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

Pat M. Fidopiastis, Carol M. Miyamoto,
Michael G. Jobling, Edward A. Meighen and
Edward G. Ruby

Summary

Vibrio fischeri is the bacterial symbiont within the light-emitting organ of the sepiolid squid *Euprymna scolopes*. Upon colonizing juvenile squids, bacterial symbionts grow on host-supplied nutrients, while providing a bioluminescence that the host uses during its nocturnal activities. Mutant bacterial strains that are unable to emit light have been shown to be defective in normal colonization. A 606 bp open reading frame was cloned from *V. fischeri* that encoded a protein, which we named LitR, that had about 60% identity to four related regulator proteins: *Vibrio cholerae* HapR, *Vibrio harveyi* LuxR, *Vibrio parahaemolyticus* OpaR and *Vibrio vulnificus* SmcR. When grown in culture, cells of *V. fischeri* strain PMF8, in which *litR* was insertionally inactivated, were delayed in the onset of luminescence induction and emitted only about 20% as much light per cell as its parent. Protein-binding studies suggested that LitR enhances quorum sensing by regulating the transcription of the *luxR* gene. Interestingly, when competed against its parent in mixed inocula, PMF8 became the predominant symbiont present in 83% of light organs. Thus, the *litR* mutation appears to represent a novel class of mutations in which the loss of a regulatory gene function enhances the bacterium's competence in initiating a benign infection.

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 5×10^5 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 3 kb 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).

1	*	****	*****	***	**	**	***	*****	*****	50
HapR	MDASIEKRPR	TRLSPQKRKL	QLMEIALEVF	AKRGIGRGGH	<u>ADIAEIAQVS</u>					
LuxR	.MDSIAKRPR	TRLSPLKRKQ	QLMEIALEVF	ARRGIGRGGH	<u>ADIAEIAQVS</u>					
OpaR	.MDSIAKRPR	TRLSPLKRKQ	QLMEIALEVF	ARRGIGRGGH	<u>ADIAEIAQVS</u>					
SmcR	.MDSIAKRPR	TRLSPLKRKQ	QLMEIALEVF	ARRGIGRGGH	<u>ADIAEIAQVS</u>					
LitR	.MDTIQKRPR	TRLSPEKRKE	QLLDIAIEVF	SQRGIGRGGH	<u>ADIAEIAQVS</u>					
51	*****	*****	***	*	*		* **	*		100
HapR	VATVFNYFPT	REDLVDDVLN	FVVRQYSNFL	TDHIDLDDLV	KTNLQTVCKE					
LuxR	VATVFNYFPT	REDLVDEVLN	HVVRQFSNFL	SDNIDLDIHA	RENIANITNA					
OpaR	VATVFNYFPT	REDLVDEVLN	HVVRQFSNFL	SDNIDLDIHA	RENIANITNA					
SmcR	VATVFNYFPT	REDLVDEVLN	HVVRQFSNFL	SDNIDLDLHA	KENIANITNA					
LitR	VATVFNYFPT	REDLVDDVLN	KVNEFHQFI	NNSISLDDLV	RSNLNTLLLN					
101		*	*****	*****	*			**	*	150
HapR	MVKLAMTDCH	<u>WLKVWFEWSA</u>	STRDEVWPLF	VSTNRTNQLL	IRNMFMKAME					
LuxR	MIELVSQDCH	<u>WLKVWFEWSA</u>	STRDEVWPLF	VTNRTNQLL	VQNMFIKAIE					
OpaR	MIELVSQDCH	<u>WLKVWFEWSA</u>	STRDEVWPLF	VSTNRTNQLL	VQNMFIKAIE					
SmcR	MIELVVQDNH	<u>WLKVWFEWSA</u>	STRDEVWPLF	VTNRTNQLL	VQNMFIKAIE					
LitR	IIDSVQTGNK	WIKVWFEWST	STRDEVWPLF	LSTHSNTNQV	IKTMFEEGIE					
151	* * *	*	***	**	* * *			* ****		200
HapR	RGELCEKHDV	DNMASLFHGI	FYSIFLQVNR	LGEQEAVYKL	ADSYLNMLCI					
LuxR	RGEVCDQHEP	EHLANLFHGI	CYSIFVQANR	SKSEAELTNL	VSAYLDMLCI					
OpaR	RGEVCDQHDS	EHLANLFHGI	CYSLFVQANR	FKGEAELKEL	VSAYLDMLCI					
SmcR	RGEVCDQHNP	EDLANLFHGI	CYSLFVQANR	TNNTAELSKL	VSSYLDMLCI					
LitR	RNEVCNDHTP	ENLTKMLHGI	CYSVFIQANR	NSSSEEMEET	ANCFLNMLCI					
201	*									
HapR	YKN...									
LuxR	YNREHH									
OpaR	YNREH.									
SmcR	YKREHE									
LitR	YK....									

