



An Expanded Transposon Mutant Library Reveals that *Vibrio fischeri* δ -Aminolevulinate Auxotrophs Can Colonize *Euprymna scolopes*

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ABSTRACT Libraries of defined mutants are valuable research tools but necessarily lack gene knockouts that are lethal under the conditions used in library construction. In this study, we augmented a *Vibrio fischeri* mutant library generated on a rich medium (LBS, which contains [per liter] 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 50 mM Tris [pH 7.5]) by selecting transposon insertion mutants on supplemented LBS and screening for those unable to grow on LBS. We isolated strains with insertions in *alr*, *glr* (*murl*), *glmS*, several heme biosynthesis genes, and *ftsA*, as well as a mutant disrupted 14 bp upstream of *ftsQ*. Mutants with insertions in *ftsA* or upstream of *ftsQ* were recovered by addition of Mg²⁺ to LBS, but their cell morphology and motility were affected. The *ftsA* mutant was more strongly affected and formed cells or chains of cells that appeared to wind back on themselves helically. Growth of mutants with insertions in *glmS*, *alr*, or *glr* was recovered with *N*-acetylglucosamine (NAG), D -alanine, or D -glutamate, respectively. We hypothesized that NAG, D -alanine, or D -glutamate might be available to *V. fischeri* in the *Euprymna scolopes* light organ; however, none of these mutants colonized the host effectively. In contrast, *hemA* and *hemL* mutants, which are auxotrophic for δ -aminolevulinate (ALA), colonized at wild-type levels, although mutants later in the heme biosynthetic pathway were severely impaired or unable to colonize. Our findings parallel observations that legume hosts provide *Bradyrhizobium* symbionts with ALA, but they contrast with virulence phenotypes of *hemA* mutants in some pathogens. The results further inform our understanding of the symbiotic light organ environment.

IMPORTANCE By supplementing a rich yeast-based medium, we were able to recover *V. fischeri* mutants with insertions in conditionally essential genes, and further characterization of these mutants provided new insights into this bacterium's symbiotic environment. Most notably, we show evidence that the squid host can provide *V. fischeri* with enough ALA to support its growth in the light organ, paralleling the finding that legumes provide *Bradyrhizobium* ALA in symbiotic nodules. Taken together, our results show how a simple method of augmenting already rich media can expand the reach and utility of defined mutant libraries.

KEYWORDS *Photobacterium*, *Aliivibrio*, symbiosis, aminolevulinic acid, hemin, photobacteria

Defined knockout mutant libraries are useful resources that have been generated to promote research in several bacterial experimental models and pathogens (1–10). In such studies, researchers have often listed essential genes, the knockouts of which were apparently lethal under the conditions of library construction and therefore not represented by mutants in the library. In order to recover insertion mutants collectively

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representing as many disrupted genes as possible, the bacteria targeted for mutation are generally grown on complex rich media, which makes many genes expendable. A popular choice for a growth medium has been LB (1–6, 9), which contains sodium chloride, tryptone as a rich source of peptides (10 g liter⁻¹), and a complex array of vitamins and common metabolites from yeast extract (5 g liter⁻¹). Despite its richness and complexity, LB needs further amendment to support growth of certain mutants, for example, those disrupted in the synthesis of certain bacterial cell wall components that are not found in yeast. Therefore, gaps in defined mutant libraries include some mutants that are missing because the respective gene is essential for growth on LB, although it could be nonessential in other media.

An effort is under way to generate a defined mutant library for *Vibrio fischeri* (11), a genetically tractable organism studied for its bioluminescence (12, 13), cell-cell signaling (14–16), interesting physiology (17, 18), biofilm formation (19–22), and light organ symbiosis with the Hawaiian bobtail squid *Euprymna scolopes* (23–26). Both this insertion mutant library, which is based on a mini-Tn5 transposon (27), and a recent complementary mariner-based InSeq study with *V. fischeri* (28) involve selection on LBS medium (29), which is a Tris-buffered high-NaCl derivative of LB (containing [per liter] 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 50 mM Tris [pH 7.5]). In this study, we sought to augment the suite of mutants being generated by screening for mutants able to grow on supplemented LBS (SLBS) but not on LBS. Moreover, we tested whether the host environment could support colonization by mutants that are unable to grow on LBS.

RESULTS AND DISCUSSION

To search for supplements that could reverse the lethality or strong negative effects of specific mutations during growth on LBS, we amended this medium with MgSO₄, riboflavin, D-alanine, D-glutamate, diaminopimelic acid (DAP), N-acetylglucosamine (NAG), ribose, succinate, glycerol, biotin, NAD⁺, folate, thymidine, cytidine, uracil, and hemin. In addition, we added the iron chelator 2,2-bipyridyl, which stimulates the uptake of hemin by *V. fischeri* (30). The choices of supplements were based on precedent, genome annotations for *V. fischeri*, and conjecture. Although some of these compounds are present in LBS, we considered the possibility that larger amounts might be necessary to support growth of some mutants. For example, thymidine and riboflavin may be present in yeast extract, but supplementing LB with larger amounts of these compounds is necessary for robust growth of certain mutants of other bacteria (31, 32).

