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LuxO controls *luxR* expression in *Vibrio harveyi*: evidence for a common regulatory mechanism in *Vibrio*

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Summary

Quorum-sensing control of luminescence in *Vibrio harveyi*, which involves an indirect autoinducer-mediated phosphorelay signal transduction system, contrasts with the prototypical quorum-sensing system of *Vibrio fischeri*, in which the autoinducer and the transcriptional activator LuxR directly activate *lux* operon expression. In *V. harveyi*, a regulator not homologous to *V. fischeri* LuxR and also designated LuxR ($LuxR_{vh}$), binds specifically to the *lux* operon promoter region and activates the expression of luminescence. A direct connection has not been identified previously between *V. harveyi* $LuxR_{vh}$ and the autoinducer-mediated phosphorelay system. Here, we demonstrate by mobility shift assays and measurement of $luxR_{vh}$ mRNA levels with $luxO^+$ and $luxO^-$ cells that the central response regulator of the *V. harveyi* phosphorelay system (LuxO) represses the level of $LuxR_{vh}$. Expression of a $luxR_{vh}$-bearing plasmid strongly stimulated luminescence of a $luxO^-$ mutant but had no effect on luminescence of wild-type $luxO^+$ cells, indicating tight regulation of $luxR_{vh}$ by LuxO. Furthermore, *luxO* null mutants of *V. fischeri* MJ-1 and two autoinducer mutants, MJ-211 ($luxI^-$) and MJ-215 ($luxI^-$ $ainS^-$), emitted more light and exhibited more elevated levels of *litR*, a newly identified *V. harveyi* $luxR_{vh}$ homologue, than their $luxO^+$ counterparts. These results suggest that activity of the autoinducer-mediated phosphorelay system is coupled to $LuxR_{vh}$/LitR control of luminescence through LuxO in *V. harveyi* and *V. fischeri*. The presence of homologues of *V. harveyi* $LuxR_{vh}$, LuxO and other phosphorelay system proteins in various *Vibrio* species and the control of $LuxR_{vh}$ and its homologues by LuxO identified here in *V. harveyi* and *V. fischeri* and recently in *Vibrio cholerae* suggest that the $luxO$–$luxR_{vh}$ couple is a central feature of this quorum-sensing system in members of the genus *Vibrio*.

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Introduction

Quorum sensing in prokaryotes has received much attention after the discovery that the *luxI/R* regulatory system controlling cell density-dependent expression of the *Vibrio fischeri lux* operon is found in many Gram-negative bacteria and acts as a global regulator for a number of functions ranging from virulence and natural competence to luminescence (Fuqua *et al*., 1994; Bassler, 1999; Swift *et al*., 1999; Joyce *et al*., 2000). The regulatory gene *luxR* is upstream and divergently transcribed from the *lux* operon, *luxICDABEG*, and directs the synthesis of a transcriptional activator (LuxR). On interaction with the autoinducer N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), the product of the acyl-homoserine lactone (acyl-HSL) synthase (LuxI), LuxR activates the *lux* operon as well as its own gene (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984). A second autoinducer, N-octanoyl-HSL (C8-HSL), the product of the acyl-HSL synthase (AinS), also interacts with LuxR to modulate luminescence via LuxR (Kuo *et al*., 1994; Gilson *et al*., 1995; Hanzelka *et al*., 1999).

A different quorum-sensing mechanism has been described in *Vibrio harveyi*, another luminescent species. The *V. harveyi* system lacks the *luxI/R* circuitry but uses a phosphorelay signal transduction network (Bassler *et al*., 1993; 1994a) with two autoinducer signals: N-(3-hydroxybutanoyl)-L-HSL (3-hydroxy-C4-HSL) (AI-1) (Cao and Meighen, 1989) and a nonacyl-compound (AI-2), recently shown to be a furanone (Schauder *et al*., 2001), specifically, a furanosyl borate ester (Chen *et al*., 2002). Syntheses of 3-hydroxy-C4-HSL and the furanosyl borate ester are dependent on LuxM and LuxS respectively. The presence of LuxS in many bacteria, both Gram-negative and Gram-positive, and its potential control of interspecies

communication, have further stimulated interest in quorum sensing (Bassler, 1999).

Parallel sensory response pathways respond to the two autoinducer signals in *V. harveyi*. The sensors for AI-1 and AI-2 are LuxN and LuxQ (Freeman and Bassler, 1999a; Freeman *et al*., 2000), respectively, which can be phosphorylated with ATP at a specific histidine residue within the sensor recognition kinase domain followed by transphosphorylation to an aspartate residue in the downstream receiver domain. The phosphate is relayed from both sensors to the histidine-containing phosphate transfer domain in LuxU (Freeman and Bassler, 1999b), a detached phosphorelay protein, and then transferred to an aspartate residue in the response domain of the central response regulator LuxO (Freeman and Bassler, 1999a). The *luxU* and *luxO* genes are closely linked with the 5′-terminal of *luxU* overlapping the 3′-terminal of *luxO*. LuxO is a σ^{54}-binding protein with a DNA-binding domain that belongs to the NtrC family of σ^{54}-binding activators (Lilley and Bassler, 2000). The phosphorylated form (P-LuxO) at low cell densities is proposed to stimulate the σ^{54}-dependent expression of an unknown factor, X, which in turn represses the expression of the *V. harveyi luxCDABEGH* operon. At high concentrations, AI-1 interacts with LuxN, and AI-2 associates with a periplasmic protein LuxP to interact with LuxQ (Bassler *et al*., 1994a; Schauder *et al*., 2001). These interactions cause the sensors to switch from kinase to phosphatase activity, leading to the dephosphorylation and inactivation of LuxO, thus permitting luminescence induction.

Conspicuously lacking in models of the quorum-sensing regulatory system of *V. harveyi* is the connection between *V. harveyi* $LuxR_{vh}$ (not related to *V. fischeri* LuxR), a critical component for expression of the *V. harveyi lux* operon, and the autoinducer-mediated phosphorelay system. $LuxR_{vh}$ is a transcriptional activator that binds directly to the *lux* operon (Martin *et al*., 1989; Showalter *et al*., 1990; Swartzman *et al*., 1992; Swartzman and Meighen, 1993). The *V. harveyi* $luxR_{vh}$ null mutant MR1130 (Martin *et al*., 1989) does not produce luminescence even though its levels of autoinducers are normal. $LuxR_{vh}$ has been suggested to be involved in the relay of the quorum-sensing signal (Miyamoto *et al*., 1996), but it is currently depicted in regulatory models for luminescence as an activator that functions independently of the quorum-sensing signals (Lilley and Bassler, 2000; Dunlap and Kita-Tsukamoto, 2001).

In this paper, we present evidence that LuxO represses $luxR_{vh}$ expression in *V. harveyi* through measurement of the levels of $luxR_{vh}$ mRNA by dot-blot hybridization, determination of $LuxR_{vh}$ production by mobility shifts in regulatory mutants and comparison of the effects of expression with and without $luxR_{vh}$ in *luxO* null (*luxO*$^-$) and wild-type cells. Recently, a parallel study of LuxO and HapR on toxicity in *Vibrio cholerae* has shown that LuxO represses *hapR* expression at early stages of growth, which in turn blocks ToxR regulon expression (Zhu *et al*., 2002). HapR is a homologue of *V. harveyi* $LuxR_{vh}$, both belonging to the TetR family of regulators (Jobling and Holmes, 1997). Because regulation of $LuxR_{vh}$ by LuxO in *V. harveyi* had not been demonstrated, Zhu *et al*. (2002) speculated that the regulation of HapR by LuxO is specifically related to the adaptation of *V. cholerae* to human host expression. We demonstrate, however, that LuxO functions by repression of not only $LuxR_{vh}$ in *V. harveyi* but also a new $LuxR_{vh}$ homologue (LitR) in *V. fischeri*. LuxO control of $LuxR_{vh}$ and its homologues may be a general regulatory mechanism in *Vibrio*.

