

# Cyclic AMP Receptor Protein Regulates Pheromone-Mediated Bioluminescence at Multiple Levels in *Vibrio fischeri* ES114

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Bioluminescence in *Vibrio fischeri* ES114 is activated by autoinducer pheromones, and this regulation serves as a model for bacterial cell-cell signaling. As in other bacteria, pheromone concentration increases with cell density; however, pheromone synthesis and perception are also modulated in response to environmental stimuli. Previous studies suggested that expression of the pheromone-dependent bioluminescence activator LuxR is regulated in response to glucose by cyclic AMP (cAMP) receptor protein (CRP) (P. V. Dunlap and E. P. Greenberg, *J. Bacteriol.* 164:45–50, 1985; P. V. Dunlap and E. P. Greenberg, *J. Bacteriol.* 170:4040–4046, 1988; P. V. Dunlap, *J. Bacteriol.* 171:1199–1202, 1989; and W. F. Friedrich and E. P. Greenberg, *Arch. Microbiol.* 134:87–91, 1983). Consistent with this model, we found that bioluminescence in *V. fischeri* ES114 is modulated by glucose and stimulated by cAMP. In addition, a  $\Delta crp$  mutant was  $\sim 100$ -fold dimmer than ES114 and did not increase luminescence in response to added cAMP, even though cells lacking *crp* were still metabolically capable of producing luminescence. We further discovered that CRP regulates not only *luxR* but also the alternative pheromone synthase gene *ainS*. We found that His-tagged *V. fischeri* CRP could bind sequences upstream of both *luxR* and *ainS*, supporting bioinformatic predictions of direct regulation at both promoters. Luminescence increased in response to cAMP if either the *ainS* or *luxR* system was under native regulation, suggesting cAMP-CRP significantly increases luminescence through both systems. Finally, using transcriptional reporters in transgenic *Escherichia coli*, we elucidated two additional regulatory connections. First, LuxR-independent basal transcription of the *luxI* promoter was enhanced by CRP. Second, the effect of CRP on the *ainS* promoter depended on whether the *V. fischeri* regulatory gene *litR* was also introduced. These results suggest an integral role for CRP in pheromone signaling that goes beyond sensing cell density.

The observation that pheromones govern bioluminescence in *Vibrio fischeri* was a fundamental discovery that shed light on bacterial cell-cell communication. It is now appreciated that diverse bacteria similarly utilize pheromones to regulate numerous processes (1); however, the study of bioluminescence and its regulation in *V. fischeri* still serves as a powerful model, enabling researchers to explore the mechanisms by which pheromone signals and their regulatory circuits are used by bacteria. In particular, strain ES114 has become a widely used experimental model for *V. fischeri*, because this isolate's bioluminescent symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes*, can be reconstituted in the laboratory.

The genes primarily responsible for bioluminescence in *V. fischeri* are separated into two divergent transcripts composed of *luxR* and *luxICDABEG* (2–4). LuxI and LuxR underpin pheromone-mediated regulation of bioluminescence. LuxI encodes a pheromone synthase that generates *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL), which is also referred to as an autoinducer (5). As the bacterial population density increases, the membrane-permeable 3-oxo-C6-HSL accumulates, and at a threshold concentration it binds to the transcriptional regulator LuxR (2, 6, 7). This LuxR-HSL complex binds the *lux* box located within the intergenic region between *luxR* and *luxI* to activate transcription of *luxICDABEG*.

Two additional autoinducer pheromones, *N*-octanoyl-HSL (C8-HSL), generated by *AinS*, and AI-2, generated by LuxS, also regulate bioluminescence (8, 9). C8-HSL and AI-2 are thought to function through distinct receptors that both act via LuxU, LuxO, Hfq, and the small RNA Qrr to increase levels of LitR, which then activates *luxR* expression (10–13). C8-HSL can also activate LuxR directly, although it is a weaker activator than 3-oxo-C6-HSL (11).

Although pheromone-mediated regulation such as that controlling bioluminescence is frequently framed in terms of cell density-dependent quorum sensing, environmentally responsive regulators often govern these systems. As a result, a high cell density may be necessary but not sufficient to achieve stimulatory pheromone levels. For example, symbiotic *V. fischeri* ES114 cells are  $\sim 1,000$ -fold brighter and produce more 3-oxo-C6-HSL than cultured cells grown to similar density (14, 15), indicating that environmental conditions are important in *luxICDABEG* expression in the squid. Several studies have suggested a role for the environmentally responsive cyclic AMP (cAMP) receptor protein (CRP) in regulating *V. fischeri* bioluminescence (16–18). CRP was originally identified in *Escherichia coli* for its role in catabolite repression (19, 20) and has since been shown to activate transcription from numerous promoters (21–23). In *E. coli*, when glucose concentrations are low, adenylate cyclase (Cya) activity and intracellular cAMP levels are relatively high, and this effector molecule associates with CRP (24). Together the cAMP-CRP complex binds target DNA and promotes transcription of many genes, particularly those involved in growth on substrates other than glucose (21, 24, 25).

Received 27 June 2013 Accepted 28 August 2013

Published ahead of print 30 August 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00751-13>.

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doi:10.1128/JB.00751-13

Although previous research supports a link between catabolite repression and pheromone-mediated regulation of luminescence, the exact relationship and mechanism have remained unclear, particularly in *V. fischeri* ES114. In transgenic *E. coli* cells carrying the *lux* operon from strain MJ-1, CRP and cAMP stimulate the induction of luminescence, while the addition of glucose causes a 1- to 2-h delay in the onset of luciferase activity (16). Analyses of transcriptional regulators further suggested that the cAMP-CRP complex activates transcription of *luxR* (16, 17), and CRP from *E. coli* showed binding to DNA upstream of *luxR* (26). Studies of *V. fischeri* have suggested strain-specific differences. Luminescence in strain MJ-1 is decreased in the presence of glucose (27); however, it was reported that in strain ES114 glucose had no effect (14). In both strains the addition of cAMP increased light production (14, 27). Furthermore, undefined *cya*- and *crp*-like mutants of MJ-1 produce low levels of luminescence, and in the *cya*-like mutant luminescence was restored by supplying cAMP exogenously (18).

In this study, we exploited genomic and genetic advances in understanding *V. fischeri* ES114 to examine the underpinnings of glucose- or cAMP-mediated effects on luminescence and to explore the regulatory role of CRP. Our results suggest a multilayered integration of carbon metabolism and pheromone-mediated regulation.

## MATERIALS AND METHODS

**Bacteria and media.** Bacterial strains used in this study are listed in Table 1. *V. fischeri* ES114 was the wild-type strain used throughout (14). Plasmids were transformed into *E. coli* strain DH5 $\alpha$  (28) or DH5 $\alpha$  *lambda*pir (29). *E. coli* was grown in LB medium (30) or brain heart infusion medium (BHI), and *V. fischeri* was grown in LBS medium (31) or SWTO medium (32). Solid media were prepared with 15 mg ml<sup>-1</sup> agar for plating. For selection of *E. coli*, ampicillin (Amp), chloramphenicol (Cam), kanamycin (Kan), and tetracycline (Tet) were added to LB at final concentrations of 100, 20, 40, and 12  $\mu$ g ml<sup>-1</sup>, respectively, and 150  $\mu$ g ml<sup>-1</sup> 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  $\mu$ g ml<sup>-1</sup>, respectively. Where specified, glucose, cyclic AMP (cAMP), and/or C8-HSL were added to cultures at final concentrations of 20 mM, 10 mM, and 250 nM, respectively.

**Molecular genetic techniques and analyses.** Plasmids (Table 1) were constructed using standard molecular techniques. DNA ligase and restriction enzymes were obtained from New England BioLabs (Beverly, MA). Oligonucleotides used for PCR and cloning (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA). PCR products were generated using KOD HiFi DNA polymerase (Novagen, Madison, WI) or Phusion high-fidelity DNA polymerase (New England BioLabs, Beverly, MA) with an iCycler (Bio-Rad Laboratories, Hercules, CA). Plasmids used in cloning were purified with the GenElute plasmid miniprep kit (Sigma-Aldrich, Inc., St. Louis, MO), and DNA from PCR, digestion, and ligation reactions was purified with the DNA clean and concentrator-5 kit (Zymo Research, Orange, CA). When specified, PCR products were cloned into the pCR-BluntII-TOPO vector using a ZeroBlunt-TOPO PCR cloning kit (Invitrogen, Carlsbad, CA), and white colonies were identified on plates containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside at a final concentration of 60  $\mu$ g ml<sup>-1</sup>. All cloned PCR products were sequenced at the University of Michigan DNA Sequencing Core Facility, and sequences were analyzed using Sequencher 4.1.2 (Gene Codes Corp., Ann Arbor, MI).

To construct a constitutively expressed *gfp* gene as a control for promoter-*gfp* reporters, oligonucleotides JBPromoter1 and JBPromoter2 were designed to anneal together, forming an *E. coli* consensus promoter and single-stranded overhangs compatible with SpeI-digested DNA. The

oligonucleotides were combined in a 1:1 ratio, heated to 100°C, and then annealed by allowing the mix to cool slowly at room temperature. This fragment was then ligated into SpeI-digested pVSV33 (33) to generate pMTV1. To construct the P<sub>*ainS*</sub>-*gfp* reporter, the 427-bp region upstream of *ainS* was PCR amplified using primers DMC2 and pr\_NL58. The PCR product was digested with KpnI and StuI and ligated into KpnI- and StuI-digested pVSV33 (33) to generate pMTV2. Plasmids pNL56 and pNL69 (Table 1) were generated for interspecies complementation of *crp* mutants between *E. coli* and *V. fischeri*. To generate pNL56, *E. coli crp* from SallI- and PvuII-digested pDCRP (34) was ligated into SallI- and PvuII-digested pVSV105 (33). To generate pNL69, *V. fischeri* ES114 *crp* from ClaI- and XhoI-digested pNL15 was ligated into ClaI- and SallI-digested pDU9 (35).

