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Engineering *Vibrio fischeri* for Inducible Gene Expression

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Abstract: The marine bacterium *Vibrio fischeri* serves as a model organism for a variety of natural phenomena, including symbiotic host colonization. The ease with which the *V. fischeri* genome can be manipulated contributes greatly to our ability to identify the factors involved in these phenomena. Here, we have adapted genetic tools for use in *V. fischeri* to promote our ability to conditionally control the expression of genes of interest. Specifically, we modified the commonly used mini-Tn*5* transposon to contain an outward-facing, LacI-repressible/IPTG-inducible promoter, and inserted the *lacI* gene into the *V. fischeri* chromosome. Used together, these tools permit the identification and induction of genes that control specific phenotypes. To validate this approach, we identified IPTG-controllable motility mutants. We anticipate that the ability to randomly insert an inducible promoter into the genome of *V. fischeri* will advance our understanding of various aspects of the physiology of this microbe.

Keywords: Genetics, inducible gene expression, motility, mutagenesis, *Vibrio fischeri.*

INTRODUCTION

Vibrio fischeri is a marine bacterium used as a model organism for studies of bioluminescence [1, 2], quorum sensing [3], biofilm formation [4, 5], and symbiosis [6, 7]. The discovery and characterization of these natural phenomena have been greatly facilitated by the development and use of genetic tools that disrupt and modify genes within the *V. fischeri* genome.

While the bioluminescent nature of *V. fischeri* was first described in the 19th century by Martinus Willem Biejerinck [8], it was not until 1992 when the first genetic manipulation was described by Dunlap and Kuo [9]. These researchers successfully introduced foreign DNA *via* conjugation and generated gene replacement mutants *via* homologous recombination. Specifically, they manipulated a plasmid-borne copy of the *V. fischeri* luminescence (*lux*) genes in *Escherichia coli* to obtain insertion and deletion mutants, then transferred the resulting constructs into *V. fischeri*, selecting for *lux* mutant recombinants. In 1994, the technique of transposon mutagenesis was applied to *V. fischeri* [10], thus permitting the investigation of uncharacterized genes and their involvement in phenotypes such as host colonization.

In subsequent years, the ability to manipulate *V. fischeri* genetically was facilitated by the identification of antibiotic resistance cassettes that functioned in this organism and, in concert, the construction and/or use of a variety of plasmids: those that were more stable were used for complementation, while those that were less stable were used for recombination [11]. Importantly, the identification of a *V. fischeri*-specific origin of replication facilitated the construction of highly stable vectors that (1) could be maintained without antibiotic selection and (2) were compatible with other commonly used plasmids [12], allowing experiments that depended on the use of two plasmids [13]. In addition, constructs used for inserting genes in single copy at the Tn*7* site of other bacteria [14] were successfully adapted for *V. fischeri* [11, 15], permitting single copy gene expression and complementation in the absence of antibiotic selection and making it possible to conduct well-controlled gain of function experiments in the context of animal colonization [16-19].

The whole genome of *V. fischeri* was sequenced and annotated in 2005 [20], allowing researchers to more readily utilize reverse genetics approaches. Facilitating such approaches, a counter-selectable suicide vector used to generate unmarked gene replacements in *Vibrio splendidus* [21] was adapted for use in *V. fischeri*. [17, 22]. This approach has permitted the reliable and rapid construction of unmarked mutations and the ability to readily generate strains with multiple unmarked mutations.

Although *V. fischeri* genetics has advanced greatly since 1992, some aspects remain under-developed. For example, little work has been done to develop inducible control over the expression of genes present in the chromosome. Although plasmid libraries have been developed [23, 24] and can be used to identify positive regulators of a given phenotype, such libraries are limited in the size of the insert and are unlikely to contain large operons intact. Here, we discuss the adaptation and use of a genetic tool for *V. fischeri* that allows for gene disruption as well as the insertion of a conditional promoter randomly into the genome, and can

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thus facilitate the identification of genes, such as positive regulators or large operons, whose induction drives a desired phenotype.

MATERIALS AND METHODS

Strains and media

The *V. fischeri* strains and plasmids constructed and/or used in this study are listed in Table **1**. *V. fischeri* strain ES114 was used as the wild-type strain in these studies. *V. fischeri* strains were grown in complex LB-salt (LBS) medium [25] and tryptone broth-seawater (TB-SW) motility medium [26]. We also used the *E. coli* strains GT115 (Invivogen, San Diego, CA), CC118 [27], and π3813 [21] for the purposes of cloning and conjugation. *E. coli* strains were grown using either LB [28] or Brain-heart infusion (BHI) (Difco) medium. The following antibiotics were added, as appropriate, at the indicated final concentrations: chloramphenicol (Cm) at 1 to 5 µg/ml for *V. fischeri* and 25 µg/ml for *E. coli*; tetracycline (Tc) at 5 µg/ml for *V. fischeri* and 15 µg/ml for *E. coli*; kanamycin (Kan) at 50 µg/ml; erythromycin (Erm) at 5 µg/ml for *V. fischeri* and 150 µg/ml for *E. coli*; and ampicillin (Ap) at 100 µg/ml. Thymidine was added to a final concentration of 300 µM for the growth of π3813 *E. coli* cells. Agar was added at a final concentration of 1.5% for solid media and 0.225% for the motility medium.

