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GENETICS AND MOLECULAR BIOLOGY

Control of Competence in *Vibrio fischeri*

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ABSTRACT *Vibrio* species, including the squid symbiont *Vibrio fischeri*, become competent to take up DNA under specific conditions. For example, *V. fischeri* becomes competent when grown in the presence of chitin oligosaccharides or upon overproduction of the competence regulatory factor TfoX. While little is known about the regulatory pathway(s) that controls *V. fischeri* competence, this microbe encodes homologs of factors that control competence in the well-studied *V. cholerae*. To further develop *V. fischeri* as a genetically tractable organism, we evaluated the roles of some of these competence homologs. Using TfoX-overproducing cells, we found that competence depends upon LitR, the homolog of *V. cholerae* master quorum-sensing and competence regulator HapR, and upon homologs of putative pilus genes that in *V. cholerae* facilitate DNA uptake. Disruption of genes for negative regulators upstream of LitR, namely, the LuxO protein and the small RNA (sRNA) Qrr1, resulted in increased transformation frequencies. Unlike LitR-controlled light production, however, competence did not vary with cell density under *tfoX* overexpression conditions. Analogous to the case with *V. cholerae*, the requirement for LitR could be suppressed by loss of the Dns nuclease. We also found a role for the putative competence regulator CytR. Finally, we determined that transformation frequencies varied depending on the TfoX-encoding plasmid, and we developed a new dual *tfoX* and *litR* overexpression construct that substantially increased the transformation frequency of a less genetically tractable strain. By advancing the ease of genetic manipulation of *V. fischeri*, these findings will facilitate the rapid discovery of genes involved in physiologically relevant processes, such as biofilm formation and host colonization.

IMPORTANCE The ability of bacteria to take up DNA (competence) and incorporate foreign DNA into their genomes (transformation) permits them to rapidly evolve and gain new traits and/or acquire antibiotic resistances. It also facilitates laboratory-based investigations into mechanisms of specific phenotypes, such as those involved in host colonization. *Vibrio fischeri* has long been a model for symbiotic bacterium-host interactions as well as for other aspects of its physiology, such as bioluminescence and biofilm formation. Competence of *V. fischeri* can be readily induced upon overexpression of the competence factor TfoX. Relatively little is known about the *V. fischeri* competence pathway, although homologs of factors known to be important in *V. cholerae* competence exist. By probing the importance of putative competence factors that control transformation of *V. fischeri*, this work deepens our understanding of the competence process and advances our ability to genetically manipulate this important model organism.

KEYWORDS competence, *Vibrio fischeri*, LitR, HapR, quorum sensing, luminescence, *Euprymna scolopes*, transformation, *Vibrio cholerae*, symbiosis

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The ability of bacteria to take up and incorporate foreign DNA into their genomes plays a vital role in bacterial adaptation. For example, bacteria can acquire genetic material that permits them to become resistant to antibiotics or to survive/thrive in a specific environment. The acquisition of extracellular DNA, or competence, can be induced under particular environmental conditions. For example, the pathogen *Vibrio cholerae*, found in brackish water and associated with chitin-containing zooplankton such as copepods, induces its competence pathway upon exposure to chitin (1, 2).

Once the cells have taken up exogenous DNA, they can recombine it into their genomes in a process known as transformation. Researchers have exploited these phenomena to rationally generate specific bacterial mutants. For example, exposing cells to DNA containing sequences up- and downstream of a gene to be disrupted along with a centrally located antibiotic resistance cassette, followed by selection for the resistance marker, results in growth of the desired mutant. In *V. cholerae*, competence and transformation have been optimized such that strains carrying multiple unmarked mutations can be engineered in a single step: exposing competent cells to multiple fragments of mutant DNA, followed by selection for a single selectable marker, can result in uptake and recombination of all of the introduced DNA fragments to produce unmarked deletions (3). This technology, termed MuGent (multiplex genome editing by natural transformation), has been applied to the study of genes that are present in multiple copies in the genome, such as those involved in carbohydrate transport (4).

The genes for competence and transformation in *V. cholerae* have been well studied (5–8). *V. cholerae* uses a type IV competence pilus to acquire environmental DNA and bring it into the periplasm. A second step results in transport of this transforming DNA (tDNA) across the inner membrane, delivering it as single-stranded DNA into the cytoplasm. Finally, the tDNA is bound by and protected by proteins, including RecA, that facilitate its recombination into the chromosome. At least 19 structural proteins contribute to these different steps of DNA uptake and recombination (7).

Competence in *V. cholerae* is positively controlled by the transcription factor HapR, which, in turn, is controlled by quorum sensing; HapR activity and competence are low at low cell densities and increase with exposure to autoinducers and/or at high cell density (Fig. 1) (2, 6, 9, 10). In *V. cholerae*, HapR activates transcription of positive competence regulators, including *comEA*, which encodes a DNA binding protein required for DNA uptake and thus competence (2, 5, 9, 11). HapR also inhibits expression of the secreted nuclease Dns, which degrades extracellular DNA, thereby preventing DNA uptake (6). A mutant defective for production of Dns exhibits increased transformation, while one lacking the gene for the competence secretin protein PilQ fails to take up DNA (6, 11). Competence is also stimulated by the chitin-induced regulator TfoX, which contributes to induction of *comEA* and other competence factors (2, 6). A complex regulatory scheme controls the production of TfoX, including chitin-sensing proteins and a small regulatory RNA, TfoR (12). Overexpression of TfoX can bypass the need for chitin (2). Finally, HapR and TfoX control downstream regulators, including the quorum-sensing and TfoX-dependent regulator QstR and the TfoX-controlled cytidine regulator CytR, that contribute to competence control (13–15).

The *V. cholerae* competence genes are largely conserved in other *Vibrio* species, including *Vibrio fischeri*, an important model for mutualistic microbe-host associations, bioluminescence, quorum sensing, and biofilm formation (5, 7, 8, 16). In 2010, Pollack-Berti et al. reported that *V. fischeri* could be made competent by growth in the presence of chitin oligosaccharides and/or by overexpression of the competence regulatory factor TfoX (17). While the reported frequency of transformation is somewhat variable, ranging as much as 100-fold between experiments (2, 17), this ability to directly introduce "naked" DNA into *V. fischeri* has facilitated numerous genetic manipulations. For example, *V. fischeri* researchers have used TfoX overexpression to (i) map a deletion in a lab strain (18), (ii) backcross mutations of interest (for an example, see reference 19), and (iii) readily make strains containing multiple mutations (for an example, see reference 20). Combined with the recent development of tools to rapidly make

