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# Construction of an Alpha Toxin Gene Knockout Mutant of *Clostridium perfringens* Type A by Use of a Mobile Group II Intron

Yue Chen,[1]* Bruce A. McClane,[2] Derek J. Fisher,[2] Julian I. Rood,[3] and Phalguni Gupta[1]

*Department of Infectious Diseases and Microbiology, Graduate School of Public Health,[1] and Department of Molecular Genetics and Biochemistry, School of Medicine,[2] University of Pittsburgh, Pittsburgh, Pennsylvania, and Department of Microbiology, Monash University, Victoria, Australia[3]*

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**In developing *Clostridium perfringens* as a safe vaccine vector, the alpha toxin gene (*plc*) in the bacterial chromosome must be permanently inactivated. Disrupting genes in *C. perfringens* by traditional mutagenesis methods is very difficult. Therefore, we developed a new strategy using group II intron-based Target-Tron technology to inactivate the *plc* gene in *C. perfringens* ATCC 3624. Western blot analysis showed no production of alpha toxin protein in the culture supernatant of the *plc* mutant. Advantages of this technology, such as site specificity, relatively high frequency of insertion, and introduction of no antibiotic resistance genes into the chromosome, could facilitate construction of other *C. perfringens* mutants.**

*Clostridium perfringens* type A, a ubiquitous intestinal microorganism, produces toxins that are responsible for its pathogenesis. The major extracellular toxin produced by all *C. perfringens* type A isolates is alpha toxin. In addition, less than 5% of *C. perfringens* type A isolates produce the sporulation-associated *C. perfringens* enterotoxin (*cpe*). In *C. perfringens*, the alpha toxin (*plc*) gene is located on the chromosome, while the *cpe* gene can be located on either the chromosome or a large plasmid (1, 13, 14). Recently the entire cytotoxic portion of the *cpe* gene cloned onto a shuttle vector (4) has been replaced, in frame, with the simian immunodeficiency virus (SIV) p27 open reading frame sequence such that expression of this viral DNA sequence is now under the control of the *cpe* promoter (2). *C. perfringens* type A ATCC 3624 transformants carrying the plasmid that contains this *cpe*-SIVp27 fusion gene are being used to develop an oral vaccine against SIV (2). Previous studies demonstrated that a *C. perfringens cpe* knockout mutant carrying a wild-type chromosomal *plc* gene does not cause any intestinal effects in animal models (12). However, because of potential harmful effects of alpha toxin, the *plc* gene must also be inactivated in the *C. perfringens* vaccine vector before it can be considered a safe oral vaccine delivery vehicle for the development of a mucosal vaccine.

In bacterial research, reverse genetics has been used as a powerful tool to insertionally inactivate a particular gene. In this study, we describe the application of reverse genetics using a group II intron to inactivate the chromosomal *plc* gene of type A *C. perfringens*. Mobile group II introns, found in bacterial and organelle genomes, are site-specific retroelements that use a mobility mechanism termed retrohoming, by which the excised intron lariat RNA inserts directly into a DNA target site and is then reverse transcribed by the associated intron-encoded enzyme protein (IEP) (10). Since the DNA target site is recognized primarily by base pairing of intron RNA, which can be modified, and a few IEP recognition positions, these introns are able to insert efficiently into any specific DNA target (3, 8, 9, 16). As a result, the mobile group II intron has been commercially developed as a genetic tool by modifying the intron RNA and then programming that RNA to insert into any desired target DNA (InGex, LLC; now Sigma-Aldrich). Since the *Lactococcus lactis L1.LtrB* intron has a wide host range and has been shown to be efficiently mobile in genomic DNA of both gram-positive and -negative bacteria (6, 9, 11), the commercially available *L1.LtrB* intron was modified in this study to specifically disrupt the *plc* gene present in the bacterial chromosome of ATCC 3624 type A in order to create a *plc* gene knockout of *C. perfringens.*

**Construction of *plc* gene knockout mutants.** A schematic diagram for construction of the shuttle plasmid containing the *plc* targetron used to construct the *C. perfringens plc* gene knockout mutant is described in Fig. 1.

