ARCA AND FNR REGULATE BIOLUMINESCENCE IN THE LIGHT-ORGAN SYMBIONT *VIBRIO FISCHERI*

by

JEFFREY L. BOSE

(Under the Direction of Eric V. Stabb)

ABSTRACT

Despite years of intense study of bioluminescence in *Vibrio fischeri*, much remains unanswered regarding its benefit to the cell and the environmental factors affecting its regulation. In this dissertation, I show that bioluminescence leads to a growth rate reduction under some culture conditions, yet it contributes to this bacterium's ability to colonize one of its symbiotic hosts, the squid *Euprymna scolopes*. Based on the reaction catalyzed by the light-producing enzyme luciferase, two conflicting hypotheses have been suggested to explain the benefit that bioluminescence provides symbiotic *V. fischeri*. One proposal is that luminescence provides an advantage by consuming oxygen and thus serving as an antioxidant to protect the cell from toxic oxygen radicals. Conversely, a second proposal suggests that by consuming excess reductant luminescence acts as an electron sink. I examined the regulation of luminescence to help distinguish between these two hypotheses, assuming that luminescence would be maximally expressed when it is most beneficial. I examined luminescence of mutants lacking the redox-responsive and oxygen-sensitive regulators ArcA and FNR, respectively. This was performed in the well-studied and naturally visibly luminescent strain MJ1 as well as in the relatively dim strain ES114, which is a natural symbiont of *E. scolopes*. ArcA is most active under reducing

conditions, and I found that it represses luminescence in both strains due to a conserved ArcA binding site in the P_{luxI} promoter, which controls the expression of the proteins necessary for light production. Similar to ArcA, FNR is active under anaerobic conditions and represses luminescence in MJ1, although no such effect is apparent in ES114. These results demonstrate that luminescence is repressed under reduced conditions and therefore my data is consistent with the antioxidant model explaining the benefits of luminescence, but are inconsistent with the electron sink model. This work is the first to use defined mutants in *V. fischeri* to elucidate a mechanism by which specific environmental factors alter the regulation of luminescence.

INDEX WORDS: *Photobacterium*, Symbiosis, Quorum sensing, Lux

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by

JEFFREY L. BOSE

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by

JEFFREY L. BOSE

Major Professor: Eric Stabb

Committee: Anna Karls
Ellen Neidle
Mark Eiteman

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I would like to dedicate this dissertation to three people, my parents and my wife Stephanie. I need to thank my parents Greg and Nancy who have lovingly supported me throughout all my years in school. I never could have made it this far without years of their support and encouragement. They have never once doubted me and have been a great support during times of discouragement and doubt. Stephanie has been around for only this leg of the journey; however, it has been one of the toughest. Her strength and perseverance going through this process herself was a great model to follow. She has been a continual source of encouragement during this time despite the stress of both of us graduating within a couple of months of each other and in the first year of marriage. Words aren't enough for thanks that I owe all three of you for your years of support.

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I want to thank the friends and family that I have outside this department, you have given me a mental break when I have needed it. It's great to have close friends and family who I can't talk science with. Particularly my brothers who are always a good source of laughs. Thanks Brent, Emily, and Shane. The three of you through your prayers, encouragment, and support, helped me stay focused when the going got rough.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Overview of Bioluminescence

Bioluminescence has been the subject of intrigue for centuries, with the first record of bioluminescence dating to approximately 1000 BC in the Chinese book Shi jing (2). The attraction to bioluminescence is easy to see, from kids collecting lightning bugs to adults who see a glowing culture of bacteria, the luminescence of dinoflagellates in crashing waves, or the flashing luminescent organs of some fish. Beyond appreciating the aesthetically captivating visual display is the intrigue that a living organism can convert chemicals to light. Even though only a small percentage of organisms can produce light, this ability can be found in diverse groups, both on the land and in the sea, and includes both multi- and unicellular organisms.

Among the bioluminescent Eukaryotes are several families of beetles, with perhaps the most well known being fireflies and glow worms (243). Almost all luminescent beetles appear to have similar luciferase enzymes that produce light using essentially the same chemistry. Other terrestrial bioluminescent organisms fall into several genera of mushrooms, *Armillaria* (honey mushrooms), *Omphalotus* (Jack O' Lantern mushroom), *Panellus*, and *Mycena* (foxfire mushrooms) (199). In addition to these terrestrial organisms, bioluminescence is also produced by several marine animals including fish such as the hatchetfish (109) and the cookie-cutter shark (239), as well as invertebrates such as the jellyfish *Aequorea victoria* and the sea pansy (243). Perhaps one of most stunning examples of bioluminescence comes from dinoflagellates.

The light produced by dinoflagellates is blue, and under the right conditions these organisms bloom and give ocean waves a bluish glow (91).

There are also several species of bacteria that make light. Bioluminescent bacteria are currently categorized in only three genera, one terrestrial and two aquatic. The only known terrestrial bioluminescent bacterium is *Photorhabdus luminescens*, which is typically found in a mutualistic association with nematodes of the family Heterorhabditidae, and this bacterium aids the nematode in killing insects, yielding dead glowing larvae (68). The luminescence of *P. luminescens* also may have been described by military doctors reporting glowing wounds (161), and this has been substantiated by the recent isolation of *P. luminescens* from a human wound (24).

Aquatic luminescent bacteria belong to the genera *Photobacterium* or *Vibrio*. Bioluminescent *Vibrio* and *Photobacterium* species can be found either as free-living cells or in association with marine animals. As free-living cells, they are widespread in the oceans and are subject to dynamic population changes with seasonal variability, although population densities are typically far too low to produce visible light (149). However, on rare occasions luminescent *V. harveyi* can grow in association with the microalga *Phaeocystis* and are believed to be the cause of the phenomenon known as "Milky seas" (115, 143). This is the name given by sailors when large swaths of the ocean glow, reports of which date back to accounts in ship's logs from the 17th century. Due to their unpredictability, only two occurrences of "Milky seas" have ever been scientifically studied (115, 143), and therefore it is not known if *V. harveyi*-*Phaeocystis* associations are the cause of all Milky seas (151). The most recent Milky seas event covered >17,000 km^2 (approximately the size of Connecticut), involved perhaps 10^{22} bacteria, and lasted

for three days (143). In contrast to these large scale luminescent events, luminescence of bacterial cells is typically only seen when the bacteria are associated with animal hosts.

Host-associated luminescent marine bacteria can be found as part of mixed bacterial communities or in mono-specific symbioses. Luminescent bacteria are often found in the guts of fish and other marine animals; reaching 10^7 cells per ml of gut contents, which in some cases constitutes 100% of the colony forming units (149). Considering the large populations these bacteria can reach in the fish gut, it is not surprising that the fish fecal pellets can be luminescent due to high numbers of bacteria (178). It is believed that the luminescence of these fecal pellets attracts fish to eat them, thereby allowing the bacteria to reach another fish gut.

In addition to such associations with marine animal gut communities, some luminescent bacteria colonize specialized host organs as single-species cultures (149, 174). These specific bacteria-animal interactions are present in at least 30 host genera encompassing 11 families of fish and at least two families of squid (82, 149). Interestingly, this is a relatively small number considering there are approximately 25 families of fish and 19 families of squid that are luminescent (149). Each of the symbiotically luminescent animals has a specialized "light organ", which contains a culture of luminescent bacteria. The location and anatomy of the light organ depends on the animal in question and the role of luminescence for that animal. These bacteria-host relationships are considered mutualistic symbioses with the bacteria receiving nutrients in a protected niche and the fish or squid receiving the use of the bacterium's light. Several species of bacteria have been found in these symbioses, including *V. fischeri* (8, 180), *V. logei* (42), *Photobacterium phosphoreum* (149), and *P. leionathi* (149). Among these, *V. fischeri* is notably widespread and can be found in association with both fish (180) and squid (8). *V. fischeri* has also been especially well characterized and is the subject of this thesis.

The biochemistry and genetics of *V. fischeri* bioluminescence were worked out largely using bright isolates such as strain MJ1 (206). Unfortunately though, the symbiosis between MJ1 and its pinecone fish host, *Monocentris japonica*, cannot be reconstituted in the lab, because the fish have not been successfully bred in captivity. In addition, MJ1 was passaged on slants for years, and the current freezer stocks of this strain vary genetically from lab to lab (see Appendix C). For these reasons, most of the *V. fischeri* research recently has focused on strain ES114, a wild-type isolate from the light organ of the Hawaiian bobtail squid, *Euprymna scolopes*. Fortunately, the most basic principles of bioluminescence are conserved in MJ1 and ES114, allowing researchers to use insight from older investigations of MJ1 while exploiting the experimental tractability of ES114 and its host.

The *Euprymna scolopes-Vibrio fischeri* Symbiosis

Studying bioluminescence in *V. fischeri* ES114 provides several advantages. First, this strain can be easily genetically manipulated and the genomic sequence of ES114 has recently become available (181). In addition, the symbiotic partner of ES114, *E. scolopes*, can be maintained in the lab and breeds readily, giving rise to aposymbiotic *Vibrio*-free eggs and hatchling juveniles, on which most experiments are conducted (236). Because juveniles hatch without any symbiotic bacteria (236), researchers can control and monitor the infection process. For example, uninoculated squid and those inoculated with *V. fischeri* have been compared to determine how the symbionts affect host tissue (37). Similarly, bacteria with fluorescent tags have been used as inocula to visualize the infection process (45, 157, 159, 215). Researchers can also compare squid infected with wild-type or mutant *V. fischeri* strains to identify bacterial factors that contribute to host colonization (34, 144, 174, 211, 233, 234, 238, 245). Finally, only

V. fischeri cells are able to maintain colonization of the *E. scolopes* light organ, which allows researchers to examine the association of a single bacterial species with a host tissue (8). For these reasons and others, the symbiosis between *V. fischeri* ES114 and *E. scolopes* has become a model system for host-bacterial interactions and allows researchers to study bioluminescence under conditions that are more ecologically relevant than growth in a shake flask.

The association of *E. scolopes* and *V. fischeri*, like other luminescent bacteria-host interactions, is a mutualistic symbiosis. During symbiosis, *V. fischeri* lives in a protected niche that is free from competitors and receives nutrients from *E. scolopes*. The chemical conditions in the light organ have been difficult to study with mechanical probes due this organ's small size, elasticity, and fragility. However, analysis of symbiotic light organs revealed the activity of anaerobic respiratory pathways, suggesting that the light organ is an oxygen-limited environment (166). The use of auxotrophic mutants of *V. fischeri* in colonization studies indicates that *V. fischeri* receives amino acids and peptides in the light organ, and this was supported by *ex vivo* analysis of light organ contents (71). Other studies have implicated mannose (136), sialomucin (157), dead host cells (158), and chitin as potential nutrients for *V. fischeri* in the light organ. Interestingly, *E. scolopes* expels ~90% of the *V. fischeri* from its light organ every morning and regrows a healthy culture of bioluminescent bacteria each day, enhancing the local *V. fischeri* population (11, 120). In exchange, *E. scolopes* receives the use of the *V. fischeri*'s light.

No conclusive studies have been reported to explain the benefit that luminescence provides *E. scolopes*. Considering how luminescence is used by other marine animals, *E. scolopes* may use the light as a defense strategy, a predatory advantage, or a means of communication (81, 82, 239). A popular theory is that *E. scolopes* uses luminescence in a camouflaging mechanism called counterillumination (137, 174). In this model, light projected

from the ventral side of the squid would be adjusted to mimic moonlight from above in order to prevent predators below from recognizing the squid. Consistent with this theory, the architecture of the light organ is structured to allow controlled ventral light emission, and one study demonstrated that *E. scolopes* alters the intensity of light emitted from the light organ in response to downwelling light (100). On the other hand, if *E. scolopes* was using luminescence for such a strategy, I would expect the light emitted to be easily observed from beneath the animal when it is swimming in the water column. However, no one has published a picture of light emitted from swimming *E. scolopes* and if you examine an adult squid sitting in a clear dish from below, you are unable to see any emitted light (personal observation).

In other species where counterillumination has been observed, the animal has multiple light-emitting photophores (81, 99). Animals with fewer points of light emission are less likely to produce an even light field and each light source must be brighter to balance out unlit regions of the animal (99). This effect is particularly important at shallow depths where downwelling light is bright. The effectiveness of counterillumination is also related to the optical acuity of the predator, because fish with high-resolution eyes are more likely to be able to discriminate between moonlight and counterillumination. Based on this information bioluminescent animals at shallow depths would be at a disadvantage when using fewer, but brighter, points of light (99). It is hard to reconcile these observations with the single light source of *E. scolopes*, although it may be explained if *E. scolopes* only benefits by hiding the ink sac that is found just above the light organ, which is the only large structure of the squid that the animals cannot make translucent.

These considerations bring into question the hypothesis of counterillumination in *E. scolopes*. A second theory, which I support, proposes that *E. scolopes* uses luminescence as a

startling tactic (137). This theory suggests that *E. scolopes* is able to emit brief, yet intense, flashes of light to startle either prey or an attacking predator. Recently, it was found that physically squeezing an adult squid caused it to produce such a bright flash (Andrew Wier, personal communication). Only proper ecological studies will be able to distinguish these theories. Whatever benefit(s) luminescence confers on the animal, it is clearly not necessary for their health in laboratory aquaria (77), facilitating research on this symbiosis by allowing comparisons of infected and uninfected animals.

The initial colonization of *E. scolopes* by *V. fischeri* has become a topic of intense research and many of the events leading to symbiotic establishment have been described. Upon hatching, ciliated appendages on either side of the light organ serve to collect and enrich *V. fischeri* from the surrounding seawater. These cilia increase water flow around the light organ (159), and they shed mucus to which bacteria adhere. This combination of increased water flow and sticky mucus promotes the aggregation of bacteria outside the light organ (157, 159). Following aggregation, an unknown signal triggers the migration of the bacterial aggregate toward six pores, three on each half of the bilobed light organ (159). The bacteria then have to make their way through outward beating cilia-lined ducts that lead to antechambers and finally into the epithelial cell-lined crypts of the light organ where a few initial colonists proliferate (135, 175, 215). Interestingly, upon successful colonization, *V. fischeri* triggers morphological changes in the host resulting in loss of the ciliated appendages (37). Other bacteria are able to aggregate outside the light organ; however, only *V. fischeri* is able to enter the crypts, proliferate to large numbers, and maintain colonization of the light organ.

Many factors contribute to the ability of *V. fischeri* to colonize the *E. scolopes* light organ successfully. First, motility is essential for *V. fischeri* to colonize and non-motile mutants are

unable to migrate into the pores and move against the current created by cilia lining the ducts (70, 159, 175). In addition, the level of motility must be tightly regulated because hyper-motile cells also have reduced colonization efficiencies (144). Recently, a protein similar to type IV-A pilin has been shown to aide in initial colonization and may be important for either twitching motility or adherence (211).

In addition to traversing physical barriers, *V. fischeri* also faces chemical obstacles. The squid produce several antimicrobial compounds, notably oxidatively reactive toxic chemicals, presumably to prevent unwanted bacteria from colonizing the light organ and perhaps to control *V. fischeri* populations as well. For example, the squid tissues in direct contact with bacteria produce halide-dependent peroxidases that produce hypohalous acid (201, 223, 237). In addition, the cells of the ciliated appendages and those lining the ducts and light organ antechambers produce nitric oxide (33), which was also detected in vesicles where bacteria aggregate on the light-organ surface. Finally, *V. fischeri* likely encounters hydrogen peroxide (234), and perhaps other oxidative species (177). Therefore *V. fischeri* must possess defensive systems to protect itself from this onslaught. Indeed, *V. fischeri* produces a catalase that contributes to colonization competitiveness, and its periplasmic localization suggests it is counteracting host-generated H_2O_2 (234). Genes encoding putative enzymes that inactivate nitric oxide have also been identified in the *V. fischeri* genome (33). Additionally, bioluminescence itself has been hypothesized to prevent the production of toxic oxidative species by depriving the host of O_2 (221, 233). If this is true, then it is not surprising that bioluminescence is required for *V. fischeri* to fully colonize *E. scolopes*, as was indicated by Visick *et al*. (233) and confirmed in Chapter 2 of this thesis. To understand the logic of this model, one must first be acquainted with the biochemical mechanisms of bacterial bioluminescence.

Bioluminescence in *V. fischeri*

The biochemical mechanisms underpinning bacterial bioluminescence have been worked out primarily in *V. fischeri* and *V. harveyi*, and they are well understood in both. In short, a heterodimeric flavin monooxygenase enzyme luciferase, composed of LuxA and LuxB, uses O_2 to oxidize a long-chain aldehyde and $FMNH_2$, generating FMN, the corresponding long-chain carboxylic acid, water, and light (Fig. 3.1) (251). This occurs in a specific sequence of steps that were worked out by Hastings and Gibson (85). First, luciferase reacts rapidly with $FMNH_2$ to form an intermediate reduced enzyme that is short lived due to oxidation by O_2 to form a second intermediate. The second intermediate then reacts with aldehyde to produce a light-emitting species. This yet unknown species releases a photon of light with a wavelength of ~490 nm.

Other interesting aspects of luciferase activity may reflect functionally important roles that are not yet fully understood. Interestingly, bacterial luciferases fall into two distinct groups with "fast" or "slow" reaction kinetics. For example, the *V. fischeri* luciferase is ~10-fold faster than that of *V. harveyi*, and while this seems significant there is no explanation for the difference (149). Luciferase can also carryout a "dark" reaction in the absence of aldehyde resulting in the generation of FMN and H_2O_2 (84). The extent to which this reaction occurs and its physiological implications are uncertain; however, it has been proposed that the dark reaction could be a mechanism for producing host-damaging H_2O_2 (59), as some pathogens do (163).

In order to keep luciferase supplied with substrates, four additional Lux proteins are involved in the (re)generation of aldehyde and $FMNH_2$. LuxC, LuxD, and LuxE are responsible for (re)generating luciferase's long chain aldehyde substrate (16). LuxD is an acyl-transferase that brings in a fatty acid that can then be activated with ATP by a synthetase, LuxE, to form an acyl-protein intermediate. This intermediate is then reduced by LuxC to form the aldehyde.

LuxG is a flavin reductase that is involved in the (re)generation of $FMNH_2$ (250); however, other enzymes in the cell also perform this function and not all bioluminescent bacteria have a dedicated LuxG. The genes encoding these proteins necessary for light production in *V. fischeri* are co-transcribed in the *luxICDABEG* operon. LuxI is not necessary for light production, but is involved in the regulation of the *luxICDABEG* operon discussed below.

The bioluminescence-producing *lux* genes are subject to cell-density-dependent regulation. This type of regulation, also called quorum sensing, has become a focus of much research in recent years and has been found in many bacteria. The earliest discoveries of quorum sensing in Gram-negative bacteria were made in studies of luminescence induction in *V. fischeri* and *V. harveyi*, and both bacteria continue to be important model systems for understanding this phenomenon. Although the specific mechanisms of quorum sensing vary between species, in many basic respects they are similar to the *V. fischeri* system described below.

In a basic model of quorum sensing, *V. fischeri* cells produce small signaling molecules referred to as autoinducers (AIs) in order to monitor population densities. For sensing of population density to occur, two proteins are necessary, an AI synthase and a regulator that responds to AI. In the *V. fischeri* Lux system, LuxI, which is part of the *luxICDABEG* operon is the synthase (188), and the divergently transcribed *luxR* encodes the AI-dependent regulator (55, 56). LuxI produces *N*-3-oxo-hexanoyl homoserine lactone (*N*-3-oxo-C6-HSL) (188) which readily diffuses out of the cell. As the density of cells increases, *N*-3-oxo-C6-HSL accumulates in the environment until a threshold concentration is reached, whereupon *N*-3-oxo-C6-HSL binds to LuxR, activating LuxR to stimulate gene expression (48, 152). Activated LuxR binds to the *luxICDABEG* promoter at a sequence called the "*lux* box" (35, 196, 228), compensating for a weak -35 promoter element and stimulating *luxICDABEG* expression. This activation of

expression is amplified by a feed-forward mechanism, because the production of LuxI leads to more *N*-3-oxo-C6-HSL, which activates LuxR and yields yet more LuxI protein expression. Initiation of this self-stimulatory system, termed "autoinduction", leads to rapidly increasing levels of luciferase and light production (152).

In contrast to this simplified model, cell density-dependent induction of luminescence in *V. fischeri* is actually far more complex. In addition to responding to *N*-3-oxo-C6-HSL, LuxR is also weakly activated by the competing autoinducer *N*-octanoyl-HSL (C8-HSL), which is made by a second AI-synthase, AinS (110, 127). These two AIs are important for coordinating the induction of luminescence with C8-HSL primarily stimulating induction at lower cell densities than *N*-3-oxo-C6-HSL (129). Aside from its direct interaction with LuxR, C8-HSL also activates a regulatory cascade apparently through AinR, LuxU, LuxO, and LitR, ultimately activating *luxR* transcription (Fig. 1.1, reviewed in (232)).

In addition, *V. fischeri* contains a third AI-synthase, LuxS (127). The autoinducer produced by LuxS, AI-2, is presumably a furanosyl borate diester (21). Unlike *N*-3-oxo-C6-HSL and C8-HSL, which are relatively specific, AI-2 is produced and recognized in many bacterial species and appears to be involved in inter-species communication (247). In *V. fischeri*, AI-2 stimulates LuxR expression first through its receptors LuxP and LuxQ and then by feeding into the same LuxU, LuxO, and LitR regulatory cascade as C8-HSL. Compared to the simplified view of quorum-sensing described above, these networks underscore the complex regulation that *V. fischer*i uses to coordinate luminescence in response to cell density.

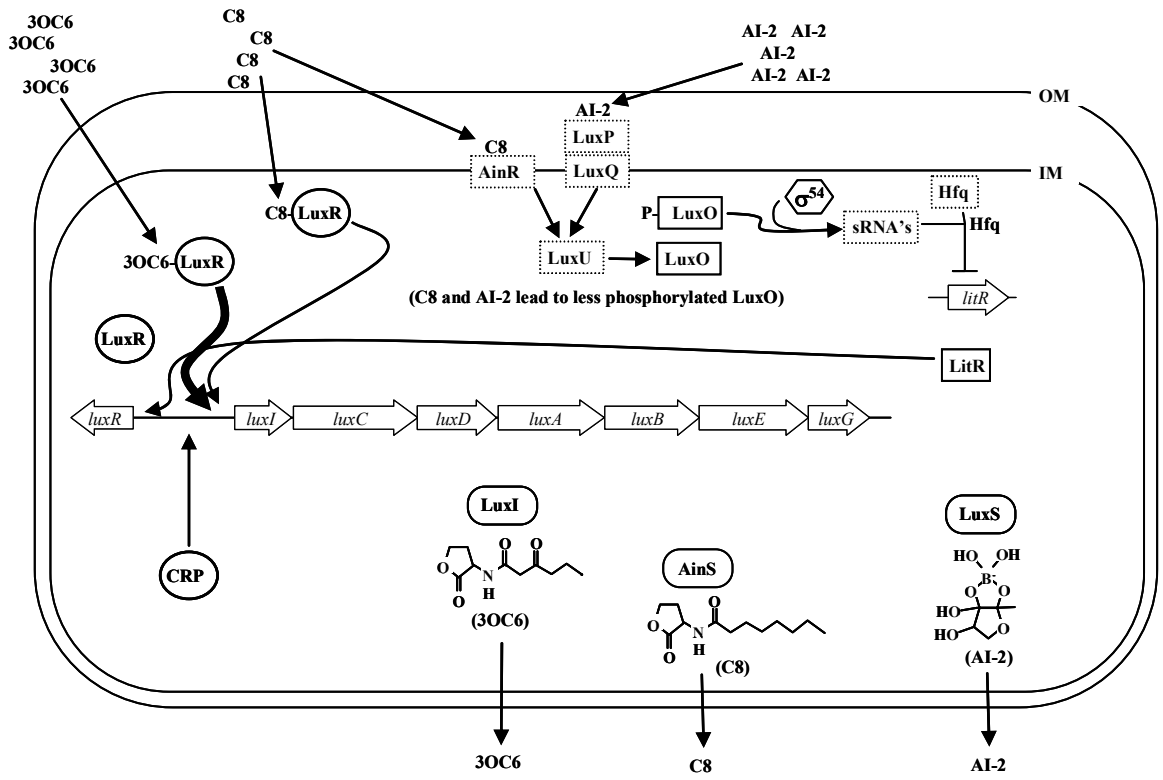

FIG. 1.1. Model of autoinducer-mediated *lux* regulation in *V. fischeri*. Elements with dashed boxes have been described in *V. harveryi* and their involvements implicated in *V. fischeri* due to homologs in the *V. fischeri* genome. The three auotinducers, *N*-3-oxo-hexanoyl-HSL, *N*-octanoyl-HSL, and presumably a furanosyl borate diester, are abbreviated as 3OC6, C8, and AI-2, respectively. (Modified from (206))

Environmental conditions have also been shown to affect the luminescence in certain *V. fischeri* strains. In strain MJ1, the addition of glucose to growth media leads to a longer lag in the induction of luminescence (62), and iron also represses luminescence in this strain (87), although neither glucose nor iron affects luminescence in strain ES114 (8). Another study found that *luxR* expression is activated by cAMP and its receptor, the regulator protein "cAMP receptor protein", or CRP (40, 41); however, these studies were performed using the MJ1 *lux* genes on a plasmid in *E. coli* and the role of CRP-mediated *lux* activation in *V. fischeri* remains uncertain. It is important to note that while cAMP increases *lux* expression in both MJ1 (62) and ES114 (8),

repression of luminescence by glucose in MJ1 cannot be rescued by addition of cAMP and therefore is apparently independent of cAMP-CRP (180).

Finally, it has been suggested that luminescence is maximally expressed in MJ1 when cultures are poorly aerated (150). Interestingly, a study using the MJ1 *lux* operon cloned in *E. coli* suggested that the low-oxygen activated regulator FNR induces luminescence (147), providing a possible mechanistic explanation for aeration-dependent *lux* regulation. On the other hand, FNR has not been investigated in *V. fischeri* and aeration apparently affects strain ES114 differently than MJ1 (8). As discussed below, these unresolved issues of aeration- and FNR-dependent *lux* regulation are topics I have addressed in this thesis.

In the section above I summarized what is known about the biochemistry, genetics, and regulation of bioluminescence in *V. fischeri*. Although many of the regulatory mechanisms controlling bioluminescence remain undetermined, it is clear that expression of bioluminescence is subject to tight control. Such regulation makes sense, because given its biochemical basis, luminescence can be an energetically expensive process. Therefore, cells have presumably evolved mechanisms for inducing luminescence only when its benefits outweigh the costs. In the following section I will briefly discuss the energetic costs of bioluminescence as well as some of the possible fitness advantages conferred by this process. I will then close this chapter by introducing the objectives of my research and revisiting the topic of regulation as it relates to understanding the functional significance of bioluminescence.

Evolution and Ecology of Bioluminescence

Before discussing the evolution and ecology of luminescence, it is appropriate to consider the cost that this process has on a light-producing bacterium. This will be discussed in greater

length in Chapter 2 and therefore only briefly here. Luciferase consumes both reducing power, ultimately from the NADH pool, and oxygen, which could theoretically compete with aerobic respiration and therefore lead to decreased ATP production. In addition to this potential opportunity cost, there is sizeable energetic input necessary to synthesize the Lux proteins (103). Finally, recycling luciferase's aldehyde substrate directly consumes ATP. These energetic considerations have led researchers to predict that luminescence should be a significant energetic drain on brightly glowing cells (149). Indeed, several studies have described dark or dim mutants arising that grow faster than their bright parents and that out-compete their parental strains in culture (86, 104, 165). Conversely, a bright mutant of ES114 was isolated and found to have slowed growth (43). A shortcoming of these studies is that they examined undefined or pleiotropic mutants, but they do suggest that luminescence leads to decreased fitness in culture. Nonetheless, the fact that these bacteria produce light suggests there must have been some evolutionary drive and selection for maintaining this costly process.

The selective advantage driving the origin and evolution of bioluminescence has been a subject of much debate. It has been proposed that luciferases evolved from general oxygenases, which simply use molecular oxygen to oxidize a substrate. This theory would put oxygenases originating first and then evolving into light-emitting luciferases due to reaction chemistry (170). However, luciferases are relatively poor oxygenases and show slower oxygen turnover than well characterized oxygenases (170). In addition, luciferases appear to be phylogenetically distinct from oxygenases and may have evolved independently in more than 30 instances (170, 243). Others have suggested that the increased fitness conferred by early luciferases was based on their oxidation of luciferin substrate, and that luminescence evolved as a way to alter electron flow (149, 170). A third proposal is that bioluminescence first evolved to detoxify oxygen as

anaerobic life forms struggled to cope with the shift from a reducing to an oxidizing atmosphere on ancient Earth (170). Luciferases may also have played a role in detoxification of mutagenic peroxidated aldehydes. Unsaturated fatty acids can be peroxidated to yield fatty acid aldehydes, some of which may be mutagenic and/or highly reactive with amino and sulfhydryl groups of proteins (49). Since one substrate for bacterial luciferase is a long-chain aldehyde, the selective advantage of the ancestral luciferase may have been to rid the cell of these toxic aldehydes in order to protect the cell from oxidative damage (170).

It has also been proposed that the light itself was beneficial to early cells, and may have allowed cells to carry out photochemistry in the dark (149). Also consistent with the notion that light is the driving force behind the evolution of luminescence, researchers have recently suggested that bioluminescence evolved as a mechanism to stimulate photolyase-mediated DNA repair to protect cells from DNA damage (30). However, a study performed in our lab demonstrated that this hypothesis is unlikely to be true and does not account for the current benefits of luminescence to *V. fischeri* in the *E. scolopes* light organ, where this strain maximally produces light (235). As is apparent in this discussion, many theories for the origin and early biological function of luciferases and bioluminescence have been proposed; however, I believe it is unlikely the selective forces that led to the emergence of light production will ever be known. Moreover, the variety of luciferases extant today probably reflects multiple evolutionary origins that may not all have arisen from the same selective advantage.

Unlike studies of the origin of luminescence, the ecology and current selective benefits of luminescence can be studied both in the lab and in the natural habitat of luminescent organisms. Studies of this nature may not answer the question of how luminescence originated, but may give insight as to why it continued to evolve and what advantage(s) light production gives now. The

rationale for depositing a large amount of energy into luminescence in eukaryotes is understandable because it may provide a defense strategy, a predatory advantage, or a means of communication (82, 239). However, the benefit to a single bacterium is harder to understand. It is unlikely that any organism can detect the luminescence of a single bacterium, and therefore it is not surprising that luminescence is a social behavior that only happens at high cell density. It is fascinating that luminescent marine bacteria have developed the complex regulatory cascade of quorum sensing to ensure that luminescence is only produced where it is needed. For example, luminescence of *V. fischeri* ES114 is necessary for complete colonization of its squid host, and light is maximally produced by this strain in the densely populated confines of the light organ. In this view, bacterial luminescence may have been maintained throughout evolution because helping the host ultimately helps the symbiont and its close relatives in the light organ. But in addition to this advantage of symbiotic mutualism, luminescence is also apparently beneficial to symbiotic bacteria for some other reason, because it is required for efficient host colonization.

Interestingly, this symbiotic benefit creates an apparent paradox for a light-producing bacterium such as *V. fischeri* in that bioluminescence is also predicted to be a significant energetic drain on the cell. Based on the reaction catalyzed by luciferase, two conflicting hypotheses have been presented to explain the advantage luminescence imparts *V. fischeri* in host colonization. First, luminescence may give *V. fischeri* a means of defending itself from toxic reactive oxygen species (ROS) (221, 233). One variant of this theory suggests that luminescence decreases the ambient oxygen concentration in the host to limit the oxygen available for the host enzymes that produce nitric oxide, hypohalous acid, hydrogen peroxide, and other ROS, thereby limiting production of these antimicrobials in the host light organ (233).

Another variant of this theory suggests that luminescence leads to a more reduced cytoplasmic environment in the bacterium, making it more resistant to oxygen radicals derived from either the host or the bacterium itself (221). This theory agrees well with proposals that luminescence originally evolved as an antioxidant to protect cells. A second completely different theory suggests that luminescence may play a central role in maintaining redox balance by consuming excess reductant (15). This theory posits that when NADH/NAD^+ ratios become too high in the cell, luminescence acts as an electron sink to regenerate NAD^+.

Little work has been done to distinguish between these opposing theories. Bourgois *et al*. used *n*-butyl malonate to inhibit and control electron input into reducing equivalents and observed an increase in *P*. *phosphoreum* luminescence when reductant availability exceeded the demand from respiration (15). Based on these findings, they suggest luminescence acts as a reducing equivalents scavenging enzyme that "dissipates the energy contained in an overflow of electronic flux". However, it has been shown that *V*. *harveyi* mutants lacking luciferase are more sensitive to several oxidants, suggesting that luminescence functions to detoxify ROS (216). Much work remains to be done to resolve these observations and to define the benefit(s) of luminescence for symbiotic cells.

Rationale and Objectives of this Study

As described above, and in greater detail in Chapter 2, luminescence is expected to be an energetic drain on brightly glowing cells, yet it apparently provides a real-world advantage by helping *V. fischeri* cells to colonize the host light organ. However, this predicted cost to cultured cells has not been clearly demonstrated in defined strains. Similarly, an attenuated and pleiotropic mutant was used to test the role of luminescence in colonization of *E*. *scolopes* (233).

Therefore, the first objective of my thesis was to use defined *lux* mutants to examine the decades-old hypothesis that luminescence has a negative affect on growth of *V. fischeri* and to retest the finding that luminescence provides an advantage during symbiosis. In Chapter 2, I demonstrate that high luminescence leads to a decrease in growth rate under some culture conditions and that a dark mutant has a competitive advantage relative to a bright parent in a mixed culture. I also confirm that luminescence contributes to persistent infection of the host, with a dark mutant reduced ~4-fold in colonization levels.

In Chapters 3 and 4 I focus my attention on the regulation of luminescence with the goal of gaining new insights into the advantage luminescence affords symbiotic cells colonizing the host. Enzymes often are regulated both in response to substrate availability and to ensure that the enzyme is abundant only when it is needed (17). Based on these common regulatory themes, and the conflicting models of luciferase functioning either as an antioxidant or an electron sink, I hypothesized that the *lux* genes are regulated by oxygen- and/or redox-dependent regulators such as ArcA and FNR.

The redox-responsive ArcAB two-component regulatory system

The ArcAB (aerobic respiration control) proteins comprise a two-component regulatory system. ArcB is the membrane-bound sensor kinase and ArcA is its cognate response regulator. The *arcA* gene was first identified and referred to as "*dye*" because mutations in this locus lead to sensitivity to dyes such as methylene blue (172) and toluidine blue (95). Since then, ArcA has also been referred to as *fexA*, *msp*, *seg*, and *sfrA* based on phenotypic analyses (3). ArcA regulates a variety of metabolic genes and was first thought to be a response regulator based on DNA sequence similarity to OmpR (95). Shortly thereafter, *arcB* was identified based on the

observation that mutations in this gene yield phenotypes similar to that of *arcA* mutants (92). Demonstration of phenotypes linking ArcB to ArcA and ArcB's similarity to other sensor kinases confirmed it as the sensor paired with ArcA in a two-component system (94).

The ArcAB system responds to the redox state of the cell. In *E. coli*, ArcB is bound to the cytoplasmic membrane where it senses the redox state of the quinone pool. Oxidized quinones bind to and inhibit ArcB autophosphorylation (65) due to dimerization of the protein (133). Shifts to more reducing conditions, artificially mimicked by the addition of reducing agents like DTT, lead to a reduction of quinones and activation of the ArcAB system (171). Additionally, effector molecules such as D-lactate, acetate, and pyruvate can stimulate ArcB activation by an unknown mechanism (64, 171). Under reducing conditions, in the absence of oxidized quinones, ArcB autophosphorylates, starting a His-292 → Asp-576 →His-717 phosphorelay (66, 111). The phosphate is then transferred to Asp-54 of ArcA forming ArcA-P (97). This activates ArcA's ability to regulate expression of target promoters.

Both ArcA and ArcA-P are able to bind to target promoters; however, ArcA binds with a lower affinity and less specificity than ArcA-P (97). Prior to DNA-binding, ArcA must form a multimer of ArcA and ArcA-P in an apparent 1:1 ratio that can include up to 8 monomers. Coinciding with these large multimers, DNase I protection assays have yielded footprints up to 100 base pairs (131). Upon multimerization, ArcA then binds to target promoters that contain a semi-conserved sequence of 5'-[A/T]GTAATTAA[A/T]-3' (131, 160).

ArcA is primarily known for controlling the expression of proteins involved in aerobic and anaerobic metabolism, but it also regulates other cellular functions (39, 93, 95, 131, 160, 198). *Vibrio cholerae arcA* mutants have decreased virulence in mice due to a decrease in the expression of cholera toxin and toxin-coregulated pilus (194). In *E. coli*, ArcA binds close to

oriC of the chromosome to limit replication initiation and therefore maintain slow growth rate anaerobically (121). In addition, ArcA mediates Xer-specific recombination at the *psi* site of some plasmids (25), alters F-pilus formation when *E. coli* is grown anaerobically (94), and represses expression of *sodA*, which encodes superoxide dismutase (83). The repression of *sodA* illustrates that ArcA can mediate responses to ROS, and consistent with this *Salmonella enterica arcA* mutants have increased sensitivity to reactive nitrogen and oxygen intermediates (126).

When all the functions controlled by ArcAB are considered, it is clear ArcAB represents a global regulatory system. Previously, thirty operons in *E. coli* had been demonstrated to be controlled by ArcA, with most of these being involved in respiratory metabolism (130). Recently, two microarray studies have been published studying an *E. coli arcA* mutant. Liu and De Wulf used a combined weight matrix and microarray approach to determine that 9% of the genome is under direct or indirect control of ArcA (124). Using a slightly different growth medium and statistical analysis, Salmon *et al*. demonstrated 175 genes are ArcA-regulated (184). However, when examining those genes expressed above background with more rigorous statistical analysis, they predict that 1139 (26%) of the 4345 genes in *E. coli* are controlled directly or indirectly by ArcA. Together, these studies emphasize that ArcA is a global regulator whose activity extends well beyond genes involved in metabolism.

