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The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host

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Summary

Bacterial quorum sensing using acyl-homoserine lactones (acyl-HSLs) as cell-density dependent signalling 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 well-described phenomenon, and its role in light organ symbiosis has been defined, little is known about the *ain* system. We have investigated the impact of the *V. fischeri* acyl-HSL synthase AinS on both luminescence and symbiotic colonization. Through phenotypic studies of *V. fischeri* mutants we have found 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, our 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, our 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 signalling molecules was originally discovered as the regulatory mechanism underlying the induction of bioluminescence in *V. fischeri* (Eberhard *et al.*, 1981). The enzymes catalysing 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 α and β subunits of the luciferase enzyme, which catalyses the reaction of reduced flavomononucleotide (FMNH2), 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 and Ruby, 1996; Visick *et al*., 2000). 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 the wild type.

Vibrio 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 (Kuo *et al*., 1994; Gilson *et al*., 1995; Kuo *et al*., 1996; Callahan and Dunlap, 2000). In *Vibrio harveyi*, the AinS-homologue 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; 1994; Freeman and Bassler, 1999; Mok *et al*., 2003).

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

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 1). In culture, the *ainS* mutant did not produce light at a detectable level; however, providing C8-HSL exogenously restored wild-type luminescence levels. In contrast, whereas the addition of 3-oxo-C6-HSL, the LuxI-synthesized signal, to the *ainS* mutant led to luminescence induction to a detectable level, luminescence was <1% of the similarly supplemented wild-type strain (Table 1). As previously shown (Visick *et al*., 2000), the *V. fischeri luxI* mutant produced a reduced (30–40% 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 1). 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 1). 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).

Table 1. Effects of acyl-HSL additions on the luminescence of *V. fischeri ainS* and *luxI* mutants.[a]

	Additions[b]		
Strain	none	C8-HSL	3-oxo-C6-HSL
wild type	3.8 (0.4)	4.8 (1.0)	2360 (370)
ainS	BD[c]	3.9 (0.3)	4 (1)
luxI	1.3 (0.1)	2.2 (0.5)	2070 (290)
ainS-luxI	BD[c]	1.4 (0.1)	1 (2)

a. Specific luminescence values (x 10^{-2} quanta s^{-1} per cell) 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.
b. Cultures were grown in SWT medium alone or supplemented with 120 nM of either 3-oxo-C6-HSL or C8-HSL.
c. Below detection ($< 2 \times 10^{-4}$ quanta s^{-1} $cell^{-1}$).

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, because of the relatively low levels of 3-oxo-C6-HSL it produces (Boettcher and Ruby, 1990;

Table 2. Luminescence expression of *V. fischeri* ES114 cultures with the addition of different acyl-HSL concentrations.[a]

	Concentration of acyl-HSL added (nM)				
Acyl-HSL	no addition	12	120	1200	12000
3-oxo-C6-HSL	1.0	550	1400	1450	1500
C8-HSL	1.0	1.6	2.2	3.6	6.2
C8-HSL (+ 3-oxo-C6-HSL[b])	420	ND[c]	120	5.0	5.5

a. 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.
b. Cultures were grown in SWT medium supplemented both with 120 nM 3-oxo-C6-HSL and with the C8-HSL concentrations indicated.
c. Not determined.

Gray and Greenberg, 1992). We were 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. We 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 2). In contrast, the maximum level of light emission continued to rise without saturation when C8-HSL was added up to a concentration of 12 μM (Table 2). However, in the presence of 120 nM 3-oxo-C6-HSL, C8-HSL had an inhibitory effect on the expression of luminescence that grew with increasing concentrations (Table 2). 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 our 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 wild-type levels (Fig. 1A). These results suggested that the reduced light emission of the *luxI* mutant, compared to wild-type *V. fischeri* observed in culture (Table 1), 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, we 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. FMNH2, aldehyde and oxygen) that can occur in living cells. When we 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. 1B). Specifically: (i) luciferase levels in the *luxI* 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. 1B). Thus, at the cell densities achieved in culture ($<10^9$ cells ml^{-1}), 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. 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% of the wild-type level, depending on the experiment and the

