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METHODS

Tools for Rapid Genetic Engineering of *Vibrio fischeri*

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ABSTRACT *Vibrio fischeri* is used as a model for a number of processes, including symbiosis, quorum sensing, bioluminescence, and biofilm formation. Many of these studies depend on generating deletion mutants and complementing them. Engineering such strains, however, is a time-consuming, multistep process that relies on cloning and subcloning. Here, we describe a set of tools that can be used to rapidly engineer deletions and insertions in the *V. fischeri* chromosome without cloning. We developed a uniform approach for generating deletions using PCR splicing by overlap extension (SOEing) with antibiotic cassettes flanked by standardized linker sequences. PCR SOEing of the cassettes to sequences up- and downstream of the target gene generates a DNA product that can be directly introduced by natural transformation. Selection for the introduced antibiotic resistance marker yields the deletion of interest in a single step. Because these cassettes also contain FRT (FLP recognition target) sequences flanking the resistance marker, Flp recombinase can be used to generate an unmarked, in-frame deletion. We developed a similar methodology and tools for the rapid insertion of specific genes at a benign site in the chromosome for purposes such as complementation. Finally, we generated derivatives of these tools to facilitate different applications, such as inducible gene expression and assessing protein production. We demonstrated the utility of these tools by deleting and inserting genes known or predicted to be involved in motility. While developed for *V. fischeri* strain ES114, we anticipate that these tools can be adapted for use in other *V. fischeri* strains and, potentially, other microbes.

IMPORTANCE *Vibrio fischeri* is a model organism for studying a variety of important processes, including symbiosis, biofilm formation, and quorum sensing. To facilitate investigation of these biological mechanisms, we developed approaches for rapidly generating deletions and insertions and demonstrated their utility using two genes of interest. The ease, consistency, and speed of the engineering is facilitated by a set of antibiotic resistance cassettes with common linker sequences that can be amplified by PCR with universal primers and fused to adjacent sequences using splicing by overlap extension and then introduced directly into *V. fischeri*, eliminating the need for cloning and plasmid conjugation. The antibiotic cassettes are flanked by FRT sequences, permitting their removal using Flp recombinase. We augmented these basic tools with a family of constructs for different applications. We anticipate that these tools will greatly accelerate mechanistic studies of biological processes in *V. fischeri* and potentially other *Vibrio* species.

KEYWORDS *Vibrio*, *Vibrio fischeri*, cellulose, genetics, motility

Vibrio fischeri is a model organism for the investigation of quorum sensing (1–5), bioluminescence (6–11), biofilm formation (12–19), and symbiosis (20–25). As a result, the ability to readily manipulate this organism genetically has the potential to impact studies of many important aspects of bacterial physiology. Historically, most genetic studies have used strain ES114, an isolate from *V. fischeri*'s symbiotic host, the Hawaiian bobtail squid *Euprymna scolopes*, although other squid isolates have been sequenced (15, 26–28) and some are beginning to be manipulated through genetic

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approaches (7, 29), thus increasing the need for tools and approaches to facilitate manipulations of *V. fischeri*.

A variety of methods have been used to generate specific mutations in ES114, most of which depend on the delivery via conjugation of a plasmid that contains specific *V. fischeri* sequences. Both replication-deficient (e.g., oriR6K based, such as pEVS122 [30]) and unstable (e.g., pEVS79 based [31]) plasmids have been used with success. The replication-deficient plasmid approach has most commonly been used for generating insertional (Campbell-type integration) mutants, which involves cloning a small 5′ end region of the gene of interest. Selection for the plasmid results in mutants that have integrated the delivery vector into the chromosome; however, these mutants carry a duplication of the cloned and native sequences that can resolve, reverting the strain to the wild-type genotype. Gene replacement/gene deletion strategies have also been used. These approaches rely on cloning up- and downstream sequences into a delivery plasmid, either a replication-deficient or an unstable vector. To obtain the desired mutant, two recombination events are required. First, the plasmid must integrate into the chromosome, an event that can be selected using an antibiotic resistance marker encoded by the plasmid. The second recombination event resolves the partial duplication generated by plasmid integration but may result in either the desired mutation or restoration of the wild-type allele. The desired mutation can be identified by selection, if the target gene of interest is replaced by an antibiotic resistance cassette, or by screening (by phenotype or with PCR), if it is unmarked. The ability to capture strains that have undergone the second recombination event can be facilitated by inducing toxin production from the integrated plasmid (e.g., CcdB [32, 33]), thus eliminating cells that retain the integrated plasmid. Numerous marked and unmarked mutations have been generated with these approaches (e.g., see references 25, 34, and 35). However, these approaches are time-consuming and require cloning, conjugation, and selection for the initial plasmid integration, followed by additional selection or screening for the desired deletion.

More recently, natural transformation has been developed as a way to introduce DNA into *V. fischeri* (36). The original report documented the ability of ES114 to take up chromosomal, plasmid, and PCR-generated DNA following exposure to chitin derivatives or in strains that overexpressed the *tfoX* gene. Although the efficiency was lowest with PCR products, the ability to introduce DNA through natural transformation relieves the dependence on plasmid-borne DNA for genetic manipulation of *V. fischeri*. Since the first report, subsequent studies used natural transformation of chromosomal DNA to move marked transposon insertion mutations between strains of *V. fischeri* (37, 38) and to map and repair a large deletion that arose in a derivative of ES114 (39). The latter study demonstrated that fragments as large as 10 kb could be transferred between strains. In *V. cholerae*, natural transformation has been used successfully to coordinately introduce multiple deletions using PCR products (40). The ability to reliably introduce even single deletions into *V. fischeri* using a PCR-based approach would greatly accelerate the rate of genetic manipulation and thus propel discovery in this marine model.

In addition to the current cloning burden, another roadblock to rapid genetic manipulation of *V. fischeri* is the limited number of antibiotic resistance markers that have been developed for use in this organism, particularly with markers that function in single copy. Specifically, erythromycin (Em) and chloramphenicol (Cm) resistance markers are the primary cassettes, followed by kanamycin (Kn). To date, tetracycline (Tc) has been used exclusively for selecting multicopy plasmids. One solution for the relative lack of useful antibiotic resistance cassettes is to make use of the Flp recombinase technology, which permits the deletion of sequences between direct repeats of FRT (FLP recognition target) sequences (41–43). With this technology, antibiotic resistance cassettes flanked by FRT sites can be used, resolved, and used again elsewhere in the chromosome. Alternatively, or in addition, new antibiotic resistance cassettes can be developed.

Here, we sought to enhance our ability to genetically manipulate *V. fischeri* using

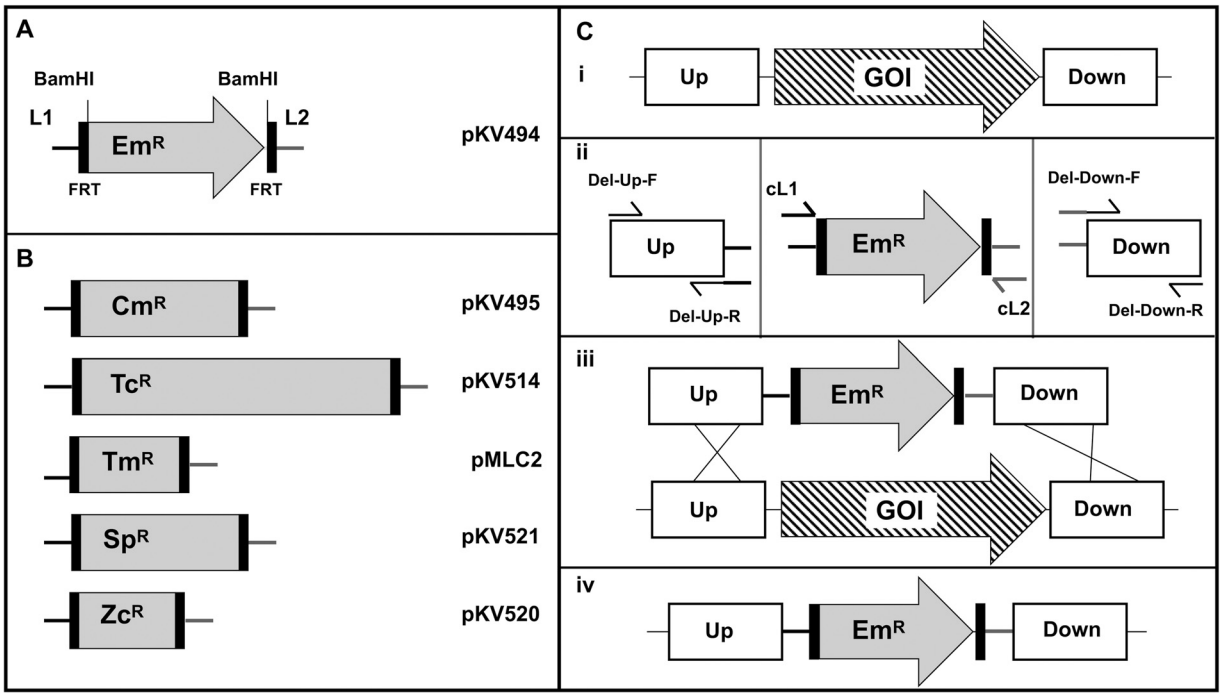

FIG 1 Scheme for rapid deletion of genes of interest. (A) Plasmid pKV494 was engineered to contain FRT sequences (black rectangles) and distinct linker sequences, L1 and L2, flanking the Em^r antibiotic resistance cassette. Em^r sequences can be removed using BamHI. (B) A family of cassettes, derived from pKV494, contain different antibiotic resistance markers. (C) Scheme for deleting a gene of interest. (i) Gene of interest, GOI, is shown with flanking up- and downstream regions. (ii) Three PCRs were carried out with the indicated primers and templates. For up- and downstream sequences, the template was typically chromosomal DNA. (iii) The PCR SOE product, at the top, recombines into the chromosome, at the bottom, as indicated by the crossed lines. (iv) The final strain with GOI replaced by the engineered sequences.

two related approaches that take advantage of natural transformation. First, we developed tools for the rapid deletion of specific genes using PCR splicing by overlap extension (SOEing) (44) of antibiotic resistance cassettes that can be resolved by the FRT/Flp recombinase system. As part of this work, we developed and validated additional antibiotic resistance cassettes to increase this toolset for *V. fischeri*. Second, we developed a method for the rapid insertion of specific genes at a benign site in the chromosome to facilitate complementation. We then expanded this toolset to include a variety of promoters for constitutive and inducible gene expression. These deletion and insertion approaches were validated using the genes for FlrA, the master flagellar regulator (25, 45), and FliQ, an uncharacterized putative flagellar protein. These studies confirmed and demonstrated roles for *flrA* and *fliQ* in motility and uncovered an unexpected connection between motility and cellulose. While this toolset was developed specifically for *V. fischeri* strain ES114, we anticipate that the ease, consistency, and speed of the engineering will be attractive to a wide audience and will likely be adapted for compatibility in other *V. fischeri* strains and other organisms. These methods will permit researchers to quickly and reliably generate strains to probe the mechanisms involved in a variety of biological processes.

