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Utility of the Clostridial Site-Specific Recombinase TnpX To Clone Toxic-Product-Encoding Genes and Selectively Remove Genomic DNA Fragments

Vicki Adams,[a] Radhika Bantwal,[b] Lauren Stevenson,[a] Jackie K. Cheung,[a] Milena M. Awad,[a] Joel Nicholson,[a] Glen P. Carter,[a] Kate E. Mackin,[a] Julian I. Rood,[a,b] Dena Lyras[a]

Department of Microbiology, Monash University, Clayton, VIC, Australia[a]; ARC Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, VIC, Australia[b]

TnpX is a site-specific recombinase responsible for the excision and insertion of the transposons Tn*4451* and Tn*4453* in *Clostridium perfringens* and *Clostridium difficile*, respectively. Here, we exploit phenotypic features of TnpX to facilitate genetic mutagenesis and complementation studies. Genetic manipulation of bacteria often relies on the use of antibiotic resistance genes; however, a limited number are available for use in the clostridia. The ability of TnpX to recognize and excise specific DNA fragments was exploited here as the basis of an antibiotic resistance marker recycling system, specifically to remove antibiotic resistance genes from plasmids in *Escherichia coli* and from marked chromosomal *C. perfringens* mutants. This methodology enabled the construction of a *C. perfringens plc virR* double mutant by allowing the removal and subsequent reuse of the same resistance gene to construct a second mutation. Genetic complementation can be challenging when the gene of interest encodes a product toxic to *E. coli*. We show that TnpX represses expression from its own promoter, P_{attCI}, which can be exploited to facilitate the cloning of recalcitrant genes in *E. coli* for subsequent expression in the heterologous host *C. perfringens*. Importantly, this technology expands the repertoire of tools available for the genetic manipulation of the clostridia.

The clostridia are a diverse group of bacteria, incorporating both pathogenic and industrially important species. Genetic manipulation of clostridia can be challenging. The recent introduction of TargeTron technology, using mobile group II introns to disrupt the gene of interest (1–3), has improved researchers' abilities to generate targeted gene disruptions; however, the tools available for use in the clostridia are still limited. Antibiotic resistance cassettes are commonly used to select for relatively rare recombination events in attempts to introduce DNA onto another DNA molecule *in vivo*. The number of antibiotic resistance markers available for use within a particular species or strain can sometimes be limited, restricting a researcher's ability to manipulate these strains. When using the TargeTron system, there are few antibiotic resistance retrotransposition-activated markers (RAMs) available, and only one is currently widely used in the clostridia (*ermB* RAM) (1, 4, 5). Without multiple resistance markers at one's disposal, the ability to remove an integrated marker for subsequent reuse becomes essential. The FLP recombinase system to remove antibiotic resistance markers (6) has been used in the nonpathogenic clostridial species *Clostridium acetobutylicum* (7, 8); however, to date, there have been no reports of this system being used on clinically important clostridial species. Here, we describe the use of an alternative system for marker recycling in the human and animal pathogen *Clostridium perfringens* based on the clostridial recombinase TnpX.

TnpX is a site-specific serine recombinase encoded by the Tn*4451*/*53* family of clostridial mobilizable transposons (9). This family includes Tn*4451* and Tn*4453*, which confer chloramphenicol resistance to *C. perfringens* (10) and *Clostridium difficile* (11, 12), respectively. TnpX is required for the transposition of Tn*4451*, excising the linear element from surrounding DNA to form a circular intermediate. TnpX then mediates the integration of the circular intermediate back into a replicating DNA molecule in a linear form (12). TnpX recognizes *attL* and *attR* sites that flank the linear transposon, leading to the excision of the element (13). The formation of the circular intermediate results in the juxtaposition of these sites to form the *attCI* site, which also results in the formation of a strong promoter for *tnpX* expression, P_{attCI} (14). TnpX binds with high affinity to *attCI* (13), thereby in effect binding to P_{attCI} (13, 14). In this study, we demonstrate the construction of a *plc* mutant using TargeTron technology, with the subsequent removal of the *ermB* RAM by TnpX. This process then allowed for the disruption of a second gene, *virR*, in the same strain by using the *ermB* RAM.

