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Symbiotic Characterization of *Vibrio fischeri* ES114 Mutants That Display Enhanced Luminescence in Culture

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***Vibrio fischeri* ES114 is a bioluminescent symbiont of the squid *Euprymna scolopes*. Like most isolates from *E. scolopes*, ES114 produces only dim luminescence outside the host, even in dense cultures. We previously identified mutants with brighter luminescence, and here we report their symbiotic phenotypes, providing insights into the host environment.**

Vibrio fischeri is a bioluminescent symbiont that colonizes the light-emitting organs of certain marine animals, including the Hawaiian bobtail squid *Euprymna scolopes* (1–3). *V. fischeri* strain ES114 is a commonly used wild type that was isolated from the light organ of an *E. scolopes* squid (4). Like most isolates from *E. scolopes*, ES114 displays dim luminescence in culture (4–6), despite being similar to bright strains with regard to the genetic arrangement of the *luxCDABEG* genes, which are responsible for bioluminescence, and the *luxI* and *luxR* genes, which underpin pheromone-mediated regulation of luminescence (7, 8). ES114's bioluminescence is a colonization factor (9, 10), and it is induced over 1,000-fold upon symbiotic infection (4, 11).

We previously probed the basis for the relative repression of the *luxICDABEG* operon in culture by screening transposon insertion mutants of ES114 for brighter luminescence on solid media (12), using an efficient mini-Tn*5* mutagenesis system (13). In that study, we identified luminescence-up mutants with transposon insertions in 14 loci (Table 1), including *arcA*, *arcB*, *acnB*, *topA*, *lonA*, *pstA*, *pstC*, *hns*, $tRNA^{Met}$, $tRNA^{Thr}$, *tfoY*, *phoQ*, *guaB*, and *ainS* (12). The *acnB* mutants gave rise to suppressors and proved difficult to examine; however, characterizing the remaining mutants gave insight into the mechanisms of *lux* regulation and the environmental parameters that influence *lux* expression, as we discussed previously (12).

In this study, we examined symbiotic phenotypes of these previously described luminescence-up mutants in the *E. scolopes* light organ. Specifically, we determined (i) colonization competence, (ii) the proficiency of the mutants at competing with ES114 for infection in mixed inocula, and (iii) whether a luminescence-up phenotype relative to ES114 was apparent in the symbiosis.

To determine the strains' colonization proficiency, aposymbiotic *E. scolopes* hatchlings were inoculated with *V. fischeri* as described previously (14, 15). To generate inocula, cultures were grown to an OD_{595} between 0.4 and 0.7 in 5 ml of SWTO medium (16) within 50-ml conical tubes at 28°C without shaking. Based on their optical densities at 595 nm (OD_{595}), cultures were diluted to ~2,000 CFU ml^{-1} in Instant Ocean (Aquarium Systems, Mentor, Ohio), and inocula were plated onto LBS medium (17). Aposymbiotic hatchlings were then placed in 5-ml inocula in 20-ml vials for 12 to 14 h before being rinsed in Instant Ocean. The number of CFU in the inoculum was determined by plate counts after incubation at 28°C. Although inocula ranged from 1,000 to 3,000 CFU ml^{-1}, within each experiment considered here, squid were exposed to similar concentrations of mutant or wild-type cells in the respective inocula. Luminescence of each squid was measured by integrating the light output over 9 s, after a 9-s delay, using an LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). One luminescence reading per squid was taken at each time point. Infected squid were homogenized at 24 or 48 h postinoculation, then serially diluted, and plated onto LBS medium (17), and the number of CFU per squid was determined.

We similarly mixed mutants 1:1 with ES114 to test their ability to compete for colonization of the host. A previous calculation suggested that under these conditions, at 48 h postinoculation symbionts have undergone no more than 30 generations in the host, assuming modest death rates (15). We therefore used a value of 30 generations to estimate a conservative per-generation relative competitive index (RCI), defined as the mutant/ES114 ratio in the animal divided by the initial 1:1 ratio. We considered the possibility that competitive defects during symbiotic infection may reflect general strain attenuation rather than a symbiosis-specific phenomenon, and we therefore also determined the competitiveness of each strain in batch cocultures with ES114 in SWTO medium, using defined dilutions and regrowth to the same OD_{595} to prescribe the number of generations in culture, as we have done previously (18).