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.

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. 3. Phylogenetic tree constructed from the amino acid sequences of LitR and five of its homologues using the neighbour-joining method (Saitou and Nei, 1987). The homologues are found in *V. harveyi* (LuxR), *V. parahaemolyticus* (OpaR), *V. vulnificus* (SmcR), *V. cholerae* (HapR), *V. fischeri* (LitR) and *Salmonella enterica* (TetR). Calculated evolutionary distances are based on the 'p-distance' or the proportion of amino acid sites at which two sequences differ. The bar indicates a p-distance of 0.1.

(Fig. 4). 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

Fig. 4. Luminescence patterns of cells of *V. fischeri* strain ES114 (squares) and strain PMF8 (circles), with (open symbols) or without (closed symbols) exposure to decanal. At regular intervals, an aliquot of each culture was removed and measured for both OD and luminescence. Decanal was then added to the aliquot, and the level of luminescence was remeasured. One luminescence unit equals 1.3×10^4 quanta $s^{-1} ml^{-1}$. Similar data were obtained in two other experiments.

Table 1. Complementation of the luminescence defect in PMF8.

Strain (with complementing plasmid)	Luminescence units per OD ^a
ES114 (<i>litR</i> ⁺)	3.5
PMF8 (<i>litR</i> ⁻)	<0.1
PMF8 (with pMF2; <i>litR</i> ⁺)	30
PMF8 (with pVO8 ^b)	<0.1
PMF8 (with pMGM150; <i>luxR</i> ^{+c})	40

a. Measurements made on cultures at an OD of 1.0; one luminescence unit equals 1.3×10^4 quanta s⁻¹ ml⁻¹.

b. pVO8 is the parent vector of pMF2.

c. *V. harveyi luxR*.

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 β -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 *Bst*BI–*Pvu*II 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

Fig. 5. Luminescence patterns of cells from *V. fischeri* strain ES114 (circles) and strain PMF8 (squares), grown in the presence (open symbols) or absence (closed symbols) of added VAI-1. One luminescence unit equals 1.3×10^4 quanta s⁻¹ ml⁻¹.

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

Test plasmid	Reporter plasmid	β -Galactosidase expression ^a
(None)	pJE455 (<i>luxR::lacZ</i>)	7
pMF7 (<i>litR</i> ⁻ ; vector control)	pJE455 (<i>luxR::lacZ</i>)	3
pMF2 (<i>litR</i> ⁺)	pJE455 (<i>luxR::lacZ</i>)	121
(None)	pJE413 (<i>luxC::lacZ</i>)	7
pMF7 (<i>litR</i> ⁻ ; vector control)	pJE413 (<i>luxC::lacZ</i>)	8
pMF2 (<i>litR</i> ⁺)	pJE413 (<i>luxC::lacZ</i>)	9

a. Fluorescence units per OD unit (5×10^8 cells) above background; values are representative of three independent experiments.

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

Fig. 7. Competition between the wild-type *V. fischeri* symbiont ES114 and its derivative, the *litR* mutant PMF8, during colonization of juvenile squid light organs. Twenty-one newly hatched squid were exposed to a mixed inoculum containing equal cell numbers of each strain. Forty-eight hours after the initial exposure to the bacterial inoculum, squid light organs were homogenized, and dilutions were spread on SWT agar medium. The ratio of mutant to parental bacterial strain in each light organ is expressed as the relative competitive index (RCI) and is indicated by a circle. Circles with an arrow indicate an RCI of >100. An RCI above 1.0 indicates that the *litR* mutant cells were competitively dominant.