RESULTS AND DISCUSSION

Constructs to facilitate rapid deletion of *V. fischeri* genes. To facilitate rapid gene deletion in *V. fischeri*, we developed a PCR-based approach that would permit reliable amplification of fragments of interest by gene SOEing. Specifically, we engineered constructs that contained two distinct linkers (L1 and L2) positioned on either side of an antibiotic resistance cassette (Fig. 1A). We also included flanking FRT sequences to provide an option for making unmarked deletions by resolving the antibiotic cassette. Initially, we engineered pKV494 (erythromycin resistant, Em^r) (Fig. 1A; see also Fig. S1 in the supplemental material). The antibiotic resistance cassette in this plasmid is

TABLE 1 Primer design for generating PCR SOE products for making deletions and insertions

Primer	Sequence[a] (from 5′ end)	Purpose[b]
Deletions		
Del-Up-F	GOI specific	Amplifying upstream sequences, F
Del-Up-R	TAGGCGGCCGCACTAAGTATGG-[20–24-nt GOI specific]	Amplifying upstream sequences, R
cL1	CCATACTTAGTGCGGCCGCCTA	Amplifying Ab^r cassette, F
cL2	CCATGGCCTTCTAGGCCTATCC	Amplifying Ab^r cassette, R
Del-Down-F	GGATAGGCCTAGAAGGCCATGG-[20–24-nt GOI specific]	Amplifying downstream sequences, F
Del-Down-R	GOI specific	Amplifying downstream sequences, R
Insertions		
Ins-Up-F	CTTGATTTATACAGCGAAGGAG	Amplifying upstream sequences, F
Ins-Up-F*	AAGAAACCGATACCGTTTACG	Use instead of Ins-Up-F for recombining at Em′
Ins-Up-R (cL2)	Same as cL2, above	Amplifying upstream sequences, R
Ins-GOI-F	GGATAGGCCTAGAAGGCCATGG-[20–24-nt GOI specific]	Amplifying GOI sequences, F
Ins-GOI-R	TAGGCGGCCGCACTAAGTATGGA-[20–24-nt GOI specific]	Amplifying GOI sequences, R
Ins-Down-F (cL3)	TCCATACTTAGTGCGGCCGCCTA	Amplifying downstream sequences, F
Ins-Down-R	GGTCGTGGGGAGTTTTATCC	Amplifying downstream sequences, R

[a]GOI specific means sequences specific to the gene of interest (GOI) or to sequences up- or downstream of the GOI; nt, nucleotide.
[b]Ab^r, antibiotic resistance; F, forward primer; R, reverse primer.

flanked by BamHI sites, so it can be replaced with other antibiotic resistance cassettes or other genes or markers of interest. We subsequently replaced the Em^r gene in pKV494 with chloramphenicol (Cm^r), tetracycline (Tc^r), trimethoprim (Tm^r), spectinomycin (Sp^r), and zeocin (Zc^r) resistance markers to generate a family of cassettes that can be used for recombination (Fig. 1B). Each cassette can be amplified from these constructs using PCR with primers complementary to the linkers, cL1 and cL2 (Table 1), and then fused via PCR SOEing to sequences up- and downstream of a gene of interest (GOI; Fig. 1C); the up- and downstream sequences would be generated using primers with 5′-end tails that incorporate sequences complementary to L1 and L2 (Del-up-F and Del-Up-R as well as Del-Down-F and Del-Down-R, respectively) (Table 1).

We then tested the system by following the scheme outlined in Fig. 1C. Specifically, we amplified ~500-bp sequences up- and downstream of two different genes positioned at the top and bottom of the flagellar hierarchy: *flrA*, which has previously been shown to function as the master regulator of flagellar biosynthesis in *V. fischeri* (25), and *fliQ* (*VF_1841*), which is predicted, but not yet shown, to be involved in motility based on homology and its position within the *fliL-R* flagellar operon (46). We fused appropriate flanking sequences to different FRT-antibiotic cassettes and introduced the composite PCR products into *V. fischeri* using natural transformation and selection for antibiotic resistance (Table 2). We evaluated motility of representative colonies that arose and found that the strains were nonmotile (Fig. 2A and B). PCR using the outermost up- and downstream primers confirmed the presence of the expected gene replacement mutations (Fig. 2C and D). These data thus indicate that both *flrA*, as previously determined, and *fliQ*, as hypothesized, are required for motility of *V. fischeri*.

TABLE 2 Antibiotics, resistance cassettes, and selection conditions

Antibiotic	Cassette size[a] (bp)	Final concn[b]	Special condition[c]	Note[e]
Erythromycin	960	2.5		
Chloramphenicol	886	1		Longer incubation required[f]
Tetracycline	2,223	2.5	No Ca^{2+} or Low Mg[d]	
Trimethoprim	691	10		Spontaneous Tm^r occurs
Spectinomycin	1,091	200		Initial lawn gives rise to colonies
Zeocin	572	10	Recover in LB	Initial lawn gives rise to colonies

[a]Cassette size includes the flanking BamHI (12 bp) and FRT (112 bp) sequences.
[b]Concentration in μg/ml in LBS, except for zeocin, for which LB is used.
[c]For natural transformation and selection.
[d]Five mM Mg is sufficient to promote growth and transformation.
[e]Observations of growth on selective media.
[f]Colonies arise about a day later than occurs for Em^r colonies.

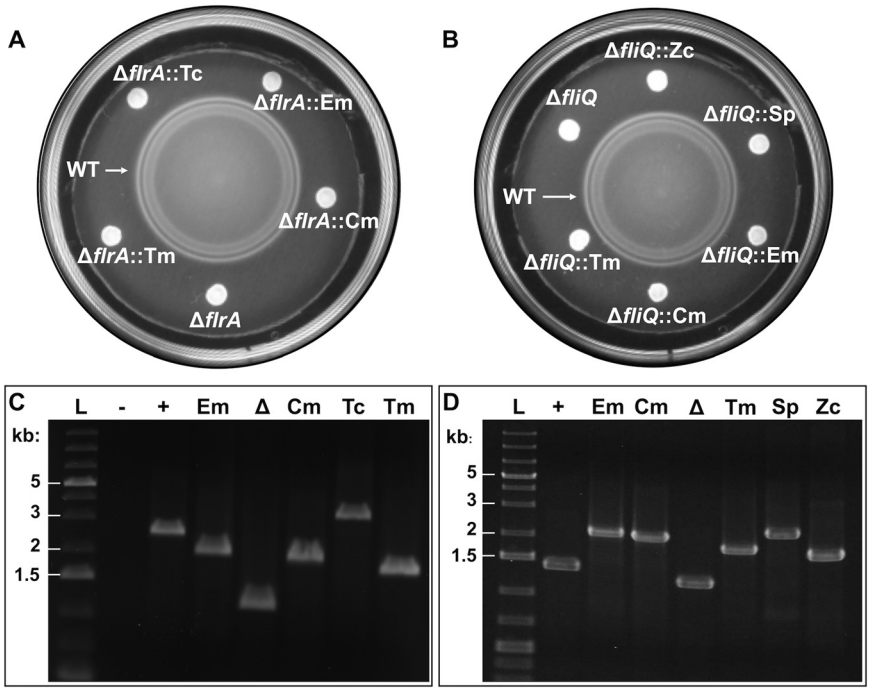

FIG 2 Assessment of Δ*flrA* and Δ*fliQ* mutants. (A and B) Motility of the indicated *flrA* (A) and *fliQ* (B) mutants and the wild-type control. (C and D) PCR amplification of the indicated *flrA* (C) and *fliQ* (D) mutants generated with primer sets 2215 and 2218 (*flrA*) or 2356 and 2359 (*fliQ*). Abbreviations for the antibiotic resistance cassettes are as indicated in the text. L, ladder; −, no template; +, ES114; Δ, deletion with the antibiotic resistance cassette resolved. Numbers on the left indicate the sizes (kb) of relevant ladder bands.

Of the antibiotic resistance cassettes tested, Em^r worked the best in our hands for natural transformation: selection for Em^r colonies resulted in relatively robust growth of resistant colonies and little background growth. Some of the other antibiotics, notably Cm^r, required longer incubation times than Em^r (up to a day longer, depending). We found that effective selection for Tc^r colonies required special conditions, namely, preparing competent cells in minimal medium that either lacked calcium chloride or contained only small amounts of magnesium sulfate (Table 2); incorporation of standard salt concentrations permitted lawns of growth for *V. fischeri* strains that lacked the Tc^r marker. Selection for either Sp^r or Zc^r initially gave rise to background growth (lawns), but with additional incubation time, the background diminished and larger colonies arose that contained the desired mutation. For Tm^r, we occasionally obtained colonies that appeared to be spontaneously resistant, but these did not grow well upon restreaking on selective medium. Thus, although not all antibiotic resistance markers worked equally well, it was possible to obtain deletion mutants using each of the cassettes presented here.

