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Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA

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Summary

Bioluminescence generated by the *Vibrio fischeri* Lux system consumes oxygen and reducing power, and it has been proposed that cells use this to counteract either oxidative stress or the accumulation of excess reductant. These models predict that *lux* expression should respond to redox conditions; yet no redox-responsive regulator of *lux* is known. We found that the *luxICDABEG* operon responsible for bioluminescence is repressed by the ArcAB system, which is activated under reducing conditions. Consistent with a role for ArcAB in connecting redox monitoring to *lux* regulation, adding reductant decreased luminescence in an *arc*-dependent manner. ArcA binds to and regulates transcription from the *luxICDABEG* promoter, and it represses luminescence both in the bright strain MJ1 and in ES114, an isolate from the squid *Euprymna scolopes* that is not visibly luminescent in culture. In ES114, deleting *arcA* increased luminescence in culture ~500-fold to visible levels comparable to that of symbiotic cells. ArcA did not repress symbiotic luminescence, but by 48 h after inoculation, ArcA did contribute to colonization competitiveness. We hypothesize that inactivation of ArcA in response to oxidative stress during initial colonization derepresses *luxICDABEG*, but that ArcAB actively regulates other metabolic pathways in the more reduced environment of an established infection.

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Introduction

Bacterial bioluminescence is a tightly regulated energy-consuming process. In *Vibrio fischeri*, luminescence is produced when luciferase, composed of LuxA and LuxB, converts $FMNH_2$, O_2 and an aliphatic aldehyde (RCHO) to FMN, water and an aliphatic acid (Ziegler and Baldwin, 1981). LuxC and LuxE recycle the aliphatic acid, which is also produced by LuxD, to regenerate the RCHO substrate (Boylan *et al*., 1989), while LuxG and other proteins in the cell reduce FMN using NADH to supply luciferase with $FMNH_2$ (Zenno and Saigo, 1994). Figure 1 outlines the biochemistry of bioluminescence in *V. fischeri* and helps illustrate the energetic commitment to this process. Energy is directly required for Lux protein synthesis, and the recycling of RCHO similarly involves the hydrolysis of ATP (Fig. 1). In addition, the consumption of oxygen and reductant, which ultimately comes from the NADH pool, appears to compete with energy-generating aerobic respiratory pathways that use the same substrates. Both theoretical calculations and experimental measurements suggest that perhaps 10% of the energy expended by bright cells is devoted to luminescence (Karl and Nealson, 1980).

Given this energetic commitment, it is not surprising that luminescence is tightly regulated. In *V. fischeri*, the *lux* genes responsible for luminescence are cotranscribed with *luxI*, and this *luxICDABEG* operon is adjacent to, but divergently transcribed from, *luxR* (Engebrecht and Silverman, 1984). LuxI and LuxR form an archetype 'quorum-sensing' regulatory circuit whereby LuxI generates a *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) autoinducer that, together with LuxR, stimulates *luxICDABEG* transcription (Nealson *et al*., 1970; Eberhard *et al*., 1981; Engebrecht *et al*., 1983). As a result, luminescence is induced when 3-oxo-C6-HSL accumulates at high cell densities, i.e. when cells reach a 'quorum'. Two other autoinducers also regulate the *V. fischeri lux* genes: *N*-octanoyl homoserine lactone (C8-HSL) produced by AinS (Hanzelka *et al*., 1999), and a compound, presumably a furanosyl borate diester (Chen *et al*., 2002), produced by LuxS (Lupp and Ruby, 2004). These signals apparently stimulate *luxR* transcription through a pathway involving LuxO and LitR (Fidopiastis *et al*., 2002; Lupp *et al*., 2003). C8-HSL can also bind to and activate LuxR, although it is less effective than 3-oxo-C6-HSL (Schaefer *et al*., 1996; Egland and Greenberg, 2000).

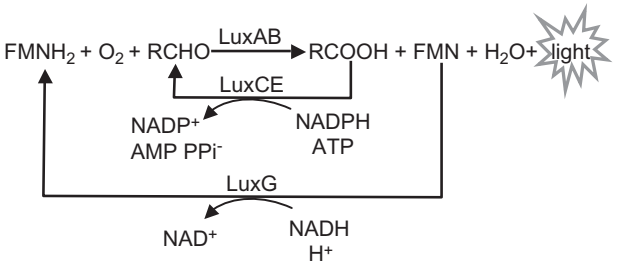

Fig. 1. Biochemistry of Lux-mediated bioluminescence in *V. fischeri*. LuxD (not shown) contributes to RCOOH synthesis.

In contrast to this detailed understanding of quorum sensing, relatively little is known about other regulators of *lux* in *V. fischeri*. Notably, no redox-dependent regulatory mechanism has been connected to *lux* expression, although conflicting reports indicate that aeration may affect *lux* (Nealson and Hastings, 1977; Boettcher and Ruby, 1990). A better understanding of *lux* regulation in response to its substrates, oxygen and reductant would help elucidate how luminescence benefits bacteria. Opposing models suggest that luminescence imparts a selective advantage on *V. fischeri* either by consuming oxygen or, conversely, by acting as a sink for excess reductant (Visick *et al*., 2000; Bourgois *et al*., 2001; Timmins *et al*., 2001). These models predict that luminescence should be activated either in response to oxygen/oxidative stress or, conversely, upon buildup of reductant. Based on redox-dependent modulation of gene expression in other bacteria, an ideal candidate to mediate such a regulatory decision would be ArcAB.

The ArcAB two-component regulatory system controls gene expression in response to the redox state of the quinone pool (Georgellis *et al*., 2001). Quinones oxidized during aerobic respiration, or perhaps by reactive oxygen species (ROS), inhibit the kinase activity of the ArcB sensor (Malpica *et al*., 2004). However, when the quinone pool shifts towards the reduced state, ArcB autophosphorylates and activates the response regulator ArcA through a phosphorelay that results in phosphorylated ArcA (ArcA-P) (Georgellis *et al*., 1997). Upon activation by such reducing conditions, ArcA-P binds DNA and regulates target promoters either positively or negatively (Lynch and Lin, 1996; Salmon *et al*., 2005).

We investigated the Arc system and its relationship to luminescence in *V. fischeri* strains MJ1 and ES114, which were isolated from the light organs of the pinecone fish *Monocentris japonica* (Ruby and Nealson, 1976) and the Hawaiian bobtail squid, *Euprymna scolopes* (Boettcher and Ruby, 1990) respectively. MJ1 is brightly bioluminescent and traditionally used in studies of the *lux* system. In contrast, ES114 is dim in culture (Boettcher and Ruby, 1990) but has become a popular model for studying luminescence under ecologically relevant conditions, because its association with *E. scolopes* can be established and observed in the laboratory (Stabb, 2006). The induction of luminescence in symbiotic ES114 cells has been attributed to quorum sensing and high cell density in the host (Boettcher and Ruby, 1995; Lupp *et al*., 2003); however, even very dense ES114 cultures are dim, suggesting that environmental cues also modulate *lux* expression. Here we report that, in culture, ArcA represses luminescence in both MJ1 and ES114, indicating that the redox state detected by ArcAB is a key environmental factor governing *lux* regulation.

Results

Identification of arcA *and* arcB *in* V. fischeri

To explore the possibility that ArcAB regulates luminescence, we searched the *V. fischeri* ES114 genome for ArcB and ArcA homologues (Ruby *et al*., 2005). We identified putative *arcB* (VF2122) and *arcA* (VF2120) genes, which encode proteins with 57% and 84% amino acid identity to *Escherichia coli* ArcB and ArcA respectively. These homologues were reciprocal best matches in comparisons between *V. fischeri* and *E. coli*, with VF2120 and VF2122 being the open reading frames (ORFs) in the ES114 genome most similar to *E. coli* ArcA and ArcB respectively, and visa versa. The putative *V. fischeri* ArcA and ArcB are similar in length to their *E. coli* counterparts, including conservation of key regions in ArcB (e.g. PAS, HisKA, HATPase and signal-receiver domains) and ArcA (e.g. winged-helix DNA-binding, signal-receiver and effector domains). Moreover, the specific residues critical for redox-sensing (ArcB: Cys-180 and Cys-241) and phosphotransfer (ArcB: His-292, Asp-576 and His-717; and ArcA: Asp-54) in *E. coli* (Georgellis *et al*., 1997; Malpica *et al*., 2004) are conserved in the *V. fischeri* homologues. The genetic context of the putative *V. fischeri arcA* and *arcB* is also conserved relative to known ArcAB systems. These genes are convergently transcribed on chromosome 1 in *V. fischeri*, as they are in *V. cholerae* (Sengupta *et al*., 2003), and the genes immediately upstream of *V. fischeri arcB* are similar to those upstream of *arcB* in both *E. coli* and *V. cholerae*. Finally, a recent global survey of response regulators in *V. fischeri* concurs with the assignment of VF2122 and VF2120 as *arcB* and *arcA* (Hussa *et al*., 2007).

In *E. coli*, the ArcAB system regulates genes involved in the TCA cycle, respiration and fermentation, among others (Salmon *et al*., 2005). One consequence of this activity is that *arc* mutants redirect electron flow and become sensitive to redox dyes such as toluidine blue (Ruiz *et al*., 2006). Therefore, to test the functional conservation of the putative *V. fischeri arcA*, we tested whether it could restore toluidine blue resistance to an

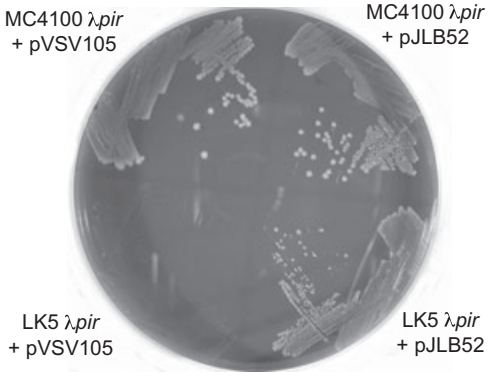

Fig. 2. *Vibrio fischeri arcA* restores toluidine blue resistance to an *E. coli arcA* mutant. MC4100λ*pir* (parent) and LK5λ*pir* (*arcA* mutant) carrying pVSV105 (vector) or pJLB52 (pVSV105 with *V. fischeri arcA*) were plated on LB containing 200 ng ml^{-1} toluidine blue and incubated overnight under white light at 37°C.