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 over-expression 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 *luxI-CDABE* 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 (Sitnikov *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 *Bst*BI–*Pvu*II DNA sequence that encompasses a portion of the 5' 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 cytotoxin (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 aldehyde and/or VAI-1; (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 LitR 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. parahaemolyticus* (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. parahaemolyticus* are reported to be more resistant to killing by oyster haemocytes (Genthner *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 symbiont 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 squids 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

Table 3. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Reference or source
<i>E. coli</i>		
DH5a	<i>V. fischeri luxR</i> promoter:: <i>lacZ</i>	Dunlap and Greenberg (1985)
BL21	T7 RNA polymerase, Ptac	Sturdivant <i>et al.</i> (1990)
<i>V. fischeri</i>		
ES114	<i>E. scolopes</i> light organ symbiont	Boettcher and Ruby (1990)
PMF8	ES114 derivative, <i>litR</i> ::Kn	This study
ES401	<i>E. scolopes</i> light organ symbiont	This study
EM17	<i>E. morsei</i> light organ symbiont	Nishiguchi <i>et al.</i> (1998)
EM24	<i>E. morsei</i> light organ symbiont	Nishiguchi <i>et al.</i> (1998)
MJ1	<i>Monocentris japonica</i> symbiont	Ruby and Nealson (1976)
ATCC 7744	<i>V. fischeri</i> type strain	American Type Culture Collection
H905	Hawaii sea-water isolate	Lee and Ruby (1994)
H906	Hawaii sea-water isolate	Lee and Ruby (1994)
<i>V. logei</i>		
MdR16	S. California sea water	Fidopiastis <i>et al.</i> (1998)
MdRD	S. California sea water	Fidopiastis <i>et al.</i> (1998)
<i>V. harveyi</i>		
B392	Sea-water isolate	Reichelt and Baumann (1973)
MR1130	B392 derivative, <i>luxR</i> null mutant	Showalter <i>et al.</i> (1990)
Plasmid		
pKSII ⁺	Bluescript, Ap ^R resistance	Stratagene
pVO8	pACYC with Cm ^R , Em ^R	Visick and Ruby (1997)
pJE413	pBR322 with <i>luxC</i> ::mini-Mu, Kn ^R	Dunlap and Greenberg (1985)
pJE455	pBR322 with <i>luxR</i> ::mini-Mu, Kn ^R	Dunlap and Greenberg (1985)
pUC4K	Kn ^R on 1.2 kb <i>Bam</i> HI fragment	Vieira and Messing (1982)
pEVS79	pBC derivative, <i>mob</i> ⁺ , Cm ^R	Stabb and Ruby (2002)
pMGM150	<i>V. harveyi luxR</i> on pKT230, Sm ^R	Miyamoto <i>et al.</i> (1990)
PMF2	<i>litR</i> on 1 kb fragment, pVO8, Cm ^R	This study
PMF6	<i>litR</i> on 3 kb <i>SacI</i> – <i>Clal</i> fragment in pEVS79, Cm ^R	This study
PMF7	PMF6, <i>litR</i> ::Kn, Cm ^R	This study
pRS205	<i>V. harveyi luxCDABE</i>	Showalter <i>et al.</i> (1990)
pQF3.1	<i>V. cholerae hap</i> promoter:: <i>lacZ</i>	Jobling and Holmes (1997)
pC1.1	<i>V. cholerae hapR</i> gene	Jobling and Holmes (1997)
pCR2.1	Cloning vector, Ap ^R , Cm ^R	Invitrogen

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 (Boettcher 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 first the *V. fischeri* *hapR* homologue: VchapR-deg5 (forward) 5' GGN ATN GGN CGN GGN GGN CAY GCN GA 3' and VchapR-deg3 (reverse) 5' CCA YTC RAA CCA NAC YTT NAR CCA 3'. PCR primers Vf litRf (forward) 5' GGC ATT GGT GGC GGT GGT CAT GCT GA 3' and Vf litRr (reverse) 5' CCA TTC AAA CCA TAC TTT GAT CCA 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' GAT TAA GGA AGA GCT GTT AAC GG 3'; and Vf litRdr (reverse): 5' GCT GCG 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 10⁹ 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 pEV579 (Stabb and Ruby, 2002) as follows. The two vectors were digested with *Clal*, 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*–*Clal* fragment. The *litR* gene in pMF6 was then disrupted by the insertion of a 1.2 kb *Bam*HI fragment encoding the kanamycin marker from pUC4K (Vieira and Messing, 1982) into a unique *Bgl*II 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). Lumi-

