RESEARCH LETTER

FNR-mediated regulation of bioluminescence and anaerobic respiration in the light-organ symbiont *Vibrio fischeri*

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Abstract

Vibrio fischeri induces both anaerobic respiration and bioluminescence during symbiotic infection. In many bacteria, the oxygen-sensitive regulator FNR activates anaerobic respiration, and a preliminary study using the light-generating *lux* genes from *V. fischeri* MJ1 cloned in *Escherichia coli* suggested that FNR stimulates bioluminescence. To test for FNR-mediated regulation of bioluminescence and anaerobic respiration in *V. fischeri*, we generated *fnr* mutants of *V. fischeri* strains MJ1 and ES114. In both strains, FNR was required for normal fumarate- and nitrate-dependent respiration. However, contrary to the report in transgenic *E. coli*, FNR mediated the repression of *lux*. ArcA represses bioluminescence, and P_{arcA}-*lacZ* reporters showed reduced expression in *fnr* mutants, suggesting a possible indirect effect of FNR on bioluminescence via *arcA*. Finally, the *fnr* mutant of ES114 was not impaired in colonization of its host squid, *Euprymna scolopes*. This study extends the characterization of FNR to the *Vibrionaceae* and underscores the importance of studying *lux* regulation in its native background.

Introduction

Vibrio fischeri is a model for investigations of bioluminescence and mutualistic symbioses, two fields connected by the importance of oxygen. O_2 is a substrate for the luminescence-producing enzyme luciferase, and luciferase may benefit *V. fischeri* by generating a more reduced environment in or near cells (Visick *et al*., 2000; Timmins *et al*., 2001). Reduction of O_2 could be especially advantageous for this facultative anaerobe when it is colonizing animal tissue and may minimize the host's ability to generate reactive oxygen species (Visick *et al*., 2000). Luminescence emanating from bacteria colonizing the symbiotic light organ of the host indicates that O_2 is present; however, evidence suggests that luciferase is O_2 limited in this environment (Boettcher *et al*., 1996) despite its high affinity ($K_m \sim 35$ nM) for O_2 (Bourgois *et al*., 2001). Moreover, anaerobic respiration is apparently induced in symbiotic *V. fischeri* (Proctor & Gunsalus, 2000), consistent with the idea that $[O_2]$ is low in the light organ.

One regulator that might control anaerobic respiration and luminescence in response to $[O_2]$ is FNR (so named for its role in fumarate and nitrate reduction). FNR regulates genes during the switch between aerobic and anaerobic growth in *Escherichia coli* and other bacteria, and it often activates genes responsible for anaerobic respiration (Browning *et al*., 2002; Reents *et al*., 2006; Fink *et al*., 2007). Although FNR is expressed during both aerobic and anaerobic growth, it is only functional under microaerobic or anaerobic conditions due to its dependence on an oxygen-labile 4Fe–4S center (Khoroshilova *et al*., 1995, 1997; Lazazzera *et al*., 1996; Kiley & Beinert, 1998). Under anaerobic conditions, [4Fe–4S]-FNR forms a functional dimer that binds DNA at a 5′-TTGAT(N_4)ATCAA-3′ FNR-box sequence (Eiglmeier *et al*., 1989), and it activates or represses transcription depending on the location of binding relative to the promoter (Wing *et al*., 1995; Meng *et al*., 1997; Marshall *et al*., 2001).

FNR was reported to activate bioluminescence in transgenic *E. coli* carrying the *V. fischeri* MJ1 *luxR-luxICDABEG* region, which encodes the autoinducer-dependent *lux* activator LuxR, the autoinducer synthase LuxI, and the Lux proteins that produce bioluminescence (Muller-Breikreutz & Winkler, 1993). Although FNR-mediated regulation of luminescence is cited frequently (Meighen, 1994; Spiro, 1994; Sitnikov *et al*., 1995; Ulitzur & Dunlap, 1995; Stevens & Greenberg, 1999), these data were only presented in

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preliminary form in a symposium report (Muller-Breikreutz & Winkler, 1993).

We have examined *fnr* in two *V. fischeri* strains: ES114 and MJ1. ES114's genome is sequenced, and its symbiosis with the squid *Euprymna scolopes* can be reconstituted in the laboratory (Ruby *et al.*, 2005; Stabb, 2006); however, like most isolates from these animals, ES114 is not visibly luminescent in culture (Boettcher & Ruby, 1990). In contrast, MJ1 has bright luminescence typical of isolates from the pinecone fish *Monocentris japonica*, but this symbiosis is not yet experimentally tractable. The genes required for luminescence and autoinduction are similar in the two strains, with the *luxICDABEG* operon adjacent to and divergently transcribed from *luxR* (Gray & Greenberg, 1992). However, there are differences in the *luxR-luxI* intergenic region, and notably there is a putative FNR box upstream of *luxR* in MJ1 that is absent in ES114. Our goals were to examine *V. fischeri* to assess FNR's regulation of luminescence and anaerobic respiration, and to determine whether FNR contributes to symbiotic competence.