Based on the redox-sensitive regulation of ArcAB, I sought to determine if *V. fischeri* possesses an ArcAB and if it contributes to *lux* regulation. In Chapter 3, I present data describing an ArcAB system in *V. fischeri* encoded by VF2120 and VF2122 that is functionally similar to ArcAB in *E. coli*. I show that this system represses luminescence in a redox-dependent manner and that this repression of *lux* is primarily due to an ArcA-binding site in the

luxICDABEG promoter. Finally, a model is presented to explain how ArcA might modulate *lux* repression in culture versus the host light organ.

The oxygen-sensitive regulator FNR

The FNR (fumarate nitrate reduction) protein is an oxygen-dependent global regulator. This protein was originally identified based on the observation that a *fnr* mutant was unable to grow anaerobically with nitrate or fumarate as sole electron acceptors (112). In *E. coli*, FNR is expressed during both aerobic and anaerobic growth, but it is only active under low-oxygen conditions (106, 205). Under aerobic conditions, FNR exists as a monomer and is unable to bind DNA (105, 116); however, FNR becomes a functionally active dimer under anaerobic conditions. This switch between inactive and active forms is mediated by an oxygen labile [4Fe-4S] center that when assembled leads to a conformational change in the protein, dimerization, and regulatory activity (29, 105, 106).

Following dimerization, FNR is able to bind target promoters and regulate gene expression. FNR recognizes a highly conserved sequence, 5'-T**TGA**TN_4A**TCA**A-3', referred to as a FNR-box (53), and upon binding FNR can either activate or repress transcription (134, 140, 244). The 5'-TGA-3' of each half-site, highlighted with bold letters above, are the nucleotides that FNR specifically interacts with and are the most important nucleotides of the recognition sequence (189, 203). The non-conserved bases, represented by "N", are less important, but do influence the degree of FNR regulation (192). FNR, like its close relative CRP, can act as either a Class I or Class II activator, depending on where it binds relative to the promoter and the specific interactions with RNA Polymerase (RNAP) (6). FNR possesses three activating regions AR1, AR2, and AR3. AR1 is necessary for contact with the RNA Polymerase α-subunit C-

terminal domain for class I activation. In class II activation, AR1, AR2, and AR3 are required for making contact with RNAP at the α-subunit C-terminal domain, the α-subunit N-terminal domain, and the σ-subunit, respectively (6, 113, 122, 125, 241). In addition to acting as a positive regulator, FNR can also repress gene expression by binding to multiple sites and bending DNA to prevent transcription initiation (140), or it can act as a simple repressor (69, 242).

FNR is a global regulator that is important in controlling the expression of many genes in the cell. Initial characterization of an *E. coli fnr* mutant identified altered levels of 125 proteins by two-dimensional electrophoresis (186). Some of these proteins are important in the tricarboxylic acid cycle, fermentation, and respiration (75). Several recent microarray studies have examined the global role of FNR in gene expression. They found that FNR affected 37 genes in 10 operons in *Bacillus subtilis* (169), 311 genes in *Salmonella enterica* (61), and 712 genes in *E. coli* (183). These numbers suggest that FNR either directly or indirectly affects the expression of 0.9%, 6.8%, and 16.6% of the entire genome for *B. subtilis*, *S. enterica*, and *E. coli*, respectively. Interestingly, many studies examining FNR-dependent promoters find that ArcA also acts at the same promoters suggesting that FNR and ArcA work in concert to fine-tune the expression of many genes (20, 27, 75, 83, 101, 224).

My goal for Chapter 4 was to determine if *V. fischeri* possesses a functional *fnr*, and if so, to test whether FNR regulates luminescence. A previous study of the MJ1 *lux* genes cloned in *E. coli* suggested that *lux* is activated by FNR in this heterologous system; therefore, I wanted to test this conclusion by examining FNR-dependent *lux* regulation in its native bacterium, *V. fischeri*. In Chapter 4, I describe the identification of FNR in *V. fischeri*, showing that it is functionally similar to FNR of *E. coli*. However, in contrast to the previous study, I found that

FNR represses luminescence in strain MJ1 when grown anaerobically and has no apparent affect on ES114 luminescence. In addition, FNR is shown to be active during, but not essential for, colonization of the *E. scolopes* light organ by *V. fischeri* ES114, giving insight into the environment of this important habitat.

Summary

Despite luminescence being a subject of intense study for several decades, the reports in Chapter 2 are the first examining the cost of luminescence to *V. fischeri* in culture using isogenic mutants. In addition, few studies have examined the regulation of luciferase in response to the compounds consumed during light production; oxygen and reducing power. Surprisingly, despite pervasive theories that luminescence benefits symbiotic cells either by consuming oxygen or by consuming excess reductant, no studies in *V. fischeri* have been published demonstrating that the *lux* genes are controlled by oxygen- or redox-monitoring regulators. The results presented in Chapters 3 and 4 are the first to identify such regulators and examine their contribution to luminescence under an ecologically relevant condition, during symbiosis. In addition, the results of these studies provide key information regarding the environment of the *E. scolopes* light organ. In Chapter 5, I discuss how my work has shed new light on the costs and potential benefits of luminescence and on the complex network that *V. fischeri* uses to regulate the *lux* genes.

CHAPTER 2

EFFECTS OF BIOLUMINESCENCE PRODUCTION ON GROWTH OF *VIBRIO FISCHERI*[1]

[1]Bose, J. L., Rosenberg, C. S., and E. V. Stabb. Submitted to *Archives of Microbiology*, May 2007.

Abstract:

The production of bioluminescence theoretically presents an energetic cost that could slow *Vibrio fischeri* growth; however, previous tests of this model were inconclusive, partly because they compared nonisogenic strains, or undefined and/or pleiotropic mutants. Therefore, to test the influence of the bioluminescence-producing *lux* operon on growth, we generated dark Δ*luxCDABEG* mutants in wild-type strains MJ1 and ES114 without disrupting the adjacent *luxR-luxI* regulatory circuit. The MJ1 Δ*luxCDABEG* mutant out-competed its visibly luminescent parent ~26% per generation in a carbon-limited chemostat. Similarly, addition of autoinducer to ES114 cultures stimulated luminescence of this otherwise dim strain and slowed growth relative to its Δ*luxCDABEG* mutant. Furthermore, when *luxCDABEG* was artificially controlled by $lacI^{q}$, induction of bioluminescence slowed growth. For each of these comparisons, some culture conditions yielded no detectable effect of luminescence on growth, indicating that luminescence is not always growth limiting; however, luminescence was never found to confer a growth advantage in culture. In contrast to this conditional disadvantage of *lux* expression, ES114 achieved ~4-fold higher populations than its Δ*luxCDABEG* mutant in the light organ of *Euprymna scolopes*. These results demonstrate that induction of *luxCDABEG* can slow *V. fischeri* growth under certain culture conditions but is a positive symbiotic colonization factor.

Introduction

Bioluminescence produced by *Vibrio fischeri* is well understood biochemically and genetically (86, 138, 225), and it results when luciferase, comprised of LuxA and LuxB, converts $FMNH_2$, O_2, and an aliphatic aldehyde (RCHO) to FMN, water, and an aliphatic acid. LuxC, LuxD, and LuxE (re)generate RCHO (16), consuming additional reductant and hydrolyzing ATP, while LuxG and other flavin reductases re-reduce $FMNH_2$ (123, 250). The genes encoding these Lux proteins are co-transcribed with *luxI*, and the *luxICDABEG* operon is adjacent to and divergently transcribed from *luxR* (55, 72, 73). Together LuxI and LuxR form a "quorum sensing" regulatory circuit that induces bioluminescence at high cell density (63).

Despite this detailed mechanistic understanding, the functional significance of bioluminescence for light-producing bacteria remains uncertain (207). Presumably, generating bioluminescence confers selective advantage(s) under some circumstances; however, this must be weighed against apparently significant energetic costs. In this context, "bioluminescence" refers to the complete process of generating light, encompassing not only the emission of photons but also the synthesis of Lux proteins and their enzymatic activities concomitant with light production. By the early 1890's Beijerinck and others observed dark mutants arising after prolonged culturing, leading to speculation that the energy devoted to bioluminescence in wild-type strains might actually hinder growth or survival (for a review see (82)). Given a modern understanding of this process there are at least three potential mechanisms of energy loss: (i) GTP hydrolysis devoted to Lux protein synthesis, (ii) ATP hydrolysis associated with regeneration of aldehyde substrate, and (iii) consumption of reducing power and oxygen without coupling this to the generation of a proton motive force to recover energy.

The net effect of these mechanisms on cellular economy and growth is unknown, but theoretical estimates suggest that the energy devoted to bioluminescence is substantial (86, 103, 225). Hastings and Nealson calculated that the energy of each photon emitted is equivalent to the hydrolysis of six ATP molecules, and that in bright cultures this corresponds to ~6 x 10^5 ATP hydrolyzed sec^{-1} $cell^{-1}$, with even more energy required if luciferase sometimes consumes substrates without producing light, as is likely the case (86). Theoretically, there is less, but still substantial, energy devoted to Lux protein synthesis, which was estimated for luciferase as equivalent to ~1 x 10^5 ATP hydrolyzed sec^{-1} $cell^{-1}$ in bright cells (103). For comparison, theoretical calculations suggest active *Escherichia coli* cells hydrolyze at least 2 x 10^6 ATP sec^{-1} $cell^{-1}$ (162), and experimentally determined values for *E. coli* were ~5 x 10^6 ATP hydrolyzed sec^{-1} $cell^{-1}$ (89, 153). Given these data, it seems reasonable to estimate that the energy devoted to bioluminescence could be equivalent to ≥10% of the ATP turnover in bright cells.

Despite such intriguing theoretical considerations, experimental attempts to address the effect(s) of luminescence on cell physiology and growth have yielded mixed results. In support of luminescence representing an energetic drain, growth in culture has been negatively correlated to luminescence, with relatively dark strains reportedly outgrowing brighter ones (30, 43, 86, 104, 165). On the other hand, one study detected no effect of luminescence on ATP pools or growth (103). These reports and the discrepancies between them are difficult to interpret because they compared non-isogenic strains, induced both luminescence and non-target genes, or used undefined or pleiotropic *lux* mutants.

Our goal was to test the effect of bioluminescence on *V. fischeri* and answer several longstanding questions: Can bioluminescence slow the growth of *V. fischeri* in culture under any circumstance? If so, is this always true or is it conditional? Finally, could we confirm a report

(233) that luminescence is a positive colonization factor for *V. fischeri* cells colonizing the light organ of the Hawaiian bobtail squid, *Euprymna scolopes*? Our use of Δ*luxCDABEG* mutants and strains where *luxCDABEG* are specifically inducible enabled us to answer these questions.

Materials and Methods

Bacteria and media. Bacterial strains, plasmids, and oligonucleotides used in this study are described in Table 2.1. Plasmids were maintained in *E. coli* strain DH5α, except plasmids containing the R6Kγ origin of replication, which were maintained in CC118λ*pir* or DH5αλ*pir*. *E. coli* was grown in LB medium (141) or Brain Heart Infusion (BHI). *V. fischeri* was grown in one of four media types: SWT, which contained 5 g of tryptone, 3 g of yeast extract, 3 ml of glycerol, and 700 ml of Instant Ocean (Aquarium Systems, Mentor, Ohio) per liter; SWTO, which was prepared by adding 170 mM NaCl to SWT to achieve an osmolarity near that of seawater; LBS, which contained, per liter of water, 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 50 mM Tris (pH 7.5); or BGMYE (modified from (173)), which contained 940 ml Instant Ocean, 50 mM Tris (pH 7.5), 19 mM NH_4Cl, 0.03% glycerol, 7 μM $FeSO_4$-$7H_20$, 570 μM K_2HPO_4, and 10 mg yeast extract per liter. We primarily used SWTO because we previously found that luminescence of *V. fischeri* was enhanced by its seawater-like osmolarity (209). Agar (15 mg ml^{-1}) was added to solidify media for plating experiments.

When added to LB medium for selection of *E. coli*, ampicillin, chloramphenicol, and kanamycin were used at concentrations of 100, 20 and 40 μg ml^{-1}, respectively. *E. coli* was grown on BHI medium for selection of resistance to 150 μg ml^{-1} erythromycin. When added to LBS medium for selection in *V. fischeri*, chloramphenicol, erythromycin, and kanamycin were used at concentrations of 2, 5, 100 μg ml^{-1}, respectively. Isopropyl-ß-D-thiogalactoside (IPTG),

5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal), and *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) were added to media at final concentrations of 2 mM, 120 µM, and 1 µM, respectively.

Table 2.1 Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid(s), or oligonucleotide	Relevant characteristics[a]	Source or reference
***V. fischeri* strains**		
ES114	wild-type isolate from *Euprymna scolopes*	(8)
ESR1	spontaneous *rifR* derivative of ES114	(70)
EVS100	ES114 Δ*luxA*::*ermR* (allele exchanged from pEVS139)	this study
EVS101	ES114 *luxR*::*ermR lacI*^{q} P_{tac}-*luxI luxCDABEG* (allele exchanged from pEVS137)	(209)
EVS102	ES114 Δ*luxCDABEG* (allele exchanged from pEVS153)	this study
EVS103	MJ1 Δ*luxCDABEG* (allele exchanged from pEVS165)	this study
JB22	ES114 *lacI*^{q} $P_{A1/34}$-*luxCDABEG* (allele exchanged from pJLB101)	this study
JB23	ES114 *lacI*^{q} $P_{A1/34}$-Δ*luxCDABEG* (allele exchanged from pJLB102)	this study
MJ1	wild-type isolate from *Monocentris japonica*	(180)
Plasmids		
pCR-BluntII-TOPO	PCR-product cloning vector; ColE1 *oriV*, *kanR*	Invitrogen
pEVS79	ColE1 *oriV*, $oriT_{RP4}$, *chmR*	(212)
pEVS94S	R6Kγ *oriV*, $oriT_{RP4}$, *ermR*	(212)
pEVS104	R6Kγ *oriV*, *kanR* RP4-derived conjugative plasmid	(212)
pEVS118	R6Kγ *oriV*, $oriT_{RP4}$, *chmR*	(44)
pEVS137	*Kpn*I-digested pEVS118 in pKV44 *Kpn*I site	this study
pEVS139	*Bgl*II-digested pEVS118 in *Bam*HI-*Bgl*II-digested pKV17	this study
pEVS141	P15A *oriV*, *kanR*	(45)
pEVS143	*lacI*^{q}, P_{tac} *Xho*I cassette (36) in *Sal*I-digested pEVS141	this study
pEVS147	PCR product (primers Lux1 and Lux2, ES114 template) in pCR-BluntII-TOPO	this study
pEVS148K	PCR product (primers Lux3 and Lux4, ES114 template) in pCR-BluntII-TOPO; *Kpn*I digest, self ligated	this study
pEVS149K	PCR product (primers Lux5 and Lux6, ES114 template) in pCR-BluntII-TOPO; *Kpn*I digest, self-ligated	this study
pEVS151	pEVS118 *Spe*I-*Avr*II in pEVS147 *Spe*I/*Nhe*I sites; *Bam*HI digested, filled, self-ligated	this study

pEVS153	*Not*I-digested pEVS151 fused with *Not*I-digested pEVS149K; *Apa*I digest, self-ligate; Δ*luxCDABEG* and flanking sequence from ES114	this study
pEVS162	PCR product (primers Lux5 and Lux6), MJ1 template, in pCR-BluntII-TOPO	this study
pEVS163	pEVS162 *Pst*I-*Kpn*I fragment in pEVS94S *Pst*I/*Kpn*I sites	this study
pEVS165	*Not*I-digested pJLB62 fused with *Not*I-digested pEVS163, Δ*luxCDABEG* and flanking sequence from MJ1	this study
pJLB62	PCR product (primers Lux1 and Lux2MJ1, MJ1 template) in *Sma*I-digested pEVS79	this study
pJLB72	*Not*I-digested pEVS151 fused with *Not*I-digested pEVS148K; *Apa*I digest, self-ligate; linker inserted between ES114 *luxI* and *luxC*	this study
pJLB97	*lacI*^{q} PCR product (primers EVS116 and EVS117, pEVS143 template) in pCR-BluntII-TOPO; *Not*I digest, self-ligate; oligo1 and oligo2 annealed, and ligated into *Eco*RI and *Spe*I sites, forming the $P_{A1/34}$ promoter[b]	this study
pJLB101	pJLB97 *lacI*^{q}-$P_{A1/34}$ *Bam*HI-*Not*I fragment in pJLB72, *lacI*^{q}-$P_{A1/34}$-*luxCDABEG*	this study
pJLB102	pJLB97 *lacI*^{q}-$P_{A1/34}$ *Bam*HI-*Not*I fragment in pEVS153, *lacI*^{q}-$P_{A1/34}$-Δ*luxCDABEG*	this study
pKV44	ColE1 *oriV*, *ampR*, *luxR*::*ermR*-*lacI*^{q}-P_{tac}::*luxI* and flanking sequence from ES114	(233)
pKV17	ColE1 *oriV*, *ampR*, Δ*luxA*::*ermR* and flanking sequence from ES114	(231)
pVSV3	pES213 *oriV*, *kanR*, *lacZ* (full) , $oriT_{RP4}$	(45)
Oligonucleotides[c]		
EVS116	GAG GCG GCC GCC TCG GTT CAA AGA GTT GGT AGC TCA GAG	this study
EVS117	CCG TGC AGT CGA TAA GCC CGG ATC AGC TTG C	this study
Lux1	GGG GTC TAG AGC TTT AGA AAT ACT TTG GCA GCG G	this study
Lux2	GGA TCC GCT AGC GCG GCC GCC TAA CTA TAT GTA TTA TGT TCG AG	this study
Lux2MJ1	GGA TCC GCT AGC GCG GCC GCC TTA GTA TTT AAA ATA AAT TAA TG	this study
Lux3	GCG GCC GCG CTA GCG GAT CCT AGG GGA AAT AAT GAT TAA ATG TAT TCC G	this study
Lux4	GGG GGG TAC CAA TTT GTC TTC TTC TAA GTA ACG CG	this study
Lux5	GCG GCC GCG CTA GCG GAT CCG CCG ATG CTT TTG CAT ACA TAT AAA GAG	this study

Lux6	GGG GGG TAC CCC AAC AAT GGC ATA AGC CCC CAC AGT CG	this study
Lux7	GTC ATC GCA TTG GTG ATA AGG AG	this study
Lux9	AGA CTT CTT ATC TCG TTG GGG TG	this study
Oligo1	AAT TTT TAT CAA AAA GAG TGT TGA CTT GTG AGC GGA TAA CAA TGA TAC TTA GAT TCA ATT GTG AGC GGA TAA CAA TTT CAC ACA G	this study
Oligo2	CTA GCT GTG TGA AAT TGT TAT CCG CTC ACA ATT GAA TCT AAG TAT CAT TGT TAT CCG CTC ACA AGT CAA CAC TCT TTT TGA TAA A	this study

[a] Drug resistance abbreviations used: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *rifR*, rifampicin resistance.
[b] The $P_{A1/34}$ promoter is a hybrid of $P_{A1/03}$ and $P_{A1/04}$ promoters (114), derived from the phage T7 A1 promoter by placing *lac* operators at the transcriptional start and between -10 and -35 elements.
[c] Oligonucleotide sequences are provided in the 5'-3' orientation.

Genetic techniques and analyses. We generated plasmids using standard molecular cloning techniques. Klenow fragment, DNA ligase, and restriction enzymes were obtained from New England Biolabs (Beverly, Mass.), and oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). Specifics of plasmid construction are outlined in Table 2.1. Plasmids were purified using Qiagen Mini-prep kits (Qiagen Inc., Valencia Calif.), and DNA was recovered from restriction and ligation reactions with the DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, Calif.). We used the ZeroBlunt-TOPO PCR Cloning Kit (Invitrogen, Carlsbad, Calif.) to clone PCR products into pCR-BluntII-TOPO. PCR was performed with an iCycler (BioRad Laboratories, Hercules, Calif.) using KOD HiFi DNA Polymerase (Novagen, Madison, Wisc.). In addition to using a high-fidelity polymerase, we sequenced the cloned PCR products to ensure that unintended base-pair alterations were not incorporated into our constructs. DNA sequencing was conducted at the University of Georgia Molecular Genetics Instrumentation Facility or at the University of Michigan DNA sequencing

Core Facility and sequences were compared using Sequencher 4.1.2 (Gene Codes Corp., Ann Arbor, Mich) and DNA Strider.

The construction of mutant alleles is described below and in more specific detail in Table 2.1. We used previously described *luxR*::*ermR*-*lacI*^{q}-P$_{tac}$::*luxI*$^{-}$ and Δ*luxA*::*ermR* alleles on pKV44 (231) and pKV17 (233), respectively, by adding mobilization origins to these plasmids. Our strategy to generate each Δ*luxCDABEG* allele was to amplify a 1.5-kb fragment containing *luxI* and part of *luxR* and fuse this to an amplified 1.5-kb fragment containing sequences downstream of *luxG*, such that a small linker region replaced the *luxCDABEG* genes in an otherwise normal gene arrangement. Because of sequence differences between MJ1 and ES114, two separate strain-specific constructs were generated. The *luxI* gene upstream from *luxC* and the transcriptional terminator downstream of *luxG* were unaltered in these constructs. To place the *luxCDABEG* genes under control of an inducible promoter without disrupting *luxI* or *luxR*, a cassette consisting of three transcriptional terminators, *lacI*^{q}, and a LacI^{q}-controlled promoter designated P$_{A1/34}$ was cloned between *luxI* and *luxC* in pJLB101. As a control, the same cassette was cloned downstream of *luxI* in a Δ*luxCDABEG* mutant generating construct pJLB102. The alleles on pJLB101 and pJLB102 were then exchanged onto the *V. fischeri* chromosome as described below.

Mutant alleles were transferred to *V. fischeri* from *E. coli* by triparental mating using conjugative helper plasmid pEVS104 (212). Recombinational marker exchange was scored by screening for the appropriate antibiotic-resistance and/or luminescence phenotypes, and was confirmed by PCR. The mutant allele from Δ*luxCDABEG* strain EVS102 was PCR amplified, cloned, and sequenced to confirm the marker exchange method. The mutant alleles on plasmids pEVS137 (*luxR*::*ermR*-*lacI*^{q}-P$_{tac}$::*luxI*$^{-}$), pEVS139 (Δ*luxA*::*ermR*), pEVS153 (Δ*luxCDABEG*),

pJLB101 (*lacI*^{q}-P$_{A1/34}$-*luxC*), and pJLB102 (*lacI*^{q}-P$_{A1/34}$-Δ*lux*) were crossed into the genome of ES114 to generate mutants EVS101, EVS100, EVS102, JB22 and JB23, respectively. The Δ*luxCDABEG* allele on pEVS165 was crossed into the MJ1 genome to generate mutant EVS103. The genotypes of these strains are illustrated in Figure 2.1.

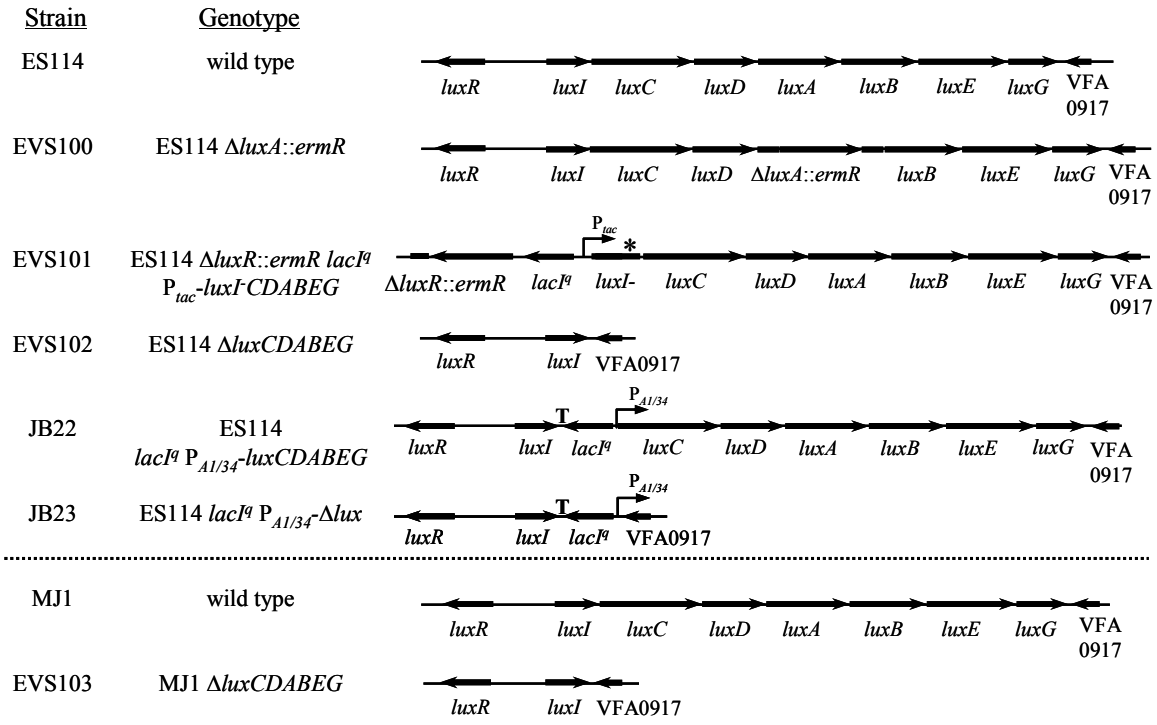

FIG. 2.1. Schematic representation of chromosomal gene organization in *lux* mutants. Black arrows indicate the direction of transcription of *lux* or other genes. An asterisk denotes location of a frame-shifting 4-bp insertion in strain EVS101 resulting in a non-functional LuxI. "T" indicates where three bidirectional transcriptional terminators were added between *luxI* and *lacI*^{q} in strains JB22 and JB23.

Growth and luminescence measurements. Unless noted otherwise, overnight cultures were diluted 1:1000 in 50 ml of SWTO in 250-ml flasks and incubated at 24^{o}C with shaking (200 rpm). 500-µl samples were taken from two or three parallel cultures at regular intervals and absorbance at 595 nm (A_{595}) was determined with a BioPhotometer (Brinkman Instruments, Westbury, NY). We calculated growth rate (min per A_{595} doubling) by plotting log(A_{595}) as a

function of time and determining the slope of the best-fit line through a particular time point and the two flanking data points. Relative luminescence was determined using a TD-20/20 or TD-20/20n luminometer (Turner Designs, Sunnyvale, Calif.), immediately after vigorous shaking to oxygenate the sample.

Mixed-strain competitions. To determine the relative competitiveness of EVS103 (Δ*luxCDABEG*) in batch co-culture with its parent MJ1, the two strains were incubated at 24°C with shaking (200 rpm) in 10 ml of SWTO in 125-ml flasks until reaching an A_{595} of ~2.0. At this point, designated generation 0, aliquots of the cultures were plated, the cultures were diluted 4-fold, re-grown through two generations to re-achieve an A_{595} of ~2.0, and plated again. This procedure of dilution and re-growth to starting A_{595} was repeated for later generations. Plates were incubated overnight, at least 200 colonies were patched onto solid media, and the patches were scored for luminescence after overnight incubation to determine the ratio of EVS103 to MJ1. The ratio of EVS103 to MJ1 at each generation was divided by this ratio at generation 0 to determine the relative competitive index (RCI).

To determine the relative competitiveness of EVS103 relative to MJ1 in a carbon-limited chemostat, a mix of the two strains was pre-grown at 24°C with shaking (200 rpm) in 25 ml of BGMYE in baffled 125-ml flasks until reaching an A_{595} between 0.11 and 0.18. This mix was diluted 1:10 in a 100-ml chemostat and incubated at 24°C with aeration (breathable air bubbled through the medium at 100 ml min^{-1} using a diffuser) until reaching an A_{595} between 0.11 and 0.16, at which point the in-flow pump was started (designated as time = 0) at a rate of ~7 ml hr^{-1}. Following 0.25 retention times (4.5 hrs), the out-flow pump was initiated and the volume returned to 100 ml. At the indicated generation, samples were removed and used for determining A_{595} and luminescence. The steady-state A_{595} varied between 0.04 and 0.15 in different

experiments, but luminescence of MJ1 and the competitiveness values were similar in each case. The mutant to wild type ratio was determined by counting the number of luminescent and non-luminescent colonies following dilution plating. Upon completion of the experiment, presence of the Δ*luxCDABEG* allele was confirmed in ten non-luminescent colonies by PCR using primers Lux7 and Lux9. We confirmed that carbon (glycerol) availability was limiting growth in this experiment by increasing glycerol concentration, resulting in faster growth.

Relative competitiveness of ES114 and EVS102 during colonization of the squid was determined by dividing the ratio of EVS102 to ES114 colonizing each animal by this ratio in the inoculum, with strain ratios determined by marking ES114 or EVS102 with pVSV3 and determining that strain's population in the mixture by plating on LBS containing X-Gal. By alternately tagging ES114 or EVS102 with pVSV3, we eliminated the possibility that pVSV3 was responsible for observed differences in competitiveness, confirming our earlier observations that pVSV3 (data not shown) and the related plasmid pVSV103 (45) do not significantly affect competitiveness. Methods for inoculating *E. scolopes* and recovering the bacteria colonizing each animal are described below. Calculations of the RCI average, confidence interval, and significance were performed on log-transformed data.

Colonization of *Euprymna scolopes.* *E. scolopes* hatchlings were infected with *V. fischeri* using previously described inoculation procedures (175, 211) and overnight exposures of squid to *V. fischeri*. Within each experiment, similar concentrations of mutant or wild-type cells were present in the respective inocula. Inoculant strains were pre-grown unshaken in 5 ml of SWT in 50-ml conical tubes at 28°C such that the A_{595} was between 0.3 and 1.0, cultures were diluted in Instant Ocean to between 1000 and 3000 total CFU ml^{-1}, and juvenile *E. scolopes* were exposed to inocula for between 12- to 14-h before being rinsed in *V. fischeri*-free Instant Ocean.

Squid were homogenized at 48 h post-inoculation, and homogenates were serially diluted and plated onto LBS or LBS supplemented with X-Gal. Following overnight incubation, colonies were counted to determine CFU per animal. For competition experiments plated on LBS with X-Gal, colonies were scored for their blue or white color to determine strain ratios.

Results

3-oxo-C6-HSL induces luminescence and slows growth of *V. fischeri* ES114. We began studying the effects of luminescence on *V. fischeri* by examining wild-type strain ES114, which was isolated from an *E. scolopes* light organ (8). Like most isolates from *E. scolopes*, ES114 is relatively dim in culture (118) and produces little 3-oxo-C6-HSL autoinducer (8), which stimulates luminescence by activating the quorum sensing transcriptional regulator LuxR. Supplementing media with 3-oxo-C6-HSL induced light production ~1500-fold (Table 2.2 and Fig. 2.2A) and also slowed the growth of ES114 (Fig. 2.2B), reflected in longer doubling times (Fig. 2.2C). This phenomenon was not specific to growth in SWTO at 24^{o}C because it also occurred when cells were grown in LBS at 28^{o}C (Fig. 2.2D).

Interestingly, in both media types the increase in doubling time was most evident when culture A_{595} was between 0.5 and 2, and was not seen when A_{595} was <0.5 (Fig. 2.2C and 2.2D). This indicates that luminescence was not growth-limiting when cultures were below 0.5 A_{595}; however, it is important to note specific luminescence did not reach its maximum until culture A_{595} was >1 (Fig. 2.2A), so these data are also consistent with a threshold luminescence required to inhibit growth. Although 3-oxo-C6-HSL was present throughout growth, quorum-sensing inhibitors present in complex media must be metabolized before luminescence is induced (47), which is consistent with this rise in specific luminescence.

Table 2.2 Relative luminescence of *V. fischeri* strains

Strain	Condition[a]	Luminescence relative to ES114[b]
ES114		1
ES114	in squid	860[c]
ES114	+ 3-oxo-C6-HSL	1500
ES114	+ IPTG	1
ESR1		0.11
EVS101		17
EVS101	+ IPTG	340
JB22		9.4
JB22	+ IPTG	4300
MJ1		13000
MJ1	C-limited chemostat	7000[d]

[a] Except as noted, strains were grown in SWTO medium at 24°C.
[b] Maximum specific luminescence (luminescence/A_{595}) relative to ES114.
[c] Specific luminescence was determined by dividing luminescence per CFU in the *E. scolopes* light organ by luminescence per CFU for ES114 cells grown in SWTO medium.
[d] Specific luminescence in BGMYE medium during continuous culture at A_{595} 0.1 ± 0.05.

It is intriguing that Figure 2.2 shows an increase in doubling time associated with 3-oxo-C6-HSL addition and brighter luminescence. This is consistent with the idea that luminescence can slow growth; however, 3-oxo-C6-HSL also activates LuxR to stimulate non-*lux* genes (19), so luminescence cannot be pinpointed as the cause of decreased growth rate.

Construction of new *lux* mutants in *V. fischeri*. To focus specifically on effects of *luxCDABEG* induction, we sought defined *lux* mutants of *V. fischeri* for comparative experiments. To our knowledge, no defined dark *lux* mutant has been reported in a wild-type *V. fischeri* strain. A Δ*luxA*::*ermR* mutant allele has been generated (231, 233), but this was crossed into ESR1 (70), which is a spontaneous rifampicin-resistant mutant of strain ES114. Rifampicin-resistant mutants often have pleiotropic phenotypes (5, 7, 98, 248), and ESR1 has notable differences from the wild type, including a reduced ability to colonize *E. scolopes* (135). We found that ESR1 has about one tenth the specific luminescence of ES114 when grown in SWTO

(Table 2.2), and out of concern that ESR1 is an inappropriate parent strain for investigations of bioluminescence we placed the *luxA*::*ermR* allele in strain ES114, generating mutant EVS100.

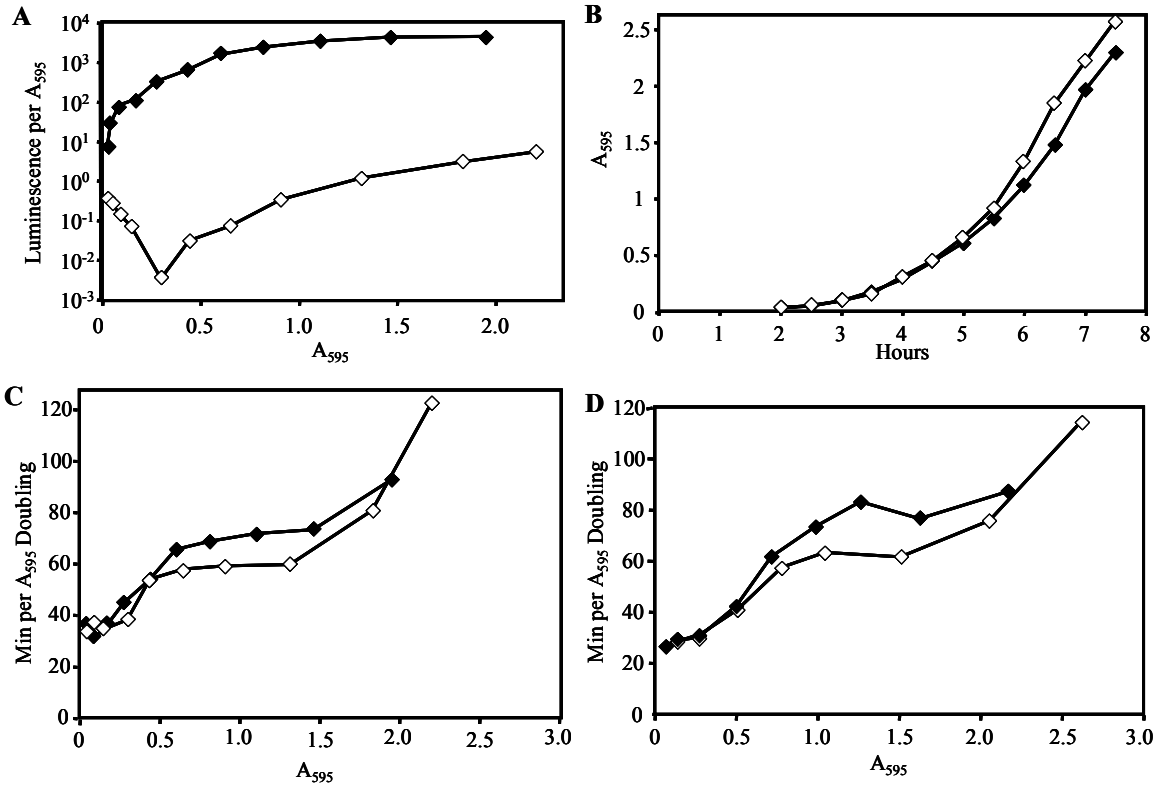

FIG. 2.2. Effect of 3-oxo-C6-HSL on the growth and luminescence of *V. fischeri* ES114 in culture. ES114 was grown with (filled symbols) or without (empty symbols) addition of 200 ng ml^{-1} 3-oxo-C6-HSL. For cells grown in SWTO at 24°C (panels A, B, and C), the data shown include the specific luminescence plotted as a function of culture density (panel A), the culture density over time (panel B), and the doubling time as a function of A_{595} (panel C). The doubling time as a function of A_{595} is also reported for cells grown in LBS at 28°C (panel D).

Surprisingly, EVS100 grew more slowly than wild type in SWTO medium, and this was slightly exacerbated by the addition of 3-oxo-C6-HSL (Fig. 2.3). This growth defect of EVS100 and influence of 3-oxo-C6-HSL also occurred in SWT and LBS media (data not shown). These data could indicate that bioluminescence confers some growth advantage, but it could also reflect deleterious effects from expressing the *ermR* marker and/or from Lux-mediated production of RCHO and $FMNH_2$ without the turnover of these products by luciferase.

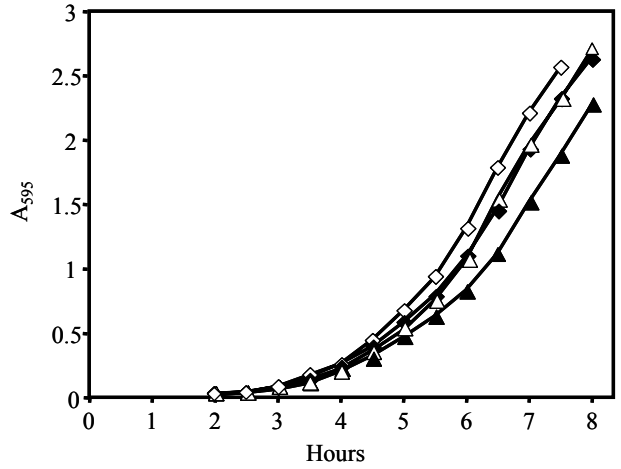

FIG. 2.3. Relative growth of strains EVS100 and ES114. The Δ*luxA*::*ermR* mutant EVS100 (triangles) and wild type ES114 (diamonds) were grown in SWTO medium at 24°C with (filled symbols) or without (empty symbols) addition of 200 ng ml^{-1} 3-oxo-C6-HSL.