In the course of these experiments, we observed that the *flrA*::Tm mutant exhibited altered colony morphology. Rather than the smooth phenotype of the wild-type strain, colonies formed by the *flrA*::Tm mutant grown on trimethoprim were bumpy, a phenotype indicative of biofilm formation (Fig. S2). Two polysaccharide loci in *V. fischeri* have been associated with altered colony morphology, *syp* (symbiosis polysaccharide) and *bcs* (cellulose) (35, 47–49). Consistent with the possibility that *bcs* was responsible for this phenotype, we found that growth of either *flrA* or *fliQ* mutants on agar containing Congo red resulted in colonies with a darker red hue than ES114 colonies, suggesting the mutants produce more cellulose (Fig. 3A and B). In addition, whereas *flrA syp* and *fliQ syp* double mutants phenocopied the *flrA* and *fliQ* single mutants, *flrA bcs* and *fliQ bcs* double mutants behaved like the *bcs* mutant, lacking colony color (Fig. 3A and B). These results thus reveal a new function for FlrA and FliQ and, likely, flagella in inhibiting cellulose production by *V. fischeri*.

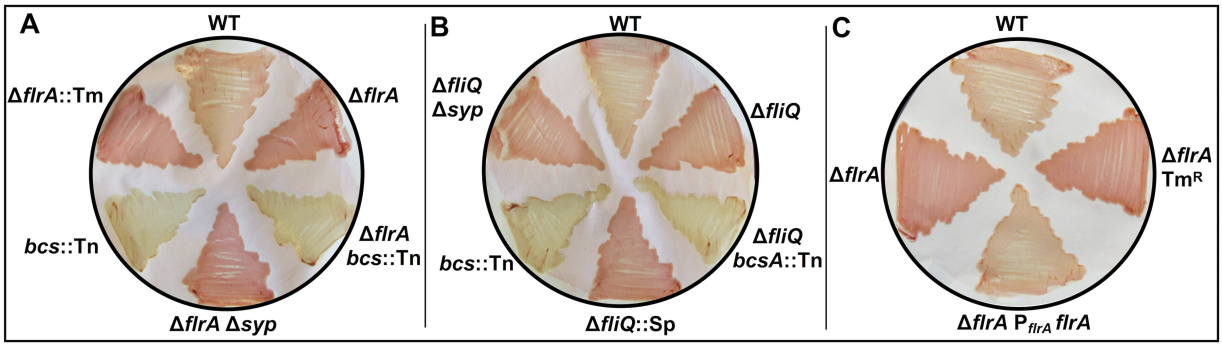

FIG 3 Congo red staining of *V. fischeri* strains. Strains were streaked onto LBS containing Congo red dye and then transferred to paper and photographed. The indicated *flrA* (A and C) and *fliQ* (B) mutants and controls were evaluated.

In summary, the use of standardized linkers and a family of cassettes that can be amplified with the same set of primers makes it possible to rapidly and reliably generate PCR fusion products. Combined with the fact that this approach requires no cloning or subcloning, mutants can similarly be obtained very rapidly, within as few as 2 to 3 days, rather than a few weeks or more. In this study, we did not explore the optimal size of the flanking sequences necessary for recombination; thus, it is possible that sizes smaller than 500 bp would work as well. Finally, this work expands the number of antibiotic resistance markers available for generating mutations from the limited set (Em^r and Cm^r) routinely used in the past (e.g., see references 30 and 31).

Removal of antibiotic resistance cassette. Despite the increased availability of useful antibiotic resistance cassettes, the presence of a specific marker limits subsequent manipulations. Thus, to facilitate the engineering of multiple deletions and/or introduction of other sequences as described below, we cloned the *flp* recombinase gene into a plasmid that could be introduced into *V. fischeri* to resolve antibiotic cassettes between FRT sites. We then introduced the resulting construct, pKV496, into the Δ*flrA*::FRT-Em and Δ*fliQ*::FRT-Cm mutants. Colonies that were Em^s and Cm^s, respectively, were obtained, and representative isolates retained the expected nonmotile phenotype (Fig. 2A and B). Sequencing of the junction revealed that the resulting unmarked mutants contained residual "scar" sequences in the chromosome of 78 bp (26 amino acids) (Fig. S3), confirming the resolution of the FRT sequences. The linker sequences were engineered such that the resulting scar could be in frame with the deleted gene to reduce the possibility of polarity on downstream genes. While neither the insertion nor the scar present in the *flrA* gene is predicted to impact any downstream genes, *fliQ* is embedded in an operon (*fliOPQR*). The potential polarity of the *fliQ* mutation was evaluated in experiments described below.

Rapid insertion of genes for complementation. Because PCR can result in unwanted mutations, even with use of a high-fidelity polymerase, and spontaneous mutations can arise, it is necessary to apply an additional approach, such as complementation, to verify that the engineered mutation is the cause of any observed phenotypes. We thus sought to develop an equally rapid method for complementing *V. fischeri* mutants. We first considered where to insert the complementation sequences. In previous work using a plasmid-based recombination approach (50), we had inserted the *lacI* gene between the *yeiR* and *glmS* genes of *V. fischeri* (*VF_2370-2372*). This location is adjacent to, but does not disrupt, the position where Tn*7* inserts (Fig. 4A); maintaining the Tn*7* site allows for simultaneous use of the many tools that rely on site-specific insertion by the Tn*7* transposase (51–53). The *lacI* insertion at the indicated position also had no demonstrable effect on important aspects of *V. fischeri* physiology, notably motility and biofilm formation (50). We therefore chose to use this location (between *yeiR* and *glmS*) as the basis for our chromosomal complementation system.

We then developed an approach that facilitates reliable insertion at that location (Fig. 4B and C). Specifically, we engineered tools that would permit a three-piece PCR

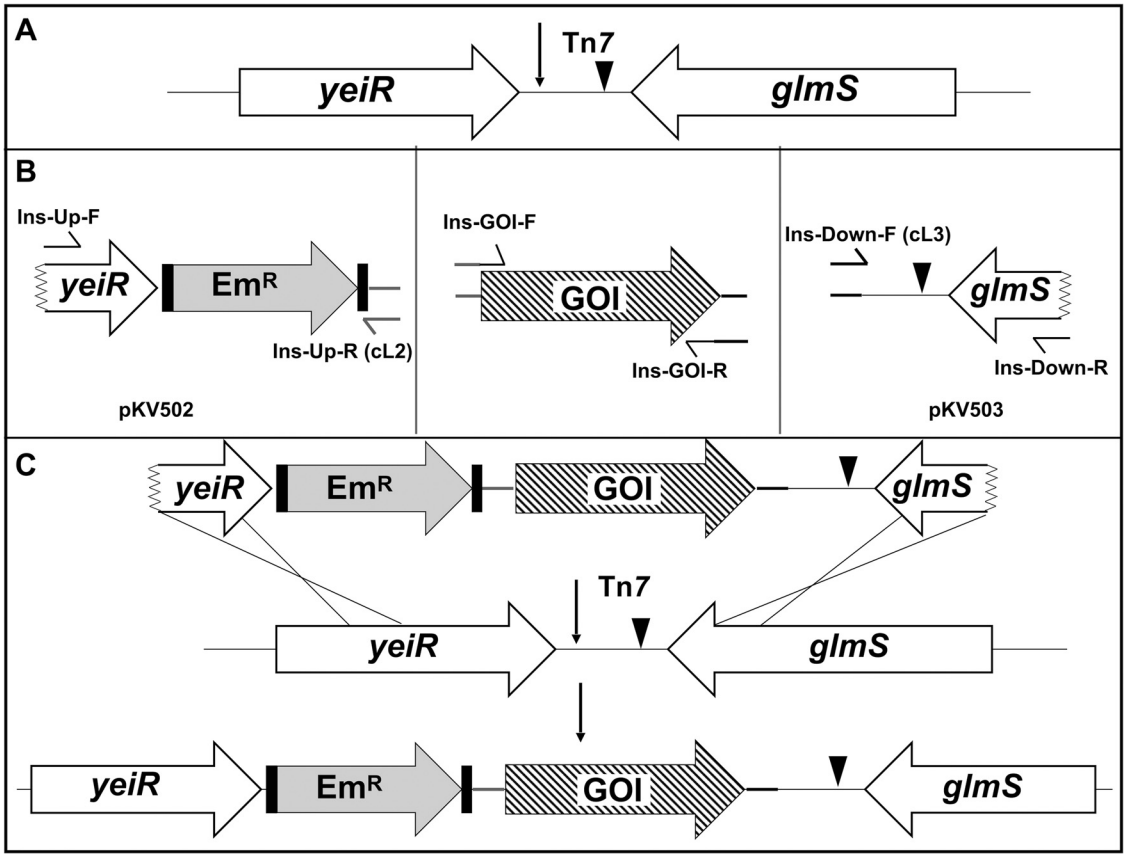

FIG 4 Scheme for rapid insertion of genes of interest. A method was developed to introduce a gene of interest (GOI) with an Em^r cassette into a benign site in the chromosome. (A) In this study, insertions were targeted to the indicated locus, the intergenic region between *yeiR* (*VF_2370*) and *glmS* (*VF_2372*). The arrow indicates the site of insertion, while the arrowhead indicates the position of the Tn*7* site. (B) The up- and downstream segments are amplified with the indicated primers using pKV502 and pKV503 as templates, as shown, or similar templates, while the GOI is typically amplified from chromosomal DNA. (C) The PCR SOE product (top) recombines into the chromosome (middle), as indicated by the crossed lines, resulting in the insertion (bottom).

SOEing reaction similar to the deletion approach. We generated a construct, pKV502, that could be used as a template to amplify the upstream (3′ end of *yeiR*) sequences together with FRT-Em^r sequences in a single reaction using a forward primer complementary to *yeiR* and a reverse primer (cL2) complementary to the linker (Fig. 4B). We also generated a construct, pKV503, that fuses a linker to the downstream sequences (3′ end of *glmS*). Amplification of pKV502 and pKV503 with appropriate primers results in PCR fragments with ends compatible with a linker-containing middle piece consisting of the gene of interest (GOI). SOEing of the three PCR fragments results in a product that is poised to be inserted into the region between *yeiR* and *glmS* (Fig. 4C).