Materials and methods

Bacteria and media

The bacterial strains used in this study are described in Table 1. *Escherichia coli* was grown in Luria–Bertani (Miller, 1992) or in M9 (Sambrook *et al.*, 1989) supplemented with 1 mg mL^{-1} casamino acids, 40 mM glycerol, and 40 mM of either sodium nitrate or sodium fumarate. *Vibrio fischeri* was grown in Luria broth plus salt (LBS) (Stabb *et al.*, 2001), sea water tryptone (SWT) (Boettcher & Ruby, 1990), wherein seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, OH), sea water tryptone at high osmolarity (SWTO) (Bose *et al.*, 2007), or in a defined salts medium (Adin *et al.*, 2009) with 40 mM glycerol as a carbon source, 1 mg mL^{-1} casamino acids, and 40 mM of sodium nitrate or sodium fumarate. Agar (15 mg mL^{-1}) was added to solidify media for plating. Anaerobic growth on plates was assessed using the GasPak EZ Anaerobic Container System from Becton, Dickinson and Company (Sparks, MD). Antibiotics were added as described previously for selection (Stabb & Ruby, 2002), and *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) autoinducer was added to the media at 140 nM.

Genetic manipulations

Cloning was performed using standard procedures, with plasmids transformed in *E. coli* strain DH5α or DH5αλ*pir*, as described previously (Bose *et al.*, 2008). Cloned PCR products were sequenced to ensure that unintended alterations were not incorporated. Sequencing was conducted at the University of Michigan DNA Sequencing Core Facility or at the University of Georgia Molecular Genetics Instrumentation Facility. Plasmids were mobilized into *V. fischeri* from *E. coli* by triparental mating using strain CC118λ*pir* with pEVS104 as a helper (Stabb & Ruby, 2002), and mutations were placed on the chromosome by allelic exchange. Parent strains and plasmids used for allelic exchange are listed in Table 1.

Key plasmids and oligonucleotides are described in Table 1, and an overview of allele construction follows. To mutate *fnr*, an ~3.3 kb region of the *V. fischeri* genome centered on *fnr* was PCR amplified with primers EVS97 and EVS98 using ES114 or MJ1 genomic DNA as a template, and the fragments were ultimately subcloned into pEVS136 and pJLB69, respectively (Table 1). We generated Δ*fnr*::*tmpR* alleles by replacing the ClaI to AvrII fragment of *fnr* with the trimethoprim-resistance gene (*tmpR*) from pJLB1 (Dunn *et al.*, 2005) on a BstBI to AvrII fragment, resulting in *tmpR* replacing an internal 255-bp fragment beginning in the middle of *fnr*, with *tmpR* in the same orientation as *fnr*. The ES114-derived Δ*fnr*::*tmpR* allele was placed in pJLB5 and pJLB70, and the MJ1-derived Δ*fnr*::*tmpR* allele was used in pCDW5. For complementation of *E. coli* with ES114 *fnr*, we ligated the *fnr*-containing BsrBI–PstI fragment from pEVS136 into SmaI- and PstI-digested pDMA5, generating pJLB6. To place *lacZ* under control of the *arcA* promoter, we PCR amplified an ~3.1-kb fragment containing an engineered *lacZ* (Tomich *et al.*, 1988) using pVSV3 (Dunn *et al.*, 2006) as a template and primers JBLACZ1 and JBLACZ2 (Table 1). We cloned this product into SmaI-digested pAJ4 and pJLB55 (Bose *et al.*, 2007), which carry regions flanking *arcA* from ES114 and MJ1, respectively, with the sequence between the start and the stop codons of *arcA* replaced by a 6-bp SmaI recognition site. The P_{arcA}-*lacZ* alleles contain the *arcA* start codon, followed by a 5′-CCC-3′ proline codon, and then the *lacZ* reporter (Tomich *et al.*, 1988) from its second codon onward. These ES114- and MJ1-derived alleles were subcloned into pAS31 and pJLB139, respectively.

Growth and luminescence

Overnight cultures in LBS were diluted 1 : 1000 into SWTO and incubated at 24 °C with shaking (200 r.p.m.). Aerobic cultures contained 50 mL of SWTO in 250-mL flasks. For anaerobic cultures, aerobically grown overnight cultures were diluted 1 : 10 in LBS before inoculation of 0.2 mL into 20 mL SWTO in 165-mL sealed bottles with a headspace containing 5% CO_2, 10% H_2, and 85% N_2. Samples (500 μL each) were removed periodically and culture optical density ($OD_{595\,nm}$) was determined using a BioPhotometer (Brinkman Instruments, Westbury, NY) or a SmartSpec 3000 (BioRad Laboratories, Hercules, CA). After measuring $OD_{595\,nm}$, cuvettes were covered with parafilm and shaken vigorously for ~10 s to aerate the sample, followed by