**(i) Insertion of a *C. perfringens*-specific promoter in front of the intron.** In order to inactivate the *plc* gene in *C. perfringens* chromosomal DNA, the intron DNA must be transcribed in *C. perfringens* to allow this RNA to perform its retrohoming function. The intron donor plasmid used was pACD3 (InGex, LLC; now Sigma-Aldrich), a chloramphenicol (CM)-resistant plasmid containing the *L1.LtrB* intron expressed from a T7lac promoter. Because it was unlikely that T7 promoters would function well in *C. perfringens*, we inserted the promoter region of the beta-2 toxin gene (*cpb2*) from a *C. perfringens* type A isolate into the pACD3 plasmid downstream of the T7lac promoter. For this purpose, *cbp2* was amplified by PCR from a *cpb2* promoter-containing plasmid, pDF(B2h1)v751 (5), using the primer pair B2PF/B2PR (B2PF, 5′ ACTAGTGAATTC CATTATTAACATGAT; and B2PR, 5′ AAGCTTTGGTTT CCCCCCTGAATTTTT), which have SpeI and HindIII sites

* Corresponding author. Mailing address: Department of Infectious Diseases and Microbiology, University of Pittsburgh, 430 Parran Hall, GSPH, 130 DeSoto Street, Pittsburgh, PA 15261. Phone: (412) 624-5398. Fax: (412) 624-4953. E-mail: cheny@pitt.edu.

