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Engineering of *FRT-lacZ* fusion constructs: induction of the *Pseudomonas aeruginosa fadAB1* operon by medium and long chain-length fatty acids

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Abstract

Without prior knowledge of the promoters of various genes in bacteria, it can be difficult to study gene regulation using reporter-gene fusions. Regulation studies of promoters are ideal at their native locus, which do not require prior knowledge of promoter regions. Based on a previous study with *FRT-lacZ-Km^R* constructs, we constructed two novel *FRT-lacZ-Gm^R* plasmids. This allows easy engineering of *P. aeruginosa* reporter-gene fusions, post-mutant construction with the Fip-*FRT* system. We demonstrate the usefulness of one of these *FRT-lacZ-Gm^R* plasmids to study the regulation of the *fadAB1* operon in *P. aeruginosa* at its native locus. The *fadAB1* operon, involved in fatty acid (FA) degradation, was significantly induced in the presence of several medium chain-length fatty acids (MCFA) and, to a lesser degree, long chain-length fatty acids (LCFA). In addition to the previous work on the *FRT-lacZ-Km^R* tools, these new constructs increase the repertoire of tools that can be applied to *P. aeruginosa* or other species and strains of bacteria where kanamycin resistance may not be appropriate.

Keywords

Pseudomonas aeruginosa; *FRT-lacZ* fusion; fatty acid degradation

Introduction

Studying gene-regulation is the foundation of understanding cellular physiology. Specifically in bacteria, gene-expression studies have become more elaborate and the technologies more complex. Gene-expression profiles can be achieved using macro- and microarrays (Firoved et al., 2004) and quantitative real-time polymerase chain reactions (qRT-PCR) (Freeman et al., 1999; Savli et al., 2003). However, these innovative technologies can prove costly and a classical approach may be more appropriate.

Many plasmid-based systems have been developed to study gene-expression by creating reporter-gene fusions, such as *lacZ* (Hand and Silhavy, 2000; Hoang et al., 2000; Manoil, 2000; Schweizer, 1991; Silhavy and Beckwith, 1985; Slauch and Silhavy, 1991), *xylE* (Hassett, 2000), the *luxAB* of (Gonzalez-Flecha, 1994), and the green fluorescent protein (gfp) (Miller,

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1997). However, they all suffer from inherent problems such as titration of regulators for high-copy plasmids, and incorrect representation of the natural regulation of the gene(s). Previously, *lacZ* fusions were introduced into *P. aeruginosa* using the mini-CTX system (Hoang et al., 2000) or the *Tn7* integration system (Choi et al., 2005), allowing site-specific *lacZ* fusions to be studied as a more representative single copy. However, these existing systems require prior knowledge of the promoter region, and fusions other than at the native locus may misrepresent the more complex natural regulatory mechanism. More recently, Ellermeier et al constructed a Flp-mediated site-specific recombination system, where *lacZ* can be introduced into the chromosome at the native locus as a transcriptional fusion, using a Flp recombination target sequence (*FRT*) (Ellermeier et al., 2002). This system requires a chromosomally located *FRT* previously introduced via the λ -Red mutant construction method (Hand and Silhavy, 2000). A plasmid harbouring *lacZ* and a kanamycin resistance (Km^R) cassette downstream of an *FRT* (*FRT-lacZ-Km^R*) was easily constructed (Ellermeier et al., 2002). When Flp was introduced, the two *FRT*s recombined, integrating the plasmid-borne *FRT-lacZ-Km^R* fusion with the chromosomally located *FRT*, to generate a *lacZ* transcriptional fusion. However, *P. aeruginosa* can have high resistance levels to kanamycin ($\geq 1000 \mu\text{g/ml}$), and a cleaner alternative selectable marker (i.e. gentamycin resistance) is more desirable.