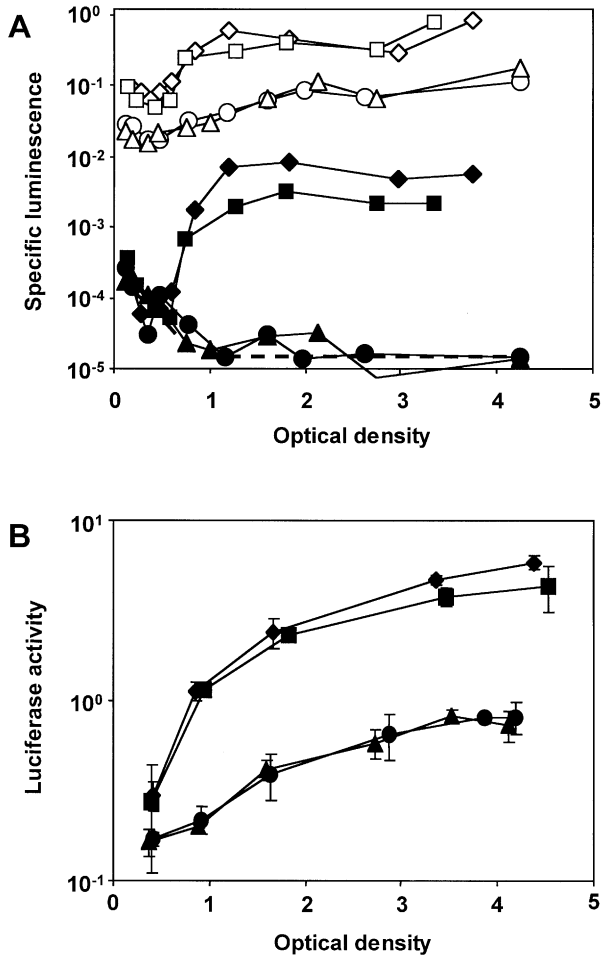

Fig. 1. Specific luminescence (quanta s^{-1} $cell^{-1}$) and luciferase activity (quanta s^{-1} $cell^{-1}$) of *V. fischeri* wild type (diamonds), *ainS* mutant (triangles), *luxI* mutant (squares) and *ainS-luxI* mutant (circles).
A. Specific luminescence of SWT medium-grown cells. Samples were taken during growth, and luminescence was measured in the presence (open symbols) or absence (closed symbols) of added decanal. The results are from a representative experiment, which was repeated twice with the same outcome. The dashed line indicates the approximate detection limit of the assay.
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. The experiment was conducted three times with the same outcome.

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 s^{-1} per cell) when associated with the animal (Fig. 2). Because *in vivo* luminescence measurements are limited to the 10^5–10^6 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 5×10^8 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, although *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 1) and in the light organ (Fig. 2).

Because decanal addition significantly decreased differences between the luminescence levels of the acyl-HSL mutants and wild type in culture, we wondered whether the reduced luminescence of animals colonized by the acyl-HSL synthase mutants might be a result of 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 two to threefold 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, we 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 sug-