Given this uncertainty, we reasoned that a more interpretable approach was to generate strains wherein all the *lux* structural genes were eliminated (Δ*luxCDABEG*) or disconnected from the LuxR/LuxI quorum-sensing regulon and placed under control of an inducible promoter. Using ES114 as a parent, we generated a Δ*luxCDABEG* mutant (EVS102), two strains in which *luxCDABEG* expression is controlled by $LacI^q$ (EVS101 and JB22), and control strain JB23, which contains $lacI^q$ and the same inducible promoter found in JB22 but placed upstream of a *luxCDABEG* deletion. We also generated a Δ*luxCDABEG* mutant (EVS103) of the visibly bright *V. fischeri* strain MJ1, an isolate from the light organ of a pinecone fish, *Monocentris japonica* (180). The genotypes of these mutants are illustrated in Figure 2.1. Each of the Δ*luxCDABEG* strains, like the Δ*luxA*::*ermR* mutant EVS100, produces no detectable luminescence, and the relative luminescence of the lux^+ strains under various conditions is listed in Table 2.2.

Specific induction of *luxCDABEG* slows growth under certain conditions. We tested the effect of specifically inducing bioluminescence in EVS101 and JB22, in which the *luxCDABEG* genes are controlled by $LacI^q$ and the P_{tac} or $P_{A1/34}$ promoters, respectively. Using

IPTG to induce luminescence in these strains offered two advantages over inducing their wild-type parent ES114 with 3-oxo-C6-HSL. First, IPTG will specifically induce *luxCDABEG* in EVS101 and JB22 without also stimulating the entire LuxR regulon as addition of 3-oxo-C6-HSL does. Second, an inhibitor of 3-oxo-C6-HSL activity present in complex media must be catabolized by cells before the autoinducer is active, but this should not affect induction with IPTG.

In the presence of IPTG, specific luminescence of EVS101 (Δ*luxR*::*ermR lacI*^{q} P_{tac}-*luxI CDABEG*) was induced 20-fold to levels ~340-fold brighter than ES114, although this was still dimmer than ES114 cells exposed to 3-oxo-C6-HSL or growing in the squid light organ (Table 2.2). When induced by IPTG, EVS101 also displayed longer doubling times when the culture was between A_{595} of 0.5 and 1.5 (Fig. 2.4A). Similarly, IPTG induced the luminescence of JB22 (*lacI*^{q} $P_{A1/34}$-*luxCDABEG*) ~460-fold and slowed growth when the A_{595} was >0.5 (Fig. 2.4B). Upon induction with IPTG, JB22 was ~13-fold brighter than EVS101 (Table 2.2) and also suffered a more dramatic growth rate reduction (Fig. 2.4). IPTG did not induce luminescence in control strain JB23 (*lacI*^{q} $P_{A1/34}$-Δ*lux*) or ES114 (Table 2.2) and did not slow growth of these strains (Fig. 2.4B).

Interestingly, neither EVS101 nor JB22 displayed a growth defect when culture A_{595} was <0.5, even though the induced P_{tac} and $P_{A1/34}$ promoters drove expression of bioluminescence at low cell densities (data not shown). This observation that luminescence only affects growth above a certain culture A_{595} is not due to LuxR/LuxI-mediated quorum sensing, because LuxR/LuxI does not regulate luminescence in these strains. Indeed, *luxR* and *luxI* are disrupted in EVS101. This phenomenon also parallels the effect of 3-oxo-C6-HSL on the growth of ES114 shown in Figure 2.2.

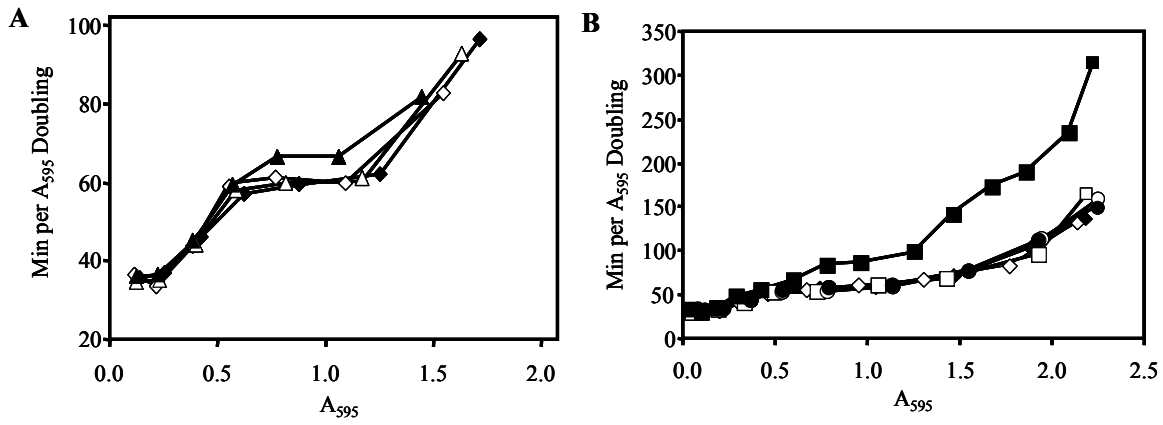

FIG. 2.4. Specific induction of bioluminescence affects growth of strains EVS101 and JB22. Doubling time is reported as a function of culture A_{595} for cells grown in SWTO at 24°C with (filled symbols) or without (empty symbols) addition of 0.5 mg ml^{-1} IPTG. (A) Growth of wild type ES114 (diamonds) and *luxR*::*ermR lacI*^{q} P_{tac}-*luxI luxCDABEG* mutant EVS101 (triangles). (B) Growth of ES114 (diamonds), the *lacI*^{q} $P_{A1/34}$-*luxCDABEG* mutant JB22 (squares), or the dark *lacI*^{q} $P_{A1/34}$-Δ*luxCDABEG* control strain JB23 (circles).

We considered the possibility that oxygen might be replete at low A_{595} but growth-limiting once A_{595} is >0.5. If this were the case, then oxygen consumption by luciferase in induced JB22 and EVS101 cells could account for their reduced growth rate once cultures reach this threshold A_{595}. However, changing the degree of culture aeration by adjusting culture volume from 25 to 100 ml per 250-ml flask did not detectably affect the A_{595} at which the growth rate of induced and uninduced cultures diverged (data not shown). We also compared the growth of JB22 and ES114 in filter-sterilized medium in which JB22 cells had previously been grown to an A_{595} of 1.0 and then removed. In such partially-spent medium, induction of luminescence in JB22 caused an immediate reduction in growth rate, even at very low optical density when the cells are well aerated (data not shown). Together, these data suggest that competition for oxygen is not what limits the growth of highly luminescent cells when batch cultures reach A_{595} >0.5.

Comparison of Δ*luxCDABEG* mutants to their wild-type parents. To test whether bioluminescence attenuates growth when the *lux* genes are under LuxR regulatory control, we compared Δ*luxCDABEG* mutants to their respective wild-type parents. Growth comparisons were made between distinct clonal cultures of mutant and wild type, and also by mixing the strains and determining their relative competitiveness. We first compared Δ*luxCDABEG* mutant EVS102 to its wild-type parent ES114. This wild type produces little light in culture unless 3-oxo-C6-HSL is added, and we saw no difference in the growth of ES114 and EVS102 in the absence of 3-oxo-C6-HSL (Fig. 2.5). However, addition of 3-oxo-C6-HSL induced luminescence and slowed the growth of ES114 but had relatively little effect on the doubling time of EVS102 (Fig. 2.5). Thus, the reduced growth rate of ES114 in the presence of 3-oxo-C6-HSL (Fig. 2.2) can be attributed to induction of *luxCDABEG*.

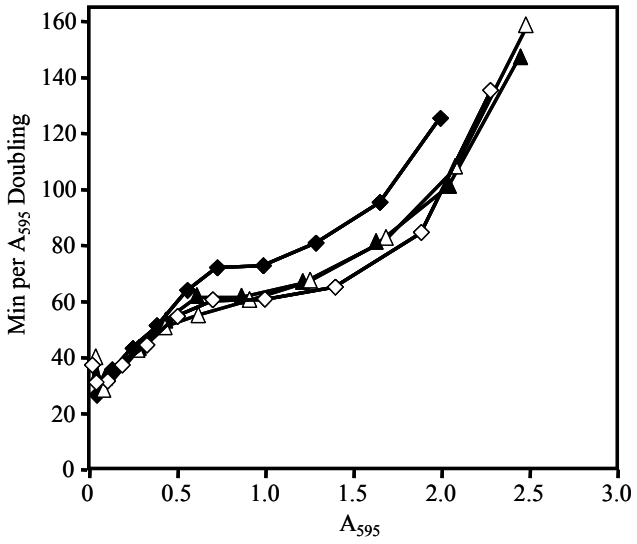

FIG. 2.5. 3-oxo-C6-HSL slows growth of ES114 but not Δ*luxCDABEG* mutant. Doubling time is reported as a function of culture A_{595} for wild type ES114 (diamonds) or Δ*luxCDABEG* mutant EVS102 (triangles) grown in SWTO at 24°C with (filled symbols) or without (empty symbols) addition of 200 ng ml^{-1} 3-oxo-C6-HSL.

We next compared Δ*luxCDABEG* mutant EVS103 to its visibly luminescent wild-type parent MJ1. Despite the bright luminescence of MJ1 (Table 2.2), we did not detect a growth

difference between MJ1 and EVS103 when the strains were cultured individually in SWTO (data not shown). However, subtle differences in growth can be detected by monitoring the ratio of two strains in co-culture, and in a mixed-culture competition in SWTO the relative competitiveness index (RCI) of EVS103 was 1.055 per generation, equal to a 5.5% growth advantage for the dark mutant (Fig. 2.6A). We speculated that this disadvantage for the luminescent wild type might be exacerbated under low-nutrient conditions, and when MJ1 and EVS103 were similarly competed in a carbon-limited chemostat, the RCI of EVS103 was 1.26 per generation, equal to a 26% growth advantage for this Δ*luxCDABEG* mutant (Fig. 2.6B).

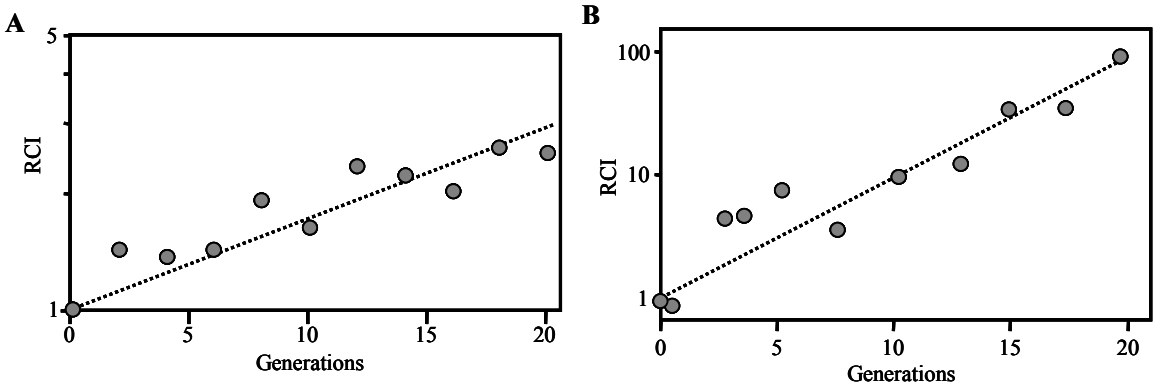

FIG. 2.6. Δ*luxCDABEG* mutant EVS103 out-competes wild-type parent MJ1. (A) Relative competitiveness (RCI) of EVS103 (Δ*luxCDABEG*) co-cultured with MJ1 in SWTO at 24°C. Data represent the combined results of two similar experiments, and the dotted line follows the best fit of the data (RCI=1.055 per generation). (B) RCI of EVS103 and MJ1 co-cultured continuously in a carbon-limited chemostat in BGMYE with 4 mM glycerol at 24°C (see Materials and Methods), conditions under which they are highly luminescent (Table 2). Data is from one representative experiment of three. The dotted line follows the best fit of the data (RCI=1.26 per generation). RCI is defined as the ratio of EVS103 to MJ1 at each generation divided by the ratio of these strains at the start of the experiment.

Bioluminescence confers an advantage during colonization of *E. scolopes*. In contrast to the conditional disadvantage of expressing *luxCDABEG* in culture described above, a previous report suggested that bioluminescence is advantageous for cells colonizing the *E. scolopes* light organ. Specifically, Visick *et al.* found that a Δ*lux*::*ermR* mutant colonizes *E. scolopes* at a

lower level than its parent, ESR1 (233); however, two recent findings led us to reconsider the significance of this result. First, McCann *et al.* found that the rifampicin-resistant ESR1 does not colonize *E. scolopes* as effectively as does its wild-type parent ES114 (135), which could indicate that the factors limiting symbiotic colonization by ESR1 and its derivatives are not representative of normal infections. Second, we found that the same Δ*luxA*::*ermR* allele used previously also attenuated growth of strain EVS100 relative to that of its wild-type parent ES114 (Fig. 2.3), raising the possibility that the symbiotic deficiency associated with this mutation reflected a general attenuation and not a symbiosis-specific defect.

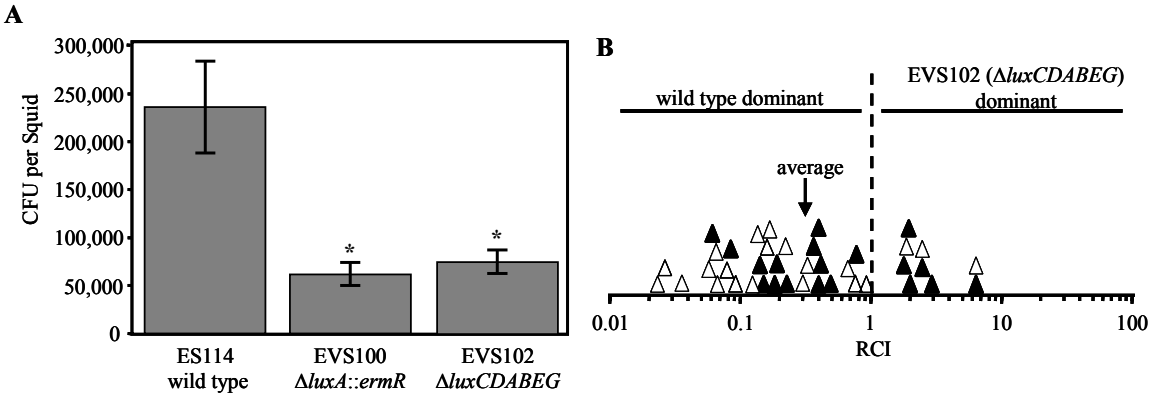

FIG. 2.7. Symbiotic colonization of *E. scolopes* by *lux* mutants and wild type. (A) Average colonization levels 48 h after inoculation with the indicated strain. Error bars represent standard error (n=17). Asterisks denote a significant ($p<0.005$) difference from wild type. (B) Competitiveness of EVS102 (Δ*luxCDABEG*) when presented in a mixed (~1:1) inoculum with wild type and recovered from squid after 48 h. Either ES114 (solid symbols) or EVS102 (empty symbols) contained pVSV3 (*lacZ*), which enabled strain identification by blue/white screening after patching on LBS plates supplemented with 50 μg/ml X-Gal. Each symbol represents the RCI determined from one squid, defined as the ratio of EVS102:ES114 in the squid divided by the ratio in the inoculum. An arrow marks the average RCI of 0.32, which was significantly <1 ($p<0.01$).

Therefore, to reexamine the importance of bioluminescence in colonization of the *E. scolopes* light organ, we compared the symbiotic competence of ES114 to mutants EVS100 (Δ*luxA*::*ermR*) and EVS102 (Δ*luxCDABEG*). We found that EVS100 and EVS102 each achieved populations three- to four-fold lower than ES114 colonization levels 48 h after

inoculation (Fig. 2.7A). Moreover, when squid were exposed to a mixed (~1:1) inoculum of EVS102 and ES114, the mutant was out-competed ~3-fold by wild type as evidenced by an average RCI ~0.3 (Fig. 2.7B). This competitive defect was significant ($p<0.01$), and a 95% confidence interval for the data presented in Figure 2.7B indicates an RCI between 0.2 and 0.5, which encompasses the RCI observed by Visick *et al.* (233). Taken together, our data support the conclusion that bioluminescence contributes to the symbiotic competence of *V. fischeri*.

Discussion

Using strains wherein the *luxCDABEG* genes are deleted or specifically inducible we have shown that luminescence can slow the growth of *V. fischeri* (Figs. 2.4 and 2.5) and put bioluminescent cells at a competitive disadvantage when mixed with a Δ*luxCDABEG* mutant (Fig. 2.6). This supports decades-old speculation that the energetic commitment to bioluminescence, encompassing both the synthesis and enzymatic activity of Lux proteins, represents an appreciable drag on culture-grown cells (reviewed in (251)). On the other hand, we also found instances where bright bioluminescence had no measurable effect on growth rate, particularly during growth in fresh, rich media. This observation that luminescence only slows growth under certain conditions is consistent with the long-standing model that the Lux system affects growth by sapping cellular energy, because the ability to generate energy will not necessarily be growth-limiting under all conditions. Also consistent with this notion we found the greatest effects of luminescence on growth in either partially-spent medium or in a carbon-limited chemostat, conditions where energy conservation may be more important for cell growth.

Karl and Nealson (103) estimated that the bioluminescence of strain MJ1 could theoretically account for 1% to 10% of cellular energy, and they noted that the real energetic

costs of bioluminescence could be higher when considering the energy committed to Lux protein synthesis and inefficiency of luciferase (i.e. substrate turnover that does not generate a photon of light). Similar to these estimates, we found that MJ1 was out-competed 5.5-25% per generation by a Δ*luxCDABEG* mutant (Fig. 2.6). We also showed that induction of luminescence in ES114 using 3-oxo-C6-HSL resulted in 15% reduction in growth rate at an A_{595} of 1.0 (Fig. 2.5).

Although Karl and Nealson did not detect a growth rate reduction attributable to bioluminescence (103), there are several possible explanations for the discrepancy between their results and ours. For example, competition studies such as those shown in Figure 2.6 can detect subtle growth differences that might be missed by growth curve comparisons. Along these lines, different factors could limit growth at different times in batch culture, so dark mutants may only have an advantage during a specific window that might easily be missed in a comparison of batch cultures. Also, Δ*luxCDABEG* mutants have not been available until now, and other mutations (e.g. Δ*luxA*::*ermR*), non-isogenic strains, or addition of autoinducer to stimulate bioluminescence may lead to secondary effects that confound efforts to understand the specific effects of bioluminescence.

A particularly striking observation in this study is that a Δ*luxCDABEG* mutant can out-compete its wild-type parent in culture by as much as 26% (Fig. 2.6). This is consistent with previous reports that spontaneous dark mutants can arise and take over chemostat cultures of bioluminescent bacteria (165). A simple explanation for these observations is that non-bioluminescent cells do not expend energy that can instead be used for other cellular processes, and that they therefore outgrow wild-type cells. An alternative explanation is that non-bioluminescent mutants somehow inhibit the growth of wild type in mixed cultures. The physiology of dark mutants might differ from that of wild type sufficiently that the release and

reuptake of metabolic products (e.g., pyruvate (179)) is coordinated differently with respect to culture conditions. This could theoretically result in an attenuation of the overall growth of wild type by a mechanism that is dependent on the presence of mutant cells. It is therefore important that we also observed an affect of luminescence on growth in clonal batch cultures (Figs. 2.2, 2.4, and 2.5), not just in co-culture (Fig. 2.6). Moreover, the growth inhibition by high luminescence was dependent on media type, with a more pronounced effect under low nutrient-conditions as would be expected if luminescence were draining resources that can be used for energy generation. For example, Δ*luxCDABEG* mutant EVS103 had a greater co-culture growth advantage in nutrient-poor BGMYE medium than it did in the rich medium SWTO. Thus, although other mechanistic interpretations can explain why a Δ*luxCDABEG* mutant out-competes its parent in co-culture, our data are consistent with a model whereby the advantage of dark mutants is manifest by a higher growth rate.

Our data also confirm that bioluminescence enhances *V. fischeri*'s ability to colonize *E. scolopes*. This indicates, not surprisingly, that there are important differences between specific culture conditions and the host environment. The properties of the light organ that render bioluminescence useful remain to be determined and warrant further investigation, but several hypotheses offer explanations for how bioluminescence might be advantageous (for a review see (207)). For example, luminescence may counteract oxidative stress imposed by the host (233), or it may act as a sink to consume excess reductant (15). In these models, the important function of the Lux proteins is to consume oxygen or reductant, respectively, and light itself can be considered a byproduct. Other hypotheses propose a central role for light itself. In one such hypothesis, the host may detect symbiotic bioluminescence, perhaps using cryptochromes, and impose some sanctions against dark infections (M. McFall-Ngai and C. Whistler, personal

communication). Some of these models can be tested, in part, by examining the relative growth of bright cells and Δ*luxCDABEG* mutants under specifically manipulated culture conditions. Culture conditions wherein luminescence attenuates growth may ultimately help elucidate how bioluminescence helps symbionts fully colonize the host.

Acknowledgements

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CHAPTER 3

BIOLUMINESCENCE IN *VIBRIO FISCHERI* IS CONTROLLED BY THE REDOX-RESPONSIVE REGULATOR ARCA[1]

[1]Bose, J. L., Kim, U., Bartkowski, W., Gunsalus, R. P., Overley, A. M., Lyell, N.L., Visick, K.L., and E. V. Stabb. 2007. Accepted by *Molecular Microbiology*, reproduced here with permission of publisher.

Abstract

Bioluminescence generated by the *Vibrio fischeri* Lux system consumes oxygen and reducing power, and it has been proposed that cells use this to counteract either oxidative stress or the accumulation of excess reductant. These models predict that *lux* expression should respond to redox conditions, yet no redox-responsive regulator of *lux* is known. We found that the *luxICDABEG* operon responsible for bioluminescence is repressed by the ArcAB system, which is activated under reducing conditions. Consistent with a role for ArcAB in connecting redox monitoring to *lux* regulation, adding reductant decreased luminescence in an *arc*-dependent manner. ArcA binds to and regulates transcription from the *luxICDABEG* promoter, and it represses luminescence both in the bright strain MJ1 and in ES114, an isolate from the squid *Euprymna scolopes* that is not visibly luminescent in culture. In ES114, deleting *arcA* increased luminescence in culture ~500-fold to visible levels comparable to that of symbiotic cells. ArcA did not repress symbiotic luminescence, but by 48 hrs after inoculation ArcA did contribute to colonization competitiveness. We hypothesize that inactivation of ArcA in response to oxidative stress during initial colonization derepresses *luxICDABEG*, but that ArcAB actively regulates other metabolic pathways in the more reduced environment of an established infection.

Introduction

Bacterial bioluminescence is a tightly regulated energy-consuming process. In *Vibrio fischeri*, luminescence is produced when luciferase, comprised of LuxA and LuxB, converts $FMNH_2$, O_2, and an aliphatic aldehyde (RCHO) to FMN, water, and an aliphatic acid (251). LuxC and LuxE recycle the aliphatic acid, which is also produced by LuxD, to regenerate the RCHO substrate (16), while LuxG and other proteins in the cell reduce FMN using NADH to supply luciferase with $FMNH_2$ (250). Figure 3.1 outlines the biochemistry of bioluminescence in *V. fischeri* and helps illustrate the energetic commitment to this process. Energy is directly required for Lux protein synthesis, and the recycling of RCHO similarly involves the hydrolysis of ATP (Fig. 3.1). In addition, the consumption of oxygen and reductant, which ultimately comes from the NADH pool, appears to compete with energy-generating aerobic respiratory pathways that use the same substrates. Both theoretical calculations and experimental measurements suggest that perhaps 10% of the energy expended by bright cells is devoted to luminescence (103).

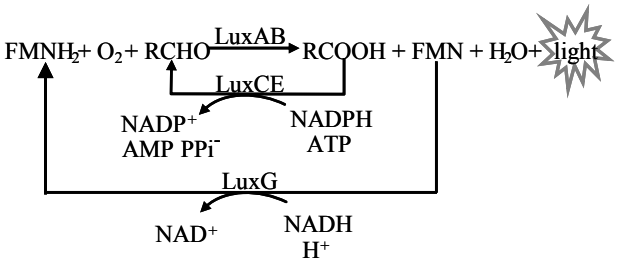

FIG. 3.1. Biochemistry of Lux-mediated bioluminescence in *V. fischeri*. LuxD (not shown) contributes to RCOOH synthesis.

Given this energetic commitment, it is not surprising that luminescence is tightly regulated. In *V. fischeri*, the *lux* genes responsible for luminescence are co-transcribed with *luxI*, and this *luxICDABEG* operon is adjacent to but divergently transcribed from *luxR* (56). LuxI

and LuxR form an archetypical "quorum-sensing" regulatory circuit whereby LuxI generates a *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) autoinducer that together with LuxR stimulates *luxICDABEG* transcription (48, 55, 152). As a result, luminescence is induced when 3-oxo-C6-HSL accumulates at high cell densities, i.e. when cells reach a "quorum". Two other autoinducers also regulate the *V. fischeri lux* genes; *N*-octanoyl homoserine lactone (C8-HSL) produced by AinS (79) and a compound, presumably a furanosyl borate diester (21), produced by LuxS (127). These signals apparently stimulate *luxR* transcription through a pathway involving LuxO and LitR (58, 129). C8-HSL can also bind to and activate LuxR, although it is less effective than 3-oxo-C6-HSL (51, 187).

In contrast to this detailed understanding of quorum sensing, relatively little is known about other regulators of *lux* in *V. fischeri*. Notably, no redox-dependent regulatory mechanism has been connected to *lux* expression, although conflicting reports indicate aeration may affect *lux* (8, 150). A better understanding of *lux* regulation in response to its substrates, oxygen and reductant, would help elucidate how luminescence benefits bacteria. Opposing models suggest that luminescence imparts a selective advantage on *V. fischeri* either by consuming oxygen or, conversely, by acting as a sink for excess reductant (15, 221, 233). These models predict that luminescence should be activated either in response to oxygen/oxidative stress or, conversely, upon buildup of reductant. Based on redox-dependent modulation of gene expression in other bacteria, an ideal candidate to mediate such a regulatory decision would be ArcAB.

The ArcAB two-component regulatory system controls gene expression in response to the redox state of the quinone pool (65). Quinones oxidized during aerobic respiration, or perhaps by reactive oxygen species (ROS), inhibit the kinase activity of the ArcB sensor (133). However, when the quinone pool shifts toward the reduced state, ArcB autophosphorylates and

activates the response regulator ArcA through a phosphorelay that results in phosphorylated ArcA (ArcA-P) (66). Upon activation by such reducing conditions, ArcA-P binds DNA and regulates target promoters either positively or negatively (131, 184).

We investigated the Arc system and its relationship to luminescence in *V. fischeri* strains MJ1 and ES114, which were isolated from the light organs of the pinecone fish *Monocentris japonica* (180) and the Hawaiian bobtail squid, *Euprymna scolopes* (8), respectively. MJ1 is brightly bioluminescent and traditionally used in studies of the *lux* system. In contrast, ES114 is dim in culture (8) but has become a popular model for studying luminescence under ecologically relevant conditions because its association with *E. scolopes* can be established and observed in the laboratory (208). The induction of luminescence in symbiotic ES114 cells has been attributed to quorum sensing and high cell density in the host (9, 129); however, even very dense ES114 cultures are dim, suggesting that environmental cues also modulate *lux* expression. Here we report that in culture ArcA represses luminescence in both MJ1 and ES114, indicating that the redox state detected by ArcAB is a key environmental factor governing *lux* regulation.

Results

Identification of *arcA* and *arcB* in *V. fischeri*. To explore the possibility that ArcAB regulates luminescence, we searched the *V. fischeri* ES114 genome for ArcB and ArcA homologs (181). We identified putative *arcB* (VF2122) and *arcA* (VF2120) genes, which encode proteins with 57% and 84% amino acid identity to *Escherichia coli* ArcB and ArcA, respectively. These homologs were reciprocal best matches in comparisons between *V. fischeri* and *E. coli*, with VF2120 and VF2122 being the ORFs in the ES114 genome most similar to *E. coli* ArcA and ArcB, respectively, and vice versa. The putative *V. fischeri* ArcA and ArcB are

similar in length to their *E. coli* counterparts, including conservation of key regions in ArcB (e.g., PAS, HisKA, HATPase, and signal-receiver domains) and ArcA (e.g., winged-helix DNA-binding, signal-receiver, and effector domains). Moreover, the specific residues critical for redox-sensing (ArcB: Cys-180 and Cys-241) and phosphotransfer (ArcB: His-292, Asp-576, and His-717, and ArcA: Asp-54) in *E. coli* (66, 133) are conserved in the *V. fischeri* homologs. The genetic context of the putative *V. fischeri arcA* and *arcB* is also conserved relative to known ArcAB systems. These genes are convergently transcribed on chromosome 1 in *V. fischeri*, as they are in *Vibrio cholerae* (194), and the genes immediately upstream of *V. fischeri arcB* are similar to those upstream of *arcB* in both *E. coli* and *V. cholerae*. Finally, a recent global survey of response regulators in *V. fischeri* concurs with the assignment of VF2122 and VF2120 as *arcB* and *arcA* (90).

In *E. coli*, the ArcAB system regulates genes involved in the tricarboxylic acid cycle, respiration, and fermentation, among others (184). One consequence of this activity is that *arc* mutants redirect electron flow and become sensitive to redox dyes such as toluidine blue (182). Therefore, to test the functional conservation of the putative *V. fischeri arcA*, we tested whether it could restore toluidine blue resistance to an *E. coli arcA* mutant. We cloned the putative *arcA* gene into pVSV105 (45), which contains the replication origin from *V. fischeri* plasmid pES213 and the R6Kγ replication origin. This shuttle vector replicates both in *E. coli* strains engineered to contain *pir* and (as described below) in *V. fischeri*. The *E. coli arcA* mutant LK5λ*pir* had a severe growth defect on toluidine blue plates compared to the isogenic $arcA^+$ strain MC4100λ*pir* when these strains carried the control vector pVSV105; however, when *V. fischeri arcA* was provided on pJLB52, LK5λ*pir* was substantially rescued from toluidine blue sensitivity (Fig. 3.2).

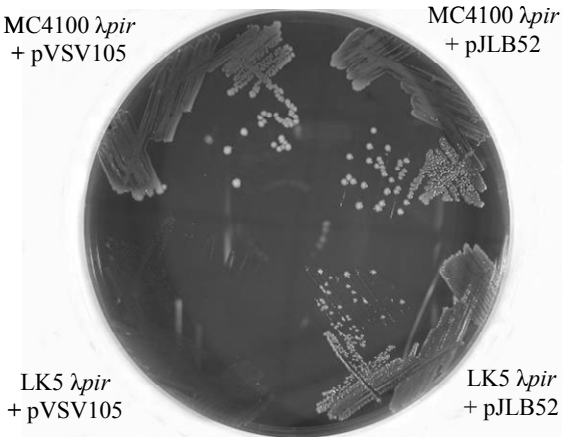

FIG. 3.2. *V. fischeri arcA* restores toluidine blue resistance to an *E. coli arcA* mutant. MC4100λ*pir* (parent) and LK5λ*pir* (*arcA* mutant) carrying pVSV105 (vector) or pJLB52 (pVSV105 with *V. fischeri arcA*) were plated on LB containing 200 ng ml^{-1} toluidine blue and incubated overnight under white light at 37°C.

Additional evidence suggests that the putative ArcAB system in *V. fischeri* functions similarly to that in *E. coli*. A recent bioinformatic analysis of *Vibrio* genomes concluded that these bacteria, including *V. fischeri*, use the *arc* system to regulate many of the same metabolic processes that are regulated by ArcAB in *E. coli* (168). To test this experimentally, we fused *lacZ* to the promoter region of succinate dehydrogenase, one system predicted to be repressed by *arc*, and compared expression of this reporter in wild type ES114 and in a mutant with an in-frame deletion of *arcA*. As predicted, we saw a higher level of expression from P_{sdh}-*lacZ* in the *arcA* mutant (Fig. 3.3). Also, as described below, we found that *arcA* and *arcB* mutants behave similarly in mediating a regulatory response to reductant, consistent with ArcA and ArcB functioning together in a redox-responsive two-component system. Taken together, these findings indicate that VF2120 and VF2122 encode an ArcAB system in *V. fischeri* that is similar to ArcA and ArcB in *E. coli*.

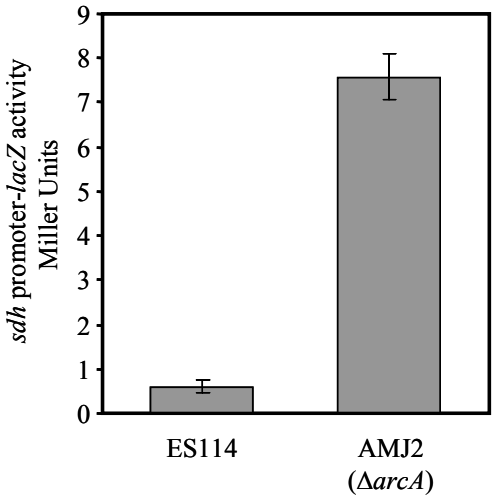

FIG. 3.3. ArcA-mediated regulation of a succinate dehydrogenase promoter-*lacZ* reporter. ß-galactosidase activity, expressed in Miller units, in cultures of ES114 or Δ*arcA* mutant AMJ2 each carrying pJLB217 (P_{sdh}-*lacZ*). The activity from a promoterless *lacZ* was subtracted for each strain. Data represent the average with standard error (n=3).

ArcA and ArcB repress luminescence in culture and respond to reductant. We next tested whether ArcA regulates luminescence in *V. fischeri*. In culture, Δ*arcA* mutant AMJ2 was ~500-fold brighter than its parent ES114 (Fig. 3.4A), a level of luminescence induction similar to that seen when ES114 infects *E. scolopes* or is exposed to exogenous 3-oxo-C6-HSL (8, 9, 129). Low luminescence was restored when *arcA* was provided *in trans* (Fig. 3.4A). Directed mutation of *arcA* to convert Asp-54 to Glu destroyed complementation activity (Fig. 3.5), confirming the importance of ArcA and the conserved putative phosphorylation site Asp-54 in the repression of luminescence. These studies used *arcA* alleles placed on shuttle vector pVSV105, which is derived from a native *V. fischeri* plasmid and is stable in this bacterium (45), so these experiments could be performed in the absence of antibiotic selection that might otherwise have indirectly affected luminescence.

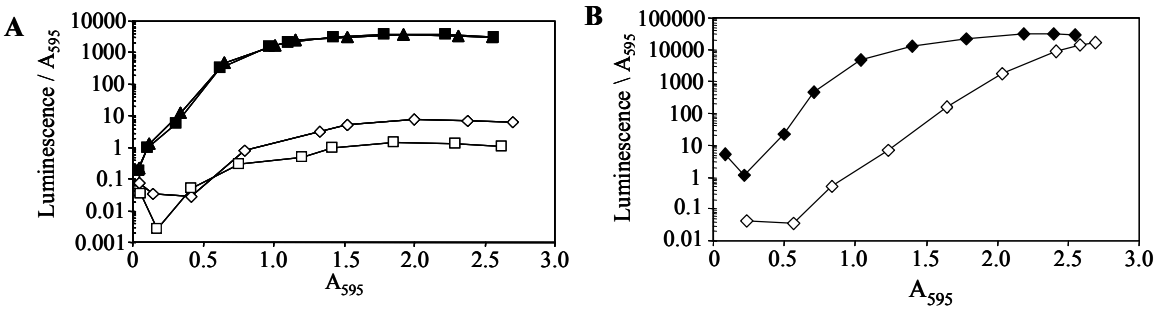

FIG. 3.4. Enhanced luminescence in Δ*arcA* mutants. (A) Specific luminescence (luminescence per A_{595}) of ES114 (open diamonds), its isogenic Δ*arcA* derivative AMJ2 (filled triangles), AMJ2 with vector pVSV105 (filled squares), and AMJ2 with *arcA*-containing plasmid pJLB52 (open squares). (B) Specific luminescence of MJ1 (open diamonds) and its isogenic Δ*arcA* derivative JB11 (solid diamonds).

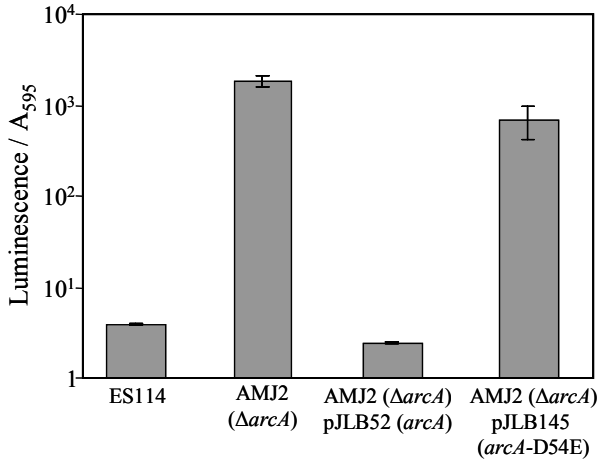

FIG. 3.5. The Asp54 residue of ArcA is required for regulation of bioluminescence. Comparison of maximal luminescence per A_{595} for ES114, Δ*arcA* mutant AMJ2, and AMJ2 bearing plasmids pJLB52 or pJLB145 with wild-type *arcA* or a mutant *arcA* allele encoding Glu instead of Asp at residue 54, respectively. Data represent the average with standard error (n=2).

Regulation of the *lux* genes varies in different *V. fischeri* isolates, and this variation is reflected in divergent *lux* promoter sequences and wide-ranging differences in luminescence output (8, 73). To test whether *arcA*-mediated regulation is specific to dim strains like ES114, we generated an *arcA* mutant in visibly luminescent strain MJ1. Here too we found that deleting *arcA* enhanced luminescence, particularly at low cell densities (Fig. 3.4B). Thus, ArcA repression of luminescence is conserved in MJ1 and ES114.