Plasmid and strain construction. Standard molecular biology, transformation, and conjugation approaches were used to generate the plasmids and strains used in this study. The primers used are shown in Table **2**. Plasmid pJMO14 was used to insert the *lacI*^{q}gene into the chromosome between *yeiR* and *glmS*, adjacent to the attTn*7* site (Fig. **2**). To generate pJMO14, upstream and downstream sequences (~500 bp) flanking the target insertion site were first amplified from the ES114 chromosome by PCR using primers 1484 and 1485 and primers 1486 and 1487, then joined using overlap extension PCR [22, 29]. The resulting DNA fragment, which consisted of the flanking sequences joined by non-native sequences including a NotI site, was ligated into suicide vector pKV363 to generate pJMO8. Finally, the *lacI*^{q} gene, which was amplified using PCR with pCA24N as a template and primers 1544 and1489, was cloned into the PCR cloning vector pJET1.2, then sub-cloned into NotI-digested pJMO8 to generate pJMO14. To insert *lacI*^{q} into the chromosome, the method of Le Roux *et al.* [21] was used as described previously [22]. The insertion of *lacI*^{q} into ES114, generating KV6576, was confirmed using PCR with primers 974 and 975.

Plasmid pJMO10, which was used to deliver the Tn*5*+ promoter (Tn*5*P) for transposon mutagenesis, was constructed as follows. Oligonucleotides 1439 and 1440, which contained sequences for the LacI-repressible $P_{A1/34}$ promoter [1, 30], were annealed and ligated into the ApaI/SpeI-digested Tn*5* delivery plasmid pEVS170 [31]. The insertion of the promoter into Tn*5* was confirmed by sequencing.

Table 1. Bacterial strains and plasmids.

Strain	**Genotype**	**Reference**
V. fischeri		
ES114	Wild-Type	[42]
KV6576	IG *(yeiR-glmS*)::*lacI*^{q}	This Study
KV7432	IG (*yeiR-glmS*)::*lacI*^{q} IG (*VF_A0340-VF_A0341*)::Tn*5*P (Erm^r)	This Study
KV7433	IG *(yeiR-glmS*)::*lacI*^{q} *cheZ*::Tn*5*P (Erm^r)	This Study
Plasmid	**Description**	**Reference**
pCA24N	*lacI*^{q} (Cm^r)	[43]
pCLD46	LacI-repressible RscS overexpression plasmid (Cm^r)	[13]
pEVS104	Conjugal helper plasmid (Kan^r)	[32]
pEVS107	Mini-Tn*7* delivery plasmid (Kan^r Erm^r)	[15]
pEVS170	Tn*5* carrier plasmid (Kan^r Erm^r)	[31]
pJET1.2	Commercial cloning vector (Amp^r)	Fermentas
pJMO8	pKV363 containing the *yeiR-glmS* IG region (Cm^r)	This Study
pJMO10	pEVS170 containing a promoter within the Tn (Tn*5*P) (Kan^r Erm^r)	This Study
pJMO14	pJMO8 containing the *lacI*^{q} allele (Cm^r)	This Study
pKV363	Mobilizable suicide vector (Cm^r)	[22]
pVSV105	Mobilizable vector (Cm^r)	[12]

Table 2. Oligonucleotides.

Primer #	Name	Sequence
908	170Ext2	GCACTGAGAAGCCCTTAGAGCC
974	Tn7_primerdma89	GCTAAAGCGGTGACGGTGGAGTAG
975	Tn7_primerdma90	CCTCACCCCAGATGGTTTGGCA
1439	Tn5-P_out Spe/Apa-F	CTAGTTTATCAAAAAGAGTGTTGACTTGTGAGCGGATAACAATGATACTTA GATTCAATTGTGAGCGGATAACAATTTCACACAGGGCC
1440	Tn5-P_out Spe/Apa-R	CTGTGTGAAATTGTTATCCGCTCACAATTGAATCTAAGTATCATTGTTATC CGCTCACAAGTCAACACTCTTTTTGATAAA
1484	VF2371 F	CTTGATTTATACAGCGAAGG
1485	VF2371 Not I R	TAGGCGGCCGCACTTAGTATGGTTTTGAAGAGTAATTAATGTTTATTG
1486	VF2372 Not I F	CATACTAAGTGCGGCCGCCTATATTGTCTCTCTTAGAACAATTATTC
1487	VF2372 R	GGTCGTGGGGAGTTTTATCC
1489	LacINotI R	GCGGCCGCCGCTCACTGCCCGCTTTCC
1544	LacIqNotI F	GCGGCCGCGACACCATCGAATGGTGCAAAAC