To test this approach, we used it to introduce cassettes containing *flrA* and *fliQ*. This approach was successful with the ~0.3-kb *fliQ* gene: *fliQ* was readily inserted into the intergenic region, as determined by PCR. Unfortunately, for *flrA*, we found that the large size of the final cassette (~3.7 kb, with ~1.4 kb upstream [+ Em^r], ~1.8 kb *flrA* [including the putative promoter region], and ~0.5 kb downstream), made the PCR SOEing reaction suboptimal. Potentially, the further optimization of the PCR and/or the use of a distinct long-range PCR enzyme would permit a more efficient reaction. Rather than continue to optimize PCR SOEing, we devised an alternative approach that would be more feasible for larger genes. We reasoned that the total size of the PCR SOEing product could be diminished by directing recombination to occur downstream of *yeiR* at inserted Em^r sequences. We thus introduced the 5′ end of the Em^r gene (a 3′ end truncation; Em′) into the intergenic region between *yeiR* and *glmS*, selecting for this insertion using an adjacent Tm^r cassette, to generate strain KV8232 (Fig. 5A). The use

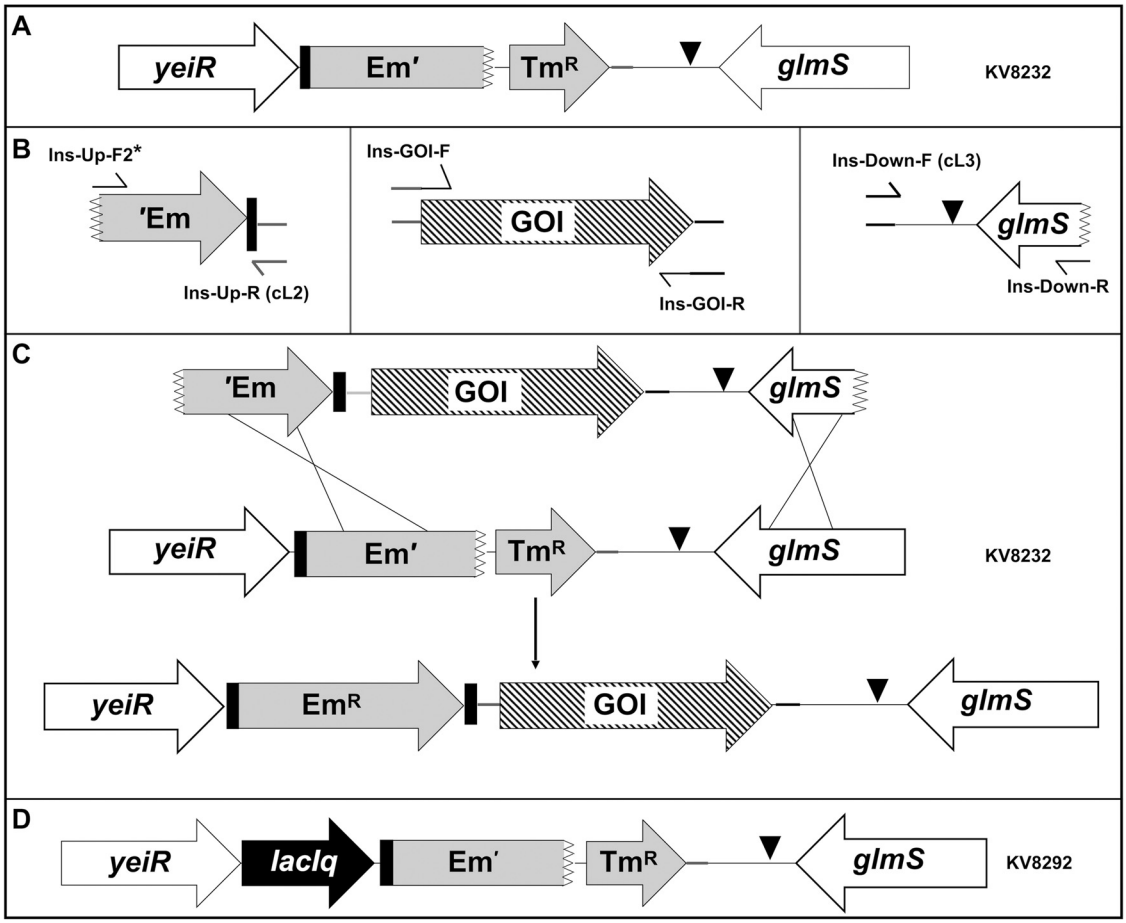

FIG 5 Solution to the problem of large PCR SOE sizes. A modified scheme was developed to facilitate insertion of larger genes/sequences. (A) A truncated Em^r (Em′) cassette was integrated into the chromosome, generating KV8232 and providing a region of homology for recombination and permitting shorter products to be generated. (B) The up- and downstream segments were amplified with the indicated primers using pKV502 and pKV503 as templates, as shown, or similar templates; amplification results in a 5′-truncated, nonfunctional EmR sequence (′Em). (C) Recombination occurs between the two truncated Em^r cassettes to restore functional Em^r. The PCR SOE product (top) recombines into the chromosome (middle), as indicated by the crossed lines, resulting in the insertion (bottom). (D) KV8292, a strain similar to KV8232 but also containing *lacI*^{q}, was engineered to facilitate IPTG-inducible gene expression.

of KV8232 as a recipient eliminated the need to amplify any *yeiR* sequence. In addition, the length of necessary Em^r sequence was reduced, diminishing the total size of the PCR SOE product (Fig. 5B). For *flrA*, the cassette was reduced to ~3 kb, which we could readily obtain. By including the missing 3′ portion of the Em^r resistance gene (′Em) in the PCR SOEing product, the Em^r gene can be reconstituted by recombination, permitting selection for colonies with the desired insertion with Em (Fig. 5C). Recombination results in the concomitant loss of Tm^r.

To more easily evaluate the efficacy of this modified approach, we moved the truncated Em′ (Tm^r) sequences into a Δ*flrA* strain. This strain (KV8281) was transformed with a PCR SOE product containing *flrA* under the control of its native promoter. A representative Em^r/Tm^s colony that arose (KV8290) exhibited motility similar to that of the wild-type strain (Fig. 6A and B). PCR-based analysis of this motile strain confirmed both the Δ*flrA* deletion and an insertion of the expected size at the *yeiR-glmS* region (Fig. 6C). Together, these data indicate that the *flrA* cassette had recombined at the desired position in the chromosome and was responsible for the restored motility of this strain. In addition, the Congo red phenotype was similarly restored to wild-type levels in this complemented strain, a result that confirms that FlrA negatively controls this phenotype (Fig. 3C).

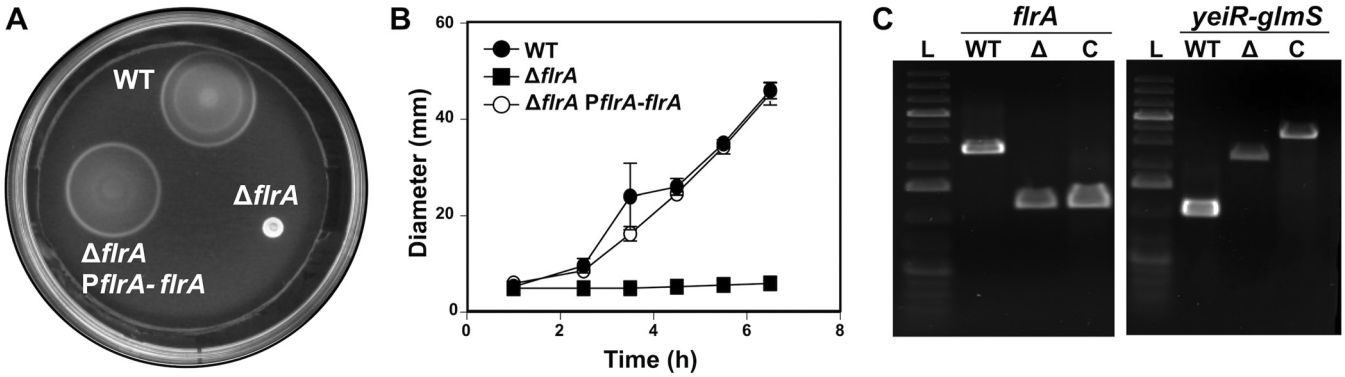

FIG 6 Analysis of *flrA* complementation strain. (A) Motility of the WT (wild type; ES114), Δ*flrA* (Δ*flrA* Em′ Tm^r; KV8281), and Δ*flrA* P*flrA-flrA* (KV8290) strains. (B) Motility of the same strains as those shown for panel A, monitored over time. (C) PCR amplification of the *flrA* and *yeiR-glmS* regions of the same strains as those shown in panel A with primer set 2215 and 2218 (*flrA*) or 2185 and 1487 (*yeiR-glmS*). L, ladder; WT, ES114; Δ, Δ*flrA* strain KV8281; C, complemented strain KV8290. Ladder is the same as that indicated in Fig. 2.

In summary, this insertion approach can facilitate rapid introduction of sequences to complement specific mutations. As with the deletion approach, we engineered the insertion tools to be versatile: the antibiotic resistance marker can be removed from the strain of interest using Flp recombinase, and/or the Em^r cassette in the template plasmid(s) can be replaced by other antibiotic resistance cassettes by digesting with BamHI. Our modification to accommodate larger genes of interest further increases the utility of this approach by reducing the overall size of the required PCR SOE product. The truncated Em′ and associated Tm^r sequences can be readily introduced into other strains of interest by selection for Tm^r (as we did for the *flrA* mutant), and/or marked mutations can be moved into this parent strain. This modified approach can be adapted to introduce Em′ sequences at other positions in the chromosome, facilitating the use of these tools (and the ones described below) for insertion elsewhere. This adaptation may be necessary if this approach is used for other strains, as other sequenced *V. fischeri* isolates appear to differ somewhat in this region of the chromosome. While we used PCR SOEing to generate the strains and deletions/insertions described here, as we could rapidly and reliably generate a variety of products of interest, it is becoming increasingly cost-effective to obtain synthetic DNA fragments; such synthetic DNA could be modeled after the tools described here to facilitate introduction/removal of sequences at any desired location. Finally, although the maximum amount of DNA that can be readily recombined into the chromosome has not been specifically addressed, the insertion of *flrA* along with associated promoter and truncated Em^r sequences totaled about 2.5 kb and should support insertion of other nonnative sequences up to at least this size.