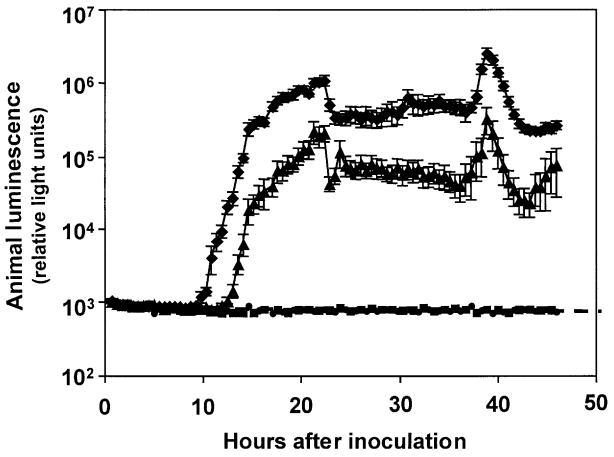

Fig. 2. Animal luminescence during the initial stages of *E. scolopes* colonization by *V. fischeri* wild type (diamonds), *ainS* mutant (triangles), *luxI* mutant (squares) and *ainS-luxI* mutant (circles). The luminescence pattern indicates the initial onset of colonization (0–20 h), followed by a changing level of light emission that reflects the diurnal behaviour of the animal (Boettcher *et al*., 1996). 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.

gest 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, we examined the pathway(s) through which it exerts its effects. By analogy to homologues in *V. harveyi* (Bassler *et al*., 1993; 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, we 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. 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. 3A). However, at lower cell densities, the *ainS-luxO* mutant was not as bright as the wild type, suggesting that the AinS-signal 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), 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 the wild type (Fig. 3A). This effect is apparent at approximately the same cell concentrations at which the luminescence levels of the *luxO* mutant are indistinguishable from the 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). Similarly, providing a wild-type copy of the *luxO* gene *in trans* restored the luminescence phenotype of the *luxO* and the *ainS luxO* mutants

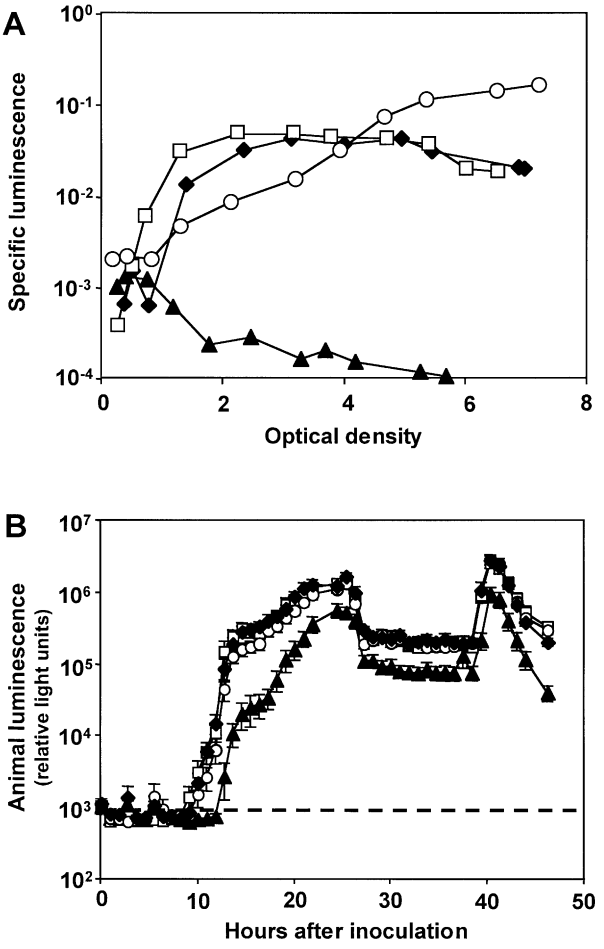

Fig. 3. Luminescence expression in culture (quanta s^{-1} $cell^{-1}$) and during the initial stages of colonization (animal luminescence) of *V. fischeri* wild type (diamonds), the *ainS* mutant (triangles), the *luxO* mutant (squares) and the *ainS-luxO* mutant (circles).
A. The four strains were grown 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.

