

## *Vibrio fischeri* lux Genes Play an Important Role in Colonization and Development of the Host Light Organ

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**The bioluminescent bacterium *Vibrio fischeri* and juveniles of the squid *Euprymna scolopes* specifically recognize and respond to one another during the formation of a persistent colonization within the host's nascent light-emitting organ. The resulting fully developed light organ contains brightly luminescing bacteria and has undergone a bacterium-induced program of tissue differentiation, one component of which is a swelling of the epithelial cells that line the symbiont-containing crypts. While the luminescence (*lux*) genes of symbiotic *V. fischeri* have been shown to be highly induced within the crypts, the role of these genes in the initiation and persistence of the symbiosis has not been rigorously examined. We have constructed and examined three mutants (*luxA*, *luxI*, and *luxR*), defective in either luciferase enzymatic or regulatory proteins. All three are unable to induce normal luminescence levels in the host and, 2 days after initiating the association, had a three- to fourfold defect in the extent of colonization. Surprisingly, these *lux* mutants also were unable to induce swelling in the crypt epithelial cells. Complementing, *in trans*, the defect in light emission restored both normal colonization capability and induction of swelling. We hypothesize that a diminished level of oxygen consumption by a luciferase-deficient symbiotic population is responsible for the reduced fitness of *lux* mutants in the light organ crypts. This study is the first to show that the capacity for bioluminescence is critical for normal cell-cell interactions between a bacterium and its animal host and presents the first examples of *V. fischeri* genes that affect normal host tissue development.**

Bioluminescent bacteria are commonly found associated with marine animal tissues, as members of the enteric consortia, as opportunistic pathogens, or most notably as essentially pure cultures colonizing the light-emitting organs of certain squids and fishes (43). In at least some of these light organ associations, normal development of host tissues requires the presence of their species-specific luminous bacterial symbionts (28), which are obtained from the surrounding seawater by the newly hatched host (41, 52). The importance of these light organs to antipredatory defense and other behaviors of the hosts has been well documented (27). In contrast, two questions that focus on the initiation and development of light organ symbioses have remained unanswered: (i) why is it that only certain strains of bacteria are able to colonize and persist in these associations, and (ii) what are the bacterial signals that induce host tissue differentiation? We report here that the capacity to bioluminescence plays a critical part in the answers to both of these questions.

While relatively little is known about the symbiotic significance of light emission, much has been published about the biochemistry and regulation of bioluminescence in luminous bacteria. Bacterial luminescence is a product of the enzyme luciferase, which uses molecular oxygen from the surrounding environment to oxidize both an aliphatic aldehyde and a reduced flavin mononucleotide (reviewed in reference 30). The final products of this reaction are the corresponding aliphatic acid, oxidized flavin, and water, and an unstable intermediate in the reaction emits a photon of blue-green light.

There are at least eight *lux* genes comprising a regulon that encodes the proteins essential for luminescence in the best-studied luminous bacterium, *Vibrio fischeri* (30). The *lux* operon (Fig. 1), encoding luciferase (*luxAB*) and proteins required to synthesize the aldehyde substrate (*luxCDE*), is controlled by a number of factors, most notably the quorum-sensing regulator LuxR (13) and the autoinducer molecule 3-oxohexanoyl L-homoserine lactone (3-oxo-C6-HSL), synthesized by LuxI (11, 20). *V. fischeri* cells also synthesize a second autoinducer molecule, octanoyl L-homoserine lactone (C8-HSL) that, under some conditions, may stimulate transcription of the *lux* genes (10, 14, 21).

The process of quorum sensing, although first discovered in *V. fischeri*, is a widespread regulatory mechanism in gram-negative bacteria, particularly among a number of pathogens, which use various autoinducer molecules to modulate genes encoding virulence factors (5, 13, 37). To date, several organisms defective in their *luxI* homolog have been constructed, and they display a reduction in virulence (39, 47); the defect can be at least partially restored by exogenous addition of synthetic autoinducer. Such studies provide strong evidence that quorum sensing plays an important role in pathogenic bacterium-host interactions. While not a pathogen, *V. fischeri* produces a persistent, benign infection of specific light-emitting tissues of a number of species of squids and fishes (43). The best studied of these cooperative bacterium-host associations is that between *V. fischeri* and the Hawaiian sepiolid squid, *Euprymna scolopes* (reviewed in references 29 and 41). The bacteria reside as a monospecific culture in epithelial cell-lined crypts of the light organ, which is used in the host's nocturnal behavior (26).

Newly hatched juvenile squid are symbiont free and must acquire an inoculum of *V. fischeri* from the surrounding seawater (42, 52). As a result of colonization by *V. fischeri*, a

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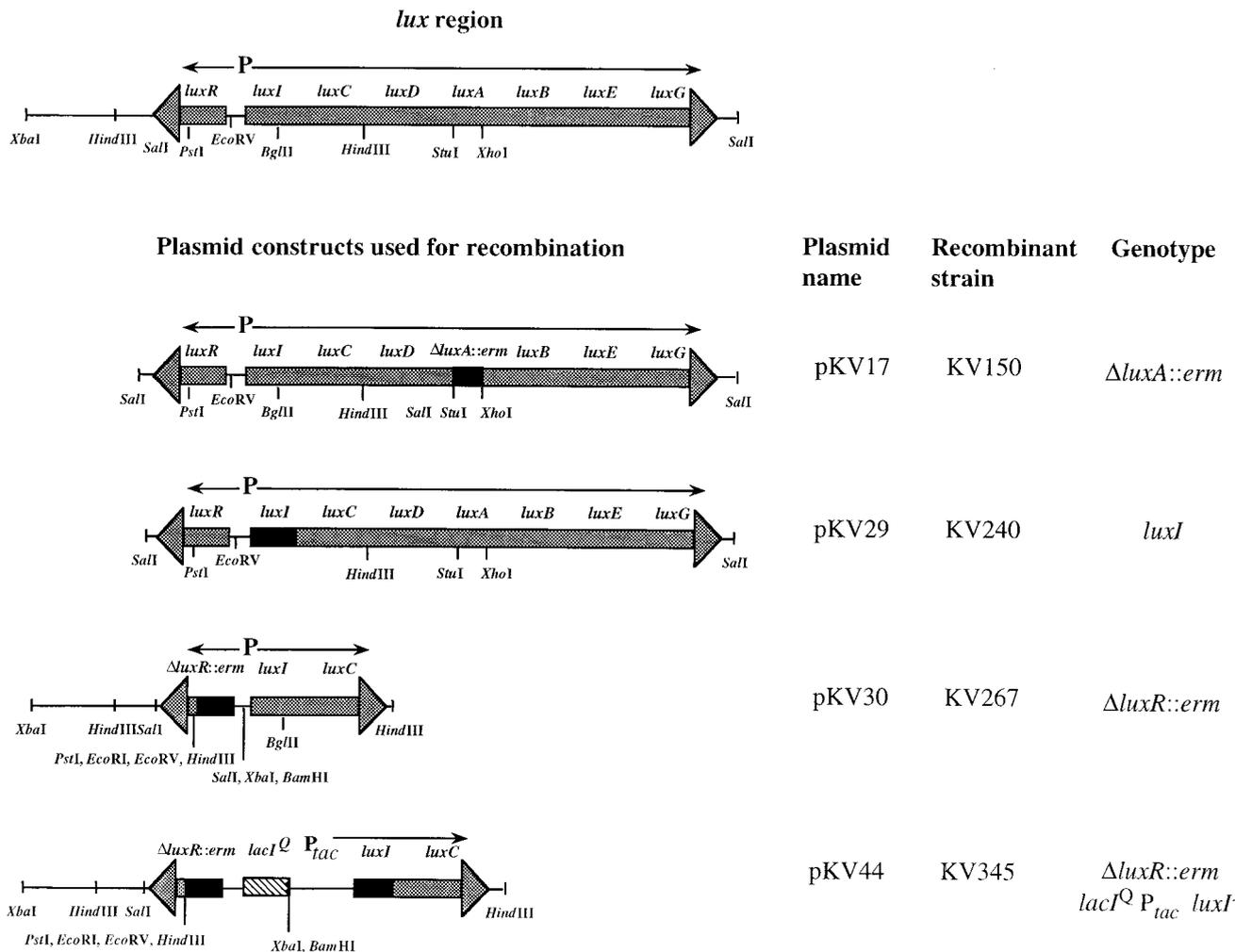