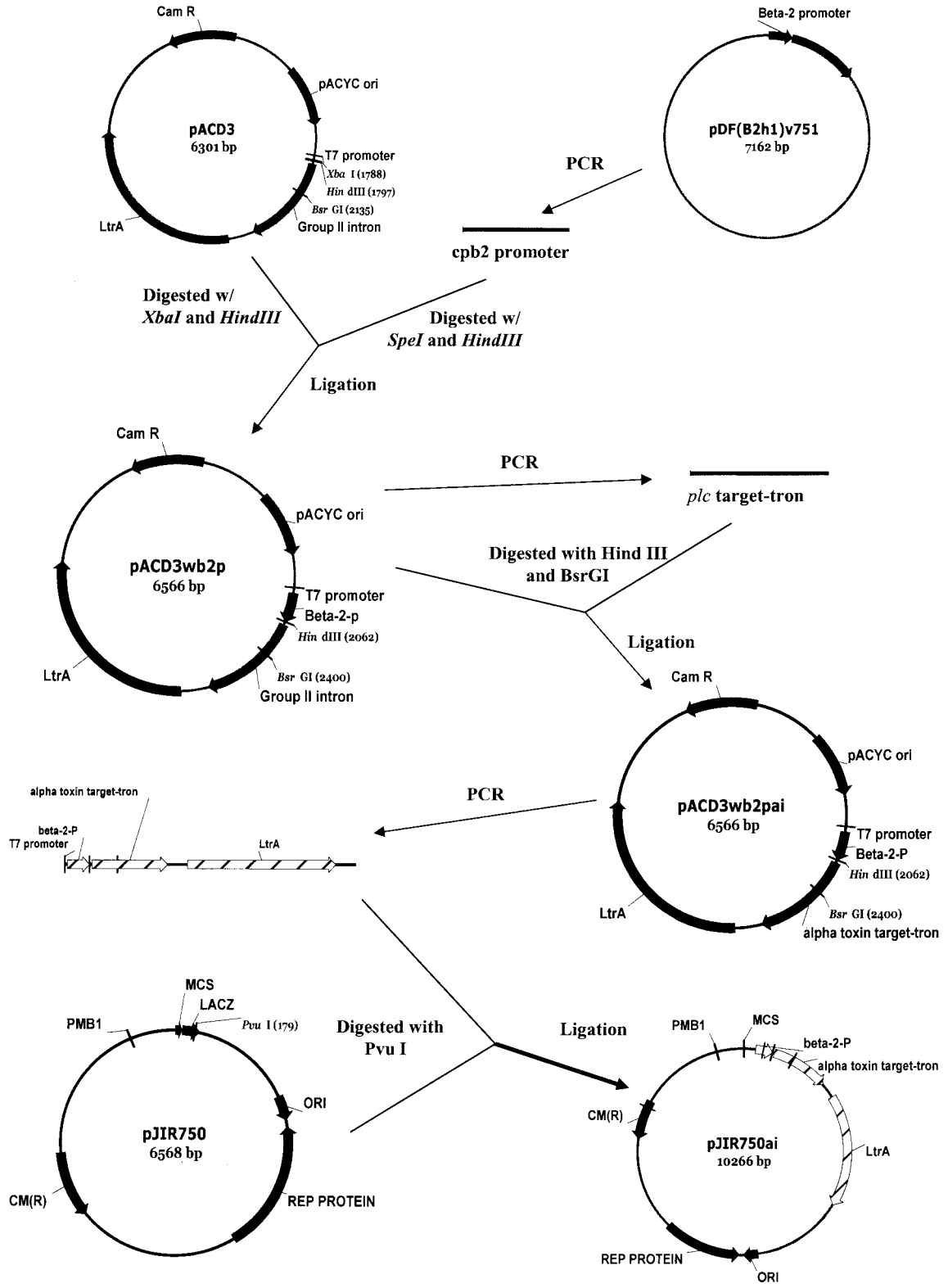


FIG. 1. Schematic diagram of construction of an *E. coli-C. perfringens* shuttle plasmid containing a *plc* targetron. See the text for a detailed description.

