 2003). Recently, the genome sequence of *V. fischeri* was completed and microarrays were constructed, allowing the investigation of global transcriptional responses to mutation or changes in environmental conditions. Finally, because *V. fischeri* is bioluminescent and expression of bioluminescence is regulated by quorum sensing, quorum-sensing activity can be easily detected by measuring light emission both *in vitro* and *in vivo* (Visick and Ruby, 1999).

# The light-organ colonization process requires multiple selective interactions between host and bacterial symbiont

*V. fischeri* colonizes the squid light organ, a specialized organ comprising two lobes surrounded by accessory tissue, located on the ventral side of the animal (McFall-Ngai and Montgomery, 1990). Colonization with *V. fischeri* is believed to provide the nocturnally active animal with light which it uses as an anti-predatory behavior (McFall-Ngai and Montgomery, 1990), while the squid light organ presents a nutrient-rich environment allowing the bacteria to grow at a maximal doubling time of approximately 30 minutes (Ruby and Asato, 1993). In the juvenile animal, three pores connect each lobe of the light organ to the environment. These pores lead through the ducts into the blind-ended crypts where cells of *V. fischeri* are housed extracellularly in close approximation to the epithelial

cells (McFall-Ngai and Montgomery, 1990).

The symbiosis is initiated every generation, when newly hatched juvenile squids collect their bacterial symbionts from the surrounding seawater (Nyholm et al., 2000). Within the first hour after hatching, bacteria present in the ambient seawater trigger the secretion of from the superficial epithelium of the squid light organ (Nyholm et al., 2002). Bacterial cells attach to the mucus, forming dense aggregates at the outside of the light organ (a process not restricted to V. fischeri). Between 3 and 6 h after hatching, the bacterial aggregates begin to move towards the pores leading into the interior of the light organ. Interestingly, this migration is V. fischeri specific and requires flagella-driven motility (Nyholm et al., 2000). After entering the pores, bacterial cells move through the ducts into the deep crypts of the light organ, where they will grow on nutrients supplied by the squid host (Ruby and Asato, 1993, Graf and Ruby, 1998). Several components of the squid's innate immunity have been identified (Margaret McFall-Ngai, personal communication), V. fischeri thus must employ strategies to survive the challenges of these stress conditions imposed by the host (Visick and Ruby, 1998; Aeckersberg et al., 2001). The colonization of the deep regions of the squid light organ causes several morphological changes, including swelling of the epithelial cells lining the crypts and an increase in microvilli density (Montgomery and McFall-Ngai, 1994; Lamarcq and McFall-Ngai, 1998). Although the mechanism underlying these host responses are as yet unknown, these observations imply that host cells specifically respond to the presence of bacterial cells. This notion is also supported by the discovery that the dense colonization of the squid light organ with V. fischeri results in a cessation of the external mucus production, impairing secondary infections (Nyholm et al., 2002).

The association between V. *fischeri* and E. *scolopes* remains dynamic throughout the lifetime of the squid. Every morning, a light cue-triggered muscle contraction causes the expulsion of 90-95% of the bacterial population, and the remaining population will grow and re-colonize the squid light organ within a couple of hours thereafter (Lee and Ruby, 1994; Graf and Ruby, 1998).

# V. fischeri lux quorum sensing is required for luminescence expression in vivo and persistent colonization

The most dramatic change in *V. fischeri* cells during the transition from the planktonic to the symbiotic state is the onset of luminescence expression. In *V. fischeri*, the transcription of the *lux* genes, which encode the enzymes responsible for the light reaction, is regulated in a cell density-dependent fashion through binding of 3-oxo-hexanoyl-HSL, the LuxI synthesized acyl-HSL, to the transcriptional activator protein LuxR (Table 1.1). The LuxR-acyl-HSL complex binds to the *lux* promoter and induces the transcription of the *lux* operon (Fuqua *et al.*, 1996). The effects of the *lux* quorum-sensing system on symbiotic competence have been investigated. A *V. fischeri* strain carrying a *luxI* mutation is defective in symbiotic light emission. Although this strain is capable of colonizing *E. scolopes* and initially appears to reach colonization levels that are indistinguishable from the wild-type parent (Table 1.1). Because mutations in *luxA*, encoding for one of the luciferase subunits, displayed an essentially identical colonization phenotype, it was concluded that the underlying cause must be the same; i.e., the light reaction catalyzed by the luciferase enzyme (Visick *et al.*, 2000).

#### **Dissertation research objectives**

More recently, it became apparent that the lux system might not be the only quorumsensing system in V. fischeri (Table 1.1). In addition to LuxI, V. fischeri possesses a second acyl-HSL synthase synthesizing octanoyl-HSL, AinS, and a putative receptor protein AinR (Kuo et al., 1994; Gilson et al., 1995; Kuo et al., 1996). The V. harveyi AinS- homolog LuxM was demonstrated to utilize a signaling cascade comprising of the phosphorelay proteins LuxU and LuxO (Miller and Bassler, 2001), both of which can be found in the V. fischeri genome (http://ergo.integratedgenomics.com/ Genomes/VFI/). In addition to this putative ain quorum-sensing system, an analysis of the V. fischeri genome sequence revealed the presence of a LuxS-homolog. LuxS together with its assumed receptor proteins LuxP and LuxQ might present a third system in V. fischeri, the AI-2-system (Table 1.1).

Research presented in this dissertation addressed four major questions:

1. Does the *Vibrio fischeri* sap locus, proposed to be regulated by quorum sensing, affect antimicrobial peptide resistance and/or symbiotic competence?

2. How do the V. fischeri quorum-sensing systems; ain, lux and AI-2, interplay to regulate luminescence gene expression?

3. What is the impact of ain and AI-2 quorum sensing on symbiotic competence of *V. fischeri*?

4. Which genes, besides the lux genes, does quorum sensing regulate in V. fischeri?

Quorum-sensing system	lux	ain	AI-2
Signal			HO B OH HO CH <sub>3</sub>
	3-oxo-hexanoyl	Octanoyl	Furanosyl borate
	homoserine lactone	homoserine lactone	diester
	(3-oxo-C6-HSL)	(C8-HSL)	(AI-2)
Signal synthase	LuxI	AinS	LuxS
Receptor protein	LuxR	AinR (?)	LuxPQ (?)
Signal transduction	-	LuxU, LuxO (?)	LuxU, LuxO (?)
Regulated functions	Luminescence	Luminescence (?) Colonization factors (?)	Luminescence (?) Colonization factors (?)
Colonization			
phenotype of	Persistence defect	?	?
mutant strains			

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# Table 1.1. V. fischeri quorum-sensing systems.

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### CHAPTER 2

# THE VIBRIO FISCHERI SAPABCDF LOCUS IS REQUIRED FOR NORMAL GROWTH, BOTH IN CULTURE AND IN SYMBIOSIS

#### ABSTRACT

Inactivation of the sapABCDF genes results in a loss of virulence in several bacterial pathogens of animals and plants. I have investigated the role of this locus in the growth physiology of Vibrio fischeri, as well as in the symbiotic colonization of the squid Euprymna scolopes. In rich medium a V. fischeri sapA insertion mutant grows at only 85% the rate of its wild-type parent. While a similar effect has been attributed to a potassiumtransport defect in sap mutants of enteric bacteria, the V. fischeri mutant grows more slowly regardless of the potassium concentration of the medium. Similarly, the growth rate defect is independent of the source of either carbon, nitrogen, or phosphorous, indicating that the V. fischeri sap genes do not encode functions required for the transport of a specific form of any of these nutrients. Finally, while a delay in colonizing the nascent light organ of the squid can be accounted for by the lower growth rate of the mutant, a small but statistically significant reduction in its final population size in the host, but not in medium, suggests that the sap genes play another role in the symbiosis. All of these phenotypic defects can be genetically complemented in trans by the sapABCDF genes, but not by the sapA gene alone, indicating that the insertion in sapA is polar to the four downstream genes in the locus. Thus, while the sap locus is important to the normal growth of V. fischeri, it plays different physiological roles in growth and tissue colonization than it does in enteric pathogens.

#### INTRODUCTION

The marine luminescent bacterium *Vibrio fischeri* is the specific light-organ symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*. This cooperative association is initiated each generation, when newly hatched juvenile squids collect an inoculum of their bacterial symbionts from the surrounding seawater (Nyholm *et al.*, 2000). The ensuing colonization process can be divided into four distinct phases: (i) an initial infection phase in which *V. fischeri* cells enter the squid light organ and grow to a maximal colonization level of about  $5 \times 10^5$  cells (0 to 12 h); (ii) the expulsion of about 95% of the bacterial symbionts from the light organ triggered by a light stimulus at sunrise (~12 h). (iii) re-growth of the remaining *V. fischeri* cells to their maximal colonization level (12 to 18 h) and, (iv) persistence in a stationary phase (18 to 36 h) (Ruby, 1993; Boettcher *et al.*, 1996). The last three phases repeat on a daily basis throughout the life of the host, creating a complex and dynamic cycle of bacterial growth in the symbiosis.

Each of the symbiotic partners can be easily maintained in the laboratory, and molecular techniques that allow the genetic manipulation of *V. fischeri* have been developed. These characteristics, mono-specificity, horizontal transfer of the bacterial partner, and availability of genetic tools, make this symbiosis an ideal model system to study cooperative microbe-host interactions on a molecular level (Ruby, 1996; Ruby, 1999). Using this system, several bacterial genes have been identified that are required to (i) initiate the symbiosis (Graf *et al.*, 1994; Visick and Skoufos, 2001; Millikan and Ruby, 2002); (ii) attain a normal level of colonization (Graf and Ruby, 1998; Aeckersberg, *et al.*, 2001); (iii) establish a persistent colonization (Graf and Ruby, 2000; Visick *et al.*, 2000) and (iv) compete effectively with the wild-type parent strain (Visick and Ruby, 1998).

The sapABCDF (sap = sensitive to antimicrobial peptides) genes were originally discovered in the intracellular pathogen Salmonella typhimurium. A transposon mutant library of S. typhimurium was screened with the antimicrobial peptide protamine, and

mutants with insertions in either the sapC or sapD gene were found to have an enhanced susceptibility to protamine and to be less virulent (Groisman *et al.*, 1992). It was later shown that a strain with a mutation in a sapA homologue of the plant pathogen *Erwinia chrysanthemi* showed a similar phenotype: its susceptibility to plant-derived antimicrobial peptides was increased, and its virulence was greatly reduced (Lopez-Solanilla *et al.*, 1998; Lopez-Solanilla *et al.*, 2001). Recently, a transposon mutagenesis approach was used to identify four *Proteus mirabilis* mutants with an enhanced susceptibility to the antimicrobial peptide polymyxin B; one of these mutants carried the transposon in a gene homologous to *sapD* of *S. typhimurium* and *Erwinia chrysanthemi* (McCoy *et al.*, 2001).

Other physiological defects have been ascribed to *sap* mutants of enteric bacterial species. A mutation in the *Escherichia coli trkE* (*sapDF*) locus conferred both a reduced rate of potassium uptake and a reduced growth rate under potassium limiting conditions. However, these changes in growth and potassium transport kinetics were not produced by mutations in other *trk* genes (Bossemeyer *et al.*, 1989; Dosch *et al.*, 1991). A subsequent study of an *E. coli*  $\Delta trkE$  mutant concluded that resistance to protamine is actually dependent on the ability of the cell to take up potassium, rather than on having a functional SapABCDF system (Stumpe and Bakker, 1997). In contrast, while *Vibrio alginolyticus* also possesses a Trk potassium uptake system, the transport of potassium by this bacterium is apparently independent of *sapDF* (Nakamura *et al.*, 1998; Harms *et al.*, 2002).

As part of an unrelated study a 500-bp fragment of V. fischeri ATCC 7744 genomic DNA was found to contain two partial open reading frames with sequence similarity to the sapD and sapF genes of various species (Chen et al., 2000). The goal of this study was to investigate the function of the sapABCDF locus in the V. fischeri - E. scolopes symbiosis by (i) locating, cloning and sequencing the genes, (ii) constructing a sapABCDF null mutant, and (iii) determining its phenotype in culture and in the host.

#### MATERIAL AND METHODS

#### **Bacterial strains and growth conditions**

Strains and plasmids used in this study are listed in Table 2.1. The symbiotic V. fischeri strain ES114 and its derivatives were grown at 28°C either in a seawater-based nutrient medium (SWT) (Boettcher and Ruby, 1990) or Luria-Bertani Salt (LBS) medium (Graf et al., 1994). Escherichia coli strains were grown at 37°C in Luria-Bertani medium (LB) (Sambrook et al., 1989). Media were solidified with 1.5% (w/v) agar as needed. Antibiotics were added to the media at the following levels, when appropriate: chloramphenicol (Cam, 2 µg/ml for V. fischeri, 20 µg/ml for E. coli), kanamycin (Kan, 100 µg/ml for both V. fischeri and E. coli). For certain growth studies a defined, artificial seawater-based minimal medium (MM) was used that contained 300 mM NaCl, 50 mM MgSO4, 10 mM CaCl<sub>2</sub>, 10 mM KCl, 0.01 mM FeSO4, 50 mM Tris-HCl pH 7.4 with either 0.33 mM K<sub>2</sub>HPO<sub>4</sub> or 0.33 mM glycerol 2-phosphate as a phosphate source. The carbon and nitrogen sources were either (i) 20 mM ribose and 0.3% casamino acids, (ii) 20 mM ribose and 10 mM NH4Cl, or (iii) 20 mM N-acetyl-D-glucosamine. Medium reagents were purchased from Difco Laboratories (Sparks, MD) and Sigma Chemical Co. (St. Louis, MO). Antimicrobial peptides CP11CN, CP26, CP28, CP29, LL37, and P-CN were provided by REW. Hancock, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada.

#### **Genetic techniques**

Genomic and plasmid DNA were extracted using Qiagen DNeasy and Qiaprep Miniprep systems (Qiagen Inc., Valencia, CA), respectively. PCR was performed according to standard protocols (Sambrook *et al.*, 1989) using AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NY). For plasmid constructions, restriction enzymes and DNA ligase were obtained from New England BioLabs, Inc. (Beverly, MA) and used according to the manufacturer's protocols. Transfer of plasmids into *E. coli* host strains was accomplished using standard techniques (Sambrook *et al.*, 1989). Triparental conjugation was used to transfer plasmids into *V. fischeri* strains (Stabb *et al.*, 2001). A Perkin-Elmer/ABI Prism automated sequencer was used (University of Hawaii Biotechnology/Molecular Biology Instrumentation and Training Facility).

#### Cloning and sequencing of the V. fischeri sapABCDF locus

A 420-bp fragment of V. fischeri ES114 genomic DNA containing partial gene sequences of sapD and sapF was amplified by PCR using the primers 5' TTACCCATTGGTTGTCG 3' and 5' GGATCCTGGAAAATCAT 3'. The primer sequence was based on a previously published 480-bp sequence of the V. fischeri strain ATCC 7744 (Chen et al., 2000). The resulting PCR product was used as a template to create a digoxigenin-labeled Southern hybridization probe (PCR DIG labeling kit, Boehringer Mannheim). Genomic DNA obtained from V. fischeri ES114 was digested with different restriction enzymes, separated on a 1% agarose gel, transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with the probe, which was detected using CDP-Star<sup>TM</sup> Chemi-Luminescence substrate (Boehringer Mannheim). A 6.9-kb fragment containing the target sequence was identified and isolated from a Xbal-Spel restriction digest of genomic DNA, and cloned into the mobilizable vector pEVS79. The clone containing pCL103 (Fig. 2.1 A) was identified by dot-blot hybridization, and both strands of the insert were sequenced by primer-walking. The sequence of the *sapABCDF* locus has been submitted to GenBank under the accession number: AF454370. Sequence analysis was carried out using the software programs Vector NTI Suite 5.5 (InforMax, North Betheseda. MD) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences other than those from V. fischeri were

obtained from GenBank (S. typhimurium, E. chrysanthemi, E. coli) and TIGR (Vibrio cholerae).

#### Construction of a *sapA* mutant and complementing strains

A 3.4-kb fragment of pCL103 was subcloned by digestion with *SacI* and religation, producing pCL105 (Table 2.1). A kanamycin-resistance marker was isolated from pUC4K by restriction digest with *PstI*, and ligated into the *NsiI* site of pCL105. The resulting plasmid pCL109 (Fig. 2.1 B) was transferred into *V. fischeri* ES114 by triparental mating, and single and double recombinants were selected as previously described (Stabb *et al.*, 2001), generating the *V. fischeri sapA* mutant strain CL10.

The sapA mutant was complemented in trans with either the complete sapABCDF locus or with the sapA gene only. A 6.0-kb Bg/II-EarI fragment carrying the sapABCDF locus and a 2.4-kb Bg/II-HpaI fragment carrying only the sapA gene were each isolated from pCL103, gel-purified and ligated into the vector pLS6 (Table 2.1). The resulting plasmids, pCL110 and pCL111 (Fig. 2.1 C and 2.1 D), as well as the vector control pLS6, were transferred into both wild-type V. fischeri and the sapA mutant CL10 (Table 2.1). Because the addition of antibiotics to maintain the complementing plasmids was not practical in some experiments, the sapA mutant was also reverse-complemented by recombining the plasmid pCL105, which carries the wild-type sapA gene, into the genome of the sapA mutant strain CL10 by triparental mating. The resulting strain CL16, a double recombinant that had become restored with a functional sapA, was identified by its kanamycin sensitivity; the loss of the kanR marker was further confirmed by analysis of the PCR of the sapA gene locus.

#### **Growth characteristics**

To determine the growth characteristics of *V. fischeri* wild-type, the *sapA* mutant and the complemented *sapA* mutant strains in media of different compositions, 10 ml of each medium were inoculated to an optical density (OD) at 600 nm of about 0.05 with cells that had been pre-grown in this medium. Cultures were kept shaking at 28°C, samples were taken during the exponential growth-phase at different times, and the ODs were measured and plotted against time. Regression analyses of the resulting growth curves were carried out using MiniTab 10 Xtra (MiniTab Inc, State College, PA), and generation times were calculated from the slope of the exponential regression line during exponential growth. To determine the growth yield, the cultures were incubated for a total of 24 h and the final OD was measured.

#### **Colonization assays**

Four assays were used to determine the colonization phenotype of wild-type V. *fischeri*, the *sapA* mutant and their derivatives. (i) Bioluminescence assay: The course of the early colonization events was monitored as previously described (Ruby and Asato, 1993). Briefly, newly hatched squids were placed into vials with 4 ml of filter-sterilized seawater containing an inoculum of approximately 4,000 colony forming units (CFU) of either wild-type V. *fischeri*, the *sapA* mutant or the reverse-complemented *sapA* mutant. Thirty-six individual animals were infected per treatment group; 12 animals served as an uninoculated control and were placed into filter-sterilized seawater without added bacteria. Bioluminescence of the animals, an indirect measure of the degree of colonization of the squids, was monitored periodically over 24 h using an automated photometer.

(ii) Level of colonization in the squid light organ: The number of CFU per squid was determined at 12, 20 and 48 h post-inoculation as previously described (Ruby, 1996).

Briefly, newly hatched squids were placed into 50 ml of filter-sterilized seawater containing about 50,000 CFU of either wild-type V. *fischeri*, the *sapA* mutant or the reverse-complemented *sapA* mutant, and incubated for 12 h. Some animals were placed in filter-sterilized seawater without added bacteria. At each of the time points, 15 animals per treatment group and 2 uninoculated animals were homogenized, and the homogenate was diluted and spread onto SWT agar. The V. *fischeri* colonies arising after an overnight incubation were counted, and the number of CFU per squid calculated.

(iii) Minimum infectious dose: Individual, newly hatched squids were placed into 4 ml of filter-sterilized seawater containing 50, 100, 200 or 400 CFU of either wild-type V. *fischeri* or the *sapA* mutant. After an incubation period of 3 h, the squids were transferred to fresh vials with 4 ml of filter-sterilized seawater containing no added bacteria. Successful colonization was indicated by the presence of animal luminescence at 48 h as detected with a TD-20/20 luminometer (Turner Design, Sunnyvale, CA).

(iv) Competitive phenotype: A presence of a competitive colonization advantage of either wild-type *V. fischeri* or the *sapA* mutant strain was determined as previously described (Visick and Ruby, 1998) with the following modifications. Briefly, about 15 newly hatched squid were placed into 50 ml of filter-sterilized seawater, containing approximately 50,000 CFU of each of the competing strains, and incubated for 12 h. A sample of the inoculated seawater was spread onto LBS agar to determine the exact ratio of the two strains in the inoculum. At 24 h post-inoculation, squid were homogenized and a dilution of the homogenate spread onto LBS agar. About 100 CFU from both the inoculum and the homogenates were patched onto antibiotic-containing and antibiotic-free LBS agar to determine the ratio of wild-type *V. fischeri* (kanamycin-sensitive) to *sapA* mutant (kanamycin-resistant).

#### Stress response assays

Four assays were used to determine the effects of a *sapA* mutation on the resistance of the bacterium to agents of cellular stress.

(i) Antimicrobial peptides: The minimal inhibitory concentration (MIC) for each of eight cationic antimicrobial peptides, CP11CN, CP26, CP28, CP29, LL37, P-CN, polymyxin sulfate, and protamine sulfate (Friedrich *et al.*, 1999; Friedrich *et al.*, 2000; Jia *et al.*, 2000; Travis *et al.*, 2000), was determined using a modified microtiter broth-dilution method (Jia *et al.*, 2000). Briefly, a solution of each peptide was diluted by serial two-fold dilutions in distilled water containing 0.01% acetic acid and 0.2% bovine serum albumin. Bacterial cultures in mid-exponential growth phase were diluted to a concentration of about  $1 \times 10^5$  cells per ml of LBS, and 200 µl of the culture were placed into 96-well microtiter dishes, to which 22 µl of the peptide dilutions were added. The dish was incubated with shaking at 22°C for between 16 to 20 h, during which time the OD of each of the cultures was periodically measured using a Perkin-Elmer HTS 7000 BioAssay plate reader. The MIC was defined as the concentration of the peptide that resulted in a 50% decrease in maximal growth rate. In each of the assays, the *V. fischeri* strain *ompU* (Aeckersberg, *et al.*, 2001) was used as a positive control.

(ii) Detergent exposure: The MIC of sodium dodecyl sulfate and dioxycholate were determined by the modified microtiter-broth dilution method as described above for the antimicrobial peptide resistance assay.

(iii) Heat shock: Mid-exponential phase SWT cultures of wild-type V. fischeri or the sapA mutant were diluted to a concentration of about 5,000 cells per ml in fresh medium, and aliquots of these cultures were placed in a 42°C water bath. After different exposure times (no exposure, 10 sec, 30 sec, 1 min, 2 min, 5 min, 10 min and 20 min), 50 μl of the cultures were spread on SWT agar. After an overnight incubation at 28°C, CFU were determined, normalized to the unexposed control, and plotted against the exposure time to estimate the time required to kill 50% of the cells.

(iv) Osmotic shock: Mid-exponential phase SWT cultures of wild-type V. fischeri or the sapA mutant were diluted to a concentration of about 2.5 x  $10^7$  cells per ml of SWT medium. A 10-µl aliquot of the diluted culture was transferred into 490 µl of sterile distilled water containing (per ml) 0, 2.5, 5, 10, 15, 20, 25, 35, 50, 75 or 100 mg of NaCl. After a 10-min incubation, 10 µl were diluted into 990 µl of sterile seawater, and 50 µl of the dilution spread onto SWT agar. CFU were determined after an overnight incubation at 28°C and normalized to the unexposed positive control to estimate the NaCl concentration required to kill 50% of the cells.

#### RESULTS

#### The sapABCDF locus in V. fischeri is homologous to sap loci in other species

To characterize the V. fischeri ES114 sapABCDF locus, a 6.9-kb Xbal-SpeI genomic DNA fragment containing the sap genes, as well as several hundred base pairs of flanking sequence, was cloned and sequenced (Fig. 2.1 A). The locus has the same gene arrangement as the homologous sapABCDF loci of V. cholerae, S. typhimurium, E. chrysanthemi and E. coli. The predicted V. fischeri Sap proteins are most closely related to those of V. cholerae. The SapD and SapF proteins, homologs of the cytoplasmic ATPases of the ABC transporter family, exhibit the highest similarity among all the species. Conserved ATP-binding motifs (Walker et al., 1982), which are the characteristic functional domains of ATPases, were identified at amino acids 40-55 and 169-184 in SapD, and at amino acids 47-62 and 161-176 of SapF in V. fischeri.

The sapABCDF genes have been shown to be transcribed as a single transcript in S. typhimurium (Parra-Lopez et al., 1993), and sequence analyses suggested that the same polycistronic operon structure is present in V. fischeri. Specifically, a strong transcriptional terminator was found downstream of the V. fischeri sapF gene, while none was detected within the sapABCDF locus. In addition, the locus contains overlapping start- and stop-codons; i.e., there are no intergenic regions.

