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Antisocial *luxO* Mutants Provide a Stationary-Phase Survival Advantage in *Vibrio fischeri* ES114

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ABSTRACT

The squid light organ symbiont *Vibrio fischeri* controls bioluminescence using two acyl-homoserine lactone pheromone-signaling (PS) systems. The first of these systems to be activated during host colonization, AinS/AinR, produces and responds to *N*-octanoyl homoserine lactone (C_8-AHL). We screened activity of a P_{ainS}-*lacZ* transcriptional reporter in a transposon mutant library and found three mutants with decreased reporter activity, low C_8-AHL output, and other traits consistent with low *ainS* expression. However, the transposon insertions were unrelated to these phenotypes, and genome resequencing revealed that each mutant had a distinct point mutation in *luxO*. In the wild type, LuxO is phosphorylated by LuxU and then activates transcription of the small RNA (sRNA) Qrr, which represses *ainS* indirectly by repressing its activator LitR. The *luxO* mutants identified here encode LuxU-independent, constitutively active LuxO* proteins. The repeated appearance of these *luxO* mutants suggested that they had some fitness advantage during construction and/or storage of the transposon mutant library, and we found that *luxO mutants survived better and outcompeted the wild type in prolonged stationary-phase cultures. From such cultures we isolated additional *luxO** mutants. In all, we isolated LuxO* allelic variants with the mutations P41L, A91D, F94C, P98L, P98Q, V106A, V106G, T107R, V108G, R114P, L205F, H319R, H324R, and T335I. Based on the current model of the *V. fischeri* PS circuit, *litR* knockout mutants should resemble *luxO** mutants; however, *luxO** mutants outcompeted *litR* mutants in prolonged culture and had much poorer host colonization competitiveness than is reported for *litR* mutants, illustrating additional complexities in this regulatory circuit.**

IMPORTANCE

Our results provide novel insight into the function of LuxO, which is a key component of pheromone signaling (PS) cascades in several members of the *Vibrionaceae*. Our results also contribute to an increasingly appreciated aspect of bacterial behavior and evolution whereby mutants that do not respond to a signal from like cells have a selective advantage. In this case, although "antisocial" mutants locked in the PS signal-off mode can outcompete parents, their survival advantage does not require wild-type cells to exploit. Finally, this work strikes a note of caution for those conducting or interpreting experiments in *V. fischeri*, as it illustrates how pleiotropic mutants could easily and inadvertently be enriched in this bacterium during prolonged culturing.

Many bacteria use pheromones to regulate group behaviors (1). These pheromone-signaling (PS) systems generally require sufficiently high cell density for pheromone accumulation and are also regulated in response to the environment (2–6). Accordingly, PS outputs depend on both population density and an appropriate context. The importance of such control is easily rationalized, given the energetic cost of many PS-activated processes, such as bioluminescence (7, 8). This cost of inducing PS-controlled systems is underscored by the observation that spontaneous signal-blind "antisocial" PS-negative mutants often appear in populations, and it is thought that these mutants are enriched as "cheaters" if their lack of participation in group behavior minimizes their own costs while exploiting the presence of PS-positive relatives (9–11).

Vibrio fischeri is an excellent model for studying PS and was central to the discovery of cell-cell communication in bacteria (12). *V. fischeri* is a bioluminescent light organ symbiont that controls luminescence and other phenotypes using three distinct but interconnected PS systems, with the signal synthase/receptor combinations LuxI/LuxR, AinS/AinR, and LuxS/LuxPQ (13–18). Luminescence is induced largely by LuxI/LuxR, which produces and responds to *N*-3-oxo-hexanoyl homoserine lactone. However, *luxR* itself is controlled in part by the other two systems, and LuxR can be activated by the AinS-produced pheromone *N*-octanoyl-homoserine lactone (C_8-AHL). AinS/AinR also controls motility and genes important for initiating colonization of *V. fischeri*'s squid host, *Euprymna scolopes* (18, 19). The LuxS/LuxPQ system, which synthesizes and responds to autoinducer 2 (AI-2) (20, 21), uses the same core signal transduction pathway as AinS/AinR, but because LuxS/AI-2 has only modest effects in *V. fischeri* under the conditions tested (17), we have focused more on AinS/AinR.

The AinS/AinR PS system controls *luxR* and other genes through a core PS circuit (Fig. 1) that is conserved in the *Vibrionaceae*. Much of this PS cascade was elucidated in *Vibrio harveyi*, and recent studies have verified parallel functions with subtle differences in *V. fischeri* (17–19, 22–26). In the model that has

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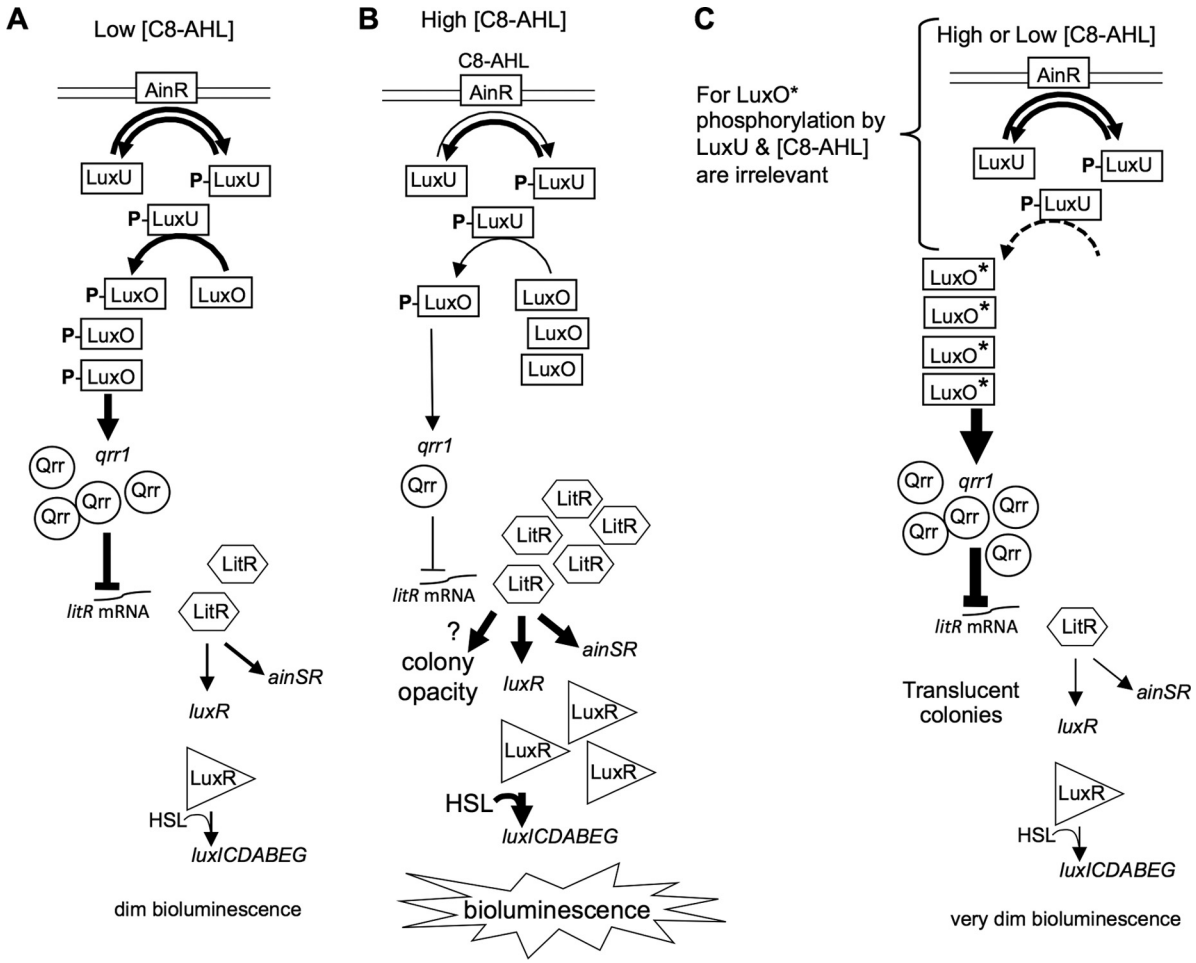

FIG 1 Model showing roles of Ain and LuxO in the pheromone regulatory circuit. The pheromone receptor AinR phosphorylates and dephosphorylates LuxU, which phosphorylates LuxO, stimulating it to activate transcription of *qrr*. The regulatory small RNA (sRNA) Qrr represses expression of the master regulator LitR. (A) At low C_8-AHL levels, LuxO-P ultimately leads to relatively low LitR levels. (B) At high C_8-AHL levels, binding of C_8-AHL to AinR reduces its kinase activity, reducing the relative amounts of phosphorylated LuxU and LuxO, resulting in more LitR and induction of the LitR regulon, including *luxR*, bioluminescence, and *ainSR*. On plates, this induction also results in colony opacity through an unknown mechanism. (C) The activity of LuxO* does not require phosphorylation, although prior to this study, the effects of LuxU were untested. LuxO* essentially locks this circuit on "off," and phenotypes resemble those at low C_8-AHL levels. The colonies of *luxO** mutants on plates remain translucent despite their high cell density.

emerged (Fig. 1A and B), at low pheromone concentrations (Fig. 1A), AinR phosphorylates LuxU, which in turn phosphorylates the σ^{54}-dependent activator LuxO. LuxO-P activates transcription of a small RNA, Qrr, which posttranscriptionally represses the PS master regulator *litR* (24, 26). In contrast, when C_8-AHL accumulates to higher levels (Fig. 1B), its binding to AinR is thought to decrease AinR's kinase activity, allowing AinR's phosphatase activity to dominate, resulting in more unphosphorylated LuxO, deactivation of *qrr*, and induction of the LitR regulon, which includes *ainSR* and *luxR*. LitR homologs are widespread master regulators of the PS-dependent phenotypes in other members of the *Vibrionaceae* (17, 22, 23). Spontaneous mutations in *Vibrio cholerae hapR* and *Vibrio parahaemolyticus opaR*, which encode their respective PS master regulators, have been enriched under some conditions (11, 27–29), but no parallel to these observations has been reported in *V. fischeri*.

