The iron-dependent regulator Fur controls pheromone-signaling systems and luminescence in the squid symbiont *Vibrio fischeri* ES114

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Running title: Fur regulates *litR* and luminescence in *V. fischeri*
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 but not in culture, even at equivalent cell density. Our goal is to better understand this environment-specific control over pheromone signaling and bioluminescence. Previous work in *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 in 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.
Introduction

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 (4, 54, 74), and it is especially common and well studied among the Proteobacteria, which use various signals including acyl-homoserine lactones (28, 32). The accumulation of pheromones to stimulatory levels often depends on high cell densities, giving rise to the term “quorum sensing” to describe such behavior (27); 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 (20, 25, 61). 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 gamma proteobacterium that monospecifically colonizes the light organ of the Hawaiian bobtail squid, *Euprymna scolopes* (51, 75). Bioluminescence is a colonization factor for *V. fischeri* (10, 73), and it is regulated in part by LuxR-LuxI pheromone-mediated regulation described below (23). 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 (66).

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) (22), which can combine with LuxR to activate expression of the lux operon (2, 15, 58). Bioluminescence in *V. fischeri* is influenced by two additional autoinducers; octanoyl-homoserine lactone (C8) (29, 40), and the product of LuxS (45), which is called 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 in *V. fischeri* (5, 12, 26, 29, 45, 46, 56, 59).

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 (24). *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* (38), *Vibrio parahaemolyticus* (50), *Vibrio vulnificus* (43), and *Vibrio harveyi* (65).

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 ~1000 times dimmer in culture than in the host (7). Moreover, *lux* expression appears heterogeneous in different light-organ microenvironments (19). Recent work has identified several regulatory inputs controlling ES114’s pheromone signal systems (9, 47, 62). Others demonstrated a link between low iron levels and increased luminescence in strain MJ1 (35). In transgenic *Escherichia coli*, the ferric uptake regulator (Fur) did not directly control the *luxR-luxICDABEG* locus (16); however, this experimental setup would not have accounted for regulation through LitR or other regulators that are absent in *E. coli*.

Low iron levels are often faced by symbiotic bacteria in host tissues, and have been implicated in the *V. fischeri*-squid symbiosis (31, 63). 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 (63). We therefore investigated the possible...
connection between iron and luminescence regulation in *V. fischeri* ES114. Strain ES114 is significantly different from MJ1 (11, 70), 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 (68), ASWT medium (63), or SWTO medium (9) at 28°C or 24°C. *E. coli* strains were grown in either LB medium (53) or Brain Heart Infusion (Difco) at 37°C. Antibiotic selection for *V. fischeri* and *E. coli* strains was performed as described previously (18). Plasmids were maintained in *E. coli* strain DH5α (34) except for plasmids with the R6Kγ origin of replication, which were maintained in strain DH5αλpir (18), or in strain CC118λpir (37) in the case of plasmid pEVS104 (69). The chelators ethylenediamine-\(N,N\)-diacetic acid (EDDA) or 2, 2′-bipyridyl, were added to *V. fischeri* cultures at final concentrations of 1 µM and 100 µM, respectively, with the latter added from a stock solution prepared at 100 mM in 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 ΔryhB mutant, sequence upstream
of *ryhB* was PCR amplified using primers prNL66 and prNL67.2 and cloned into pCR-Blunt II-TOPO resulting in plasmid pNL35. Sequence downstream of *ryhB* was PCR amplified using primers prNL68 and prNL69 and cloned into Smal-digested pEVS122 resulting in plasmid pNL36. AvrII-digested pNL35 was ligated to 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 Δfur *litR* double mutant, the *litR::erm* allele on plasmid pJLB96 was exchanged into *V. fischeri* Δfur 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 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*_<i>litR</i>-*lacZ* transcriptional reporter plasmid pAS120. To construct the *V. vulnificus* C7184 *smcR* promoter reporter plasmid, a region containing 391 bp upstream of the ATG 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*_<i>smcR</i>-*lacZ* transcriptional reporter plasmid pAS123. To construct the *V. cholerae* *hapR* promoter reporter plasmid, a region containing 295 bp upstream of the ATG 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 cloned into the same restriction sites of pAKD701 to generate the *P*_<i>hapR</i>-*lacZ* transcriptional reporter plasmid pAS128. Sequences for the fragment upstream of *smcR* and *hapR* were deposited in Genbank, with accession numbers of JX519291 and JX519292, respectively.
Luminescence assays