#### The growth rate of the V. fischeri sapA insertion mutant is reduced

Because of its apparent operon structure, the strategy chosen to create a *sapABCDF* null mutant strain was to insert a kanamycin-resistance (*kanR*) marker into the *sapA* gene. A clone was obtained that carried a 3.7-kb XbaI-SacI fragment comprised of 0.9 kb of upstream sequence, as well as complete *sapA* and *sapB*, and partial *sapC* sequences. The *kanR* marker was inserted 900 bp downstream of the putative transcriptional start site of the *sapA* gene in this clone, producing pCL109 (Fig. 2.1 B). The V. fischeri sapA mutant strain

CL10 was obtained by homologous recombination of pCL109 into the chromosome of strain ES114 (Table 2.1). The insertion of the *kanR* marker into the expected location of the genome was confirmed by Southern-blot analysis. Its orientation was opposite that of the *sapA* gene as determined by sequencing the flanking region of the insert using primers from sites within the *kanR* gene (data not shown). Control experiments performed in another study have shown that carriage of this *kanR* gene has no detectable effect on the growth of *V. fischeri* cells either in culture or in the symbiosis (Stabb and Ruby, 2001).

After an overnight incubation at  $28^{\circ}$ C on nutrient agar the *V. fischeri sapA* mutant produced colonies that were visibly smaller than the wild-type strain. To further investigate this observation, I determined the generation times of both *V. fischeri* wild type and the *sapA* mutant carrying *in trans*: (i) no complementing plasmids, (ii) pLS6, the vector control, (iii) pCL111, the vector containing *sapA*, or (iv) pCL110, the vector containing *sapABCDF* (Table 2.1; Fig. 2.1 C and 2.1 D). None of the strains displayed a lag phase, but the *V. fischeri sapA* mutant grew significantly more slowly than the wild-type strain, except when it was complemented by the entire *sapABCDF* locus (Fig. 2.2 A and 2.2 B). Thus, the *sapA* mutation appears to have a polar effect on the expression of one or more of the downstream *sap* genes. Interestingly, the final growth yields of all of the strains were the same, indicating that in culture medium the *sap* mutation does not limit the extent of growth (Fig. 2.2 A, and data not shown).

These complementation data provide evidence that the observed reduction of growth rate is due to the interruption of *sapA* and not to a secondary effect, such as the expression of a truncated SapA protein, a secondary mutation elsewhere in the genome, or a downstream effect on genes outside the putative *sapABCDF* operon. The observation that complementation with *sapA* alone did not restore the wild-type phenotype also supports the hypothesis that the *sapABCDF* genes are co-transcribed.

#### The V. fischeri sapA mutant is less effective in colonizing the host squid

The initial events in the colonization process of *E. scolopes* by either *V. fischeri* wild type, the *sapA* mutant, or the reverse-complemented *sapA* mutant were monitored for the first 24 h post-inoculation (Fig. 2.3). The time that light emission is first detectable, which is a function of both bacterial population size and autoinducer concentration (Boettcher and Ruby 1990), was delayed by about 2 h in animals colonized by the *sapA* mutant, and the level of light produced was generally lower thereafter. The colonization phenotype of the *sapA* mutant could be complemented by reverse complementation, demonstrating that the observed phenotype is a result of the mutation on the *sap* locus, and not to any secondary effect. Because luminescence is only an indirect measure of the degree of colonization, the number of *V. fischeri* CFU per light organ was also determined at 12, 20 and 48 h post-inoculation (Table 2.2). These data confirmed that the number of symbionts is significantly lower at 12 h and 24 h in animals colonized by the *sapA* mutant, and that this decreased level of colonization by the *sapA* mutant continues for at least 48 h post-inoculation.

A possible explanation for the delay in colonization was that the *sapA* mutant requires a higher level of inoculum than the wild-type strain. However, for both the wild-type and the *sapA* mutant strains the minimal inoculum that resulted in colonization of 50% of the animals under the standard assay conditions was about 100 CFU per animal (data not shown).

The competitive phenotype of the *sapA* mutant was examined to determine whether there was evidence that a factor other than its growth defect might contribute to the observed colonization phenotype. Newly hatched squids were incubated with a mixed inoculum consisting of an equal proportion of wild-type *V. fischeri* and *sapA* mutant cells, and the ratio of the two strains was determined in the resulting light organ population 24 h postinoculation (Fig. 2.4). The *sapA* mutant was significantly reduced in its ability to compete
with the wild-type strain during colonization: the ratio of *V. fischeri sapA* mutant to wild type decreased by a factor of >15 in the light organ as soon as 24 h post-inoculation. In contrast, genetic complementation with the *sapABCDF* genes *in trans* allowed the *sapA* mutant to compete well with the wild-type strain, essentially maintaining its inoculation ratio during growth in the light organ.

# The growth defect of the *sapA* mutant is not related to either the concentration of potassium or the source of carbon, nitrogen or phosphorus in the medium

It has been reported that a mutation in the *sapDF* (*trkE*) genes confers a potassiumdependent growth defect on *E. coli* cells (Dosch *et al.*, 1991). Therefore, I compared the growth rates of the *V. fischeri sapA* mutant and the wild-type strain in minimal medium containing 0.1, 1, 10 or 100 mM potassium chloride. Neither strain grew in medium with a potassium ion concentration of 0.1 mM or less. However, the relative growth rates of the mutant and the wild type were the same at the other three potassium concentrations (data not shown), indicating that the growth defect of the *sapA* mutant is unlikely to be due to potassium starvation.

In an attempt to identify a possible substrate for the putative Sap transporter, the growth rates of wild-type V. *fischeri* and the *sapA* mutant were compared in defined minimal media containing different compounds as sole carbon, nitrogen or phosphorous sources. I reasoned that if the *sap* locus encodes a permease system for a specific form of one of these nutrients, then the *sapA* mutant might have a relative growth defect in medium that contained this substrate as the sole source of an essential chemical element; however, there should be no such defect when this substrate was replaced by a chemically distinct source of the element. Because the carriage of complementing plasmids reduces the growth rate substantially in minimal medium, the reverse-complemented *sapA* mutant was used as a control in this set of experiments.

Regardless of the nature of the nutrient source tested, the *sapA* mutant grew more slowly than the wild-type strain (Fig. 2.5); however, there was no detectable difference in the final growth yield (data not shown). Reverse complementation could restore the wild-type growth rate in each case. Each of the classes of elemental nutrients (C, N or P) could be excluded as being the source of a limiting substrate for the possible transporter because substitution with a different chemical form of the element did not relieve the growth-rate defect of the *sapA* mutant.

The SapABCDF proteins also do not appear to be involved in the transport of iron because the addition of a high concentration (100  $\mu$ M) of Fe<sup>3+</sup> to LBS medium had no effect on the growth rate of either the *V. fischeri sapA* mutant strain or wild type (data not shown).

#### The V. fischeri sapA mutant withstands cell-membrane stress agents normally

While light microscopy revealed no differences in cell morphology between the wild-type and the *sapA* mutant strain, it remained possible that the reduced growth rate of the mutant was caused by a general membrane disturbance resulting from the absence of the putative cytoplasmic membrane proteins SapB and SapC. To explore this possibility, I investigated the responses of *V. fischeri* wild type and the *sapA* mutant to four different stress conditions that target membrane integrity: cationic peptides, detergents, heat, and osmolarity.

The presence of functional *sap* genes has been implicated in the ability of enteric bacteria to withstand cationic antimicrobial peptides (Parra-Lopez *et al.*, 1993; Lopez-Solanilla *et al.*, 1998). Thus, I determined for wild-type *V. fischeri* and the *sapA* mutant strain the MICs of a variety of cationic antimicrobial peptides with diverse amino acid sequences and structures. Although there were differences between the actual values of the MICs for wild-type *V. fischeri* and for *Vibrio anguillarum* or *S. typhimurium* (Jia *et al.*,

2000) the relative levels of sensitivities to the eight peptides were generally the same under my assay conditions (data not shown). Most significantly, for each of the tested peptides, the MICs were identical for the *sapA* mutant and wild-type *V. fischeri*, suggesting that a functional SapABCDF system in *V. fischeri* does not confer an increased resistance to antimicrobial peptides. The *V. fischeri* strain *ompU*, which was used as a control in these experiments, displayed an enhanced susceptibility to each of these peptides. Similarly, under each of the other three stress conditions tested I could not detect a significant difference between the responses of wild-type *V. fischeri* and the *sapA* mutant during growth or in survival of stationary phase (data not shown).

#### DISCUSSION

The sapABCDF genes in the pathogenic bacterial species S. typhimurium and E. chrysanthemi have been reported to contribute to the ability of these bacteria to colonize their hosts (Parra-Lopez et al., 1993; Lopez-Solanilla et al., 1998). A recently published 500-bp genomic sequence from the V. fischeri strain ATCC 7744 that had a high level of identity to the 3'end of the sapD gene and the 5'end of the sapF gene (Chen et al., 2000) indicated that these genes might be present in this species as well. If they were, I wanted to know whether the V. fischeri sap locus encodes functions that might be important in the symbiotic colonization of the light organ of the Hawaiian bobtail squid, E. scolopes.

I identified and cloned the *sapABCDF* locus of *V. fischeri* strain ES114, and its sequence revealed high similarity to these genes in other bacteria. I constructed a *sapA* mutant in *V. fischeri* and compared its growth and symbiotic colonization phenotypes with those of the wild-type strain. As has been reported for a *E. chrysanthemi sapA* deletion mutant (Lopez-Solanilla *et al.*, 1998), and *S. typhimurium sapC* and *sapD* transposon mutants (Groisman *et al.*, 1992), the *V. fischeri sapA* mutant is less effective at colonizing its host. During its colonization of the light organ *V. fischeri* attains a sufficient population density to induce bioluminescence, which increases to a maximum level at about 12 h post-inoculation (Ruby and Asato, 1993). The times at which luminescence is first detected, and at which it reaches a maximum, are both delayed in squids that are colonized by the *sapA* mutant. However, because the *V. fischeri sapA* mutant also displays a growth rate defect in culture (Fig. 2.1), explanation of any defect in colonization rate does not require a specific role of the *sap* operon in symbiosis.

At each of three times during the first 48 h of colonization I found that there were fewer bacteria in animals colonized by the *sapA* mutant then by the wild type (Table 2.2). This result indicates that not only the rate but also the extent of colonization by the *sapA* mutant is significantly impaired. Because the growth yield of the *sapA* mutant is unaffected in culture medium, there is no simple explanation for its failure to reach a normal level of colonization in the host, suggesting that this yield defect may be specific to the conditions of the symbiosis. Such a phenotype is similar to that reported for certain auxotrophic mutants of *V. fischeri* (Graf and Ruby, 1998) and indicates that the *sap* locus may play an analogous, but as yet unknown, function when the symbionts are colonizing the host light organ.

Little is known about the physiological effects caused by sap gene mutations in other bacteria. Because an *E. coli sapD* mutant (also called  $\Delta trkE$ ) grows more slowly under potassium-limiting conditions, a link between the sap genes and potassium transport has been proposed (Bossemeyer et al., 1989; Dosch et al., 1991; Harms et al., 2002). However, I detected no difference between the growth rates of the V. fischeri wild type and the sapA mutant in media containing either low or high potassium concentrations. Thus, if the V. fischeri sapA mutation does disrupt the expression of the rest of the locus, then it appears that the sapD gene of this bacterium does not contribute to potassium transport in the same way as its homologue does in E. coli. It has been proposed that, unlike E. coli, the V. alginolyticus Trk potassium-uptake system utilizes ATPase subunits from transporters other than the SapABCDF proteins (Nakamura et al., 1998; Harms et al., 2002). Thus, it is possible that in Vibrio species the Trk system is independent of sapD, which would explain the potassium-insensitive growth rate defect of the V. fischeri sapA mutant. If, as proposed for E. coli (Stumpe and Bakker, 1997), a sufficiency of potassium transport is integral to antimicrobial peptide resistance, then the V. fischeri sapABCDF genes may not contribute to antimicrobial peptide resistance because they are not required for potassium transport.

To better understand the physiological function of the V. fischeri SapABCDF proteins I investigated the observed growth defect in more detail. Because of the growthrate defect of the V. fischeri sapA mutant in tryptone-containing medium, as well as the sequence similarity of SapA to peptide transporters like DppA and OppA (Detmers et al., 2001), it seemed reasonable that the SapABCDF proteins might play a role in the nutritional uptake of peptides. However, I found that the growth defect of the *sapA* mutant was also expressed in defined minimal media containing structurally distinct sources of carbon, nitrogen and phosphorous (Fig. 2.5). Thus it is unlikely that the SapABCDF proteins are required for the specific uptake of an external nutrient.

Similarly, I found that under my assay conditions the responses of the *sapA* mutant to several stress conditions (i.e., cationic peptides, detergents, heat, and osmolarity) were indistinguishable from those of the wild-type strain, suggesting that the absence of the SapABCDF proteins does not create a general membrane defect in culture. Preliminary studies suggest that *E. scolopes* tissues produce antimicrobial peptides (W. Crookes and M. McFall-Ngai, pers. comm.). While the *sapA* mutant is not more sensitive to the cationic peptides tested in this study, it remains possible that the *V. fischeri sapA* mutant will prove to be more sensitive to such host peptides.

### TABLES

 Table 2.1. Strains and plasmids used in this study.

Strains and	Characteristics	Reference or source
plasmids		
<i>E. coli</i> strains		
DH5a	Cloning strain	(Stabb <i>et al.</i> , 2001)
CC118Apir	Host strain for plasmid pEVS104	(Visick and Ruby, 1997)
V. fischeri strains		1777)
ES114	V. fischeri wild-type isolate from E. scolopes	(Boettcher and Ruby, 1990)
CL10	<i>V. fischeri sapA</i> mutant; <i>sapA</i> gene disrupted by <i>kanR</i> marker	This study
CL16	CL10 that has been reverse-complemented $(san A^{\dagger})$	This study
Plasmide	(supr )	
pEVS79	Allelic exchange vector	(Stabb and Ruby,
pEVS104	Conjugal helper plasmid	(Stabb and Ruby, 2001)
pLS6	V. fischeri cloning vector, carrying a chloramphenicol-resistance marker (camR)	(Visick and Ruby
PCR2.1	PCR-product cloning vector	Invitrogen
pUC4K	Origin of the kanamycin-resistance marker ( <i>kanR</i> )	(Messing and Vieira, 1982)
pCL103	pEVS79 with a 5.9-kb XbaI-SpeI fragment carrying the V. fischeri ES114 sapABCDF locus, 900 bp of upstream and 700 bp of downstream sequence	This study
pCL105	pEVS79 with a 3.7-kb XbaI-SacI fragment carrying the V. fischeri ES114 sapA, sapB and partial sapC genes, and 900 bp of upstream sequence	This study
pCL109	pCL105 with a kanR insertion at the NsiI site in the sanA gene	This study
pCL110	pLS6 with a 6.0-kb <i>Bgl</i> II- <i>Ear</i> I fragment from <i>V</i> . <i>fischeri</i> ES114 genomic DNA carrying the <i>sapABCDF</i> locus with 600 bp of upstream and downstream sequence	This study
pCL111	pLS6 with a 2.4-kb <i>BgI</i> II- <i>Hpa</i> I fragment from <i>V. fischeri</i> ES114 genomic DNA, carrying <i>sapA</i> with 600 bp of upstream sequence and a partial <i>sapB</i> sequence	This study

Table 2.2. Colonization effectiveness of V. fischeri strains.<sup>a</sup>

	CFU per light organ (x $10^4$ ) at		
Inoculating strain	12 h	20 h	48 h
ES114 (wild type)	0.21 (± 0.033)	12.8 (± 1.9)	9.1 (± 1.3)
CL10 (sapA mutant)	0.02 (± 0.003)	2.4 (± 0.9)	6.0 (± 1.2)
CL16 (reverse-complemented <i>sapA</i> mutant)	not determined	14.9 (± 3.2)	13.5 (± 1.7)

<sup>a</sup> The number of CFU per squid of *Vibrio fischeri* wild type, the *sapA* mutant and the reverse-complemented *sapA* mutant was determined at 12, 20 and 48 h post-inoculation. The values represent the mean ( $\pm$  standard error of the mean) of 15 animals per treatment, for each time point after inoculation; no CFU could be detected in light organs of animals that were maintained in uninoculated seawater.

#### FIGURES



Fig. 2.1. The V. fischeri sapABCDF locus. A The V. fischeri sapABCDF genes were cloned on plasmid pCL103 carrying a 6.9-kb XbaI-SpeI chromosomal fragment. **B** The sapA mutant was constructed by homologous recombination using the plasmid pCL109, which carries a kanR cassette inserted into the NsiI site, disrupting the sapA gene 900 bp downstream of the predicted transcriptional start site. **C** Plasmids pCL110 and **D** pCL111, carrying sapABCDF and sapA, respectively, as well as 600 bp of upstream sequence, were used in complementation studies.



**Fig. 2.2.** Relative growth rates of *V. fischeri* wild type, *sapA* mutant and complemented *sapA* mutant strains. A Growth curves of *V. fischeri* wild type strain ES114 (closed circles) and the *sapA* mutant CL10 (open triangles) grown in SWT medium. Maximum growth rates were determined on the early exponential portion of the curves (usually the first 2 h). **B** The *V. fischeri* wild-type (black bars), and the *sapA* mutant (hatched bars) were grown either (i) carrying the vector plasmid (control), (ii) carrying the vector containing *sapA* or (iii) carrying the vector containing *sapABCDF*. Presented are the results of a single experiment, normalized to wild-type levels, standard error bars are indicated.



Fig. 2.3. Development of bioluminescence in squids during colonization by V. fischeri strains. Newly hatched juveniles of E. scolopes were incubated with either V. fischeri wild-type strain ES114 (circles), the sapA mutant CL10 (diamonds), or the reverse-complemented sapA mutant CL16 (triangles). Animal bioluminescence was detected as a measure of the degree of colonization. Each data point represents the average bioluminescence level of 36 animals, with the indicated standard errors of the mean. The luminescence of uninoculated animals was equal to the background level (data not shown). The same results were obtained when the experiment was repeated.



Fig. 2.4. Colonization phenotype of the *sapA* mutant when competed against the wild-type strain. Juvenile animals were incubated with an approximately 1:1 ratio of either the *sapA* mutant CL10 and the wild-type strain ES114 (open circles) or the *sapA* mutant and the wild-type strain, each carrying pCL110 *sapABCDF in trans* (closed circles). At 24 h after inoculation, 100 bacterial colonies from the homogenate of each squid light organ were identified, and the ratio of mutant to wild-type cells in the population determined. Each circle represents the competitive index determined from an individual animal, expressed as the relative proportion of *sapA* mutant cells present in the light organ at 24 h post-inoculation. Circles with an arrow indicate animals with a mutant competitive index below the detection level (<0.01). The experiments were repeated twice with the same results.





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### CHAPTER 3

## THE VIBRIO FISCHERI QUORUM-SENSING SYSTEMS AIN AND LUX SEQUENTIALLY INDUCE LUMINESCENCE GENE EXPRESSION AND ARE IMPORTANT FOR PERSISTENCE IN THE SQUID HOST

#### ABSTRACT

Bacterial quorum sensing using acyl-homoserine lactones (acyl-HSLs) as celldensity dependent signaling molecules is important for the transcriptional regulation of many genes essential in the establishment and the maintenance of bacteria-host associations. Vibrio fischeri, the symbiotic partner of the Hawaiian bobtail squid Euprymna scolopes, possesses two distinct acyl-HSL synthase proteins, LuxI and AinS. Whereas the cell density-dependent regulation of luminescence by the LuxI-produced signal is a welldescribed phenomenon, and its role in light organ symbiosis has been defined, little is known about the ain system. I have investigated the impact of the V. fischeri acyl-HSL synthase AinS on both luminescence and symbiotic colonization. Phenotypic studies of V. fischeri mutants demonstrated that the AinS-signal is the predominant inducer of luminescence expression in culture, whereas the impact of the LuxI-signal is apparent only at the high cell densities occurring in symbiosis. Furthermore, my studies revealed that ainS regulates activities essential for successful colonization of E. scolopes, i.e. the V. fischeri ainS mutant failed to persist in the squid light organ. Mutational inactivation of the transcriptional regulator protein LuxO in the *ainS* mutant partially or completely reversed all the observed phenotypes, demonstrating that the AinS-signal regulates expression of downstream genes through the inactivation of LuxO. Taken together, my results suggest that the two quorum-sensing systems in V. fischeri, ain and lux, sequentially induce the expression of luminescence genes and possibly other colonization factors.

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#### INTRODUCTION

The symbiosis between the marine, luminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*, represents an ideal experimental system for the study of molecular processes underlying the colonization of host tissue (Ruby, 1999). The symbiosis is exclusive, but not essential, and each of the partners can be maintained individually under laboratory conditions, allowing a comparison of the symbiotic condition with both the planktonic (bacteria) or aposymbiotic (squid) life-styles (Ruby, 1996). The association begins shortly after the juvenile squid hatches and becomes inoculated by *V. fischeri* cells from the surrounding seawater. These bacteria subsequently colonize a specialized host structure, the squid light organ (McFall-Ngai and Montgomery, 1990; Nyholm *et al.*, 2000). The horizontal transfer of *V. fischeri* between generations has made it possible to develop colonization assays to investigate the early events of the symbiotic relationship (Ruby, 1996).

A hallmark of the colonization process is the onset of light emission induced by acyl-homoserine lactone (acyl-HSL) quorum sensing (Visick and Ruby, 1999). Quorum sensing comprises regulatory mechanisms that allow host-associated bacteria to selectively induce colonization-related genes when their products are advantageous to the bacterial community growing within the host. As a result, constitutive production of low levels of quorum-sensing signals allows the bacteria to sense the ambient cell density and to induce the expression of specific genes once a certain threshold concentration of the signal is achieved (reviewed in Fuqua *et al.*, 2001; Whitehead *et al.*, 2001). Quorum sensing using acyl-HSL signaling molecules was originally discovered as the regulatory mechanism underlying the induction of bioluminescence in *V. fischeri* (Eberhard *et al.*, 1981). The enzymes catalyzing bacterial light emission are encoded by the *lux* operon, which, in *V. fischeri*, consists of two divergently transcribed units, the *luxICDABEG* operon and the *luxR* gene. The *luxA* and *luxB* genes encode the  $\alpha$  and  $\beta$  subunits of the luciferase enzyme,

which catalyzes the reaction of reduced flavomononucleotide (FMNH<sub>2</sub>), long-chain aliphatic aldehyde and oxygen, producing oxidized flavomononucleotide (FMN), aliphatic acid, water and light. The *luxC*, *luxD* and *luxE* genes encode the aliphatic acid reductase complex that recycles the acid to aldehyde, and *luxG* is believed to participate in FMN metabolism. In *V. fischeri* the expression of these genes 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 binding to the transcriptional activator protein LuxR (Engebrecht and Silverman, 1984; Meighen, 1991). The LuxR-acyl-HSL complex binds to the *lux* promoter and induces the transcription of the *luxICDABEG* locus (Fuqua *et al.*, 1996). The *V. fischeri* transcriptional regulator LitR also participates in luminescence regulation by inducing the transcription of *luxR*, particularly at low cell densities (Fidopiastis *et al.*, 2002).

Derivatives of the symbiotic strain V. *fischeri* ES114 carrying mutations in either the luciferase gene *luxA* or the regulatory genes *luxI* and *luxR* do not produce light at detectable levels when colonizing their squid hosts (Visick *et al.*, 2000; Visick and Ruby, 1996). Although these mutants are capable of initiating *E. scolopes* colonization, and initially appear to reach colonization levels indistinguishable from the wild-type parent, by 48 h post-inoculation the mutant colonization levels are only 25-30% that achieved by wild type.

*V. fischeri* possesses a second acyl-HSL synthase protein, AinS, which synthesizes *N*-octanoyl-homoserine lactone (C8-HSL) and was originally discovered and characterized in the *V. fischeri* strain MJ1, a fish light organ symbiont (Gilson *et al.*, 1995; Kuo *et al.*, 1994; Kuo *et al.*, 1996; Callahan and Dunlap, 2000). In *Vibrio harveyi*, the AinS-homolog LuxLM synthesizes *N*-3-hydroxybutanoyl-HSL, which, together with the LuxS-derived 'autoinducer-2', induces luminescence by activating a phosphorylation cascade that results in the inactivation of LuxO, a repressor of luminescence gene expression (Bassler *et al.*, 1993; Bassler *et al.*, 1994; Freeman and Bassler, 1999; Mok *et al.*, 2003).

In the work described here I investigated the impact of the AinS-signal on luminescence and colonization competence of the squid light organ symbiont V. fischeri ES114. My results show that AinS is important not only for luminescence regulation, but also for successful host colonization.

#### MATERIALS AND METHODS

#### **Bacterial strains and growth conditions**

Medium reagents were purchased from Difco Laboratories (Sparks, MD) and Sigma Chemical Co. (St. Louis, MO). Strains and plasmids used in this study are listed in Table 3.1. *V. fischeri* strains were grown at 28 °C either in a seawater-based nutrient medium (SWT) (Boettcher and Ruby, 1990) or Luria-Bertani Salt (LBS) medium (Graf *et al.*, 1994). *E. coli* strains were grown at 37°C in Luria-Bertani medium (LB) (Sambrook *et al.*, 1989). Media were solidified with 1.5% (w/v) agar as needed. Antibiotics were added to the media at the following concentrations when appropriate: chloramphenicol (Cam; 2.5  $\mu$ g/mL for *V. fischeri*, 20  $\mu$ g/mL for *E. coli*), kanamycin (Kan; 100  $\mu$ g/mL for *V. fischeri* and *E. coli*), erythromycin (Erm; 5  $\mu$ g/mL for *V. fischeri* and 150  $\mu$ g/mL for *E. coli*). 3oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) was obtained from Sigma Chemical Co.; octanoyl-L-homoserine lactone (C8-HSL) was obtained from Aurora Biosciences (Coralville, IA).