The *V. fischeri* AinS/AinR system is activated early during colonization of its symbiotic host squid and is responsible for priming LuxI/LuxR-based symbiotic luminescence (18). Given that luminescence is only weakly induced outside the host and that AinS/AinR apparently sits atop the PS hierarchy early in infection, regulatory controls over *ainSR* may reveal important elements of the host environment encountered during symbiosis establishment. Only cyclic AMP receptor protein (CRP) and LitR are known to activate *ainSR* (17, 22, 30), and the goal of this study was to discover new regulators of *ainSR*. However, we unwittingly isolated spontaneous mutants in the core PS circuitry, providing insight into its function and revealing conditions under which such mutants can be enriched.

MATERIALS AND METHODS

Bacteria, growth media, and reagents. Bacterial strains are listed and briefly described in Table 1. *V. fischeri* ES114 was the wild-type strain used throughout (31). Plasmids were transformed into *Escherichia coli* strain DH5α (32) or DH5αλ*pir* (33) in the case of plasmids with the R6K origin of replication. *E. coli* was grown in LB medium (34) or brain heart infusion (BHI) medium (Bacto), and *V. fischeri* was grown in LB salt (LBS) medium (35), seawater-tryptone marine-osmolarity (SWTO) medium (36),

TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s)[a]	Source or reference
E. coli strains		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	41
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	32
DH5α λ*pir*	DH5α lysogenized with λ*pir*	33
V. fischeri strains		
AKD100	ES114 Tn*7-ermR*	47
AKD200	ES114 Tn*7-camR*	48
CG103	Wild-type isolate from *Cleidopus gloriamaris*	84
CL59	ES114 *luxO*(*D47E*)	19
DC22	C_8-AHL bioreporter: ES114 Δ*ainS* Δ*luxR-luxI*, mutant *luxR* (MJ1-T33A, R67M, S116A, M135I), P_{luxI}-*luxCDABEG*	22
DM131	ES114 *flaJ*::*kanR*	48
EM17	Wild-type isolate from *Euprymna morsei*	85
ES114	Wild-type isolate from *E. scolopes*	31
ES12	Wild-type isolate from *E. scolopes*	86
ES213	Wild-type isolate from *E. scolopes*	86
ES401	Wild-type isolate from *E. scolopes*	85
ET101	Wild-type isolate from *Euprymna tasmanica*	87
H905	Planktonic isolate, Hawaii	76
KV1319	ES114 with 2-bp insertion in *celG*	51
KV5005	ES114 Δ*rpoN*	43
JB18	ES114 *litR*::*ermR*	22
JHK057	ES114 *luxO*(*V106A*)	This study
JHK061	ES114 *luxO*(*T335I*)	This study
JHK062	ES114 *luxO*(*V106A*) Δ*rpoN*	This study
JHK063	ES114 *luxO*(*T335I*) Δ*rpoN*	This study
JHK065	ES114 *luxO*(*V106A*) *luxU*::pEVS122	This study
JHK066	ES114 *luxO*(*T335I*) *luxU*::pEVS122	This study
JHK068	ES114 *luxU*::pEVS122	This study
JHK069	ES114 *luxO*(*D47E*) *luxU*::pEVS122	This study
JHK070	ES114 *luxO*(*V106G*)	This study
JHK073	ES114 *luxO*(*V106A*) *litR*::*ermR*	This study
JHK074	ES114 *luxO*(*A91D*)	This study
JHK075	ES114 *luxO*(*P41L*)	This study
JHK076	ES114 *luxO*(*A91D*) *luxU*::pEVS122	This study
JHK077	ES114 *luxO*(*P41L*) *luxU*::pEVS122	This study
JHK087	ES114 *luxO*(*V106G*) *luxU*::pEVS122	This study
JHK105	ES114 *luxO*(*T335I*) Δ*qrr*	This study
NL60	ES114 Δ*ainS*	4
PMF8	ES114 *litR*::*kanR*	23
PP3	Planktonic isolate, Hawaii	76
TIM305	ES114 Δ*qrr*	24
TIM306	ES114 Δ*luxO*	24
TME014F5	ES114 *mtsA*::mini-Tn*5 ermR*	This study
TME014B6	ES114 *znuC2*::mini-Tn*5 ermR*	This study
TME021D9	ES114 *yfhD*::mini-Tn*5 ermR*	This study
VFS002F6	ES114 *vf0295*::mini-Tn*5 ermR*	C. Whistler
VFS002F6-T[b]	ES114 *vf0295*::mini-Tn*5 luxO*(*A91D*) *ermR*	C. Whistler
VFS012E9	ES114 *yfbQ*::mini-Tn*5 ermR*	C. Whistler
VFS012E9-T	ES114 *yfbQ*::mini-Tn*5 luxO*(*P41L*) *ermR*	C. Whistler
VFS014F5-T	ES114 *mtsA*::mini-Tn*5 luxO*(*V106A*) *ermR*	C. Whistler
VFS014B6	ES114 *znuC2*::mini-Tn*5 ermR*	C. Whistler
VFS014B6-T	ES114 *znuC2*::mini-Tn*5 luxO*(*V106G*) *ermR*	C. Whistler
VFS021D9-T[c]	ES114 *yfhD*::mini-Tn*5 luxO*(*T335I*) *ermR*	C. Whistler
VLS2	Wild-type isolate from *E. scolopes*	84
WH1	Planktonic isolate, Massachusetts	84
WTTG0	ES114 *luxO*(*T107R*)	This study
WTTG1	ES114 *luxO*(*F94C*)	This study
WTTG2	ES114 *luxO*(*R114P*)	This study
WTTG3	ES114 *luxO*(*H324R*)	This study
WTTG4	ES114 *luxO*(*P98Q*)	This study
WTTG5	ES114 *luxO*(*R113L*)	This study
WTTG12	ES114 *luxO*(*P98L*)	This study
WTTG17	ES114 *luxO*(*V108G*)	This study

(Continued on following page)

TABLE 1 (Continued)

Strain, plasmid, or oligonucleotide	Relevant characteristic(s)[a]	Source or reference
WTTG20	ES114 *luxO*(*V106G*)	This study
WTTG21	ES114 *luxO*(*V106G*)	This study
WTTG22	ES114 *luxO*(*H319R*)	This study
WTTG24	ES114 *luxO*(*L205F*)	This study
Plasmids[d]		
pAKD701	Promoterless *lacZ*, pES213 R6Kγ $oriT_{RP4}$ *kanR*	37
pEVS79	pBC SK (+) *oriT camR*	40
pEVS104	Conjugative helper plasmid, R6Kγ $oriT_{RP4}$ *kanR*	40
pEVS122	R6Kγ $oriT_{RP4}$ *ermR lacZ*α	33
pEVS170	Mini-Tn*5*-*ermR* R6Kγ $oriT_{RP4}$ *kanR*	50
pHK10	P_{ainS}-*lacZ* reporter in pAKD701, pES213 R6Kγ $oriT_{RP4}$ *kanR*	This study
pHK11	*mtsA* in pVSV104, *kanR*	This study
pHK12	P_{ainS}-*gfp* P_{con}-mCherry in pJLS27, pES213 R6Kγ $oriT_{RP4}$ *kanR camR*	This study
pHK20	P_{qrr}-*lacZ* reporter in pAKD701, pES213 R6Kγ $oriT_{RP4}$ *kanR*	22
pHK29	*znuC2* in pVSV104, *kanR*	This study
pHK45	ES114 *luxO* in pVSV105, *camR*	This study
pHK70	*luxO*(*V106A*) in pVSV105, *camR*	This study
pHK71	*luxO*(*D47E*) in pVSV105, *camR*	This study
pHK73	*luxO*(*T335I*) in pVSV105, *camR*	This study
pHK77	*luxO*(*V106A*) and flanking sequence in pEVS79, *camR*	This study
pHK78	*luxO*(*T335I*) and 1.5 kbp flanking sequence in pEVS79, *camR*	This study
pHK79	228-bp *luxU* fragment in pEVS122, *ermR*	This study
pHK80	*luxO*(*V106G*) and flanking sequence in pEVS79, *camR*	This study
pHK82	*luxO*(*V106G*) in pVSV105, *camR*	This study
pHK83	*luxO*(*A91D*) in pVSV105, *camR*	This study
pHK84	*luxO*(*P41L*) in pVSV105, *camR*	This study
pHK85	*luxO*(*P41L*) and flanking sequence in pEVS79, *camR*	This study
pHK86	*luxO*(*A91D*) and flanking sequence in pEVS79, *camR*	This study
pHK87	*luxO*(*T335I*) and 1.1-kbp flanking sequence in pEVS79, *camR*	This study
pJLB95	*litR*::*ermR* (opposite), ColE1 *camR*	This study
pJLS27	Promoterless *gfp*, P_{con}-mCherry pES213 R6Kγ $oriT_{RP4}$ *kanR*	38
pLosTfoX	*tfoX* in pEVS79, *camR*	42
pMSM28	Δ*rpoN* allele, R6Kγ $oriT_{RP4}$ *camR*	43
pMulTfoX	*tfoX* in pVSV104, *kanR*	42
pTM267	*kan gfp* P_{tetA}-mCherry in pVSV105, *camR*	24
pTM268	P_{qrr}-*gfp* P_{tetA}-mCherry in pVSV105, *camR*	24
pVSV104	Shuttle vector; pES213 R6Kγ $oriT_{RP4}$ *kanR lacZ*α	39
pVSV105	Shuttle vector; pES213 R6Kγ $oriT_{RP4}$ *camR lacZ*α	39
Oligonucleotides[e]		
pr_HK03	GGGGCATGCAGAACCAAGACCTGCTCGTGCTAA	This study
pr_HK04	GGCGCTAGCCATCAGTTGTTGAAGTAAATTAAAATTCTGCG	This study
pr_HK05	CATGGTACCATATAGCCGTCTAGATGTAAACATTTCCAAACCG	This study
pr_HK06	CATCCTAGGTTATGGCGCCTCTGTTAAATTAGTACTTTGTTTT	This study
pr_HK07	CATGGTACCAAAATTAATTGTTATGTTATAACATAACAATTAAATAGCC	This study
pr_HK08	CATCCTAGGCATCCAACATTCAGTACACTCCC	This study
pr_HK29	GGCTCTAGACATCAGTTGTTGAAGTAAATTAAAATTCTGCG	This study
pr_HK73	CATGGCATGCATATACCTATTGCAGGGAGCGTGC	This study
pr_HK74	CATGGGTACCCAGCGATTTGATTAACATACTGACTCACGATAG	This study
pr_HK93	CATGGGATCCAGACTATTTATGTCTCAGCCACACC	This study
pr_HK94	CATGGGTACCATCGCCAAATCATGATTGG	This study
pr_HK95	CATGGGATCCTCGGAAGTTGCAGAAGAAGG	This study
pr_HK96	CATGGGTACCTGGTTCCACAGGCCGTATAC	This study
pr_HK101	CATGGGATCCGGGACTATCGTGAGTCAGTA	This study
pr_HK102	CATGGGATCCTGCCATTGTTGCAAGCTTATCT	This study
pr_HK110	CATGGGATCCTGCAAATTGCGTTTTGCG	This study
pr_HK111	CATGGGTACCGCTGTCAAACAAGCGGATTTAATA	This study