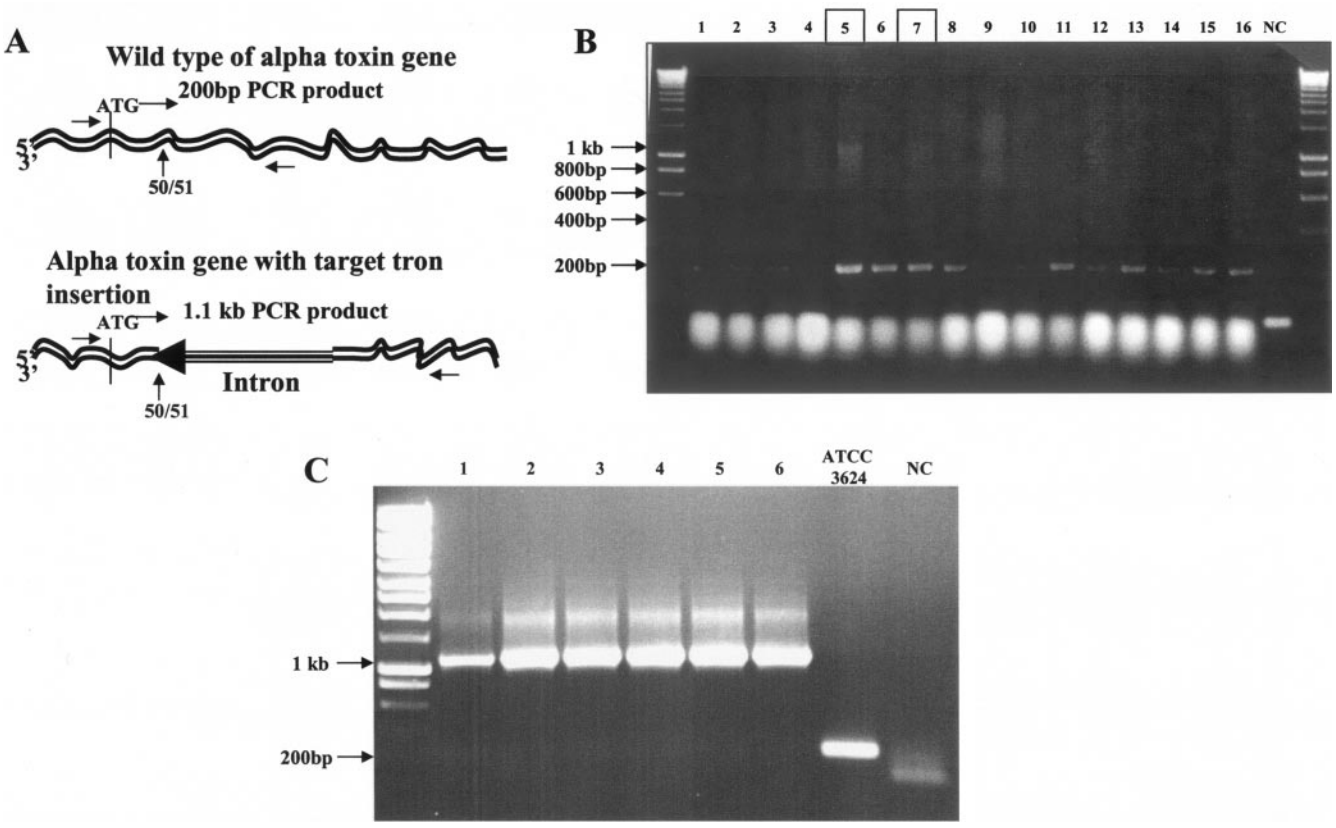


FIG. 2. Screening of *plc* gene knockout *C. perfringens* mutants. (A) Diagram of the *plc* gene with and without insertion of the targetron. →, forward primer; ←, reverse primer. (B) Screening of CM-resistant *C. perfringens* colonies for targetron insertion by PCR. The numbers at the top are bacterial colony numbers. NC, negative control. Colonies 5 and 7 (lanes 5 and 7 showing both 1.1-kb and 200-bp bands) have been used for subsequent characterization of the *plc* knockout. (C) Confirmation of *plc* gene inactivation in *plc* mutant clones by PCR. The numbers at the top are numbers of bacterial clones lacking a white halo.

5′ of the forward and reverse primers, respectively. After digestion of the PCR product with SpeI and HindIII and pACD3 with XbaI and HindIII followed by ligation, the digested *cpb2* promoter fragment was cloned into pACD3 downstream of the T7lac promoter but upstream of the *Ll.LtrB* intron to create pACD3wb2p.

**(ii) Modification of an *Ll.LtrB* group II intron for targeting to the *plc* gene.** To target the intron to *plc*, the *L1.LtrB* intron sequence in pACD3wb2p was modified based on the sequences of predicted insertion sites in the *plc* gene using the InGex Intron Prediction Program to accompany Target-Tron products (www.Sigma-Aldrich.com/Targetronaccess). The program predicted 10 intron insertion sites across the 1,197-bp *plc* gene. For optimal gene interruption and stable insertion, the insertion site in the antisense strand at position 50/51 from the initial ATG was chosen for intron modification. There are three short sequence elements involved in the base pairing interaction between the DNA target site (IBS1, IBS2, and δ′) and intron RNA (EBS1, EBS2, and δ). Modifications of intron RNA sequences (EBS1, EBS2, and δ) to base pair with the *plc* target site sequences were introduced via PCR. The first two rounds of PCR used IBS1/IBS2 (AAAAAAGCTTATAATTATCCTTACCAGCCCATAGGGTGCGCCCAGATAGGGTG) and LtrBAsEBS2 (CGAAATTAGAAACTTGCGTTCAGTAAAC) primer pairs to generate a 257-bp amplicon and EBS1/delta (CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATAGGCTTAACTTACCTTTCTTTGT) and EBS2 (TGAACGCAAGTTTCTAATTTCGGTTGCTGGTCGATAGAGGAAAGTGTCT) to amplify a 117-bp amplicon, respectively. The 257-bp and 117-bp amplicons, having 21 overlapping nucleotides, were then used as templates for the second round of PCR with IBS1/IBS2 and EBS1/delta primers to generate a 353-bp *plc* targetron. The amplified 353-bp fragment was then digested with HindIII and BsrGI and ligated into the pACD3wb2p vector digested with the same two restriction enzymes. The *plc* targetron together with the *cpb2* promoter was recloned into the *Escherichia coli-C. perfringens* shuttle vector pJIR750 (ATCC 87015) by PCR amplification from pACD3w62pai with a PvuI site at the 5′ end of each primer. After digestion of the PCR product with PvuI, the 3.7-kb fragment, containing the *cpb2* promoter, *plc* targetron, and downstream *LtrA*, was ligated into PvuI-digested pJIR750 to construct a recombinant plasmid carrying the *plc* targetron driven by a *cpb2* promoter (pJIR750ai).