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

Strain or plasmid	Relevant characteristics*	References
Bacterial strains		
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ*pir*	Herrero *et al*. (1990)
DH5α	F − *F80dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
DH5αλ*pir*	DH5α lysogenized with λ*pir*	Dunn *et al*. (2005)
MC4100	F- *araD139* Δ(*argF-lac*) U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Silhavy *et al*. (1984)
PC2	MC4100 Δ*fnr*	Cotter & Gunsalus (1992)
V. fischeri		
AMJ2	ES114 Δ*arcA*	Bose *et al*. (2007)
ANS23	ES114 Δ*arcA*::*lacZ* (allele exchanged from pAS31 into ES114)	This study
ANS24	ES114 *fnr*::*tmpR*Δ *arcA*::*lacZ* (allele exchanged from pAS31 into JB1)	This study
ANS25	ES114 *fnr*::*tmpR* $lacI^q$ $P_{A1/34}$-*luxCDABEG* (allele exchanged from pJLB5 into JB22)	This study
ES114	Wild-type isolate from *E. scolopes*	Boettcher & Ruby (1990)
EVS102	ES114 Δ*luxCDABEG*	Bose *et al*. (2008)
EVS601	MJ1 Δ*fnr*::*tmpR* (allele exchanged from pCDW5 into MJ1)	This study
JB1	ES114 Δ*fnr*::*tmpR* (allele exchanged from pJLB5 into ES114)	This study
JB2	*fnr* restored in JB1 (wild-type allele exchanged from pEVS136 into JB1)	This study
JB8	ES114 *fnr*::*tmpR* Δ*arcA* (allele exchanged from pJLB70 into AMJ2)	This study
JB11	MJ1 Δ*arcA* (allele exchanged from pJLB76 into MJ1)	Bose *et al*. (2007)
JB12	MJ1 *fnr*::*tmpR* Δ*arcA* (allele exchanged from pJLB76 into EVS601)	This study
JB22	ES114 $lacI^q$ $P_{A1/34}$-*luxCDABEG*	Bose *et al*. (2008)
JB28	MJ1 Δ*arcA*::*lacZ* (allele exchanged from pJLB139 into MJ1)	This study
JB29	MJ1 *fnr*::*tmpR* Δ*arcA*::*lacZ* (allele exchanged from pJLB139 into EVS601)	This study
JB27	*fnr* restored in EVS601 (wild-type allele exchanged from pJLB69 into EVS601)	This study
MJ1	Wild-type isolate from *Monocentris japonica*	Ruby & Nealson (1976)
VCW2G7	ES114 $luxI^-$ (frameshift mutation)	Lupp *et al*. (2003)
Select plasmids†		
pAS31	R6Kγ, ColE1, *chmR*, *ampR*, ES114 Δ*arcA*::*lacZ* allele	This study
pCDW5	R6Kγ, ColE1, *chmR*, *kanR*, MJ1 Δ*fnr*::*tmpR* allele	This study
pDMA5	p15A *oriV*, $oriT_{RP4}$, *lacZ*α, *chmR*	Dunn *et al*. (2005)
pEVS136	R6Kγ, *ermR*, ES114 *fnr*	This study
pJLB5	R6Kγ, *ermR*, ES114 Δ*fnr*::*tmpR* allele	This study
pJLB6	p15A, *chmR*, ES114 *fnr*	This study
pJLB69	R6Kγ, ColE1, *chmR*, *kanR*, MJ1 *fnr*	This study
pJLB70	R6Kγ, ColE1, *ermR*, *kanR*, ES114 Δ*fnr*::*tmpR* allele	This study
pJLB76	R6K, ColE1, *chmR*, *ampR*, MJ1 Δ*arcA*	Bose *et al*. (2007)
pJLB139	R6Kγ, ColE1, *chmR*, *ampR*, MJ1 Δ*arcA*::*lacZ* allele	This study
Oligonucleotides‡		
AS1310RTF2	TAT TGG TTA AAG AGC GCC CAT GG	This study
AS1310RTR2	CAC TTC AGC GAA ATA GAT GGC	This study
EVS97	CCG GGT ACC ATG GTT GGT GAT GGA ATA AAT GAT GC	This study
EVS98	CCG GGT ACC TTT TGA AGC TTA TTG AAA TTG TAT TG	This study
JBLACZ1	CTG ACT CTG GGT AAC ACT ACT TCT TCT GTG	This study
JBLACZ2	TTA TTT TTG ACA CCA GAC CAA CTG GTA ATG G	This study

*Drug resistance abbreviations: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tmpR* trimethoprim resistance (*dfr*).

†All plasmids listed contain the RP4 origin of transfer. Replication origin(s) are denoted as p15A, R6Kγ, and/or ColE1.

‡Oligonucleotide sequences are provided in the 5′–3′ orientation.

determination of luminescence using a GLOMAX 20/20 luminometer (Promega, Madison, WI).

Quantitative reverse transcriptase (RT)- PCR

Triplicate aerobic cultures of ES114 and JB1 were grown in LBS to an $OD_{595\,nm}$~2.1. Samples (1 μL each) were removed, added to microcentrifuge tubes containing 1/5 volume 5% (v/v) phenol, pH 4.3, with 95% (v/v) ethanol, and placed on ice for 30 min. Samples were centrifuged and the pellets were stored at −80 °C overnight. Pellets were thawed, and RNA was isolated using Absolutely RNA Mini-preps (Stratagene, La Jolla, CA). RNA was treated using the Turbo DNA-free kit (Applied Biosystems, Foster City, CA),

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and RNA quantity and purity were assessed using a Biotek Synergy 2 plate reader with Take3 Multi-Volume Plate and software (Winooski, VT). RNA was then stored at $-80\,^{\circ}C$. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), and reactions were cleaned using a DNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA). cDNA was quantified using the Synergy 2 plate reader. Real-time PCR was performed using the MyIQ Single-Color Real-Time PCR Detection System (BioRad Laboratories), and reactions were set up using the BioRad IQ SYBR Green Supermix. Primers AS1310RTF2 and AS1310RTR2 were used to determine the level of VF1310 cDNA. ES114 genomic DNA was used to generate a standard curve. Real-time PCR data were analyzed using BioRad IQ^{TM}5 software.

lacZ reporter expression

To determine P_{arcA}-*lacZ* reporter expression, strains were grown overnight in LBS and diluted 1 : 1000 in 20 mL SWTO in 250-mL baffled flasks and grown at 24 °C with shaking to an OD of $\sim$0.1. Four hundred microliters were removed to inoculate 20 mL SWTO in anaerobic bottles. These were incubated at 24 °C with shaking until peak luminescence was reached. Strains were also grown aerobically in 20-mL SWTO in 250-mL baffled flasks and incubated at 24 °C with shaking until peak luminescence was reached. Culture samples were taken, cells were pelleted, the supernatant was discarded, and the pellet was frozen at $-20\,^{\circ}C$. The next day, the pellet was thawed and resuspended in Z-buffer for determination of β-galactosidase activity expressed as Miller units as described previously (Miller, 1992).

