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Bright Mutants of *Vibrio fischeri* ES114 Reveal Conditions and Regulators That Control Bioluminescence and Expression of the *lux* Operon▽

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***Vibrio fischeri* ES114, an isolate from the *Euprymna scolopes* light organ, produces little bioluminescence in culture but is ~1,000-fold brighter when colonizing the host. Cell-density-dependent regulation alone cannot explain this phenomenon, because cells within colonies on solid medium are much dimmer than symbiotic cells despite their similar cell densities. To better understand this low luminescence in culture, we screened ~20,000 mini-Tn*5* mutants of ES114 for increased luminescence and identified 28 independent "luminescence-up" mutants with insertions in 14 loci. Mutations affecting the Pst phosphate uptake system led to the discovery that luminescence is upregulated under low-phosphate conditions by PhoB, and we also found that *ainS*, which encodes an autoinducer synthase, mediates repression of luminescence during growth on plates. Other novel luminescence-up mutants had insertions in *acnB*, *topA*, *tfoY*, *phoQ*, *guaB*, and two specific tRNA genes. Two loci, *hns* and *lonA*, were previously described as repressors of bioluminescence in transgenic *Escherichia coli* carrying the light-generating *lux* genes, and mutations in *arcA* and *arcB* were consistent with our report that Arc represses *lux*. Our results reveal a complex regulatory web governing luminescence and show how certain environmental conditions are integrated into regulation of the pheromone-dependent *lux* system.**

Vibrio fischeri is a valuable model for examining bioluminescence, pheromone signaling, and symbiotic bacteria-animal interactions. Studies of *V. fischeri*'s mutualistic interactions have gained momentum since the discovery that this bacterium's light organ symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes*, can be reconstituted in the laboratory (54, 70, 83). Moreover, the bioluminescence induced by *V. fischeri* in the host light organ is a pheromone-mediated behavior, making this an attractive system for examining environmental influences on bacterial pheromone signaling in a natural infection. Largely because of interest in this symbiosis, strain ES114, which was isolated from the *E. scolopes* light organ, has become the experimental strain of choice for many studies of *V. fischeri*.

The genetic basis of bioluminescence in *V. fischeri* ES114 is fundamentally similar to that of other characterized *V. fischeri* strains (31, 32). The *lux* genes responsible for bioluminescence, *luxABCDE* and *-G*, are found together with the regulatory genes *luxR* and *luxI* and are arranged with *luxR* divergently transcribed from the *luxICDABEG* operon, as shown in Fig. 1 (24, 25, 56). Light is generated when luciferase, comprised of LuxA and LuxB, binds to $FMNH_2$, O_2, and an aliphatic aldehyde, and then converts these substrates to FMN, water, and an aliphatic acid (35, 76). LuxC, LuxD, LuxE, and LuxG (re)generate luciferase's aldehyde and $FMNH_2$ substrates (12, 64).

The remaining genes in this cluster, *luxI* and *luxR*, underlie a pheromone-mediated regulatory mechanism often referred to as quorum sensing. LuxI generates the membrane-permeable autoinducer pheromone *N*-3-oxohexananoyl-L-homoserine lactone (3-oxo-C6-HSL) (23, 41). As cell density increases, 3-oxo-C6-HSL accumulates until reaching a threshold concentration, whereupon it binds LuxR and together they activate transcription of *luxICDABEG* (24, 25, 77). As a result, the *lux* genes are most highly expressed at high cell densities, when the bacteria have reached a "quorum" (28). Like many bacterial quorum-sensing systems, the *lux* operon constitutes a positive feedback circuit, because the 3-oxo-C6-HSL produced by LuxI leads to increased transcription of *luxICDABEG*. As a result, environmental regulatory inputs to the *lux* system can be amplified and spread in a population.

V. fischeri generates two additional autoinducers: *N*-octanoyl-L-homoserine lactone (C8-HSL) produced by AinS (44) and AI-2 (46), which may be a furanosyl borate diester as it is in *Vibrio harveyi* (13). As Fig. 1 illustrates, AI-2 and C8-HSL presumably function through distinct receptors that both act via LuxU and LuxO, Hfq, and a small RNA to increase levels of LitR, which in turn increases *luxR* expression (26, 47, 58). C8-HSL can also activate LuxR directly (47). Although C8-HSL is a weaker activator of LuxR than 3-oxo-C6-HSL (67), ES114 produces more C8-HSL in broth cultures (73), and under these conditions it is the main activator of bioluminescence in ES114 (47).

Interestingly, despite conserved *lux* circuitry, ES114 and other isolates from *E. scolopes* are much dimmer in culture than previously studied *V. fischeri* strains (8). ES114 colonies on solid medium are not visibly luminescent, and cells in these colonies produce ~1,000-fold less luminescence than do symbiotic ES114 cells, despite achieving similar high population densities. Thus, cell density alone cannot account for the dim luminescence of ES114 in culture, and environmentally re-

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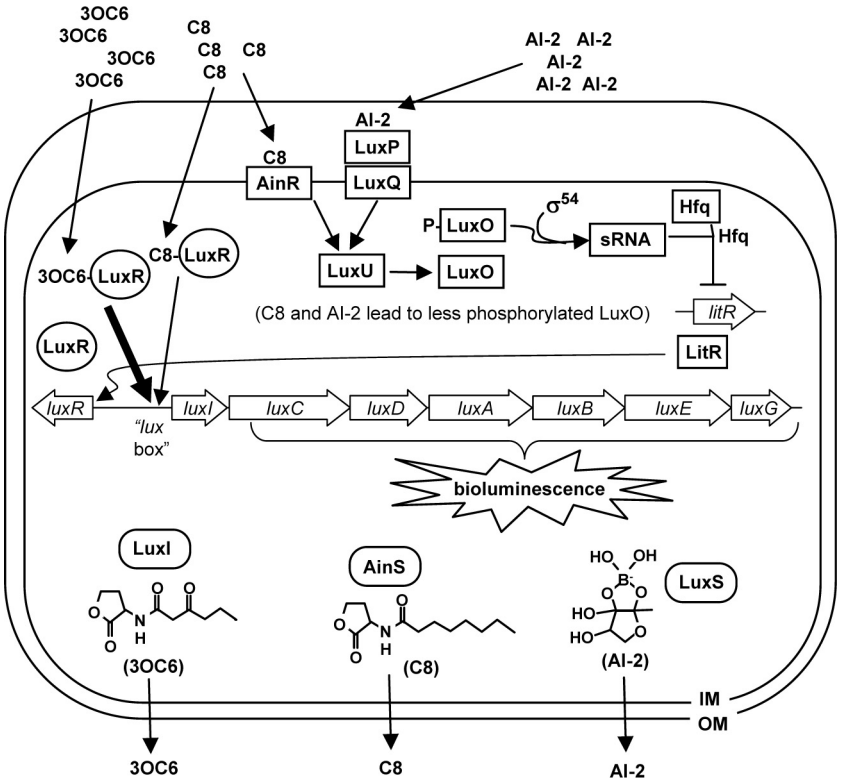


FIG. 1. Model of autoinducer-mediated regulation of bioluminescence in *V. fischeri*. Labeled open arrows correspond to genes, and the structures of three autoinducer molecules are presented with their respective synthases. The AI-2 structure is inferred from studies of *V. harveyi* (13) but has not been identified in *V. fischeri*. Interactions between autoinducers, proteins, genes, and small RNA (sRNA) are indicated. For simplicity, multimerization of proteins is not shown. This model is derived in part from experimental results but in some aspects relies on genomic predictions. For details see reference 73.

sponsive regulators must also play a critical role in regulating the *luxICDABEG* operon.

Relatively little is known about environmental influences on *luxICDABEG* expression in *V. fischeri*, particularly in ES114. CRP-mediated regulation of *lux* in response to glucose has been documented by using transgenic *lux*-containing *Escherichia coli* (18–20), although the response of *V. fischeri* to glucose is less clear and may be strain specific (8, 27). Similarly, the luminescence of strain MJ1 is inhibited by iron (36), but iron does not affect ES114 luminescence (8). We found that the redox-responsive ArcA/ArcB system represses *lux* (10); however, as we report here, ArcA/ArcB does not account for aeration-dependent regulation of luminescence (73). Other genes affecting luminescence in *V. fischeri* ES114 have been reported (38, 79, 84), but to date no directed study has screened for regulators that could account for the relatively low *lux* expression by ES114 in culture or its induction/derepression upon entering the host.

