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Effects of *luxCDABEG* induction in *Vibrio fischeri*: Enhancement of symbiotic colonization and conditional attenuation of growth in culture

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Abstract

Production of bioluminescence theoretically represents a cost, energetic or otherwise, that could slow *Vibrio fischeri* growth; however, bioluminescence is also thought to enable full symbiotic colonization of the *Euprymna scolopes* light organ by *V. fischeri*. Previous tests of these models have proven inconclusive, partly because they compared nonisogenic strains, or undefined and/or pleiotropic mutants. To test the influence of the bioluminescence-producing *lux* operon on growth and symbiotic competence, we generated dark Δ*luxCDABEG* mutants in strains MJ1 and ES114 without disrupting the *luxR-luxI* regulatory circuit. The MJ1 Δ*luxCDABEG* mutant out-competed its visibly luminescent parent ~26% per generation in a carbon-limited chemostat. Similarly, induction of luminescence in the otherwise dim ES114 strain slowed growth relative to Δ*luxCDABEG* mutants. 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 an 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 *E. scolopes*. These results demonstrate that induction of *luxCDABEG* can slow *V. fischeri* growth under certain culture conditions and is a positive symbiotic colonization factor.

Keywords

Photobacterium; *Aliivibrio*; autoinduction; competition; evolution

Introduction

Vibrio fischeri serves as a model system for studies of both bioluminescence and symbiotic bacteria-animal interactions. This bacterium's bioluminescence has been examined since the late 19th century (Harvey 1952), whereas studies of *V. fischeri*'s symbiotic interactions have gained recent momentum from the ability to reconstitute its symbiosis with the Hawaiian bobtail squid *Euprymna scolopes* in the laboratory (Wei and Young 1989; Stabb 2006). *V. fischeri*'s bioluminescence and symbiotic interactions are intimately interrelated. For example, the most obvious symbiotic role of *V. fischeri* is to generate bioluminescence in

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specialized light emitting organs of hosts, and bioluminescence is induced upon colonization – an effect that is particularly pronounced in symbionts of *E. scolopes* (Boettcher and Ruby 1990; Boettcher and Ruby 1995). Most interestingly, Visick et al. (2000) reported that bioluminescence acts as a colonization factor, and that without it *V. fischeri* does not fully colonize *E. scolopes*. Unfortunately, the parent strain used in that study was subsequently found to be attenuated in colonization (McCann et al. 2003), and we have now shown that the mutation yielding the dark phenotype also caused unexpected and negative pleiotropic effects. Thus, this role of bioluminescence as a symbiosis-specific colonization factor could be questioned.

If bioluminescence does enable *V. fischeri* to fully colonize the host, this would be especially interesting juxtaposed the seemingly paradoxical belief that bioluminescence has a negative impact on cells. This is a controversial topic with a long history. 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 (Harvey 1952)). 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 (Keynan and Hastings 1961; Hastings and Nealson 1977; Dunlap et al. 1995; Czyz et al. 2003; Pooley et al. 2004). On the other hand, one especially thorough study detected no effect of luminescence on ATP pools or growth (Karl and Nealson 1980). 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.

Although this topic remains unresolved, hypotheses predicting positive or negative effects of bioluminescence on *V. fischeri* can draw on a firm understanding of the biochemistry and genetics underlying this process (Hastings and Nealson 1977; Meighen 1994; Tu and Mager 1995). Bioluminescence 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 (Boylan et al. 1989), consuming additional reductant and hydrolyzing ATP, while LuxG and other flavin reductases regenerate $FMNH_2$ (Zenno and Saigo 1994; Lin et al. 1998). The genes encoding these Lux proteins are co-transcribed with *luxI*, and the *luxICDABEG* operon is adjacent to and divergently transcribed from *luxR* (Engebrecht et al. 1983; Gray and Greenberg 1992b; Gray and Greenberg 1992a). Together LuxI and LuxR form a "quorum sensing" regulatory circuit that induces bioluminescence at high cell density (Fuqua et al. 1994).

In light of this detailed mechanistic understanding, any potential functional advantages of bioluminescence for light-producing bacteria must be weighed against apparently significant energetic costs (Stabb 2005). 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. 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 latter mechanism, which amounts to competition between luminescence and energy-generating respiratory pathways, has gained support from the observation that luminescence consumes a significant (>10%) share of the oxygen reduced in *V. fischeri* cells (Makemson 1986). As discussed below, hypotheses explaining potential symbiotic advantages of producing bioluminescence have also drawn on our understanding of the biochemistry underlying this process.

Our goal in this study 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 the report by Visick et al. (2000) 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 1. Plasmids were maintained in *Escherichia coli* strain DH5α, except plasmids containing the R6Kγ origin of replication, which were maintained in CC118λ*pir* or DH5αλ*pir*. *E. coli* was grown at 37°C in LB medium (Miller 1992) or Brain Heart Infusion (BHI) (Becton, Dickinson, and Co, Sparks Maryland). *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 (Rosson and Nealson 1981)), which contained 940 ml Instant Ocean, 50 mM Tris (pH 7.5), 19 mM NH_4Cl, 0.03% glycerol, 7 μM $FeSO_4$-$7H_2O$, 570 μM K_2HPO_4, and 10 mg yeast extract per liter. In most experiments we used SWTO, because we previously found that luminescence of *V. fischeri* was enhanced by its seawater-like osmolarity (Stabb et al. 2004). 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-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-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.