Wrinkled colony formation. Strains were grown overnight in LBS containing CM at 28°C with agitation, then subcultured into fresh medium and grown until they reached exponential phase. Cultures were then diluted to an optical density at 600 nm (OD_{600}) of 0.2, concentrated by centrifugation, and re-suspended in fresh medium. 10 µl aliquots of each culture were spotted onto an agar plate and incubated at room temperature. Images of the wrinkled spots were captured at the indicated times.

Tn*5*P mutagenesis and identification of IPTG-inducible motility mutants. Parental strain KV6576 was mutagenized with Tn*5*P by performing a tri-parental conjugation [32] with two *E. coli* strains, one carrying pJMO10 and other carrying pEVS104 [32] and selecting for the insertion of the transposon using Erm-containing LBS (LBS-erm) plates. Resulting colonies were inoculated into TB-SW motility plates lacking or containing 1.75 mM IPTG. Putative motility mutants with IPTG-controlled phenotypes were purified from motility plates by streaking onto LBS-erm plates. These strains were then evaluated a second time to verify their motility phenotypes.

Identification of Tn*5*P insertion sites. To determine the site of the Tn*5*P insertion in the motility mutants, chromosomal DNA was isolated and digested using the HhaI restriction enzyme as previously described [31]. Digested DNA was then self-ligated and transformed into CC118 cells, and clones were selected on Erm-containing BHI agar. The resulting plasmids were sequenced using primer 908.

Motility Assays. Motility mutants were grown overnight in LBS and the cultures were diluted to an OD_{600} of 0.4. Cells were pelleted, washed, and re-suspended in LBS and 5 µl aliquots were inoculated on the surface of TB-SW motility plates that contained the indicated amounts of IPTG. Representative images were captured and the diameters of the outer ring of the swimming cells were measured using a ruler.

RESULTS AND DISCUSSION

Generation of Tn*5*P, a Tn*5* transposon with an outward-facing promoter. One genetic tool that remained to be developed for *V. fischeri* was a method for randomly inserting an inducible promoter into the genome. To develop this tool, we chose to use the LacI-repressible promoter A1/34 [1]. This promoter contains two LacI binding sites, one between the -35 and -10 sites and another that overlaps the transcriptional start site (Fig. **1**), and has been shown previously to function as a strong promoter in *V. fischeri* when it was inserted directly upstream of an operon [1]. We then engineered the mini-Tn*5* delivery vector pEVS170 [31] to contain this promoter within the transposable element in an outward-facing position; the insertion of the promoter was confirmed by sequencing. This transposon will be referred to as Tn*5*P, for Tn*5* plus Promoter.

Insertion of the *lacI* gene into *V. fischeri* and phenotypic assessment. *V. fischeri* does not contain the *lac* operon or the *lacI* repressor gene. Therefore, to control expression from the promoter within Tn*5*P, it was necessary to introduce the *lacI* gene into *V. fischeri*. One location traditionally used to insert genes within the *V. fischeri* genome is the Tn*7* site, which is positioned between *yeiR* and *glmS* (Fig. **2**) [15, 33]. However, because this site is heavily utilized for single copy complementation (*e.g.*, [34]), we chose to leave this site intact for future manipulations. Instead, we targeted the insertion of $lacI^q$, a more highly transcribed allele of the *lacI* gene, to a region immediately adjacent to the Tn*7* site (between *yeiR* and the Tn*7* site) (Fig. **2**). The result was a strain, KV6576, that contains $lacI^q$, retains an intact Tn*7* site, and remains unmarked, allowing for the use of an antibiotic resistance marker in future manipulations.

With the generation of the $lacI^q$-containing *V. fischeri*, two questions arose: (i) Is the $lacI^q$ allele functional (*e.g.*, does it control gene expression in *V. fischeri*?); and (ii) is the