Symbiotic colonization assays

Inoculant strains were grown unshaken in 5 mL of SWT in 50-mL conical tubes at 28 °C to an $OD_{595\,nm}$ of 0.3–1.0, and cultures were diluted in Instant Ocean to a density no higher than 1700 CFU mL^{-1}. In each experiment, the inoculant density of wild-type and mutants strains was equivalent, and this was checked by plating the inocula on LBS. Hatchling squid were placed in these inocula for up to 14 h before being rinsed in *V. fischeri*-free Instant Ocean. To study infection kinetics, the squid were placed in 5 mL of inoculant in scintillation vials, and the onset of luminescence was monitored using an LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). For mixed-strain competitions, hatchlings were exposed to an inoculum containing an $\sim$1 : 1 ratio of wild type and mutant. At 48-h postinoculation, individual squid were homogenized and dilution plated on LBS. The resulting colonies were patched onto LBS with added trimethoprim to determine the ratio of strains in each animal. Inocula were similarly plated and patched to determine the starting ratio. The relative competitiveness index (RCI) was determined by dividing the mutant to wild-type ratio in each animal by the ratio of these strains in the inoculum. The mean RCI was calculated from log-transformed data.

Results

Identification of *V. fischeri fnr*

BLAST searches (Altschul *et al*., 1990) of the *V. fischeri* ES114 genome revealed the similarity of ORFs VF1308 and VF1309 to the N and C termini of *E. coli* FNR, respectively (Fig. 1a). We suspected that a sequencing error had led to the misannotation of *fnr* as two genes, and we therefore cloned and sequenced the region spanning VF1308 and VF1309. We found five errors in the genome database, leading to an erroneously predicted truncation of VF1308, which we corrected in GenBank (Mandel *et al*., 2008). In the revised sequence, VF1308 encodes a protein that is the same length as, and shares 84% identity with, *E. coli* FNR. This ES114 FNR is identical to the previously deposited *V. fischeri* MJ1 FNR (accession no. CAE47558). Importantly, the residues necessary for interactions with RNA polymerase (Williams *et al*., 1997; Lonetto *et al*., 1998; Blake *et al*., 2002; Lamberg *et al*., 2002), 4Fe–4S center assembly (Spiro & Guest, 1988; Kiley & Beinert, 1998), and DNA recognition (Spiro *et al*., 1990) in *E. coli* are conserved in *V. fischeri* FNR. Using TransTermHP (Kingsford *et al*., 2007), we also found a likely Rho-independent transcriptional terminator downstream of *fnr* (Fig. 1a and b). Given the 142-bp spacing and strong putative terminator between *fnr* and VF1310 (Fig. 1b), it seems likely that these are expressed on separate transcripts. Using quantitative RT-PCR, we found that the *fnr*∷*tmpR* allele in mutants described below did not affect the transcript levels for VF1310.

We next generated mutants disrupted in the putative *fnr* in *V. fischeri* ES114 and MJ1. We did not observe any attenuation of these strains under aerobic growth conditions, consistent with the role of FNR in other bacteria. *Escherichia coli fnr* mutants do not grow anaerobically with nitrate or fumarate as an electron acceptor (Lambden & Guest, 1976), and we found that *V. fischeri fnr* mutants were similarly attenuated. Specifically, when grown with minimal medium under anaerobic conditions, ES114 and MJ1 displayed nitrate- or fumarate-dependent growth on a nonfermentable carbon source (glycerol) that was lacking in the *fnr* mutants (e.g. Fig. 1c). Restoring *fnr* by replacing the *fnr*∷*tmpR* allele with the wild-type allele by a crossover exchange back into these mutants recovered the ability to respire anaerobically. We restored the wild-type *fnr* allele on the chromosome in this way (replacing *fnr*∷*tmpR*) rather than providing it *in trans* due to concerns that *fnr* provided in multicopy can show uncharacteristic effects such as gene