We isolated 25 mini-Tn5 transposon insertion mutants that grew on SLBS but not on LBS. The chromosomal location of the transposon insertion and the supplement requirement for each mutant are summarized in Table 1. We used the DOOR Database of Prokaryotic Operons (33) to assess which transposon insertions might occur in larger multigene transcripts, and these results are also listed in Table 1. Some of the mutants, for example, *hemC* mutants KP1 and SV1, are likely siblings, because they have insertions in identical locations and were isolated from the same mating to introduce the transposon. On the other hand, although three of the *hemG* mutants carry transposon insertions in the same location, only mutants AN2 and SV13 (and not EP1) were isolated from the same mating. In all, our screen yielded at least 22 independent mutants of ES114, with insertions corresponding to 13 different genes. Each of these genes was previously designated “putative essential” for growth on LBS in a recent InSeq analysis (28), although it should be noted that when assigning genes as putative essential, Brooks et al. (28) considered the possibility that insertions toward the 3' end of genes might not eliminate function, whereas some of our insertion mutations may lead to partial gene function, as discussed below.

Five of the supplements in SLBS restored growth to particular mutants when added to LBS: MgSO₄, NAG, D-alanine, D-glutamate, and hemin/bipyridyl. Representative mutants were characterized with respect to ability to colonize *E. scolopes*, and in some cases we further defined their auxotrophy and/or other defects.

TABLE 1 Mutants isolated in this study

Strain(s)	Disrupted gene ^a	Insertion after bp	Gene length (bp)	Putative function ^b	Component that best restores growth ^c	Putative operon structure ^d
RP5	<i>ftsA</i> (VF_2197)	894	1,263	Cell division	Mg ²⁺ or Ca ²⁺	<i>ftsQAZ-lpxC</i> (VF_2199–VF_2195)
SV11	<i>ftsQ</i> (VF_2199)	–14	522	Cell division	Mg ²⁺ or Ca ²⁺	<u>Tn-ftsQAZ-lpxC</u> (VF_2199–VF_2195) Monocistronic
NL81	<i>glmS</i> (VF_2372)	686	1,830	Glucosamine-6P synthesis	NAG or glucosamine	Monocistronic
AN4, DD1, RM1	<i>alr</i> (VF_0302)	246, 465, 134	1,080	Alanine racemase	D-Ala	<i>dnaB-alr-hyp</i> (VF_0301–VF_0303) Monocistronic
AN3, SV8, SV14	<i>glr</i> (<i>murl</i>) (VF_2433)	154, 431, 431	786	Glutamate racemase	D-Glu	Monocistronic
RP4	<i>hemA</i> (VF_0767)	894	1,257	Glutamyl-tRNA reductase	Hemin or ALA	<i>hemA-prfA-prmC-hyp-ycaA-kdsA</i> (VF_0767–VF_0772)
NL67, RP1	<i>hemL</i> (VF_2135)	475, 342	1,296	ALA synthase	Hemin or ALA	Monocistronic
DD2, NP18, NP19	<i>hemB</i> (VF_0053)	880, 872, 322	1,017	ALA dehydratase	Hemin	Monocistronic
KP1, SV1	<i>hemC</i> (VF_0066)	346, 346	936	PBG deaminase	Hemin	<i>hemCDXY</i> (VF_0066–VF_0063)
DD3	<i>hemD</i> (VF_0065)	189	762	Uroporphyrinogen III synthase	Hemin	<i>hemCDXY</i> (VF_0066–VF_0063)
AN6	<i>hemE</i> (VF_2402)	596	1,068	Uroporphyrinogen III decarboxylase	Hemin	<i>hemE-yhgl</i> (VF_2402–VF_2401)
AN2, EP1, NL66, SV13	<i>hemG</i> (VF_0028)	245, 245, 238, 245	522	Protoporphyrinogen oxidase	Hemin	<i>yigZ-trkH-hemG</i> (VF_0026–VF_0028)
AN5, RP3	<i>hemH</i> (VF_0794)	895, 329	969	Ferrochelatase	Hemin	<i>adk-hemH</i> (VF_0793–VF_0794)

^aThe gene designation is followed by the ORF number (VF number) from the *V. fischeri* ES114 genome.

^bALA, δ -aminolevulinic acid; PBG, porphobilinogen.

^cThis column lists the component of SLBS that best restores growth in LBS, as well as other supplements that enable growth (see the text). The iron chelator 2,2-bipyridyl was required to stimulate uptake of hemin in these auxotrophs.

^dOperon composition predicted by the DOOR Database of Prokaryotic Operons (33). Underlined genes were categorized as “putative essential” (possibly due to polar effects) in an InSeq analysis (28). The disrupted gene is in bold, except in the case of SV11, where the Tn insertion is upstream of the operon (indicated by Tn-). “hyp” indicates “hypothetical” when no gene designation is provided by the genome annotation.

