LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization

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**Introduction**

An increasing awareness of the role that benign bacterial associations play in the normal development and health of animals has driven an interest in the mechanisms by which a host initially obtains and subsequently maintains its specific bacterial symbionts (McFall-Ngai, 1999; Hooper and Gordon, 2001). Within hours of hatching, juveniles of the squid *Euprymna scolopes* acquire within the epithelium-lined crypts of their nascent light organ a monospecific population of the luminous bacterium *Vibrio fischeri*. This symbiotic infection initially stabilizes at a level of about $5 \times 10^5$ cells, but eventually reaches about $10^9$ cells in the fully grown adult (Ruby and Asato, 1993). Within the crypt spaces of the light organ, *V. fischeri* cells induce their *lux* genes, which results in the emission of a bright luminescence that is believed to assist the squid in its nocturnal activities (McFall-Ngai, 1990). During the first few hours to days following this benign infection, a number of morphological changes occur in the polarized epithelial cells lining the light organ crypts, e.g. both the volume and the microvillar density of the cells increase fourfold, and they become more cuboidal in shape (McFall-Ngai, 1994; Lamarcq and McFall-Ngai, 1998). These changes are triggered only in the presence of *V. fischeri* cells and presumably enhance the intimacy of the symbionts with one another and with the crypt epithelium.

The light organ is a dynamic environment for the bacteria: each morning, about 95% of the *V. fischeri* population is vented into the surrounding sea water, leaving the remaining 5% to repopulate the organ by the subsequent nightfall (Lee and Ruby, 1994a). The ability to induce this venting behaviour experimentally has facilitated morphological and biochemical analyses of the light organ contents (Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998). Graf and Ruby (1998) concluded that the dense material surrounding the bacteria within the crypts consists largely of peptides that are capable of supporting the growth of auxotrophic mutants of *V. fischeri*. In addition, before entering the external pores that lead into the crypts, *V. fischeri* cells in the ambient sea water must aggregate on and move through a mucous matrix produced by the host (Nyholm et al., 2000).

Recent results have begun to identify bacterial activities such as catalase and bioluminescence that are
required for successful colonization and/or the initiation of host development (Visick and McFall-Ngai, 2000). Preliminary evidence (Fidopiastis and Ruby, 1999) has suggested that V. fischeri produces an extracellular proteolytic activity similar to that exhibited by the Vibrio cholerae Hap (Finkelstein et al., 1983), Vibrio vulnificus Vvp (Nishina et al., 1992) or Vibrio anguillarum EmpA (Garcia et al., 1997) proteins. This activity might allow symbiosis-competent cells of V. fischeri to (i) move through the mucous barrier outside the light organ pores (Nyholm et al., 2000) and/or (ii) gain access to host-derived peptides in the crypts (Graf and Ruby, 1998). The expression of all three of these other Vibrio spp. proteases has been shown to be dependent on TetR family regulator proteins [i.e. HapR (Jobling and Holmes, 1997); SmcR (McDougald et al., 2001; Shao and Hor, 2001); and VanT (Milton et al., 1999) respectively]. In addition, two other homologues have been described: OpaR, which controls colony opacity in Vibrio parahaemolyticus (McCarter, 1998), and LuxR, which is required for luminescence in Vibrio harveyi (Showalter et al., 1990). To date, there have been no reports of a homologous regulatory protein in V. fischeri. To understand better the control of both protease activity and luminescence in V. fischeri, and to examine how these activities might be modulated in the symbiosis, we searched for a gene that might encode a member of this family of regulators.

We report here the discovery in V. fischeri of litR, a gene that encodes a protein with high sequence identity to the other TetR family transcriptional regulators present in Vibrio spp. Its product, designated LitR, not only has functional characteristics that are like those reported for some of the other homologues, but also an unexpected activity. Specifically, we provide evidence that LitR is important for the normal induction of luminescence, and also plays a novel role in modulating the ability of V. fischeri to colonize juvenile squid.

Results

Homology of litR to other regulatory proteins in Vibrio species

Using degenerate primers based on highly conserved coding regions between the V. cholerae hapR and the V. harveyi luxR gene sequences, a 255 bp product was amplified from eight strains of V. fischeri isolated from diverse sources, as well as from two Vibrio logei strains (Fig. 1). No product was obtained from the slightly more distantly related species Photobacterium profundum SS9. The peptide encoded by the polymerase chain reaction (PCR) product from V. fischeri strain ES114 was 67% identical to that encoded by the corresponding region of V. cholerae hapR. Using this PCR product as a probe, the complete litR gene was detected within a 12 kb EcoRI fragment of V. fischeri strain ES114 genomic DNA and further localized to a 3 kb SacI–ClaI fragment. The 3kb fragment was sequenced, and a putative ribosome-binding sequence ‘AAGGA’ was detected 9 bases upstream of the start of the predicted open reading frame (ORF); however, we did not find consensus –35 (TTGACC) or –10 (TACACT) sequences to be present in