**Mutant strain construction.** Plasmids carrying mutant alleles were transferred from *E. coli* into *V. fischeri* by triparental matings using conjugative helper strain CC118 $\lambda$ pir pEV5104 (36). Recombinational insertion and marker exchange were identified by screening for antibiotic resistance, and putative mutants were tested by PCR. To generate a  $\Delta$ *ainS* allele, the 1,833-bp region downstream of ES114 *ainS* was PCR amplified using primers pr\_NL78.2 and pr\_NL79. This product was cloned into SmaI-digested pEV5122 (29) to generate pNL53. Plasmid pNL31, which contains the region upstream of *ainS* (37), and pNL53 were linearized with AvrII and ligated together to generate pNL62, which contains the upstream and downstream sequences fused at the  $\Delta$ *ainS* allele, with the *ainS* start and stop codons separated by the 6-bp AvrII recognition sequence. The  $\Delta$ *ainS* allele from pNL62 was crossed into ES114 and CL53 (9) to generate strains NL60 and NL62, respectively, and the deletion of *ainS* was confirmed by PCR. To generate DC1, the  $\Delta$ *crp* allele on pJLB117 (32) was crossed into the genome of JB22 (38) and allelic exchange was confirmed by PCR and by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) inducibility of luminescence in the resulting strain.

**Luminescence and fluorescence measurements.** Overnight cultures of *V. fischeri* were diluted 1:1,000 in 20 or 50 ml of SWTO in 125- or 250-ml flasks, respectively, and then incubated with shaking (200 rpm) at 24°C. At regular intervals, 500- $\mu$ l samples were removed to measure optical density at 595 nm (OD<sub>595</sub>) with a BioPhotometer (Brinkman Instruments, Westbury, NY). Relative luminescence was measured with a Glo-max TD-20/20 luminometer (Promega, Madison, WI) immediately following shaking to aerate the sample (38). Specific luminescence was calculated as the luminescence per OD<sub>595</sub> unit. Fluorescence expressed from reporter plasmids carrying *gfp* was measured with a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using excitation and emission filters of 486 and >510 nm, respectively. Fluorescence of strains carrying the promoterless vector pVSV33 (33) was subtracted as the background. Specific fluorescence was calculated as the fluorescence per unit of OD<sub>595</sub> from cells growing in SWTO supplemented with 20 mM glucose, and the mean specific fluorescence is reported for cultures at an OD<sub>595</sub> of ~2.0.

**Quantitative PCR and transcript analysis.** Overnight cultures of *V. fischeri* ES114 or JB24 were diluted 1:1,000 in 20 ml of SWTO in 125-ml flasks and then incubated with shaking (200 rpm) at 24°C. At an OD<sub>595</sub> of ~0.5, 5-ml samples were removed and a 1/5 volume 5% acidic phenol and 95% ethanol (vol/vol) was added. Samples were incubated on ice for 30 min, and RNA was isolated using the Stratagene absolutely RNA miniprep kit (Agilent Technologies, La Jolla, CA). Isolated RNA was further treated with Ambion Turbo DNase (Applied Biosystems, Foster City, CA). cDNA was generated from 1  $\mu$ g of DNase-treated RNA using a Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). Reverse transcription-PCRs (RT-PCRs) were completed with iQ SYBR green Supermix (Bio-Rad, Hercules, CA), and 10 ng cDNA was analyzed using the MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA). The *luxR* cDNA was amplified using primers pr\_NL54 and pr\_NL55, and *ainS* cDNA was amplified using primers pr\_NL108 and pr\_NL109. The *lpxA* cDNA was amplified using primers pr\_NL94 and pr\_NL95. All RNA and cDNA concentrations were determined with a Synergy 2 plate reader and Gen5 software (BioTek, Winooski, VT).

TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence <sup>a</sup>	Source or reference
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80 <i>dlacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	28
DH5 $\alpha$ <i>lpir</i>	DH5 $\alpha$ lysogenized with <i>lpir</i>	29
M182	$\Delta$ ( <i>lacIPOZY</i> )X74 <i>galK galU rpsL150 (strA) mel</i>	43
M182 $\Delta$ <i>crp</i>	M182 $\Delta$ <i>crp</i>	42
UQ3811	K12 $\Delta$ ( <i>lacIPOZY</i> ), <i>crp::camR</i> , <i>ilv::Tn10 cya</i>	40
<i>V. fischeri</i>		
ES114	Wild-type isolate from <i>E. scolopes</i>	14
CL53	ES114 $\Delta$ <i>luxR::ermR</i>	9
DC1	ES114 $\Delta$ <i>crp lacI<sup>q</sup> P<sub>A1/34</sub>-luxCDABEG</i>	This study
DC22	C8-HSL bioreporter; ES114 $\Delta$ <i>ainS</i> $\Delta$ <i>luxR-luxI</i> , mutant <i>luxR</i> (MJ1-T33A, R67M, S116A, M135I), <i>P<sub>luxI</sub>-luxCDABEG</i>	This study
JB22	ES114 <i>lacI<sup>q</sup> P<sub>A1/34</sub>-luxCDABEG</i>	38
JB24	ES114 $\Delta$ <i>crp</i>	32
NL60	ES114 $\Delta$ <i>ainS</i>	This study
NL62	ES114 $\Delta$ <i>luxR::ermR</i> , $\Delta$ <i>ainS</i>	This study
Plasmids <sup>b</sup>		
pCL149	$\Delta$ <i>luxR::ermR</i> allele; ColE1, <i>oriT<sub>RP4</sub></i> , <i>camR</i>	9
pCR-BluntII-TOPO	PCR product cloning vector; ColE1, <i>kanR</i>	Invitrogen
pDCRP	<i>E. coli crp</i> in pBR322 derivative; ColE1, <i>ampR</i>	34
pDCRP TA158	<i>E. coli crp</i> variant TA158 in pBR322 derivative; ColE1, <i>ampR</i>	34
pDCRP HL159	<i>E. coli crp</i> variant HL159 in pBR322 derivative; ColE1, <i>ampR</i>	44
pDCRP HY19	<i>E. coli crp</i> variant HY19 in pBR322 derivative; ColE1, <i>ampR</i>	45
pDCRP KE101	<i>E. coli crp</i> variant KE101 in pBR322 derivative; ColE1, <i>ampR</i>	45
pDU9	$\Delta$ <i>crp</i> in pBR322 derivative; ColE1, <i>ampR</i>	35
pEVS122	Suicide vector; R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>ermR</i>	29
pEXT20	<i>P<sub>tac</sub></i> expression vector; ColE1, <i>ampR</i>	39
pJLB36	<i>P<sub>luxR-gfp</sub></i> reporter in pVSV33; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>kanR</i>	32
pJLB113	<i>luxR</i> in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pJLB117	<i>V. fischeri</i> $\Delta$ <i>crp</i> allele; ColE1, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>camR</i> , <i>kanR</i>	32
pJLB123	<i>P<sub>orf1-luxR</sub></i> ; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>kanR</i>	32
pMTV1	<i>P<sub>con-gfp</sub></i> reporter in pVSV33; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>kanR</i>	This study
pMTV2	<i>P<sub>ainS-gfp</sub></i> reporter in pVSV33; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>kanR</i>	This study
pNL12	<i>crp</i> in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pNL15	<i>P<sub>orf1-crp</sub></i> in pVSV105; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>camR</i>	This study
pNL19	<i>P<sub>tac-crp</sub></i> (His <sub>6</sub> tag) in pEXT20; ColE1, <i>ampR</i>	This study
pNL31	<i>ainS</i> upstream fragment in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	37
pNL53	<i>ainS</i> downstream fragment in pEVS122; R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>ermR</i>	This study
pNL56	<i>E. coli crp</i> in pVSV105; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>camR</i>	This study
pNL62	$\Delta$ <i>ainS</i> allele; ColE1, R6K $\gamma$ , <i>oriV</i> , <i>oriT<sub>RP4</sub></i> , <i>kanR</i> , <i>ermR</i>	This study
pNL66	129–269 bp of <i>luxR</i> in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pNL69	<i>V. fischeri crp</i> in pDU9; ColE1, <i>ampR</i>	This study
pNL73	<i>P<sub>luxR-lacZ</sub></i> reporter in pRW50; <i>oriV</i> , <i>tetR</i>	This study
pNL76	<i>P<sub>ainS-lacZ</sub></i> reporter in pRW50; <i>oriV</i> , <i>tetR</i>	This study
pNL81	98–260 bp of <i>lpxA</i> in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pNL82	<i>P<sub>luxI-lacZ</sub></i> reporter in pRW50; <i>oriV</i> , <i>tetR</i>	This study
pNL84	<i>P<sub>luxI-lacZ</sub></i> reporter in pRW50; <i>oriV</i> , <i>tetR</i> , <i>luxR</i>	This study
pNL85	<i>litR</i> in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pNL86	<i>P<sub>ainS-lacZ</sub></i> reporter in pRW50; <i>oriV</i> , <i>tetR</i> , <i>litR</i>	This study
pNL90	91–244 bp of <i>ainS</i> in pCR-BluntII-TOPO; ColE1, <i>kanR</i>	This study
pRW50	Promoterless <i>lacZYA</i> ; <i>oriV</i> , <i>tetR</i>	41
pVSV33	Promoterless <i>camR-gfp</i> ; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>kanR</i>	33
pVSV105	Shuttle vector; pES213, R6K $\gamma$ , <i>oriT<sub>RP4</sub></i> , <i>camR</i> , <i>lacZ<math>\alpha</math></i>	33
Oligonucleotides <sup>c</sup>		
DMC2	GGC GGT ACC AGA ACC AAG ACC TGC TCG TGC TAA	This study
JBCRP4	TGC AGG GCA ACG TTG TAC TTG TGC	32
JBCRP5	GCA TCC TCC AGC AGC CAT TAA GAC C	32
JBLUXR1	GAA GGA GAT ATA CAT ATG AAC ATT AAA AAT ATA AAT GC	32
JBLUXR2	CGC CAA GAT TTT ATG GAA ATG TAT GAG	32