[a] Genes for drug resistance are as follows: *camR*, chloramphenicol resistance; *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*).
[b] Strains designated with the prefix "VFS" and the suffix "-T" were isolated from a mapped-transposon mutant library and represent the translucent (T) *luxO**-bearing derivatives of the original transposon mutant parent. Strains designated with "WTTG" were isolated in a series of independent static culture survival experiments.
[c] VFS021D9-T lacked the Kan resistance gene from the transposon delivery vector but displayed some Kan resistance.
[d] All alleles cloned in this study are from *V. fischeri* strain ES114. Replication origins of the vectors are listed as R6Kγ, ColE1, *oriV*, and/or pES213. Plasmids based on pES213 are stable and do not require antibiotic selection for maintenance (39).
[e] All oligonucleotides are shown 5′ to 3′. Restriction enzyme recognition sequences are underlined.

or Fischeri minimal medium (FMM) (4). Solid media were prepared with 15 g liter^{-1} agar. For selection of *E. coli*, chloramphenicol (Cam) and kanamycin (Kan) were added to LB at final concentrations of 20 and 100 μg ml^{-1}, respectively, and erythromycin (Erm) was added to BHI at a final concentration of 150 μg ml^{-1}. For selection of *V. fischeri* on LBS, the concentrations of Cam, Erm, and Kan used were 2, 5, and 100 μg ml^{-1}, respectively. For colorimetric screening of β-galactosidase activity, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to LBS at 100 μg ml^{-1}. C_8-AHL was obtained from Sigma-Aldrich (St. Louis, MO).

Molecular genetics and sequence analysis. Oligonucleotides and plasmids are listed in Table 1, and the latter were constructed using standard techniques and materials as described previously (22). Genomic DNA (gDNA) for genome resequencing and natural transformation was purified using the Easy-DNA gDNA purification kit (Life Technologies, Grand Island, NY).

The P_{ainS}-*lacZ* transcriptional reporter plasmid pHK10 was generated by PCR amplifying 428 bp upstream of *ainS* using primers pr_HK03 and pr_HK04, digesting the resulting amplicon with SphI and NheI, and cloning this fragment between the SphI and NheI sites of pAKD701 (37). To generate the P_{ainS}-*gfp* transcriptional reporter, pHK12, the same promoter region used in pHK10 was amplified using primers pr_HK03 and pr_HK29, digested with SphI and XbaI, and ligated into similarly digested pJLS27 (38). To generate pHK45, pHK70, pHK71, pHK73, pHK82, pHK83, and pHK84, *luxO* was amplified from ES114, VFS014F5-T, CL59 (19), VFS021D9-T, VFS014B6-T, VFS002F6-T, and VFS012E9-T, respectively, using primers pr_HK73 and pr_HK74. The resulting amplicons were digested with SphI and KpnI and ligated into SphI- and KpnI-digested pVSV105 (39). To generate pHK11, *mtsA* was amplified from ES114 using primers pr_HK05 and pr_HK06. The resulting amplicon was digested with KpnI and AvrII and ligated into KpnI- and AvrII-digested pVSV104 (39). To generate pHK29, *znuC2* was amplified from ES114 using primers pr_HK07 and pr_HK08. The resulting amplicon was digested with KpnI and AvrII and ligated into KpnI- and AvrII-digested pVSV104.

Mutant alleles were transferred from *E. coli* into *V. fischeri* on plasmids by triparental matings using the conjugative helper strain CC118λ*pir* pEVS104 (40, 41). Recombination and marker exchange were identified by screening for antibiotic resistance, and putative mutants were tested by PCR. Transposon insertions were placed in new strain backgrounds using competence induced by overexpression of *tfoX* from pMulTfoX or pLosTfoX, followed by plasmid curing, as previously described (42). Strains TME014F5, TME014B6, and TME021D9 were generated using DNA from transposon mutants VFS014F5-T, VFS014B6-T, and VFS021D9-T, respectively, using *tfoX*-mediated transformation (42). To place spontaneous *luxO* mutations in fresh strain backgrounds, 1.5-kbp regions flanking the mutation sites were PCR amplified using primer sets pr_HK93 and pr_HK94 (P41L, A91D, V106A, and V106G alleles) or pr_HK95 and pr_HK96 (T335I allele), respectively. Amplicons were digested with KpnI and BamHI and ligated into similarly digested pEVS79 (40) to create pHK77 [*luxO*(*V106A*)], pHK78 [*luxO*(*T335I*)], pHK80 [*luxO*(*V106G*)], pHK85 [*luxO*(*P41L*)], and pHK86 [*luxO*(*A91D*)]. Strains JHK057, JHK061, JHK070, JHK074, and JHK075 were generated by exchanging the mutated *luxO* variants on pHK77, pHK78, pHK80, pHK85, and pHK86, respectively, into ES114. To generate the *luxO** Δ*qrr* double mutant, 1.1-kbp regions flanking the mutation site of *luxO**(*T335I*) were PCR amplified using primers pr_HK110 and pr_HK111. This amplicon was digested with KpnI and BamHI and ligated into similarly digested pEVS79 to create pHK87. The insert on pHK87 is smaller than that of pHK78 and does not overlap the *qrr* sequence. Strain JHK105 was generated by exchanging the mutated *luxO* variant on pHK87 into the Δ*qrr* strain TIM305.

To generate *luxU* mutants, an internal 228-bp fragment of *luxU* was amplified using primers pr_HK101 and pr_HK102. The resulting amplicon was digested with BamHI and ligated into BamHI-digested pEVS122 (33) to generate pHK79. The *luxU*::pEVS122 allele of pHK79 was introduced into JHK057, JHK061, ES114, CL59, JHK070, JHK074, and JHK075 to generate strains JHK065, JHK066, JHK068, JHK069, JHK087, JHK076, and JHK077, respectively. To generate *rpoN* mutants, the Δ*rpoN* allele on plasmid pMSM28 (43) was introduced into strains JHK057 and JHK061 to generate strains JHK062 and JHK063, respectively. To generate the *luxO** *litR*::*erm* double mutant, the *litR*::*erm* allele on pJLB95 (22) was introduced into JHK057 to generate strain JHK073.

Genome resequencing. Ten micrograms of gDNA was fragmented by sonication in iced Tris-EDTA buffer five times for 1 min with 1-s pulses at 40% duty using a W-380 ultrasonic processor (Heat Systems-Ultrasonics, Inc.) to produce ~200-bp fragments for Illumina library construction following the Illumina Tru-seq manufacturer's protocol (San Diego, CA), except that genomic DNA adaptors were diluted 1:100 prior to ligation. Sequencing was performed using an Illumina Hi-Seq 2000 genome analyzer at the University of Missouri DNA Core Laboratory. Assembled reads were compared to the resequenced and published ES114 genomes (44, 45) as references using the Integrative Genomics Viewer (46).

Luminescence measurements. Overnight *V. fischeri* cultures were diluted 1:1,000 in 25 ml SWTO medium in 125-ml flasks and incubated with shaking (200 rpm) at 24°C. At regular intervals, the optical density at 595 nm (OD_{595}) was measured for 500-μl samples using a BioPhotometer (Brinkman Instruments, Westbury, NY). Relative luminescence was measured with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) immediately following shaking to aerate the sample. Specific luminescence was calculated as the luminescence per unit of optical density at 595 nm (OD_{595}).

***lacZ* and *gfp* reporter assays.** Strains harboring the P_{ainS}-*lacZ* or P_{qrr}-*lacZ* reporter plasmids pHK10 and pHK20 (22), respectively, or the promoterless parent vector pAKD701, were grown overnight in LBS, subcultured 1:300 into 1.5 ml of SWTO medium in 24-well microtiter plates, and incubated with shaking (200 rpm) at 24°C. Cells were collected at an OD_{595} of ~2.5 by centrifugation, the supernatant was discarded, and the cell pellet was stored overnight at −80°C. β-Galactosidase assays were then performed as previously described (7). Strains harboring the P_{ainS}-*gfp* or P_{qrr}-*gfp* reporter plasmids pHK12 and pTM268 (24), respectively, or the promoterless parent vectors pJLS27 and pTM267 were grown overnight in LBS, subcultured 1:1,000 into flasks containing 25 ml SWTO medium, and incubated with shaking (200 rpm) at 24°C. At regular intervals, 200-μl samples were aliquoted into clear-bottomed, black-walled, 96-well plates, where green fluorescence and OD_{595} were measured using a Synergy 2 plate reader (BioTek).

Motility assays. Motility was determined by diluting overnight cultures 1:1,000 in SWTO medium and growing to an OD_{595} of ~0.5. Five-microliter aliquots of culture were then spotted on the surface of 0.25% agar FMM plates containing 2.2 mM *N*-acetylglucosamine (19). The diameters of areas visibly covered by swimming cells were measured after 24 h of incubation at 28°C. The Erm-resistant ES114 derivative AKD100 and the ES114 *flaJ*::*aph* strain DM131 were used as positive and negative motility controls, respectively (47, 48).