In *E. coli*, the ArcAB system is activated by the reducing agent dithiothreitol (DTT) (133), and we therefore predicted DTT would stimulate Arc-dependent regulation of luminescence. Addition of 5 mM DTT caused a ~22-fold decrease in luminescence of ES114; however, DTT had only a minor transient effect on luminescence in the *arcA* mutant (Fig. 3.6A). Similarly, the *arcB*::miniTn*5* mutant NL3 was brighter than ES114 and did not regulate luminescence in response to DTT (Fig. 3.6B), consistent with ArcA mediating DTT-responsive regulation in combination with ArcB. Thus, DTT lowered luminescence in *V. fischeri* in an ArcA- and ArcB-dependent manner, indicating that the ArcAB system mediates stronger repression of luminescence in response to more reducing conditions.

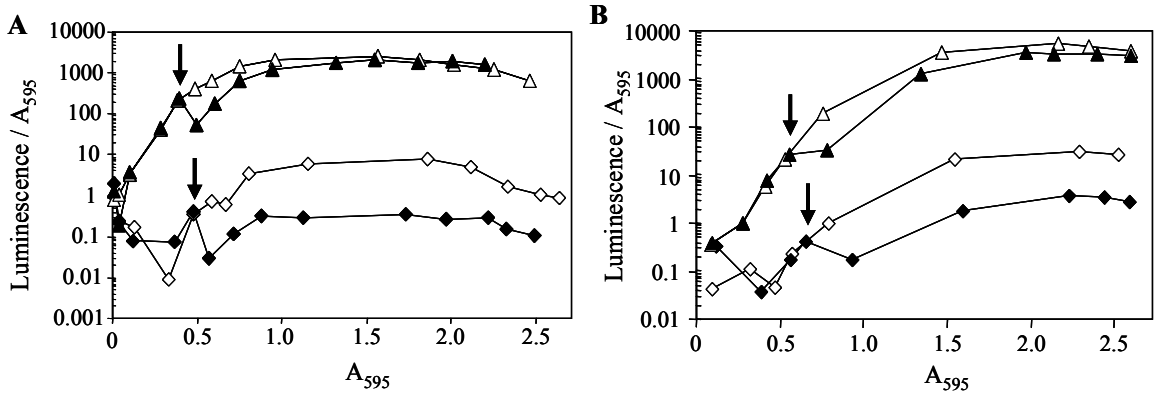

FIG. 3.6. Addition of DTT represses luminescence in an ArcA- and ArcB-dependent manner. Specific luminescence (luminescence per A_{595}) with (filled symbols) or without (open symbols) DTT addition for ES114 (diamonds) and *arc* mutants (triangles). The *arc* mutants were (A) AMJ2 (Δ*arcA*) or (B) NL3 (*arcB*::Tn). Arrows indicate time of DTT addition.

Although the *arcA* and *arcB* mutants sometimes appeared to grow slightly slower than ES114, growth rate cannot account for the differences in luminescence shown in Figures 3.4 and 3.6. For example, there was no discernable difference between the growth of wild type and the *arc* mutants in aerated cultures when the A_{595} was <0.5 (data not shown); however, enhancement of luminescence was evident even at this cell density (Figs. 3.4 and 3.6). We also found that DTT had only a minor effect on growth that was similar both in wild-type and *arc* mutants (data

not shown), yet DTT had a large and ArcAB-dependent effect on luminescence (Fig. 3.6). Furthermore, in our experience several mutants with minor to pronounced growth defects do not exhibit such altered luminescence phenotypes relative to ES114. From these observations we concluded that the effect of ArcAB on luminescence may be direct or indirect but is not simply due to altered growth of the *arc* mutants.

***luxI* promoter derepressed in *arcA* mutant.** Enhanced luminescence in an *arcA* mutant could theoretically result from increased availability of luciferase's substrates (e.g. oxygen or $FMNH_2$) or from regulation of the *lux* genes. Two lines of evidence support the latter model. First, deleting *arcA* did not enhance luminescence when the *lux* genes were expressed from the non-native P_{tac} promoter (Fig. 3.7), indicating that enhanced luminescence in the absence of ArcA is specific to the native *lux* promoter. Second, specific fluorescence from $P_{luxICDABEG}$-*gfp* transcriptional reporter plasmids was controlled by *arcA*. For example, the ES114-derived *luxI* promoter driving *gfp* expression on pJLB38 yielded 19-fold greater fluorescence in the *arcA* mutant than in ES114 (Fig. 3.8A). We considered the possibility that placing the *gfp* reporter on a multi-copy plasmid might affect regulation; however, we observed a similar *arcA*-dependent repression of *gfp* when it was placed on the ES114 chromosome between *luxI* and *luxC*, and this derepression of *gfp* was complemented by reintroducing *arcA in trans* (Fig. 3.8B). These results show that the native $P_{luxICDABEG}$ promoter is derepressed in an *arcA* mutant.

ArcA also mediated repression of the MJ1 $P_{luxICDABEG}$ promoter, and the MJ1 $P_{luxICDABEG}$-*gfp* reporter on pJLB37 yielded 5-fold greater fluorescence in the *arcA* mutant than in parent strain ES114 (Fig. 3.8A). Although the bright luminescence of MJ1 caused high background readings in the fluorometer, epifluorescence microscopy confirmed that the MJ1 $P_{luxICDABEG}$-*gfp* reporter was derepressed and produced more GFP in the *arcA* mutant of MJ1 (data not shown).

Thus, although the intergenic *lux* region has diverged between ES114 and MJ1, ArcA mediates repression of the *lux* promoter in both strains.

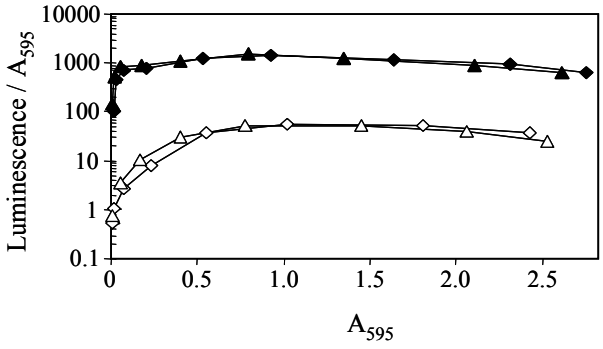

FIG. 3.7. Luminescence is not increased by Δ*arcA* when *luxCDABEG* is expressed from a non-native promoter. Specific luminescence (luminescence per A_{595}) of *luxR*::*ermR lacI*^{q}-P_{tac}::*luxI CDABEG* strain EVS101 (diamonds) and its Δ*arcA* derivative AMJ3 (triangles) with (solid symbols) or without (open symbols) 0.5 mg ml^{-1} IPTG.

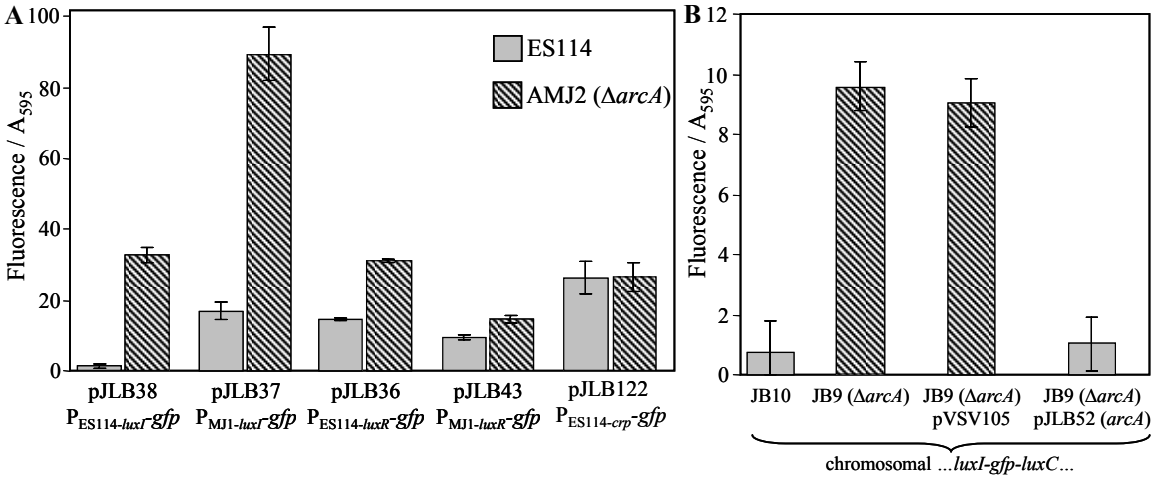

FIG. 3.8. Effect of *arcA* on *gfp* reporters. (A) Specific fluorescence generated from P_{luxR}-, $P_{luxICDABEG}$-, or P_{crp}-*gfp* reporter plasmids harbored in ES114 (gray bars) or Δ*arcA* mutant AMJ2 (hatched bars). The source of the promoter, from strain ES114 or MJ1, is indicated as a subscript (e.g. $P_{MJ1\text{-}luxI}$-*gfp* indicates that the *luxI* promoter from strain MJ1 is driving *gfp* expression). Data represent the average specific fluorescence with standard error (n=10 or 11). (B) Specific fluorescence generated from a chromosomal insertion of *gfp* between *luxI* and *luxC* in ES114 derivatives JB10 and JB9 (Δ*arcA*), or in JB9 carrying the insertless control vector pVSV105 or complemented with the *arcA*-containing plasmid pJLB52. In both panels data represent the average specific fluorescence when culture was between A_{595} 2.0 and 2.8; a range in which specific luminescence is constant for strain ES114. Bars indicate standard error (n=10 or 11).

Relationship of ArcA to known regulators of luminescence. We used the more genetically tractable strain ES114 to test whether repression of luminescence by ArcA could be indirect and dependent on ArcA modulating LitR, LuxO, CRP, AinS, or LuxR, which are regulators known to affect expression of *luxICDABEG* either in *V. fischeri* (58, 110, 129, 145, 146, 233), or for CRP in transgenic *E. coli* (40, 41). We found that ArcA influences luminescence even when *litR*, *luxO*, *crp*, or *ainS* are absent or artificially expressed. For example, deleting *arcA* still greatly enhanced luminescence even in *litR* and *luxO* mutant backgrounds (Fig. 3.9). Comparisons of Δ*crp* mutant JB24 and Δ*crp*Δ*arcA* mutant JB25 were uninterpretable, because deleting *crp* severely inhibited growth (data not shown); however, when *crp* was expressed from the P_{lac} promoter rather than from its native promoter, deletion of *arcA* enhanced luminescence (Fig. 3.9). Moreover, expression of the P_{crp}-*gfp* reporter on pJLB122 was not affected by *arcA* (Fig. 3.8). An *ainS* mutant, which does not produce C8-HSL autoinducer (129), was dim, and its luminescence was enhanced only about 10-fold in an *arcA* backround; however, when the C8-HSL product of AinS was added to *ainS* cells, bright luminescence was restored and the Δ*arcA* allele enhanced luminescence (Fig. 3.9). Thus, repression of luminescence by ArcA cannot be accounted for by transcriptional regulation of *litR*, *luxO*, *crp*, or *ainS*.

A *luxR* mutant produced a low level of luminescence, and this was not detectably enhanced by deleting *arcA* (Fig. 3.9). Thus, derepression of luminescence in an *arcA* mutant is largely dependent on *luxR*, which could indicate that ArcA represses luminescence by inhibiting LuxR from activating $P_{luxICDABEG}$ or that ArcA regulates *luxR*. P_{luxR}-*gfp* reporters on plasmids pJLB36 and pJLB43 displayed 1.5- to 2-fold greater fluorescence in *arcA* mutant AMJ2 than in its parent ES114 (Fig. 3.8), indicating that ArcA does mediate repression of *luxR*. However,

when *luxR* lacking its native promoter was expressed from a plasmid, deletion of *arcA* greatly enhanced luminescence (Fig. 3.9). Therefore, the full repressive effect of ArcA cannot be accounted for by the modest regulatory effect ArcA has on the native *luxR* promoter.

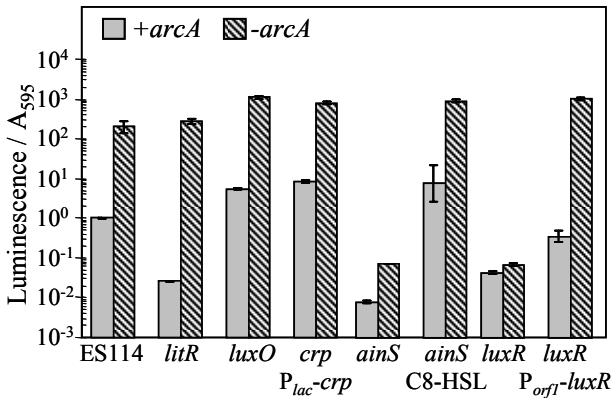

FIG. 3.9. Repression of luminescence by ArcA does not depend on regulation of *litR*, *luxO*, *crp*, *ainS*, or *luxR*. Comparison of maximal luminescence per A_{595} for ES114 and strains harboring various mutations, indicated by gene designations under the corresponding bars, with either the wild-type *arcA* allele (gray bars) or the Δ*arcA* mutation (hatched bars). "P_{lac}-*crp*" and "P_{orf1}-*luxR*" indicate that strains harbored plasmids pJLB146 or pJLB123 wherein the *crp* or *luxR* coding sequences, lacking their native promoter regions, were placed downstream of the *E. coli* P_{lac} promoter or the plasmid pES213 *orf1* promoter (44), respectively. "C8-HSL" indicates that cultures were supplemented with 100 nM C8-HSL. Strains compared were ES114 (wild type), AMJ2 (Δ*arcA*); JB19 (*litR*::*ermR*), JB21 (Δ*arcA litR*::*ermR*); JB13 (*luxO*::pAIA3), JB14 (Δ*arcA luxO*::pAIA3); JB24 (Δ*crp*) pJLB146 (P_{lac}-*crp*), JB25 (Δ*arcA* Δ*crp*) pJLB146 (P_{lac}-*crp*); CL21 (Δ*ainS*::*chmR*), EVS21 (Δ*arcA* Δ*ainS*::*chmR*); and CL53 (Δ*luxR*::*ermR*) and JB5 (Δ*arcA* Δ*luxR*::*ermR*) with or without pJLB123 (P_{orf1}-*luxR*).

An ArcA binding site in the lux promoter region is required for ArcA-mediated repression of *lux*. To test whether phosphorylated ArcA might bind to and regulate the *lux* promoter directly, we performed DNA gel retardation assays using *E. coli* ArcA-P. Although dephosphorylation of ArcA-P is unavoidable over time, resulting in a mix of ArcA-P and ArcA, care was taken to work quickly with the phosphylated form and we refer here to our purified protein simply as ArcA-P. Titration of the intergenic *luxR-luxI* promoter region with ArcA-P revealed binding to both the ES114- and MJ1-derived sequences (Fig. 3.10). ArcA-P exhibits some non-specific DNA binding, particularly with AT rich DNA like that of *V. fischeri*, and we

therefore used the promoter region for the ES114 16S rRNA gene as a comparative control that is not expected to bind ArcA-P specifically. The 16S rRNA gene promoter did form a DNA-protein complex with ArcA-P, but at a weaker affinity than the *lux* promoter region as evidenced by higher concentrations of ArcA-P required to shift the mobility of this DNA fragment (Fig. 3.10).

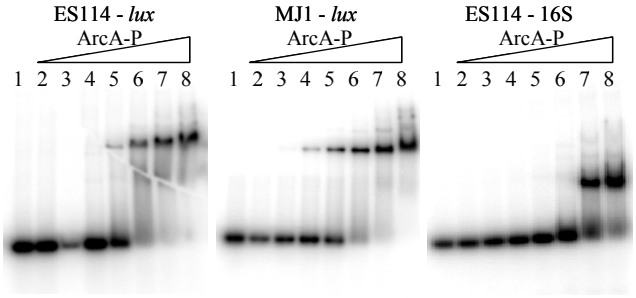

FIG. 3.10. Gel retardation of *lux* or 16S rRNA promoters by ArcA-P. Radiolabeled DNA fragments were incubated with increasing amounts of ArcA-P. Protein concentrations used in lanes 1-8 were; 1, none; 2, 0.031 μM; 3, 0.063 μM; 4, 0.125 μM; 5, 0.25 μM; 6, 0.5 μM; 7, 1.0 μM; and 8, 2.0 μM.

We next examined ArcA binding to the *lux* promoter region more precisely using footprint analysis. ArcA-P protected a region designated "site 1" from DNase I in both the ES114 (Fig. 3.11A) and MJ1 (data not shown) *luxI* promoters, proximal to the "*lux* box" site (35, 213) for LuxR-mediated activation of the *luxICDABEG* promoter (Fig. 3.11B). ArcA-P binding also caused increased DNase I sensitivity at certain bases (indicated by arrows in Fig. 3.11A), suggestive of DNA conformational changes. Within site 1 there is a 5'-TTAACAT-3' found in both ES114 and MJ1 (Fig. 3.11B), and the complementary 5'-ATGTTAA-3' sequence matches the most conserved core of the ArcA-P binding consensus (124). "Site 2" was protected by ArcA-P in the ES114 *lux* fragment (Fig. 3.11A) but not in the MJ1-derived fragment (data not shown). Site 2 overlaps the CRP binding site proposed to be involved in transcriptional activation of *luxR* in *V. fischeri* (18, 196), which may account for ArcA's negative regulation of

P_{luxR} from ES114 (Fig. 3.8A). Both site 1 and site 2 footprints were consistent in size with other ArcA-P footprinting analyses (28, 198).

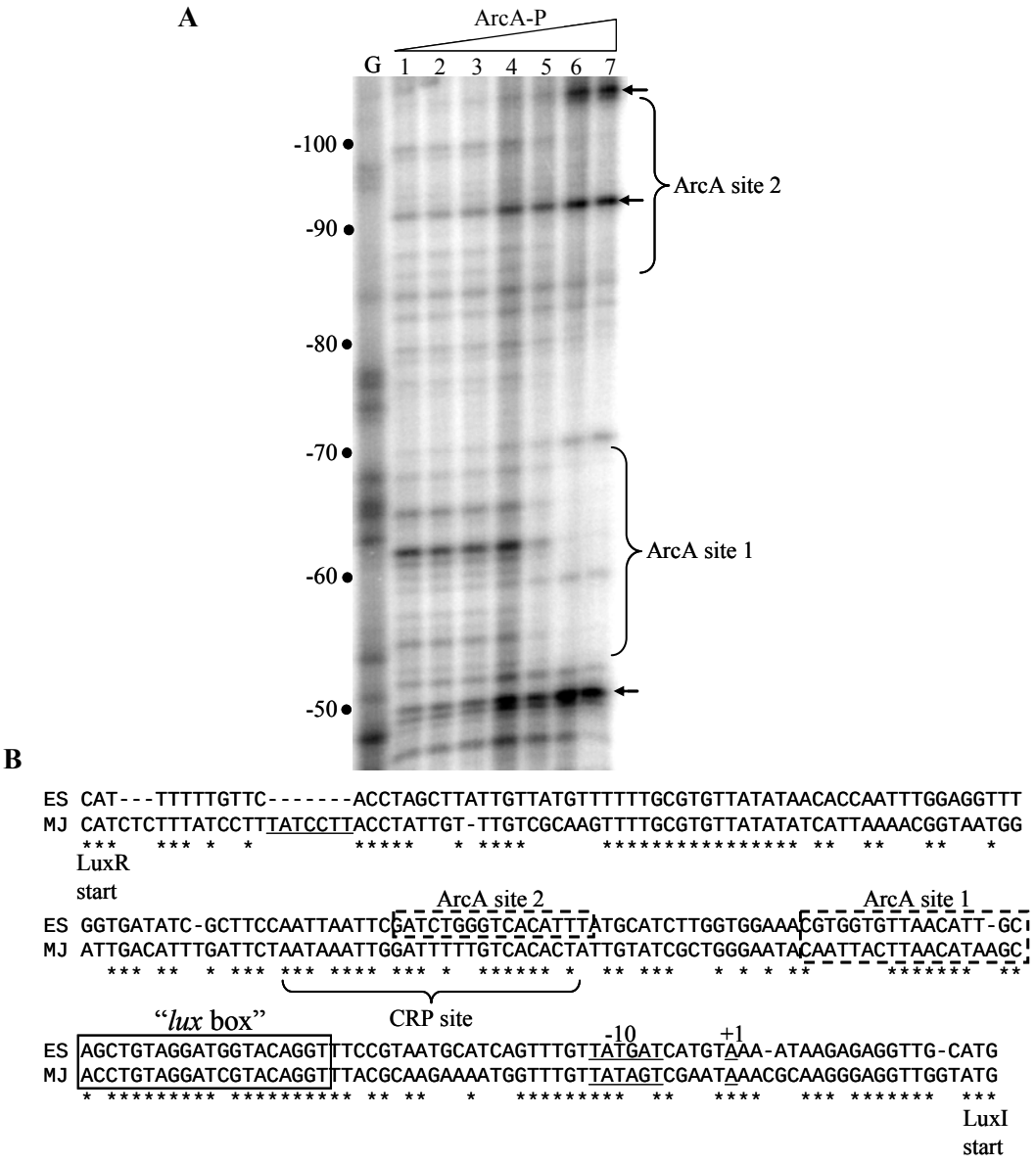

FIG. 3.11. ArcA binding to the *lux* promoter region. (A) DNase I footprint analysis of ArcA-P binding to the ES114 *lux* promoter region, using the sense strand with respect to *luxI*. Numbers correspond to position with respect to the *luxI* transcriptional start. ArcA-P Protein concentrations used in lanes 1-7 were; 1, none; 2, 0.063 μM; 3, 0.125 μM; 4, 0.25 μM; 5, 0.5 μM; 6, 1.0 μM; and 7, 2.0 μM. Lane "G" shows the Maxam-Gilbert G ladder. Arrows point to bases with increased sensitivity to DNase I cleavage upon binding of ArcA-P. (B) Alignment of ES114 and MJ1 *luxR-luxI* intergenic promoter region, labeled "ES" and "MJ" respectively, showing the bases protected by ArcA-P in panel A in dashed boxes. Asterisks indicate bp's conserved in ES114 and MJ1. The transcriptional start for *luxI* and the -10 promoter element

(52) and the predicted binding site for CRP (18, 196) are shown, although the importance of the latter *in vivo* in *V. fischeri* is uncertain. The underlined 7-bp direct repeat near the LuxR start is present in our copy of MJ1 but absent from the sequence for this strain deposited by others in Genbank (Y00509).

To test the importance of "site 1" in *arcA*-mediated regulation of luminescence, we generated mutants where the "site 1" sequence on the chromosome (Fig. 3.11B) was deleted and a six-base pair *Nhe*I recognition site incorporated in its place. We found that this replacement of ArcA "site 1" resulted in an increase in luminescence equivalent to that observed in a Δ*arcA* mutant (Fig. 3.12). Moreover, in the absence of "site 1" adding the Δ*arcA* allele did not change the maximal luminescence of the strain (Fig. 3.12). We did see a small but reproducible difference between the "site 1" mutant and the Δ*arcA* "site 1" double mutant, with the latter being slightly brighter at low cell densities (Fig. 3.12). This may be due to ArcA binding at "site 2" or to indirect effects of ArcA modulating other *lux* regulators such as *luxR*. However, the ability of ArcA to repress luminescence is largely dependent on "site 1".

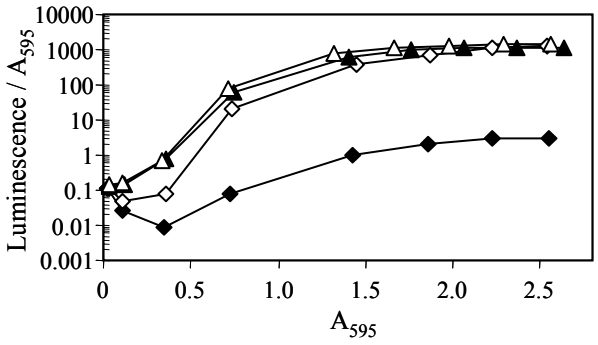

FIG. 3.12. ArcA-mediated repression of *lux* is dependent on ArcA binding site 1 in the *lux* promoter. Specific luminescence (luminescence per A_{595}) of strains JB35 (empty diamonds) and JB36 (empty triangles), which have site-directed alterations to the ArcA site 1 sequence on the chromosome, and their respective parents ES114 (solid diamonds) and Δ*arcA* mutant AMJ2 (solid triangles).

ArcA does not repress symbiotic luminescence but contributes to colonization competitiveness. *V. fischeri* ES114 is much brighter in its symbiotic association with *E. scolopes* than it is in culture (8), and we therefore examined whether ArcA represses luminescence in this symbiosis. The onset of luminescence during infection was similar for ES114 and the Δ*arcA* mutant (Fig. 3.13A), and these strains achieved similar populations in the host (Fig. 3.13B). In contrast to the derepression of luminescence in the Δ*arcA* mutant in culture, luminescence per CFU of the Δ*arcA* mutant and ES114 was similar in the squid (Fig. 3.13C). Furthermore, the amount of light produced per CFU in culture by the Δ*arcA* mutant was similar to that produced by either wild type or the Δ*arcA* mutant in the squid (Fig. 3.13C). Thus, the increase in luminescence observed when cells begin to colonize the host could reflect deactivation of ArcA in this environment. The dramatic difference between the luminescence of cultured and symbiotic cells can be accounted for by differential regulation by ArcA and is not necessarily due to different cell densities affecting quorum-sensing.

Once a symbiotic infection is established *V. fischeri* cells become densely packed, and it seems unlikely that the light organ crypt environment would be replete with molecular oxygen. This is supported by the observation that *V. fischeri* appears to induce its anaerobic respiratory pathways when colonizing the host (166). Such metabolism suggests the ArcAB system might ultimately be important for symbionts. We therefore tested the competitiveness of an *arcA* mutant relative to wild type by inoculating these two strains in a ~1:1 mix and then measuring the ratio in established infections 48h later. We found that ES114 significantly ($p<0.01$) out-competed the *arcA* mutant for light organ colonization, and it was about 4-fold more competitive than the *arcA* mutant in these mixed infections (Fig. 3.13D). We similarly found that *arcB* mutant NL3 was out-competed by wild type (data not shown). This suggests that the ArcAB

system is activated in the first 48h of the symbiosis, although as discussed below it does not appear to turn off the feed-forward regulatory cascade governing *lux* once cells become luminescent.

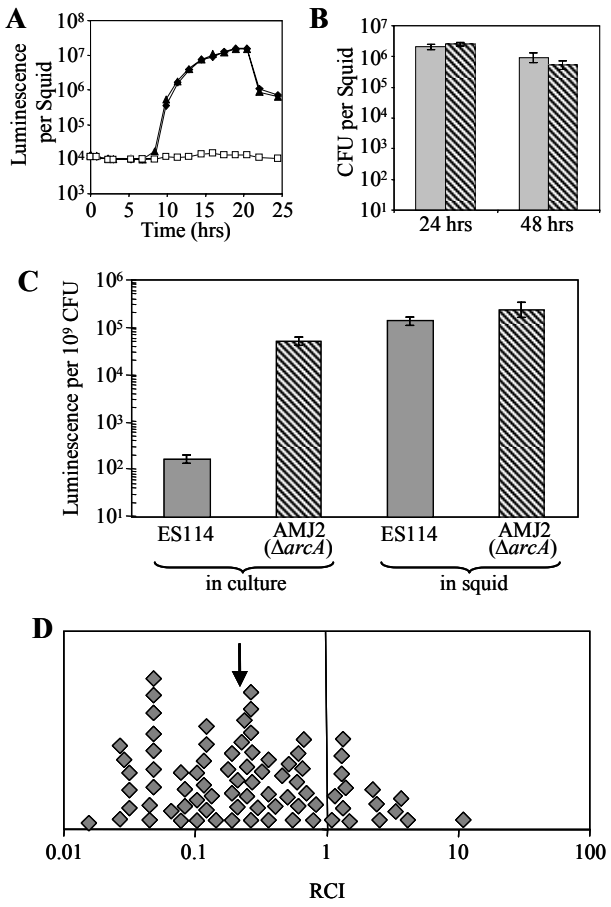

FIG. 3.13. Symbiotic phenotypes of *arcA* mutants. (A) Onset of symbiotic luminescence in *E. scolopes* hatchlings inoculated with ES114 (solid diamonds), Δ*arcA* mutant AMJ2 (solid triangles), or no *V. fischeri* (open squares). Data represent the mean (n=22). (B) Colonization levels of ES114 (gray bars) and AMJ2 (hatched bars) 24 and 48 h post-inoculation. Data show the mean and standard error (n=8). (C) Luminescence per CFU of ES114 and AMJ2 in culture and in the host. Data show the mean and standard error (n=4 for culture, n=17 or 18 in the host). (D) Competitiveness of AMJ1 (Δ*arcA*::*ermR*) when presented in a mixed (~1:1) inoculum with wild type and recovered from squid after 48 h. Each symbol (n=80) represents the RCI determined from one squid, defined as the ratio of AMJ1:ES114 in the squid divided by the ratio of these strains in the inoculum. An arrow marks the average RCI of 0.22, which was significantly <1 ($p<0.01$).

Discussion

Despite intense interest in the autoinducer-mediated quorum sensing regulation of luminescence in *V. fischeri* (74, 142), little is known about how *lux* expression is modulated in response to luciferase's substrates, oxygen and reductant. We have now shown that ArcA, which is activated under reducing conditions, dramatically inhibits luminescence and mediates repression of the $P_{luxICDABEG}$ promoter in *V. fischeri*. Moreover, in contrast to environmental factors such as glucose (62) and iron (87) that repress luminescence in strain MJ1 but not in strain ES114 (8), ArcA strongly repressed luminescence in each of these strains (Fig. 3.4), suggesting an important conserved relationship between redox monitoring and luminescence in *V. fischeri*.

This ArcA-mediated repression of *lux* has important implications for the functional utility of bioluminescence. It has been proposed that luminescence provides a means of lowering intra- or extra-cellular oxygen concentrations as an anti-oxidant defense mechanism (221, 233), but it has also been suggested that the important function of luminescence is to consume excess reductant (15). The finding that ArcA represses *lux* expression in response to reductant is inconsistent with the hypothesis that luminescence provides an advantage by consuming excess reductant, but is consistent with the idea that luminescence acts as a counterbalance to oxidative conditions. Moreover, this finding together with the observation that ArcA does not repress luminescence during host colonization is consistent with the hypotheses proposed by Visick *et al* (2000) and Timmins *et al.* (2001) in which luminescence contributes to symbiotic competence by decreasing oxygen levels in the light organ, thereby protecting cells from host-derived oxidants.

Our results also offer a mechanistic explanation for how ArcA affects luminescence. A simple model consistent with our data is that ArcA blocks the LuxR-autoinducer activator from

binding with RNA polymerase to the "*lux* box" and stimulating $P_{luxICDABEG}$ (35, 213). Binding of ArcA to "site 1" in the *lux* promoter roughly centers on a 5'-ATGTTAA-3' oriented on the minus strand relative to *luxICDABEG* just upstream of the "*lux* box", potentially close enough for ArcA to interfere with LuxR or RNA polymerase binding (Fig. 3.10B). This sequence not only resembles an ArcA binding consensus (124), it is also conserved in the *lux* promoters of strains MJ1, ES114, and ATCC7744. Interestingly, Devine *et al.* (1989) previously noted a *cis*-acting negative regulatory element upstream of the "*lux* box" in strain ATCC7744, and our data are consistent with the possibility that the negative regulation they observed was mediated by ArcA.

ArcA binding to this "site 1" in the *lux* promoter appears to be the primary mechanism of *arc*-mediated *lux* regulation, and it is notable that ArcA's ability to repress luminescence is almost entirely abolished when the "site 1" sequence was removed from the *lux* promoter (Fig 3.12). However, a small degree of *arcA*-mediated *lux* repression was evident at low culture density even in the strain lacking "site 1" (Fig. 3.12). The mechanism for this repression is not apparent, but could be due to the modest effect of *arcA* on regulation of the *luxR* promoter (Fig. 3.8), assuming that this is not also mediated by ArcA binding to "site 1", which remains a possibility. ArcA might regulate *luxR* by binding to "site 2" (Fig. 3.10B). ArcA binding to this site was not clearly evident in the MJ1 promoter (data not shown), and ArcA also had a less pronounced effect on P_{luxR} from MJ1 (Fig. 3.8A). Given the conservation of both "site 1" and the *lux* box in ES114 and MJ1, the differences in ArcA's regulation of *lux* expression in these strains probably stems from other more variable factors such as "site 2".

One of the interesting variations between MJ1 and ES114 is that the effect of ArcA on quorum sensing is notably different in the two strains. In MJ1, a bright strain isolated from the

light organ of a pinecone fish, ArcA represses luminescence 100- to 1000-fold specifically at low cell density, and repression is eventually overcome at high cell density (Fig. 3.4B). Therefore ArcA appears to raise the threshold "quorum" required for *lux* activation in MJ1 without preventing dense cultures from fully inducing *lux*. The effect of ArcA on quorum sensing appears to be quite different in ES114, a dim isolate typical of those isolated from the *E. scolopes* light organ. In ES114 ArcA also represses luminescence 100- to 1000-fold, but unlike in MJ1 this repression is evident even in dense cultures (Fig. 3.4A).

The direct control of *luxICDABEG* by ArcA and the observation that even very high cell density cannot overcome this ArcA-mediated repression in *V. fischeri* ES114 (Fig. 3.4A) lead us to question our perception of 3-oxo-C6-HSL's role in this strain. Interestingly, in culture ES114 is relatively dim and C8-HSL is the primary signal controlling luminescence, whereas in the *E. scolopes* light organ the cells are bright and stimulated primarily by 3-oxo-C6-HSL (129). This discrepancy has previously been attributed to differences in cell density and the idea that 3-oxo-C6-HSL mediates responses to very dense populations (9, 129). However, even cells in ES114 colonies on plates are dim despite being as densely packed as cells in the light organ, and it therefore seems clear that 3-oxo-C6-HSL cannot be simply considered a census-taking molecule. In light of our data (Fig. 3.13C), the difference in luminescence between cultured and symbiotic cells could be accounted for by ArcA-mediated repression of the *lux* genes in culture and derepression of *lux* by deactivation of the ArcAB system in the squid. Although cell density influences luminescence in ES114, we hypothesize that this is a secondary requisite for signal amplification while the primary role for 3-oxo-C6-HSL is to act as a signal in response to ArcA-mediated redox cues from the environment.

In considering the function of 3-oxo-C6-HSL and other homoserine lactone signals, it is worth remembering two elements of this autoinducer that are conserved in other systems. First, 3-oxo-C6-HSL generated by LuxI autoactivates production of more LuxI, so inputs activating *luxICDABEG* can be amplified by a feed-forward mechanism. Second, 3-oxo-C6-HSL is diffusible and transmitted between cells (102). Both this feed-forward signal amplification and diffusibility are features common among other autoinducers (80, 193). Moreover, it is becoming increasingly clear that autoinducer synthases are regulated in response to specific environmental cues (46, 107, 190), much as *luxI* is regulated by ArcAB. Thus, these systems are set up such that environmental inputs could result in amplified responses shared among neighboring cells. In this sense, 3-oxo-C6-HSL and other autoinducers might be functioning as alarm pheromones that transmit information about local environmental conditions across larger populations.

The role of ArcAB in sensing symbiotic conditions is not entirely clear; however, in Figure 3.14 we present a preliminary model consistent with our data. In culture, the ArcAB system controls metabolic genes such as *sdh* and represses luminescence, which is stimulated primarily by LuxR and its weak activator C8-HSL (Fig. 3.14A). Early in the onset of symbiotic infection there was no detectable difference in luminescence of animals infected with ES114 or the Δ*arcA* mutant (Fig. 3.13A), and we propose that this phenomenon is due to deactivation of ArcAB system either prior to or immediately upon colonizing the light-organ crypts (Fig. 3.14B). *E. scolopes* produces oxidatively reactive compounds including NO (33), HOCl (201), H_2O_2 (234), and perhaps others (177) that the bacteria encounter during infection. Theoretically, some of these could directly oxidize reduced quinones (or menaquinones), resulting in deactivation of the Arc system and concomitant derepression of the *lux* genes. This regulatory scheme seems more likely than ArcB sensing a more highly aerobic environment in the host. In either case, the

derepression of the *luxICDABEG* operon should result in more production of 3-oxo-C6-HSL, and this strong activator would contribute to high luminescence levels (Fig. 3.14B).

Our model for later infection (Fig. 3.14C) must reconcile the observations that by 48 hr post-inoculation ArcA does not appear to repress luminescence (Fig. 3.13C) but does seem to be active insofar as it contributes to colonization competitiveness (Fig. 3.13D). In our model, we suggest that oxygen consumption by respiration and luminescence in the crowded infection results in relatively reducing conditions that activate the ArcAB system (Fig. 3.14C). However, while ArcA may be actively regulating metabolic genes such as *sdh*, we propose that it is no longer able to repress luminescence effectively due to the accumulation of 3-oxo-C6-HSL. Essentially, we propose that the feed-forward amplification mechanism of the LuxI circuitry makes this system inherently different from other ArcA-regulated genes, rendering derepression of *lux* a more difficult regulatory switch to reverse.

The model presented above and in Figure 3.14 can be integrated with previous views of bioluminescence functioning as an antioxidant and with our hypothesis that 3-oxo-C6-HSL acts as an alarm pheromone. Visick *et al.* (2000) proposed that bioluminescence aids symbiotic bacteria by lowering the local ambient oxygen concentration, thereby depriving the host of a substrate used in the production of antimicrobial ROS. Consistent with that view, we suggest that the ArcAB system may derepress *lux* in response to ROS (Fig. 3.14B), providing a regulatory connection between *lux* expression and this proposed benefit for luminescence in symbionts. If in fact the bacteria can effectively deprive the host of oxygen, it seems reasonable that this might require a concerted group effort, and in this respect it makes sense for cells experiencing oxidative stress to use 3-oxo-C6-HSL as a signal to stimulate luminescence in other nearby *V. fischeri* cells, which may not be experiencing this stress yet themselves.