Table 3. Effect of acyl-HSL additions on the luminescence of *V. fischeri luxO* mutants.[a]

	Additions[b]		
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)

a. Specific luminescence values (x10^{-2} quanta s^{-1} $cell^{-1}$) 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.
b. Cultures were grown in SWT medium, with or without 120 nM C8-HSL or 3-oxo-C6-HSL.
c. Below detection ($< 2 \times 10^{-4}$ quanta s^{-1} $cell^{-1}$)

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. 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. 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. 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 ≤ 0.01), and this persistence defect continued through 72 h (mean values of about 20% the wild-type level; *P*-values ≤ 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 ≥0.40). The decreased colonization levels of the *luxI* mutant are in agreement with previous observations (Visick *et al*., 2000), and our 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).

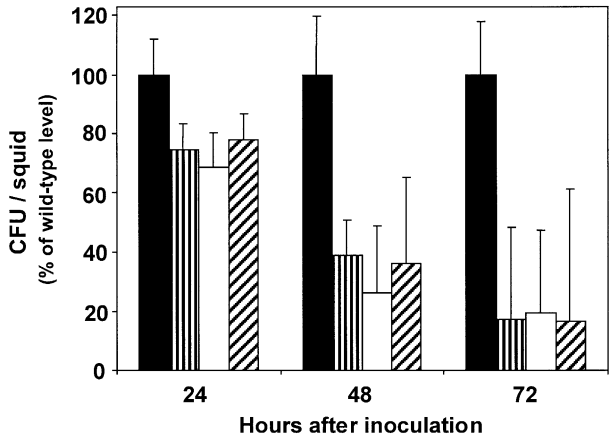

Fig. 4. Colonization levels of the *ainS* mutant (striped bars), *luxI* mutant (white bars), and *ainS luxI* mutant (hatched bars) relative to *V. fischeri* wild type (black bars) at 24, 48 and 72 h post inoculation. Each bar represents the mean value of 15 animals with the associated standard errors. The experiment was conducted three times with the same outcome.

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 the 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 1).

The introduction of a *luxO* mutation into an *ainS* mutant background could fully relieve the luminescence phenotype of the *ainS* mutant *in vivo* (Fig. 3B), indicating that the colonization defect of the *ainS* mutant might also be relieved by inactivation of LuxO. To test this hypothesis, we 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. 5). As in previous experiments (Fig. 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, we have investigated the impact of the *V. fischeri* acyl-HSL synthase AinS on luminescence and symbiotic colonization. Our results demonstrate that: (i) the AinS-synthesized signal plays an important role in luminescence regulation in *V. fischeri* especially at cell

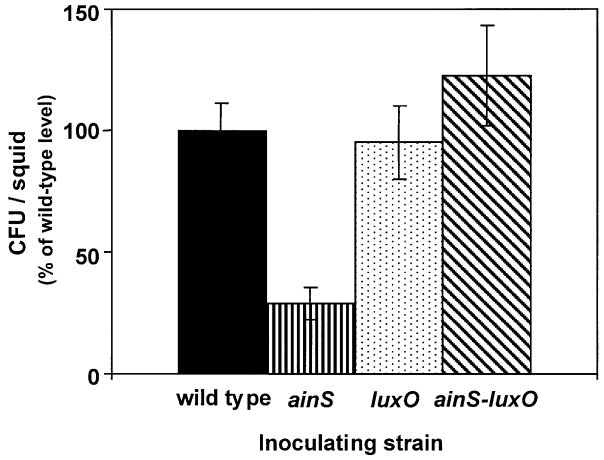

Fig. 5. Colonization levels of the *ainS* mutant (striped bars), *luxO* mutant (dotted bars), and *ainS-luxO* mutant (hatched bars) relative to *V. fischeri* wild type (black bars) at 72 h post inoculation. Each bar represents the mean value of 15 animals with the associated standard errors. The experiment was conducted twice with the same outcome.