FIG. 1. Plasmids used for construction of *lux* mutants. The region of the chromosome containing the *lux* regulon is shown, with arrows demonstrating the direction of transcription of the two transcriptional units. Relevant restriction enzyme sites are indicated. Plasmid constructs that were used to make mutations in the chromosomal copy of the *lux* genes are also shown. Black boxes indicate the location of the mutation resulting from either a deletion and gene replacement with the *erm* gene or a frameshift mutation (see Materials and Methods); the box with diagonal stripes depicts the location of a *lacI*<sup>Q</sup>/*P*<sub>tac</sub> cassette.

number of morphological and biochemical changes are triggered in the nascent light organ, leading to the development of the functional adult structure (32, 33). Among these changes is a dramatic swelling of the epithelial cells that line the bacterium-containing crypts (8, 32). The swelling, as well as several other developmental events, does not occur in the absence of colonization by *V. fischeri* cells (32).

This study presents evidence that the bioluminescence of *V. fischeri* cells plays an important role during the development of a successful light organ association. Specifically, we have examined *luxA*, *luxI*, and *luxR* mutants of a symbiosis-competent strain of *V. fischeri* for their relative abilities to (i) colonize and persist in the squid light organ and (ii) induce normal host developmental morphogenesis. Our results indicate a direct role of bacterial luciferase and/or its bioluminescence activity in both the normal induction of host development and symbiotic persistence in this cooperative bacterial association.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *V. fischeri* strain ESR1 (13), a rifampin-resistant derivative of wild-type strain ES114 (1), was used as the parent strain for all mutant constructions. *Escherichia coli* strain DH5 $\alpha$  was used as the recipient for

cloning experiments, and plasmids were passaged through a *dam* mutant *E. coli* strain (49) prior to introduction into *V. fischeri*.

*V. fischeri* strains were grown in either SWT (1), which contains 0.5% tryptone, 0.3% yeast extract, and 0.3% glycerol in 70% seawater, or LBS (9), which contains 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 2% NaCl, and 0.3% (vol/vol) glycerol in 50 mM Tris-HCl (pH 7.5). *E. coli* strains were grown in LB (6) or Difco brain heart infusion medium. Conditioned medium (CM) was prepared as follows. *V. harveyi* strain B392, which removes inhibitory compounds from rich media without producing interfering autoinducers, was inoculated into 500 ml of luminous medium (34) (50 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, 300 mM NaCl, 10 mM KCl, 333  $\mu$ M K<sub>2</sub>HPO<sub>4</sub>, 18.7 mM NH<sub>4</sub>Cl, 0.5% yeast extract, 0.5% tryptone, and 0.3% glycerol in 50 mM Tris-HCl [pH 7.5]) and grown to an optical density at 600 nm (OD<sub>600</sub>) of approximately 0.8. Cells were removed from the spent medium by centrifugation and filtration through a 0.22- $\mu$ m-pore-size membrane filter. Agar was added to a concentration of 1.5% to solidify media. Antibiotics and other supplements were added to media where appropriate to the following final concentrations: ampicillin, 100  $\mu$ g/ml; erythromycin, 150  $\mu$ g/ml for *E. coli* and 5  $\mu$ g/ml for *V. fischeri*; chloramphenicol, 25  $\mu$ g/ml for *E. coli* and 2.5  $\mu$ g/ml for *V. fischeri*; isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), 570  $\mu$ g/ml; 3-oxo-C6-HSL, 200 to 600 ng/ml; and C8-HSL, 15 to 45 ng/ml.

**Plasmid construction.** All cloning was performed using standard molecular biology techniques as follows. DNA fragments obtained by restriction digest were physically separated by agarose gel electrophoresis, and the desired fragments were extracted from gel slices using GeneClean (Bio101, Inc., Vista, Calif.). T4 DNA ligase was used to join two fragments together, the ligated fragments were transformed into DH5 $\alpha$  cells made competent by CaCl<sub>2</sub> treatment, and plasmid-carrying strains were isolated on selective media. Enzymes

TABLE 1. Effects of autoinducer additions on the bioluminescence of *V. fischeri* ESR1 and its *lux* mutant derivatives

Strain	Luminescence (quanta/s/cell at OD <sub>600</sub> of 0.1–1.5)	Alteration of luminescence by the addition of <sup>a</sup> :	
		3-oxo-C6-HSL	C8-HSL
ESR1	24	4,400 ± 1,400	2.4 ± 0.89
KV150 ( <i>luxA</i> )	<0.01	<0.01	<0.01
KV240 ( <i>luxI</i> )	8.7	3,600 ± 1,500	1.7 ± 0.76
KV267 ( <i>luxR</i> )	17	1.0 ± 0.11	0.74 ± 0.06

<sup>a</sup> Ratio of the level of luminescence per cell for each strain grown in the presence and in the absence of the specified autoinducer: mean of three measurements, ± 1 standard deviation.

were obtained from either Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.). Plasmid pKV29 (Fig. 1) was constructed by insertion of the 8.8-kb *SalI* fragment from pHV2001<sup>-</sup> (38) containing the *lux* genes from *V. fischeri* strain ES114 (17) into the *SalI* site of pBC (Stratagene, La Jolla, Calif.). The *luxI* gene in that construct carries a 2-bp frameshift mutation near the end of the gene, rendering the protein product inactive (38). Because this strain regains luminescence activity with the addition of 3-oxo-C6-HSL, the product of LuxI (Table 1), the mutation does not have any significant polar effects on downstream *lux* genes.