Additional tools for other outcomes and analyses. While the tools described above were a great advance, they remained limited. For example, genes positioned in the middle of an operon, such as *fliQ*, rely on promoters located upstream. Thus, complementing the *fliQ* mutant with a single gene, rather than an operon, requires additional engineering, such as another PCR SOEing event, to include the native promoter. To overcome this problem, as well as to further increase the utility of this approach, we expanded these tools to support a variety of experimental outcomes (Fig. 7 and Fig. S4), as described below.

Constitutive promoters. To facilitate expression of genes such as *fliQ* that lack immediately adjacent promoters, we engineered constructs with two different constitutive promoters fused to the *yeiR*-FRT-Em sequences: one (pKV506) incorporated the *V. fischeri* promoter for the *nrdR* gene, P*nrdR*, which has previously been shown to be a strong promoter (37), and one (pKV519) contained P*ma*, a modified version of P*A1/34*, a synthetic promoter generated by Bose et al. (6). When either promoter was fused to promoterless *fliQ* and introduced into a *fliQ* mutant, the resulting strains were motile,

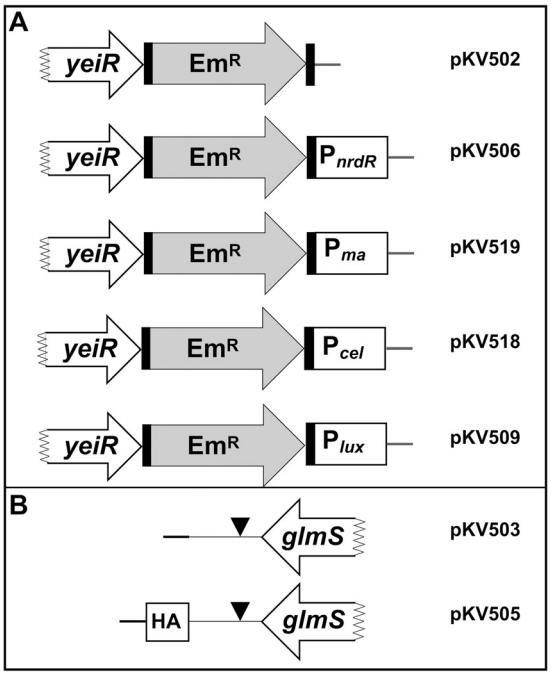

FIG 7 Constructs designed to support a variety of outcomes. (A) A family of cassettes was designed to be similar to base plasmid pKV502. They contain the FRT-flanked Em^r gene and different promoters as indicated. (B) A derivative of pKV503 was engineered to contain an HA epitope tag (pKV505).

albeit less so than the wild-type strain (Fig. 8B). Surprisingly, however, when a similar approach was used to complement a *flrA* mutant, the strains were nonmotile (Fig. 8A). Further inspection revealed that *flrA* lacks an obvious ribosome-binding site (RBS) (Fig. S5); thus, we considered the possibility that the failure stemmed from a lack of protein production rather than a lack of transcription. To test this possibility, we engineered the upstream primer to contain RBS sequences upstream of the start codon for *flrA* and repeated the experiment. These RBS-containing *flrA* complementation cassettes restored motility to the *flrA* mutant, albeit not to the level of the wild type (Fig. 8A). It is unclear why neither promoter restored full motility to the *flrA* or *fliQ* mutants, but it is likely due to the relative strength of the engineered promoters and/or their activity

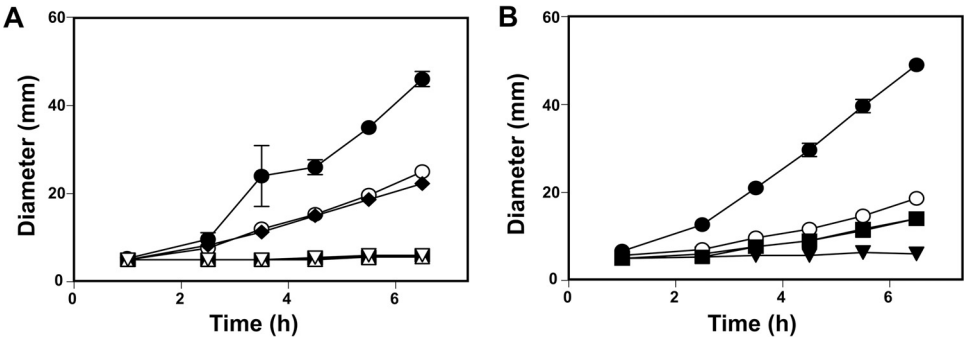

FIG 8 Motility of strains expressing *flrA* or *fliQ* from constitutive promoters. (A) Motility of a *flrA* mutant that expresses *flrA* under the control of different promoters positioned in the *yeiR-glmS* intergenic region. We assessed the wild type (ES114; filled circles), Δ*flrA* strain (KV8281; filled squares), and Δ*flrA* strains expressing the following *flrA* alleles: P*ma-flrA* (KV8293; triangles), P*nrdR-flrA* (KV8294; inverted triangles), P*ma* RBS-*flrA* (KV8364, filled diamonds), and P*nrdR*-RBS-*flrA* (circles; KV8365). (B) Motility of a *fliQ* mutant that expresses *fliQ* under the control of different promoters positioned in the *yeiR-glmS* intergenic region. We assessed the wild type (ES114; filled circles), the Δ*fliQ* strain (KV8376; filled triangles), and Δ*fliQ* strains expressing the following *fliQ* alleles: promoterless *fliQ* (KV8397; filled squares), P*ma-fliQ* (KV8398; open circles), and P*nrdR-fliQ* (KV8294; gray squares). Data shown in panel A for wild-type and KV8281 strains are the same as those shown in Fig. 6B.

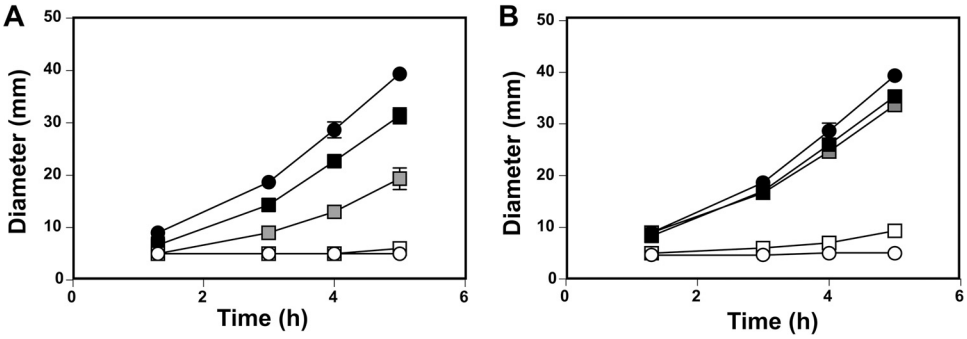

FIG 9 Motility of strains expressing *flrA* or *fliQ* from the cellobiose-inducible promoter. (A) Motility of a *flrA* mutant that expresses *flrA* under the control of the cellobiose-inducible promoter P*cel* in medium lacking or containing cellobiose. The following strains were assessed: the wild type (ES114; filled circles), the Δ*flrA* strain (KV8281; filled squares), and the Δ*flrA* strain expressing P*cel-flrA* in medium lacking cellobiose (open squares) or containing 0.001% (gray-filled squares) or 0.1% (black-filled squares) cellobiose. (B) Motility of a *fliQ* mutant that expresses *fliQ* under the control of the cellobiose-inducible promoter P*cel* in medium lacking or containing cellobiose. The following strains were assessed: the wild type (ES114; filled circles), the Δ*fliQ* strain (KV8298; open circles), and the Δ*fliQ* strain expressing P*cel-fliQ* in medium lacking cellobiose (open squares) or containing 0.001% (gray-filled squares) or 0.1% (black-filled squares) cellobiose. For both panels A and B, motility of wild-type strain ES114 was unaffected by cellobiose; shown are the results for the no-cellobiose control.

under these conditions. Additional, stronger promoters could be generated with a similar approach. The sequences that naturally promote translation of *flrA* remain to be determined.

Inducible promoters. We also developed tools for inducible expression by engineering constructs and strains to permit induction by (i) cellobiose, (ii) isopropyl-β-D-thiogalactopyranoside (IPTG), or (iii) 3-oxo-C6-homoserine lactone (HSL) (*V. fischeri* autoinducer, or AI). For induction via cellobiose addition, we engineered pKV518, which contains the cellobiose-inducible promoter P*cel* (34). We fused this promoter upstream of *flrA* and *fliQ* and inserted it at the *yeiR-glmS* region of the *flrA* and *fliQ* mutants, respectively. We then evaluated motility in the presence of two concentrations of cellobiose. Motility was observed with as little as 0.001% cellobiose, and for *flrA*, additional cellobiose (0.1%) increased motility (Fig. 9). For the *flrA* construct, motility was not observed (within the time frame of the experiment) unless cellobiose was added, and the larger amount of cellobiose yielded an increase level of motility relative to that of the smaller amount (Fig. 9A). For *fliQ*, a low basal level of activity was observed in the absence of cellobiose, and both amounts of cellobiose promoted robust motility, approaching that of the wild-type control (Fig. 9B). These data indicate that the P*cel* promoter can be used for inducible expression, but the basal level and range of induction may vary depending on the GOI and/or the exact construction. These data also demonstrate that the *fliQ* deletion was not substantially polar on the downstream *fliR* gene, which is required for motility (46).