nescence reporters were also used to determine the role of *litR*. Plasmid pPMF2 was electroporated into an *E. coli* strain carrying pRS205 or conjugated (Stabb *et al.*, 2001) into *V. fischeri luxI* and *luxR* mutants (Visick *et al.*, 2000) before luminescence measurement. *E. coli* reporter strains were streaked on LB agar medium with the appropriate antibiotics to maintain the plasmids and incubated overnight at 37°C. Confluent growing cells from the plates were scraped 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, *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 *E. coli* strains was confirmed by PCR.

The results of *litR* complementation experiments with the *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 *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 *V. fischeri* or *V. harveyi*, plasmids pMF2 and pMGM150 (*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.

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 *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.

Colony appearance, protease activity, siderophore production and motility

The protease activities of *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 K₂HPO₄ 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% HgCl₂ solution (acidified with HCl) and incubated overnight. This overlying solution was then replaced with a 1% aqueous Coomassie blue stain and incu-

bated 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 Zincov (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.

Colonization assays

The ability of the *V. fischeri litR* mutant strain to colonize juvenile *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 *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).

Acknowledgements

We thank C. Lupp for help with several experiments, and K. Visick, D. Millikan and E. Stabb for valuable advice and technical assistance throughout the course of this study. M. Urbanowski generously provided the plasmid pJE413. This work was supported by National Institutes of Health grant RR12294 to E.G.R. and M. McFall-Ngai, and by National Science Foundation grant IBN 9904601 to M. McFall-Ngai and E.G.R.