To assay luminescence, *V. fischeri* cultures were grown overnight in LBS and diluted 1:1000 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 FeSO₄, or with 20 mM trisodium citrate, as indicated. Cultures were incubated at 24°C with shaking at 200 rpm. At indicated time points, 0.5 ml samples were removed and the cell density measured at 595 nm wavelength (OD₅₉₅) using a BioPhotometer (Brinkman Instruments, Westbury, NY). The cuvette was then shaken to aerate the sample and luminescence was measured using a GLOMAX 20/20 luminometer (Promega, Madison, WI) with a 10 sec integration setting. Luminescence values were normalized to OD₅₉₅.

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 OD₅₉₅ of ~1.0, while strains harboring reporter plasmids pAS120 (P₅ᵢᵣ), pAS123 (Pₑₛᵣ), or pAS128 (P₇₉₉₉) were harvested at an OD₅₉₅ 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 units as described previously (10). All β-galactosidase assays were performed in *V. fischeri*.

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 a ~3-4-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 (67), 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 the alternative iron chelators 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 (49, 71), 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 citrate addition was a useful non-toxic 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 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 in members of the *Vibrionaceae* have found that Fur mediates many responses to iron limitation (13, 42, 52). 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 (3). To test whether 20 mM exogenous citrate addition 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 in low-iron conditions in a Fur-dependent manner (63). This reporter showed greater activity in the Δfur mutant than in the wild type, and as we predicted neither citrate nor iron supplementation affected the reporter in the Δfur background (Fig 3). In contrast, in the wild-type background the reporter was derepressed in medium containing citrate, and this elevated expression was reversed by supplementation with 2 mM iron (Fig 3). These data indicate that supplementing the medium with citrate results in 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 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, Δfur mutant cultures showed enhanced induction of luminescence at low (less than 1.0) OD$_{595}$, similar to that observed for wild-type cultures supplemented with citrate (Fig 4A). The luminescence of Δfur mutant cultures did not attain the same maximal luminescence level as that of the wild type at higher OD$_{595}$; however, citrate had little effect on luminescence in the Δfur mutant at any cell
density (Fig 4A). Citrate did not alter the timing of luminescence induction in the Δfur mutant (Fig 4A), and addition of 2 mM FeSO₄ to citrate-supplemented Δfur mutant cultures did not affect luminescence (Fig 4B).

In eight out of eleven experiments we observed a small (8 to 29%) but statistically significant (p<0.05) increase in peak luminescence for the Δfur 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 Figures 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 though, such a <30% fur-independent effect of citrate on luminescence would appear too small to account for the >300% effect in wild type. Moreover, the results of adding iron suggest that any small increase in luminescence observed in citrate-supplemented Δfur mutant cultures is not an iron-mediated effect. Taken together, the data above indicate that decreased iron availability induces bright 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 (14, 48, 60), and we therefore wanted to determine whether iron limitation influences luminescence indirectly through RyhB. To test this, we assayed the effect of citrate addition on luminescence of a ΔryhB 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 control of a constitutive non-native 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 two to three orders of magnitude below its maximal luminescence capacity (10), 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 (57) 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 \( \text{litR} \) appeared embedded between sequences that matched reasonable -10 and -35 transcriptional promoter elements (Fig 6B). Because LitR is a transcriptional activator of \( \text{luxR} \) (Fig 1), we further investigated a possible role for LitR in Fur-mediated regulation of luminescence.