Results

Dependence of $luxR_{vh}$ *mRNA levels on LuxO*

Previously, disruption of the *V. harveyi luxO* gene was shown to lead to a high constitutive level of luminescence independent of autoinducers. One explanation is that LuxO, as a σ^{54} activator, regulates the synthesis of a σ^{54}-dependent repressor affecting the expression of the *lux* operon. Alternatively, LuxO might block $LuxR_{vh}$ activation of the *lux* operon or inhibit synthesis or stability of $luxR_{vh}$ mRNA (Lilley and Bassler, 2000). Accordingly, we investigated the level of $luxR_{vh}$ mRNA in *V. harveyi* wild-type and *luxO*$^-$ strains, comparing luminescence and levels of $luxR_{vh}$ and *luxC* mRNA. As was shown previously (Freeman and Bassler, 1999a), luminescence intensity was higher in *luxO*$^-$ mutant than in wild-type cells early in growth (Fig. 1). The inhibition of light emission at low cell density in the *luxO* mutant reflects repression by components in the medium unrelated to quorum-sensing signals (Miyamoto *et al*., 2000; Chatterjee *et al*., 2002). Early in growth (Fig. 1, samples a–c), *luxO*$^-$ cells contained more $luxR_{vh}$ and *luxC* mRNA than the wild-type cells, whereas late in growth (Fig. 1, sample d, $A_{660} > 1$), the mutant and wild-type cells contained the same levels of each of these transcripts. The levels of *luxC* and $luxR_{vh}$ mRNA cannot be compared directly as different labelled probes were used. Approximately seven times more *luxC* mRNA and three times more $luxR_{vh}$ mRNA were produced by the *luxO*$^-$ cells than by the wild-type cells at low cell densities ($A_{660} \geq 0.6$) (Table 1). As a control, the mRNA for cyclic AMP receptor protein (CRP) (Chatterjee *et al*., 2002) was also assayed in wild-type and *luxO*$^-$ cells at different stages of growth (Table 1). The levels of *crp* mRNA were equivalent in both wild-type and *luxO*$^-$ cells at all stages of growth, representing constitutive production of message for CRP in contrast to that of *luxC and* $luxR_{vh}$ in wild-type cells. Moreover, the same level found for *crp* mRNA in the *luxO* null mutant as in the wild-type cells contrasts

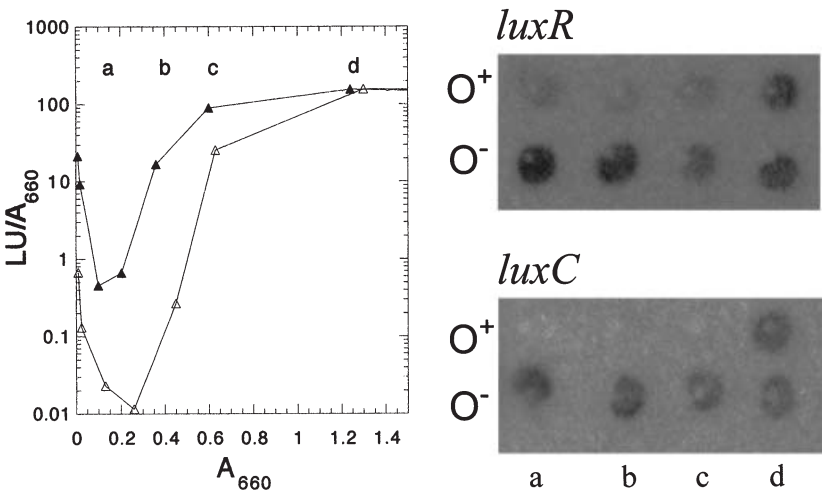

Fig. 1. Luminescence of *V. harveyi* wild type and the *luxO* null mutant and the levels of *luxR*$_{vh}$ and *luxC* mRNA at various stages of cellular growth. Luminescence per unit A_{660} (LU/A_{660}) as a function of cellular density (A_{660}) for *V. harveyi* wild type (open triangles) and the *luxO*$^-$ mutant (closed triangles) grown in complex medium is shown on the left. Periodically (a, b, c, d), cells were collected. After RNA extraction, RNA dot blots were prepared, and hybridization using ^{32}P-labelled probes for *luxR*$_{vh}$ and *luxC* was conducted. See *Experimental procedures* for further details. The autoradiogram of the resultant RNA dot blots of both wild-type and LuxO$^-$ phenotypes is shown on the right.

with the elevated levels of *luxC* and *luxR*$_{vh}$ mRNA in the *luxO* null mutant. Thus, disruption of *luxO* results in higher levels of *lux* mRNA including *luxR*$_{vh}$ mRNA at early stages of growth.

We next examined the *V. harveyi* autoinducer mutant D1 (Cao and Meighen, 1993), which lacks 3-hydroxy-C4-HSL and furanosyl borate ester (Bassler *et al*., 1997; Chen *et al*., 2002) and produces a decreased level of *luxR*$_{vh}$ mRNA (Miyamoto *et al*., 1996). Disruption of *luxO* in D1 (D1O$^-$) dramatically affected the level of luminescence, increasing it almost 3×10^5-fold (Fig. 2) to a level surpassing that of wild-type cells. Consistent with these results, D1O$^-$ contained 6.7-fold more *luxR*$_{vh}$ mRNA than the D1 strain (Table 1). Furthermore, characteristic of *luxO* null mutants (Bassler *et al*., 1994b), exogenous addition of 3-hydroxy-C4-HSL had no effect on the luminescence of D1O$^-$, whereas the luminescence of D1 and wild-type was stimulated by this addition (Fig. 2).

Measurement of LuxR$_{vh}$ levels by mobility shifts

To confirm that *luxR*$_{vh}$ mRNA was being translated into active LuxR$_{vh}$, we used mobility shift assays of crude extracts from cells grown in LB medium to $A_{660} = 0.6$ with labelled *luxR*$_{vh}$ promoter DNA to assess LuxR$_{vh}$ binding. Two bands migrating more slowly than the *luxR*$_{vh}$ DNA can readily be observed (Fig. 3A), reflecting the two LuxR$_{vh}$ binding sites in the *luxR*$_{vh}$ promoter (Chatterjee *et al*., 1996). These mobility shifts resulted from LuxR$_{vh}$ binding, as Fig. 3B (lane 2) shows that a crude extract of MR1130, the *luxR*$_{vh}$ null mutant of *V. harveyi* BB7, gave rise to a minor bandshift, which mirrored that seen using the control extract of *Escherichia coli* bearing the plasmid pT7 (Fig. 3B, lane 5). Using a positive control of an *E. coli* extract bearing *luxR*$_{vh}$ in pT7 (pT7/luxR$_{vh}$), two major bandshifts attributed to LuxR$_{vh}$ binding to its own promoter (Chatterjee *et al*., 1996) migrate to the same positions in the gel as the reference sample (Fig. 3B, lane 8), which is reproduced in Fig. 3A (lane 2). The stronger mobility shifts with extracts of the *luxO*$^-$ mutants than with extracts of wild-type *V. harveyi* cells indicated that a higher level of LuxR$_{vh}$ was present in the *luxO*$^-$ mutant, consistent with the relative levels of *luxR*$_{vh}$ mRNA (Fig. 1). Using D1 extracts, the mobility shifts were even more dramatic; migration of very little DNA was retarded with 1 μg of the D1(*luxO*$^+$) extract, whereas most of the DNA migrated

Table 1. Quantification of mRNAs in various *luxO*$^+$ *and luxO*$^-$ cells *of V. harveyi* and *V. fischeri* at different stages of growth[a].