![Fig. 1. PCR amplification of a fragment of a V. cholerae hapR homologue from strains of V. fischeri and V. logei. PCR primers recognizing V. cholerae hapR sequences were mixed in a PCR with genomic DNA from a number of strains, and a product of the indicated size was amplified. V. logei MdR16 and MdRD are sea-water isolates from southern California; V. fischeri EM17 and EM24 are light organ symbionts of Euprymna morsei (Ruby and Asato, 1993); V. fischeri ES114 and ES401 are light organ symbionts of Euprymna scolopes (Boettcher and Ruby, 1994); V. fischeri H905 and H906 are sea-water isolates from Kaneohe Bay, Hawaii (Lee and Ruby, 1994); V. fischeri MJ1 is a light organ symbiont of Monocentris japonica (Ruby and Nealson, 1976); and P. profundum SS9 is a deep-ocean sea-water isolate (Welch and Bartlett, 1998).](image-url)
the same location as the promoter regions of hapR, luxR, opaR and smcR (Shao and Hor, 2001).

The litR sequence aligned across its entire coding region with the sequences of hapR, luxR, opaR and smcR. In addition, partial sequences of the ORFs flanking litR shared significant (>80%) identity to the hpt (5’ of litR) and lpd (3’ of litR) genes of V. parahaemolyticus. The litR gene encodes a 201-amino-acid protein that shares significant identity (58–60%) with HapR, V. harveyi LuxR, V. parahaemolyticus OpaR and V. vulnificus SmcR (Fig. 2). The amino-terminal domain residues (numbers 6–69) share particularly high identity, with over 90% conserved in the other homologues. In addition, this region showed significant conservation when compared with a consensus sequence derived from the helix–turn–helix (HTH) DNA-binding domain of genes in the TetR family of negative transcriptional regulators. A phylogenetic comparison of the amino acid sequences of the homologues revealed them all to be within the TetR family of regulatory proteins (Fig. 3). LuxR, OpaR and SmcR form the most closely related grouping, whereas LitR appeared to have branched off earliest during the evolution of the Vibrio clade of TetR proteins.

Luminescence defect in the litR mutant strain PMF8

The growth rates of ES114 and PMF8 were essentially the same when cells were cultured in SWT medium at 28°C. and PMF8 grew only slightly more slowly at 22°C (data not shown). Interestingly, under either of these growth conditions, PMF8 produced no detectable luminescence until the culture reached an optical density (OD) at 600 nm of >1.2. In contrast, the parent strain ES114 always induced luminescence at or before an OD of 1.0

Fig. 2. Amino acid sequence alignment of LitR homologues. Residues that are conserved in all the five proteins are marked with an asterisk. Residues that are encoded by the nucleotides used to construct the degenerate PCR primers are underlined. Numbering refers to the residue order in the HapR molecule.
Subsequently, the level of luminescence (per cell) emitted by PMF8 remained <20% that of ES114. This phenotypic defect could be relieved by the addition of a wild-type copy of either litR or V. harveyi luxR carried in trans (Table 1). These data confirm that litR is responsible for the luminescence defect, and that V. harveyi luxR can not only functionally complement the litR mutation but also, on a multicopy plasmid, leads to a level of luminescence expression that was even greater than that produced by the wild-type strain. In contrast, the presence of pMF2, which encodes a wild-type copy of litR, could not restore luminescence to a V. harveyi luxR mutant (data not shown).

**Regulation of luminescence by LitR enhancement of V. fischeri luxR expression**

To determine how the litR::Kan mutation results in a depression of the onset of light emission, we first asked whether the normal pattern of luminescence could be restored by the addition of either the aliphatic aldehyde decanal (a substrate of the luciferase reaction) or the V. fischeri quorum-sensing autoinducer 3-oxo-hexanoyl homoserine lactone (VAI-1). Supplementing cultures with decanal did not fully restore luminescence of PMF8 to the levels emitted by the wild type, ES114 (Fig. 4). Instead, the addition of aldehyde stimulated luminescence proportionally, with PMF8 cells continuing to emit on average <20% of the luminescence of ES114 cells. Similarly, although the addition of VAI-1 greatly enhanced light emission of both PMF8 and ES114, it did not fully restore PMF8 luminescence to the level emitted by strain ES114 grown in the presence of VAI-1 (Fig. 5). In fact, when compared with the wild-type parent, the absence of a functional litR resulted in a significant delay in the response of PMF8 to either VAI-1 (Fig. 5) or VAI-1 and aldehyde together (data not shown). We interpreted these data to suggest that this small but reproducible difference indicates that the mutant litR allele does not act simply through a direct effect on the levels of either of these
components of the luminescence system in *V. fischeri*. Instead, these data were consistent with the notion that the wild-type allele of *litR* enhances expression of the *V. fischeri luxR* gene (note that although they have the same gene name, *V. fischeri luxR* and *V. harveyi luxR*, the *V. fischeri litR* homologue, encode completely unrelated proteins). We reasoned that, by increasing the basal level of the *V. fischeri* LuxR, the presence of a functional LitR might lead to a more rapid induction of luminescence in response to added VAI-1. In support of this hypothesis, expression of *litR* in trans led to a four- to fivefold increase in the expression of *b-galactosidase* from the *V. fischeri luxR* promoter (Table 2) when assayed in an *Escherichia coli* strain carrying pJE455 (luxR::*lacZ*). No such enhancement by LitR of the expression of the luxICDABE operon was noted by a similarly constructed luxC::*lacZ* fusion (Table 2). Furthermore, mobility shift analyses indicated that LitR binds to a 427 bp BstBI–PvuII fragment that includes the 5’ region of the *luxR* coding sequence and the complete *luxR* promoter region (Fig. 6).