Plasmid pKV30 (Fig. 1) was constructed as follows. A 3-kb *SalI*-*HindIII* DNA fragment carrying the *luxR*, *luxI*, and *luxC* genes was cloned into pUC19 (54) that had been digested with *SalI* and *HindIII*. The *luxR*-encoding portion of the resulting plasmid, pVO13, was removed by a partial digestion with *EcoRI* and *PstI* and replaced with the gene for erythromycin resistance (*erm*) contained on a 1.2-kb *SmaI*-*PstI* fragment from pKV25 (50), resulting in pVO15. To facilitate the subsequent recombination of the *luxR::erm* mutation into the chromosome of *V. fischeri*, the region of the chromosome downstream of the *luxR* gene was cloned (K. Visick and E. G. Ruby, unpublished data). A *PstI*-*SalI* fragment carrying a portion of the *luxR* gene and approximately 2.5 kb of DNA downstream of *luxR* was cloned into pVO15 digested with *PstI* and *XbaI*, resulting in pKV30 (Fig. 1).

Plasmid pKV44 (51) was obtained by first cloning a 1.6-kb *XhoI* fragment (carrying the *lacI<sup>q</sup>* gene and the *tac* promoter [P<sub>tac</sub>]) from WM2194 (7) into pKV30 digested with *SalI* (Fig. 1). The plasmid construct was then digested with *BglII*, treated with T4 DNA polymerase to fill in the overhanging ends of the DNA, and self-ligated using T4 DNA ligase. In the resulting plasmid, pKV44, the *luxR* gene has a large deletion, the *luxI* gene is disrupted by a 2-bp frameshift mutation at the *BglII* site, and transcription of *luxCDABE* is under the control of P<sub>tac</sub> (Fig. 1). Construction of pKV19, which carries constitutively expressed copies of the wild-type *luxA* and *luxB* genes of *V. harveyi*, has been described previously (49).

**Construction of *V. fischeri* mutants.** Construction of the *V. fischeri lux* mutants was begun by electroporation of the plasmid DNA carrying the *lux* mutation into strain ESR1, followed by antibiotic selection, as described previously for the *luxA* mutant (49). Strains that had undergone a single recombination event, i.e., had in their chromosome one wild-type and one mutant copy of either *luxI* or *luxR*, were identified by their increased levels of luminescence due to the carriage of duplicate copies of one of these regulatory genes. Strains that had undergone the second recombination event, i.e., had replaced the wild-type copy in the chromosome with the mutant *luxI* or *luxR* allele, were identified by a screen for decreased luminescence levels. The *luxR* mutant alleles retained erythromycin resistance, while the *luxI* mutant lost the chloramphenicol resistance encoded by the vector. Strain KV345 was similarly constructed using plasmid pKV44 (Fig. 1) as the donor DNA.

**Southern analysis.** The putative mutations in strains KV240 (*luxI*), KV267 (*luxR*), and KV345 (*luxR luxI* P<sub>tac</sub>) were confirmed by Southern analysis (45) performed as previously described (49). Chromosomal DNA from either KV240 or its parent strain ESR1 was isolated and digested with *SalI*, *SalI/BglII*, and *BglII*. As expected, the *SalI* fragment that carries the *lux* operon was the same in the two strains, but the *SalI/BglII* and *BglII* fragments from KV240 were larger for the mutant strain, which carries a 2-bp frameshift at the *BglII* site. Chromosomal DNA from KV267 or ESR1 was digested with *HindIII* and *SalI*. The *lux* probe-hybridizing fragment from the *HindIII* digest of KV267 DNA was approximately 1 kb shorter than that from the wild-type parent, due to the insertion of a *HindIII* site downstream of the *erm* cassette, and the *SalI lux* fragment from KV267 was shorter by about 1 kb due to the insertion of a *SalI* fragment upstream of the *erm* cassette. Similarly, the *SalI* digests of chromosomal DNA from KV345 yielded fragments of *lux* DNA that were 1 kb larger than those obtained with the wild type, and the *BamHI* fragment was approximately 1.8 kb larger than that of KV267, due to the insertion of the *lacI<sup>q</sup>* cassette.

**Luminescence assays.** *V. fischeri* strains were grown in CM at 28°C in either the presence or absence of added autoinducer (either 3-oxo-C6-HSL or C8-HSL) (21). At approximately 30-min intervals, 1-ml aliquots were removed from the

culture flask, and both bioluminescence and OD levels were quantified. The level of bioluminescence was measured with a Turner (Sunnyvale, Calif.) 20/20 luminometer.

**Squid colonization assays.** Juveniles of *E. scolopes* were inoculated within 4 h of hatching as described previously (41) and exposed for at least 12 h either to single strains of *V. fischeri* or to pairs of competing strains. After this exposure, the animals were transferred to symbiont-free seawater and maintained for up to 3 days. At specified times after inoculation, the juveniles were homogenized, and dilutions of the homogenates were spread on SWT agar plates to determine the number of CFU in the homogenate. In mixed inoculation experiments, the medium contained erythromycin to distinguish between parent and *lux* mutant CFU. Luminescence measurements of juvenile squid colonized by either wild-type or *lux* mutant strains of *V. fischeri* were performed using either the Turner 20/20 luminometer or a scintillation counter modified to detect luminescence by single-photon counting.

**Host developmental events.** Juveniles infected with wild-type or *lux* mutants were assayed for two of the benchmarks of squid development known to be triggered by the presence of the symbiotic bacteria: (i) apoptotic cell death and (ii) swelling of the epithelial cells lining the crypts of the light organ (32). Animals were exposed to *V. fischeri* cells and, at 14 h postinoculation, monitored for the normal pattern of apoptotic cell death. Briefly, the squid were first anesthetized in a 1:1 mixture of filter-sterilized seawater and 7.5% MgCl<sub>2</sub> and then stained for 1 min in a solution containing 5 ng of acridine orange per ml of seawater. Following a ventral dissection, the exposed light organs were visualized by epifluorescence microscopy to determine whether the typical pattern of apoptotic cell death was evident.

For a determination of epithelial cell swelling, squid light organ tissues were prepared and analyzed with transmission electron microscopy (TEM) (31). Juvenile squid were placed in a 0.1 M sodium cacodylate–0.45 M NaCl buffer (pH 7.4) containing 2.5% glutaraldehyde and 2.5% paraformaldehyde and incubated for 12 h at 23°C. This fixation was followed with three 15-min washes with buffer alone, and the samples were then dehydrated in a graded ethanol series. Uranyl acetate (2%) was added to the 70% ethanol wash to increase contrast of the ultrathin sections. Next, the animals were infiltrated with 100% propylene oxide for 15 min and then placed in a 1:1 ratio of propylene oxide and unaccelerated Spurr embedding medium (46) for 30 min. After infiltration, specimens were left for 3 days in unaccelerated Spurr medium and then moved to accelerated Spurr medium for overnight incubation at 23°C. The animals were then embedded within freshly prepared accelerated Spurr medium at 62°C for 24 h. Ultrathin sections were stained first with a solution of 5% uranyl acetate for 10 min and then with a 0.3% solution of lead citrate for 5 min (31).