(Continued on following page)

TABLE 1 (Continued)

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence <sup>a</sup>	Source or reference
JBPromoter1	CTA GTT GAC ATG ATA GAA GCA CTC TAC TAT ATT	This study
JBPromoter2	CTA GAA TAT AGT AGA GTG CTT CTA TCA TGT CAA	This study
Lux1	GGG GTC TAG AGC TTT AGA AAT ACT TT	38
Lux2	GGA TCC GCT AGG GCG GCC GCC TAA CT	38
pr_MTV1	CGC GGA ATT CAG GAA CTA TAA ACT ATG GTT CTA GGT AAA CCT CAA ACA	This study
pr_MTV2 <sup>d</sup>	GCG CAA GCT TAT TAA TGG TGA TGG TGA TGG TGA TGA TAA CGA TGA CC GTA AAC TAC AAT	This study
pr_NL54	CGC TAT TAT CTA TCC TCA CTC AAT AAT TAA ACC TGA TG	This study
pr_NL55	GAA TGA TGG GAC TTA GAG TAA TCG ACT ACA GGG TC	This study
pr_NL58	GGC AGG CCT CAT CAG TTG TTG AAG TAA ATT AAA ATT CTG CG	This study
pr_NL78.2	GCG CCC TAG GTG ACT TTT ATA TAA ATG TTA ACT ACT TTA C	This study
pr_NL79	CCT CGA CT AAT CGA ATA GAT ATA GAA CTT TTA T	This study
pr_NL80	GCG CGA ATT CCA TGA TCA TAA CAA ACT GAT GCA TTA CGG	This study
pr_NL81	GCG CAA GCT TGT TCA TTT TTT GTT CAC CTA GCT TAT TGT TA	This study
pr_NL86	GCG CGA ATT CAG AAC CAA GAC CTG CTC GTG CTA A	This study
pr_NL87	GCG CAA GCT TGA TCA GTT GTT GAA GTA AAT TAA AAT TCT GCG	This study
pr_NL94	CTG GTA ATG TAA CGA TTG GCG AAG GTA CGG AAG TG	This study
pr_NL95	CCG ATA ACA ACT GTT GTT GCT TCA CCA CCA TAT TTC	This study
pr_NL96	GCG CGA ATT CCA CCA ATT TGG AGG TTT GGT GAT ATC GC	This study
pr_NL97	GCG CAA GCT TTA TCA TTA CAG CCA TGC AAC CTC TCT TAT TT	This study
pr_NL100	GCG CCC TAG GGG TTT CCC GAC TGG AAA GCG GGC	This study
pr_NL101	GCG CTC TAG AGG TTT CCC GAC TGG AAA GCG GGC	This study
pr_NL102	CTG TTG AAT ATA TAA GCA GCC TTA TGT AAA GTT ATT GAG	This study
pr_NL103	TCA GCG GTT TTT TAT TAT TAT CAT TTC ATG AAT CCT G	This study
pr_NL108	GGC GGA ACG ATT GGA AAT TTG GAA TAC TTA TTT TCA ACA TC	This study
pr_NL109	CAG TAC TGC ATT TCA AAA GAC AAC CAA AAA CTT TGA TAG CC	This study

<sup>a</sup> Drug resistance gene designations: *ampR*, ampicillin resistance (*bla*); *camR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tetR*, tetracycline resistance (*tetM*).

<sup>b</sup> All alleles cloned in this study are from *V. fischeri* strain ES114. The replication origins of each vector are listed as R6Kγ, ColE1, *oriV*, and/or pES213. Plasmids based on pES213 are stable and do not require antibiotic selection for maintenance (33).

<sup>c</sup> All oligonucleotides are shown 5' to 3'.

<sup>d</sup> Underlined bases indicate the C-terminal His<sub>6</sub> tag used in protein purification and a newly created stop codon.

To generate standard curves, the primer sets described above (pr\_NL54 and pr\_NL55, pr\_NL108 and pr\_NL109, and pr\_NL94 and pr\_NL95) were used to PCR amplify *luxR*, *ainS*, and *lpxA*, respectively, using ES114 DNA as a template. The blunt *luxR*, *ainS*, and *lpxA* PCR products were ligated into the pCR-BluntII-TOPO vector to generate pNL66, pNL90, and pNL81, respectively. These plasmids were utilized as standards in real-time PCR analyses. The efficiency of each primer set was calculated to be ~98, ~96, and ~95% using the standard curves generated from serial dilutions of pNL66, pNL90, and pNL89, respectively. To ensure that chromosomal DNA contamination was not affecting the real-time analyses, control samples lacking reverse transcriptase were also examined using each primer set, and no-template controls were included in each assay.

**Purification of *V. fischeri* CRP.** For CRP purification, *V. fischeri crp* was PCR amplified using primers JBCRP4 and JBCRP5, and the blunt product was cloned into pCR-BluntII-TOPO to generate pNL12. The wild-type *crp* allele from pNL12 was then used as a template for PCR with primers pr\_MTV1 and pr\_MTV2. The latter primer mutated the *crp* stop codon, added a C-terminal His<sub>6</sub> tag, and created a new stop codon (Table 1). The PCR product was digested with HindIII and EcoRI and then ligated into HindIII- and EcoRI-digested pEXT20 expression vector (39) to generate pNL19.

*V. fischeri crp* was expressed from pNL19 in *E. coli* strain UQ3811 (40). Overnight cultures were diluted 1:100 and grown with shaking (200 rpm) in LB at 37°C to an OD<sub>595</sub> of ~0.5. At this OD<sub>595</sub>, CRP overexpression was induced by adding IPTG to a final concentration of 1 mM. Following induction, cultures were incubated for 5 h and then cells were pelleted by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl (pH 8.0), 500 mM KCl, and 1 mM EDTA and lysed using a French pressure

apparatus. Cell debris was pelleted by centrifugation at 10,000 × *g* for 10 min. The cell lysate was loaded onto a Ni-MAC cartridge (Novagen, EMD Chemicals Inc., Gibbstown, NJ), the column was washed with 10 volumes of 50 mM Tris-HCl (pH 8.0), 500 mM KCl, and 50 mM imidazole, and then it was eluted with 50 mM Tris-HCl (pH 8.0), 500 mM KCl, and 250 mM imidazole in 1-ml fractions. To remove imidazole, elution fractions containing protein were dialyzed overnight in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, and 1 mM EDTA.

**Fluorescence polarization DNA binding assays.** DNA binding assays were completed using 5'-fluorescein-labeled oligonucleotides annealed to unlabeled complementary oligonucleotides, both synthesized by Sigma-Aldrich (St. Louis, MO). Oligonucleotides were resuspended to a final concentration of 1 mM and then combined in a 1:1 ratio, boiled for 5 min, and allowed to anneal by cooling slowly at room temperature. Specified concentrations of purified CRP were added to binding assays, which contained a final concentration of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 15 nM oligonucleotide probe, and 20 ng ml<sup>-1</sup> salmon sperm DNA. Binding assay mixtures were incubated at room temperature for 15 min, and then samples were excited at 485 nm and emission measured at 528 nm using a Synergy 2 plate reader (BioTek) set to read the extent of fluorescence polarization. Each sample was assayed two additional times, after 25 and 35 min, and all three measurements were averaged to generate the anisotropy values reported. The data shown below reflect two biological replicates, each of which was assayed three times to determine a value.

**Beta-galactosidase transcriptional reporter assays.** Beta-galactosidase transcriptional reporters were constructed using the promoterless *lacZ* plasmid pRW50 (41). The *luxR* promoter was amplified with primers pr\_NL80 and pr\_NL81, the *ainS* promoter was amplified with primers pr\_NL86 and pr\_NL87, and the *luxI* promoter was amplified

with primers pr\_NL96 and pr\_NL97. The PCR products were digested with EcoRI and HindIII and then ligated into EcoRI- and HindIII-digested pRW50 to generate pNL73, pNL76, and pNL82, respectively.

Because these reporters were used in *E. coli*, which lacks specific key regulators found in *V. fischeri*, the complete *luxR* and *litR* genes were added to the  $P_{luxI}$ -*lacZ* and  $P_{ainS}$ -*lacZ* reporter plasmids, respectively. To construct pNL84, *luxR* was amplified using primers JBLUXR1 and JBLUXR2, and then the blunt PCR product was ligated into the pCR-BluntII-TOPO vector, generating pJLB113. The vector-derived *lacZ* promoter and *luxR* insert from pJLB113 were amplified with primers pr\_NL100 and pr\_NL101, and the PCR product was digested with AvrII and ligated into AvrII-digested pNL76. To construct pNL86, *litR* was amplified using primers pr\_NL102 and pr\_NL103, and the blunt PCR product was ligated into the pCR-BluntII-TOPO vector, generating pNL85. Both *litR* and the vector-derived *lacZ* promoter upstream of it were amplified from pNL85 using primers pr\_NL100 and pr\_NL101, and the PCR product was digested with AvrII and ligated into AvrII-digested pNL76.

All reporter plasmids were cotransformed into *E. coli* strain  $\Delta crp$  (42), which is a *crp* mutant of *E. coli* M182 (43), along with pDCRP (34), pDCRP-TA158 (34), pDCRP-HL159 (44), pDCRP-HY19 (45), pDCRP-KE101 (45), or pDU9 (35). Cotransformants were tested in beta-galactosidase assays. Overnight cultures of cotransformants were subcultured 1:100 into 20 ml of LB containing Amp and Tet in 125-ml flasks and then incubated with shaking (200 rpm) at 28°C. At an  $OD_{595}$  of  $\sim 0.5$  for strains with pNL73, pNL82, and pNL84 and  $\sim 1.0$  for strains with pNL76 and pNL86, 1-ml samples were removed, pelleted, and stored at  $-80^\circ\text{C}$ . For beta-galactosidase assays, pelleted cells were resuspended in 1 ml of Z buffer and 0.5 ml of each resuspension was assayed as previously described (30).