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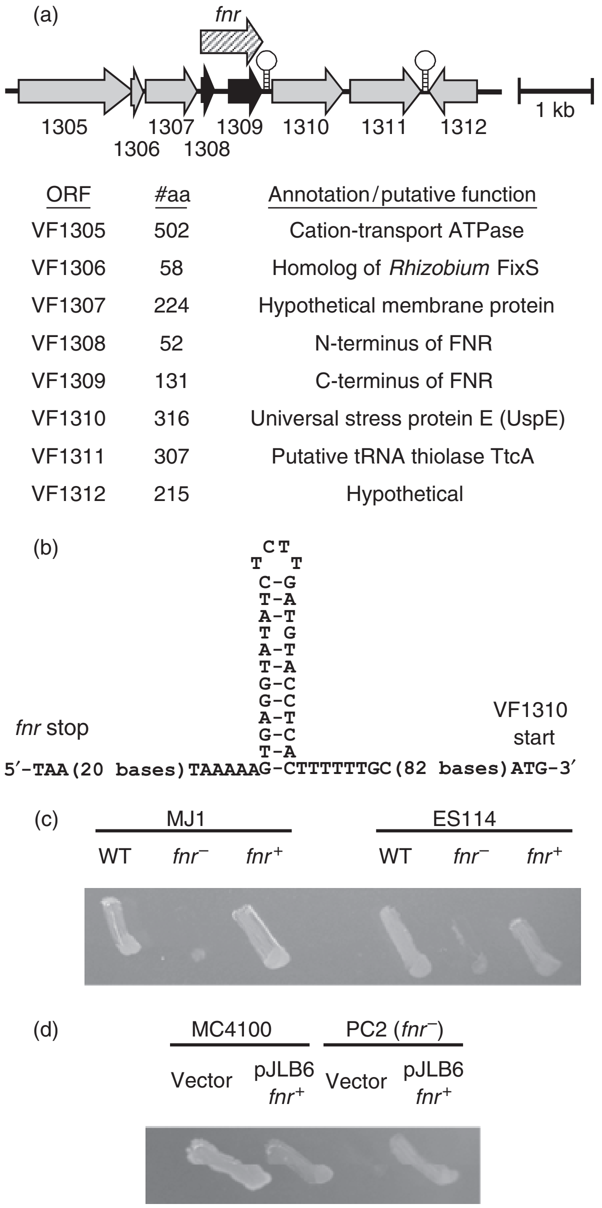

Fig. 1. Genomic context and function of *fnr* in *Vibrio fischeri*. (a) Gene arrangement around *fnr* in *V. fischeri* ES114. Numbers represent the corresponding VF#### ORF designation. Stem-loop icons indicate the positions of Rho-independent transcriptional terminators predicted using TransTermHP (Kingsford *et al*., 2007), with a confidence score of 100 in each case. '#aa' indicates the number of amino acids encoded by each ORF. VF1308 and VF1309 (black arrows) indicate ORFs with similarity to the N and C termini of *Escherichia coli* FNR, respectively. The striped arrow shows the complete *fnr* based on our sequence revision. (b) The predicted Rho-independent transcriptional terminator between *fnr* and VF1310. (c) Growth of *V. fischeri* MJ1, *fnr* mutant EVS601, and restored *fnr*$^+$ strain JB27 along with ES114, *fnr* mutant JB1, and restored *fnr*$^+$ strain JB2 on defined medium with glycerol and fumarate, incubated in anaerobic jars at 28 °C. (d) *Escherichia coli* MC4100 and *fnr* mutant PC2 with vector pDMA5 or pJLB6, which contains the *V. fischeri* ES114 *fnr*, grown on M9 medium with glycerol and nitrate in anaerobic jars at 37 °C.

activation under aerobic conditions (Reyes-Ramirez & Sawers, 2006) and a narrowing of the difference between better and poorer FNR activation sites (Scott *et al*., 2003). However, because our *V. fischeri*-derived allele-replacement constructs were not appropriate (homologous) for exchange into *E. coli*, we provided the putative *fnr* of *V. fischeri* ES114 to *E. coli in trans* on plasmid pJLB6, which restored anaerobic respiration of *E. coli fnr* mutant PC2 on nitrate (Fig. 1d). Taken together, our results indicate that the putative *V. fischeri* FNR is similar in both sequence and function to *E. coli* FNR.

Repression of luminescence by FNR

We tested whether FNR regulates *lux* expression by monitoring the luminescence of strains grown aerobically or anaerobically (Fig. 2a and b). The luminescence of the *fnr* mutants was similar to that of their parent strains under aerobic conditions (Fig. 2a). FNR is inactivated by oxygen, and we therefore also assessed *lux* expression anaerobically. Luciferase uses oxygen as a substrate, and so anaerobic cultures do not luminesce; however, as with all luminescence measurements, samples removed from anaerobic bottles were shaken for ∼10 s to saturate luciferase with oxygen before measuring luminescence. When grown anaerobically, luminescence was higher in *fnr* mutant EVS601 than in MJ1 (Fig. 2b). The magnitude of this difference varied between 1.5- and 20-fold, and averaged eightfold, in five experiments. The luminescence of ES114 and *fnr* mutant JB1 was below the background, appearing the same as a dark Δ*luxCDABEG* strain (data not shown), which raised the possibility that FNR regulates *lux* in ES114, but that the overall luminescence is below detection. To test this possibility, we added the luminescence-stimulating autoinducer 3-oxo-C6-HSL to anaerobic cultures of ES114 and its *fnr* mutant JB1. 3-oxo-C6-HSL stimulated the luminescence of ES114 and JB1, and under these conditions, JB1 was brighter than ES114 (Fig. 2c).

We considered the possibility that increased luminescence in *V. fischeri fnr* mutants could result from increased availability of luciferase's substrates due to the physiological effects of this global regulator. To test this possibility, we disrupted *fnr* in a background where the *luxCDABEG* genes are under the control of LacI^q and a non-native promoter. In this background, FNR had no significant effect ($P > 0.05$) on luminescence (Fig. 2c). Thus, the repressive effect of FNR on luminescence is dependent on the native *lux* promoter.

The *luxICDABEG* operon can be subject to positive feedback regulation, because the autoinducer synthase LuxI generates 3-oxo-C6-HSL, which, in combination with LuxR, stimulates *luxICDABEG* transcription. Given the amount of 3-oxo-C6-HSL added exogenously to the cultures (Fig. 2c), we predicted that endogenously produced autoinducer

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would have no further stimulatory effect, and therefore the effect of FNR on luminescence in this experiment would not have a significant LuxI-mediated positive-feedback component. We examined *luxI* point mutant VCW2G7 and found that, as predicted, it achieved the same luminescence as the wild type under anaerobic conditions with added 3-oxo-C6-HSL (data not shown).

