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

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

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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

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

36 We previously probed the basis for the relative repression of the *luxICDABEG* operon in
37 culture by screening transposon-insertion mutants of ES114 for brighter luminescence on solid
38 media (22), using an efficient mini-Tn5 mutagenesis system (23). In that study, we identified
39 luminescence-up mutants with transposon insertions in fourteen loci (Table 1), including *arcA*,
40 *arcB*, *acnB*, *topA*, *lonA*, *pstA*, *pstC*, *hns*, tRNA^{Met}, tRNA^{Thr}, *tfoY*, *phoQ*, *guaB*, and *ainS* (22).
41 The *acnB* mutants gave rise to suppressors and proved difficult to examine; however,
42 characterizing the remaining mutants gave insight into the mechanisms of *lux* regulation and the
43 environmental parameters that influence *lux* expression, as we discussed previously (22).

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

49 To determine the strains' colonization proficiency, aposymbiotic *E. scolopes* hatchlings
50 were inoculated with *V. fischeri* as described previously (25, 29). To generate inocula, cultures

51 were grown to an OD_{595} between 0.4 and 0.7 in 5 ml of SWTO medium (4) within 50-ml conical
52 tubes at 28°C without shaking. Based on their OD_{595} , cultures were diluted to ~ 2000 CFU ml^{-1}
53 in Instant Ocean (Aquarium Systems, Mentor, Ohio), and inocula were plated onto LBS medium
54 (28). Aposymbiotic hatchlings were then placed in 5-ml inocula in 20-ml vials for 12 to 14
55 hours before being rinsed in Instant Ocean. CFU in the inoculum was determined by plate
56 counts after incubation at 28 degrees C. Although inocula ranged from 1,000 to 3,000 CFU ml^{-1} ,
57 within each experiment considered here, squid were exposed to similar concentrations of mutant
58 or wild-type cells in the respective inocula. Luminescence of each squid was measured by
59 integrating the light output over 9 seconds, after a 9-second delay, using a LS 6500 Scintillation
60 Counter (Beckman Coulter, Fullerton, Calif.). One luminescence reading per squid was taken at
61 each time point. Infected squid were homogenized at 24 or 48 h post-inoculation, then serially
62 diluted and plated onto LBS medium (28), and the CFU per squid was determined.

63 We similarly mixed mutants 1:1 with ES114 to test their ability to compete for
64 colonization of the host. A previous calculation suggested that under these conditions, 48 hr
65 post-inoculation symbionts have undergone no more than 30 generations in the host assuming
66 modest death rates (29). We therefore used a value of 30 generations to estimate a conservative
67 per-generation relative competitive index (RCI) defined as the mutant:ES114 ratio in the animal
68 divided by the initial 1:1 ratio. We considered the possibility that competitive defects during
69 symbiotic infection may reflect general strain attenuation rather than a symbiosis-specific
70 phenomenon, and we therefore also determined the competitiveness of each strain in batch co-
71 cultures with ES114 in SWTO medium, using defined dilutions and regrowth to the same OD_{595}
72 to prescribe the number of generations in culture as we have done previously (10).

73 The strains tested and the genes disrupted in these mutants are listed in Table 1. Five loci
74 were represented by multiple, independent insertion mutants in our original screen (Table 1 and
75 (22)), and all of the independent mutants were tested for symbiotic competence. Distinct
76 mutants with insertions in the same locus gave similar results, and we present the
77 characterization of one representative mutant for each locus (Table 1). Because the *acnB*
78 mutants were not stable, giving rise rapidly to suppressors (22), we did not analyze them here.
79 The symbiotic phenotypes of *ainS* mutants are not reported, because *ainS* mutants have
80 previously been characterized in the symbiosis (19-21).

81 Our results should be considered with some caveats. We cannot rule out the possibility
82 that the phenotypes of our mutants are related to polar effects of the transposon, although it
83 seems less likely for insertions in *arcA*, *hns*, *tfoY*, and *phoQ*, because the ORF immediately
84 downstream of each of these is oriented in the other direction. The genes downstream of *guaB*,
85 *pstA*, and *pstC*, are involved in similar functions to the upstream gene, so even if polarity affects
86 downstream genes, interpretations of the results would remain largely or entirely unchanged.
87 We also cannot rule out the possibility that phenotypes of these mutants are due to second-site
88 mutations unrelated to the transposon insertion. The multiple mutants tested for *arcB*, *topA*,
89 *lonA*, *tfoY*, and *phoQ* mutants (Table 1) not only provide additional replication, but because they
90 were isolated independently it seems less likely that the phenotypes conserved across mutants in
91 a locus are due to spontaneous second-site mutations. Nonetheless, in instances where mutants
92 appeared to diverge from wild type, future work using defined, non-polar (e.g. in-frame) mutants
93 and complementation analyses might better delineate causality.