**(iii) Creation of a *plc* gene knockout with the *plc* targetron.** pJIR750ai was electroporated into *C. perfringens* ATCC 3624 (which is *plc* positive and *cpe* negative). CM (15 μg/ml CM on a brain heart infusion [BHI] agar plate)-resistant colonies were screened for insertion of the targetron by PCR using two *plc* primers, F (5′GTGAGGTTATGTTAATTATATGGTATA

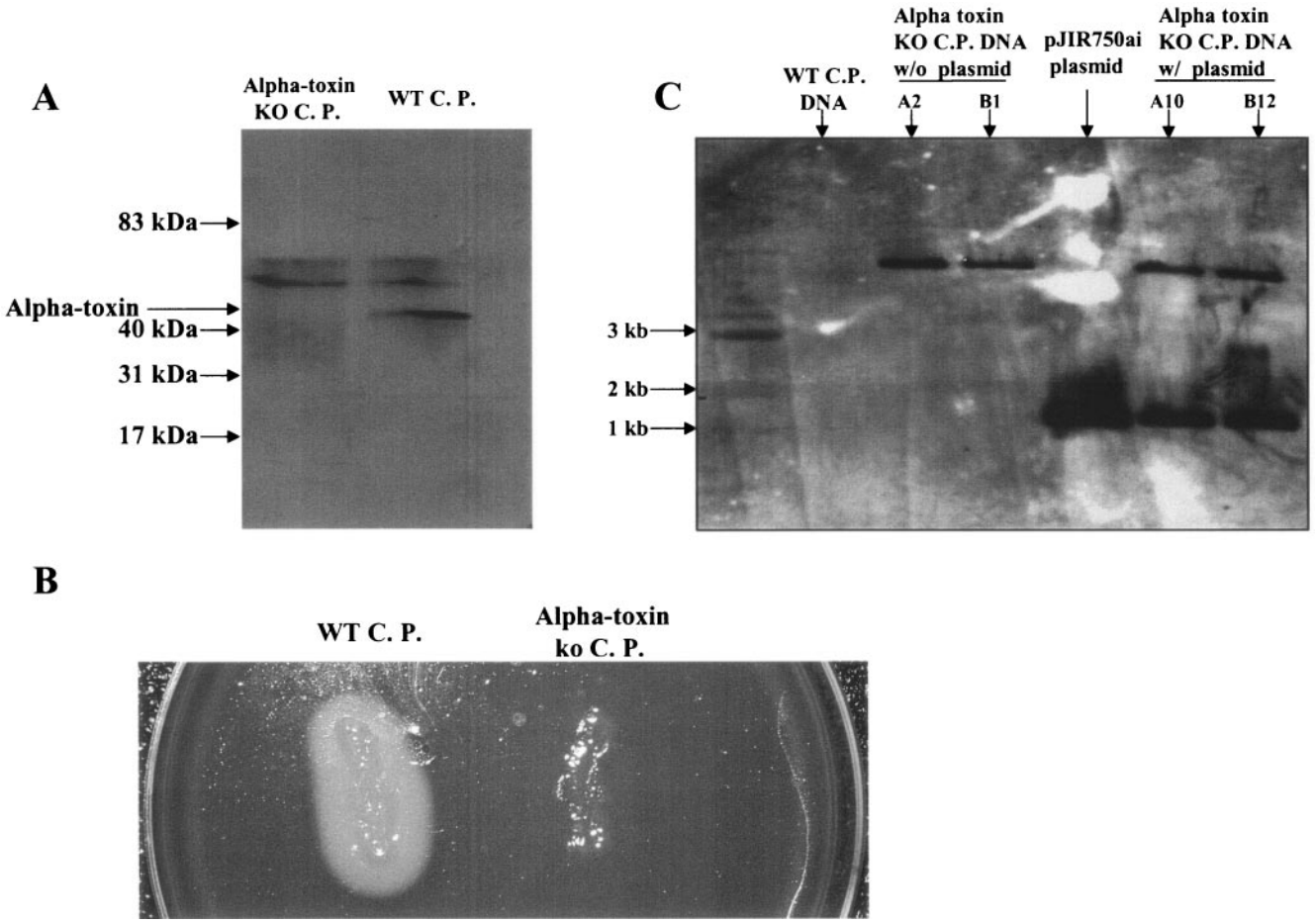


FIG. 3. Characterization of *plc* knockout *C. perfringens* (KO C.P.). (A) Western blot analysis of alpha toxin production in bacterial culture of wild-type (WT C.P.) or *plc* mutant *C. perfringens*. (B) Growth of wild-type and *plc* mutants on egg yolk BHI agar plates after 24 h of incubation in anaerobic conditions. (C) Southern blot analysis of chromosomal DNA of *C. perfringens* with and without (w/o) the targetron insertion and the plasmid containing the targetron.