Fatty acid degradation (Fad) is an essential pathway for bacteria to utilize lipids and other fatty acid-associated compounds. In *E. coli*, Fad has been well characterized (Black and DiRusso, 1994; Clark and Cronan Jr., 1996), where several of the genes, *fadA*, *fadB*, *fadD*, *fadE* and *fadL*, are negatively regulated by FadR in the absence of external fatty acids (FA) $\geq C_{12:0}$. However, virtually nothing is known about Fad in *P. aeruginosa*, and the regulatory mechanism remains an enigma. With a genome of 6.3Mb (Stover et al., 2000), *P. aeruginosa* has 6–8 fold more genes involved in Fad than *E. coli*, suggesting increased complexity and redundancies in the Fad pathway. *P. aeruginosa* is capable of growing on short ($C_{4:0}$ – $C_{8:0}$), medium ($C_{10:0}$ – $C_{14:0}$) and long chain-length FAs ($\geq C_{16:0}$), and the involvement of one Fad operon has been identified as *fadABI* (PA1737 and PA1736, respectively) (Son et al., 2003).

Here, we report the engineering of two *FRT-lacZ-Gm^R* plasmids, one of which was utilized to generate a chromosomal *P_{fadABI}-FRT1-lacZ* transcriptional fusion. This fusion was characterized in *P. aeruginosa* by monitoring the regulation of the *fadABI*-promoter at its native locus. *P. aeruginosa* containing this *P_{fadABI}-FRT1-lacZ* fusion was grown and characterized in minimal glucose media with different chain-length FAs (short chain-length FAs (SCFA), medium chain-length FAs (MCFA) and long chain-length FAs (LCFA)). These constructs add to the repertoire of *FRT*-based tools previously reported (Ellermeier et al., 2002) to study non-essential gene function and regulation, because these genes are often involved in virulence and pathogenesis.

Materials and Methods

Bacterial Strains and Media

Strains and plasmids used and constructed in this study are shown in Table 1. *E. coli* DH5 α (Liss, 1987) was used for all molecular cloning steps. *E. coli* SM10 (Simon et al., 1983) was the donor strain for conjugation to introduce the plasmid pEX18T Δ *fadABI::FRT-Gm^R-FRT* into wild-type *P. aeruginosa* PAO1 to create a mutant PAO1 Δ *fadABI::FRT-Gm^R-FRT*. For tri-parental mating to introduce the *FRT1-lacZ* transcriptional fusion into PAO1 Δ *fadABI::FRT*, *E. coli* strains ER2566*mob-pir116/pFRT1-lacZ* and ER2566*mob-pir116/pCD13SK-Flp-oriT* were used as donors. All *E. coli* strains were grown in LB broth (Difco, Sparks, MD), with gentamycin (15 $\mu\text{g/ml}$) or streptomycin (25 $\mu\text{g/ml}$) (Teknova, Halfmoon Bay, CA) for plasmid maintenance. *P. aeruginosa* strains were grown in *Pseudomonas* Isolation Broth (PIB) or *Pseudomonas* Isolation Agar (PIA) (Difco, Sparks, MD), supplemented with gentamycin (150 $\mu\text{g/ml}$) for selection, unless otherwise stated.

Growth curves were conducted in 1x M9 media (1x M9, 1% Brij-58, 0.5 mM MgSO₄, 0.02 mM CaCl₂) + 40 mM glucose ± 0.1% (w/v) of the individual saturated FAs (butyric acid, C_{4:0}; n-caproic acid, C_{6:0}; caprylic acid, C_{8:0}; capric acid, C_{10:0}; lauric acid, C_{12:0}; myristic acid, C_{14:0}; palmitic acid, C_{16:0}) or an unsaturated FA (oleic acid, C_{18:1}^{Δ9}) (Sigma, St. Louis, MO). 3% stock solutions of FAs were made in equimolar KOH and 1% Brij-58. Cell cultures were diluted 1:4 in 4% Brij-58 prior to taking OD₅₄₀ measurements.

