AEM Accepts, published online ahead of print on 1 February 2013
Appl. Environ. Microbiol. doi:10.1128/AEM.03111-12

Version 2: AEM03111-12

Applied and Environmental Microbiology (short form format)

Symbiotic characterization of *Vibrio fischeri* ES114 mutants that display enhanced luminescence in culture

Noreen L. Lyell and Eric V. Stabb*

Department of Microbiology, University of Georgia, Athens GA 30602

*Corresponding author. Mailing address: University of Georgia, Department of Microbiology, 120 Cedar Street, Athens, GA 30602. Phone: (706) 542-2414; Fax: (706) 542-2674. Email estabb@uga.edu

Running Title (<54 characters): Symbiotic phenotypes of bright *V. fischeri* mutants

Keywords: *Photobacterium*, *Aliivibrio*, *topA*, *pstA*, *pstC*, *guaB, hns, lonA*

22 **Abstract**

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

27

Vibrio fischeri is a bioluminescent symbiont that colonizes the light-emitting organs of certain marine animals, including the Hawaiian bobtail squid *Euprymna scolopes* (26, 27, 32). *V. fischeri* strain ES114 is a commonly used wild type that was isolated from the light organ of an *E. scolopes* squid (2). Like most isolates from *E. scolopes*, ES114 displays dim luminescence in culture (2, 6, 18), despite being similar to bright strains in the genetic arrangement of the *luxCDABEG* genes responsible for bioluminescence and the *luxI/luxR* genes that underpin pheromone-mediated regulation of luminescence (12, 13). ES114's bioluminescence is a colonization factor (5, 31), and it is induced over a 1,000-fold upon symbiotic infection (2, 3).

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 (22), using an efficient mini-Tn*5* mutagenesis system (23). In that study, we identified luminescence-up mutants with transposon insertions in fourteen loci (Table 1), including *arcA*, *arcB*, *acnB*, *topA*, *lonA*, *pstA*, *pstC*, *hns*, $tRNA^{Met}$, $tRNA^{Thr}$, *tfoY*, *phoQ*, *guaB*, and *ainS* (22). 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 (22).

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) mutant proficiency 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 (25, 29). To generate inocula, cultures

were grown to an OD_{595} between 0.4 and 0.7 in 5 ml of SWTO medium (4) within 50-ml conical tubes at 28˚C without shaking. Based on their OD_{595}, cultures were diluted to ~2000 CFU ml^{-1} in Instant Ocean (Aquarium Systems, Mentor, Ohio), and inocula were plated onto LBS medium (28). Aposymbiotic hatchlings were then placed in 5-ml inocula in 20-ml vials for 12 to 14 hours before being rinsed in Instant Ocean. CFU in the inoculum was determined by plate counts after incubation at 28 degrees 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 seconds, after a 9-second delay, using a LS 6500 Scintillation Counter (Beckman Coulter, Fullerton, Calif.). One luminescence reading per squid was taken at each time point. Infected squid were homogenized at 24 or 48 h post-inoculation, then serially diluted and plated onto LBS medium (28), and the 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, 48 hr post-inoculation symbionts have undergone no more than 30 generations in the host assuming modest death rates (29). 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 co-cultures 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 (10).

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 and (22)), 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 (22), we did not analyze them here. The symbiotic phenotypes of *ainS* mutants are not reported, because *ainS* mutants have previously been characterized in the 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 ORF immediately downstream of each of these is oriented in the other direction. The genes downstream of *guaB*, *pstA*, and *pstC*, are involved in similar functions to 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. The multiple mutants tested for *arcB*, *topA*, *lonA*, *tfoY*, and *phoQ* mutants (Table 1) not only provide additional replication, but 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 wild type, future work using defined, non-polar (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

96 competition with the parental wild-type ES114 over 48 hours (Table 2). Mutants also showed
97 varying degrees of attenuation during growth in culture, and not all the attenuated growth
98 phenotypes could be considered symbiosis-specific, as discussed below. The *arcA, arcB*,
99 tRNAs, and *tfoY* mutants did not show any colonization attenuation in clonal infections (Fig. 1),
100 their competitive disadvantage in the symbiosis was moderate, with estimated RCI per
101 generation values of 0.95-0.99 (Table 2), and they were no less competitive in the symbiosis than
102 they were in culture (Table 2). Mutants with insertions in these loci are not discussed further.