Complementation is another important aspect of classical mutagenesis studies, particularly when attempting to decipher gene function. Often, complementation may be achieved through cloning the gene of interest, either with or without its native promoter, onto a plasmid and introducing this construct into the mutant. However, attempts to clone clostridial genes in *Escherichia coli* are occasionally unsuccessful, as the products of these genes may be toxic to *E. coli*. The use of a controlled inducible expression system can overcome these difficulties. One such system is a tetracycline-inducible system developed for *C. difficile* which involves promoter repression to control gene expression; the addition of tetracycline relieves repression, allowing the expression of the gene of interest (15). However, if the complemented strain is then to be

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Address correspondence to Dena Lyras, dena.lyras@monash.edu.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strains		
E. coli		
CB454	F$^-$ Δ*lacZ lacY*$^+$ *galK rpsL thi recA56*; Sm^r	17
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA argF*)*U169 endA1 recA1 hsdR17* (r_K^- m_K^-) *deoR thi-1 supE44 gyrA96 relA1*	Bethesda Research Laboratories
DH12S	ϕ80d*lacZ*ΔM15 *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *araD139* Δ(*ara, leu*)*7697* Δ(*lacX74 galU galK rpsL* (Str^r) *nupG recA1*/F′ *proAB*$^+$ *lacI*^{q}*Z*ΔM15	Life Technologies
C. perfringens		
JIR325	Strain 13; Rif^r Nal^r	18
JIR12483	JIR325 *plc*::TargeTron; Em^r	This study
JIR12531	JIR12483(pDLL3); Em^r Cm^r	This study
JIR12564	JIR12531 cured of pDLL3; Em^r Cm^s	This study
JIR12594	JIR12483(pDLL3), second independent derivative; Em^r Cm^r	This study
JIR12597	JIR12594 cured of pDLL3; Em^r Cm^s	This study
JIR12600	JIR12594 *virR*::TargeTron (*plc viR* double mutant); Em^r	This study
JIR12128	JIR325 (pCW3Δ*tcpG*::*ermQ*)	19
Plasmids		
pCR-Blunt II-TOPO	Cloning vector; Kn^r	Invitrogen
pCW3	Conjugative plasmid from *C. perfringens*; Tc^r	20
pSU39	Cloning vector; Kn^r	21
pJIR750	*E. coli-C. perfringens* shuttle vector; Cm^r	22
pJIR1456	*E. coli-C. perfringens* mobilizable shuttle vector; Cm^r	23
pJIR1457	*E. coli-C. perfringens* mobilizable shuttle vector; Em^r	23
pJIR1635	pJIR1457 carrying *tnpX*; Em^r	13
pJIR1835	pCB182Ω*attCI* from Tn*4453a*; Ap^r	14
pJIR1872	pSU39Ω*tnpX*; Kn^r	12
pJIR2085	pSU39Ω$tnpX_{S15L}$; Kn^r	24
pJIR2816	*E. coli-C. perfringens* mobilizable shuttle vector; Cm^r	25
pJIR3422	pJIR750Ω*attCI*; Cm^r	This study
pJIR3562	TargeTron vector targeted to interrupt *C. perfringens plc*; *erm* RAM; Cm^r	26
pJIR3589	pCR-Blunt II-TOPOΩ*attL-catP-attR* fragment generated by SOE-PCR; Cm^r Kn^r	This study
pJIR3607	pJIR3678 retargeted to *virR* of *C. perfringens*; Cm^r	This study
pJIR3629	pJIR3422Ω*tcpG*; Cm^r	19
pJIR3678	pJIR3562 containing *erm* RAM with flanking *attL* and *attR* sites; Cm^r	This study
pJIR3683	pJIR3422Ω*feoB*; Cm^r	This study
pDLL3	TnpX subcloned from pJIR1635 into pJIR2816; Cm^r	This study

[a] Cm^r, chloramphenicol resistant; Cm^s, chloramphenicol sensitive; Ap^r, ampicillin resistant; Kn^r, kanamycin resistant; Sm^r, streptomycin resistant; Rif^r, rifampin resistant; Nal^r, nalidixic acid resistant.

used in an animal infection model, induction of the gene of interest becomes more difficult. We experienced toxicity problems when attempting to clone the gene encoding a *C. perfringens* peptidoglycan hydrolase, TcpG, in *E. coli* and a gene involved in ferrous iron uptake by *C. perfringens*, *feoB*, and developed a new technique to overcome this barrier. Repression of transposon-encoded recombinase promoters by the recombinase itself is a mechanism used by mobile elements to minimize deleterious effects on the host by limiting recombinase expression and, thus, element movement (16). In this study, we demonstrate that TnpX is able to repress expression from its own promoter, P_{attCI}. We then used this ability to repress expression from P_{attCI} to facilitate the cloning of *tcpG* and *feoB* in *E. coli*. By placing these genes under the control of P_{attCI} and repressing their expression with TnpX, we avoided the production of toxic products in *E. coli* and were able to generate the required recombinant plasmids for complementation. Subsequent transfer of the cloned genes into *C. perfringens tcpG* or *feoB* mutants resulted in the expression of the target gene for successful complementation analysis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were grown at 37°C in 2× YT medium (27) and supplemented with antibiotic selection where appropriate, using ampicillin (100 μg/ml), kanamycin (20 μg/ml), or chloramphenicol (30 μg/ml). *C. perfringens* strains were cultured at 37°C in TPG (Trypticase-peptone-glucose) broth (28), FTG (fluid thioglycolate) medium (Difco/BD, Sparks, MD, USA), or nutrient agar (NA) (29) supplemented with chloramphenicol (30 μg/ml), erythromycin (50 μg/ml), or tetracycline (10 μg/ml) when required. *C. perfringens* cultures were grown in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 37°C in anaerobic jars (Oxoid, Basingstoke, United Kingdom).