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 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 1. We used previously described *luxR::ermR-lacI*^{q}-P$_{tac}$::*luxI*- and Δ*luxA::ermR* alleles on pKV44 (Visick and Ruby 1996) and pKV17 (Visick et al. 2000), 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 (Stabb and Ruby 2002). 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 (*lacIq*-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 1.

Growth, OD_{595}, 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°C with shaking (200 rpm). 500-μl samples were taken from two or three parallel cultures at regular intervals and optical density at 595 nm (OD_{595}) was determined on these undiluted samples with a BioPhotometer (Brinkman Instruments, Westbury, NY). The OD_{595} of undiluted samples diverges somewhat from a linear relationship with cell density, leading to an underestimation of cell density that becomes more pronounced at higher OD_{595} (e.g. OD_{595} >1). For example, an undiluted culture with an OD_{595} of 2.0 when diluted and measured in the linear range has an actual OD_{595} of ~2.4. We compared the OD_{595} of cultures grown in SWTO at 24°C before and after ten-fold dilution, and found that the relationship between measured OD_{595} of an undiluted culture (X) and the OD_{595} calculated from diluted culture (Y) can be described as $Y=0.042X^3 + 0.02X^2 + X$ ($r^2=0.98$). We used this conversion to calculate the OD_{595} values presented (and those used in growth rate calculation) from values taken on undiluted samples. Strain comparisons are not affected by this conversion, nor are any of our conclusions altered.

We calculated growth rate (min per OD_{595} doubling) by plotting log(OD_{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. Each luminescence value was determined by integration over a 10 sec measurement, and kinetic analyses of a dense (OD_{595}=3) bright culture of strain MJ1 for several consecutive 1 sec measurements indicated that there was no significant decay in luminescence over a >10 sec period. Moreover, diluting this culture sample ten fold results in a ~ten-fold reduction in the luminescence reading, further suggesting that high density samples do not become self-limiting by consuming oxygen over the course of these measurements.

Mixed-strain competitions

To determine the relative competitiveness of EVS103 (Δ*luxCDABEG*) in batch co-culture with its parent MJ1, we used methodology similar to that described previously (Stabb and Ruby 2003; Dunn et al. 2006; Hussa et al. 2007). The two strains were incubated at 24°C with shaking (200 rpm) in 10 ml of SWTO in 125-ml flasks until reaching an OD_{595} of ~2.4. 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 OD_{595} of ~2.4, and plated again. This procedure of dilution and re-growth to starting OD_{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 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 OD_{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 OD_{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 OD_{595} and luminescence. The steady-state OD_{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 (Table 1). We confirmed that carbon (glycerol) availability was limiting growth in this experiment by increasing glycerol concentration, resulting in faster growth until a higher cell density was reached.

Relative competitiveness of ES114 and EVS102 during colonization of the squid was assessed essentially as described previously (Visick et al. 2000; Fidopiastis et al. 2002; Lupp et al. 2002; McCann et al. 2003; Millikan and Ruby 2004; Dunn et al. 2006; Bose et al. 2007; Hussa et al. 2007). RCI values were 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 (Dunn et al. 2006) 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 (Ruby and Asato 1993; Stabb and Ruby 2003) and overnight exposures of squid to *V. fischeri*. Inoculant strains were pre-grown unshaken in 5 ml of SWT in 50-ml conical tubes at 28°C such that the OD_{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. Within each experiment, similar concentrations of mutant or wild-type cells were present in the respective inocula. 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. In the case of competition experiments plated on LBS with X-Gal, colonies were scored for their blue or white color to determine strain ratios (see mixed strain competitions above).

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 (Boettcher and Ruby 1990). Like most isolates from *E. scolopes*, ES114 is relatively dim in culture (Lee and Ruby 1994) and produces little 3-oxo-C6-HSL autoinducer (Boettcher and Ruby 1990), which stimulates luminescence by activating the quorum sensing transcriptional regulator LuxR. Supplementing media with 3-oxo-C6-HSL induced maximal light production ~1500-fold (Fig. 2a and Table 2) and also slowed the growth of ES114 (Fig. 2b), reflected in longer doubling times (Fig. 2c). This phenomenon was not specific to growth in SWTO at 24 °C because it also occurred when cells were grown in LBS at 28°C (Fig. 2d).

Interestingly, in both media types the increase in doubling time was most evident when culture OD_{595} was between 0.5 and 2.4, and was not seen when OD_{595} was <0.5 (Fig. 2c and 2d). This indicates that luminescence was not growth-limiting when cultures were below 0.5 OD_{595}; however, it is important to note that specific luminescence also did not reach its maximum until culture OD_{595} was >1 (Fig. 2a). Although 3-oxo-C6-HSL was present throughout growth, quorum-sensing inhibitors present in complex media must be metabolized before luminescence is induced (Eberhard 1972), which is consistent with this rise in specific luminescence. In experiments described below with strains JB22 and EVS101, IPTG induced luminescence from the beginning of growth, yet luminescence-dependent attenuation of growth rate was similarly dependent on the growth stage of the culture (i.e. OD_{595}>0.5), so the lag in luminescence induction seen in Figure 2 may not be related to the observation that luminescence-dependent growth attenuation only became evident after cultures reached an OD_{595}>0.5.