Analysis of FNR boxes

It was suggested that a putative FNR box upstream of *luxR* might underpin the FNR-mediated regulation of luminescence in MJ1 (Muller-Breikreutz & Winkler, 1993); however, attempts to define a footprint using FNR*, an *E. coli* FNR derivative that is active aerobically (Kiley & Reznikoff, 1991), failed to show binding to this site (A.M. Stevens, pers. commun.). To further explore how FNR might affect luminescence, we conducted a 'Virtual Footprint' analysis with the PRODORIC database (Munch *et al.*, 2005), searching the *V. fischeri* genome for FNR boxes using a weighted consensus matrix based on data from *E. coli*. As expected, high Position Weight Matrix (PWM) scores ($\geq$7.0) were skewed toward intergenic regions. Such putative FNR boxes numbered in the hundreds, consistent with FNR's global role in *E. coli*, and these included intergenic regions upstream of genes involved in anaerobic metabolism (e.g. upstream of nitrate and nitrite reductase genes). However, the best FNR box matches in the *lux* intergenic region of MJ1 and ES114 returned scores of 6.73 and only 5.88, respectively. To put this in perspective, > 25 000 sites with no skew toward intergenic regions returned scores $\geq$5.9. Although we cannot rule out the possibility that FNR directly binds to the *lux* intergenic region, we believe this model is unlikely, especially in strain ES114.

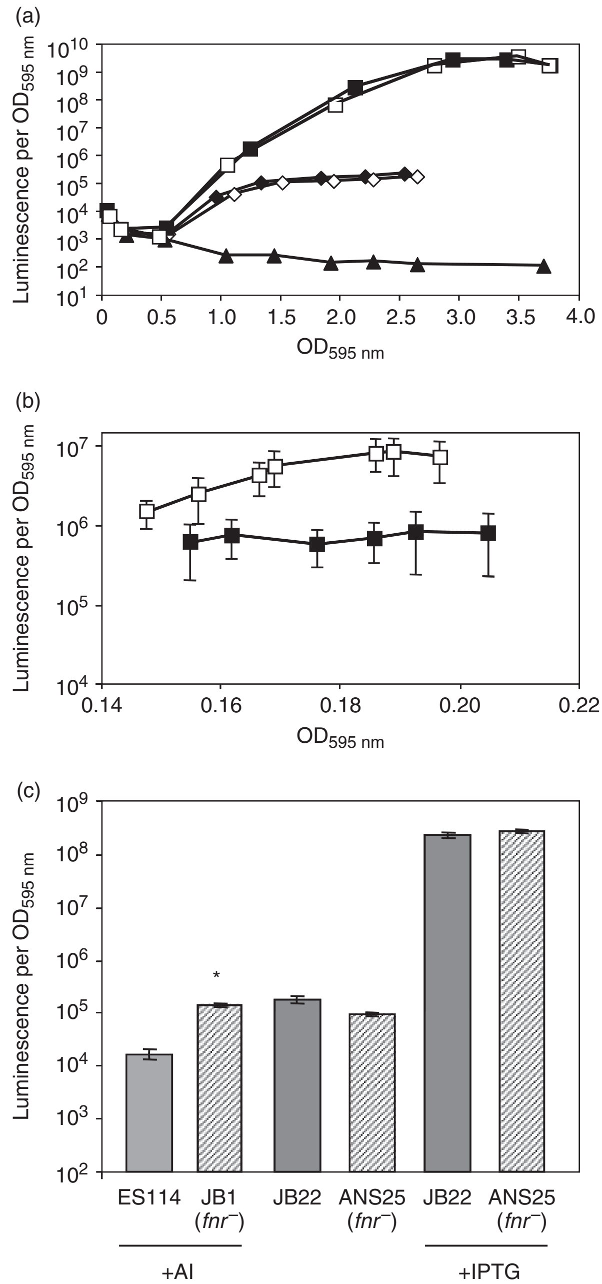

Fig. 2. Luminescence per $OD_{595\,nm}$ of *fnr* mutants. (a, b) Specific luminescence is shown at different culture densities for *Vibrio fischeri* ES114 (solid diamonds), ES114 *fnr* mutant JB1 (empty diamonds), MJ1 (solid squares), MJ1 *fnr* mutant EVS601 (empty squares), and dark Δ*luxCDABEG* mutant EVS102 (solid triangles) grown in batch cultures that were (a) aerobic (50 mL medium in 250-mL flask) or (b) anaerobic (20 mL medium in 165-mL bottles with anaerobic headspace) at 24 °C with shaking (200 r.p.m.). ES114, JB1, and EVS102 were excluded from(b), because luminescence was not detected above the background for these strains under these conditions. Bars in (b) indicate the SD ($n = 5$). Error bars were excluded in (a), because they were generally smaller than (and never extended above) the data symbols. (c) ES114 (wild type), JB22 (*lacI^q* $P_{A1/34}$-*lux*), and their respective *fnr* mutants (represented by hatched bars) JB1 and ANS25, respectively (Table 1), were grown under anaerobic conditions. 'AI' indicates supplementation with 140 nM 3-oxo-C6-HSL autoinducer, and 'IPTG' indicates that isopropyl-β-D-thiogalactoside was added to 2 mM to induce *luxCDABEG* expression in strains containing *lacI^q* $P_{A1/34}$-*lux*. Data are the average peak luminescence per $OD_{595\,nm}$ with SD ($n = 2$). Asterisks indicate that the *fnr* mutant was significantly ($P < 0.01$) brighter than the corresponding isogenic *fnr*-positive strain. Other comparisons were not significant ($P > 0.05$).