ATTTCAATGC) and 100R (5′AGTTACAATCATAGCATG AGTTCCTGTTCC), which hybridize to flanking sequences of the insertion site. When the *plc* targetron in the plasmid is transcribed and excised out and the intron-encoded protein (IEP) is produced in the transformants, the IEP and excised intron lariate RNA should carry out the targetron insertion into the *plc* gene. When a single transformed bacterium replicates to form a colony, the targetron insertion occurs in some of the progeny bacteria but not all of them. As a result, a single colony coming from one transformed cell contains some bacteria with the targetron-inserted *plc* genes and some bacteria with wild-type *plc* genes. Analysis of 38 CM-resistant *C. perfringens* colonies for *plc* insertion showed that at least two colonies contained both a wild-type (200-bp PCR product) and intron-inserted (1,100-bp PCR product) *plc* gene (Fig. 2A and B). Since alpha toxin activity can be monitored by observing the white halo around colonies on BHI agar containing egg yolk (4% [vol/vol] egg yolk) (1), the bacteria from these two colonies were plated on egg yolk BHI agar plates in the presence of CM. Ten percent of the colonies obtained showed no white halo around them.

**Confirmation of *plc* gene inactivation.** PCR analysis using the F and 100R primers confirmed the presence of the targetron in the middle of the *plc* gene in *C. perfringens* colonies lacking a white halo (Fig. 2C). Sequence analysis of the 1,100-bp PCR products from these colonies indicates that the intron had inserted in the antisense strand at the 50/51 site of *plc*.

To further confirm the absence of alpha toxin production in *C. perfringens* carrying the intron insertion, Western blot analysis was performed. For this purpose, a 10-ml *C. perfringens* BHI culture was sonicated, and the resulting supernatant was lyophilized for concentration. The dried powder was then reconstituted in 500 μl of water and subjected to alpha toxin Western blot analysis. As shown in Fig. 3A, no production of alpha toxin was detected from the putative *plc* mutants of ATCC 3624. As expected, these *plc* mutants also showed no white halo around their colonies when grown on egg yolk BHI agar plates, further confirming their inability to produce alpha toxin (Fig. 3B).

Southern blot analysis of EcoRI (which does not cut in the intron sequence)-digested DNA from the *C. perfringens* 3624 mutant carrying the targetron insertion indicated a single intron insertion (Fig. 3C).

**Characterization of the *plc* mutants.** We next tested the stability of the targetron insertion in the ATCC 3624 *plc* knockout mutant. Although intron insertional mutagenesis requires an intron donor plasmid to initiate intron integration, the donor plasmid is not required for maintaining the integration. Therefore, it was crucial for the mutants to lose the donor plasmid and restore their sensitivity to selective antibiotics before testing the stability of the intron insertion. To this end, the *plc* mutants were serially subcultured daily for 10 days in FTG medium lacking CM. Those cultures were then plated onto BHI agar. Colony PCR with donor plasmid-specific primers 4931F (TCAATAAGGTCAAAAATCTTAAAGGCA AAGAAAA) and pJIR750R (CAGATGCGTAAGGAGAAA ATACCGC) demonstrated that 82% of colonies tested had lost their donor plasmid and had become sensitive to CM after 10 days of culture in FTG without this antibiotic. To assess whether this 10-day culture process might lead to spontaneous loss of virulence genes, *C. perfringens* F4969 (a type A isolate carrying both a chromosomal *plc* gene and a plasmid *cpe* gene) was similarly grown in FTG for 10 days with daily subculture. A multiplex PCR assay (7) that amplifies both *plc* and *cpe* genes was performed on the F4969 culture prior to and after the 10-day culture period. Colony PCR results (data not shown) confirmed that 100% of these bacteria maintained their *plc* and *cpe* genes throughout the 10 days of daily subculture without selection.