General Molecular Methods and Reagents

All restriction enzymes, *Taq* DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) and used according to manufacturer recommendations. Plasmids were recovered using the Zippy Plasmid Miniprep Kit (Zymo Research, Orange, CA) and gel purification of DNA bands were performed using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). All primers were from IDT Technologies (Coralville, IA).

Construction of *fadAB1::FRT* Mutant

The *fadAB1* operon (3.7kb) was PCR amplified using forward primer 5'-CGAAAGCTTGCATGGTGCTATCTTCC-3' and reverse primer 5'-GCGGAATTCGCCCTACCCGTGGCG-3'. PCR was assembled in 50 μl reactions containing 10 ng PAO1 chromosomal DNA, 30 pmoles of each forward and reverse primer, 0.2 mM dNTP, 1x ThermoPol reaction buffer, and 5 units of *Taq* DNA polymerase. After thirty-four cycles (95°C for 1 min, 58°C for 45 sec, 72°C for 3 min), a final extension at 72°C for 10 min was performed. The product was gel purified, digested with *HindIII* + *BamHI*, and cloned into the vector pEX18T, digested with the same restriction enzymes, to yield pEX18T*fadAB1*.

To create the final gene-replacement vector pEX18TΔ*fadAB1::FRT*-Gm^R-*FRT*, the 1.1 kb *SmaI* digested *FRT*-Gm^R-*FRT* cassette from plasmid pPS856 (Hoang et al., 1998) was ligated into pEX18T*fadAB1*, previously digested with *PstI* + *BamHI* and blunt-ended. pEX18TΔ*fadAB1::FRT*-Gm^R-*FRT* was conjugated from *E. coli* SM10 into PAO1 and unmarked mutations constructed as previously described (Hoang et al., 1998).

pCD13SK-Flp-oriT Helper Plasmid Construction

The 2.5 kb *BamHI*-*SacI* fragment containing *flp* and the λ-repressor from pFlp2 was cloned into pCD13SK cut with the same enzymes, yielding pCD13SK-Flp. The 660 bp *oriT* from pTZ120 was removed with *NarI* + *NdeI*, and blunt-ended. This fragment was cloned into pCD13SK-Flp at the *SmaI* site to give pCD13SK-Flp-oriT.

pFRT1-lacZ and pFRT2-lacZ Plasmid Construction

Plasmid pTZ120 was digested with *SapI* and *NarI*, blunt-ended and self-ligated to remove the *ori1600-rep*; this gave a final plasmid size of 5.7 kb. The *FRT*-Gm^R-*FRT* cassette (1.1 kb) was excised from plasmid pPS856 using *BamHI*. This fragment was then cloned into pTZ120, also digested with *BamHI*, yielding pDTN100::*FRT1*-Gm^R-*FRT1* and pDTN100::*FRT2*-Gm^R-*FRT2*, which contains the Gm^R-cassette in the opposite orientation, along with the *lacZ* fusion. *FRT1-lacZ* and *FRT2-lacZ* have identical sequences but differ in the orientation of the *FRT* relative to the *lacZ*. These plasmids were transformed into a Flp expression strain (DH5α-*λattB::pCD13SK-Flp*), to remove the Gm^R-cassette, yielding pDTN100::*FRT1-lacZ* and pDTN100::*FRT2-lacZ*. The 2.3 kb *FRT1-lacZ* and *FRT2-lacZ* fragments were then excised from pDTN100::*FRT1-lacZ* and pDTN100::*FRT2-lacZ*, respectively, using *XhoI* + *AflIII* and blunt-ended. These fragments were ligated with the Gm^R-R6K *γori-T₁T₂* fragment (2.6 kb) from plasmid pXR6KΔS, which was digested with *XmnI* + *PvuII*. This ligation gave the final plasmids pFRT1-lacZ and pFRT2-lacZ (Figure 1A).