To develop a system for IPTG-inducible gene expression, we began with promoter P*ma*, which we used above for constitutive expression. This promoter contains two binding sites for the IPTG-inducible regulator LacI (6). We then engineered a new *V. fischeri* strain that contains *lacI*^{q} in the *yeiR-glmS* region, along with the 3′-truncated Em′ sequences, to facilitate recombination (Fig. 5D). Using this approach, we generated a LacI-expressing Δ*flrA* mutant strain that contains P*ma-flrA*. The resulting strain was nonmotile in the absence of IPTG and became motile upon IPTG addition (Fig. 10A). This tight control is similar to that observed by Bose et al. (6).

Finally, to develop an AI-responsive promoter system, we engineered pKV509, which contains the AI-responsive *lux* promoter. P*lux* is activated by LuxR bound by AI, primarily but not exclusively the 3-oxo-C6-HSL produced by LuxI. *V. fischeri* is an underproducer of AI (54), thus it was not surprising that cells expressing *flrA* from P*lux* were only poorly motile. To determine if motility could be increased by overexpression of LuxR and/or induced by the LuxI-produced AI, we introduced a multicopy plasmid

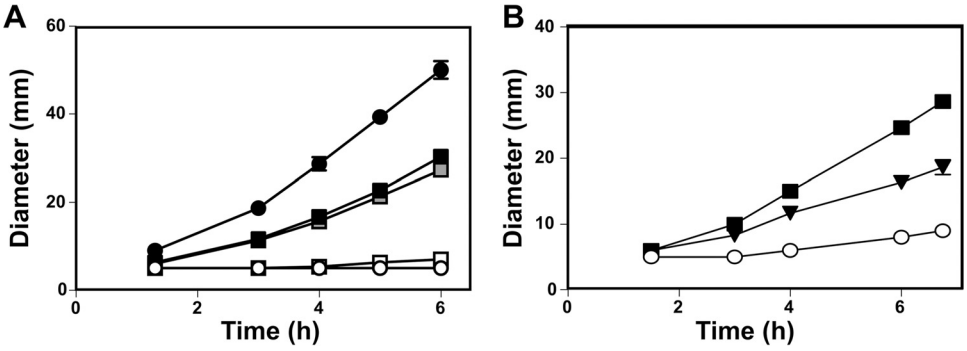

FIG 10 Motility of strains expressing *flrA* from IPTG-inducible and autoinducer-inducible promoters. (A) Motility of a *flrA* mutant that expresses *flrA* under the control of the IPTG-inducible promoter P*ma* in medium lacking or containing IPTG. The following strains were assessed: the wild type (ES114; filled circles), the Δ*flrA* strain (KV8281; open circles), and the Δ*flrA* strain expressing P*ma-flrA* in medium lacking IPTG (open squares) or containing 0.1 mM (gray filled squares) or 10 mM (black filled squares) IPTG. (B) Motility of a *flrA* mutant that expresses *flrA* under the control of the *lux* promoter P*lux*. The ability of *lux* regulators LuxR and LuxI to induce P*lux* was assessed by overexpressing these regulators in Δ*flrA* P*lux-flrA* strain KV8416. Motility of KV8416 carrying vector pVO8 (open circles), plasmid pKV27 (*luxI*), and plasmid pKV31 (*luxR*; open squares) was assessed in medium that contained Cm.

that encodes either LuxR or LuxI. Motility of these strains was greatly increased relative to that of the vector control (Fig. 10B). Thus, P*lux* is responsive to LuxR and AI. This particular tool could be useful for experiments designed to control/induce a gene during symbiotic colonization, as the LuxI-controlled luminescence phenotype is highly induced under those conditions (54, 55).

A tool for evaluating protein production. Another useful tool is the ability to evaluate protein production using an epitope tag. To facilitate this application, we incorporated the sequences for the hemagglutinin (HA) epitope tag into the downstream construct to generate pKV505 (Fig. 7B). Fusion of a gene of interest lacking its stop codon to PCR generated using pKV505 as a template should result in an HA-tagged protein that can be evaluated by Western blotting. To test this tool, we amplified *flrA* without its stop codon sequences under the control of P*cel*. We fused these sequences to the PCR product generated with template pKV505 and introduced the resulting products into the Δ*flrA*::FRT mutant, selecting for Em^r colonies. Motility of the resulting P*cel*-RBS-*flrA* derivative depended on the presence of cellobiose, consistent with the motility of the untagged version (data not shown). Importantly, HA-tagged FlrA was produced in the presence of cellobiose, as detected by Western immunoblotting using an anti-HA antibody (Fig. 11). Thus, this tool permits the rapid generation of strains that produce an epitope-tagged protein either constitutively or upon induction. Additional epitope tags or even sequences for fusion proteins, such as green fluorescent protein (GFP), or transcriptional reporters, such as *lacZ*, could be incorporated into the downstream construct to further increase their versatility.

Concluding remarks. This work presents tools for the rapid generation of deletions and insertions and demonstrates their utility using *flrA* and *fliQ*. This toolbox includes a set of cassettes with identical linkers that makes genetic manipulation of *V. fischeri* an easy and reliable method that requires few primers and does not depend on plasmid conjugation. In addition, the incorporation of BamHI restriction sites outside the

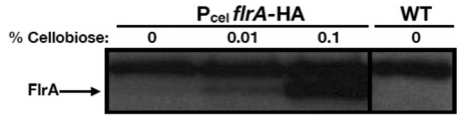

FIG 11 Detection of FlrA protein. Western blot analysis was used to detect HA epitope-tagged FlrA protein produced from P*cel*-RBS-*flrA*-HA. The amount of cellobiose added to overnight cultures of *V. fischeri* is indicated. The FlrA-HA band is indicated with an arrow. The nonspecific larger band observed in all lanes serves as a loading control.

antibiotic resistance markers readily permits the construction of plasmids with additional antibiotic resistance markers, and the use of FRT sequences further increases the versatility of the system. These tools provide a foundation that can be adapted to include other promoters, epitope tags, and even reporters such as *gfp* to meet the experimental needs of the scientific community. Insertions are not limited to the *yeiR-glmS* region; they can be facilitated by the introduction of a truncated Em^r cassette anywhere in a strain of interest. We anticipate that these tools and approaches will propel genetic manipulation and, thus, discovery in *V. fischeri*. Furthermore, we expect that these tools, while developed for ES114, can also be used to facilitate the study of other *V. fischeri* isolates of interest and/or adapted for use in other species.

MATERIALS AND METHODS

Strains and media. *V. fischeri* strains used or generated in this study are listed in Table 3. Genetic manipulations were performed in *V. fischeri* strain ES114 and its derivatives. ES114 was also used as a template for PCR designed to amplify *V. fischeri* genes of interest. *Escherichia coli* strains TAM1 (Active Motif), TAM1 λ *pir* (Active Motif), DH5α, and π3813 (33) were used for cloning. For routine culturing, *V. fischeri* strains were grown in LBS (56, 57), while *E. coli* strains were grown in LB (58), in some cases supplemented with thymidine. For selection of *E. coli* on Em, brain heart infusion agar (Difco) was used. LBS agar containing Congo red and Coomassie blue dyes (40 μg ml^{-1} and 15 μg ml^{-1}, respectively) was used to evaluate polysaccharide production by motility mutants. For natural transformation of *V. fischeri*, Tris minimal medium (TMM) (100 mM Tris, pH 7.5, 300 mM NaCl, 0.1% ammonium chloride, 10 mM *N*-acetylglucosamine, 50 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$, 0.0058% K_2HPO_4, 10 μM ferrous ammonium sulfate) was used. Soft-agar motility medium contained tryptone (1%), NaCl (2%), agar (0.25%), and $MgSO_4$ (35 mM). Antibiotics were added as appropriate at the following final concentrations: ampicillin, 100 μg ml^{-1}; Cm, 1 μg ml^{-1}; Em, 2.5 μg ml^{-1} (*V. fischeri*) or 150 μg ml^{-1} (*E. coli*); kanamycin (Kn), 100 μg ml^{-1} (*V. fischeri*) or 50 μg ml^{-1} (*E. coli*); Tc, 2.5 μg ml^{-1}; Tm, 10 μg ml^{-1}; Sp, 200 μg ml^{-1}; and Zc, 10 μg ml^{-1}. For Zc, LB was used as the medium instead of LBS for outgrowth and plating.

Plasmid construction. Plasmids used or generated in this study are listed in Table 4. To generate the tools used in this study, PCR products were cloned into the pJET plasmid using the CloneJET PCR cloning kit (Fisher Scientific) according to the manufacturer's instructions and as described in Table 4. In some cases, PCR products were purified using the DNA Clean & Concentrator kit (Zymo Research) or extracted from a gel using a Zymoclean gel DNA recovery kit (Zymo Research). To generate pKV496, the *flp* gene was amplified using primers 2116 and 2117, using pCP20 as a template, and cloned into pJET. The resulting clone was digested with KpnI and SacI and ligated to KpnI/SacI-digested plostfoX-Kan (37), resulting in the insertion of *flp* in place of *tfoX*. Either plasmid pKV494, which contains the Em^r cassette flanked by BamHI, FRT, and linker sequences, or pKV495 (the Cm^r equivalent) was used as the base plasmid for the generation of other linker- and FRT-flanked cassettes with different antibiotic resistance markers. Plasmid pKV494 (or pKV495) was cut with BamHI, as were the donor pJET derivatives that contained BamHI-flanked antibiotic resistance cassettes. The resulting DNA fragments were gel purified with a Zymoclean gel DNA recovery kit (Zymo Research) and ligated using T4 DNA ligase (Promega).