Although our model presented in Figure 3.14 and our proposed function for 3-oxo-C6-HSL as a redox-responsive signal are consistent with our results, there are other plausible explanations. Notably, we cannot rule out the possibility that ArcA remains activated during early infection but that another regulatory system overrides its effect on *lux* expression. Similarly, despite obvious parallels to ArcAB in *E. coli*, it is possible that ArcAB in *V. fischeri* responds to somewhat different environmental cues. It is also possible that the "feed-forward" regulatory effect of LuxI may not play an important role in *arcA*-mediated *lux* regulation as we have proposed. Distinguishing between these and other possibilities awaits further studies.

Fortunately, our models present many hypotheses that can be tested experimentally. For example, we hypothesize that *arcA* mutants should stimulate luminescence in wild-type cells when the cell types are mixed, and that this signaling as well as the bright luminescence of *arcA* mutants will depend on *luxI*. These predictions can be tested by a combination of further mutant and reporter analyses. Our model (Fig. 3.14) also predicts that under some conditions one or more ROS will stimulate luminescence in ES114, which may be testable in cultured cells. Interestingly, we recently found that *lux* expression lags behind colonization in "crypt 3" of the light organ (45), which is late to develop in the squid. This is consistent with a model whereby a host-derived environmental cue absent in crypt 3 is important for triggering bioluminescence induction, and confocal microscopy combined with P_{lux}-*gfp* reporters and *arcA* mutants could be used to test the possibility that ArcA is actively repressing *lux* in this light organ microenvironment. These and other experimental approaches promise to further our understanding of *V. fischeri* with respect to its Arc regulon, regulation of *lux*, and symbiotic associations.

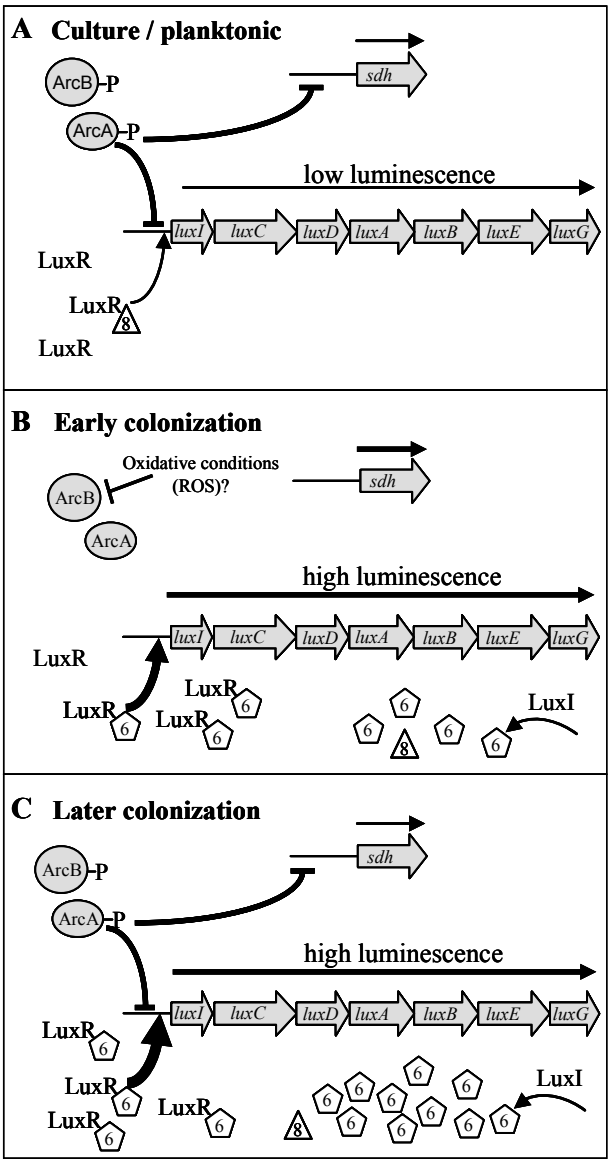

Fig. 3.14. Hypothetical model of ArcAB-mediated regulation of *lux* in *V. fischeri*. Repression of the succinate dehydrogenase (*sdh*) gene is included as one example of several non-*lux* genes presumably regulated (positively or negatively) by ArcAB. (A) In planktonic/cultured cells, LuxR is stimulated primarily by the weak inducer C8-HSL (triangles) (129), the ArcAB system is active, and ArcA-P binds the *lux* promoter adjacent to the Lux box effectively repressing luminescence. (B) During initial infection, oxidative conditions, possibly related to host-generated ROS, are recognized by ArcAB leading to derepression of *lux* and feed-forward autoinduction due to increased LuxI production and concomitant production of the stronger LuxR inducer 3-oxo-C6 HSL (pentagons). (C) Later in an established infection, the tightly packed oxygen-consuming symbionts generate more reducing conditions, leading to reactivation of ArcAB and its regulatory control over *sdh* and other non-*lux* genes. However, luminescence remains high due to the high levels of 3-oxo-C6 HSL activating LuxR.

For decades, *V. fischeri* has served as a powerful model organism for studies of bioluminescence, quorum-sensing gene regulation, and beneficial bacteria-animal interactions. Our discovery that ArcA is a major regulator of the *lux* genes responsible for bioluminescence has provided new insights into each of these lines of research, as discussed above. Perhaps most importantly, this discovery adds to growing evidence that autoinducer-mediated "quorum-sensing" systems can themselves be strongly influenced by input from environmentally responsive regulators such as ArcA.

Experimental Procedures

Bacteria and media. Plasmids were maintained in *E. coli* strain DH5α (76) except conjugative helper plasmid pEVS104 (212), which was maintained in CC118λ*pir* (88), and other plasmids containing the R6Kγ replication origin, which were maintained in DH5αλ*pir* (44). Other bacterial strains used in this study are described in Table 3.1. *E. coli* was grown in LB medium (141) or Brain Heart Infusion. *V. fischeri* was grown in LBS medium (210), in SWT (8) wherein seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, OH), or in SWTO, which was prepared by adding 150 mM NaCl to SWT to achieve an osmolarity near that of seawater. Agar (15 mg ml^{-1}) was added to solidify media for plating. Antibiotics were added as previously described (44), and resistance to 200 ng ml^{-1} toluidine blue was selected on LB plates that were wrapped in parafilm and incubated under constant illumination. To add C8-HSL to cultures, a stock solution of C8-HSL was dissolved in ethyl acetate, an appropriate amount was added to flasks, and the ethyl acetate was allowed to evaporate before adding medium.

Molecular genetic techniques and analysis. Plasmids were constructed using standard cloning procedures and methods described previously (44). Descriptions of select plasmids are

provided in Table 3.1, and details of plasmid construction along with primer sequences are provided in Table 3.2. DNA ligase, Klenow fragment, and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). PCR was performed using KOD HiFi high-fidelity DNA polymerase (Novagen, Madison, WI), and PCR products were cloned into pCR-BluntII-TOPO or pCRBlunt (Invitrogen, Carlsbad, CA) and sequenced to ensure that unintended mutations were not incorporated. DNA sequencing was conducted at the University of Michigan DNA Sequencing Core Facility, and sequences were analyzed using Sequencher 4.1.2 (Gene Codes Corp., Ann Arbor, MI). Primers JBARC7 and JBARC10 enabled us to fill sequencing gaps in *arcA* inserts, and primer JBCRP5 was similarly used to sequence *crp* clones.

Mutant Construction. Mutant alleles were constructed in *E. coli* and transferred to *V. fischeri* from *E. coli* by triparental mating using pEVS104 as a conjugative helper plasmid (212). Recombinational insertion and marker exchange were scored by screening for antibiotic resistance where possible, and confirmed by PCR. The in-frame Δ*arcA* and Δ*crp* alleles were generated such that the open reading frame between the start and stop codons was replaced with a six-bp restriction enzyme recognition site. Due to sequence variations between MJ1 and ES114, separate Δ*arcA* alleles were generated for these strains. We have deposited the MJ1 *arcA* sequence (GenBank DQ834371). Mutant alleles of *luxR* (127), and *ainS* (129) have partial deletions, as described previously. We disrupted *litR* in much the same manner as previously described (58), except that an *ermR* cassette was cloned into a unique *Stu*I site within *litR* in pMF6. Mutants JB35 and JB36 contain a deletion of the ArcA "site 1" (Fig. 3.10B), which was replaced with the 5'-GCTAGC-3' *Nhe*I restriction enzyme recognition sequence, allowing rapid screening for incorporation of this allele by PCR amplifying the *luxIR* intergenic region and

digesting products with *Nhe*I. We replaced wild-type with mutant alleles by marker exchange except in *luxO* mutants JB13 and JB14, for which an internal *luxO* fragment was cloned into suicide vector pEVS122, such that mobilization of the resulting construct pAIA3 and selection for recombinational insertion inactivated *luxO*. We identified the *arcB*::miniTn*5* mutant NL3 in a screen of random mutants as part of another study.

Table 3.1. Select bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
MC4100λ*pir*	F- *araD139* Δ(*argF-lac*) U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* λ*pir*	this study
LK5λpir	same as MC4100λ*pir* but *arcA*$^-$	this study
V. fischeri		
ES114	wild-type isolate from *E. scolopes*	(8)
AMJ1	ES114 Δ*arcA*::*ermR* (allele exchanged from pAJ7 into ES114)	this study
AMJ2	ES114 Δ*arcA* (allele exchanged from pAJ4 into AMJ1)	this study
AMJ3	ES114 Δ*arcA luxR*::*ermR lacI*^{q}-P$_{tac}$::*luxΓluxCDABEG* (allele exchanged from pEVS137 into AMJ2)	this study
CL21	ES114 Δ*ainS*::*chmR*	(129)
CL53	ES114 Δ*luxR*::*ermR*	(127)
EVS21	ES114 Δ*arcA* Δ*ainS*::*chmR* (allele exchanged from pMU106 into AMJ2)	this study
EVS101	ES114 *luxR*::*ermR lacI*^{q}-P$_{tac}$::*luxΓluxCDABEG*	(209)
JB5	ES114 Δ*arcA* Δ*luxR*::*ermR* (allele exchanged from pCL149 into AMJ2)	this study
JB9	ES114 Δ*arcA luxI-gfp-luxCDABEG* (allele exchanged from pJLB73 into AMJ2)	this study
JB10	ES114 *luxI-gfp-luxCDABEG* (allele exchanged from pJLB73 into ES114)	this study
JB11	MJ1 Δ*arcA* (allele exchanged from pJLB76 into MJ1)	this study
JB13	ES114 *luxO*::pAIA3	this study
JB14	ES114 Δ*arcA luxO*::pAIA3 (pAIA3 into AMJ2)	this study
JB19	ES114 *litR*::*ermR* (allele exchanged from pJLB96 into ES114)	this study
JB21	ES114 Δ*arcA litR*::*ermR* (allele exchanged from pJLB96 into AMJ2)	this study
JB24	ES114 Δ*crp* (allele exchanged from pJLB117 into ES114)	this study

JB25	ES114 Δ*arcA* Δ*crp* (allele exchanged from pJLB117 into AMJ2)	this study
JB35	ES114 with ArcA "site 1" in *lux* promoter replaced (allele exchanged from pJLB212 into ES114)	this study
JB36	ES114 Δ*arcA* with ArcA "site 1" in *lux* promoter replaced (allele exchanged from pJLB212 into AMJ2)	this study
MJ1	wild-type isolate from *Monocentris japonica*	(180)
NL3	ES114 *arcB*::mini-Tn5-*ermR*	this study
Plasmids [b]		
pAIA3	internal *luxO* fragment; R6Kγ, *ermR*	this study
pAJ4	Δ*arcA* allele; R6Kγ, *chmR*	this study
pAJ7	Δ*arcA*::*ermR* allele; R6Kγ, *chmR*	this study
pCL149	Δ*luxR*::*ermR*; ColE1, *chmR*	(127)
pEVS137	*luxR*::*ermR*-*lacI*^{q}-P_{tac}::*luxI*; R6Kγ, ColE1, *chmR*, *ampR*	(12)
pJLB36	P_{luxR}-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	this study
pJLB37	MJ1 P_{luxI}-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	this study
pJLB38	P_{luxI}-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	this study
pJLB43	MJ1 P_{luxR}-*gfp* reporter in pVSV33; pES213, R6Kγ, *kanR*	this study
pJLB52	*arcA* in shuttle vector pVSV105; pES213, R6Kγ, *chmR*	this study
pJLB73	*luxI*-*gfp*-*luxC* allele; R6Kγ, ColE1, *chmR*, *kanR*	this study
pJLB76	MJ1 Δ*arcA* allele; R6Kγ, ColE1, *chmR*, *ampR*	this study
pJLB96	*litR*::*ermR* allele; ColE1, *chmR*	this study
pJLB117	Δ*crp* allele; R6Kγ, ColE1, *chmR*, *kanR*	this study
pJLB122	P_{crp}-*gfp* in pVSV33; pES213, R6Kγ, *kanR*	this study
pJLB123	P_{orf1}-*luxR* in shuttle vector pVSV104; pES213, R6Kγ, *kanR*	this study
pJLB145	*arcA* D54E in shuttle vector pVSV105; pES213, R6Kγ, *chmR*	this study
pJLB146	P_{lac}-*crp* in shuttle vector pVSV105; pES213, R6Kγ, *chmR*	this study
pJLB212	*luxR*-*luxI* region with ArcA "site 1" replaced by *Nhe*I site; R6Kγ, ColE1, *chmR*, *ampR*	this study
pJLB217	P_{sdh}-*lacZ* reporter; pES213, R6Kγ, *chmR*	this study
pMU106	Δ*ainS*::*chmR*; R2K, *tetR*	(129)
pVSV33	promoterless *chmR*-*gfp*; pES213, R6Kγ, *kanR*	(45)
pVSV104	Shuttle vector; pES213, R6Kγ, *kanR*, *lacZα*	(45)
pVSV105	Shuttle vector; pES213, R6Kγ, *chmR*, *lacZα*	(45)

[a] Drug resistance abbreviations used: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tetR*, tetracycline resistance (*tetM*).

[b] *V. fischeri* alleles are from strain ES114 unless denoted "MJ1". All plasmids listed contain the RP4 origin of transfer. Replication origin(s) present on each vector are listed as RK2, R6Kγ, ColE1, and/or pES213. Plasmids based on pES213, which is native to *V. fischeri*, are stable in this bacterium and do not require constant antibiotic selection for maintenance in the population (44, 45). Additional details of plasmid composition and construction can be found in supplemental Table 3.2.

Luminescence, fluorescence, and ß-galactosidase measurements in culture. Overnight cultures grown in LBS were diluted 1:1000 into 50 ml of SWTO in 250-ml flasks, incubated at 24°C with shaking (200 rpm). Unless noted otherwise, data represent the mean of two parallel cultures. 500-µl samples were removed at regular intervals and culture absorbance (A_{595}) was determined using a BioPhotometer (Brinkman Instruments, Westbury, NY). After vigorous shaking to oxygenate the sample, luminescence was determined using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Where maximal luminescence per A_{595} is reported, this represents the highest specific luminescence observed during growth in batch culture from low to high turbidity (A_{595} <0.1 to ~3.0). In all cases, maximal luminescence was achieved when the culture A_{595} was between 1.5 and 2.5. Fluorescence was measured in a TD-700 fluorometer (Turner Designs) using excitation and emission filters of 486 nm and >510 nm, respectively. Fluorescence data reflect averaged measurements when culture was between A_{595} 2.0 and 2.8; a range in which specific luminescence is nearly constant for strain ES114. Fluorescence of strains carrying promoterless-*gfp* constructs was subtracted as background. To measure β-galactosidase, cultures were grown to A_{595}= 0.2, diluted 1:100 and re-grown to A_{595} ~0.2 (to minimize carryover of activity from the inoculum culture), 1-ml was pelleted, the supernatant was discarded, and the pellet frozen at -20°C. The next day the frozen pellet was thawed and resuspended in 1-ml of Z-buffer, and 750 µl used for determination of β-galactosidase activity expressed as Miller units (141). β-galactosidase activity of strains carrying a promoterless-*lacZ* construct was subtracted as background.

Gel retardation and DNA footprinting assays. DNA was PCR amplified using primer sets JBELUX1/JBELUX2, JBMLUX1/JBMLUX2, or JB16S1/JB16S2, for the ES114 *lux* promoter, the MJ1 *lux* promoter, and the ES114 16S rRNA promoter, respectively, and the

products were cloned and sequenced in pJLB151, pJLB152, and pJLB141, respectively (Table 3.2). The sequences of these oligonucleotides were (5'-3'): JBELUX1, CGG AAT TCC ATT TTT TGT TCA CCT AGC TTA TTG TTA TGT; JBELUX2, GCT CTA GAC ATG CAA CCT CTC TTA TTT TAC ATG ATC; JBMLUX1, CGG AAT TCC ATC TCT TTA TCC TTT ATC CTT ACC TAT TGT; JBMLUX2, GCT CTA GAC ATA CCA ACC TCC CTT GCG TTT ATT C; JB16S1, CGG AAT TCT AAG CAA CGC TTA GTT TTG AGC TC; and JB16S2, GCT CTA GAT CAA TTA AAG TTT TTT TGG TTG CTC TGT C. The cloned fragments were again PCR amplified, the products were cleaned using Zymo Research (Orange, CA) Clean and Concentrator kit, digested with *Eco*RI or *Xba*I, and gel purified using the Zymo Research Gel-Extraction kit. The digested fragments were end-labeled with [α-^{32}P]-dATP (MP Biomedical, Irvine, CA) using Klenow fragment, and labeled DNA was separated from dNTP's using a G-25 Sephadex column (Roche, Indianapolis, IN). ArcA-P was purified as described (198).

For gel retardation assays with ArcA-P (198), aliquots of each labeled DNA fragment were incubated with varying amounts of purified ArcA-P that had been diluted in a solution of 50 mM Tricine buffer (pH 8.0), 200 mM KCl, 20 mM $MgCl_2$, and 0.5 mM DTT. Each reaction was performed for 10 min at room temperature in 15 µl with 2 nM target DNA, 1 mM Tris buffer (pH 7.5), 5 mM KCl, 0.7 mM $CaCl_2$, 8.6% glycerol, 57 µg ml^{-1} bovine serum albumin, and 1.4 µg ml^{-1} poly-(dI-dC) (Sigma, St Louis, MO). The samples were loaded onto a 4-20% polyacrylamide TBE gel (Invitrogen) and run at 200 V for 85 min at 4C. The gels were then transferred to filter paper, dried under vacuum and exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) to visualize bands.

DNase I footprinting of the *lux* promoter region was performed as described previously (198) but with addition of 1.67µg ml^{-1} poly-(dI-dC) to the reaction mixtures. The DNA-ArcA-P

complex was digested for 8 min with 1.67 μg ml^{-1} of DNase I, and digestion was terminated by adding 0.94 μg ml^{-1} poly-(dI-dC), 18.8 mM EDTA and 0.3 M sodium acetate.

***E. scolopes* colonization assays.** *V. fischeri* was grown unshaken in 5 ml of SWT in 50-ml conical tubes at 28°C until the A_{595} was between 0.3 and 1.0, the cultures were diluted in Instant Ocean to a density no higher than 1500 CFU ml^{-1}, and *E. scolopes* hatchlings were exposed to inocula for up to 14 h before being rinsed in *V. fischeri*-free Instant Ocean. To study infection kinetics, hatchlings were placed in 5 ml of inoculant in 20-ml scintillation vials, and luminescence was monitored using a LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). To determine CFU per squid, infected animals were homogenized in 700 μl Instant Ocean in 1.5-ml tubes, and the homogenates were dilution plated onto LBS. To assess the specific luminescence of symbionts, luminescence of each animal was determined with a TD-20/20 luminometer prior to homogenization and plating. In parallel, luminescence was measured for cultured cells, and cultures were dilution plated to determine CFU ml^{-1}. For competition experiments, ES114 and AMJ1 (Δ*arcA*::*ermR*) were mixed at a ~1:1 ratio, and squid were inoculated with this mix. The ratio of these strains in the inoculum and in each infected animal was determined by plating on LBS and then patching colonies onto LBS supplemented with erythromycin. The relative competitiveness index (RCI) was calculated by dividing the ratio of the strains in each animal by the ratio in the inoculum. Three independent experiments were combined, and log-transformed data were used to calculate the average RCI and to determine statistical significance.

Table 3.2. Plasmid construction and oligonucleotides

Plasmid or oligonucleotide	Relevant characteristics[a]	Source or reference
Plasmids		
pAIA3	internal *luxO* PCR product (primers LuxOintF and LuxOintR, ES114 template) *Bam*HI- and *Kpn*I-digested, in same sites of pEVS122	this study
pAJ1	ES114 *arcA* region on PCR product (primers JBARC1 and JBARC2), digested with *Ava*I and *Avr*II, in same sites in pEVS118	this study
pAJ4	PCR product (primers JBARC3 and JBARC4, pAJ1 template) *Sma*I-digested, self-ligated; ES114 Δ*arcA* allele	this study
pAJ6	*arcA* PCR product (primers JBARC5 and JBARC6, pAJ1 template) in pCR-BluntII-TOPO; source of *arcA* for complementation	this study
pAJ7	*ermR* from *Eco*RV-digested pEVS94 in *Sma*I site of pAJ4; ES114 Δ*arcA*::*ermR* allele	this study
pAKD702	Transcriptional terminators, MCS, and promoterless *lacZ* (PCR amplified from *E. coli* MG1655 with primers JLBlacZF and JLBlacZR) in *Not*I site of pVSV105	A. Dunn
pBluescript	ColE1 *oriV*, *ampR*	Stratagene
pCL149	ColE1 *oriV*, $oriT_{RP4}$, *chmR* Δ*luxR*::*ermR*	(127)
pCR-Blunt	PCR-product cloning vector; ColE1 *oriV*, *kanR*	Invitrogen
pCR-BluntII-TOPO	PCR-product cloning vector; ColE1 *oriV*, *kanR*	Invitrogen
pEVS94	R6Kγ *oriV*, $oriT_{RP4}$, *ermR*	(212)
pEVS118	R6Kγ *oriV*, $oriT_{RP4}$, *chmR*	(44)
pEVS122	R6Kγ *oriV*, $oriT_{RP4}$, *ermR, lacZα*	(44)
pEVS137	R6Kγ *oriV* , $oriT_{RP4}$, *chmR*, ColE1 *oriV*, *ampR*, *luxR*::*ermR*-$lacI^q$-P_{tac}::*luxI*	(12)
pEVS148K	ES114 *luxC* region cloned in pCR-BluntII-TOPO backbone	(12)
pEVS151	R6Kγ *oriV*, $oriT_{RP4}$, *chmR*; ES114 *luxI* and upstream region	(12)
pJLB29	ES114 *luxI-luxR* intergenic region in pCR-Blunt	(45)
pJLB30	MJ1 *luxI-luxR* intergenic region on PCR product (primers EVS109 and EVS110) in pCR-Blunt	this study
pJLB36	pJLB29 *Avr*II fragment containing *lux* intergenic region in *Avr*II site of pVSV33; ES114 P_{luxR}-*gfp* promoter-reporter	this study
pJLB37	pJLB30 *Avr*II fragment containing *lux* intergenic region in *Avr*II site of pVSV33; MJ1 P_{luxI}-*gfp* promoter-reporter	this study
pJLB38	pJLB29 *Avr*II fragment containing *lux* intergenic region in *Avr*II site of pVSV33; ES114 P_{luxI}- *gfp* promoter-reporter	this study
pJLB43	pJLB30 *Avr*II fragment containing *lux* intergenic region in *Avr*II site of pVSV33; MJ1 P_{luxR}-*gfp* promoter-reporter	this study
pJLB45	MJ1 *arcA* region on PCR product (primers JBARC1 and JBARC2) in pCR-BluntII-TOPO	this study

pJLB52	*arcA* from pAJ6 *Spe*I- and *Bsr*GI- fragment in same sites of pVSV105	this study
pJLB54	pJLB45 *Ava*I and *Avr*II fragment containing MJ1 *arcA* region in same sites of pEVS118	this study
pJLB55	*Sma*I-digested, self-ligated PCR product (primers JBARC3 and JBARC4M, pJLB54 template); MJ1 Δ*arcA* allele	this study
pJLB72	Fusion of *Not*I-digested pEVS151 and pEVS148K; *Apa*I digest, self-ligate; *luxR luxI*-(*Not*I-*Nhe*I-*Bam*HI)-*luxC*	this study
pJLB73	*gfp* on *Not*I- and *Bam*HI-digested PCR product (primers JBGFP1 and DMA26, pQBI63 template) in same sites of pJLB72; *luxR luxI gfp luxC* insert	this study
pJLB76	Fusion of *Xho*I-digested pJLB55 and pBluescript; ColE1 *oriV* and MJ1 Δ*arcA* allele	this study
pJLB96	*ermR* on pEVS94 *Eco*RV fragment in *Stu*I-digested pMF6; *litR*::*ermR* allele	this study
pJLB108	Region upstream of *crp* on PCR product (primers JBCRP1 and JBCRP2, ES114 template) in pCR-BluntII-TOPO	this study
pJLB109	Region downstream of *crp* on PCR product (primers JBCRP3 and JBCRP4, ES114 template) in pCR-BluntII-TOPO	this study
pJLB116	Fusion of *Kpn*I-digested pEVS118 and pJLB109, Δ*Xba*I fragment; subclone of region downstream of *crp* into pEVS118	this study
pJLB117	pJLB108 and pJLB116, fused at *Nhe*I sites; Δ*crp* allele	this study
pJLB122	pJLB108 *Xba*I and *Nhe*I fragment containing *crp* promoter into *Xba*I- and *Avr*II-digested pVSV33; P_{crp}-*gfp* reporter plasmid	this study
pJLB123	*luxR* PCR product (primers JBLUXR1 and JBLUXR2, ES114 template) in *Hpa*I-digested pVSV104; *Nde*I digest, self-ligate; P_{orf1}-*luxR* plasmid	this study
pJLB135	PCR product (primers JBARCAMUT3 and JBARCAMUT4, pAJ6 template), generates D54E *arcA* mutant allele	this study
pJLB141	PCR product (primers JB16S1 and JB16S2, ES114 template) in pCR-BluntII-TOPO; template for 16S promoter used in gel shifts	this study
pJLB145	*Nhe*I-*Xmn*I fragment containing *arcA* D54E allele from pJLB135 in same sites of pJLB52	this study
pJLB146	PCR product (primers JBCRP8 and JBCRP9, ES114 template) in *Sma*I-digested pVSV105; P_{lac}-*crp* plasmid	this study
pJLB151	PCR product (primers JBELUX1 and JBELUX2, pJLB29 template) in pVSV105; template for ES114 *lux* promoter used in gel shifts	this study
pJLB152	PCR product (primers JBMLUX1 and JBMLUX2, pJLB30 template) in pVSV105; template for MJ1 *lux* promoter used in gel shifts	this study
pJLB211	PCR product (primers JBNOARC1 and JBNOARC2, pEVS151 template), *Nhe*I-digested and self-ligated; P_{luxI} with ArcA "site 1" replaced by *Nhe*I site	this study

pJLB212	Fusion of *Spe*I-digested pJLB211 and pBluescript	this study
pJLB217	*Avr*II-digested PCR product (primers JBSDH1 and JBSDH2, ES114 template) in *Nhe*I-digested pAKD702; P_{sdh}-*lacZ* reporter	this study
pMF6	ColE1 *oriV*, $oriT_{RP4}$, *chmR*, *litR*	(58)
pMU106	R2K *oriV*, $oriT_{RP4}$, *tetR*, Δ*ainS*::*chmR*	(129)
pQBI63	ColE1 *oriV*, *ampR*, *gfp*	Quantum Biotech.
pVSV33	pES213 *oriV*, R6Kγ *oriV*, $oriT_{RP4}$, *kanR; promoterless chmR-gfp*	(45)
pVSV104	pES213 *oriV*, R6Kγ *oriV*, $oriT_{RP4}$, *kanR*, *lacZα*	(45)
pVSV105	pES213 *oriV*, R6Kγ *oriV*, $oriT_{RP4}$, *chmR*, *lacZα*	(45)
Oligonucleotides[b]		
DMA26	CGG GCT TTG TTA GCA GCC GGA TCC	this study
EVS109	CCG CCC TAG GTT ATT CAG ATA AGC ATT GAT TAA TAT C	(45)
EVS110	CCG CCC TAG GGC ATG CTT AAC CTC TAT ACT CCT CCG ATG GAA T	(45)
JB16S1	CGG AAT TCT AAG CAA CGC TTA GTT TTG AGC TC	this study
JB16S2	GCT CTA GAT CAA TTA AAG TTT TTT TGG TTG CTC TGT C	this study
JBARC1	ACG GCC TAG GAA TCA GAA CCA TTA CGG CCA AGA G	this study
JBARC2	TCC CTC GAG ATA TGG ATA AGT TCG ATC GCC GCA	this study
JBARC3	TCC CCC GGG CAT GGG CGG TAC CTA ACT AAC TGG TTA AA	this study
JBARC4	TCC CCC GGG TAA GAA TCT CTT CTA TCT GAC T	this study
JBARC4M	TCC CCC GGG TAA GAA CCT CTT CTT ATA TGA C	this study
JBARC5	GCT GTA CAA ATC TCG CCA TAA AAA CAC GCC TTC CC	this study
JBARC6	GCT CTA GAA AAA TGC CCA GCT ATC ACT AGC TGG GC	this study
JBARC7	TTT GGC GGG AAG GCG TGT TTT TAT GGC GAG	this study
JBARC10	CGA GCC TGC TGC ACC TTT CAT TTT ATG CGT	this study
JBARCAMUT3	CTT AAT CTT GTG ATT ATG GAG ATT AAC CTG CCA GGT AAA	this study
JBARCAMUT4	TTT ACC TGG CAG GTT AAT CTC CAT AAT CAC AAG ATT AAG	this study
JBCRP1	GGC GTG GAA ATC ATG GCT CCT CC	this study
JBCRP2	CTA GCT AGC CCA TAC TTT ATA CTT CCT CTG CGT TTG CC	this study
JBCRP3	CTA GCT AGC CTC GTT AAT TTA ACG TCG TTA CCA TAA GC	this study
JBCRP4	TGC AGG GCA ACG TTG TAC TTG TGC	this study
JBCRP5	GCA TCC TCC AGC AGC CAT TAA GAC C	this study

JBCRP8	AGA GGA AGT ATA AAG TAT GGT TCT AGG TAA ACC	this study
JBCRP9	GCT TAA CGA GTA CCG TAA ACT ACA ATT GTT TTA CCG	this study
JBELUX1	CGG AAT TCC ATT TTT TGT TCA CCT AGC TTA TTG TTA TGT	this study
JBELUX2	GCT CTA GAC ATG CAA CCT CTC TTA TTT TAC ATG ATC	this study
JBGFP1	GCG CGG CCG CGA AGG AGA TAT ACA TAT GGC TAG CAA AGG	this study
JBLUXR1	GAA GGA GAT ATA CAT ATG AAC ATT AAA AAT ATA AAT GC	this study
JBLUXR2	CGC CAA GAT TTT ATG GAA ATG TAT GAG	this study
JBMLUX1	CGG AAT TCC ATC TCT TTA TCC TTT ATC CTT ACC TAT TGT	this study
JBMLUX2	GCT CTA GAC ATA CCA ACC TCC CTT GCG TTT ATT C	this study
JBNOARCA1	GGC TAG CTT CCA CCA AGA TGC ATA AAT GTG ACC CAG ATC	this study
JBNOARCA2	CGC TAG CAG CTG TAG GAT GGT ACA GGT TTC CG	this study
JBSDH1	GCC CTA GGC CAT TCG CTC CTA ATT TAC GCA CAT CG	this study
JBSDH2	GCC CTA GGC GGA TCG TTT GTA GAT CTA GGT TGA CAG G	this study
JLBlacZF	AAC GCG GCC GCA GGA CAA GTT TTG GTG AC	this study
JLBlacZR	AAC GCG GCC GCC GCA GAC ATG GCC TGC CCG GTT A	this study
LacZlink1	TCG ACG AAC TAG TTC GCT AGC GGG CC	this study
LacZlink2	CTA GGG CCC GCT AGC GAA CTA GTT CG	this study
LuxOintF	AAA AAG GTA CCC GGC TCA TGG CTC AAT TG	this study
LuxOintR	TTT TTG GAT CCT CTA GCC AAG GGT CTC GG	this study

[a] Drug resistance abbreviations used: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tetR*, tetracycline resistance (*tetM*).

[b] Oligonucleotides sequences are provided in the 5'-3' orientation.

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CHAPTER 4

FNR-MEDIATED REGULATION OF BIOLUMINESCENCE AND ANAEROBIC RESPIRATION IN THE LIGHT-ORGAN SYMBIONT *VIBRIO FISCHERI*[1]

[1]Bose, J. L., A. K. Dunn, and E. V. Stabb. To be submitted to the *Journal of Bacteriology*.

Abstract

Oxygen is a substrate for the bioluminescence-producing luciferase in *Vibrio fischeri*, and a previous report indicated that the oxygen-sensitive regulator FNR stimulates the *lux* genes necessary for light production. This conclusion was drawn from analyses of the *lux* genes cloned in *Escherichia coli*, and to test for FNR-mediated regulation of luminescence in the native organism we generated *fnr* mutants of *V. fischeri*. In contrast to the results in *E. coli*, we found that FNR represses *lux* expression under anaerobic conditions in *V. fischeri* strain MJ1 and does not detectably affect luminescence in *V. fischeri* strain ES114. In both strains FNR activated anaerobic respiratory systems, which are thought to be induced in symbiotic *V. fischeri* cells. Using an FNR-dependent promoter-*gfp* reporter we determined that FNR is active in ES114 during infection of the light organ of it host squid, *Euprymna scolopes*, suggesting that the light organ crypts have a relatively low oxygen concentration despite having sufficient oxygen to support luminescence. However, the FNR mutant was not impaired in colonization. This study provides insight into the symbiotic environment of the *E. scolopes* light organ, adds to our understanding of the complex mechanisms controlling bioluminescence, and underscores the importance of studying *lux* regulation in its native background.

Introduction

Vibrio fischeri is an important model for investigations of bioluminescence, mutualistic symbioses, and acyl-homoserine lactone (HSL) mediated signaling known as quorum sensing. These three areas are intimately interrelated, as illustrated by the observations that HSL signaling strongly regulates bioluminescence, that bioluminescence contributes to symbiotic competence (12, 233), and that the relative importance of different HSL signals varies between growth in the host and in culture (127, 128).

Studies of bioluminescence, symbiosis, and HSL signaling in *V. fischeri* are also unified by the relevance of oxygen. O_2 is a substrate for the luminescence-producing luciferase, and this enzyme may benefit *V. fischeri* by generating a more reduced environment in or near cells (221, 233), which could be particularly advantageous for this facultative anaerobe when it is colonizing animal tissue and may minimize the host's ability to generate reactive oxygen species (233). Although luminescence emanating from the host's light organ indicates that O_2 is present, evidence suggests that luciferase is O_2-limited in this environment (11) despite its relatively high affinity (K_m ~35 nM) for O_2 (15). Moreover, anaerobic respiration is induced in symbiotic *V. fischeri*, consistent with the idea that [O_2] is low in the light organ (166). We recently found that luminescence is regulated by the redox-responsive ArcAB two-component regulatory system (14), but, this does not account for the aeration-dependent control of luminescence that we and others have observed (150, 180, 206). One candidate regulator that might control luminescence and other symbiotic factors in response to oxygen concentration is FNR.

FNR alters gene expression during the switch between aerobic and anaerobic growth in *Escherichia coli* and other proteobacteria (61, 130, 169, 183). 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 (106, 205). In the presence of oxygen, the 4Fe-4S center dissociates leading to the inactive form; however, in the absence of oxygen, FNR forms a functional dimer that binds target promoters (29, 105, 106, 116). Active FNR binds a 5'-TTGAT(N_4)ATCAA-3' FNR-box sequence (53) and can activate or repress transcription depending on the location of binding relative to the promoter (69, 134, 140, 244). Interestingly, an examination of transgenic *E. coli* carrying the *V. fischeri* MJ1 *luxR-luxICDABEG* region, which encodes the HSL-dependent *lux* activator LuxR, the HSL synthase LuxI, and the Lux proteins that produce bioluminescence, suggested that FNR activates luminescence, and attributed this to a potential FNR box in the *luxR* promoter (147).

In this study, we examined FNR's regulation of luminescence in *V. fischeri*. We also tested whether FNR's role in regulating anaerobic respiration is conserved in *V. fischeri* and whether FNR is active during host colonization. We tested this using two distinct *V. fischeri* strains: ES114 and MJ1. ES114's genome sequence has been published, and its symbiosis with the squid *E. scolopes* can be reconstituted in the laboratory (8, 174, 181, 208); however, like most strains isolated from these animals ES114 is not visibly luminescent in culture (8). In contrast, MJ1 has bright luminescence typical of isolates from the pinecone fish *Monocentris japonica*, but this symbiosis is not amenable to experimentation. Organization of the genes required for luminescence and HSL-mediated autoinduction are similar in the two strains, with the *luxICDABEG* operon adjacent to and divergently transcribed from the *luxR*, but the *luxR-luxI* intergenic region has diverged significantly. By generating and examining *fnr* mutants in MJ1 and ES114 we found that FNR represses luminescence anaerobically in strain MJ1, but does not appear to control luminescence in strain ES114. We also show that FNR regulates anaerobic

respiration and is active in ES114 during symbiotic infection, although FNR is not required for colonization of *E. scolopes*.

Materials and Methods

Bacteria and media. Select bacterial strains used in this study are described in Table 4.1. Plasmids were maintained in *E. coli* strain DH5α (76), except those containing the R6Kγ origin of replication, which were maintained in CC118λ*pir* (88) or DH5αλ*pir* (44). *E. coli* was grown in LB medium (141) or M9 basal medium (185) supplemented with 1 mg ml^{-1} casamino acids, 40 mM glycerol and 40 mM of either sodium nitrate or sodium fumarate. *V. fischeri* was grown in LBS medium (210), SWT medium (8) wherein seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, OH), SWTO medium (14), or in fischeri minimal medium (FMM), which contained 0.378 mM $NaPO_4$ pH7.5, 50 mM Tris pH 7.5, 0.011 mM $FeSO_4$-$7H_2O$, 13.8 mM $MgSO_4$-$7H_2O$, 11 mM NH_4Cl, 11 mM KCl, 334 mM NaCl, 10 mM $CaCl_2$, 40 mM glycerol, 1 mg ml^{-1} casamino acids, and 40 mM of either sodium nitrate or sodium fumarate. Agar (15 mg ml^{-1}) was added to solidify media for plating. Anaerobic growth on solid media was assessed using the GasPak EZ Anaerobic Container System from Becton, Dickinson and Company (Sparks, MD). When added to LB for selection of *E. coli*, ampicillin, chloramphenicol, kanamycin, and trimethoprim were used at 100, 20, 40, and 10 μg ml^{-1}, respectively. When added to LBS for selection or screening in *V. fischeri*, chloramphenicol, kanamycin, and trimethoprim were typically used at concentrations of 2, 100, and 10 μg ml^{-1}, respectively.