\( \text{litR} \) is repressed by Fur and is required for luminescence induction in response to iron limitation

We hypothesized that Fur represses \( \text{litR} \) under iron-rich conditions, but when iron is limiting Fur-mediated repression of \( \text{litR} \) is relieved resulting in elevated levels of LitR, increased \( \text{luxR} \) expression, and bright 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 \( \text{luxO} \) mutant cultures resulted in an increase in luminescence similar to what was observed for wild-type cultures (Fig 5), indicating the effect of citrate on luminescence is downstream of LuxO. Next we added citrate to \( \text{litR} \) mutant cultures and found no change in luminescence (Fig 5), indicating the effect of citrate requires \( \text{litR} \) as well as \( \text{fur} \).

To test if Fur regulates \( \text{litR} \) expression we constructed a \( \text{lacZ} \)-based \( \text{litR} \) promoter reporter plasmid (pAS120), and assayed for \( \text{fur} \)-dependent regulation. We found elevated \( P_{\text{luxR}-\text{lacZ}} \) expression in the \( \Delta\text{fur} \) mutant relative to the wild type (Fig 7A), suggesting Fur represses \( \text{litR} \) 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 \( \text{luxR} \). To test this possibility, we assayed \( \text{luxR} \) promoter activity in wild type, \( \Delta\text{fur} \), \( \text{litR} \), and \( \Delta\text{fur} \ \text{litR} \) mutants. Consistent with our prediction, we observed elevated expression of a \( P_{\text{luxR}-\text{lacZ}} \) reporter in the \( \Delta\text{fur} \) mutant compared to its
expression in the wild type, and this increase was dependent on litR (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* (1). Based on the presence or absence of predicted Fur binding sites determined by Ahmad et al. (2009), 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 Δfur* mutant. Although the promoters in our constructs were cloned from different strains than those analyzed by Ahmad et al. (2009), 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 in the Δfur mutant relative to the wild type (Fig 7C), however, $P_{hapR}$-lacZ expression was unaffected by Fur (Fig 7C). These data suggest Fur-mediated repression of pheromone-signaling master regulators is not limited to control of LitR in *V. fischeri*, and that the consensus Fur binding site described 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 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 (9, 47, 62). Both the pheromone synthase (LuxI) and its cognate receptor (LuxR) are regulated in response to environmental conditions, as are LuxI and LuxR homologs in other bacteria. Expanding on previous findings (16, 35), 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 LitR quorum-sensing regulator (Fig 7A), which influences luxR expression (Fig 7B). Because luxI is co-transcribed 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 indicates that the squid light organ has low iron availability (31, 63). We speculate that the Fur- and LitR-dependent response described above might contribute to luminescence induction in symbiotic cells. Fidopiastis et al. showed that while a litR mutant achieved wild-type levels of colonization and luminescence in juvenile squid 24 hrs post-inoculation, the litR mutant displayed a 1 hr delay in the onset of detectable
luminescence compared to wild type during squid colonization (24). 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 (63), we speculate that Fur-mediated control of litR might contribute to the onset of symbiotic luminescence during initial infection.

This model of Fur’s role 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 in iron-rich culture conditions but not in the host, the symbiotic phenotype of the Δfur 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 (24). A litR mutant outcompeted wild type in a squid co-infection assay, and it also had altered colony morphology (24). 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, 33, 36, 38, 43, 44, 55, 64, 76, 77). Interestingly, work by Ahmad et al. identified putative Fur binding sites upstream of *litR, smcR* and *opaR*, but not *hapR* (1), and transcriptional reporter assays shown here using wild-type and Δfur *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 in *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.
environment. Interestingly, in *V. parahaemolyticus*, a microarray analysis of transcripts regulated by OpaR included genes that appear to be involved in iron transport (30); however, these were a small portion of the total regulon. Moreover, an iron transport system in *V. vulnificus* was identified in a genome-wide search using a consensus SmcR-binding sequence (41). 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 (30, 64). 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 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 (39). A separate study demonstrated a *lasI* and *lasR*-dependent increase in expression of the *lasI* pheromone synthase gene when iron was limited (8). Moreover, work in *Streptococcus pneumoniae* demonstrated the autoinducer synthase LuxS mediates iron-dependent regulation of biofilm formation and competence (72). While these bacteria do not have LitR homologs, the connection between iron and pheromone-mediated regulation is intriguing and suggests iron levels may be a conserved density-independent regulator of pheromone systems in organisms outside of 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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References