Control of bacterial pheromone systems such as *lux* by environmentally responsive regulators is widespread and has important functional implications, yet the significance of this phenomenon remains obscure. Therefore, our goal is to elucidate regulators and environmental contingencies that control induction of the *lux* operon. In this study, we screened for "luminescence-up" mutants of *V. fischeri* ES114, which led to the identification of conditions and regulators that affect bioluminescence and expression of the *lux* operon.

MATERIALS AND METHODS

Bacteria and media. *V. fischeri* strain ES114, originally isolated from an *E. scolopes* light organ (8), was the wild-type strain used throughout this study. *V. fischeri* mutants KV2801 (79), KV2850 (79), KV1651 (38), and KV2655, with disruptions in *ptsI*, *crr*, *phoB*, and *phoU*, respectively, were obtained from Karen Visick. *E. coli* strains DH5α (34) or DH5αλ*pir* (21) were transformed by plasmids in the cloning steps outlined below. Transfer of plasmids to *V. fischeri* was accomplished by triparental matings using conjugative helper strain CC118λ*pir* pEVS104 as previously described (72). *E. coli* was grown in LB medium (57) or brain heart infusion (BHI) medium (Bacto), and *V. fischeri* strains were grown in one of three medium types: (i) LBS, which contained, per liter of water, 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 50 mM Tris (pH 7.5); (ii) SWTO, which contained, per liter of total volume, 5 g of tryptone, 3 g of yeast extract, 3 ml of glycerol, 700 ml of Instant Ocean mixed to 36 ppt (Aquarium Systems, Mentor, OH), and an additional 170 mM NaCl; (iii) FMM, which contained, per liter, 950 ml of water, 378 μl of 1 M $NaPO_4$ (pH 7.5), 50 ml of 1 M Tris (pH 7.5), 3 mg of $FeSO_4 \cdot 7H_2O$, 13.6 g of $MgSO_4 \cdot 7H_2O$, 0.59 g of NH_4Cl, 0.83 g of KCl, 19.5 g of NaCl, 1.62 g of $CaCl_2 \cdot 2H_2O$, 1 g of Casamino Acids, and 3 ml of glycerol. Solid media were prepared with 15 mg ml^{-1} agar for plating. For selection of *E. coli*, chloramphenicol (CAM) and kanamycin (KAN) were added to LB at final concentrations of 20 and 40 μg ml^{-1}, respectively, and 150 μg ml^{-1} erythromycin (ERM) was added to BHI. For selection of *V. fischeri* on LBS, CAM, ERM, and KAN were used at concentrations of 2, 5, and 100 μg ml^{-1}, respectively. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropyl-β-D-thiogalactoside (IPTG) were added to media at final concentrations of 60 and 50 μg ml^{-1}, respectively.

Transposon mutagenesis. Transposon mutants were generated as previously described (1, 48). Briefly, pEVS170 was transferred to ES114 by triparental matings (72). Following overnight incubation, mating spots were resuspended in LBS and dilution plated onto LBS supplemented with ERM. Plates were incubated overnight at ~24°C, and images were generated using a Fluor-S Max-2 imager (Bio-Rad Laboratories, Hercules, CA) set to high-sensitivity chemilumi-

nescence mode with a 20-min exposure. Colonies that were more luminescent than JRM100, an ERM-resistant derivative of ES114 (52), were patched onto LBS supplemented with ERM and imaged following overnight incubation to confirm their luminescence phenotype. To ensure that *ermR* colonies carried transposon insertions and were not harboring pEVS170, mutants were screened for kanamycin resistance, which is present on pEVS170 outside the transposon, and KAN-resistant mutants were discarded.

Genetic techniques and analyses. Plasmids were generated using standard techniques. DNA ligase and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). PCR was performed with an iCycler (Bio-Rad Laboratories) using KOD DNA polymerase (Novagen, Madison, WI) or Phusion high-fidelity polymerase (Finnzymes, Finland). Plasmids used for cloning were purified using Qiagen miniprep kits (Valencia, CA) or the GenElute plasmid miniprep kit (Sigma-Aldrich, Inc., St. Louis, MO). For supercoiling analyses, plasmids were purified using the ChargeSwitch-ProDNA plasmid miniprep kit (Invitrogen, Carlsbad, CA). DNA was purified from PCR, digestion, and ligation reactions with the DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA). To clone PCR products into the pCR-BluntII-TOPO plasmid, we used the ZeroBlunt-TOPO PCR cloning kit (Invitrogen) and screened for white colonies on plates containing X-Gal. Cloned PCR products were sequenced at the University of Michigan DNA Sequencing Core Facility, and sequences were compared to the ES114 genomic database using Sequencher 4.1.2 (Gene Codes Corp., Ann Arbor, MI) to ensure that no unintended base pair changes were incorporated.

Plasmids pJLB52 (10), pJLB114, pAS5, pNL4, pNL18, pNL75, pNL6, pNL72, and pCL112 (47) were used to mobilize native copies of *arcA*, *arcB*, *acnB*, *topA*, *lonA*, *pstA*, *hns*, *phoQ*, and *ainS*, respectively, into mutants with transposon insertions in these genes. A description of new complementation plasmid construction follows. In each case the cloned gene was expressed by including ~500 bp of upstream sequence and/or by a promoter(s) on the vector. For example, the *pstA* and *phoQ* genes appear to be toward the distal ends of operons and were cloned by themselves such that they would be expressed by the *lacZ* promoter on the vector. *arcB* was PCR amplified using primers ARC B1 (5′ CCG CCC TAG GCA GGG TAT GTT TAT GAA GCA GTT AA 3′) and ARC B2 (5′ GGG GTA CCG TGT TGC GGC AAA TAG TAC CTT CTT C 3′), and the blunt product was cloned into pCR-BluntII-TOPO to generate pJLB106. pJLB106 was linearized with AvrII and ligated to XbaI-digested pVSV105 (22), generating pJLB110, which was then digested with KpnI and self-ligated to excise the pCR-BluntII-TOPO vector, generating pJLB114. *acnB* was PCR amplified using primers JBACNB5 (5′ GCG CCA TAA GTC GTA TGT TGT TTG TTG TGG G 3′) and JBACNB7 (5′ CCA GCC CT TAA ATA AAA AAG CAG CCA ATT GCC 3′), and the blunt product was ligated directly into HpaI-digested pJLB103 to generate pJLB130. The vector pJLB103, which contains the R6K origin of replication and encodes KAN resistance, was derived from pVSV104 (22) by deleting the pES213 origin on a BamHI fragment. pJLB130 was digested with AvrII and KpnI, and the fragment containing *acnB* was ligated into XbaI- and KpnI-digested pVSV105 (22) to generate pAS5. *topA* was PCR amplified using primers pr_NL3 (5′ CAG CCT CAG AAA TGG ATT TTT TAT CGC TCA TAA G 3′) and pr_NL4 (5′ GAG CCG CAT TTC TGC AGC TCT TTC 3′), and the blunt product was cloned into pCR-BluntII-TOPO to generate pNL2. pNL2 was digested with EcoRV, and the *topA*-containing fragment was ligated into SmaI-digested pVSV105 (22) to generate pNL4. *lonA* was PCR amplified using primers pr_NL1 (5′ GGC CGT TTA CCT GTA ACA ACA ACG GAC 3′) and pr_NL2 (5′ GAG TGA CAA GTC ATT TCG ACT TGT CAG CCC 3′), and the blunt product was cloned into pCR-BluntII-TOPO to generate pNL3. pNL3 was digested with XbaI, and the *lonA*-containing fragment was ligated into SpeI-digested pVSV105 (22) to generate pNL18. *hns* was PCR amplified using primers pr_NL11 (5′ GCC AAA CCC AGA GCT ATA AGC GGG GGC 3′) and pr_NL12 (5′ TTT CGA GCA ATA ATA CGT TTC TAA ATG TAA TAA AAT GAA A 3′). *pstA* was PCR amplified using primers pr_NL13 (5′ GCG CTA GTT GTT GGC ATT GCA ATG GGA GCT GC 3′) and pr_NL14 (5′ AGA CAG GTG GTT AAC ATC CAT CGG TGA TAG 3′). *phoQ* was PCR amplified using primers pr_NL84 (5′ GCC TAA CGG TAC TAA AAA GCA TTC TGT ATG 3′) and pr_NL85 (5′ GAT GAA GAG CAT GAT TAT TAT TCT GAT GGA GAG ATA TTG G 3′). The blunt *hns*-, *pstA*-, and *phoQ*-containing products were cloned into SmaI-digested pVSV105 (22) to generate pNL6, pNL75, and pNL72, respectively.