#### Genetic techniques

Genomic and plasmid DNA were extracted using Qiagen DNeasy and Qiaprep Miniprep systems (Qiagen Inc., Valencia, CA), respectively. PCR was performed according to standard protocols (Sambrook *et al.*, 1989) using AmpliTaq DNA polymerase (Perkin-Elmer Inc., Branchburg, NY). For plasmid constructions, restriction enzymes and DNA ligase were obtained from New England Biolabs, Inc. (Beverly, MA) and used according to the manufacturer's protocol. Transfer of plasmids into *E. coli* host strains was accomplished using standard techniques (Sambrook *et al.*, 1989). Triparental conjugation was used to transfer plasmids into *V. fischeri* strains (Stabb *et al.*, 2001). 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 the V. fischeri mutant strains

To generate the *ainS*, the *ainS-luxI* and the *ainS-luxO* mutant strains, plasmid pMU106 was transferred into *V. fischeri* ES114, *luxI* and *luxO* mutant strains (see below) by triparental mating. Single and double recombinants were selected as previously described (Stabb *et al.*, 2001), generating the *V. fischeri ainS* and *ainS luxI* and the *ainS-luxO* mutant strains (CL21, CL24 and CL63, respectively). Introduction of the *ainS* mutation into the genome of *V. fischeri* was confirmed by PCR analysis. The *ainS* mutant did not produce detectable C8-HSL activity in a bioassay (Schaefer *et al.*, 2000), whereas a similarly treated, but 1000-fold diluted sample of the wild-type parent strain exhibited activity. The *ainS*-complementing plasmid pCL112 was generated by subcloning a 2.1-kb *Hae*III fragment carrying the intact *ainS* gene from pMU105 into the *V. fischeri* cloning vector pVO8 (Table 3.1).

To generate the *luxO* mutant strain, a 1.8-kb fragment carrying the *luxO* gene was PCR-amplified based on the *V. fischeri* genomic sequence provided by Integrated Genomics Inc. (Chicago, IL) at <u>http://ergo.integratedgenomics.com/Genomes/VFI/</u>. The fragment was cloned into the mobilizable vector pEVS79 (Table 3.1), generating pCL145. A 1.2-kb kanamycin resistance (*kanR*) gene from pUC4K (Table 3.1) was inserted into the *Nsi*I site located approximately 300-bp downstream of the *luxO* gene's start site. The resulting plasmid, pCL146, was transferred into *V. fischeri* ES114 by triparental mating, and single and double recombinants were selected as previously described (Stabb *et al.*, 2001), generating the *V. fischeri luxO* mutant strain CL42. The *luxO*-complementing plasmid pCL126 was generated by cloning the 1.8-kb PCR product into the *V. fischeri* cloning vector pVO8 (Table 3.1).

#### Luminescence in culture

To determine the luminescence characteristics of *V. fischeri* wild-type and mutant strains, 10 mL of SWT, or SWT containing either 120 nM 3-oxo-C6-HSL, 120 nM C8-HSL, or both, were inoculated to an optical density at 600 nm (OD) of about 0.05 with cells that had been pre-grown in SWT and washed three times to eliminate any natural acyl-HSL carry-over. Cultures were kept shaking at 28°C, samples were taken at various times during growth, and both luminescence and OD were measured. Where indicated an aqueous decanal (Sigma Chemical Co.) solution was added to the sample to a final concentration of 0.01% before light emission was measured. Maximum specific luminescence (luminescence/OD) values were averaged for measurements made between OD 1.0 and 5.0. The results presented are from a representative experiment, which was repeated twice with the same outcome. Growth rate and growth yield were also determined during these experiments by plotting OD as a function of time.

#### Luciferase assay

Bacterial luciferase activity was measured as previously described (Nealson, 1978). Briefly, during growth in SWT medium, culture samples (3x1 mL) of appropriate strains were harvested by centrifugation, and the resulting cell pellets were frozen at - $80^{\circ}$ C. The pellets were thawed on ice and suspended in 100 µL of ice-cold lysis buffer (10 mM Na-EDTA, 1 mM dithiothreitol, pH 7.0). Aliquots of the lysate were added to 1 mL of assay buffer (10 mM sodium phosphate buffer, pH 7.1), and supplemented with 10 µL of a 1% aqueous decanal solution. Light emission was assayed three times after injecting 0.5 mL of 50 µM FMNH<sub>2</sub> solution into the mixture in a light-tight chamber, and the specific light emission (quanta sec<sup>-1</sup> cell<sup>-1</sup>) was calculated. The mean of the three samples and their appropriate three measurements was determined and plotted as a function of OD, and the standard errors of the mean were calculated and indicated as error bars. The experiment was conducted three times with the same outcome.

#### **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 (Ruby and Asato, 1993). Briefly, newly hatched squids were placed into vials with 4 mL of filter-sterilized seawater containing an inoculum of approximately 1,000 colony-forming units (CFUs) 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 filter-sterilized seawater without added bacteria. Animal bioluminescence, an indirect measure of the degree of squid colonization, was monitored periodically over 48 h using a modified Packard Tri-Carb 2100TR scintillation counter (Packard Instruments Inc., Meriden, CT) as a photometer.

(ii) Colonization level in the squid light organ: The number of CFU per squid was determined at 24, 48 and 72 h post-inoculation following a previously described method (Ruby, 1996). Newly hatched squids were placed into 50 mL of filter-sterilized seawater containing about 1,000 CFU of the indicated strain per mL, and incubated for 12 h. Some animals were placed into filter-sterilized seawater 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. Statistical analysis was carried out using a two-sample, equal-variance Student's t-test.

(iii) Competitive phenotype: The ability of bacterial symbionts to compete during host colonization under conditions of co-inoculation was tested for the *V. fischeri* wild-type and *ainS* mutant strains, using a previously described approach (Visick and Ruby, 1998)

with the following modifications. About 15 newly hatched squid were placed into 50 mL of filter-sterilized seawater 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 number and exact ratio of the two strains in the inoculum. At 48 h post-inoculation, squids were homogenized and a dilution of the homogenate spread onto LBS agar. Approximately 100 CFU from the inoculum and each of the homogenates were patched onto antibiotic-containing and antibiotic-free LBS agar to determine the ratio of *V. fischeri* wild type (Cam-sensitive) to *ainS* mutant (Cam-resistant) cells.

#### RESULTS

# Light emission and growth characteristics of V. *fischeri ainS* and *ainS-luxI* mutants in culture

The light emission patterns of culture-grown cells of V. fischeri ainS and ainS-luxI mutants were determined and compared to those of their parent strains, the wild-type strain ES114, and the *luxI* mutant, respectively (Table 3.2). In culture, the *ainS* mutant did not produce light at a detectable level; however, providing C8-HSL exogenously restored wildtype luminescence levels. In contrast, while the addition of 3-oxo-C6-HSL, the LuxIsynthesized signal, to the *ainS* mutant led to luminescence induction to a detectable level, luminescence was <1% of the similarly supplemented wild-type strain (Table 3.2). As previously shown (Visick et al., 2000), the V. fischeri luxI mutant produced a reduced (30 to 40% of wild type) but detectable level of light emission in culture. This defect could be completely relieved by 3-oxo-C6-HSL addition, whereas C8-HSL had no significant effect on luminescence of the *luxI* mutant (Table 3.2). These data are consistent with an additive effect of the two acyl-HSLs. As expected from the data obtained with the single mutants, the ainS-luxI mutant was dark in culture. When grown in media supplemented with C8-HSL, luminescence of the ainS-luxI mutant was indistinguishable from that of the luxI mutant. Similarly, the addition of 3-oxo-C6-HSL to the double mutant enhanced luminescence to the level observed with the ainS single mutant (Table 3.2). The wild-type and three mutant strains all displayed luminescence levels that were indistinguishable from each other when both acyl-HSLs were added to the culture medium; the luminescence level of a wild-type culture with both acyl-HSLs added was comparable to one with only 3-oxo-C6-HSL added (data not shown).

The inability of the V. fischeri ES114 ainS mutant to produce light in culture contrasts with the luminescence phenotype reported for a V. fischeri MJ1 ainS mutant. When compared to the wild-type parent, luminescence of an ainS mutant of strain MJ1 was

induced at lower cell densities, suggesting that C8-HSL can competitively inhibit 3-oxo-C6-HSL-binding to LuxR (Kuo et al., 1996). However, V. fischeri MJ1 produces sufficient levels of 3-oxo-C6-HSL in laboratory culture to become brightly luminous, whereas V. fischeri ES114 emits only little light in laboratory culture, due to the relatively low levels of 3-oxo-C6-HSL it produces (Boettcher and Ruby 1990; Gray and Greenberg, 1992). I was curious to determine whether V. fischeri ES114 luminescence could be repressed by C8-HSL in the presence of exogenous 3-oxo-C6-HSL, thereby mimicking the acyl-HSL production of V. fischeri MJ1 in culture. I exogenously supplied increasing concentrations of 3-oxo-C6-HSL to growing cells of V. fischeri ES114 and found that 120 nM 3-oxo-C6-HSL was sufficient to induce maximal luminescence (Table 3.3). In contrast, the maximum level of light emission continued to rise without saturation when C8-HSL was added up to a concentration of  $12 \,\mu\text{M}$  (Table 3.3). However, in the presence of  $120 \,\text{nM}$  3-oxo-C6-HSL, C8-HSL had an inhibitory effect on the expression of luminescence that grew with increasing concentrations (Table 3.3). Thus, the positive effect of C8-HSL on luminescence is only apparent when 3-oxo-C6-HSL is limiting, that is, at lower cell densities that precede 3-oxo-C6-HSL accumulation. Because 3-oxo-C6-HSL rapidly accumulates to high levels in V. fischeri MJ1 (Gray and Greenberg, 1992), this signal is likely not limiting in culture.

The growth rates of the three acyl-HSL mutant strains were indistinguishable from the wild-type strain when they were cultured in the presence or absence of added acyl-HSLs (data not shown). However, the *ainS* and the *ainS-luxI* mutant strains consistently reached only about 75% of the growth yield of wild-type ES114, whereas a mutation in *luxI* alone did not affect the final growth yield. This growth yield defect in the *ainS* mutant strains was eliminated when 12 nM C8-HSL were supplied exogenously (data not shown). The reason for the premature growth termination by strains lacking a functional *ainS* gene is as yet unknown, but such a phenotype demonstrates that this regulatory gene plays a role in the control of more than light emission. Genetic complementation of the *ainS* mutant with a wild-type copy of *ainS* restored both wild-type luminescence levels and growth yields (data not shown), indicating that the observed defects are due to the inactivation of the *ainS* gene and not to either a polar genetic effect or a secondary mutation.

#### Luciferase enzyme activity of the V. fischeri ainS and ainS-luxI mutants

The addition of decanal, a substrate of the luciferase reaction, to culture-grown luminous bacteria has been previously shown to increase luminescence levels significantly, especially at low cell densities (Nealson et al., 1970), and the same effect was recently demonstrated for V. fischeri ES114 (Fidopiastis et al., 2002). Under my experimental culture conditions, decanal addition increased luminescence expression of V. fischeri ES114 about 100-fold. Interestingly, this addition restored the luminescence phenotype of the luxI mutant to wild-type levels and increased the light emission per cell of both the *ainS* and the ainS-luxI mutant from <1% (i.e., below the detection limit) to approximately 10% of wildtype levels (Fig. 3.1 A). These results suggested that the reduced light emission of the *lux1* mutant, compared to wild-type V. fischeri observed in culture (Table 3.2), is due primarily to aldehyde limitation and not to a difference in luciferase expression. Secondly, because light emission of the *ainS* mutant could be partially restored by exogenous decanal addition, I hypothesized that the amount of luciferase enzyme in the ainS mutant was no less than 10% that of wild-type V. fischeri. In vitro measurements of total luciferase enzyme activity are not subject to the substrate limitation (e.g., FMNH<sub>2</sub>, aldehyde, and oxygen) that can occur in living cells. When I performed assays to determine the luciferase contents of the four strains, the relative enzyme activities correlated with the light emission levels in culture when decanal was added (Fig. 3.1 B). Specifically, (i) luciferase levels in the luxl mutant and its wild-type parent were indistinguishable, and (ii) the ainS and the ainS-luxI mutants both produced a significant amount of luciferase in culture, at about 10% the levels of wild-type V. fischeri ES114 cells (Fig. 3.1 B). Thus, at the cell densities achieved in culture (<  $10^9$  cells/ml), quorum-sensing control of *lux* gene expression in *V. fischeri* ES114 is the result of the activity of *ainS* rather than of *luxI*.

#### Symbiotic light emission of the V. fischeri ainS and ainS-luxI mutants

The ability of the ainS, luxI, and ainS-luxI mutant strains to produce light when associated with the squid host was monitored during the first 48 h of the colonization process (Fig. 3.2). In contrast to culture conditions, the ainS mutant produced detectable light levels in the squid light organ. However, luminescence expression of animals colonized by the *ainS* mutant varied between 10 and 40% the level of wild type, depending on the experiment and the time point of colonization. Normal luminescence levels were restored when the ainS mutation was complemented with the wild-type ainS gene (data not shown). As predicted from previous work (Visick et al., 2000), both the luxI and the ainS *luxI* mutant strains did not produce detectable luminescence levels (> 0.1 quanta sec<sup>-1</sup> cell<sup>-1</sup>) when associated with the animal (Fig. 3.2). Because in vivo luminescence measurements are limited to the 10<sup>5</sup> to 10<sup>6</sup> cells present in the juvenile squid light organ, the detection limit of bacterial luminescence in the squid is more than 100-fold higher than in culture, which can reflect up to  $5 \times 10^8$  cells. Therefore, it is possible that the *luxI* mutant is as luminous in symbiosis as it is in culture, but appears 'dark' in the animal because it is below the light detection level. In any case, while *luxI* has little effect on luminescence in culture, it is critical for normal symbiotic light emission. In contrast, the presence of ainS is required for full luminescence levels both in culture (Table 3.2) and in the light organ (Fig. 3.2).

Because decanal addition significantly decreased differences between the luminescence levels of the acyl-HSL mutants and wild type in culture, I wondered whether the reduced luminescence of animals colonized by the acyl-HSL synthase mutants might be due to an aldehyde limitation in the symbiosis. To address this question, squid were colonized with either wild-type V. fischeri, the ainS mutant, the luxI mutant or the ainS-luxI

mutant. Squid light organs were then homogenized 24 h post-inoculation, and the light emission of the released bacteria was immediately measured, both before and after the addition of decanal. Although light emission of all four strains was stimulated about 2- to 3-fold upon decanal addition, the relative differences between wild-type *V. fischeri* and the mutant strains did not change (data not shown). To determine whether luminescence was not fully induced upon aldehyde addition in symbiotic bacteria because of an inhibitory factor present in host tissue, I added squid homogenate to cell extracts of cultured *V. fischeri* (data not shown). Luciferase enzyme activity in these extracts was the same when measured either before or after addition of the homogenate. Taken together, these data suggest that the diminished light levels of bacteria growing in the animal are due primarily to decreased luciferase production as a result of the *ainS* and/or *luxI* mutation(s).

#### **Regulation of luminescence by the AinS-synthesized acyl-HSL**

Having determined that *ainS* plays a role in luminescence expression both in culture and in symbiosis, I examined the pathway(s) through which it exerts its effects. By analogy to homologs in *V. harveyi* (Bassler *et al.*, 1993, Bassler *et al.*, 1994), the AinS-synthesized C8-HSL may function with a cognate receptor, AinR (Gilson *et al.*, 1995), to bring about the inactivation of LuxO, a transcriptional-regulator protein that negatively regulates luminescence (Miyamoto *et al.*, 2000). To determine whether such a pathway might function in *V. fischeri* ES114, I compared the luminescence phenotype of the *ainS* mutant with those of a *luxO* and an *ainS-luxO* mutant. Inactivation of *V. fischeri luxO* resulted in an acceleration of the onset of luminescence in culture (Fig. 3.3A), a response similar to that seen with *luxO* mutants of *V. harveyi* (Bassler *et al.*, 1994) and *V. fischeri* MJ1 (Miyamoto *et al.*, 2000). Furthermore, introducing the *luxO* mutation into the *ainS* mutant strain partially relieved the luminescence defect of the *ainS* mutant, consistent with the hypothesis that the AinS-signal inactivates the negative regulator LuxO (Fig. 3.3 A). However, at lower cell densities, the *ainS-luxO* mutant was not as bright as wild type, suggesting that the AinSsignal activity not only leads to the repression of LuxO, but also contributes an additional positive effect on luminescence that is independent of LuxO. Interestingly, the addition of 3-oxo-C6-HSL to the *luxO* and the *ainS-luxO* strains resulted in the same levels of enhanced luminescence (Table 3.4), indicating that high levels of 3-oxo-C6-HSL override this additional positive effect of the AinS-signal on luminescence. In contrast, at very high cell densities, luminescence expression of the *ainS-luxO* mutant was higher than that of wild type (Fig. 3.3A). This effect is apparent at approximately the same cell concentrations at which the luminescence levels of the *luxO* mutant are indistinguishable from wild type, suggesting that in the absence of LuxO inhibition, the AinS-signal can negatively affect luminescence expression at these cell concentrations. The effects of the *ainS* mutation on luminescence could be biochemically complemented by the exogenous addition of C8-HSL (Table 3.4). Similarly, providing a wild-type copy of the *luxO* mutants to wild type and *ainS* mutant levels, respectively (data not shown).

Whereas an *ainS* mutation led to a reproducible decrease in symbiotic luminescence, both the *luxO* and the *ainS-luxO* mutants expressed luminescence levels in the juvenile squid that were essentially equal to that of *V. fischeri* wild type throughout colonization (Fig. 3.3B). Thus, unlike in culture, the *luxO* mutation in symbiosis simply relieves the luminescence defect of the *ainS* mutant. Taken together these data indicate that (i) *ainS* functions to alleviate LuxO repression and, (ii) the positive effect of the AinS-signal on luminescence late in culture (Fig. 3.3A) is not a significant factor in the symbiosis.

The growth rates of the four strains, wild type, *ainS* mutant, *luxO* mutant and *ainS-luxO* mutant were indistinguishable in culture. As in previous experiments, the *ainS* mutant displayed a growth-yield defect, whereas both the *luxO* and the *ainS-luxO* mutant strains

reached wild-type growth yields (data not shown). These data further demonstrate that cellular functions other than luminescence are regulated through the AinS-LuxO pathway.

#### Symbiotic competence of the V. fischeri acyl-HSL mutants

The colonization ability of the *luxI*, *ainS* and *ainS-luxI* mutants was investigated by comparing the number of CFU per squid that were present at 24, 48 and 72 h post-inoculation relative to the levels achieved by wild-type *V. fischeri* (Fig. 3.4). At 24 h post-inoculation, all three acyl-HSL mutant strains colonized their squid hosts to about 75% of the wild-type level. However, by 48 h post-inoculation the number of cells per light organ for each mutant significantly decreased (mean values of about 30% the wild-type level; p-values  $\leq 0.01$ ), and this persistence defect continued through 72 h (mean values of about 20% the wild-type level; p-values  $\leq 0.01$ ). There was no significant difference between the colonization levels of the *luxI*, *ainS* or *ainS-luxI* mutants at any of these times (p-values  $\geq 0.40$ ). The decreased colonization levels of the *luxI* mutant are in agreement with previous observations (Visick *et al.*, 2000), and my results further demonstrate that *V. fischeri* requires not only a functional *luxI* but also an intact *ainS* gene for persistent colonization of the host. When the *ainS* mutant was genetically complemented with a functional copy of the *ainS* gene, wild-type colonization levels were restored (data not shown).

When juvenile squid were exposed to seawater containing a 1:1 ratio of *ainS* mutant to wild-type cells, examination of the population in the light organ 48 h post-inoculation showed this ratio was decreased to between 0.4:1 and 0.6:1. Interestingly, squid that were colonized by both the wild type and the *ainS* mutant emitted light levels indistinguishable from squid colonized by wild type only (data not shown), suggesting that wild-type cells are providing sufficient C8-HSL for the entire bacterial population to complement the luminescence defect when the two strains are in close contact within the interior of the light organ. This hypothesis is supported by the facts that acyl-HSLs can diffuse freely through

bacterial cell membranes (Kaplan and Greenberg, 1985; Boettcher and Ruby, 1995) and that in culture the *ainS* mutation can be complemented by providing exogenous C8-HSL (Table 3.2).

The introduction of a *luxO* mutation into an *ainS* mutant background could fully relieve the luminescence phenotype of the *ainS* mutant *in vivo* (Fig. 3.3B), indicating that the colonization defect of the *ainS* mutant might also be relieved by inactivation of LuxO. To test this hypothesis, I determined the number of bacterial cells in the squid light organ of animals colonized by wild-type *V. fischeri*, the *ainS* mutant, the *luxO* mutant, and the *ainS-luxO* mutant at 72 h post inoculation (Fig. 3.5). As in previous experiments (Fig. 3.4), *ainS* mutant-colonized animals displayed reduced numbers of bacterial cells in the light organ when compared to wild type-colonized animals. However, colonization levels of both the *luxO* mutant and the *ainS-luxO* mutant were indistinguishable from wild-type levels, again consistent with the notion that the AinS-regulated activity operates through inactivation of LuxO-repression.
#### DISCUSSION

In this study, I have investigated the impact of the *V. fischeri* acyl-HSL synthase AinS on luminescence and symbiotic colonization. My results demonstrate that (i) the AinS-synthesized signal plays an important role in luminescence regulation in *V. fischeri* especially at cell densities preceding LuxI-dependent regulation of gene expression, (ii) AinS regulates functions in *V. fischeri* that are important for successful host colonization and (iii) luminescence, as well as other putative functions, are regulated by AinS through a signaling cascade involving the transcriptional regulator LuxO.

# The impact of C8-HSL on luminescence expression is apparent at lower cell densities than that of 3-oxo-C6-HSL

A comparison of the luminescence phenotypes of the *ainS* and the *ainS-luxI* mutants to those of their parent strains revealed that *ainS* plays a major role in the regulation of luminescence in *V. fischeri* ES114 (Table 3.2). While a mutation in the *luxI* gene affected light emission in culture only slightly, and did not significantly decrease the level of luciferase synthesized, a mutation in *ainS* resulted in a dark phenotype and the synthesis of only 10-20% of wild-type luciferase activity (Fig. 3.1 A and 3.1 B). This result implies that the AinS-synthesized C8-HSL is active at lower cell densities than the LuxI-synthesized 3-oxo-C6-HSL. It has been shown previously that the 1000-fold increase in luminescence expression occurring when *V. fischeri* colonizes its squid host (Boettcher and Ruby, 1990), relies on the LuxI-synthesized signal. That is, animals colonized by a *luxI* mutant do not express light at a detectable level as a result of the absence of normal *lux* operon induction (Visick *et al.*, 2000). The light emission of squid colonized by the *ainS* mutant was decreased to between10 and 40% of wild type (Fig. 3.2, and data not shown), which can, at least in part, be attributed to the presence of only 20 to 80% of bacterial cell numbers in the squid relative to wild type (Fig. 3.4). Unfortunately, due to high variability of luminescence

expression between individual animals, I was unable to determine whether the specific luminescence levels of the *ainS* mutant in the squid are equal to wild-type levels throughout the first 48 h of colonization. However, although addition of 3-oxo-C6-HSL to a culture of the *ainS* mutant stimulated light production in culture, luminescence levels were only 1% that of a similarly induced wild-type culture. These data suggest that synthesis of both of the acyl-HSLs is necessary for maximal *lux* operon induction. Taken together, my results demonstrate that the impact of the AinS-synthesized signal, at least on *lux* gene expression, is evident at cell concentrations occurring in culture, and continues to be important at the higher densities reached in the squid host. In contrast, the cell density that is necessary for LuxI-signal induction is apparently only reached when *V. fischeri* colonizes the squid host.

#### A model for luminescence regulation in V. fischeri ES114

Based on these and previous studies, I propose a model of luminescence gene expression in *V. fischeri* ES114 (Fig. 2.6). For simplicity, my model is limited to the effects of quorum sensing on luminescence gene expression and does not include other physiological and genetic factors known to be involved (for review see Sitnikov *et al.*, 1995). Furthermore, specific luminescence of cells expelled from the squid light organ express higher luminescence levels than culture-grown cells supplemented with exogenous 3-oxo-C6-HSL suggesting that the squid light-organ environment provides quorum sensing-independent factors capable of stimulating luminescence expression (Boettcher and Ruby, 1990).

The model depicts a regulatory scheme that involves sequential quorum sensing in which the effects of the C8-HSL signal precede those of 3-oxo-C6-HSL. As a result, luminescence is repressed under low cell-density conditions ( $<10^8$  cells/ml, as when bacteria exist planktonically in seawater; Lee and Ruby 1994), becomes partially induced at

moderate cell densities ( $10^8$  to  $10^9$  cells/ml, as occurs in culture), and becomes fully induced under the high cell-density conditions found during symbiosis (> $10^{10}$  cells/ml; Ruby, 1996).

