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The Iron-Dependent Regulator Fur Controls Pheromone Signaling Systems and Luminescence in the Squid Symbiont *Vibrio fischeri* ES114

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Bacteria often use pheromones to coordinate group behaviors in specific environments. While high cell density is required for pheromones to achieve stimulatory levels, environmental cues can also influence pheromone accumulation and signaling. For the squid symbiont *Vibrio fischeri* ES114, bioluminescence requires pheromone-mediated regulation, and this signaling is induced in the host to a greater extent than in culture, even at an equivalent cell density. Our goal is to better understand this environment-specific control over pheromone signaling and bioluminescence. Previous work with *V. fischeri* MJ1 showed that iron limitation induces luminescence, and we recently found that ES114 encounters a low-iron environment in its host. Here we show that ES114 induces luminescence at lower cell density and achieves brighter luminescence in low-iron media. This iron-dependent effect on luminescence required ferric uptake regulator (Fur), which we propose influences two pheromone signaling master regulators, LitR and LuxR. Genetic and bioinformatic analyses suggested that under low-iron conditions, Fur-mediated repression of *litR* is relieved, enabling more LitR to perform its established role as an activator of *luxR*. Interestingly, Fur may similarly control the LitR homolog SmcR of *Vibrio vulnificus*. These results reveal an intriguing regulatory link between low-iron conditions, which are often encountered in host tissues, and pheromone-dependent master regulators.

Many bacteria transmit diffusible pheromone signals within and between species to coordinate group functions such as biofilm formation, antibiotic production, and infection. Such signaling is widespread among diverse bacteria (1–3), and it is especially common and well studied among the *Proteobacteria*, which use various signals, including acyl-homoserine lactones (4, 5). The accumulation of pheromones to stimulatory levels often depends on high cell densities, giving rise to the term "quorum sensing" to describe such behavior (6); however, environmentally responsive regulators control both the synthesis of pheromones and responsiveness to these signals, rendering such signaling dependent on environmental context as well as cell density (7–9). We have sought to elucidate this interconnection of environment-specific regulation and pheromone signaling in the model symbiont *Vibrio fischeri*.

V. fischeri is a bioluminescent gammaproteobacterium that monospecifically colonizes the light organ of the Hawaiian bobtail squid, *Euprymna scolopes* (10, 11). Bioluminescence is a colonization factor for *V. fischeri* (12, 13), and it is regulated in part by LuxR-LuxI pheromone-mediated regulation, as described below (14). This highly tractable symbiosis serves as a model system for studying host-microbe interactions and how bacterial pheromone-mediated gene regulation functions during a natural infection (15).

In *V. fischeri*, the *luxCDABEG* genes underlie bioluminescence and are downstream of *luxI* in the *lux* operon (Fig. 1). LuxI produces the pheromone autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3OC6) (16), which can combine with LuxR to activate expression of the *lux* operon (17–19). Bioluminescence in *V. fischeri* is influenced by two additional autoinducers; octanoyl-homoserine lactone (C8) (20, 21) and the product of LuxS (22), which is called autoinducer-2 (AI-2). Figure 1 illustrates a current model of the interconnected signaling cascades of 3OC6, C8, and AI-2, based on homology to other systems and studies of *V. fischeri* (20, 22–28).

In *V. fischeri*, LuxR and LitR are considered pheromone-dependent master regulators. Based on the current model (Fig. 1), LuxR activates transcription of the *lux* operon and other genes in response to 3OC6 and, to a lesser extent, in response to C8. LitR levels are enhanced by elevated levels of C8 or AI-2, and LitR activates transcription of *luxR* and other genes (29). *V. fischeri* LuxR-type regulators are absent from most *Vibrio* species, but LitR is a homolog of the quorum-sensing master regulators in *Vibrio cholerae* (30), *Vibrio parahaemolyticus* (31), *Vibrio vulnificus* (32), and *Vibrio harveyi* (33).

The influence of environmental context on pheromone-dependent regulation is dramatically evident in *V. fischeri* ES114, a strain typical of isolates from the *E. scolopes* light organ. Even at similar high cell densities, ES114 cells produce less 3OC6 and are ~1,000 times dimmer in culture than in the host (34). Moreover, *lux* expression appears heterogeneous in different light organ microenvironments (35). Recent work has identified several regulatory inputs controlling ES114's pheromone signal systems (36–38). Others demonstrated a link between low iron levels and increased luminescence in strain MJ1 (39). In transgenic *Escherichia coli*, the ferric uptake regulator (Fur) did not directly control the *luxR-luxICDABEG* locus (40); however, this experimental setup would not have accounted for regulation through LitR or other regulators that are absent from *E. coli*.

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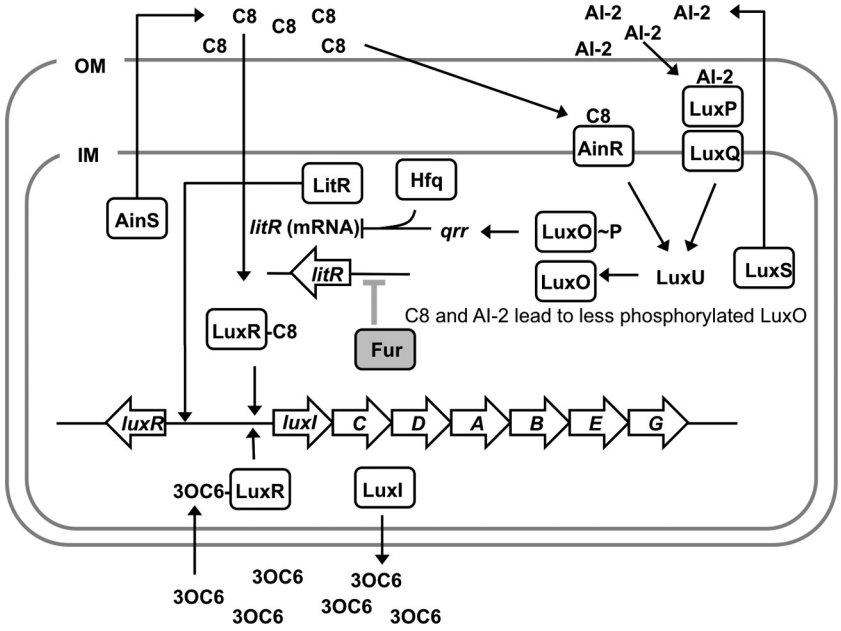