The *lacI*^{q}-$P_{A1/34}$-*luxC* allele on pJLB101 was moved into the transposon mutants as described previously (11). Allelic exchange was confirmed by PCR and by IPTG inducibility of luminescence in the resulting strains. The *lacI*^{q}-$P_{A1/34}$-*luxC* allele from plasmid pJLB101 was crossed into the genomes of mutants EMH3, EMH5, EMH6, EMH7, EMH9, EMH12, EMH13, SLV4, SLV5, SLV10, SLV15, SLV16, SLV20, SLV29, SLV30, SLV32, SLV33, SLV41, SLV42, SLV43, NL1, NL3, NL4, NL6, and NL8 to generate strains NL18, NL19, NL20, NL21, NL22, NL23, NL24, NL25, NL26, NL27, NL28, NL29, NL30, NL31, NL32, NL33, NL34, NL35, NL36, NL37, NL38, NL39, NL40, NL41, and NL42, respectively. Similarly, the Δ*litR*::*kanR* allele on plasmid pMF7 (26) was crossed into the genome of NL2 by using two-step allelic exchange to generate the mutant NL11.

To generate a Δ*ainR* allele on plasmid pNL30, the 1,350-bp region upstream of *ainR* was PCR amplified using primers pr_NL27 (5′ GTA CTC ATA ACA CCA CTA CCT ATT TTT ACT ATA CTG 3′) and pr_NL28.3 (5′ GGG CCT AGG CAT TTA TAT AAA ACT CAC TGA TTT CGA AGT TT 3′), and the product was cloned into pCR-BluntII-TOPO plasmid to generate pNL28. The 1,480-bp region downstream of ES114 *ainR* was PCR amplified using primers pr_NL29 (5′ GGG GCC TAG GTA ACA CCG ATA AAA AAA TAG CCA GAA C 3′) and pr_NL30 (5′ CCC CAC TAG TCA TGA CTC TGT TGC GGG TCT TGA TGA AGC T 3′). AvrII and SpeI sites incorporated into the PCR product on primers pr_NL29 and pr_NL30 were digested with these enzymes, and this fragment was ligated into AvrII-digested pEVS118 (21) to generate pNL29. Plasmids pNL28 and pNL29 were linearized with AvrII and ligated together to generate pNL30, which contains upstream and downstream sequences fused at the Δ*ainR* allele, with the *ainR* start and stop codons separated only by the 6-bp AvrII recognition sequence. The mutant strain NL43 (Δ*ainR*) was generated by crossing the mutant Δ*ainR* allele from plasmid pNL30 into ES114. Replacement of *ainR* with the Δ*ainR* allele in NL43 was confirmed by PCR using primers pr_NL35 (5′ GAG TCC GTT AGC AAG GTC ACA CTT TGT TG 3′) and pr_NL36 (5′ ACC CAA AAC GTA AGA CCA TTG GTA TGC G 3′).

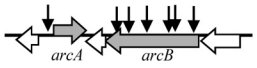
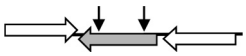
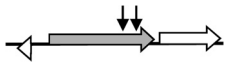
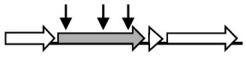
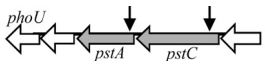
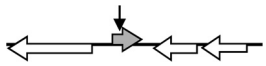
The Δ*ainSR* allele was generated on plasmid pNL32 by PCR amplification of the 1,430-bp region upstream of *ainS* using primers pr_NL62 (5′ GGC GCT TTA CCG TTT GGT GAA AAC TTA CTT C 3′) and pr_NL63 (5′ GGG CCT AGG CTA CTC TTT TAT AAA TTC ATA TTG CAG GTT TT 3′). This product was cloned into pCR-BluntII-TOPO plasmid to generate pNL31. Plasmids pNL29 and pNL31 were linearized with AvrII and ligated together to generate pNL32, which contains the upstream and downstream sequences fused at the Δ*ainSR* allele, with the *ainS* start and *ainR* stop codons separated by the 6-bp AvrII recognition sequence. Crossing the mutant Δ*ainSR* allele from plasmid pNL32 into ES114 generated the mutant strain NL55 (Δ*ainSR*). Replacement of *ainSR* with the Δ*ainSR* allele was confirmed by PCR using primers DMC2 (5′ GGC GGT ACC AGA ACC AAG ACC TGC TCG TGC TAA 3′) and pr_NL36.

Luminescence and fluorescence measurements in culture. Overnight *V. fischeri* cultures were diluted 1:1,000 in 50 ml of LBS or SWTO in 250-ml flasks and then incubated with shaking (200 rpm) at 24°C. At regular intervals, 500-μl samples were removed and the optical density at 595 nm (OD_{595}) was measured with a BioPhotometer (Brinkman Instruments, Westbury, NY). Relative luminescence was measured with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) immediately following shaking to aerate the sample. Specific luminescence was calculated as the luminescence per OD_{595} unit. Luminescence images of strains patched on plates and incubated overnight at ~24°C were obtained with a Bio-Rad Fluor-S Max2 imager. Fluorescence of green fluorescent protein (GFP) expressed from reporter plasmids pJLB36 and pJBL38 (10) was measured with a TD-700 fluorometer (Turner Designs) with excitation and emission filters of 486 nm and >510 nm, respectively. Fluorescence values for strains carrying the promoterless vector pVSV33 (22) were subtracted as background. Unless otherwise indicated, the reported mean fluorescence for cultures was between OD_{595} values of 2.0 and 2.8, a range in which ES114-specific luminescence is approximately constant.

RESULTS

Isolation of luminescence-up mutants of *V. fischeri* ES114. We screened ~20,000 mini-Tn*5* mutants of ES114 for increased luminescence and isolated 30 candidate luminescence-up clones that displayed consistent and significant increases in luminescence. The chromosomal location of the transposon insertions in these strains and their luminescence phenotypes are summarized in Table 1. Three of these mutants are likely siblings, because they have insertions in the same location in *hns* and originated from the same mating. Thus, our screen yielded 28 independent luminescence-up mutants of ES114 distributed over several loci (Table 1).

TABLE 1. Analysis of luminescence-up transposon mutants[a]

Insertion diagram	Strain	Disrupted gene (ORF)	Location of insertion (after indicated bp)	Image on solid medium		Fold brighter than ES114 in broth	
				LBS	SWTO	LBS	SWTO
	ES114	None		NV		1.00	1.00
	SLV41	*arcA* (VF2120)	−35			5.43 ± 0.92	1,190 ± 713
	SLV19	*arcB* (VF2122)	709			0.71 ± 0.09	1,570 ± 385
	NL5		1283			2.28 ± 0.22	807 ± 463
	NL3		1290			2.24 ± 0.42	870 ± 562
	SLV36		1725			3.39 ± 1.13	934 ± 758
	NL6		1903			2.33 ± 0.24	883 ± 604
	SLV33		1993			3.12 ± 0.88	994 ± 578
	SLV4	*acnB* (VF2158)	318	VAR	VAR	1.45 ± 0.36	427 ± 300
	SLV5		2362	VAR	VAR	3.31 ± 1.11	844 ± 321
	EMH12	*topA* (VF1051)	2139			35.8 ± 11.9	37.8 ± 9.56
	EMH13		2345			29.9 ± 9.15	48.0 ± 13.0
	SLV39	*lonA* (VF0798)	218			1.30 ± 0.07	18.8 ± 4.74
	SLV32		1469			2.12 ± 0.03	42.3 ± 9.44
	EMH6		2177			1.66 ± 0.34	29.1 ± 11.1
	SLV10	*pstA* (VF1984)	49			21.7 ± 5.69	6.72 ± 1.65
	SLV30	*pstC* (VF1985)	59			32.2 ± 10.1	9.23 ± 1.93
	SLV15	*hns* (VF1631)	456			10.9 ± 0.86	2.93 ± 1.03
	SLV20		456			17.3 ± 2.80	2.51 ± 0.51
	SLV29		456			13.0 ± 1.87	2.67 ± 0.82
	EMH7	$tRNA^{Met}$ (VFIRNA222)	24			1.71 ± 0.44	6.19 ± 1.11
	NL4	$tRNA^{Thr}$ (VFIRNA003)	52			1.12 ± 0.18	6.34 ± 1.00
	NL1	*tfoY* (VF1573)	2			0.93 ± 0.16	4.14 ± 1.38
	EMH9		517			1.08 ± 0.07	4.17 ± 1.33
	SLV43	*phoQ* (VF1397)	576			2.36 ± 0.45	0.41 ± 0.14
	SLV16		656			2.77 ± 0.49	0.73 ± 0.16
	EMH3		974			2.37 ± 0.49	0.64 ± 0.16
	EMH5	*guaB* (VF0637)	677	†		0.60 ± 0.29	2.28 ± 0.68
	SLV42	*ainS* (VF1037)	477			0.13 ± 0	0.12 ± 0.07
	NL8		705			0.14 ± 0.02	0.43 ± 0.39
	NL2		855			0.20 ± 0.06	0.28 ± 0.24