103 Mutants with disruptions in *pstA* and *pstC,* which encode a high-affinity phosphate
104 uptake system (17, 30, 33), showed a delay in the onset of symbiotic luminescence relative to
105 ES114 (Fig. 1A), although these mutants colonized at levels equivalent to that of ES114 by 48 hr
106 (Fig 1A and data not shown). The attenuated colonization of these mutants would be consistent
107 with the symbiotic environment being low in available inorganic phosphate. Moreover, we
108 previously reported that luminescence is induced in a PhoB-dependent manner in low-phosphate
109 medium (22), raising the possibility that a low-phosphate symbiotic environment contributes to
110 luminescence induction. On the other hand, *pstA* and *pstC* mutants had doubling times ~25%
111 longer than that of ES114 in aerobic batch cultures, and they appeared to be outcompeted by
112 ES114 in culture even more severely than they were in the host (Table 2), so the effect of these
113 mutations is not symbiosis-specific. Future work will help determine whether phosphate
114 scavenging is induced and important in the light organ.

115 The *topA* mutants also were attenuated in the symbiosis. For example, *topA* mutant
116 EMH12 colonized to levels ~4-fold lower than that of ES114 after 48 hr (Fig. 1B), and was
117 outcompeted by ES114 (Table 2). Similarly, onset of luminescence was slower in EMH12-
118 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 wild type in aerobic batch culture than in the symbiosis (Table 2). This mutant has increased DNA superhelicity (22), which can function in global responses to conditions such as osmolarity and aeration (7, 8, 14, 15). 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 (9), 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. 2C), although 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 providing essentially no detectable symbiotic luminescence. As with the *phoQ* mutants however, the attenuation was also evident in co-culture (Table 2). In clonal batch cultures, the *phoQ* and *hns* mutants had doubling times 25 and 100% longer than ES114, respectively. H-NS has broad regulatory effects (1), 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 virulence of *Vibrio cholerae* (11, 16). However, it appears that at least in *V. fischeri* the *hns* mutation has severe effects on cells under all the conditions tested, indicating that it is important generally and not specifically in the 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 1 D), 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 inosine monophosphate 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 ranging from 3- to 9-fold higher than ES114. Therefore, whatever mechanism is responsible for the repressive effect of *guaB* on luminescence, this mechanism appears to be functioning during growth both in SWTO and inside the host. None of the other mutants displayed a luminescence-up phenotype relative to 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 co-culture in SWTO, suggesting that *lonA* plays a more important role in symbiotic cells. Although the connection of LonA to symbiotic luminescence remains uncertain, it has been implicated in turnover of the key pheromone-dependent regulator LuxR (24).

Conclusions

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 (22). 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 the relative contributions of the respective genes are not symbiosis specific. Attempting to quantify RCI per generation both inside and outside of 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 betwee *V. fischeri* and *E. scolopes*.

Acknowledgements

We thank Susan L. Vescovi, Dawn M. Adin and Jeffrey L. Bose for technical assistance. This research was supported by the National Science Foundation (NSF) under grants CAREER MCB-0347317, IOS-0841480, and IOS-1121106.

References

1. **Atlung, T., and H. Ingmer.** 1997. H-NS: a modulator of environmentally regulated gene expression. Mol. Microbiol. **24:**7-17.
2. **Boettcher, K. J., and E. G. Ruby.** 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J. Bacteriol. **172:**3701-3706.
3. **Boettcher, K. J., and E. G. Ruby.** 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. J. Bacteriol. **177:**1053-8.
4. **Bose, J. L., U. Kim, W. Bartkowski, R. P. Gunsalus, A. M. Overley, N. L. Lyell, K. L. Visick, and E. V. Stabb.** 2007. Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. Mol. Microbiol. **65:**538-553.
5. **Bose, J. L., C. S. Rosenberg, and E. V. Stabb.** 2008. Effects of *luxCDABEG* induction in *Vibrio fischeri*: enhancement of symbiotic colonization and conditional attenuation of growth in culture. Arch. Microbiol. **190:**169-183.
6. **Bose, J. L., M. S. Wollenberg, D. M. Colton, M. J. Mandel, A. N. Septer, A. K. Dunn, and E. V. Stabb.** 2011. Contribution of rapid evolution of the luxR-luxI intergenic region to the diverse bioluminescence outputs of *Vibrio fischeri* strains isolated from different environments. Appl. Environ. Microbiol. **77:**2445-2457.