**Other phenotypes affected by the *litR* mutation**

Colonies of *V. fischeri* ES114 grown on SWT agar medium are normally golden yellow and opaque. In contrast, colonies of PMF8 are almost entirely translucent, except for a slight opacity in the centre of the colony. This appearance is similar to that reported for colonies of an SmcR mutant strain of *V. vulnificus* (Shao and Hor, 2001). These findings suggest that *litR*, like *opaR* (McCarter, 1998), *hapR* (Jobling and Holmes, 1997) and *smcR* (Shao and Hor, 2001), may also control opacity. Opacity has also been associated with the ability of *V. vulnificus* to produce siderophores (Reddy et al., 1992). However, PMF8 showed no defect in its ability to sequester iron on CAS agar when compared with the parent strain (data not shown).

The *litR* homologues *hapR* and *smcR* have been reported to control protease production in *V. cholerae* (Jobling and Holmes, 1997) and *V. vulnificus* (McDougald et al., 2001; Shao and Hor, 2001) respectively. However, we were unable to detect any alteration in either protease or mucinase activities in strain PMF8 (data not shown). The protease activities of PMF8 and ES114 were equivalent, and both were reduced to undetectable levels by the addition of Zincov, suggesting that the major detectable extracellular agent of proteolysis in each of these strains is a zinc-requiring metalloprotease(s). Furthermore, overexpression of *litR* in a strain of *E. coli* carrying a *hap* promoter that controlled *lacZ* expression did

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**Table 1. Complementation of the luminescence defect in PMF8.**

<table>
<thead>
<tr>
<th>Strain (with complementing plasmid)</th>
<th>Luminescence units per OD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES114 (<em>litR</em>)</td>
<td>3.5</td>
</tr>
<tr>
<td>PMF8 (<em>litR</em>)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>PMF8 (with pMF2; <em>litR</em>)</td>
<td>30</td>
</tr>
<tr>
<td>PMF8 (with PV08)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>PMF8 (with pMGM150; luxR*)</td>
<td>40</td>
</tr>
</tbody>
</table>

a. Measurements made on cultures at an OD of 1.0; one luminescence unit equals $1.3 \times 10^4$ quanta s$^{-1}$ ml$^{-1}$.

b. PV08 is the parent vector of pMF2.

c. *V. harveyi luxR*.

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**Table 2. *litR*-enhanced expression of *b-galactosidase* from a *V. fischeri luxR* promoter–reporter.**

<table>
<thead>
<tr>
<th>Test plasmid</th>
<th>Reporter plasmid</th>
<th><em>b-Galactosidase expression</em> a</th>
</tr>
</thead>
<tbody>
<tr>
<td>(None)</td>
<td>pJE455 (<em>luxR</em>:lacZ)</td>
<td>7</td>
</tr>
<tr>
<td>pMF7 (<em>litR</em> ; vector control)</td>
<td>pJE455 (<em>luxR</em>:lacZ)</td>
<td>3</td>
</tr>
<tr>
<td>pMF2 (<em>litR</em>)</td>
<td>pJE455 (<em>luxR</em>:lacZ)</td>
<td>121</td>
</tr>
<tr>
<td>(None)</td>
<td>pJE413 (<em>luxC</em>:lacZ)</td>
<td>7</td>
</tr>
<tr>
<td>pMF7 (<em>litR</em> ; vector control)</td>
<td>pJE413 (<em>luxC</em>:lacZ)</td>
<td>8</td>
</tr>
<tr>
<td>pMF2 (<em>litR</em>)</td>
<td>pJE413 (<em>luxC</em>:lacZ)</td>
<td>9</td>
</tr>
</tbody>
</table>

a. Fluorescence units per OD unit ($5 \times 10^8$ cells) above background; values are representative of three independent experiments.
not result in the induction of β-galactosidase activity (data not shown). Taken together, these data suggest that a mutation in \( \text{litR} \) does not affect protease production in \( V.\ fischeri \), and that \( \text{litR} \) is not a functional homologue for this activity of \( \text{hapR} \). In addition to its potential role in protease regulation, \( \text{smcR} \) may also negatively regulate motility (McDougald et al., 2001). However, PMF8 and ES114 were equally motile when assayed in semi-solid agar (data not shown).