E. coli arcA mutant. We cloned the putative *arcA* gene into pVSV105 (Dunn *et al*., 2006), which contains the replication origin from *V. fischeri* plasmid pES213 and the R6Kγ replication origin. This shuttle vector replicates both in *E. coli* strains engineered to contain *pir* and (as described below) in *V. fischeri*. The *E. coli arcA* mutant LK5λ*pir* had a severe growth defect on toluidine blue plates compared with the isogenic *arcA*$^+$ strain MC4100λ*pir* when these strains carried the control vector pVSV105; however, when *V. fischeri arcA* was provided on pJLB52, LK5λ*pir* was substantially rescued from toluidine blue sensitivity (Fig. 2).

Other evidence also suggests that the putative ArcAB system in *V. fischeri* functions similarly to that in *E. coli*. A recent bioinformatic analysis of *Vibrio* genomes concluded that these bacteria, including *V. fischeri*, use the *arc* system to regulate many of the same metabolic processes that are regulated by ArcAB in *E. coli* (Ravcheev *et al*., 2007). To test this experimentally, we fused *lacZ* to the promoter region of succinate dehydrogenase, one system predicted to be repressed by *arc*, and compared expression of this reporter in wild-type ES114 and in a mutant with an in-frame deletion of *arcA*. As predicted, we saw a higher level of expression from P_{sdh}-*lacZ* in the *arcA* mutant (Fig. 3). Also, as described below, we found that *arcA* and *arcB* mutants behave similarly in mediating a regulatory response to reductant, consistent with ArcA and ArcB functioning together in a redox-responsive two-component system. Taken together, these findings indicate that VF2120 and VF2122 encode an ArcAB system in *V. fischeri* that is similar to ArcA and ArcB in *E. coli*.

ArcA and ArcB repress luminescence in culture and respond to reductant

We next tested whether ArcA regulates luminescence in *V. fischeri*. In culture, Δ*arcA* mutant AMJ2 was ~500-fold brighter than its parent ES114 (Fig. 4A), a level of luminescence induction similar to that seen when ES114 infects *E. scolopes* or is exposed to exogenous 3-oxo-C6-HSL (Boettcher and Ruby, 1990; 1995; Lupp *et al*., 2003). Low luminescence was restored when *arcA* was provided

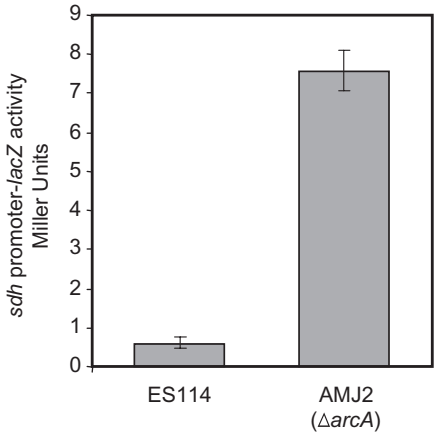

Fig. 3. ArcA-mediated regulation of a succinate dehydrogenase promoter-*lacZ* reporter. β-Galactosidase activity, expressed in Miller units, in cultures of ES114 or Δ*arcA* mutant AMJ2 each carrying pJLB217 (P_{sdh}-*lacZ*). The activity from a promoterless *lacZ* was subtracted for each strain. Data represent the average with standard error ($n = 3$).

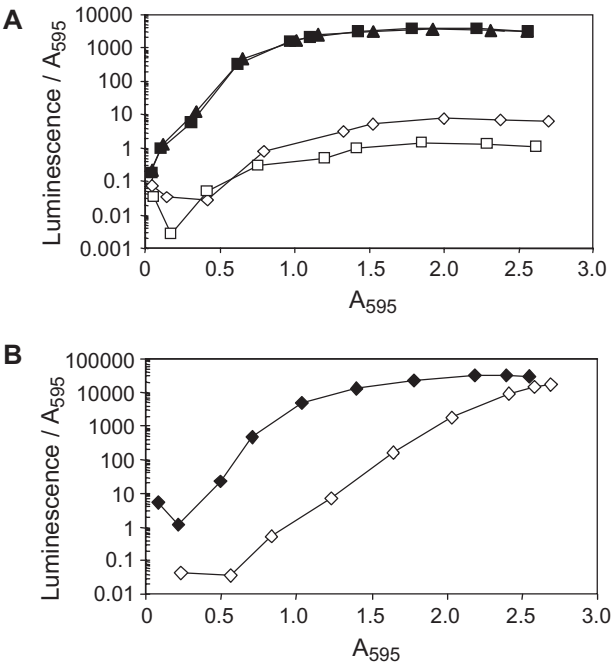

Fig. 4. Enhanced luminescence in Δ*arcA* mutants.
A. Specific luminescence (luminescence per A_{595}) of ES114 (open diamonds), its isogenic Δ*arcA* derivative AMJ2 (filled triangles), AMJ2 with vector pVSV105 (filled squares), and AMJ2 with *arcA*-containing plasmid pJLB52 (open squares).
B. Specific luminescence of MJ1 (open diamonds) and its isogenic Δ*arcA* derivative JB11 (solid diamonds).

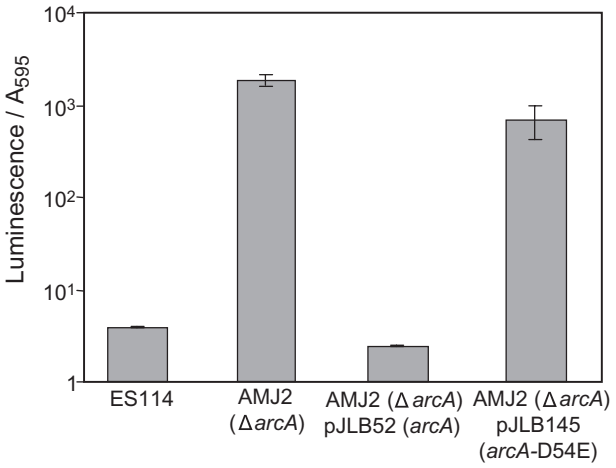

Fig. 5. The Asp-54 residue of ArcA is required for regulation of bioluminescence. Comparison of maximal luminescence per A_{595} for ES114, Δ*arcA* mutant AMJ2, and AMJ2 bearing plasmids pJLB52 or pJLB145 with wild-type *arcA* or a mutant *arcA* allele encoding Glu instead of Asp at residue 54 respectively. Data represent the average with standard error ($n = 2$).

in trans (Fig. 4A), and directed mutation of *arcA* to convert Asp-54 to Glu destroyed complementation activity (Fig. 5), confirming the importance of ArcA and the conserved putative phosphorylation site Asp-54 in the repression of luminescence. These studies used *arcA* alleles placed on shuttle vector pVSV105, which is derived from a native *V. fischeri* plasmid and is stable in this bacterium (Dunn *et al*., 2006), so these experiments could be performed in the absence of antibiotic selection that might otherwise have indirectly affected luminescence.

Regulation of the *lux* genes varies in different *V. fischeri* isolates, and this is reflected in divergent *lux* promoter sequences and wide-ranging differences in luminescence output (Boettcher and Ruby, 1990; Gray and Greenberg, 1992). To test whether *arcA*-mediated regulation is specific to dim strains like ES114, we generated an *arcA* mutant in visibly luminescent strain MJ1. Here too we found that deleting *arcA* enhanced luminescence, particularly at low cell densities (Fig. 4B). Thus, ArcA repression of luminescence is conserved in MJ1 and ES114.

In *E. coli*, the ArcAB system is activated by the reducing agent dithiothreitol (DTT) (Malpica *et al*., 2004), and we therefore predicted DTT would stimulate Arc-dependent regulation of luminescence. Addition of 5 mM DTT caused a ~22-fold decrease in luminescence of ES114; however, DTT had only a minor transient effect on luminescence in the *arcA* mutant (Fig. 6A). Similarly, the *arcB*::miniTn*5* mutant NL3 was brighter than ES114 and did not regulate luminescence in response to DTT (Fig. 6B), consistent with ArcA mediating DTT-responsive regulation in combination with ArcB. Thus, DTT lowered luminescence in *V. fischeri* in an ArcA- and ArcB-dependent manner, indicating that the ArcAB system mediates stronger repression of luminescence in response to more reducing conditions.

Although the *arcA* and *arcB* mutants sometimes appeared to grow slightly slower than ES114, growth rate cannot account for the differences in luminescence shown in Figs 4 and 6. For example, there was no discernible difference between the growth of wild-type and the *arc* mutants in aerated cultures when the A_{595} was < 0.5 (data not shown); however, enhancement of luminescence was evident even at this cell density (Figs 4 and 6). We also found that DTT had only a minor effect on growth that was similar in both wild-type and *arc* mutants (data not shown); yet DTT had a large and ArcAB-dependent effect on luminescence (Fig. 6). Furthermore, in our experience, several mutants with minor to pronounced growth defects do not exhibit such altered luminescence phenotypes relative to ES114. From these observations we concluded that the effect of ArcAB on luminescence may be direct or indirect but is not simply due to altered growth of the *arc* mutants.