It is intriguing that Figure 2 shows an increase in doubling time associated with 3-oxo-C6-HSL addition and brighter luminescence. This is consistent with the idea that the process of producing luminescence can slow growth; however, 3-oxo-C6-HSL also activates LuxR to stimulate non-*lux* genes (Callahan and Dunlap 2000), 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 (Visick and Ruby 1996; Visick et al. 2000), but this was crossed into ESR1 (Graf et al. 1994), which is a spontaneous rifampicin-resistant mutant of strain ES114. Rifampicin-resistant mutants often have pleiotropic phenotypes (Yanofsky and Horn 1981; Jin and Gross 1989; Blanc-Potard et al. 1995; Björkman et al. 1998) and ESR1 has notable differences from the wild type, including a reduced ability to colonize *E. scolopes* (McCann et al. 2003). We found that ESR1 has about one tenth the specific luminescence of ES114 when grown in SWTO (Table 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.

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. 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. Given this uncertainty, we believe that the influence of the *luxA::ermR* allele on growth of *V. fischeri* is not interpretable, and we sought alternative genetic approaches so that we would not have to base any conclusions about the importance of bioluminescence on this pleiotropic mutant.

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. We generated such strains using as parent strains either ES114 or the visibly bright *V. fischeri* strain MJ1, an isolate from the light organ of a pinecone fish, *Monocentris japonica* (Ruby and Nealson 1976). The genotypes of these mutants are illustrated in Figure 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.

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). When induced by IPTG, EVS101 also displayed longer doubling times when the culture was between OD_{595} of 0.5 and 1.5 (Fig. 4a). Similarly, IPTG induced the luminescence of JB22 (*lacI*^{q} $P_{A1/34}$-*luxCDABEG*) ~460-fold and slowed growth when the OD_{595} was >0.5 (Fig. 4b). Upon induction with IPTG, JB22 was ~13-fold brighter than EVS101 (Table 2) and also suffered a more dramatic growth rate reduction (Fig. 4). IPTG did not induce luminescence in control strain JB23 (*lacI*^{q} $P_{A1/34}$-Δ*lux*) or ES114 (Table 2) and did not slow growth of these strains (Fig. 4b).

Interestingly, neither EVS101 nor JB22 displayed a growth defect when culture OD_{595} was <0.5, even though the IPTG-induced P_{tac} and $P_{A1/34}$ promoters drove expression of

bioluminescence at such low cell densities (Fig 4c). This observation that luminescence only affects growth above a certain culture OD_{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.

We considered the possibility that oxygen might be replete at low OD_{595} but growth-limiting once OD_{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 OD_{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 OD_{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 OD_{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 OD_{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. 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. 5). Thus, the reduced growth rate of ES114 in the presence of 3-oxo-C6-HSL (Fig. 2) can be attributed to induction of *luxCDABEG*.

We next compared Δ*luxCDABEG* mutant EVS103 to its visibly luminescent wild-type parent MJ1. Despite the bright luminescence of MJ1 (Table 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. 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. 6b).

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 (Visick et al. 2000); 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 (McCann et al. 2003), 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. 3), raising the possibility that the symbiotic deficiency associated with this mutation reflected a general attenuation and not a symbiosis-specific defect.

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 48h after inoculation (Fig. 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. 7b). This competitive defect was significant ($p<0.01$), and a 95% confidence interval for the data presented in Figure 7B indicates an RCI between 0.2 and 0.5, which encompasses the RCI observed by Visick *et al.* (2000). Taken together, our data support the conclusion that bioluminescence contributes to the symbiotic competence of *V. fischeri*.

Discussion

By generating and characterizing *V. fischeri* mutants wherein the *luxCDABEG* genes are deleted or specifically inducible, we have demonstrated two apparently antithetical effects of bioluminescence induction in *V. fischeri*. Using a fully colonization-proficient wild-type strain, we have shown that bioluminescence contributes to the ability of *V. fischeri* to colonize *E. scolopes* (Fig 7). This confirms an earlier report by Visick et al. (2000) that reached a similar conclusion but used a symbiotically attenuated parent strain and a deleteriously pleiotropic *luxA::ermR* mutation. We also show that the poor colonization by the Δ*luxCDABEG* mutant is symbiosis-specific and cannot be attributed to a general attenuation associated with the loss of luminescence. On the contrary, we demonstrate that in culture luminescence can slow the growth of *V. fischeri* (Figs. 4 and 5) and put luminescent cells at a competitive disadvantage when mixed with a Δ*luxCDABEG* mutant (Fig. 6). This is consistent with decades-old speculation that the energetic commitment to bioluminescence, encompassing both the synthesis and enzymatic activity of Lux proteins, might represent an appreciable drag on culture-grown cells (reviewed in (Ziegler and Baldwin 1981)), although it may alternatively (or in addition) reflect a negative impact of luminescence on cells independent of energetic considerations.