When V. fischeri cells are in low abundance neither of the acyl-HSL signals accumulates; thus, light expression is not detectable in seawater or at low cell concentrations in culture (Fig. 3.1 A). By analogy to the luminescence regulatory cascade in V. harveyi (Miller and Bassler, 2001), I predict that V. fischeri LuxO represses lux operon transcription at these low cell densities (Fig. 2.6 A). Consistent with this hypothesis, I observed that a V. fischeri ES114 luxO mutant induced luminescence at lower cell densities than wild type (Fig. 3.3 A), a phenotype reported for luxO mutants in other Vibrio strains (Bassler et al., 1994; et al., 2000). In V. harveyi, LuxO is believed to activate an unknown factor ('X') that represses transcription of luxR (not a homolog of V. fischeri luxR) (Lilley and Bassler, 2000). The fact that LitR, the V. fischeri homolog of V. harveyi LuxR, has been shown to increase luminescence expression through up-regulation of V. fischeri luxR transcription (Fidopiastis et al., 2002, Miyamoto et al., 2003), provides evidence that a similar cascade may function in V. fischeri as well. However, both V. fischeri luxR and litR mutants express detectable luminescence in culture (Visick et al., 2000, Fidopiastis et al., 2002), whereas the ainS mutant is dark (Table 3.2), suggesting that the ainS mutation results in an additional depression of lux operon expression that is independent of the litR-luxR-pathway. The mechanism of this depression is not understood, but is likely to operate through LuxO, because the ainS mutant phenotype can be almost completely relieved by the luxO mutation, both in culture and during colonization of the squid host (Fig. 3.3 A and 3.3 B).

As V. fischeri ES114 grows in culture, I propose that C8-HSL accumulates and activates a signaling pathway through binding to its cognate receptor AinR, a homolog of V. harveyi LuxN (Gilson et al., 1995, Bassler et al., 1993). A phosphorelay cascade involving a V. fischeri LuxU homolog (Freeman and Bassler 1999; http://ergo.integratedgenomics.com/Genomes/VFI/) is predicted to result in the inactivation

of LuxO, thereby relieving the repression of luminescence (Fig. 2.6 B). I propose that at these moderate cell-density conditions, 3-oxo-C6-HSL has not accumulated sufficiently to bind to LuxR and induce luminescence gene expression. This hypothesis is consistent with the fact that a V. fischeri ES114 luxI mutant is not significantly impaired in its ability to express luminescence in culture (Table 3.2, Visick et al., 2000). Therefore a lowerefficiency binding of the more abundant C8-HSL to LuxR may occur, which can weakly stimulate expression of the *luxICDABEG* operon (Fig. 2.6 B). The capability of C8-HSL to bind to LuxR in the absence of 3-oxo-C6-HSL, and to subsequently induce lux operon transcription, has been previously demonstrated (Schaefer et al., 1996; Egland and Greenberg 2000), and is consistent with the inhibitory effect of excess C8-HSL shown here (Table 3.3). Furthermore, previous studies revealed that the stimulatory effect of C8-HSL on luminescence requires a functional luxR gene (Kuo et al., 1994; Visick et al., 2000). Finally, the inability of the *luxO* mutation to completely reverse the effect of the *ainS* mutation at low cell densities (Fig 3 A), supports the hypothesis of a direct positive effect of the AinS-signal on luminescence gene expression.

Eventually, at the very dense bacterial concentrations found in the light organ, 3-oxo-C6-HSL accumulates to significant levels and binds to LuxR, leading to enhanced luminescence expression (Fig. 2.6 C). Therefore, both *luxI* and *luxR* mutants are dark when colonizing the juvenile squid, whereas mutants involved in the AinS-pathway, *ainS*, *luxO*, and *litR*, display relatively mild or no luminescence phenotypes in symbiosis (Figs. 3.2, and 3.3B; Fidopiastis *et al.*, 2002).

In summary, the AinS-synthesized signal, C8-HSL, apparently has two major functions: (i) relieving the LuxO-modulated inhibition of luminescence expression through a phosphorelay cascade, and (ii) stimulating *lux* operon transcription through direct interaction with the LuxR protein. These pathways function in addition to the well-

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characterized LuxI-LuxR system, and apparently operate in a sequential manner to modulate luminescence gene expression over a large range of bacterial densities.

#### The symbiotic competence of acyl-HSL mutants is compromised

The most striking finding of this study is that the colonization levels of all three acyl-HSL mutant strains, ainS, luxI and ainS-luxI, were significantly decreased when compared to the wild type. Previous work has shown that V. fischeri mutant strains luxR, luxI, and luxA produce a non-detectable (i.e., <1% of wild type) level of luminescence when colonizing the squid host (Visick et al., 2000). Because the colonization defect in each of these mutant strains was essentially the same, it was concluded that the underlying cause was their common luminescence deficiency. Engineering the *luxR* mutant strain to produce 10% of the wild-type light levels in the squid restored its ability to colonize the host normally (Visick et al., 2000). Thus, as little as 10% of the normal luminescence level was sufficient to relieve the symbiotic defect of dark strains. Although I was unable to quantitatively determine the specific luminescence of the ainS mutant in vivo, I never detected luminescence levels that were below 10% that of wild type (Figs. 3.2 and 3.3 B; data not shown). Thus, while the *ainS* mutant may have a reduced level of luminescence while in the symbiosis, this phenotype can not fully explain its colonization defect. Taken together these data suggest either that the colonization defect is due to a qualitative, rather than quantitative, difference in luminescence expression by the ainS mutant, or that the V. fischeri C8-HSL signal induces other activities required for symbiosis. Regardless of what these activities might be, the fact that an *ainS-luxO* mutant is not defective in its colonization ability suggests that they may be regulated through the AinS-LuxO pathway. This assumption is not without precedence: LuxO homologs in other Vibrio species regulate multiple phenotypes including biofilm formation, siderophore production, and virulence gene expression in addition to luminescence (Lilley and Bassler, 2000; Zhu et al., 2002; Vance et al. 2003).

The identity of other possible factors underlying the reduced colonization levels of the *V. fischeri ainS* mutant remains undetermined. However, neither siderophore production nor motility, which are both essential for colonization and persistence (Graf *et al.*, 1994; Graf and Ruby, 2000; Millikan and Ruby, 2002), were reduced in any of the acyl-HSL mutants (data not shown). Nevertheless, the fact that the *ainS* and the *ainS-luxI* mutant strains were unable to grow in culture beyond 75% of the wild-type growth yield, while the *luxI* mutant strain reached a normal yield, supports the hypothesis that C8-HSL regulates cellular functions other than luminescence. These as yet unknown cellular functions may prove to be the cause of the observed colonization defects of the *ainS* mutant.

In conclusion, as with *V. fischeri*, an increasing number of other bacterial species have been found to possess more than one quorum-sensing system (Fuqua *et al.*, 2001). Thus, one might expect that they, too, will employ these systems in a step-wise, cell density-dependent manner like that hypothesized here (Fig. 2.6). Because most of these species are host-associated, the biological significance of a sequential regulation of quorum signaling may not be fully revealed simply by growing cells in culture, but may require the study of the relative importance of these signals at different stages during colonization of their hosts.

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## TABLES

Table 3.1. Bacterial	strains	and	plasmids.
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Strains and plasmids	Description	Reference or source
E. coli		
C118λpir	$\Delta(ara$ -leu) araD $\Delta lac X74$ galE	(Herrero et al., 1990)
-	galK phoA20 thi-1 rpsE rpoB	
	argE(Am) recAI, lysogenized with	
	λpir	
DH5a	$F \Phi 80 dlac Z \Delta M15 \Delta (lac ZYA-$	(Hanahan, 1983)
	argF)U169 deoR supE44 hsdR17	(,,
	recA1 gyrA96 thi-1 felA1	
V. fischeri		
wild type	Strain ES114, isolate from E.	(Boettcher and Ruby,
	scolopes light organ	1990)
ainS mutant CL21	ainS gene partially deleted and	This study
	replaced by a chloramphenicol-	
	resistance (cat) marker	
<i>luxI</i> mutant VCW2G7	<i>luxI</i> gene inactivated by a frameshift	C. Whistler, University
	mutation	of Hawaii
ainS-luxI mutant CL24	double mutant carrying mutations as	This study
	described above	
luxO mutant CL42	luxO gene inactivated by insertion of	This study
	a kanamycin-resistance marker	
ain & lun O mutant CI 64	( <i>kank</i> )	This study
ains-taxo mutani CL04	double mutant carrying mutations as	This study
Plasmide	described above	
pCL 112	2.1 kb W finahari ES114 DNA with	This study
PCL112	the girs gone cloned into pVO8	This study
pCI 126	1 8-kb V fischeri ES114 DNA with	This study
pellizo	the <i>luxO</i> gene cloned into nVO8	This study
nCL145	1 8-kb V fischeri ES114 DNA with	This study
politie	the <i>luxO</i> gene cloned into nEVS79	
pCL146	pCL145 with <i>luxO</i> gene inactivated	This study
F	by a insertion of kanR-marker into	
	the Nsil site	
pEVS79	Allelic exchange vector	(Stabb and Ruby,
•	<b>C</b>	2002)
pMU106	Allelic exchange vector carrying	M. Urbanowski,
-	partially deleted ainS gene replaced	University of Iowa
	by a chloramphenicol-resistance	-
	marker ( <i>cat</i> )	
pUC4K	Origin of the kanamycin-resistance	(Messing and Vieira
	marker (kanR)	1982)
pVO8	V. fischeri cloning vector, ermR	(Visick and Ruby,
		1997)

	Additions <sup>b</sup>					
Strain	none	C8-HSL	3-oxo-C6-HSL			
wild type	3.8 (0.4)	4.8 (1.0)	2,360 (370)			
ainS	BD <sup>c</sup>	3.9 (0.3)	4 (1)			
luxI	1.3 (0.1)	2.2 (0.5)	2,070 (290)			
ainS-luxl	BD℃	1.4 (0.1)	1 (2)			

 Table 3.2. Effects of acyl-HSL additions on the luminescence of V. fischeri ainS and luxI

 mutants.<sup>a</sup>

<sup>a</sup> Specific luminescence values (x  $10^{-2}$  quanta sec<sup>-1</sup> cell<sup>-1</sup>) are the means of cultures with an optical density (at 600 nm) of between 1.0 and 5.0; standard errors of the means are indicated in parentheses. The results are from a representative experiment, which was repeated twice with the same outcome.

<sup>b</sup> Cultures were grown in SWT medium alone or supplemented with 120 nM of either 3oxo-C6-HSL or C8-HSL.

<sup>c</sup> Below detection ( $< 2 \times 10^{-4}$  quanta sec<sup>-1</sup> cell<sup>-1</sup>)

Table	3.3.	Luminescence	e expression	of V	. fischeri	ES114	cultures	with	the	addition	of
differe	nt acy	yl-HSL conce	ntrations. <sup>a</sup>								

	Concentration of acyl-HSL added (nM)				
Acyl-HSL	no addition	12	120	1,200	12,000
3-oxo-C6-HSL	1.0	550	1,400	1,450	1,500
C8-HSL	1.0	1.6	2.2	3.6	6.2
C8-HSL					
(+ 3-oxo-C6-HSL <sup>b</sup> )	420	ND <sup>c</sup>	120	5.0	5.5

<sup>a</sup> Values shown are fold-induction compared to the control with no addition of either acyl-HSL. The results are from an experiment that was repeated with the same outcome.

<sup>b</sup> Cultures were grown in SWT medium supplemented both with 120 nM 3-oxo-C6-HSL and with the C8-HSL concentrations indicated.

<sup>c</sup> not determined

	Additions <sup>b</sup>					
Strain	none	C8-HSL	3-oxo-C6-HSL			
wild type	1.2 (0.3)	2.6 (0.4)	1280 (150)			
ainS	$BD^{c}$	1.8 (0.1)	2 (0)			
luxO	2.3 (0.3)	4.9 (0.5)	2100 (220)			
ainS-luxO	1.4 (0.8)	5.9 (0.3)	2010 (150)			

Table 3.4. Effect of acyl-HSL additions on the luminescence of V. fischeri luxO mutants.<sup>a</sup>

<sup>a</sup> Specific luminescence values (x  $10^{-2}$  quanta sec<sup>-1</sup> cell<sup>-1</sup>) are the means of cultures with an optical density (at 600 nm) of between 1.0 and 5.0; standard errors of the means are indicated in parentheses. The results are from a representative experiment, which was repeated twice with the same outcome.

<sup>b</sup> Cultures were grown in SWT medium, with or without 120 nM C8-HSL or 3-oxo-C6-HSL.

<sup>c</sup> Below detection (<  $2 \times 10^{-4}$  quanta sec<sup>-1</sup> cell<sup>-1</sup>)

**FIGURES** 



Fig. 3.1. Specific luminescence (quanta sec<sup>-1</sup> cell<sup>-1</sup>) and luciferase activity (quanta sec<sup>-1</sup> cell<sup>-1</sup>) of *V. fischeri* wild-type (diamonds), *ainS* mutant (triangles), *luxI* mutant (squares) and *ainS-luxI* mutant (circles). A Specific luminescence of SWT medium-grown cells. Samples were taken during growth, and luminescence was measured in the presence (open symbols) or absence (closed symbols) of added decanal. B Luciferase activity of cell culture lysates. For each time point three samples were taken, and the luciferase activity of each sample was measured in triplicate. The mean of the nine measurements was determined, and the standard errors of the mean were calculated and indicated as error bars.



Fig. 3.2. Animal luminescence during the initial stages of *E. scolopes* colonization by *V. fischeri* wild-type (diamonds), *ainS* mutant (triangles), *luxI* mutant (squares) and *ainS-luxI* mutant (circles). The luminescence pattern indicates the initial onset of colonization (0 to 20 h), followed by a changing level of light emission that reflects the diurnal behavior of the animal. The dashed line indicates the approximate detection limit of the assay. Mean values were calculated and standard errors of the mean are indicated. The experiment was conducted twice with the same outcome.



Fig. 3.3. Luminescence expression in culture (quanta sec<sup>-1</sup> cell<sup>-1</sup>) and during the initial stages of colonization (animal luminescence) of V. fischeri wildtype (diamonds), the ainS mutant (triangles), the luxO mutant (squares) and the ainS-luxO mutant (circles). A The four strains were grown in SWT medium, samples were taken and luminescence was measured at different times during the growth curve. The results are from a representative experiment, which was repeated twice with the same outcome. B The mean luminescence expression of 24 animals colonized by the four strains was measured during the first 48 h of colonization. Standard errors of the mean are indicated. The experiment was performed twice with the same outcome. The dashed line indicates the approximate detection limit of the assay.



**Fig. 3.4.** Colonization levels of the *ainS* mutant (striped bars), *luxI* mutant (white bars), and *ainS luxI* mutant (hatched bars) relative to V. fischeri wild type (black bars) at 24, 48 and 72 h post-inoculation. Each bar represents the mean value of 15 animals with the associated standard errors. The experiment was conducted three times with the same outcome.



**Fig. 3.5.** Colonization levels of the *ainS* mutant (striped bars), *luxO* mutant (dotted bars), and *ainS-luxO* mutant (hatched bars) relative to *V*. *fischeri* wild type (black bars) at 72 h post-inoculation. Each bar represents the mean value of 15 animals with the associated standard errors. The experiment was conducted twice with the same outcome.

A Low cell densities (seawater)



**B** Intermediate cell densities (culture)



C High cell densities (squid light organ)



Fig. 3.6. Proposed model of luminescence regulation in V. fischeri ES114. A At low cell densities, B in culture, and C when colonizing the squid host (C). See the Discussion section for an explanation. C6 = 3-0x0-C6-HSL; C8 = C8-HSL.

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### CHAPTER 4

# VIBRIO FISCHERI LUXS AND AINS: TWO SIGNAL SYNTHASES, ONE PATHWAY, AND TWO OUTCOMES

#### ABSTRACT

Vibrio fischeri, the bacterial light-organ symbiont of the Hawaiian squid Euprymna scolopes, has been demonstrated to utilize two quorum-sensing systems, ain and lux, for the cell-density dependent transcriptional regulation of luminescence and possibly other colonization gene expression. The recently sequenced genome of V. fischeri revealed the presence of a putative third system, consisting of LuxS, LuxP and LuxQ. In this study, I investigated the impact of this system on luminescence expression and colonization competence of V. fischeri. A V. fischeri luxS mutant expressed approximately 70% wild-type luminescence levels in culture, whereas luminescence expression in symbiosis was not affected. The inactivation of luxS alone did not compromise symbiotic competence of V. fischeri; however, colonization levels of an ainS-luxS mutant were reduced to 50% that of the ainS mutant. The introduction of a luxO mutation into the luxS and ainS-luxS background could relieve the observed defects, suggesting that V. fischeri LuxS regulates both luminescence and colonization factor expression through LuxO, similar to what has been previously demonstrated for AinS. Although my findings suggest that both the LuxS and the AinS signals exert their downstream effects through the same pathway, the quantitative contribution of the two signals to gene regulation appears to be different; i.e., the defects displayed by a *luxS* mutant are minor compared to those of an ainS mutant. I found major differences in both the patterns of LuxS and AinS signal production and in the transcriptional regulation of the luxS and ainS genes. My data demonstrate that the concentration of the AinSsynthesized signal sharply increased at around the cell density at which luminescence is induced, and that signal concentration is controlled by transcriptional autoregulation of

the *ainS* gene. In contrast, although LuxS signal concentration changed with increasing cell density, the observed rate of change was gradual, and did not correlate with luminescence expression. Furthermore, my data did not supply evidence for a transcriptional regulation of the *luxS* gene. Taken together, under the conditions investigated in this study; i.e., culture medium and the squid light organ, and in comparison to the AinS signal, the *V. fischeri* LuxS signal does not significantly impact either luminescence expression or colonization competence.

#### INTRODUCTION

LuxS, the enzyme synthesizing autoinducer-2 (AI-2), a furanosyl borate diester (Chen *et al.*, 2002), was originally discovered in *Vibrio harveyi* as part of the quorumsensing circuit regulating luminescence gene expression (Surette *et al.*, 1999). Many bacterial species, Gram-negative and Gram-positive, possess *luxS* homologs and have since been found to produce AI-2 activity (Xavier and Bassler, 2003). Therefore, unlike acyl-homoserine lactone signals in Gram-negative bacteria, or peptide signals in Grampositive bacteria, AI-2 is not species-specific, which has led to the hypothesis that AI-2 serves as an interspecies communication signal (Miller and Bassler, 2001).

In V. harveyi, luminescence gene expression is coordinately controlled both by the autoinducer-1 (AI-1), N-3-hydroxybutanoyl homoserine lactone, synthesized by LuxM, and by the LuxS-derived AI-2 (Mok et al., 2003). Both signals bind to cognate hybrid two-component sensor kinase proteins, LuxN and LuxPQ, respectively (Bassler et al., 1994a; Freeman et al., 2000), which transduce information through a phosphorelay cascade involving a protein designated LuxU (Freeman and Bassler, 1999b), leading to the inactivation of the transcriptional regulator LuxO (Bassler et al., 1994b; Freeman and Bassler, 1999a). Inactivation of LuxO results in increased transcription of LuxR, a positive regulator of luminescence gene expression in V. harveyi (Miyamoto et al., 2003). Recent studies have demonstrated the involvement of homologous proteins of this regulatory cascade in virulence factor expression of Vibrio cholerae (Miller et al., 2002; Zhu et al., 2002), Vibrio anguillarum (Milton et al., 2001; Croxatto et al., 2002) and Vibrio vulnificus (McDougald et al., 2001; Shao and Hor, 2001; Kim et al., 2003), and both Vibrio alginolyticus and Vibrio parahaemolyticus have been demonstrated to possess AI-2 activity (Bassler *et al.*, 1997). While the complete regulatory system remains to be determined for most of these species, the accumulating data are suggesting a common mechanism in *Vibrio spp.* (McDougald *et al.*, 2003; Miyamoto *et al.*, 2003).

I have previously demonstrated that Vibrio fischeri, the bacterial light-organ symbiont of the Hawaiian squid Euprymna scolopes, utilizes two quorum-sensing systems whose relative importance is dependent on cell density (Lupp et al., 2003): (i) the well-known lux system comprising LuxI, the acyl-homoserine lactone (acyl-HSL) synthase producing 3-oxo-hexanoyl-HSL (3-oxo-C6-HSL), and the transcriptional regulator LuxR (Fuqua et al., 2001), and (ii) the ain system including the LuxLM homolog AinS (Lupp et al., 2003). The AinS-synthesized signal, N-octanoyl homoserine lactone (C8-HSL), was found to regulate luminescence and colonization factor expression through the inactivation of LuxO similar to the AI-1 pathway in V. harveyi (Lupp et al., 2003). Inactivation of LuxO results in increased transcription of litR; LitR in turn positively regulates luxR transcription, thereby linking the two quorum-sensing systems ain and lux in V. fischeri (Lupp et al., 2003; Miyamoto et al., 2003).

Analysis of the recently sequenced V. fischeri genome (http://ergo. integratedgenomics.com/Genomes/VFI/) revealed the presence of not only the *luxS* gene, but also homologs of the putative AI-2 receptor genes *luxP* and *luxQ*. This discovery, together with the fact that the AI-1 pathway is apparently conserved in V. harveyi and V. fischeri, led me to speculate that an AI-2 pathway similar to V. harveyi and other Vibrio spp. might operate in V. fischeri. Besides the potential impact of AI-2 on luminescence expression, I was curious to know whether this signal is important in colonization competence of V. fischeri, as AI-2 has been implicated in colonization factor-regulation in a variety of bacterial species [for review see (Xavier and Bassler, 2003)]. Furthermore, the V. fischeri ainS mutant is defective in colonizing its squid host, suggesting that ainS regulates the expression of genes important in symbiosis (Lupp et al., 2003). If the AI-2 pathway functions in V. fischeri, those factors might then also be coordinately regulated by ainS and luxS.

#### MATERIALS AND METHODS

#### **Bacterial strains and growth conditions**

Strains and plasmids used in this study are listed in Table 4.1. Medium reagents were purchased from Difco Laboratories (Sparks, MD) and Sigma Chemical Co. (St. Louis, MO). Strains and plasmids used in this study are listed in Table 4.1. *V. fischeri* strains were grown at 28 °C either in a seawater-based nutrient medium (SWT) (Boettcher and Ruby, 1990) or Luria-Bertani Salt (LBS) medium (Graf *et al.*, 1994). *E. coli* strains were grown at 37°C in Luria-Bertani medium (LB) (Sambrook *et al.*, 1989). Media were solidified with 1.5% (w/v) agar as needed. Antibiotics were added to the media at the following concentrations when appropriate: chloramphenicol (Cam; 2.5  $\mu$ g/mL for *V. fischeri*, 20  $\mu$ g/mL for *E. coli*), kanamycin (Kan; 100  $\mu$ g/mL for *V. fischeri* and *E. coli*), erythromycin (Erm; 5  $\mu$ g/mL for *V. fischeri* and 150  $\mu$ g/mL for *E. coli*). 3-oxo-C6-HSL was obtained from Sigma Chemical Co.; C8-HSL was obtained from Aurora Biosciences (Coralville, IA).

#### **Genetic techniques**

Genomic and plasmid DNA were extracted using Qiagen DNeasy and Qiaprep Miniprep systems (Qiagen Inc., Valencia, CA), respectively. PCR was performed according to standard protocols (Sambrook *et al.*, 1989) using AmpliTaq DNA polymerase (Perkin-Elmer Inc., Branchburg, NY). For plasmid constructions, restriction enzymes and DNA ligase were obtained from New England Biolabs, Inc. (Beverly, MA) and used according to the manufacturer's protocol. Transfer of plasmids into *E. coli* host strains was accomplished using standard techniques (Sambrook *et al.*, 1989). Triparental conjugation was used to transfer plasmids into V. fischeri strains (Stabb et al., 2001). 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 based on the *V. fischeri* genomic sequence provided by Integrated Genomics Inc. (Chicago, IL) at http://ergo.integratedgenomics.com/ Genomes/VFI/. The fragment was cloned into the mobilizable vector pEVS79 (Table 4.1), generating pCL115. Plasmid pCL115 was randomly mutagenized using the EZ::TN <Kan-2> Insertion Kit (Epicentre, Madison WI). One clone, designated pCL122, was identified carrying the <Kan-2> cassette insertion 70 bp downstream of the translational start site of the *luxS* gene. Plasmid pCL122 was transferred into *V. fischeri* ES114 and *ainS* mutant strains by triparental mating. Single and double recombinants were selected as previously described (Stabb *et al.*, 2001), 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 4.1).

To generate the *luxS-luxO* and the *ainS-luxS-luxO* mutants, the *luxO* gene carried on pCL145 (Table 4.1) was mutagenized by inserting a 1.2-kb erythromycin resistance (*ermR*) gene from pEVS94 (Table 4.1) into the *Nsi*I site located approximately 300-bp downstream of the *luxO* gene's 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 (Stabb *et al.*, 2001), 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 strains CL91. Strain CL91 did not produce C8-HSL activity in a previously described bioassay (Schaefer *et al.*, 2000), confirming introduction of the *ainS* mutation into the genome.