Mutants rescued by magnesium. Two mutants, RP5 and SV11, were rescued by adding MgSO₄ to LBS (Table 1), and in each case MgCl₂ also rescued growth, indicating that Mg²⁺ likely underlies this growth recovery. Because Mg²⁺ rescues the growth of certain *V. fischeri* mutants with multiple defects in lipid A biosynthesis (34), we speculate that Mg²⁺ bridging lipopolysaccharide (LPS) molecules structurally promotes outer membrane integrity and counteracts mutations or conditions that might compromise the outer membrane. Alternatively, dramatic regulatory effects of Mg²⁺ have been observed in *V. fischeri* (35–37), and Mg²⁺ may restore growth to mutants through a regulatory mechanism. Mutants RP5 and SV11 could also be rescued by supplementing LBS with 20 mM Ca²⁺, which has regulatory and structural effects distinct from but similar to those of Mg²⁺ (38, 39).

Unexpectedly, we recovered mutants with insertions in or near *ftsA* and *ftsQ*, which play a critical role in cell division in *E. coli* (40) and were previously designated essential in *V. fischeri* (28). Each of these mutants isolated in this study may retain partial function. The Tn insertion in SV11 is just upstream of *ftsQ* and does not disrupt the open reading frame (ORF) itself, while the insertion in *ftsA* leaves nearly the first three quarters of the protein intact (Table 1). It should also be noted that *ftsQ* (VF_2199) was originally misannotated as two genes (VF_2199 and VF_2198), which explains why its ORF number is two apart from that of *ftsA* (VF_2197) despite these being adjacent genes (41). The DOOR operon prediction database predicts that *ftsQAZ* is cotranscribed with *lpxC*; however, over 100 bp separate *ftsZ* from *lpxC*. We complemented mutant RP5 with *ftsQAZ* in *trans* on pJLS234, which restored growth on LBS without Mg²⁺, whereas the parent vector, pVSV105, did not (data not shown).

These two *fts* mutants were phenotypically similar to each other, with the *ftsA* mutant displaying more severe deviations from the wild type. Both grew detectably in LBS, but even with growth enhanced by Mg²⁺ supplementation, the mutants grew somewhat slower than the wild type. Growth of the mutants was likewise improved in SWTO medium (see Materials and Methods) with its marine salts, including Mg²⁺, although it still did not match that of the wild type. The *fts* mutants also had altered

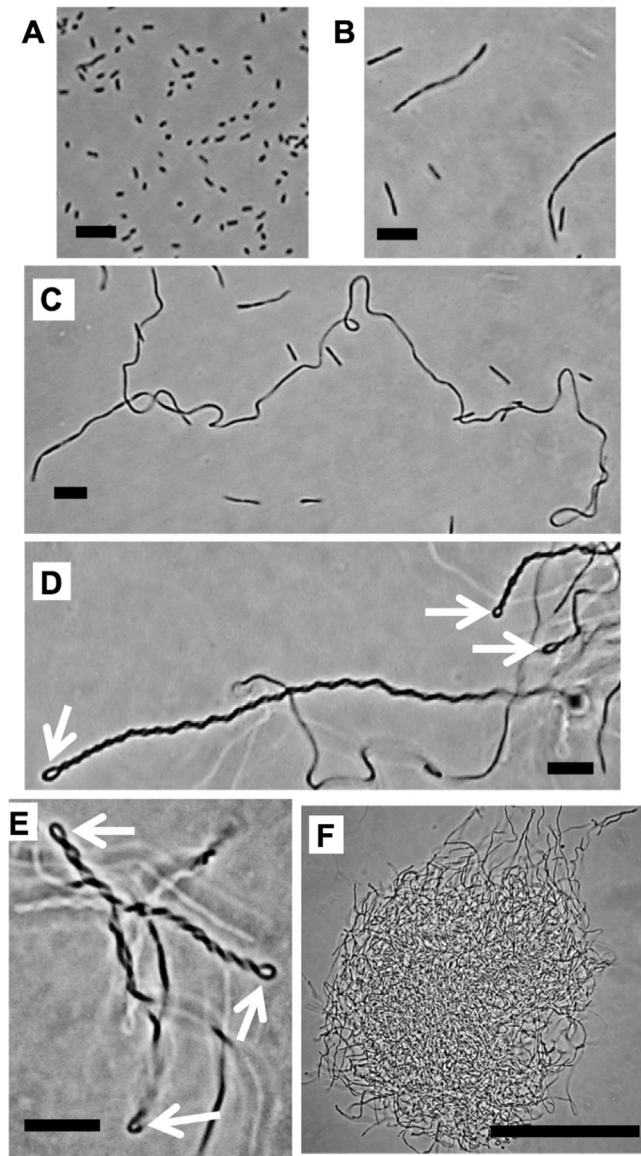


FIG 1 Cell morphology of *fts* mutants. Phase-contrast images show wild-type strain ES114 (A), Tn-*ftsQ* mutant SV11 (B and C), and *ftsA*::Tn mutant RP5 (D, E, and F). Bars indicate size standards of 10 μm (A to E) and 100 μm (F). White arrows in panels D and E indicate where mutant cells or strings of cells have looped back on themselves. Cells were grown to mid-log phase in SWTO at 28°C with shaking at 200 rpm.