[a] Horizontal arrows indicate disrupted genes (shaded) and flanking (open) open reading frames (ORFs). Vertical arrows correspond to locations of transposon insertions, and the insertion location is provided in bp relative to the A of the ATG start codon. All mutants were patched onto LBS and SWTO plates, and representative negative images of patches are shown, such that darker patches indicate greater bioluminescence. †, the contrast was increased to visualize the luminescence phenotype of mutant patches. NV, patches were not visible even with enhanced contrast. VAR, variable result as discussed in the text. All mutants were also grown in LBS and SWTO broth, and the average maximum luminescence/OD_{595} relative to ES114 ± standard error ($n \geq 2$) is reported.

The mutants displayed a wide range of luminescence phenotypes that were from 2-fold to more than 1,000-fold brighter than their wild-type parent (Table 1). When grown in broth, all of the mutants still displayed cell-density-dependent regulation of luminescence (data not shown); however, with the exception of *ainS* mutants, they achieved brighter luminescence than the wild type in broth and some induce luminescence at lower cell densities (data not shown). Most mutants' luminescence phenotypes varied depending on whether the cells were grown on plates or in broth and whether they were grown in LBS or SWTO. Both LBS and SWTO contain tryptone and yeast extract; however, LBS is buffered and supplemented with NaCl, whereas SWTO is unbuffered, supplemented with marine salts and glycerol, and is near marine osmolarity (71). Mutants with insertions in *arcA*, *arcB*, *acnB*, *lonA*, *tfoY*, *guaB*, and specific tRNA genes showed greater increases in luminescence relative to ES114 when grown in SWTO than in LBS (Table 1). In contrast, mutants with insertions in *pstA*, *pstC*, *hns*, and *phoQ* were comparatively brighter than ES114 when cultured in LBS (Table 1). Only the *topA* mutants showed similar luminescence-up phenotypes in both LBS and SWTO (Table 1). We found an unexpected result, discussed below, in that *ainS* mutants were much brighter than ES114 on plates (Table 1), despite being dimmer than ES114 in broth (Table 1), as was previously shown (47).

Comprehensiveness of the screen. Based on previous work with mini-Tn*5* derivatives (1, 39), we estimated that the ~20,000 mutants screened should be approaching saturation of nonessential genes. Consistent with this prediction, multiple independent mutants were isolated at most of the loci identified, including all of the loci where dramatic and easily detected luminescence-up effects were observed (Table 1). On the other hand, some mutants of *V. fischeri* previously identified as having luminescence-up phenotypes in broth were not found in our study. These strains were patched onto LBS to test whether they would have been detected in our screen. Strains CL42 (47) and KV2801 (79), containing mutations within *luxO* and *ptsI* (E1), respectively, were dim on plates and would not have been identified in our screen (data not shown). A *crr* mutant (KV2850) yielded patches that were only marginally brighter on plates than ES114 (data not shown), and the phenotype was inconsistent; therefore, it is not surprising that a *crr* mutant was not isolated in our screen. A *phoU* mutant (KV2655) was brighter than ES114 on plates (data not shown), and this was missed in our screen; however, insertions were identified in *pstA* and *pstC*, which are upstream of *phoU* in what appears to be a phosphate transport operon. Taken together, these results suggest that continuing to screen additional transposon mutants under these conditions would yield relatively few additional new insights, although screening under different growth conditions might reveal novel mutants missed here.

Complementation of mutants. To confirm that the luminescence-up phenotypes resulted from the disruption of the genes identified, we genetically complemented the *arcA*, *arcB*, *acnB*, *topA*, *lonA*, *pstA*, *phoQ*, and *hns* mutants by providing the respective native genes in *trans* on low-copy-number shuttle plasmids (Fig. 2). In all strains except the *pstA* and *phoQ* mutants, wild-type luminescence was restored following reintroduction of the native gene. The *pstA* mutant maintained its

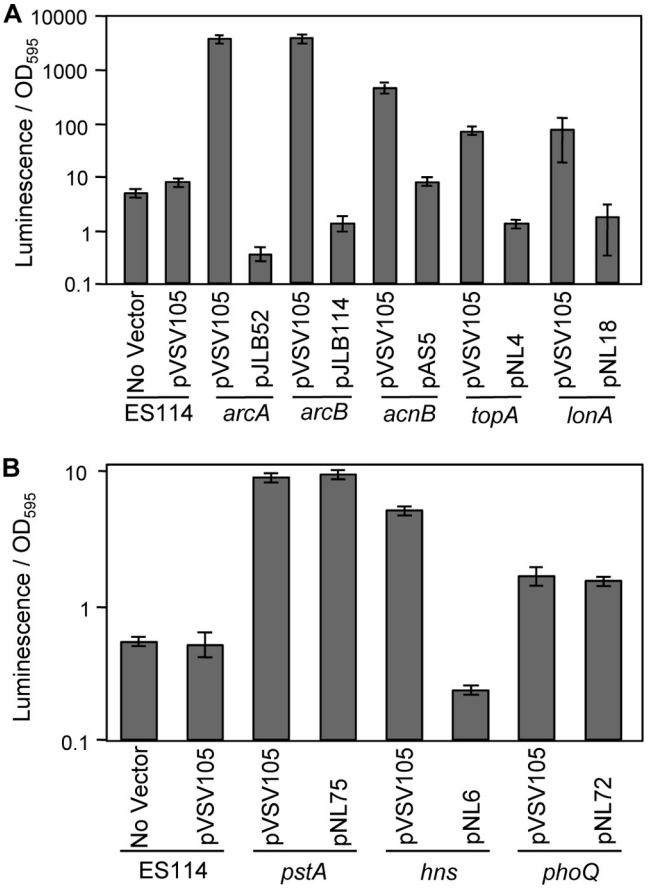


FIG. 2. Genetic complementation of transposon mutants. Luminescence-up mutants were complemented with the respective complete gene carried on shuttle vector pVSV105. ES114 and mutants carrying the empty pVSV105 vector were included as controls. (A) Complementation of mutants with increased luminescence in SWTO. (B) Complementation of mutants with increased luminescence in LBS. Data for one representative mutant from each locus are shown, including SLV41 (*arcA*), NL3 (*arcB*), SLV4 (*acnB*), EMH12 (*topA*), SLV32 (*lonA*), SLV10 (*pstA*), SLV15 (*hns*), and SLV16 (*phoQ*). Error bars represent standard errors ($n = 2$).

luminescence-up phenotype (Fig. 2B), and this was likely due to polar effects on the downstream *phoU*. The relationship between this phosphate uptake system and luminescence is explored further below. Although the luminescence phenotype of the *phoQ* mutant was not complemented in LBS medium (Fig. 2B), it was complemented by *phoQ* in *trans* during growth in minimal medium (see below). There is no apparent cotranscribed gene downstream of *phoQ* (Table 1), making polar effects of this insertion unlikely. We further describe the effects of *phoQ* and Mg^{2+} on luminescence below. Because the *ainS* mutants only displayed a luminescence-up phenotype on plates, we evaluated genetic in *trans* complementation of *ainS* mutants in patches by using pCL112 (47), which restored wild-type-like dim luminescence (data not shown). We did not attempt to complement mutants with insertions in *tfoY*, *guaB*, or tRNA genes, which had relatively modest luminescence-up phenotypes. Given the apparent lack of cotranscribed genes downstream of *tfoY* (Table 1), it seems unlikely that the phenotype of this insertion mutant is caused by polar effects.