The *luxR* mutant was constructed by subcloning a 4.1-kb *Hae*III fragment from pKV30 (Table 4.1) into the mobilizable vector pEVS79 (Table 4.1). This fragment carries the *lux R* gene from *V. fischeri* ES114, which has been inactivated by partial deletion and insertion of an erythromycin resistance cassette (Visick *et al.*, 2000). The resulting plasmid pCL149 was transferred into the chromosome of *V. fischeri* ES114 by triparental mating, and single and double recombinants were selected as previously described (Stabb *et al.*, 2001), 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 previously described *V. fischeri luxR* mutant strains. Specifically, luminescence in culture was decreased to approximately 10-20% of wild type. Whereas wild-type luminescence was induced several 100-fold upon exogenous addition of 120 nM 3-oxo-C6-HSL, a similar addition did not change luminescence expression of the *luxR* mutant.

#### Luminescence in culture

To determine the luminescence characteristics of *V. fischeri* wild type and mutant strains, 10 mL of SWT, or 10 mL of SWT containing either 120 nM 3-oxo-C6-HSL or 120 nM C8-HSL, or 9 mL SWT and 1 mL over-night culture supernatant of *V. harveyi* BB152, were inoculated to an optical density at 600 nm (OD) of about 0.05. Cultures were kept shaking at 28°C, samples were taken at various times during growth, and both luminescence and OD were measured. Maximum specific luminescence (luminescence/OD) values were averaged for measurements made between OD 1.0 and 5.0. Growth rate and growth yield were also determined during these experiments by plotting 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 (Ruby and Asato, 1993). Briefly, newly hatched squids were placed into vials with 4 mL of filter-sterilized seawater containing an inoculum of approximately 1,000 colony-forming units (CFUs) 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 filter-sterilized seawater without added bacteria. Animal bioluminescence, an indirect measure of the degree of squid colonization, was monitored periodically over 48 h using a modified Packard Tri-Carb 2100TR scintillation counter (Packard Instruments Inc., Meriden, CT) as a photometer. (ii) Colonization level in the squid light organ: The number of CFU per squid was determined at 24 and 48 h post-inoculation following a previously described method (Ruby, 1996). Newly hatched squids were placed into 50 mL of filter-sterilized seawater containing about 1,000 CFU of the indicated strain per mL, and incubated for 12 h. Some animals were placed into filter-sterilized seawater 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 co-inoculation was tested for the *V. fischeri* wildtype and *ainS* mutant strains, using a previously described approach (Visick and Ruby, 1998) with the following modifications. About 15 newly hatched squid were placed into 50 mL of filter-sterilized seawater 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 number and exact ratio of the two strains in the inoculum. At 48 h post-inoculation, squids were homogenized and a dilution of the homogenate spread onto LBS agar. Approximately 100 CFU from the inoculum and each of the homogenates were patched onto antibiotic-containing and antibiotic-free LBS agar to determine the ratio of *V. fischeri* wild type (Kan-sensitive) to *luxS* mutant (Kan-resistant) cells.

#### LuxS and AinS-signal activities of V. fischeri cultures

To determine the ability of V. fischeri to produce LuxS-signal activity, I used a previously described assay (Bassler et al., 1997). Briefly, V. fischeri strains were grown to the indicated optical density in SWT medium. The medium was separated from the cells by centrifugation and subsequent filtration through a 0.2-µm filter. Overnight cultures of the V. harveyi reporter strains BB170 and BB886 (Table 4.1) were diluted 1:5000 in autoinducer bioassay (AB) medium (Greenberg et al., 1979), and the V. fischeri conditioned broth added to a final concentration of 10%. Luminescence of 1 mL V. harveyi cultures was periodically measured using a TD, 20/20 luminometer (Turner design, Sunnyvale, CA). Unconditioned medium served as a negative control, and culture supernatants of either an overnight culture of wild-type V. harveyi BB120 or the AI-1 mutant V. harveyi BB152 (Table 4.1) were used as positive controls. I report here only a 2 to 5-fold induction of the V. harveyi biosensor strain, whereas many studies report induction of several 100-fold. I used supernatants of V. harveyi wild type and AI-1 mutant strains as positive controls in the experiment. Because supernatants of both V. fischeri and V. harveyi yielded comparable levels of activity, and I was able to detect differences between strains and at different ODs, I believe that this discrepancy may arise because I determined luminescence in a vial-reader rather than a plate-reader luminometer.

The concentration of C8-HSL produced by *V. fischeri* cultures grown in AB medium was determined at the same time. Supernatants of cultures at different optical densities were extracted with acidified acyl acetate, and concentrated using evaporation.

The amount of C8-HSL in the concentrated samples was determined using a previously described bioassay (Schaefer *et al.*, 2000).

Signal extractions were carried out three times from independent V. *fischeri* cultures, and from each extraction, the induction of luminescence expression of the AI-2 reporter strain and induction of  $\beta$ -galactosidase expression in the C8-HSL reporter strain was determined in triplicate.

#### Transcriptional activity of *luxS* and *ainS*

Two plasmids were generated to determine the transcriptional activity of the *luxS* and *ainS* genes in *V. fischeri*. The *lacZ* gene was isolated from plasmid pKV124 (Table 4.1) by *BamH*I restriction digest and blunt ended using Klenow fragment. To obtain the transcriptional fusions *ainS::lacZ* and *luxS::lacZ*, the *lacZ* gene was ligated (i) into the *EcoRV* site located approximately 200 bp downstream of the translational start site of the *ainS* gene carried on pCL112 (Table 4.1) creating pCL152 and (ii) into the *EcoRV* site located approximately, 200 bp downstream of the translational start site of the *luxS* gene carried on pCL125 (Table 4.1), creating pCL154. Each plasmid 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 SWT Erm or SWT Cm and the transcriptional activity of *ainS* and *luxS* was determined by assaying  $\beta$ -galactosidase activity of the cultures at different time points during growth using a standard ONPG method (Sambrook *et al.*, 1989).  $\beta$ -galactosidase assays were conducted in triplicate.
### RESULTS

## LuxS-signal activity in V. fischeri

To investigate whether V. fischeri produces a signal activity that is dependent on a functional luxS gene, I tested the ability of V. fischeri wild type and the luxS mutant culture supernatants to induce luminescence in the V. harveyi reporter strains BB170 and BB886 (Table 4.1). Supernatants harvested from mid-log phase cultures of V. fischeri wild type, but not of the *luxS* mutant, induced luminescence in the AI-1 receptor mutant V. harveyi BB170, similar to supernatants harvested from wild-type V. harveyi BB120 (Fig. 4.1). Complementation of the *luxS* mutation by providing a functional *luxS* gene in trans restored the ability to induce luminescence (Fig.4.1). 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 V. fischeri wild type nor the luxSmutant induced luminescence in the AI-2 receptor mutant V. harveyi BB886, whereas medium conditioned by wild-type V. harveyi BB120 did (Fig. 4.1), demonstrating that the V. fischeri LuxS-activity is, as V. harveyi AI-2, dependent on the presence of the AI-2 receptor protein. Taken together, my data indicate that V. fischeri produces a LuxSsignal in culture, which is likely to be identical to V. harveyi AI-2.

## Luminescence expression of V. fischeri luxS mutants in culture

Having established that V. fischeri possesses LuxS-signal activity, I asked whether this signal is important for luminescence expression in V. fischeri, similar to what has been shown in V. harveyi. I determined the luminescence levels of V. fischeri wild type, the luxS, ainS and ainS-luxS mutants in culture with and without the addition of culture supernatant of V. harveyi BB152, an acyl-HSL synthase mutant, synthetic C8-HSL or 3-oxo-C6-HSL (Table 4.2). Luminescence expression of the *luxS* mutant was slightly decreased (60-80%) compared to the wild-type strain (Table 4.2). When the growth medium contained 10% culture supernatant of V. harveyi BB152, the luminescence expression of wild-type V. fischeri and the luxS mutant were indistinguishable (Table 4.2), providing further evidence that V. harvevi and V. fischeri LuxS synthesize identical compounds capable of cross-inducing luminescence expression. The luminescence levels of both these cultures were lower than that of the wild-type control with no addition. However, luminescence expression of V. fischeri is highly dependent on medium composition, in particular on the ionic content of the medium; i.e., luminescence levels are highest in medium containing natural seawater (data not shown). The fact that cultures supplemented with V. harveyi supernatant contained 10% non-seawater based medium (AB medium) might explain the lower absolute luminescence levels. Exogenous addition of the AinS-synthesized signal C8-HSL increased luminescence expression, but not to wild-type levels (Table 4.2). In contrast, when the LuxI-derived 3-oxo-C6-HSL was supplied exogenously, luminescence levels of *luxS* mutant and wild type were indistinguishable (Table 4.2).

Both the *ainS* and the *ainS-luxS* mutant were not detectably luminous in culture (Table 4.2). The addition of C8-HSL could restore luminescence expression of the *ainS* and the *ainS-luxS* mutants to nearly wild-type and *luxS* mutant levels, respectively, and luminescence levels were indistinguishable from the parent strains when 3-oxo-C6-HSL was supplied exogenously (Table 4.2). Because complementation of both the *luxS* and *ainS* mutation with functional genes *in trans* could restore luminescence to wild-type

levels (data not shown), these mutations are unlikely to exert a significant downstream effect.

In V. harveyi, AI-2 activates a phosphorylation cascade resulting in the inactivation of the transcriptional regulator LuxO, a negative modulator of luminescence (Bassler *et al.*, 1994b; Freeman and Bassler, 1999a). I asked whether this pathway is conserved in V. *fischeri* and determined the luminescence expression of a *luxS-luxO* double mutant in comparison to a *luxO* mutant. Consistent with the hypothesis, I found that luminescence expression of these two strains was essentially indistinguishable with and without the addition of V. *fischeri* acyl-HSLs (Table 4.2). In conclusion, as has been demonstrated for the AinS-signal C8-HSL (Lupp *et al.*, 2003), my data suggest that V. *fischeri* LuxS produces a signal that regulates luminescence expression through inactivation of LuxO,

# Symbiotic luminescence expression and colonization competence of V. *fischeri luxS* mutants

I next asked whether symbiotic competence of V. fischeri is compromised by a mutation in *luxS*. I monitored the luminescence expression of juvenile animals colonized by V. fischeri wild type, or the *luxS*, ainS and ainS-luxS mutants during the first 24 h of colonization (Fig. 4.2). As previously shown (Lupp et al., 2003), ainS mutant-colonized animals expressed approximately 10% wild-type luminescence levels. In contrast, the luminescence of *luxS* mutant-colonized animals was indistinguishable from wild type-colonized animals (Fig. 4.2). However, luminescence expression of animals colonized by the ainS-luxS mutant was only 50% compared to animals colonized by the ainS single

mutant (Fig. 4.2), suggesting that either luminescence expression or colonization levels were decreased.

To differentiate between these two possibilities, I determined the number of bacterial cells in the light organs of animals colonized by wild-type *V. fischeri*, the *luxS*, *ainS* or *ainS luxS* mutants at 24 and 48 h (Fig. 4.3). While I could not detect a significant difference in colonization ability between the *luxS* mutant and wild-type *V. fischeri*, I found that the *ainS-luxS* double mutant colonized the juvenile squid to only 50-75% of the level reached by the *ainS* single mutant (Fig. 4.3), correlating with the reduced level of luminescence expression in the animal (Fig. 4.2). The fact that specific luminescence of the *ainS* and the *ainS-luxS* mutants in symbiosis does not appear to be different, yet the colonization ability does, suggests that genes encoding activities other than luminescence are regulated by these signals. Complementation of the *ainS-luxS* mutant with an intact *luxS* gene *in trans* restored *ainS* mutant luminescence and colonization levels (data not shown). When squid were colonized with an *ainS-luxS-luxO* triple mutant, colonization levels were indistinguishable from wild type (Fig. 4.3), indicating that both the LuxS- and AinS-signals regulate colonization gene expression through LuxO.

To ensure that the observed limitation in colonization level is not a result of a general metabolic defect, I determined 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 observed with the *ainS* mutant (Lupp *et al.*, 2003), suggesting that *luxS* does not impact growth characteristics of *V. fischeri*.

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To determine whether the *luxS* mutation would reduce the competitive competence of *V. fischeri*, I performed mixed-inoculum colonization experiments (Appendix A). I could not detect a significant defect in competitive colonization ability of either the *luxS* mutant or the *ainS-luxS* mutant. Because the *ainS-luxS* mutant displayed a colonization defect one might expect to observe this defect as well in a mixed inoculum experiment. However, because both LuxS and AinS synthesize small molecules, which presumably freely diffuse, it is possible 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 (Whitehead *et al.*, 2001). To determine whether synthesis of either the *V. fischeri* LuxS signal or the AinS-derived C8-HSL signal, which both positively influence luminescence expression (Table 4.2), correlate with luminescence expression, I determined both signal activities during growth (Fig. 4.4 A). While C8-HSL signal concentration mirrored luminescence expression in culture, increasing more than 100-fold between the optical densities of 0.5 to 1.0 at which luminescence is induced (<0.1 nM (detection limit) to > 10 nM), AI-2 activity increased only slightly from 1.5 to 2.5 in the same range of optical densities (Fig. 4.4 A and B).

## **Trancriptional activity of** *luxS* and *ainS*

The transcriptional autoregulation of the quorum-sensing signal synthase gene, as occurs in the *V. fischeri lux* system (Fuqua *et al.*, 1996), is one mechanism to reach the threshold concentration of quorum-sensing signal at a particular cell density. To determine whether *luxS* and/or *ainS* transcription are autoregulated, I determined the transcriptional activity of these two genes throughout culture growth using *lacZ* transcriptional fusions (Fig. 4.5). The rate of *ainS* transcription changed significantly during growth, with the highest rate observed at cell densities immediately preceding luminescence induction (Fig. 4.5, Fig. 4.4 B), and coinciding with C8-HSL signal production (Fig. 4.5), correlating with the relatively constant production of the signal (Fig. 4.4 A) and consistent with a constitutive expression of this gene. Furthermore, the relative transcription rates of the two genes differed considerably; i.e., transcription levels of the *ainS* gene were low compared to those of the *luxS* gene (Fig. 4.5).

I was curious to know whether proteins involved in the three V. fischeri quorumsensing systems lux (i.e., LuxR and LuxI), ain (i.e., AinS, LuxO and LitR), and/or LuxS, regulate transcription of *luxS* and *ainS*. I determined the transcriptional activity of these two genes in quorum-sensing mutant strains carrying transcriptional *lacZ* fusions *in trans* (Table 4.3). The inactivation of either of these genes did not influence *luxS* gene transcription (Table 4.3), 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 by both introducing a *luxO* mutation into the *ainS* mutant background and supplying C8-HSL exogenously (Table 4.3). These data suggest that the *ainS* gene induces its own transcription through the synthesis of the AinS-signal and that this regulation involves inactivation of LuxO. A similarly decreased level of *ainS* transcription could also be observed in a *litR* mutant, but not in a *luxR* or a *luxI* mutant, implying that the *ain* quorum-sensing system autoregulates itself independent of the *lux* system. Neither a mutation in *luxS* nor *luxO* changed transcription levels of *ainS* significantly (Table 4.3).

## DISCUSSION

In this study, I investigated the impact of V. fischeri LuxS on luminescence and colonization ability in comparison to AinS. My work suggests that (i) V. fischeri LuxS synthesizes a compound that is indistinguishable from V. harveyi AI-2, (ii) the inactivation of the luxS gene results in a defect in luminescence expression in culture, but not in symbiosis, (iii) mutation in V. fischeri luxS decreases the ability to colonize the squid host when AinS is also absent (iv) the LuxS and AinS signals exert their effects through the same downstream cascade; i.e., through LuxO, and (v) luxS transcription and LuxS signal production are constitutive, whereas ainS transcription and C8-HSL signal concentration correlate to luminescence expression in culture, which is accomplished by a positive feedback autoregulatory loop.

In V. harveyi, both the LuxM derived AI-1, N-3-hydroxybutanoyl homoserine lactone, and the LuxS-derived AI-2 regulate the expression of luminescence and other genes through the inactivation of the transcriptional regulator LuxO (Bassler *et al.*, 1994b; Freeman and Bassler, 1999a; Mok *et al.*, 2003). Similarly, my results demonstrate that the inactivation of the V. *fischeri luxS* gene causes a defect in luminescence expression and colonization competence, and that this defect could be relieved by the inactivation of LuxO (Table 4.2). These data, together with my previous findings that the signal synthesized by the LuxM homolog AinS, operates through LuxO (Lupp *et al.*, 2003), suggest that both the AI-1 and AI-2 pathways are conserved between V. *fischeri* and V. harveyi (Fig. 4.6). However, it appears that in V. *fischeri* the AinSsynthesized signal C8-HSL is of greater importance for luminescence expression than the LuxS-derived signal, because the inactivation of *ainS* resulted in a dark mutant, whereas a luxS mutant expressed approximately 70% wild-type luminescence levels (Table 4.2, Lupp et al., 2003). In addition to its inactivation of the negative regulator of luminescence LuxO, I proposed that the AinS-signal induces luminescence through binding to LuxR (Lupp et al., 2003; Fig. 4.6). It is therefore possible that the relative effect of the V. fischeri LuxS-signal on inactivation of LuxO is stronger than my data imply (Table 4.2), but that luminescence induction by C8-HSL-LuxR is dominant. Nevertheless, it seems unlikely that the quantitative input of the LuxS-signal is equal to that of C8-HSL because, similar to the luminescence phenotype, my results demonstrate that V. fischeri LuxS does not significantly affect host colonization, whereas a mutation in ainS does (Fig. 4.2 and 4.3). Specifically, a luxS mutation affected colonization ability only when AinS-signal was absent; i.e., colonization levels of the *ainS-luxS* double mutant were decreased to 50% that of the *ainS* single mutant (Fig. 4.3). While these data supply further indication that these two signals feed into the same downstream cascade (Fig. 4.6), they also demonstrate the negligible impact of the LuxS-signal on colonization. The absence of a strong defect in colonization ability of luxS mutants is not without precedence. Although the regulation of niche-specific genes has been demonstrated for many pathogens (Xavier and Bassler, 2003), colonization competence of *luxS* mutants has been investigated in only a few bacterial species and observed defects were in most cases either small or not detectable (McNab and Lamont, 2003).

In V. harveyi, both AI-1 and AI-2 were reported to act synergistically in the induction of luminescence and other gene expression; i.e., the absence of either of the signals results in a dramatic decrease in luminescence expression (Mok *et al.*, 2003). In contrast, a recent study demonstrated that V. cholerae possesses a regulatory cascade

similar to that of V. harveyi, and that at least two signals feed into this cascade, but that the quantitative signal input differs (Miller *et al.*, 2002). The two signals are the LuxSderived AI-2 and an as yet to be identified signal synthesized by an enzyme designated CqsA. Similar to my results, a *cqsA* mutant was found to be defective in expressing luminescence from the V. harveyi lux genes in trans, whereas a mutation in luxS did not have a significant effect (Miller *et al.*, 2002). These and my data suggest that even though a complete regulatory circuit is conserved, there can be significant differences in the importance of each of the involved genes and proteins on the induction of downstream genes.

In a further attempt to dissect the impact of the LuxS and AinS-derived signals, I determined the concentration of the two signals and the transcriptional activity of luxS and ainS during growth in rich medium. Although AI-2 is produced in a growth-phase dependent manner in many bacterial species (Surette and Bassler, 1998; Joyce *et al.*, 2000; Burgess *et al.*, 2002; Dove *et al.*, 2003; Kim *et al.*, 2003), nutrient composition of the medium and other physiological factors appear to impact AI-2 production more than cell density (Surette *et al.*, 1999; Burgess *et al.*, 2002; Hardie *et al.*, 2003; Kim *et al.*, 2003). It has therefore been proposed that AI-2 activity is dependent on the metabolic state of the cell and not on cell density (Surette *et al.*, 1999; Beeston and Surette, 2002). I did not extensively study the environmental conditions in which *V. fischeri* produces AI-2, but chose growth conditions that resulted in a >1000-fold difference in luminescence output, a phenotype regulated by *luxS*. Under these conditions, neither the LuxS-signal concentration nor *luxS* transcription, correlated convincingly to luminescence expression (Fig. 4.4 and 4.5). In contrast, the pattern of both C8-HSL concentration and

transcriptional activity correlated well with luminescence expression; i.e., both were highest at cell densities at which luminescence is induced (Fig. 4.4 and 4.5). However, it should be noted that while the concentration of C8-HSL can be determined directly 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. Nevertheless, even at cell densities that preceded luminescence induction (i.e., OD < 0.5), LuxS-signal and *luxS* transcription were comparably high (Fig. 4.4 and 4.5), suggesting an absence of correlation between signal production and regulated phenotype. Furthermore, *V. harveyi* supernatants, presumably containing high levels of AI-2 did not stimulate *V. fischeri* wild type luminescence at cell densities preceding luminescence induction (Table 4.2 and data not shown), indicating that the signal is saturating for luminescence expression even at low cell densities. Finally, as expected by the constitutive transcription levels, *luxS* gene transcription was not affected by mutations of known proteins of the *V. fischeri* quorum-sensing system (Table 4.3).

Because the change in rate of C8-HSL signal production and transcriptional activity of *ainS* correlated, it is likely that *ainS* gene transcription rate directly determines signal concentration. The fact that *ainS* gene transcription was not detectable in the absence of C8-HSL (Table 4.3), suggests that regulation of *ainS* gene transcription, and therefore the change in C8-HSL signal concentration during growth, relies on an autoregulatory mechanism. The inactivation of the transcriptional regulator LitR abolished *ainS* gene transcription, implying that LitR either directly or through another transcriptional protein positively regulates *ainS* gene transcription (Fig. 4.6). C8-HSL has been proposed to activate a phosphorelay cascade resulting in the inactivation of

LuxO, which in turn is a negative regulator of LitR (Lupp *et al.*, 2003; Miyamoto *et al.*, 2003). Therefore, inactivation of LuxO should result in increased levels of *litR* and therefore increased levels of *ainS*; however, *ainS* transcriptional levels were indistinguishable between wild type and the *luxO* mutant (Table 4.3). 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* is below my detection levels. Further evidence for this notion is that the positive autoregulatory circuit operates through the LuxO-LitR pathway is that *ainS* transcription levels were indistinguishable from wild type when measured in an *ainS-luxO* mutant background (Table 4.3). Mutations in neither *luxR* nor *luxI* affected *ainS* gene transcription, demonstrating that the regulation is independent of the LuxR-I quorum sensing system.

Because the effects of the LuxS-signal on luminescence and colonization competence could be relieved by the introduction of a *luxO* mutation (Table 4.2 and Fig. 4.3), this signal appears to be transduced through the LuxO-LitR cascade. Thus, one would expect decreased *ainS* transcription levels in a *luxS* mutant as well. However, *ainS* gene transcription levels were indistinguishable between the *luxS* mutant and wild type, providing additional evidence that the relative effect of the LuxS-signal in this regulatory cascade is much lower than that of the AinS-signal.

Since LuxS was discovered in *V. harveyi*, the question of why a bacterium would utilize two inputs into the same regulatory cascade has led to many speculations. It was originally proposed that the species-specific acyl-HSL signal senses cell density of the bacterium's own species, whereas the unspecific AI-2 senses the cumulative cell density of all bacterial species (Bassler *et al.*, 1997). The discovery that AI-2 signal production is dependent on the metabolic state of the cell rather than cell density per se has later led to the hypothesis that AI-2 reflects a change in environmental conditions through a change in the metabolic activity of the bacterial population (Xavier and Bassler, 2003). However, one might argue that a highly dense culture is indicative of good growth conditions by itself, and the fact that a change in environment can be detected through conditions that provide more specific information about the environment; i.e., the presence of mechanisms for sensing and responding to ambient nutrients, pH, Mg<sup>2+</sup> concentration or others, might mean that AI-2's rather unspecific measure of 'metabolic state' was redundant. In any case, 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 byproduct of a common detoxifying pathway (Winzer *et al.*, 2002).

My study unfortunately provides very little evidence to support or refute either of these theories. The inactivation of *luxS* did not result in a growth defect either in culture or in symbiosis (data not shown and Fig. 4.2), suggesting that the absence of LuxS activity does not significantly affect metabolic processes, at least under my experimental conditions. My data demonstrating that the effects of a *luxS* mutation could be relieved by a *luxO* mutation (Table 4.2 and Fig. 4.3) provide evidence for a signal function exerting its effect through a transduction cascade. However, this putative signal does not appear to be important for the transition of *V. fischeri* cells from a planktonic state in seawater to the rich environment in the squid light organ, because a mutation in *luxS* did not decrease colonization competence by itself (Fig. 4.2 and 4.3). In addition to monospecific light-organ associations, *V. fischeri* is part of the multi-species community in the guts of fishes (Ruby, 1977; Haygood, 1993; Makemson and Hermosa, 1999). Based on

the interspecies communication theory, one might speculate, that in these environments the *V. fischeri* LuxS-pathway becomes important and AI-2 produced by other bacteria might have an impact on colonization competence.