Negative effect of luminescence on growth in culture

Our demonstration that expression of *luxCDABEG* can slow growth and our measurements of this effect are consistent with previous theoretical estimates of the energy devoted to bioluminescence in relation to the energy economy of the cell (Hastings and Nealson 1977; Karl and Nealson 1980; Tu and Mager 1995), although as discussed below such comparisons must be viewed cautiously. Hastings and Nealson (1977) 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 ~6×10^5 ATP hydrolyzed sec^{-1} $cell^{-1}$, with even more energy required if luciferase sometimes consumes substrates without producing light, as is likely the case. Theoretically, there is less, but still substantial, energy devoted to Lux protein synthesis, which was estimated for luciferase as equivalent to ~1×10^5 ATP hydrolyzed sec^{-1} $cell^{-1}$ in bright cells (Karl and Nealson 1980). For comparison, theoretical calculations suggest active *E. coli* cells hydrolyze at least 2×10^6 ATP sec^{-1} $cell^{-1}$ (Peterson and Moller 2000), and experimentally determined values for *E. coli* were ~5×10^6 ATP hydrolyzed sec^{-1} $cell^{-1}$ (Holms et al. 1972; Neidhardt et al. 1990). Based on these estimates, the energy devoted to bioluminescence could be equivalent to ≥10% of the ATP turnover in bright cells. Karl and Nealson (1980) similarly estimated that the bioluminescence of strain MJ1 could account for 1% to 10% of cellular energy, and noted that the real energetic costs could be higher when considering the energy committed to Lux protein synthesis and inefficiency of luciferase. Our results were in line with these calculations, as we found that bright strain MJ1 was out-competed 5.5–26% per generation by a Δ*luxCDABEG* mutant (Fig. 6).

While such theoretical estimates may bring a useful perspective to the data, the fraction of cellular energy devoted to luminescence provides at best an overly simplified view of far more complex biology, and at worst it can be misleading. Similarly, it is tempting to look for a direct relationship between bioluminescence output and the extent of growth inhibition, but such a relationship, if it exists, should not be expected to hold across different growth conditions or even strain backgrounds. A key issue in this regard is that the ability of cells to generate energy will not be growth-limiting under all conditions, and if the longstanding model is correct that the energy committed to luminescence is responsible for luminescence-dependent growth inhibition, there may be no growth inhibition when energy is not growth limiting. So, for example, one might question why strain EVS101 has the same luminescence output when OD_{595} is 0.25 or 1.0, yet luminescence only inhibits growth in the latter instance. A simple explanation consistent with the model described above is that cell growth is limited by different parameters at these different growth stages. If something other than the ability to generate energy, for example translation, limits growth at low optical density (where growth rates are highest), this could account for the observation that ES114, EVS101, and JB22 did not display any luminescence-dependent growth inhibition at OD_{595} <0.5. This point highlights a limitation of the current study: most of the experiments were performed in batch culture where the factor limiting growth rate is an ever-moving target. At best one might expect bioluminescence output and the extent of growth inhibition to correlate for near-isogenic strains at the same growth phase in batch culture under the same conditions, and indeed it does appear to hold true that at OD_{595} of 1.0 in SWTO the relative luminescence output of ES114, EVS101, and JB22 (Table 2) corresponds with the

relative magnitude of luminescence-dependent growth inhibition in these strains (Figs. 4 and 5). One might expect given the much brighter luminescence of strain MJ1 (Table 2) that it would suffer a greater luminescence-dependent growth inhibition than ES114; however, MJ1 and its derivatives grew more slowly than ES114 and its derivatives (data not shown), so again the factors governing growth may be different making such comparisons invalid.

With these caveats in mind, our data do seem consistent with the hypothesis that the energy committed to luminescence is responsible for luminescence-dependent growth inhibition. Specifically, the growth inhibition by luminescence was more pronounced under low nutrient conditions as would be expected if luminescence were draining resources away from ATP generation. For example, no luminescence-dependent growth inhibition was seen during growth in fresh rich media, but it became evident once the medium was partially spent. Even more strikingly Δ*luxCDABEG* mutant EVS103 had a greater co-culture growth advantage in a carbon-limited chemostat than it did in the rich medium SWTO.

It remains possible that *luxCDABEG* expression imposes some burden on cells apart from the loss of energy that is reflected in emitted photons and Lux protein synthesis, and such an alternative mechanism may be responsible for the slowing of growth that we have observed when bioluminescence is induced. For example, production of reactive oxygen species by luciferase or other stresses associated with expression of the *lux* operon might limit growth. However, microarray comparisons of mRNA from ES114 and the Δ*luxCDABEG* do not reveal clear differences reflective of a specific identifiable stress (e.g., relative induction of oxidative stress responses) (E.G. Ruby, personal communication). Perhaps a more plausible alternative model is that growth slows not because of the energy devoted to luminescence per se, but rather because luminescence disrupts the physiological balance that cells must maintain while growing and generating energy. This might be expected in particular in instances where luminescence was artificially induced (e.g. in strains EVS101 and JB22), because in these circumstances luminescence might be imposed on cells that under natural induction conditions would modify their physiology to accommodate the luminescence process but do not do so under these artificial induction conditions.