**Volumetric measurements of crypt epithelial cells.** Stained ultrathin sections were visualized on a LEO 912 transmission electron microscope, and TEM images were captured with a Proscan-CCD frame transfer camera. The epithelial cells selected for measurement were taken from at least five different sections for each animal, all located deep in the interior of the crypt spaces. Previous studies have reported that the shape of crypt epithelial cells in juvenile squid is essentially columnar (8, 32); thus, we calculated whole-cell volumes from two-dimensional areas (height times width) by assuming that the width of the cell along the axis parallel to basal lamina is equal to its depth. The volume of the nucleus of each cell was similarly measured; unlike the case for total-cell volume (see below), no differences were observed in these values under any of the conditions reported in this study (data not shown). We therefore subtracted the nuclear volume from the whole-cell volume to obtain a calculated cytoplasmic volume.

**Statistical analyses of cytoplasmic volumes.** Crypt cell cytoplasmic volumes were compared between groups of animals colonized by different bacterial strains. The volumes of each group were shown to be normally distributed, and a one-way analysis of variance was performed using a Tukey pairwise comparison at the 95% confidence level to determine whether significant differences in cell volumes occurred between the various groups.

## RESULTS

**Construction and luminescence phenotypes of *V. fischeri lux* mutants.** To investigate the role of luminescence in the light organ association of *V. fischeri* with *E. scolopes*, mutants defective either for *luxA*, encoding one of the subunits of luciferase, or for *luxI* or *luxR*, two regulatory genes controlling *lux* gene expression, were constructed in the symbiosis-competent strain ESR1. Some characteristics of the *luxA* mutant have been described previously (49). The other two mutations were made in plasmid-borne copies of the *luxI* and *luxR* genes (Fig. 1) and recombined into strain ESR1. The genetic and physiological nature of the resultant mutants was confirmed both by Southern analysis (see Materials and Methods) and by their luminescence phenotype. In laboratory culture (SWT or CM), the growth rates of the *lux* mutants were indistinguishable from that of their parent, strain ESR1. In contrast, when cultures

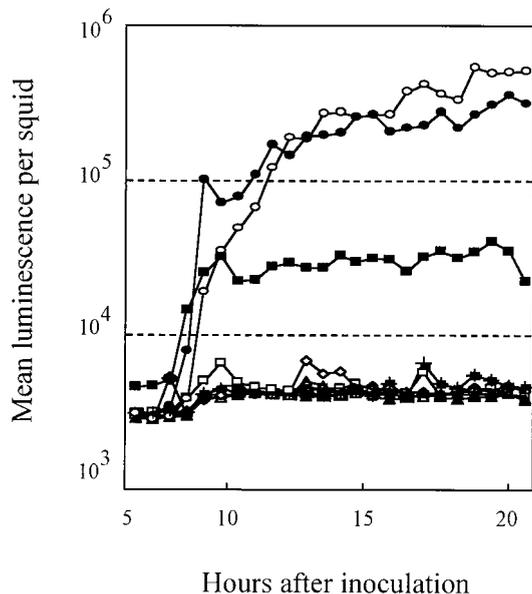


FIG. 2. Relative luminescence over time of newly hatched *E. scolopes* juveniles exposed to either the parent strain ESR1 (○), the *luxA* mutant strain KV150 (■), the *luxI* mutant strain KV240 (◆), the *luxR* mutant strain KV267 (◇), or the *luxR luxI P<sub>tac</sub>* strain KV345 (□). A subset of the squid exposed to KV345 (■) were treated with IPTG (see Materials and Methods) to induce luminescence genes. ESR1-exposed animals treated with IPTG (●) served as a control for IPTG effects on the association.

were grown with an addition of 3-oxo-C6-HSL to achieve the full level of induction that occurs in the light organ (3), those strains that produced a very high level of luminescence (ESR1 and the *luxI* mutant) exhibited a slightly lower rate of growth than those whose light emission remained low or absent (the *luxA* or *luxR* mutants) (data not shown). These results are consistent with previous reports that the synthesis of luciferase by highly induced cells requires a significant energy commitment (30).

Luminescence levels of the parent and the three *lux* mutants were assayed by growing the cells in media that either did or did not contain additions of an autoinducer (either 3-oxo-C6-HSL or C8-HSL). *V. fischeri* strain ESR1, like its wild-type parent (1), produces low concentrations of 3-oxo-C6-HSL in culture and thus emits a relatively low level of luminescence at cell densities below about  $10^8$  per ml (i.e.,  $OD_{600} < 1$ ). Thus, as expected, the luminescence of ESR1 was stimulated several thousand-fold by the addition of 3-oxo-C6-HSL (Table 1); in contrast, cells of ESR1 that were supplemented with C8-HSL increased their luminescence level only about twofold. Because the *luxA* mutant produces no active luciferase, it remains non-luminescent in the presence or absence of either autoinducer (Table 1). In the absence of a functional *luxI* gene, *V. fischeri* cells produced a reduced, basal level of luminescence; however, upon addition of 3-oxo-C6-HSL, light emission of this strain was induced to essentially the same extent as it is in its parent strain (Table 1). Also like the parent strain, the *luxI* mutant showed only a slight induction of bioluminescence in response to the addition of C8-HSL. Interestingly, the *luxR* mutant produced a level of luminescence in culture that was very close to the uninduced level of the parent (Table 1). The addition of 3-oxo-C6-HSL to this mutant strain did not significantly alter bioluminescence levels, suggesting that the absence of LuxR prevented the cells from responding to the added autoinducer. Curiously, addition of C8-HSL to the cul-

ture caused a small decrease in the level of luminescence (Table 1); whether this effect is a significant one awaits further investigation.

**Levels of colonization by the *lux* mutant strains.** The *lux* mutants were assayed for the ability to colonize newly hatched juvenile *E. scolopes*. Measurements of bioluminescence, a central product of the symbiotic association, typically provide a noninvasive but indirect measure of colonization (42). Juvenile squid exposed to strain ESR1 showed the typical induction of luminescence, which becomes apparent between 12 and 24 h postinfection. However, animals colonized by either the *luxA* (luciferase) or the *luxR* or *luxI* (regulatory) mutant failed to produce detectable levels of luminescence (Fig. 2). Although in the light organ the *luxI* and *luxR* mutants are likely to produce some small level of luminescence that is masked by the surrounding animal tissue, additional measurements with a sensitive photometer (data not shown) showed that this activity was undetectable, thus representing less than 0.1% of that produced by the parent strain. These data indicate that the *luxI* and *luxR* mutants are uninduced in the light organ and that it is the bacterium, not the host tissue, that is the primary source of any quorum-sensing inducers.