Taken together, under my experimental conditions and compared to AinS, V. *fischeri* LuxS does not have a significant impact on either luminescence expression or colonization competence.

## TABLES

 Table 4.1. Bacterial strains and plasmids.

Strains and plasmids	Description	Reference
V. fischeri		
Wild type	Strain ES114, isolate from E. scolopes	(Boettcher and
	light organ	Ruby, 1990)
ainS mutant CL21	ainS gene partially deleted and replaced	(Lupp et al., 2003)
	by a chloramphenicol-resistance (cat)	-
	marker	
<i>litR</i> mutant PMF8	<i>litR</i> gene inactivated by insertion of a	(Fidopiastis et al.,
	kanamycin-resistance marker (kanR)	2002)
luxI mutant VCW2G7	<i>luxI</i> gene inactivated by a frameshift	(Lupp et al., 2003)
	mutation	
luxO mutant CL42	<i>luxO</i> gene inactivated by insertion of a	(Lupp et al., 2003)
	kanamycin-resistance marker (kanR)	
luxR mutant CL53	luxR gene inactivated by insertion of a	This study
	erythromycin-resistance marker (ermR)	
huxS mutant CL39	luxS gene inactivated by insertion of a	This study
	kanamycin-resistance marker (kanR)	
ainS-luxS mutant CL41	Double mutant carrying mutations as	This study
	described above	
luxS-luxO mutant CL90	Double mutant carrying mutations as	This study
	described above	
ainS-luxS-luxO mutant	Triple mutant carrying mutations as	This study
CL91	described above	
V. harveyi		
BB120	Wild-type strain	(Bassler et al.,
		1997)
BB152	luxLM mutant, AI-1 deficient	(Bassler et al.,
		1997)
BB170	LuxN mutant, AI-1 receptor deficient	(Bassler et al.,
		1997)
BB886	LuxPQ mutant, AI-2 receptor deficient	(Bassler et al.,
		1 <b>997</b> )

Table 4.1.	continued.	

Strains and plasmids	Description	Reference
Plasmids		
pCL112	2.1 kb <i>Hae</i> III fragment carrying the <i>ainS</i> gene in pVO8	(Lupp et al., 2003)
pCL115	2.7-kb V. fischeri ES114 DNA with the luxS gene cloned into pEVS79	This study
pCL122	pCL115 with random TnKan insertion 70 bp downstream of the transcriptional start site	This study
pCL125	1.1-kb V. fischeri ES114 DNA with the luxS gene cloned into pVO8	This study
pCL145	1.8-kb V. fischeri ES114 DNA with the luxO gene cloned into pEVS79	(Lupp et al., 2003)
pCL149	Allelic exchange vector pEVS79 carrying partially deleted <i>luxR</i> gene replaced by a erythromycin-resistance ( <i>erm</i> ) marker	This study
pCL152	pCL112 with the <i>lacZ</i> gene from pKV124 cloned into the <i>Eco</i> RV site approximately 200 bp downstream of the translational start site	This study
pCL154	pCL125 with the <i>lacZ</i> gene from pKV124 cloned into the <i>Eco</i> RV site approximately 200 bp downstream of the translational start site	This study
pCL155	pCL145 with <i>luxO</i> gene inactivated by a insertion of <i>ermR</i> -marker into the <i>Nsi</i> I site	This study
pEVS79	Allelic exchange vector	(Stabb and Ruby, 2002)
pEVS94	Origin of the ermR cassette	(Stabb and Ruby, 2002)
рКV30	Cloning vector carrying partially deleted <i>luxR</i> gene replaced by a erythromycin-resistance ( <i>erm</i> ) marker	(Visick et al 2000)
pKV124	Origin of the $lacZ$ gene	(Visick and Skoufus, 2001)
pMU106	Allelic exchange vector carrying partially deleted <i>ainS</i> gene replaced by a chloramphenicol-resistance marker	(Lupp et al., 2003)
pVO8	V. fischeri cloning vector, ermR	(Visick and Ruby, 1997)

	Additions <sup>b</sup>			
Strain	none	V. harveyi	C8-HSL	3-oxo-C6-HSL
		B152		
Wild type	5.7 (0.1)	3.1 (0.1)	8.2 (1.1)	17,000 (3,900)
luxS	4.2 (0.2)	2.9 (0.2)	5.1 (0.4)	22,000 (3,600)
ainS	₿D°	$ND^d$	4.3 (0.2)	5 (1)
ainS-luxS	BD	ND	2.6 (0.1)	4 (1)
luxO	6.8 (0.8)	ND	12.2 (2.9)	24,000 (4,000)
luxS-luxO	8.3 (1.1)	ND	17.2 (4.0)	37,000 (1,500)

Table 4.2. Luminescence expression of V. fischeri luxS mutants.<sup>a</sup>

<sup>a</sup> Specific luminescence values (x  $10^{-2}$  quanta s<sup>-1</sup> cell<sup>-1</sup>) are the means of cultures with an optical density (at 600 nm) of between 1.0 and 5.0; standard errors of the means are indicated in parentheses. The results shown are from a representative experiment.

<sup>b</sup> Cultures were grown in SWT medium without additions or with the addition of 10% over-night culture supernatant of *V. harveyi* BB152, or 120 nM C8-HSL, or 120 nM 3-oxo-C6-HSL.

<sup>c</sup> Below detection ( $< 2 \times 10^{-4}$  quanta s<sup>-1</sup> cell<sup>-1</sup>)

<sup>d</sup>Not determined

## Table 4.3. Transcriptional activity of V. fischeri luxS and ainS.\*

Strain	luxS	ainS
Wild type	205 (46)	17.8 (4.4)
ainS	189 (15)	2.0 (0.1)
ainS + C8-HSL	ND <sup>b</sup>	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)

<sup>a</sup> Transcriptional activity of *ainS* and *luxS* in different genetic backgrounds.  $\beta$ -galactosidase activity (Miller Units) was measured during growth and averaged for optical densities between 1 and 5. Shown is a representative experiment, with standard errors in parentheses.

<sup>b</sup> Not determined



Fig. 4.1. LuxS activity of V. fischeri. The V. harveyi reporter strains BB170 (AI-1 receptor mutant, black bars) and BB886 (AI-2 receptor mutant, striped bars) were incubated in medium containing 10% (v/v) culture supernatants of either V. fischeri wild type or the *luxS* mutant. In each case, the V. fischeri strain carried in trans either a vector control or a functional copy of the *luxS* gene on pCL125 (Table 4.1). The addition of wild-type V. harveyi BB120 supernatant served as a positive control. Shown is the fold-difference in luminescence expression of each strain over the negative control (the reporter strain incubated in medium) of a representative experiment. Standard errors are indicated.



Fig. 4.2. Luminescence expression of *luxS* mutants in symbiosis. Animal luminescence was monitored during the initial stages of *E. scolopes* colonization by *V. fischeri* wild-type (diamonds), *ainS* mutant (squares), *luxS* mutant (triangles) and *ainS-luxS* mutant (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. 4.3.** Colonization competence of *luxS* mutants. Colonization levels of the *ainS* mutant (striped bars), *luxS* mutant (white bars), *ainS luxS* mutant (hatched bars) and the *ainS-luxS-luxO* mutant (grey bars) relative to *V*. *fischeri* wild type (black bars) were measured at 24 and 48 h post-inoculation. Each bar represents the mean value of 15 animals with the associated standard errors. The experiment was conducted twice with the same outcome.



**Fig. 4.4.** Relationship between LuxS-signal and AinS-signal activity and luminescence expression during growth of *V. fischeri* in culture. A LuxS-signal activity (closed diamonds) is presented as fold-induction of luminescence expression of the *V. harveyi* reporter strain BB170 over the negative control. The concentration of the AinS-signal, C8-HSL (open diamonds), was determined using synthetically produced C8-HSL as standard. **B** Luminescence expression of a *V. fischeri* culture during growth. Shown is a representative experiment; standard deviation bars were smaller than the symbols.



Fig. 4.5. Transcriptional activity of *ainS* and *luxS* in culture.  $\beta$ -galactosidase activity of a *V. fischeri* wild-type cells carrying either a *luxS::lacZ* fusion (closed diamonds) or an *ainS::lacZ* fusion (open diamonds) *in trans* on plasmids pCL152 and pCL154, respectively, were measured during growth. Shown is the cumulative data of three independent cultures; standard deviation bars were smaller than the symbols.



**Fig. 4.6.** Model for the regulation of luminescence and colonization genes by *V. fischeri* LuxS and AinS. See Discussion for explanation.

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## CHAPTER 5

## THE VIBRIO FISCHERI QUORUM-SENSING SYSTEM AIN NEGATIVELY REGULATES MOTILITY AND IS IMPORTANT FOR EARLY STAGES OF COLONIZATION

## ABSTRACT

Vibrio fischeri possesses at least two quorum-sensing systems, ain and lux, involved in the regulation of luminescence and possibly other colonization factors. I have previously demonstrated that the ain system activates luminescence gene expression at cell densities that precede lux system activation and that both systems are required for persistent colonization of the squid host, Euprymna scolopes. In this study, a recently developed V. fischeri microarray was used to identify genes specifically regulated by the ain system. I found that flagellins and flagellar basal body genes were repressed by ain quorum sensing while genes homologous to a Vibrio vulnificus extracellular polysaccharide (EPS) biosynthesis locus were positively regulated. Neither putative iron uptake systems nor genes coding for metalloproteases, both affected by quorum sensing in other Vibrio species, were differentially regulated in V. fischeri. Consistent with the microarray data, I was able to demonstrate a nutrient-dependent motility defect, and a difference in both biofilm formation and colony morphology of quorum-sensing mutants in vitro. Furthermore, the inactivation of the ain, but not the lux, system resulted in a delay in initiation of the symbiotic relationship between V. fischeri and E. scolopes. Specifically, V. fischeri strains carrying mutations in the signal synthase gene ainS colonized the juvenile squid to only 50% that of the levels reached by wild type or the luxI mutant at 12 h post inoculation. In addition, I found that the lux system is not fully active until 12 h post inoculation, which together with the fact that the V. fischeri luxI mutant colonized as well as wild type implies that this system is not required until later stages of colonization. Taken together, my data suggest the ain system is essential for the regulation of colonization factors important in the initial stages of colonization, where cell densities are below the threshold density of luxdependent quorum sensing.

#### INTRODUCTION

The association between the marine luminescent bacterium Vibrio fischeri and the Hawaiian bobtail squid, *Euprymna scolopes*, represents a model system for the study of mutualistic bacteria-host interactions that has been successfully used for the investigation of the processes underlying the initiation, accommodation and persistence of symbioses (Ruby, 1996; Ruby, 1999). Several genetic factors required for the different stages of the cooperative association have been identified, for example (i) motility is essential for the initiation of the symbiosis (Graf *et al.*, 1994; Visick and Skoufos, 2001; Millikan and Ruby, 2002), (ii) the ability to acquire iron is important for the attainment of a normal level of colonization (Graf and Ruby, 2000), (iii) bacterial defense against oxidative stress is necessary to compete effectively with the wild-type parent strain (Visick and Ruby, 1998), (iv) luminescence expression is required for maintenance of a persistent colonization (Visick *et al.*, 2000), and (v) the regulation of colonization-gene expression is important for each of these stages (Visick *et al.*, 2000; Visick and Skoufos, 2001; Lupp *et al.*, 2003; Millikan and Ruby, 2003).

The coordinated expression of colonization-related traits can be established by a process termed quorum sensing, which relies on the secretion of small signal molecules accumulating in the surrounding environment. Once the bacterial population reaches a threshold cell density, the concentration of the quorum-sensing signal becomes sufficient to induce gene expression either through directly interacting with a transcriptional regulator or by induction 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 non-permissive environments. In Gram-negative bacteria the most commonly utilized signals are either acylated homoserine lactones (acyl-HSLs) synthesized by enzymes belonging to one of two families, the LuxI and the LuxM type; or a furanosyl borate diester, synthesized by the LuxS enzyme. While acyl-HSLs are

species-specific due to differences in the acyl side chain, the LuxS-signal appears to be ubiquitous; i.e., many Gram-negative and Gram-positive species produce and respond to this signal molecule (Whitehead *et al.*, 2001).

Quorum sensing based on a LuxR-I system was first described in V. fischeri as the process regulating luminescence expression (Eberhard et al., 1981). 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 binding to the transcriptional activator protein LuxR (Engebrecht and Silverman, 1984; Meighen, 1991). The LuxR-acyl-HSL complex binds to the *lux* promoter and induces the transcription of the *luxICDABEG* locus (Fuqua et al., 1996). The V. fischeri lux system is required for both luminescence expression in vivo and persistence of the bacterial symbiont in the squid light organ (Visick et al., 2000).

A second type of acyl-HSL quorum sensing, also utilized for the regulation of luminescence gene expression, was later discovered in *Vibrio harveyi* (Bassler *et al.*, 1993). This bacterium produces two quorum-sensing signals coordinately regulating luminescence gene expression, the *V. harveyi* autoinducer-1, *N*-3-hydroxybutanoyl homoserine lactone, synthesized by LuxM, and the LuxS-derived autoinducer-2 (Mok *et al.*, 2003). The signals bind to cognate hybrid two-component sensor kinase proteins, LuxN and LuxPQ, respectively (Bassler *et al.*, 1994a; Freeman *et al.*, 2000), which transduce information through a phosphorelay cascade involving a protein designated LuxU (Freeman and Bassler, 1999b), and leading to the inactivation of the transcriptional regulator LuxO (Bassler *et al.*, 1994b; Freeman and Bassler, 1999a). The inactivation of LuxO results in increased transcription of LuxR, a positive regulator of luminescence gene expression in *V. harveyi* (Miyamoto *et al.*, 2003).

I have recently demonstrated that V. fischeri possesses two systems homologous to the autoinducer-1 and autoinducer-2 systems in V. harveyi (Lupp et al., 2003; Chapter 4). The LuxM homolog AinS synthesizes a *N*-octanoyl homoserine lactone signal (C8-HSL), which was found to regulate luminescence and colonization factor expression through the inactivation of LuxO (Lupp *et al.*, 2003). Inactivation of LuxO results in an increased transcription of *litR*; LitR, a homolog of *V. harveyi* LuxR, in turn positively regulates transcription of *V. fischeri luxR* (not homologous to *V. harveyi luxR*) (Miyamoto *et al.*, 2003). My data suggested that the ain and lux systems sequentially induce the expression of luminescence and possibly other colonization genes (Lupp *et al.*, 2003). Furthermore, as in *V. harveyi*, the *V. fischeri* LuxS-signal was shown to regulate luminescence expression and colonization competence through LuxO. However, compared to the *ain* and the *lux* quorum-sensing systems the impact of the LuxS-signal on luminescence expression and colonization competence was minor (Chapter 4).

Several other Vibrio species have been found to possess homologs of components of the autoinducer-1 and autoinducer-2 regulatory cascades that are linked to colonization factor expression (McDougald et al., 2001; Milton et al., 2001; Shao and Hor, 2001; Croxatto et al., 2002; Miller et al., 2002; Zhu et al., 2002). For example, besides its effects on luminescence, V. harveyi LuxO has been shown to be involved in siderophore production and a mutation results in altered colony morphology (Lilley and Bassler, 2000). Similarly, a mutation in Vibrio cholerae LuxO affects tcp operon expression, HA protease activity, motility and biofilm formation (Zhu et al., 2002; Vance et al., 2003). The Vibrio anguillarum LitR homolog VanT regulates metalloprotease activity and pigment production as well as biofilm formation, which might be related to a defect in extracellular polysaccharide (EPS) production (Croxatto et al., 2002). Similarly, the Vibrio parahaemolyticus LitR homolog OpaR regulates colony opacity and biofilm formation through EPS production (McCarter, 1998; Guevener and McCarter, 2003). Finally, a mutation in the Vibrio vulnificus LitR homolog SmcR reduces extracellular metalloprotease activity (Shao and Hor, 2001).
In this study I aimed to identify genes, besides those involved in luminescence, that are regulated by the *V. fischeri* ain quorum-sensing system. My data suggest that motility is repressed by the ain system, whereas EPS production is positively regulated. Furthermore, I found that the *ainS* mutant is delayed in initiating colonization of *E. scolopes*, a phenotype that is consistent with its hypermotile phenotype. Finally, I demonstrate that the lux system is not fully active until 12 h post inoculation, while the effects of the ain system are already apparent at this time point, indicating that the two systems are important at different stages of colonization.

#### MATERIALS AND METHODS

#### **Bacterial strains and growth conditions**

Medium reagents were purchased from Difco Laboratories (Sparks, MD) and Sigma Chemical Co. (St. Louis, MO). Strains and plasmids used in this study are listed in Table 5.1. *V. fischeri* strains were grown at 28 °C either in a seawater-based nutrient medium (SWT) (Boettcher and Ruby, 1990) or Luria-Bertani Salt (LBS) medium (Graf *et al.*, 1994). *E. coli* strains were grown at 37°C in Luria-Bertani medium (LB) (Sambrook *et al.*, 1989). Media were solidified with 1.5% (w/v) agar as needed. Antibiotics were added to the media at the following concentrations when appropriate: chloramphenicol (Cam; 2.5 µg/mL for *V. fischeri*, 20 µg/mL for *E. coli*), kanamycin (Kan; 100 µg/mL for *V. fischeri* and *E. coli*). 3-oxo-C6-HSL was obtained from Sigma Chemical Co.

## **Genetic techniques**

Genomic and plasmid DNA were extracted using Qiagen DNeasy and Qiaprep Miniprep systems (Qiagen Inc., Valencia, CA), respectively. PCR was performed according to standard protocols (Sambrook *et al.*, 1989), using AmpliTaq DNA polymerase (Perkin-Elmer Inc., Branchburg, NY). For plasmid constructions, restriction enzymes and DNA ligase were obtained from New England Biolabs, Inc. (Beverly, MA) and used according to the manufacturer's protocol. Transfer of plasmids into *E. coli* host strains was accomplished using standard techniques (Sambrook *et al.*, 1989). Triparental conjugation was used to transfer plasmids into *V. fischeri* strains (Stabb *et al.*, 2001). Sequencing was carried out on a Perkin-Elmer ABI Prism automated sequencer at the University of Hawaii Biotechnology/Molecular Biology Instrumentation and Training Facility.

#### **Mutant construction**

The LuxOD47E mutant was constructed using the QuikChange<sup>TM</sup>Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene, LaJolla, CA). pCL145 (Table 5.1), carrying the intact *luxO* gene in the allelic exchange vector pEVS79 (Table 5.1) served as a template to create the site-directed mutation. The aspargine <u>GAT</u> codon in position 47 of the LuxO protein was changed into a glutamine <u>GAG</u> codon by introducing a T->G point mutation using the primers: CL59 5' CCT GAT CTT GTG TTA CTA <u>GAG</u> TTG CGC CTG CCT GAC 3' and CL60 5' GTC AGG CAG GCG CAA <u>CTC</u> TAG TAA CAC AAG ATC AGG 3'. The resulting plasmid, pCL150, was transferred into the *V. fischeri luxO* mutant CL42 by triparental mating, and single and double recombinants that had lost kanamycin resistance were selected as previously described (Stabb *et al.*, 2001), generating the *V. fischeri* LuxOD47E mutant strain CL59. The introduction of the point mutation into the genome was confirmed by sequence analysis of the *luxO* gene. The luminescence and colonization phenotypes of the LuxOD47E mutant are presented in Appendices B to F.

#### Microarray comparison

The V. fischeri luxO and LuxOD47E mutants were grown in SWT medium to an optical density (OD600) of 2.5. RNA extractions were carried out as previously described (Schuster *et al.*, 2003). Reverse transcription, labeling of cDNA, and hybridization of the microarray slide were carried out according to a protocol developed by Amy Schaefer, University of Hawaii (Appendix G and H). Data analysis was carried out using Microsoft Excel.

#### Siderophore and biofilm production

The ability of *V. fischeri* strains to produce siderophores was determined using chrome azurol S (CAS) medium agar plates as previously described (Lee and Ruby, 1994).

To visualize biofilm production, *V. fischeri* strains were inoculated into SWT medium to an optical density of 0.01 and incubated at 28 °C for 24 h in new borosilicate tubes. Cultures were removed and the tubes extensively washed with de-ionized water. Tubes were incubated with a 1% crystal violet solution for 15 min at 25 °C, and again washed with de-ionized water to remove excess dye. The extent of biofilm present correlates with dye intensity.

### Motility and chemotaxis assays

Motility and chemotaxis behavior of *V. fischeri* was determined by growing wildtype and mutant strains in SWT liquid medium to an optical density of approximately 0.4. Three microliters of the culture were spotted on the surface of a fresh agar plate, and the relative diameters of the colony and the expanding migration rings were determined after 6 to 8 h incubation at 25 °C. Assays were carried out on (i) SWT medium containing 0.4 or 0.7% agar, or (ii) defined medium containing 0.25% agar, 10 mM NH4Cl, 0.33 mM K2HPO4, 300 mM NaCl, 50 mM MgSO4, 10 mM CaCl<sub>2</sub>, 10 mM KCl, 0.01 mM FeSO4, and 50 mM Tris-HCl (pH 7.4) to which was added either 1 % (w/v) tryptone and 2 mM serine, or 0.3% (w/v) casamino acids as carbon sources.

#### **Colonization assays**

Two 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 (Ruby and Asato, 1993). Briefly, newly hatched squids

were placed into vials containing 4 mL of filter-sterilized (0.2 µm pore size) seawater to which an inoculum of approximately 4,000 colony-forming units (CFUs) of the indicated strain was added. Twelve to 24 individual animals were infected per treatment group; 6 animals served as uninoculated controls and were placed into filter-sterilized seawater without added bacteria. When appropriate, squid were transferred into seawater containing 120 or 1200 nM 3-oxo-C6-HSL after 6 h incubation. Animal bioluminescence, an indirect measure of the degree of squid colonization, was monitored periodically over 24 h using a Packard Tri-Carb 2100TR scintillation counter (Packard Instruments Inc., Meriden, CT) modified to be an automated photometer.

(ii) Colonization level in the squid light organ: The number of CFU per squid was determined at 12 h post-inoculation following a previously described method (Ruby, 1996). Fifteen 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 expulsion of bacteria upon sunrise (Lee and Ruby, 1994; Graf and Ruby, 1998), 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.

#### RESULTS

## Microarray comparison of V. fischeri LuxOD47E and luxO

I have previously demonstrated that the *ain* quorum-sensing system, similar to the *lux* system, is important for colonization competence of *V. fischeri*, and that this effect is likely to be unrelated to the inability of the mutant to express maximal light *in vivo* (Visick *et al.*, 2000; Lupp *et al.*, 2003). To begin to identify colonization factors regulated by *ainS* that could be responsible for the observed colonization defect, I performed a microarray study. Because the AinS-signal, C8-HSL, and the LuxI-synthesized 3-oxo-C6-HSL are very similar, they are likely, at least to some extent, to cross-feed into each other's signaling cascades. To separate the effect of the *ain* system on gene regulation from those of the *lux* system I chose to compare the transcriptomes of the *luxO* deletion mutant to that of a LuxOD47E mutant grown in culture. The LuxOD47E amino acid substitution was previously shown to lock the *V. harveyi* LuxO protein in its active, phosphorylated form, mimicking the state of the protein in the absence of quorum-sensing signals (Freeman and Bassler, 1999a).

A selected summary of the microarray study is presented in Tables 5.2 to 5.6. As expected from the luminescence phenotype, the *lux* operon was repressed in the LuxOD47E mutant, although not to a high degree (1.2 to 2.1-fold, Table 5.2). The relative level of *luxI* gene transcription could not be assessed due to high standard deviation. I have proposed a model of luminescence gene regulation in *V. fischeri* in which the AinS-synthesized signal, C8-HSL, activates a signaling cascade resulting in the inactivation of LuxO, a negative regulator of luminescence expression (Lupp *et al.*, 2003). LuxO has been demonstrated to exert its effects through negatively regulating *litR* transcription (Miyamoto *et al.*, 2003), and LitR was shown to positively regulate *luxR* transcription (Fidopiastis *et al.*, 2002). Consistent with this model, both *litR* and *luxR* transcription were suppressed in the LuxOD47E mutant (Table 5.2). I have previously shown (Chapter 4) that *ainS* positively

regulates its own transcription, unfortunately, the auto-regulation could neither be confirmed nor rejected, due to high variability of the data (Table 5.2). Interestingly, *ainR*, coding for the putative C8-HSL receptor protein and located immediately downstream of *ainS*, is repressed in the LuxOD47E mutant (Table 5.2). Data presented in Chapter 4 suggested a constitutive transcription of the *luxS* gene, accordingly, neither transcription of *luxS* nor of the putative LuxS-signal receptor genes *luxP* and *luxQ* were affected by a *luxO* mutation (Table 5.2). The absence of the complete *luxO* mRNA in the *luxO* deletion mutant was confirmed by an apparent up-regulation in the LuxOD47E mutant (Table 5.2).

The most striking finding was that motility genes were differentially regulated in the two strains. Table 5.3 presents the transcriptional regulation of all genes annotated to be involved in flagellar synthesis, export and chemotaxis. It is notable that only structural components, flagellins and flagellar basal body proteins are up-regulated in the LuxOD47E mutant, while motility regulators, motors and export apparatus do not seem to be affected (Table 5.3). Similarly, none of the 31 putative methyl-accepting chemotaxis proteins were differentially regulated in the two mutants (data not shown).