DNA isolation and molecular techniques. Plasmid DNA from *E. coli* was extracted by using QIAprep spin miniprep columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA from *C. perfringens* was prepared from FTG cultures grown overnight, as described previously (25).

Transformation of *E. coli* (30) and *C. perfringens* (31) was performed as described previously. Standard methods for the digestion, ligation, and analysis of plasmid DNA and PCR products were used (30).

TABLE 2 Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5′–3′)	Purpose	Reference
JRP3803	GCGCGCAAGCTTTGCGAAGAAGCCTTTGAATAC	Amplification of P_{attCI} for cloning into pJIR750	This study
JRP3804	GCGCGCGCATGCAGTCCTTGACATTTTTCTTAT	Amplification of P_{attCI} for cloning into pJIR750	This study
JRP3820	GCGCGGATCCCCTTAAACTATGAGGATGTT	Amplification of *tcpG* and the upstream region; includes the BamHI site	19
JRP3821	GCGCGGTACCTTATTCTACGACTCTTCTTACA	Amplification of *tcpG* and the upstream region; includes the KpnI site	19
JRP4321	GCGGTACCTTCCTTTTGAAGCACATCCACTG	Amplification of *feoB* and the upstream region; includes the KpnI site	This study
JRP4322	CGGGATCCGAATAATTTAATTAGGGGGAAAACTAATGAC	amplification of *feoB* and the upstream region; includes the BamHI site	This study
JRP4313	TTGATGCAGGACGATTTGATTG	Forward oligonucleotide for *tcpG* qRT-PCR analysis	This study
JRP4314	TTTCTCTTGGCCCTAACTGTATTCC	Reverse oligonucleotide for *tcpG* qRT-PCR analysis	This study
JRP2479	CCATCTGTTTTTATATCTGCTCCAGTA	*rpoA*, forward oligonucleotide for qRT-PCR analysis	32
JRP2480	GGAAGGTGAAGGACCAAAAACTATT	*rpoA*, reverse oligonucleotide for qRT-PCR analysis	32
JRP5715	GTCGGAGAATACAAAATCTC	Amplification of *attL* from Tn*4451* for SOE-PCR	This study
JRP5716	CTTCTTGAACACTTGCCGACCTTTCCGTATGTATTTTTTC	Amplification of *attL* and SOE-PCR to splice together the *attL* site with the 5′ end of *catP*	This study
JRP5717	GAAAAAATACATACGGAAAGGTCGGCAAGTGTTCAAGAAG	SOE-PCR to splice together the *attL* site with the 5′ end of *catP*	This study
JRP4071	GTCCGTAAAGAAATCGCCCATTAGTTACAGACAAACCTGAAG	SOE-PCR to splice together the *attR* site with the 3′ end of *catP*	This study
JRP4072	CTTCAGGTTTGTCTGTAACTAATGGGCGATTTCTTTACGGAC	SOE-PCR to splice together the *attR* site with the 3′ end of *catP*	This study
JRP5720	CACCTATTTTAAAGGTTCC	Amplification of *attR* from Tn*4451* for SOE-PCR	This study
JRP2142	CTCAGTACTGAGAGGGAACTTAGATGGTAT	*catP* probe for Southern blotting	33
JRP2143	CCGGGATCCTTAGGGTAACAAAAAACACC	*catP* probe for Southern blotting	33
JRP2980	AATAAGTAAACAGGTAACGTCT	*ermB* probe for Southern blotting	34
JRP2981	GCTCCTTGGAAGCTGTCAGTAG	*ermB* probe for Southern blotting	34
JRP3947	GGGAACGAAACGAAAGCG	TargeTron probe for Southern blotting	26
JRP3948	CGTAATAAATATCTGGAC	TargeTron probe for Southern blotting	26

PCR was performed by using *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany), unless otherwise stated, and 0.5 μM each primer. Denaturation (94°C for 30 s), annealing (50°C for 30 s), and extension (72°C for 3 to 5 min) steps were carried out for 30 to 35 cycles. DNA sequencing was carried out by using a Prism Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems/Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The oligonucleotide primers used in this study are listed in Table 2. Sequence analysis was carried out on an Applied Biosystems 3730S genetic analyzer and by using Sequencher 3.1 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Construction of the repression shuttle plasmid pJIR3422. The P_{attCI} promoter fragment was generated by PCR amplification using oligonucleotide primers JRP3803 and JRP3804 to generate a 225-bp product flanked with HindIII and SphI sites. Plasmid pJIR3422 was constructed by cloning the digested PCR product into HindIII/SphI-digested pJIR750 DNA. The plasmid was confirmed by restriction and sequence analysis.