**Characterization of light organ colonization by the \( \text{litR} \) mutant**

The three criteria of efficiency, extent and competitiveness were used to assess the ability of the \( \text{litR} \) mutant to colonize the juvenile squid light organ. The colonization efficiency of strain PMF8 at low cell inocula was indistinguishable from its parent; that is 100% of juveniles became infected when they were exposed to sea water containing either strain at concentrations as low as 400 cells ml\(^{-1}\) (data not shown). Interestingly, although there was a 1 h delay in the onset of detectable luminescence, the levels of light emitted by these squids at 12, 24 and 48 h after infection were equivalent for both strains. These results indicated that the mutant was able to reach an essentially fully induced state in the light organ, even without a functioning \( \text{litR} \) gene.

The second criterion examined was the size of the symbiont population attained by each strain. At 24 h after infection, the average number of cfus present in light organs colonized by the parent strain (\( 2.3 \pm 0.7 \times 10^{5} \)) was not significantly different from the number in mutant-colonized animals (\( 1.7 \pm 0.5 \times 10^{5} \)). This similarity was observed in the symbiotic populations of light organs after 48 h as well.

Thirdly, we determined whether either the parent or the mutant strain was able to outcompete the other when they simultaneously colonized the same light organ. Forty-eight hours after exposure of 21 juvenile squids to sea water containing a 1:1 mixture of ES114 and PMF8 cells, the \( \text{litR} \) mutant was the predominant symbiont isolated from 18 (86%) of the light organs (Fig. 7). In a second experiment, the mutant again outcompeted its parent in 79% (11 out of 14) of the juvenile squid (data not shown). Thus, not only can the \( \text{litR} \) mutant colonize the juvenile light organ as well as the wild-type strain, but it also expresses a competitive advantage during at least the first 48 h after infection. Because neither strain exhibited a growth advantage when competed against the other in a mixed inoculum of SWT medium, some condition(s) specific to the light organ environment is apparently responsible for eliciting the differential effect.

**Discussion**

During colonization of host tissue, the expression of sets of bacterial genes can be under the control of specific transcriptional regulators (Cotter and DiRita, 2000). Although most of these regulators have been described in bacteria that initiate pathogenic infections, a few have been found to play a role in the more commonly occurring phenomena of benign bacterial colonization (van Rhijn and Vanderleyden, 1995; Graf and Ruby, 2000). We report here a gene encoding such a regulator in the symbiotic luminous bacterium \( V.\ fischeri \). This gene, designated \( \text{litR} \), is a member of a family of genes found in at least five other \( \text{Vibrio} \) species that are animal pathogens (Showalter et al., 1990; Jobling and Holmes, 1997; McCarter, 1998; Milton et al., 1999; McDougald...
et al., 2000). Our studies with a litR mutant strain of V. fischeri have resulted in two discoveries: (i) a new level at which luminescence is regulated in this species; and (ii) a novel genotype that increases symbiotic competency.

Genetic analysis of the litR gene locus has revealed that it is flanked by gene homologues of hpt (upstream) and lpd (downstream); these two genes also flank V. parahaemolyticus opaR and V. vulnificus smcR, and occur in the same orientation (McCarter, 1998; McDougald et al., 2000). The hpt gene is also found upstream of V. cholerae hapR and V. harveyi luxR, but the downstream flanking genes in each of these species are not related either to lpd or to each other (Jobling and Holmes, 1997). LitR shares about 60% identity to V. harveyi LuxR, V. cholerae HapR, V. parahaemolyticus OpaR and V. vulnificus SmcR. A comparison of the amino acid sequence of these homologues revealed several regions of particularly high identity, including the consensus HTH DNA-binding domain characteristic of the TetR family of negative transcriptional regulators (PROSITE accession number PS01081). Taken together, these similarities suggest that the homologues are derivatives of an ancestral gene that was acquired before these Vibrio species diverged (Fig. 3).

The high sequence similarity of these regulators suggested that they might be able to complement each other genetically. Previous work has shown that V. harveyi LuxR and V. cholerae HapR are functionally interchangeable (Jobling and Holmes, 1997) and, when carried in trans, either OpaR or SmcR could activate the expression of the V. harveyi lux operon in E. coli (McCarter, 1998). In the work reported here, expression of V. harveyi LuxR in trans alleviated the luminescence deficiency of strain PMF8 (Table 1). In contrast, expression of LitR in trans did not similarly restore luminescence to either V. harveyi strain MR1130 (luxR) or an E. coli strain carrying plasmid pRS205 (V. harveyi luxR; luxCDABE); in fact, cell growth was severely inhibited in transconjugants of both these strains (data not shown). These results are consistent with previous reports (Chatterjee et al., 1996), in which overexpression of V. harveyi luxR in certain strains of V. harveyi and E. coli produced a cytotoxic affect, and suggested possible gene dosage effects on other unknown genes.

In most cases, the litR gene homologues in Vibrio species encode a protein that controls the expression of one or more phenotypes believed to be important in the successful colonization of animal tissue (e.g. motility, protease secretion, siderophore production, etc.). Interestingly, although there is some overlap in these phenotypes, the particular set of regulated genes is distinct in each species. In V. fischeri, a litR mutant has two distinct phenotypes in culture: (i) diminished luminescence, similar to but not as great as that of a V. harveyi luxR mutant (Martin et al., 1989); and (ii) reduced colony opacity, like that of a V. parahaemolyticus opaR mutant (McCarter, 1998).