cell morphology (elongating and chaining) consistent with decreased or disrupted function of *ftsQAZ* (Fig. 1). The Tn-*ftsQ* mutant formed chains of cells (Fig. 1B) some reaching 100 μm or more in length (Fig. 1C). The *ftsA*::Tn mutant also formed chains (Fig. 1D and E), and these tended to become tangled into large aggregates of cells (Fig. 1F), some of which precipitated from culture. Interestingly, elongated cells or chains of cells often looped back and wound around themselves in a helical structure, a phenotype that was especially common in the *ftsA*::Tn mutant (Fig. 1D and E). A similar hairpin turn leading to a double helical structure was reported for an *mreB* mutant of the curved alphaproteobacterium *Caulobacter crescentus* (42). Wild-type *V. fischeri* forms slightly comma-shaped rods (43), but a spiral aspect to its cell shape has not been described, nor is it evident in our mutants except where cells wind around themselves. We speculate that the wild type may have a slight and nonrigid helical pitch to its comma-shaped cells, although it remains to be tested whether the *fts* mutations allow

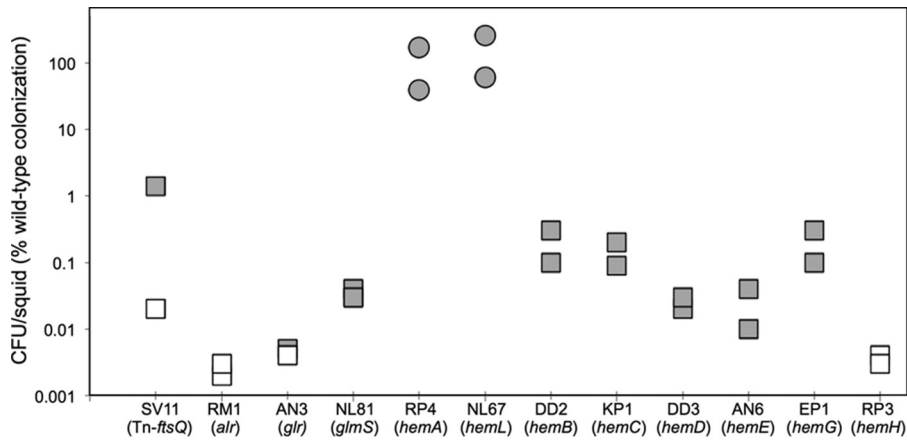


FIG 2 Colonization of squid by representative mini-Tn5 insertion mutants. Squid were inoculated in parallel with either a representative mutant or E5114, and the CFU in each of the squid was determined 48 h later. Colonization by E5114 ranged from 0.7×10^5 to 3.8×10^5 CFU/squid across experiments, and mutant CFU/squid data were therefore normalized to those for the wild type and presented as the percentage of the wild-type CFU/squid in the same experiment. Each symbol indicates results (average CFU/squid for multiple squid) of an independent experiment. Each mutant was tested on at least 7 and no more than 19 squid in each experiment. Open boxes indicate the limit of detection in experiments where no mutant colonies were recovered from inoculated squid. Boxes indicate that mutant colonization and wild-type colonization were significantly different ($P < 0.01$) in a Mann-Whitney test, and circles indicate that the values for the mutant and wild type were not significantly different ($P > 0.01$).

a natural cell shape to be seen or perhaps actually cause it in deviation from the wild type.

The Tn-*ftsQ* mutant was severely attenuated in colonizing the host (Fig. 2), and the *ftsA::Tn* mutant was difficult to test because the cells clumped so severely in the inoculum (e.g., Fig. 1F). In either case, a colonization defect could simply reflect a lack of motility. Motility is a key colonization factor for *V. fischeri* (44, 45), and nonmotile mutants are unable to colonize *E. scolopes*. Although microscopic examination of these mutants revealed a few swimming cells, the strains were virtually nonmotile in swim plate motility assays (data not shown). It seems likely that the *fts* mutants can be exploited to learn more about *V. fischeri* cell architecture, but they seem unlikely to inform our understanding of the light organ environment.

Mutants affected in biosynthesis of peptidoglycan precursors. Multiple independent mutants were isolated with insertions in the *alr* and *glr* (*murl*) genes, encoding alanine and glutamate racemases, respectively, and one mutant had an insertion in *glmS*, which encodes glucosamine-6-phosphate synthase (Table 1). These enzymes are required to make D-Ala, D-Glu, and NAG, respectively, which are key components of peptidoglycan. In Gram-negative bacteria, these genes often are essential unless the medium is appropriately supplemented (46, 47). Accordingly, *alr*, *glr* (*murl*), and *glmS* mutants required the D-Ala, D-Glu, or NAG supplements from SLBS to grow in LBS. Growth of the *glmS* mutant could alternatively be restored with glucosamine rather than NAG.