Instability of *acnB* mutants. The observable phenotypes of the luminescence-up mutants remained stable with the notable exception of mutants with insertions in *acnB*, which encodes the tricarboxylic acid cycle enzyme aconitase. There is no other aconitase gene apparent in the *V. fischeri* genome, and *acnB* mutants all grew relatively poorly on aerobic streak plates. Faster-growing suppressors arose frequently and had dim luminescence, like that of the wild type. We were able to confirm complementation of an *acnB* mutant in *trans* (described above) by curing the complementing plasmid with concomitant restoration of slow growth and bright luminescence. However, we found further genetic manipulation of these strains difficult due to the rapid appearance of suppressors. We have therefore omitted *acnB* mutants from the genetic analyses below and will describe the influence of *acnB* on luminescence in greater detail once the nature of the suppressors is more fully understood.

Dependence of luminescence-up phenotypes on the native *luxICDABEG* promoter. We hypothesized that the luminescence-up phenotype of the mutants in Table 1 was due to regulatory effects on *lux* gene expression; however, the bright phenotypes of these mutants might alternatively be due to metabolic effects caused, for example, by increasing the availability of the $FMNH_2$ or O_2 substrates for LuxAB. To test this possibility, we placed a nonnative IPTG-inducible promoter construct, *lacI*^{q}-$P_{A1/34}$ between *luxI* and *luxCDABEG* (11), into the chromosomes of the mutant strains, with the exception of *acnB* mutants. Transposon insertions in *arcA*, *arcB*, *lonA*, *pstA*, *pstC*, *hns*, *tfoY*, *phoQ*, *guaB*, *ainS*, and the tRNAs had no effect on luminescence when *luxCDABEG* was expressed from this nonnative promoter, either with or without addition of IPTG (data not shown). Thus, the luminescence-up phenotype associated with most insertions was dependent on the native *lux* promoter and transcript.

Only *topA* mutations yielded an increased level of luminescence in the *lacI*^{q}-$P_{A1/34}$-*luxC* background. *topA* mutations in the *lacI*^{q}-$P_{A1/34}$-*luxC* background led to enhanced luminescence at low ODs (Fig. 3A) but did so to a lesser extent than the ~35- to 40-fold effect seen in the native *lux* promoter background (Table 1 and Fig. 3B). This suggests the *topA* mutants' luminescence-up phenotype may be due to combined effects that are both dependent and independent of the native *lux* promoter.

To test whether the luminescence-up mutations enhanced transcription from the *luxI* or *luxR* promoters, we moved reporter plasmids pJLB36 (P_{luxR}-*gfp*) and pJLB38 (P_{luxI}-*gfp*) into each mutant. Fluorescence data for these reporters were determined by growing the mutant strains in the medium (SWTO or LBS) that showed the greatest luminescence-up phenotype relative to the wild type. Consistent with our previous report (10), the P_{luxR}-*gfp* and P_{luxI}-*gfp* reporters yielded ~2- and ~15-fold greater fluorescence, respectively, in either the *arcA* or *arcB* mutants than in parental strain ES114 (Fig. 4A and B). These reporters also yielded higher fluorescence in *topA* mutants than in ES114, although the relative influence on the P_{luxR}-*gfp* was greater than in the *arcA* or *arcB* backgrounds (Fig. 4A and B). The P_{luxI}-*gfp* reporter yielded ~4- and ~8-fold greater fluorescence in the *pstC* and *hns* backgrounds, respectively, although these mutations had no effect on the P_{luxR}-*gfp* reporter (Fig. 4C and D). None of the other transposon insertions, with the possible exception of those in unstable *acnB* mutants (data not shown), had a significant ($P < 0.05$) effect on either reporter under these conditions. This lack of effect in most mutants may reflect limitations of these reporters. It is worth noting that *arc* and *topA* mutants have much larger effects on luminescence (Table 1) than on *gfp* reporter activity (Fig. 4), and similar results were obtained with *lacZ* reporters (data not shown). These plasmid-borne reporters are maintained at ~9.4 copies per genome (21), potentially titrating out regulators, although at least in the case of ArcA effects of similar magnitude were seen with *lux-gfp* reporters on a plasmid or on the chromosome in single copy (10). Taken together, it is perhaps not surprising that mutations with moderate effects on luminescence have no discernible influence on these reporters.

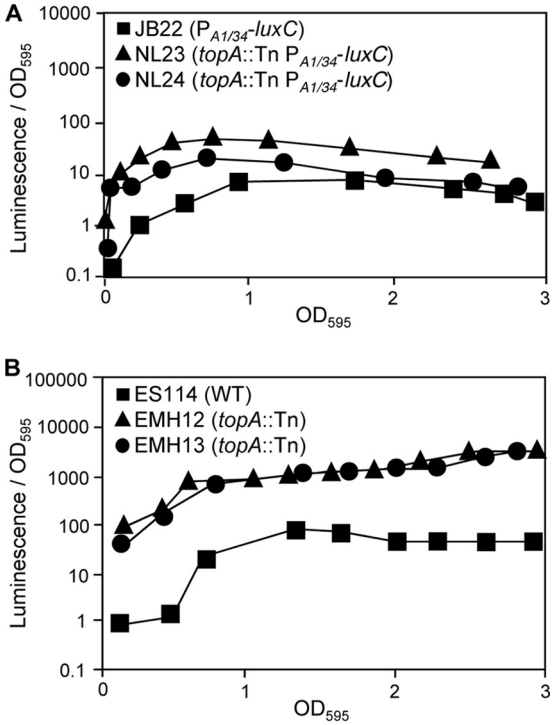


FIG. 3. Luminescence of *topA* mutants in SWTO. (A) Specific luminescence phenotype of JB22 as well as *topA* mutants NL23 and NL24, each of which have the *luxCDABEG* genes controlled by *lacI*^{q} and the $P_{A1/34}$ promoter. JB22 has the $P_{A1/34}$-*luxCDABEG* allele in an otherwise-wild-type (ES114) background. No IPTG was added. (B) Specific luminescence of wild-type (WT) ES114 as well as *topA* mutants EMH12 and EMH13. Averages of two replicate flasks are shown.

Role of *ainS*-mediated signaling in repression of luminescence. The luminescence-up phenotype of *ainS* mutants on plates was unexpected given their previously reported diminished luminescence in broth (47), and we examined these mutants further. The AinS-produced pheromone C8-HSL is thought to act as a signal through two pathways (Fig. 1). In one pathway C8-HSL binds to LuxR and activates transcription, much like the LuxI product 3-oxo-C6-HSL. C8-HSL is a weaker activator of LuxR (67) and can apparently compete with the stronger activator 3-oxo-C6-HSL to dim luminescence (47). In the second pathway, C8-HSL is thought to be sensed by AinR (30), which as illustrated in Fig. 1 ultimately leads to an increase in the regulator LitR (26, 47, 58).