The structure and regulation of the luminescence (lux) genes of V. fischeri have been the subjects of extensive investigation for over 25 years (Nealson, 1999). These genes are arranged as two divergent operons, with luxICDABE comprising the rightward operon, and luxR (not a homologue of V. harveyi luxR) comprising the leftward operon (Engebracht and Silverman, 1987). The luxI product encodes the synthase of the primary V. fischeri autoinducer (VAI-1). The luxC, luxD and luxE genes encode products necessary to synthesize an aliphatic aldehyde, a substrate for luciferase. The α and β subunits of luciferase are encoded by luxA and luxB respectively. In the intergenic sequence between luxR and luxI is a regulatory region of about 150bp that contains a 20bp inverted repeat (called the lux box), to which V. fischeri...
LuxR binds when it is conjugated with VAI-1. The result of this binding is an enhanced transcription of the rightward operon and a concomitant induction of luminescence emission (Devine et al., 1989). LuxR and VAI-1 comprise the primary regulatory components modulating luxICDABE expression (Sinitnikov et al., 1995) and are required for the normal cell density-sensing luminescence response exhibited by V. fischeri in the squid light organ (Visick et al., 2000).

The V. harveyi LuxR protein does not use a quorum-sensing cofactor in its regulation of luminescence gene expression, although a V. harveyi autoinducer has been reported to enhance the transcription of V. harveyi luxR itself (Miyamoto et al., 1996). We propose a similarly direct mechanism to explain the regulation of luminescence by V. fischeri LitR. Mobility shift studies presented here support this hypothesis by identifying a region in the V. fischeri lux operon to which LitR can bind (Fig. 6). This region is located within a 427 bp BstBI–PvuII DNA sequence that encompasses a portion of the S' end of V. fischeri luxR as well as 70 bp of the luxR promoter region: the lux box is not included in this apparent LitR binding region (Fig. 6). Activation of the expression of a promoterless β-galactosidase gene fused to the V. fischeri luxR promoter (but not one fused to luxC) further indicated that LitR not only bound a region of DNA within the luxR promoter, but was also a positive regulator of that promoter’s activity. It is important to note that LitR may have other more complex regulatory roles in V. fischeri. LitR homologues have been reported to express dual positive and negative regulatory roles for both luminescence control by V. harveyi LuxR (Chatterjee et al., 1996) and the modulation of protease (positive) and cytolysin (negative) expression by V. vulnificus SmcR (Shao and Hor, 2001).

Taken together, the following evidence supports a model in which LitR plays its greatest role in luminescence induction as a transcriptional activator of V. fischeri LuxR: (i) PMF8 is delayed in luminescence induction and reduced in luminescence level; (ii) this defect in luminescence behaviour was not fully alleviated by additions of DNA that encompassed the promoter; (iii) LitR was able to bind a region of DNA that encompassed the V. fischeri luxR promoter; and (iv) LitR activated expression of a reporter gene that was controlled by a V. fischeri luxR promoter fusion. As a result of LuxR activating luxR expression, LuxR becomes available to bind to VAI-1 and induce the expression of luxICDABE. When the ambient concentration of VAI-1 reaches a critical level, this mechanism for sensing cell density will dramatically induce the level of light production (Nealson, 1977).

In addition to the role in luminescence regulation played by both V. fischeri LitR and V. harveyi LuxR (Showalter et al., 1990), defects in protease activity, motility and/or siderophore production have also been associated with mutations in LitR homologues from several other Vibrio species (Reddy et al., 1992; Jobling and Holmes, 1997; McDougald et al., 2001). However, although V. fischeri cells normally express all these latter activities, a litR mutation apparently affects none of them. In contrast, LitR does seem to be involved in a fourth associated phenotype, the regulation of opacity; i.e. colonies of PMF8 were noticeably less opaque than those of the parent strain. In V. cholerae (Jobling and Holmes, 1997), V. para-haemolyticus (McCarter, 1998) and V. vulnificus (Reddy et al., 1992), colony opacity or rugosity is controlled by the presence of their LitR homologues and has been associated with the production of an extracellular polysaccharide. In V. vulnificus, opacity has also been correlated with an enhanced virulence resulting from the presence of an antiphagocytic capsular polysaccharide (Wright et al., 1990). Similarly, opaque strains of V. para-haemolyticus are reported to be more resistant to killing by oyster haemocytes (Gentther et al., 1999). When colonizing the squid, V. fischeri cells must face phagocytic host haemocytes in the light organ crypts (Nyholm and McFall-Ngai, 1998), a challenge that they avoid more effectively than other Vibrio species. It will be interesting to see whether the V. fischeri LitR mutant is more or less resistant to this particular host defence; i.e. is the surface chemistry associated with opacity a positive colonization factor for pathogens, but a negative one for co-operative infections?