Cells	mRNA	A_{660}[b]	O$^+$ (c.p.m.)	O$^-$ (c.p.m.)	O$^-$/O$^+$
V. harveyi	*luxC*	0.02–0.6	79 ± 19	570 ± 83	7.2
V. harveyi	*luxC*	1.0–1.8	567 ± 104	584 ± 92	1.0
V. harveyi	*luxR*	0.02–0.6	169 ± 41	507 ± 124	3.0
V. harveyi	*luxR*	1.0–1.8	557 ± 81	656 ± 93	1.2
V. harveyi	*crp*	0.08–0.5	495 ± 47	641 ± 160	1.3
V. harveyi	*crp*	0.6–2.2	395 ± 134	431 ± 20	1.1
V. harveyi D1	*luxR*	0.6–2.0	112 ± 54	748 ± 212	6.7
V. fischeri	*litR*	0.3–1.2	387 ± 109	1220 ± 479	3.2
V. fischeri	*litR*	1.3–2.5	2693 ± 616	2172 ± 369	0.8

a. After growth in complex medium as in Fig. 1, cells were obtained and RNA prepared. RNA dot-blot hybridizations were quantified using the Fuji Bioimager, and c.p.m. after background subtraction is given. Three to six samples were used in each case.
b. A range of A_{660} was used as the samples had similar amounts of radioactive probe bound.

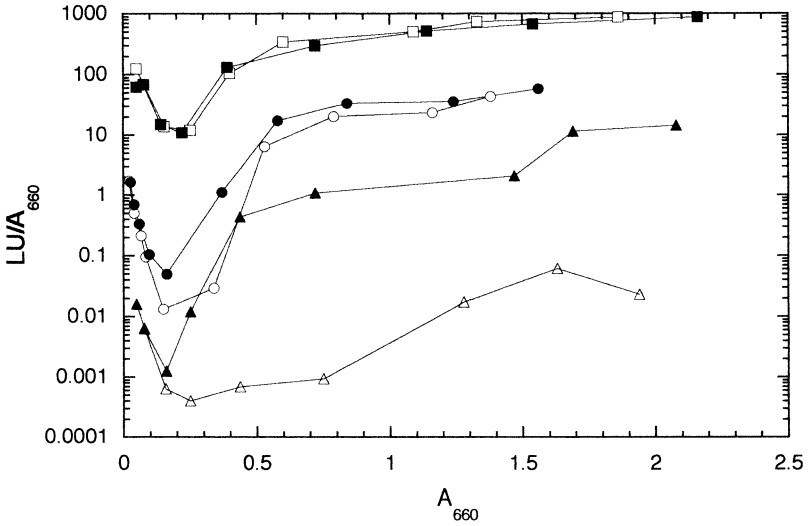

Fig. 2. Luminescence of D1O⁻, D1 and *V. harveyi* wild type during growth with and without added 3-hydroxy-C4-HSL. Wild-type *V. harveyi*, the dark mutant D1 of *V. harveyi* and the *luxO* null mutant, D1O⁻, were grown in complex medium to obtain the plot of luminescence (LU/A_{660}) with cell density (A_{660}) in the presence and absence of 3-hydroxy-C4-HSL at 10 μg ml^{-1}. Wild type without 3-hydroxy-C4-HSL (open circles), with 3-hydroxy-C4-HSL (closed circles); D1 without 3-hydroxy-C4-HSL (open triangles), with 3-hydroxy-C4-HSL (closed triangles); D1O⁻ without 3-hydroxy-C4-HSL (open squares), with 3-hydroxy-C4-HSL (closed squares).

more slowly with 1 μg of the D1O⁻ extract. These results demonstrate a direct correlation between higher levels of *luxR*$_{vh}$ mRNA and higher levels of $LuxR_{vh}$ (see Table 1). Finally, when *V. harveyi* MR1130, the *luxR*$_{vh}$ null phenotype, was made *luxO*⁻ by gene replacement (*Experimental procedures*) and examined for light production, the dark phenotype of the mutant remained unchanged (data not shown). In Fig. 3B, an extract of the *luxO*⁻*luxR*⁻$_{vh}$ mutant (MRO⁻) subjected to mobility shift analysis with *luxR*$_{vh}$ promoter DNA gave rise to the same minor band as seen in both extracts of MR1130 and *E. coli*(pT7) cells; with five times more MRO⁻ extract, the intensity of this minor band remained unchanged. These negative findings for luminescence and $LuxR_{vh}$ in MRO⁻ suggest that no other component that may be under LuxO control appears to enable luminescence when $LuxR_{vh}$ is absent.

Expression of $luxR_{vh}$ in trans *in* luxO *null mutants*

Previously, we demonstrated that the presence of *luxR*$_{vh}$ *in trans* in D1 restored luminescence to wild-type levels bypassing the requirement for the addition of autoinducers (Miyamoto *et al.*, 1996). To examine the effect of multiple copies of *luxR*$_{vh}$ in the absence of LuxO, we transferred the *luxR*$_{vh}$-bearing plasmid (pMGM150) into both wild-type and *luxO*⁻ strains. Cells were grown in LB, which is necessary to maintain good growth with a plasmid present (Fig. 4). The typical repression of luminescence early in growth was observed, which was not alleviated by the presence of pMGM150 in wild-type cells, but the weaker repression of luminescence observed in *luxO*⁻ cells was almost completely overcome in the presence of pMGM150. Indeed, at earlier stages of growth, luminescence was 10 times higher in the *luxO*⁻ (+*luxR*$_{vh}$) cells than in the *luxO*⁻ cells and 100 times higher than in wild-type (±*luxR*$_{vh}$) cells. To ensure that the *luxR*$_{vh}$ plasmid was being expressed in both cell types, extracts (from cells grown to A_{660} = 0.6 in LB medium) were assayed for their ability to cause the $LuxR_{vh}$ mobility shifts using labelled *luxR*$_{vh}$ promoter DNA (Fig. 3B). When the *luxR*$_{vh}$ plasmid was present in wild-type and *luxO*⁻ cells, both extracts

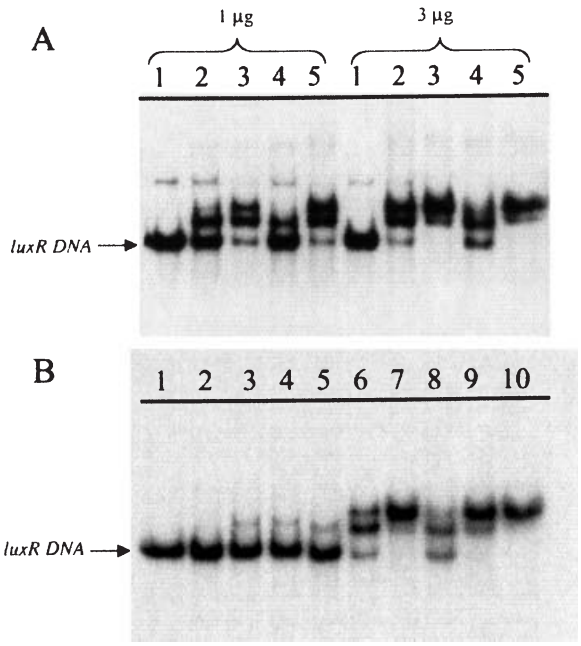

Fig. 3. Levels of $LuxR_{vh}$ as determined by mobility shifts. Autoradiograms after 5% (w/v) PAGE of *V. harveyi* 5′ ^{32}P-labelled *luxR*$_{vh}$ promoter DNA (400 bp, as described in *Experimental procedures*) mixed with (A) 1 or 3 μg of protein lysate: 1, no addition; 2, *V. harveyi* wild type; 3, VhO⁻, the *luxO* null mutant; 4, D1; 5, D1O⁻; (B) 1 μg of protein lysate for all except lane 4 (5 μg), lane 6 (0.01 μg) and lane 7 (0.05 μg): 1, no addition; 2, MR1130; 3, MRO⁻; 4, MRO⁻; 5, *E. coli* BL21(pT7); 6, *E. coli* BL21(pT7/*luxR*$_{vh}$); 7, *E. coli* BL21(pT7/*luxR*$_{vh}$); 8, *V. harveyi* wild type; 9, *V. harveyi luxO*⁺ (+*luxR*$_{vh}$ [pMGM150]); 10, *V. harveyi luxO*⁻ (+*luxR*$_{vh}$ [pMGM150]). All lysates were prepared from cells grown in LB medium to A_{660} = 0.6.