FIG 1 Model of pheromone-mediated regulation of the *lux* operon in *V. fischeri* ES114. Large block arrows correspond to genes including *luxR* (encoding a pheromone-dependent transcriptional regulator [VF_A0925]), *luxI* (encoding an acyl-homoserine lactone synthase [VF_A0924]), genes for bioluminescence (*luxCDABEG* [VF_A0918 to VF_A0923]), and *litR* (encoding another pheromone-controlled regulator [VF_2177]). 3OC6 and, to a lesser extent, C8 bind LuxR and enable it to stimulate transcription of the *lux* operon (among other genes). C8 and AI-2 are thought to be detected by AinR and LuxP/LuxQ, respectively. When C8 and AI-2 levels are elevated, AinR and LuxQ initiate a regulatory cascade via LuxU, resulting in less phosphorylation of LuxO. Phosphorylated LuxO (LuxO-P) increases transcription levels of the regulatory RNA Qrr, which, together with Hfq, represses expression of LitR. LitR activates transcription of LuxR, among other genes. Thus, C8 and AI-2 lead to increased levels of LitR in a pheromone signaling circuit conserved in many *Vibrio* species. This model is derived from experimental data, genomic predictions, and work with related bacterial species (see the text) (reviewed in reference 44). The putative role of Fur in the regulatory circuit, as described in this paper, is highlighted in gray. OM, outer membrane; IM, inner membrane. (Reprinted from reference 37.)

Low iron levels are often faced by symbiotic bacteria in host tissues and have been implicated in the *V. fischeri*-squid symbiosis (41, 42). For example, we recently demonstrated that the heme uptake system in *V. fischeri* ES114 is repressed by Fur but is induced under low-iron conditions and during symbiotic colonization (42). We therefore investigated the possible connection between iron and luminescence regulation in *V. fischeri* ES114. Strain ES114 is significantly different from MJ1 (43, 44), which was isolated from a fish, and the response of ES114 to iron is not well understood. Here we describe how changes in iron levels influence luminescence through Fur-mediated regulation of LitR.

MATERIALS AND METHODS

Media and growth conditions. *V. fischeri* strains were grown in either LBS medium (45), ASWT medium (42), or SWTO medium (36) at 28°C or 24°C. *E. coli* strains were grown in either LB medium (46) or brain heart infusion broth (Difco) at 37°C. Antibiotic selection for *V. fischeri* and *E. coli* strains was performed as described previously (47). Plasmids were maintained in *E. coli* strain DH5α (48), except for plasmids with the R6Kγ origin of replication, which were maintained in strain DH5αλpir (47) or in strain CC118λpir (49), in the case of plasmid pEVS104 (50). As a chelator, ethylenediamine-*N*,*N*′-diacetic acid (EDDA) or 2,2′-bipyridyl was added to *V. fischeri* cultures at a final concentration of 1 μM or 100 μM, respectively, with the latter added from a stock solution prepared at 100 mM in dimethyl sulfoxide (DMSO).

Strain and plasmid construction. Bacterial strains, plasmids, and oligonucleotides used in this study are presented in Table 1. For constructing *V. fischeri* mutants, plasmids bearing mutant alleles were mobilized into *V. fischeri* by triparental mating using CC118λpir pEVS104 as a conjugative helper. Transconjugants were selected with appropriate antibiotics and screened for allelic exchange using PCR and antibiotic resistance markers. To construct the Δ*ryhB* mutant, the sequence upstream of *ryhB* was PCR amplified by using primers prNL66 and prNL67.2 and cloned into pCR-Blunt II-TOPO, resulting in plasmid pNL35. The sequence downstream of *ryhB* was PCR amplified by using primers prNL68 and prNL69 and cloned into SmaI-digested pEVS122, resulting in plasmid pNL36. AvrII-digested pNL35 was ligated into AvrII-digested pNL36, resulting in plasmid pNL49. The Δ*ryhB* allele on plasmid pNL49 was exchanged into wild-type *V. fischeri* ES114, resulting in strain NL58. To construct the Δ*fur litR* double mutant, the *litR*::*erm* allele on plasmid pJLB96 was exchanged into *V. fischeri* Δ*fur* strain YLM111, resulting in strain ANS63. To construct the *V. fischeri* ES114 *litR* promoter-reporter plasmid, a region containing 374 bp upstream of the ATG start codon and 71 bp into the coding region of *litR* in *V. fischeri* ES114 was PCR amplified with primers ASlitRP2 and ASlitRP3. This product was digested with SphI and NheI and then cloned into the same restriction sites of pAKD701 to generate the P_{litR}-*lacZ* transcriptional reporter plasmid pAS120. To construct the *V. vulnificus* C7184 *smcR* promoter reporter plasmid, a region containing 391 bp upstream of the ATG start codon and 19 bp into the coding region of *smcR* in *V. vulnificus* C7184 was PCR amplified with primers ASvv1634P1 and ASvv1634P2. This product was digested with SphI and NheI and then cloned into the same restriction sites of pAKD701 to generate the P_{smcR}-*lacZ* transcriptional reporter plasmid pAS123. To construct the *V. cholerae hapR* promoter reporter plasmid, a region containing 295 bp upstream of the ATG start codon and 24 bp into the coding region of *hapR* in *V. cholerae* CB98-41 was PCR amplified with primers ASvcA0115P1 and ASvcA0115P2. This product was digested with SphI and NheI and then