Perhaps the most striking finding in this work is that, when squid were exposed to an inoculum containing equal cell numbers of both the mutant and the parent strain, PMF8 became the predominant cell type in the symbiotic population. Although surprising, evidence for enhanced infection of a host by bacteria that are mutated for a particular wild-type activity is not without precedent (Campbell et al., 1998; Lee et al., 2001; Shao and Hor, 2001). Nevertheless, our data are unexpected in view of previous work showing that a knock-out mutation in the V. fischeri luxR gene, which is positively regulated by LitR (Table 3), results in both an inability to produce normal levels of luminescence in the light organ and a significantly decreased symbiont population size (Visick et al., 2000). Juvenile squid infected by PMF8 were as luminous at 12, 24 and 48 h after initial infection as were those squid infected with the parent strain. Thus, even in the absence of LitR, luxR transcription and its eventual effect on induction of luminescence still occur. This conclusion is supported by a previous report that the level of VAI-1 in the light organ is over 40-fold higher than that required for maximal luminescence induction (Boettcher and Ruby, 1995), and data presented here showing that cells of
PMF8 growing in culture medium containing VAI-1 rapidly attained levels of luminescence that were only slightly below those reached by the parent strain (Fig. 5). The result is that there is no evidence that the role of LitR in luminescence induction has a significant role in symbiotic colonization.

What then might be the basis of the competitive advantage expressed by the *V. fischeri* litR mutant? The altered opacity of colonies produced by strain PMF8 suggested that this strain might have a different cell surface chemistry. Thus, we hypothesized that such a difference might allow the mutant to adhere to the walls of the light organ crypt more effectively than its wild-type parent. Because ~95% of the symbiotic cells in the light organ are expelled each morning (Lee and Ruby, 1994a), an initially mixed (wild type + litR mutant) population might eventually tend towards dominance by the mutant. When this hypothesis was tested by examining the ratio of mutant and wild-type cells present in the light organ before and after an expulsion event, no evidence for a differential adherence was detected (data not shown). Thus, the mechanism underlying the competitive advantage of the litR mutant, at least during the first few days of colonization, must await future efforts, such as the identification of the genetic loci that LitR regulates. Perhaps a more complete model for LitR-mediated transcriptional activation in *V. fischeri* will provide important clues to how the critical balance that is required for an animal host and its bacterial symbionts maintains a persistent benign infection.

**Experimental procedures**

**Bacterial strains, plasmids and growth media**

The *Vibrio* spp. strains and *E. coli* strains and plasmids used in this study are listed in Table 3. All strains were stored at ~70°C until needed. *V. fischeri* strains were grown in a sea water–tryptone–yeast extract medium (SWT) as described previously (Boettcher and Ruby, 1990). Measurements of culture luminescence were performed in this medium rather than in a conditioned medium (Boettcher and Ruby, 1995) to exclude any possible influence of the *V. harveyi* luxS auto-
inducer. Antibiotics were used at the following concentrations: ampicillin (Ap) 100 μg·ml⁻¹; chloramphenicol (Cm) 30 μg·ml⁻¹; kanamycin (Kn) 100 μg·ml⁻¹; and streptomycin (Sm) 50 μg·ml⁻¹. Xgal indicator was used in Luria–Bertani (LB) agar medium at 40 μg·ml⁻¹.

**PCR, cloning and sequencing of V. fischeri litR**

Cells of *V. fischeri* strain ES114 were grown with shaking in SWT medium at 28°C overnight to an optical density (OD) at 600 nm of between 1.0 and 1.2 (Boet chter and Ruby, 1990). Cells were collected by centrifugation, and genomic DNA was extracted using the Gnome DNA kit (Bio101) in accordance with manufacturer’s instructions. An aliquot containing 100–200 ng of *V. fischeri* genomic DNA was mixed with PCR primers to amplify *litR* in a PCR as described previously (Fidopiastis et al., 1998). The following degenerate PCR primers were used to detect the first the *V. fischeri* hapR homologue: Vchapor-deg5 (forward) 5′ GGN ATN GGN CGN GGN GGN CAY GCN GA 3′ and Vchapor-deg3 (reverse) 5′ CCA TTC AAA CCA TAC TTT GAT 3′. PCR primers Vf litRf (forward) 5′ GGG ATT GGT GGC GGT GAT GCT GA 3′ and Vf litRr (reverse) 5′ CCA TTC AAA CCA TAC TTT GAT 3′ were designed based on the sequence of the homologous product and used to locate the *litR* gene within a 12 kb fragment carried in a plasmid library of EcoRI-cut *V. fischeri* genomic DNA. The *litR* locus was localized on the fragment and sequenced. Using this sequence, PCR primers Vf litRdf (forward) 5′ GGT TAA AGA AGA GGT GAA A 3′ and Vf litRdr (reverse) 5′ GGT AGA GAA GTA TTT GAA GG 3′ were designed to amplify 1 kb of DNA containing the entire *litR* gene with additional flanking genomic DNA. PCR products were ligated into the vector PCR2.1 and cloned into *E. coli* using the TA cloning kit (Invitrogen). The 1 kb PCR product was cut from pCR2.1 with EcoRI and ligated into the mobilizable vector pVO8 (Visick and Ruby, 1997) to create pMF2 (*litR*). Plasmid DNA was purified from several *E. coli* clones using the Perfect Prep plasmid kit (5 prime-3 prime), cut with the appropriate restriction enzymes and loaded into separate wells of a 1% agarose gel. Inserts of the predicted size were sequenced at the Biotechnology Molecular Biology Instrumentation Facility, University of Hawai‘i, Manoa. Double-stranded sequence of the *litR* coding region and single-stranded sequence of flanking DNA extending into the neighbouring upstream and downstream genes were obtained by primer walking. Sequence data were analysed using DNA STRIDER version 1.2, BLAST (Altschul et al., 1990) and VECTOR NTI SUITE software (InforMax). The nucleotide sequence of the 606 bp *litR* coding region has been submitted to the GenBank databases under accession number AF378100.