The strains tested and the genes disrupted in these mutants are listed in Table 1. Five loci were represented by multiple, independent insertion mutants in our original screen (Table 1) (12), and all of the independent mutants were tested for symbiotic competence. Distinct mutants with insertions in the same locus gave similar results, and we present the characterization of one representative mutant for each locus (Table 1). Because the *acnB* mutants were not stable, giving rise rapidly to suppressors (12), we did not analyze them here. The symbiotic phenotypes of *ainS* mutants are not reported, because *ainS* mutants have previously been characterized in this symbiosis (19–21).

Our results should be considered with some caveats. We cannot rule out the possibility that the phenotypes of our mutants are related to polar effects of the transposon, although it seems less likely for insertions in *arcA*, *hns*, *tfoY*, and *phoQ*, because the open reading frame (ORF) immediately downstream of each of these is oriented in

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TABLE 1 Luminescence-up mutants

Disrupted gene (ORF)	Independent mutant(s)[a]	Putative function[b]
arcA (VF2120)	SLV41	Regulator, redox-responsive TCRS
arcB (VF2122)	NL3, SLV41, SLV19, NL5, SLV36, NL6, SLV33	Sensor, redox-responsive TCRS
acnB (VF2158)	NR[c]	Aconitase (TCA cycle enzyme)
topA (VF1051)	EMH12, EMH13	Topoisomerase I (relieves negative supercoils)
lonA (VF2352)	SLV32, SLV39, EMH6	ATP-dependent protease
pstA (VF1984)	SLV10	High-affinity phosphate transport
pstC (VF1985)	SLV30	High-affinity phosphate transport
hns (VF1631)	SLV15	Nucleoid-associated DNA-binding protein, global repressor
$tRNA^{Met}$ (VFIRNA222)	EMH7	Methionine tRNA
$tRNA^{Thr}$ (VFIRNA003)	NL4	Threonine tRNA
tfoY (VF1573)	NL1, EMH9	Regulator of transformation competence
phoQ (VF1397)	SLV16, SLV43, EMH3	Sensor, Mg^{2+}-responsive TCRS
guaB (VF0637)	EMH5	Inositol-5-monophosphate dehydrogenase (purine metabolism)
ainS (VF1037)	NR	Octanoyl homoserine lactone synthase

[a] Mutants previously identified as brighter than wild type in culture (12). Underlining indicates the representative strain for each locus reported here.
[b] TCRS, two-component regulatory system; TCA, tricarboxylic acid.
[c] NR, not reported, either due to a high rate of suppressor mutations (*acnB* mutants) or because symbiotic phenotypes were previously published (*ainS* mutants).

the other direction. The genes downstream of *guaB*, *pstA*, and *pstC* are involved in functions similar to those of the upstream gene, so even if polarity affects downstream genes, interpretations of the results would remain largely or entirely unchanged. We also cannot rule out the possibility that phenotypes of these mutants are due to second-site mutations unrelated to the transposon insertion. Not only do the multiple mutants tested for *arcB*, *topA*, *lonA*, *tfoY*, and *phoQ* mutants (Table 1) provide additional replication, but also, because they were isolated independently, it seems less likely that the phenotypes conserved across mutants in a locus are due to spontaneous second-site mutations. Nonetheless, in instances where mutants appeared to diverge from the wild type, future work using defined, nonpolar (e.g., in-frame) mutants and complementation analyses might better delineate causality.