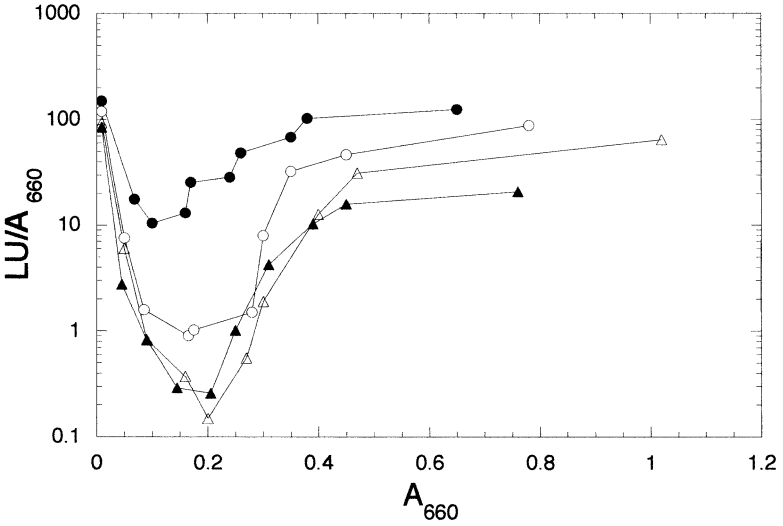

Fig. 4. Luminescence with cellular growth of *V. harveyi* wild type and the *luxO* null mutant with and without *in trans* addition of *V. harveyi luxR$_{vh}$*. The mobilizable plasmids pKT/*luxR$_{vh}$* (pMGM150) or pKT230 were introduced to wild-type and *luxO*$^-$ cells, and the luminescence (LU/A$_{660}$) was measured at various times during growth (A$_{660}$) in LB medium: wild type + pKT230 (open triangle), wild type + pMGM150 (closed triangle); *luxO*$^-$ + pKT230 (open circle); *luxO*$^-$ + pMGM150 (closed circle).

caused a very strong mobility shift of the *luxR$_{vh}$* promoter DNA, indicating high levels of LuxR$_{vh}$, with the *luxO*$^-$ extract showing the strongest binding (Fig. 3B). Although the levels of functional LuxR$_{vh}$ *in vivo* are difficult to define, particularly with the presence of multicopy plasmid capable of binding LuxR$_{vh}$, the ability of *luxR$_{vh}$ in trans* to stimulate luminescence in *luxO*$^-$ cells and not in *luxO*$^+$ cells provides strong support that *luxO* regulates *luxR$_{vh}$*.

Polyhydroxybutyrate production in luxO *null mutants*

In *V. harveyi*, it has been demonstrated that LuxR$_{vh}$ controls the production of polyhydroxybutyrate (PHB) at late stages of growth (Miyamoto *et al*., 1998). To test whether a phenotype controlled by LuxR$_{vh}$ other than luminescence is also affected when LuxO is absent, we analysed D1 and MR1130 cells and their respective *luxO* null cells for PHB (*Experimental procedures*). Both D1 and MR1130 have been determined to lack PHB because of the insufficiency or absence, respectively, of LuxR$_{vh}$. As expected, the level of crotonic acid produced by oxidation of PHB was not detectable in D1 or MR1130 cells (Fig. 5). However, when MRO$^-$ and D1O$^-$ cells were tested, PHB could still not be detected in the *luxR*-negative MRO$^-$ cells, whereas a large amount of PHB was produced in the D1O$^-$ cells. These results are consistent with the removal of the repression of *luxR$_{vh}$* by disrupting *luxO* and demonstrate that the *luxO–luxR$_{vh}$* regulatory couple controls multiple phenotypes.

LuxO regulation of LitR, a V. harveyi *LuxR$_{vh}$ homologue in* V. fischeri

LuxO has been identified in *V. fischeri* as well, where it

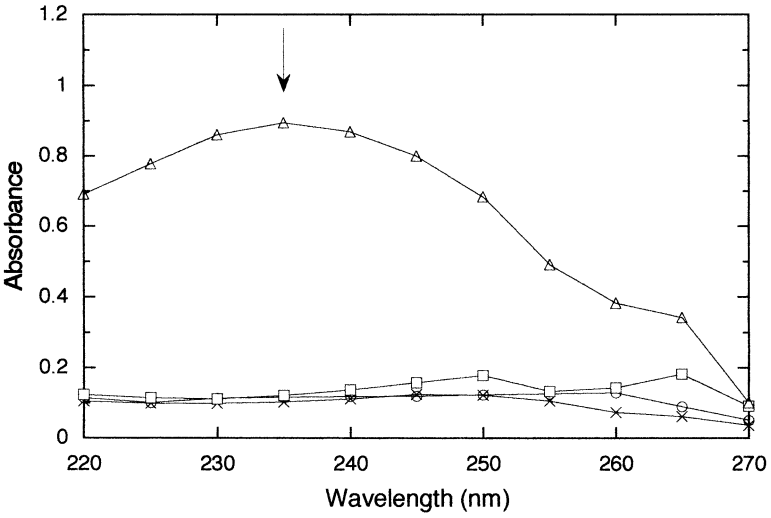

Fig. 5. PHB production in MR1130, D1 and their *luxO*$^-$ counterparts. The oxidation of PHB into crotonic acid was measured as absorbance vs. wavelength. The maximum wavelength for crotonic acid (235 nm) is indicated. The scans of absorbance from 220 to 270 nm were measured in a Beckman DU 640 spectrophotometer: MR1130 (×); MRO$^-$ (circle); D1 (square); D1O$^-$ (triangle).

also controls luminescence (Miyamoto *et al*., 2000). The quorum-sensing systems controlling luminescence in *V. harveyi* and *V. fischeri*, however, appear to be distinctly different, leading to questions on the mechanism by which LuxO operates in *V. fischeri*. The recent discovery of a *V. harveyi* $luxR_{vh}$ homologue, *litR*, in *V. fischeri* ES114 (Fidopiastis *et al*., 2002) allowed us to address this issue. Specifically, to determine whether LuxO operates via LitR in *V. fischeri*, we quantified *litR* mRNA in *V. fischeri* MJ-1 (wild type) and its *luxO* mutant. Levels of *litR* mRNA were threefold higher in the *luxO* null mutant compared with MJ-1 in the early stages of growth ($A_{660} < 1.3$), whereas *litR* mRNA levels were similar in these strains at higher cell densities (Table 1). The similarity of these results to those obtained with *V. harveyi* (Table 1) suggests that *litR* in *V. fischeri* and $luxR_{vh}$ in *V. harveyi* carry out the same function. To assess this possibility further, we mobilized the *V. harveyi* $luxR_{vh}$ gene into *V. fischeri*. The presence of $luxR_{vh}$ strongly stimulated luminescence in the *V. fischeri luxO* null mutant but had no effect in MJ-1 (Fig. 6). These results again are very similar to those observed for the effect of $luxR_{vh}$ in *V. harveyi* (Fig. 4).