References

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., and

- Lipmann, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.
- Boettcher, K.J., and Ruby, E.G. (1990) Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. *J Bacteriol* **172**: 3701–3706.
- Boettcher, K.J., and Ruby, E.G. (1994) Occurrence of plasmid DNA in the sepiolid squid symbiont *Vibrio fischeri*. *Curr Microbiol* **29**: 279–286.
- Boettcher, K.J., and Ruby, E.G. (1995) Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. *J Bacteriol* **177**: 1053–1058.
- Campbell, E.L., Brahamsha, B., and Meeks, J.C. (1998) Mutation of an alternative sigma factor in the cyanobacterium *Nostoc punctiforme* results in increased infection of its symbiotic plant partner, *Anthoceros punctatus*. *J Bacteriol* **180**: 4938–4941.
- Chatterjee, J., Miyamoto, C.M., and Meighen, E.A. (1996) Autoregulation of *luxR*: the *Vibrio harveyi* *lux*-operon activator functions as a repressor. *Mol Microbiol* **20**: 415–425.
- Cotter, P.A., and DiRita, V.J. (2000) Bacterial virulence gene regulation: an evolutionary perspective. *Annu Rev Microbiol* **54**: 519–565.
- Devine, J.H., Shadel, G.S., and Baldwin, T.O. (1989) Identification of the operator of the *lux* regulon from *Vibrio fischeri* ATCC 7744. *Proc Natl Acad Sci USA* **86**: 5688–5692.
- Dunlap, P.V., and Greenberg, E.P. (1985) Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic AMP and cyclic AMP receptor protein. *J Bacteriol* **164**: 45–50.
- Engelbracht, J., and Silverman, M. (1987) Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence. *Nucleic Acids Res* **15**: 10455–10467.
- Fidopiastis, P.M., and Ruby, E.G. (1999) The role of the homologue of the *Vibrio cholerae* *hapR* gene in the symbiotic colonization of a squid by *Vibrio fischeri*. *Abstr Gen Meet Am Soc Microbiol* **99**: 461–462.
- Fidopiastis, P.M., von Boletzky, S., and Ruby, E.G. (1998) A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepioida*. *J Bacteriol* **180**: 59–64.
- Fidopiastis, P.M., Sørum, H., and Ruby, E.G. (1999) Cryptic luminescence in the cold-water fish pathogen *Vibrio salmonicida*. *Arch Microbiol* **171**: 205–209.
- Finkelstein, R.A., Boesman-Finkelstein, M., and Holt, P. (1983) *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F. M. Burnet revisited. *Proc Natl Acad Sci USA* **80**: 1092–1095.
- Fried, M.G., and Crothers, D.M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res* **9**: 6505–6525.
- Garcia, T., Otto, K., Kjelleberg, S., and Nelson, D.R. (1997) Growth of *Vibrio anguillarum* in salmon intestinal mucus. *Appl Environ Microbiol* **63**: 1034–1039.
- Genthner, F.J., Volety, A.K., Oliver, L.M., and Fischer, W.S. (1999) Factors influencing *in vitro* killing of bacteria by hemocytes of the Eastern oyster (*Crassostrea virginica*). *Appl Environ Microbiol* **65**: 3015–3020.
- Graf, J., and Ruby, E.G. (1998) Host-derived amino acids support the proliferation of symbiotic bacteria. *Proc Natl Acad Sci USA* **95**: 1818–1822.
- Graf, J., and Ruby, E.G. (2000) Novel effects of a transposon insertion in the *Vibrio fischeri* *glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. *Mol Microbiol* **37**: 168–179.
- Hooper, L.V., and Gordon, J.I. (2001) Commensal host-bacterial relationships in the gut. *Science* **292**: 1115–1118.
- Jobling, M.G., and Holmes, R.K. (1997) Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi* *luxR* gene. *Mol Microbiol* **26**: 1023–1034.
- Lamarco, L.H., and McFall-Ngai, M.J. (1998) Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri*. *Infect Immun* **66**: 777–785.
- Lee, K.H., and Ruby, E.G. (1994a) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microbiol* **60**: 1565–1571.
- Lee, K.H., and Ruby, E.G. (1994b) Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. *J Bacteriol* **176**: 1985–1991.
- Lee, S.H., Butler, S.M., and Camilli, A. (2001) Selection for *in vivo* regulators of bacterial virulence. *Proc Natl Acad Sci USA* **98**: 6889–6894.
- McCarter, L.L. (1998) OpaR, a homolog of *Vibrio harveyi* LuxR, controls opacity of *Vibrio parahaemolyticus*. *J Bacteriol* **180**: 3166–3177.
- McDougald, D., Rice, S.A., and Kjelleberg, S. (2000) The marine pathogen *Vibrio vulnificus* encodes a putative homologue of the *Vibrio harveyi* regulatory gene, *luxR*: a genetic and phylogenetic comparison. *Gene* **248**: 213–221.
- McDougald, D., Rice, S.A., and Kjelleberg, S. (2001) SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus*. *J Bacteriol* **183**: 758–762.
- McFall-Ngai, M. (1990) Cypsis in the pelagic environment. *Am Zool* **30**: 175–188.
- McFall-Ngai, M.J. (1994) Evolutionary morphology of a squid symbiosis. *Am Zool* **34**: 554–561.
- McFall-Ngai, M.J. (1999) Consequences of evolving with bacterial symbionts: insights from the squid-vibrio associations. *Annu Rev Ecol Syst* **30**: 235–256.
- Martin, M., Showalter, R., and Silverman, M.R. (1989) Identification of a locus controlling expression of luminescence genes in *Vibrio harveyi*. *J Bacteriol* **171**: 2406–2414.
- Milton, D., Hope, V., Camara, M., and Williams, P. (1999) Metalloprotease production in *Vibrio anguillarum* is regulated by VanS, a homolog of *Vibrio harveyi* LuxR. *Abstr Gen Meet Am Soc Microbiol* **99**: 366.
- Miyamoto, C.M., Meighen, E.A., and Graham, A.F. (1990) Transcriptional regulation of *lux* genes transferred into *Vibrio harveyi*. *J Bacteriol* **172**: 2046–2054.
- Miyamoto, C.M., Chatterjee, J., Swartzman, E., Szittner, R., and Meighen, E.A. (1996) The role of *lux* autoinducer in regulating luminescence in *Vibrio harveyi*: control of *luxR* expression. *Mol Microbiol* **19**: 767–775.
- Nealson, K.H. (1977) Autoinduction of bacterial luciferase: occurrence, mechanism, and significance. *Arch Microbiol* **112**: 73–79.