After 15 days of further daily subculture of the *plc* mutants lacking the donor plasmid, two analyses were performed: (i) PCR with primers flanking the insertion sites and (ii) detection of alpha toxin activity using egg yolk BHI agar plates. Both approaches confirmed that the chromosomal insertion of the targetron into *plc* is very stable. After an additional 30 rounds of vegetative culture and more than 10 rounds of sporulation-inducing culture, the mutants maintained their intron-inserted *plc* gene without any indication of reversion to the wild-type *plc* gene.

Since endospore formation and transformability are very important for construction of a *C. perfringens* vaccine vector using *cpe* promoter-driven expression, the *plc* mutant of ATCC 3624 was then electroporated with cp27, a shuttle plasmid containing the SIV p27 gene driven by the *cpe* promoter (2). A comparable number of transformants was achieved with the *plc* mutant and wild-type ATCC 3624. Furthermore, upon induction of sporulation by growth in modified Duncan-Strong media, transformants of the *plc* knockout mutant still showed a high percentage of sporulation. Consistent with that observation, Western blot analysis demonstrated similar levels of SIV p27 expression in sporulating cultures of the *plc* mutant versus wild-type ATCC 3624 (Fig. 4). PCR analysis of the insertion sites and monitoring for lack of alpha toxin activity on egg yolk BHI agar plates confirmed that the targetron was retained when the *plc* mutant transformants expressing SIV p27 were cultured for five vegetative sporulation cycles.

**Discussion.** Alpha toxin, a phospholipase C, is the most important toxin involved in gas gangrene resulting from *C. perfringens* invasion into traumatic wounds or deoxygenated tissues (1, 15). Since *C. perfringens* is now being used as an antigen delivery vehicle for an oral vaccine, it is necessary to inactivate the *plc* gene in this vector for safety reasons. In this report we modified Target-Tron technology for application in *C. perfringens*. This involved constructing a shuttle plasmid containing a modified *L. lactis Ll.LtrB* intron and then selecting insertional mutants after introducing the plasmid into *C. perfringens.*

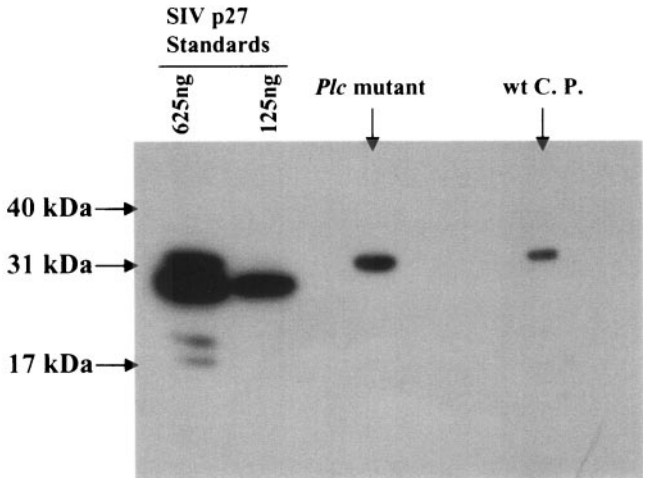


FIG. 4. Western blot analysis of SIV p27 expression in transformed *plc C. perfringens* mutants. wt C.P., wild-type *C. perfringens.*

The retrohoming reaction of the *L. lactis Ll.LtrB* intron is carried out by a ribonucleoprotein complex of an intron-encoded protein (IEP) and excised intron lariate RNA. The IEP first recognizes a small number of "fixed" positions in the flanking regions of the DNA target site and facilitates local DNA unwinding, enabling the intron RNA to base pair to a 14- to 16-nucleotide target region. The intron RNA then inserts directly into one strand of the DNA by reverse splicing, while the IEP cleaves the opposite strand and uses the cleaved 3′ end as a primer for reverse transcription of the inserted intron RNA. Host DNA recombination or repair enzymes integrate the resulting intron cDNA. Since the preferred positions recognized by the IEP are few (T −23, G −21, A −20, and T +5 from the intron insertion site) and flexible, multiple intron target sites can be easily identified in any gene by an intron prediction program (InGex, LLC). By modifying the intron to base pair with the target sequence, the modified intron could insert into any target gene.