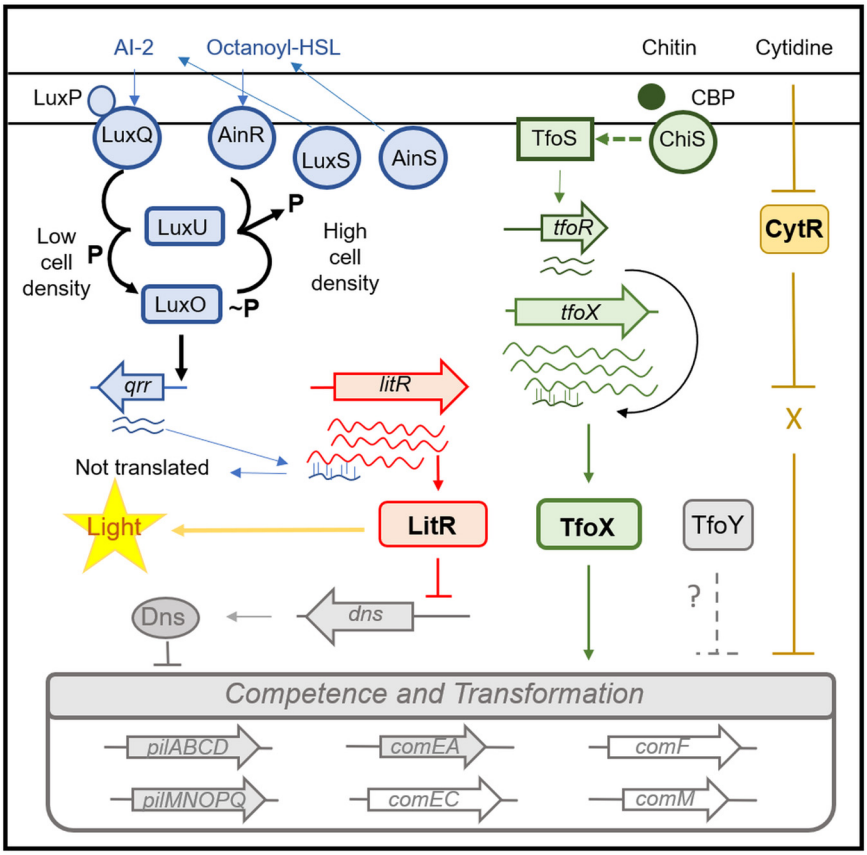

FIG 1 Model for competence and transformation of *V. fischeri*. Major regulatory pathways and other factors predicted, based on studies of *V. cholerae*, to control competence and transformation of *V. fischeri* are shown. In the top left (blue), quorum sensing regulators are shown. At low cell density, two kinases, LuxQ and AinR, function as kinases, promoting phosphorylation, via LuxU, of the response regulator LuxO, which, in turn, activates transcription of the gene for small RNA Qrr1. Qrr1 activity results in decreased levels of LitR (orange), a positive regulator of competence that promotes light production; in *V. cholerae*, the LitR homolog HapR controls transcription of *dns*, which encodes a nuclease that interferes with DNA uptake. At high cell densities, autoinducers made by LuxS and AinS switch the activity of LuxQ and AinR to phosphatases, thereby decreasing Qrr1 levels and increasing LitR levels and light production (yellow). Another major regulator of competence is TfoX (middle, in green). In *V. cholerae*, TfoX is controlled at the level of translation by the sRNA TfoR, which, in turn, is controlled by chitin-sensing regulators ChiS and TfoS. In *V. fischeri*, competence is readily induced by overproduction of TfoX, even in the absence of chitin. Another important regulator appears to be the cytidine-responsive regulator CytR (right, in brown), which activates competence via an unknown mechanism. The role of TfoY, if any, in competence remains unknown. At the bottom (in gray and white), a few of the structural genes known to be important for competence in *V. cholerae* are shown; gray coloring indicates factors assessed in this study.

unmarked deletions using splicing by overlap extension PCR (SOE PCR) and FLP (flippase)-dependent recombination technology, the approach of using TfoX-induced competence has advanced the development of *V. fischeri* as a model genetic organism by facilitating the rapid and targeted manipulation of this microbe (17, 18, 21, 22).

Beyond the finding that TfoX overexpression promotes competence, however, little is known about the regulatory pathway(s) that controls competence in *V. fischeri*. Given the conservation of competence genes, we hypothesized that the competence pathway of *V. fischeri* is similar to that of *V. cholerae* (Fig. 1). Indeed, we found that transformation of *V. fischeri* depends on the HapR homolog LitR (23), as well as on genes predicted to encode structural proteins required for competence. We also identified a role in transformation for the putative cytidine-responsive regulator CytR and determined that the use of specific conditions or plasmids impacts transformation frequency. Finally, we developed a new dual *tfoX* and *litR* overexpression plasmid that

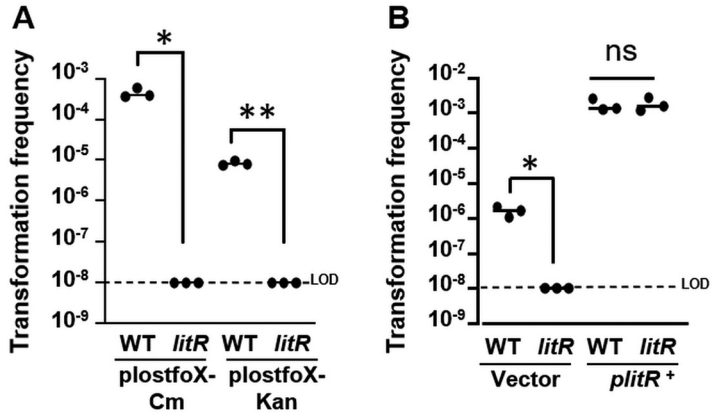

FIG 2 LitR is required for transformation of *V. fischeri*. Shown is the transformation frequency (total Trim^r CFU/viable CFU) of *V. fischeri* cells exposed to gDNA from KV8300 (*fliQ*::Trim). (A) Transformation frequencies of plostfoX-Cm- or plostfoX-Kan-containing ES114 (WT) and *litR* strains PMF8 (*litR*::Kan) and JB19 (*litR*::Erm) containing plostfoX-Cm and plostfoX-Kan, respectively. (B) Transformation frequencies of ES114 and JB19 containing plostfoX-Kan and either vector control (pVSV105) or *litR* expression plasmid p*litR* (pPMF5). Both *litR* mutants PMF8 and JB19 exhibited transformation frequencies below the limit of detection (LOD). *, $P < 0.05$; **, $P < 0.005$; ns, not significant.

could induce transformation about 100- to 1,000-fold higher than the original *tfoX* plasmid in a strain of *V. fischeri* with low genetic tractability.

RESULTS

LitR is required for *V. fischeri* competence. Our interest in *V. fischeri* competence was prompted when two independently generated *litR* mutants failed to incorporate a marked allele into their genomes following multiple transformation attempts. These results suggested that *litR* might be required for competence of *V. fischeri*, as is the case for the LitR homolog HapR in *V. cholerae* (2, 23). However, because the initial study on *V. fischeri* competence reported a wide range in transformation frequencies (17), we first sought to obtain consistent and reliable data by optimizing and standardizing the conditions used to obtain transformants. As described in Materials and Methods, we used fresh minimal medium made from the component reagents to avoid precipitation of the component parts and thus altered growth. We found that reducing the overnight growth of the *tfoX*-overproducing cells to approximately 14 h (rather than a more typical 16 h or longer) substantially diminished the subsequent lag phase following subculture on the next day, resulting in a more consistent outgrowth with cells generally reaching an optical density (OD) of about 0.5 in 5 or 6 h. Finally, we used a consistent amount (500 ng) of genomic DNA (gDNA) and a consistent recovery time of 90 min to permit better comparisons between experiments.