94 Each of the mutants colonized juvenile squid; however, mutants in six loci showed some
95 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,

119 based on an estimated RCI per generation, EMH12 was vastly more attenuated relative to wild
120 type in aerobic batch culture than in the symbiosis (Table 2). This mutant has increased DNA
121 superhelicity (22), which can function in global responses to conditions such as osmolarity and
122 aeration (7, 8, 14, 15). Our results are consistent with a model whereby TopA activity is less
123 important in the symbiosis than in culture, and where a transition to more negative DNA
124 superhelicity during infection influences gene expression, including luminescence induction.
125 Others have hypothesized that supercoiling may be an important global regulator for pathogens,
126 commensals, and mutualists (9), and *V. fischeri* should provide a good model for testing this
127 hypothesis, particularly as new methods for assessing supercoiling in vivo become available.

128 Mutants with disruptions in *phoQ* colonized *E. scolopes* ~2-fold less than did ES114
129 (Fig. 2C), although symbiotic luminescence per cell was unchanged. The *phoQ* mutant SLV16
130 was also outcompeted by ES114, but based on estimated RCI per generation it was outcompeted
131 similarly in culture and in the host (Table 2). Mutants with insertions in *hns* showed the most
132 severe colonization defect (Fig. 1D), while also providing essentially no detectable symbiotic
133 luminescence. As with the *phoQ* mutants however, the attenuation was also evident in co-culture
134 (Table 2). In clonal batch cultures, the *phoQ* and *hns* mutants had doubling times 25 and 100%
135 longer than ES114, respectively. H-NS has broad regulatory effects (1), and considering the
136 severe defect in colonization of the *hns* mutant, it might be tempting to speculate that H-NS
137 plays an important symbiotic role, much as it has been suggested that *hns* contributes to
138 virulence of *Vibrio cholerae* (11, 16). However, it appears that at least in *V. fischeri* the *hns*
139 mutation has severe effects on cells under all the conditions tested, indicating that it is important
140 generally and not specifically in the symbiosis. Given the broad number of genes affected by

141 PhoQ and H-NS and the general attenuation of these mutants, it will take future work focused on
142 their respective regulons to interpret their phenotypes meaningfully.

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

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

162 Although *lonA* mutants such as SLV32 colonized the host as well as did ES114 in clonal
163 infections (Fig. 1F), they were outcompeted more severely in the host than in culture. The

164 estimated RCI per generation of SLV32 was 0.88 in the host, whereas the RCI was 0.96 per
165 generation during batch co-culture in SWTO, suggesting that *lonA* plays a more important role in
166 symbiotic cells. Although the connection of LonA to symbiotic luminescence remains uncertain,
167 it has been implicated in turnover of the key pheromone-dependent regulator LuxR (24).

168

169 *Conclusions*

170 The symbiotic phenotypes of previously isolated luminescence-up mutants have provided
171 insights into the *V. fischeri*-*E. scolopes* symbiosis. For example, the ability of a *guaB* mutant to
172 colonize the host suggests that symbionts have a source of guanine, or an appropriate guanine
173 precursor, in the symbiosis. Symbiotic attenuation of *pst* mutants could suggest that the light
174 organ is a low-phosphate environment, and this is consistent with other data showing that
175 luminescence is induced in the host and by low phosphate in culture (22). It should be noted
176 though that *pst* mutants were also attenuated in growth in a complex medium (SWTO). Indeed,
177 our results highlight the importance of considering symbiotic attenuation juxtaposed with a
178 similar analysis outside the host. As illustrated in Table 2, mutants can be similarly attenuated in
179 the host and in batch cultures, meaning the relative contributions of the respective genes are not
180 symbiosis specific. Attempting to quantify RCI per generation both inside and outside of the
181 host provides a firmer basis for generating new testable hypotheses. For example, the *topA*
182 mutants were attenuated under all conditions tested, but their RCI per generation values were far
183 lower in culture than in the host (Table 2), consistent with a new hypothesis that increased
184 negative DNA supercoiling may mediate a global regulatory response during symbiotic infection
185 by *V. fischeri*. The results here provide the basis for prioritizing more in depth analyses of these
186 loci, and their effects on the symbiosis between *V. fischeri* and *E. scolopes*.