LuxO has been demonstrated to be involved in siderophore production in *V. harveyi* (Lilley and Bassler, 2000); however, transcription levels of putative iron-uptake systems were identical between *luxO* and LuxOD47E (Table 5.4). In agreement with these results, the *V. fischeri ainS*, *luxI*, *luxS*, *luxO*, LuxOD47E, *luxR* and *litR* mutants as well as the *ainS luxI*, *ainS-luxS* and *ainS-luxO* double mutants produced siderophore activity indistinguishable from wild type when assayed on CAS agar plates (data not shown).

Mutations in LuxO or LitR homologs have further been demonstrated to affect colony morphology and biofilm formation in several *Vibrio* species (McCarter, 1998; Lilley and Bassler, 2000; Shao and Hor, 2001; Croxatto *et al.*, 2002; Zhu *et al.*, 2002), which appear to correlate to EPS production (McCarter, 1998; Croxatto *et al.*, 2002). Although EPS production by *V. fischeri* has not yet been characterized, a locus homologous to a *V.* 

vulnificus EPS biosynthesis locus was repressed in the LuxOD47E mutant (Table 5.5). The V. fischeri litR mutant was reported to display a more translucent colony morphology and to produce more biofilm than wild type (Fidopiastis *et al.*, 2002; P. Fidopiastis, personal communication) and I found that V. fischeri ainS displayed a similar phenotypes (data not shown, Fig. 5.1). Whether these phenotypes are related to EPS production remains to be determined.

The production of extracellular metalloprotease activity appears to be a common feature regulated by LuxO and LitR homologs in *V. cholerae* (Vance *et al.*, 2003), *V. vulnificus* (Shao and Hor, 2001) and *V. anguillarum* (Croxatto *et al.*, 2002). While the *V. fischeri* genome does not reveal homologs of either *V. cholerae* HapA, *V. anguillarum* EmpA or *V. vulnificus* Vvp, other metalloproteases were identified by the genome annotation. None of these appear to be differentially regulated in the two LuxO mutants (Table 5.6).

Lastly, a previous study reported the regulation of the sap locus by quorum sensing (Chen *et al.*, 2000), however, there was no difference in transcription levels between the two LuxO mutants (data not shown).

#### Motility and chemotaxis phenotype of V. fischeri quorum-sensing mutants

Because of the unexpected finding that flagellin and flagellar basal body genes appear to be differentally regulated in the two LuxO mutants, I determined motility and chemotaxis behavior of several quorum-sensing mutants. As I had previously reported (Lupp *et al.*, 2003), swimming of the *ainS* and the *luxI* mutant on 0.4% and 0.7% SWT plates was indistinguishable from wild type (data not shown). However, when defined, 0.25% agar plates containing either tryptone/serine or casamino acids as the carbon sources were used, differences in motility behavior between mutant and wild-type strains became apparent (Fig. 5.2 and 5.3). The effects of mutations in quorum-sensing genes on motility were minor, but observable, in medium containing tryptone and serine. The *luxO* deletion mutant produced slightly smaller migration ring diameters, while the LuxOD47E mutant migrated faster, and a mutation in *ainS* resulted in the absence of a colony at the inoculation site (Fig. 5.2). Interestingly, the differential effects on motility observed on casamino acid plates were more dramatic (Fig. 5.3). Specifically, and in agreement with the microarray study, the *ainS*, the *litR* and the LuxOD47E mutant strains displayed hyperswimmer phenotypes in casamino acids-containing media (Fig. 5.3 A, B and C), whereas the *luxO* deletion mutant did not migrate from the inoculation site (Fig. 5.3 D). The *luxO* mutant phenotype was dominant over that of the *ainS* mutant phenotype, suggesting that AinS regulates motility, like luminescence (Lupp *et al.*, 2003), through inactivation of LuxO (Fig. 5.3 D). Mutations in *luxS* and *luxI* increased migration speed as well, however, not to the same extent as a mutation in *ainS*, whereas the *luxR* phenotype was indistinguishable from wild type. Each of the tested strains grew on casamino acid medium agar plates over-night, implying that the observed phenotypes were not due to a general metabolic defect.

#### Initiation of colonization by V. fischeri quorum-sensing mutants

The results of the previous sections illustrate that a mutation in *ainS* affects motility of *V. fischeri* (Table 5.3, Fig. 5.2 and 5.3). Motility has been demonstrated to be an essential colonization factor; i.e., non-motile and hypermotile mutants are significantly impaired in initiating light-organ colonization (Graf *et al.*, 1994; Millikan and Ruby, 2002). To determine whether either of the *V. fischeri* quorum-sensing systems is important for the initial stages of colonization I monitored light expression of squid colonized by either *V. fischeri* wild type, the *ainS*, the *luxI*, or the *ainS-luxI* mutants during the first 12 h of colonization (Fig. 5.4 A). As previously shown (Visick *et al.*, 2000), *luxI* mutant-colonized animals did not express luminescence at detectable levels, whereas *ainS* mutant-colonized animals were detectable luminous, expressing about 10% of wild-type luminescence levels (Lupp *et al.*, 2003). Such decreased light levels can either be due to a luminescence defect or to a lower number of total bacterial cells colonizing the squid light organ. To differentiate between these two possibilities, I determined the number of CFUs in squid light organs colonized by *V. fischeri* wild type, the *ainS*, the *luxI*, or the *ainS-luxI* mutants at 12h post inoculation (Fig. 5.4 B). While the dark *luxI* mutant colonized to wild-type levels, suggesting that a defect in light expression does not affect colonization efficiency during the initiation of colonization, the colonization levels of the *ainS* and the *ainS luxI* mutant were only 50% that of wild type (Fig. 5.4 B). Consequently, and in agreement with the microarray data, these data suggest that *ainS* regulates factors distinct from luminescence that are important for colonization. Because the *ainS* mutant expresses only 10% wild-type luminescence levels, but colonizes to 50% the wild-type number of bacterial cells in the squid light organ, luminescence expression is also affected by a mutation in *ainS* early in colonization.

#### Onset of lux quorum sensing

Having established that *luxI*-dependent quorum sensing does not impact the ability of *V. fischeri* to initiate colonization, I was curious to know whether this system is fully active during the early stages of colonization. Because the *lux* system is required for detectable light expression *in vivo* (i.e., *luxI* mutants do not express light), *lux* activity can be easily monitored by measuring symbiotic light output. In culture, where the *lux* system is inactive, the exogenous addition of saturating concentration of the LuxI-synthesized 3-oxo-C6-HSL stimulates light expression more than 1000-fold (Lupp *et al.*, 2003). I used a similar approach to determine *lux* activity *in vivo* by measuring luminescence expression of wild-type-colonized juvenile squid exposed to seawater containing (i) no addition, (ii) 120 nM 3-oxo-C6-HSL or (iii) 1200 nM 3-oxo-C6-HSL during the first 24 h of colonization (Fig. 5.5). To minimize any possible effects of 3-oxo-C6-HSL on colonization phenotypes

other than luminescence expression, juvenile squid were incubated with V. fischeri wild type 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 detectable luminous (Fig. 5.5). This positive effect of 3-oxo-C6-HSL on luminescence expression was apparent until approximately 12 h post inoculation; beyond 12 h, luminescence levels were indistinguishable between animals exposed to exogenous 3oxo-C6-HSL and the no-addition control (Fig. 5.5). Exposure of wild type-colonized squid to 1200 nM of the AinS-synthesized signal C8-HSL had no significant effect on the development of luminescence expression (data not shown). Because acyl-HSL are susceptible to hydrolysis, it was a concern that the absence of any positive effect on luminescence at time points beyond 12 h is due to degradation of the compound. However, because luminescence expression of animals exposed to 10-times the saturating concentration, 1200 nM 3-oxo-C6-HSL, was indistinguishable from animals exposed to 120 nM 3-oxo-C6-HSL (Fig. 5.5), it is unlikely that degradation of 3-oxo-C6-HSL affected the outcome of this experiment.

These data demonstrate, that the *luxI* system is not maximally induced until approximately 12 h post inoculation, coinciding with the time at which the internal spaces of the squid light organ become densely colonized with *V. fischeri* cells (Nyholm *et al.*, 2000).

#### DISCUSSION

Using microarray comparisons of transcriptional activity in conjunction with *in vitro* phenotype studies of quorum-sensing mutants, I identified novel genes regulated by ain quorum sensing in *V. fischeri*. I found that motility genes were repressed and a putative EPS biosynthesis locus was positively regulated, while siderophore production and metalloprotease gene expression were unaffected. Consistent with its motility phenotype, the *ainS* mutant displayed a defect in colonization initiation. In contrast, my data suggest that the lux system is neither required nor fully active during the first 12 h of colonization.

Several, if not all, *Vibrio* species possess regulatory systems involved in colonization factor expression similar to the here described ain system (McDougald *et al.*, 2001; Milton *et al.*, 2001; Shao and Hor, 2001; Croxatto *et al.*, 2002; Miller *et al.*, 2002; Zhu *et al.*, 2002; Kim *et al.*, 2003), suggesting that not only the signaling cascade but also its purpose; i.e., the regulation of host-associated traits, is conserved in the genus *Vibrio*. The colonization factors regulated in *Vibrio* species include toxins, metalloproteases, siderophores, exopolysaccharide production, luminescence and motility; some of these also appear to be regulated by quorum sensing in *V. fischeri* (Table 5.7). As expected from the symbiotic nature of its relationships with animal hosts (Ruby, 1977; Haygood, 1993; Makemson and Hermosa, 1999), *V. fischeri* is not known to express toxins nor does the genome reveal homologs to metalloproteases of *V. cholerae*, *V. anguillarum* or *V. vulnificus*, all of which are involved in pathogenicity (Milton *et al.*, 1992; Shao and Hor, 2000; Silva *et al.*, 2003).

While quorum sensing regulates siderophore production in *V. harveyi*, my data provide no evidence for a similar regulatory mechanism in *V. fischeri* (Table 5.7). However, genome analysis reveals a minimum of 5 iron uptake systems (Table 5.4), demonstrating the importance of iron acquisition in the ecology of *V. fischeri*. Besides the possibility that the lux system is involved in regulation of iron uptake, it is feasible that, as in many other

bacteria, the intracellular Fe(II) concentration regulates the expression of iron uptake systems through the transcriptional regulator Fur (Ratledge and Dover, 2000).

Interestingly, exopolysaccharide (EPS) production appears to be positively regulated by the ain quorum sensing system (Table 5.5). The function of EPS expression in bacteriahost association is diverse, involving functions such as (i) prevention of desiccation, which promotes transmission and survival, (ii) enhanced adherence to surfaces, and (iii) protection from challenges of the innate immunity, including complement-mediated phagocytosis and killing (Roberts, 1996). While the role of *V. fischeri* extracellular polysaccharides remains to be determined, EPS might play a role in either adherence to mucus secreted by the juvenile squid or in promoting survival of cells exposed to the innate immune defenses of the squid. An increase in EPS production has been correlated to an increase in biofilm formation; however, my data show an opposite effect; i.e., the *ainS* mutant produces a thicker biofilm than wild type (Fig. 5.1). An alternative explanation for this observation is that the enhanced expression of flagellin genes in the *ainS* mutant causes the increased attachment to glass surfaces (Table 5.3), as it has been demonstrated that flagella can promote adherence (Watnick and Kolter, 1999).

When planktonic V. fischeri cells colonize juvenile E. scolopes, both the animal host and the bacterial symbiont undergo dramatic developmental changes (McFall-Ngai and Ruby, 1998). Two of the most apparent differences between planktonic and symbiotic V. fischeri cells are the onset of luminescence expression and the loss of flagella upon colonization (Ruby and Asato, 1993). Flagellar motility was the first phenotype demonstrated to affect colonization competence of V. fischeri; i.e., non-motile mutants are unable to initiate the symbiotic relationship with E. scolopes (Graf et al., 1994). More recently, the specifics of colonization initiation were investigated (Nyholm et al., 2000). The first encounter between the host and potential bacterial symbionts occurs within the first hour after hatching, when V. fischeri cells adhere to mucus secreted by the epithelium of the juvenile squid light organ (Nyholm et al., 2002). The attachment results in the formation of dense bacterial aggregates, a process that is neither specific to V. fischeri nor dependent on motility. Between 4 and 6 h after hatching, only motile V. fischeri cells begin to migrate towards the pores leading into the interior of the light organ (Nyholm et al., 2000). Not only non-motile mutants, but also hyperswimmer mutants fail to initiate colonization, suggesting that the correct expression of motility is essential for the initial stages of colonization as well (Millikan and Ruby, 2002). It is therefore likely that the initiation defect of the ainS mutant observed here is directly related to its hypermotile phenotype (Fig. 5.2 to 5.4). In later stages of colonization, the majority of bacterial cells will have lost their flagella, implying that swimming motility is not essential for maintenance of the symbiosis (Ruby and Asato, 1993). In contrast, the ability to express luminescence becomes important only at later stages of symbiosis; i.e., luminescence mutants are capable of initiating symbiosis but display a persistence defect (Fig. 5.4, Visick et al., 2000). While the lux quorum-sensing system is the major player in regulation of V. fischeri luminescence expression in vivo (Visick et al., 2000), the data presented here suggest that the ain quorumsensing system might be responsible for negative regulation of flagella expression when V. fischeri begins to colonize the squid (Table 5.3).

Many Gram-negative bacteria possess multiple quorum-sensing systems (Whitehead *et al.*, 2001), raising the question of the usefulness of this apparent redundancy. My results provide a possible explanation; i.e., the utilization of multiple quorum-sensing systems allows the induction (or repression) of colonization factors important for specific phases of the colonization process. The fact that the ain system is functional and essential at stages of colonization where the lux system is neither required nor fully induced (Fig. 5.4 and 5.5), suggests that the ain system is operative at a lower threshold cell density than the lux system, providing a possible mechanism for this hypothesis. Further support for this notion is the finding that the effect of the ain system on luminescence is apparent at bacterial

concentrations occurring in culture  $(10^8 \text{ to } 10^9 \text{ cells ml}^{-1})$ , whereas the lux system is the predominant inducer of luminescence expression at the very high cell densities found within the squid light organ (>10<sup>10</sup> cells ml<sup>-1</sup>) (Lupp *et al.*, 2003). Future studies will show whether multiple quorum-sensing systems possessed by other bacteria (Whitehead *et al.*, 2001) serve the same purpose.

# TABLES

Table 5.1. Bacterial strains and plasmids.

Strains and plasmids	Description	Reference or source
E. coli		
CC118Apir	Cloning strain	(Herrero et al., 1990)
DH5a	Cloning strain	(Hanahan, 1983)
V. fischeri		
wild type	Strain ES114, isolate from <i>E. scolopes</i>	(Boettcher and Ruby, 1990)
ainS mutant CL21	<i>ainS</i> gene partially deleted and replaced by a chloramphenicol-resistance ( <i>cat</i> ) marker	(Lupp et al., 2003)
litR mutant PMF8	<i>litR</i> gene inactivated by insertion of a kanamycin-resistance marker (kanR)	(Fidopiastis <i>et al</i> , 2002)
luxI mutant VCW2G7	<i>luxI</i> gene inactivated by a frameshift mutation	(Lupp et al., 2003)
<i>luxO</i> mutant CL42	<i>luxO</i> gene inactivated by insertion of a kanamycin-resistance marker ( <i>kanR</i> )	
LuxOD47E mutant CL59	LuxO protein carrying a T->G point mutation, resulting in a D->E substitution in position 47	This study
luxR mutant CL53	<i>luxR</i> gene inactivated by insertion of a erythromycin-resistance marker ( <i>ermR</i> )	Chapter 4
luxS mutant CL39	<i>luxS</i> gene inactivated by insertion of a kanamycin-resistance marker ( <i>kanR</i> )	Chapter 4
ainS-luxI mutant CL24	Double mutant carrying mutations as described above	(Lupp et al., 2003)
ainS-luxO mutant CL64	Double mutant carrying mutations as described above	(Lupp et al., 2003)
ainS-luxS mutant CL41	Double mutant carrying mutations as described above	Chapter 4
Plasmids		
pCL145	pEVS79 carrying a 1.8-kb V. fischeri ES114 DNA with the luxO gene	(Lupp et al., 2003)
pCL150	pCL145, LuxO mutated by a D47E amino acid substitution	This study
pEVS79	Allelic exchange vector	(Stabb and Ruby, 2002)

FIAT # <sup>b</sup>	Name	Annotated function	ratio <sup>c</sup> LuxOD47E: <i>luxO</i>	std. dev.
1061	RVFI03416	Flavin reductase LuxG	-1.6	0.2
1062	RVFI03417	Long-chain-fatty-acid-luciferin-component ligase LuxE (EC 6.2.1.19)	-1.5	0.0
1063	RVF103418	Alkanal monooxygenase beta chain LuxB (EC 1.14.14.3)	-1.6	0.2
1064	RVFI03419	Alkanal monooxygenase alpha chain LuxA (EC 1.14.14.3)	-2.1	0.3
1065	RVFI03420	Acyl transferase LuxD (EC 2.3.1)	-1.2	0.2
1066	RVF103421	Acyl-CoA reductase LuxC (EC 1.2.1.50)	-1.0	0.1
1067	RVFI03422	Autoinducer synthesis protein LuxI	1.7	1.2
1068	RVFI03423	Transcriptional regulator LuxR	-3.0	1.2
3245	RVFI01721	Transcriptional regulator LitR	-3.2	0.1
4420	RVFI01670	Acyl-Homoserine lactone synthase AinS	-1.2	0.3
4421	RVFI01669	Sensor protein AinR (EC 2.7.3)	-2.9	0.7
531	RVFI01104	Autoinducer-2 production protein LuxS (EC 3.3.1.3)	-1.4	0.3
4756	RVF103610	LuxP protein precursor	-1.2	0.2
4755	RVFI03609	Sensor protein LuxQ (EC 2.7.3)	-1.3	0.1
4524	RVFI05252	Transcriptional regulator LuxO	1.8	0.1

Table 5.2. Microarray data: quorum-sensing genes.<sup>a</sup>

<sup>a</sup> The *V. fischeri* microarray carries three replicates of 95% of the open reading frames of the genome. For each gene presented in the table, the average ratio of the three replicates was calculated with the appropriate standard deviation. Greater than 2-fold differences in transcription levels are indicated by bold letters and numbers. The experiment was repeated and showed a similar outcome, however, due to low fluorescence numbers of one of the dyes, the absolute values of the ratios were problematic and therefore not included in these tables.

<sup>b</sup> The FIAT number corresponds to the approximate linear position of the gene in the genome of *V. fischeri* 

<sup>c</sup> Negative values represent repression

FIAT	Name	Annotated function	ratio <sup>c</sup>	std. dev.
# <sup>b</sup>			LuxOD47E:luxO	
296	RVFI00346	Flagellar protein FliL	1.1	0.2
557	RVFI05132	Flagellar biosynthesis protein FlhF	-1.1	0.1
837	RVFI00977	Chemotaxis protein CheV (EC 2.7.3)	-1.3	0.3
939	RVFI01729	Chemotaxis protein CheV (EC 2.7.3)	1.3	0.4
1506	RVFI03203	Chemotaxis MotA protein	1.2	0.4
1507	RVFI03202	Chemotaxis MotB protein	-1.3	0.5
3105	RVFI01765	Sodium-type polar flagellar protein MotX	1.5	0.3
3349	RVFI01363	Flagellin	3.9	0.3
3559	RVFI02331	Negative regulator of flagellin synthesis FlgM	1.3	0.1
3560	RVFI02330	Flagella basal body P ring formation protein FlgA	1.0	0.1
3561	RVFI04616	Chemotaxis protein CheV (EC 2.7.3)	1.0	0.0
3562	RVFI01049	Pili chemotaxis protein CheR (EC 2.1.1.80)	1.1	0.2
3563	RVFI01048	Flagellar basal-body rod protein FlgB	4.1	0.9
3564	RVFI01047	Flagellar basal-body rod protein FlgC	4.7	0.3
3565	RVFI01046	Basal-body rod modification protein FlgD	2.9	0.2
3566	RVFI01045	Flagellar hook protein FlgE	2.3	0.4
3567	RVFI01044	Flagellar basal-body rod protein FlgF	1.6	0.1
3568	RVFI01043	Flagellar basal-body rod protein FlgG	2.1	0.2
3569	RVFI01376	Flagellar L-ring protein FlgH	2.0	0.1
3570	RVFI01375	Flagellar P-ring protein FlgI	1.0	0.1
3571	RVF101374	Flagellar protein FlgJ	1.7	0.0
3572	RVFI01373	Flagellar hook-associated protein 1	1.4	0.3
3573	RVFI01372	Flagellar hook-associated protein 3	2.0	0.3
3574	RVFI04615	Flagellin	3.9	1.6
3575	RVFI01759	Flagellin	1.9	0.6
3576	RVFI01758	Flagellin	2.5	0.2
3577	RVFI01757	Flagellin	3.9	0.8
3578	RVFI01756	Flagellin	6.1	0.8
3579	RVFI01755	Flagellin	1.7	0.7
3580	RVF105202	Flagellar hook-associated protein 2	1.5	0.3
3581	RVFI00342	Flagellar rod protein FlaI	1.2	0.4
3582	RVFI01016	Flagellar protein FliS	1.7	0.3
3584	RVFI01017	Sigma-54-dependent transcriptional activator	-1.2	0.3
3585	RVFI01018	Two-component sensor kinase FleS (EC 2.7.3)	1.4	0.1
3586	RVFI05201	Transcriptional regulatory protein	1.6	0.1
3587	RVFI01020	Flagellar hook-basal body complex protein	2.3	0.3
3588	RVFI04613	FILE Flagellar M-ring protein FliF	12	0.1
3589	RVFI01127	Flagellar motor switch protein FliG	1.7	0.1

Table 5.3. Microarray data: motility and chemotaxis.<sup>a</sup>

Table	5.3.	continue	ed.