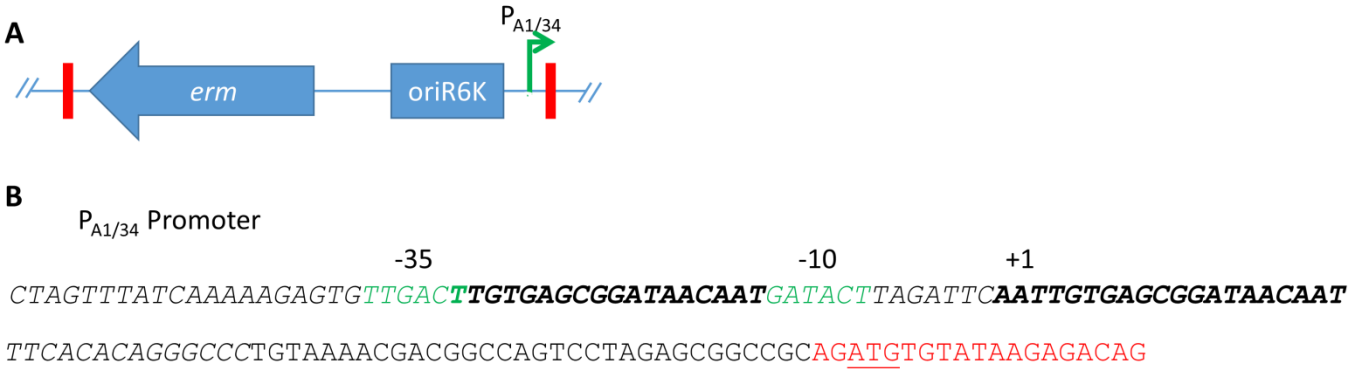

Fig. (1). Construction of the Tn*5*P transposon. A) The Tn*5* from pEVS170 contains an erythromycin resistance gene and an origin of replication (blue arrow and box) within the Tn*5* ends (red rectangles). pEVS170 was modified to contain an outward-facing, LacI-repressible/IPTG-inducible promoter, $P_{A1/34}$ (green arrow) [1]. (**B**) The $P_{A1/34}$ promoter region (italics) contains two LacI binding sites (bold). The binding of LacI to these sites hinders access to the -35 and -10 sites (green), thus repressing transcription. The Tn*5* end (red) contains a start codon (underlined).

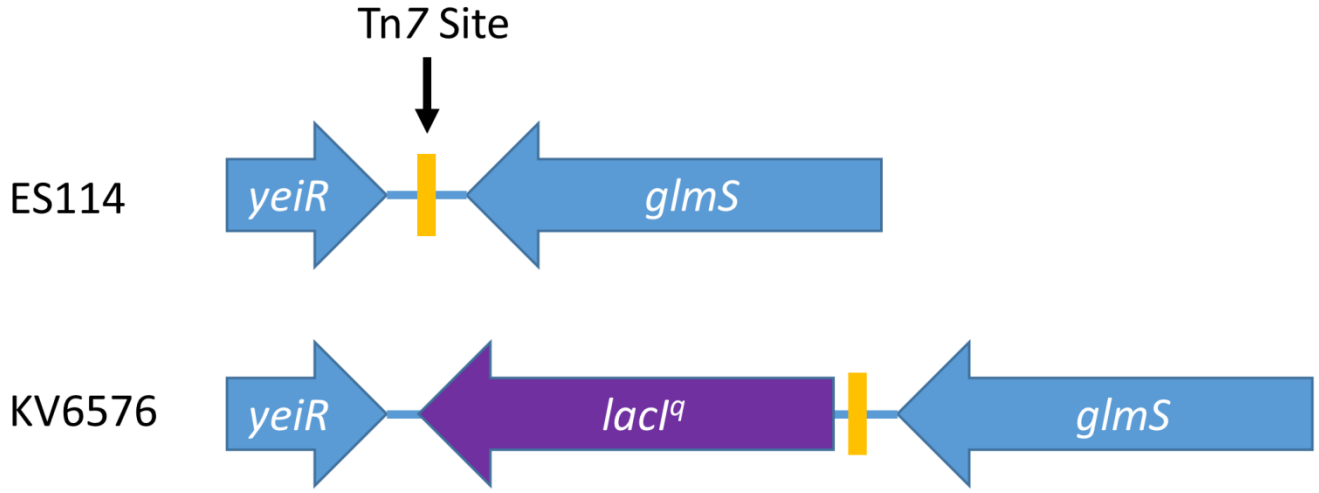

Fig. (2). Construction of a *lacI*-expressing *V. fischeri* strain. ES114 contains a Tn*7* site (yellow rectangle) between *yeiR* and *glmS*. KV6576 was engineered to contain the *lacI* overexpression allele (*lacI*^{q}, purple arrow) near, but not disrupting, the Tn*7* site.