β-Galactosidase assays. β-Galactosidase assays were conducted as previously described (14, 27). The optical density at 600 nm (OD_{600}) values of cultures grown overnight were adjusted to 0.6. An equal volume of Z buffer (500 μl) (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol [pH 7.0]) was added to the diluted cultures, followed by 2 drops from a Pasteur pipette of chloroform and 1 drop of 0.1% (wt/vol) SDS. The sample was mixed, and 100-μl aliquots were added to a microtiter plate and incubated at room temperature. After a 15-min incubation, 200 μl of a 0.7-mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (in Z buffer) solution was added to the microtiter plate. Optical density measurements were made at 410 nm and 570 nm for up to 5 h. Miller units were calculated as $\{1{,}000 \times [OD_{410} - (1.75 \times OD_{570})]\}/(\text{time} \times \text{volume} \times OD_{600})$, as previously described (14).

Determination of dependence on the presence of TnpX for stable maintenance of the TcpG expression plasmid. In separate experiments, plasmid DNA was extracted from strains DH12S(pJIR3422, pJIR2085), DH12S(pJIR3683, pJIR2085), and DH12S(pJIR3629, pJIR2085) in triplicate. Plasmid DNA from each strain was introduced into DH5α, and transformants were selected on chloramphenicol-containing medium. The resultant colonies were patched onto medium containing kanamycin and chloramphenicol, and the number of chloramphenicol- and kanamycin-resistant colonies was calculated.

RNA isolation and qRT-PCR. Total RNA was obtained from *C. perfringens* strain JIR325 derivatives grown in 20 ml TPG to an optical density (at 600 nm) of 0.9 to 1.0. Cells were pelleted and stored in RNAwiz (Ambion/Life Technologies, Carlsbad, CA, USA) overnight. RNA extraction was performed by using a Ribopure Bacteria kit (Ambion) according to the manufacturer's instructions. RNA was treated for DNA contamination by using Turbo DNase (Ambion) and was determined to be DNA free by the failure to amplify the *tet*(A)P gene present on pCW3 (data not shown). cDNA was amplified from 4 μg of RNA by using random hexamers, as described previously (32, 33, 35). Quantitative PCR using cDNA was performed as described previously (32, 33, 35). The oligonucleotides used for reverse transcription-quantitative PCR (qRT-PCR) are listed in Table 2.

Splicing by overlap extension PCR. Splicing by overlap extension PCR (SOE-PCR) (36) was used to generate *att-catP* and *att-ermB* resistance cassettes. The oligonucleotide primer sequences used for SOE-PCR are described in Table 2. Phusion polymerase (Finnzymes/New England BioLabs, Ipswich, MA, USA) was used for all SOE-PCRs, according to the manufacturer's instructions. Primary PCR mixtures were gel purified by using the QIAquick gel extraction kit (Qiagen) before use as the templates in secondary PCRs. Secondary PCRs were performed by using 0.1 pmol of each of the purified primary PCR mixtures as the template.

Oligonucleotide primers were designed to splice together the *attL* site from Tn*4451* and the 5′ end of the *catP* gene and then the 3′ end of the *catP* gene and *attR* from Tn*4451*. This product was subsequently cloned into pCR-Blunt II-TOPO (Invitrogen/Life Technologies, Carlsbad, CA, USA) to generate pJIR3589.

To introduce *attL* and *attR* sites flanking the *ermB* RAM into pJIR3678, SOE-PCR was used to introduce an MluI site between the fused *attL* and *attR* sites, which were also flanked by AscI sites. The AscI-*attL*-MluI-*attR*-AscI fragment was introduced into the vector pJIR3562 by digestion with MluI to remove the existing *ermB* RAM in this vector. The *ermB* RAM was subsequently reinserted between the *att* sites by digestion with MluI and ligation of the MluI *ermB* RAM fragment from pJIR3562 to construct pJIR3678.