These optimized conditions resulted in a relatively consistent transformation frequency for the control strains but did not yield any transformants for the *litR* mutants (Fig. 2A). Complementation using *litR* expressed from a plasmid restored transformation frequencies to the level achieved by the control strain (Fig. 2B). Together, these data confirm a role for LitR in controlling transformation of *V. fischeri*.

These experiments also revealed an unexpected finding: *V. fischeri* exhibited over a 10-fold higher transformation frequency when it overproduced TfoX from plostfoX (Cm^r) (termed plostfoX-Cm here for clarity) (17) relative to plostfoX-Kan (Kan^r) (21) (Fig. 2A). The cause of this discrepancy is unknown, as the plasmids are identical except for the antibiotic resistance cassette, and resequencing of the respective *tfoX* cassettes revealed no differences. Thus, it is possible that selection for Cm^r maintains the plasmid better than does selection for Kan^r. In support of this possibility, we observed a similar difference in transformation frequencies when we evaluated newly generated plasmids (pJJC2 [Cm^r] and pJJC3 [Kan^r]) in which we substituted *tfoX* for an arabinose-inducible

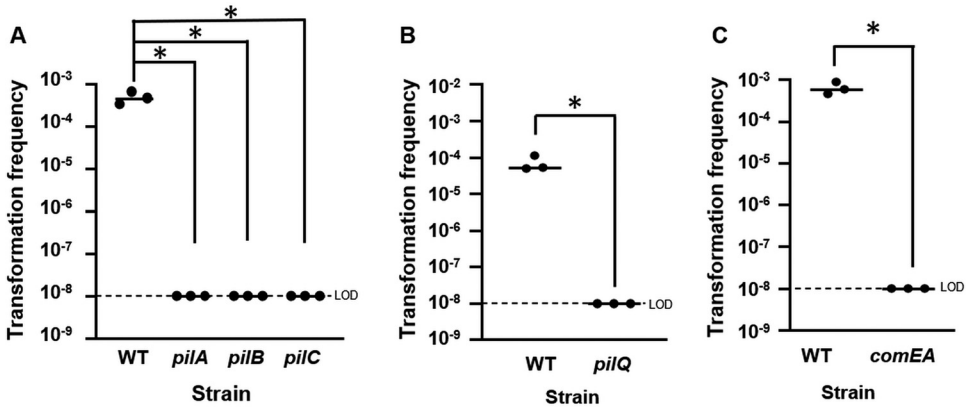

FIG 3 *V. fischeri* transformation requires *pil* and *comEA* genes. Shown are transformation frequencies (Trim^r CFU/viable CFU) of plostfoX-containing wild-type and mutant *V. fischeri* cells exposed to gDNA from KV8300 (*fliQ*::Trim). (A) Transformation frequencies of plostfoX-Cm-containing ES114 (WT) and *pil* Tn mutant strains KV8875 (*pilA*), KV8876 (*pilB*), and KV8877 (*pilC*). (B) Transformation frequencies of plostfoX-Kan-containing ES114 and KV8879 (*pilQ*). (C) Transformation frequencies of plostfoX-Cm-containing ES114 and KV9219 (*comEA*). The *pilA*, *pilB*, *pilC*, *pilQ*, and *comEA* mutants each exhibited a transformation frequency below the LOD. *, $P < 0.05$.

tfoX cassette in the same two backbones (see Fig. S1 in the supplemental material). As a result of the differential transformation frequencies that result from the use of plostfoX-Cm and plostfoX-Kan, we generally chose to use plostfoX-Cm for experiments in which a high transformation frequency was needed (for example, to evaluate the roles of structural factors expected to have a severe competence defect), while we used plostfoX-Kan to better assess subtler defects that might be overcome by the use of plostfoX-Cm.

Putative competence genes required for *V. fischeri* transformation. The finding that LitR is a critical factor in competence prompted us to determine the requirement of other genes predicted in the literature to function in competence in *V. fischeri* and/or known to be important for *V. cholerae* competence (Fig. 1) (for examples, see references 5, 7, and 8). Namely, we tested the requirement for the following putative pilus genes, homologous to *V. cholerae* type IV pili components: *pilA*, *pilB*, *pilC*, and *pilQ* (*VF_2185* to *VF_2187* and *VF_2293*, respectively), for which transposon (Tn) insertion mutants were available. We also generated a deletion mutant for putative competence gene *comEA* (*VF_0801*) and tested its role. Whereas *tfoX*-overexpressing wild-type cells were transformed at high levels (a transformation frequency of $\sim 10^{-3}$ to 10^{-4}, or about 1 transformant per 1,000 to 10,000 CFU), *tfoX*-overexpressing mutants defective for the *pil* genes *pilA*, *pilB*, *pilC*, and *pilQ* failed to be transformed (Fig. 3A and B and Fig. S2A). Similarly, the *comEA* mutant produced no transformants (Fig. 3C and Fig. S2B). These data suggest that the structural proteins demonstrated as competence factors in *V. cholerae* (7) likely function in the same manner in *V. fischeri*.

Neither TfoR nor TfoY is required when TfoX is overexpressed. We next tested roles for two putative competence factors, TfoR and TfoY. In *V. cholerae*, TfoX translation is activated by the sRNA TfoR, which binds the 5′ end of the *tfoX* mRNA (12). We thus hypothesized that TfoR activity could be necessary for optimal TfoX production in *V. fischeri*, even when the latter is overproduced from a multicopy plasmid. However, we observed no impact of *tfoR* deletion on transformation frequency (Fig. S3).

TfoY is a distant homolog of TfoX whose role in competence has been evaluated in two studies (17, 24). In the original study of TfoX-mediated competence in *V. fischeri*, Pollack-Berti et al. reported that chitobiose-induced competence depended on TfoY (17). A more recent report, however, found no role for TfoY in TfoX-dependent activities in *V. fischeri* or *V. cholerae*. Instead, the authors concluded that the two proteins function independently of one another and that TfoY functioned primarily in a type VI secretion pathway responsible for releasing extracellular DNA by killing competitor bacteria in the environment (25). In an attempt to address this discrepancy, we