TABLE 1 Strains, plasmids, and oligonucleotides used in this work

Strain, plasmid, or oligonucleotide	Relevant characteristic(s)[a]	Reference or source
Strains		
Escherichia coli		
DH5α	F′ *endA1 hsdR17 glnV44 thi-1 recA1 gyrA96* (Nx^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR*[ϕ80d*lacI*Δ(*lacZ*)M15]	48
DH5αλpir	λ*pir* derivative of DH5α	47
CC118λpir	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	49
Vibrio vulnificus C7184	Wild-type strain	Brett Macey
Vibrio cholerae CB98-41	Wild-type strain	Christopher J. Grim
Vibrio fischeri		
ANS63	ES114 *litR*::*erm* Δ*fur*	This study
ES114	Wild-type isolate from *E. scolopes* light organ	51
JB13	ES114 *luxO*::*erm*	36
JB19	ES114 *litR*::*erm*	36
JB22	ES114 *lacI*^{q} $P_{A1/34}$-*luxCDABEG*	12
NL58	ES114 Δ*ryhB*	this study
YLM111	ES114 Δ*fur*	42
Plasmids		
pAKD701	Promoterless *lacZ*; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Kn^r	53
pAKD702	Promoterless *lacZ*; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Cm^r	43
pAKD912	pAKD701 containing the ES114 VF_1225 promoter region; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Kn^r	42
pAS120	pAKD701 containing the ES114 *litR* promoter region; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Kn^r	This study
pAS123	pAKD701 containing the *V. vulnificus* C7184 *smcR* promoter region; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Kn^r	This study
pAS128	pAKD701 containing the *V. cholerae* CB98-41 *hapR* promoter region; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Kn^r	This study
pEVS104	Conjugative helper; $oriV_{R6K\gamma}$ *oriT* Kn^r	50
pEVS122	$oriV_{R6K\gamma}$ *oriT* Erm^r	47
pJLB96	*litR*::*erm* allele; $oriV_{ColE1}$ *oriT* Erm^r Cm^r	36
pJLB170	pAKD702 containing the ES114 *luxR* promoter region; $oriV_{R6K\gamma}$ $oriV_{pES213}$ *oriT* Cm^r	43
pNL49	Δ*ryhB* allele; $oriV_{R6K\gamma}$ $oriV_{ColE1}$ *oriT* Erm^r Kn^r	This study
Oligonucleotides[b]		
prNL66	GGCGGTAATGCTGCCTGTTGCCCAAGGCATAAA	This study
prNL67.2	GGCCCCTAGGAAATAGTGCGGATAACTCCGTGTGCGTATTCCCT	This study
prNL68	GGCCCCTAGGAGCAGTGGTGGACGTACAAACGTATTACCA	This study
prNL69	CCAATAAGGTTCGCCACCATGTAATCTAAACTATCGGTTTC	This study
ASlitRP2	TAGCTAGCATATCAAGTAATTGTTCTTTGC	This study
ASlitRP3	TAGCATGCACTATCTCACTTATTCGTTG	This study
ASvv1634P1	TAGCATGCACTGTACTCAATGTTTTATAGTTGC	This study
ASvv1634P2	TAGCTAGCTCTTTGCGATTGAGTCCATAG	This study
ASvcA0115P1	TAGCATGCACCATTCTCGTTGTGTTGG	This study
ASvcA0115P2	TAGCTAGCGCGTTTTTCGATTGATGCG	This study

[a] Kn^r, kanamycin resistance; Cm^r and *cat*, chloramphenicol resistance; Erm^r and *erm*, erythromycin resistance; Nx^r, nalidixic acid resistance. Plasmid replication origins are designated *oriV* with a subscript, indicating the source, and *oriT* indicates the RP4 origin of transfer.
[b] Oligonucleotides are in the 5′-to-3′ orientation, with introduced restriction sites underlined.

cloned into the same restriction sites of pAKD701 to generate the P_{hapR}-*lacZ* transcriptional reporter plasmid pAS128.

Luminescence assays. To assay luminescence, *V. fischeri* cultures were grown overnight in LBS medium and diluted 1:1,000 into either 25 ml SWTO medium in 125-ml flasks or 50 ml SWTO medium in 250-ml flasks. Media were supplemented with 43 μM or 2 mM $FeSO_4$ or with 20 mM trisodium citrate, as indicated. Cultures were incubated at 24°C with shaking at 200 rpm. At the indicated time points, 0.5-ml samples were removed, and the cell density was measured at a 595-nm wavelength (optical density at 595 nm [OD_{595}]), using a BioPhotometer (Brinkman Instruments, Westbury, NY). The cuvette was then shaken to aerate the sample, and luminescence was measured by using a Glomax 20/20 luminometer (Promega, Madison, WI) with a 10-s integration setting. Luminescence values were normalized to the OD_{595}.