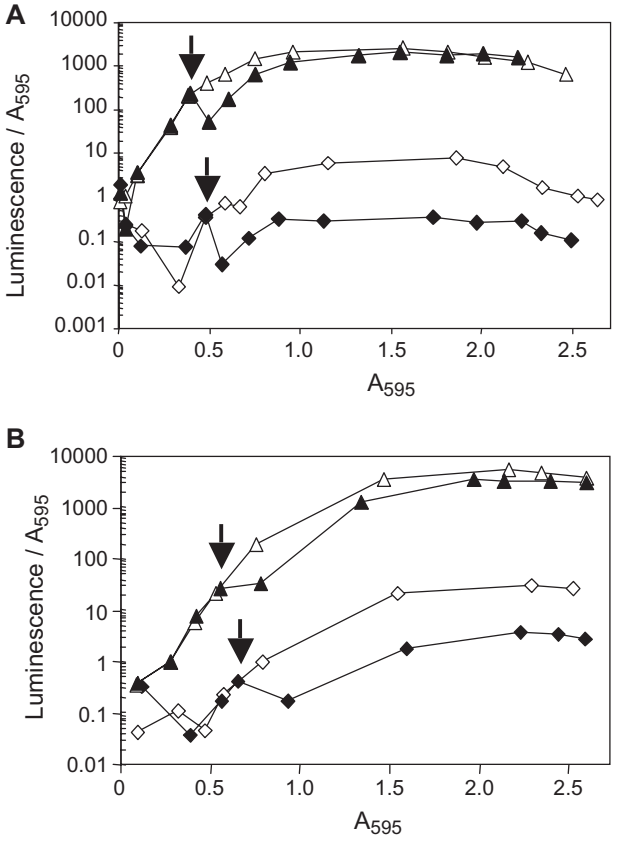

Fig. 6. Addition of DTT represses luminescence in an ArcA- and ArcB-dependent manner. Specific luminescence (luminescence per A_{595}) with (filled symbols) or without (open symbols) DTT addition for ES114 (diamonds) and *arc* mutants (triangles). The *arc* mutants were (A) AMJ2 (Δ*arcA*) or (B) NL3 (*arcB*::Tn). Arrows indicate time of DTT addition.

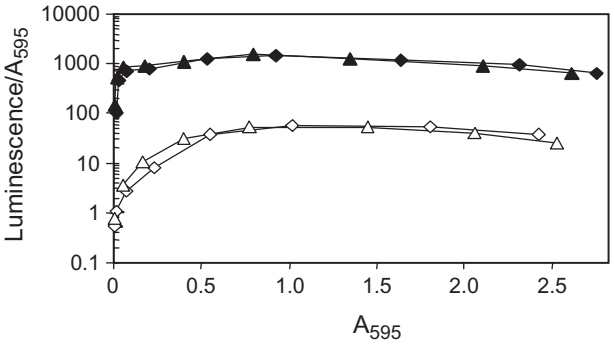

Fig. 7. Specific luminescence (luminescence per A_{595}) of *luxR*::*ermR lacI*^{q}-P_{tac}::*luxI*$^{-}$*CDABEG* strain EVS101 (diamonds) and its Δ*arcA* derivative AMJ3 (triangles) with (solid symbols) or without (open symbols) 0.5 mg ml^{-1} IPTG.

luxI *promoter derepressed in* arcA *mutant*

Enhanced luminescence in an *arcA* mutant could theoretically result from increased availability of luciferase's substrates (e.g. oxygen or $FMNH_2$), or from regulation of the *lux* genes. Two lines of evidence support the latter model. First, deleting *arcA* did not enhance luminescence when the *lux* genes were expressed from the non-native P_{tac} promoter (Fig. 7), indicating that enhanced luminescence in the absence of ArcA is specific to the native *lux* promoter. Second, specific fluorescence from $P_{luxICDABEG}$-*gfp* transcriptional reporter plasmids was controlled by *arcA*. For example, the ES114-derived *luxI* promoter driving *gfp* expression on pJLB38 yielded 19-fold greater fluorescence in the *arcA* mutant than in ES114 (Fig. 8A). We considered the possibility that placing the *gfp* reporter on a multicopy plasmid might affect regulation; however, we observed a similar *arcA*-dependent repression of *gfp* when it was placed on the ES114 chromosome between *luxI* and *luxC*, and this derepression of *gfp* was complemented by reintroducing *arcA in trans* (Fig. 8B). This shows that the native $P_{luxICDABEG}$ promoter is derepressed in an *arcA* mutant.

ArcA also mediated repression of the MJ1 $P_{luxICDABEG}$ promoter, and the MJ1 $P_{luxICDABEG}$-*gfp* reporter on pJLB37 yielded fivefold greater fluorescence in the *arcA* mutant than in parent strain ES114 (Fig. 8A). Although the bright luminescence of MJ1 caused high background readings in the fluorometer, epifluorescence microscopy confirmed that the MJ1 $P_{luxICDABEG}$-*gfp* reporter was derepressed and produced more GFP in the *arcA* mutant of MJ1 (data not shown). Thus, although the intergenic *lux* region has diverged between ES114 and MJ1, ArcA mediates repression of the *lux* promoter in both strains.

Relationship of ArcA to known regulators of luminescence

We used the more genetically tractable strain ES114 to test whether repression of luminescence by ArcA could be indirect and dependent on ArcA modulating LitR, LuxO, CRP, AinS or LuxR, which are regulators known to affect expression of *luxICDABEG* either in *V. fischeri* (Kuo *et al*.,

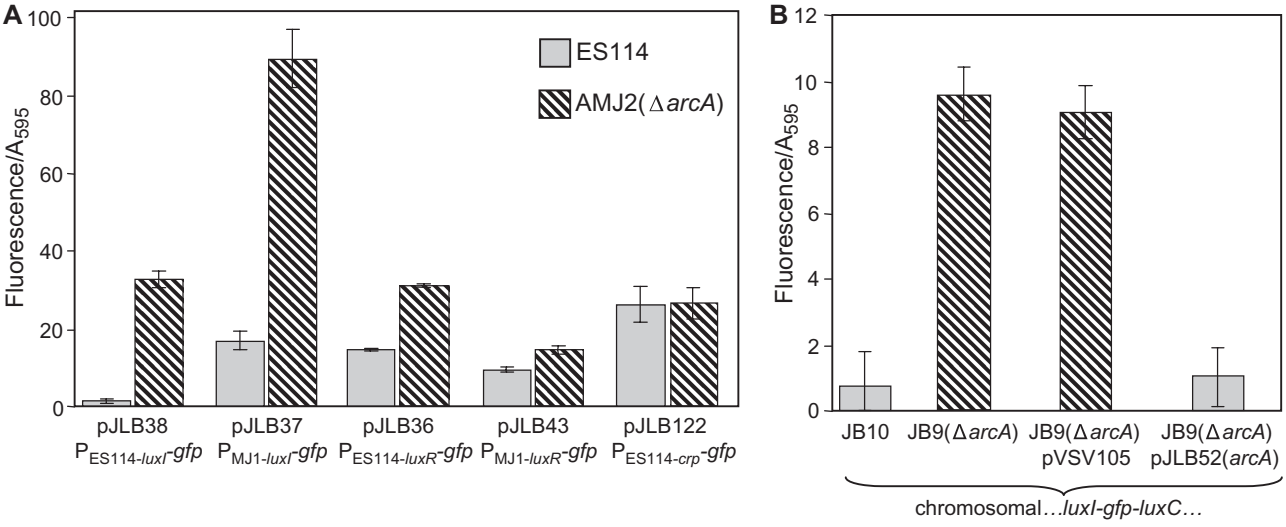

Fig. 8. Effect of *arcA* on *gfp* reporters.
A. Specific fluorescence generated from P_{luxR}-, $P_{luxICDABEG}$-, or P_{crp}-*gfp* reporter plasmids harboured in ES114 (grey bars) or Δ*arcA* mutant AMJ2 (hatched bars). The source of the promoter, from strain ES114 or MJ1, is indicated as a subscript (e.g. $P_{MJ1\text{-}luxI}$-*gfp* indicates that the *luxI* promoter from strain MJ1 is driving *gfp* expression). Data represent the average specific fluorescence with standard error ($n = 10$ or 11).
B. Specific fluorescence generated from a chromosomal insertion of *gfp* between *luxI* and *luxC* in ES114 derivatives JB10 and JB9 (Δ*arcA*), or in JB9 carrying the insertless control vector pVSV105 or complemented with the *arcA*-containing plasmid pJLB52. In both panels data represent the average specific fluorescence when culture was between A_{595} 2.0 and 2.8, a range in which specific luminescence is constant for strain ES114. Bars indicate standard error ($n = 10$ or 11).

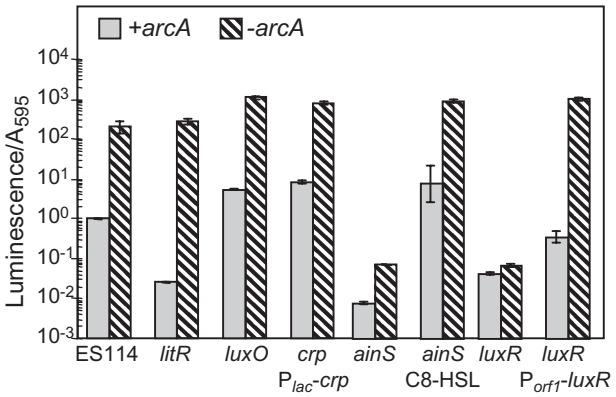

Fig. 9. Repression of luminescence by ArcA does not depend on regulation of *litR*, *luxO*, *crp*, *ainS* or *luxR*. Comparison of maximal luminescence per A_{595} for ES114 and strains harbouring various mutations, indicated by gene designations under the corresponding bars, with either the wild-type *arcA* allele (grey bars) or the Δ*arcA* mutation (hatched bars). 'P_{lac}-*crp*' and 'P_{orf1}-*luxR*' indicate that strains harboured plasmids pJLB146 or pJLB123 wherein the *crp* or *luxR* coding sequences, lacking their native promoter regions, were placed downstream of the *E. coli* P_{lac} promoter or the plasmid pES213 *orf1* promoter (Dunn *et al*., 2005) respectively. 'C8-HSL' indicates that cultures were supplemented with 100 nM C8-HSL. Strains compared were: ES114 (wild type), AMJ2 (Δ*arcA*); JB19 (*litR*::*ermR*), JB21 (Δ*arcA litR*::*ermR*); JB13 (*luxO*::pAIA3), JB14 (Δ*arcA luxO*::pAIA3); JB24 (Δ*crp*) pJLB146 (P_{lac}-*crp*), JB25 (Δ*arcA* Δ*crp*) pJLB146 (P_{lac}–*crp*); CL21 (Δ*ainS*::*chmR*), EVS21 (Δ*arcA* Δ*ainS*::*chmR*); and CL53 (Δ*luxR*::*ermR*) and JB5 (Δ*arcA* Δ*luxR*::*ermR*) with or without pJLB123 (P_{orf1}-*luxR*).