Tn*7* site, which is adjacent to the site of insertion of the *lacI*^{q} gene, still permissive for insertion events? To assess whether the *lacI*^{q} allele in KV6576 was functional, we investigated the ability of this strain to impact expression of a *lac* promoter-controlled gene. Specifically, we introduced into KV6576 a plasmid, pCLD46, that contains the *rscS* gene driven by the *lac* promoter, or pVSV105, the empty vector from which pCLD46 was derived. When pCLD46 is introduced into wild-type *V. fischeri*, RscS protein is made and induces biofilm formation [13]. One biofilm phenotype that can be readily assessed is the formation of wrinkled colonies. We anticipated that, if the *lacI*^{q} allele in KV6576 were functional, then LacI would repress expression of *rscS*, resulting in a strain that either fails to form wrinkled colonies or does so after a delay. Indeed, we found that this strain failed to wrinkle after 24 hours of growth in the absence of IPTG (Fig. **3**). Moreover, when we grew the strain in the presence of IPTG, which should inactivate LacI, wrinkled colonies developed with a timing indistinguishable from the control (Fig. **3** and data not shown). These data indicate that functional LacI was made and was responsive to IPTG. We note, however, that the repression of *rscS* expression from pCLD46 was not complete: at later times, the strain exhibited a modest wrinkling phenotype in the absence of IPTG (Fig **3**). We attribute this result to the inability of LacI expressed from the chromosome to fully repress a promoter present on a multi-copy plasmid.

To verify that the Tn*7* site near the site of *lacI*^{q} insertion in KV6576 remained amenable to manipulation, we used pEVS107, a Tn*7* delivery vector that targets the Tn*7* site [15], to introduce an Erm resistance cassette at that location. pEVS107 contains an Erm resistance cassette within the Tn*7* ends and a kanamycin resistance cassette outside. Erythromycin-resistant strains were readily isolated and exhibited sensitivity to kanamycin, as expected when the Tn*7* cassette inserts at the Tn*7* site (data not shown).

Identification of motility mutants. Our data above indicate that the *lacI* gene inserted into the chromosome is functional to repress transcription of a *lac* promoter-controlled gene. However, the question remained, does the *lacI*^{q} allele control transcription from the $P_{A1/34}$ promoter