**Mobility shift assays**

Production and partial purification of *V. fischeri* LitR and *V. harveyi* LuxR proteins were performed using a previously described protocol (Swartzman and Meighen, 1993). Plasmids (encoding Ap resistance) containing *litR* or *luxR* under T7 promoter control were transformed into *E. coli* BL21 (DE3), a strain with T7 RNA polymerase encoded within the chromosome under IPTG-inducible control. The recombinant *E. coli* strains were inoculated to an OD of 0.05 in LB medium containing Ap and grown at 37°C. At an OD of 0.5, IPTG was added to a final concentration of 1 mM to induce T7 polymerase and thereby overexpress the plasmid-encoded gene products. After the cultures had reached an OD of between 1 and 2, the cells were harvested by centrifugation. One hundred microlitres of lysis buffer (Swartzman and Meighen, 1993) was added to a pellet containing about 10⁶ cells, and the suspension was disrupted with a Branson ultrasonicator at a setting of 30, using three 10 s treatments. Cellular debris was removed by centrifugation, and the protein content in the lysis supernatants was estimated spectrophotometrically. Mobility shift assays were performed with the lysates according to established procedures (Fried and Crothers, 1981; Swartzman and Meighen, 1993), using restricted *V. fischeri* DNA as the binding target.

**Mutation of *litR***

The *litR* gene was centred within a 5 kb *PstI–SacI* fragment of DNA from the *EcoRi*-cut *V. fischeri* genomic library, which was subcloned into pBluescript KS (Stratagene), creating plasmid pMF5. The *litR* gene and flanking DNA from pMF5 was moved into the mobilizable vector pEVS79 (Stabb and Ruby, 2002) as follows. The two vectors were digested with *ClaI*, then fused by ligating the two major fragments produced. This hybrid plasmid was restricted with *SacI* and self-ligated to remove a 3 kb fragment of unnecessary DNA. The resulting vector, pMF6, carried *litR* on a 3 kb *SacI–ClaI* fragment. The *litR* gene in pMF6 was then disrupted by the insertion of a 1.2 kb BamHI fragment encoding the kanamycin marker from pUC4K (Vieira and Messing, 1982) into a unique *BglII* site located 180 bases downstream of the predicted *litR* start codon, creating pMF7.

The mutant allele was then introduced into the *V. fischeri* ES114 chromosome by triparental conjugation as described previously (Stabb et al., 2001). The addition of chloramphenicol to growth media selected for single recombination events between pMF7 and the *V. fischeri* genome. Double recombinants that arose upon subsequent transfer, and that no longer retained vector sequences, were identified by both their chloramphenicol sensitivity and stable expression of kanamycin resistance (Visick and Ruby, 1996). The presence of the mutant allele in the chromosome of recombinant strain PMF8 was confirmed by PCR using primer sets Vf litRf and Vf litRr, or Vf litRdf and Vf litRdr, which both flank the site of kanamycin gene insertion.

**Functional complementation of the *litR* mutation**

Plasmid pMF2 (*litR*) or its parent vector pVO8 were electroporated into *E. coli* strains carrying reporter plasmids in which promoters of either *hap* (pQF3.1) (Jobling and Holmes, 1997) or *V. fischeri* luxR (Dunlap and Greenberg, 1985) drive lacZ expression. Plasmid pMF2 was also electroporated into an *E. coli* strain carrying pRS205 (*V. harveyi* luxR; luxCDABE; Showalter et al., 1990) or conjugated (as described previously) into *V. fischeri* luxR and luxI mutants (Visick et al., 2000), and the level of luminescence of the resultant strains was determined. Electroporation was performed at 2.5 kV and 400 Ω using a GenePulser apparatus (Bio-Rad). Lumina...
necescore reporters were also used to determine the role of \textit{litR}. Plasmid pPMF2 was electroporated into an \textit{E. coli} strain carrying pRS205 or conjugated (Stabb et al., 2001) into \textit{V. fischeri luxR} and \textit{luxR} mutants (Visick et al., 2000) before luminescence measurement. \textit{E. coli} reporter strains were streaked on LB agar medium with the appropriate antibiotics to maintain the plasmids and incubated overnight at 37°C. Confluently growing cells from the plates were scrapped and added to separate microfuge tubes. Cells were washed three times in 10% glycerol and resuspended in 100 μl of 10% glycerol to which vector (pVO8), a positive control (pC1.1, \textit{hapR}) (Jobling and Holmes, 1997) or pMF2 was added just before electroporation. Colonies expressing the appropriate antibiotic resistances were expected to contain both the reporter and the experimental plasmids. The presence of pMF2 in the appropriate \textit{E. coli} strains was confirmed by PCR.