187

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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
<i>arcA</i> (VF2120)	<u>SLV41</u>	Regulator, redox-responsive TCRS ^b
<i>arcB</i> (VF2122)	<u>NL3</u> , SLV41, SLV19, NL5, SLV36, NL6, SLV33	Sensor, redox-responsive TCRS
<i>acnB</i> (VF2158)	NR ^b	Aconitase (TCA cycle enzyme)
<i>topA</i> (VF1051)	<u>EMH12</u> , EMH13	Topoisomerase I (relieves negative supercoils)
<i>lonA</i> (VF2352)	<u>SLV32</u> , SLV39, EMH6	ATP-dependent Protease
<i>pstA</i> (VF1984)	<u>SLV10</u>	High-affinity phosphate transport
<i>pstC</i> (VF1985)	<u>SLV30</u>	High-affinity phosphate transport
<i>hns</i> (VF1631)	<u>SLV15</u>	nucleoid-associated DNA-binding protein, global repressor
tRNA ^{Met} (VFIRNA222)	<u>EMH7</u>	Methionine transfer RNAs
tRNA ^{Thr} (VFIRNA003)	<u>NL4</u>	Threonine transfer RNA
<i>tfoY</i> (VF1573)	<u>NL1</u> , EMH9	Regulator of transformation competence
<i>phoQ</i> (VF1397)	<u>SLV16</u> , SLV43, EMH3	Sensor, Mg ²⁺ -responsive TCRS ^b
<i>guaB</i> (VF0637)	<u>EMH5</u>	Inositol-5-monophosphate dehydrogenase (purine metabolism)
<i>ainS</i> (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	In Squid	In Culture ^b
		48 Hour RCI	RCI/Generation ^c	RCI/Generation
SLV41	<i>arcA</i>	0.49	0.98	0.90
NL3	<i>arcB</i>	0.25	0.95	0.93
EMH12	<i>topA</i>	0.03	0.89	<0.05
SLV32	<i>lonA</i>	0.02	0.88	0.96
SLV10	<i>pstA</i>	0.12	0.93	0.78
SLV30	<i>pstC</i>	0.04	0.90	0.78
SLV15	<i>hns</i>	<0.05	<0.05	<0.05
EMH7	<i>tRNA^{Met}</i>	0.55	0.98	0.86
NL4	<i>tRNA^{Thr}</i>	0.63	0.99	0.90
NL1	<i>tfoY</i>	0.48	0.98	1.02
SLV16	<i>phoQ</i>	0.30	0.96	0.94
EMH5	<i>guaB</i>	0.40	0.97	0.93
NL2	<i>ainS</i>	ND ^d	ND ^d	0.95

291 ^aAll data calculated for n \geq 10 animals.

292 ^bCo-culture data collected from cultures grown in SWTO and calculated from n=2

293 ^cEstimated by assuming 30 generations for 48 hrs in squid host, which was previously published
294 as a high estimate under these conditions (29).

295 ^d‘ND’ indicates experiment was not done, because results previously published (21).

296 **Fig. 1:** Symbiotic phenotypes of representative mutants. (A) Onset of symbiotic luminescence
297 in aposymbiotic squid (white squares) and those colonized by strains ES114 (wild type) (black
298 squares), SLV10 (*pstA*) (grey circles), and SLV30 (*pstC*) (grey triangles). (B-H) Ability of
299 mutant strains EMH12 (*topA*), SLV16 (*phoQ*), SLV15 (*hns*), EMH5 (*guaB*), EMH7 (tRNA^{Met}),
300 NL4 (tRNA^{Thr}), SLV32 (*lonA*), NL1 (*tfoY*), SLV41 (*arcA*), and NL3 (*arcB*) to individually
301 colonize the squid host relative to ES114 48 h post-inoculation. Data in each panel show one
302 representative experiment from among at least three independent colonization experiments
303 comparing wild type to each of the respective mutants. Error bars represent standard error for
304 $n \geq 9$ animals. Asterisk a significant difference between wild type and mutant colonization
305 ($p \leq 0.05$) using a Student's t test.