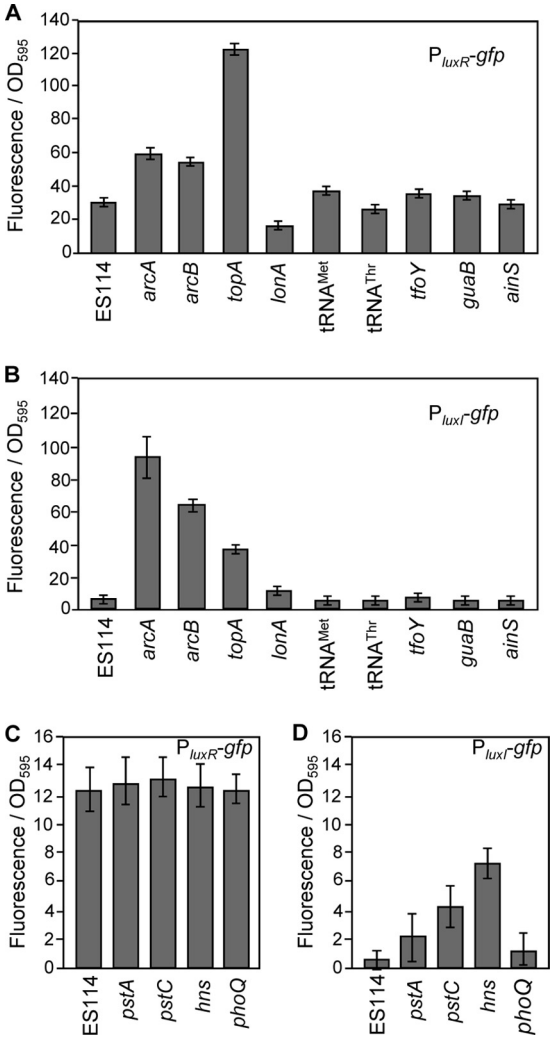


FIG. 4. Effects of transposon mutations on *luxR* and *luxI* promoter-*gfp* reporters. Specific fluorescence generated from reporter plasmids pJLB36 (P_{luxR}-*gfp*) (A and C) or pJLB38 (P_{luxI}-*gfp*) (B and D) harbored in ES114 or in luminescence-up mutants is shown. Reporters were assayed in mutants grown in either SWTO (A and B) or LBS (C and D), depending on which medium yielded the greatest effect on luminescence. Fluorescence from ES114 harboring the promoterless parent vector pVSV33 was subtracted as background. Reporters were tested in each mutant described in Table 2, and data for one representative mutant from each locus are shown, including SLV41 (*arcA*), NL3 (*arcB*), EMH12 (*topA*), SLV32 (*lonA*), SLV10 (*pstA*), SLV30 (*pstC*), SLV15 (*hns*), EMH7 (*tRNA-Met*), NL4 (*tRNA-Thr*), NL1 (*tfoY*), SLV16 (*phoQ*), EMH5 (*guaB*), and NL2 (*ainS*). In all panels, data represent the average specific fluorescence when the culture OD_{595} was between 2.0 and 2.8, a range in which specific luminescence is constant for these strains. Averages and standard errors were calculated from 8 to 12 distinct samples taken from two independent replicate flasks of each examined strain. Mutants with insertions at the same locus were analyzed together in two separate experiments with similar results, and data for one representative mutant from one experiment are shown.

To explore which C8-HSL-responsive pathway is responsible for the luminescence-up phenotype of *ainS* mutants on plates, we used targeted mutants lacking components of these signaling pathways and compared their luminescence in patches on solid medium. Table 2 shows our results, which include the following: (i) the luminescence-up phenotype associated with loss of *ainS* was independent of *ainR*, indicating that this phenotype is not simply due to a lack of C8-HSL-AinR; (ii) the luminescence-up phenotype of the *ainS* mutant was dependent on *luxI*, indicating a key role for 3-oxo-C6-HSL in generating the bright luminescence; (iii) the luminescence-up phenotype of *ainS* mutants was also dependent on both *luxS* and *litR*, and such dependence could be relieved by inactivating *luxO*, even though a *luxO* mutation itself did not result in bright luminescence on plates (data not shown). When taken together and compared with the model of signaling presented in Fig. 1, these data are consistent with the luminescence-up phenotype of *ainS* mutants on plates resulting from the removal of competition for LuxR activation between C8-HSL and the stronger inducer 3-oxo-C6-HSL. Moreover, under these conditions it appears that LuxS and AI-2 are sufficient for the signaling through LuxO necessary to generate LitR, rendering AinS and C8-HSL dispensable in this regard.

Roles of PhoB and phosphate in regulation of luminescence. The luminescence-up phenotypes of the *phoU*, *pstA*, and *pstC* mutants (Table 1 and data not shown) suggested a role for inorganic phosphate (P_i) in the regulation of luminescence. In *E. coli*, the *pst* genes encode a high-affinity P_i transport system that is active under low-P_i conditions (86), and mutations in the *pst* genes result in increased expression of genes associated with the *pho* regulon in a mechanism dependent on the PhoR/PhoB two-component regulatory system (74). At low P_i, the sensor PhoR phosphorylates the response regulator PhoB, which then activates the transcription of specific genes within the *pho* regulon (49, 50). PhoU, which is encoded at the distal end of the *pst* operon, counteracts PhoB when [P_i] is high (59).

To test whether luminescence and *lux* regulation are tied to the *pho* regulon, luminescence was measured for ES114 as well as *pst*, *phoU*, and *phoB* transposon-insertion mutants cultured at relatively high and low P_i concentrations (Fig. 5A). In a medium with reduced P_i levels, the luminescence of ES114 was ~10-fold higher at an OD_{595} of 0.5, and this response to low P_i was absent in a *phoB* mutant (Fig. 5A). Moreover, mutants with insertions in *phoU*, *pstA*, or *pstC* displayed constitutively high levels of luminescence regardless of [P_i]. These data sug-

TABLE 2. Luminescence analysis of mutants involved in the AinS signaling pathways

Strain	Reference	Genotype	Patches on LBS plates[a]
ES114	8	Wild type	−
NL2	This study	ES114 *ainS*::mini-Tn*5-ermR*	+
NL43	This study	ES114 Δ*ainR*	−
NL55	This study	ES114 Δ*ainSR*	+
CL24	47	ES114 *luxS*::*kanR*	−
NL11	This study	ES114 *ainS*::mini-Tn*5-ermR litR*::*kanR*	−
CL39	46	ES114 *luxI-ainS*::*catR*	−
CL41	46	ES114 *ainS*::*catR luxS*::*kanR*	−
CL64	47	ES114 *ainS*::*catR luxO*::*kanR*	+
CL90	46	ES114 *luxS*::*kanR luxO*::*kanR*	−
CL91	46	ES114 *ainS*::*catR luxS*::*kanR luxO*::*kanR*	+

[a] All strains were patched onto LBS plates, and the results of the negative images are reported as follows: +, patches were bright; −, patches were not bright (compared to *ainS* mutant patches [Table 1]).

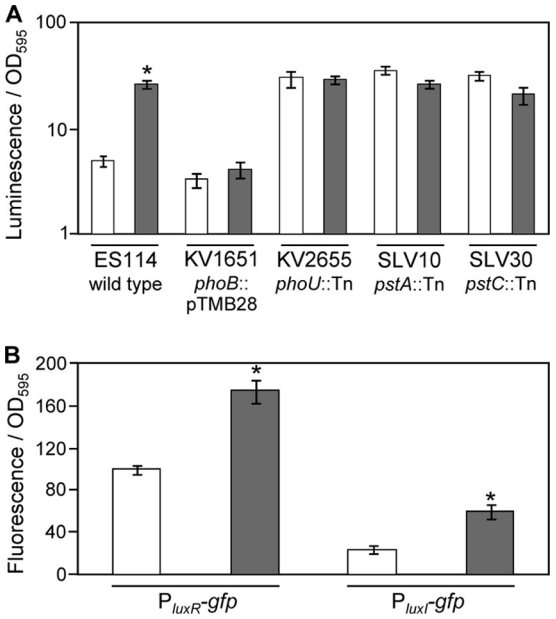


FIG. 5. Effects of P_i concentration on luminescence and *lux* promoter-reporters. (A) Specific luminescence at OD_{595} of 0.5 for strains ES114 (wild type), KV1651 (*phoB*::pTMB28), KV2655 (*phoU*::mini-Tn*5*-*ermR*), SLV10 (*pstA*::mini-Tn*5*-*ermR*), and SLV30 (*pstC*::mini-Tn*5*-*ermR*) grown in FMM containing 378 μM (open bars) or 37.8 μM (gray bars) phosphate. (B) Specific fluorescence from P_{luxR}-*gfp* and P_{luxI}-*gfp* reporters on plasmids pJLB36 and pJLB38, respectively, harbored in ES114 grown in FMM containing 378 μM (open bars) or 37.8 μM (gray bars) phosphate. Fluorescence from ES114 harboring promoterless parent vector pVSV33 was subtracted as background. Data represent average specific fluorescence when culture OD_{595} values were ~0.5. Bars indicate standard errors ($n = 2$). *, significant difference between high and low P_i conditions as determined with Student's *t* test ($P \leq 0.01$).