Conjugation and Selection

All strains were grown to mid-log phase for conjugation. Conjugation was performed by mixing 500 μ l of each donor strain ER2566*mob-pir116/pFRT1-lacZ* and ER2566*mob-pir116/pCD13SK-Flp-oriT*, with 500 μ l of the recipient strain PAO1 Δ *fadAB1::FRT1*. Cells were pelleted sequentially and the media decanted leaving ~40 μ l in the final pelleting step. The conjugation mixture was resuspended in the remaining media and spotted onto cellulose-acetate filters (Satorius, Goettingen, Germany) on LB plates and incubated overnight at 37°C. Filters were resuspended in 1 ml of 1x M9 (Sambrook and D.W., 2001) and plated on PIA containing gentamycin. Positive clones were screened for a 1.2kb PCR product using the Gm^R-specific forward primer 5'-CATACGCTACTTGCATTACAG-3' and the *fadAB1*-specific reverse primer 5'-GCGGAATTTCGCCCTACCCGTGGCG-3'.

Growth Curve and β -Galactosidase Assay

PAO1 was grown simultaneously, as a control, with PAO1 Δ *fadAB1::FRT1-lacZ*. Cultures were grown for 14 hours in PIB and the cells were washed twice with 1x M9 media. A 1:100 dilution was inoculated into 50 ml 1x M9 minimal media supplemented as described in Bacterial Strains and Media. Both strains were grown in 1x M9 + 40 mM glucose as controls. Cultures were diluted as described in Bacterial Strains and Media and cell densities were measured in a Beckman-DU7500 spectrophotometer, taking dilution factors into account. Standard β -galactosidase assays were conducted in triplicate as described previously (Lederberg, 1950; Miller, 1972) and mean values determined with S.E.M.

GenBank Accession Numbers

The plasmids *pFRT1-lacZ*, *pFRT2-lacZ* and *pCD13SK-Flp-oriT* have been deposited in GenBank with accession numbers EU034636, EU034637 and EU034638, respectively.

Results and Discussion

When used in conjunction with the gene-replacement system (Hoang et al., 1998), the integration of the *FRT-lacZ* fusion makes it possible to study the regulation of non-essential genes, eliminating the arduous task of identifying promoters. Using this fusion involves two steps: first, the construction of the mutant containing a single *FRT*; and second, introduction of the *FRT-lacZ* fusion through tri-parental mating.

In this study, a *fadAB1* mutant was constructed in *P. aeruginosa*, by inserting a Gm^R-cassette flanked by *FRT*s into the operon, as described previously (Hoang et al., 1998). The Gm^R-cassette and a single *FRT* were then excised by introducing a *flp*-containing plasmid, leaving behind a single *FRT* at the allelic replacement site. This chromosomally located *FRT* is the subsequent insertion site of the *FRT-lacZ* fusion.

The two *FRT1-lacZ* and *FRT2-lacZ* fusions were generated on the suicidal plasmids *pFRT1-lacZ* and *pFRT2-lacZ*, respectively (Figure 1A). Although two *FRT-lacZ* fusion plasmids were created to accommodate the two orientations of the *FRT*s, only *pFRT1-lacZ* was used in this regulation study. During tri-parental mating, the fusion plasmid *pFRT1-lacZ* (Figure 1A) is introduced into the *P. aeruginosa* recipient along with the helper plasmid *pCD13SK-Flp-oriT* (Figure 1B). This helper plasmid encodes Flp, which catalyzes the recombination of the *FRT1* on *pFRT1-lacZ* fusion and the chromosomally located *FRT1* within the inactivated *fadAB1* operon (Figure 1C). This resulting transcriptional fusion is downstream of the *fadAB1*-promoter and therefore expression of the *fadAB1* operon can be directly correlated to β -galactosidase activity (Figure 1D).