Table 4.1. Select bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Bacterial strains		
E. coli		
MC4100	F- *araD139* Δ(*argF-lac*) U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	(200)
PC2	MC4100 Δ*fnr*	(27)
V. fischeri		
ES114	wild-type isolate from *E. scolopes*	(8)
EVS102	ES114 Δ*luxCDABEG*	(12)
EVS601	MJ1 Δ*fnr*::*tmpR* (allele exchanged from pCDW5)	this study
JB1	ES114 Δ*fnr*::*tmpR* (allele exchanged from pJLB5)	this study
JB2	*fnr* restored in JB1 (wild-type allele exchanged from pEVS136)	this study
JB3	ES114 with putative FNR-box replaced by corresponding MJ1 sequence (allele exchanged from pJLB58)[b]	this study
JB4	ES114 Δ*fnr*::*tmpR* with putative FNR-box replaced by corresponding MJ1 sequence (allele exchanged from pJLB61 into JB1)[b]	this study
JB6	MJ1 Δ*fnr*::*tmpR*, putative FNR-box replaced by corresponding ES114 sequence (allele exchanged from pJLB64 into EVS601)[b]	this study
JB7	MJ1 with putative FNR-box replaced by corresponding ES114 sequence (allele exchanged from pJLB69 into JB6)[b]	this study
JB27	*fnr* restored in EVS601 (wild-type allele exchanged from pJLB69)	this study
MJ1	wild-type isolate from *Monocentris japonica*	(180)
Select plasmids[b]		
pAKD50	pES213, *kanR*, $P_{yfiD1/2}$-*cat-gfp*	this study
pCDW5	R6Kγ, ColE1, *chmR*, *kanR*, MJ1 Δ*fnr*::*tmpR* allele	this study
pDMA5	p15A *oriV*, $oriT_{RP4}$, *lacZα*, *chmR*	(44)
pEVS136	R6Kγ, ColE1, *ermR*, *kanR*, ES114 *fnr*	this study
pJLB5	R6Kγ, ColE1, *ermR*, *kanR*, ES114 Δ*fnr*::*tmpR* allele	this study
pJLB6	p15A, *chmR*, ES114 *fnr*	this study
pJLB58	R6Kγ, *chmR*, ES114 *luxR-luxI* with MJ1 FNR-box allele	this study
pJLB61	R6Kγ, ColE1, *ampR*, *chmR*, ES114 *luxR-luxI* with MJ1 FNR-box allele	this study
pJLB64	ColE1, *chmR*, MJ1 *luxR-luxI* with ES114 FNR-box allele	this study
pJLB69	R6Kγ, ColE1, *chmR*, *kanR*, MJ1 *fnr*	this study

[a] Drug resistance abbreviations: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tmpR* trimethoprim resistance (*dfr*). FNR box alleles are shown in Figure 4.5.

[c] All plasmids contained the RP4 origin of transfer. Replication origin(s) are listed as p15A, R6Kγ, ColE1, and/or pES213.

Molecular genetic techniques and sequence analysis. Cloning was performed using standard techniques. Select plasmids are described in Table 4.1, and details of plasmid construction are given in Table 4.1. Plasmids were purified using Qiagen Mini-prep kits (Qiagen Inc., Valencia, CA). PCR products were cloned into pCR-BluntII-TOPO using the ZeroBlunt-TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA). Oligonucleotides (Table 4.1) were synthesized by Integrated DNA Technologies (Coralville, IA). Klenow fragment, DNA ligase, and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Between restriction and ligation reactions, DNA was recovered with the DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, CA). PCR was performed with an iCycler (BioRad Laboratories, Hercules, CA) using KOD HiFi DNA polymerase (Novagen, Madison, WI) or *Taq* polymerase (Sigma, St. Louis, MO). Cloned PCR products were sequenced to ensure that unintended alterations were not incorporated. DNA sequencing was conducted at the University of Michigan DNA Sequencing Core Facility or at the University of Georgia Molecular Genetics Instrumentation Facility. Sequences were analyzed using Sequencher 4.1.2 (Gene Codes Corp., Ann Arbor, MI) and DNA Strider.

The details of *fnr* mutant generation are outlined in Table 4.1. In short, the putative *fnr* gene and flanking DNA were cloned and an internal 255 bp of *fnr* was replaced by a trimethoprim resistance gene. Due to sequence differences, separate constructs were generated for strains ES114 and MJ1. The resulting Δ*fnr*::*tmpR* alleles were then crossed into ES114 and MJ1 to replace wild-type *fnr* generating mutants JB1 and EVS601, respectively.

Determination of luminescence expression in cultures with varied degrees of aeration. Overnight cultures grown in LBS were diluted 1:1000 into SWTO and incubated at 24^{o}C with shaking (200 rpm). Cultures designated “well-aerated” and “poorly-aerated”

contained 50 or 200 ml SWTO, respectively in 250-ml flasks. For anaerobic cultures, aerobically-grown overnight cultures were diluted 1:10 in LBS prior to 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. 500-µl samples were removed at regular intervals and culture absorbance (A_{595}) was determined using a BioPhotometer (Brinkman Instruments, Westbury, NY). Immediately following vigorous shaking to aerate each sample fully, luminescence was determined using a GLOMAX 20/20 luminometer (Promega, Madison, WI).

Determination of P_{yfiD}-*gfp*-reporter expression. To determine $P_{yfiD1/2}$-*gfp* reporter expression in cultured cells, ES114 or JB1 carrying plasmid pAKD50 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 at 200 rpm. To minimize carryover of GFP from the overnight inoculum culture in our final samples, cultures were diluted multiple times and kept in log-phase growth. Specifically, strains were grown until the A_{595} was 0.1, diluted 1:100 and re-grown to an A_{595} of 0.1. This culture was then diluted 1:100 into 20 ml SWTO in 250-ml baffled flasks for aerobic cultures or 165-ml sealed bottles for anaerobic cultures. Parallel aerobic and anaerobic cultures were grown to an A_{595} of ~0.2 at which point samples were removed and examined by epifluorescence microscopy. To assay GFP expression during symbiosis, squid were inoculated (as described below) and examined at 24 h post-inoculation. Images were obtained using a Nikon (Melville, NY) Eclipse E600 epifluorescence microscope through a Chroma (Rockingham, VT) 41017 Endow GFP filter for visualizing GFP or a Chroma 51004v2 FITC/TRITC filter for visualizing GFP and squid tissue autofluorescence. Following imaging, squid were homogenized and dilution plated to ensure that animals were colonized with similar numbers of *V. fischeri*.

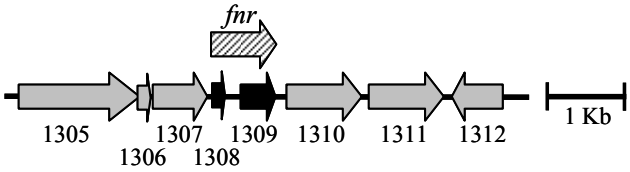
Colonization Assays. *E. scolopes* hatchlings were infected with *V. fischeri* as described previously (175, 210). Inoculant strains were grown unshaken in 5 ml of SWT (8) in 50-ml conical tubes at 28°C until the A_{595} was between 0.3 and 1.0, and cultures were diluted in Instant Ocean to a density no higher than 1500 CFU ml^{-1}. 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 a LS6500 scintillation counter (Beckman Coulter, Fullerton, CA).

For mixed-strain competition experiments, juvenile squid were exposed to an inoculum containing a ~1:1 ratio of wild type and mutant. At 48 h post-inoculation, individual squid were homogenized and dilution plated on LBS. Resulting colonies were patched onto LBS supplemented with trimethoprim to determine the ratio of mutant to wild type for each animal. Inocula were similarly plated and patched to determine the starting ratio of the strains. 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. Mean RCI was calculated from log-transformed data.

Results

Identification of *fnr* in *V. fischeri*. BLAST searches of the *V. fischeri* ES114 genome revealed similarity of ORF's VF1308 and VF1309 to the N and C termini of the *E. coli* FNR, respectively (Fig. 4.1). We suspected that a sequencing error archived in the ES114 genome database had led to the misannotation of *fnr* as two genes, and to test this possibility we cloned and sequenced the region spanning VF1308 and VF1309 in ES114. Our analysis of this sequence shows that there are five errors in the genome database (Fig. 4.2), and one of these

leads to an erroneously predicted truncation of VF1308. Based on the corrected sequence, VF1308 and VF1309 encode a 250 amino acid protein that is the same length as, and shares 84% identity with, *E. coli* FNR. This putative FNR in ES114 is also identical to the previously deposited sequence of *V. fischeri* MJ1 FNR (NCBI accession number CAE47558). Importantly, the amino acids necessary for interactions with RNA polymerase (6, 113, 122, 125, 241), iron-sulfur center assembly (106, 139, 197, 204), and promoter recognition (203) in *E. coli* FNR are conserved in the proposed *V. fischeri* FNR.



ORF	#aa	Annotation/Putative Function
VF1305	502	Cation-transport ATPase
VF1306	58	Homolog of *Rhizobium* FixS
VF1307	224	Hypothetical membrane protein
VF1308	52	N terminus of FNR
VF1309	131	C terminus of FNR
VF1310	316	Universal stress protein
VF1311	307	ATPase of the PP superfamily
VF1312	215	hypothetical

FIG. 4.1. Genome arrangement around predicted *fnr* in *V. fischeri* ES114. Numbers represent the corresponding VF#### ORF designation in the genome database (http://ergo.integratedgenomics.com/Genomes/VFI). "#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 *E. coli* FNR, respectively. Striped arrow shows the complete *fnr* gene based on our sequence revision.

```
                    …R  K  K  P  Y  *
ES114-databse       CGTAAAAAACCCTATTAAAAAGGCCAAGA
ES114-this study    CGTAAAAAGCCTATTCAAAAAGGCCAAGA
MJ1                 CGTAAAAAGCCAATTCAAAAAGGCCAAGA
                    …R  K  K  P  I  Q  K  G  Q  E…
```

FIG. 4.2. Corrected sequence of *fnr* in *V. fischeri* ES114. Alignment (starting with base-pair 142 of VF1308) of ES114 genome database (accession number CP000020, row 1), our revised ES114 sequence (this study, row 2), and MJ1 *fnr* (accession number CAE47558, row 3). Underlined nucleotides indicate mistakes in the genome database. Letters above row 1 show the predicted amino acid sequence for the ES114 genome in database (asterisk indicates a stop codon) and letters below row 3 show the amino acids encoded by both the corrected ES114 and MJ1 sequences.

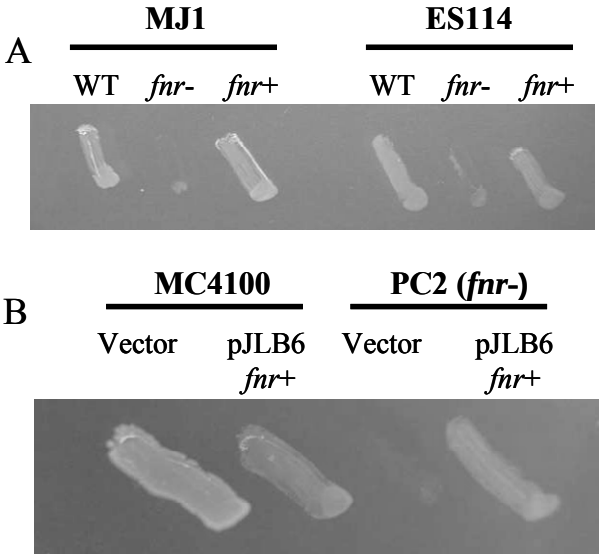


FIG. 4.3. *V. fischeri* FNR is functionally similar to *E. coli* FNR. (A) *V. fischeri* MJ1, *fnr* mutant EVS601, and restored *fnr*+ strain JB27 along with ES114, *fnr* mutant JB1, and restored *fnr*+ strain JB2 grown on FMM medium with glycerol and fumarate, incubated in anaerobic jars at 28°C. (B) *E. coli* MC4100 and *fnr* mutant PC2 with vector pDMA5 or pJLB6, which contains the *V. fischeri* ES114 *fnr* grown on M9 medium with nitrate in anaerobic jars at 37°C.

We next generated mutants lacking this putative *fnr* gene in *V. fischeri* strains ES114 and MJ1. *E. coli fnr* mutants are unable to grow anaerobically with nitrate or fumarate as terminal electron acceptors (112), and we found that *V. fischeri fnr* mutants were similarly attenuated in their ability to grow anaerobically when forced to respire using nitrate (not shown) or fumarate

(Fig. 4.3A). Restoring the wild-type *fnr* allele by replacing the *fnr*::*tmpR* allele with the wild-type allele in these *fnr* mutants recovered the ability to respire anaerobically (e.g., Fig. 4.3A). Additionally, the putative *fnr* of *V. fischeri* ES114 provided on plasmid pJLB6 restored anaerobic growth of *E. coli fnr* mutant PC2 on nitrate (Fig. 4.3B) and fumarate (not shown). Thus, the putative *V. fischeri* FNR is similar in both sequence and function to *E. coli* FNR.

Strain-dependent repression of luminescence by FNR. Having confirmed the presence of a *fnr* homolog in both ES114 and MJ1, we tested whether it regulates *lux* expression by monitoring luminescence of wild-type and *fnr* mutant strains grown with different levels of aeration. To examine these strains under well-aerated or poorly-aerated conditions we grew cultures in 250-ml flasks containing 50 or 200 ml SWTO, respectively. As seen in Figure 4.4A and B, the luminescence of the *fnr* mutants was similar to that of their parent strains under these conditions. In some experiments, *fnr* mutant EVS601 appeared as much as 4-fold brighter than MJ1 under poorly-aerated conditions, but this was not reproducible, and the lack of difference between MJ1 and EVS601 shown in Figure 4.4B is representative of most experiments.

FNR should be most active under anaerobic conditions, and we therefore also assessed *lux* expression in these strains grown anaerobically. Luciferase uses oxygen as a substrate, and anaerobic cultures do not luminesce; however, as with the experiments described above, samples removed from these anaerobic bottles were briefly shaken to saturate luciferase with oxygen prior to placing the sample in a luminometer. Thus, this luminescence reading gives an indication of the amount of *lux* expression in an anaerobic culture.

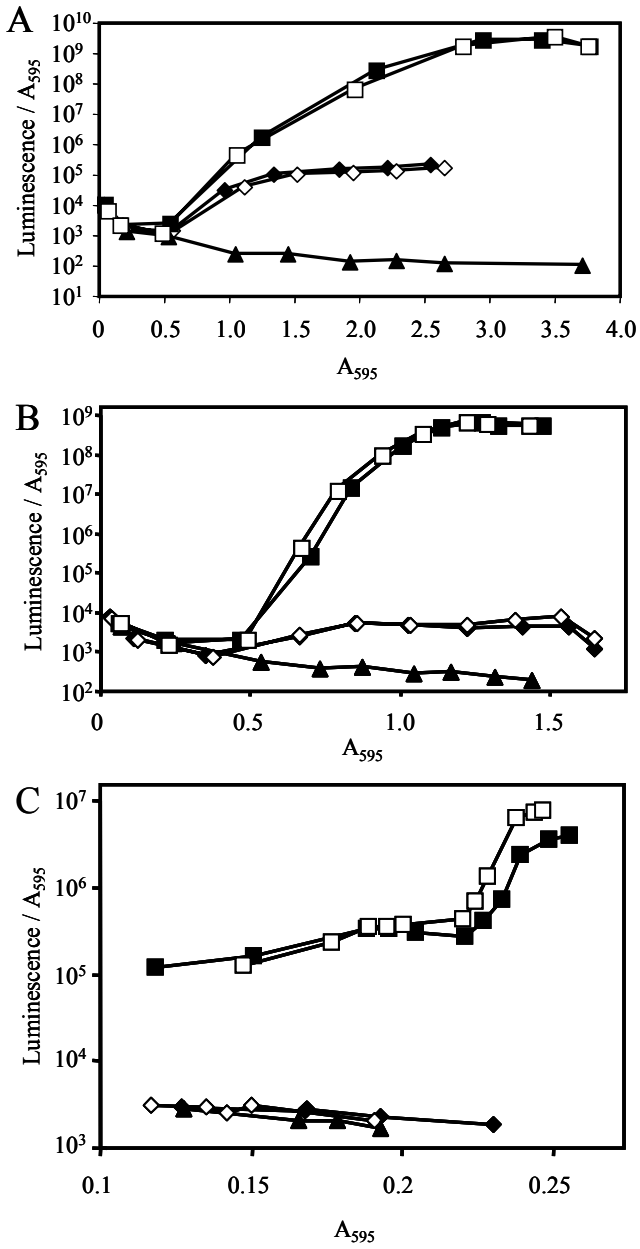


FIG. 4.4. Luminescence per A_{595} of ES114 and MJ1 *fnr* mutants. *V. fischeri* ES114 (solid diamonds), ES114 *fnr* mutant JB1 (empty diamonds), MJ1 (solid squares), MJ1 *fnr* mutant EVS601 (empty squares), and dark Δ*luxCDABEG* mutant EVS102 (solid triangles) grown in cultures that were: (A) well-aerated (50 ml media in 250-ml flask), (B) poorly-aerated (200 ml media in 250-ml flask), or (C) anaerobic (20 ml media in 165-ml bottles with anaerobic headspace) at 24°C with shaking (200 rpm).

When grown anaerobically, ES114 and *fnr* mutant JB1 were indistinguishable and did not display any *lux* expression above background levels, as seen by comparison to the dark

Δ*luxCDABEG* strain (Fig. 4.4C). However, luminescence was consistently higher in the MJ1 *fnr* mutant EVS601 relative to its parent, indicating that FNR mediates repression of *lux* expression in this strain. The magnitude of this difference varied between 1.5- to 20-fold in individual experiments. In five experiments, EVS601 yielded an average of 8-fold more *lux* expression than its parent MJ1, and a 2-fold difference is shown in Figure 4.4C. Interestingly, this is the opposite of the result generated in a heterologous system wherein an *fnr* mutant of *E. coli* carrying the MJ1 *lux* genes had lower luminescence than its fnr^+ parent (147).

Potenetial importance of a FNR-box upstream of *luxR* in FNR-mediated repression of luminescence. We next examined whether sequence differences in ES114 and MJ1 upstream of *luxR* could explain the different regulation of *lux* by FNR in these strains. In this region there is a near-consensus (9/10 match) FNR-box present in MJ1 that is not conserved in ES114 (Fig. 4.5). The corresponding sequence in ES114 has two additional mismatches in the putative FNR-box, and these are both in base pairs important for FNR-DNA binding (189, 203). To determine if interstrain differences in the putative FNR-box upstream of *luxR* are important for *lux* repression, we interchanged these sequences between MJ1 and ES114. For example, the MJ1 *lux* promoter was altered so that the FNR-box was replaced with the corresponding sequence from ES114 while leaving the rest of this intergenic region intact, resulting in strain JB7 (Fig. 4.5). Similarly, the MJ1 FNR-box replaced the corresponding sequence in ES114, generating strain JB3 (Fig. 4.5), and *fnr* mutants with each of these *lux* promoter backgrounds were also generated.

Examination of these strains revealed the importance of this putative FNR-box sequence in determining luminescence levels, and possibly in FNR-mediated anaerobic regulation of *lux* expression (Fig. 4.6). Interchanging the "FNR-box" of ES114 and MJ1 altered the overall

brightness of these strains independent of FNR. Specifically, mutant JB3 which has the MJ1 FNR-box in an otherwise ES114-like *lux* promoter had higher luminescence than ES114, and conversely, mutant JB7 which has the ES114 FNR-box in an otherwise MJ1-like *lux* promoter produces less luminescence than MJ1. Also, intriguingly, only those strains containing the near-consensus MJ1 FNR-box displayed FNR-dependent regulation of luminescence expression. As described above, the *fnr* mutant EVS601 yielded more *lux* expression than its parent MJ1, and we also found that the *fnr* mutant JB4, which contains the MJ1 FNR-box in an otherwise ES114-like background, had significantly higher *lux* expression than JB3 (Fig. 4.6). In contrast, expression of luminescence in ES114 and JB7 which contains the weak ES114 FNR-box in an otherwise MJ1-like background were not detectably affected by *fnr*; however, these were also at background levels, i.e. not significantly different from a Δ*luxCDABEG* mutant (not shown). Thus, it remains possible that FNR regulates *lux* expression in strains ES114 and JB7, but that the overall luminescence is too low to detect these differences.

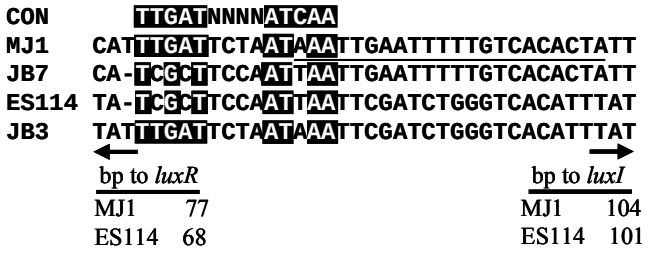


FIG. 4.5. Putative FNR-box sequence upstream of *luxR*. Alignment of *lux* intergenic sequences from strains MJ1, ES114, JB7 (MJ1 background with ES114 FNR-box), and JB3 (ES114 background with MJ1 FNR-box), respectively. The sequence is given 5' to 3'. White letters with black background indicate bases matching the *E. coli* FNR consensus (CON). For reference, the MJ1 sequence similar to a CRP binding-site is underlined and the distances from the ends of the sequence to the ATG's of *luxR* and *luxI* are given.

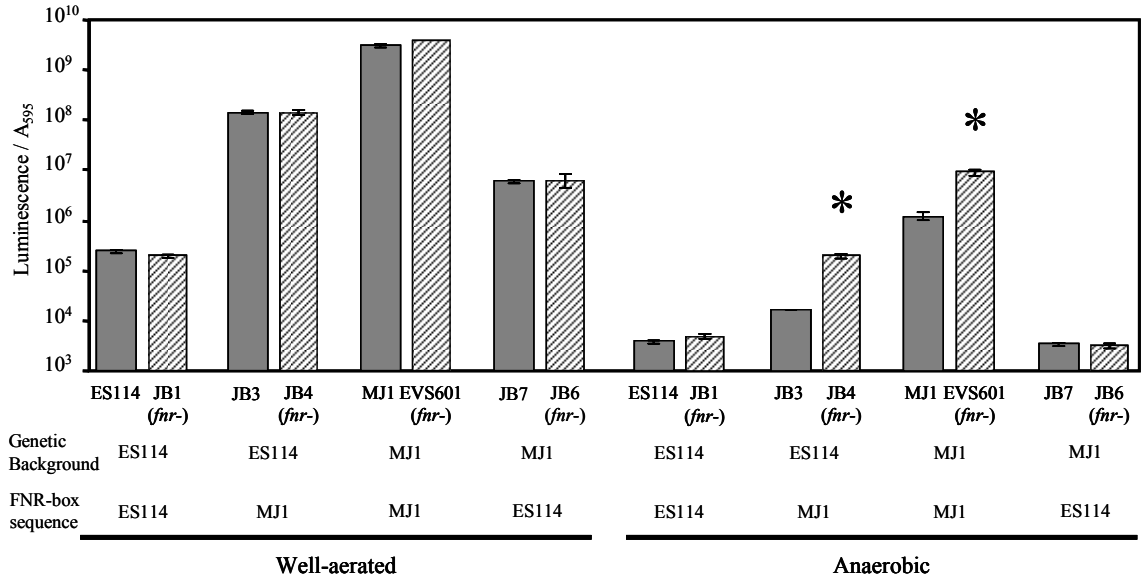


FIG. 4.6. FNR-mediated *lux* repression depends on anaerobic conditions and the MJ1-*lux* FNR-box sequence. ES114, JB3, MJ1, JB7, and their Δ*fnr*::*tmpR* mutants JB1, JB4, EVS601, and JB6, respectively, grown in SWTO under well-aerated (50 ml media in 250-ml flask) or anaerobic (20 ml media in 165-ml bottles with anaerobic headspace) conditions at 24°C with shaking (200 rpm). Data is the average peak luminescence per A_{595} with standard error (n=2 for aerobic or 3-5 for anaerobic cultures, respectively). Asterisks indicate that the *fnr* mutant was significantly ($p<0.01$) brighter than the corresponding isogenic *fnr*$^+$ strain.

FNR is active in *V. fischeri* ES114 during symbiotic colonization of *E. scolopes*. Although FNR does not appear to regulate luminescence ES114, we were nonetheless interested in whether FNR is active in a relevant niche for this strain, the *E. scolopes* light organ. We focused on the interaction with *E. scolopes* and not the symbiosis between MJ1 and *M. japonica*, because the latter cannot be established and monitored in the lab and MJ1 does not effectively colonize *E. scolopes*. We recently used promoter-*gfp* transcriptional fusions on a stable *V. fischeri*-derived replicon to observe promoter activity during growth in the host light organ (45), and we sought to develop a FNR-activated *gfp* reporter. Several candidate promoters in the ES114 genome were analyzed with the goal of identifying one where FNR-controlled elements

could be separated from regions controlled by other regulators, such that we might generate a reporter specifically responsive to FNR. *V. fischeri yfiD* yielded a promising candidate promoter.

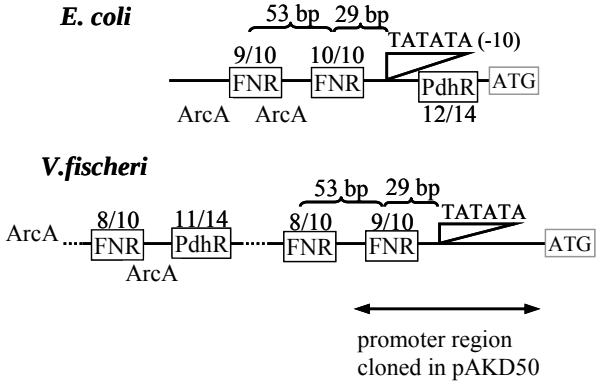


FIG. 4.7. Schematic comparison of *E. coli* and *V. fischeri yfiD* promoter regions. Locations of potential binding sites for the regulators FNR (53), ArcA (131, 160), and PdhR (167) are shown upstream of the ATG start codon of *yfiD*. Fractions refer to the match relative to the FNR or PdhR consensus binding sequence (e.g., 9/10 indicates nine bases match the 10 base FNR consensus). Putative ArcA sites are indicated without reference to this regulator's relatively degenerate consensus binding sequence. Dotted lines indicate large (100 bp) gaps. The transcriptional start site (+1) has been characterized in *E. coli*, allowing assignment of a TATATA sequence as the -10 promoter element. The double headed arrow corresponds to the promoter and regulatory region cloned from *V. fischeri* to generate reporter plasmid pAKD50.

The sequence upstream of *yfiD* in *V. fischeri* closely resembles the corresponding region in *E. coli*, with the important difference that FNR regulatory elements appeared separable from ArcA and PdhR binding sites (Fig. 4.7). In *E. coli* the position and relative affinities of two FNR sites tune *yfiD* to express maximally microaerobically (134). A higher affinity (i.e. nearer to consensus) FNR box overlapping a –35 promoter region stimulates transcription in response to low oxygen, and as $[O_2]$ decreases further a lower-affinity FNR site is filled, repressing transcription. ArcA sites overlapping the distal FNR site contribute to *yfiD* regulation, as does PdhR, which regulates the transcript in response to pH (246). *V. fischeri yfiD* has FNR sites with

identical spacing relative to each other and to a conserved TATATA, which serves as the –10 promoter region in *E. coli yfiD* (Fig. 4.7). As in *E. coli*, the FNR binding site proximal to the promoter is a closer match to the FNR consensus than is the distal FNR site (Fig. 4.7). *V. fischeri* possesses a PdhR homolog, and sequences upstream of *yfiD* match the consensus PdhR binding site (167), but in *V. fischeri* the putative PdhR site is well upstream of the FNR sites, as were the only putative ArcA sites we identified near *yfiD* (Fig. 4.7).

In *E. coli*, a truncated construct with only the promoter-proximal stimulatory FNR site is not tuned to microaerobic conditions but is simply induced by FNR as O_2 is lowered (134). We cloned the corresponding sequence from *V. fischeri* (Fig. 4.7) upstream of a *gfp* reporter to generate pAKD50, predicting that this reporter would be activated by FNR at low O_2. As predicted, expression of *gfp* was evident in wild-type cells grown in anaerobic bottles, but not in well-aerated cultures or in the *fnr* mutant JB1 (Fig. 4.8). GFP was also evident in ES114 cells colonizing the host light organ, but was either absent or expressed at a lower level in cells of the *fnr* mutant (Fig. 4.8). The latter observation was not due to lower colonization by the *fnr* mutant (see below), nor had these cells lost the reporter plasmid (data not shown). The bioluminescence of symbiotic cells demonstrates that the symbionts are not in an anaerobic environment; however, our reporter data suggest that [O_2] is sufficiently low in the light organ for FNR to function as a transcriptional activator.

FNR is not necessary for host colonization. Given the evidence that FNR is active in the symbiosis (Fig. 4.8) and the previous suggestion that anaerobic respiration may be important for *V. fischeri* in the light organ (166), we examined whether FNR was an important colonization factor. Using luminescence to monitor the onset of symbiosis, we found that infection kinetics were similar for ES114 and *fnr* mutant JB1 and that these strains achieved similar levels of

colonization (Fig. 4.9A). Sometimes, subtle colonization defects are only detected when mutants are forced to compete with wild type (211, 234); therefore, we mixed ES114 and JB1 in a ~1:1 ratio and examined the competitiveness of these two strains. ES114 and JB1 showed equal competitiveness with an average RCI of 1.0 (Fig. 4.9B). These data demonstrate that FNR is not necessary for host colonization during the first two days of a symbiotic infection, despite the fact that FNR is an active regulator in this environment.

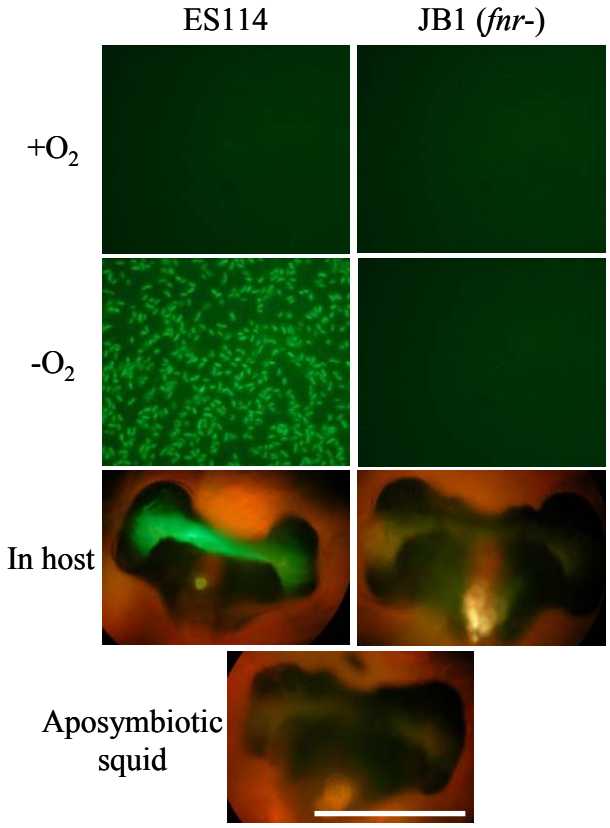


FIG. 4.8. FNR is active during colonization of the *E. scolopes* light organ. Fluorescence images of ES114 and *fnr* mutant JB1 carrying a $P_{yfiD1/2}$-*gfp* reporter grown under well-aerated (+O_2) or anaerobic (-O_2) culture conditions and during *E. scolopes* colonization (In host). Images were taken using a green filter (+O_2 and –O_2) at A_{595} = 0.2 for cultures, or through a red/green filter to distinguish symbionts (green) from *E. scolopes* tissue (red/orange/black). For comparison, an aposymbiotic squid, i.e. no *V. fischeri* inoculum, is shown. White scale bar for squid light organ images is equal to 0.5 mm.

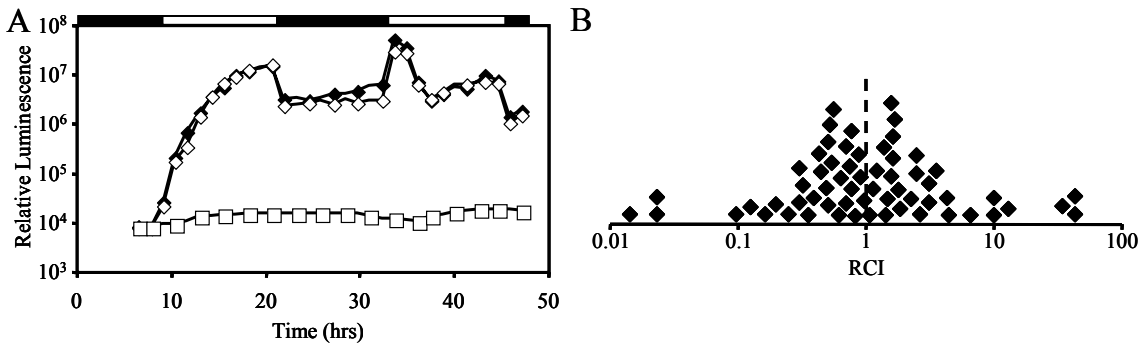


FIG. 4.9. Colonization of *E. scolopes* by *fnr* mutant and wild type. (A) Symbiotic luminescence in *E. scolopes* hatchlings inoculated with ES114 (solid diamonds), *fnr* mutant JB1 (empty diamonds), or no *V. fischeri* (empty squares). Data represent the mean (n=14 for ES114 and JB1). Bars above graph indicate periods of light (empty bar) and darkness (solid bar). (B) 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 is presented. The dashed line represents equal competitiveness and in this case is also the mean RCI (n=60).

Discussion

In this study we investigated the oxygen-sensitive regulator FNR in *V. fischeri*. *V. fischeri fnr* complemented an *E. coli fnr* mutant, and like its counterpart in *E. coli* it activated fumarate- and nitrate-dependent anaerobic respiration (Fig. 4.3). Moreover, the functional complementation of an *E. coli* FNR mutant strongly suggests that the consensus FNR-box recognition site is conserved in *V. fischeri*, and this is supported by a recent bioinformatic analysis of putative FNR boxes in *V. fischeri* (168), and by our results with a predicted FNR-regulated promoter fused to a *gfp* reporter (Figs. 4.7 and 4.8).

Interestingly, we found that FNR represses expression of the *lux* genes responsible for luminescence in *V. fischeri* MJ1. This FNR-mediated repression was consistently observed in anaerobic cultures, and the magnitude of this effect averaged ~8-fold (e.g., Fig. 4.6). Repression of the *lux* genes anaerobically may help minimize production of the oxygen-utilizing luciferase when its O_2 substrate is unavailable. This is consistent the recent finding that luminescence is

also repressed by the redox-responsive ArcAB two-component regulatory system, which is similarly active under relatively reduced conditions (14). Thus it appears that ArcA and FNR work in concert to ensure that luminescence is most highly expressed under relatively oxidative conditions. There could be other regulators controlling *V. fischeri* luminescence in response to oxygen and/or redox state of the cell as well. While it intuitively makes sense that the *lux* genes responsible for bioluminescence would be controlled in response to oxygen availability, it is important to remember that these genes are co-transcribed and co-expressed with the autoinducer synthase gene *luxI*. Therefore, autoinducer and the other genes it induces in concert with LuxR are also controlled by these oxygen- and redox-responsive regulators, adding a puzzling new component to the LuxI-LuxR quorum sensing regulatory system.

The FNR-mediated repression of luminescence in MJ1 correlated with a FNR box upstream of *luxR*. A simple model to explain this effect is that FNR binding to this site represses *luxR* transcription, possibly by interfering with CRP-mediated activation of *luxR*, which is thought to require an overlapping binding site (Fig. 4.5). There is precedence for FNR acting as a simple repressor by binding a single site (69, 242); however, FNR-dependent repression can be complex and involve binding to multiple sites and DNA bending (134, 140). We cannot rule out the possibility that FNR regulation of luminescence involves another FNR-box in the *luxR-luxI* intergenic region; however, given our results (Figs. 4.5 and 4.6) this other hypothetical site must be conserved in the MJ1 and ES114 backgrounds, and no such sequence is apparent. Exactly how FNR might repress *luxR* depends on the location of this gene's promoter, and unfortunately, the *luxR* transcriptional start in *V. fischeri* has not been mapped. An attempt to accomplish this using the *V. fischeri* ATCC7744 *lux* genes expressed in *E. coli* was not conclusive (195), and our

attempts in MJ1 or ES114 using rapid amplification of 5' complementary DNA ends were unsuccessful (data not shown), possibly because this regulator is not highly expressed.

We also found that the putative FNR-box sequence upstream of *luxR* conferred brighter luminescence independent of FNR (Fig. 4.6), and a competely different interpretation of our results is that this brighter luminescence allows us to detect an effect of FNR on luminescence that is completely independent of FNR binding to this particular site. Strains with the ES114-like sequence (Fig. 4.5) grown anaerobically do not produce luminescence above background levels (Fig. 4.6), so we cannot say with certainty whether or not FNR is affecting their *lux* expression. One way to address this would be to add 3-oxo-C6-HSL to cultures to stimulate luminescence of ES114 and its *fnr* mutant above background levels. If FNR does regulate luminescence through a mechanism independent of the putative FNR box upstream of *luxR*, this might be due to its proposed regulation of *arcA* (168), which is a known regulator of the *luxICDABEG* operon (Chapter 3).