Construction of *plc* TargeTron mutants and removal of the *att-ermB* cassette. Strains containing the group II intron derived from pJIR3678 were constructed as described previously (26). To remove the *att-ermB* cassette from the integrated group II intron, the *tnpX* gene, carried by plasmid pDLL3, was introduced into a *plc*-negative, erythromycin-resistant strain, designated JIR12483, via electroporation (37). The resultant strains, JIR12531 and JIR12594, were plated onto NA containing chloramphenicol to select for pDLL3 and subsequently onto NA containing erythromycin or chloramphenicol. Erythromycin-susceptible derivatives were obtained and passaged in the absence of chloramphenicol to cure the strains of pDLL3. Loss of the plasmid was determined by patching onto medium containing chloramphenicol to identify strains that were no longer resistant. Southern hybridization analysis was subsequently conducted to illustrate the genomic content of the derived strains at each stage. A *plc virR* double mutant was constructed by introducing plasmid pJIR3607 into the strains and selecting for erythromycin-resistant colonies. Further characterization involved antibiotic resistance screening to demonstrate chloramphenicol sensitivity and PCR to confirm the interruption of the *virR* gene and the loss of pJIR3607.

Southern hybridization. Randomly digoxigenin (DIG)-labeled probes were generated by PCR according to the manufacturer's instructions (Roche), using the oligonucleotide primers listed in Table 2. Genomic DNA was extracted from *C. perfringens* cultures, digested with HaeII, and separated by electrophoresis at a low voltage of 30 V overnight on a 0.8% agarose gel, alongside DIG-labeled HindIII-digested Lambda standards. DNA transfer and high-stringency washes were conducted as described previously (30). Detection of DIG-labeled probes was performed according to the manufacturer's instructions (Roche), using CDP-Star (Roche) as the chemiluminescent substrate.

RESULTS AND DISCUSSION

TnpX-mediated excision of antibiotic resistance cassettes allows for the reuse of resistance markers. TnpX-mediated excision of Tn*4451* relies on the recognition of *attL* and *attR* sites flanking the transposon (13, 14). Thus, we introduced the *attL* and *attR* sites on either side of the *catP* gene, which confers chloramphenicol resistance, by using splicing by overlap extension PCR (SOE-PCR). The resultant *att-catP* resistance cassette was cloned into pCR-Blunt II-TOPO (Invitrogen) (encoding kanamycin resistance) to produce pJIR3589. In the presence of TnpX, we would expect to see a loss of chloramphenicol resistance, as TnpX excises the *att-catP* cassette from pJIR3589. To demonstrate this property, we initially performed an excision assay with the *E. coli* host before attempting this strategy in *C. perfringens*. The TnpX-carrying vector pJIR1635 (encoding erythromycin resistance) was introduced into DH5α(pJIR3589). The resultant strain was grown overnight in the presence of kanamycin and erythromycin, to allow for the loss of the *att-catP* fragment, and plasmid DNA was then extracted. This plasmid DNA was then reintroduced into DH5α, selecting transformants on kanamycin only. Colonies were patched onto chloramphenicol and kanamycin to determine the percent loss of the *att-catP* cassette. This experiment was also performed by using pJIR1457 instead of pJIR1635; pJIR1457 encodes erythromycin resistance but does not carry the *tnpX* gene. In the absence of TnpX, there was no detectable loss of chloramphenicol resistance. However, in the presence of TnpX, the frequency of loss of chloramphenicol resistance was 10.7% ± 3.7%, demonstrating TnpX-mediated excision of the *att-catP* cassette.

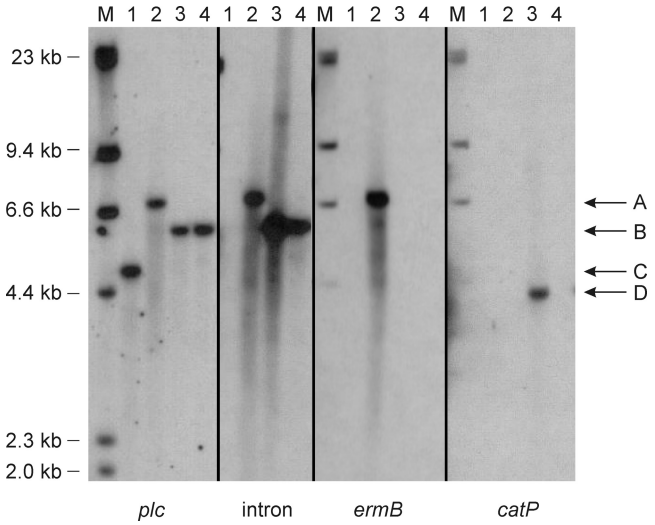

FIG 1 Southern hybridization analysis of *C. perfringens plc* mutants. Genomic DNA was prepared from *C. perfringens* strains and digested with HaeII. Digested DNA was separated by electrophoresis in a 0.8% agarose gel, transferred, and probed with the respective probes indicated below each panel. M indicates the DIG-labeled HindIII-digested lambda marker lanes, and sizes are indicated on the left. Lanes contained DNA from the following strains: JIR325 (wild type) (lane 1), JIR12483 (*plc* mutant) (lane 2), JIR12531 (*plc* mutant with the *att-erm* cassette excised by TnpX, encoded by pDLL3) (lane 3), and JIR12531 cured of pDLL3, designated JIR12564 (lane 4). The lettered arrows on the right indicate the *plc* gene containing the intron with the *ermB* gene (A), the *plc* gene containing the inserted intron without the *ermB* gene (B), the wild-type *plc* gene (C), and the pDLL3 fragment containing the *catP* gene (D).