Using the *P. aeruginosa* mutant, we were able to demonstrate the utility of the *FRT1-lacZ* fusion to study the induction of the *fadAB1* operon by the different chain-length FAs. The mutant carrying the *lacZ*-reporter fusion was grown in 1x M9 minimal media containing 40 mM glucose supplemented with or without FA. To address the effects of the different chain-length FAs on gene-expression levels of the *fadAB1* operon, growth rates and β -galactosidase activity were measured. The growth rates of the PAO1 Δ *fadAB1::FRT1-lacZ* mutant carrying the fusion were compared to that of wild-type PAO1 (Figure 2A and 2B), since growth rate differences can affect gene-expression. Figures 2A and 2B show that the growth rates were identical when the mutant was grown in the same media as wild-type PAO1, and that the addition of FAs both increased the length of stationary phase and decreased the rate of death. In addition, the same growth rates were observed when the mutant was grown in glucose $\pm$ individual FAs (Figure 2B). These identical growth rates allowed appropriate comparison of the *fadAB1* operon expression level in response to the different FA, by measuring β -galactosidase activities during three different growth phases (early-, mid-, and late-log) for each FA chain-length (Figure 2C). In the presence of short chain-length FAs (SCFA) (C_{4:0}–C_{8:0}), the *fadAB1* operon was not induced, as the β -galactosidase activities were relatively equal to that grown in the absence of FA (Figure 2C). The lack of gene induction by the SCFA and the absence of any growth defect in the Δ *fadAB1* mutant were observed throughout all growth phases. Our laboratory has identified two other *fadAB* operons that are also involved in Fad, and future studies are required to show if these other two operons are involved in SCFA degradation.

The *fadAB1* operon was most significantly induced when the mutant was grown in the presence of medium chain-length FAs (MCFA) (C_{10:0} to C_{14:0}). In all growth phases, the effects of the MCFA were similar, with C_{12:0} causing slightly higher induction levels throughout. In early-log, the basal activity in glucose alone was only 7.3 Miller units, where C_{10:0} (59.7 Miller units), C_{12:0} (65.5 Miller units) and C_{14:0} (40.1 Miller units) induced the *fadAB1* operon 8.2-, 9.0- and 5.5-folds, respectively. This trend was mirrored at slightly higher levels in mid-log, where basal activity in glucose was at 7.8 Miller units and induction levels reached 10.1-, 12.7- and 8.5-folds, for C_{10:0} (79.2 Miller units), C_{12:0} (99.1 Miller units) and C_{14:0} (66.4 Miller units), respectively. The most significant increase in *fadAB1* operon induction was observed when going from mid- to late-log. In late-log phase, basal levels in glucose alone were at 4.1 Miller units and C_{10:0} induced the operon up to 26.2-folds (108 Miller units), C_{12:0} up to 27.6-folds (113.9 Miller units), and C_{14:0} up to 28.8-folds (118.5 Miller units).

Although *P. aeruginosa* can metabolize and reach high cell densities when grown solely on long chain-length FA (LCFA) (C_{16:0} and C_{18:1} ^{Δ 9}) (unpublished data), the *fadAB1* operon was not induced to high levels during early-log (Figure 2C). At early-log, the β -galactosidase activity with these LCFA was 18.9 and 17.3 Miller units for C_{16:0} and C_{18:1} ^{Δ 9}, respectively. However, as the cells reached mid-log phase, induction rose to 45.2 Miller units and 39.9 Miller units by C_{16:0} and C_{18:1} ^{Δ 9}, respectively, (5.8- and 5.1-folds respectively, compared to glucose alone). As the cells reached late-log, induction rose further up to 69.5 Miller units and 60.2 Miller units by C_{16:0} and C_{18:1} ^{Δ 9}, respectively, (16.9- and 14.6-folds respectively, compared to glucose alone). *fadAB1* is most responsive to the MCFA in all three growth phases, but also responds to LCFA to a lesser degree.