1994; Miyamoto *et al*., 2000; 2003; Visick *et al*., 2000; Fidopiastis *et al*., 2002; Lupp *et al*., 2003), or for CRP in transgenic *E. coli* (Dunlap and Greenberg, 1985; 1988). We found that ArcA influences luminescence even when *litR*, *luxO*, *crp* or *ainS* is absent or artificially expressed. For example, deleting *arcA* still greatly enhanced luminescence even in *litR* and *luxO* mutant backgrounds (Fig. 9). Comparisons of Δ*crp* mutant JB24 and Δ*crp*Δ*arcA* mutant JB25 were uninterpretable, because deleting *crp* severely inhibited growth (data not shown); however, when *crp* was expressed from the P_{lac} promoter rather than from its native promoter, deletion of *arcA* enhanced luminescence (Fig. 9). Moreover, expression of the P_{crp}-*gfp* reporter on pJLB122 was not affected by *arcA* (Fig. 8). An *ainS* mutant, which does not produce C8-HSL autoinducer (Lupp *et al*., 2003), was dim, and its luminescence was enhanced only about 10-fold in an *arcA* background; however, when the C8-HSL product of AinS was added to *ainS* cells, bright luminescence was restored and the Δ*arcA* allele enhanced luminescence (Fig. 9). Thus, repression of luminescence by ArcA cannot be accounted for by transcriptional regulation of *litR*, *luxO*, *crp* or *ainS*.

A *luxR* mutant produced a low level of luminescence, and this was not detectably enhanced by deleting *arcA* (Fig. 9). Thus, derepression of luminescence in an *arcA* mutant is largely dependent on *luxR*, which could indicate that ArcA represses luminescence by inhibiting LuxR from activating $P_{luxICDABEG}$ or that ArcA regulates *luxR*. P_{luxR}-*gfp* reporters on plasmids pJLB36 and pJLB43 displayed 1.5- to 2-fold greater fluorescence in *arcA* mutant AMJ2 than in its parent ES114 (Fig. 8), indicating that ArcA does mediate repression of *luxR*. However, when *luxR* lacking its native promoter was expressed from a plasmid, deletion of *arcA* greatly enhanced luminescence (Fig. 9). Therefore, repression of luminescence by ArcA cannot be accounted for by the modest regulatory effect that ArcA has on the native *luxR* promoter.

An ArcA binding site in the lux *promoter region is required for ArcA-mediated repression of* lux

To test whether ArcA-P might bind to and regulate the *lux* promoter directly, we performed DNA gel retardation assays using *E. coli* ArcA-P. Although dephosphorylation of ArcA-P is unavoidable over time, resulting in a mix of ArcA-P and ArcA, care was taken to work quickly with the phosphylated form and we refer here to our purified protein simply as ArcA-P. Titration of the intergenic *luxR*–*luxI* promoter region with ArcA-P revealed binding to both the ES114- and MJ1-derived sequences (Fig. S1). ArcA-P exhibits some non-specific DNA binding, particularly with AT-rich DNA like that of *V. fischeri*, and we therefore used the promoter region for the ES114 16S rRNA gene as a comparative control that is not expected to bind ArcA-P specifically. The 16S rRNA gene promoter did form a DNA–protein complex with ArcA-P, but at a weaker affinity than the *lux* promoter region as evidenced by higher concentrations of ArcA-P required to shift the mobility of this DNA fragment (Fig. S1).

We next examined ArcA binding to the *lux* promoter region more precisely using footprint analysis. ArcA-P protected a region designated 'site 1' from DNase I in both the ES114 (Fig. 10A) and MJ1 (data not shown) *luxI* promoters, proximal to the '*lux* box' site (Devine *et al*., 1989; Stevens *et al*., 1994) for LuxR-mediated activation of the *luxICDABEG* promoter (Fig. 10B). ArcA-P binding also caused increased DNase I sensitivity at certain bases (indicated by arrows in Fig. 10A), suggestive of DNA conformational changes. Within site 1 there is a 5′-TTAACAT-3′ found in both ES114 and MJ1 (Fig. 10B), and the complementary 5′-ATGTTAA-3′ sequence matches the most conserved core of the ArcA-P binding consensus (Liu and De Wulf, 2004). 'Site 2' was protected by ArcA-P in the ES114 *lux* fragment (Fig. 10A), but not in the MJ1-derived fragment (data not shown). Site 2 overlaps the CRP binding site proposed to be involved in transcriptional activation of *luxR* in *V. fischeri* (Shadel *et al*., 1990; Busby and Ebright, 1999), which may account for ArcA's negative regulation of P_{luxR} from ES114 (Fig. 8A). Both site 1 and site 2 footprints were consistent

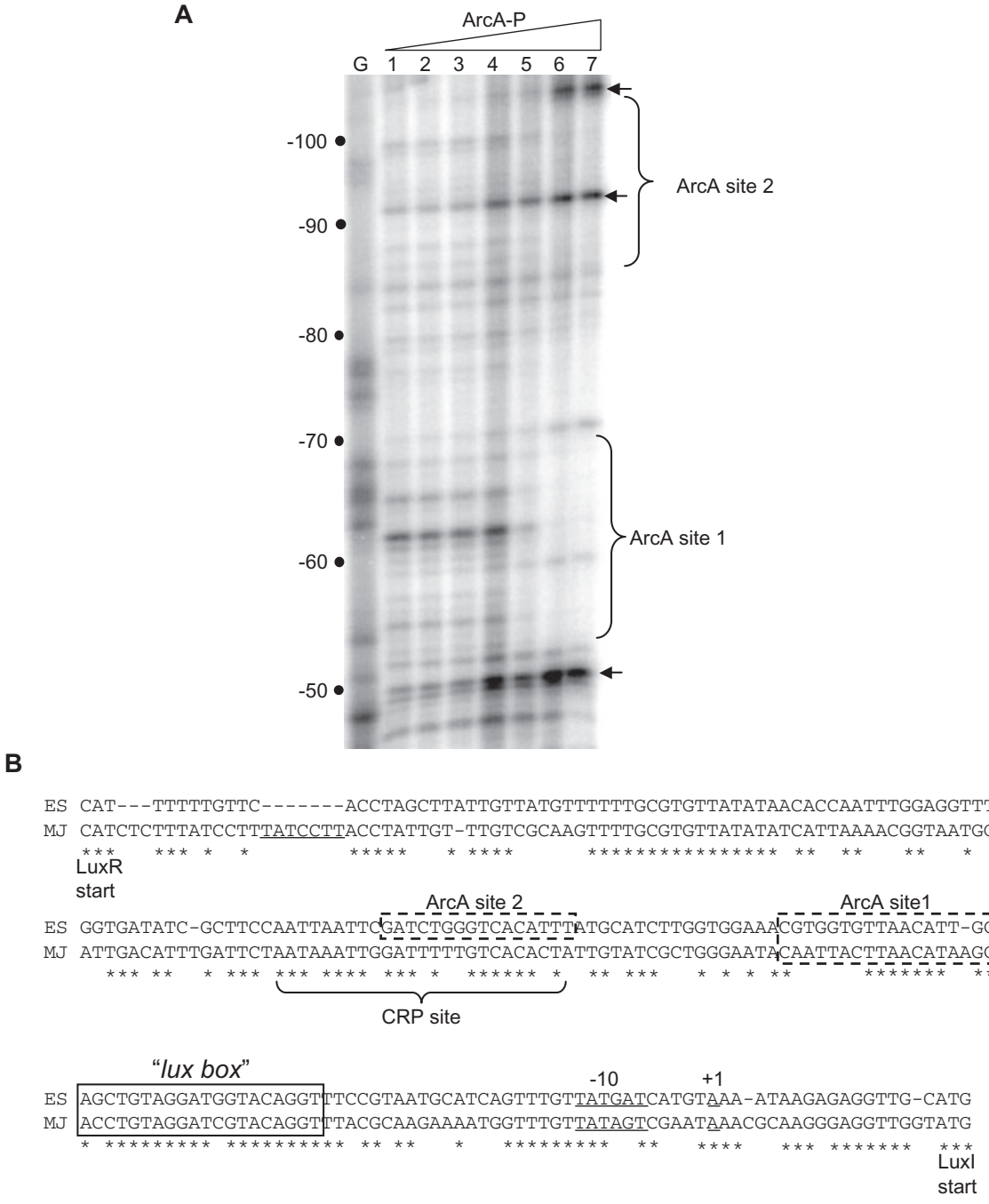

Fig. 10. ArcA binding to the *lux* promoter region.
A. DNase I footprint analysis of ArcA-P binding to the ES114 *lux* promoter region, using the sense strand with respect to *luxI*. Numbers correspond to position with respect to the *luxI* transcriptional start. ArcA-P protein concentrations used in lanes 1–7 were: (1) none; (2) 0.063 μM; (3) 0.125 μM; (4) 0.25 μM; (5) 0.5 μM; (6) 1.0 μM; and (7) 2.0 μM. Lane 'G' shows the Maxam-Gilbert G ladder. Arrows point to bases with increased sensitivity to DNase I cleavage upon binding of ArcA-P.
B. Alignment of ES114 and MJ1 *luxR–luxI* intergenic promoter region, labelled 'ES' and 'MJ' respectively, showing the bases protected by ArcA-P in panel A in dashed boxes. Asterisks indicate bps conserved in ES114 and MJ1. The transcriptional start for *luxI* and the −10 promoter element (Egland and Greenberg, 1999), and the predicted binding site for CRP (Shadel *et al.*, 1990; Busby and Ebright, 1999), are shown, although the importance of the latter *in vivo* in *V. fischeri* is uncertain. The underlined 7 bp direct repeat near the LuxR start is present in our copy of MJ1 but absent from the sequence for this strain deposited by others in GenBank (Y00509).