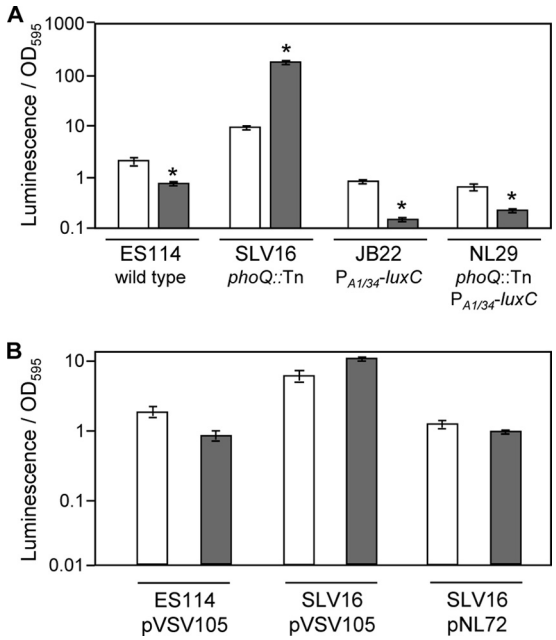


FIG. 6. Effect of Mg^{2+} concentration on luminescence. (A) Specific luminescence of *V. fischeri* strains ES114 (wild type), SLV16 (*phoQ*::mini-Tn*5*-*ermR*), JB22 (ES114 *lacI*^{q}-$P_{A1/34}$-*luxC*), and NL29 (SLV16 *lacI*^{q}-$P_{A1/34}$-*luxC*). (B) Specific luminescence of ES114 and SLV16 carrying the empty pVSV105 vector and SLV16 carrying pNL72, which contains the native *phoQ* allele. Both panels report data from cultures grown in FMM, which contains 55 mM magnesium (open bars), or FMM with low (0.03 mM) Mg^{2+} (gray bars). *, significant difference in luminescence for that strain grown in low-Mg^{2+} medium relative to normal FMM as determined with Student's *t* test ($P \leq 0.05$).

gest that luminescence is controlled in part by the PhoR/PhoB system in response to low P_i.

To further examine the mechanism of P_i-mediated *lux* regulation, ES114 cells containing P_{luxR}-*gfp* and P_{luxI}-*gfp* reporter plasmids were grown in high- and low-P_i media (Fig. 5B). Under conditions with lower P_i levels, the activities of P_{luxR}-*gfp* and P_{luxI}-*gfp* each increased ~2-fold. As a control we tested a constitutive promoter-*gfp* reporter and found no relative effect of high and low P_i (data not shown). Thus, low P_i leads to an increase in activity from the *lux* promoters, which may account for the luminescence-up phenotypes observed in *phoU*, *pstA*, and *pstC* mutant strains.

Effect of PhoQ and Mg^{2+} on luminescence. We also identified luminescence-up mutants with insertions in *phoQ* (Table 1). In other bacteria PhoQ together with the response regulator PhoP act as a two-component regulatory system that responds to low Mg^{2+} (42, 63, 78). Based on a simple model drawn from the luminescence-up phenotype of *phoQ* mutants and the function of PhoQ in other systems, we predicted a PhoQ-dependent repression of luminescence when Mg^{2+} was relatively low.

To further examine the effects of PhoP/PhoQ and Mg^{2+} on luminescence, we measured the luminescence of wild-type ES114, the constitutive *lux*-expressing strain JB22 (11), and their respective *phoQ* mutants, SLV16 and NL29, grown in a defined medium with differing concentrations of Mg^{2+}. Consistent with our prediction, low Mg^{2+} led to decreased luminescence in ES114 but not in the *phoQ* mutant (Fig. 6A). Providing *phoQ* in *trans* restored low luminescence to the *phoQ* mutant (Fig. 6B). However, we also observed an unexpected increase in luminescence at low Mg^{2+} in the *phoQ* mutant (Fig. 6), suggesting a PhoQ-independent mechanism that functions counter to PhoQ to increase luminescence at low Mg^{2+}. Moreover, low Mg^{2+} also decreased luminescence in both strains JB22 and NL29, in which luminescence is expressed from the constitutive nonnative $P_{A1/34}$-*luxC* promoter (Fig. 6). These data indicate that luminescence is influenced by Mg^{2+} through multiple mechanisms that are both PhoQ dependent and PhoQ independent.

Effect of GuaB and guanine on luminescence. GuaB and GuaA are required for the synthesis of GMP (55, 75), and we predicted that the insertion disrupting *guaB* would disrupt guanine synthesis. As predicted, in FMM medium a severe growth defect of the *guaB* mutant was observed that was recovered by adding 0.25 mM guanine (data not shown). Furthermore, luminescence of the *guaB* mutant was restored to dimmer, wild-type levels by adding 0.15 mM guanine to SWTO (data not shown).

Regulation of luminescence in response to aeration is *topA* dependent. When ES114 is poorly aerated, its expression of luminescence is repressed (73); however, no mechanism for this regulation has yet been shown. The isolation of luminescence-up mutants with insertions in *arcA*, *arcB*, *acnB*, and *topA*

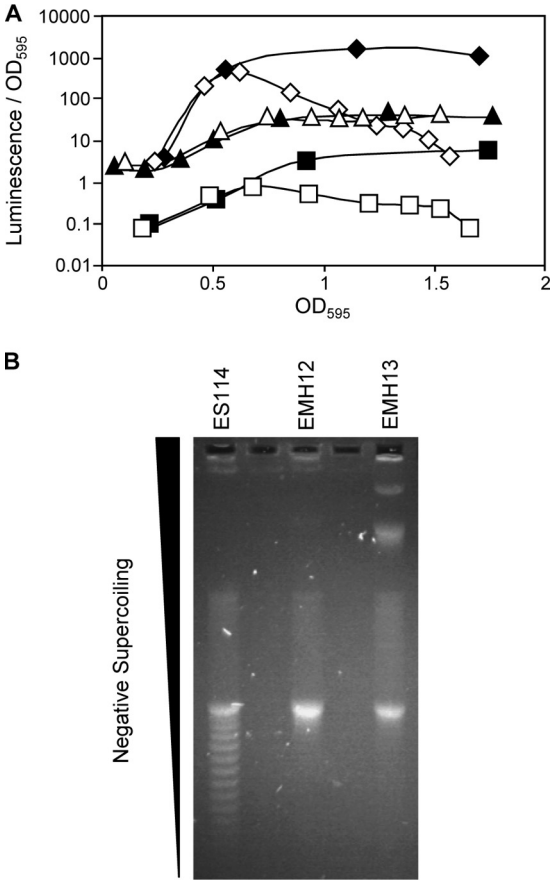


FIG. 7. Effect of *topA* mutations on aeration-dependent regulation of luminescence and supercoiling. (A) Effect of *topA* mutation on aeration-mediated luminescence. Specific luminescence of wild-type ES114 (squares), *topA* mutant EMH12 (triangles), and *arcA* mutant AMJ2 (diamonds) in well-aerated (closed) or poorly aerated (open) cultures. Poor aeration conditions were created by growing cultures in 250-ml flasks containing 200 ml of SWTO, and well-aerated conditions consisted of 50 ml of SWTO in 250-ml flasks. (B) Supercoiling of plasmid DNA isolated from ES114 or *topA* mutants was assayed by electrophoresis through 0.8% agarose containing 20 μg/ml chloroquine. DNA that is less negatively supercoiled prior to loading will migrate more rapidly at this concentration of chloroquine (29). Laddering on the gel is indicative of topoisomers of the same plasmid that differ by one linking number. The gel was washed in distilled H_2O for 2 h, stained in 1 μg/ml ethidium bromide for 1 h, and then imaged.

(Table 1) suggested possible mechanisms for regulation of luminescence in response to culture aeration, because ArcA/ArcB, aconitase, and DNA supercoiling, which is mediated in part by TopA, have been implicated in redox-dependent control of gene expression in *E. coli* (2, 6, 33). Due to the problems with suppressors arising in cultures of *acnB* mutants as mentioned above, we limited our investigation to possible effects of ArcA/ArcB or TopA on aeration-dependent regulation of luminescence.

The redox-responsive ArcA/ArcB system represses luminescence presumably in response to reducing conditions (10). However, we found that ArcA cannot account for the repression of luminescence when cultures are grown in poorly aerated broth. In both the wild type and a Δ*arcA* mutant, the luminescence of cells grown in poorly aerated flasks began to diverge from that of well-aerated cells at an OD_{595} of ~0.7 (Fig. 7A). Thus, although the Δ*arcA* mutant is brighter than ES114 under both conditions, the effect of poor aeration on luminescence is similar in both strains.

We next examined the topoisomerase I (*topA*) mutants. In *E. coli*, DNA is more negatively supercoiled in anaerobically grown cultures, leading to changes in gene expression (17, 37). Similarly, we found that plasmid supercoiling in *V. fischeri* is affected by culture aeration (data not shown). Topoisomerase I removes negative supercoils from DNA (43, 80), and we hypothesized that *topA* mutations might prevent cells from using genomic supercoiling as part of their global response to aeration. We confirmed the supercoiling phenotypes of *topA* mutants, assaying the level of negative supercoiling by isolating plasmid DNA from the wild type and the *topA* mutant strain and examining them in chloroquine gels. As predicted, supercoiling was affected in a *topA* mutant background (Fig. 7B). We also examined the luminescence phenotype of a *topA* mutant grown under different aeration conditions. In contrast to the wild type and *arcA* mutants, the luminescence of the *topA* mutant remained similar in both well-aerated and poorly aerated cultures (Fig. 7A). This suggests that a *topA*-dependent effect on the level of negative supercoiling may regulate luminescence in response to aeration.