Mutants RM1 (*alr*) and AN3 (*glr*) grew and swam similarly to the wild type when appropriately supplemented (data not shown), suggesting that they should be able to colonize *E. scolopes* if the host provides the corresponding D-amino acid. Interestingly, D-amino acids and D-Ala in particular have been found in tissues of marine animals (48, 49), leading us to speculate that at least the *alr* mutant might colonize squid. However, both RM1 and AN3 were severely attenuated in colonization (Fig. 2), so it appears likely either that D-Ala and D-Glu are not abundant osmolytes in this cephalopod or that they are restricted from the symbiotic environment of the light organ.

The *glmS* mutant was similarly unable to colonize *E. scolopes* (Fig. 2), although it grew at a rate similar to that of the wild type in LBS when provided 1 mM NAG or glucosamine (data not shown). This result is consistent with recently published studies

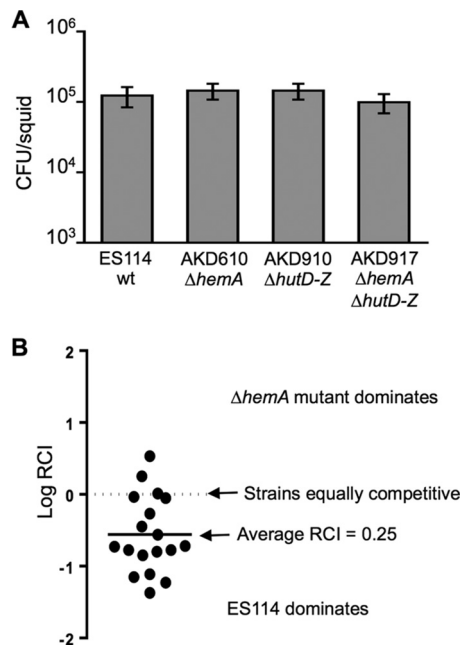


FIG 3 Effects of a $\Delta hemA$ mutation on squid colonization. (A) CFU per squid 72 h after inoculation with *V. fischeri* ES114 (wt), AKD610 ($\Delta hemA$), AKD910 ($\Delta hutD-hutZ$), or AKD917 ($\Delta hemA \Delta hutD-hutZ$). Bars indicate standard errors of the means for colonization levels in 20 animals ($n = 20$). A one-way analysis of variance (ANOVA) indicated that there was no statistical difference in colonization levels for the tested strains ($P > 0.05$). (B) Relative symbiotic competitiveness of $\Delta hemA$ mutant AKD610:ES114 ratio in an individual animal divided by the ratio of these strains in the inoculum. The average log RCI of -0.6 (RCI = 0.25) indicated a statistically significant competitive disadvantage for the mutant ($P < 0.0001$).

suggesting that NAG becomes a significant carbon source for symbiotic *V. fischeri* well after initial infection (50, 51) and therefore would not be expected to be supplied by the host in sufficient amounts to support colonization in our experiments.

Mutants affected in heme biosynthesis. Several mutants auxotrophic for hemin were isolated, and each of them contained insertions in *hem* genes involved in hemin (heme) biosynthesis (Table 1). *V. fischeri* appears to use the typical bacterial C_5 biosynthetic pathway (i.e., the Beale pathway) for heme, with HemA and HemL responsible for making the first committed tetrapyrrole precursor, δ -aminolevulinic acid (ALA), HemB catalyzing conversion of ALA to porphobilinogen, and several enzymes, including HemC, HemD, HemE, HemF, HemN, HemG, and HemH, sequentially forming protoporphyrin IX and inserting iron to generate heme (52). Using a representative mutant for each locus that was disrupted (Table 1), we found no evidence that growth or motility was attenuated relative to those of the wild type in media with hemin and 2,2-bipyridyl (data not shown). However, mutants with insertions in *hemB*, *hemC*, *hemD*, *hemE*, *hemG*, and *hemH* were severely attenuated in host colonization (Fig. 2). Induction of transcriptional *gfp* reporters fused to the promoters of hemin-uptake genes was visualized during squid colonization, and a $\Delta hutD-hutZ$ mutant lacking this hemin uptake system was outcompeted by the wild type 3-fold over 4 days (30), indicating a symbiotic role for hemin uptake. However, the data reported here suggest that hemin uptake in the light organ is insufficient to support robust colonization if *de novo* synthesis is blocked. Given the Fur-dependent upregulation of hemin uptake in response to low-iron conditions (30), it seems likely that iron acquisition is the symbiotic function of hemin scavenging by *V. fischeri* in the squid light organ.

Interestingly, mutants with transposon insertions in *hemA* and *hemL* appeared to colonize *E. scolopes* as well as the wild type (Fig. 2). A mutant engineered with an in-frame deletion of *hemA* was similarly able to colonize the host light organ (Fig. 3A), although it displayed a 4-fold defect in competition with the wild type (Fig. 3B). The