β-Galactosidase assays. *V. fischeri* strains harboring *lacZ*-based transcriptional reporter plasmids were grown as described above for luminescence assays. For strains containing reporter plasmids pAKD912 and pJLB170, cells were harvested at an OD_{595} of ~1.0, while strains harboring reporter plasmid pAS120 (P_{litR}), pAS123 (P_{smcR}), or pAS128 (P_{hapR}) were harvested at an OD_{595} of ~0.5. Cells were collected by centrifugation, and the supernatant was discarded. Cell pellets were frozen at −20°C overnight, and β-galactosidase assays were performed to determine Miller

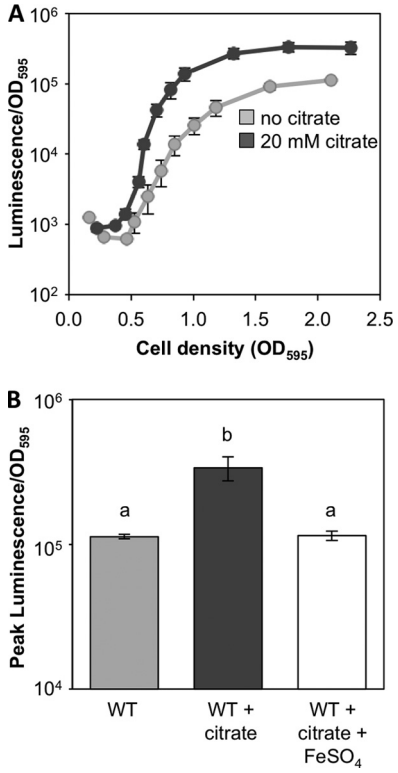

FIG 2 Effect of citrate on luminescence. Shown is luminescence as a function of cell density (A) or peak luminescence per OD_{595} (B) for wild-type (WT) ES114 cultures grown in aerobic shake flasks in SWTO medium supplemented with 43 μM $FeSO_4$ without further additions or supplemented with 20 mM citrate or with 20 mM citrate and 2 mM additional $FeSO_4$. In panel B, lowercase letters shared between bars indicate no statistically significant difference ($P > 0.9$), whereas different letters indicate a significant difference ($P < 0.001$), based on a one-way analysis of variance and Tukey's honestly significant difference test. Data are representative of at least three independent experiments. Error bars (some too small to visualize) indicate standard deviations ($n = 3$) for the one experiment shown in each panel.

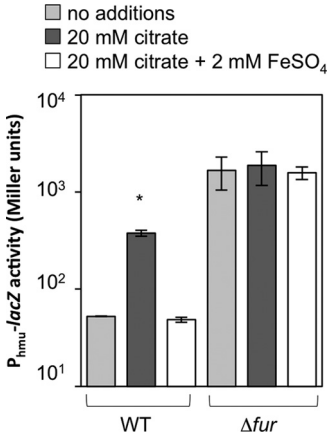

FIG 3 Citrate addition affects expression of a Fur-regulated reporter. Shown is β-galactosidase reporter activity for wild-type and Δ*fur* mutant *V. fischeri* strains with the Fur-dependent P_{VF_1225}-*lacZ* reporter plasmid pAKD912 grown in SWTO medium supplemented with 43 μM $FeSO_4$ without further additions or supplemented with 20 mM citrate or with 20 mM citrate and 2 mM additional $FeSO_4$. Cells were harvested at an OD_{595} of ~1.0. The asterisk indicates a significant difference from other medium conditions within a strain ($P < 0.001$), based on an analysis of variance and Tukey's honestly significant difference test. Data are representative of at least three independent experiments. Error bars indicate standard deviations ($n = 2$).

units, as described previously (12). All β-galactosidase assays were performed with *V. fischeri*.

Nucleotide sequence accession numbers. Sequences for the fragments upstream of *smcR* and *hapR* were deposited in GenBank under accession numbers JX519291 and JX519292, respectively.

RESULTS

Iron limitation affects luminescence in *V. fischeri* ES114. To manipulate the iron available to *V. fischeri* ES114, we supplemented the medium with a chelator and/or ferrous sulfate. In medium supplemented with 20 mM trisodium citrate as an iron chelator, ES114 induced luminescence at a lower OD_{595} and displayed an approximately 3- to 4-fold increase in peak luminescence (Fig. 2A). To test whether this effect on luminescence was the result of sodium ions or their influence on osmolarity (54), 60 mM NaCl was added to cultures, which had no effect on growth or luminescence under these conditions (data not shown).

Supplementing the medium with an alternative iron chelator, either EDDA or 2,2′-bipyridyl, also resulted in earlier luminescence by ES114 (data not shown). However, addition of 2,2′-bipyridyl or EDDA inhibited ES114 growth, possibly owing to these chelators' reported cell permeability (55, 56), and we found it difficult to reproducibly limit iron without restricting growth severely. In addition to acting as a chelator, citrate can also be used as a carbon source by ES114; however, we found that citrate had similar effects on luminescence in a citrate synthase and aconitase double mutant that cannot metabolize citrate (data not shown), suggesting that the citrate addition was a useful nontoxic approach to manipulate availability of extracellular iron for luminescence assays.

To test further whether the effect of citrate on luminescence was due to iron limitation, we added iron to the medium along with citrate. The brighter luminescence of wild-type cultures supplemented with citrate as a chelator was reversed by additional supplementation with 2 mM $FeSO_4$ (Fig. 2B). These data suggest that 20 mM citrate leads to an increase in luminescence in ES114 as a result of citrate's chelating effect lowering iron availability.