We also cannot rule out the possibility that altogether different mechanisms account for the competitiveness of dark mutants over wild type in mixed cultures. Some of our most striking data show that a Δ*luxCDABEG* mutant can out-compete its wild-type parent in a chemostat by as much as 26% (Fig. 6), similar to previous reports that spontaneous dark mutants can take over chemostat cultures of bioluminescent bacteria (Pooley et al. 2004). 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 (Ruby and Nealson 1977)) 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, 4, and 5), not just in co-culture (Fig. 6).

Although Karl and Nealson (1980) did not detect a growth rate reduction attributable to bioluminescence, there are several possible explanations for the discrepancy between their results and ours. For example, competition studies such as those shown in Figure 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. Indeed, as discussed above, we found that the effect of *lux* expression on growth was conditional. Another potential reason that Karl and Nealson did not see an effect of bioluminescence on growth is that 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.

Bioluminescence as a colonization factor: potential benefits of expressing *luxICDABEG*

Our demonstration that bioluminescence enhances the ability of wild-type *V. fischeri* to colonize *E. scolopes* is intriguing, especially in light of the neutral or negative effects of bioluminescence that we have now documented in culture. Strains of many host-associated *Vibrio* species produce bioluminescence, raising the possibility that bioluminescence acts as a colonization factor in other host-microbe interactions as well. For example, it was recently reported that a *Vibrio salmonicida* mutant harboring a suicide-plasmid insertion in *luxA* was attenuated in the rate at which it caused mortality in Atlantic salmon (Nelson et al. 2007); however, our finding that a *luxA* insertion mutant of *V. fischeri* has unexpected pleiotropic effects might indicate that the effect on pathogenesis seen with *V. salmonicida* is due to similar unexpected factors. In the future, deletion mutants cleanly lacking the full complement of *lux* bioluminescence genes will be useful in determining whether *lux* plays a role in other bacteria-host interactions.

In the case of *V. fischeri*, the properties of the *E. scolopes* light organ that render bioluminescence useful remain to be determined and warrant further investigation. Several hypotheses offer explanations for how bioluminescence might be advantageous (for a review see (Stabb 2005)). One model proposed that luminescence might substitute for cytochrome-dependent respiration under conditions where this system cannot operate (Makemson and Hastings 1986), such as iron limitation, which might be experienced in some host tissues. However, an essential prediction of this model was that energy would be recovered during the reduction of FMN from the NADH pool, but it now seems clear that neither LuxG nor the other enzymes catalyzing this step are tied to ATP generation or Na^+/H^+ pumping (Duane and Hastings 1975; Watanabe and Hastings 1982; Lei et al. 1994; Lin et al. 1998). Other hypotheses suggest that luminescence may counteract oxidative stress imposed by the host (Visick et al. 2000), or it may act as a sink to consume excess reductant (Bourgois et al. 2001). 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. Our recent finding that luminescence in *V. fischeri* is repressed under more reducing conditions by the ArcAB system is more consistent with the notion that luminescence is used as a response to oxidative stress (Bose et al. 2007).

Other hypotheses propose a central role for light itself. In one such hypothesis, it was proposed that luminescence could stimulate photolyase-mediated DNA repair (Czyz et al. 2000; Czyz et al. 2003; Kozakiewicz et al. 2005); however, we have demonstrated that this is not the mechanism by which luminescence benefits symbiotic *V. fischeri* cells in the *E. scolopes* light organ (Walker et al. 2006). It has also been proposed that the host may detect symbiotic bioluminescence, perhaps using cryptochromes, and impose some sanctions against dark infections (M. McFall-Ngai and C. Whistler, personal communication). Sanctions could be imposed by withholding nutrients or by increasing the production of antimicrobial peptides or reactive oxygen species, which the squid produce (Weis et al. 1996; Davidson et al. 2004). This model has evolutionary appeal, in that it provides a mechanism to assure that the host receives benefits from the symbionts.

Some of these hypotheses attempting to explain the symbiotic benefit of bioluminescence can be tested, in part, by examining the relative growth of bright cells and Δ*luxCDABEG* mutants under specifically manipulated culture conditions. While it is clear that in this study culture conditions do not reflect those in the host environment, in the future, culture conditions wherein luminescence attenuates growth may ultimately serve to elucidate the how bioluminescence helps symbionts fully colonize the host.