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 signalling 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 1). Whereas 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. 1A and 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. 2, and data not shown), which can, at least in part, be attributed to the presence of only 20–80% of bacterial cell numbers in the squid relative to the wild type (Fig. 4). Unfortunately, because of the high variability of luminescence expression between individual animals, we were 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% 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, our 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, we propose a model of luminescence gene expression in *V. fischeri* ES114 (Fig. 6). For simplicity, our 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^{-1}, as when bacteria exist planktonically in seawater; Lee and Ruby, 1994), becomes partially induced at moderate cell densities (10^8–10^9 cells ml^{-1}, as occurs in culture), and becomes fully induced under the high cell-density conditions found during symbiosis ($>10^{10}$ cells ml^{-1}; 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. 1A). By analogy to the luminescence regulatory cascade in *V. harveyi* (Miller and Bassler, 2001), we predict that *V. fischeri* LuxO represses *lux* operon transcription at these low cell densities (Fig. 6A). Consistent with this hypothesis, we observed that a *V. fischeri* ES114 *luxO* mutant induced luminescence at lower cell densities than the wild type (Fig. 3A), a phenotype reported for *luxO* mutants in other *Vibrio* strains (Bassler

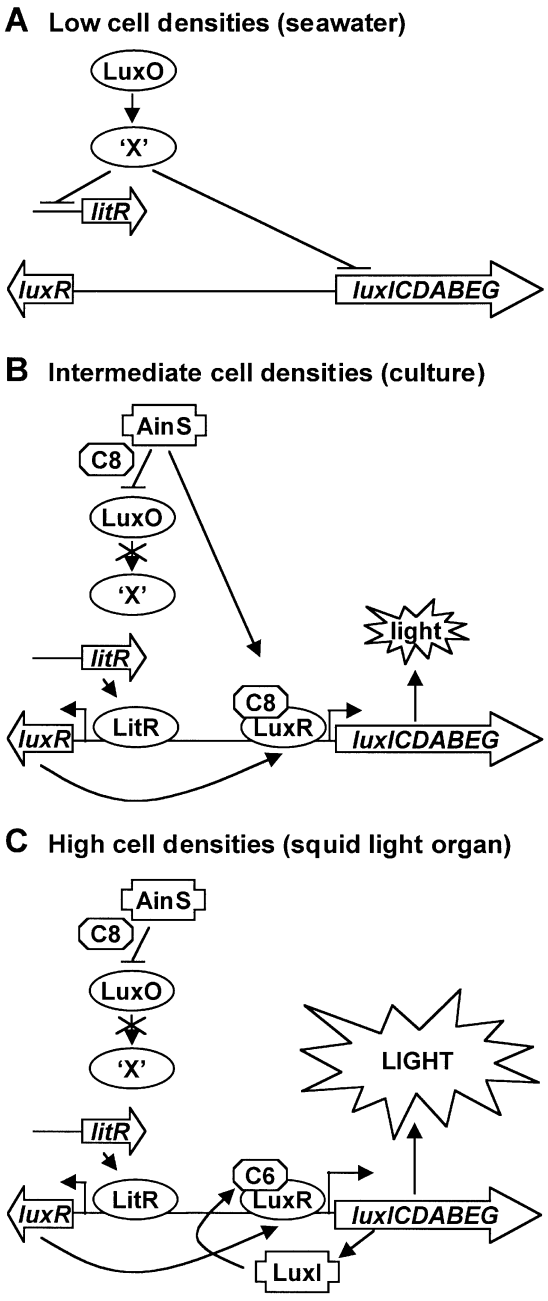

Fig. 6. Proposed model of luminescence regulation in *V. fischeri* ES114 at low cell densities (A), in culture (B), and when colonizing the squid host (C). See the *Discussion* for an explanation. C6 = 3-oxo-C6-HSL; C8 = C8-HSL.

et al., 1994; Miyamoto *et al*., 2000). In *V. harveyi*, LuxO is believed to activate an unknown factor ('X') that represses transcription of *luxR* (not a homologue of *V. fischeri luxR*) (Lilley and Bassler, 2000). The fact that LitR, the *V. fischeri* homologue of *V. harveyi* LuxR, has been shown to increase luminescence expression through upregulation 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 1), 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. 3A and B).