C_8-AHL bioassays. C_8-AHL accumulation was assessed as previously described (22). Briefly, culture supernatants were extracted with acidified ethyl acetate, extracts were dried and resuspended in SWTO medium, and C_8-AHL levels were determined by comparison to standards using the bioassay strain DC22 (22, 49).

Transposition frequency. The frequency of recovering transposon mutants following conjugative introduction of pEVS170 (50) was determined for ES114 and the *luxO* mutant JHK057, each mixed 1:1 with the *celG* mutant KV1319, which is phenotypically wild type except that it can be readily distinguished as white colonies on LBS plates containing 10 mM D-cellobiose and X-Gal (51). Performing transposon (Tn) mutagenesis on 1:1 mixtures of ES114 plus KV1319 or of JHK057 plus KV1319 enabled us to normalize values to transposition frequency in KV1319 and to test whether a *luxO* mutant such as JHK057 could be enriched from a mixed population during transposon mutagenesis. Conjugation spots were in-

cubated at 28°C for 6 h before being resuspended in 500 μl Instant Ocean (Aquarium Systems, Mentor, OH), dilution plated to determine recipient strain ratios, and then stored overnight at −80°C in glycerol stocks. Stocked conjugations were thawed at room temperature and plated on LBS containing D-cellobiose, X-Gal, and Erm to select transposon-containing strains and to use blue/white screening to distinguish each mutant's parent. Erm-resistant colonies were patched on Kan plates to eliminate mutants with the Kan marker outside the transposon on pEVS170.

Viability in static culture. To monitor survival rates of *V. fischeri* strains singly, overnight *V. fischeri* cultures were inoculated into LBS and grown to an OD_{595} of ~2.5 to simulate the inoculum density present in the construction of the transposon mutant library. These cultures were then diluted 1:1,000 in fresh LBS and grown statically in 24-well microtiter plates at 28°C. CFU were determined for the initial inoculum and after 24, 48, and 96 h. To determine mixed-culture viability, strains were grown separately as described above, and at an OD_{595} of ~2.0, cultures were mixed in ratios of 1:1, 10:1, or 100:1 and cocultured until an OD_{595} of ~2.5 was reached, at which point they were subcultured, grown, and sampled as described above. Strains were differentiated by colony opacity (ES114 versus the *luxO** or *litR*::*ermR* strain), Erm resistance (the *luxO** strain versus the *litR*::*ermR* or *luxO** *litR*::*erm* strain), or Cam resistance (AKD200 versus the *luxO** or *luxO** Δ*qrr* strain).

Squid colonization. Competitive colonization of *E. scolopes* hatchlings was determined as previously described (51). Briefly, hatchlings were exposed to an ~1:1 mixture of strains for 12 h, and the ratio of colonizing strains was determined 48 h after initial exposure to the inoculum. The relative competitive index (RCI) was determined by dividing the final mutant-to-wild-type ratio by the ratio of the strains in the inoculum. Unmarked *luxO* mutants were competed against the Cam^r-marked *V. fischeri* strain AKD200, which has wild-type colonization competitiveness (48, 52).

RESULTS

In an attempt to identify regulators of *ainSR*, we used blue/white screening on plates containing X-Gal to test activity of a P_{ainS}-*lacZ* reporter in a library of *V. fischeri* transposon mutants. Collectively, this library has representative mutants with insertions in approximately 2,100 distinct genes (R. Foxall and C. Whistler, personal communication). Mutants VFS021D9-T, VFS014B6-T, and VFS014F5-T, with Tn insertions in *yfhD* (VF_A0984), *znuC2* (VF_2367), and *mtsA* (VF_1566), respectively, had markedly lower P_{ainS}-*lacZ* reporter activity. The Tn insertions from these three mutants were reintroduced into the ES114 wild-type background, and these backcrossed Tn mutants were compared to the original mutants, ES114, and the Δ*ainS* mutant NL60, with respect to P_{ainS}-*gfp* reporter activity and three other phenotypes associated with loss of *ainS*: (i) decreased luminescence in broth, (ii) increased swimming motility, and (iii) lowered production of C_8-AHL (18, 19). VFS021D9-T, VFS014B6-T, and VFS014F5-T displayed decreased P_{ainS}-*gfp* expression (Fig. 2A), relatively dim luminescence (Fig. 2B), hypermotility (Fig. 2C), and low C_8-AHL output (Fig. 2D); however, none of these phenotypes were associated with the Tn insertions backcrossed into ES114 (Fig. 2). Moreover, wild-type copies of *mtsA* and *znuC2* provided in *trans* failed to complement the mutant phenotypes of strains VFS014F5-T and VFS014B6-T (data not shown), further indicating that the Tn insertions in these mutants were not the cause of the *ainS*-related phenotypes.

The genome sequences of VFS014F5-T, VFS014B6-T, and VFS021D9-T confirmed their respective transposon locations and also revealed that each mutant has a unique point mutation in *luxO*, resulting in V106A, V106G, and T335I LuxO variants (Table 2). No other deviations from the wild-type sequence were discovered in the mutants. Given the current model of PS (Fig. 1), the phenotypes of these mutants would be consistent with constitutive LuxO activity, leading to high Qrr expression, low LitR levels, and decreased activation of *ainSR*. A *luxO* mutant with a D47E allele that effectively mimics phosphorylated LuxO shows similar phenotypes (17, 53). The mutants also displayed translucent colony morphology similar to that of *litR* mutants, and we appended a "T" to their strain names to indicate translucent variants. As discussed below, we eventually discovered that there were corresponding Tn library mutants that were not translucent, and adopting this strain nomenclature distinguishes between, for example, original library mutant VFS014F5 (*mtsA*::Tn) and what is presumably its derivative, VFS014F5-T [*mtsA*::Tn *luxO*(*V106A*)].

To test whether the *luxO* alleles have the predicted downstream effect on the core PS circuit, we cloned the spontaneous *luxO* point mutations from the Tn-mutant backgrounds, moved them into ES114 by allelic exchange, and compared P_{qrr}-*gfp* activities in the resulting strains and the original mutants. Consistent with the model in Fig. 1, the mutants encoding V106A, V106G, or T335I LuxO variants, as well as a previously described mutant encoding the LuxO(D47E) variant, yielded higher P_{qrr}-*gfp* expression than the wild type (Fig. 3). In contrast, strains where the Tn insertions had been backcrossed into ES114 with wild-type *luxO* displayed wild-type expression of P_{qrr}-*gfp* (Fig. 3). Thus, the *luxO* mutations and not the Tn insertions were causal to this mutant phenotype. Below, mutant alleles that apparently encode constitutively active LuxO are designated *luxO**, consistent with the nomenclature in previous reports of similar alleles in other vibrios (54).

We explored whether LuxO* variants bypass elements of the PS regulatory circuitry (Fig. 1), first by testing whether the *luxO** mutants were still sensitive to C_8-AHL. As shown in Fig. 1, transcription of *qrr* should decrease as C_8-AHL levels increase. To test whether *luxO** mutants also responded to C_8-AHL, we assayed the activity of a P_{qrr}-*gfp* transcriptional reporter in the wild type and five *luxO** mutants (some of which were isolated in experiments described below). Supplementation of cultures with C_8-AHL resulted in a significant ($P < 0.05$) decrease in P_{qrr}-*gfp* activity in ES114 but not in any of the *luxO** mutants (Fig. 4), demonstrating that they are blind to addition of the C_8-AHL signal.

We further examined whether LuxO* variants are independent of the core PS circuitry (Fig. 1) by examining whether their activity required *luxU* or *rpoN*, which encode LuxU and σ^{54}, respectively. LuxU is the only known phosphoryl donor for LuxO, which in turn activates *qrr* as a σ^{54} enhancer (Fig. 1). To test the role of *luxU*, we introduced a *luxU*::pEVS122 allele, which inactivated LuxU function, as indicated by the bright luminescence of the ES114-derived *luxU*::pEVS122 strain JHK068 (data not shown). Even with *luxU* disrupted, the three *luxO** mutations described above, as well as the D47E mutation described previously, increased P_{qrr}-*gfp* expression (Fig. 5) and decreased P_{ainS}-*gfp* expression (see Fig. S1 in the supplemental material). In contrast, deletion of *rpoN* eliminated the effect of the *luxO**(*V106A*) and *luxO**(*T335I*) variants. For example, *luxO** Δ*rpoN* double mutants had low P_{qrr}-*gfp* activity similar to that of the ES114 Δ*rpoN* mutant KV5005 (43) (see Fig. S2 in the supplemental material). Thus, as discussed below, at least for the *luxO** alleles tested, their activity is independent of LuxU but remains dependent on σ^{54}.