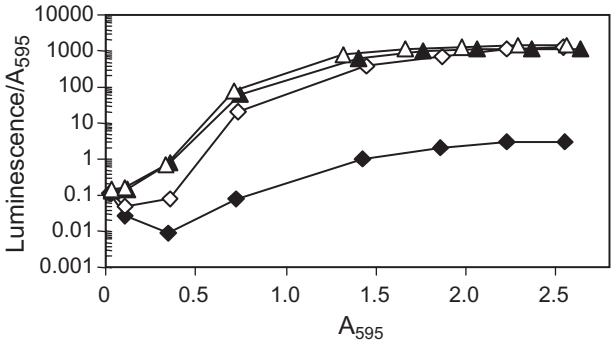

Fig. 11. ArcA-mediated repression of *lux* is dependent on ArcA binding site 1 in the *lux* promoter. Specific luminescence (luminescence per A_{595}) of strains JB35 (empty diamonds) and JB36 (empty triangles), which have site-directed alterations to the ArcA site 1 sequence on the chromosome, and their respective parents ES114 (solid diamonds) and Δ*arcA* mutant AMJ2 (solid triangles).

in size with other ArcA-P footprinting analyses (Cotter *et al*., 1997; Shen and Gunsalus, 1997).

To test the importance of 'site 1' in *arcA*-mediated regulation of luminescence, we generated mutants where the 'site 1' sequence on the chromosome (Fig. 10B) was deleted and an NheI recognition site incorporated in its place. We found that this replacement of ArcA 'site 1' resulted in an increase in luminescence equivalent to that observed in a Δ*arcA* mutant (Fig. 11). Moreover, in the absence of 'site 1', adding the Δ*arcA* allele did not change the maximal luminescence of the strain (Fig. 11). We did see a small, but reproducible difference between the 'site 1' mutant and the Δ*arcA* 'site 1' double mutant, with the latter being slightly brighter at low cell densities (Fig. 11). This may be due to ArcA binding to at 'site 2', or to indirect effects of ArcA modulating other *lux* regulators, such as *luxR*. However, the ability of ArcA to repress luminescence is largely dependent on 'site 1'.

ArcA does not repress symbiotic luminescence but contributes to colonization competitiveness

Vibrio fischeri ES114 is much brighter in its symbiotic association with *E. scolopes* than it is in culture (Boettcher and Ruby, 1990), and we therefore examined whether ArcA represses luminescence in this symbiosis. The onset of luminescence during infection was similar for ES114 and the Δ*arcA* mutant (Fig. 12A), and these strains achieved similar populations in the host (Fig. 12B). In contrast to the derepression of luminescence in the Δ*arcA* mutant in culture, luminescence per colony-forming unit (cfu) of the Δ*arcA* mutant and ES114 was similar in the squid (Fig. 12C). Furthermore, the amount of light produced per cfu in culture by the Δ*arcA* mutant was similar to that produced by either wild-type or the Δ*arcA* mutant in the squid (Fig. 12C). Thus, the increase in luminescence observed when cells begin to colonize the host could reflect deactivation of ArcA in this environment, and the dramatic difference between the luminescence of cultured and symbiotic cells can be accounted for by differential regulation by ArcA and is not necessarily due to different cell densities affecting quorum sensing.

Once a symbiotic infection is established, *V. fischeri* cells become densely packed, and it seems unlikely that the light-organ crypt environment would be replete with

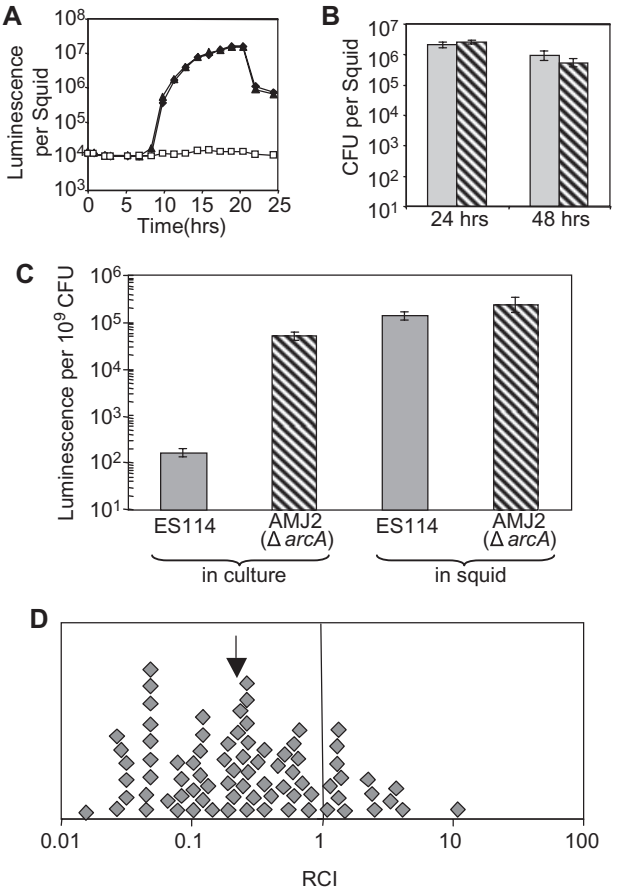

Fig. 12. Symbiotic phenotypes of *arcA* mutants.
A. Onset of symbiotic luminescence in *E. scolopes* hatchlings inoculated with ES114 (solid diamonds), Δ*arcA* mutant AMJ2 (solid triangles), or no *V. fischeri* (open squares). Data represent the mean ($n = 22$).
B. Colonization levels of ES114 (grey bars) and AMJ2 (hatched bars) 24 and 48 h post inoculation. Data show the mean and standard error ($n = 8$).
C. Luminescence per cfu of ES114 and AMJ2 in culture and in the host. Data show the mean and standard error ($n = 4$ for culture, $n = 17$ or 18 in the host).
D. Competitiveness of AMJ1 (Δ*arcA*::*ermR*) when presented in a mixed (~1:1) inoculum with wild type and recovered from squid after 48 h. Each symbol ($n = 80$) represents the RCI determined from one squid, defined as the ratio of AMJ1 to ES114 in the squid divided by the ratio of these strains in the inoculum. An arrow marks the average RCI of 0.22, which was significantly < 1 ($P < 0.01$).

molecular oxygen. This is supported by the observation that *V. fischeri* appears to induce its anaerobic respiratory pathways when colonizing the host (Proctor and Gunsalus, 2000). Such metabolism suggests that the ArcAB system might ultimately be important for symbionts. We therefore tested the competitiveness of an *arcA* mutant relative to wild type by inoculating these two strains in a ~1:1 mix and then measuring the ratio in established infections 48 h later. We found that ES114 significantly ($P < 0.01$) outcompeted the *arcA* mutant for light-organ colonization, and was about fourfold more competitive than the *arcA* mutant in these mixed infections (Fig. 12D). We similarly found that *arcB* mutant NL3 was outcompeted by wild type (data not shown). This suggests that the ArcAB system is activated in the first 48 h of the symbiosis although, as discussed below, it does not appear to turn off the feedforward regulatory cascade governing *lux* once cells become luminescent.

Discussion

Despite intense interest in the autoinducer-mediated quorum-sensing regulation of luminescence in *V. fischeri* (Greenberg, 1997; Miller and Bassler, 2001), little is known about how *lux* expression is modulated in response to luciferase's substrates, oxygen and reductant. We have now shown that ArcA, which is activated under reducing conditions, dramatically inhibits luminescence and mediates repression of the $P_{luxICDABEG}$ promoter in *V. fischeri*. Moreover, in contrast to environmental factors such as glucose (Friedrich and Greenberg, 1983) and iron (Haygood and Nealson, 1985) that repress luminescence in strain MJ1 but not in strain ES114 (Boettcher and Ruby, 1990), ArcA strongly repressed luminescence in each of these strains (Fig. 4), suggesting an important conserved relationship between redox monitoring and luminescence in *V. fischeri*.

This ArcA-mediated repression of *lux* has important implications for the functional utility of bioluminescence. It has been proposed that luminescence provides a means of lowering intra- or extracellular oxygen concentrations as an antioxidant defence mechanism (Visick *et al*., 2000; Timmins *et al*., 2001), but it has also been suggested that the important function of luminescence is to consume excess reductant (Bourgois *et al*., 2001). The finding that ArcA represses *lux* expression in response to reductant is inconsistent with the hypothesis that luminescence provides an advantage by consuming excess reductant, but is consistent with the idea that luminescence acts as a counterbalance to oxidative conditions. Moreover, this finding, together with the observation that ArcA does not repress luminescence during host colonization, is consistent with the hypotheses proposed by Visick *et al*. (2000) and Timmins *et al*. (2001), in which luminescence contributes to symbiotic competence by decreasing oxygen levels in the light organ, thereby protecting cells from host-derived oxidants.