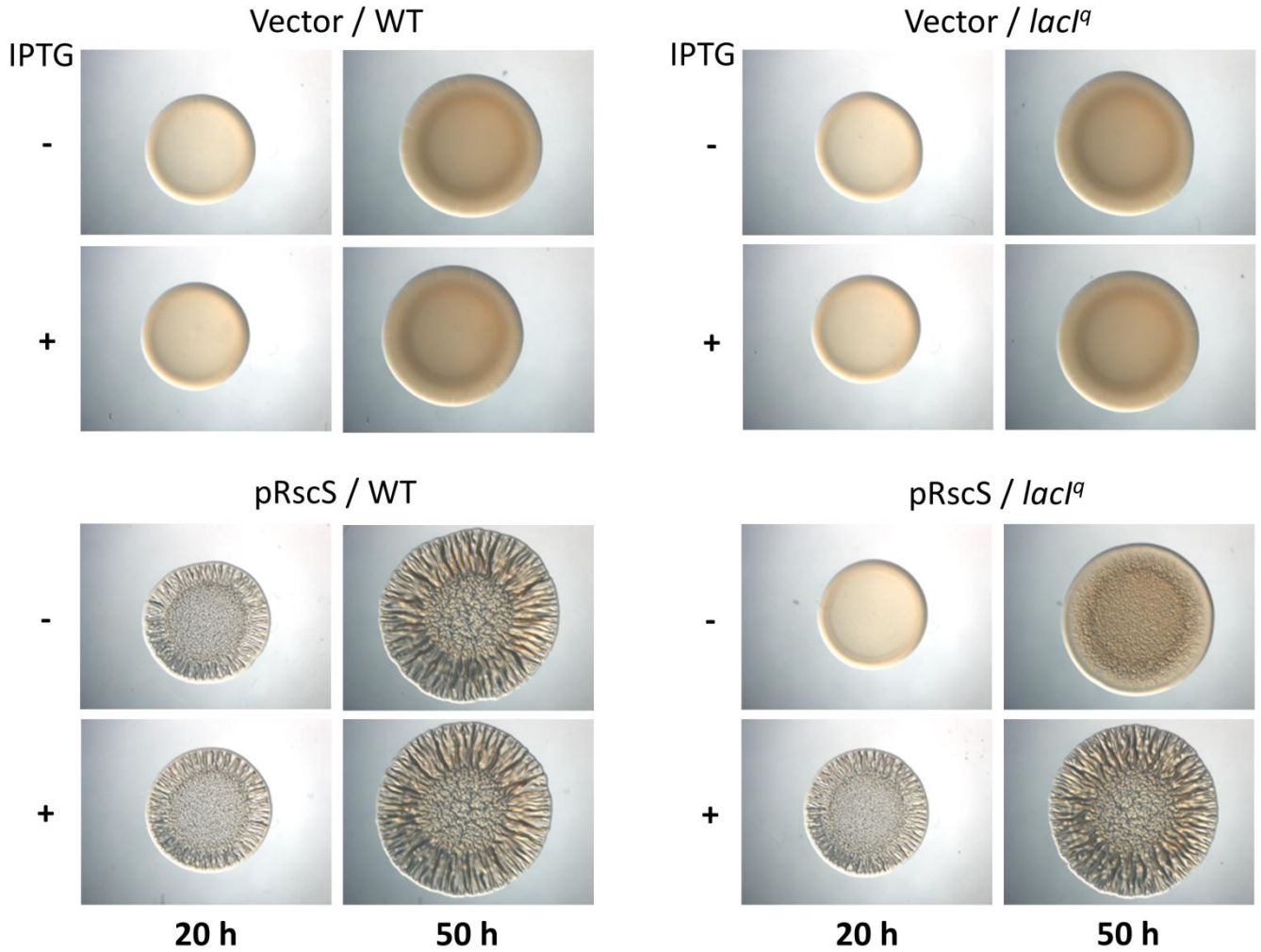

Fig. (3). *lac* promoter-driven biofilm formation by WT and *lacI* expressing strains. Cultures of wild-type (ES114) and *lacI*^{q} (KV6576) strains containing either the empty vector pVSV105 or RscS expression plasmid pCLD46 were grown in LBS containing Cm. Aliquots were diluted to an OD_{600} of 0.2, spotted onto LBS-Cm medium containing or lacking 1.75 mM IPTG, and incubated at room temperature. Wrinkled colony formation was assessed at 20 and 50 h post inoculation.

contained within Tn*5*P? Specifically, we wondered whether we could induce or repress native *V. fischeri* genes in KV6576 containing insertions of Tn*5*P. To address this question, we chose to evaluate a readily assessable phenotype, motility. *V. fischeri* contains a number of genes known [35-38] or predicted [39] to impact motility. We hypothesized that Tn*5*P insertions upstream of such genes could result in strains with inducible or repressible motility. We thus introduced Tn*5*P into KV6576 and assessed mutant motility on soft agar plates that contained or lacked IPTG. From a screen of about 2000 mutants, we identified about 20 strains with potential IPTG-dependent motility phenotypes and confirmed the phenotypes of a subset of these mutants. Of these, our focus was drawn to two strains with opposing phenotypes (Fig. **4**). One strain, KV7432, had IPTG-repressible motility: it exhibited near wild-type motility in the absence of IPTG but greatly diminished motility in the presence of IPTG (Fig. **4B**). In contrast, IPTG did not impact motility of the control strain (Fig. **4A**). The second strain of interest, KV7433, had IPTG-inducible motility: it was non-motile in the absence of IPTG but regained a wild-type motility phenotype in the presence of IPTG (Fig. **4C**). Because of their strong yet opposite phenotypes, we chose these two strains for additional characterization.

We first identified the sites of insertion of Tn*5*P as described in Materials and Methods. The mutant with IPTG-repressible motility, KV7432, contained an insert within the intergenic region between *VF_A0340* and *VF_A0341*, with the promoter of Tn*5*P oriented toward *VF_A0341* (Fig. **5A**). In this orientation, the promoter appears positioned to drive expression of the nearby three-gene operon consisting of *VF_A0342*, *VF_A0343*, and *VF_A0344*. These three genes are predicted to encode proteins with GGDEF or EAL domains. These domains are found in diguanylate cyclase and phosphodiesterase proteins, which synthesize and degrade, respectively, the second messenger cyclic-di-GMP (c-di-GMP) [40]. High levels of cellular c-di-GMP inhibit motility in a variety of bacteria [41]. Therefore, we hypothesize that IPTG-mediated expression from the transposon promoter increases the levels of c-di-GMP in the cell and thus, inhibits motility. It also remains possible that