FIAT			ratio <sup>c</sup>	
# <sup>b</sup>	Name	Annotated function	LuxOD47E:luxO	std. dev.
3590	RVFI01128	Flagellar assembly protein FliH	1.2	0.4
3591	RVFI05200	Flagellum-specific ATP synthase (EC 3.6.3.14)	1.4	0.5
3592	RVFI01131	Flagellar protein FliJ	1.3	0.1
3593	RVFI01132	Flagellar hook-length control protein	1.3	0.1
3594	RVFI01133	Flagellar protein FliL	1.9	0.1
3595	RVFI01134	Flagellar motor switch protein FliM	-1.4	0.6
3596	RVF100401	Flagellar protein FliO	1.9	0.4
912	RVF100402	Flagellar motor switch protein FliN	1.7	0.5
3598	RVFI04612	Flagellar biosynthetic protein FliP	-1.5	0.2
3599	RVFI01519	Flagellar biosynthetic protein FliQ	1.0	0.1
3600	RVFI01520	Flagellar biosynthetic protein FliR	1.2	0.1
3601	RVFI01521	Flagellar biosynthetic protein FlhB	low hybridization	value
3602	RVFI01522	Hypothetical protein	-1.2	0.4
3603	RVFI01523	Flagellar biosynthetic protein FlhA	1.2	0.1
3604	RVFI01524	Flagellar biosynthesis protein FlhF	2.0	0.3
3605	RVFI04201	Flagellar synthesis regulator FleN	-1.4	0.3
3606	RVFI00752	RNA polymerase sigma factor flagellar operon FliA	1.3	0.1
3607	RVFI00751	Chemotaxis protein CheY	1.5	1.1
3608	RVFI00750	Chemotaxis protein CheZ	1.5	0.4
3609	RVFI04906	Pili chemotaxis protein CheA (EC 2.7.3)	1.2	0.0
3610	RVFI01640	Protein-glutamate methylesterase (EC 3.1.1.61)	1.1	0.1
3611	RVFI01639	Chromosome partitioning protein ParA	1.0	0.1
3612	RVF105199	Hypothetical protein	1.1	0.1
3613	RVFI01638	CheW-like domain	1.2	0.1
3614	RVFI01637	Chemotaxis protein CheW	-1.3	0.1
4535	RVFI04646	Sodium-type flagellar protein MotY precursor	1. <b>1</b>	0.1
4748	RVFI03602	Chemotaxis MotB protein	1.3	0.0
4749	RVFI03603	Chemotaxis MotA protein	1.8	0.2

FIAT	Name	Annotated function	ratio <sup>c</sup>	std. dev.
# <sup>b</sup>			LuxOD47E:lux	
3597	RVFI00400	Ferrichrome transport system permease protein FhuB	1.3	0.4
919	RVFI04552	Ferrichrome-binding protein	1.1	0.4
920	RVF102857	Ferrichrome transport ATP-binding protein FhuC	1.1	0.4
921	RVFI02858	Ferrioxamine receptor precursor	-1.2	0.2
961	RVFI02657	Ferric anguibactin transport ATP-binding protein	-1.5	0.3
962	RVF102658	Ferric anguibactin transport system permease protein FatC	1.0	0.3
963	RVFI05133	Ferric anguibactin transport system permease protein FatD	-1.3	0.4
964	RVFI02660	Ferric anguibactin-binding protein	-1.4	0.6
1479	RVFI02461	Ferrichrome transport ATP-binding protein fhuC	-1.2	0.1
1480	RVFI02460	Ferrichrome-binding protein	-1.8	0.6
1481	RVFI02459	Ferrichrome transport system permease protein fhuB	1.3	0.2
1482	RVFI02458	Siderophore biosynthesis protein	1.0	0.0
1483	RVFI02457	Siderophore biosynthesis protein	-1.2	0.3
1484	RVFI04716	Siderophore biosynthesis protein	1.0	0.6
3275	RVFI01219	Iron(III)-binding protein	-1.3	0.3
3276	RVFI01218	Iron(III)-transport system permease protein sfuB	1.0	0.6
40	RVFI05043	ABC transporter ATP-binding protein	-1.1	0.1
4628	RVFI02672	Ferrous iron transport protein B	1.2	0.8
4629	RVFI02671	Ferrous iron transport protein A	1.1	0.1

Table 5.4. Microarray data: iron uptake systems.<sup>a</sup>

FIAT # <sup>b</sup>	Name	Annotated function	ratio <sup>c</sup> LuxOD47E:lux	std. dev.
321	RVFI01650	Hypothetical protein	-2.1	0.9
322	RVFI01651	Glycosyltransferases I	-1.7	0.2
323	RVFI01652	Lipopolysaccharide N- acetylglucosaminyltransferase	-2.3	0.3
324	RVFI01653	Glycosyl transferase (EC 2.4.1)	-2.2	0.6
325	RVFI01654	Hypothetical protein	low hybridization value	
326	RVFI02839	Sensor protein UhpB (EC 2.7.3)	low hybridization value	
327	RVFI02838	Succinoglycan biosynthesis transport protein ExoP	-4.4	1.9
328	RVFI02837	Putative capsule polysaccharide export protein	~1.7	0.4
329	RVFI02836	Transcriptional regulator, TetR family	-4.2	1.3
330	RVFI02835	Undecaprenyl-phosphate glucosephosphotransferase (EC_2.7.8)	-2.2	0.5

Table 5.5. Microarray data: EPS biosynthesis locus.<sup>a</sup>

FIAT # <sup>b</sup>	Name	Annotated function	ratio <sup>c</sup> LuxOD47E:lux	std. dev.
528	RVFI01107	Zinc protease (EC 3.4.99)	-1.6	0.2
1026	RVFI02469	Zinc metalloprotease (EC 3.4.24)	low hybridization	value
1603	RVFI01909	ATP-dependent Zn protease	-1.5	0.2
3485	RVFI05208	Membrane metalloprotease	1.0	0.1
3518	RVFI03360	Zinc metalloprotease (EC 3.4.24)	-1.8	0.2
4243	RVFI01188	Membrane metalloprotease	-1.2	0.2
4515	RVFI01298	ATP-dependent Zn proteases	1.3	<b>0.</b> 1
4972	RVFI03058	Zinc protease (EC 3.4.99)		_0.4

Table 5.6. Microarray data: metalloproteases.<sup>a</sup>

Species	Lumines- cence	Motility	Siderophore	Protease	Biofilm/ EPS
V. fischeri	+	+	-	-	÷
V. harveyiª	+	-	+	+	+
V. cholerae <sup>b</sup>	$\mathbf{NA}^{\mathrm{f}}$	+	ND	+	+
V. anguillarum <sup>c</sup>	NA	ND <sup>g</sup>	ND	+	+
V. vulnificus <sup>d</sup>	NA	ND	ND	+	+
V. parahaemolyticus <sup>e</sup>	NA	-	ND	÷	+

Table 5.7. Phenotypes regulated by quorum sensing in Vibrio spp.

<sup>a</sup> Lilley and Bassler, 2000; Mok et al., 2003

<sup>b</sup> Zhu et al., 2002; Vance et al., 2003

<sup>c</sup> Croxatto *et al.*, 2002

<sup>d</sup> Shao and Hor, 2001

<sup>e</sup> McCarter, 1998; Guevener and McCarter, 2003

<sup>f</sup> Not applicable

<sup>8</sup> Not determined

## **FIGURES**



**Fig. 5.1.** Biofilm formation of *V. fischeri* wild type and the *ainS* mutant. Cultures were grown for 24 h at 28 C in SWT medium. Cells adhering to the glass surface were stained with a 1% crystal violet solution.



Fig. 5.2. Motility and chemotaxis behavior of V. fischeri wild type and quorum-sensing mutants on tryptone/serine medium 6 h after inoculation. A The V. fischeri wild type, as well as the *luxI*, the *luxS* and the *luxR* mutants, produced indistinguishable migration phenotypes on tryptone/ serine agar. The outer ring had a diameter of 3.5 cm, the inner ring of 2.0 cm. B In the V. fischeri ainS single mutant, as well as the ainS-luxI and ainS-luxS double mutants, diameters of both rings were indistinguishable from wild type, however the middle colony was missing. C The V. fischeri luxO and ainS-luxO mutants migrated more slowly than wild type, the diameter of the outer ring was 2.75 cm, and that of the inner ring was 1.0 cm. D The V. fischeri LuxOD47E mutant migrated faster than all other strains, the diameter of the outer ring was 4.0 cm, and that of the inner ring was 2.5 cm.



Fig. 5.3. Motility and chemotaxis behavior of V. fischeri wild type and quorum-sensing mutants on casamino acid medium 8 h after inoculation. A The V. fischeri wild type and the luxR mutant produced indistinguishable migration patterns, both had a single migration ring with a diameter of 2.5 cm. The appearance of other mutants was similar to that of wild type, except that the ring diameters of the luxI, the luxS, and the LuxOD47E mutants were increased to 3.0 cm. B The V. fischeri ainS mutant also migrated faster than wild type, with a ring diameter of 4.0 cm, and the central colony was missing. The double mutants ainS-luxI and ainS-luxS displayed the same ring structure as ainS, but migrated even faster with a ring diameter of 4.5 cm. C The litR mutant produced a fainter inner colony than wild type, and the diameter of the migration ring was 3.5 cm. D The luxO and ainS-luxO did not migrate on casamino acid agar, but formed a colony.



Fig. 5.4. Initiation of colonization by V. fischeri quorum-sensing mutants. A Animal luminescence during the initial stages of E. scolopes colonization by V. fischeri wild-type (diamonds), ainS mutant (triangles), luxI mutant (squares) and ainS-luxI mutant (circles). Mean values of 12 squid were calculated, and standard errors of the mean are indicated. The experiment was conducted twice with the same outcome. B Colonization levels of the ainS mutant (striped bars), luxI mutant (white bars), and ainS luxI mutant (hatched bars) relative to V. fischeri wild type (black bars) at 12 h post-inoculation. Each bar represents the mean value of 15 animals with the associated standard errors.



Fig. 5.5. Effect of 3-oxo-C6-HSL addition on luminescence expression 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 (open circles), or 1200 nM 3-oxo-C6-HSL (crosses).

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## CHAPTER 6

## SUMMARY AND CONCLUSIONS
Dissertation research objective #1: Does the Vibrio fischeri sap locus, proposed to be regulated by quorum sensing, affect antimicrobial peptide resistance and/or symbiotic competence?

The sapABCDEF locus encodes an ABC transporter involved in bacterial resistance to host-derived antimicrobial peptides in both Salmonella typhimurium and Erwinia chrysanthemi (Groisman et al., 1992; Lopez-Solanilla et al., 1998; Lopez-Solanilla et al., 2001). A homologous locus in V. fischeri was discovered through a screen aimed to identify quorum sensing-regulated genes (Chen et al., 2000). My work demonstrated that the V. fischeri sap locus was essential for maximal growth, but that it was required for neither antimicrobial peptide resistance nor responses to other stress conditions (Chapter 2). These data suggest a function in V. fischeri that is distinct from that of other bacterial species. Furthermore, transcription of the sap locus was not affected by ain quorum sensing (Chapter 5) indicating that the initial screen was not specific for quorum sensingregulated genes. Interestingly, the extent to which a sapA mutation affected the ability of V. fischeri to colonize the squid light organ was greater than its growth defect in culture. It is therefore still possible that these genes are specifically involved in host colonization.

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# Dissertation research objective #2: How do the *V. fischeri* quorum-sensing systems; ain, lux and AI-2, interplay to regulate luminescence gene expression?

The lux quorum-sensing system was considered the predominant regulatory mechanism for luminescence expression in *V. fischeri* (Fuqua *et al.*, 1996). However, the results of my study suggest that, at least in the *V. fischeri* strain ES114, the lux system is only fully active at the very high cell densities occurring in the squid light organ (Chapter 3 and 5). At cell densities below the lux quorum-sensing threshold, e. g. in culture, luminescence expression depends on ain quorum sensing (Chapter 3). Furthermore, the effects of the ain and lux systems are interconnected; i.e., the ain system is required to relieve luminescence gene repression and appears to initiate expression of LuxI, the signal synthase of the lux system (Chapter 3). These data suggest a sequential induction of luminescence gene expression with increasing cell density by these two systems.

Based on the data presented in Chapter 3, I proposed a model of gene regulation by the ain and lux quorum-sensing systems. One unexplained observation was that both a *litR* mutant and a *luxR* mutant expressed significant luminescence levels in culture, while the light level of the *ainS* mutant was not detectable. Because this result was inconsistent with the fact that *litR* and *luxR* operate downstream of *ainS*, I suggested a second activity of *ainS* at low cell densities; i.e., repression of luminescence genes operating downstream of *luxR*. However, I later discovered that the *ainS* gene is autoregulated through a pathway involving LitR (Chapter 4). Thus, a mutation in *litR* will not only decrease *luxR* but also *ainS* transcription and therefore reduce AinS signal production, consistent with the very low light expression of a *litR* mutant (Fidopiastis *et al.*, 2002). Another unexplained result was that a *luxR* mutant expressed more light in culture than a *litR* mutant (Appendix I). A possible explanation for this phenomenon is that LuxR is a repressor of luminescence gene expression in the absence of acyl-HSL. Consistent with this hypothesis is the finding that an *ainS-luxR* double mutant expresses light levels very similar to a *luxR* mutant (Appendix I) indicating that LuxR exerts a dominant negative effect on luminescence gene expression. Another layer of regulation absent in the model presented in Chapter 3 is the effect of the AI-2 system. Based on my results, the LuxS signal regulates luminescence gene expression through the same pathway as the ain system, although its effect is less than that of the AinS signal (Chapter 4).

# Dissertation research objective #3: What is the impact of ain and AI-2 quorum sensing on symbiotic competence of V. fischeri?

It has been previously shown that the inactivation of both the *luxA* gene and the regulatory genes luxI and luxR decreases the ability of V. fischeri to persist in the squid light organ (Visick et al., 2000). Because the defects of the three mutants were similar, it was concluded that the underlying cause must be the same; i.e., their defect in luminescence expression (Visick et al., 2000). The inactivation of the V. fischeri ain quorum-sensing system resulted in a persistence defect indistinguishable from that of the lux mutants (Chapter 3). Because the ain system also affects light expression in the squid, although to a lesser extent than the lux system, the persistence defect might be directly related to the luminescence defect. Furthermore, the inactivation of the ain, but not the lux, quorum-sensing system resulted in a delay of colonization initiation (Chapter 5), indicating that ain quorum sensing regulates cell functions other than luminescence. The result of the previous section; i.e., the ain quorum-sensing system is active at lower cell densities than the lux quorum-sensing system, provides a possible mechanism for this colonization-stage specificity. The AI-2 system had a minor effect on colonization ability of V. fischeri in the absence of the ain system (Chapter 4), indicating that these two systems feed into the same downstream cascade, and that the effects of the AI-2 system are minor compared to those of ain system.

# Dissertation research objective #4: Which genes, besides the *lux* genes, does quorum sensing regulate in *V. fischeri*?

The results obtained in the previous section suggested that the ain quorum-sensing system specifically regulates genes important for early colonization stages. I used two *V*. *fischeri* strains carrying mutations in the central, ain-specific, regulator LuxO and compared their transcriptomes in culture using a microarray. The results of this study suggested that ain quorum sensing represses flagellin and flagellar basal body genes, and positively regulates a putative exopolysaccharide biosynthesis locus (Chapter 5). Consistent with the microarray data, ain quorum-sensing system mutants were altered in motility behavior, colony morphology and biofilm formation (Chapter 5). Because motility has been demonstrated to be important for the early stages of colonization (Graf et al., 1994; Millikan and Ruby, 2002; Millikan and Ruby 2003), it is likely that this phenotype is the cause of the observed initiation defect. These data confirm the hypothesis that the ain quorum-sensing system regulates functions important for early stages of colonization.

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Strains	Ratio inoculum	Ratio symbiosis	RCI <sup>b</sup>
wild type : <i>luxS</i>	1:0.8	1:0.7	0.9
	1:0.9	1:0.7	0.8
wild type : <i>ainS-lux</i> .	s 1:0.7	1:0.1	0.1
·····	1:0.8	1:0.7	0.9
	1:1.1	1:1.0	1.0
ainS : ainS-luxS	1:1.1	1:1.3	1.2
	1:1.1	1:0.5	0.5
luxS : ainS-luxS	1:0.7	1:0.6	0.5
	1:1.1	1:0.5	0.4

Appendix A.	Competition com	petence of V.	fischeri luxS	' mutants.ª
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<sup>a</sup> About 15 newly hatched squid were placed into 50 mL of filter-sterilized seawater 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 number and exact ratio of the two strains in the inoculum. At 48 h post-inoculation, squids were homogenized and a dilution of the homogenate spread onto LBS agar. Approximately 100 CFU from the inoculum and each of the homogenates were patched onto antibiotic-containing and antibiotic-free LBS agar to determine the ratio of V. *fischeri* wild type (Cam and Kansensitive) to *luxS* mutant (Kan-resistant) or *ainS* mutant (Cam-resistant) cells. Shown is the average of 10 to 15 animals for each experiment.

<sup>b</sup> Relative competitive index; RCI = ratio symbiosis divided by the ratio inoculum



Appendix B. Growth and luminescence characteristics of the V. fischeri luxO mutants in comparison to the ainS mutant.

To determine A growth and B specific luminescence of V. *fischeri* wildtype (diamonds), the *ainS* mutant (triangles), the *luxO* mutant (circles) and the LuxOD47E mutant (crosses), 10 mL of SWT were inoculated to an optical density at 600 nm (OD) of about 0.05 with cells that had been pregrown in SWT. Cultures were kept shaking at 28°C, samples were taken at various times during growth, and both luminescence (LU) and OD were measured. Specific luminescence is defined as LU/OD.



**Appendix C.** Luminescence characteristics of the *V. fischeri luxO* mutants in comparison to the *ainS* mutant with addition of acyl-HSLs.

To determine specific luminescence of V. fischeri wild-type (diamonds), the ainS mutant (triangles), the luxO mutant (circles) and the LuxOD47E mutant (crosses), 10 mL of SWT containing either A 120 nM 3-oxo-C6-HSL or B 120 nM C8-HSL, were inoculated to an optical density at 600 nm (OD) of about 0.05 with cells that had been pre-grown in SWT. Cultures were kept shaking at 28°C, samples were taken at various times during growth, and both luminescence (LU) and OD were measured. Specific luminescence is defined as LU/OD.

**Appendix D.** Early colonization kinetics of V. fischeri luxO mutants in comparison to the ainS mutant.



For each strain, V. fischeri wild type (diamonds), the ainS mutant (triangles), the *luxO* mutant (circles) and the LuxOD47E mutant (crosses). newly hatched squids were placed into vials with 4 mL of filter-sterilized seawater containing an inoculum of approximately 1,000 colony-forming units (CFUs) 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 filter-sterilized seawater without added bacteria. Animal bioluminescence, an indirect measure of the degree of squid colonization, was monitored periodically over 48 h.

Appendix E. Colonization levels of the V. fischeri luxO mutants in comparison to the ainS mutant.



Newly hatched squids were placed into 50 mL of filter-sterilized seawater containing about 50,000 CFU of *V. fischeri* wild type (black bars), the *ainS* mutant (striped bars), the *luxO* mutant (white bars) or the LuxOD47E mutant (hatched bars) and incubated for 12 h. At three subsequent times post-inoculation, 15 animals per treatment group were homogenized, and the homogenate was diluted and spread onto SWT agar. The number of colonies was determined after overnight incubation, and CFU/squid calculated and normalized to wild-type colonization levels.

Strains	Ratio inoculum	Ratio symbiosis	RCI <sup>b</sup>
wild type : <i>luxO</i>	1:1.9	1:0.3	0.1
	1:1.0	1:0.7	0.7
	1:1.7	1:0.8	0.5
<i>luxO</i> : LuxOD47E	1:1.0	1:0.4	0.4
	1:1.0	1:0.9	0.9
wild type : ainS	1:1.3	1:0.2	0.1
	1:1.0	1:0.3	0.3
	1:1.0	1:0.6	0.6
	1:1.0	1:0.4	0.4
	1:0.9	1:0.6	0.7

Appendix F. Competition competence of V. fischeri luxO mutants in comparison to the ainS mutant.<sup>a</sup>

<sup>a</sup> About 15 newly hatched squid were placed into 50 mL of filter-sterilized seawater 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 number and exact ratio of the two strains in the inoculum. At 48 h post-inoculation, squids were homogenized and a dilution of the homogenate spread onto LBS agar. Approximately 100 CFU from the inoculum and each of the homogenates were patched onto antibiotic-containing and antibiotic-free LBS agar to determine the ratio of V. *fischeri* wild type or LuxOD47E mutant (Cam-resistant) cells. Shown is the average of 10 to 15 animals for each experiment.

<sup>b</sup> Relative competitive index; RCI = ratio symbiosis divided by the ratio inoculum

Appendix G. Reverse transcription and amino-allyl coupling of cDNA.

### A. RT Reaction

1. To anneal primer, mix 10-15  $\mu$ g total RNA with 15  $\mu$ g of random decamer primer in a total volume of 17  $\mu$ l.

2. Heat to 70 °C for 10 min. Cool on ice for 5 min.

3. Add 11  $\mu$ l of nucleoside master mix to each of the Cy3 and Cy5 reactions.

 master mix:

 Rnasin
 0.5 μl

 5X Buffer
 6.0 μl

 0.1 M DTT
 3.0 μl

 25 mM dNTPs
 0.6 μl

 H2O
 0.9 μl

4. Add 1.5  $\mu$ l SuperscriptII, incubate 1-1.5 hours at 42 °C; add additional 1  $\mu$ l of SuperscriptII and continue incubation for another hour.

# **B.** Hydrolysis

1. Degrade RNA by addition of 10  $\mu l$  of 1N NaOH, 10  $\mu l$  0.5M EDTA. Incubate at 65 °C for 10 min.

2. Neutralize by addition of 25  $\mu$ l 1M Tris pH 7.4

3. Add 450 µl H20 to each reaction.

# C. Clean-up

1. Add the 500  $\mu$ l neutralized, diluted reaction mix to a Microcon-30 filter

2. Spin at 10,000 rpm for 7-12 min

3. Repeat process two more times, refilling original filter with 450  $\mu$ l water. Concentrate to approximately 20  $\mu$ l. Take concentrate and speed-vac to dryness. Samples can now be stored at -20 °C indefinitely.

# **D.** Coupling and Quenching

1. Resuspend cDNA in 4.5  $\mu$ l water, add 4.  $\mu$ l of 0.1 M carbonate buffer pH 9. Incubate 10-15 min.

2. Add cDNA mixture to aliquot of Cy3 and Cy5 dye (equilibrate unopened dye package to RT, resuspend dye in 45  $\mu$ l of high-purity DMSO, completely solubilize pellet; aliquot ten 4.5  $\mu$ l-aliquots in 0.2 ml tubes, dry in speed-vac, store dried and dessicated in the dark at 2-8 °C), incubate in the dark at RT for 1h.

3. Add 4.5 µl 4M hydroxylamine, incubate 15 min in dark at RT.

# E. Cleanup II

1. Add 35  $\mu l$  of 100 mM NaOAc pH 5.2 to the cDNA.

2. Add 250 µl Buffer PB from Qiagen PCR Purification kit, mix well.

3. Apply cDNA sample to a Qiaquick PCR purification column, place in a collection tube, and spin in a microcentrifuge for 1 minute at 14,000 rpm.

4. Discard flow through.

5. Add 750 µl of Buffer PE from the Qiagen PCR purification kit.

6. Spin 1 min. in microcentrifuge 14,000, discard flow-through.

7. Repeat the wash and spin in steps 5 and 6 above.

8. Transfer the column to a clean collection tube and add 30  $\mu$ l of water, let stand for 1 min, spin to elute; repeat with an additional 30  $\mu$ l (60  $\mu$ l final volume).

### F. Quantitation of cDNA and labeling

1. Set up spec program to read 260 nm, 550 nm, and 650 nm. Blank with water at 260 nm; read absorbance of water at 550 nm and 650 nm.

2. Measure the absorbance of each labeled cDNA sample (the entire 60  $\mu$ l sample) using quartz cuvette at 260 nm, 550 nm, and 650 nm. Calculate pmol of nucleotides, cy-dye, and the nucleotide:cy-dye ratio:

pmol nucleotides =  $[A260 * vol in \mu]$  \* 37 \* 1000]/324.5 pmol Cy3=  $[A550* vol (in \mu l)]/0.15$ pmol Cy5= $[A650*vol(in \mu l)]/0.25$ 

the synthesis is considered to be of good quality if pmol of nucleotides > 10,000 and the nucleotide:cy-dye ratio is < 50

3. Either continue on to the hybridization protocols or dry products to completion in the speed vac. Samples can be stored at 4 °C overnight.

Appendix H. Hybridization of microarray slides.

#### A. Slide preparation

1. Boil 500 ml of MQ-H2O, dip slide into boiling water for 5-10 seconds, lift slide from the water avoiding excess water drop retention.

2. Make up pre-hybridization solution (40 ml per slide) in a 50-ml disposable tube (use purchased SSC and SDS solutions):

10 ml 20X SSC 30 ml MQ-H2O 0.4 ml 10% SDS 0.2 g BSA

3. Insert slide into the 50-ml tube containing the pre-hybridization solution and incubate for 45 minutes at 42 °C in the hybridization oven. Rinse slide by dipping into RT MQ-H2O and then rinse with ethanol or isopropanol and allow to air dry (or gently blow dry with an air can).

#### **B.** Preparing probes for hybridization

1. Combine purified probes and concentrate to 10  $\mu$ l using a Microcon-30 column.

2. Add 3.5  $\mu$ l each of salmon sperm DNA (1 mg/ml stock) and yeast tRNA (1 mg/ml stock) and 18  $\mu$ l of Sigmahyb to 35  $\mu$ l total volume.

3. Denature at 99 °C for 1.5 minutes. Do NOT put mixture on ice.

#### C. Hybridization

1. Add probe mix a cloverslip and press DNA chip onto the probe solution.

2. Place slide into the hybridization chamber (add water to bottom for moisture) and incubate 14-18 hours at 42 °C.

3. Notify DNA facility that you will be using the scanner the next day.

#### D. Washing (40 ml for each slide in 50-ml disposable tube)

Wash Buffer 1:	Wash Buffer 2:	Wash Buffer 3:
2 ml 5X SSC	0.2 ml 5X SSC	
0.4 ml 1% SDS	0.4 ml 1% SDS	0.4 ml 1% SDS
38 ml H2O	39 ml H2O	39.6 ml H2O

1. Dunk the slide several times into Buffer 1 until the cover slide is loosened and gently slide off. Incubate 5 min. (cover in alumninum foil) at RT with shaking.

2. Transfer slide to Buffer 2, incubate on rocker at RT for 5 min.

3. Transfer slide to Buffer 3, incubate on rocker at RT for 5 min.

4. Transfer slide to 40 ml of H2O, incubate on rocker at RT for 5 min.

5. Gently dribble ~1 ml of ethanol over slide surface and let air dry or dry with air can. Place in hybridization chamber and take to campus for scanning.

		Additions <sup>b</sup>	
Strain	none	C8-HSL	3-oxo-C6-HSL
wild type	2.6 (0.2)	3.7 (0.3)	1,400 (300)
litR	0.1 (0.0)	$ND^{c}$	ND
ainS	$\mathrm{BD}^{\mathrm{d}}$	2.1 (0.3)	4.0 (1)
luxR	0.4 (0.2)	0.4 (0.1)	0.4 (0)
ainS-luxR	0.2 (0.1)	0.4 (0.1)	0.3 (0)

Appendix I. Luminescence characteristics of the V. fischeri ainS-luxR mutant.

<sup>a</sup> Specific luminescence values (x  $10^{-2}$  quanta sec<sup>-1</sup> cell<sup>-1</sup>) are the means of cultures with an optical density (at 600 nm) of between 1.0 and 5.0; standard errors of the means are indicated in parentheses. The results are from a representative experiment, which was repeated twice with the same outcome.

<sup>b</sup> Cultures were grown in SWT medium alone or supplemented with 120 nM of either 3oxo-C6-HSL or C8-HSL.

<sup>c</sup> Not determined

<sup>d</sup> Below detection ( $\leq 2 \times 10^{-4}$  quanta sec<sup>-1</sup> cell<sup>-1</sup>)