Group II intron retrohoming mobility presents unique opportunities for site-specific chromosomal insertion. The modified group II introns from the *L. lactis Ll.LtrB* intron have been used for efficient targeted gene disruption in both gram-negative and gram-positive bacteria, such as *Lactococcus lactis* and *E. coli* (16), as well as in human cells (8). However, there have been no previous reports of application of group II intron mobility to disrupt the genes in a *Clostridium* species, which are gram-positive, endospores-forming rods. In the entire 1,197-bp *plc* gene, 10 recognition sites (5 sites in the sense strand and 5 sites in antisense strand) for the intron IEP were predicted by computer analysis. The E values (expected number of false positives) of the 10 target sites range from 0.005 to 0.561. To completely block alpha toxin synthesis, the site (50/51; E value, 0.064) closest to the *plc* initiation ATG codon in the antisense strand was chosen for the insertion. A shuttle plasmid was constructed that contains a *cpb2* promoter in front of the *plc*

targetron in order to assure transcription of the intron and expression of IEP in *C. perfringens* ATCC 3624. This construct worked well, since a relatively high frequency (0.5%) of intron insertion into the *plc* gene was detected. Southern blot analysis confirmed there was only a single targetron insertion in each *C. perfringens* chromosome. This specificity is important as it assures that important housekeeping genes of *C. perfringens* are not disrupted. The IEP open reading frame was deleted from within the intron carried by the parental targetron (9) and was expressed from a downstream position to promote intron splicing and mobility. After insertion into the *plc* gene, the targetron would not be able to splice out due to the targetron insertion in the antisense orientation and the absence of the IEP. Furthermore, alpha toxin expression was disrupted by the presence of multiple stop codons. As a result, a stable insertional inactivation of the *plc* gene was observed even in the absence of selection.

The relatively high frequency (0.5%) of stable gene inactivation observed in this study using intron-mediated technology represents a potentially major technical advance for conducting reverse genetics in *C. perfringens* for the following reasons. (i) Inactivation of the *C. perfringens* gene was achieved without antibiotic selection. In contrast, conventional homologous recombination methods for inactivating bacterial genes involves introduction of antibiotic resistance genes onto the chromosome or plasmids during gene inactivation. (ii) It is possible to construct multiple gene disruptions in the same bacterial cell using the same shuttle plasmid carrying different targetrons. This is an important advance for constructing *C. perfringens* knockouts, since only a few natural antibiotic resistance genes have been identified, i.e., it has not been possible to construct *C. perfringens* mutants carrying more than two inactivated genes (14). (iii) The relatively high frequency of intron insertion into targeted genes, as observed in this study for the *plc* gene, should considerably shorten the time needed for constructing *C. perfringens* knockout mutants. (iv) Intron insertion is stable. This stability is particularly important for our goal of constructing *C. perfringens* vaccine vectors, where reversion to toxin expression is not acceptable.

One important characteristic of *C. perfringens* ATCC 3624 for vaccine development is that it is electroporatable and expresses high levels of foreign proteins from the *cpe* promoter cloned onto a shuttle plasmid (2). After the *plc* gene was inactivated by the targetron, a *C. perfringens plc* mutant derived from the ATCC 3624 strain maintained the same characteristics and expressed a level of SIV p27 protein as high as that of the wild-type bacteria when the cp27 plasmid was introduced into the bacteria by electroporation.

In conclusion, we report disruption of the *plc* gene in *C. perfringens* using a new, improved method of group II intron technology. Advantages of this technology, such as site specificity, relatively high frequency of insertion, and introduction of no antibiotic resistance genes onto the chromosome, could facilitate construction of other *C. perfringens* mutants and/or persistent expression of foreign antigens by a *C. perfringens* vaccine vector. Although not tested, it is also possible that the modified intron prepared in this study may also work in other pathogenic *Clostridium* species, where genetic manipulation techniques are generally less well established than they are for *C. perfringens.*

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