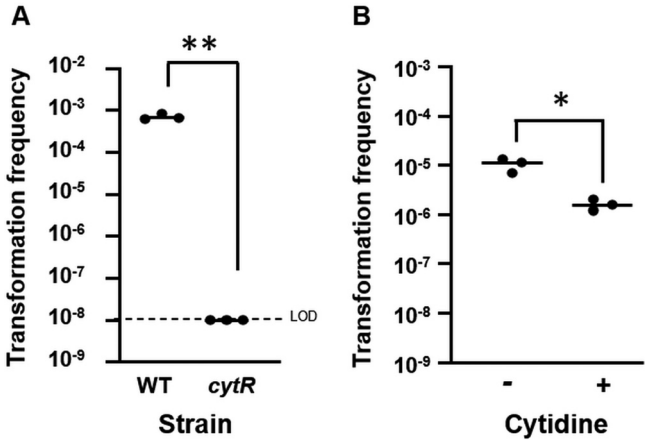

FIG 4 *V. fischeri* transformation is activated by CytR and inhibited by cytidine. (A) Transformation frequencies (total Trim^r CFU/viable CFU) of plostfoX-Cm-containing wild-type strain ES114 (WT) and Δ*cytR* mutant (KV8840) cells exposed to gDNA from KV8300 (*fliQ*::Trim). (B) Transformation frequencies of plostfoX-Kan-containing ES114 (WT) exposed to 100 mM cytidine or left untreated during incubation with KV8300 gDNA. The *cytR* mutant exhibited a transformation frequency below the LOD. *, $P < 0.05$; **, $P < 0.005$.

generated a deletion mutant of *tfoY* and determined the transformation frequency. In multiple independent experiments, we observed either no difference or a small but statistically insignificant increase in the transformation frequency of the *tfoY* mutant relative to the wild-type control (Fig. S4). Thus, under our TfoX overproduction conditions, TfoY appears to play no role in the competence pathway.

In *V. cholerae*, TfoY also controls motility (25, 26). Thus, we assessed the impact of *tfoY* deletion on motility of *V. fischeri*; we found that it caused a severe decrease in migration of this organism through soft agar (Fig. S4). We conclude that *V. fischeri* TfoY appears to function similarly in the two *Vibrio* species with respect to motility.

Role for CytR. We next assessed the role of CytR, a putative cytidine-responsive regulator that promotes *V. cholerae* competence through an as-yet-unknown mechanism (Fig. 1) (14, 15). To test the role of CytR in *V. fischeri*, we generated a *cytR* deletion mutant. This strain failed to produce transformants (Fig. 4A and Fig. S5). Because CytR is inhibited by cytidine (14), these data indicate that *V. fischeri* competence may be negatively controlled by cytidine. To test this possibility, cytidine was added to the cells together with DNA at the start of the 30-min incubation period. The presence of cytidine decreased the transformation frequency about 10-fold (Fig. 4B). Thus, cytidine is inhibitory, indicating that *V. fischeri* and *V. cholerae* share this aspect of competence control.

Transformation is controlled by regulators of *litR*. LitR production and thus activity is controlled by quorum-sensing regulators (Fig. 1) (27–30). Thus, we hypothesized that disruption of regulators that positively and negatively control LitR would exert opposite effects on transformation. Indeed, a double mutant lacking the autoinducer synthases AinS and LuxS, which positively control *litR* expression, exhibited decreased transformation (Fig. 5A). A similar result was seen for an *ainS* single mutant, while a *luxS* single mutant was indistinguishable from the wild-type strain (Fig. 5A). These data parallel those seen for the control of *V. fischeri* bioluminescence, in which AinS plays the more important role (27, 31, 32).

LitR is negatively regulated by sRNA Qrr1, which in turn is activated by LuxO, and thus mutation of either *qrr1* or *luxO* results in increased LitR levels (29) (Fig. 1). Consistent with their roles in controlling LitR levels, the loss of Qrr1 or LuxO caused as much as a 20-fold increase in competence (Fig. 5B). This increase did not overcome the necessity for *litR*, as *qrr1 litR* and *luxO litR* double mutants failed to transform (Fig. 5C). Together, these data demonstrate that LitR and its regulators control competence,

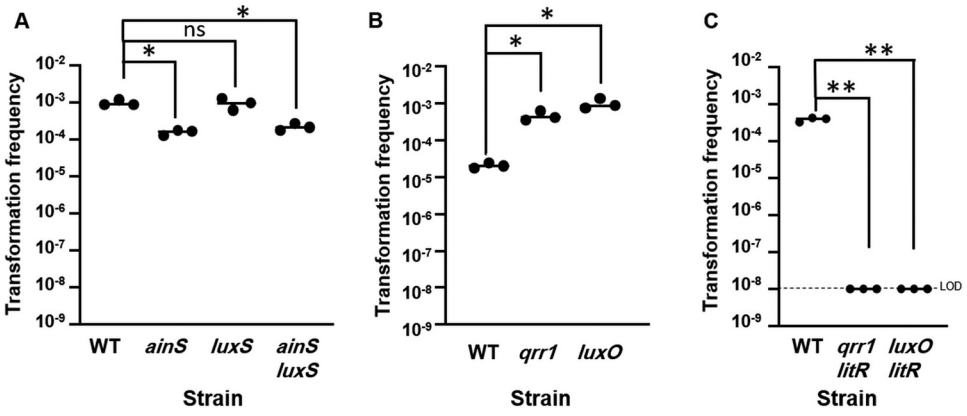

FIG 5 Quorum-sensing regulators control *V. fischeri* transformation. (A) Transformation frequencies (Trim^r CFU/viable CFU) of plostfoX-Cm-containing wild-type (WT) and *ainS* (NL60), *luxS* (CL39), and *ainS luxS* (KV9420) mutant strains. (B) Transformation frequencies of plostfoX-Kan-containing wild-type (WT) and *qrr1* (TIM305) and *luxO* (KV5467) mutant strains. (C) Transformation frequencies of plostfoX-Cm-containing wild-type and *qrr1 litR* (KV8790) and *luxO litR* (KV8791) mutant strains. All strains were exposed to gDNA from KV8300 (*fliQ*::Trim). The *qrr1 litR* and *luxO litR* mutants each exhibited a transformation frequency below the LOD. *, $P < 0.05$; **, $P < 0.005$.

similar to HapR of *V. cholerae* (2), and reveal strains (Δ*qrr1* and Δ*luxO*) with increased competence that can be used as tools when a high transformation frequency is critical.

Requirement for LitR can be partially suppressed by nuclease mutation. In *V. cholerae*, a key role for HapR is to inhibit transcription of the gene encoding Dns, a nuclease that degrades extracellular DNA, preventing its uptake (6). Deletion of *dns* is sufficient to largely restore competence to a *hapR* mutant. To test whether the same is true for *V. fischeri*, we identified and deleted the *dns* homolog, *VF_0437*, and found that the loss of Dns significantly increased the transformation frequency (~50-fold) (Fig. 6A). We then combined the *dns* mutation with a *litR* mutation and found that the resulting double mutant exhibited over a 3-log increase in transformation frequency relative to that of the *litR* mutant (Fig. 6B). The ability of the *dns* mutation to suppress the *litR* transformation defect suggests that the requirement for LitR can be largely attributed to the activity of Dns, which presumably mediates degradation of extracellular DNA.