Our results also offer a mechanistic explanation for how ArcA affects luminescence. A simple model consistent with our data is that ArcA blocks the LuxR-autoinducer activator from binding with RNA polymerase to the '*lux* box' and stimulating $P_{luxICDABEG}$ (Devine *et al*., 1989; Stevens *et al*., 1994). Binding of ArcA to 'site 1' in the *lux* promoter roughly centres on a 5′-ATGTTAA-3′ oriented on the minus strand relative to *luxICDABEG* just upstream of the '*lux* box', potentially close enough for ArcA to interfere with LuxR or RNA polymerase binding (Fig. 10B). This sequence not only resembles an ArcA binding consensus (Liu and De Wulf, 2004), it is also conserved in the *lux* promoters of strains MJ1, ES114 and ATCC7744. Interestingly, Devine *et al*. (1989) previously noted a *cis*-acting negative regulatory element upstream of the '*lux* box' in strain ATCC7744, and our data are consistent with the possibility that the negative regulation they observed was mediated by ArcA.

ArcA binding to this 'site 1' in the *lux* promoter appears to be the primary mechanism of *arc*-mediated *lux* regulation, and it is notable that ArcA's ability to repress luminescence is almost entirely abolished when the 'site 1' sequence was removed from the *lux* promoter (Fig. 11). However, a small degree of *arcA*-mediated *lux* repression was evident at low culture density even in the strain lacking 'site 1' (Fig. 11). The mechanism for this repression is not apparent, but could be due to the modest effect of *arcA* on regulation of the *luxR* promoter (Fig. 8), assuming that this is not also mediated by ArcA binding to 'site 1', which remains a possibility. ArcA might regulate *luxR* by binding to 'site 2' (Fig. 10B). ArcA binding to this site was not clearly evident in the MJ1 promoter (data not shown), and ArcA also had a less pronounced effect on P_{luxR} from MJ1 (Fig. 8A). Given the conservation of both 'site 1' and the *lux* box in ES114 and MJ1, the differences in ArcA's regulation of *lux* expression in these strains probably stem from other more variable factors such as 'site 2'.

One of the interesting variations between MJ1 and ES114 is that the effect of ArcA on quorum sensing is notably different in the two strains. In MJ1, a bright strain isolated from the light organ of a pinecone fish, ArcA represses luminescence 100- to 1000-fold specifically at low cell density, and repression is eventually overcome at high cell density (Fig. 4B). Therefore, ArcA appears to raise the threshold 'quorum' required for *lux* activation in MJ1 without preventing dense cultures from fully inducing *lux*. The effect of ArcA on quorum sensing appears to be quite different in ES114, a dim isolate typical of those isolated from the *E. scolopes* light organ. In ES114, ArcA also represses luminescence 100- to 1000-fold, but,

unlike in MJ1, this repression is evident even in dense cultures (Fig. 4A).

The direct control of *luxICDABEG* by ArcA, and the observation that even very high cell density cannot overcome this ArcA-mediated repression in *V. fischeri* ES114 (Fig. 4A), lead us to question our perception of 3-oxo-C6-HSL's role in this strain. Interestingly, in culture, ES114 is relatively dim and C8-HSL is the primary signal controlling luminescence, whereas in the *E. scolopes* light organ, the cells are bright and stimulated primarily by 3-oxo-C6-HSL (Lupp *et al*., 2003). This discrepancy has previously been attributed to differences in cell density and the idea that 3-oxo-C6-HSL mediates responses to very dense populations (Boettcher and Ruby, 1995; Lupp *et al*., 2003). However, even cells in ES114 colonies on plates are dim despite being as densely packed as cells in the light organ, and it therefore seems clear that 3-oxo-C6-HSL cannot be simply considered a census-taking molecule. In light of our data (Fig. 12C), the difference in luminescence between cultured and symbiotic cells could be accounted for by ArcA-mediated repression of the *lux* genes in culture and derepression of *lux* by deactivation of the ArcAB system in the squid. Although cell density influences luminescence in ES114, we hypothesize that this is a secondary requisite for signal amplification while the primary role for 3-oxo-C6-HSL is to act as a signal in response to ArcA-mediated redox cues from the environment.

In considering the function of 3-oxo-C6-HSL and other homoserine lactone signals, it is worth remembering two elements of this autoinducer that are conserved in other systems. First, 3-oxo-C6-HSL generated by LuxI autoactivates production of more LuxI, so inputs activating *luxICDABEG* can be amplified by a feedforward mechanism. Second, 3-oxo-C6-HSL is diffusible and transmitted between cells (Kaplan and Greenberg, 1985). Both this feedforward signal amplification and diffusibility are features common among other autoinducers (Seed *et al*., 1995; Hao and Burr, 2006). Moreover, it is becoming increasingly clear that autoinducer synthases are regulated in response to specific environmental cues (Kim *et al*., 2005; Schuster and Greenberg, 2006; Dunn and Stabb, 2007), much as *luxI* is regulated by ArcAB. Thus, these systems are set up such that environmental inputs could result in amplified responses shared among neighbouring cells. In this sense, 3-oxo-C6-HSL and other autoinducers might be functioning as alarm pheromones that transmit information about local environmental conditions across larger populations.

The role of ArcAB in sensing symbiotic conditions is not entirely clear; however, in Fig. 13 we present a preliminary model consistent with our data. In culture, the ArcAB system controls metabolic genes such as *sdh* and represses luminescence, which is stimulated primarily by LuxR and its weak activator C8-HSL (Fig. 13A). Early in

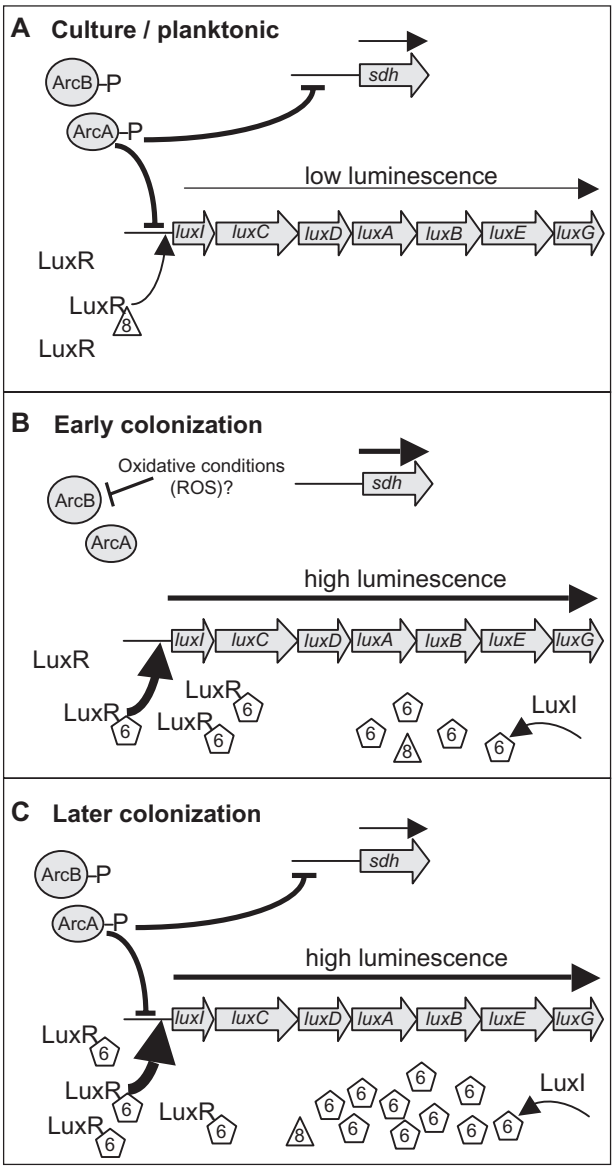

Fig. 13. Hypothetical model of ArcAB-mediated regulation of *lux* in *V. fischeri*. Repression of the succinate dehydrogenase (*sdh*) gene is included as one example of several non-*lux* genes presumably regulated (positively or negatively) by ArcAB.
A. In planktonic/cultured cells, LuxR is stimulated primarily by the weak inducer C8-HSL (triangles) (Lupp *et al*., 2003), the ArcAB system is active, and ArcA-P binds the *lux* promoter adjacent to the Lux box effectively repressing luminescence.
B. During initial infection, oxidative conditions, possibly related to host-generated ROS, are recognized by ArcAB, leading to derepression of *lux* and feedforward autoinduction due to increased LuxI production and concomitant production of the stronger LuxR inducer 3-oxo-C6 HSL (pentagons).
C. Later in an established infection, the tightly packed oxygen-consuming symbionts generate more reducing conditions, leading to reactivation of ArcAB and its regulatory control over *sdh* and other non-*lux* genes. However, luminescence remains high due to the high levels of 3-oxo-C6-HSL activating LuxR.

the onset of symbiotic infection, there was no detectable difference in luminescence of animals infected with ES114 or the Δ*arcA* mutant (Fig. 12A), and we propose that this phenomenon is due to deactivation of ArcAB system either prior to or immediately upon colonizing the light-organ crypts (Fig. 13B). *E. scolopes* produces oxidatively reactive compounds, including NO (Davidson *et al*., 2004), HOCl (Small and McFall-Ngai, 1999), H_2O_2 (Visick and Ruby, 1998), and perhaps others (Ruby and McFall-Ngai, 1999) that the bacteria encounter during infection. Theoretically, some of these could directly oxidize reduced quinones (or menaquinones), resulting in deactivation of the Arc system and concomitant derepression of the *lux* genes. This seems more likely than ArcB sensing a more highly aerobic environment in the host. In either case, the derepression of the *luxICDABEG* operon should result in more production of 3-oxo-C6-HSL, and this strong activator would contribute to high luminescence levels (Fig. 13B).

Our model for later infection (Fig. 13C) must reconcile the observations that, by 48 h post inoculation, ArcA does not appear to repress luminescence (Fig. 12C) but does seem to be active insofar as it contributes to colonization competitiveness (Fig. 12D). In our model, we suggest that oxygen consumption by respiration and luminescence in the crowded infection results in relatively reducing conditions that activate the ArcAB system (Fig. 13C). However, while ArcA may be actively regulating metabolic genes such as *sdh*, we propose that it is no longer able to effectively repress luminescence due to the accumulation of 3-oxo-C6-HSL. Essentially, we propose that the feed-forward amplification mechanism of the LuxI circuitry makes this system inherently different from other ArcA-regulated genes, rendering derepression of *lux* a more difficult regulatory switch to reverse.