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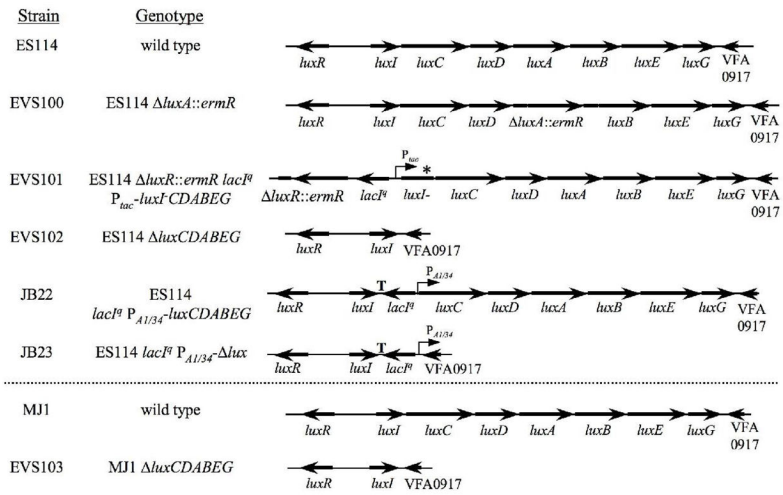

Fig. 1.

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.

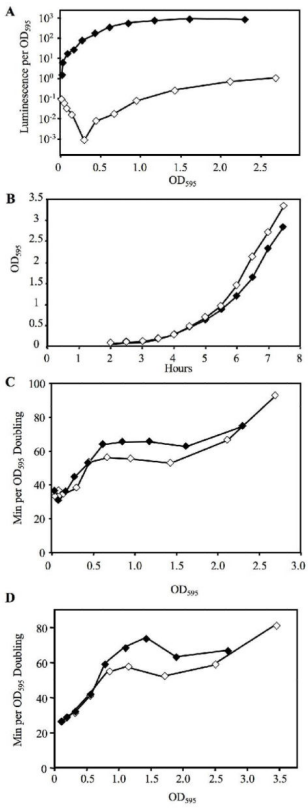

Fig. 2.

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 1 μM 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 OD_{595} (panel **c**). The doubling time as a function of OD_{595} is also reported for cells grown in LBS at 28°C (panel **d**).

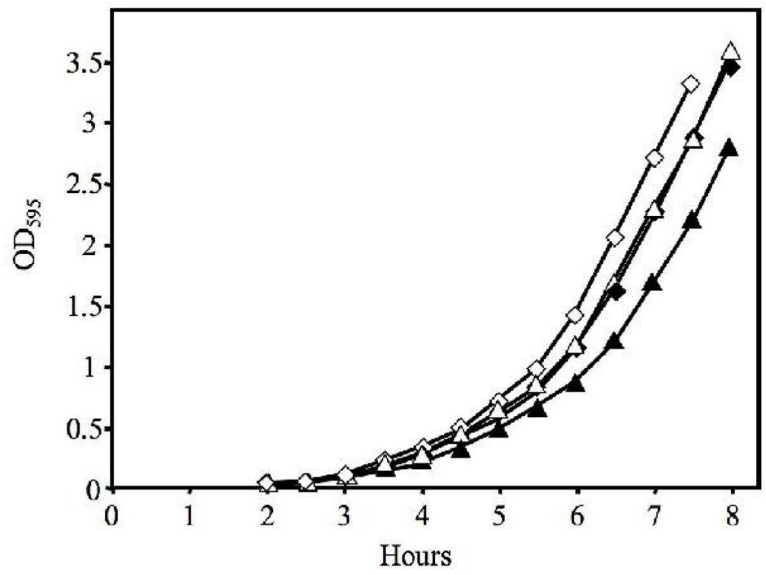

Fig. 3.

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 1 μM 3-oxo-C6-HSL.

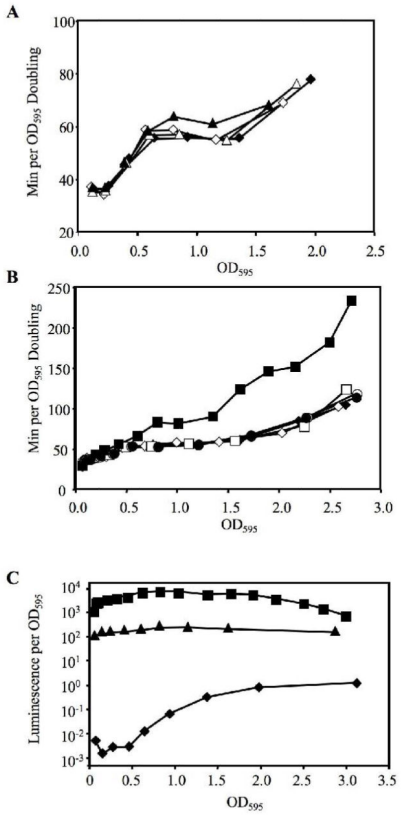

Fig. 4.

Specific induction of bioluminescence affects growth of strains EVS101 and JB22. Doubling time is reported as a function of culture OD_{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). **c** Specific luminescence of ES114 (diamonds), EVS101 (triangles), and JB22 (squares).