We next examined the effect of *luxO* in *V. fischeri* in the presence and absence of quorum-sensing signals to assess the possibility that LuxO may function in this bacterium in a manner analogous to its autoinducer-mediated activity in *V. harveyi*. The *V. fischeri* acyl-HSL synthase mutants, MJ-211 (*luxI*), which does not produce 3-oxo-C6-HSL but produces C8-HSL, and MJ-215 (*luxI*, *ainS*), which produces neither 3-oxo-C6-HSL nor C8-HSL (Kuo *et al*., 1994; 1996), were made $luxO^-$ by gene replacement (*Experimental procedures*). MJ-211 produces ≈100-fold more light than MJ-215 because of the ability of C8-HSL, a competitive inhibitor of 3-oxo-C6-HSL, to activate LuxR weakly (Kuo *et al*., 1996). We found that the *luxO* mutants of MJ-211 and MJ-215 in each case produced 10–50 times more light than their parent strains (Fig. 7). These results demonstrate that LuxO represses luminescence in *V. fischeri* in the absence of either 3-oxo-C6-HSL or both 3-oxo-C6-HSL and C8-HSL.

As AinS is a homologue of LuxM that produces 3-hydroxy-C4-HSL to overcome repression of luminescence by LuxO in *V. harveyi*, we added the AinS inducer, C8-HSL, to deduce whether it could stimulate light not only by interaction with LuxR but by overcoming the repression of *litR* expression caused by LuxO. As expected, C8-HSL stimulated luminescence in all cell lines (Fig. 7). Moreover, in both $luxI^-$ and $luxI^-ainS^-$ cell lines, the degree of luminescence stimulation by C8-HSL is larger for the $luxO^+$ cells than for the $luxO^-$ cells, with the same level of luminescence being reached for the $luxO^+$ and $luxO^-$ strains of the $luxI^-ainS^-$cells. This result may suggest a link between LuxO repression and C8-HSL activation of luminescence, although interpretation of these data is complicated by the stimulation of luminescence by the interaction of C8-HSL with LuxR.

To examine this possible link more directly, we quantified *litR* mRNA in MJ-1 (wild-type), MJ-211 and MJ-215 and their *luxO* null mutants grown in the presence and absence of C8-HSL. In the absence of added autoinducer, a higher stimulation of *litR* mRNA (O^-/O^+) was observed for MJ-1 in complex medium (threefold, Table 1) than in the ASH medium (twofold, Table 2). A similar fold increase was observed with MJ-215 and its $luxO^-$ mutant in ASH medium (1.8-fold), whereas the increase in the amount of *litR* mRNA in $luxO^-$ cells in MJ-211 was relatively low and possibly not significant. Unfortunately, MJ-211 and MJ-215 only exhibit high luminescence in ASH medium and not in complex medium, in which wild-type cells gave the highest change in *litR* mRNA levels in response to the *luxO* mutation. For MJ-1, addition of C8-HSL, or elimination of LuxO, resulted in a twofold increase in *litR* mRNA;

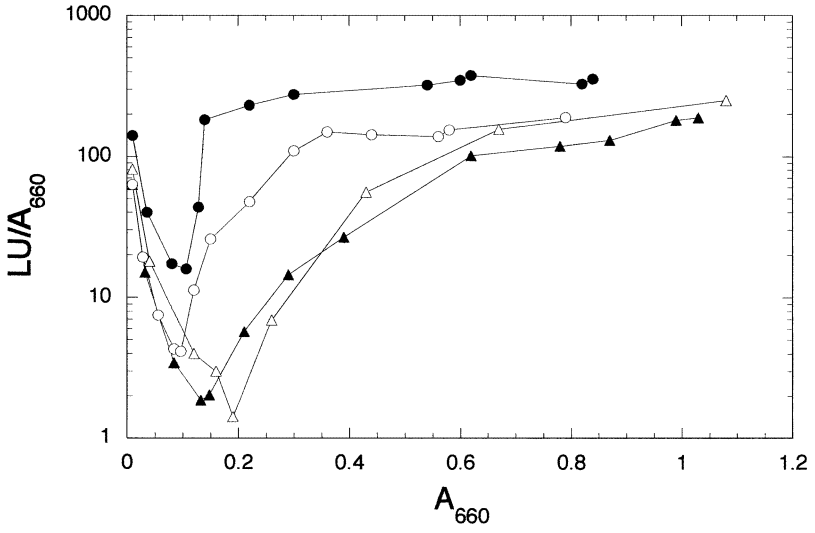

Fig. 6. Effect of *in trans* addition of *V. harveyi* $luxR_{vh}$ to *V. fischeri* wild-type and $luxO^-$ cells on cell density-dependent luminescence. Growth and luminescence were done as described in the legend to Fig. 4. *V. fischeri* wild type + pKT230 (open triangle), wild type + pMGM150 (closed triangle); $luxO^-$ + pKT230 (open circle), $luxO^-$ + pMGM150 (closed circle).

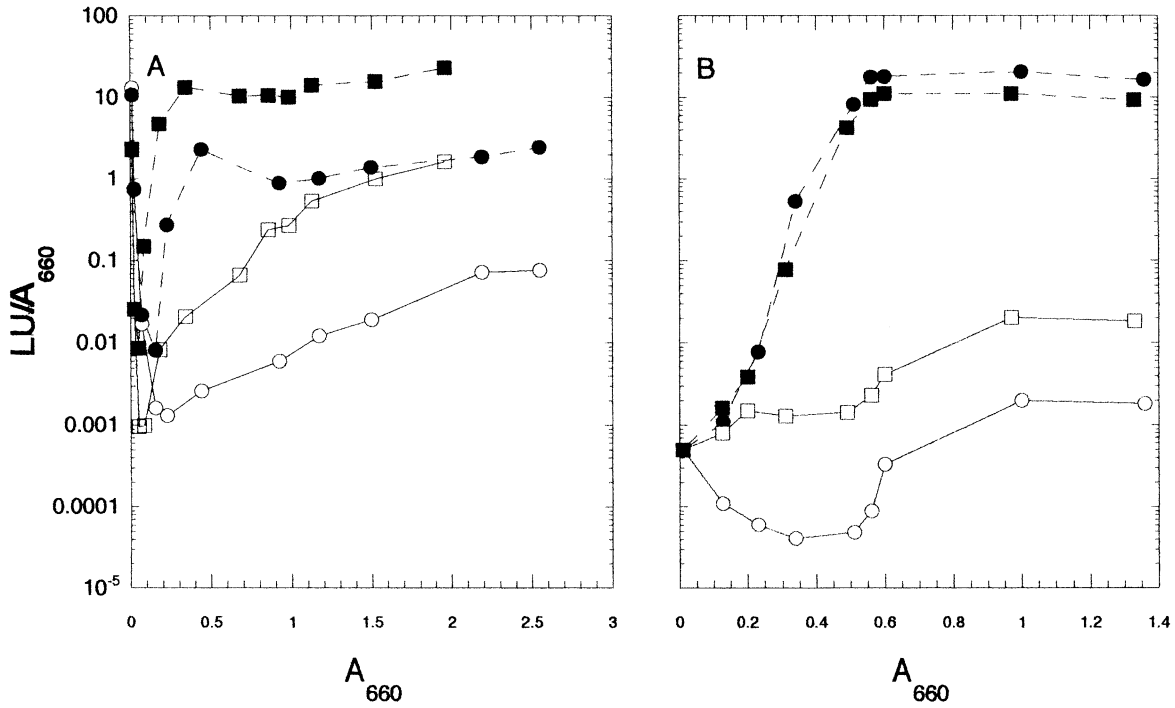

Fig. 7. Luminescence profiles during growth in ASH medium of *V. fischeri* autoinducer mutants (*luxO*$^+$ and *luxO*$^-$) in the presence and absence of C8-HSL.
A. Without addition of C8-HSL, MJ-211(*luxO*$^+$) (open circle, solid line) and MJ-211(*luxO*$^-$) (open square, solid line); in the presence of 2 mg ml^{-1} C8-HSL, MJ-211 (*luxO*$^+$) (closed circle, dashed line) and MJ-211(*luxO*$^-$) (closed square, dashed line).
B. Without addition of C8-HSL, MJ-215(*luxO*$^+$) (open circle, solid line) and MJ-215(*luxO*$^-$) (open square, solid line); in the presence of 2 mg ml^{-1} C8-HSL, MJ-215 (*luxO*$^+$) (closed circle, dashed line) and MJ-215 (*luxO*$^-$) (closed square, dashed line).

however, the addition of C8-HSL had no effect on the *luxO*$^-$ mutant of MJ-1 (Table 2). The lack of an effect of C8-HSL on the levels of *litR* mRNA in the three *luxO* null mutants of *V. fischeri* indicates that LuxO may be necessary for the response to this quorum-sensing signal. These results are very similar to those obtained for the effects of LuxO and 3-hydroxy-C4-HSL on luminescence (Bassler *et al*., 1994b) and *luxR$_{vh}$* mRNA in *V. harveyi* (Miyamoto *et al*., 1996).