The model presented above and in Fig. 13 can be integrated with previous views of bioluminescence functioning as an antioxidant and with our hypothesis that 3-oxo-C6-HSL acts as an alarm pheromone. Visick *et al*. (2000) proposed that bioluminescence aids symbiotic bacteria by lowering the local ambient oxygen concentration, thereby depriving the host of a substrate used in the production of antimicrobial ROS. Consistent with that view, we suggest that the ArcAB system may derepress *lux* in response to ROS (Fig. 13B), providing a regulatory connection between *lux* expression and this proposed benefit for luminescence in symbionts. If in fact the bacteria can effectively deprive the host of oxygen, it seems reasonable that this might require a concerted group effort, and in this respect, it makes sense for cells experiencing oxidative stress to use 3-oxo-C6-HSL as a signal to stimulate luminescence in other nearby *V. fischeri* cells, which may not be experiencing this stress yet themselves.

Although our model presented in Fig. 13 and our proposed function for 3-oxo-C6-HSL as a redox-responsive signal are consistent with our results, there are other plausible explanations. Notably, we cannot rule out the possibility that ArcA remains activated during early infection, but that another regulatory system overrides its effect on *lux* expression. Similarly, despite obvious parallels to ArcAB in *E. coli*, it is possible that ArcAB in *V. fischeri* responds to somewhat different environmental cues. It is also possible that the 'feed-forward' regulatory effect of LuxI may not play an important role in *arcA*-mediated *lux* regulation as we have proposed. Distinguishing between these and other possibilities awaits further studies.

Fortunately, our models present many hypotheses that can be tested experimentally. For example, we hypothesize that *arcA* mutants should stimulate luminescence in wild-type cells when the cell types are mixed, and that this signalling as well as the bright luminescence of *arcA* mutants will depend on *luxI*. These predictions can be tested by a combination of further mutant and reporter analyses. Our model (Fig. 13) also predicts that under some conditions one or more ROS will stimulate luminescence in ES114, which may be testable in cultured cells. Interestingly, we recently found that *lux* expression lags behind colonization in 'crypt 3' of the light organ (Dunn *et al*., 2006), which is late to develop in the squid. This is consistent with a model whereby a host-derived environmental cue absent in crypt-3 is important for triggering bioluminescence induction, and confocal microscopy combined with P_{lux}-*gfp* reporters and *arcA* mutants could be used to test the possibility that ArcA is actively repressing *lux* in this light-organ microenvironment. These and other experimental approaches promise to further our understanding of *V. fischeri* with respect to its Arc regulon, regulation of *lux*, and symbiotic associations.

For decades, *V. fischeri* has served as a powerful model organism for studies of bioluminescence, quorum-sensing gene regulation, and beneficial bacteria–animal interactions. Our discovery that ArcA is a major regulator of the *lux* genes responsible for bioluminescence has provided new insights into each of these lines of research, as discussed above. Perhaps most importantly, this discovery adds to growing evidence that autoinducer-mediated 'quorum-sensing' systems can themselves be strongly influenced by input from environmentally responsive regulators such as ArcA.

Experimental procedures

Bacteria and media

Plasmids were maintained in *E. coli* strain DH5α (Hanahan, 1983) except conjugative helper plasmid pEVS104 (Stabb and Ruby, 2002), which was maintained in CC118λ*pir* (Herrero *et al*., 1990), and other plasmids containing the

R6Kγ replication origin, which were maintained in DH5αλ*pir* (Dunn *et al*., 2005). Other bacterial strains used in this study are described in Table 1. *E. coli* was grown in Luria–Bertani (LB) medium (Miller, 1992) or brain heart infusion. *V. fischeri* was grown in LBS medium (Stabb *et al*., 2001), in SWT (Boettcher and Ruby, 1990), wherein seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, OH), or in SWTO, which was prepared by adding 150 mM NaCl to SWT to achieve an osmolarity near that of seawater. Agar (15 mg ml^{-1}) was added to solidify media for plating. Antibiotics were added as previously described (Dunn *et al*., 2005), and resistance to 200 ng ml^{-1} toluidine blue was selected on LB plates that were wrapped in parafilm and incubated under constant illumination. To add C8-HSL to cultures, a stock solution of C8-HSL was dissolved in ethyl acetate, an appropriate amount was added to flasks, and the ethyl acetate was allowed to evaporate before adding medium.

Molecular genetic techniques and analysis

Plasmids were constructed using standard cloning procedures and methods described previously (Dunn *et al*., 2005). Descriptions of select plasmids are provided in Table 1, and details of plasmid construction along with primer sequences are provided in Table S1. DNA ligase, Klenow fragment and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Polymerase chain reaction (PCR) was performed using KOD HiFi high-fidelity DNA polymerase (Novagen, Madison, WI), and PCR products were cloned into pCR-BluntII-TOPO or pCRBlunt (Invitrogen, Carlsbad, CA) and sequenced to ensure that unintended mutations were not incorporated. DNA sequencing was conducted at the University of Michigan DNA Sequencing Core Facility, and sequences were analysed using Sequencher 4.1.2 (Gene Codes, Ann Arbor, MI). Primers JBARC7 and JBARC10 enabled us to fill sequencing gaps in *arcA* inserts, and primer JBCRP5 was similarly used to sequence *crp* clones.

Mutant construction

Mutant alleles were constructed in *E. coli* and transferred to *V. fischeri* from *E. coli* by triparental mating using pEVS104 as a conjugative helper plasmid (Stabb and Ruby, 2002). Recombinational insertion or marker exchange was scored by screening for antibiotic resistance where possible, and confirmed by PCR. The in-frame Δ*arcA* and Δ*crp* alleles were generated such that the ORF between the start and stop codons was replaced with a 6 bp restriction enzyme recognition site. Due to sequence variations between MJ1 and ES114, separate Δ*arcA* alleles were generated for these strains. We have deposited the MJ1 *arcA* sequence (GenBank DQ834371). Mutant alleles of *luxR* (Lupp and Ruby, 2004) and *ainS* (Lupp *et al*., 2003) have partial deletions, as described previously. We disrupted *litR* in much the same manner as previously described (Fidopiastis *et al*., 2002), except that an *ermR* cassette was cloned into a unique StuI site within *litR* in pMF6. Mutants JB35 and JB36 contain a deletion of the ArcA 'site 1' (Fig. 10B), which was replaced with the 5′-GCTAGC-3′ NheI restriction enzyme recognition sequence, allowing rapid screening for incorporation of this allele by PCR amplifying the *luxIR* intergenic region and digesting products with NheI. We replaced wild-type with mutant alleles by marker exchange except in *luxO* mutants JB13 and JB14, for which an internal *luxO* fragment was cloned into suicide vector pEVS122, such that mobilization of the resulting construct pAIA3 and selection for recombinational insertion inactivated *luxO*. We identified the *arcB*::miniTn*5* mutant NL3 in a screen of random mutants as part of another study.

Luminescence, fluorescence and β-galactosidase measurements in culture

Overnight cultures grown in LBS were diluted 1:1000 into 50 ml of SWTO in 250 ml flasks, incubated at 24°C with shaking (200 r.p.m.). Unless noted otherwise, data represent the mean of two parallel cultures. Samples (500 μl each) were removed at regular intervals, and culture absorbance (A_{595}) was determined using a BioPhotometer (Brinkman Instruments, Westbury, NY). After vigorous shaking to oxygenate the sample, luminescence was determined using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Where maximal luminescence per A_{595} is reported, this represents the highest specific luminescence observed during growth in batch culture from low to high turbidity ($A_{595} < 0.1$ to ~3.0). In all cases, maximal luminescence was achieved when the culture A_{595} was between 1.5 and 2.5. Fluorescence was measured in a TD-700 fluorometer (Turner Designs) using excitation and emission filters of 486 nm and > 510 nm respectively. Fluorescence data reflect averaged measurements when culture was between A_{595} 2.0 and 2.8, a range in which specific luminescence is nearly constant for strain ES114. Fluorescence of strains carrying promoterless-*gfp* constructs was subtracted as background. To measure β-galactosidase, cultures were grown to $A_{595} = 0.2$, diluted 1:100 and regrown to A_{595} ~0.2 (to minimize carryover of activity from the inoculum culture), 1 ml was pelleted, the supernatant was discarded, and the pellet was frozen at −20°C. The next day, the frozen pellet was thawed and resuspended in 1 ml of Z-buffer, and 750 μl used for determination of β-galactosidase activity expressed as Miller units (1992). β-galactosidase activity of strains carrying a promoterless-*lacZ* construct was subtracted as background.