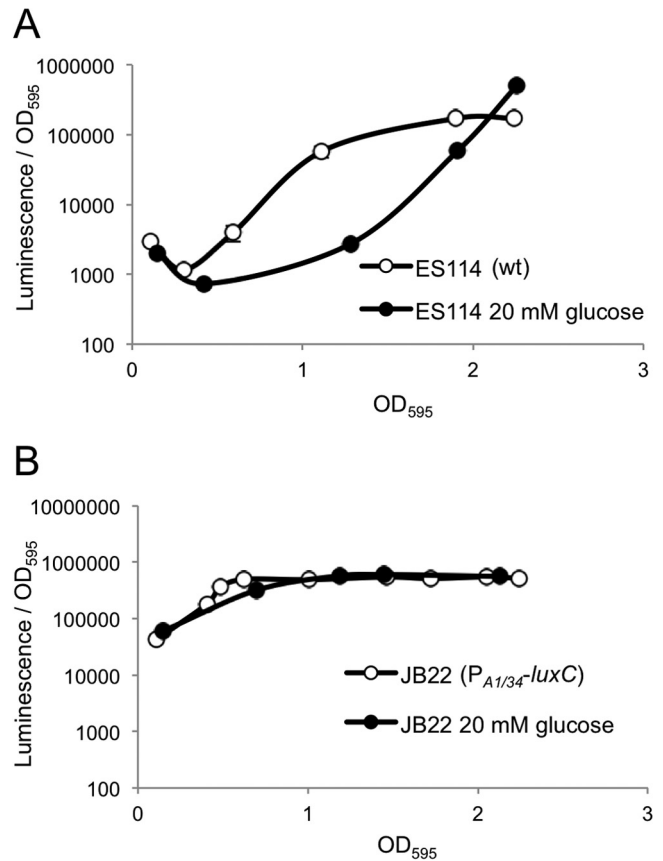
**C8-HSL bioassays.** To assess C8-HSL production by particular strains, overnight cultures were diluted 1:1,000 in 15 ml SWTO medium in 125-ml flasks and grown at 24°C with shaking (200 rpm) to an  $OD_{595}$  of 2.4 to 2.5. Cells were pelleted by centrifugation, and supernatants were filter sterilized before adding an equal volume of acidified ethyl acetate (1:1,000 glacial acetic acid:ethyl acetate) and incubating for 30 min on a rotary shaker at 55 rpm to extract acyl-homoserine lactones (AHLs). Three-ml samples of the organic phase were removed and added to sterile glass beakers, and the ethyl acetate was allowed to evaporate overnight. Extracts were dissolved in 3 ml SWTO, and 200- $\mu\text{l}$  samples were added to alternating wells of a 96-well microplate. Wells were then inoculated with the C8-HSL bioreporter strain DC22 (Table 1), which will be described in greater detail elsewhere. It was engineered to lack the pheromone synthases encoded by *luxI* and *ainS* and to have expression of the *luxCDABEG* genes activated by a mutant LuxR derivative (LuxR<sub>MJ1</sub> T33A, R67M, S116A, M135I) that is responsive to C8-HSL but not to 3-oxo-C6-HSL (46). C8-HSL standards were likewise prepared, and C8-HSL concentration was determined by measuring luminescence of DC22 using a Synergy 2 plate reader (BioTek).

**Experimental repetitions and statistical analyses.** Each figure represents data from one experiment but is representative of at least three experimental repetitions. Means, standard errors, and significance in Student's *t* tests were calculated using Microsoft Excel.

## RESULTS

**Luminescence in ES114 is influenced by glucose.** To determine the effect of glucose on *V. fischeri* ES114 luminescence, we examined cultures grown in SWTO alone and with 20 mM glucose (Fig. 1A). We found that glucose decreased luminescence when the culture  $OD_{595}$  was below 2.0, with a maximum effect of  $\sim 15$ -fold. Later, a brief peak of brighter luminescence was observed in the glucose-supplemented culture at an  $OD_{595}$  of  $\sim 2.3$ . At this peak, during a brief but reproducible window in cell density, cultures grown with glucose were  $\sim 3$ -fold brighter than cultures grown without glucose (Fig. 1A).

The effect of glucose on the luminescence of ES114 in Fig. 1A



**FIG 1** Effect of glucose on luminescence. Specific luminescence is shown for wild-type ES114 (A) or strain JB22 ( $lacI^P$ - $P_{A1/34}$ -*luxC*) (B) grown in SWTO either without (open circles) or with (filled circles) 20 mM glucose added. Averages and standard errors were calculated from replicate flasks ( $n = 2$ ). Many error bars are smaller than the circles used to mark the means and are difficult to visualize.

might be due to regulation of genes involved in light production, or growing on glucose might directly affect the luminescence output of cells, for example, simply by altering the availability of substrates for luciferase. To distinguish between these possibilities, we examined the luminescence of mutant JB22, which contains the constitutive promoter,  $lacI^P$ - $P_{A1/34}$ , inserted between *luxI* and *luxC*, resulting in constitutive expression of *luxCDABEG* (38). In the  $lacI^P$ - $P_{A1/34}$ -*luxC* background, glucose had no effect on luminescence below an  $OD_{595}$  of  $\sim 2.5$  (Fig. 1B). Because constitutive *luxCDABEG* expression eliminated glucose-mediated repression of luminescence, these data suggest that at these culture densities glucose affects ES114 luminescence by controlling gene expression rather than by altering the physiological capacity of cells to produce luminescence.

In both ES114 and JB22, luminescence declines rapidly at cell densities around an  $OD_{595}$  of 2.5 to 2.7, and the data shown in Fig. 1 precede this rapid decline in light production, which is observed roughly coinciding with a rapid drop in pH (data not shown), possibly owing to fermentation of the glucose.

**Luminescence in ES114 is affected by cAMP and *crp*.** Previous work using transgenic *E. coli* carrying the *lux* genes from *V. fischeri* strain MJ-1 suggested that cAMP-CRP activated *luxR* transcription and luminescence (16, 17). In *E. coli*, cAMP levels are rela-

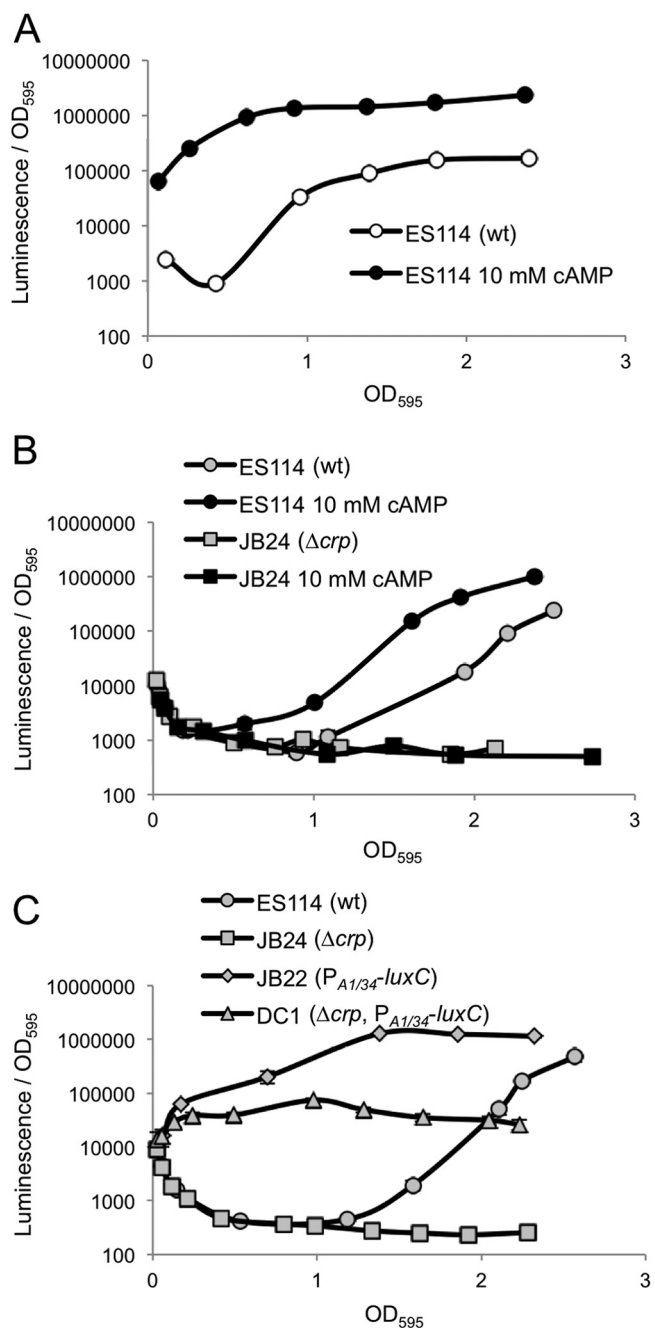
tively low in the presence of glucose, but exogenous addition of 4 mM cAMP to *lux*-containing *E. coli* stimulated luminescence (16), an effect attributed to enough cAMP entering cells to activate CRP. Similarly, it was reported that addition of 10 mM cAMP stimulates luminescence in *V. fischeri* cultures (14, 18).

We likewise found that addition of cAMP to ES114 stimulated luminescence ~10-fold (Fig. 2A). It was difficult to interpret the effects of glucose on luminescence in a *V. fischeri*  $\Delta$ *crp* mutant, because this strain grew very poorly without glucose; however, we could test the effect of cAMP addition on both ES114 and the  $\Delta$ *crp* mutant in media with added glucose. In glucose-containing media we found that the  $\Delta$ *crp* mutant was both dimmer than the wild type and unresponsive to cAMP (Fig. 2B).