PCRs. PCR was carried out using primers listed in Table 5. For making insertions and deletions, KOD high-fidelity polymerase (Fisher Scientific) was used per the manufacturer's instructions, with an annealing temperature of 55°C. The resulting products were examined using agarose gel electrophoresis, staining with ethidium bromide, and visualization with UV light. PCR products were separated from primers and other components of the PCR using a DNA Clean & Concentrator kit (Zymo Research) and quantified using a NanoDrop instrument. To splice PCR products together, 100 to 200 ng of each product was used as the templates in a PCR SOE reaction in the absence of primers for 20 cycles. An aliquot (5 to 10 μl) from the SOE reaction mixture was used in a subsequent amplification reaction in the presence of the outermost primers. In some cases, the concentration of primers was reduced from the recommended 0.4 μM to obtain a cleaner full-length product. PCRs were also used to evaluate the deletion or insertion of sequences of interest using whole cells or chromosomal DNA that was extracted using either the DNeasy blood and tissue kit (Qiagen) or the Quick-DNA miniprep plus kit (Zymo Research; purchased from Genesee) with Promega GoTaq Flexi DNA polymerase (Fisher Scientific) per the manufacturer's instructions.

Natural transformation. Natural transformation was carried out using established protocols (36, 37) with PCR products or chromosomal DNA. Briefly, *V. fischeri* strains carrying plostfoX (36) or plostfoX-Kan (37) were grown overnight in TMM containing Cm or Kan, respectively, subcultured into the same medium, and grown at 24°C to an optical density at 600 nm (OD_{600}) of between 0.2 and 1.1. Cells were incubated with DNA for 30 min at room temperature, and then 0.5 ml of LBS broth was added. The cultures were incubated with shaking for at least 90 min, and then 200-μl aliquots were spread onto LBS plates containing the appropriate antibiotics. In some cases, a second aliquot was spread onto plates following overnight incubation of the cultures statically at room temperature.

Conjugation. Plasmids, such as pKV496 or plostfoX-Kan, were introduced into strains of interest using triparental conjugation as previously described (59).

Bioinformatics. The locations of putative promoters were predicted using BPROM (60).

Motility assays. Bacteria were grown in LBS at 28°C overnight and standardized to a uniform optical density at 600 nm (OD_{600}) of between 0.2 and 0.3. Aliquots (10 μl) were inoculated on the surface of fresh soft-agar motility plates and incubated at 28°C. Measurements of the outer diameter of the band of

TABLE 3 Strains generated or used in this study

Strain	Genotype[a]	Derivation[b]	Reference or source
ES114	WT	NA	54
KV4607	Δ*binA bcsA*::Tn*5*	NA	47
KV6576	IG::*lacI*^{q}	NA	50
KV8111	Δ*flrA*::FRT-Em^r	NT ES114 with SOE using primers 2215 and 2216 (ES114), 2089 and 2090 (pKV494), and 2217 and 2218 (ES114)	This study
KV8140	Δ*flrA*::FRT-Cm^r	NT ES114 with SOE using primers 2215 and 2216 (ES114), 2089 and 2090 (pKV495), and 2217 and 2218 (ES114)	This study
KV8148	Δ*flrA*::FRT	KV8111 with Em^r cassette removed via pKV496	This study
KV8183	Δ*flrA*::FRT-Tm^r	NT ES114 with SOE using primers 2215 and 2216 (ES114), 2089 and 2090 (pMLC2), and 2217 and 2218 (ES114)	This study
KV8191	Δ*sypQ*::FRT-Em^r	NT ES114 with SOE using primers 443 and 1188 (ES114), 2089 and 2090 (pKV494), and 2174 and 2175 (ES114)	This study
KV8225	Δ*fliQ*::FRT-Sp^r	NT ES114 with SOE using primers 2356 and 2357 (ES114), 2089 and 2090 (pKV521), and 2358 and 2359 (ES114)	This study
KV8232	IG::Em′ Tm^r	NT ES114 with SOE using primers 2185 and 2292 (pKV502), 2293 and 2294 (Tm^r), and 2089 and 1487 (pKV503)	This study
KV8244	Δ*flrA*::FRT *bcsA*::Tn*5*	NT KV8148 with KV4607 chDNA	This study
KV8245	Δ*flrA*::FRT Δ*sypQ*::FRT-Em^r	NT KV8148 with KV8191 chDNA	This study
KV8268	Δ*flrA*::FRT-Tc^r	NT ES114 with SOE using primers 2215 and 2216 (ES114), 2089 and 2090 (pKV514), and 2217 and 2218 (ES114)	This study
KV8281	Δ*flrA*::FRT IG::Em′ Tm^r	NT KV8148 with KV8232 chDNA	This study
KV8290	Δ*flrA*::FRT IG::P*flrA-flrA*	NT KV8281 with SOE using primers 2097 and 2098 (pEVS170), 2265 and 2254 (ES114), and 2196 and 1487 (pKV503)	This study
KV8292	IG::*lacI*^{q}-Em′ Tm^r	NT KV6576 with SOE using primers 2324 and 2325 (pKV507) and 2326 and 1487 (KV8232)	This study
KV8293	Δ*flrA*::FRT IG::P*ma-flrA*	NT KV8281 with SOE using primers 2290 and 2090 (pKV519) and 2219 and 1487 (KV8290)	This study
KV8294	Δ*flrA*::FRT IG::P*nrdR-flrA*	NT KV8281 with SOE using primers 2290 and 2090 (pKV506) and 2219 and 1487 (KV8290)	This study
KV8295	Δ*flrA*::FRT IG::P*cel-flrA*	NT KV8281 with SOE using primers 2290 and 2090 (pKV518) and 2219 and 1487 (KV8290)	This study
KV8298	Δ*fliQ*::FRT-Em^r	NT ES114 with SOE using primers 2356 and 2357 (ES114), 2089 and 2090 (pKV494), and 2358 and 2359 (ES114)	This study
KV8299	Δ*fliQ*::FRT-Cm^r	NT ES114 with SOE using primers 2356 and 2357 (ES114), 2089 and 2090 (pKV495), and 2358 and 2359 (ES114)	This study
KV8300	Δ*fliQ*::FRT-Tm^r	NT ES114 with SOE using primers 2356 and 2357 (ES114), 2089 and 2090 (pMLC2), and 2358 and 2359 (ES114)	This study
KV8364	Δ*flrA*::FRT IG::P*ma*-RBS-*flrA*	NT KV8281 with SOE using primers 2290 and 2090 (KV8293) and 2355 and 1487 (KV8290)	This study
KV8365	Δ*flrA*::FRT IG::P*nrdR*-RBS-*flrA*	NT KV8281 with SOE using primers 2290 and 2090 (KV8294) and 2355 and 1487 (KV8290)	This study
KV8366	Δ*flrA*::FRT IG::P*cel*-RBS-*flrA*	NT KV8281 with SOE using primers 2290 and 2090 (KV8295) and 2355 and 1487 (KV8290)	This study
KV8368	Δ*flrA*::FRT IG::P*ma-fliQ*	NT KV8281 with SOE using primers 2290 and 2090 (KV8293), 2360 and 2361 (ES114), and 2196 and 1487 (pKV503)	This study
KV8369	Δ*flrA*::FRT IG::P*nrdR-fliQ*	NT KV8281 with SOE using primers 2290 and 2090 (KV8294), 2360 and 2361 (ES114), and 2196 and 1487 (pKV503)	This study
KV8370	Δ*flrA*::FRT IG::P*cel-fliQ*	NT KV8281 with SOE using primers 2290 and 2090 (KV8295), 2360 and 2361 (ES114), and 2196 and 1487 (pKV503)	This study
KV8376	Δ*fliQ*::FRT	KV8299 with Cm^r cassette removed using pKV496	This study
KV8377	IG::*fliQ*	NT ES114 with SOE using primers 2185 and 2090 (pKV502), 2360 and 2361 (ES114), and 2196 and 1487 (pKV503)	This study
KV8378	Δ*flrA*::FRT IG::P*lux*-RBS-*flrA*	NT KV8281 with SOE using primers 2290 and 2090 (pKV509) and 2355 and 1487 (KV8290)	This study
KV8380	IG::P*cel*-RBS-*flrA*-HA	NT KV8281 with SOE using primers 2290 and 2220 (KV8366) and 2196 and 1487 (pKV505)	
KV8384	Δ*flrA*::FRT IG::*lacI*^{q}-Em′ Tm^r	NT KV8148 with KV8292 chDNA	This study
KV8397	Δ*fliQ*::FRT IG::*fliQ*	NT KV8376 with KV8377 chDNA	This study
KV8398	Δ*fliQ*::FRT IG::P*ma-fliQ*	NT KV8376 with KV8368 chDNA	This study
KV8399	Δ*fliQ*::FRT IG::P*nrdR-fliQ*	NT KV8376 with KV8369 chDNA	This study
KV8400	Δ*fliQ*::FRT IG::P*cel-fliQ*	NT KV8376 with KV8370 chDNA	This study
KV8407	Δ*fliQ*::FRT *bcsA*::Tn*5*	NT KV8376 with KV4607 chDNA	This study
KV8408	*bcsA*::Tn*5*	NT ES114 with KV4607 chDNA	This study
KV8411	Δ*fliQ*::FRT Δ*sypQ*::FRT-Em^r	NT KV8376 with KV8191 chDNA	This study

(Continued on next page)

TABLE 3 (Continued)

Strain	Genotype[a]	Derivation[b]	Reference or source
KV8417	Δ*flrA*::FRT IG::P*cel*-RBS-*flrA*-HA	NT KV8281 with KV8380 chDNA	This study
KV8430	Δ*fliQ*::FRT-Zc^r	NT ES114 with SOE using primers 2356 and 2357 (ES114), 2089 and 2090 (pKV520), and 2358 and 2359 (ES114)	This study
KV8435	Δ*flrA*::FRT IG::*lacI*^{q} P*ma*-RBS-*flrA*	NT KV8384 with KV8364 chDNA	This study

[a]IG, intergenic region between *yeiR* and *glmS*; except for KV6576 and where indicated as Em^r Tm^r, all strains showing an IG designation contain the Em^r marker.
[b]Natural transformation (NT) was performed using *tfoX*-expressing derivatives of the indicated strains; for the indicated PCR or PCR SOE reactions, templates are indicated in parentheses. chDNA, chromosomal DNA; NA, not applicable.

swimming cells were taken over time. Error bars represent standard deviations from three biological replicates; when no error bars can be observed, the error bars are smaller than the symbol. Data are representative of experiments performed on at least two separate days. Images were taken with a Canon XS170 IS camera.