We next decided to test this system further in *C. perfringens*, coupled with TargeTron technology. The *attL* and *attR* sites were introduced on either side of the *ermB* RAM to produce an *att-ermB* cassette. The *ermB* RAM is used to select for the integration of a TargeTron derivative into a gene of interest. Following the construction of the mutant via TargeTron insertion, the introduction of the TnpX-encoding gene onto a plasmid allows the *att-ermB* cassette to be removed. The plasmid encoding TnpX can then be lost via passage, while the group II intron (disrupting the gene of interest) is still present and stable but no longer marked with an antibiotic resistance gene. We used this method to introduce mutations into both the *plc* and *virR* genes of *C. perfringens* strain JIR325. The *plc* gene was chosen as it encodes alpha-toxin, a phospholipase C; colonies that no longer express functional alpha-toxin can be screened for a loss of phospholipase activity on egg yolk agar (EYA) (37). The *virR* gene encodes a global regulator that controls the expression of the cholesterol-dependent cytolysin perfringolysin O (PFO), and mutants of this gene no longer demonstrate hemolytic activity on horse blood agar (HBA) (26, 38).

Initially, the *plc* gene in strain JIR325 was disrupted with the TargeTron insertion containing the *att-ermB* RAM. Potential mutants were screened for erythromycin resistance and for a loss of phospholipase C activity. The integration of the TargeTron insertion into *plc* and the loss of the TargeTron vector were subsequently confirmed by Southern hybridization (strain designated JIR12483) (Fig. 1, lanes 1 and 2 in each panel). Plasmid pDLL3,

carrying the *tnpX* gene, was introduced into this *plc* mutant via electroporation. Transformants were selected on chloramphenicol (resistance encoded by *catP* on pDLL3) and erythromycin (resistance encoded on the TargeTron vector). Colonies were then selected and grown overnight before plating out for single colonies on nutrient agar (NA) with chloramphenicol. To check for the loss of the *att-ermB* cassette, single colonies were patched onto NA with and without erythromycin. The frequency of excision of *att-ermB* was 1%. One strain found to be erythromycin sensitive was confirmed by Southern hybridization (JIR12531) (Fig. 1, third panel, lane 3). This strain was further passaged to select for chloramphenicol-sensitive colonies cured of pDLL3 and confirmed by Southern hybridization (JIR12564) (Fig. 1, third panel, lane 4). An identical independent unmarked *plc* mutant was also constructed and confirmed by using the same methodology, designated JIR12597 (data not shown), and used for subsequent experiments.

The unmarked *plc* mutant strain JIR12597 was used for experiments in which a second TargeTron mutation was introduced, into the regulator gene *virR*. Putative mutants were isolated and confirmed by PCR and Southern analyses (data not shown). Plasmid pDLL3 was then introduced into the double mutant strain for TnpX-mediated excision of the *att-ermB* RAM from the inactivated *virR* gene. The frequency of excision of *att-ermB* from the double mutant was 5%, providing proof of principle that the TnpX-mediated excision strategy employed here resulted in the construction of an unmarked double mutant, designated JIR12600. Patching onto HBA confirmed the loss of hemolytic activity in the double mutant compared to single mutant strain JIR12597, as expected (data not shown).

In this study, we have used the excision properties of TnpX to facilitate the removal of antibiotic resistance cassettes, flanked by *attL* and *attR* sites, from a plasmid in *E. coli* and from chromosomal mutants constructed in *C. perfringens*. While providing evidence that TnpX-mediated excision can be used to remove antibiotic resistance markers, the initial proof of principle using *E. coli* also demonstrated that the system works outside a clostridial genetic background. This result suggests that the TnpX-mediated excision system could be applied to species other than clostridia, provided that TnpX can be introduced and expressed in that strain background. Although the frequency of excision was not high in *C. perfringens* (1 to 5%), it was sufficient to ensure that arduous amounts of screening were not required to obtain colonies in which excision of the resistance element had occurred.

TnpX represses expression from its own promoter, P_{attCI}. Attempts to clone clostridial genes in *E. coli* are occasionally unsuccessful, as the products of these genes may be toxic to *E. coli*. Recombinases may repress transcription from their own promoter, in order to limit recombinase expression and, thus, element movement, which may be detrimental to the host cell (16). Whether TnpX represses its own expression was not known. However, we hypothesized that if TnpX did repress expression from P_{attCI}, we could exploit this repression to enable the cloning of genes encoding toxic products in *E. coli*.