***tfoX*-induced competence peaks at mid-exponential growth.** Because quorum-sensing regulation changes over time, we sought to determine if a specific point in the

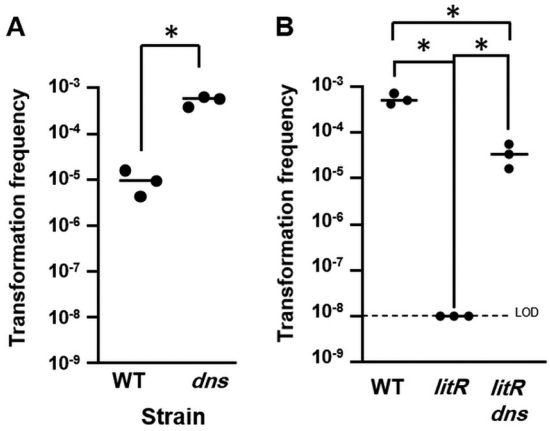

FIG 6 Dns inhibits *V. fischeri* transformation. Shown are transformation frequencies (Trim^r CFU/viable CFU) of strains exposed to gDNA from KV8300 (*fliQ*::Trim). (A) plostfoX-Kan-containing wild-type (WT; ES114) and *dns* mutant (KV8807) strains. (B) plostfoX-Cm-containing wild-type (WT; ES114) and *litR* (PMF8) and *litR dns* (KV9352) mutant strains. The *litR* mutant, but not the *litR dns* mutant, exhibited a transformation frequency below the LOD. *, $P < 0.05$.

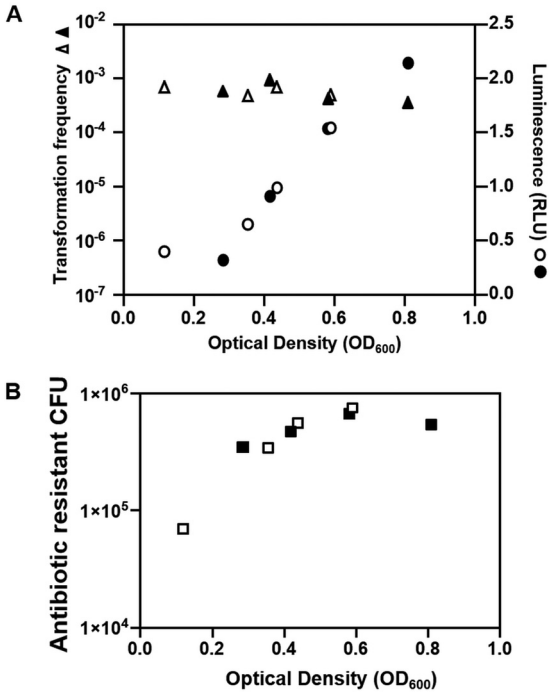

FIG 7 Transformation frequencies during *V. fischeri* growth. (A) Transformation frequencies (Trim^r CFU/viable CFU) and relative light units (RLU) of plostfoX-Cm-containing ES114 assessed at the indicated optical densities during growth of *V. fischeri* in minimal medium. (B) The same transformation experiment as shown in panel A, plotted instead as numbers of transformants at each optical density (not normalized to total cell number). Cells were exposed to gDNA from KV8300 (*fliQ*::Trim). This experiment was performed by sampling from two flasks, indicated by the open and closed symbols.

cell cycle was optimal for transformation by assessing transformation frequencies at different times during liquid growth. Furthermore, we reasoned that another quorum-sensing-controlled phenotype, luminescence, could provide an easy marker for LitR activation (Fig. 1): increased LitR levels should correspond to increased luminescence and increased competence, and thus, luminescence measurements might have predictive value. Surprisingly, however, under our standard TfoX overproduction conditions, the transformation frequencies were consistently at the same approximate level regardless of the cell density (Fig. 7A and Fig. S6). Furthermore, competence could not be predicted by light production: while relatively little light was produced under these minimal medium conditions (which are not the typical conditions used for assessing light production by *V. fischeri*), it did increase modestly over time. However, even at low cell densities when there was little light production, the cells achieved high competence. These data indicate that levels of LitR are only one factor governing competence in TfoX-overexpressing cells and/or that additional levels of control over luminescence (e.g., LuxR and ArcA [33, 34]) impact the timing of luminescence relative to that of competence. We note, however, that with increased OD and thus increased cell number, increased numbers of transformants were obtained (i.e., the overall numbers of transformants, not normalized for total cell number) (Fig. 7B). Thus, the OD of culture used for transformations may ultimately affect the ability to obtain a desired mutant.

Co-overexpression of TfoX and LitR promotes KB2B1 transformation. Finally, we sought to apply the knowledge gleaned in this study to further increase transformation frequencies of *V. fischeri*. We hypothesized that dual overexpression of the two major regulators, TfoX and LitR, might result in higher levels of transformation. To test this possibility, we generated pJJC4, which contains both genes in the Cm^r vector

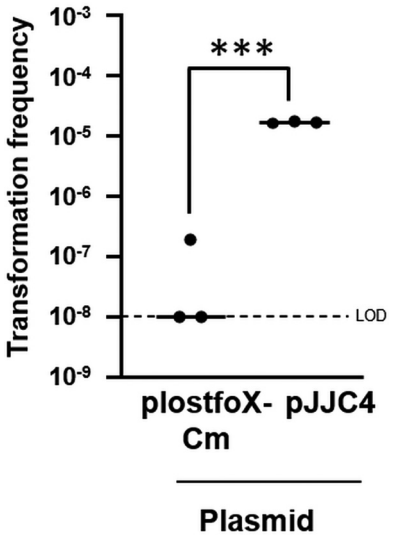

FIG 8 Co-overexpression of *tfoX* and *litR* promotes transformation of KB2B1. Shown are transformation frequencies (Trim^r CFU/viable CFU) of plostfoX-Cm- and pJJC4-containing KB2B1 strains exposed to gDNA from KV8300 (*fliQ*::Trim). Two of the three replicate samples of KB2B1 resulted in transformation frequencies below the LOD. ***, $P < 0.0005$.

backbone. When we compared the transformation frequencies of pJJC4- versus plostfoX-Cm-containing cells of wild-type *V. fischeri* strain ES114, however, we observed no difference (Fig. S7). Because ES114 is already highly transformable with overexpression of *tfoX* alone, we turned our attention to KB2B1, another isolate of *V. fischeri* (35) that, in our hands, is only poorly transformable. We compared the transformation frequencies of KB2B1 derivatives that carried either plostfoX-Cm or pJJC4. We found that there was over a 100-fold increase upon dual overexpression of LitR and TfoX (Fig. 8). Thus, competence and/or transformation of otherwise less transformable *V. fischeri* isolates can be further induced by co-overexpression of these two positive regulators.

DISCUSSION

Chitin- and TfoX-inducible competence of *V. fischeri* was first described in 2010 (17), yet to date, little has been reported about the mechanisms underlying this phenomenon in this bacterium. Bioinformatic analysis by Antonova and Hammer (5) indicated that *V. fischeri* possesses homologs for 22 of 23 genes known or predicted to be important for *V. cholerae* transformation. While our work did not analyze all of those homologs, our results support the model that *V. fischeri* uses similar factors and at least some underlying mechanisms (Fig. 1), providing a foundation for future work characterizing transformation in this microbe.