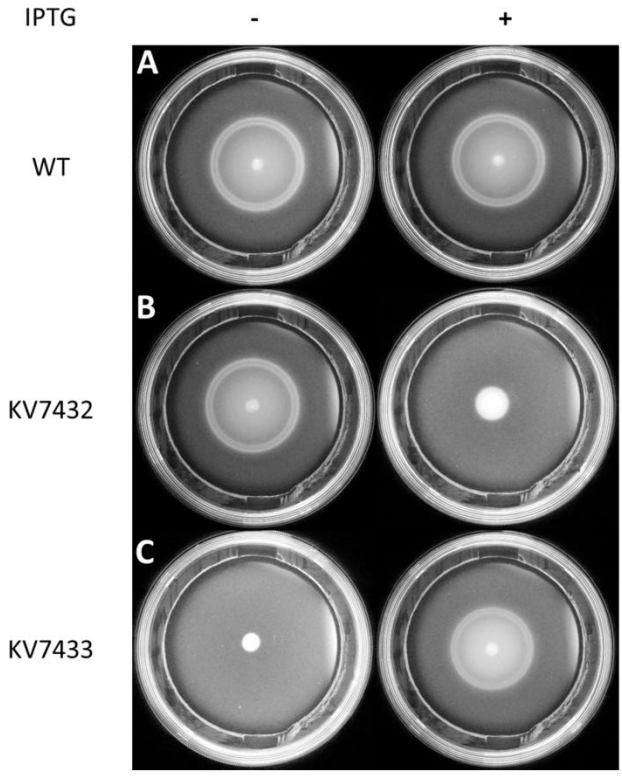

Fig. (4). Migration of mutant strains on soft agar. KV6576 (**A**), KV7432 (**B**), and KV7433 (**C**) were grown overnight in LBS. Cultures were diluted to an OD_{600} of 0.4 prior to inoculation on TB-SW motility medium containing or lacking 1.75 mM IPTG. The images depict migration after 5.5 h of incubation at 28°C.

expression from the Tn*5*P promoter decreases the expression of *VF_A0341*, which encodes a hypothetical protein with no conserved domains.

The mutant with IPTG-inducible motility, KV7433, carried the Tn*5*P insertion within the *cheZ* gene, with the transposon's promoter oriented with the *che* operon (Fig. **5B**). In the absence of IPTG, the transposon insertion should interrupt transcription of *cheZ* as well as the downstream *che* genes which coordinate chemotaxis and are required for motility in *V. fischeri* [38]. Thus, it was not surprising that, in the absence of IPTG, this mutant exhibited a motility defect. Given that the Tn insertion was within the *cheZ* gene, however, it was unexpected that the addition of IPTG would restore near wild-type motility (Fig. **4**). Upon closer investigation of the insertion site, we noted that (1) the Tn is inserted near the beginning of *cheZ*, and (2) an ATG start codon within the Tn*5*P transposon end is in frame with the *cheZ* open reading frame (Fig. **1B** and data not shown). Based on these observations, we hypothesize that expression from the transposon's promoter and translation from the ATG within the transposon end results in the production of a hybrid CheZ protein with an altered N-terminus that is functional to promote motility.

Assessment of IPTG Induction. Our previous experiments used a single concentration of IPTG, 1.75 mM, to induce transcription from the Tn*5*P promoter. However, it was unclear whether this high amount of IPTG was necessary to obtain full repression/induction of motility by our strains. Thus, to determine the sensitivity of the Tn*5*P promoter to IPTG, we assessed the mutants' motility phenotypes in the presence of a range of IPTG concentrations. KV7433 exhibited a dose-dependent increase in motility within a wide range of IPTG concentrations between 3.5 µM and 175 µM IPTG that was not further increased with additional IPTG (Fig. **6**). The other mutant, KV7432, similarly exhibited a dose-dependent change. In this case, the impact on motility required higher IPTG concentrations, above 35 µM; at the highest amount tested, 1.75 mM, motility was not fully repressed (Fig. **6**). These data further support our conclusion that expression from the Tn*5*P promoter is induced by the addition of IPTG. Additionally, because we obtained different ranges of IPTG addition required for a transition from the motile to non-motile phenotype in the two strains, we conclude that it may