After evaluating the characteristics of the newly discovered *luxO** variants, we were curious about their relatively high repre-

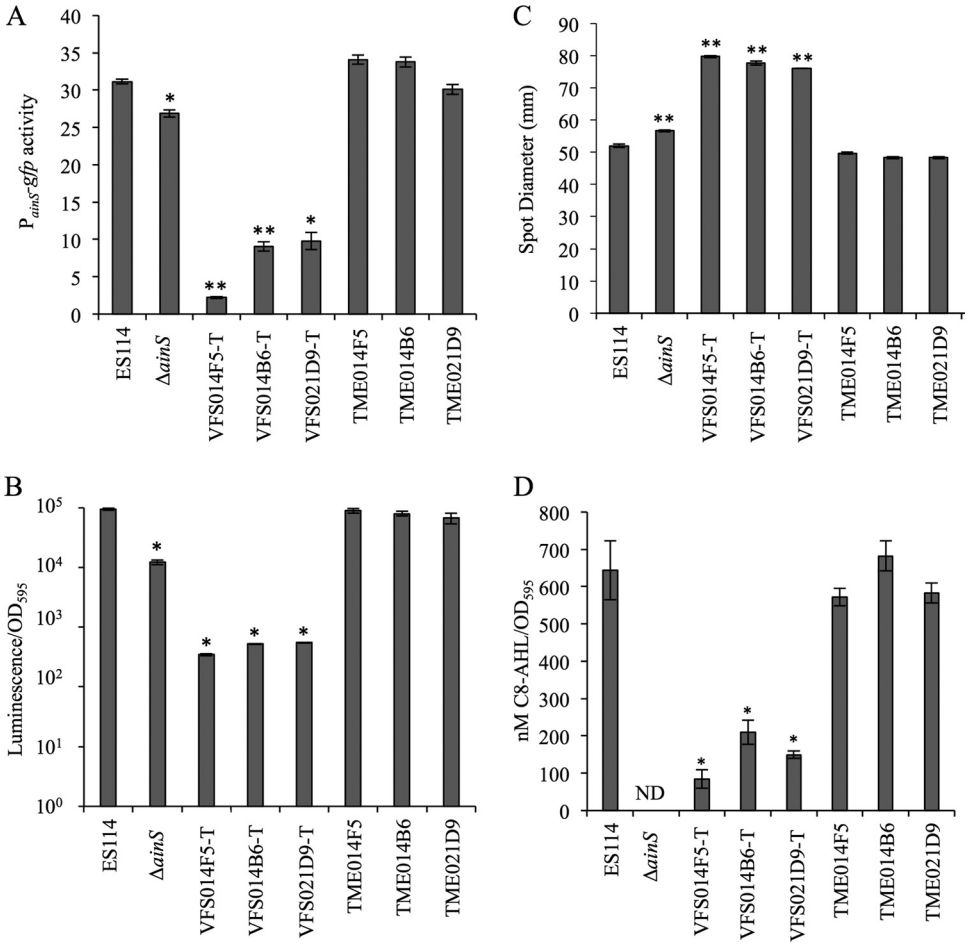

FIG 2 Transposon library mutants (VFS strains), but not their backcrossed-Tn counterparts (TME strains), have phenotypes consistent with deficient *ainS* pheromone signaling. (A) P_{ainS}-*gfp* transcriptional reporter activity in cultures grown in SWTO medium to an OD_{595} of ~2.5. (B) Peak specific luminescence of cultures grown in SWTO medium. (C) Strain motility as measured by increase in spot diameter (on 0.25% agar plates) over 24 h. (D) C_8-AHL extracted and bioassayed from cultures grown in SWTO medium. ND, none detected. In each panel, data are from single representative experiments of three independent experiments. Error bars on all panels indicate standard errors ($n = 3$). *, $P < 0.01$, and **, $P < 0.001$, compared to ES114.

TABLE 2 Summary of *luxO** mutants described in this study

Strain	*luxO** mutation	LuxO* amino acid substitution	*luxO** crossed into ES114	*luxO** function assessed in *trans*[a]
VFS002F6-T	C272A	A91D	X	X
VFS012E9-T	C122T	P41L	X	X
VFS014F5-T	T317C	V106A	X	X
VFS014B6-T	T317G	V106G	X	X
VFS021D9-T	C1004T	T335I	X	X
WTTG0	C320G	T107R		
WTTG1	T281G	F94C		
WTTG2	G341C	R114P		
WTTG3	A971G	H324R		
WTTG4	C293A	P98Q		
WTTG5	G338T	R113L		
WTTG12	C293T	P98L		
WTTG17	T323G	V108G		
WTTG20	T317G	V106G		
WTTG21	T317G	V106G		
WTTG22	A956G	H319R		
WTTG24	C613T	L205F		

[a] *luxO* genes were cloned into the low-copy-number vector pVSV105, and their ability to affect *ainS*-controlled phenotypes (luminescence as well as P_{ainS} and P_{qrr} reporter activity) in the Δ*luxO* strain TIM306 was confirmed (24).

sentation in the transposon mutant library. Gain-of-function mutations resulting in constitutively active LuxO were recovered from three mutants in a library of less than 3,000, which seems remarkably frequent. Moreover, such *luxO** mutations presumably ought to happen far less frequently than simple loss-of-function mutations in *litR*, yet based on our simplified working model (Fig. 1), both should theoretically have similar phenotypic characteristics. We therefore hypothesized that *luxO** mutations provided some benefit during Tn library construction, storage, and/or propagation, thereby resulting in their enrichment. Furthermore, we speculated that this *luxO** phenotype must be different than that of *litR* loss-of-function mutants.

To test this hypothesis, we assessed the *luxO** V106A strain JHK057 for its ability to outcompete the wild type under conditions mimicking library development. For example, we tested relative viability after freezing overnight at −80°C and the frequency of transposon insertion following conjugative transfer of pEVS170 in a mixed culture. Only in the latter assay did we see a difference in the *luxO** mutant (Fig. 6); however, this difference was relatively minor, and our results actually suggest that *luxO** mutants are somewhat poorer recipients for conjugation-based

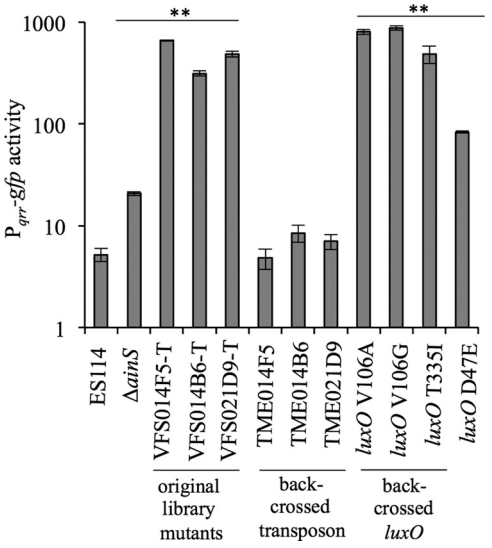

FIG 3 P_{qrr}-*gfp* transcriptional reporter activity is elevated in strains with *luxO** mutations. GFP activity was expressed from strains harboring the P_{qrr}-*gfp* reporter on pTM268, grown in SWTO medium, and assayed at an OD_{595} of ~2.5. Data are from a single representative experiment of three independent experiments. Error bars indicate standard errors ($n = 3$). **, $P < 0.001$ compared to wild-type ES114.

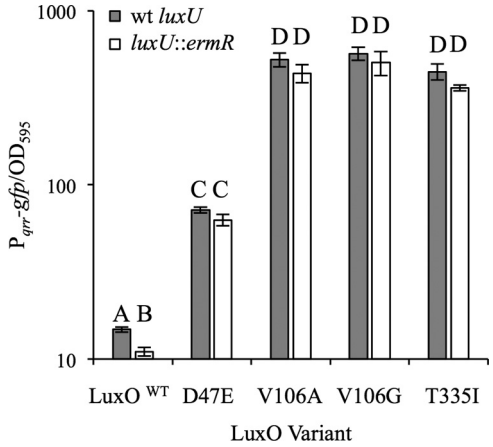

FIG 5 The inactivation of *luxU* does not eliminate the effect of different *luxO** variants on P_{qrr}-*gfp* reporter activity. GFP expression in strains harboring the P_{qrr}-*gfp* transcriptional reporter pTM268 and grown in SWTO medium was assayed at an OD_{595} of ~2.5. Values with the same letter are not statistically significantly different ($P > 0.05$), whereas different letters indicate significant differences ($P < 0.01$), based on a one-way analysis of variance (ANOVA) and *post hoc* testing using Student's *t* test. Data are from a single representative experiment of three independent experiments. Error bars indicate standard errors ($n = 3$).

Tn mutagenesis and if anything should be modestly enriched against as parents for Tn mutagenesis.

We discovered evidence that the spontaneous *luxO** mutations occurred after Tn mutagenesis. The well occupied by VFS014B6 in the 96-well plates housing the mutant library contained a mix of translucent and opaque CFU. PCR and sequencing revealed that all colonies from this well contained the Tn insertion, but only the translucent colonies carried the *luxO** mutation. Two other Tn mutants, VFS002F6 [*luxO**(*A91D*)] and VFS012E9 [*luxO**(*P41L*)], were similarly stocked as a mix of *luxO** and wild-type *luxO* strains in a uniform Tn-mutant background. Each of these *luxO* mutants (Table 2) possesses phenotypes similar to those described above for the initial *luxO** mutants, and these *luxO** alleles are similarly independent of *luxU* (data not shown). Additionally, a mixture of opaque and translucent colonies in the well containing VFS014F5, but not the other Tn mutants, was discovered in the

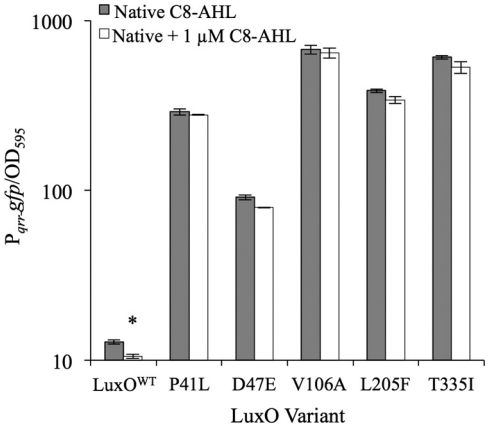

FIG 4 Strains bearing *luxO** variants are insensitive to C_8-AHL addition. GFP expression in strains ES114, JHK075 [*luxO*(*P41L*)], CL59 [*luxO*(*D47E*)], JHK057 [*luxO*(*V106A*)], WTTG24 [*luxO*(*L205F*)], and JHK061 [*luxO*(*T335I*)] harboring the P_{qrr}-*gfp* reporter plasmid pTM268, grown in SWTO medium with and without 1 μM added C_8-AHL, was assayed at an OD_{595} of ~2.0. Data are from a single representative experiment of three independent experiments. Error bars indicate standard errors ($n = 2$). *, $P < 0.05$ compared to the strain without added C_8-AHL, as determined using Student's *t* test.