To determine the effect of TnpX on P_{attCI}, we used a β-galactosidase reporter assay. In a previous study, we cloned P_{attCI} into the promoter probe vector pCB182, constructing pJIR1835 (14). To introduce TnpX into this system, we used pJIR2085, which contains a $tnpX_{S15L}$ allele, the product of which is catalytically inert (24), as the use of a plasmid expressing wild-type TnpX in *E. coli* resulted in the integration of the plasmid into the *E. coli* chromosome, disrupting P_{attCI} (data not shown). The two plasmids pJIR1835 and pJIR2085 were introduced into *E. coli* CB454 (17), and β-galactosidase assays were performed as described previously (14, 27). In the absence of $TnpX_{S15L}$, activity from P_{attCI} resulted in 4,600 ± 290 Miller units of β-galactosidase activity; however, when $TnpX_{S15L}$ was introduced into this system, the levels of β-galactosidase activity decreased to 34 ± 21 Miller units (Fig. 2). These data suggest that TnpX very efficiently represses expression from its own promoter.

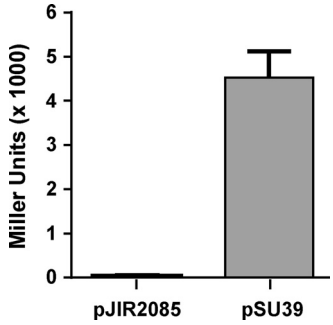

FIG 2 TnpX-mediated repression of P_{attCI}. β-Galactosidase assays were performed by using strains CB454(pJIR2085, pJIR1835), labeled pJIR2085, and CB454(pSU39, pJIR1835), labeled pSU39. Assays were performed as described previously (14), with data represented in Miller units. Data are the averages of three biological replicates. Error bars represent standard deviations.

The reporter experiments described here show that the catalytically inactive derivative $TnpX_{S15L}$ repressed expression from P_{attCI} and support our previous findings; strong binding of TnpX to the P_{attCI} region presumably blocks RNA polymerase binding to the promoter and results in reduced transcription initiated from this promoter.

$TnpX_{S15L}$-mediated repression can facilitate cloning of recalcitrant genes in *E. coli*. Having established that $TnpX_{S15L}$ represses expression from P_{attCI}, we moved on to test whether this system could be used for the cloning of recalcitrant genes in *E. coli*. We tested this approach for the cloning of *tcpG*, which encodes a peptidoglycan hydrolase from *C. perfringens* (19). For classical mutagenesis studies, a *tcpG* mutant was made, and complementation was required to confirm the mutant phenotype. Many attempts at cloning *tcpG* had been made, but all were unsuccessful, resulting in the isolation of truncated or mutated DNA fragments (data not shown), suggesting that the production of TcpG was not tolerated by *E. coli*.

The TnpX-repressible P_{attCI} fragment was cloned into the clostridial vector pJIR750 to construct shuttle plasmid pJIR3422. The *tcpG* gene, together with 25 bp of upstream DNA that included the ribosome binding site, was amplified by PCR. A ligation mixture containing the *tcpG* PCR product and pJIR3422 was introduced into *E. coli* strain DH12S, which already contained $TnpX_{S15L}$ plasmid pJIR2085. The resultant TcpG expression plasmid was designated pJIR3629 and was confirmed by restriction digestion and sequence analysis (data not shown).

To investigate the maintenance of pJIR3629 in the absence of TnpX in *E. coli*, plasmid DNA was extracted from DH12S(pJIR3629, pJIR2085) and used to transform DH5α. Transformants were selected on chloramphenicol to select for transformants carrying TcpG plasmid pJIR3629. Subsequent colonies were then patched onto medium containing either chloram-

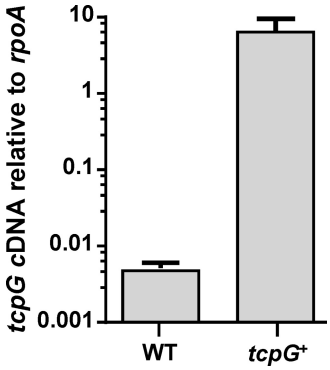

FIG 3 Transcriptional analysis of *tcpG* expression. Transcription of mRNA from the *tcpG* gene was quantified by using qRT-PCR, in comparison to *rpoA* expression levels from each corresponding strain. WT is wild-type strain JIR325(pCW3), and *tcpG*$^+$ is complemented *tcpG* mutant strain JIR325 (pCW3*tcpG*, pJIR3629). Results are the averages of three biological replicates, and the error bars represent standard deviations.