Table 2. Levels of *litR* mRNA in *luxO*$^+$ *and luxO*$^-$ genotypes of various *V. fischeri* cells at mid-log phase of growth in the presence and absence of C8-HSL.

Cells[a]	O$^+$ (c.p.m.)	O$^-$/	O^{+b}
MJ-1[c]	1656 ± 355	3261 ± 397	2.0
MJ-1 + C8-HSL	3764 ± 1439	3468 ± 681	0.9
MJ-211	1951 ± 308	2524 ± 389	1.3
MJ-211 + C8-HSL	2184 ± 504	1882 ± 411	0.9
MJ-215	1064 ± 138	1928 ± 279	1.8
MJ-215 + C8-HSL	1534 ± 177	1628 ± 176	1.1

a. Samples of cells grown in ASH medium from A_{660} = 0.3–1.0 were used; when required, C8-HSL was added at 2 μg ml^{-1}.
b. Ratio of *litR* mRNA in *luxO*$^-$ and *luxO*$^+$ cells.
c. MJ-1 was used instead of the parent MJ-100 as both have identical phenotypes.

Discussion

The systems regulating luminescence in bacteria are of great interest as they represent the prototypes for acyl-HSL-based quorum-sensing systems found in many pathogenic bacteria controlling secondary metabolism. The quorum-sensing system in *V. harveyi* involves signal transduction via phosphorelay proteins that transmit the signal from two different autoinducers, 3-hydroxy-C4-HSL and a furanosyl borate ester. Although LuxR$_{vh}$ is the primary activator of the *lux* operon in *V. harveyi* binding to two sites in the *luxCDABEGH* promoter region (Swartzman *et al*., 1992; Miyamoto *et al*., 1994), its connection, if any, to the quorum-sensing system has been unclear. Previously, the strongest data supporting the involvement of LuxR$_{vh}$ in quorum sensing were the twofold stimulation of *luxR$_{vh}$* expression by 3-hydroxy-C4-HSL (Miyamoto *et al*., 1996); however, these results did not establish its linkage to the phosphorelay components involved in the quorum-sensing regulatory system even though it has been speculated that both LuxN and LuxO may be able to transmit signals to *luxR$_{vh}$* (Bassler *et al*., 1993; 1994a).

In the present paper, we have shown that *luxR$_{vh}$* expression is repressed by LuxO in *V. harveyi* and provide evi-

dence that a similar regulatory control also occurs for *litR* in *V. fischeri*. Enhanced *luxR*$_{vh}$ mRNA production (threefold) and LuxR$_{vh}$ levels were clearly evident in the *luxO*$^{-}$ mutant of *V. harveyi* at low cell density compared with those in wild-type cells, and paralleled the level of *luxC* mRNA in *luxO*$^{-}$ cells, which was about sevenfold higher. Luminescence was restored in an autoinducer-negative mutant (D1) with a *luxO* knock-out (D1*luxO*$^{-}$) and, at mid-log phase, the level of *luxR*$_{vh}$ mRNA in D1*luxO*$^{-}$ was seven times higher than that of D1, the *luxR*$_{vh}$ mRNA of which is lower than that of wild type. The larger increase in *luxR*$_{vh}$ message in D1O^{-} compared with the twofold increase in *luxR*$_{vh}$ mRNA observed on adding 3-hydroxy-C4-HSL to D1 cells (Miyamoto *et al*., 1996) is consistent with the absence of LuxO in D1*luxO*$^{-}$ vs. the only partial inactivation of LuxO by dephosphorylation on addition of 3-hydroxy-C4-HSL. In addition, introduction of an excess of *luxR*$_{vh}$ to wild-type cells had little effect on luminescence, indicating that LuxO maintained a tight regulatory control on expression of *luxR*$_{vh}$ even when there were multiple copies of *luxR*$_{vh}$. In contrast, the cells produced more light (up to 10-fold) when *luxR*$_{vh}$ was added to the already strongly luminous *luxO* null mutants, consistent with loss of regulation of LuxO repression of *luxR*$_{vh}$. These results indicate that the quorum-sensing signals are transmitted via LuxO to *luxR*$_{vh}$. When MR1130 (*luxR*$^{-}$$_{vh}$) was made *luxO*$^{-}$, the dark phenotype remained unchanged: no other product necessary for luminescence appears to be repressed by LuxO. As LuxO is a σ^{54} regulator (Lilley and Bassler, 2000), it is unlikely that it can bind directly to the *luxR*$_{vh}$ promoter. Whether or not a putative repressor (X), synthesis of which may be controlled by LuxO, interacts directly with the *luxR*$_{vh}$ promoter is unknown; however, experiments are under way to detect proteins other than LuxR$_{vh}$ that bind strongly to the *luxR*$_{vh}$ promoter. The possibility that LuxO may destabilize *luxR*$_{vh}$ mRNA cannot be ruled out, as both message and protein of *luxR*$_{vh}$ would increase in a *luxO*$^{-}$ mutant under these conditions. To determine whether another phenotype known to be under the control of LuxR$_{vh}$, namely PHB production late in growth, is associated with LuxO, MR1130 and D1 and their *luxO* null counterparts were analysed for the presence of crotonic acid, the oxidation product of PHB. Indeed, only D1O^{-} produced PHB.

The possibility that LuxO control of *luxR*$_{vh}$ expression is a general regulatory mechanism was tested, because LuxO has been discovered to have similar effects on luminescence in *V. fischeri* (Miyamoto *et al*., 2000). Moreover, a LuxR$_{vh}$ homologue, depicted as LitR, was recently isolated from *V. fischeri* ES114 (Fidopiastis *et al*., 2002). LitR is believed to upregulate LuxR, and the *litR* mutant PMF-2 has one-fifth the luminescence expression of wild-type cells. Similar results were obtained for *litR* levels in *V. fischeri* wild type and the *luxO*$^{-}$ mutant compared with those with the same phenotypes from *V. harveyi*, with the *litR* mRNA being three times higher in *luxO*$^{-}$ than in wild-type *V. fischeri* (Table 1). When *V. harveyi luxR*$_{vh}$ was transferred into the *luxO*$^{-}$ and wild-type *V. fischeri* cells, luminescence was stimulated only in the *luxO*$^{-}$ cells in an analogous pattern to that found for the *luxO*$^{-}$ mutant of *V. harveyi*. The *luxO*$^{-}$ mutants of both MJ-211 and MJ-215 exhibited 10- to 50-fold higher luminescence as well as higher levels of *litR* mRNA than their isogenic parents, further substantiating the repressive role of LuxO in *V. fischeri* on luminescence and *litR* production.