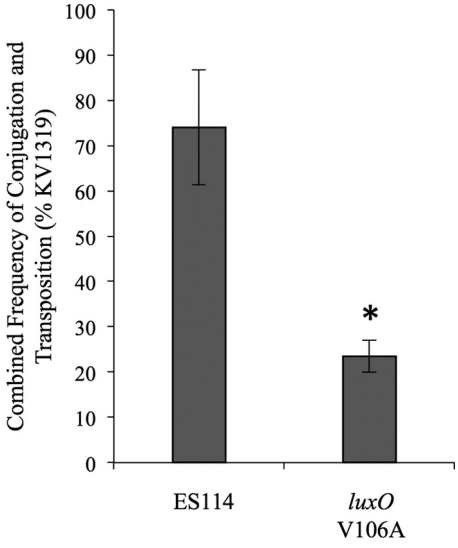

FIG 6 A *luxO** mutation does not enhance frequency of conjugation-based transposon mutagenesis. The transposon-containing vector pEVS170 was conjugated into mixed recipient cultures of KV1319 (*celG*) combined ~1:1 with either JHK057 [*luxO*(*V106A*)] or the wild type. Following conjugation, nonselective plating determined the ratio of recipient strains, and plating on Erm was used to determine the number of Tn mutants. By including 10 mM D-cellobiose and X-Gal, we differentiated recipients as either blue (JHK057 or ES114) or white (KV1319). Data are from one representative experiment of three independent experiments. Error bars indicate standard errors ($n = 5$). *, $P < 0.01$ relative to KV1319.

master Tn library, a copy of which was sent to our lab (Foxall and Whistler, personal communication). Taken together, our results suggested the *luxO** mutants had arisen after transposon insertion, that freezing did not favor these mutants, and that the enrichment of *luxO** mutants relative to their parent strains likely resulted during library outgrowth.

To test whether *luxO** mutants could have been enriched during growth and storage of the Tn mutant library, we first grew ES114 and JHK057 [*luxO*(*V106A*)] individually during prolonged incubation in static cultures of LBS medium. The strains initially grew similarly and reached cell numbers (CFU per milliliter) at 24 h that were not significantly different from one another ($P > 0.05$); however, from 24 to 96 h, ES114 suffered greater decreases in CFU (see Fig. S3 in the supplemental material). Moreover, translucent colonies began to appear in ES114-inoculated wells after 48 h and eventually dominated ES114-inoculated cultures. Most of these new spontaneous translucent mutants harbored *luxO** alleles (see strains with the prefix "WTTG" in Table 2); however, a small subset possessed characteristics similar to those of *luxO** mutants while having wild-type *luxO* sequences. Interestingly, nine translucent mutants maintained wild-type P_{qrr}-*gfp* activity (and clumped in static broth culture), suggesting that there are multiple paths to translucent colony morphology and static-culture survival, including at least one independent of the central PS network.

We next competed strains to approximate the conditions under which a *luxO** mutant might be enriched after arising in a wild-type culture. When JHK057 [*luxO*(*V106A*)] was grown with ES114 in static coculture at initial ES114/mutant ratios of 1:1, 10:1, or 100:1, the ratio of JHK057 to ES114 increased as much as 10-fold after 24 h (Fig. 7A to D). This change in strain ratios was most dramatic in cultures where JHK057 had initially been the most outnumbered (Fig. 7A to D). Moreover, the number of CFU per milliliter of the *luxO** mutant actually increased, even during periods when the CFU/ml of ES114 dropped. In each case, the final strain ratio reflected a similar small numerical advantage for the *luxO** mutant, regardless of how outnumbered it was in the initial inoculum (Fig. 7D). We observed a similar competitive advantage for JHK057 and JHK061 [*luxO*(*T335I*)] when these strains were competed against the Cam^r-marked ES114 derivative AKD200 (data not shown; also, see Fig. S4 in the supplemental material).

The advantage of *luxO** mutants appeared to be dependent on prolonged stationary-phase culturing. When cultures were kept in log phase ($OD_{595} < 0.5$) for 30 generations by repeated subculturing, translucent derivatives did not appear in wild-type cultures, and in mixed competitions, the *luxO** mutant was not enriched relative to the wild type (data not shown).

Given that *luxO** mutants should lead to strong repression of *litR* (Fig. 1C), and that both *litR* and *luxO** mutants share a translucent colony phenotype, we considered the possibility that *litR* mutants would have the same advantage relative to ES114 in mixed cultures; however, this was not the case (data not shown). Although translucent colonies began to dominate mixed cultures of ES114 and the *litR* mutant, upon closer examination, most translucent colonies lacked the erythromycin resistance of the *litR*::*ermR* mutant and were instead spontaneous mutants of ES114. These results, along with those demonstrating differences in growth and luminescence in shaking culture and P_{qrr}-*gfp* reporter activity (see Fig. S5 in the supplemental material), indicated that *luxO** and *litR* mutants do not phenocopy.

To further explore the difference between *luxO** and *litR* mutants, we competed JB18 (*litR*::*ermR*) and JHK057 [*luxO**(*V106A*)] in static cultures. Here too, the *luxO** mutant dominated when mixed with *litR* mutants (Fig. 7E to H). Similar to its competition with ES114, JHK057 outcompeted the *litR* mutant at all three initial strain ratios, and the experiments ended at relatively similar strain ratios, with a slight dominance by the *luxO** mutant, regardless of the starting ratio (Fig. 7D and H). Similarly, the *luxO** *litR* mutant JHK073 outcompeted the *litR* mutant PMF8 (data not shown) (23). Taken together, the data indicated that in prolonged static broth LBS culture, a *luxO** mutant can outcompete a *litR* mutant, and that a competitive advantage for a *luxO** mutant does not require *litR*. We next tested the *litR* mutant JB18 and the Δ*qrr* mutant TIM305 for their abilities to give rise to spontaneous *luxO** mutants when grown in static culture. We grew strains carrying the P_{qrr}-*lacZ* reporter pHK20 in static culture for up to 96 h, periodically plating on LBS containing X-Gal, and screening for blue colonies, but we were unable to isolate any mutants with increased P_{qrr}-*lacZ* activity in the JB18 or TIM305 backgrounds, indicating that *litR* and *qrr* are required for cells carrying a *luxO** mutation to arise and dominate cultures. In the case of *litR*, this result contrasts with the observation above that a *luxO** *litR* mutant could outcompete a *litR* mutant when they were coinoculated (data not shown).

Although the importance of *litR* was somewhat unclear, we were most interested in whether the *luxO** survival advantage acts through Qrr, as *qrr* is the only described regulatory target of LuxO. We competed AKD200 (Cam^r-marked ES114) against JHK105 [*luxO**(*T335I*) Δ*qrr*] and found that the initial strain ratios were maintained through 96 h (Fig. 8). In this instance, it was necessary to use a marked derivative of ES114, because the JHK105 (*luxO** Δ*qrr*) lacked the distinctive translucent colony morphology of *luxO** mutants. Although we used a *luxO**(*V106A*) mutant in many of the experiments described above, we used the T335I allele here because the mutation was further from *qrr*, which allowed us to move the allele into a Δ*qrr* background without also exchanging in wild-type *qrr*. As in other experiments, translucent mutants began to appear within 48 h, but these were solely derived from AKD200 (data not shown). The results in Fig. 8 show that the competitive advantage of the *luxO** allele requires *qrr*.

In contrast to its competitive advantage in broth culture (see Fig. S4 in the supplemental material), mutant JHK061 [*luxO**(*T335I*)] was significantly ($P < 0.001$) outcompeted by the Cam^r-marked ES114 derivative AKD200 during host infection and colonization (Fig. 9). The competitive defect of the *luxO** mutant in symbiosis does not stem from differential survival of the inoculum in artificial seawater (data not shown). The results shown in Fig. 9 are another example where a *luxO** mutant phenotype is dissimilar from that of *litR* mutants, which actually outcompete the wild type during colonization of *E. scolopes* (23). Our results are consistent with the attenuated colonization previously reported for the *luxO*(*D47E*) mutant CL59 (19), but the effects appear to be more severe, potentially owing to the higher relative activity of the *luxO** T335I allele in JHK061 than of the *luxO**(*D47E*) allele in CL59 (Fig. 3, 4, and 5).

Given the identification of *luxO** alleles other than the previously reported D47E allele, we were curious if certain sites were more prone to mutation than others and/or if additional allelic