Strains, plasmids, and oligonucleotides used in this work

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| DH5\(\alpha\)                          | \(F'/endA1\ hsdR17\ glnV44\ thi-1\ recA1\ gyrA96\ (N\(\chi\)\) \(relA1\) \\
\(\Delta(lacIZYA-argF)U169deoR(f80dlacI\Delta(lacZ)M15)\) | (34)                |
| DH5\(\alpha\)\(\lambda\)pir           | \(\lambda\)pir derivative of DH5\(\alpha\) | (18)                |
| CC118\(\lambda\)pir                    | \(\Delta(ara-leu)\ araD\ \Delta lac74\ galE\ galK\ phoA20\ thi-1\ rpsE\ rpsB \\
argE(Am) \ recA \ \lambda\)pir | (37)                |
| **Vibrio strains**                     |                                 |                     |
| *Vibrio vulnificus*                    | wild type C7184                 | Brett Macey         |
| *Vibrio cholerae*                      | wild-type strain CB98-41        | Christopher J. Grim |
| *Vibrio fischeri*                      |                                 |                     |
| ANS63                                  | ES114 \(litR::erm, \Delta fur\) | this study          |
| ES114                                  | wild-type isolate from *E. scolopes* light organ | (6)                |
| JB13                                   | ES114 \(luxO::erm\)            | (9)                 |
| JB19                                   | ES114 \(litR::erm\)            | (9)                 |
| JB22                                   | ES114 \(lacP^\chi\ \ Pa\Delta\gamma luxCDABEG\) | (10)               |
| NL58                                   | ES114 \(\Delta yhB\)           | this study          |
| YLM111                                 | ES114 \(\Delta fur\)           | (63)                |
| **Plasmids**                           |                                 |                     |
| pAKD601B                               | \(lac^I\) and IPTG-inducible promoter, \(oriV_{R\&K}\gamma, \(oriV\)_{R\&K}\psi213, \(oriT\), | (17)               |

\(^a\) Characteristics for *Vibrio fischeri* strains were previously published in [16].
Kn\(^R\)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAKD701</td>
<td>promoterless lacZ, ori(<em>{R6K\gamma}), ori(</em>{pES213}), ori(_T), Kn(^R) (21)</td>
</tr>
<tr>
<td>pAKD702</td>
<td>promoterless lacZ, ori(<em>{R6K\gamma}), ori(</em>{pES213}), ori(_T), Cm(^R) (11)</td>
</tr>
<tr>
<td>pAKD912</td>
<td>pAKD701 containing the ES114 VF_1225 promoter region, ori(<em>{R6K\gamma}), ori(</em>{pES213}), ori(_T), Kn(^R) (63)</td>
</tr>
<tr>
<td>pAS120</td>
<td>pAKD701 containing the ES114 litR promoter region, ori(_V) this study</td>
</tr>
<tr>
<td>pAS123</td>
<td>pAKD701 containing the (V.\ vulnificus) C7184 smcR promoter region, ori(<em>{R6K\gamma}), ori(</em>{pES213}), ori(_T), Kn(^R) this study</td>
</tr>
<tr>
<td>pAS128</td>
<td>pAKD701 containing the (V.\ cholerae) CB98-41 hapR promoter region, ori(<em>{R6K\gamma}), ori(</em>{pES213}), ori(_T), Kn(^R) this study</td>
</tr>
<tr>
<td>pEVS104</td>
<td>conjugative helper, ori(_{R6K\gamma}), ori(_T), Kn(^R) (69)</td>
</tr>
<tr>
<td>pEVS122</td>
<td>ori(_{R6K\gamma}), ori(_T), Erm(^R) (18)</td>
</tr>
<tr>
<td>pJLB96</td>
<td>litR::erm allele; ori(_{ColE1}), ori(_T), Erm(^R), Cm(^R) (9)</td>
</tr>
<tr>
<td>pJLB170</td>
<td>pAKD702 containing the ES114 luxR promoter region, ori(<em>{R6K\gamma}), ori(</em>{pES213}), ori(_T), Cm(^R) (11)</td>
</tr>
<tr>
<td>pNL49</td>
<td>ΔryhB allele; ori(<em>{R6K\gamma}), ori(</em>{ColE1}), ori(_T), Erm(^R), Kn(^R) this study</td>
</tr>
</tbody>
</table>