Citrate supplementation causes derepression of the Fur-regulated heme uptake system. Previous studies of members of the *Vibrionaceae* found that Fur mediates many responses to iron limitation (57–59). Typically, under iron-replete conditions, coordination of one Fe^{2+} to each Fur monomer allows dimerized Fur to bind DNA at a "Fur box" and repress transcription, while low-iron conditions result in derepression of Fur-regulated genes (60). To test whether the addition of 20 mM exogenous citrate causes derepression of the Fur regulon, we assayed expression of the Fur-repressed heme uptake gene cluster promoter using the *lacZ* transcriptional reporter on plasmid pAKD912. This transcriptional reporter was previously shown to have elevated activity under low-iron conditions in a Fur-dependent manner (42). This reporter showed greater activity in the Δ*fur* mutant than in the wild type, and, as we predicted, neither citrate nor iron supplementation affected the reporter in the Δ*fur* background (Fig. 3). In contrast, in the wild-type background, the reporter was derepressed in medium containing citrate, and this elevated expression level was reversed by supplementation with 2 mM iron (Fig. 3). These data indicate that supplementing the medium with citrate results in

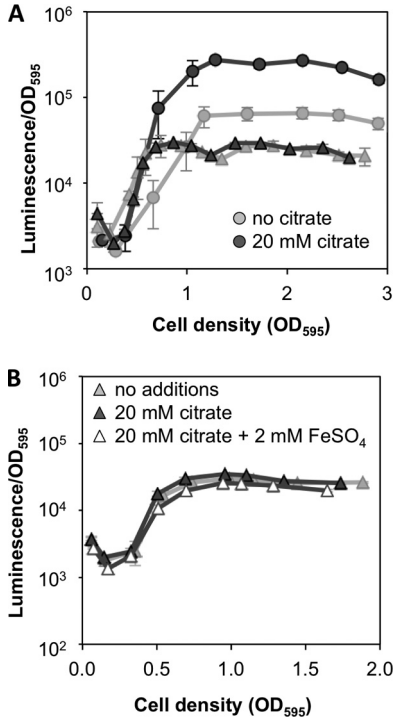

FIG 4 Role of Fur in the response of luminescence to low iron. (A) Cultures of wild-type ES114 (circles) or the Δ*fur* mutant YLM111 (triangles) were grown in aerobic shake flasks in SWTO medium supplemented with 43 μM $FeSO_4$ and either 20 mM citrate or no citrate. Error bars indicate standard deviations ($n = 2$). (B) Cultures of Δ*fur* mutant strain YLM111 were grown in SWTO medium supplemented with 43 μM $FeSO_4$ without further additions or supplemented with 20 mM citrate or with 20 mM citrate and 2 mM additional $FeSO_4$. Error bars (some too small to visualize) indicate standard deviations ($n = 3$). The data in each panel are from one experiment representative of at least three independent experiments.

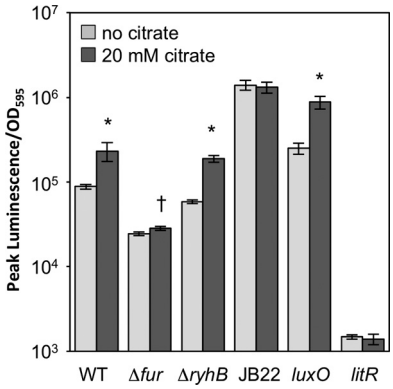

FIG 5 Regulatory determinants of luminescence induction in response to low iron. *V. fischeri* cultures were grown in aerobic shake flasks in SWTO medium supplemented with 43 μM $FeSO_4$ either without or with 20 mM citrate. Error bars indicate standard deviations ($n = 3$). Asterisks indicate a significant effect of citrate addition on luminescence of the strain ($P < 0.03$), as determined by using Student's *t* test, while the dagger indicates variable statistical significance (*P* value ranged from 0.0001 to 0.73 in 11 experiments [see the text for details]). In the representative experiment shown, citrate addition to Δ*fur* mutant cultures resulted in a small (16%) but statistically significant ($P < 0.05$) increase in peak luminescence. Data shown were collected from the same experiment and are representative of at least three independent experiments for each strain.

derepression of Fur-regulated transcripts, such as those encoding the heme uptake system.

The effect of iron limitation on luminescence is largely Fur dependent. Given the results described above and the prominent role of Fur in other members of the *Vibrionaceae*, we hypothesized that Fur may modulate luminescence in response to iron levels, repressing luminescence when cells are iron replete. Consistent with our hypothesis, Δ*fur* mutant cultures showed enhanced induction of luminescence at a low OD_{595} (less than 1.0), similar to that observed for wild-type cultures supplemented with citrate (Fig. 4A). The luminescence of Δ*fur* mutant cultures did not attain the same maximal luminescence level as that of the wild-type cultures at a higher OD_{595}; however, citrate had little effect on luminescence in the Δ*fur* mutant at any cell density (Fig. 4A). Citrate did not alter the timing of luminescence induction in the Δ*fur* mutant (Fig. 4A), and addition of 2 mM $FeSO_4$ to citrate-supplemented Δ*fur* mutant cultures did not affect luminescence (Fig. 4B).

In 8 out of 11 experiments, we observed a small (8 to 29%) but statistically significant ($P < 0.05$) increase in peak luminescence for the Δ*fur* mutant in the presence of citrate. The magnitude of this difference is so small that it may not be apparent on the log-scale *y* axes of Fig. 4 and 5, despite statistical significance. Thus, taken together, our data suggest the likely possibility of a *fur*-independent effect of citrate on luminescence. Importantly, however, such a <30% *fur*-independent effect of citrate on luminescence would appear too small to account for the >300% effect in the wild type. Moreover, the results of adding iron suggest that any small increase in luminescence observed for citrate-supplemented Δ*fur* mutant cultures is not an iron-mediated effect. Taken together, the data described above indicate that decreased iron availability induces brighter luminescence in ES114 when iron chelators are added to cultures and that this response requires Fur-mediated regulation.