phenicol or kanamycin (resistance encoded by *tnpX*-carrying plasmid pJIR2085) to determine the proportion of transformants containing pJIR3629 alone or both pJIR3629 and pJIR2085. All of these colonies were resistant to both kanamycin and chloramphenicol, demonstrating that they all contained both pJIR3629 and $TnpX_{S15L}$ plasmid pJIR2085. No colonies that contained the TcpG expression plasmid alone were isolated. As a control, plasmid DNA from DH12S(pJIR3422, pJIR2085) was also used to transform DH5α, with selection on chloramphenicol for the vector pJIR3422 and subsequent patching onto chloramphenicol and kanamycin. All of the colonies were chloramphenicol resistant, while 67% ± 5% were resistant to both chloramphenicol and kanamycin, suggesting cotransformation of both plasmids in this proportion of transformants. These data suggest that TcpG expression plasmid pJIR3629 can be stably maintained only in the presence of $TnpX_{S15L}$-mediated repression, while the base vector pJIR3422 can be maintained in the absence of *tnpX*-carrying plasmid pJIR2085. This finding further demonstrates the toxicity of TcpG to *E. coli*, which is consistent with its function as a peptidoglycan hydrolase (19).

Plasmid pJIR3629 was then introduced into a *C. perfringens tcpG* mutant, which showed a decrease in the conjugative transfer of *C. perfringens* plasmid pCW3 compared to the wild type. The introduction of pJIR3629 into this mutant, without $TnpX_{S15L}$-mediated repression, restored the conjugative transfer levels back to that of the wild-type strain (19). To confirm the expression of *tcpG* from pJIR3629 in the complemented derivative, the production of *tcpG* mRNA was examined by using qRT-PCR. RNA was extracted from the wild-type strain [JIR325(pCW3)] and the *tcpG*-complemented strain [JIR325(pCW3Δ*tcpG*, pJIR3629)]. Levels of *tcpG* transcript were determined relative to *rpoA* transcript levels (Fig. 3). The results indicated that *tcpG* transcript levels were 6.2 ± 2.5 times those of *rpoA* in the complemented mutant strain, demonstrating high-level transcription of *tcpG* from pJIR3629 in *C. perfringens*. In the wild-type strain, where *tcpG* is carried on plasmid pCW3, *tcpG* transcript levels were 0.005 ± 0.0008 relative to those of *rpoA* (Fig. 3).

Having demonstrated the effectiveness of the $TnpX_{S15L}$ repression system in the cloning of *tcpG*, we subsequently used the system to clone another clostridial gene, *feoB*, the product of which is involved in the uptake of ferrous ions (39, 40). Again needed for complementation studies, many unsuccessful attempts were made to clone the gene, with or without its native promoter. However, the gene was successfully cloned here by using pJIR3422 in the presence of $TnpX_{S15L}$. The requirement of $TnpX_{S15L}$ for the maintenance of FeoB expression plasmid pJIR3683 in *E. coli* was assessed as described above for pJIR3629. All colonies were resistant to both kanamycin and chloramphenicol, demonstrating that all colonies contained both pJIR3683 and pJIR2085. Thus, as we observed for the TcpG expression plasmid, FeoB plasmid pJIR3683 was successfully introduced into DH5α only in the presence of $TnpX_{S15L}$ encoded on pJIR2085. In contrast, DH5α transformed with the vector control pJIR3422 demonstrated resistance to kanamycin only 76% ± 9% of the time, indicating that pJIR2085 was not cotransformed every time in the absence of selection and that pJIR3422 could be stably maintained in the absence of $TnpX_{S15L}$. The FeoB plasmid was subsequently shown to complement a *C. perfringens* strain JIR325 *feoB* mutant and to restore ferrous iron uptake (data not shown) (M. M. Awad, J. K. Cheung, D. Lyras, and J. I. Rood, unpublished results).

The exploitation of TnpX-mediated repression for successful cloning of otherwise recalcitrant genes represents an important addition to the *C. perfringens* genetic arsenal. Coupled with the ability of TnpX to specifically excise fragments such as antibiotic resistance markers, we have developed tools that will further enable the genetic modification of the clostridia. The construction of a *plc virR* double mutant in *C. perfringens* and the cloning of the *tcpG* and *feoB* genes into *E. coli* provide direct practical examples of how this technology can be used for the construction and subsequent analysis of clostridial mutants. One clear advantage of this system is that it does not require the expression of the cloned gene to be induced in the clostridial background. While other systems which facilitate the control of gene expression via the controlled alleviation of promoter repression are available (15, 41, 42), they often require the addition of specific compounds to cultures to achieve this outcome. This requirement may not be ideal and may interfere with experimental protocols, such as when conducting infection studies with animal models. The technology described here provides new options that may be suitable for these purposes. Furthermore, while we have demonstrated proof of principle that this TnpX-centered strategy is effective for clostridial genes, this system should provide a feasible method for the cloning of recalcitrant genes from other bacterial species.

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