We considered the possibility that pleiotropic physiological effects associated with the  $\Delta$ *crp* mutation underlie the low luminescence of this mutant. Therefore, we tested the effect of the  $\Delta$ *crp* allele in the JB22 strain background with *lux* expressed from a constitutive nonnative  $P_{A1/34}$  promoter. We found that deleting *crp* led to an ~15-fold decrease in luminescence in the constitutive *lux* background (Fig. 2C); however, this does not match the ~1,000-fold decrease in luminescence seen when *crp* is deleted from ES114 (Fig. 2C). Moreover, the  $\Delta$ *crp* constitutive *lux* strain DC1 is ~100-fold brighter than the  $\Delta$ *crp* mutant JB24. This phenotype of DC1 indicates that cells with the  $\Delta$ *crp* mutation are metabolically capable of much brighter luminescence than that displayed by the  $\Delta$ *crp* mutant JB24. Taken together, our data suggest that the attenuation of luminescence caused by the  $\Delta$ *crp* allele in JB24 in large part reflects a limitation of *lux* gene expression with a smaller contribution arising from another, possibly metabolic, mechanism.

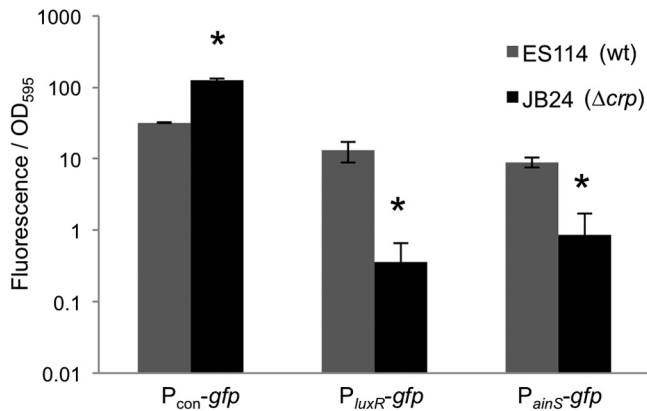
**CRP influences *luxR* and *ainS* transcription.** To identify genes involved in luminescence that may be controlled by cAMP-CRP, we used Virtual Footprint to analyze the *V. fischeri* ES114 genome using that program's position weight matrix default setting for CRP, which is derived from *E. coli* K-12 CRP binding sites (47). As expected based on previous studies, a putative CRP binding site was identified upstream of *luxR*. More surprisingly, a potential CRP binding site was also identified upstream of *ainS* which encodes the autoinducer synthase responsible for generating C8-HSL (8). In fact, the putative CRP binding site upstream of *ainS* was a better match to the weighted consensus CRP binding site than the site in the *lux* intergenic region, as Virtual Footprint returned position weight matrix scores of 7.0 and 7.6 for putative CRP sites upstream of *luxR* and *ainS*, respectively. Both sites are similar matches to the core CRP binding consensus of 5'-TGTGA(N6)TCACA-3' (see Fig. 5A).

We next examined the effect of CRP on the expression of the *luxR* and *ainS* promoters using  $P_{luxR}$ -*gfp* and  $P_{ainS}$ -*gfp* promoter-reporters. Fluorescence expressed from each reporter was measured in the wild type and the  $\Delta$ *crp* mutant (Fig. 3). The  $P_{luxR}$ -*gfp* and  $P_{ainS}$ -*gfp* reporters yielded a statistically significant ( $P \leq 0.05$ ) ~15- and ~10-fold decrease in fluorescence in the  $\Delta$ *crp* mutant, respectively. A constitutively active promoter-reporter with a consensus promoter driving *gfp* expression was also examined in the wild type and *crp* mutant to ensure that the  $\Delta$ *crp* allele did not decrease fluorescence of all *gfp* reporters. Fluorescence of the  $P_{con}$ -*gfp* reporter was actually significantly higher in the  $\Delta$ *crp* mutant, suggesting that, if anything, the effects of *crp* on the  $P_{luxR}$ - and  $P_{ainS}$ -*gfp* reporters underestimate the positive effects of *crp* on transcription of these genes.



**FIG 2** Effect of cAMP on luminescence. (A) Specific luminescence of ES114 (wild type) grown in SWTO without (open circles) or with (filled circles) 10 mM cAMP added. (B) Specific luminescence of ES114 and  $\Delta$ *crp* mutant JB24 grown in SWTO containing 20 mM glucose without (gray circles) or with (filled circles) 10 mM cAMP. (C) Specific luminescence of ES114, JB22 ( $lacI^q$ - $P_{A1/34}$ -*luxC*),  $\Delta$ *crp* mutant JB24, and  $\Delta$ *crp*  $lacI^q$ - $P_{A1/34}$ -*luxC* mutant DC1 grown with 20 mM glucose. Averages and standard errors were calculated from replicate flasks ( $n = 2$ ).

To measure directly whether CRP controls the transcript levels of *luxR* and *ainS*, we utilized quantitative real-time PCR to determine the copy number of both *luxR* and *ainS* transcripts in *V. fischeri* ES114 and in the  $\Delta$ *crp* mutant (Fig. 4A and B). The copy number of the *luxR* and *ainS* transcripts was decreased by ~250- and ~7-fold, respectively. To test whether all transcripts appear



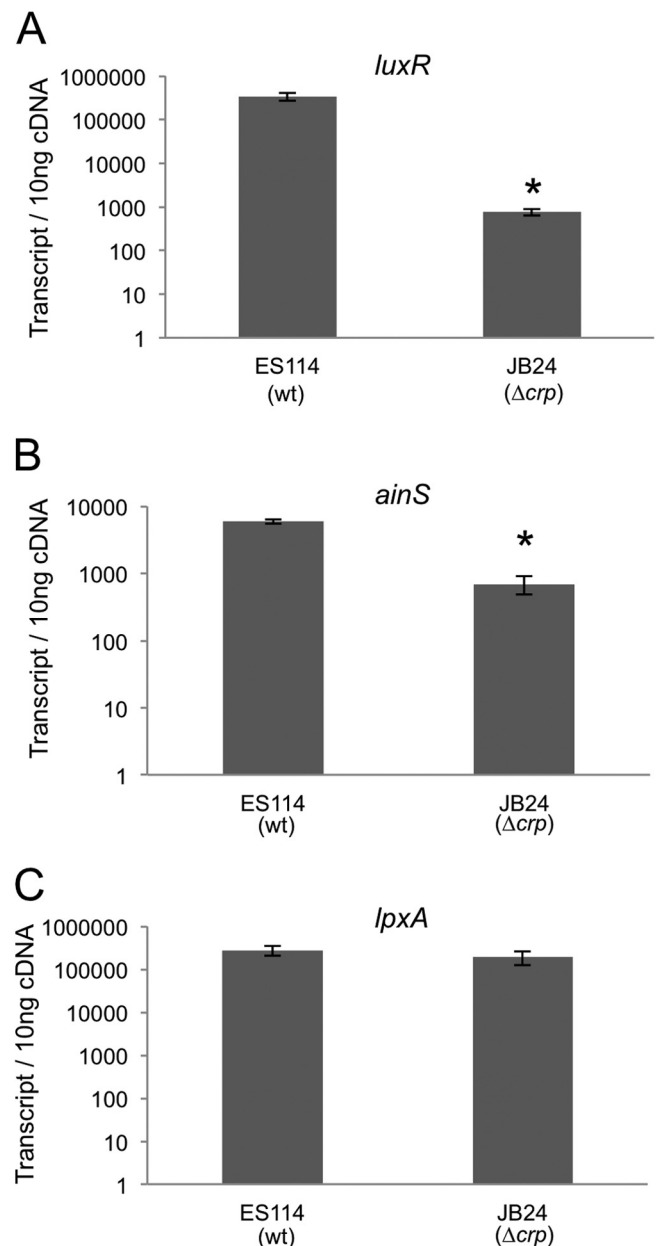
**FIG 3** CRP regulation of *luxR* and *ainS* promoter activity. Specific fluorescence was generated from the reporters  $P_{con-gfp}$  (pMTV1),  $P_{luxR-gfp}$  (pJLB36), and  $P_{ainS-gfp}$  (pMTV2) carried within ES114 (gray bars) or *crp* mutant JB24 (black bars) grown in SWTO with 20 mM glucose. Fluorescence from each strain harboring the promoterless vector pVSV33 was subtracted as the background. Data represent the average specific fluorescence when the culture  $OD_{595}$  was  $\sim 2.0$ . Averages and standard errors were calculated from replicate flasks ( $n = 2$ ). An asterisk indicates a significant difference between fluorescence in reporters expressed from ES114 or JB24 cultures, as determined by a Student's *t* test ( $P \leq 0.05$ ).

decreased in the  $\Delta crp$  mutant, we also examined the *lpxA* transcript as a control. *LpxA* is involved in lipid A biosynthesis in Gram-negative bacteria and is an essential housekeeping gene (48) that is not part of the CRP regulon in *E. coli* (49). The transcript level of *lpxA* was equivalent in both the wild type and the  $\Delta crp$  mutant (Fig. 4C). These results support the model that CRP regulates *luxR* and *ainS* expression.

**CRP binds upstream of *luxR* and *ainS*.** Previous work showed that *E. coli* CRP can bind upstream of *luxR* from *V. fischeri* strain ATCC 7744 (26), and we sought to test whether this would also be true for CRP from *V. fischeri* binding the ES114 *lux* promoter and whether binding upstream of *ainS* could be detected. Using purified His-tagged *V. fischeri* CRP, we tested *in vitro* binding to 5' fluorescein-labeled DNA fragments (Fig. 5A). The transcriptional start sites for *luxR* and *ainS* in *V. fischeri* remain uncertain, but the sequences upstream of these genes along with the target binding sequences are highlighted in Fig. S1 in the supplemental material. A *melR* promoter target region was previously shown to associate with CRP (40), and we used it in this study as a positive control (Fig. 5A). The *melR* shuffled target contains the same nucleotides as the *melR* target, but they were randomized, thereby serving as a negative control for binding. With increased anisotropy serving as a measure of target binding, we observed cAMP-dependent binding of CRP to the *melR* target and no binding to the *melR* shuffled target (Fig. 5B and C).