**Oligonucleotides**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>prNL66</td>
<td>GGCGGATAATGCTGCTGTGGCCCAAGGCATAAA this study</td>
</tr>
<tr>
<td>prNL67.2</td>
<td>GGCCCTTAGGAATAATGTCGGGATAACTCCGTGCGGTAT this study</td>
</tr>
<tr>
<td>prNL68</td>
<td>TCCCT this study</td>
</tr>
<tr>
<td>prNL69</td>
<td>GGCCCTTAGGAGCGATGTTGGAGCTACAACGTATTACC A this study</td>
</tr>
<tr>
<td></td>
<td>CCAATAAGGTTCCGCCACCATGTAATCTAAACTATCGGGTTT C this study</td>
</tr>
</tbody>
</table>
ASlitRP2  TAGCTAGCATATCAAGTAATTGTTCTTTGC  this study
ASlitRP3  TAGCATGCACTATCTCCTTTCTGTTG  this study
ASvv1634P1 TAGCATGCACTGTACTCAATGTTTTATAAGATGTTG  this study
ASvv1634P2 TAGCTAGCTCTTTCGATTTGAGTCCATAG  this study
ASvcA0115P1 TAGCATGCACTTTCTCGTTGTGTGTTGG  this study
ASvcA0115P2 TAGCTAGCGGTTTTTCGATTGAGTGGCG  this study

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aKnR, Kanamycin resistance; CmR and cat, Chloramphenicol resistance; ErmR and erm,
Erythromycin resistance; AmpR, Ampicillin resistance; NxR, Nalidixic acid resistance. Plasmid
replication origins are designated oriV with a subscript indicating the source, and oriT indicates
the RP4 origin of transfer.

bOligonucleotides are in the 5' to 3' orientation with introduced restriction sites underlined.
Figure 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-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. LuxO-P increases transcription 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 in related bacterial species (see text and review in (70)). The putative role of Fur in the regulatory circuit, as described in this paper, is highlighted in gray. Figure modified from (47).

Figure 2. The effect of citrate on luminescence. Luminescence as a function of cell density (panel A) or peak luminescence per OD595 (panel B) for wild-type ES114 cultures grown in aerobic shake flasks in SWTO medium supplemented with 43 µM FeSO4 without further additions (light gray circles/bars), or supplemented with 20 mM citrate (dark gray circles/bars) or with 20 mM citrate and 2 mM additional FeSO4 (white bars). In Panel B, lowercase letters shared between bars indicates no statistically significant difference (*P* > 0.9), whereas different letters indicates a significant difference (*P* < 0.001), based on a one-way ANOVA and Tukey’s
Figure 3. Citrate addition affects expression of a Fur-regulated reporter. β–galactosidase reporter activity for wild-type and Δfur mutant *V. fischeri* strains with the Fur-dependent P*VF*_{1225}-lacZ reporter plasmid pAKD912 grown in SWTO medium supplemented with 43 µM FeSO₄ without further additions (light gray), or supplemented with 20 mM citrate (dark gray) or with 20 mM citrate and 2 mM additional FeSO₄ (white). Cells were harvested at an OD₅₉₅ of ~1.0. The asterisk indicates a significant difference from other media conditions within a strain (*P* < 0.001) based on an ANOVA and Tukey’s HSD test. Data are representative of at least three independent experiments. Error bars indicate standard deviation (n=2).