Each of the mutants colonized juvenile squid; however, mutants in six loci showed some defect in clonal symbiotic colonization (Fig. 1), and all of the mutants were attenuated in competition with the parental wild-type ES114 over 48 h (Table 2). Mutants also showed various degrees of attenuation during growth in culture, and not all the attenuated growth phenotypes could be considered symbiosis specific, as discussed below. The *arcA*, *arcB*, tRNA, and *tfoY* mutants did not show any colonization attenuation in clonal infections (Fig. 1), their competitive disadvantage in the symbiosis was moderate, with estimated RCI-per-generation values of 0.95 to 0.99 (Table 2), and they were no less competitive in the symbiosis than they were in culture (Table 2). Mutants with insertions in these loci are not discussed further.

Mutants with disruptions in *pstA* and *pstC*, which encode a high-affinity phosphate uptake system (22–24), showed a delay in the onset of symbiotic luminescence relative to ES114 (Fig. 1A), although these mutants colonized at levels equivalent to that of ES114 by 48 h (Fig. 1A and data not shown). The attenuated colonization of these mutants would be consistent with the symbiotic environment being low in available inorganic phosphate. Moreover, we previously reported that luminescence is induced in a PhoB-dependent manner in low-phosphate medium (12), raising the possibility that a low-phosphate symbiotic environment contributes to luminescence induction. On the other hand, *pstA* and *pstC* mutants had doubling times ~25% longer than that of ES114 in aerobic batch cultures, and they appeared to be outcompeted by ES114 in culture even more severely than they were in the host (Table 2), so the effect of these mutations is not symbiosis specific. Future work will help determine whether phosphate scavenging is induced and important in the light organ.

The *topA* mutants also were attenuated in the symbiosis. For example, *topA* mutant EMH12 colonized to levels ~4-fold lower than that of ES114 after 48 h (Fig. 1B) and was outcompeted by ES114 (Table 2). Similarly, the onset of luminescence was slower in EMH12-infected animals (data not shown). EMH12 also grew more slowly outside the host. Indeed, based on an estimated RCI per generation, EMH12 was vastly more attenuated relative to the wild type in aerobic batch culture than in symbiosis (Table 2). This mutant has increased DNA superhelicity (12), which can function in global responses to conditions such as osmolarity and aeration (25–28). Our results are consistent with a model whereby TopA activity is less important in the symbiosis than in culture and where a transition to more negative DNA superhelicity during infection influences gene expression, including luminescence induction. Others have hypothesized that supercoiling may be an important global regulator for pathogens, commensals, and mutualists (29), and *V. fischeri* should provide a good model for testing this hypothesis, particularly as new methods for assessing supercoiling *in vivo* become available.

Mutants with disruptions in *phoQ* colonized *E. scolopes* ~2-fold less than did ES114 (Fig. 1C), although the level of symbiotic luminescence per cell was unchanged. The *phoQ* mutant SLV16 was also outcompeted by ES114, but based on estimated RCI per generation, it was outcompeted similarly in culture and in the host (Table 2). Mutants with insertions in *hns* showed the most severe colonization defect (Fig. 1D) while also exhibiting essentially no detectable symbiotic luminescence. As with the *phoQ* mutants, however, the attenuation was also evident in coculture (Table 2). In clonal batch cultures, the *phoQ* and *hns* mutants had doubling times 25 and 100% longer than that of ES114, respectively. H-NS has broad regulatory effects (30), and considering the severe defect in colonization of the *hns* mutant, it might be tempting to speculate that H-NS plays an important symbiotic role, much as it has been suggested that *hns* contributes to the virulence of *Vibrio cholerae* (31, 32). However, it appears that at least in *V. fischeri*, the *hns* mutation has severe effects on cells under all the conditions tested,