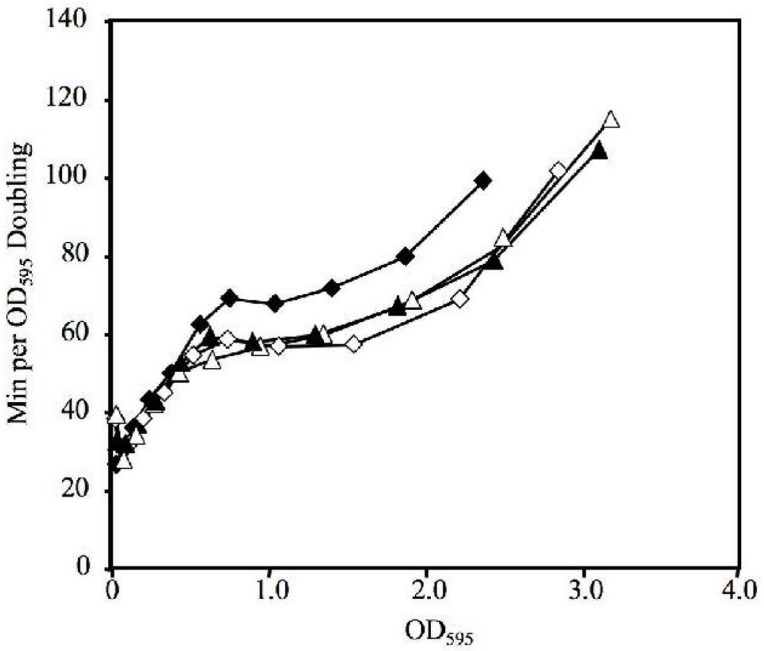

Fig. 5.

3-oxo-C6-HSL slows growth of ES114 but not Δ*luxCDABEG* mutant. Doubling time is reported as a function of culture OD_{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 1 μM 3-oxo-C6-HSL.

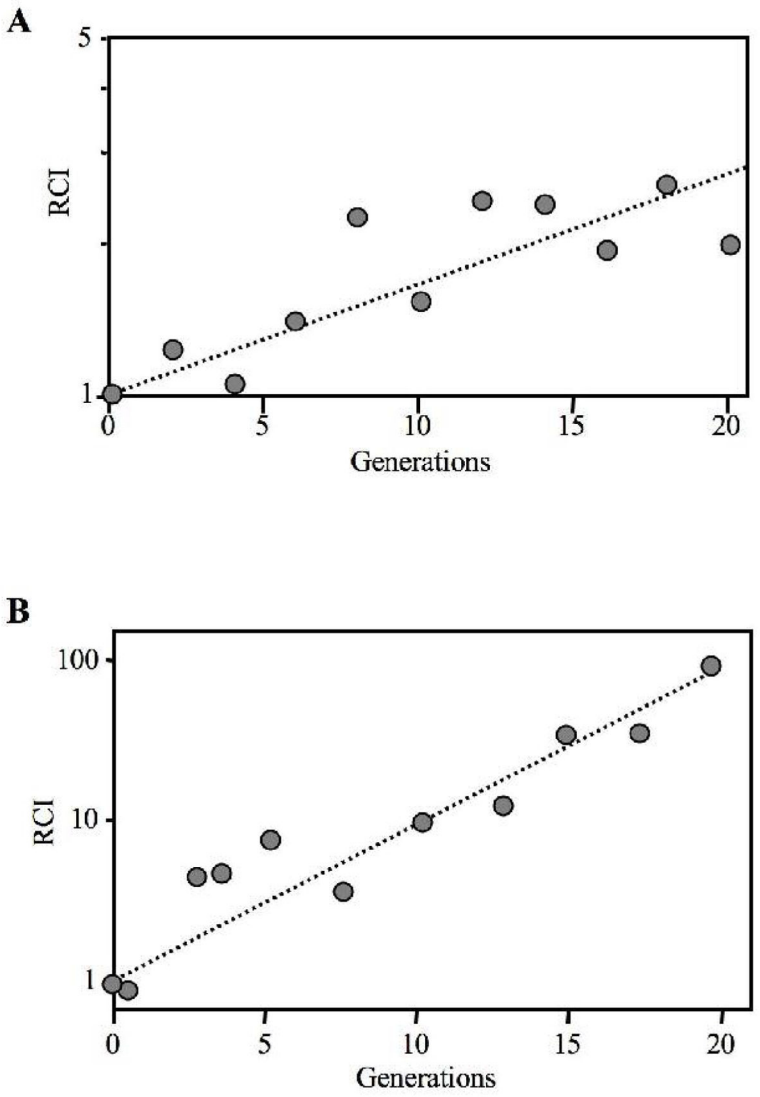

Fig. 6.

Δ*luxCDABEG* mutant EVS103 out-competes wild-type parent MJ1. In each panel data is from one representative experiment of three. **a** Relative competitiveness (RCI) of EVS103 (Δ*luxCDABEG*) co-cultured with MJ1 in SWTO at 24°C. The dotted line follows the best fit of the data (RCI=1.05 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). 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.