A direct measure of the extent of colonization that was achieved by each of the strains was obtained by determining the number of CFU present in juvenile light organs at various times after the association had been initiated. Approximately 24 h after initiation, all three of the mutants (*luxA*, *luxI*, and *luxR*) achieved colonization levels that were indistinguishable from that of the parent strain (Fig. 3). However, by about 48 h postinfection, the three *lux* mutant strains exhibited a three- to fourfold reduction in the level of colonization compared to that of the parent strain. This reduced level of colonization remained unchanged for at least another 24 h (data not shown). There is no evidence that the reduction in CFU results from the death of a significant number of the *lux* mutant bacteria in the light organ: an examination of both *luxA* mutant and parent cells released from the light organ crypts after 48 h

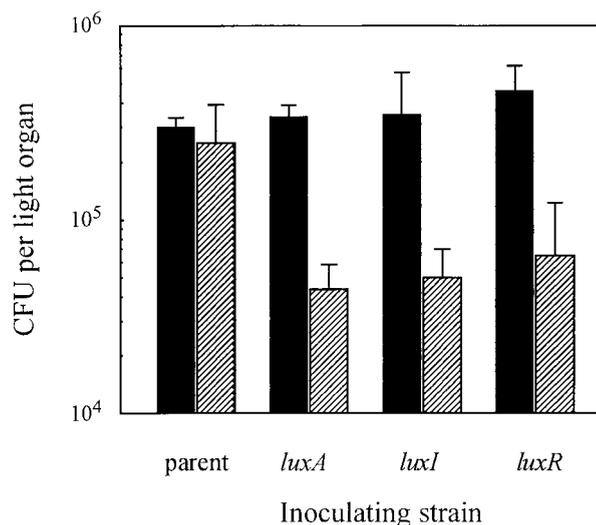


FIG. 3. Symbiotic colonization levels achieved by *lux* mutant strains of *V. fischeri* and their parent, strain ESR1. The number of CFU present in the light organs of juvenile *E. scolopes* exposed to either *lux* mutant *V. fischeri* strains or the parent strain was determined at two times after inoculation, 24 h (black bars) or 48 h (striped bars). Each bar represents an average value obtained with at least four animals (standard error of the mean ranges are indicated). Similar results were obtained in three other independent trials.

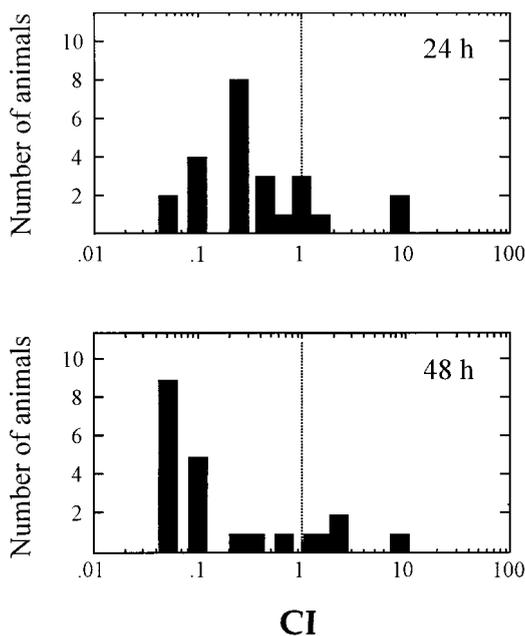


FIG. 4. Colonization of the *E. scolopes* light organ by a mixed inoculum of *lux*<sup>+</sup> and *luxA* mutant *V. fischeri* cells. Forty-eight newly hatched *E. scolopes* juveniles were coinoculated with a 1:1 mixture of the *luxA* mutant (KV150) and its parent (ESR1). At two subsequent time points (24 and 48 h postinfection), the numbers of the two strains in the light organs of each of 24 animals were determined. The competitive index (CI) of the *luxA* mutants was calculated by dividing the number of KV150 mutant cells present in each organ by the number of ESR1 cells present. The number of animals with a given CI value is indicated by the bars. Symbiont populations of animals with a CI of <1 are dominated by *lux*<sup>+</sup> cells.

showed that at least 90% of the cells of both groups were viable in the symbiosis (data not shown). Because the common characteristic of these mutants is that, unlike the parent strain, they do not produce induced levels of luminescence in the squid light organ (Fig. 2), we hypothesized that (i) luminescence is a requirement for the normal persistence of *V. fischeri* in the symbiosis and (ii) because of the role of the *luxI* and *luxR* genes in regulating luminescence induction, they are essential to the normal symbiotic competence of *V. fischeri*.

**Competitive colonization defects of the *V. fischeri lux* mutants.** The ability of the *luxA* mutant strain to colonize the light organs of juvenile squid in coinoculations with the parent (*lux*<sup>+</sup>) strain was also investigated. When presented to the host animal in a 1:1 ratio with parent strain ESR1, the *luxA* mutant showed signs of being outcompeted by the parental strain as early as 24 h postinoculation (Fig. 4). This competitive advantage of ESR1 over the *luxA* mutant occurred in spite of the relatively slower growth of induced ESR1 cells discussed above. Not surprisingly, at 48 h the mutants continued to exhibit a significant competitive defect. A similar defect was observed in the *luxR* mutant when it was competed against the parent strain (data not shown). Because both of these mutants were constructed by the insertion of an *erm* cassette (Fig. 1), we were concerned that the carriage of the cassette was itself the basis for the defect; however, insertions of this cassette into other chromosomal loci had essentially no effect in competition experiments (data not shown). Taken together, these data provided support for the idea that luminescence plays a significant role in the ability of *V. fischeri* to colonize the light organ of *E. scolopes* juveniles and that this effect manifests itself as early as 24 h after colonization has been initiated.

**Defects in the induction of host development by *V. fischeri lux* mutants.** We also tested whether carriage of the *luxA*, *luxI*, or *luxR* mutation resulted in a defect in the ability of the bacteria to trigger normal symbiont-induced changes in the host's program of light organ development. No differences were observed in the mutants' ability to induce the normal temporal and spatial pattern of apoptotic cell death in the ciliated surface of the light organ (data not shown). In contrast, defects were observed in the ability to trigger another morphological event that typically occurs in response to *V. fischeri* colonization, i.e., an increase in cytoplasmic volume, or cell swelling, within the epithelia that line the light organ crypt spaces. This swelling, or edema, results in the transformation of the initially columnar epithelial cells into more cuboidal ones. Cells of *V. fischeri* strain ESR1 (*lux*<sup>+</sup>) that infect juvenile squid induce the swelling within 48 h of inoculation, while the crypt epithelia of uninfected (aposymbiotic) juveniles remain unswollen (Fig. 5A and reference 32). In juvenile animals infected with either the *luxA*, *luxI*, or *luxR* mutant, the mean volume of the crypt cells in these animals was indistinguishable from that of uninfected animals (Fig. 5), demonstrating that all of the *lux* mutants are defective in triggering this specific host developmental event.