Unexpectedly, our results appear to contradict a previous report showing that FNR is an activator of luminescence (147). However, that study used the MJ1 *lux* genes cloned in *E. coli* whereas we examined *fnr* mutants of *V. fischeri*. It seems likely that *lux* expression may be quite different in transgenic *E. coli* and in *V. fischeri*, and this offers several possible explanations for the discrepancy between the studies. For example, LitR is an important transcriptional activator of *luxR* in *V. fischeri*, but LitR is absent in *E. coli* (58). In addition, *E. coli* carried the *lux* genes on a multi-copy plasmid, which may titrate regulators or reflect the contribution of plasmid-borne promoters. Such differences could result in *luxR* expression from a non-native promoter and thereby lead to different regulatory effects of FNR. It is also possible that simple differences in culture conditions account for the disparate outcomes of the two studies, but all things

considered it is not surprising that a *lux* expression study in transgenic *E. coli* might yield different results than a parallel experiment in *V. fischeri*.

In contrast to our results with strain MJ1, FNR apparently does not regulate *lux* in ES114, which also lacks the near-consensus FNR-box upstream of *luxR*. This differential regulation of *lux* by FNR in ES114 and MJ1 may reflect their specific niches and uses of luminescence. Luminescence is a positive colonization factor for ES114 (12, 233), and we have now shown that FNR is active in the *E. scolopes* light organ. This might suggest that in ES114 *lux* regulation has evolved such that FNR does not affect *lux* expression, allowing for high luminescence in the squid light organ even though FNR is active. On the other hand, the FNR-box sequence upstream of *luxR* also confers brighter luminescence in addition to FNR-mediated control of luminescence (Fig. 4.6), and another possibility is that this region diverged in MJ1 and ES114 based on selection for relative brightness. ES114 and other isolates from *E. scolopes* are considerably dimmer than MJ1 and isolates from fish, perhaps because squid use their symbionts in a dim counterillumination light whereas fish use their symbionts to produce a brighter visible light. If ES114 evolved from a brighter ancestor, it might have lost FNR-mediated regulation as a secondary consequence of becoming dimmer.

For multiple reasons we were surprised that the FNR mutant had no discernable attenuation in colonizing *E. scolopes* (Fig. 4.9). Proctor and Gunsalus found that *V. fischeri* induces anaerobic respiration in the symbiosis, and they suggested that this could be important in the squid light organ environment where symbionts may encounter low oxygen conditions (166). Other research also suggested that luminescence is oxygen-limited in the light organ (11), although it has not been possible to test [O_2] in this environment with mechanical probes owing to the small size, elasticity, and delicate nature of this tissue. Using a fluorescent *gfp*-based

reporter we showed that FNR is indeed active in the *E. scolopes* light organ (Fig. 4.8), supporting the prediction that oxygen concentration is low. Considering this information, we predicted that FNR would be important for robust colonization of the host, as it is in *Salmonella* (63); however, that was not the case. Our results also suggest that oxygen-independent respiration may be less important in this symbiosis than thought, although our colonization experiments monitored early infection events whereas previous work demonstrating induction of anaerobic respiration sampled tissues from adult animals with more established infections.

V. fischeri is a cosmopolitan member of marine communities and is found in both fish gut tracts and sediments where oxygen concentrations may be quite low. FNR may be most important for *V. fischeri* in one of these environments outside the light organ of its symbiotic host. In the future, microarray studies comparing the *fnr* mutant to wild type may provide clues regarding where and when the FNR regulon is stimulated, and this would shed light on the ecological significance of FNR in *V. fischeri*.

Table 4.2. Plasmid construction and oligonucleotides

Plasmid or oligonucleotide	Relevant characteristics[a]	Source or reference
Plasmids		
pAKD50	PCR product (primers 141F and 141R, pEVS141yfidhalf template) with partial *yfiD* promoter digested with *Not*I and *Sma*I in same sites of pES213Kn; $P_{yfiD1/2}$-*cat-gfp*	this study
pBluescript	ColE1 *oriV*, *ampR*	Stratagene
pCDW1	*fnr*-containing PCR product (primers EVS97 and EVS98, MJ1 template) in pCRBluntII-TOPO	this study
pCDW4	pJLB1 *Bst*BI/*Avr*II fragment containing *tmpR* in *Cla*I/*Avr*II-digested pCDW1	this study
pCDW5	Fusion of *Spe*I-digested pCDW4 and pEVS118	this study
pCR-BluntII-TOPO	PCR-product cloning vector; ColE1 *oriV*, *kanR*	Invitrogen
pDMA5	p15A *oriV*, $oriT_{RP4}$, *lacZα*, *chmR*	(44)
pES213Kn	pES213 *oriV*, *kanR*, $oriT_{RP4}$	(45)

pEVS118	R6Kγ *oriV*, *oriT*$_{RP4}$, *chmR*	(44)
pEVS122	R6Kγ *oriV*, *oriT*$_{RP4}$, *ermR*, *lacZα*	(44)
pEVS133	*fnr*-containing PCR product (primers EVS97 and EVS98, ES114 template) in pCR-BluntII-TOPO	this study
pEVS136	*fnr*-containing *Kpn*I fragment from EVS133 in same site of pEVS122	this study
pEVS141	P15A *oriV*, *oriT*$_{RP4}$, *kanR*, promoterless *chmR-gfp*	(45)
pEVS141yfiDhalf	PCR product (oligos yfid1/2F and yfid1/2R), *Sal*I and *Avr*II digested in same sites of pEVS141	this study
pEVS147	ColE1 *oriV*, *kanR*, *luxR-luxI*	(12)
pEVS150	pEVS147 *luxR-luxI*-containing *Spe*I-*Nhe*I fragment in same sites of pEVS118	this study
pJLB1	ColE1 *oriV*, *kanR*, *tmpR*	(44)
pJLB5	pJLB1 *Bst*BI/*Avr*II fragment containing *tmpR* in *Cla*I/*Avr*II-digested pEVS136	this study
pJLB6	Fusion of *Bsr*BI-digested pEVS136 and *Sma*I-digested pDMA5, Δ*Pst*I	this study
pJLB58	Self-ligated PCR product (primers, JBESMUT1 and JBESMUT2, pEVS150 template)	this study
pJLB61	Fusion of *Spe*I-digested pBluescript and pJLB58	this study
pJLB62	ColE1 *oriV*, *chmR*, *oriT*$_{RP4}$, MJ1 *luxR-luxI*	(12)
pJLB64	Self-ligated PCR product (primers JBMJMUT1 and JBMJMUT2, pJLB62 template)	this study
pJLB69	Fusion of *Xba*I-digested pCDW1 and pEVS118	this study
Oligonucleotides[b]		
141F	AAT AGC GGC CGC AGG ACA AGT TTT GGT GAC	(45)
141R	ATT ACC CGG GGC CAA CAT AGT AAG CCA G	(45)
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
JB1	GCG CTT CGA ACT CTG AGG AAG AAT TGT G	(44)
JB2	GCG CCC TAG GTT AGT TAG TTA ACC CTT TTG CCA GA	(44)
JBESMUT1	P- TAA TAA ATT CGA TCT GGG TCA CAT TTA TGC ATC TTG	this study
JBESMUT2	P- GAA TCA AAT ATC ACC AAA CCT CCA AAT TGG TG	this study
JBMJMUT1	P- CAA TTA ATT GGA TTT TTG TCA CAC TAT TGT ATC GC	this study

JBMJMUT2	P- GAA GCG ATG TCA ATC CAT TAC CGT TTT AAT G	this study
LUX1	GGG GTC TAG AGC TTT AGA AAT ACT TTG GCA GCG G	(12)
LUX2MJ1	GGA TCC GCT AGC GCG GCC GCC TTA GTA TTT AAA ATA AAT TAA TG	(12)
yfiD1/2F	GCG CGG TCG ACG TTT TTT GAC ATA TAT CAA TAT ACA AAG TAA GGG	this study
yfid1/2R	GGG CCT AGG TCA CAT TAT ATA CAT GAT ACC CTT ACT TTG TAT A	this study

[a] Drug resistance abbreviations used: *ampR*, ampicillin resistance (*bla*); *chmR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); and *tmpR* trimethoprim resistance (*dfr*).
[b] Oligonucleotide sequences are provided in the 5'-3' orientation, and "P-" indicates 5' phosphorylation.

Acknowledgements

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this dissertation was to examine the costs and benefits of bioluminescence for *V. fischeri*. My approach to this was two-pronged. My first goal was to test whether luminescence affects the fitness of *V. fischeri* in culture and in a symbiotic host, in order to establish whether any costs or benefits exist. To accomplish this goal, in Chapter 2 I confirmed the decades-old prediction that bioluminescence can inhibit the growth of *V. fischeri* in culture and confirmed that luminescence is a positive colonization factor. My second goal was to gain insight into the conditions that render luminescence beneficial by determining the environmental inputs governing *lux* gene regulation, assuming that luminescence will be maximally expressed when it is most beneficial. In Chapters 3 and 4 I demonstrated that luminescence is repressed by the redox-responsive and oxygen-sensitive transcriptional regulators ArcA and FNR, respectively. Each of these studies was consistent with the view that luminescence is used in response to oxidative conditions. In this chapter, I will discuss how these studies expand and change what is known about the regulation of luminescence in *V. fischeri*, and I will suggest directions for future research.

Previous studies of the regulation of luminescence focused primarily on the cell-density dependent regulation known as quorum sensing. An interest in how *Vibrio* rapidly increases luciferase production upon reaching a critical cell density ultimately led to the identification of three autoinducers and many components of their regulatory cascades in *V. fischeri* (Fig. 5.1,

reviewed in (206)), although the interplay of LuxI, its product 3-oxo-C6-HSL, LuxR, and the *luxICDABEG* promoter is by far the best understood aspect of quorum sensing in *V. fischeri*.

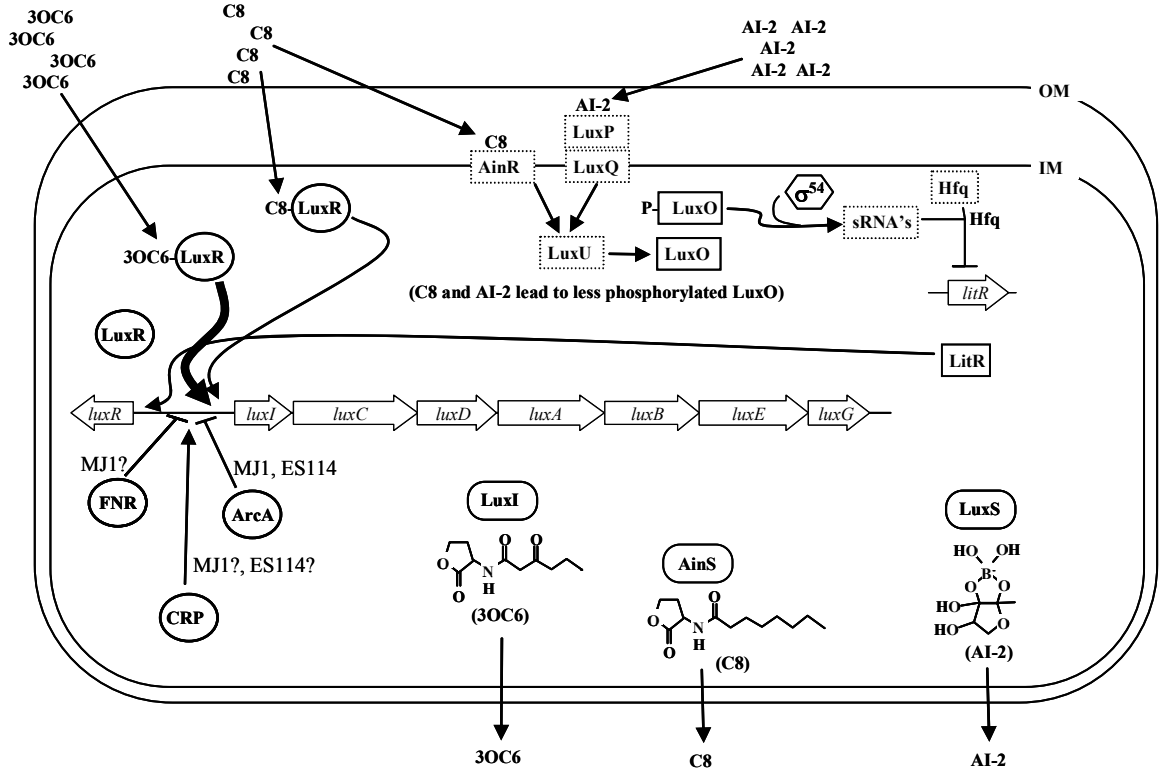

FIG. 5.1. Model of autoinducer-mediated *lux* regulation in *V. fischeri*. Elements with dashed boxes have been described in *V. harveyi* and their involvements implicated in *V. fischeri* based on the presence of homologs in the *V. fischeri* genome. The three auotinducers, *N*-3-oxo-hexanoyl-HSL, *N*-octanoyl-HSL, and presumably a furanosyl borate diester, are abbreviated as 3OC6, C8, and AI-2, respectively. Regulatory contributions of FNR and ArcA in strains MJ1 or ES114 have been added relative to Fig. 1.1. (Modified from (206)).

Regulation of luminescence in response to environmental factors such as culture aeration, glucose, and iron have also been described (62, 87, 150), although this has been less intensely studied and few regulatory mechanisms have been defined. Thus, despite keen interest in luminescence, there are significant gaps in our understanding of how *lux* is modulated in response to environmental factors. In light of these unanswered regulatory questions, and the hypothesis that luciferase benefits symbiotic cells by serving an antioxidant function, I decided

to examine the regulation of *lux* by oxygen- and redox-dependent regulators. Given their important regulatory roles in other bacteria, I focused on two global regulators, ArcA and FNR. Based on the data presented in Chapters 3 and 4, we can add ArcA and FNR to the list of known *lux* regulators (Fig. 5.1). ArcA has a profound effect on *lux* regulation, repressing luminescence by more than two orders of magnitude in both strain ES114 and strain MJ1. FNR has a more moderate effect on *lux* regulation that is only apparent in MJ1; however, both regulators serve to repress *lux* expression in response to relatively reducing conditions. Although mechanisms of *lux* regulation were previously found using the *V. fischeri lux* genes cloned in *E. coli*, these studies are the first to define how environmentally-responsive regulators alter luminescence in *V. fischeri*.

In light of my work, regulation of the *luxI* promoter is more complicated than previously appreciated. The *luxR-luxI* intergenic region is ~220 bp and includes binding sites for LuxR (35) and LitR (58), and based on analysis of the *lux* genes in *E. coli* CRP is also thought to bind in this region (18, 196). One study highlighted a putative FNR binding site that might direct FNR-mediated *lux* regulation (147), and in Chapter 4 I presented evidence that this FNR-box in P_{luxR} may direct FNR-mediated repression of luminescence in MJ1 but not in ES114. This FNR-box sequence also enhanced luminescence independent of FNR, and it is possible that this brightening of luminescence allowed us to measure an effect of FNR mediated at another site. Future work will distinguish between these possibilities. In Chapter 3 we identified ArcA-binding sites in both the MJ1 and ES114 *lux* promoters, one of which is conserved and is the major source of Arc-mediated *lux* repression. Interestingly, some of these sites may overlap each other (Fig. 5.2). For example, Arc site 2 in ES114 and the FNR-box in MJ1 both partly overlap

the predicted CRP site. Others, such as ArcA site 1 and the *lux* box are immediately adjacent to each other. The proximity of sites could lead to inhibition or synergism between regulators.

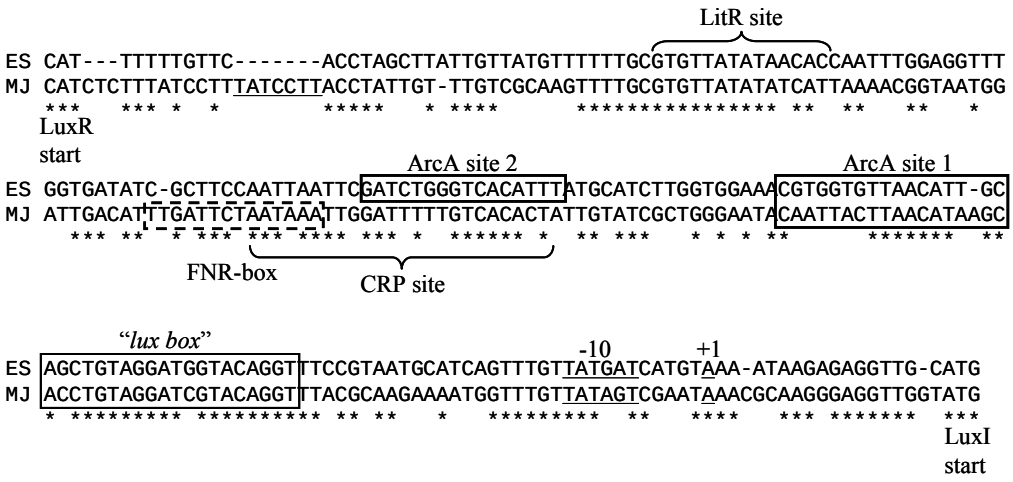

FIG. 5.2. Positions of binding sites for known and predicted *lux* regulators. Alignment of ES114 and MJ1 *luxR-luxI* intergenic, labeled ES and MJ1, respectively. Asterisks denote bp conservation in ES114 and MJ1. The underlined sequence near the LuxR start in MJ1 is found in our stock strain, but is absent from the sequence deposited in GenBank. Promoter elements and regulatory sites with solid-lined boxes have been demonstrated in *V. fischeri*. The putative CRP site is based on the CRP recognition sequence, footprint analysis, and other studies of the *V. fischeri lux* promoter in *E. coli* (18, 196). The putative LitR site is predicted based on a sequence protected during footprinting by its homolog, LuxR, in *V. harveyi* (214).

I also now question the view that there is a clear spatial distinction between the promoters controlling *luxR* and *luxI*, with LitR, CRP, and FNR binding sites in the *luxR* promoter and LuxR binding in the *luxI* promoter (Fig. 5.2). My preliminary data (not shown) suggests that the *luxI* promoter extends past the ArcA site1. Specifically, I generated a partial P_{luxI}-*gfp* reporter plasmid that extends from *luxI* up to and including ArcA site 1, but excludes further upstream *luxR-luxI* intergenic sequence. Unlike the native promoter construct on pJLB38 used in Chapter 3, no GFP production was detected from this construct compared to a promoterless *gfp*. This suggests that sequences upstream of Arc site 1 are essential for proper P_{luxI} expression. In addition, one report has suggested that CRP binding may partially inhibit LuxR activation of P_{luxI} (213). This has significant implications regarding the structure of these promoters. There may

be less distinction between the promoters of *luxR* and *luxI* than we originally thought, and perhaps this sequence has evolved such that the promoters are intimately linked. On the other hand, it is important to remember that studies of CRP-mediated *lux* activation utilized the *lux* genes on a plasmid in *E. coli* and could yield misleading information with regard to regulation in *V. fischeri*. The regulation of *lux* by CRP warrants further investigation in *V. fischeri*.

Luminescence has been predicted to benefit symbiotic cells by either consuming oxygen to prevent damage by toxic reactive oxygen species or by consuming excess reductant to maintain redox balance (15, 221, 233). I have found that the *lux* genes are repressed by both ArcA and FNR. Both of these proteins are most active in anaerobic/reduced environments, therefore *lux* expression is repressed under these relatively reducing conditions. Consistent with this, in Appendix A I demonstrate that luminescence is maximally produced under highly aerobic conditions in ES114. Assuming that luminescence is maximally produced when it is most beneficial to the cell, my data support a model whereby luminescence benefits cells by consuming oxygen and are inconsistent with the idea that luminescence acts as a sink for excess reductant. Taken together, my work and that of others shows that *V. fischeri* has evolved a complex regulatory network to ensure that this energetically expensive process is only active when needed. In nature, *V. fischeri* is brightest during its symbiotic association with the host, and luminescence is necessary for efficient colonization. We speculate that oxidative stresses in the host may in part trigger the regulatory response that induces luminescence in the light organ.

In this dissertation, I have identified one redox-responsive and one oxygen-sensitive regulator that control the expression of luminescence, ArcA and FNR, respectively. We targeted these regulators based on their ability to regulate genes in other bacteria, but there are several additional regulators known that also respond to oxygen or redox, and homologs of some of

these are found in the *V. fischeri* genome (Table 5.1). It is possible that other such regulators control *lux* expression, and this seems likely based on the data presented in Appendix A and B showing that expression of luminescence is controlled in response to aeration even in *arcA* and *fnr* mutants.

Table 5.1. Presence or absence of putative oxygen- or redox-sensitive regulators in *V. fischeri* other than ArcAB or FNR

Name of regulator	Type organism	Reference	Potential *V. fischeri* homolog
CrtJ	*Rhodobacter capslatus*	(108)	none
Fur	*Escherichia coli*	(31)	VF0810
IscR	*Escherichia coli*	(67)	VF0616
NifL	*Azotobacter vinelandii*	(132)	none
NorR	*Ralstonia eutropha*	(32)	VF1783 and VFA0862
OxyR	*Escherichia coli*	(96)	VF229 and VF1974
PpsR	*Rhodobacter sphaeroides*	(108)	none
RegBA	*Rhodobacter sphaeroides*	(54)	none
SoxRS	*Escherichia coli*	(96)	none

One candidate regulator is Fur. In *E. coli* Fur regulates proteins necessary for iron acquisition, some metabolic proteins, and superoxide dismutase (78). Fur is active as a dimer when bound to Fe^{2+} (57); however, this interaction appears to be sensitive to ROS, which inactivate Fur (230). *V. fischeri* has a Fur homolog, and a Fur-box binding sequence is known from studies in *E. coli*, but no Fur-box is apparent in the *lux* promoters.

A second protein of interest, IscR, belongs to the Mar/SoxS/Rob protein family and also appears to be present in the *V. fischeri* genome. In *E. coli*, IscR regulates proteins involved in Fe-S cluster assembly, hydrogenases, a nitrate reductase, and two proteins involved in oxidative stress response, SodA and SoxS (67, 191). Similar to FNR, IscR contains a 2Fe-2S center (191) and this site is likely the basis for oxygen sensing. Unfortunately, the mechanism of IscR-DNA

interaction is not well understood, nor has an obvious consensus IscR binding site been described, making it difficult to predict whether this regulates the *lux* genes.

V. fischeri also possesses two NorR homologs, and in *Ralstonia eutropha* and *E. coli* NorR responds to nitric oxide and regulates proteins necessary for nitric oxide detoxification (32, 202). NorR is a σ^{54}-dependent enhancer binding protein that is activated by the formation of a mono-nitrosyl complex at a mono-nuclear iron center, the generation of which is aided by NO (32, 202). There are several matches to the NorR consensus (226) in the *lux* promoter; however, since the proposed recognition sequence is GCTA(N_3)TGAC or GT(N_7)AC, partial matches are readily found in random *V. fischeri* sequences. A transposon insertion mutant in one *norR* homolog, VFA0862, was recently isolated in our lab during a search for mutants with increased luminescence. However, further analysis determined that luminescence was not appreciably increased in this mutant and therefore this potential NorR homolog has at most minor effects on *lux* regulation (Noreen Lyell, personal communication).

Finally, OxyR belongs to the LysR family of transcriptional regulators (22) and controls the expression of several proteins such as catalase that protect the cell from oxidative stress (96, 202). OxyR is activated during oxidative stress due to the generation of intramolecular disulfide bonds (164). *V. fischeri* possesses two potential *oxyR* genes, and Visick *et al*. (1998) proposed that OxyR may control catalase expression in this bacterium. However, no match to the proposed OxyR consensus binding site (222) is apparent in the *lux* promoters.

Fur, IscR, NorR, and OxyR are oxygen/redox-sensitive transcriptional regulators and are primarily involved in protecting cells from toxic reactive oxygen or nitrogen species and therefore would make good candidates for potential *lux* regulators. Although consensus binding sites are not obvious for these proteins in the *luxR-luxI* intergenic region, their influence could

extend beyond this promoter region and may work indirectly through other *lux* regulators. Testing the role of these regulators could be performed by directly mutating candidate genes and testing the luminescence of mutants. Another approach would be to identify randomly generated transposon mutants that no longer regulate luminescence in response to aeration. Through such a screen, perhaps new and novel oxygen- or redox-sensitive regulators would be identified.

In addition to the activity of such regulators, DNA topology may also influence *lux* expression. DNA negative supercoiling is increased in anaerobic *E. coli* due to decreased topoisomerase I activity (26), and changes in supercoiling are known to alter expression of many genes in *E. coli*, including the aerobic respiratory *cydAB* operon (4). Supercoiling might similarly alter the binding efficiency and activity of *lux* regulators and provide another link between anaerobiosis and *lux* repression. Recently our lab isolated topoisomerase mutants in a screen for transposon mutants with increased luminescence, suggesting that a similar type of regulation as that controlling *cydAB* may also influence *lux* expression (Erica Hall and Noreen Lyell, personal communication).

In addition to transcriptional regulation, studies of the Lux proteins in *E. coli* suggest multiple levels of post-transcriptional regulation of luminescence. One study describes early induction of luminescence in the absence of the LonA protease (227), and LuxR may need GroEL for proper folding and stability (38). However, as illustrated by our results with FNR in Chapter 4, studies in *E. coli* may not accurately represent regulation of luminescence in *V. fischeri*. Therefore the involvement of these proteins in luminescence in *V. fischeri* warrants examination. The random-transposon mutagenesis suggested above would be an ideal way to further these studies. It would be important during this screen to examine mutants that had increased luminescence and conversely, those with decreased luminescence. This would

encompass both positive and negative regulation of luminescence and therefore may be the most inclusive technique to expand our understanding of luminescence regulation.

In this dissertation I have found a previously unidentified layer of *lux* regulation. Given this discovery and the questions yet to be answered about the effects of environmental conditions on luminescence, there is no doubt that continued studies will expand our knowledge and find as yet unidentified regulators of luminescence.

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APPENDIX A

AERATION AFFECTS THE EXPRESSION OF LUMINESCENCE IN *VIBRIO FISCHERI*[1]

[1]Portions of this appendix are submitted to:
Stabb, E.V., A. Schaefer, J.L. Bose, and E.G. Ruby, Quorum Signaling and Symbiosis in the Marine Luminous Bacterium *Vibrio fischeri*, *In* S. C. Winans and B. L. Bassler (eds.), *Chemical Communication Among Microbes*. ASM Press, Washington, D.C. (Submitted 2007)

Two previous reports (8, 150) indicated that the level of aeration of *V. fischeri* cultures affects *lux* expression. First, Nealson and Hastings demonstrated that culture aeration altered luminescence of several bacteria due to changes in luciferase synthesis (150). Subsequently, Boettcher and Ruby reported that *V. fischeri* ES114 does not show such aeration-dependent regulation of luciferase expression (8). Both studies varied culture volume to limit aeration. For example, Nealson and Hastings used 15, 30, 60, 120, or 240 ml of broth in 300-ml flasks, shaken at 150 rpm to generate relatively high (15 ml media volume) to low (250 ml media volume) levels of aeration. They reported that *V. fischeri* MJ1 produced maximal luminescence under intermediate aeration, when grown in 300-ml flasks with 120 ml of media as opposed to 15, 30, 60 or 240 ml of media (150). Unfortunately, the data for these experiments are not shown in either report (8, 150), so although it appears that *V. fischeri* strains MJ1 and ES114 differ in their regulation of luminescence in response to aeration, it is difficult to compare and interpret these results.

Given these conflicting reports and the uncertainty of the data not shown, we decided to repeat these experiments to determine if aeration does affect *V. fischeri* luminescence expression and if this varies between strains. Overnight *V. fischeri* cultures grown in LBS were diluted 1:1000 into 50, 100, or 200 ml SWTO or NM broth in 250-ml flasks. These two media types were tested because NM was used previously by Nealson and Hastings (150), and because SWTO is similar to the SWT used by Boettcher and Ruby (8) but has an osmolarity close to seawater and results in higher maximal luminescence (209). At regular intervals, 500-µl samples were removed and used to measure optical density (595 nm) and luminescence. Samples were shaken vigorously to oxygenate the sample immediately prior to luminescence determination to

ensure that any differences in luminescence are not simply due to limited oxygen availability for luciferase.

Using this methodology adapted from previous studies (8, 150), I found that aeration affects expression of luminescence in both MJ1 and ES114 and that media type (NM and SWTO) did not qualitatively affect the results (Figs. A.1 and A.2). As previously reported, the response of luminescence to aeration differed significantly between MJ1 (Figs. A.1A and A.2A) and ES114 (Figs. A.1B and A.2B). In the case of MJ1, aeration had little effect on maximum luminescence (Fig. A.1A), but it did influence the cell density at which luminescence was induced. Specifically, luminescence induction and peak luminescence occurred at a lower density in the less-well aerated cultures. These data conflict with the results of Nealson and Hastings, who reported that the amplitude of luminescence differed depending on aeration (150).

ES114 demonstrated a different aeration-dependent modulation of luminescence expression. Unlike in MJ1, aeration had little effect on the cell density at which luminescence was induced in ES114, but it dramatically altered the maximum luminescence as well as the density at which luminescence peaked (Figs. A.1B and A.2B). Clearly, luminescence expression by ES114 decreased with decreasing aeration. It would appear that all ES114 cultures initiate *lux* expression at the same cell density, but as the culture grows and oxygen becomes limited, induction terminates and luminescence expression begins to decrease. This is different from the results of Boettcher and Ruby, who reported that specific activity of luminescence in ES114 is unaffected by culture aeration (8).

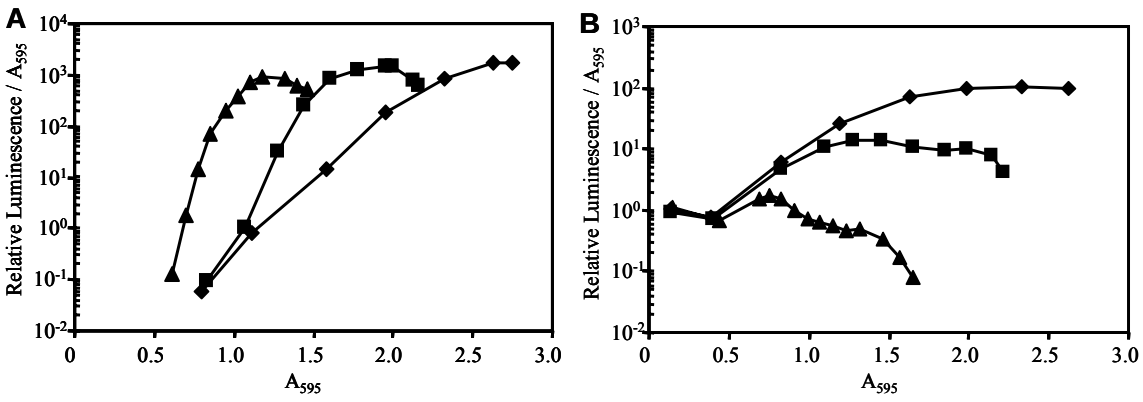

FIG. A.1. Relative luminescence per A_{595} of *V. fischeri* strains in SWTO. Luminescence of (A) MJ1 or (B) ES114 grown in 50 (diamonds), 100 (squares), or 200 (triangles) ml SWTO in 250-ml flasks at 24°C with shaking (200 rpm). At intervals, 500-µl samples were removed to determine luminescence and absorbance (A_{595}). Data represent the average (n=2).

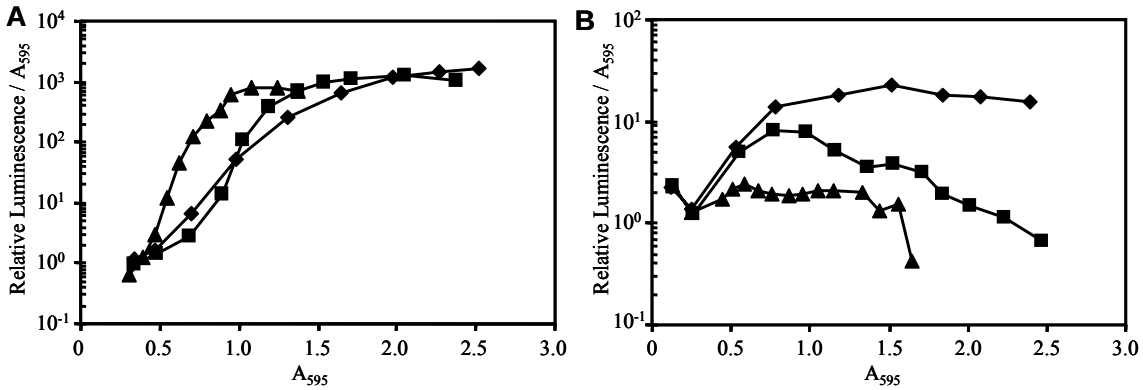

FIG. A.2. Relative luminescence per A_{595} of *V. fischeri* strains in NM broth. Luminescence of (A) MJ1 or (B) ES114 grown in 50 (diamonds), 100 (squares), or 200 (triangles) ml of NM in 250-ml flasks at 24°C with shaking (200 rpm). At intervals, 500-µl samples were removed to determine luminescence and absorbance (A_{595}). Data represent the average (n=2).

It is not clear why my data contrasts sharply with the previous reports. A possible explanation for the discrepancy between my data and the study by Nealson and Hastings is that there may be differences between our stock of strain MJ1 and the MJ1 stock they used. MJ1 was originally maintained on slants and my own data demonstrate that there are genetic and phenotypic differences between MJ1 isolates in different labs (see Appendix C). Such problems

with strain integrity are an unlikely explanation for the data concerning ES114, because the ES114 isolates used should be identical, having presumably come from the same frozen stock.

Another possible explanation for the differences between these studies is that contolling aeration by limiting head-space volume is probably somewhat shaker- or flask-dependent. Both flask shape and the diameter of shaker rotation would likely affect the movement of the media in each flask and the level of aeration. In my case, I used a long-neck Kimax 250-ml flask and a shaker with a 1 inch diameter circular orbit at 200 rpm, whereas Nealson and Hastings used 150 rpm. I do not know exactly what types of flasks or shaker were used in the earlier studies and the level of aeration achieved in my setup may not reflect those described previously.

One could argue that the discrepancies between these studies were due to slight differences between my media and the NM media used by Nealson and Hastings (150) or the SWT used by Boettcher and Ruby (8). However, I followed the recipe provided for NM and SWTO only differs from SWT in NaCl concentration. Considering that I saw similar effects in both media types, it seems unlikely that media differences resulted in the inconsistencies between my data and the previous studies.

The results above provide strong evidence that aeration affects luminescence expression of both *V. fischeri* MJ1 and ES114. Therefore, it seems plausible that luminescence is regulated by oxygen or redox-sensing regulators. Interestingly, aeration-dependent effects on luminescence are very different between these two strains, suggesting that the regulatory mechanisms used by each particular strain may be different.

Because expression of luminescence in *V. fischeri* appears to be aeration dependent, we reasoned that mutants lacking specific oxygen- and/or redox-sensitive regulators might no longer modulate luminescence expression in response to culture aeration. Therefore, we examined

mutants lacking the redox-sensitive regulator ArcA or the oxygen-sensitive regulator FNR to determine if removing one of these proteins alleviates aeration-dependent regulation of luminescence. As shown in Figure A.3A, deletion of *arcA* leads to an increase in luminescence (as discussed in Chapter 3), but this mutant still responds to culture aeration in a manner similar to the response of ES114. The role of FNR in regulating *lux* in response to culture aeration in ES114 and MJ1 is described in Chapter 4 but is also provided here briefly. My results demonstrate that FNR is relatively inactive under these microaerobic growth conditions and its effect on luminescence is undetectable (Fig. A.3B and A.3C). Taken together, these data demonstrate that the roles ArcA and FNR play in *lux* regulation, outlined in Chapters 3 and 4, are not sufficient to explain how aeration affects luminescence expression under these conditions.

In light of these data, it appears that there must be other oxygen- or redox-sensing regulators other than ArcA or FNR that control luminescence. As described in Appendix B below, one potential redox-sensitive regulator, AcnB, has been recently found to regulate the *lux* genes. Future studies paralleling those described in this appendix will need to be performed on the *acnB* mutant to delineate its contribution to aeration-dependent regulation. In addition, other regulators may also have to be examined (discussed in Chapter 5). Given the differences in how ES114 and MJ1 respond to aeration, it would not be surprising to find that different regulators contribute to altered luminescence in these strains. It would appear that luminescence is controlled by a complex oxygen- and redox-sensing regulatory system of which ArcA, FNR, and potentially AcnB are just three components.

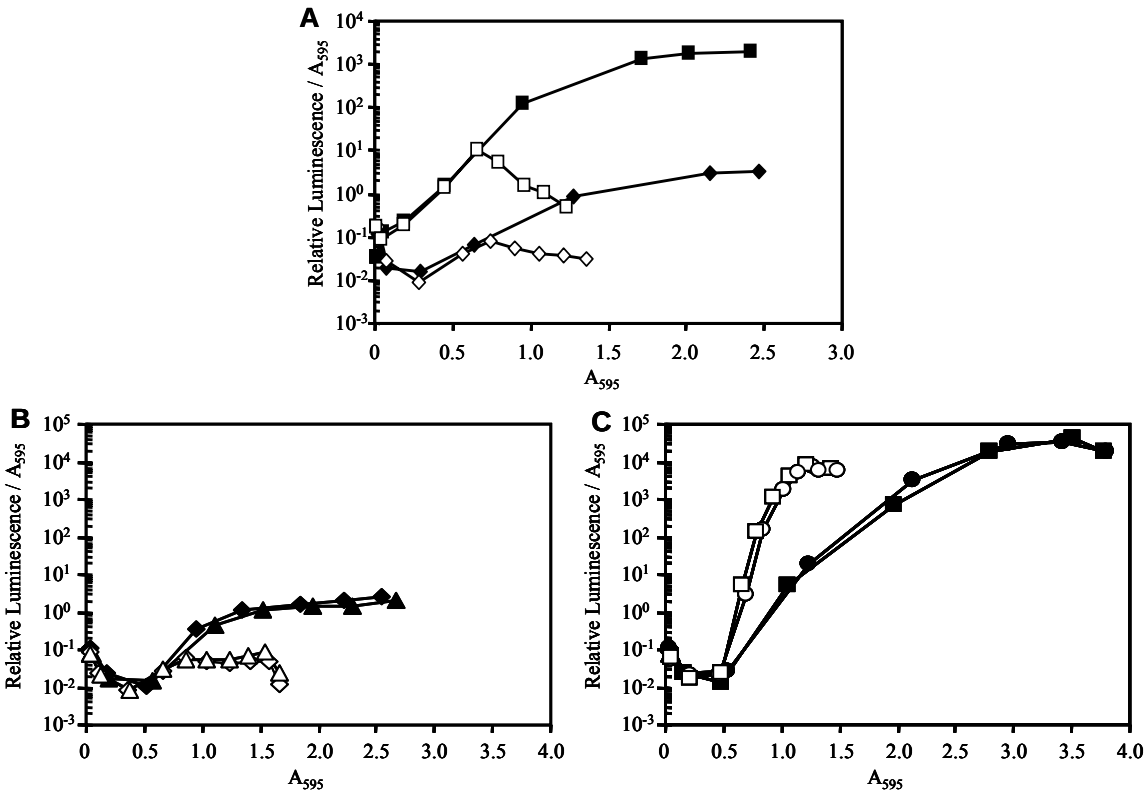

FIG. A.3. Relative luminescence per A_{595} of *V. fischeri* mutants in SWTO. Experiments were conducted in 250-ml flasks with 50 (solid symbols) or 200 (empty symbols) ml SWTO. Luminescence of (A) ES114 (diamonds) or Δ*arcA* mutant (squares), (B) ES114 (diamonds) and *fnr* mutant (triangles), or (C) MJ1 (squares) and *fnr* mutant (circles) at 24°C with shaking (200 rpm). At intervals, 500-µl samples were removed for determination of luminescence and absorbance (A_{595}). Data represent the average (n=2).