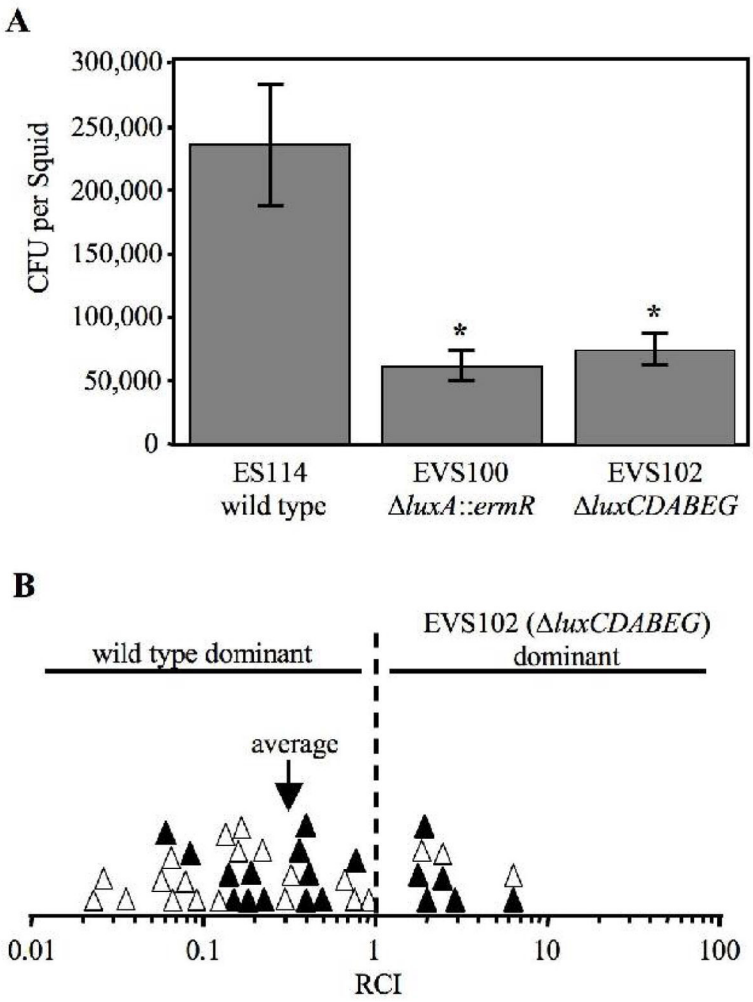

Fig. 7.

Symbiotic colonization of *E. scolopes* by *lux* mutants and wild type. **a** Average colonization levels 48h 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 ($p<0.01$).

Table 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*	(Boettcher and Ruby 1990)
ESR1	spontaneous *rifR* derivative of ES114	(Graf et al. 1994)
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)	(Stabb et al. 2004)
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*	(Ruby and Nealson 1976)
Plasmids		
pCR-BluntII-TOPO	PCR-product cloning vector; ColE1 *oriV, kanR*	Invitrogen
pEVS79	ColE1 *oriV*, $oriT_{RP4}$, *chmR*	(Stabb and Ruby 2002)
pEVS94S	R6Kγ *oriV*, $oriT_{RP4}$, *ermR*	(Stabb and Ruby 2002)
pEVS104	R6Kγ *oriV, kanR* RP4-derived conjugative plasmid	(Stabb and Ruby 2002)
pEVS118	R6Kγ *oriV*, $oriT_{RP4}$, *chmR*	(Dunn et al. 2005)
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*	(Dunn et al. 2006)
pEVS143	*lacI*^{q}, P_{tac} *Xho*I cassette (Diederich et al. 1994) 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*H I 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

Strain, plasmid(s), or oligonucleotide	Relevant characteristics[a]	Source or reference
pKV44	ColE1 *oriV*, *ampR*, *luxR::ermR-lacI*^{q}-P$_{tac}$::*luxI*$^{-}$ and flanking sequence from ES114	(Visick et al. 2000)
pKV17	ColE1 *oriV*, *ampR*, Δ*luxA::ermR* and flanking sequence from ES114	(Visick and Ruby 1996)
pVSV3	pES213 *oriV*, *kanR*, *lacZ* (full), *oriT*$_{RP4}$	(Dunn et al. 2006)
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] Derived from the phage T7 A1 promoter by placing *lac* operators at the transcriptional start and between −10 and −35 elements, the P*A1/34* promoter is a hybrid of P*A1/03* and P*A1/04* promoters (Lanzer and Bujard 1988).

[c] Oligonucleotide sequences are provided in the 5′-3′ orientation.

Table 2

Relative luminescence *V. fischeri* of strains

Strain	Condition[a]	Luminescence relative to ES114[b]	Photons/sec per cell[c]
ES114		1	3.3
ES114	in squid	860[d]	2800
ES114	+ 3-oxo-C6-HSL	1500	5000
ES114	+ IPTG	1	3.3
ESR1		0.11	0.36
EVS101		17	56
EVS101	+ IPTG	340	1100
JB22		9.4	31
JB22	+ IPTG	4300	14000
MJ1		13000	43000
MJ1	C-limited chemostat	7000[e]	23000

[a] Except as noted, strains were grown in SWTO medium at 24°C.

[b] Maximum specific luminescence (luminescence/OD_{595}) relative to ES114.

[c] Maximum luminescence converted to photons/sec per cell based on the maximal luminescence of ES114 (3.3 photons/sec per cell) reported in similar conditions (Boettcher and Ruby 1990).

[d] 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.

[e] Specific luminescence in BGMYE medium during continuous culture at OD_{595} 0.1 ± 0.05.