FNR-mediated repression of *arcA*

Virtual Footprinting did suggest a possible indirect effect of FNR on luminescence. The highest PWM score returned in this analysis (7.67) was found in six intergenic regions, one of which was upstream of *arcA*. In *E. coli*, FNR activates *arcA* (Compan & Touati, 1994), and in ES114, ArcA strongly represses the *lux* operon (Bose *et al.*, 2007). If FNR activates *arcA* in *V. fischeri*, this might explain FNR's repressive effect

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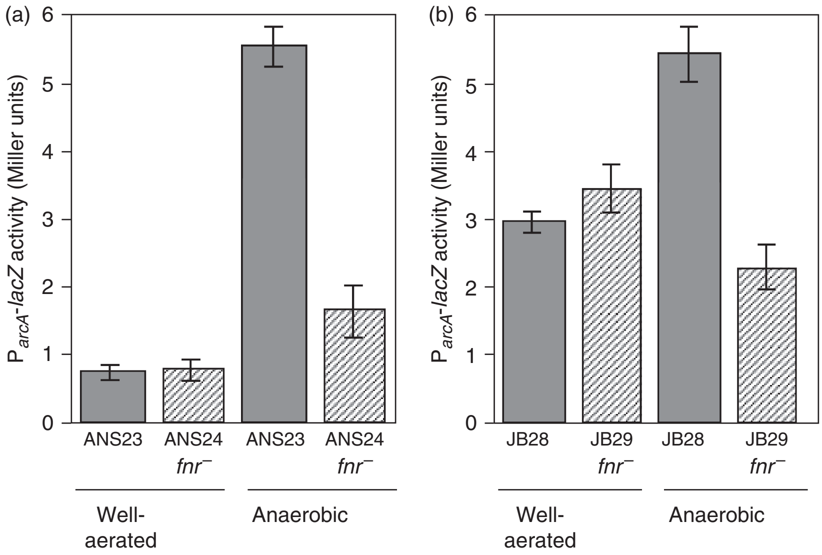

Fig. 3. FNR-mediated regulation of *arcA* promoter-*lacZ* reporters. LacZ reporter activity expressed in Miller units for (a) ES114 derivatives ANS23 (Δ*arcA*::*lacZ*) and ANS24 (Δ*arcA*::*lacZ* Δ*fnr*::*tmpR*), or (b) the MJ1 derivatives JB28 (Δ*arcA*::*lacZ*) and JB29 (Δ*arcA*::*lacZ* Δ*fnr*::*tmpR*). Culture conditions (aerobic or anaerobic) are as described in Fig. 2. Averages with SD are indicated ($n = 3$). The LacZ reporter activity shown is approximately 100-fold above the background determined using strains ES114 and JB1, which lack the Δ*arcA*::*lacZ* allele.

on luminescence. Using P_{arcA}-*lacZ* transcriptional reporters, we found that *fnr* was responsible for an ∼2–4-fold activation of the *arcA* promoter(s) anaerobically in ES114 and MJ1 backgrounds (Fig. 3).

FNR is not necessary for host colonization

We tested whether FNR was important for symbiotic colonization by ES114 using established measures of symbiotic competence (Adin *et al*., 2009). The onset of symbiotic luminescence (Fig. 4a), colonization levels (Fig. 4b), and colonization competitiveness (Fig. 4c) were similar for ES114 and *fnr* mutant JB1 during the first 2 days of infection. The *fnr* mutant was also equally competitive up to 90 h after inoculation (data not shown). Furthermore, the *fnr* mutation did not appear to affect the symbiosis in a Δ*arcA* mutant background (data not shown). We conclude that FNR is not necessary for colonization during the first days of a symbiotic infection.

Discussion

In this study, we investigated the oxygen-sensitive regulator FNR in *V. fischeri*. *Vibrio fischeri fnr* complemented an *E. coli fnr* mutant, and like *fnr* in *E. coli*, it is required for fumarate- and nitrate-dependent anaerobic respiration. Moreover, our data and another recent bioinformatic analysis (Ravcheev *et al*., 2007) suggest that the FNR-box recognition site is conserved in *V. fischeri*. For example, we observed *fnr*-mediated regulation of reporters for *arcA* (Fig. 3), *dmsA* (Dunn & Stabb, 2008), *torE* (Dunn & Stabb, 2008), and *yfiD* (data not show), which have predicted FNR boxes upstream. Taken together, FNR's function in *V. fischeri* appears to be similar to that in its fellow gammaproteobacterium *E. coli*. As the first experimental examination of FNR in the *Vibrionaceae*, this study should underpin future efforts to understand FNR-mediated regulation in this important bacterial family.

We initiated this study largely because FNR is cited as an activator of luminescence in *V. fischeri* (e.g. see Meighen, 1994; Spiro, 1994; Sitnikov *et al*., 1995; Ulitzur & Dunlap, 1995; Stevens & Greenberg, 1999). However, that paradigm was based on a preliminary study that used the MJ1 *lux* genes cloned in *E. coli* (Muller-Breikreutz & Winkler, 1993). Our results appear to contradict that report, showing instead that FNR mediates repression of the luminescence-generating *lux* system in *V. fischeri* under anaerobic conditions (Fig. 2). It is perhaps not surprising that *lux* regulation should be different in transgenic *E. coli* than in *V. fischeri*. For example, LitR, which activates *luxR* transcription, is absent in *E. coli* (Fidopiastis *et al*., 2002). It is also possible that FNR does activate luminescence in *V. fischeri* under conditions different from those tested here, and that the discrepancy between our study and previous work simply reflects methodological differences.