Despite the fact that the two *V. fischeri* autoinducers, 3-oxo-C6-HSL and C8-HSL, interact directly with *V. fischeri luxR* to activate *lux* operon transcription, we demonstrate here that the amount of *litR* mRNA was increased in the presence of additional C8-HSL in *V. fischeri* wild type, whereas the *luxO*$^{-}$ counterpart remained elevated whether or not exogenous C8-HSL was present. In the autoinducer mutants, however, the effect of C8-HSL addition was less evident; however, their *luxO*$^{-}$ counterparts had higher levels of *litR* mRNA, which were not altered in the presence of exogenous C8-HSL. These results are consistent with the autoinducer signal, at least in the case of C8-HSL, also being transduced through LuxO to inactivate LuxO repression of LitR in *V. fischeri*, in much the same way as 3-hydroxy-C4-HSL inactivates LuxO repression of LuxR$_{vh}$ in *V. harveyi*. Consequently, a parallel system, as suggested by the homology between AinS and LuxM and the discovery of *luxO* in *V. fischeri* (Gilson *et al*., 1995; Miyamoto *et al*., 2000; Dunlap and Kita-Tsukamoto, 2001), appears to be present in both species.

It seems likely that other *Vibrio* species including non-luminescent species also use the LuxO–LuxR$_{vh}$ regulatory system; recent evidence shows that *V. cholerae* LuxO represses HapR, the homologue of LuxR$_{vh}$, which in turn blocks *tcpP* gene expression and leads to inhibition of ToxR regulon expression. (Zhu *et al*., 2002). From independent searches (NCBI BLAST; Altschul *et al*., 1990) for LuxR$_{vh}$, LuxO and LuxU as well as a consensus region for LuxU and the C-terminus of LuxO, only the one *Vibrio* genome (*V. cholerae*) in the database was found to contain LuxR$_{vh}$ and the LuxO–LuxU couple, which suggests that this system may be limited to bacteria in the genus *Vibrio*. Other *Vibrio* species, the genomes of which are not listed in the database, do have LuxR$_{vh}$ homologues, including OpaR of *Vibrio parahaemolyticus* (McCarter, 1998), SmcR *of Vibrio vulnificus* (McDougald *et al*., 2000; 2001; Shao and Hor, 2001), VanT *of Vibrio anguillarum* (Croxatto *et al*., 2002), as well as LitR of *V. fischeri*. With our demonstration of a link between *luxR*$_{vh}$ and LuxO, it seems likely that these species will also be found to have LuxOU, which would raise the possibility that *luxO* is controlled by autoinducers produced by genes homologous to those of the *V. harveyi* system.

Table 3. Bacteria used in this study.

	Strain	Relevant features	Source or reference
V. harveyi	B392	*luxR$_{vh}$*$^+$ *luxO*$^+$	Our laboratory
V. harveyi	BB7	*luxR$_{vh}$*$^+$ *luxO*$^+$	Gift from M. Silverman (Martin *et al*., 1989)
V. harveyi	MR1130	*luxR$_{vh}$*$^-$ of BB7	Gift from M. Silverman (Martin *et al*., 1989)
V. harveyi	D1	Autoinducer mutant of B392	Cao and Meighen (1989)
V. harveyi	VhO$^-$	*luxO*$^-$ of B392	Miyamoto *et al*. (2000)
V. harveyi	D1O$^-$	*luxO*$^-$ of D1	This study
V. harveyi	MRO$^-$	*luxO*$^-$ of MR1130	This study
V. fischeri	MJ-1	*luxIR*$^+$, *ainS*$^+$, *luxO*$^+$	Gift from B. L. Bassler
V. fischeri	MJ-100	*luxIR*$^+$, *ainS*$^+$, *litR*$^+$, *luxO*$^+$	P. V. Dunlap (Kuo *et al*., 1994)
V. fischeri	ES114	*luxIR*$^+$, *ainS*$^+$, *litR*$^+$, *luxO*$^+$	E. Ruby (Fidopiastis *et al*. 2002)
V. fischeri	VfO$^-$	*luxO*$^-$ of MJ-1	Miyamoto *et al*. (2000)
V. fischeri	MJ-211	*luxI*$^-$ of MJ-100	P. V. Dunlap (Kuo *et al*., 1994)
V. fischeri	MJ-215	*luxI*$^-$, *ainS*$^-$ of MJ-100	P.V. Dunlap (Kuo *et al*., 1994)
E. coli	BL21(DE3)	Contains T7 RNA polymerase	Studier *et al*. (1990)

Experimental procedures

Bacteria, plasmids and media

Table 3 and Table 4 list the bacteria and plasmids, respectively, that were used in this study, their features and their sources or references. The marine bacteria studied were *V. harveyi* B392 (in our laboratory) and *V. fischeri* MJ-1 (a gift from B. L. Bassler). *Escherichia coli* BL-21(DE3) (Studier *et al*., 1990) transformed with either pT7 (Tabor and Richardson, 1985) or *luxR$_{vh}$* of *V. harveyi* B392 in pT7 (pT7/luxR$_{vh}$) (Miyamoto *et al*., 1996) or *crp* in pT7 (pT7/crp) (Chatterjee *et al*., 2002) was grown in LB medium and treated with IPTG (Sigma) to induce the gene product placed after the pT7 promoter as described previously (Studier *et al*., 1990). The media used, LB or complex (Miyamoto *et al*., 2000), contained either 1% (w/v) NaCl for *V. harveyi* B392 and its isogenic mutants as well as *E. coli*, or 2% NaCl for *V. harveyi* BB7 and its isogenic mutants as well as *V. fischeri*. When indicated, MJ-1, MJ-211 (*luxI*$^-$; Kuo *et al*., 1994) and MJ-215 (*luxI*$^-$*ainS*$^-$; Kuo *et al*., 1996) were grown in ASH medium (Dunlap and Kuo, 1992). As the strains studied here have been determined to be resistant to ampicillin, the antibiotic was routinely added at 100 μg ml^{-1}. The growth temperature was maintained at 28°C with good aeration. Spectrophotometry at A_{660} was used to measure growth, and a custom-built luminometer measured luminescence where one light unit (LU) = 4×10^9 quanta s^{-1} (Hastings and Weber, 1963).

D1, an autoinducer mutant of *V. harveyi* B392, has been described previously (Cao and Meighen, 1989) as has MR1130, a *luxR$_{vh}$* null mutant of *V. harveyi* BB7, a gift from M. Silverman (Martin *et al*., 1989). MJ-100 and MJ-211 are the isogenic wild-type and *luxI*$^-$ mutant, respectively, of *V. fischeri* (Kuo *et al*., 1994). As both MJ-100 and MJ-1 had the same phenotype, only data from MJ-1 are reported here. Likewise, MJ-215 (*luxI*$^-$*ainS*$^-$) was derived from MJ-211 (Kuo *et al*., 1996).

Synthesis and purification of synthetic autoinducers

N-3-hydroxybutanoyl-HSL (3-hydroxy-C4-HSL) was synthesized as described previously (Cao and Meighen, 1989). For *N*-octanoyl-HSL (C8-HSL), 1 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodimide (Sigma), 1 g of α-amino-γ-butyrolactone hydrobromide (Aldrich), 0.6 ml of octanoic acid (Aldrich, neat 0.91 g ml^{-1}) and 0.756 ml of 5 M NaOH were mixed and adjusted to 10 ml with H_2O. After overnight mixing at room

Table 4. Plasmids used in this study.