Figure 4. The role of Fur in the response of luminescence to low iron. (Panel A) Cultures of wild-type ES114 (circles) or the Δfur mutant YLM111 (triangles) were grown in aerobic shake flasks in SWTO medium supplemented with 43 µM FeSO₄ and either 20 mM citrate (dark gray) or no citrate (light gray). Error bars indicate standard deviation (n=2). (Panel B) Cultures of the Δfur mutant YLM111 were grown in SWTO medium supplemented with 43 µM FeSO₄ without further additions (light gray), or supplemented with 20 mM citrate (dark gray) or with 20 mM citrate and 2 mM additional FeSO₄ (white). The data in each panel are representative of at least three independent experiments. Error bars (some too small to visualize) indicate standard deviation (n=3) for the one experiment shown in each panel.
Figure 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 FeSO₄ either without (light gray) or with (dark gray) 20 mM citrate. Error bars indicate standard deviation (n=3). Asterisks indicate a significant effect of citrate addition on luminescence of the strain (*P* < 0.03) as determined using a Student’s t-test while a dagger indicates variable statistical significance (*P* ranged from 0.0001 to 0.73 in eleven experiments, see text for details).

In the representative experiment shown, citrate addition to Δfur mutant cultures resulted in a small (16%) but statistically significant (*P* < 0.05) increase in peak luminescence. Data shown were collected in the same experiment and are representative of at least three independent experiments for each strain.

Figure 6. Virtual footprint analysis of possible Fur binding sites. (Panel 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 x-axis indicates the Fur box nucleotide position. The putative Fur binding site upstream of VF_1225 is located 44 base pairs upstream of the ATG and has a score of 16.22 while the site upstream of *litR* is located 40 base pairs upstream of the ATG with a score of 19.63. Nucleotides that are identical between the two putative binding sites are in bold and nucleotides identical to bases in the PWM sequence logo are shaded with the corresponding nucleotide color. (Panel B) Position of the putative Fur binding site upstream of *litR*. The *litR* translational start site is in
bold, the putative Fur binding site is highlighted in yellow, and possible -10 and -35 sequences
are underlined.

Figure 7. The effects of Fur on *litR*, *luxR*, *smcR*, and *hapR* transcriptional reporters. In all
panels, *V. fischeri* cultures of ES114 (wild type), YLM111 (Δfur), JB19 (*litR*::erm), or ANS63
(Δfur *litR*::erm) were grown in aerobic shake flasks in SWTO medium supplemented with 43
μM FeSO₄. In Panels A and C, for *litR*, *smcR*, and *hapR* transcriptional reporters, cells harboring
pAS120, pAS123, and pAS128 were harvested at an OD₅₉₅ of ~0.5. In Panel B for the *luxR*
transcriptional reporter, cells harboring pJLB170 were harvested at an OD₅₉₅ of ~1.0. Error bars
indicate standard deviation (n=4 for Panels A and B, n=3 for Panel C). Asterisks in Panels A
and C indicate a significant difference of the indicated pair-wise comparison (*P* < 0.005) using a
Student’s t-test, while the comparison labeled “ns” was not significant (*P* > 0.05). In panel B
lowercase letters shared between bars indicates no statistically significant difference (*P* > 0.2),
whereas different letters indicates a significant difference (*P* < 0.001), based on a one-way
ANOVA and Tukey’s HSD test. Data in each panel are representative of at least three
independent experiments.
A

B

AGTTATGAGAATAATAATAAATACCCCTATAAAATATAATACATG...