In summary, we constructed two plasmids for the simultaneous study of promoter activity in conjunction with mutational analysis using the Flp-*FRT* system. Furthering the existing method of creating unmarked deletions in *P. aeruginosa* using the gene-replacement system (Hoang et al., 1998), we capitalize on the presence of the *FRT* left behind after excision of the Gm^R-cassette with Flp as previously performed for *E. coli* (Ellermeier et al., 2002). We have constructed a simple *FRT-lacZ* fusion reporter system that allows one to study the regulation of a particular gene of interest. The utility of this reporter fusion was demonstrated using the

fadAB1 operon of *P. aeruginosa* as a model. The regulation of this operon has not been characterized, and using the *FRT1-lacZ* fusion, we demonstrated its regulation by MCFA and LCFA. This study would be difficult in *P. aeruginosa* with the previously reported *FRT-lacZ-Km^R* fusion (Ellermeier et al., 2002), because of high kanamycin resistance levels. However, these novel *FRT-lacZ* fusion constructs with the Gm^R-cassette add to the existing repertoire of gene fusion tools for bacteria.

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Figure 1. Schematic of pFRT-lacZ plasmid, helper plasmid, and chromosomal target gene of interest with FRT

(A) pFRT1-lacZ or pFRT2-lacZ is conjugated with a (B) helper plasmid, pCD13SK-Flp-oriT, into the recipient *P. aeruginosa* strain containing an FRT (*FRT1*) in the same orientation as the fusion. (C) The chromosomal $\Delta fadAB1::FRT$ is the locus where the FRT-lacZ fusion will recombine catalyzed by the Flp protein expressed on the helper plasmid. (D) The final recombinant product is shown with the fusion integrated into the chromosome at the FRT site. The FRT-lacZ plasmids contain several key features that make this system appropriate for these studies: i) T_1 and T_2 immediately downstream of the FRT-lacZ fusion prevent any read through; ii) a suicidal R6K γ ori origin of replication that can only replicate in the presence of a π protein or a strain expressing the *pir* gene; iii) a Gm^R-cassette to allow for selection of positive recombinations in *P. aeruginosa*; iv) a Flp expressing R6K γ ori suicidal helper plasmid, pCD13SK-Flp-oriT. The expression of Flp is under the control of the λ -promoter, which is repressed by cI857. **Abbreviations:** cI857, temperature sensitive λ -repressor; *flp*, Flp protein driven by the λ -promoter; FRT, Flp recognition target; Gm^R, gentamycin resistance cassette; *lacZ*, β -galactosidase; *oriT*, origin of transfer; P_{fadAB1} , *fadAB1* promoter; R6K γ ori, suicidal origin of replication; Sp^R, streptomycin resistance cassette; T_1T_2 , transcriptional terminators.

Figure 2. Growth curves of wild-type PAO1 and PAO1ΔfadAB1::FRT1-lacZ fusion strain grown in glucose and various fatty acids, showing induction of the *fadAB1* operon by fatty acids

Growth curves of (A) wild-type PAO1 and (B) PAO1ΔfadAB1::FRT1-lacZ fusion strain in glucose media supplemented with and without fatty acids, showing no growth defects in the mutant fusion strain. (C) The *fadAB1* operon is regulated by the medium to long chain-length FAs, as indicated by the higher β-galactosidase activity of the *FRT1-lacZ* fusion. The three growth phases monitored were early-, mid- and late-log growth, and β-galactosidase activities measured in triplicate and displayed as an average. The points chosen at early-log (EL), mid-log (ML) and late-log (LL) for β-galactosidase measurements are indicated in (B).