Congo red assays. Bacteria were grown overnight at 24°C on LBS plates containing Congo red and Coomassie blue as indicated above. To better visualize color differences of the resulting growth, cells were transferred onto white paper in a replica plating-like approach by briefly smoothing the paper onto the agar plate and then lifting it off. The result was photographed with a Canon XS170 IS camera.

Western immunoblotting. *V. fischeri* cells were grown overnight in LBS lacking or containing cellobiose (0.01% or 0.1%). Cultures were normalized to an OD_{600} of 3. One-ml aliquots were pelleted and lysed in 200 μl 2× sample buffer (4% SDS, 40 mM Tris, pH 6.3, 10% glycerol). Proteins were separated by SDS-PAGE (12% acrylamide) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with dry milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 0.05% Tween 20), the membrane was treated with rabbit anti-HA antibody (Sigma-Aldrich), followed by exposure to a secondary antibody, goat anti-rabbit IgG antibody (Fisher Scientific) conjugated to horseradish peroxidase. Finally, to visualize HA-tagged FlrA, the membrane was incubated with Super-Signal West Pico plus chemiluminescent substrate (Thermo Fischer Scientific, Rockford, IL) and exposed to autoradiography film (Dot Scientific), which was developed in an autoprocessor.

TABLE 4 Plasmids used in this study

Plasmid	Description	Derivation[a]	Reference
pCP20	*flp*$^+$, Ap^r	NA	61
pEVS170	Vector, Em^r	NA	62
pJMO13	*lacI*^{q}, Ap^r	NA	50
pKV27	pVO8 + *luxI*	pVO8 SmaI + 1.8-kb EcoRV (*luxI*$^+$) from pHV200 (63)	This study
pKV31	pVO8 + *luxR*	pVO8 SalI/SmaI + 1-kb SalI/PvuII (*luxR*$^+$) from pHV200 (63)	This study
pKV69	Vector, Tc^r, Cm^r	NA	64
pKV494	pJET + FRT-Em^r	pJET + SOE product generated with primers 2097 and 2098 (pEVS170)	This study
pKV495	pJET + FRT-Cm^r	pKV494 BamHI + BamHI-digested pJET-Cm^r generated with primers 2087 and 2088 (pKV69)	This study
pKV496	pEVS79-Kn^r + *flp*$^+$	plostfoX-Kan KpnI/SacI + KpnI/SacI-digested pJET-*flp*$^+$ generated with primers 2116 and 2117 (pCP20)	This study
pKV502	pJET + *yeiR*-FRT-Em^r	pJET + SOE product generated with primers 2185 and 2186 (ES114) and 2187 and 2188 (pKV494)	This study
pKV503	pJET + *glmS*	pJET + PCR product generated with primers 2191 and 1487 (ES114)	This study
pKV505	pJET + HA-*glmS*	pJET + PCR product generated with 2198 and 1487 (pKV503)	This study
pKV506	pJET + *yeiR*-FRT-Em^r-P*nrdR*	pJET + SOE product generated with primers 2185 and 2240 (pKV502) and 2189 and 2190 (ES114)	This study
pKV509	pJET + *yeiR*-FRT-Em^r-P*lux*	pJET + SOE product generated with primers 2185 and 2240 (pKV502) and 2260 and 2261 (ES114)	This study
pKV510	pJET + Tc^r	pJET + PCR product generated with primers 2159 and 2160 (pKV69)	This study
pKV514	pJET + FRT-Tc^r	pKV495 BamHI + BamHI-digested pKV510	This study
pKV518	pJET + *yeiR*-FRT-Em^r-P*cel*	pJET + SOE product generated with primers 2185 and 2240 (pKV502) and 2251 and 2253 (ES114)	This study
pKV519	pJET + yeiR-FRT-Em^r-P*ma*	pJET + SOE product generated with primers 2185 and 2240 (pKV502) and 2185 and 2327 (pKV506)	This study
pKV520	pJET + FRT-Zc^r	pKV494 BamHI + BamHI-digested pJET-Zc^r generated with primers 2343 and 2344 (Zc^r)	This study
pKV521	pJET + FRT-Sp^r	pKV494 BamHI + BamHI-digested pJET-Sp^r generated with primers 2345 and 2346 (Sp^r)	This study
plostfoX	*tfoX*$^+$, Cm^r	NA	36
plostfoX-Kan	*tfoX*$^+$, Kn^r	NA	37
pMLC1	pJET + Tm^r	pJET + PCR product generated with primers 2278 and 2279 (Tm^r)	This study
pMLC2	pJET + FRT-Tm^r	pKV495 BamHI + BamHI-digested pMLC1	This study
pVO8	Vector, Em^r, Cm^r	NA	53

[a]Derivation of plasmids generated in this study.

TABLE 5 Primers used in this study

Primer no.	Sequence[a]
443	CGGTAATACTCCATAAGTTCTTTCAC
1188	GGTAATGCTGGGCGACTAG
1487	GGTCGTGGGGAGTTTTATCC
2087	GGATCCGATCGGCACGTAAGAGGTTC
2088	GGATCCTTACGCCCCGCCCTGCCACTC
2089	CCATACTTAGTGCGGCCGCCTA
2090	CCATGGCCTTCTAGGCCTATCC
2097	ccatacttagtgcggccgcctagaagttcctattctctagaaagtataggaacttcggatcCTAAGAGTGTGTTGATAGTGCAG
2098	ccatggccttctaggcctatccgaagttcctatactttctagagaataggaacttcggatcCTTATTTCCTCCCGTTAAATAATAG
2116	agagctcTCTGGCGGTGATAATGGTTG
2117	aggtaccGGCATAGTGCGTGTTTATGC
2159	ggatccGGGCTTAGTTTTTTGACCCAG
2160	ggatccATTACACTAAGTTATTTTATTGAAC
2174	taggcggccgcactaagtatggaGCATGATGGTATATTACGATACC
2175	ggataggcctagaaggccatggTGGACTCGAGTTCAAAAATAACA
2185	CTTGATTTATACAGCGAAGGAG
2186	cctatactttctagagaataggaacttcGTTTTGAAGAGTAATTAATGTTTATTG
2187	caataaacattaattactcttcaaaacGAAGTTCCTATTCTCTAGAAAG
2188	gcaaccgataaatgtatataccGAAGTTCCTATACTTTCTAGAGAATAGG
2189	ctctagaaagtataggaacttcGGGTATATACATTTATCGGTTGC
2190	ccatggccttctaggcctatccTACTTGCCTCCTAGTTTTTTCAATTAG
2191	ccatacttagtgcggccgcctaTATTGTCTCTCTTAGAACAATTATTC
2196	tccatacttagtgcggccgccta
2198	TCCATACTTAGTGCGGCCGCCTATTATCCATATGATGTTCCAGATTATGCATAATATTGTCTCTCTTAGAACAATTATTC
2215	TGGCTGGAGCTATTGAGCG
2216	taggcggccgcactaagtatggCTTTGCTAAACCTTGCATAGATG
2217	ggataggcctagaaggccatggAAAGATGATATGCGTTAATACTC
2218	TACCTAATGAAGATAGTCGTTG
2219	ggataggcctagaaggccatggTTAAGGCATATCCACATCTATG
2220	taggcggccgcactaagtatggaACGCATATCATCTTTATTTAAATTA
2240	gcaaccgataaatgtatataccGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGATCCTTATTTCCTCCCG
2251	gggtatatacatttatcggttgcCTTTTTCTAGGCCATCATTATC
2253	ccatggccttctaggcctatcccCATTGTTCACCCCTAATTAG
2254	taggcggccgcactaagtatggaTTAACGCATATCATCTTTATTTAAATTA
2260	gggtatatacatttatcggttgcGTTCATTTTTTGTTCACCTAGC
2261	ccatggccttctaggcctatcccACCTCTATACTCCTCTGATGG
2265	ggataggcctagaaggccatggTGCAGAATTTCATAACCTTACC
2278	ggatccTTAACGGTAAGCATCAGC
2279	ggatccCGAATCCGTTGCTGCCAC
2290	AAGAAACCGATACCGTTTACG
2292	gctgatgcttaccgttaattaattaGGCGTGTTTCATTGCTTGATG
2293	catcaagcaatgaaacacgcctaattaaTTAACGGTAAGCATCAGC
2294	taggcggccgcactaagtatggCGAATCCGTTGCTGCCAC
2324	CCCATTAAGTTCTGTCTCGGC
2325	ctttctagagaataggaacttcCGCTCACTGCCCGCTTTCC
2326	GGAAAGCGGGCAGTGAGCGGAAGTTCCTATTCTCTAGAAAG
2327	ccatggccttctaggcctatccAATTGTTATCCGCTCACAATTGAATCTAAGTATCATTGTTATCCGCTCACAAGTcgcaaccgataaatgtatataccc
2343	ggatccGTGTTGACAATTAATCATCGGC
2344	ggatccAATTCTCAGTCCTGCTCCTC
2345	ggatccAGAAAGCAGGTAGCTTGCAG
2346	ggatccCCCCTTATAATTTTTTTAATCTG
2355	ggataggcctagaaggccatggaggaggtCCACATCTATGCAAGGTTTAGC
2356	CGTTGTGATGTCGATTTTGCG
2357	taggcggccgcactaagtatggTTCAGGGGTCATGATTGCTC
2358	ggataggcctagaaggccatggCCAACGGTACTCTATTGACG
2359	GATAATCCACACTCGTGAGC
2360	ggataggcctagaaggccatggTAAGGAGCAATCATGACCCC
2361	taggcggccgcactaagtatggaCGTCAATAGAGTACCGTTGG

[a]Lowercase letters indicate nonnative or tail sequences.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00850-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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