7. **Dorman, C. J.** 1996. Flexible response: DNA supercoiling, transcription and bacterial adaptation to environmental stress. Trends Microbiol **4:**214-216.

8. **Dorman, C. J., G. C. Barr, N. N. Bhriain, and C. F. Higgins.** 1988. DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. J. Bacteriol. **170:**2816-2826.

9. **Dorman, C. J., and C. P. Corcoran.** 2009. Bacterial DNA topology and infectious disease. Nucleic Acids Res. **37:**672-678.

10. **Dunn, A. K., D. S. Millikan, D. M. Adin, J. L. Bose, and E. V. Stabb.** 2006. New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression in situ. Appl. Environ. Microbiol. **72:**802-810.

11. **Ghosh, A., K. Paul, and R. Chowdhury.** 2006. Role of the histone-like nucleoid structuring protein in colonization, motility, and bile-dependent repression of virulence gene expression in *Vibrio cholerae*. Infect. Immun. **74:**3060-3064.

12. **Gray, K. M., and E. P. Greenberg.** 1992. Physical and functional maps of the luminescence gene cluster in an autoinducer-deficient *Vibrio fischeri* strain isolated from a squid light organ. J. Bacteriol. **174:**4384-4390.

13. **Gray, K. M., and E. P. Greenberg.** 1992. Sequencing and analysis of *luxR* and *luxI*, the luminescence regulatory genes from the squid light organ symbiont *Vibrio fischeri* ES114. Molecular Marine Biology and Biotechnology **1:**414-419.

14. **Hsieh, L. S., R. M. Burger, and K. Drlica.** 1991. Bacterial DNA supercoiling and [ATP]/[ADP]. Changes associated with a transition to anaerobic growth. J. Mol. Biol. **219:**443-50.

15. **Hsieh, L. S., J. Rouviere-Yaniv, and K. Drlica.** 1991. Bacterial DNA supercoiling and [ATP]/[ADP] ratio: changes associated with salt shock. J. Bacteriol. **173:**3914-7.

16. **Krishnan, H. H., A. Ghosh, K. Paul, and R. Chowdhury.** 2004. Effect of anaerobiosis on expression of virulence factors in *Vibrio cholerae*. Infect. Immun. **72:**3961-3967.

17. **Lamarche, M. G., B. L. Wanner, S. Crepin, and J. Harel.** 2008. The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol. Rev. **32:**461-473.

18. **Lee, K.-H., and E. G. Ruby.** 1994. Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. J. Bacteriol. **176:**1985-1991.

19. **Lupp, C., and E. G. Ruby.** 2004. *Vibrio fischeri* LuxS and AinS: Comparative study of two signal synthases. J. Bacteriol. **186:**3873-3881.

20. **Lupp, C., and E. G. Ruby.** 2005. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. J. Bacteriol. **187:**3620-3629.

21. **Lupp, C., M. Urbanowski, E. P. Greenberg, and E. G. Ruby.** 2003. The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol. Microbiol. **50:**319-331.

22. **Lyell, N. L., A. K. Dunn, J. L. Bose, and E. V. Stabb.** 2010. Bright mutants of *Vibrio fischeri* ES114 reveal conditions and regulators that control bioluminescence and expression of the *lux* operon. J. Bacteriol. **192:**5103-5114.

23. **Lyell, N. L., A. K. Dunn, J. L. Bose, S. L. Vescovi, and E. V. Stabb.** 2008. Effective mutagenesis of *Vibrio fischeri* by using hyperactive mini-Tn*5* derivatives. Appl. Environ. Microbiol. **74:**7059-7063.

24. **Manukhov, I. V., V. Kotova, and G. B. Zavil'genskii.** 2006. [Role of GroEL/GroES chaperonin system and Lon protease in regulation of expression *Vibrio fischeri lux* genes in *Escherichia coli* cells]. Mol Biol (Mosk) **40:**277-283.