- Nealson, K.H. (1999) Early observations defining quorum-dependent gene expression. In *Cell-Cell Signaling in Bacteria*. Dunny, G.M., and Winans, S.C. (eds). Washington, DC: American Society for Microbiology Press, pp. 277–289.
- Nishiguchi, M.K., Ruby, E.G., and McFall-Ngai, M.J. (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in Sepiolid squid–vibrio symbioses. *Appl Environ Microbiol* **64**: 3209–3213.
- Nishina, Y., Miyoshi, S., Nagase, A., and Shinoda, S. (1992) Significant role of an exocellular protease in utilization of heme by *Vibrio vulnificus*. *Infect Immun* **60**: 2128–2132.
- Nyholm, S.V., and McFall-Ngai, M.J. (1998) Sampling the microenvironment of the *Euprymna scolopes* light organ: description of a population of host cells with the bacterial symbiont *Vibrio fischeri*. *Biol Bull* **195**: 89–97.
- Nyholm, S.V., Stabb, E.V., Ruby, E.G., and McFall-Ngai, M.J. (2000) Establishment of an animal–bacterial association: recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* **97**: 10231–10235.
- Reddy, G.P., Hayat, U., Abeygunawardana, C., Fox, C., Wright, A.C., Maneval, D.R., et al. (1992) Purification and determination of the structure of capsular polysaccharide of *Vibrio vulnificus* M06-24. *J Bacteriol* **174**: 2620–2630.
- Reichelt, J.L., and Baumann, P. (1973) Taxonomy of the marine, luminous bacteria. *Arch Mikrobiol* **94**: 283–330.
- van Rhijn, P., and Vanderleyden, J. (1995) The *Rhizobium*–plant symbiosis. *Microbiol Rev* **59**: 124–142.
- Ruby, E.G., and Asato, L.M. (1993) Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch Microbiol* **159**: 160–167.
- Ruby, E.G., and Nealson, K.H. (1976) Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*; a model of symbiosis based on bacterial studies. *Biol Bull* **151**: 574–586.
- Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.
- Shao, C.-P., and Hor, L.-I. (2001) Regulation of metalloprotease gene expression in *Vibrio vulnificus* by a *Vibrio harveyi* LuxR homologue. *J Bacteriol* **183**: 1369–1375.
- Showalter, R.E., Martin, M.O., and Silverman, M.R. (1990) Cloning and nucleotide sequence of *luxR*, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. *J Bacteriol* **172**: 2946–2954.
- Sitnikov, D.M., Schineller, J.B., and Baldwin, T.O. (1995) Transcriptional regulation of bioluminescence genes from *Vibrio fischeri*. *Mol Microbiol* **17**: 801–812.
- Stabb, E.V., and Ruby, E.G. (2002) New RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol* (in press).
- Stabb, E.V., Reich, K.A., and Ruby, E.G. (2001) *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD⁺-glycohydrolases. *J Bacteriol* **183**: 309–317.
- Sturdier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubenhoff, J.W. (1990) The use of T4 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**: 60–89.
- Swartzman, E., and Meighen, E.A. (1993) Purification and characterization of poly (dA–dT) *lux*-specific DNA-binding protein from *Vibrio harveyi* and identification as LuxR. *J Biol Chem* **268**: 16706–16716.
- Vieira, J., and Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259–268.
- Visick, K.L., and McFall-Ngai, M.J. (2000) An exclusive contract: specificity in the *Vibrio fischeri*–*Euprymna scolopes* partnership. *J Bacteriol* **182**: 1779–1787.
- Visick, K.L., and Ruby, E.G. (1996) Construction and symbiotic competence of a *luxA*-deletion mutant of *Vibrio fischeri*. *Gene* **175**: 89–94.
- Visick, K.L., and Ruby, E.G. (1997) New genetic tools for use in the marine bioluminescent bacterium *Vibrio fischeri*. In *Bioluminescence and Chemiluminescence*. Hastings, J.W., Kricka, L.J., and Stanley, P.E. (eds). New York: John Wiley and Sons, pp. 119–122.
- Visick, K.L., Foster, J., Doino, J., McFall-Ngai, M., and Ruby, E.G. (2000) *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. *J Bacteriol* **182**: 4578–4586.
- Welch, T.J., and Bartlett, D.H. (1998) Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9. *Mol Microbiol* **27**: 977–985.
- Wright, L., Simpson, L.M., Oliver, J.D., and Morris, J.G. (1990) Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. *Infect Immun* **47**: 446–451.