Iron-mediated regulation of luminescence is independent of RyhB. In other organisms, many of Fur's effects are mediated by its regulation of the small regulatory RNA RyhB (61–63), and we therefore wanted to determine whether iron limitation influences luminescence indirectly through RyhB. To test this possibility, we assayed the effect of citrate addition on luminescence of a Δ*ryhB* mutant and found that citrate addition increased luminescence similar to the increased brightness observed in wild-type cultures (Fig. 5). This result indicates that Fur influences luminescence in response to citrate independently of RyhB.

Chelator-mediated luminescence induction requires native LuxR-LuxI regulation. We considered the possibility that citrate-mediated iron limitation and derepression of the Fur regulon might influence luminescence through metabolic changes influencing bioluminescence rather than by affecting expression of the *lux* operon. To test whether native LuxR-LuxI regulation of luminescence was required for citrate-mediated enhancement of luminescence, we used strain JB22, which has the genes directly responsible for bioluminescence (*luxCDABEG*) under the control of a constitutive nonnative promoter. Addition of citrate to JB22 cultures did not result in any change in luminescence (Fig. 5), indicating that this effect of citrate is dependent on regulation of the native *luxI* promoter, which requires LuxR-mediated activation. Although JB22 is brighter than ES114 under these conditions, the luminescence of JB22 is still 2 to 3 orders of magnitude

FIG 6 Virtual footprint analysis of possible Fur binding sites. (A) Comparison of the sequence logo of the *E. coli* Fur binding site position weight matrix (PWM) used in the virtual footprint analysis of the *V. fischeri* ES114 genome (http://prodoric.tu-bs.de/vfp/) and putative Fur binding sites identified upstream of the Fur-regulated heme uptake cluster gene VF_1225 and the *litR* gene. For the weighted matrix, the *y* axis indicates bit scores for each nucleotide, and the *x* axis indicates the Fur box nucleotide position. The putative Fur binding site upstream of VF_1225 is located 44 bp upstream of the ATG codon and has a score of 16.22, while the site upstream of *litR* is located 40 bp upstream of the ATG codon, with a score of 19.63. Nucleotides that are identical between the two putative binding sites are in boldface type, and nucleotides identical to bases in the PWM sequence logo are shaded with the corresponding nucleotide color. (B) Position of the putative Fur binding site upstream of *litR*. The *litR* translational start site is in boldface type, the putative Fur binding site is highlighted in yellow, and possible −10 and −35 sequences are underlined.

lower than its maximal luminescence capacity (12), suggesting that if citrate mediated a luminescence-enhancing effect independent of native *lux* transcription, we would still see enhanced luminescence in JB22 despite its higher basal luminescence.

Bioinformatic analysis identifies a putative Fur binding site upstream of *litR*. To investigate the mechanism of Fur-mediated regulation of luminescence, we performed a virtual footprint analysis (64) to locate putative Fur binding sites in the *V. fischeri* genome, searching for matches to a weighted 18-bp Fur box determined in *E. coli* (Fig. 6A). As a frame of reference, this analysis returned a position weight matrix (PWM) score of 16.22 for a putative Fur box upstream of the heme uptake/utilization cluster (i.e., upstream of VF_1225), which is known to be Fur regulated (e.g., see reporter data in Fig. 3). Among other putative Fur binding sites elsewhere in the *V. fischeri* genome, we identified a site in the sequence upstream of the *litR* gene with a PWM score of 19.63 (Fig. 6A), a better match than in the Fur box of our Fur-dependent reporter. Moreover, the putative Fur box upstream of *litR* appeared embedded between sequences that matched reasonable −10 and −35 transcriptional promoter elements (Fig. 6B). Because LitR is a transcriptional activator of *luxR* (Fig. 1), we further investigated a possible role for LitR in Fur-mediated regulation of luminescence.

***litR* is repressed by Fur and is required for luminescence induction in response to iron limitation.** We hypothesized that Fur represses *litR* under iron-rich conditions, but when iron is limiting, Fur-mediated repression of *litR* is relieved, resulting in elevated levels of LitR, increased *luxR* expression levels, and brighter luminescence. Two pheromone signaling pathways converge at LuxO (Fig. 1), which is upstream of LitR in the regulatory hierarchy. Consistent with our hypothesis, addition of citrate to *luxO* mutant cultures resulted in an increase in luminescence similar to what was observed for wild-type cultures (Fig. 5), indicating that the effect of citrate on luminescence is downstream of LuxO. Next, we added citrate to *litR* mutant cultures and found no change in luminescence (Fig. 5), indicating that the effect of citrate requires *litR* as well as *fur*.

To test if Fur regulates *litR* expression, we constructed a *lacZ*-based *litR* promoter reporter plasmid (pAS120) and assayed for *fur*-dependent regulation. We found elevated P_{litR}-*lacZ* expression levels in the Δ*fur* mutant relative to the wild type (Fig. 7A), suggesting that Fur represses *litR* expression under iron-rich conditions. Based on our model of the pheromone-mediated regulatory hierarchy in *V. fischeri* (Fig. 1), we predicted that Fur's ultimate effect on luminescence is mediated by LitR's activation of *luxR*. To test this possibility, we assayed *luxR* promoter activity in the wild type and the Δ*fur*, *litR*, and Δ*fur litR* mutants. Consistent with our prediction, we observed elevated expression levels of a P_{luxR}-*lacZ* reporter in the Δ*fur* mutant compared to its expression levels in the wild type, and this increase was dependent on *litR*