Repression of the *lux* genes anaerobically may minimize the production of luciferase when its O_2 substrate is unavailable. This is consistent with the finding that luminescence is repressed by the ArcAB two-component regulatory system, which is more active under relatively reduced conditions (Bose *et al*., 2007). The observation that *arcA*::*lacZ* reporters showed a lower expression in the absence of *fnr* (Fig. 3) suggests that the effect of FNR on bioluminescence may at least in part be indirect and mediated by FNR's stimulation of *arcA*. Consistent with this idea, *fnr* did not exert much influence on luminescence in *arcA* mutant backgrounds, although *arcA fnr* double mutants were noticeably attenuated in anaerobic growth (data not shown). We speculate that FNR may amplify the

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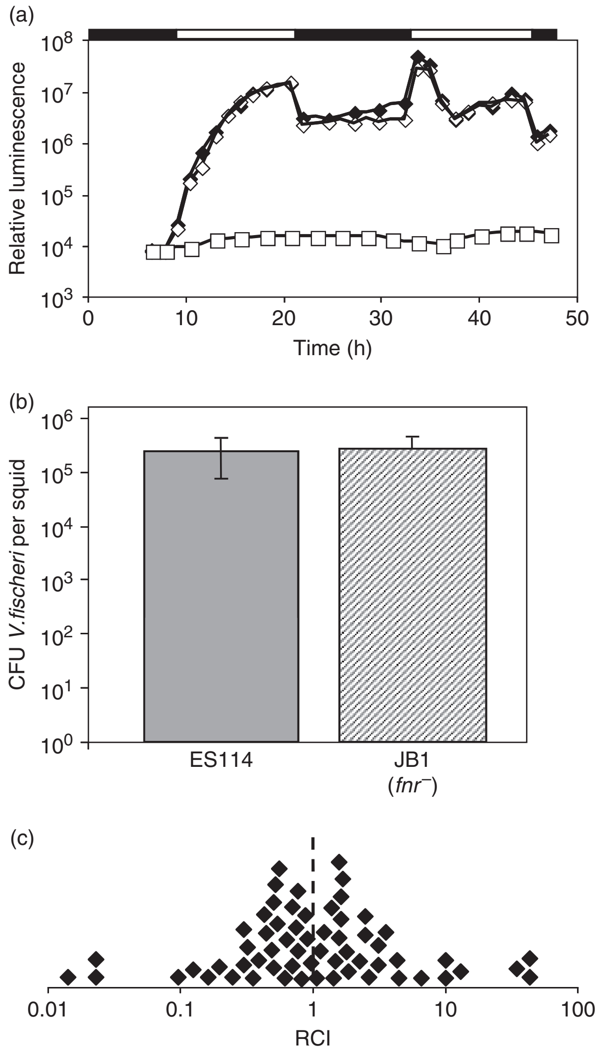

Fig. 4. Colonization of *Euprymna scolopes* by the *fnr* mutant and wild type. (a) Average symbiotic luminescence in *E. scolopes* hatchlings inoculated with ES114 (solid diamonds) or the *fnr* mutant JB1 (empty diamonds) ($n = 14$). Control squid receiving no *Vibrio fischeri* inoculum (empty squares) did not yield any bioluminescence ($n = 4$). Bars above the graph indicate periods of ambient light (empty bar) and darkness (solid bar). (b) Average colonization levels in CFU *V. fischeri* per squid 36 h after inoculation with ES114 (solid bar) or JB1 (hatched bar). Treatments are not significantly different ($P = 0.7$). Bars indicate SD ($n = 14$ for ES114 and 13 for JB1). (c) Competitiveness of JB1 when presented in a mixed (~1 : 1) inoculum with wild type and recovered from squid after 48 h. Each symbol represents the RCI determined from one squid, defined as the ratio of JB1 : ES114 in the squid divided by the ratio in the inoculum. Combined data from three experiments are presented. The dashed line represents equal competitiveness and in this case is also the mean RCI ($n = 60$).

repressive effect of ArcA on luminescence under reduced conditions. Although we cannot rule out the possibility that FNR exerts a direct effect by binding the *lux* region, as described above, we believe this model is unlikely. In either case, FNR apparently contributes to regulation that effectively turns off expression of the *lux* genes under ES114 under anaerobic conditions, which is easily rationalized, given that luciferase requires O_2 to generate light.

Given the suggestion that anaerobic respiration is important for symbiotic *V. fischeri* (Proctor & Gunsalus, 2000), and the fact that FNR can contribute to virulence factor production and/or colonization by pathogens (Baltes *et al*., 2005; Bartolini *et al*., 2006; Fink *et al*., 2007; Zigha *et al*., 2007), we hypothesized that *fnr* would play a role in the symbiotic light organ. However, the *fnr* mutant had no discernable attenuation in colonizing *E. scolopes* during the first 90 h of infection. *Vibrio fischeri*, like other members of the *Vibrionaceae* family, is a cosmopolitan member of marine communities that is found in fish gut tracts and sediments where $[O_2]$ is low. Future studies may show the ecological relevance of FNR for *V. fischeri* in such environments outside *E. scolopes*.

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Authors' contribution

J.L.B. and A.N.S. contributed equally to this work.

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