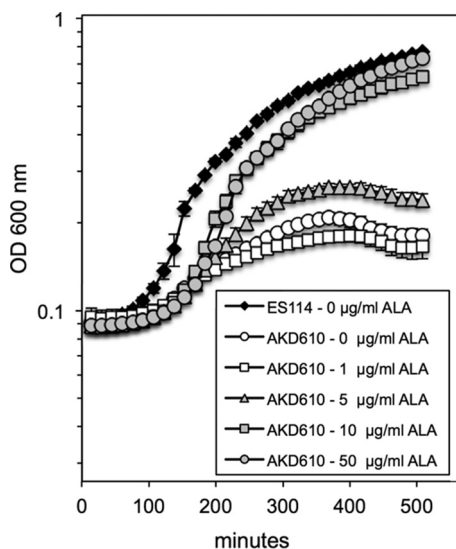


FIG 4 Concentration-dependent recovery of $\Delta hemA$ mutant growth by ALA. Wild-type strain ES114 and the $\Delta hemA$ mutant AKD610 were grown in LBS supplemented with ALA as indicated. Bars indicate standard errors ($n = 3$). For clarity, cultures of ES114 with added ALA were not graphed, but addition of ALA to ES114 did not affect its growth significantly.

hemA and *hemL* genes encode enzymes for the first two dedicated steps in heme biosynthesis, leading to production of ALA, and growth of the *hemA* and *hemL* mutants could be supported by supplementing the medium with ALA. We hypothesized that the *hemA* and *hemL* mutants are able to colonize the light organ using ALA obtained from the host; however, we considered the alternative possibility that their growth in the light organ is actually supported by heme. This alternative seemed unlikely given that other heme-dependent mutants (e.g., a *hemB* mutant) impaired in the heme biosynthetic pathway downstream of ALA cannot colonize the host (Fig. 2), but this observation could potentially be explained if accumulation of heme pathway intermediates from blocked *de novo* synthesis were more toxic to cells during growth in the host, or if heme uptake was accompanied by breakdown and recycling to ALA in the host. We therefore generated and tested a $\Delta hemA$ mutant that also has a $\Delta hutD-hutZ$ (VF_1220 to VF_1228) deletion of the heme uptake locus, and we found that it, too, can colonize the *E. scolopes* light organ (Fig. 3A). Thus, it seems more likely that ALA, and not heme, is available from the host and supports colonization by the *hemA* and *hemL* mutants.

To put these results into context, we tested how much ALA is required to restore growth to cells in culture (Fig. 4). We saw a dose-dependent response to a range of ALA concentrations, from 50 $\mu\text{g ml}^{-1}$ of ALA, which restored nearly wild-type growth, to 1 $\mu\text{g ml}^{-1}$ of ALA, which had no detectable effect (Fig. 4). Intermediate growth recovery was observed with 5 or 10 $\mu\text{g ml}^{-1}$ of ALA. When the $\Delta hemA$ mutant was cocultured with the wild type for 20 generations in LBS containing 50 $\mu\text{g ml}^{-1}$ of ALA, the relative competitiveness index (RCI) was between 0.97 and 0.98 per generation, whereas the RCI during competitive colonization of the host (Fig. 3B), similarly normalized to a per-generation metric by estimating 20 to 30 generations in the host, was between 0.93 and 0.95. A recent survey of ALA in foods indicated that the amount of ALA in squid is near or below the lower end of the range we tested, although the squid species and tissue were not reported (53). Given the amounts of ALA in various animal tissues, it seems neither obvious nor impossible that ALA should be available in sufficient quantity to support growth in the *E. scolopes* light organ.

Our results are remarkably reminiscent of the *Bradyrhizobium*-legume symbiosis, where the symbionts' *hemA* is dispensable, *hemB* is not, and ALA provided by the host is taken up by the colonizing bacteria (54–56). Such availability of ALA does not seem

to be universal in host-associated bacteria. For example, a *hemA* mutant of *Vibrio cholerae* was significantly (10-fold) attenuated in colonizing the infant mouse gut (57), and a *Staphylococcus aureus hemA* mutant was similarly reduced in virulence and colonization of hearts and livers in a mouse model (58). In the case of *V. fischeri*, symbionts in the light organ are provisioned with many amino acids and peptides from the host (59), which may prime cells for uptake of ALA via peptide transport systems (60). The observation that a $\Delta hemA$ mutant is slightly outcompeted by the wild type in mixed inoculations could indicate that *de novo* synthesis of ALA allows somewhat faster growth than uptake, that the supply of ALA is limiting for the mutant in the light organ, or that the availability of ALA is not spatially and temporally uniform in the different microenvironments of this symbiotic tissue.

Conclusions of this mutagenic screen. In conclusion, by simply amending a rich medium with extra ingredients, we identified new and interesting mutants that have provided insights that would be missed in the defined mutant library generated on LBS. The same approach could likewise extend the gains made from defined mutant libraries of other bacteria, although the specific additives that prove useful may differ. It should be noted that our screen is not entirely saturated, and we know at least two additional mutants could be isolated by this approach. Specifically, a hemin-dependent *hemN* mutant was isolated but was removed from the study when it was found to contain integrated vector sequences outside the transposon, and a Mg^{2+} -dependent *gspE* mutant disrupted in the type II general secretion apparatus (61) was inadvertently discarded from our freezer stocks. However, as we have now isolated independent mutants in some of the same loci multiple times (Table 1), the screen is nearing saturation. Future screening for mutants with mutations of conditionally essential genes is more likely to reveal new insights if done under different conditions, for example, screening for mutants that can grow anaerobically but not aerobically. In any case, this study has yielded new insights and provided strains that will facilitate further studies of *Vibrio fischeri*'s cell biology and symbiotic lifestyle.