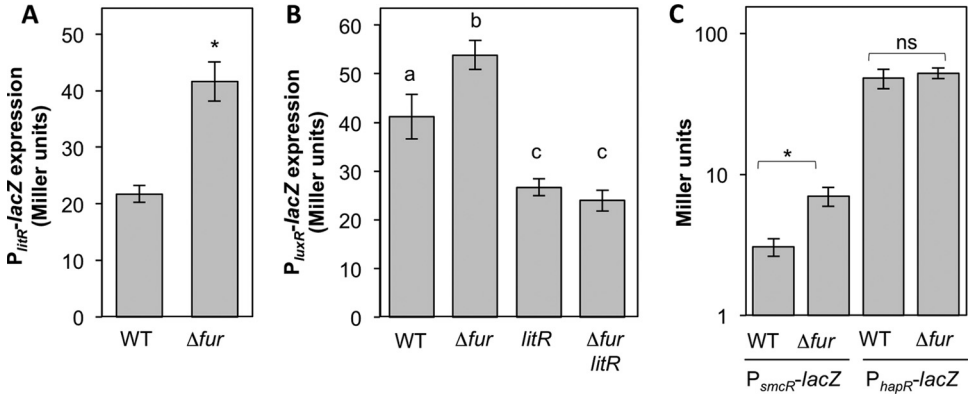

FIG 7 Effects of Fur on *litR*, *luxR*, *smcR*, and *hapR* transcriptional reporters. In all panels, *V. fischeri* cultures of ES114 (wild type), YLM111 (Δ*fur*), JB19 (*litR*::*erm*), or ANS63 (Δ*fur litR*::*erm*) were grown in aerobic shake flasks in SWTO medium supplemented with 43 μM $FeSO_4$. In panels A and C, for *litR*, *smcR*, and *hapR* transcriptional reporters, cells harboring pAS120, pAS123, and pAS128 were harvested at an OD_{595} of ~0.5. In panel B, for the *luxR* transcriptional reporter, cells harboring pJLB170 were harvested at an OD_{595} of ~1.0. Error bars indicate standard deviations ($n = 4$ [A and B] and $n = 3$ [C]). Asterisks in panels A and C indicate a significant difference of the indicated pairwise comparison ($P < 0.005$) by Student's *t* test, while the comparison labeled "ns" was not significant ($P > 0.05$). In panel B, lowercase letters shared between bars indicate no statistically significant difference ($P > 0.2$), whereas different letters indicate a significant difference ($P < 0.001$), based on a one-way analysis of variance and Tukey's honestly significant difference test. Data in each panel are representative of at least three independent experiments.

(Fig. 7B). These data indicate that the iron-dependent regulator Fur ultimately modulates expression of both key pheromone-dependent transcriptional activators, LitR and LuxR, in *V. fischeri*.

Fur also represses expression of the *Vibrio vulnificus litR* homolog *smcR*. Because Fur and LitR homologs are widespread in the *Vibrionaceae*, we asked whether this Fur-mediated regulation of a pheromone signaling master regulator is conserved. Interestingly, a previous study examining the Fur regulons in five sequenced *Vibrio* species identified putative Fur binding sites upstream of genes encoding LitR homologs in *V. parahaemolyticus* and *V. vulnificus* but not in *Vibrio salmonicida* or *V. cholerae* (65). Based on the presence or absence of predicted Fur binding sites determined previously by Ahmad et al. (65), we hypothesized that Fur would repress expression of *V. vulnificus smcR* but not influence expression of *V. cholerae hapR*. To test this, we constructed *lacZ*-based promoter reporter plasmids for *V. vulnificus smcR* (pAS123) and *V. cholerae hapR* (pAS128) and assayed for *fur*-dependent regulation of these reporters in wild-type *V. fischeri* and the *V. fischeri* Δ*fur* mutant. Although the promoters in our constructs were cloned from different strains than those analyzed by Ahmad et al. (65), the presence or absence of putative Fur binding sites was conserved between strains within each species. Consistent with the predictions by Ahmad et al., we found elevated P_{smcR}-*lacZ* expression levels in the Δ*fur* mutant relative to those in the wild type (Fig. 7C); however, P_{hapR}-*lacZ* expression was unaffected by Fur (Fig. 7C). These data suggest the Fur-mediated repression of pheromone signaling master regulators is not limited to the control of LitR in *V. fischeri* and that the consensus Fur binding site described previously by Ahmad et al. is effective at predicting Fur binding sites in members of the *Vibrionaceae*.

DISCUSSION

The accumulation of bacterial pheromones may be influenced by high cell density, but pheromone-mediated regulatory circuits in bacteria are also influenced by environmental factors, indicating that they are not simply census-taking systems. For example, in *V. fischeri*, the LuxR-LuxI pheromone-dependent regulatory system is also controlled by density-independent factors (36–38). Both the pheromone synthase (LuxI) and its cognate pheromone receptor (LuxR) are regulated in response to environmental conditions, as are LuxI and LuxR homologs in other bacteria. Expanding on previous findings (39, 40), we have now shown that iron limitation leads to derepression of Fur-regulated genes (Fig. 3), resulting in a *fur*- and *litR*-dependent increase in luminescence (Fig. 2A and 4A). Based on our data, we propose that this effect is due to a Fur-dependent increase in the level of the LitR quorum-sensing regulator (Fig. 7A), which influences *luxR* expression (Fig. 7B). Because *luxI* is cotranscribed with the genes directly underlying light production, it is likely that this enhanced luminescence parallels an effect on 3OC6 synthesis as well. Thus, elements of the *V. fischeri* pheromone (3OC6-mediated) regulatory circuit are modulated by Fur and iron availability.

