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Construction of an *Escherichia coli-Clostridium perfringens* Shuttle Vector and Plasmid Transformation of *Clostridium perfringens*

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A stable shuttle vector which replicates in *Escherichia coli* and *Clostridium perfringens* was constructed by ligating a 3.6-kilobase (kb) fragment of plasmid pBR322 with *C. perfringens* plasmid pHB101 (3.1 kb). The marker for this shuttle plasmid originated from the 1.3-kb chloramphenicol resistance gene of plasmid pHR106. The resulting shuttle vector, designated pAK201, is 8 kb in size and codes for resistance to 20 μg of chloramphenicol per ml in both *E. coli* and *C. perfringens*. Following shuttle vector construction in *E. coli*, plasmid pAK201 was transformed into *E. coli* HB101 and *C. perfringens* ATCC 3624A, using intact cell electroporation. The transformation frequencies were 10^6 and 10^4 transformants per μg of DNA in *E. coli* and *C. perfringens*, respectively. Restriction enzyme analysis of the chimera isolated from transformants of both microorganisms suggested that the plasmids were identical. Reciprocal transformation experiments in *E. coli* and *C. perfringens* indicated no difference in transformation frequency. Plasmid pAK201 was stable in *C. perfringens* following repeated transfer in the absence of chloramphenicol pressure. The restriction map of plasmid pAK201 shows six unique cut sites which should be useful for future genetic analysis and *C. perfringens* gene library construction.

Clostridium perfringens is a ubiquitous pathogenic anaerobic bacterium responsible for causing gastroenteritis, clostridial myonecrosis, uterine infection, and bacteremia. In light of the extensive characterization of *C. perfringens* plasmids (17, 18) together with the construction of various *C. perfringens* vector systems (1, 2, 16, 19), *C. perfringens* can be studied as a representative model for clostridial genetics. Several transformation methods for clostridial species have been developed in recent years. Reid et al. (15) transformed bacteriophage CA-1 into lysozyme-derived protoplasts of *C. acetobutylicum*. Lin and Blaschek (11) transformed plasmid pUB110, a *Staphylococcus*-derived plasmid, into heat-treated protoplasts of *C. acetobutylicum* SA-1. Squires et al. (19) constructed shuttle vectors and demonstrated their transferability into *Escherichia coli* and L-phase variants of *C. perfringens*. *C. perfringens* L-phase variant transformation was described by Heefner et al. (9) and, more recently, Mahony et al. (12). Roberts et al. (16) recently described an *E. coli-C. perfringens* shuttle vector (pHR106) which was transformed into L-phase variants of *C. perfringens*. Attempts at regenerating the cell wall of L-phase variants of *C. perfringens* have not been successful (9, 12, 16, 19). However, the cell wall of lysozyme-induced protoplasts of *C. perfringens* can be regenerated at low frequency (20).

Electroporation was introduced by Potter et al. (14). It was initially designed for mammalian cell (14) and plant protoplast transfection (22) experiments. During this process, the cell is exposed to an electrical field which allows for the uptake of nucleic acids (14, 22). The application of electroporation to bacteria has been limited by the requirement for a relatively high electrical field strength and a highly resistant electroporation menstruum. Recently, electroporation was applied to the gram-positive microorganism *Lactobacillus casei*, which does not have an efficient transformation system (6). Lelie et al. (10) suggested that a pretreatment to remove the cell wall of the gram-positive microorganism *Streptococcus cremoris* was required to increase transformation frequency.

The objective of this work was to construct a stable shuttle vector containing a *C. perfringens* origin of replication from the small, nonconjugative pHB101 plasmid (4), a plasmid which is stably maintained in *C. perfringens* in the absence of selection pressure. The efficiency of electroporation-induced intact cell transformation of *E. coli* and *C. perfringens* strains with the developed shuttle vector was examined.

MATERIALS AND METHODS

Bacterial strains and plasmid DNA. The bacterial strains and plasmids used in this study are shown in Table 1. *C. perfringens* 3626B contains the caseinase activity-encoding plasmid pHB101, as well as cryptic plasmids in the range of 14 to 100 kilobases (kb) (3). A chloramphenicol-sensitive, plasmid-free strain of *C. perfringens* (ATCC 3624A) was used as the recipient in transformation studies.

E. coli K-12 strain HB101 was also used in this study. Plasmid pBR322 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was transformed into *E. coli* HB101 by electroporation (see below). Plasmid pHR106 containing the clostridial chloramphenicol resistance gene originating from plasmid pIP401 (1, 2, 5) was a kind gift of Phillip Hylemon of the Medical College of Virginia.