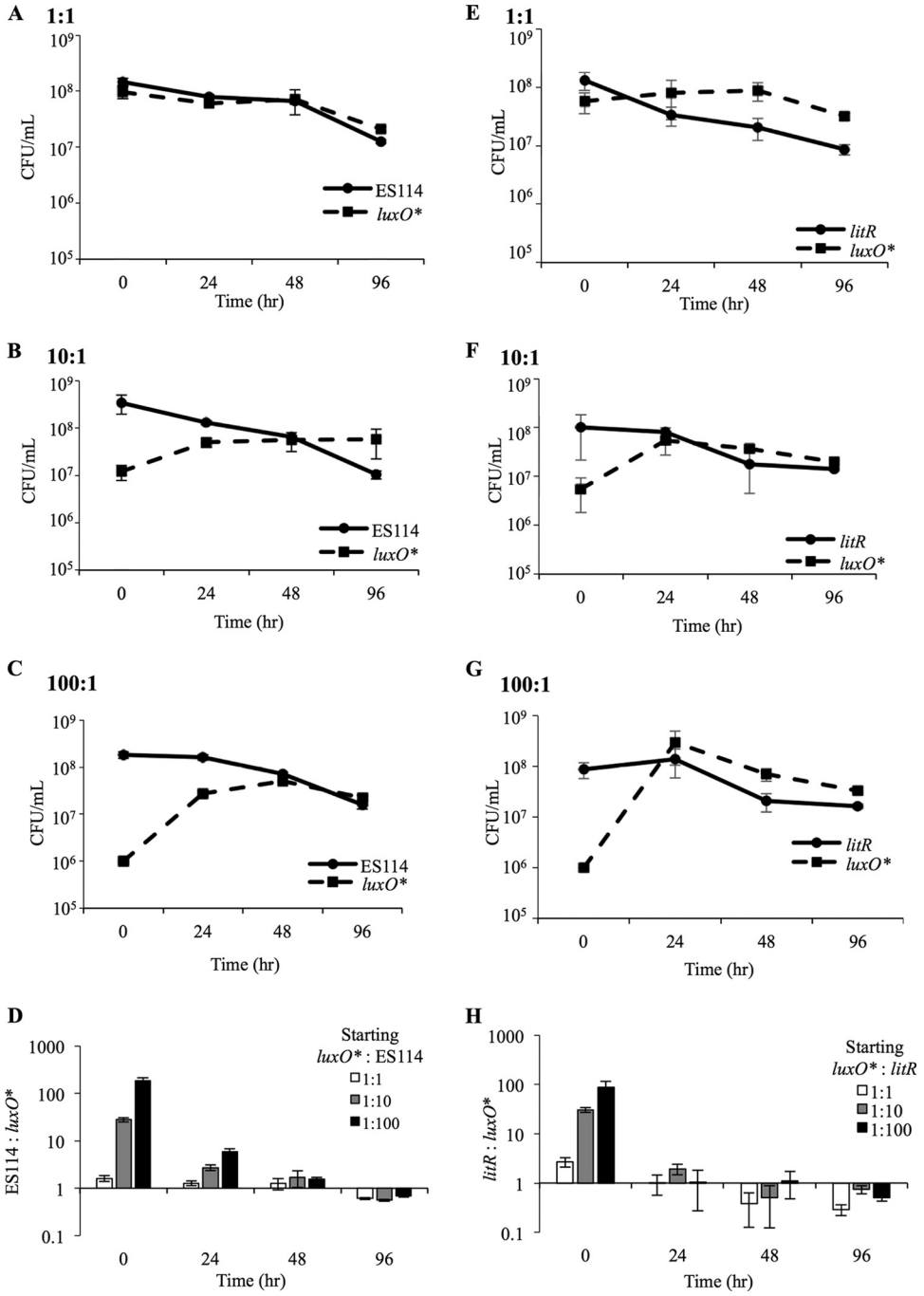

FIG 7 Competitive advantage of *luxO** mutants relative to ES114 and *litR* mutants during prolonged coculture. ES114 (A to D) or *litR* mutant JB18 (E to H) were mixed with JHK057 [*luxO*(*V106A*)] in ratios of 1:1, 10:1, or 100:1, with JHK057 always at a disadvantage. Mixes were diluted in 24-well microtiter plates and grown statically for 96 h. At intervals of 0, 24, 48, and 96 h, wells were thoroughly mixed and dilution plated to determine the ratios of viable CFU for each strain in the mixture based on translucence (JHK057 versus ES114) or Erm resistance (JHK057 versus JB18). Data are from a single representative experiment of three independent experiments. Error bars indicate standard errors ($n = 3$).

variants were possible. Using the strategy of static-culture *luxO** enrichment, we grew 26 independent cultures for 48 h, dilution plated, and picked translucent colonies for *luxO* sequencing. In all, 10 more *luxO** alleles were identified (F94C, P98L, P98Q, T107R, V108G, R113L, R114P, L205F, H319R, and H324R alleles) and *luxO**(*V106G*) was recovered two more times in our screen (Fig. 10). Taken together, these data show that alleles were distributed across LuxO, with the exception of the DNA-binding domain.

Finally, given that some components of the PS circuitry, including *ainSR* and *luxIR*, have diverged between *V. fischeri* lineages (55), we wondered whether spontaneous *luxO** mutants, or other mutants that upregulate *qrr*, would be similarly enriched during prolonged growth in static culture of other wild-type

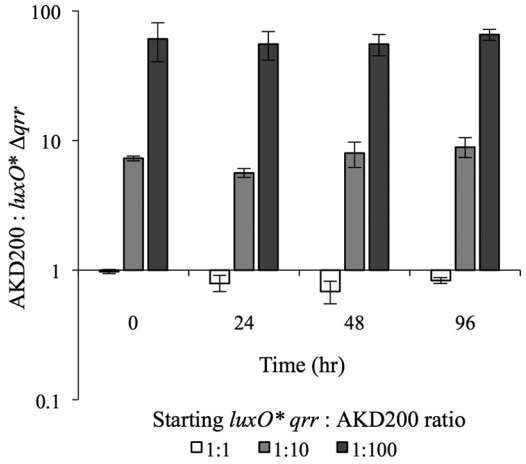

FIG 8 Qrr is required for a *luxO**-mediated survival advantage. Cam-marked ES114 (AKD200) and JHK105 [*luxO*(*T335I*) Δ*qrr*] were grown overnight and subcultured into fresh LBS medium until reaching an OD_{595} of ~2.0, at which time they were mixed in ratios of 1:1, 1:10, or 1:100, with JHK061 always at a disadvantage. Mixes were outgrown to an OD_{595} of ~2.5 and then diluted 1:1,000 in fresh LBS medium in 24-well microtiter plates grown statically for 96 h. At intervals of 0, 24, 48, and 96 h, wells were thoroughly mixed and dilution plated to determine the ratios of viable cells of each mixture by Cam resistance. Data are from a single representative experiment of two independent experiments. Error bars indicate standard errors ($n = 3$).

strains besides ES114. We tested wild-type *V. fischeri* isolates CG103, ES12, ES114, ES213, ES401, EM17, ET101, H905, PP3, VLS2, and WH1 (Table 1), by growing strains carrying the P_{qrr}-*lacZ* reporter pHK20 in static culture for up to 96 h, as described above. We further tested blue colonies by displacing pHK20 with the P_{qrr}-*gfp* reporter pTM268 and screening for high fluorescence to ensure that mutants had genomic changes that increased *qrr* reporter expression. Strains ES12, ES114, ES213, ES401, EM17, and ET101 gave rise to mutants with increased *qrr* expression.

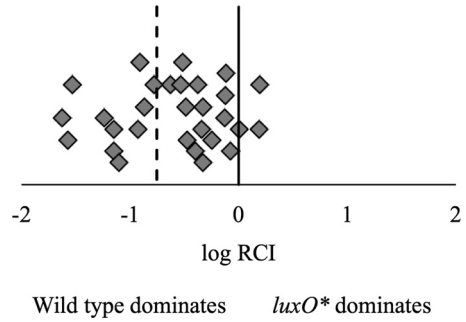

FIG 9 The *luxO** strain JHK061 shows a defect when competed 1:1 against the wild-type ES114 derivative AKD200 during colonization of *E. scolopes*. Newly hatched squid ($n = 29$) were exposed to a mixed inoculum of JHK061 [*luxO**(*T335I*)] and AKD200 (~2,000 CFU) in filter-sterilized Instant Ocean for 12 h with subsequent water change at 24 h. After 48 h, squid were homogenized, dilution plated on LBS agar, and patched to determine Cam resistance. The log relative competitive index (RCI) was calculated as the log of the ratio of JHK061 to AKD200 in the host homogenate to the ratio of the two strains in the inoculum. The solid line represents an equal ability to colonize the host; the dashed line represents the mean log RCI of −0.75. Data from a single representative experiment of three independent experiments is shown. Statistical significance was determined using a one-sample Student's *t* test where the null hypothesis is a mean log RCI of 0. Each diamond represents the log RCI in one hatchling.

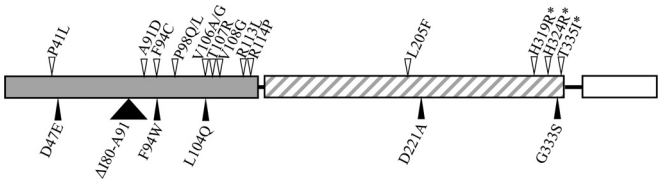

FIG 10 Distribution of *luxO** variants obtained in this study (open triangles) and those from previous studies in other vibrios aligned to the *V. fischeri* sequence (filled triangles) (53, 54, 57, 58, 60). Rectangles represent LuxO domains, including the regulatory (gray), AAA^+/RpoN interaction (hatched), and DNA-binding (white) domains. The asterisk indicates a potential site of interaction with LuxU (78).

DISCUSSION

In this study, we sought to identify regulators of the *ainSR* PS system using a library of Tn insertion mutants; however, the effects we observed on *ainSR* expression were unrelated to the Tn insertions and were instead due to spontaneous mutations in the core PS-signaling circuit (Fig. 1C). The most notable mutants had constitutively active variants of the regulator LuxO and are referred to as *luxO** mutants (54), which outcompeted the wild type during prolonged static coculture in LBS medium (e.g., see Fig. 7).

Our data suggest that these *luxO** mutations arose after Tn mutagenesis and that mutants were probably enriched in subsequent steps. Although the 96-h duration of our competition experiments is longer than that of any incubations used during library construction and propagation, >10-fold enrichment of *luxO** mutants was observed in as little as 24 h (Fig. 7), and three successive rounds of 12-h static culture incubations followed by subculturing was sufficient to generate translucent mutants (data not shown). Moreover, overnight incubations in LBS were used in stocking the original Tn mutants prior to sequencing, in generating the defined master library, and finally in propagating the library prior to introduction of the P_{ainS}-*lacZ* reporter. Knowing now that *luxO** (or phenotypically similar) mutants are prevalent in most cultures of ES114 after 48 h of incubation, and that *luxO** mutants are significantly enriched by 24 h, it seems plausible that during construction and handling of the Tn mutant library, three in approximately 2,000 wells became significantly contaminated by a *luxO** derivative. In a similar vein, Petrun and Lostroh reported a growth advantage during stationary phase for 7-day-old cultures of *V. fischeri* over 1-day-old cultures (56), and it would be interesting to assess such cultures for the presence of *luxO** variants. Overall, our findings serve as a cautionary tale, encouraging the prompt use of cultures and the verification of phenotypes using complementation and/or backcrossing. In this circumstance, examining the opacity or translucence of newly made strains also provides a convenient screen against these unexpected mutations.