MATERIALS AND METHODS

Bacteria and media. *V. fischeri* strain ES114, which was isolated from an *E. scolopes* light organ (62), was the wild-type strain used in this study. Plasmids were transformed into *Escherichia coli* strain DH5 α (63) or DH5 α λ pir (64), with the latter used for plasmids with the R6K replication origin. Plasmids were transferred to *V. fischeri* via triparental mating using *E. coli* strain CC118 λ pir (65) carrying the conjugative helper plasmid pEV5104 as previously described (66).

E. coli was grown at 37°C in LB medium (67) or brain heart infusion (BHI) medium. *V. fischeri* was grown at 28°C (unless otherwise stated). The media used for *V. fischeri* include SWTO (68) and ASWT (30), both of which contain yeast extract (3 g liter⁻¹), tryptone (5 g liter⁻¹), and glycerol (3 ml liter⁻¹) and have 700 ml liter⁻¹ of Instant Ocean mixed to 36 ppt (Aquarium Systems, Mentor, OH) in place of seawater in the recipe Boettcher and Ruby used for SWT (62), with SWTO also including extra NaCl (~4.4 g liter⁻¹) so that its osmolarity approximated that of seawater. Most culturing was performed in LBS, which contains (per liter) 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 50 mM Tris (pH 7.5) (29), or SLBS, which consisted of LBS with added riboflavin (400 μ g ml⁻¹), D-alanine (400 μ g ml⁻¹), D-glutamate (400 μ g ml⁻¹), diaminopimelic acid (DAP; 100 μ g ml⁻¹), N-acetylglucosamine (NAG; 1 mM), ribose (2 mM), succinate (5 mM), glycerol (5 mM), hemin (20 μ g ml⁻¹), 2,2-bipyridyl (16 μ g ml⁻¹), biotin (1 μ g ml⁻¹), NAD (NAD⁺; 25 μ g ml⁻¹), folate (15 μ g ml⁻¹), thymidine (50 μ g ml⁻¹), cytidine (50 μ g ml⁻¹), uracil (50 μ g ml⁻¹), and MgSO₄ (20 mM). These supplements, as well as 50 μ g ml⁻¹ of δ -aminolevulinic acid (ALA), 20 mM MgCl₂, or 1 mM glucosamine, were also added to LBS or SWTO individually at the concentrations indicated above to test their ability to support growth of particular mutants.

Solid media were prepared with 15 mg ml⁻¹ of agar for plating. Selection of *E. coli* was accomplished using chloramphenicol (CAM) or kanamycin (KAN) in LB at final concentrations of 20 and 40 μ g ml⁻¹, respectively, or using erythromycin (ERM) in BHI at a final concentration of 150 μ g ml⁻¹. Selection of *V. fischeri* was accomplished using CAM, KAN, and ERM in LBS at final concentrations of 2, 100, and 5 μ g ml⁻¹, respectively.

Transposon mutagenesis. We used pEV5170 to deliver a mini-Tn5 transposon that includes the R6K origin of replication, two bidirectional rho-independent transcriptional terminators, and a gene encoding resistance to ERM (27). Mutants were generated as previously described (27), except that mutants were selected by plating onto SLBS (rather than LBS) containing ERM. Plates were incubated overnight at ~25°C, individual colonies were patched onto both LBS and SLBS containing ERM, and these patch plates were incubated overnight at ~25°C. Patches that grew on SLBS, but not on LBS, were examined further. To confirm that the ERM-resistant clones contained transposon insertions and not the entire pEV5170 plasmid, mutants were screened for KAN resistance, which is encoded outside the transposon on pEV5170, and KAN-resistant mutants were disregarded. Transposon insertion sites were determined by

cloning insertions from genomic DNA and sequencing across the genome-transposon junctions as previously described (27, 35). For this cloning, DNA ligase and restriction enzymes were acquired from New England Biolabs (Beverly, MA), the GenElute plasmid miniprep kit (Sigma-Aldrich, Inc., St. Louis, MO) was used to purify plasmids, and the DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA) was used to purify DNA from digestion and ligation reactions. Sequencing was performed at the University of Michigan DNA Sequencing Core Facility.