One possible reason that the *lux* mutants were unable to induce host cell swelling was their reduced level of colonization. Thus, we determined the cell volumes of crypt epithelia from squid colonized by an amino acid auxotroph of *V. fischeri*. As described previously (16), we found that squid infected by this auxotrophic mutant contained 5 to 10% of the bacteria present in organs colonized by the parent strain, but unlike the *lux* mutants, the auxotroph produced easily detected levels of luminescence in the light organ. Colonization by the auxotroph induced a degree of epithelial cell swelling that was as great as that observed with the parent (Fig. 5A); thus, the defect in cell swelling exhibited by the *lux* mutants is more likely to be related to their failure to produce a normal level of luminescence than to their inability to achieve a normal extent of colonization.

**Complementation of the *luxA* mutant colonization phenotype.** Because of the nature of the *lux* regulon and the mutations we had created in it, the possibility existed that the symbiotic defects described above were due to effects of the *luxI* or *luxR* mutations on some gene(s) other than those responsible for luminescence. To eliminate that possibility, we constructed a *luxR luxI* double mutant, strain KV345, in which transcription of the structural genes of the *lux* operon (*luxCDABE*) was placed under the control of *P<sub>lac</sub>* in the chromosome (Fig. 1). In culture, the level of luminescence of KV345 was induced >100-fold by the addition of IPTG (51); when this strain was used to infect juvenile *E. scolopes*, IPTG addition to the surrounding seawater produced a >50-fold increase in bioluminescence emission from the squid (Fig. 2). The level of colonization established by KV345, in the presence and absence of IPTG, was assayed 48 h after the initiation of the association. While IPTG addition restored luminescence capability and wild-type levels of colonization to this strain, it did not affect the colonization levels of either the parent strain or the *luxR* mutant (Fig. 6A). These results suggest that the *luxR* and/or *luxI* gene products may not play a significant role in early symbiotic colonization, apart from their requirement for the normal induction of the luminescence genes (Fig. 2).

We were concerned that a gene downstream of the *luxCDABE* genes might be playing a role in the observed colonization phenotypes as a result of a polar effect caused by the *luxA* mutation. To look for evidence of polarity, we complemented this mutation *in trans* with a wild-type copy of the *luxA*

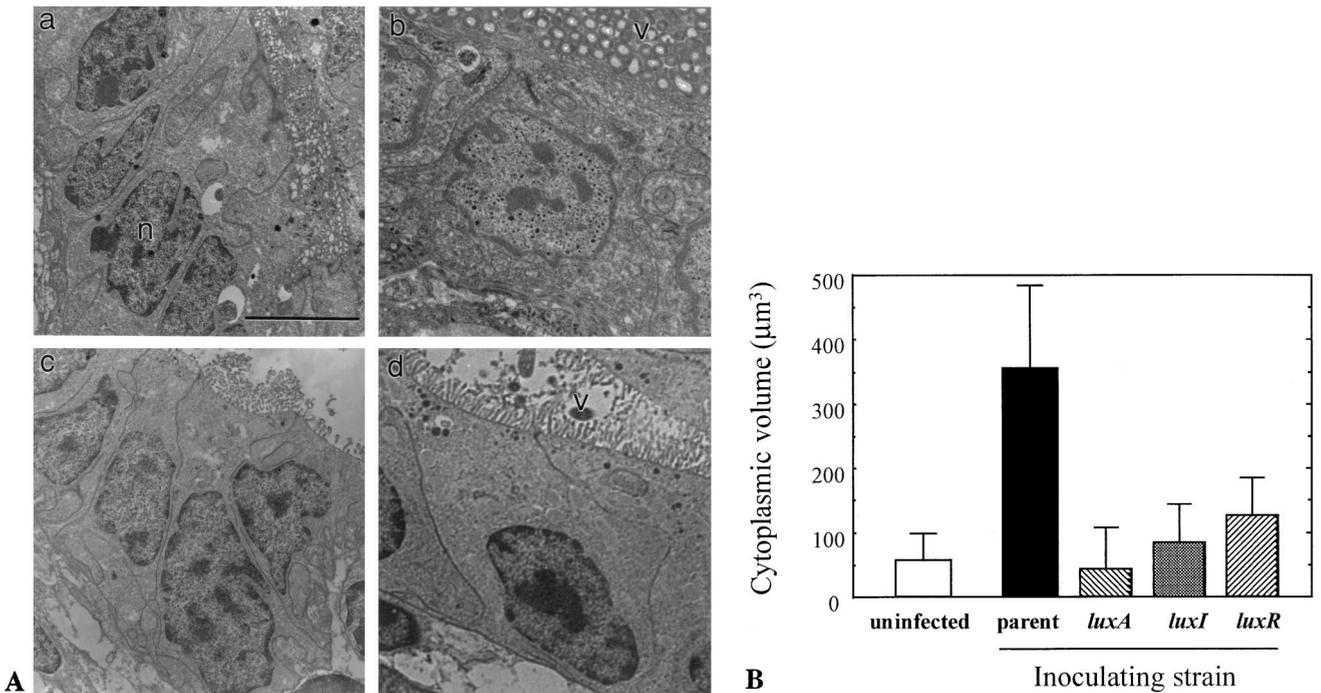


FIG. 5. Effect of colonization by *lux* mutants on host epithelial cell morphology. (A) TEMs illustrating the ultrastructural morphology of crypt epithelial cells 48 h after inoculation with wild-type and mutant strains of *V. fischeri* (v, bacterial cells). (a) Epithelial cells of uninoculated aposymbiotic animals have a narrow, columnar shape (n, nucleus). (b) Epithelial cells of animals exposed to *V. fischeri* strain ESR1 have become swollen and cuboidal. (c) Epithelial cells exposed to the *V. fischeri luxA* mutant have retained a narrow, columnar shape. (d) Epithelial cells exposed to a serine auxotrophic mutant, which is defective in colonizing the light organ at normal levels, nevertheless become swollen and cuboidal. (Bar = 10  $\mu\text{m}$ ) (B) Cytoplasmic volumes of light organ epithelial cells in *E. scolopes* infected by either *lux*<sup>+</sup> or *lux* mutant *V. fischeri*. Uninfected (aposymbiotic) *E. scolopes*, and those that had been exposed to *lux* mutants or their *lux*<sup>+</sup> parent, were fixed for TEM (Materials and Methods) after 48 h of symbiotic infection. The average cytoplasmic volume of the epithelial cells flanking the symbiont population was determined for each condition of inoculation. Measurements from at least 10 epithelial cells were used to determine the average cytoplasmic volume (error bars indicate 95% confidence limits). Similar results were obtained in two other experiments.

gene carried on pKV19 (49). The presence of pKV19, but not the parent vector alone, restored both the luminescence phenotype of the complemented *luxA* mutant strain (data not shown) and normal levels of symbiotic colonization at 48 h (Fig. 6B). These data do not support the hypothesis of polar effects but instead provide evidence for the importance of

luminescence itself in the ability of *V. fischeri* to successfully persist in the juvenile squid light organ.