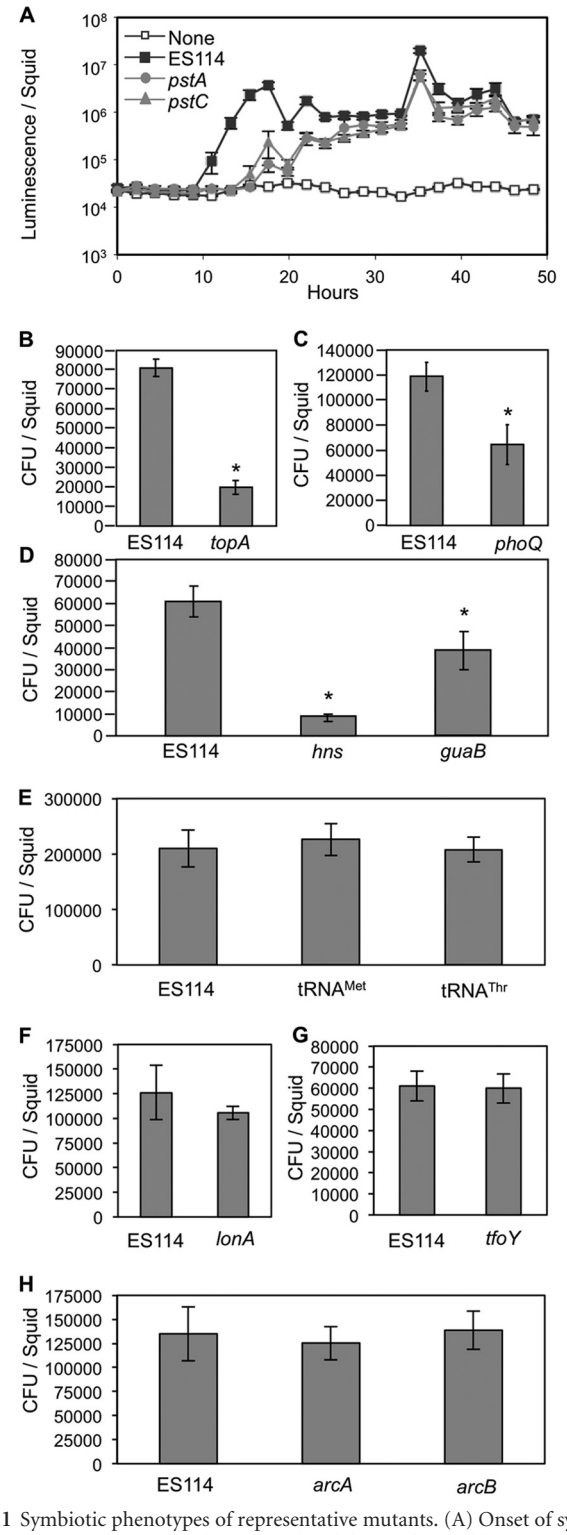

FIG 1 Symbiotic phenotypes of representative mutants. (A) Onset of symbiotic luminescence in aposymbiotic squid and those colonized by strains ES114 (wild type), SLV10 (*pstA*), and SLV30 (*pstC*). (B to H) Ability of mutant strains EMH12 (*topA*), SLV16 (*phoQ*), SLV15 (*hns*), EMH5 (*guaB*), EMH7 (tRNA^{Met}), NL4 (tRNA^{Thr}), SLV32 (*lonA*), NL1 (*tfoY*), SLV41 (*arcA*), and NL3 (*arcB*) to individually colonize the squid host relative to ES114 48 h postinoculation. Data in each panel are from one representative experiment among at least three independent colonization experiments comparing the wild type to each of the mutants. Error bars represent standard error for ≥9 animals. *, $P \leq 0.05$ compared to the wild-type value using Student's *t* test.

indicating that it is important generally and not specifically in symbiosis. Given the broad number of genes affected by PhoQ and H-NS and the general attenuation of these mutants, it will take future work focused on their respective regulons to interpret their phenotypes meaningfully.

We found the symbiotic phenotypes of the *guaB* mutant more clearly revealing. This mutant contains an insertion that should disrupt inosine-5′-monophosphate dehydrogenase, it colonized *E. scolopes* ~2-fold less than ES114 (Fig. 1D), and as expected it is a guanine auxotroph in culture. Despite its auxotrophy, the observation that this mutant is able to colonize the host light organ suggests that the host provides guanine to symbiotic *V. fischeri*, either directly or as a metabolite that cells can convert to guanine. The observation that the *guaB* mutant is reduced 2-fold in colonization relative to the wild type suggests that *de novo* guanine synthesis may be important, despite the supplies from the host; however, there are alternative explanations for this observation. For example, in a *guaB* mutant the relative levels of purines or their metabolic intermediates might be affected, starting with the IMP substrate for GuaB, having broader-ranging pleiotropic effects on metabolism.