Name	Features	Source or reference
pMF-2	*litR* of *V. fischeri* ES114, *luxR$_{vh}$* homologue	Fidopiastis *et al*. (2002)
pT7	pT7 promoter plasmid	Tabor and Richardson (1985)
pT7/luxR$_{vh}$	*luxR$_{vh}$* of *V. harveyi* B392 in pT7	Miyamoto *et al*. (1996)
pT7/crp	*crp* of *V. harveyi* B392 in pT7	Chatterjee *et al*. (2002)
pT7.litR	*litR* of *V. fischeri* ES114 in pT7	Fidopiastis *et al*. (2002)
pKT230	*mob*$^+$, broad-host-range vector, source of *kn^R*	Bagdasarian *et al*. (1981)
pMGM150	*luxR$_{vh}$* of *V. harveyi* B392 in pKT230	Miyamoto *et al*. (1996)
pJQ200	*mob*$^+$, *sacB*, suicide vector	Quandt and Hynes (1993)
pBluescript SK+	cloning vector	Stratagene
pBlue/luxC	promoter region of the *V. harveyi lux* operon in pBluescript	This study
pVHO-2	*V. harveyi luxO* with *kn^R* interrupt in pJQ200	Miyamoto *et al*. (2000)
pVHO-4	*luxO* PCR product of *V. harveyi* BB7 in pBluescript	This study
pVHO-5	pVHO-4 with *kn^R* marker, *luxO* interrupt	This study
pVHO-6	VHO-5 in pJQ200	This study
pVFO-4	*V. fischeri luxO* with *kn^R* interrupt in pT7	Miyamoto *et al*. (2000)
pVFO-7	VFO-4 in pJQ200	This study

temperature, the sample was extracted three times using an equal volume of methylene chloride each time. After drying down, the sample was resuspended in 5% (v/v) methanol in methylene chloride, followed by redrying. Methylene chloride (10 ml) was added to the sample, which was applied to a 10 cm × 2 cm diameter column of Kieselgel 60 (Merck), prewashed with methylene chloride. Flash chromatography was performed using stepwise additions of 1%, 2% and 5% (v/v) methanol in methylene chloride, and fractions of 10 ml were collected. The C8-HSL was found in 1% methanol after assays on thin-layer chromatography (TLC). The same fraction also gave rise to increased luminescence in MJ-211. After concentration, the synthetic C8-HSL was stored at –20°C in methylene chloride at 600 μg ml^{-1}.

Gene replacement to create luxO *null mutants*

All molecular microbiological techniques were performed according to Maniatis *et al*. (1982). The *luxO*$^-$ mutants *V. harveyi* B392 and *V. fischeri* MJ-1 were isolated previously (Miyamoto *et al*., 2000). The *luxO*$^-$ mutant of D1 was obtained with the use of pVHO-2 (Miyamoto *et al*., 2000). For the *luxO*$^-$ mutant of MR1130 (MRO$^-$), the polymerase chain reaction (PCR) product using two primers already described (Miyamoto *et al*., 2000) was first obtained using chromosomal DNA from *V. harveyi* BB7. The forward primer starts 272 bp upstream of the ATG codon of *luxO*, and the reverse primer starts 10 codons from the end of the gene (Bassler *et al*., 1994b). The PCR product (after blunt-end digestion and then *Bam*HI restriction) was ligated into pBluescript SK+ (Stratagene) at the *Eco*RV and *Bam*HI sites (pVHO-4). The kanamycin resistance (*kn*^{R}) marker was obtained from pKT230 after *Bam*HI and *Dra*III and blunt-end digestions and ligated into pVHO-4 at the unique *Nco*I site (blunt-ended), about 0.2 kbp after the 5′-ATG start site of *luxO* (pVHO-5). The entire *luxO* and *kn*^{R} interrupt was then excised with *Xba*I and *Sal*I of the polylinker of pVHO-5 and ligated into the corresponding sites, dephosphorylated, of the gentamicin-resistant, *sacB* suicide vector pJQ200 (Quandt and Hynes, 1993) to create pVHO-6, which was then used to perform *luxO* gene replacement in MM1130. In order to create the *luxO*$^-$ mutant of MJ-211 and MJ-215, the plasmid pVFO-4, also described earlier, was restricted at the *Xba*I and *Sac*I sites to release the *luxO* fragment interrupted by the *kn*^{R} marker, which was ligated to pJQ200, which had also been restricted at the same sites and treated with alkaline phosphatase. The desired plasmid pVFO-7 was mobilized into MJ-211 to yield MJ-211*luxO*$^-$. Southern blot hybridization analyses of all *luxO*$^-$ mutants confirmed successful gene replacement of the *luxO* allele with *luxO* DNA interrupted by the *kn*^{R} cassette and the loss of the DNA vector. For growth of the *luxO* null mutants, kanamycin (40 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) were routinely added to media.

Addition of luxR$_{vh}$ in trans *to Vibrio*s

Complementation of *luxR*$_{vh}$ *in trans* was carried out in *V. harveyi* and *V. fischeri* with pMGM150, which carries the *luxR*$_{vh}$ of *V. harveyi* B392 in the mobilization vector pKT230 (Bagdasarian *et al*., 1981) as described previously (Miyamoto *et al*., 1996). As controls, pKT230 alone was transferred into the same strains. To maintain selective pressure, the LB medium included streptomycin at 40 μg ml^{-1}.

RNA dot-blot hybridization

RNA was extracted from cellular pellets using the RNeasy kit (Qiagen) followed by DNase I digestion for 15 min at 37°C (Gibco BRL cat. no.18047-019). After phenol extraction and ethanol precipitation, RNA dot blots using the same amount of RNA for each sample were prepared (Miyamoto *et al*., 1996). DNA, ^{32}P-labelled with random primers and [α-^{32}P]-dCTP (Amersham) in a Klenow reaction according to the manufacturer's specifications (NEB), was used as hybridization probe after heat denaturation. *V. harveyi* B392 *luxR*$_{vh}$ in pT7 (pT7/luxR$_{vh}$) was the source for the *luxR*$_{vh}$ probe. The plasmid pMF-2 (*litR*$^+$) was constructed by M. Fidopiastis of E. Ruby's laboratory (Fidopiastis *et al*., 2002). The *Eco*RI fragment containing *litR* was excised and inserted into pT7 (Tabor and Richardson, 1985), and the resultant plasmid (pT7/*litR*) was used as the probe for *litR* mRNA. For *luxC* DNA, the restricted DNA insert (*Sac*I and *Cla*I) from cloned *lux* DNA in our laboratory (Swartzman and Meighen *et al*., 1993) was subcloned into pBluescript SK+ at the corresponding restriction sites (pBlue/*luxC*). The *luxC* DNA (0.45 kbp) encompasses the promoter and transcriptional start site of the *lux* operon and 62 bp of the *luxC* gene (Swartzman *et al*., 1992). Finally, the *crp* probe was obtained using pT7/crp (Chatterjee *et al*., 2002). The hybridized blots were visualized using the Fuji Bioimager. A control using *E. coli* RNA on each blot typically gave a background reading of <70 c.p.m., and the background was subtracted from the raw results of each set of blots. It should be noted that the specific activities of the probes varied with their base content and size, and comparison of intensity levels of mRNA among them cannot be done.

Mobility shift analysis

The plasmid pT7/luxR$_{vh}$ was restricted with *Sac*I and *Mfe*I to provide a 400 bp DNA fragment that contains the promoter region of *luxR*$_{vh}$ (Chatterjee *et al*., 1996). After a Klenow fill-in reaction (NEB) with [α-^{32}P]-dATP (Amersham) at the *Mfe*I site, the DNA was subjected to mobility shift analysis after mixing with various cell-free crude lysates prepared from cellular pellets of cells grown in LB to a density of A_{660} = 0.6, as described previously (Swartzman and Meighen, 1993). Protein determinations were performed using the Bio-Rad protein assay kit.

Detection of polyhydroxybutyrate (PHB)

A constant amount of cells (10 A_{660} units) grown in complex medium was harvested at high cell densities (A_{660} = 3) and processed for PHB using NaOCl oxidation as described previously (Sun *et al*., 1994; Miyamoto *et al*., 1998). Wavelength scans were obtained using the Beckman DU-640 spectrophotometer.

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