Our finding that LitR functions as a key regulator of competence in *V. fischeri* may not be considered surprising given the importance of its homolog in controlling competence in *V. cholerae*. However, recent work studying competence in other *Vibrio* species demonstrated that the LitR/HapR homolog OpaR is not required for competence of *V. parahaemolyticus* and that the *V. campbellii* homolog LuxR is not critical for transformation of at least one strain of that species (36). That work underscores the need to test predictions based on bioinformatic analyses, as we have begun to do in this study. It is of note that one *V. cholerae* homolog known to be missing in *V. fischeri* is the gene for QstR, an intermediate positive regulator found in *V. cholerae* (13, 37). The lack of a *qstR* homolog in the genome signifies that *V. fischeri* has made some adjustments to its competence pathway that will be of interest to uncover.

This new role for LitR in *V. fischeri* competence expands our understanding of this

regulator, whose function has been primarily associated with luminescence control and, to a lesser extent, motility (23, 29, 32). Due to the substantial role of LitR in controlling luminescence, we had hypothesized that we could use luminescence, which is readily measured in real time, to predict when cells might be competent. Unfortunately, our work indicated no relationship between light production and competence under the conditions used, namely, TfoX overproduction and growth in a minimal medium (Fig. 7). It was perhaps a naive view that competence and light production would correspond, given the known complex control over light production that would prevent such a straightforward assessment of LitR induction and thus competence. Alternatively, or in addition, the use of *tfoX* overexpression could disconnect competence from light production. Regardless, researchers in the field who wish to use this method to generate mutants may benefit from the knowledge that while TfoX-induced transformation frequency in *V. fischeri* is not OD dependent, the numbers of transformants increase with increasing OD (Fig. 7); thus, performing the transformation at higher ODs may be superior for obtaining a rare transformant due to, for example, a low concentration of DNA containing the marker/mutation of interest.

We had also hoped to resolve the controversy in the field over the requirement for TfoY. We found that the loss of TfoY failed to significantly impact transformation frequencies, consistent with the findings obtained by others (17, 24). In the original report of competence in *V. fischeri*, TfoY was shown to be necessary for full competence (17, 24). The discrepancy could lie in the use by Pollack-Berti et al. of chitohexose, which was omitted in this study. In *V. cholerae*, TfoY appears to function to induce a type VI secretion system (T6SS), which, in turn, promotes killing of other bacteria and thus release of free DNA in the environment (24). To date, no T6SS-mediated killing capability has been observed for ES114 (38), leaving unanswered the question of if/how TfoY contributes to competence in this microbe. Of note, TfoY does appear to play a critical, positive role in motility in *V. cholerae*, and it has been suggested to play a similar role in *V. fischeri* by overexpression studies (25, 26), which we confirmed in this study. This gene was not discovered in a wide-scale transposon mutagenesis search for genes involved in motility and chemotaxis (39), possibly because its relatively small size (588 bp) may limit insertions at that location. Alternatively, differences in how the motility experiment was conducted in this study relative to that of Brennan et al. (39) could account for this discrepancy; in *V. cholerae*, decreasing the nutrient content of the growth medium alters the motility phenotype of the *tfoY* mutant (26).

A major underlying goal of this work was to increase transformation frequencies to facilitate genetic manipulations of the classical wild-type strain ES114, with the expectation that lessons learned might also be leveraged to promote studies of less genetically tractable *V. fischeri* strains. Indeed, our work revealed mutant strains, namely, *qrr1*, *luxO*, and *dns* mutants, with increased transformation frequencies that can be used as tools for low-efficiency transformations, such as when the DNA derives from an SOE PCR approach, which can yield low levels of the correct product. Given that a marked mutation of interest can be subsequently moved from, for example, a *qrr1* mutant into a wild-type (or other) strain background by transformation, these strains are valuable tools. In some instances, however, it may be necessary or desirable to have a high transformation frequency of a specific strain. Thus, it was of interest that the two published versions of the *tfoX* expression plasmid resulted in different transformation efficiencies, with plasmid carrying the Cm^r marker yielding more transformants than those obtained with the Kan^r plasmid. Because we observed a similar phenomenon using derivatives with a newly introduced, arabinose-inducible *tfoX* gene, we conclude that the difference depends on the resistance marker and/or the stronger selective pressure of the Cm antibiotic. The disparity is substantial, and those seeking optimal conditions for transformation will thus need to consider the vector "backbone" as a factor—and whether these plasmids behave the same way when carried by a distinct isolate as they do in ES114. Finally, we developed pJJC4, which overexpresses *tfoX* and *litR* in the context of the better Cm^r plasmid, to further enhance transformation frequency. While

TABLE 1 Strains constructed and/or used in this study

Strain	Genotype	Derivation[a]	Reference
CL39	*luxS*::Kan	NA	27
ES114	Wild type	NA	50
JB19	*litR*::Erm	NA	34
KV5467	Δ*luxO*	Conjugation of ES114 with pVAR36-containing *E. coli*	This study
KV8300	Δ*fliQ*::FRT-Trim	NA	22
KV8790	Δ*qrr1 litR*::Erm	TT TIM305 with gJB19	This study
KV8791	Δ*luxO litR*::Erm	TT KV5467 with gJB19	This study
KV8807	Δ*dns*::FRT-Erm	TT ES114 with SOE PCR with primers 2504 and 2505 (ES114), 2089 and 2090 (pKV494), and 2506 and 2507 (ES114)	This study
KV8840	Δ*cytR*::FRT-Erm	TT ES114 with SOE PCR with primers 2512 and 2513 (ES114), 2089 and 2090 (pKV494), and 2514 and 2515 (ES114), amplified with primers 2512 and 2515	This study
KV8875	*pilA*::mTn5	mTn*5* mutagenesis of ES114, backcrossed into ES114	This study
KV8876	*pilB*::mTn5	mTn*5* mutagenesis of ES114, backcrossed into ES114	This study
KV8877	*pilC*::mTn5	mTn*5* mutagenesis of ES114, backcrossed into ES114	This study
KV8879	*pilQ*::mTn5	mTn*5* mutagenesis of ES114, backcrossed into ES114	This study
KV9219	Δ*comEA*::FRT-Erm	TT ES114 with SOE PCR with primers sets 2628 and 2629 (ES114), 2089 and 2090 (pKV494), and 2630 and 2631 (ES114)	This study
KV9352	Δ*dns*::FRT-Erm *litR*::Kan	TT KV8807 with gPMF8	This study
KV9420	Δ*ainS luxS*::Kan	TT NL60 with gCL39	This study
NL60	Δ*ainS*	NA	51
PMF8	*litR*::Kan	NA	23
TIM305	Δ*qrr1*	NA	29

[a]TfoX-induced transformation (TT) was performed with *tfoX* overexpression versions of the indicated strains using either SOE PCR DNA or genomic DNA (gDNA) from the indicated strain. NA, not applicable; mTn*5*, mini-Tn*5*.

this plasmid did not increase transformation of ES114, it permitted a higher level of transformation of KB2B1, a *V. fischeri* isolate that is less genetically tractable. KB2B1 is of interest due to its "dominance" in colonizing its squid host (35). Consequently, these findings may be expected to advance the investigation of this strain and potentially other *V. fischeri* strains that are less amenable to genetic manipulation.