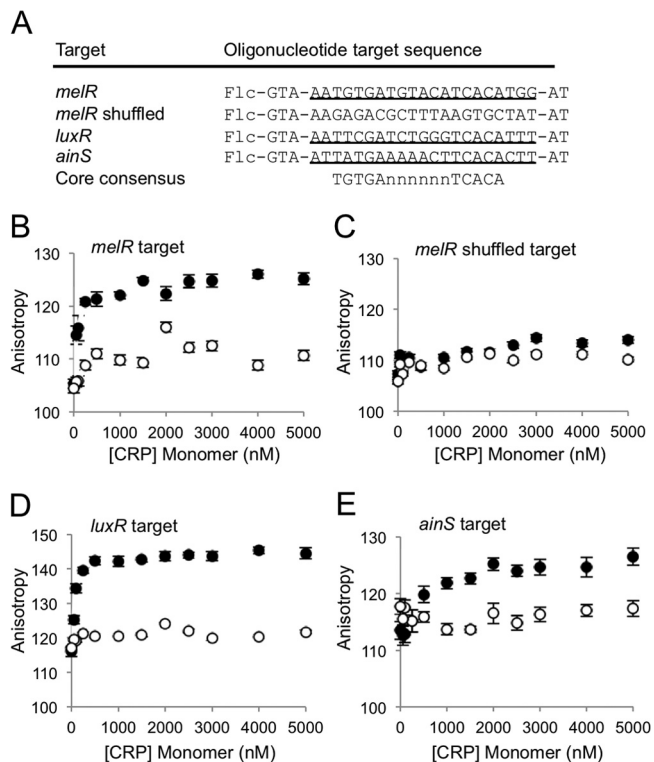
We next tested if CRP binds the predicted CRP target sequences upstream of *luxR* and *ainS*. As with *melR*, we observed a cAMP-dependent increase in anisotropy as CRP was added to both the *luxR* and *ainS* targets, indicating that CRP binds sequences found upstream of these genes (Fig. 5D and E). These results support the model that cAMP-CRP binds near the promoters of *luxR* and *ainS*.

**Regulation of both *luxR* and *ainS* may contribute to cAMP activation of luminescence.** Although CRP binds upstream of both *ainS* and *luxR*, it remained possible that one or the other of



**FIG 4** RT-PCR transcript analysis of *luxR* and *ainS*. Copy number of *luxR* (A), *ainS* (B), and *lpxA* (C) transcripts in 10 ng total cDNA generated from ES114 (wild type) or *crp* mutant JB24 RNA. Bars indicate standard errors ( $n = 3$ ). An asterisk indicates a significant difference in transcript number between wild-type and *crp* mutant transcript copy numbers as determined by a Student's *t* test ( $P \leq 0.0005$ ).

these CRP sites was solely responsible for the effect of cAMP-CRP on luminescence in ES114. To explore whether regulation of the *luxR* promoter alone could account for the effect of cAMP-CRP on luminescence, we determined the effect of cAMP on luminescence of a *luxR* mutant (CL53) (9) complemented by *luxR* constitutively expressed in *trans* (pJLB123) (32) (Fig. 6). By using this construct, we eliminated expression of *luxR* from the CRP-regulated native promoter and maintained constitutive expression of *luxR*. When 10 mM cAMP was added to this strain, luminescence nonetheless increased  $\sim 15$ -fold (Fig. 6). Thus, the cAMP-medi-

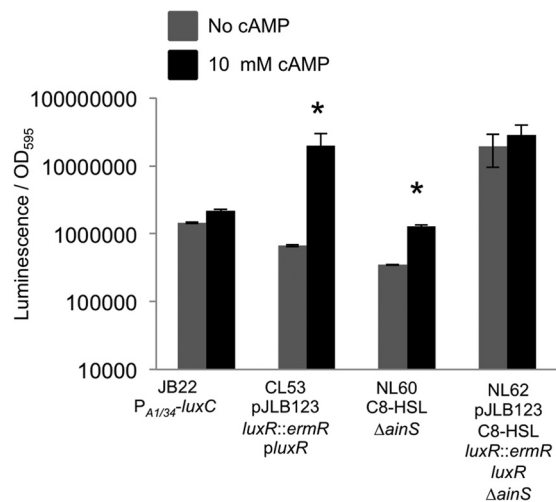


**FIG 5** Fluorescence polarization analysis of CRP binding to the *luxR* and *ainS* promoter regions. (A) Fluorescein-labeled oligonucleotides utilized for binding analyses. Flc indicates the position of fluorescein modification at the 5' end of the oligonucleotide target sequences. The *melR* sequence was previously shown to bind CRP (40) and is used in this study as a positive control. Target oligonucleotides are each 26 bp long, encompassing the putative CRP binding site, and each is flanked by 5'-GTA-target-AT-3'. The *melR* shuffled sequence was generated using the *melR* sequence and the shuffle DNA application from The Sequence Manipulation Suite (<http://www.bioinformatics.org/sms/>). The core, most heavily conserved portion of the CRP binding consensus, 5'-TGTGA(N6)TCACA, is shown for comparison. Fluorescence polarization curves without (open circles) or with (filled circles) 10 mM cAMP added are shown for the *melR* target (B), shuffled *melR* target (C), *luxR* target (D), and *ainS* target (E). Averages and standard errors were calculated from replicate samples ( $n = 2$ ) as described in Materials and Methods.

ated induction of luminescence is not due solely to CRP regulation of the native *luxR* promoter.

We also examined the luminescence of an *ainS* mutant supplemented with C8-HSL (NL60) alone or with 10 mM cAMP (Fig. 6). With constant C8-HSL, the *ainS* mutant's luminescence increased ~5-fold when 10 mM cAMP was added (Fig. 6). Thus, as with *luxR*, the cAMP-mediated induction of luminescence appears to not be solely due to CRP regulation of *ainS*.

We then tested the effect of cAMP on a *luxR ainS* double mutant (NL62) complemented with pJLB123 and supplemented with C8-HSL. Using this strain under these conditions, neither C8-HSL nor LuxR levels should be subject to direct regulatory control by cAMP-CRP over the *ainS* or *luxR* promoters. In this setup, a cAMP-mediated increase in luminescence was not observed (Fig. 6). Although this strain supplemented with C8-HSL was also the brightest treatment in the experiment, it is more than an order of magnitude dimmer than the luminescence capacity of ES114 (e.g., supplemented with 3-oxo-C6-HSL), suggesting that it would be possible to see a cAMP-mediated increase in luminescence if one



**FIG 6** Regulation of both *ainS* and *luxR* contributes to the effect of cAMP on luminescence. Maximal specific luminescence is shown for JB22 ( $lact^3$ - $P_{A1/34}$ -*luxC*), a *luxR* mutant complemented with constitutive *luxR* in trans (CL53 pJLB123), an *ainS* mutant biochemically complemented with 250 mM C8-HSL (NL60 C8-HSL), and a *luxR ainS* double mutant complemented with *luxR* in trans and 250 mM C8-HSL (NL62 pJLB123 C8-HSL). Cultures were grown in SWTO alone (gray bars) or with 10 mM cAMP added (black bars). Standard errors were calculated from two replicates. An asterisk indicates significant differences between cultures grown without and with cAMP as determined by a Student's *t* test ( $P \leq 0.05$ ).

existed. Taken together, these results suggest that cAMP-CRP control of both *ainS* and *luxR* is significant in determining the luminescence of *V. fischeri* ES114.

**CRP activates transcription of *luxR*, *luxI*, and *ainS* promoters through different mechanisms in transgenic *E. coli*.** CRP usually regulates transcription by recruiting RNA polymerase (RNAP) to promoters following binding to a target sequence (21). CRP-dependent promoters are grouped into three classes. Class I-type promoters require one point of contact with RNAP at activating region I (ARI). Class II-type promoters require two points of contact between CRP and RNAP at both activating region I and activating region II (ARI and ARII). Finally, class III-type promoters involve multiple regulators (i.e., more than one CRP or CRP and another regulator), and either these require ARI or ARII or, at some class III-type promoters, CRP exerts an effect on a second regulatory protein through DNA binding and protein-protein interaction, in which case ARI and ARII may not be required (21, 50–52).

*V. fischeri* promoters were examined using a reporter system developed in *E. coli* wherein known ARI and ARII *crp* mutations are used to classify CRP-regulated promoters (34, 35, 45). In this dual plasmid system, promoter-*lacZ* reporters are generated in pRW50 and *crp* alleles are expressed from a compatible plasmid (41). Plasmid pDCRP contains the wild-type *E. coli crp* and is used as a positive control, whereas plasmid pDU9 lacks *crp* and is used as a negative control. The *crp* alleles on pDCRP-TA158 and pDCRP-HL159 are mutated at ARI, and the *crp* alleles pDCRP-HY19 and pDCRP-KE101 are mutated at ARII. To ensure that *E. coli* CRP activates transcription from *V. fischeri* promoters, we tested whether the wild-type *E. coli crp* would complement the growth and luminescence defect of the *V. fischeri crp* mutant. When the *E. coli* wild-type *crp* allele was expressed in trans on



pNL56, luminescence and growth in the absence of glucose were restored in the *V. fischeri* *crp* mutant (data not shown). We similarly observed that *crp* from ES114 on plasmid pNL69 could complement *E. coli* mutant M182 $\Delta$ *crp* (data not shown).

Expression from the  $P_{luxR}$ -*lacZ* (pNL73) promoter-reporter increased ~100-fold in *E. coli* cells cotransformed with the wild-type CRP allele relative to cotransformation with the empty vector control (Fig. 7A). This increase in activity is also evident in cells cotransformed with ARII variant alleles but not in cells cotransformed with ARI variant alleles, indicating that the effect of CRP on *luxR* expression requires only ARI, consistent with a CRP class I-type promoter.

We next examined the *ainS* promoter using a  $P_{ainS}$ -*lacZ* reporter. Because previous research suggested a role for LitR in *ainS* expression (11), *litR* was cloned into the  $P_{ainS}$ -*lacZ* reporter plasmid. In the presence of *litR*, the wild-type CRP allele increased beta-galactosidase activity from the  $P_{ainS}$ -*lacZ* reporter ~2-fold relative to cotransformation with the empty vector control (Fig. 7B). This increase in activity was also observed in cells cotransformed with either the ARI or ARII variant allele, indicating that the activating regions that define class I and class II promoters are not required for this regulatory effect.