DISCUSSION

In many bacteria, achieving a high cell density quorum is necessary but not sufficient to elicit full induction of pheromone-dependent behaviors. Therefore, understanding the biological significance of pheromone-mediated regulation in bacteria will require examining the environmentally responsive regulators that govern these systems. Only by elucidating the environmental contingencies required for induction of pheromone-controlled regulons will we be able to fully appreciate their functions in nature and contributions to bacterial fitness. In *V. fischeri*, environmental conditions clearly play an important role in modulating *lux* expression, and the ~1,000-fold increase in luminescence of *V. fischeri* upon colonizing *E. scolopes* cannot be explained by cell density (8, 9). However, in contrast to a detailed understanding of pheromone-mediated activation of luminescence (Fig. 1), control of the *luxICDABEG* operon by environmentally responsive regulators has been less thoroughly explored.

In this study we initiated a systematic examination of the regulatory mechanisms accounting for the dim luminescence of *V. fischeri* ES114 in culture in order to elucidate the conditions that promote upregulation of luminescence and autoinducer synthesis. Using transposon mutagenesis, we isolated 28 independent luminescence-up mutants with insertions in 14 loci. Through the characterization of these mutants along with subsequent analyses of ES114 in different media, we have shown that luminescence in ES114 is responsive to a broad regulatory web influenced by specific environmental conditions. Given that the autoinducer synthase gene *luxI* is cotranscribed with the genes responsible for luminescence, these data support the idea that the quorum-sensing circuitry is regulated in response to the environment through multiple regulatory systems.

Environment-dependent *lux* regulation. The importance of environmental context in luminescence regulation was under-

scored by simple analyses of the luminescence-up mutants. For example, in all mutants except *topA* strains, the luminescence-up phenotype was medium specific (Table 1). In some instances, these medium-dependent effects could be rationalized, given the genetic analysis of the mutants. For instance, mutants SLV16, SLV43, and EMH3 displayed a larger luminescence-up effect relative to ES114 when grown in LBS medium as opposed to SWTO (Table 1). These mutants have transposon insertions in *phoQ*, encoding the response regulator of the PhoPQ two-component regulatory system, which responds to environmental signals, including low [Mg^{2+}] (42, 63, 78). Given the much lower levels of Mg^{2+} in LBS than in SWTO, the medium-dependent phenotype of these mutants was what one would predict if PhoQ repressed luminescence in response to low Mg^{2+}. This model was tested further and validated by showing a *phoQ*-dependent repressive effect of Mg^{2+} on luminescence (Fig. 6). Although in other systems PhoPQ also responds to [Ca^{2+}] (78), antimicrobial peptides (4), and pH (5), these factors did not show clear PhoQ-dependent effects on luminescence in *V. fischeri* (data not shown). Hussa et al. previously reported multiple homologs of *phoP* in *V. fischeri* (38), and the corresponding regulation may be more complex than in *E. coli*. Interestingly, our data indicate PhoQ-independent effects on luminescence, and taken together Mg^{2+} appears to influence luminescence through multiple mechanisms.

Similarly, analysis of luminescence-up mutants led to the identification of P_i as a key environmental factor governing luminescence. Mutations in *pstA*, *pstC*, or *phoU* led to increased luminescence (Table 1 and Fig. 5), and by analogy to *E. coli*, mutants in this P_i uptake operon (16, 69) would be expected to display the PhoB-dependent P_i starvation response (81). Consistent with this model, luminescence in ES114 is elevated in response to low P_i levels in a PhoB-dependent manner (Fig. 5). Interestingly, the "low Pi" concentrations utilized in this work appear to be much higher than levels found in the Hawaiian coastal waters inhabited by *E. scolopes*. Assays completed to determine the [P_i] (14) of seawater showed phosphate levels of $<$100 nM (data not shown), which is consistent with previous studies (3, 65, 68). However, this does not account for organic phosphate sources, which along with phosphate availability in the host light organ should be considered in future work.

Finally, the identification of luminescence-up mutations in *topA*, *arcA*, *arcB*, and *acnB* hinted at an important role for redox in luminescence regulation, as each of these loci has been connected to redox-dependent regulation in other systems. The degree of aeration influences regulation of *lux* expression in *V. fischeri* (62, 73), and here we show that this is dependent on *topA*, suggesting a role for supercoiling in this regulatory phenomenon (Fig. 7). Ongoing studies are aimed at understanding whether the luminescence-up mutations in *arc* or *acnB* reflect other environmental conditions that influence the cellular redox state.

Future experiments will also be aimed at understanding whether environmental conditions influence regulation mediated by genes such as *ainS*, *lonA*, *hns*, and *tfoY* (Table 1). Preliminary work suggests that at least *ainS* is subject to regulation in response to the environment, and the other genes may similarly have greater or lesser effects on luminescence in a context-dependent manner.

Mechanisms of luminescence regulation. Understanding the mechanism(s) underlying the regulation of luminescence in luminescence-up mutants is important for at least two reasons. First, regulation of the native *luxICDABEG* promoter is likely to affect LuxI synthase expression and 3-oxo-C6-HSL synthesis in addition to affecting luminescence, leading to positive feedback and the potential for cell-cell signaling. Most mutations reported here did exert their effects on luminescence through mechanisms dependent on the native *lux* promoter, and therefore such regulation has the potential for population-wide 3-oxo-C6-HSL-mediated effects. Second, it will be important to ascertain whether regulators act directly on the *lux* promoter or indirectly, for example, by modulating one of the direct regulators. This distinction will be important for building robust predictive models of luminescence regulation. For example, interdependence of different regulators would imply that multiple environmental conditions must be sensed simultaneously to elicit a regulatory response.

Little is yet known about the specific mechanisms by which the regulators identified here control *lux* operon expression. It is thought that Lon targets LuxR (51), and we have previously shown that ArcA binds directly to the *lux* promoter (10), but mechanisms for the other regulators are less clear and await testing. For example, although *luxR* promoter activity is increased under low-P_i conditions, a search of the *lux* intergenic region did not reveal a clear *pho* box typical of a PhoB binding site, suggesting that it may be regulating luminescence indirectly via an unknown mechanism.

For some mutants, the underlying regulatory mechanism is perplexing. Notably, transposon insertions in $tRNA^{Met}$ and $tRNA^{Thr}$ produced mutants with an ~6-fold increase in luminescence during growth in SWTO; however, the significance of this finding is unclear. These interrupted tRNAs and those downstream from them are not unique in the *V. fischeri* genome. There are several loci for both $tRNA^{Met}$ and $tRNA^{Thr}$ annotated within the genome with at least one additional locus having 100% identity to the mutated genes. Thus, although we cannot rule out a model of regulation by rare tRNAs, such a mechanism is not immediately apparent.

Other implications for pheromone signaling research. Our discovery of regulators that respond to environmental stimuli and control *lux* expression has important implications. For example, autoinducer-mediated regulation in *V. fischeri* has often been used as a model system for mathematically describing a genetic regulatory circuit; however, the effects of environmental regulation on *lux* expression have been largely overlooked in these studies. Rather, many mathematical models of *lux* regulatory circuitry either omit environmental regulation entirely (40, 45, 53, 60, 61, 66, 82, 85) or consider only CRP-mediated regulation in response to glucose (7, 15). In the future, components of the environmentally responsive regulators connected to *lux* should be incorporated into the models of this regulatory circuit.

This work will also direct research aimed at understanding the environment experienced by *V. fischeri* inside its host, *E. scolopes.* With a better appreciation of the conditions and environmentally responsive regulators that lead to dim luminescence in culture, we can now develop clear hypotheses

regarding the environmental cues that lead to *lux* induction during colonization. For example, the studies described above have prompted greater interest in examining redox, Mg^{2+}, and P_i levels in the light organ. Our ability to reconstitute and observe this symbiosis offers a unique opportunity to assess the dynamics and regulation of pheromone-mediated signaling in a context that is ecologically relevant for the bacterium.

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