In summary, this work forms the foundation for the construction of a competence pathway by testing hypotheses based on bioinformatics and identifying roles for *litR* and other putative competence factors. Furthermore, it delivers insight into conditions, strains, and plasmids that promote transformation of *V. fischeri*. We anticipate that these findings will accelerate the construction of mutants in various strain backgrounds and promote the study not only of competence but also of other phenomena of interest.

MATERIALS AND METHODS

Strains and media. *V. fischeri* strains, plasmids, and primers used in this study are listed in Tables 1 to 3, respectively. *Escherichia coli* strains TAM1 or TAM1 λ *pir* (Active Motif, Carlsbad, CA), π3813 (40), GT115 (InvivoGen, San Diego, CA), and DH5α were used for plasmid maintenance, cloning, and conjugation purposes. For routine growth, maintenance, or selection for transformants, *V. fischeri* was grown in the complex medium LBS (41, 42), which contains 1% tryptone, 0.5% yeast extract, 2% sodium chloride,

TABLE 2 Plasmids constructed and/or used in this study

Plasmid	Description	Resistance marker	Reference
pEVS104	Conjugal plasmid	Kan	44
pJJC4	plostfoX-Cm + *litR*	Cm	This study
pKV363	Suicide vector	Cm	47
pKV494	Carries FRT-Erm^r cassette	Ap, Erm	22
pLostfoX-Cm[a]	pEVS79 + *tfoX*	Cm	17
pLostfoX-Kan	plostfoX with Kan^r in place of Cm^r	Kan	21
pVAR36	pKV363 + sequences flanking *luxO*	Cm	52
pVSV105	Stable *Vibrio* plasmid	Cm	48
pPMF5	pVSV105 containing *litR*	Cm	This study

[a]plostfoX-Cm was published (17) as pLostfoX; we use the plostfoX-Cm terminology here for clarity. This plasmid contains the *tfoX* gene with ~150 bp of upstream sequence that may contain its promoter and is oriented in the same direction as the *lac* promoter within the pEVS79 (44) vector.

TABLE 3 Primers used in this study

Primer no.	Purpose	Sequence[a]
908	Check Tn*5* insertions	GCACTGAGAAGCCCTTAGAGCC
1319	Delete *luxO*	taggcggccgcacttagtatgGGAAGCAGTATCTTCTACCAT
1320	Delete *luxO*	catactaagtgcggccgcctaTGGAATGAAAGATAAGGGGAC
1344	Delete *luxO*	CGTAAAGTTGTTGCACCTAAG
1345	Delete *luxO*	GCAGGTAAGATGGATCATAGG
2089	Amplify antibiotic resistance cassettes by PCR	CCATACTTAGTGCGGCCGCCTA
2090	Amplify antibiotic resistance cassettes by PCR	CCATGGCCTTCTAGGCCTATCC
2504	Delete *dns*	ACCCCACTTCAACAAGCATC
2505	Delete *dns*	taggcggccgcactaagtatggGAGCCAATAGCGATAATGCT
2506	Delete *dns*	ggataggcctagaaggccatggTGTCGCAAATAGACATAATTAGAG
2507	Delete *dns*	TCAGGAACAGTATTACGTCCAC
2512	Delete *cytR*	GTGCAGGGCGTGCAAGTAAA
2513	Delete *cytR*	taggcggccgcactaagtatggTCCTGCCGATAAAGCAACGT
2514	Delete *cytR*	ggataggcctagaaggccatggGTTAGAGAGAGTACGGCGTCC
2515	Delete *cytR*	CACTGCATAATGTAAGGGATCTC
2531	Coexpress *tfoX* and *litR*	ggtaccGATTAAGGAAGAGCTGTTAAC
2532	Coexpress *tfoX* and *litR*	ggtaccGCTGCGGAAGTATTTGAAGG
2626	Check putative *pilQ*::Tn*5* insertion	ggtaccCACCTAAAGGGAGTGTGATTAAAGT
2627	Check putative *pilQ*::Tn*5* insertion	gagctcGGCACCATATTCCTCACAGATAT
2628	Delete *comEA*	GGAAGCCCTAGAACTTGCTC
2629	Delete *comEA*	taggcggccgcactaagtatggACTGAGTAAAGTGAGTATGCG
2630	Delete *comEA*	ggataggcctagaaggccatggATTGGGGATAAACTGGTTGAG
2631	Delete *comEA*	AGTTAAATTTGCGATGGCTC
2859	Check putative *pilA*::Tn*5* insertion	TCACCCGATTCATCTTGGAG
2860	Check putative *pilA*::Tn*5* insertion	GATAGAGGCGAGGTTGGTGTGC
2861	Check putative *pilB*::Tn*5* insertion	TATCCCAGGCTGTCAAGGCC
2862	Check putative *pilB*::Tn*5* insertion	CTGAACTGTTAATGCCACGCCA
2863	Check putative *pilC*::Tn*5* insertion	GAAGATGGCACCACCAGCTTAC
2864	Check putative *pilC*::Tn*5* insertion	GGGGCAAGATCTGGAAAGCA
litR-ndeI F	Amplify *litR* and ligate into pVSV105	ccccatatgGATTAAGGAAGAGCTGTTAACGG
litR-ndeI R	Amplify *litR* and ligate into pVSV105	ccccatatgGCTGCGGAAGTATTTGAAGG

[a]Lowercase letters indicate primer "tails."

and 50 mM Tris (pH 7.5). For transformations, *V. fischeri* was grown in Tris minimal medium (TMM), which contains 300 mM sodium chloride, 50 mM magnesium sulfate, 0.33 mM potassium phosphate dibasic, 10 μM ferrous ammonium sulfate, 0.1% ammonium chloride, 10 mM *N*-acetylglucosamine, 10 mM potassium chloride, 10 mM calcium chloride, and 100 mM Tris (pH 7.5). This medium was made fresh from concentrated and autoclaved stocks, with the exception that Tris was added from a concentrated, filter-sterilized stock. *E. coli* was grown in the complex medium LB (43), which contains 1% tryptone, 0.5% yeast extract, and 1% sodium chloride. Agar (1.5%) was used for solid media. As appropriate, antibiotics were added as follows (final concentrations in parentheses): for *V. fischeri*, chloramphenicol (Cm; 1 μg ml^{-1} for single-copy selection or 5 μg ml^{-1} for plasmid maintenance), erythromycin (5 μg ml^{-1}), kanamycin (Kan; 100 μg ml^{-1}), and trimethoprim (Trim; 10 μg ml^{-1}); for *E. coli*, ampicillin (100 μg ml^{-1}), Cm (12.5 μg ml^{-1}), and Kan (50 μg ml^{-1}).