The prevalence of *luxO in the *Vibrionaceae*.** LuxO is part of a PS network conserved throughout the *Vibrionaceae* that has been well studied over the last 3 decades. Other *luxO** mutants have been reported in the *Vibrionaceae*, isolated both from natural populations and during laboratory experiments (54, 57–61). Recently, phenotypic discrepancies between two Δ*luxU* mutants of *V. cholerae* were discovered to be due to one strain harboring a previously undetected *luxO** mutation (58, 59), which is reminiscent of our own discovery of spontaneous *luxO** causing phenotypes we initially attributed to defined transposon insertions. In

another parallel to our study, *luxO** mutants were recovered in strains subjected to transposon mutagenesis (54, 58, 62, 63); however, we found that *luxO** mutants of *V. fischeri* were not enriched by this procedure specifically (Fig. 6), and *luxO** mutants were readily recovered from strains that had not been genetically manipulated or subjected to Tn mutagenesis. If our observations in *V. fischeri* hold true for other *Vibrio* species, it may simply be that rounds of growth into stationary phase during genetic manipulations can give rise to *luxO** mutants. In this regard, if we consider the reports by Keynan and Hastings (64) and Silverman et al. (65) of "luminescence variation" in *V. harveyi* resulting in genetically stable dim and dark mutants in old, statically grown cultures, it is tempting to speculate that at least some of these may have been *luxO** mutants. One such dark mutant was used in the first description of the *luxO* locus (66), raising the possibility that a spontaneous *luxO** mutant contributed to the discovery of *luxO* almost 30 years ago (65).

Are LuxO* mutants cheaters? One area of recent interest in the study of bacterial PS and group behaviors is the appearance of cheaters in populations. These cheaters are PS-negative mutants that benefit from the PS-induced behaviors of nearby cells while minimizing their own costs and thus are enriched in the population (9–11). Given that *luxO** mutants make little C_8-AHL and remain locked in a noninduced state (e.g., dim) (Fig. 1C and 2) but can also take over mixed cultures with the wild type (Fig. 7), such mutants might fit the description of PS cheaters. Although such cheating mutants have been described in a variety of bacterial systems, to our knowledge, this would be the first description of cheaters arising spontaneously in *V. fischeri* and may shed light on a more general method of survival among vibrios and the importance of tight regulation of the pheromone systems of *V. fischeri* in its natural habitats.

On the other hand, *luxO** mutants did show some survival benefits even in the absence of the wild type (see Fig. S3 in the supplemental material), and they may simply be better adapted to broth culture environments without needing to exploit a nearby wild-type population. In this sense, it seems possible that these mutants, and perhaps others, are "antisocial" without being cheaters. In situations where social behaviors have costs without benefits, being antisocial could simply be viewed as "prudent" or "efficient." The terminal or master regulators of LuxO-containing PS circuits (e.g., LitR in *V. fischeri*) control a diverse array of phenotypes, including opacity (67), swarming (68), protease production (29), biofilm formation (27), symbiosis (23), and luminescence (69). These behaviors may present fitness costs without compensatory benefits under certain circumstances, and *luxO** mutants would have the effect of damping expression of these costly systems.

If *luxO** mutations are advantageous due to their repression of PS master regulators like *litR*, this raises the question of why presumably rarer gain-of-function mutations in *luxO* should dominate instead of loss-of-function mutations in *litR*. In fact, such loss-of-function mutations to these regulators have repeatedly been observed in other members of the *Vibrionaceae*. For example, in *V. cholerae*, mutants disrupted in the *litR* homolog *hapR* account for cheaters arising in populations of that bacterium and have been frequently isolated (11, 27, 29). One reason *luxO** mutants may be enriched rather than *litR* mutants may be that, in some instances, fitness is maximized by decreasing the master regulator without completely eliminating it. At least in *V. fischeri*, in terms of static culture survival, *luxO** mutants outcompete either their wild-type parents or *litR* mutants (Fig. 7). Thus, while *luxO** mutations may be more rare, in this circumstance they confer greater fitness. Others have suggested that PS systems may have evolved in ways that make the appearance of cheaters difficult (70, 71), and perhaps the *V. fischeri* system has similarly evolved such that the simple loss of *litR* is not advantageous.

Insights into the LuxO-Qrr regulatory module. Based on a simple model of the LuxO-containing PS regulatory circuit (Fig. 1A and B), *luxO** mutants and *litR* loss-of-function mutants should have a similar phenotype, with the caveat noted above that LuxO* should diminish but may not eliminate LitR. In addition to *luxO** mutants outcompeting *litR* mutants (Fig. 7), prolonged growth in stationary phase selected for *luxO** mutants but not *litR* mutants, and a *luxO* litR* double mutant outcompeted a *litR* mutant (data not shown). Furthermore, *luxO** and *litR* mutants do not phenocopy with respect to their ability to compete with the wild type in host colonization (Fig. 9), growth and luminescence in broth culture (see Fig. S5A and B in the supplemental material), and P_{qrr}-*gfp* reporter activity (see Fig. S5C in the supplemental material). On the other hand, consistent with our model of the PS circuitry (Fig. 1), the survival advantage of *luxO** mutations is Qrr dependent (Fig. 8). Given these observations, we speculate that the Qrr regulon extends beyond *litR*. There is precedent for such regulation, as Qrr has multiple targets in other *Vibrio* species (72, 73), although there are also usually multiple semiredundant copies of *qrr*, whereas *V. fischeri* has only one *qrr* gene (24). Our data seem consistent with a model where Qrr regulates an unknown target that affects survival and competitiveness during prolonged culturing.

Our results also point to mutants that could help reveal alternative targets for Qrr and perhaps novel inputs to *qrr* control. As noted above, prolonged culturing of ES114 yields not only *luxO** mutants but also other translucent-colony mutants with wild-type *luxO*. These mutants fell into two classes. One set was relatively abundant, quickly settled in clumps out of static broth culture, and had wild-type P_{qrr}-*gfp* activity. Analyses of these mutants might provide insight into the key targets for stationary-phase survival downstream of Qrr. The second set was less common and had high P_{qrr}-*gfp* activity, similar to *luxO** mutants. These mutants might reveal other regulatory inputs into *qrr* control. While we have shown that our *luxO** mutants act independently of their only known phospho-donor, LuxU (Fig. 5), this does not preclude the existence of some hitherto-unknown input into the system acting at or downstream of LuxU. Alternative inputs into LuxU have been shown in *V. harveyi* and *V. cholerae* through HqsK and VpsS, respectively, and perhaps there exist similar pheromone-independent inputs phosphorylating LuxO (74, 75).

Symbiotic phenotypes of *luxO and *litR* mutants.** Our data are also consistent with previous reports that suggest *luxO** and *litR* mutants have different symbiotic phenotypes. Fidopiastis et al. showed a *litR* mutant outcompeted its wild-type parent (23); however, Lupp and Ruby reported a colonization defect at 12 h postinoculation for a *luxO*(D47E)* mutant (19). Understanding the role of each gene was further complicated by findings that minor competition defects for *luxO* and *qrr* mutants are not rescued by the addition of a *litR* mutation (24). This observation suggested a requirement for *luxO* and *qrr* for full colonization of the host. Our results are consistent with those of Lupp and Ruby,

and it also seems possible that the more active the *luxO** allele is (e.g., see Fig. 3), the greater the competitive defect is.

Taken together, the results suggest that increased *qrr* expression may be detrimental to *V. fischeri*'s ability to colonize *E. scolopes*. In that regard, *litR* mutants did not display increased *qrr* reporter expression (see Fig. S5C in the supplemental material), despite LitR feedback regulating the *ainSR* pathway (17, 22, 30). Given the symbiotic disadvantage of *luxO** mutations, it seems unlikely that they would be enriched in natural symbiotic populations of *V. fischeri*, although they might be found in free-living populations or in mixed-species communities in the guts of fishes. At least in the shallow sandy reefs of Hawaii, where symbiosis apparently enhances *V. fischeri* populations (76, 77), symbiotic fitness presumably filters out *luxO** mutants.

New insights into LuxO structure and function. The many different *luxO** alleles that we found (Fig. 10) indicate that there are multiple paths to LuxO* activity. Classical D47E mutations alter the amino acid (D47) that is usually phosphorylated to activate the protein, and presumably the longer side chain at this site in some way mimics the conformational changes imposed by phosphorylation at this residue. This study contributes to a body of work describing mutations showing that less intuitive mechanisms of generating LuxO* variants are possible (Fig. 10). These mechanisms could include deactivation of negative autoregulation by the N-terminal receiver domain, modulation of the predicted site of interaction with the phosphor-donor LuxU (78), promotion of new interactions with other phosphate donors, or other mechanisms. Previous studies of *luxO* and other σ^{54}-dependent regulators have described similar effects of mutations in approximately the same positions within the protein as we isolated (53, 79). LuxO belongs to a subclass of σ^{54}-dependent regulators wherein the N-terminal receiver domain represses the catalytic activity of the central AAA^+ domain, and deletion of the receiver domain results in constitutive activity of the protein (53, 79). Furthermore, the majority of the variant mutations we isolated were clustered within a putative helix that, when LuxO dimerizes, serves to stabilize the "off" state conformation in other systems (79–81). Mutagenesis within these helices, like phosphorylation of the conserved aspartate residue, may destabilize this interaction, promoting a more active state (79–82). It is noteworthy, and perhaps surprising, that each of the alleles we tested appeared to be active independently of *luxU* and was more active than a D47E variant (Fig. 5). However, by allowing these mutations to arise under natural selective pressures, our enrichment was biased toward mutations that provide a competitive advantage for the bacterium and may therefore serve as a more powerful tool for revealing strong effects on protein function than targeted mutagenesis alone.

A dearth of *ainSR* regulators. We initially sought to identify novel controls of *ainSR*, and the fact that we did not identify any unknown regulators of *ainS* raises the question of whether there are any such regulators of *ainSR* beyond what is known for CRP and LitR (17, 22, 30), other than the possible regulation of *ainSR* by LuxR (22, 83). Our lack of success in identifying new regulators might reflect limitations of our original P_{ainS}-*lacZ* reporter-based screen. Moreover, the transposon library is not comprehensive and will also inherently miss any regulators of the system that are essential for *V. fischeri*'s survival. In this regard, it is worth noting that while they are not essential, neither *crp* nor *litR* is among the genes disrupted in this transposon mutant library. Finally, regulators may become apparent only under different conditions (e.g., during growth in different media) or with other approaches. It seems likely that the regulation of *ainSR* is far less complex than that of *luxI* and *luxR*, which could reflect the function of the former being more general and the latter more context dependent.

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