This connection between the iron-dependent regulator Fur and pheromone-mediated regulation could be relevant in a natural environment for *V. fischeri*, the host light organ. Previous work studying the *Vibrio*-squid symbiosis indicated that the squid light organ has low iron availability (41, 42). We speculate that the Fur- and LitR-dependent response described above might contribute to luminescence induction in symbiotic cells. Fidopiastis et al. showed previously that while a *litR* mutant achieved wild-type levels of colonization and luminescence in juvenile squid at 24 h postinoculation, the *litR* mutant displayed a 1-h delay in the onset of detectable luminescence compared to the wild type during squid colonization (29). Thus, while LitR-mediated regulation of the LuxR-LuxI regulatory system is not required for luminescence induction in symbiotic cells, given that the light organ appears to be a low-iron environment resulting in derepression of Fur-regulated genes (42), we speculate that Fur-mediated control of *litR* might contribute to the onset of symbiotic luminescence during initial infection.

This model of the role of Fur in symbiotic luminescence induction would be easier to test if it invoked Fur activating *litR* rather than relieving repression of *litR*, because in that case, a *fur* mutant would be predicted to have a phenotype similar to that of a *litR* mutant. Because our model proposes a role for Fur in repressing *litR* under iron-rich culture conditions but not in the host, the symbiotic phenotype of the Δ*fur* mutant is not helpful in testing our model. Future experiments examining the role(s) and levels of LitR in early and late colonization will help elucidate whether the regulatory connection between Fur and LitR has symbiotic significance.

Although LitR regulates bioluminescence through its role as an activator of *luxR* transcription, LitR clearly regulates additional genes, some of which appear to have symbiotic relevance (29). A *litR* mutant outcompeted the wild type in a squid coinfection assay, and it also had altered colony morphology (29). In this study, we noticed modest growth effects of the *litR* mutation, further suggesting that LitR regulates other genes of physiological importance and possibly related to adaptation to low-iron environments.

While the squid light organ has low iron levels, this environmental factor is likely not host specific, because seawater also can be iron limiting. However, while both of these low-iron environments may lead to Fur-mediated derepression of *litR* in *V. fischeri*, only conditions leading to sufficiently high concentrations of 3OC6 pheromone would result in LuxR activation and enhanced luminescence. Therefore, we speculate that when *V. fischeri* is free-living or in the host, these low-iron conditions derepress the Fur regulon, including *litR*, which regulates other functions in addition to *luxR* expression. In free-living cells, pheromone diffuses away; however, in the squid light organ, pheromone levels accumulate to stimulatory levels due to high cell density and other host factors promoting pheromone synthesis, resulting in activation of LuxR and bright luminescence. Future work focused on elucidating LitR-regulated genes in *V. fischeri* may help reveal the connection between iron and LitR and its regulatory role in free-living versus symbiotic cells.

In other vibrios, LitR homologs similarly control a number of functions, and our findings here demonstrate that Fur-mediated regulation of LitR homologs could have implications beyond *V. fischeri*. Most other species of *Vibrio* lack the LuxR-LuxI system, and instead, a LitR homolog acts as the master regulator for pheromone-mediated behaviors. As examples, the LitR homologs in *V. vulnificus* (SmcR), *V. harveyi* ($LuxR^{Vh}$), *V. parahaemolyticus* (OpaR), and *V. cholerae* (HapR) control a range of behaviors and systems, including biofilm formation, type III secretion, toxins, and virulence factors (30, 32, 66–73). Interestingly, previous work by Ahmad et al. identified putative Fur binding sites upstream of *litR*, *smcR*, and *opaR* but not *hapR* (65), and transcriptional reporter assays shown here using wild-type and Δ*fur V. fischeri*

strains demonstrate that Fur represses expression of *litR* and *smcR* but not *hapR* (Fig. 7C). It will be interesting to determine how iron levels and Fur influence the SmcR regulon in *V. vulnificus* and whether Fur also regulates OpaR in *V. parahaemolyticus*. In any case, the reach of these regulons and the evidence for *V. fischeri* suggest that modulation of LitR by Fur could have impacts well beyond the luminescence phenotype described here.

The connection of LitR to Fur begs the question of why the LitR regulon, and possibly the other LitR homologs, would be modulated in response to iron availability in the local environment. Interestingly, for *V. parahaemolyticus*, a previous microarray analysis of transcripts regulated by OpaR included genes that appear to be involved in iron transport (66); however, these were a small portion of the total regulon. Moreover, an iron transport system in *V. vulnificus* was identified previously in a genome-wide search using a consensus SmcR binding sequence (74). While it is intriguing to think that LitR and/or homologs like OpaR and SmcR could be involved in modulating a response to low iron, these regulators also control factors involved in host colonization (66, 71). Thus, Fur might modulate these regulons to enhance expression in response to low iron availability, which is a characteristic typical of many host tissues.

There is similar evidence of iron levels regulating pheromone-mediated signaling in non-*Vibrio* species. For instance, in response to iron limitation, *Pseudomonas aeruginosa* increased transcription levels of *lasR*, which encodes an acyl-homoserine lactone-dependent transcriptional activator homologous to *V. fischeri* LuxR, and LasR-regulated proteins were also significantly modulated by iron limitation (75). A separate study demonstrated a *lasI*- and *lasR*-dependent increase in expression levels of the *lasI* pheromone synthase gene when iron was limited (76). Moreover, work with *Streptococcus pneumoniae* demonstrated that the autoinducer synthase LuxS mediates iron-dependent regulation of biofilm formation and competence (77). While these bacteria do not have LitR homologs, the connection between iron- and pheromone-mediated regulation is intriguing and suggests that iron levels may be a conserved density-independent regulator of pheromone systems in organisms outside the *Vibrionaceae*. Further studies of the connection between Fur and pheromone signaling in *V. fischeri* may elucidate properties that can be generalized to a broader range of bacteria.

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