**Complementation of the epithelial cell swelling defect.** The formal possibility existed that the factors responsible for normal colonization and induction of host cell swelling are distinct. For example, the inability to induce host cell swelling

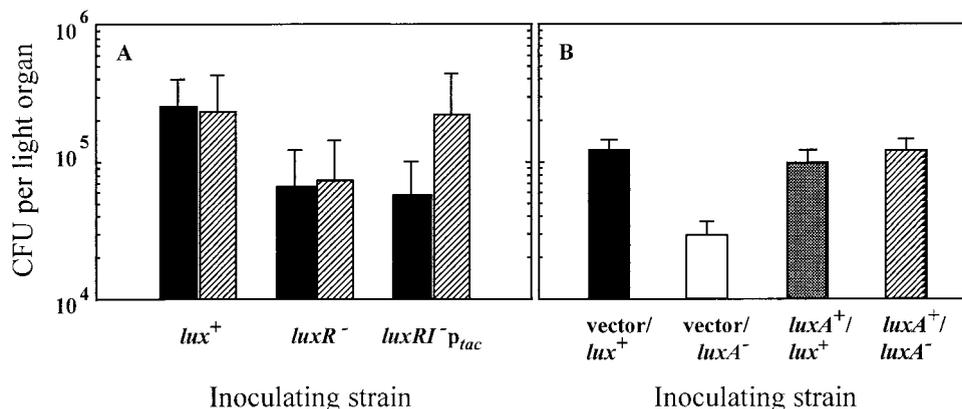


FIG. 6. Complementation of the luminescence defect. (A) The average level of colonization of juvenile *E. scolopes* by the *lux*<sup>+</sup> parent strain (ESR1), the *luxR* mutant (KV267), and the *luxR luxI* double mutant in which *luxCDABE* is under the control of P<sub>tac</sub> (KV345) was determined after 46 h of infection. Throughout the course of the symbiotic infection, eight animals were exposed to IPTG (see Materials and Methods) (striped bars) and eight were not (black bars). (B) *E. scolopes* juveniles were exposed to one of four *V. fischeri* strains: a *luxA* mutant or its *lux*<sup>+</sup> parent, carrying either a plasmid-borne copy of a complementing *luxA*<sup>+</sup> gene or the parent vector alone. The number of CFU present in the light organs of 10 animals from each group was determined 48 h after inoculation. The bars represent the mean level of colonization (standard error of the mean ranges are indicated).

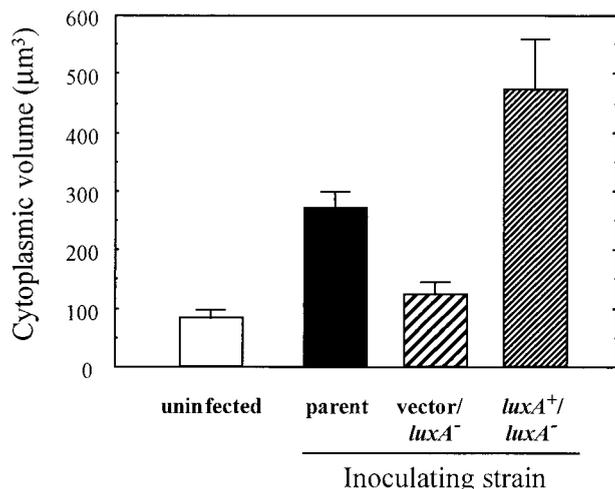


FIG. 7. Cytoplasmic volumes of light organ epithelial cells in *E. scolopes* juveniles infected by a complemented *lux* mutant of *V. fischeri*. Uninfected (aposymbiotic) *E. scolopes* juveniles or those that had been exposed to either the *lux*<sup>+</sup> parent, a *luxA* mutant carrying a *luxA*<sup>+</sup>-complementing plasmid, or the vector alone were fixed for TEM after 48 h of symbiotic infection. The average cytoplasmic volume of the epithelial cells flanking the symbiont population was determined for each condition of inoculation. Measurements from at least 10 epithelial cells were used to determine the average cytoplasmic volume (error bars indicate 95% confidence limits).

may have resulted from a downstream polar effect of the *lux* mutations. Thus, we tested the ability of the *luxA*<sup>+</sup> plasmid to also compensate for the deficiency of the *luxA* mutant in inducing host cell swelling. Measurements of the cytoplasmic volume of host light organ epithelial cells demonstrated that, in contrast to the *luxA* mutant strain, the complemented *luxA* strain was now proficient at inducing cell swelling to at least the same extent as its *lux*<sup>+</sup> parent (Fig. 7). These data provide further evidence of a linkage between bacterial light emission and host cell swelling, and thus that the ability to bioluminesce is essential for the development of a normal symbiotic association between *V. fischeri* and *E. scolopes*.

## DISCUSSION

Three important conclusions can be drawn from this study. First, when colonizing the *E. scolopes* light organ, *luxA*, *luxI*, or *luxR* mutants of *V. fischeri* express less than 0.1% of the luminescence of their *lux*<sup>+</sup> parent strain. Second, as a result of this defect, these mutants cannot maintain a normal population size within the symbiotic association and are unable to successfully compete with a *lux*<sup>+</sup> *V. fischeri* strain. Finally, and most strikingly, colonization by *luxA*, *luxI*, or *luxR* mutants fails to elicit cytoplasmic swelling in the epithelial cells that line the light organ crypts. This third finding constitutes the first report of *V. fischeri* genes that are required to induce a portion of the normal program of bacterium-triggered host differentiation in the light organ. While it has been long suspected that normal microbiota play an important role in the development of the tissues of their hosts (28), the genetic factors that underlie some of these relationships have only recently begun to be revealed (19). Similarly, we are just beginning to learn how these developmental events are related to the mechanisms by which such associations are maintained with fidelity.

Light organ symbioses are remarkably species specific: only *V. fischeri* and the closely related *Vibrio logei* are capable of initiating a light organ symbiosis with *E. scolopes* (12, 41). In

addition to this taxon level of specificity, different *V. fischeri* isolates have various colonization efficiencies (24, 35), suggesting that selectivity is active at even more subtle levels. Our studies of the symbionts of *E. scolopes* have resulted in the intriguing observation that none of the thousands of strains of *V. fischeri* that have been isolated from light organs has been found to be a nonluminescent variant (41). Thus, this specificity apparently extends to the selection of particular genotypes of *V. fischeri*, i.e., those with intact *lux* genes. A competitive disadvantage for *V. fischeri* strains that are *lux* mutants is unexpected because luminescence is not an essential trait of cells growing in laboratory culture, and in fact, light emission requires a considerable energy commitment by these bacteria (26). Thus, the absence of dark variants in symbiotic populations is surprising because the light organ is an environment in which different strains compete for dominance (24), and one would predict that by gaining a mutation in luminescence activity, a dark strain of *V. fischeri* might achieve a slight growth advantage.