As *V. fischeri* ES114 grows in culture, we propose that C8-HSL accumulates and activates a signalling pathway through binding to its cognate receptor AinR, a homologue of *V. harveyi* LuxN (Bassler *et al*., 1993; Gilson *et al*., 1995). A phosphorelay cascade involving a *V. fischeri* LuxU homologue (Freeman and Bassler, 1999; http://ergo.integratedgenomics.com/Genomics/VFI/) is predicted to result in the inactivation of LuxO, thereby relieving the repression of luminescence (Fig. 6B). We 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 1, Visick *et al*., 2000). Therefore a lower-efficiency binding of the more abundant C8-HSL to LuxR may occur, which can weakly stimulate expression of the *luxICDABEG* operon (Fig. 6B). 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 2). 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. 3A), 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. 6C). 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 (Fig 2 and Fig. 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-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 the 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 we are unable to quantitatively determine the specific luminescence of the *ainS* mutant *in vivo*, we never detected luminescence levels that were below 10% that of the wild type (Fig. 2 and Fig 3B; data not shown). Thus, whereas 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 homologues 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, whereas 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, we might expect that they, too, will employ these systems in a step-wise, cell density-dependent manner like that hypothesized here (Fig. 6). Because most of these species are host-associated, the biological significance of a sequential regulation of quorum signalling 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.

Experimental procedures

Bacterial strains and growth conditions

Medium reagents were purchased from Difco Laboratories (Sparks, MD) and Sigma Chemical (St Louis, MO). Strains and plasmids used in this study are listed in Table 4. *Vibrio 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). *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 concentrations when appropriate: chloramphenicol (Cam; 2.5 μg ml^{-1} for *V. fischeri*, 20 μg ml^{-1} for *E. coli*), kanamycin (Kan; 100 μg ml^{-1} for *V. fischeri* and *E. coli*), erythromycin (Erm; 5 μg ml^{-1} for *V. fischeri* and 150 μg ml^{-1} for *E. coli*). 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) was obtained from Sigma Chemical; 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, Valencia, CA), respectively. Polymerase chain reaction 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 (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.

Table 4. Bacterial strains and plasmids.

Strains and plasmids	Description	Reference or source
E. coli		
C118λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ*pir*	Herrero *et al.* (1990)
DH5α	F^- Φ_{80} *dLacZΔM15* Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17 recA1 gyrA96 thi-1 felA1*	Hanahan (1983)
V. fischeri		
wild type	Strain ES114, isolate from *E. scolopes* light organ	Boettcher and Ruby (1990)
ainS mutant CL21	*ainS* gene partially deleted and replaced by a chloramphenicol-resistance (*cat*) marker	This study
luxI mutant VCW2G7	*luxI* gene inactivated by a frameshift mutation	C. Whistler
ainS-luxI mutant CL24	Double mutant carrying mutations as described above	This study
luxO mutant CL42	*luxO* gene inactivated by insertion of a kanamycin-resistance marker (*kanR*)	This study
ainS-luxO mutant CL64	Double mutant carrying mutations as described above	This study
Plasmids		
pACYC184	Origin of the chloramphenicol-resistance (*cat*) marker	Chang and Cohen (1978)
pCL112	2.1 kb *V. fischeri* ES114 DNA with the *ainS* gene cloned into pVO8	This study
pCL126	1.8 kb *V. fischeri* ES114 DNA with the *luxO* gene cloned into pVO8	This study
pCL145	1.8 kb *V. fischeri* ES114 DNA with the *luxO* gene cloned into pEVS79	This study
pCL146	pCL145 with *luxO* gene inactivated by a insertion of *kanR*-marker into the *Nsi*I site	This study
pEVS79	Allelic exchange vector	Stabb and Ruby (2002)
pKV29	8.8 kb *V. fischeri* ES114 DNA containing the *lux* operon with the *luxI* gene inactivated by a frameshift mutation	Visick *et al.* (2000)
pKV69	Origin of the *mob-tetM* region	K. Visick
pMP7	Dual transcription terminator cloning vector	Hershberger *et al.* (1995)
pMU105	3.6 kb *V. fischeri* ES114 DNA with the *ainS* gene cloned into pTR100	This study
pMU106	pMU105, partially deleted *ainS* gene replaced by a chloramphenicol-resistance marker (*cat*)	This study
pTR100	R2K cloning vector	Weinstein *et al.* (1992)
pUC4K	Origin of the kanamycin-resistance marker (*kanR*)	Messing and Vieira (1982)
pVCW2A6	8.8 kb *V. fischeri* ES114 DNA from pKV29, subcloned into pEVS79	This study
pVO8	*V. fischeri* cloning vector, *ermR*	Visick and Ruby (1997)