Table 1

Bacterial strains and plasmids

		Genotype	Reference
Strains <i>E. coli</i>	DH5 α	(ϕ 80 <i>dlacZ</i> Δ M15) Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>glnV44</i> λ^- <i>thi-1 gyrA96 relA1</i>	(Liss, 1987)
	SM10 ER2566 <i>mob-pir116</i>	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km^R F⁻ λ phuA2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(<i>mcrC-mrr</i>) 114::IS10 R(<i>mcr-73::miniTn10</i>)2 R(<i>zgb-120::Tn10</i>)1 <i>endA1 [dcm] recA::RP4-2Tc::Mu Km pir116-Tet^R</i> (ϕ80<i>dlacZ</i> ΔM15) Δ(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>glnV44 thi-1 gyrA96 relA1</i></i>	(Simon et al., 1983) This study
	DH5 α - λ <i>attB</i> :pCD13SK-Flp	λ <i>attB</i> :pCD13SK-Flp Sp ^R	This study
<i>P. aeruginosa</i>	PAO1	Prototroph	(Stover et al., 2000)
	PAO1 Δ <i>fadAB1::FRT</i> -Gm ^R - <i>FRT</i>	Gm ^R , PAO1 with <i>fadAB1</i> operon deletion and <i>FRT</i> -Gm ^R - <i>FRT</i> insertion	
	PAO1 Δ <i>fadAB1::FRT</i>	PAO1 Δ <i>fadAB1::FRT</i> -Gm ^R - <i>FRT</i> with Gm ^R -cassette removed	This study
Plasmids	PAO1 Δ <i>fadAB1::FRT1-lacZ^a</i>	Gm ^R , PAO1 with <i>fadAB1</i> operon deletion and <i>FRT1-lacZ</i> insertion	This study
	pPS856	Ap ^R , Gm ^R , plasmid with <i>FRT</i> -Gm ^R - <i>FRT</i> cassette	(Hoang et al., 1998)
	pEX18T	Ap ^R , <i>oriT⁺</i> , <i>sacB⁺</i> , gene replacement vector	(Hoang et al., 1998)
	pEX18T <i>fadAB1</i>	Ap ^R , pEX18T with wild-type <i>fadAB1</i> operon	This study
	pEX18T Δ <i>fadAB1::FRT</i> -Gm ^R - <i>FRT</i>	Ap ^R , Gm ^R , pEX18T with Δ <i>fadAB1</i> operon with <i>FRT</i> -Gm ^R - <i>FRT</i> insertion	This study
	pFlp2	Ap ^R , <i>sacB⁺</i> , plasmid with <i>flp</i> gene	(Hoang et al., 1998)
	pTZ120	Ap ^R , <i>lacZ</i> operon fusion vector	(Schweizer and Chuanshuen, 2001)
	pXR6KAS	Gm ^R , <i>sacB⁻</i> , suicidal vector	This study
	pCD13SK	Sp ^R , λ <i>attB</i> integration vector	(Platt et al., 2000)
	pCD13SK-Flp	Sp ^R , pCD13SK with <i>flp</i> gene and <i>ci857</i>	This study
	pCD13SK-Flp- <i>oriT</i>	Sp ^R , pCD13SK-Flp with <i>oriT</i>	This study
	p <i>FRT1-lacZ^a</i>	Gm ^R , <i>FRT1-lacZ</i> fusion containing suicidal vector	This study
	p <i>FRT2-lacZ^a</i>	Gm ^R , <i>FRT2-lacZ</i> fusion containing suicidal vector	This study
	pDTN100:: <i>FRT1</i> -Gm ^R - <i>FRT1^a</i>	Gm ^R , pTZ120 backbone with <i>FRT1</i> -Gm ^R - <i>FRT1</i>	This study
	pDTN100:: <i>FRT2</i> -Gm ^R - <i>FRT2^a</i>	Gm ^R , pTZ120 backbone with <i>FRT2</i> -Gm ^R - <i>FRT2</i>	This study
	pDTN100:: <i>FRT1-lacZ^a</i>	Gm ^R , pDTN100:: <i>FRT1</i> -Gm ^R - <i>FRT1</i> with Gm ^R -cassette excised	This study
	pDTN100:: <i>FRT2-lacZ^a</i>	Gm ^R , pDTN100:: <i>FRT2</i> -Gm ^R - <i>FRT2</i> with Gm ^R -cassette excised	This study

^a *FRT1* and *FRT2* refer to opposite orientations of the *FRT* relative to *lacZ* (refer to Figure 1A)