Interestingly, the *guaB* guanine auxotroph is the only mutant examined that maintains a luminescence-up phenotype relative to ES114 in the host, with luminescence per CFU within the light organ being 3- to 9-fold higher than that in ES114. Therefore, whatever mechanism is responsible for the repressive effect of *guaB* on luminescence, this mechanism appears to function during growth both in SWTO and inside the host. None of the other mutants displayed a luminescence-up phenotype relative to the wild type during symbiosis. Thus, natural luminescence induction during infection could reflect multiple effects, but a decrease in GuaB activity does not seem to reflect one of these mechanisms.

Although *lonA* mutants such as SLV32 colonized the host as well as did ES114 in clonal infections (Fig. 1F), they were outcompeted more severely in the host than in culture. The estimated RCI per generation of SLV32 was 0.88 in the host, whereas the RCI was 0.96 per generation during batch coculture in SWTO, suggesting that *lonA* plays a more important role in symbiotic cells. Although

TABLE 2 Ability of mutants to compete with ES114 in symbiotic infections and cocultures

Strain	Disrupted gene	RCI in squid (48 h)[a]	RCI/generation[b]	
			In squid	In culture[c]
SLV41	*arcA*	0.49	0.98	0.90
NL3	*arcB*	0.25	0.95	0.93
EMH12	*topA*	0.03	0.89	<0.05
SLV32	*lonA*	0.02	0.88	0.96
SLV10	*pstA*	0.12	0.93	0.78
SLV30	*pstC*	0.04	0.90	0.78
SLV15	*hns*	<0.05	<0.05	<0.05
EMH7	tRNA^{Met}	0.55	0.98	0.86
NL4	tRNA^{Thr}	0.63	0.99	0.90
NL1	*tfoY*	0.48	0.98	1.02
SLV16	*phoQ*	0.30	0.96	0.94
EMH5	*guaB*	0.40	0.97	0.93
NL2	*ainS*	ND[d]	ND	0.95

[a] All data were calculated for ≥10 animals.
[b] Estimated by assuming 30 generations for 48 h in the squid host, which was previously published as a high estimate under these conditions (15).
[c] Coculture data were collected from cultures grown in SWTO ($n = 2$).
[d] ND, not done (results were published previously [21]).

the connection of LonA to symbiotic luminescence remains uncertain, it has been implicated in turnover of the key pheromone-dependent regulator LuxR (33).

The symbiotic phenotypes of previously isolated luminescence-up mutants have provided insights into the *V. fischeri-E. scolopes* symbiosis. For example, the ability of a *guaB* mutant to colonize the host suggests that symbionts have a source of guanine, or an appropriate guanine precursor, in the symbiosis. Symbiotic attenuation of *pst* mutants could suggest that the light organ is a low-phosphate environment, and this is consistent with other data showing that luminescence is induced in the host and by low phosphate in culture (12). It should be noted, though, that *pst* mutants were also attenuated in growth in a complex medium (SWTO). Indeed, our results highlight the importance of considering symbiotic attenuation juxtaposed with a similar analysis outside the host. As illustrated in Table 2, mutants can be similarly attenuated in the host and in batch cultures, meaning that the relative contributions of the respective genes are not symbiosis specific. Attempting to quantify RCI per generation both inside and outside the host provides a firmer basis for generating new testable hypotheses. For example, the *topA* mutants were attenuated under all conditions tested, but their RCI-per-generation values were far lower in culture than in the host (Table 2), consistent with a new hypothesis that increased negative DNA supercoiling may mediate a global regulatory response during symbiotic infection by *V. fischeri*. The results here provide the basis for prioritizing more in-depth analyses of these loci and their effects on the symbiosis between *V. fischeri* and *E. scolopes*.

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