Gel retardation and DNA footprinting assays

DNA was PCR amplified using primer sets JBELUX1/JBELUX2, JBMLUX1/JBMLUX2 or JB16S1/JB16S2 for the ES114 *lux* promoter, the MJ1 *lux* promoter and the ES114 16S rRNA promoter respectively, and the products were cloned and sequenced in pJLB151, pJLB152 and pJLB141 respectively (Table S1). The sequences of these oligonucleotides were (5′–3′): JBELUX1, CGG AAT TCC ATT TTT TGT TCA CCT AGC TTA TTG TTA TGT; JBELUX2, GCT CTA GAC ATG CAA CCT CTC TTA TTT TAC ATG ATC; JBMLUX1, CGG AAT TCC ATC TCT TTA TCC TTT ATC CTT ACC TAT TGT; JBMLUX2, GCT CTA GAC ATA CCA ACC TCC CTT GCG TTT ATT C; JB16S1, CGG AAT TCT AAG CAA CGC TTA GTT TTG AGC TC; and JB16S2, GCT CTA GAT CAA TTA AAG TTT TTT TGG TTG CTC TGT C. The cloned frag-

Table 1. Select bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
MC4100λ*pir*	F-*araD139* Δ(*argF-lac*) U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* λ*pir*	This study
LK5λpir	Same as MC4100λ*pir* but *arcA*$^-$	This study
V. fischeri		
ES114	Wild-type isolate from *E. scolopes*	Boettcher and Ruby (1990)
AMJ1	ES114 Δ*arcA*::*ermR* (allele exchanged from pAJ7 into ES114)	This study
AMJ2	ES114 Δ*arcA* (allele exchanged from pAJ4 into AMJ1)	This study
AMJ3	ES114 Δ*arcA luxR*::*ermR lacI*^{q}-P$_{tac}$::*luxI*$^-$*luxCDABEG* (allele exchanged from pEVS137 into AMJ2)	This study
CL21	ES114 Δ*ainS*::*chmR*	Lupp *et al*. (2003)
CL53	ES114 Δ*luxR*::*ermR*	Lupp and Ruby (2004)
EVS21	ES114 Δ*arcA* Δ*ainS*::*chmR* (allele exchanged from pMU106 into AMJ2)	This study
EVS101	ES114 *luxR*::*ermR lacI*^{q}-P$_{tac}$::*luxI*$^-$*luxCDABEG*	Stabb *et al*. (2004)
JB5	ES114 Δ*arcA* Δ*luxR*::*ermR* (allele exchanged from pCL149 into AMJ2)	This study
JB9	ES114 Δ*arcA luxI-gfp-luxCDABEG* (allele exchanged from pJLB73 into AMJ2)	This study
JB10	ES114 *luxI-gfp-luxCDABEG* (allele exchanged from pJLB73 into ES114)	This study
JB11	MJ1 Δ*arcA* (allele exchanged from pJLB76 into MJ1)	This study
JB13	ES114 *luxO*::pAIA3	This study
JB14	ES114 Δ*arcA luxO*::pAIA3 (pAIA3 into AMJ2)	This study
JB19	ES114 *litR*::*ermR* (allele exchanged from pJLB96 into ES114)	This study
JB21	ES114 Δ*arcA litR*::*ermR* (allele exchanged from pJLB96 into AMJ2)	This study
JB24	ES114 Δ*crp* (allele exchanged from pJLB117 into ES114)	This study
JB25	ES114 Δ*arcA* Δ*crp* (allele exchanged from pJLB117 into AMJ2)	This study
JB35	ES114 with ArcA 'site 1' in *lux* promoter replaced (allele exchanged from pJLB212 into ES114)	This study
JB36	ES114 Δ*arcA* with ArcA 'site 1' in *lux* promoter replaced (allele exchanged from pJLB212 into AMJ2)	This study
MJ1	Wild-type isolate from *M. japonica*	Ruby and Nealson (1976)
NL3	ES114 *arcB*::mini-Tn5-*ermR*	This study
Plasmids[b]		
pAIA3	internal *luxO* fragment; R6Kγ, *ermR*	This study
pAJ4	Δ*arcA* allele; R6Kγ, *chmR*	This study
pAJ7	Δ*arcA*::*ermR* allele; R6Kγ, *chmR*	This study
pCL149	Δ*luxR*::*ermR*; ColE1, *chmR*	Lupp and Ruby (2004)
pEVS137	*luxR*::*ermR-lacI*^{q}-P$_{tac}$::*luxI*$^-$; R6Kγ, ColE1, *chmR*, *ampR*	J.L. Bose (unpubl. results)
pJLB36	P$_{luxR}$-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	This study
pJLB37	MJ1 P$_{luxI}$-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	This study
pJLB38	P$_{luxI}$-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	This study
pJLB43	MJ1 P$_{luxR}$-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	This study
pJLB52	*arcA* in shuttle vector pVSV105; pES213, R6Kγ, *chmR*	This study
pJLB73	*luxI-gfp-luxC* allele; R6Kγ, ColE1, *chmR*, *kanR*	This study
pJLB76	MJ1 Δ*arcA* allele; R6Kγ, ColE1, *chmR*, *ampR*	This study
pJLB96	*litR*::*ermR* allele; ColE1, *chmR*	This study
pJLB117	Δ*crp* allele; R6Kγ, ColE1, *chmR*, *kanR*	This study
pJLB122	P$_{crp}$-*gfp* in pVSV33; pES213, R6Kγ, *kanR*	This study
pJLB123	P$_{orf1}$-*luxR* in shuttle vector pVSV104; pES213, R6Kγ, *kanR*	This study
pJLB145	*arcA* D54E in shuttle vector pVSV105; pES213, R6Kγ, *chmR*	This study
pJLB146	P$_{lac}$-*crp* in shuttle vector pVSV105; pES213, R6Kγ, *chmR*	This study
pJLB212	*luxR-luxI* region with ArcA 'site 1' replaced by NheI site; R6Kγ, ColE1, *chmR*, *ampR*	This study
pJLB217	P$_{sdh}$-*lacZ* reporter; pES213, R6Kγ, *chmR*	This study
pMU106	Δ*ainS*::*chmR*; R2K, *tetR*	Lupp *et al*. (2003)
pVSV33	promoterless *chmR-gfp*; pES213, R6Kγ, *kanR*	Dunn *et al*. (2006)
pVSV104	Shuttle vector; pES213, R6Kγ, *kanR*, *lacZ*α	Dunn *et al*. (2006)
pVSV105	Shuttle vector; pES213, R6Kγ, *chmR*, *lacZ*α	Dunn *et al*. (2006)

a. Drug resistance abbreviations used: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tetR*, tetracycline resistance (*tetM*).
b. *Vibrio fischeri* alleles are from strain ES114 unless denoted 'MJ1'. All plasmids listed contain the RP4 origin of transfer. Replication origin(s) present on each vector are listed as RK2, R6Kγ, ColE1 and/or pES213. Plasmids based on pES213, which is native to *V. fischeri*, are stable in this bacterium and do not require constant antibiotic selection for maintenance in the population (Dunn *et al*., 2005; 2006). Additional details of plasmid composition and construction can be found in Table S1.

ments were again PCR amplified, the products were cleaned using Zymo Research (Orange, CA) Clean and Concentrator kit, digested with EcoRI or XbaI, and gel was purified using the Zymo Research Gel-Extraction kit. The digested fragments were end-labelled with [α-^{32}P]-dATP (MP Biomedical, Irvine, CA) using Klenow fragment, and labelled DNA was separated from dNTPs using a G-25 Sephadex column (Roche, Indianapolis, IN). ArcA-P was purified as described (Shen and Gunsalus, 1997).

For gel retardation assays with ArcA-P (Shen and Gunsalus, 1997), aliquots of each labelled DNA fragment were incubated with varying amounts of purified ArcA-P that had been diluted in a solution of 50 mM Tricine buffer (pH 8.0), 200 mM KCl, 20 mM $MgCl_2$ and 0.5 mM DTT. Each reaction was performed for 10 min at room temperature in 15 μl with 2 nM target DNA, 1 mM Tris buffer (pH 7.5), 5 mM KCl, 0.7 mM $CaCl_2$, 8.6% glycerol, 57 μg ml^{-1} bovine serum albumin, and 1.4 μg ml^{-1} poly (dI-dC) (Sigma, St Louis, MO). The samples were loaded onto a 4–20% polyacrylamide TBE gel (Invitrogen) and run at 200 V for 85 min at 4°C. The gels were then transferred to filter paper, dried under vacuum and exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) to visualize bands.

DNase I footprinting of the *lux* promoter region was performed as described previously (Shen and Gunsalus, 1997) but with addition of 1.67 μg ml^{-1} poly (dI-dC) to the reaction mixtures. The DNA–ArcA-P complex was digested for 8 min with 1.67 μg ml^{-1} of DNase I, and digestion was terminated by adding 0.94 μg ml^{-1} poly (dI-dC), 18.8 mM EDTA and 0.3 M sodium acetate.

Euprymna scolopes *colonization assays*

Vibrio fischeri was grown unshaken in 5 ml of SWT in 50 ml conical tubes at 28°C until the A_{595} was between 0.3 and 1.0, the cultures were diluted in Instant Ocean to a density no higher than 1500 cfu ml^{-1}, and *E. scolopes* hatchlings were exposed to inocula for up to 14 h before being rinsed in *V. fischeri*-free Instant Ocean. To study infection kinetics, hatchlings were placed in 5 ml of inoculant in 20 ml scintillation vials, and luminescence was monitored using a LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). To determine cfu per squid, infected animals were homogenized in 700 μl Instant Ocean in 1.5 ml tubes, and the homogenates were dilution plated onto LBS. To assess the specific luminescence of symbionts, luminescence of each animal was determined with a TD-20/20 luminometer prior to homogenization and plating. In parallel, luminescence was measured for cultured cells, and cultures were dilution plated to determine cfu ml^{-1}. For competition experiments, ES114 and AMJ1 (Δ*arcA*::*ermR*) were mixed at a ratio of ~1:1, and squid were inoculated with this mix. The ratio of these strains in the inoculum and in each infected animal was determined by plating on LBS and then patching colonies onto LBS supplemented with erythromycin. The relative competitiveness index (RCI) was calculated by dividing the ratio of the strains in each animal by the ratio in the inoculum. Three independent experiments were combined, and log-transformed data were used to calculate the average RCI and to determine statistical significance.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Gel retardation of *lux* or 16S rRNA promoters by ArcA-P. Radiolabelled DNA fragments were incubated with increasing amounts of ArcA-P. Protein concentrations used in lanes 1–8 were: (1) none; (2) 0.031 μM; (3) 0.063 μM; (4) 0.125 μM; (5) 0.25 μM; (6) 0.5 μM; (7) 1.0 μM; and (8) 2.0 μM.

Table S1. Plasmid construction and oligonucleotides.

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