25. **Ruby, E. G., and L. M. Asato.** 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch. Microbiol. **159:**160-167.

26. **Ruby, E. G., and M. J. McFall-Ngai.** 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science **254:**1491-1494.

27. **Stabb, E. V.** 2006. The *Vibrio fischeri-Euprymna scolopes* light organ symbiosis, p. 204-218. *In* F. L. Thompson, B. Austin, and J. Swings (ed.), The Biology of Vibrios. ASM Press, Washington D.C.

28. **Stabb, E. V., K. A. Reich, and E. G. Ruby.** 2001. *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD^+-glycohydrolases. J. Bacteriol. **183:**309-317.

29. **Stabb, E. V., and E. G. Ruby.** 2003. Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. Appl. Environ. Microbiol. **69:**820-826.

30. **Steed, P. M., and B. L. Wanner.** 1993. Use of the *rep* technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon. J. Bacteriol. **175:**6797-809.

31. **Visick, K. L., J. Foster, J. Doino, M. McFall-Ngai, and E. G. Ruby.** 2000. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J. Bacteriol. **182:**4578-4586.

32. **Wei, S. L., and R. E. Young.** 1989. Development of symbiotic bacterial bioluminescence in a nearshore cephalopod, *Euprymna scolopes*. Marine Biology **103:**541-546.

278 33. **Willsky, G. R., and M. H. Malamy.** 1980. Characterization of two genetically separable
279 inorganic phosphate transport systems in *Escherichia coli*. J. Bacteriol. **144:**356-65.
280
281
282

283 **Table 1:** Luminescence-up mutants

Disrupted Gene (ORF)	Independent mutants[a]	Putative Function
arcA (VF2120)	**SLV41**	Regulator, redox-responsive TCRS[b]
arcB (VF2122)	**NL3**, SLV41, SLV19, NL5, SLV36, NL6, SLV33	Sensor, redox-responsive TCRS
acnB (VF2158)	NR[b]	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 transfer RNAs
$tRNA^{Thr}$ (VFIRNA003)	**NL4**	Threonine transfer RNA
tfoY (VF1573)	**NL1**, EMH9	Regulator of transformation competence
phoQ (VF1397)	**SLV16**, SLV43, EMH3	Sensor, Mg^{2+}-responsive TCRS[b]
guaB (VF0637)	**EMH5**	Inositol-5-monophosphate dehydrogenase (purine metabolism)
ainS (VF1037)	NR[c]	Octanoyl homoserine lactone synthase

284 [a] Mutants previously identified as brighter than wild type in culture (22). Boldface and

285 underlined font indicates the representative strain for each locus reported here.

286 [b] TCRS indicates "two-component regulatory system"

287 [c] 'NR' indicates mutant symbiotic phenotypes are not reported, either due to high rate of

288 suppressor mutations (*acnB* mutants) or because symbiotic phenotypes were previously

289 published (*ainS* mutants).

290 **Table 2:** Ability of mutants to compete with ES114 in symbiotic infections and co-cultures

Strain	Disrupted Gene	In Squid[a] 48 Hour RCI	In Squid RCI/Generation[c]	In Culture[b] RCI/Generation
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[d]	0.95

[a]All data calculated for n≥10 animals.

[b]Co-culture data collected from cultures grown in SWTO and calculated from n=2

[c]Estimated by assuming 30 generations for 48 hrs in squid host, which was previously published as a high estimate under these conditions (29).

[d]'ND' indicates experiment was not done, because results previously published (21).

Fig. 1: Symbiotic phenotypes of representative mutants. (A) Onset of symbiotic luminescence in aposymbiotic squid (white squares) and those colonized by strains ES114 (wild type) (black squares), SLV10 (*pstA*) (grey circles), and SLV30 (*pstC*) (grey triangles). (B-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 post-inoculation. Data in each panel show one representative experiment from among at least three independent colonization experiments comparing wild type to each of the respective mutants. Error bars represent standard error for $n \geq 9$ animals. Asterisk a significant difference between wild type and mutant colonization ($p \leq 0.05$) using a Student's t test.