We also examined expression of the *ainS* reporter in the absence of *litR* (Fig. 7C). These experiments showed higher expression of  $P_{ainS}$ -*lacZ* when *litR* is present (Fig. 7B and C), consistent with the proposed role of LitR in *ainS* regulation. Surprisingly, when *litR* was absent we observed higher  $P_{ainS}$ -*lacZ* activity in the presence of the vector control or vectors carrying CRP with ARI mutant alleles (Fig. 7C). These results suggest an additional layer of regulation, at least in *E. coli*, whereby CRP mediates negative regulation of *ainS*.

Finally, we used this reporter system to test the effect of CRP on the *luxI* promoter. The CRP binding site upstream of *luxR* likewise is upstream of the divergently transcribed *luxI*. Previous work, also in transgenic *E. coli*, suggested that in the absence of *luxR* CRP has a negative effect on expression of the *luxI* promoter from *V. fischeri* strain MJ-1 (16). We found that our  $P_{luxI}$ -*lacZ* reporter derived from strain ES114 increased ~3-fold in expression in the presence of the wild-type *crp* or with the ARII mutants (Fig. 7D), suggesting that CRP activates *luxI*-independent basal expression of *luxI* through a mechanism that requires ARI but not ARII. We also added *luxR* to the  $P_{luxI}$ -*lacZ* reporter plasmid under the control of a nonnative constitutive promoter and observed the expected autoinducer-dependent increase in  $P_{luxI}$ -*lacZ* expression (data not shown); however, when the 3-oxo-C6-HSL autoinducer combined with *luxR* to enhance transcription, no further increase was seen when *crp* was present (data not shown).

**C8-HSL levels affected by glucose.** Based on the data described above, we proposed that the effect of glucose on ES114 luminescence (Fig. 1) is due in part to lower C8-HSL levels in glucose-grown cells, reflecting regulation of *ainS* by cAMP-CRP in response to the carbon source. As predicted, we saw decreased levels of C8-HSL in ES114 cultures supplemented with glucose (Fig. 8). However, contrary to our prediction, when cultures were supplemented with glucose we also saw higher C8-HSL levels in the  $\Delta$ *crp* mutant than in the wild type (Fig. 8), a phenomenon that will be discussed below.

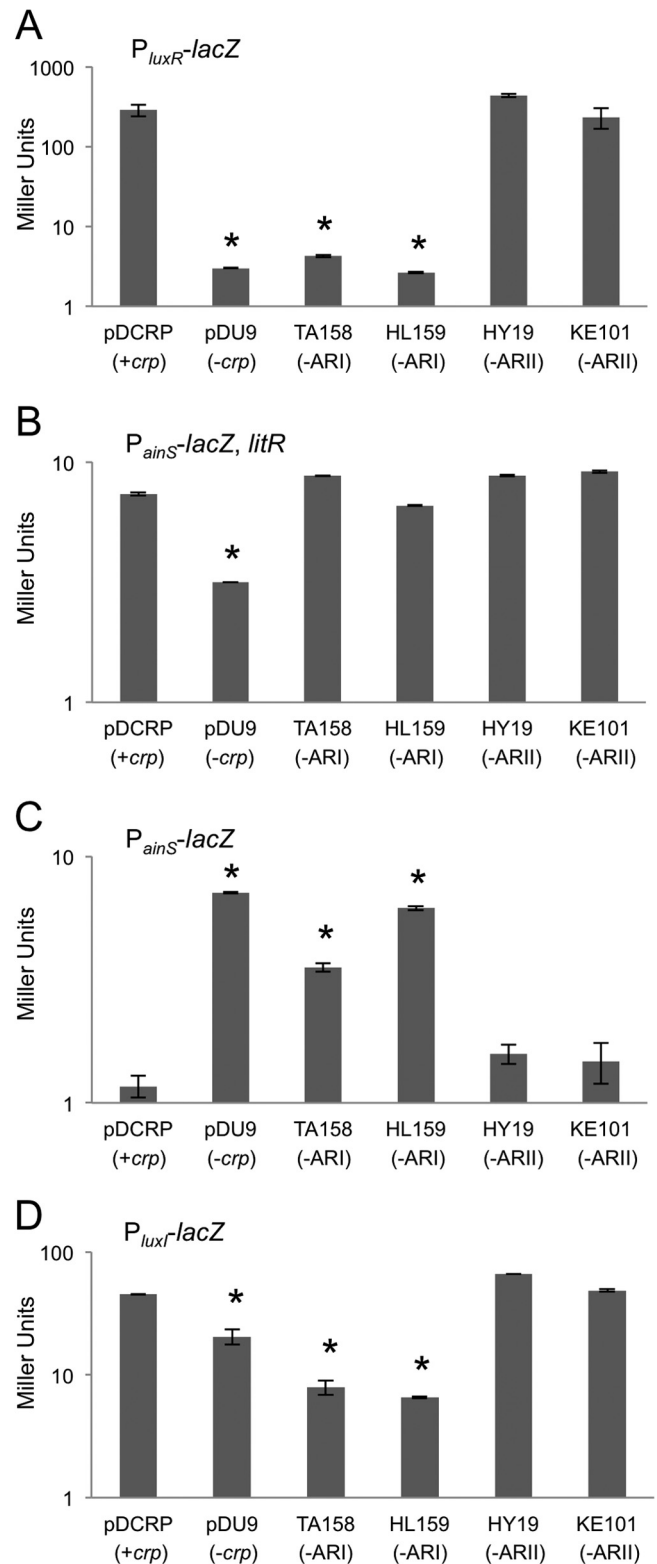
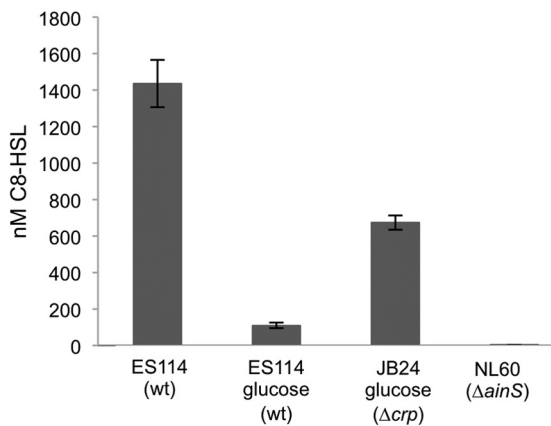


FIG 7 Effects of ARI and ARII *crp* mutations on *luxR*, *ainS*, and *luxI* promoter activity in *E. coli*. Data are given as Miller units generated from the reporters  $P_{luxR}$ -*lacZ* (pNL73) (A),  $P_{ainS}$ -*lacZ* (pNL76) (B),  $P_{ainS}$ -*lacZ*, *litR* (pNL76) (C), and  $P_{luxI}$ -*lacZ* (pNL82) (D). Bars indicate standard errors ( $n = 2$ ). An asterisk indicates a significant difference from pDCRP as determined by a Student's *t* test ( $P \leq 0.05$ ).



**FIG 8** Effects of glucose and  $\Delta crp$  mutation on C8-HSL accumulation. C8-HSL was extracted and estimated from cultures of ES114, the  $\Delta crp$  mutant JB24, or the  $ainS$  mutant NL60. The C8-HSL synthase mutant NL60 serves as a negative control. Cultures were grown to an  $OD_{595}$  of 2.4 to 2.5 in SWTO either amended with 20 mM glucose or left unamended, as indicated. C8-HSL levels were determined by bioassays of ethyl acetate-extracted cultures, and bars indicate standard errors ( $n = 3$ ).

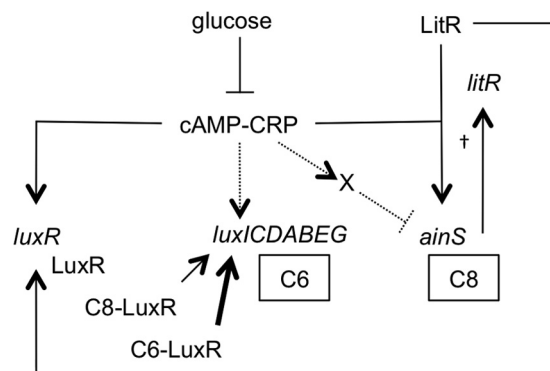
## DISCUSSION

*V. fischeri* ES114 controls luminescence using pheromone-mediated regulation, and like many other bacterial pheromone systems, this signaling is both cell density dependent and regulated in response to the environment. Although high cell density may be necessary for *lux* induction in ES114, it is not sufficient to achieve stimulatory pheromone levels. Some environmentally responsive regulators must also control *luxICDABEG* expression and are critical for symbiotic induction of luminescence (14, 15, 37). Previous reports suggested a role for glucose and CRP-mediated regulation of *V. fischeri* bioluminescence (14, 16–18, 26). However, the model that emerged of cAMP-CRP regulating *luxR* predated the development of genetic and genomic tools in *V. fischeri* or the discovery of key regulatory elements, such as the *AinS/AinR* pheromone system.

Our goal in this study was to test and define the role of glucose and CRP in *lux* regulation in *V. fischeri* isolate ES114. Recently, ES114 has become a popular experimental model, and the genetic tools developed for this strain allowed us to explore this area in ways that were not possible when the effects of glucose and CRP on *lux* were first examined. It seemed especially prudent not to assume that previous conclusions about CRP's role in *lux* regulation held for ES114, because the *lux* system in ES114 has diverged significantly from that in strains like MJ-1 (53). Moreover, it was reported that ES114 does not respond to glucose in the way MJ-1 does (14).

We found that CRP regulates the *ainS* pheromone synthase system, which is absent from studies with transgenic *E. coli*, underscoring the value of incorporating experiments in ES114 itself. We also generated support for the previous conclusion that CRP regulates *luxR* in ES114. Taken together, our data suggest that the regulatory mechanisms connecting CRP, pheromone signaling, and luminescence are more complex than previously appreciated. A new model for this CRP-mediated regulation of luminescence is presented in Fig. 9.