Strain construction. Mutant *V. fischeri* strains were generated by introducing a *tfoX* overexpression plasmid, such as plostfoX-Cm (17) or plostfoX-Kan (21), from *E. coli* by triparental conjugation with pEVS104-containing (44) *E. coli* and the appropriate *V. fischeri* recipient as described previously (45). The resulting strains were used in transformations with DNA derived from SOE PCR (46) amplifications with primers as indicated in Tables 1 and 3. High-fidelity PCR enzyme EMD Millipore KOD DNA polymerase

was used for PCR amplifications to generate fragments containing a central antibiotic resistance cassette and ~500-bp flanking sequences homologous to regions up- and downstream of the target gene as described previously (22). As a result of using the often-inefficient SOE PCR to obtain a desired PCR product, the amount of the DNA added during these strain construction transformations was variable and generally much less than described in "*V. fischeri* transformation" below. In addition, no assessments of total CFU were performed during strain constructions. Putative mutants that arose on selective medium were purified by streaking, first on the same selective medium and then nonselectively to permit loss of the *tfoX*-containing plasmid. The presence of the desired mutation was confirmed by PCR with outside primers. In some cases, the mutation was moved from an original recipient strain to a different background by isolating genomic DNA and using it to transform a new strain of interest. The unmarked Δ*luxO* strain KV5467 was generated as previously described (40, 47) using pVAR36.

Plasmid construction and analysis. Previously unpublished plasmids pJJC4 and pPMF5 (Table 2) were used in this study and were constructed as follows. For plasmid pJJC4, a PCR product containing *litR* with flanking KpnI sequences (obtained with primers 2531 and 2532) was first cloned into pJET1.2 using the Thermo Scientific CloneJET PCR cloning kit, prior to subcloning into KpnI-digested plostfox-Cm (retaining *tfoX*). pPMF5 was generated by amplifying *litR* using PCR with primers *litR*-NdeI F and *litR*-NdeI R and cloning NdeI-digested DNA into pVSV105 (48). To evaluate potential differences in the *tfoX* gene between plostfoX-Cm and plostfoX-Kan, the plasmids were purified and the *tfoX* gene was sequenced using M13 forward and reverse primers supplied by ACGT, Inc. (Wheeling, IL). No differences in sequence between the two plasmids were observed.

***V. fischeri* transformation.** *V. fischeri* strains carrying a *tfoX* overexpression plasmid were grown in TMM, made as described in "Strains and media" above, containing 100 μg ml^{-1} of Kan or 5 μg ml^{-1} of Cm. Cultures of ES114 derivatives were grown overnight in 5 ml of medium in 18- by 150-mm tubes at 28°C with shaking for 13 to 16 h, then subcultured by diluting the culture to a starting culture density (optical density at 600 nm [OD_{600}]) of 0.05 in 20 ml of the same medium in 125-ml baffled flasks, and grown with shaking at 24°C; for KB2B1 derivatives, cultures were subcultured to a starting OD_{600} of 0.1. For most experiments, cultures were grown until the OD_{600} was approximately equal to 0.5. Then, in triplicate, a 0.5-ml aliquot was transferred into a 2-ml Eppendorf tube. The cells were then exposed to DNA, or left unexposed for the negative control, and then incubated for 30 min at room temperature. For assessing transformation frequencies for the experiments, 500 ng of genomic DNA derived from strain KV8300 (Δ*fliQ*::FRT-Trim) (22) was used. Genomic DNA was prepared using the Quick-DNA Miniprep Plus kit (Zymo Research, obtained from Genesee Scientific) and normalized to a concentration of 100 ng per μl. Following the incubation period, a 0.5-ml aliquot of LBS broth was added to each culture and the 1-ml mixtures were transferred to 18- by 150-mm test tubes. The test tubes were incubated with shaking at 28°C for 90 min to permit recovery and expression of the antibiotic resistance gene. At that time, the cells were (i) diluted (or left undiluted) and spread onto selective medium (LBS plus trimethoprim) to assess the number of transformants that had recombined the DNA of interest and (ii) diluted and spotted in 10-μl aliquots on a nonselective medium (LBS) to determine the total CFU. In a few cases, cells that had completed the 90-min recovery were held on ice for a short period prior to dilution and plating; we observed no substantial difference in numbers of transformants or total CFU before or after ice exposure. Transformation frequency was calculated as the number of antibiotic-resistant CFU (multiplied by the dilution factor) divided by the number of total CFU (multiplied by the dilution factor). A similar calculation was performed to determine the limit of detection (LOD), using an approximate average total CFU of 1×10^9. Occasionally, selection for Trim^r yielded spontaneously resistant colonies; these colonies appeared more translucent than true transformants and retained an ability to swim in soft agar, unlike true transformants, which became nonmotile due to acquiring the mutation in flagellar gene *fliQ* (Δ*fliQ*::FRT-Trim^r). The phenotype of each mutant strain was evaluated in the manner described above at least twice in duplicate or triplicate, with similar outcomes.

Transformation and light production. For measurements of transformation frequency over time, and corresponding luminescence levels, we used the following procedure. *V. fischeri* strain ES114 carrying plostfoX-Cm was grown in TMM containing Cm overnight with shaking at 28°C for no longer than 14 h. The cells were then subcultured into each of two 125-ml baffled flasks to an OD of 0.05 in 20 ml of fresh medium (TMM containing Cm) and incubated with shaking at 24°C. The OD_{600} of the samples were measured at 1-h intervals until an OD of 0.2 was reached. Then, samples were taken every 30 min to assess growth, luminescence, and transformation, until an OD of about 0.4 was reached, and then every 15 min for the remainder of the experiment, which we performed until the cells reached an OD_{600} of around 1, which took about 6 or 7 h. In this medium, the cells reach a final OD_{600} of between 1.2 and 1.5. Samples were taken from alternating flasks throughout the experiment to reduce the decrease in culture volume from any one flask. At these time points, 1 ml was pipetted into a sterile scintillation vial, aerated in a consistent manner by pipetting before using a Turner Designs TD-20/20 luminometer to measure relative light units (RLU). A portion (0.5 ml) was then transferred to an Eppendorf tube containing DNA for transformation, as outlined above, while the remaining 0.5 ml was transferred to a cuvette to measure the OD_{600}. The remainder of the transformation experiment was carried out as described above in "*V. fischeri* transformation."

***pil* mutants.** The *pil* mutants were derived from a transposon (mini-Tn*5* (mTn*5*) mutant library collection. To reduce the concern that an unlinked mutation could be responsible for any observed phenotype, the *pil* mutants (*pilA*::mTn*5*, *pilB*::mTn*5*, *pilC*::mTn*5*, and *pilQ*::mTn*5*) were backcrossed into *tfoX*-containing ES114. In addition, the position of the Tn insertion was verified both by sequencing (49) and by applying PCR to amplify a segment of DNA using a Tn-specific primer and a gene-specific primer.

Statistics. Statistics were performed on GraphPad using Student's *t* test with Welch's correction.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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