APPENDIX B

CHARACTERIZATION OF A Δ*ACNB* MUTANT OF *VIBRIO FISCHERI*

Introduction

In Chapter 3 I showed that the redox-sensing ArcAB two-component system is an important repressor of luminescence in culture for *V. fischeri* strains ES114 and MJ1. In Chapter 4, I demonstrated that the oxygen-sensing regulator FNR represses luminescence in MJ1 but not ES114. However, luminescence was regulated by aeration even in *arcA* and *fnr* mutants (Appendix A), suggesting that one or more other redox-sensing systems may also play a role in *lux* regulation.

One possible redox-sensitive regulator of luminescence is Aconitase (Acn). Acn is an important TCA cycle enzyme that interconverts citrate and isocitrate; however, it also acts as a post-transcriptional regulator. The presence of an iron-sulfur center held by three cysteine residues forms an active site that is necessary for enzymatic function (218, 240). Under conditions of oxidative stress (217, 218) or iron limitation (229), the iron-sulfur center is lost and the Acn protein can act as a post-transcriptional regulator. *V. fischeri* has a homolog (VF2158) of the *E. coli* aconitase AcnB, which like eukaryotic cytoplasmic aconitases and iron-regulatory proteins (240) binds directly to mRNA (217, 218), resulting in enhanced or lowered protein expression. Interestingly, *E. coli* possesses two aconitases, but VF2158 appears to be the only aconitase in *V. fischeri*.

In its role as a regulator, Acn stabilizes mRNA when bound to the 3' untranslated region (UTR) (217), but it can also bind the 5' UTR of mRNA leading to decreased protein expression, presumably by inhibiting translation (1). Acn acts on specific transcripts; however, little is known about the feature(s) present on mRNA that Acn recognizes. Some data suggest that aconitase binds to loop structures in mRNA, but the length and role of potential conserved nucleotides in those loops is uncertain (1, 217). Despite this lack of detailed mechanistic

understanding, Acn has been shown to regulate sporulation in *Bacillus subtilis* (1), motility in *Salmonella enterica* (219), and superoxide dismutase (220) as well as the aconitases AcnA and AcnB in *E. coli* (217).

During another study to identify repressors of luminescence, we identified a transposon insertion in the *V. fischeri acnB* gene that resulted in enhanced luminescence. Based on the redox-sensing activity of aconitase and its ability to alter gene expression post-transcriptionally in other bacteria, we considered it a possible candidate for regulating luminescence in response to oxidative stress. This led me to generate and characterize a Δ*acnB* mutant in *V. fischeri*. I examined the effect of this mutation on luminescence, *lux* expression, and host colonization.

Materials and Methods

The materials and methods used to manipulate DNA, generate mutants, culture cells, and to measure bacterial growth, luminescence and fluorescence were the same as those described in Chapter 3. The plasmids used to generate the Acn mutant are described in Table B.1. In short, the DNA upstream and downstream of *acnB* were amplified and fused such that a mutant *acnB* allele including only the start and stop codons with a *Nhe*I recognition site (5'-GCTAGC-3') between them is generated. Replacing the wild-type allele on the chromosome with this in-frame deletion by allelic exchange resulted in the Δ*acnB* mutant JB26. The presence of the Δ*acnB* allele was examined by PCR using oligonucleotides JBACNB5 and JBACNB6, which amplified the predicted 1700 bp product in JB26 as opposed to the 4300 bp fragment amplified from wild type.

Table B.1. *V. fischeri* strains, plasmids, and oligonucleotides

Strain, plasmid(s), or oligonucleotide	Relevant characteristics[a]	Source or reference
Bacterial strains		
V. fischeri		
ES114	wild-type isolate from *E. scolopes*	(8)
JB22	ES114 *lacI*^{q} $P_{A1/34}$-*luxCDABEG*	(12)
JB26	ES114 Δ*acnB* (allele exchanged from pJLB124)	this study
JB26.1	ES114 Δ*acnB*, fast-growing variant of JB26	this study
JB30	ES114 Δ*acnB lacI*^{q} $P_{A1/34}$-*luxCDABEG* (allele exchanged from pJLB101 into JB26)	this study
Plasmids		
pCR-BluntII-TOPO	PCR-product cloning vector; ColE1 *oriV*, *kanR*	Invitrogen
pEVS104	R6Kγ *oriV*, *kanR* RP4-derived conjugative plasmid	(212)
pEVS118	R6Kγ *oriV*, $oriT_{RP4}$, *chmR*	(44)
pJLB36	pVSV33 with P_{luxR}-*gfp*	(14)
pJLB38	pVSV33 with P_{luxI}-*gfp*	(14)
pJLB101	R6Kγ *oriV*, ColE1 *oriV*, $oriT_{RP4}$, *chmR*, *kanR*, *luxR luxI lacI*^{q} $P_{A1/34}$-*luxC*	(12)
pJLB121	*acnB* upstream PCR product (primers JBACNB1 and JBACNB2, ES114 template) and *acnB* downstream PCR product (primers JBACNB3 and JBACNB6, ES114 template) digested with *Nhe*I, ligated, and cloned into pCR-BluntII-TOPO	this study
pJLB124	Fusion of *Xho*I-digested pJLB121 and pEVS118; R6Kγ *oriV*, ColE1 *oriV*, $oriT_{RP4}$, *chmR*, *kanR*; Δ*acnB* allele	this study
pVSV33	pES213 *oriV*, R6Kγ *oriV*, $oriT_{RP4}$, *kanR*, promoterless *chmR-gfp*	(45)
Oligonucleotides[b]		
JBACNB1	AGT GCC GAC GAA CTC GAA GAA CAT ATT C	this study
JBACNB2	CTA GCT AGC CAC GAC TTA ATT CCT CAC ATT TTG GC	this study
JBACNB3	CTA GCT AGC TAA CCT TAT CTT CAT AAA TAC AAA AAG CAG CC	this study
JBACNB5	GCG CCA TAA GTC GTA TGT TGT TTG TTG TGG G	this study
JBACNB6	GCA TCA ACA ACC GCA GGA ACT GCC G	this study

[a] Drug resistance abbreviations used: *chmR*, chloramphenicol resistance (*cat*); and *kanR*, kanamycin resistance (*aph*).
[b] Oligonucleotide sequences are provided in the 5'-3' orientation.

Results

Generation and characterization of a Δ*acnB* mutant. During a transposon mutagenesis screen to identify repressors of bioluminescence, two independent insertions were mapped to VF2158, the ORF predicted to encode Aconitase B. To test whether the bioluminescence phenotype of these mutants was due to polar effects of the transposon insertions, I generated the in-frame Δ*acnB* mutant JB26. This mutant was 260-fold brighter than its wild-type parent ES114 (Fig. B.1A), which together with the recent complementation of an *acnB*::Tn mutant (Noreen Lyell, personal communication) confirms that loss of *acnB* causes the enhanced luminescence phenotype. Interestingly, the maximal luminescence of JB26 was higher than that of wild type, yet the cell density at which luminescence was induced was similar in the two strains (Fig. B.1A). In addition to producing more light than ES114, JB26 also has a reduced growth rate (Fig. B.1B). Although luminescence can slow growth (Chapter 2), it is unlikely that the increased luminescence of JB26 causes the decrease in its growth rate, because other strains (e.g. Δ*arcA* mutants) produce larger amounts of light with less of a growth defect or no growth defect at all at low A_{595} (see Chapters 2 and 3).

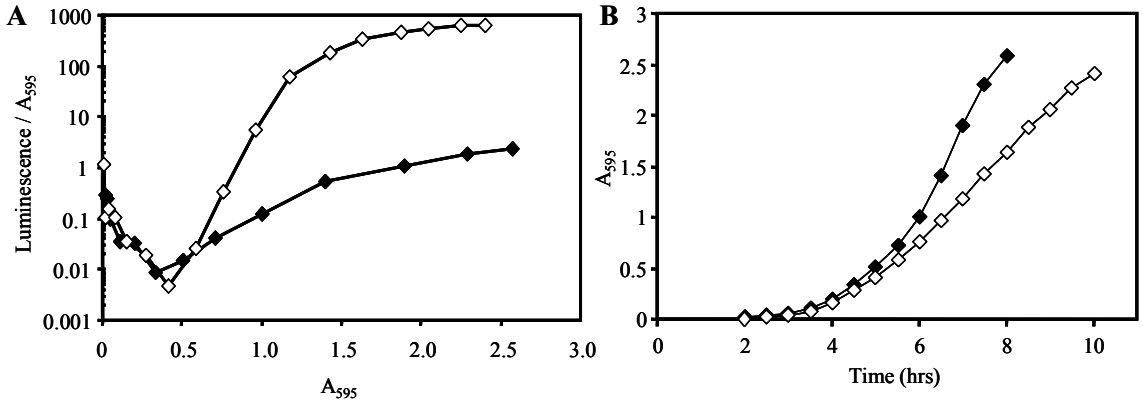

FIG. B.1. Luminescence and growth of Δ*acnB* mutant JB26. (A) Luminescence per A_{595} of ES114 (solid symbols) and Δ*acnB* mutant JB26 (empty symbols) grown in 50 mls SWTO at 24°C. Data represent the average (n=2). (B) Data plotted as culture density (A_{595}) versus time.

Expression of the *luxR* and *luxI* promoters is increased in the Δ*acnB* mutant. The brighter luminescence of Δ*acnB* mutant JB26 could be the result of more transcription from the *lux* promoters, enhanced stability and translation of the *lux* mRNA, or increased substrate availability for luciferase. To help distinguish between these possibilities, I generated strains in which the *luxCDABEG* genes responsible for producing light are disconnected from the *luxI* promoter and the *luxI* transcript, and are controlled by the IPTG-inducible promoter $P_{A1/34}$. When luminescence was expressed from the $P_{A1/34}$-*luxCDABEG* allele, deleting *acnB* had no effect on luminescence (Fig. B.2A), demonstrating that the enhanced luminescence in the absence of AcnB is dependent on the native P_{luxI} promoter and/or co-transcription of *luxI* with *luxCDABEG*. This also shows that enhanced luminescence is not due to an increase in substrates for the Lux proteins. To determine if Δ*acnB* alters *lux* regulation, I used P_{luxR}- and P_{luxI}-*gfp* reporter plasmids to examine expression from these promoters. Expression of GFP from P_{luxR} and P_{luxI} was enhanced 6- and 21-fold, respectively, in the Δ*acnB* mutant (Fig. B.2B). Because LuxR activates *luxI* expression, but LuxI does not increase *luxR* expression, a reasonable model to explain these data is that increased luminescence in the Δ*acnB* mutant results from enhanced transcription or translation of *luxR* (and P_{luxR}-*gfp*) mRNA, leading to more LuxR, which activates P_{luxI}.

Host colonization by the Δ*acnB* mutant. To determine if AcnB is important for symbiotic luminescence and colonization of the *E. scolopes* light organ, juvenile squid were exposed to ES114 or JB26, and the resulting infections were monitored by measuring luminescence and by plating animals to enumerate CFUs. Onset of luminescence was delayed in animals inoculated with JB26, although within forty hours of inoculation animals that had been infected with JB26 were as bright as ES114-infected animals (Fig. B.3).

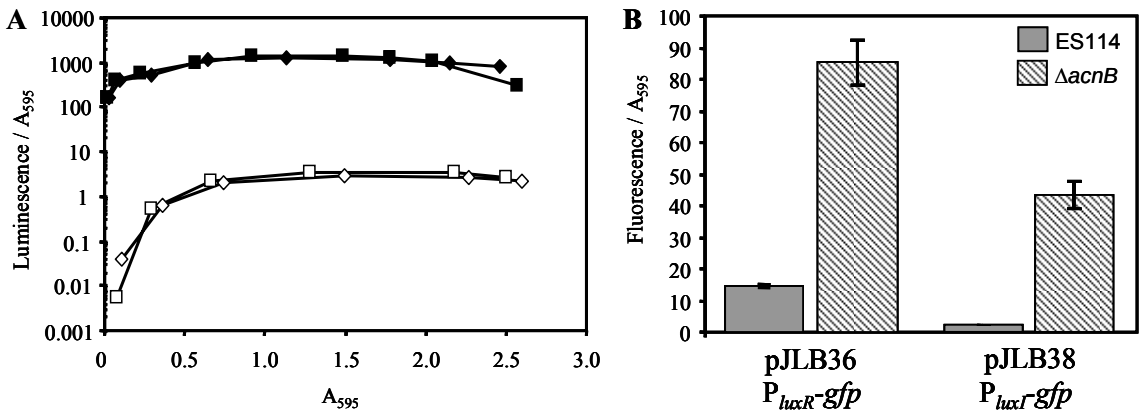

FIG. B.2. Relationship between Δ*acnB* allele, luminescence, and the native *lux* promoters. (A) Luminescence per A_{595} of *lacI*^{q}-P$_{A1/34}$-CDABEG strain JB22 (diamonds) and Δ*acnB lacI*^{q}-P$_{A1/34}$-CDABEG strain JB30 (squares) with (solid symbols) or without (open symbols) 0.5 mg ml^{-1} IPTG. (B) Specific fluorescence generated from P$_{luxR}$- or P$_{luxICDABEG}$-*gfp* reporter plasmids pJLB36 and pJLB38, respectively, harbored in ES114 (gray bars) or its Δ*acnB* mutant JB26 (hatched bars). Data represent the average specific fluorescence (between A_{595} of 2.0 and 2.7) with standard error (n=9 to 16).

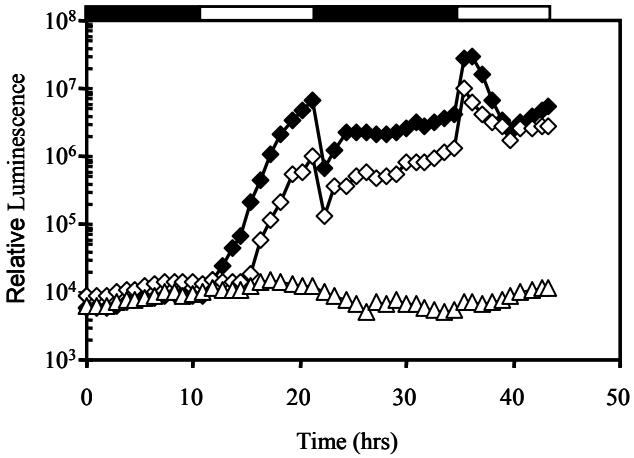

FIG. B.3. Symbiotic establishment of Δ*acnB* mutant. Symbiotic luminescence in *E. scolopes* hatchlings inoculated with ES114 (solid diamonds), Δ*acnB* mutant JB26 (empty diamonds), or no *V. fischeri* (open triangles). Data represent the mean (n=12). Black and white boxes represent periods of night and day, respectively.

This delay in the onset of luminescence may indicate that AcnB is necessary in the initiation of colonization or it may be the result of the decreased growth rate of this strain. However, in a similar but separate experiment, only 4/15 squid inoculated with the Δ*acnB* mutant ever achieved detectable luminescence, whereas 15/15 animals inoculated with ES114 became detectably

luminescent (data not shown), suggesting that JB26 has a defect in initiating the symbiosis and is not simply a slow-growing colonist. In the course of these experiments, I isolated a spontaneous mutant of JB26 with wild-type-like growth and luminescence. Upon completion of the experiment shown in Figure B.3, squid were homogenized and dilution plated to determine colonization levels, and platings from the JB26-inoculated animals had both small and large colonies similar to colonies of JB26 and ES114, respectively. One large colony, isolate JB26.1, had growth rate (not shown) and luminescence restored to levels comparable to ES114 (Fig. B.4). PCR of the *acnB* locus in JB26.1 identified a 1700 bp product, consistent with Δ*acnB* as opposed to the wild-type 4300 bp fragment, confirming that JB26.1 has the Δ*acnB* allele and therefore is a spontaneous mutant that has acquired a secondary mutation that suppresses the growth and luminescence phenotypes caused by the Δ*acnB* allele. This further suggests that AcnB is important for colonization of the host and that there is selective pressure favoring strains with compensatory suppressor mutations in the Δ*acnB* mutant that restore the ability to colonize the light organ.

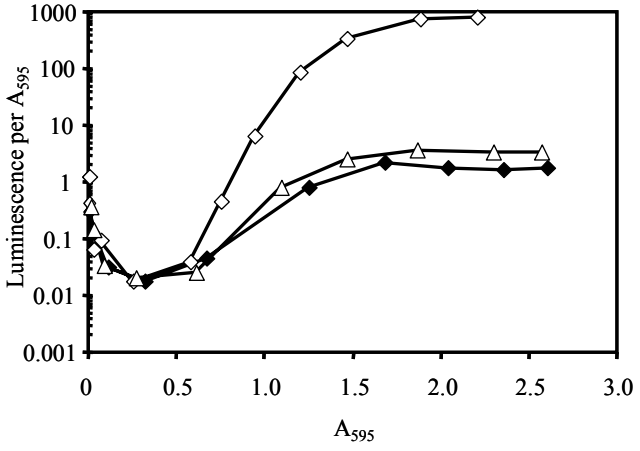

FIG. B.4. Luminescence per A_{595} of suppressor mutant JB26.1. Luminescence of ES114 (solid diamond), Δ*acnB* mutant JB26 (empty diamonds), and mutant JB26.1 (empty triangle) grown in 50 mls SWTO at 24°C.

Discussion

In this appendix, I showed that loss of AcnB, a TCA-cycle enzyme and post-transcriptional regulator in other bacteria, leads to an increase in luminescence in *V. fischeri* ES114. Increased luminescence in the Δ*acnB* mutant was not due to an increased availability of substrates for luciferase, but it did coincide with increased expression of P_{luxR}-*gfp* and P_{luxI}-*gfp* reporters.

Several scenarios could explain how AcnB might act as a post-transcriptional regulator to repress luminescence. AcnB could bind to the 5' end of either the *luxICDABEG* or *luxR* transcripts and prevent translation of the Lux structural proteins or the LuxR activator, respectively. In other systems, AcnB appears to repress translation by binding to a stem loop in the 5' UTR of the mRNA. The untranslated 5' portion of the *luxICDABEG* mRNA only contains 17 nucleotides, and it has no apparent secondary structure making this transcript an unlikely target for AcnB. This UTR could potentially create a stem loop with part of the *luxI* transcript, or perhaps AcnB recognizes secondary structure within the translated portion of *luxI*. With so little known about how AcnB specifically targets transcripts, it is difficult to rule out *luxICDABEG* as a potential target for AcnB regulation by sequence analysis. However, increased expression of *luxICDABEG* would not be expected to have a major effect on the *luxR* promoter or P_{luxR}-*gfp*, such as I observed. I therefore believe it is unlikely that AcnB regulates luminescence by acting directly on the *luxI* UTR.

It seems more probable that AcnB suppresses luminescence by binding to the 5' end of the *luxR* mRNA, and that enhanced luminescence in the *acnB* mutant is the result of increased LuxR and therefore LuxR-mediated activation of *luxICDABEG*. This is supported by the finding that P_{luxR}-*gfp* expression is increased six-fold in the Δ*acnB* mutant (Fig. B.2). Increased LuxR

activator levels would be predicted to result in increased P_{luxI}-*gfp* expression, as I observed. Unfortunately, the transcriptional start site for *luxR* has not been determined, and attempts to do so using the *V. fischeri lux* genes in *E. coli* were not conclusive (195), so it is difficult to predict how AcnB might act on this transcript directly.

It is also possible that AcnB exerts post-transcriptional control of luminescence indirectly by affecting a regulator that controls LuxR expression. In this case, AcnB may suppress production of *lux*-activators such as LitR binding the 5' end of their mRNA, suppressing translation. Conversely AcnB may increase the production of repressors such as LuxO by binding the 3' end of their transcript leading to mRNA stabilization and enhanced translation. Future studies will be necessary to determine if AcnB regulates luminescence through any of these post-transcriptional mechanisms.

Alternately, the effect of AcnB may be directly related to its metabolic and enzymatic function. Increased luminescence is not the result of increased substrates for luciferase (Fig. B2A); however, AcnB plays an important role in the TCA cycle, converting citrate to isocitrate, and disruption of this step might lead to a shift in the redox state of the cell. Such a change in cell physiology could affect activation of other redox regulators, such as ArcA, that regulate luminescence. This model may explain the phenotypes of the suppressor mutant JB26.1, which despite having the Δ*acnB* allele has regained wild-type luminescence and growth. It seems plausible that one or more secondary mutations in JB26.1 could lead to an increase in the levels or efficiency of an enzyme that allows the cell to bypass or complete the TCA-cycle step that AcnB performs. One such potential enzymatic substitute for AcnB is the two-subunit 3-isopropylmalate isomerase encoded by VF0292 (*leuD*) and VF0293 (*leuC*), which carries out a similar reaction as aconitase by interconverting 3-isopropylmalate and 2-isopropylmalate in

leucine biosynthesis (249). Perhaps if LeuCD is altered or overexpressed, it can provide enough aconitase activity to restore growth and luminescence to normal levels.

Although it seems less likely, we cannot rule out the possibility that a secondary mutation might provide a replacement for the specific post-transcriptional regulatory function of AcnB. The heterodimeric LeuCD protein complex and AcnB have similar domains and since the regulatory elements of AcnB have not been determined, it may be possible for LeuCD to have a similar, yet previously unknown, regulatory function. Perhaps a mutation can confer regulatory activity on LeuCD. Either scenario would be an unexpected but exciting finding.

The examination of other mutants will help to address whether the cause of increased luminescence in Δ*acnB* strains is due to enzymatic activity or post-transcriptional regulation. For example, a variant of AcnB in *E. coli* that lacks the cysteines necessary for iron-sulfur center formation is catalytically inactive, yet still binds mRNA (218). A similar mutant could be made in *V. fischeri* and tested for increased luminescence, and this would help determine if it is the regulatory or enzymatic function of AcnB that alters luminescence.

V. fischeri luminescence has been long studied as a model for quorum sensing and for host-microbe interactions. Here I identify a previously unknown effect of AcnB on luminescence. If luminescence is enhanced due to the regulatory function of AcnB, this would be the first identification in *V. fischeri* of post-transcriptional regulation of the *lux* genes. Alternatively, future studies of AcnB could find a previously unknown link between regulation of luminescence and metabolism if the effect of AcnB on luminescence is due to its enzymatic function. Regardless, studies of this protein and its regulation of luminescence will expand our knowledge of an interesting multi-functional protein and add new aspects to our understanding of luminescence regulation.

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APPENDIX C

ANALYSIS OF THE *LUXR-LUXI* INTERGENIC SEQUENCE AND LUMINESCENCE IN A DIVERSE COLLECTION OF *VIBRIO FISCHERI* STRAINS

For reasons that are not well understood, the brightness of different wild-type *V. fischeri* strains can vary greatly (8, 148). For example, a comparison of strains MJ1 and ES114 demonstrated that MJ1 has ~10,000-fold higher luminescence (8). Interestingly, saturating ES114 with exogenously added autoinducer will not increase the luminescence of ES114 to the levels of MJ1 (see Chapter 2), and ES114 produces little endogenous autoinducer even when *luxICDABEG* is fully induced by exogenously added autoinducer (72). These data demonstrate that different strains appear to produce not only varying levels of luminescence, but also have different luminescence capacities. Despite these intriguing observations, little work has gone into understanding why there is such strain to strain variation in luminescence. The explanation typically offered is that some strains are brighter because they produce more 3-oxo-C6-HSL autoinducer (8, 72). However, this model arguably invokes a circular explanation, because the 3-oxo-C6-HSL synthase gene is co-transcribed with the *luxCDABEG* genes responsible for light production. A more useful model would be one describing a mechanism that leads to higher *luxICDABEG* expression, simultaneously resulting in more 3-oxo-C6-HSL from LuxI and more luminescence from the other Lux poteins. Several plausible mechanisms could accomplish this, and one simple model is that the strength of the promoter upstream of *luxI* determines how much light and 3-oxo-C6-HSL a given strain produces.

In this study I sought to examine the luminescence of *V. fischeri* isolates from several sources, and to determine if there is a correlation between the sequence of the *lux* promoters in these strains and the amount of light produced. To examine this, I chose a collection of *V. fischeri* isolates from fish such as *Cleidopus glomarius* and *Monocentris japonica*, squid of both the *Euprymna* and *Sepiola* genera, as well as several planktonic isolates (Table C.1). I included two MJ1 isolates, because I found sequence and phenotypic differences between our stock of

MJ1, originally obtained from Dr. Edward Ruby, and the MJ1 from the collection of Dr. E. Peter Greenberg, which I designated as MJ1PG. I started by examining strains grown on SWTO solid media at 24^{o}C to classify each strain as producing non-visible (NV), visible (V), or highly-visible (HV) luminescence (Table C.1). These studies rely on human perception of luminescence intensity and therefore the strains were examined independently by six individuals to obtain a consensus evaluation of light output. Quantitative assessments of light output are described below.

In order to compare luminescence levels to *lux* promoter sequences, I cloned and sequenced the *luxR-luxI* intergenic region from each of these strains. This information was used to generate a neighbor-joined tree to determine relatedness, and this tree was rooted with the *luxR-luxI* intergenic sequence from *V. salmonicida* (154). The *luxR-luxI* intergenic sequences of these strains were aligned using MUSCLE (50) and viewed using Jalview 2.1 (23). The sequences were then converted using ReadSeq and the tree generated using PAUP* 4.0b10 (Sinauer Associates, Inc., Sunderland, Mass.). The resulting tree was viewed using TreeView and is shown in Figure C.1. Maximum-likelihood analysis resulted in similar tree topology (data not shown).

Examination of the tree shows two distinct clades but three interesting groupings. Not surprisingly, almost all of the isolates from the Hawaiian bobtailed squid, *Euprymna scolopes*, group together with the Hawaiian planktonic isolate PP3, which was previously shown to resemble isolates from *E. scolopes*. A second grouping includes isolates ET401, EM17, ET101 from other *Euprymna* species found in Japan or Australia, as well as two Hawaiian isolates, VLS2 and H905 that are unusual for that area and not proficient colonizers of *E. scolopes* (119). Interestingly, this second grouping includes all but one V-class strain. The third grouping, which

forms a distinct clade from the others, includes all the fish isolates, *Sepiola* squid isolates, and the planktonic strain WH1. This group encompasses all the strains classified as HV. Thus, the strains broke down into three groups, and luminescence levels were relatively consistent within each group.

Table C.1. *V. fischeri* strains and plasmids used in this study

Strain	Source	Reference	Luminescence Class[a]	Intergenic *lux* clone[b]	P_{luxR}-*lacZ* reporter[c,d]	P_{luxI}-*lacZ* reporter[c,d]
CG101	*Cleidopus gloriamaris*	(176)	HV	pJLB156	pJLB180	pJLB181
CG103	*Cleidopus gloriamaris*	(117)	HV	pJLB26	pJLB180	pJLB181
EM17	*Euprymna morsei*	(176)	V	pJLB165	pJLB196	pJLB197
ES114	*Euprymna scolopes*	(8)	NV	pJLB29	pJLB170	pJLB171
ES12	*Euprymna scolopes*	(10)	NV	pJLB161	pJLB188	pJLB189
ES213	*Euprymna scolopes*	(10)	NV	pJLB166	pJLB188	pJLB189
ES401	*Euprymna scolopes*	(176)	NV	pJLB167	pJLB198	pJLB199
ET101	*Euprymna tasmanica*	(156)	V	pJLB163	pJLB192	pJLB193
ET401	*Euprymna tasmanica*	(155)	V	pJLB164	pJLB194	pJLB195
H905	Hawaii planktonic	(119)	V	pJLB168	pJLB200	pJLB201
MJ1	*Moncentris japonica*	(180)	HV	pJLB30	pJLB172	pJLB173
MJ11	*Moncentris japonica*	(176)	HV	pJLB157	pJLB172	pJLB173
MJ1PG	*Moncentris japonica*	(180)	HV	pJLB79	pJLB204	pJLB205
PP3	Hawaii planktonic	(119)	V	pJLB25	pJLB202	pJLB203
SA1	*Sepiola affinis*	(60)	HV	pJLB159	pJLB184	pJLB185
SR5	*Sepiola robusta*	(60)	HV	pJLB162	pJLB190	pJLB191
VLS2	*Euprymna scolopes*	(117)	V	pJLB158	pJLB182	pJLB183
WH1	Mass. planktonic	(117)	HV	pJLB160	pJLB186	pJLB187

[a] Strains were designated as producing non-visible (NV), visible (V), or highly visible (HV) luminescence on SWTO plates based on the consensus of observations by six people.
[b] *lux* intergenic regions were PCR amplified using primers EVS109 and EVS110 (45) and inserted into pCR-Blunt (pJLB25-pJLB30) or pCR-BluntII-TOPO (pJLB79-pJLB168) for sequencing.
[c] The *Avr*II fragment containing *luxR-luxI* intergenic region from the intergenic *lux* plasmid was cloned into the *Avr*II site of pAKD702 (13) to generate reporters.
[d] Some strains had identical *luxR-luxI* intergenic sequences and only one P_{luxR}- and P_{luxI}-*lacZ* reporter plasmid was made for each unique sequence, therefore the same reporter plasmid may represent more than one strain.

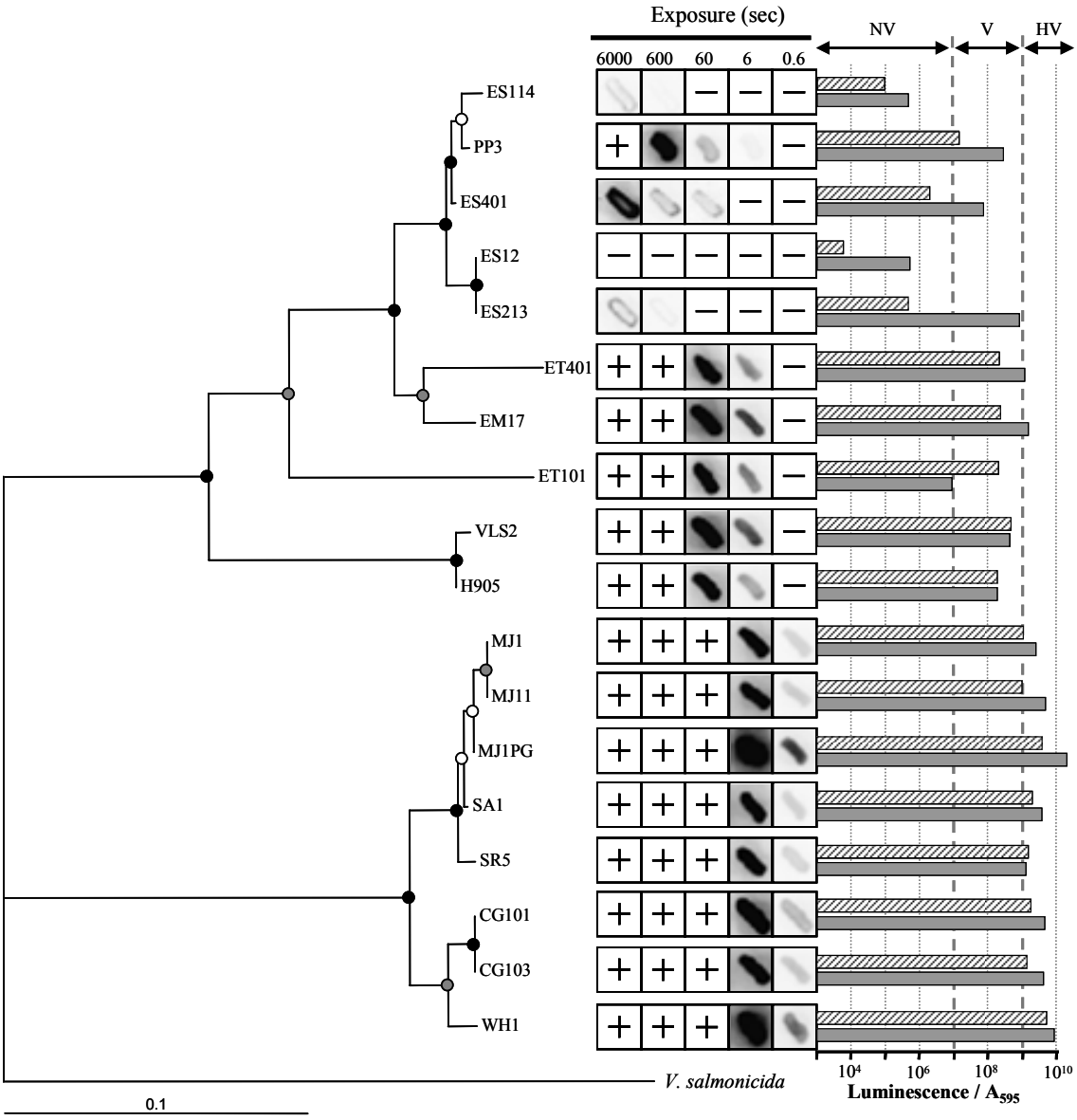

FIG. C.1. Relatedness of the *luxR-luxI* intergenic sequence in *V. fischeri* isolates. The tree was generated using the neighbor-joining method with 10,000 bootstrap replicates. Black, grey, and white circles represent branch points supported by bootstrap values of >90%, >70%, and >50%, respectively. Branches without circles are unresolved (bootstrap values of <50%). The tree was rooted with the *luxI-luxR* intergenic region of *Vibrio salmonicida* (GenBank accession number AF452135). The bar represents 0.1 substitutions per site. Patches were grown overnight on SWTO at 24°C and the negative image for given exposures are shown. A "+" or "-" indicates that luminescence was too high or low to see clearly, respectively. Horizontal bars display the luminescence per A_{595} of each strain grown on plates (hatched bars, n=3) or in broth (solid bars). Dashed vertical lines indicate approximate cutoffs distinguishing strains that produce non-visible (NV), visible (V), or highly-visible (HV) luminescence on SWTO plates.

We next examined the luminescence of these strains on solid media. Colonies were patched onto SWTO plates and luminescence imaged following incubation overnight at 24°C. To quantify luminescence triplicate patches were incubated at 24°C for 18 hrs, re-suspended separately in Instant Ocean, and optical density (A_{595}) and luminescence were measured for each suspension. Not surprising, there was a strong correlation with luminescence intensity on solid media and luminescence classification (Fig. C.1). It appears that the amount of light produced necessary to be in the V classification falls somewhere between the bioluminescence produced by PP3 (V class) and that of ES401 (NV class). This can be seen in the comparison of the patches of these two strains and corresponds to ~10^7 relative luminescence units per A_{595}. The HL class of strains produced a minimum of ~2.5-fold higher luminescence than the V class on solid media.

Luminescence of the strains was also examined in broth culture. Overnight cultures were diluted 1:500 in SWTO media and incubated at 24°C. Growth and luminescence were monitored as described in Chapter 3 and maximal luminescence of each strain is reported in Figure C.1. Generally, luminescence was higher in broth culture than when cells were grown on solid media; however, VLS2, H905, and SR5 produced similar luminescence under both conditions, and ET101, had higher luminescence on solid media than in broth.

Several other interesting observations are apparent in Figure C.1. ES12 and ES213 have identical *luxR*-*luxI* intergenic regions, yet ES213 produces 100-fold and 1000-fold more light on plates and in broth, respectively. Thus two strains having the same *luxR* and *luxICDABEG* promoters can make very different amounts of light. This may be due altered levels of other *lux*-regulating proteins, post-transcriptional regulation, or perhaps differences in cell physiology leading to altered luminescence.

One surprising observation from this analysis is that the MJ1 strains from our lab and from Dr. E. Peter Greenberg's lab have different *luxR-luxI* intergenic sequences, and the latter (MJ1PG) produces more light than our MJ1. Our strain is identical in *lux* promoter sequence to the more recently isolated MJ11, and contains a 7-bp repeat just upstream of the translational start of LuxR. MJ1PG is missing this 7-bp sequence, which may account for the differences in luminescence between these strains.

Together, the analysis of the luminescence produced by these eighteen strains demonstrates that luminescence can differ by as much as ~100,000-fold between different isolates. In addition, the strains express luminescence differently depending on whether they are grown in liquid or on solid media. Importantly, my results also suggest that strains with related *lux* promoter sequences produce similar amounts of luminescence. This is consistent with the model that increased luminescence is due to higher activity of the *luxICDABEG* promoter; however, it could result from a stronger *luxR* promoter yielding increased levels of the LuxR activator, or it could be that differences in the *lux* intergenic sequences are unrelated to the strength of either promoter and simply reflect phylogeny and genetic drift.

To begin distinguishing between these possibilities, I constructed P_{luxI}-*lacZ* and P_{luxR}-*lacZ* reporter plasmids for each strain (Table C.1). Is expression more closely associated with strain background or with specific promoters? Have the *lux* promoters evolved to express maximally in their native strains? Does P_{luxI}-*lacZ* or P_{luxR}-*lacZ* expression, or both, correlate with luminescence intensity? Does PGMJ1 produce greater amounts of *luxR* transcript and therefore more light than MJ1 due to the 7-bp repeat found in MJ1? The construction of these plasmids will enable us to address such questions in future studies.

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