**Regulation by CRP or similar proteins is integral to pheromone circuitry in diverse systems.** Interestingly, the role of CRP



**FIG 9** Proposed model of CRP-mediated regulation in *V. fischeri* luminescence. Direct activation of *luxI* and of a potential negative regulator of *ainS* (indicated by an "X") are based on data from transgenic *E. coli* (Fig. 7) and are shown with dashed lines. It should be noted that this potential negative regulator need not exist if cAMP-CRP acts as a simple repressor of *ainS*, and other mechanisms are likewise viable. The autoinducers 3-oxo-C6-HSL and C8-HSL are abbreviated as C6 and C8, respectively. The dagger indicates that C8-HSL functions through a phosphorelay involving *AinR*, *LuxU*, *LuxO*, *Hfq*, and the small RNA *qrr* that increases levels of *LitR* to activate *luxR* expression (10, 13). C8-HSL can also activate *LuxR* directly, although it is a weaker activator than 3-oxo-C6-HSL (11), and this is represented by the relative weights of the arrows from C8-LuxR and C6-LuxR.

in modulating the pheromone signaling circuitry of *V. fischeri* apparently illustrates a widespread trend. Several bacteria with mechanistically diverse pheromone signaling systems integrate CRP, CRP-like proteins, or catabolite repression into regulation of their pheromone signaling systems. For example, *Vibrio cholerae* and *Vibrio harveyi* lack the *V. fischeri* *LuxI/LuxR* system, but the quorum-sensing master regulator *HapR* in *V. cholerae* and its homolog in *V. harveyi* are regulated by CRP (54–56). *V. cholerae* mutants defective in CRP express lower levels of *hapR*, resulting in increased production of cholera toxin and enhanced biofilm formation (54, 55). Similarly, CRP indirectly regulates the pheromone synthase *luxS* in *E. coli* (57), and CRP activates transcription of the acyl homoserine lactone-dependent regulators encoded by *lasR* and *expR* in *Pseudomonas aeruginosa* and *Erwinia chrysanthemi*, respectively (58, 59). Further afield taxonomically from *V. fischeri*, in *Staphylococcus aureus* the *Agr* pheromone signaling system regulates virulence and antibiotic resistance and is modulated by carbon-catabolite protein A (CcpA) (60).

Taken together, these studies strongly suggest that pheromone signaling in diverse bacteria communicates carbon source availability as well as cell density. The trend appears to be upregulation of pheromone synthases and/or receptors when the preferred carbon source, glucose, is absent. This phenomenon is easily rationalized in the many instances where pheromones control expression of exoenzymes that help cells scavenge for alternative growth substrates, but it is less clear why, for example, regulation of luminescence would be tied to growth in the absence of glucose. Importantly, pheromone-controlled genes are not simply coregulated by CRP; rather, pheromone signaling itself is controlled. Thus, it is clear that pheromone levels will reflect environmental context as well as cell density, and future studies should take this fact into account when building models to explain the biological role(s) of these systems.

**Role of glucose in luminescence regulation.** The regulatory role of glucose in *V. fischeri* has been difficult to interpret due in

part to potential strain-specific effects. Previous research showed that luminescence in strain MJ-1 is decreased when glucose is added (27), yet glucose was reported to have no effect in ES114 (14). We found that glucose modulates luminescence in *V. fischeri* ES114, and this phenomenon appears to involve gene regulation and not simply metabolic changes. Our results also show that depending on when a culture is sampled, one might see positive, negative, or no apparent effect at all of glucose on ES114 (Fig. 1). We found that C8-HSL accumulation was lower when ES114 was grown with glucose (Fig. 8), and the opposite effects of glucose on luminescence at low or high cell densities (Fig. 1) might reflect the contrary roles of C8-HSL. In ES114, at low to moderate cell densities, luminescence expression is primarily driven by LuxR combined with C8-HSL (11); however, as cell density increases, C8-HSL competes with the stronger activator 3-oxo-C6-HSL for binding with LuxR, such that C8-HSL becomes effectively an inhibitor of luminescence (11, 37), as was seen in MJ-1 (8, 61). We speculate that in ES114 grown with glucose, there is less active cAMP-CRP to promote expression of *ainS*, leading to less C8-HSL production and opposing effects on luminescence in the absence or presence of stimulatory 3-oxo-C6-HSL at low or high OD, respectively. Such a scenario would lead to low C8-HSL levels throughout growth with effects on luminescence that vary depending on cell density.

With respect to this model, one confounding result was the observation of higher C8-HSL levels in a  $\Delta crp$  mutant than in ES114 when both were grown on glucose (Fig. 8). These data seem most directly in conflict with the results shown in Fig. 3 and 4, which indicate decreased expression of *ainS* in the  $\Delta crp$  mutant, leading us to predict less C8-HSL in the  $\Delta crp$  mutant, not more. Alternative explanations for this apparent contradiction include cAMP-CRP activating (i) a protease involved in AinS protein turnover, (ii) an unknown enzyme capable of C8-HSL degradation, and/or (iii) pathways competing with AinS for the same substrates. These three scenarios, and probably others, could result in increased AinS and/or C8-HSL accumulation in a  $crp$  mutant despite lower *ainS* transcription. In interpreting these results, it is important to keep in mind that while glucose modulates cAMP-CRP levels, the  $\Delta crp$  mutation has more profound effects by eliminating CRP altogether. Thus, while deleting *crp* is useful for elucidating regulatory connections, the  $\Delta crp$  mutant is probably unlike the wild type on any carbon source. We believe it remains possible that the effect of glucose on C8-HSL levels in ES114 reflects *ainS* regulation by cAMP-CRP; however, we cannot rule out the possibility of another glucose-responsive system being involved in AinS regulation and/or C8-HSL accumulation.

**CRP and regulation of *luxR*.** Our data support the previous model (16, 17, 26) that cAMP-CRP directly regulates *luxR* (Fig. 3, 4, and 5). The intergenic *lux* region has diverged significantly between ES114, MJ-1, and other *V. fischeri* strains, and while a putative CRP binding site is evident in the same location in a variety of strains, the exact sequence of the site does vary (53). The sequence revealed in CRP footprinting analyses of *V. fischeri* ATCC 7744 DNA by Shadel et al. (26) aligns with the ES114 sequence used in our binding studies (Fig. 5), although there are several differences in the sequences, particularly in the variable central region of the binding site (53). In addition to demonstrating binding, using a transgenic *E. coli* system we found that the effect of CRP on *luxR* requires only the CRP-activating region I, consistent with this effect being mediated by a class I-type promoter interac-

tion. Defining the *luxR* promoter(s) and transcriptional start site(s) in *V. fischeri* ES114 would provide a more detailed picture of the mechanism by which CRP regulates this gene.

**CRP and regulation of *ainS*.** We were surprised to find that when *luxR* expression was artificially held constant, addition of cAMP still influenced luminescence (Fig. 6), leading us to investigate other luminescence regulators that might be controlled by CRP. Bioinformatic analysis revealed a potential CRP binding site upstream of *ainS*, and we subsequently found evidence that CRP regulates *ainS*. In addition, we show that LitR is important for CRP-dependent *ainS* expression, and this effect appears independent of CRP-activating region I or II (Fig. 7B). It is tempting to speculate that these data reflect a class III promoter interaction involving LitR, although such a mechanism remains to be tested. Moreover, at least in *E. coli*, an additional layer of CRP- and ARI-dependent regulation represses *ainS* expression (Fig. 7C). These new wrinkles in the *ainS* and *lux* regulatory circuitry are depicted in Fig. 9, although some, particularly the mechanism of CRP-dependent *ainS* repression, remain speculative. We hope in the future to further elucidate regulators of *ainS*, which may also inform our understanding of CRP's role at this promoter.

**CRP and regulation of *luxI*.** Interestingly, we also found that CRP may directly regulate transcription of *luxI*. In transgenic *E. coli* carrying the MJ-1 *lux* genes, CRP appeared to inhibit *luxI* transcription in the absence of LuxR (17); however, in our transgenic *E. coli* system using an ES114-derived *lux* promoter, we observed the opposite effect (Fig. 7D). The spacing of the CRP binding site to the *luxI* promoter differs by one nucleotide between strains ES114 and MJ-1 (53) (see Fig. S1 in the supplemental material). We do not know whether direct regulation of *luxI* by CRP is relevant in *V. fischeri*, where the effects of CRP on *luxR* and *ainS* may overpower direct modulation of the *luxICDABEG* promoter. If cAMP-CRP has a significant and direct effect on the *luxI* promoter, it would probably be at very low cell densities where a lack of pheromone accumulation renders LuxR inactive and effects on *ainS* expression irrelevant. In the future, studies focused on low cell densities may reveal if CRP plays a role in modulating the basal LuxR-independent expression of the *luxICDABEG* operon.

**Studying cAMP-CRP in the ES114 model.** We have found that cAMP-CRP regulates luminescence and pheromone signaling in *V. fischeri* ES114. It remains to be determined exactly how environmental conditions are connected to cAMP and CRP levels in this bacterium or if there are other regulatory connections between either CRP or carbon source and pheromone signaling. By laying the groundwork using strain ES114, it should be possible to explore these areas and examine the relevance of cAMP-CRP-mediated regulation in a natural symbiotic infection.

## ACKNOWLEDGMENTS

We thank Anne Weeks and Alecia N. Septer for technical assistance and Stephen J. W. Busby for *E. coli* strains M182 and M182 $\Delta crp$ , the pRW50 promoterless *lacZ $\alpha$*  vector, and promoter-reporter constructs. We also thank Gary P. Roberts for the pEXT20 expression vector and *E. coli* strain UQ3811, utilized in the purification of CRP.

This research was supported by the National Science Foundation (NSF) under grants CAREER MCB-0347317, OCE-0929081, and IOS-1121106.

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