Construction of the V. fischeri *mutant strains*

To generate the *ainS,* the *ainS-luxI* and the *ainS-luxO* mutant strains, the *ainS* gene and approximately 2.5 kb of flanking DNA from *V. fischeri* ES114 was PCR-amplified based on the genomic sequence provided by Integrated Genomics (Chicago, IL) at http://ergo.integratedgenomics.com/Genomics/VFI/. The obtained 3.6 kb fragment was cloned into the mobilizable vector pTR100 (Table 4) resulting in plasmid pMU105. A 0.7 kb *Pme*I-*Cla*I fragment within the *ainS* gene was deleted and replaced with the 1.1 kb chloramphenicol resistance (*cat*) gene from plasmid pACYC184 (Table 4). The resulting 4 kb *Sac*I-*Kpn*I fragment containing the Δ*ainS*::*cat* allele was transferred into the *colE*1 vector pMP7 (Table 4). Subsequent deletion of the pMP7 *bla* gene using *Fsp*I, and its replacement with a 5 kb *Bst*BI fragment containing the mobilization (*mob*) and tetracycline resistance (*tetM*) region from plasmid pKV69 (provided by K. Visick, Loyola University Chicago), resulted in the *colE*1-based mobilizable plasmid pMU106. 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 4).

The *luxI* mutant was constructed by subcloning an 8.8 kb *Sal*I fragment from pKV29 into the mobilizable vector pEVS79 (Table 4). This fragment carries the *lux* operon from *V. fischeri* ES114, in which the *luxI* gene has been inactivated by a 2 bp frameshift mutation (Pearson *et al.*, 1994). The resulting plasmid pVCW2A6, 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 luxI* mutant strain VCW2G7. The introduction of the frameshift mutation into the *V. fischeri* genome was confirmed by sequencing a PCR-amplified and cloned genomic DNA fragment of the *luxI* region. An acyl-HSL bioassay (Schaefer *et al.*, 2000) was performed to ensure that the mutation in the *luxI* gene resulted in an inactive enzyme. In this assay the wild-type strain ES114 produced 0.2 nM 3-oxo-C6-HSL, whereas the concentration of this compound in the *luxI* mutant was below detection (<0.0125 nM).

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 (Chicago, IL) at http://ergo.integratedgenomics.com/Genomics/VFI/. The fragment was cloned into the mobilizable vector pEVS79 (Table 4), generating pCL145. A 1.2 kb kanamycin resistance (*kanR*) gene from pUC4K (Table 4) 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 4).

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 pregrown 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) 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. 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 (1 ml) of appropriate strains were harvested by centrifugation, and the resulting cell pellets were frozen at −80°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 after injecting 0.5 ml of 50 μM $FMNH_2$ solution into the mixture in a light-tight chamber, and the specific light emission (quanta s^{-1} per cell) was calculated and plotted as a function of OD.

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 1000 colony-forming units (CFUs) of the indicated strain per ml. Twenty-four individual animals were infected per treatment group; six 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, 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 1000 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 two 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 1000 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.

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