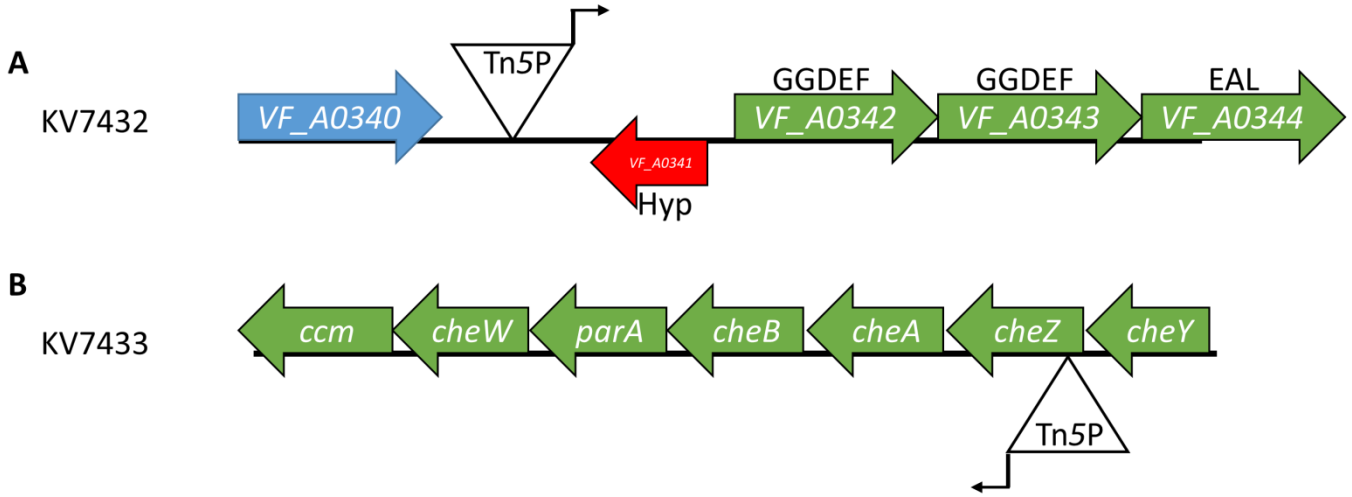

Fig. (5). The location and orientation of the Tn*5*P insertions in two motility mutants. The insertion in KV7432 is located in the intergenic region between *VF_A0340* and *VF_A0341* with the A1/34 promoter oriented toward *VF_A0341* (**A**). The insertion in KV7433 is located within the 5' end of *cheZ* with the A1/34 promoter oriented toward *cheA* (**B**).

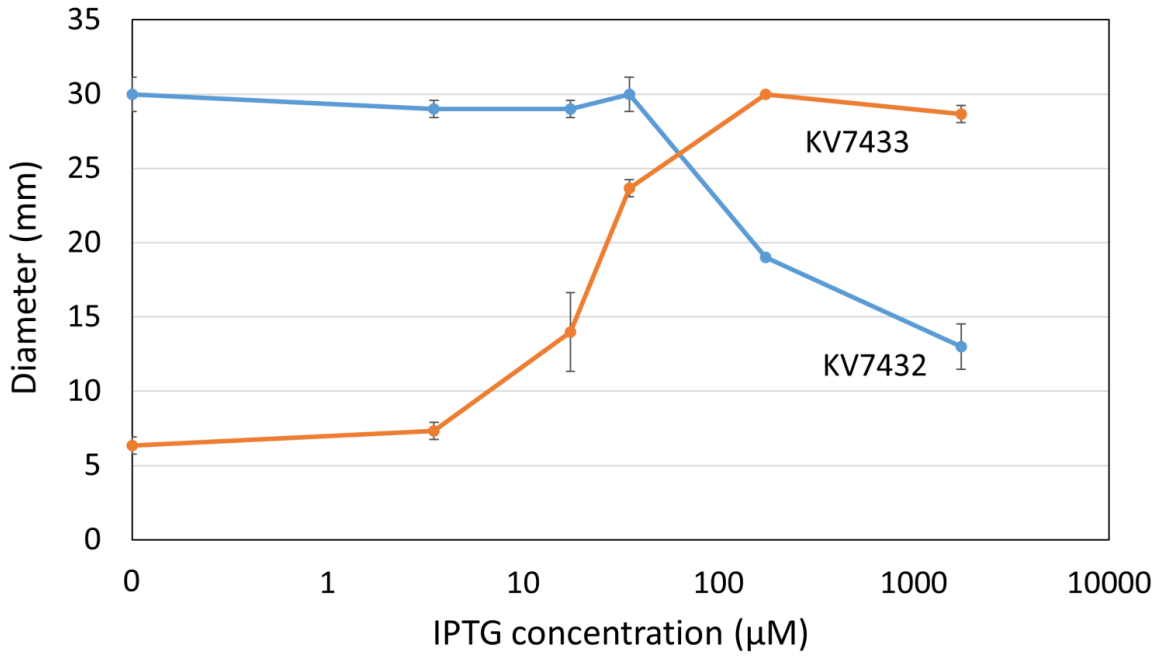

Fig. (6). Dependence of mutant motility phenotypes on IPTG. Motility mutants KV7432 and KV7433 were grown at 28°C in TB-SW motility medium containing the indicated concentrations of IPTG. The average diameter of migration of triplicate samples after 5 hours is shown. Standard deviation is indicated by error bars. Error bars smaller than the plotted points are occluded by the points and not visible.

be necessary to experimentally determine the optimal expression of a particular gene obtained using this experimental set-up (*lacI*^{q}/Tn*5*P) by titrating the concentration of IPTG in the medium against the phenotype being tested.

CONCLUDING REMARKS

We have developed a means to randomly insert and control an inducible promoter within the chromosome of *V. fischeri* by (1) generating a mini-Tn*5* modified to contain an outward-facing, LacI-repressible promoter and (2) engineering a background strain that expresses LacI to control the promoter. Insertion of the Tn*5*P within a gene or locus will result in disruption of the gene or locus and has the additional ability to conditionally induce transcription of downstream genes, thus reducing the problem of polarity typically associated with Tn insertions. Finally, our evidence indicates that the Tn*5*P end may, in some cases, generate and express proteins with N-terminal truncations. Thus, this genetic tool increases the number of phenotypes *V. fischeri* researchers can screen and has the potential to greatly increase our understanding of the physiology of this model organism.

CONFLICT OF INTEREST

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