Targeted mutant construction. We generated a targeted $\Delta hemA$ allele, with an in-frame deletion of the gene formed by placing an NheI site between the second codon and the stop codon. Sequence upstream of *hemA* and including the first two codons was PCR amplified using primers 5'-ATGTCAGGCACGGTGGAAA CCT-3' (hemAmut1F) and 5'-ACAGCTAGCTGACATTCGGCAAACGGACCAAT-3' (hemAmut1R), and the amplicon was directly blunt-end cloned into SmaI-digested pEV5122 (64). Sequence from the *hemA* stop codon and extending downstream of *hemA* was PCR amplified using primers 5'-ACAGCTAGCTAAGCGTAGCGT TCGTACCAAC-3' (hemAmut2F) and 5'-AGCGGTTTGGGTTCAAACGAACAT (hemAmut2R), and the amplicon was directly cloned into pCRBlunt-II-TOPO (Invitrogen, Carlsbad, CA). The plasmids with these sequences flanking *hemA* were linearized with NheI and fused to generate pAKD610, which contains the *hemA* deletion allele. The $\Delta hemA$ allele on pAKD610 was exchanged into ES114 and the hemin uptake-defective $\Delta hutD-hutZ$ (VF_1220-VF_1228) mutant AKD910 (30) to generate mutants AKD610 and AKD917, respectively. The cloned regions in pAKD610 were confirmed by sequencing, and allelic-exchange mutants were confirmed by PCR.

Complementation with *ftsQAZ*. The *ftsQAZ* locus and a small fragment of the downstream *lpxC* gene were PCR amplified from ES114 genomic template using primers 5'-CGCAAGCGCTGGAGATGTTGG-3' (JLSftsQF2) and 5'-GGATCCGACGGAAATCAACTGG-3' (JLSftsZR2), and the resulting amplicon was cloned into pCRBlunt-II-TOPO using the Zero Blunt Topo PCR cloning kit (Invitrogen), generating pJLS233. A portion of the insert containing only *ftsQAZ* was then PCR amplified from pJLS233 using primers 5'-GTAAAACGACGGCCAG-3' (M13F) and 5'-TCAGGGTACCAGATTAATTAGTCAGCTTGGCGT-3' (JLSftsZR; the underlined sequence is a KpnI site), the amplicon was digested with SphI and KpnI, and the resulting fragment was ligated into SphI- and KpnI-digested pVSV105, which is a stable shuttle vector derived from a plasmid native to *V. fischeri* (64, 69). The resulting plasmid, pJLS234, and its pVSV105 parent were transferred into *V. fischeri* in conjugative matings performed on LBS plates supplemented with 20 mM MgCl₂.

Microscopy. Bacteria were viewed by phase-contrast microscopy using a Nikon (Melville, NY) Eclipse E600 microscope. Images were captured with a Nikon Coolpix 5000 camera and edited using the recolor, contrast, brighten, and sharpen features in PowerPoint for Mac 2011 (Microsoft, Redmond, WA). Scale was determined by comparison to an image taken of a micrometer slide.

Dose-dependent reliance of *hemA* mutant on ALA. To assay restoration of growth to the *hemA* mutant with ALA, three independent cultures each of ES114 and AKD610 were grown overnight in LBS medium and in LBS medium containing 50 μ g/ml of ALA, respectively. A 100- μ l aliquot of culture was pelleted, the supernatant was removed, and the pellets were resuspended in 1 ml of filter-sterilized Instant Ocean. Two-microliter volumes of the resuspended pellets were used to inoculate 200 μ l of LBS medium containing 50, 10, 5, 1, or 0 μ g ml⁻¹ of ALA in a 96-well plate. The plate was incubated with shaking (double orbital shaking 282 cpm [3 mm]) in a Synergy H1 microplate reader (BioTek, Winooski, VT), with absorbance readings (600 nm) taken approximately every 15 min. The experiment was repeated three times, with results of one representative experiment shown in Fig. 4.

Motility assays. Semisolid media for assessing swimming motility was prepared by adding 2.5 mg ml⁻¹ of agar to ASWT medium, amended as necessary depending on requirements of the mutants. Overnight cultures were diluted and spotted onto motility plates, and the diameters of areas visibly covered by swimming cells were measured periodically to determine a swimming rate. The motile but ERM resistance-marked ES114 derivative AKD100 (30) and the nonmotile *flaJ::aph* mutant DM131 (70) were used as positive and negative motility controls, respectively.

Colonization of *E. scolopes*. Aposymbiotic *E. scolopes* hatchlings were inoculated with *V. fischeri* using previously described procedures (71, 72). Briefly, to prepare inocula, cultures were grown to an optical density at 595 nm (OD₅₉₅) between 0.4 and 0.7 in 5 ml of ASWT containing the required supplement in 50-ml conical tubes at 28°C without shaking. Cultures were diluted to ~3,000 CFU ml⁻¹ in Instant Ocean, and hatchling squid were added to 5-ml aliquots of this inoculum individually in 20-ml vials. After 12 to 14 h, hatchlings were rinsed into *V. fischeri*-free Instant Ocean. Although inocula ranged from 1,500 to 5,000 CFU ml⁻¹, within each experiment squid were exposed to similar concentrations of mutant or wild-type cells in the respective inocula. Infected squid were homogenized at the desired time postinoculation and then serially diluted and plated onto LBS containing the supplement required by the mutant being tested. Plates were incubated overnight at 28°C, and the colonies were counted to calculate CFU per squid. For competition experiments, the mutant and wild-type strains were mixed close to a 1:1 ratio, and the relative competitiveness index (RCI) was calculated as the ratio of mutant to wild type in each animal divided by the ratio of these strains in the inoculum. The results reported either are from multiple experiments or are representative of multiple independent colonization assays.

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