For this reason the apparent selectivity for *lux*<sup>+</sup> strains in nature suggested to us that maintaining the ability to luminesce might be of value not only to the host but to the bacterial symbionts as well. Indeed, the results presented here show that the symbiotic relationship in *E. scolopes* is profoundly affected by the inability of a colonizing symbiont to luminesce at normal levels. *V. fischeri* mutants defective for either structural (*luxA*) or regulatory (*luxI* or *luxR*) luminescence genes exhibited a three- to fourfold decrease in the extent of colonization within 48 h of initiating the symbiotic association. In fact, the defect could be detected as soon as 24 h under the competitive conditions of a mixed inoculum with the *lux*<sup>+</sup> parent. While the basis for this more rapid appearance of the colonization defect is unknown, one possibility is that there may be a host challenge that is produced only as a response to the presence of luminescing cells. Colonization by the *lux* mutants alone would not elicit this additional challenge but a mixed infection would, exacerbating their defect.

These competition results further indicate that the presence in the symbiotic organ of wild-type, light-emitting cells does not complement the colonization defect of nonluminescing cells. Therefore, it is the luminescence activity of individual bacterial cells that is important, not any net effect of the symbiotic population as a whole. A similar competitive defect has been reported in experiments with a mutant defective for a periplasm-localized catalase enzyme (*KatA*), which is not rescued by the presence of wild-type *V. fischeri* cells (50). How this apparent cell-level selectivity is achieved remains to be determined.

A recent report has suggested that the products of *luxI* and *luxR* control the expression of several non-*lux* loci, including one required for *V. fischeri* to remain competitive in mixed symbiotic infections (4). Whether the *luxI/R*-dependent induction of these loci observed in culture occurs in the symbiosis, and/or is itself required for symbiotic competency, remains to be directly demonstrated. Our results indicate that the function of *luxI* and *luxR* during the establishment of a normal light organ colonization lies primarily with their role in the induction of bioluminescence. Examination of colonization by a *luxI luxR* double mutant that carried a *tac* promoter to control *lux* structural gene expression revealed that at least in juvenile squid, essentially wild-type levels of colonization could be achieved by this mutant as long as luminescence was artificially induced with IPTG. The direct role of the *luxA* gene was further substantiated by complementation studies that did not indicate a polar effect on any downstream gene critical for colonization. Taken together, these data suggest that in nature,

*V. fischeri* cells that have lost their luminescence capability will be unlikely to sustain a symbiotic relationship, particularly in the face of coinfecting wild-type *V. fischeri*. Population biology theory would predict that the host would gain a benefit from imposing such a restriction (40); however, no evidence directly linking bioluminescence capacity to symbiotic success has been previously reported.

Perhaps the most novel and intriguing result presented in this report is the observation that the *lux* mutants exhibited defects in the ability to trigger a normal host tissue response. Upon colonization by wild-type *V. fischeri*, light organ epithelial cells in direct contact with bacterial symbionts initiate a program of differentiation, including a significant increase in cytoplasmic volume (23, 32). One consequence of crypt epithelial cell swelling is to effectively reduce the volume of the crypt spaces, thus forcing a greater percentage of the bacterial population to be in direct contact with the host epithelial surface. This change is bacterium induced; in the absence of *V. fischeri*, the epithelial cells do not swell, and if the colonization is cured, the swelling subsides (8). All three of the *lux* mutants that were examined were deficient in inducing epithelial edema. Complementation studies with the *luxA* mutant demonstrated that the presence of an intact copy of this gene coordinately restored both luminescence and normal epithelial development. *V. fischeri* cells that carry amino acid auxotrophy mutations making them unable to fully colonize the light organ (16), but that are *lux*<sup>+</sup>, were capable of inducing cell swelling (Fig. 5B). Thus, we believe that the *lux* mutations' effect on host development is due to a loss of luminescence function rather than to a reduced colonization level. We anticipate that there may be a number of bacterial activities that contribute to the induction of swelling and other host developmental events, and that the presence of luciferase and/or its luminescence activity is just one of them.

How does a *lux* mutation result in defects in both the effectiveness of colonization and the ability to induce host edema? Are these two symbiotic phenotypes linked and, if so, in what way? We hypothesize that the *lux* mutants express these symbiotic defects because they are less able to severely reduce the concentration of oxygen around them (44). Bacterial luciferase has an unusually high affinity for molecular oxygen, which is converted to water in the luminescence reaction. Specifically, its affinity constant ( $K_m$ ) for oxygen is in the nanomolar range, several orders of magnitude below that typical of cytochrome oxidase (44). Under the oxygen-limiting conditions in the symbiotic light organ (2), the high affinity of luciferase will give this enzyme an advantage over other oxidases for their required substrate. Thus, brightly luminescing bacteria can rapidly lower the ambient oxygen concentration (26), probably to a level below that resulting from the activities of nonluminescing bacteria. By creating a hypoxic condition around themselves, luminescing *V. fischeri* cells could potentially accomplish two goals: (i) reduce their exposure to host-generated reactive oxygen species (ROS) and (ii) gain access to host nutrients by a mechanism of hypoxia-induced epithelial exocytosis.

The presence of a host halide peroxidase in the light organ crypts (51), as well as the importance to *V. fischeri* of a periplasmic catalase (50), a bacterial defense against host-generated ROS, supports the hypothesis that the light organ environment is oxidatively stressful (48). Thus, it would not be surprising that symbionts might use the action of their luciferase to lower the ambient oxygen concentration, thereby reducing the potential for ROS generation. A mutant deficient in luciferase-catalyzed oxygen utilization might experience an increased steady-state oxygen concentration and become more exposed to damage by ROS (44).

The second phenotype of the *lux* mutants, failure to induce host epithelial cell edema, may also result because the typical hypoxic state is not being created in the light organ crypts by a normal level of luciferase activity. Hypoxia has been demonstrated to have an edemic effect on epithelial cells in other systems (18, 25) and to result in exocytosis of cytoplasmic material (36). Such exocytosed material may be a source of nutrients to support the growing population of luminous symbiotic bacteria colonizing the crypt surfaces. If such a mechanism does, in fact, supply host-derived nutrients to light organ symbionts, then *V. fischeri lux* mutants may face a growth disadvantage because they are unable to create the severe hypoxic conditions that lead to an attendant swelling of the crypt epithelium.

These predictions are being currently tested using a *V. fischeri* strain carrying an altered luciferase enzyme that consumes normal levels of oxygen but produces no luminescence. Such studies should further our understanding of the patterns of cell-cell signaling that occurs between *V. fischeri* and *E. scolopes*. In addition, they will encourage an examination of whether symbiont-induced hypoxia is a general mechanism used by other luminous, or highly respiratory, bacteria as they establish cooperative or pathogenic associations with their hosts.

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