The results of \textit{litR} complementation experiments with the \textit{hap} promoter reporter were indicated by the blue or white colour of colonies arising on LB agar medium supplemented with Xgal and the appropriate antibiotics. Confirmation of complementation of the \textit{V. fischeri luxR} promoter reporter was performed using the Ima Gene Green C12 FDG lacZ gene expression kit (Molecular Probes), according to the manufacturer’s instructions. Fluorescence emission was measured using an HTS 7000 fluorimeter (Perkin-Elmer). To determine their effects on light production in either \textit{V. fischeri} or \textit{V. harveyi}, plasmids pMF2 and pMGM150 (\textit{V. harveyi luxR}; Miyamoto et al., 1996) were conjugated into the recipient Vibrio species as described previously (Stabb et al., 2001). The resulting strains were grown in SWT broth, and their levels of luminescence were determined at an OD of 1.0.

\textbf{Bacterial bioluminescence assays}

The level of bacterial luminescence in culture, with or without either exposure to 3 mM decyl aldehyde (decanal) or the addition of the \textit{V. fischeri} autoinducer, 3-oxohexanoyl L-homoserine lactone (VAI-1) to a concentration of 200 ng ml$^{-1}$, was determined as described previously (Fidopiastis et al., 1998; 1999). Bacterial strains were inoculated to an OD of 0.01 in flasks containing 15 ml of SWT medium and grown with shaking at either 22°C or 28°C. The OD and luminescence of aliquots of the cultures were measured at regular intervals throughout the exponential phase of growth.

\textbf{Colony appearance, protease activity, siderophore production and motility}

The protease activities of \textit{V. fischeri} strains ES114 and PMF8 were determined using cells grown either on solid medium or in broth culture. Protease activity on plates was assayed by streaking bacterial strains on a basal medium consisting of 50 mM Tris-HCl buffer (pH 7.4), 340 μM K$_2$HPO$_4$ and 15 g of agar per litre of artificial sea water (Reichelt and Baumann, 1973) to which 10 g of porcine mucin (Sigma Chemical) were added per litre as the sole carbon and nitrogen source. After inoculation, plates were incubated for up to 96 h at 28°C and then flooded with a 15% HgCl$_2$ solution (acidified with HCl) and incubated overnight. This overlying solution was then replaced with a 1% aqueous Coomassie blue stain and incubated overnight again. When the stain solution was removed, the absence of a blue colour staining in the medium around the area of bacterial growth indicated the extent of mucin digestion. Protease activity was also assayed on cells grown to exponential phase with shaking in SWT medium. An aliquot of cells was mixed in a reaction buffer (200 mM Tris-HCl, pH 7.8) containing 2 mM sodium azide and 5 μM L-leucine 7-amido-4-methyl-coumarin protease substrate (Sigma Chemical). At intervals of 8 min, fluorescence emission by the product of the proteolytic cleavage of this substrate was determined in the HTS 7000 fluorimeter. Some of the reactions also contained 500 μM Zinc (CalBiochem), a specific zinc-metalloprotease inhibitor.

Siderophore production was determined by streaking strains on an agar medium containing the iron-chelation indicator chrome azurol S (CAS), prepared as described previously (Lee and Ruby, 1994b). The degree of motility of bacterial strains was determined by stabbing cells into SWT medium solidified with 0.4% agar and monitoring over time the diameter of the halo of migrating cells moving outwards from the point of inoculation.

\textbf{Colonization assays}

The ability of the \textit{V. fischeri} \textit{litR} mutant strain to colonize juvenile \textit{E. scolopes} squid was determined as described previously (Ruby and Asato, 1993). Briefly, cells of PMF8 and ES114 were inoculated into natural sea water to concentrations of between 400 and 2200 cells ml$^{-1}$, either as the individual strains or as a 1:1 mixture of the two. Newly hatched, uninfected (aposymbiotic) juvenile squid were then placed in this sea water and maintained at 22°C for up to 48 h. The colonization process was monitored at 12, 24 and 48 h after initial exposure to the bacteria by measuring the amount of bacterial bioluminescence emitted from the squid. At either 24 or 48 h after initial exposure to the bacteria, squid light organs were homogenized and plated on SWT agar. Total colony-forming-units (cfu) were calculated and, if the light organs had been colonized by a mixture of the two strains, about 100 colonies were patched onto both LBS agar and LBS agar containing Kn to determine the ratio of \textit{litR} mutant to wild-type cells in each light organ. This ratio was then divided by the ratio (usually between 1.0 and 1.2) of mutant to wild type in the inoculum used to infect squid, and the resulting value was termed the mutant’s relative competitive index (RCI).

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