Growth conditions and culture media. *C. perfringens* 3626B and 3624A were inoculated at the 10% level into Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-glucose-yeast extract (TGY) medium (Trypticase, 30 g; glucose, 20 g; yeast extract, 10 g; L-cysteine, 1 g; per liter of distilled water). For solid-agar plate growth, Bacto-Agar (1.3%, wt/vol; Difco Laboratories, Detroit, Mich.) was added to TGY medium. Growth of *C. perfringens* in broth was carried out at 37°C. Stock cultures were prepared by overnight incubation in cooked-meat medium (Difco) and stored at 4°C. For selective purposes, TGY medium was supplemented with 20 μg of chloramphenicol per ml. Following the electroporation process, the cell cultures were plated on selective

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TABLE 1. Bacterial strains and plasmids used in this study

Microorganism	Plasmid	Phenotype	Reference
C. perfringens			
3626B	pHB101	Caseinase	4
3624A	None		18
E. coli			
HB 101	pBR322	Amp^r-Tet^r	21; this study
HB 101	None		8
HB 101	pHR106	Chl^r	This study

plates and subsequently transferred to an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2; Coy Laboratory Products Inc., Ann Arbor, Mich.). *E. coli* HB101 was grown in Luria-Bertani (LB) broth (13) or LB agar. Broth cultures were agitated during growth at 37°C. Stock cultures were prepared by using the dry-ice freezing method (13) in which cell suspensions containing 15% (vol/vol) added glycerol were stored at −20°C. Stock cultures were maintained under appropriate antibiotic pressure. For selective purposes, the LB agar plate was supplemented with 50 μg of ampicillin and/or 2 μg of tetracycline per ml (LB-Amp/Tet, LB-Amp, LB-Tet) or 20 μg of chloramphenicol per ml (LB-Chl).

Plasmid isolation. Plasmid DNA from *C. perfringens* and *E. coli* was isolated by using a modified alkaline lysis method (13). Cell cultures (200 ml) at stationary phase (optical density at 600 nm, 1.0) were centrifuged for 10 min at 5,000 × *g*. The cell pellet was washed with 50 ml of glucose-TE buffer (50 mM glucose, 25 mM Tris hydrochloride, 10 mM EDTA, pH 8). The washed cell pellet was suspended in 20 ml of glucose-TE buffer containing lysozyme (2 mg/ml) and incubated for 30 min at room temperature. Alkaline lysis buffer (30 ml of 1% [wt/vol] sodium dodecyl sulfate in 0.2 N NaOH solution) was added to the cell suspension, and the mixture was incubated for 10 min on ice. Mixing was done by gentle inversion until a viscosity change occurred. The lysed cell suspension was added to 40 ml of 5 M potassium acetate buffer (pH 4.8), and the mixture was incubated for 2 h on ice to precipitate chromosomal DNA and proteins. Plasmid DNA was recovered following 15 min of centrifugation at 5,000 × *g* and extracted once with Tris buffer-saturated phenol (pH 8) followed by two chloroform (chloroform-isoamyl alcohol, 24:1) extractions. Plasmid DNA was precipitated by adding 2 volumes of cold ethanol and incubating overnight at −20°C. After washing with 70% ethanol and drying in a vacuum oven at room temperature, the DNA pellet was suspended in TE buffer (pH 8). Plasmid DNA was purified by cesium chloride-ethidium bromide dye-buoyant density gradient centrifugation at 55,000 rpm for 19 h followed by a 1-h, 40,000-rpm relaxation spin. The purified plasmid was suspended in TE buffer to give the desired concentration.

Miniprep plasmid isolation was carried out by scaling down the large-scale plasmid isolation method and eliminating cesium chloride-ethidium bromide purification steps. This protocol was used for screening transformants and for preparing DNA for restriction enzyme digestion analysis and electroporation-induced transformation. Cells (1 ml) of both *E. coli* and *C. perfringens* transformants, grown overnight in 1.5-ml Eppendorf tubes, were centrifuged for 5 min at room temperature, using an Eppendorf centrifuge, and subsequently dried at room temperature by inverting the tubes for 5 min. The dried cell pellet was suspended in glucose-TE buffer containing lysozyme. The incubation times for potassium acetate-induced protein precipitation and ethanol precipitation of plasmid DNA were decreased to 30 min and 2 h, respectively. The DNA pellet was suspended in TE buffer containing 50 μg of RNase per ml.

DNA manipulation. Restriction enzyme digestion was carried out as described by Maniatis et al. (13). All restriction enzymes were purchased from Bethesda Research Laboratories or Sigma Chemical Co., St. Louis, Mo. Restriction enzyme mapping of plasmids was performed by using either single- or double-enzyme digestions. DNA fragments were analyzed by 2% (wt/vol) agarose gel electrophoresis for small-sized fragments or 0.7% (wt/vol) agarose gel electrophoresis for large-sized fragments, using Tris-acetate running buffer (pH 8) at 100-V constant voltage for 3 h. The gel was stained in an ethidium bromide (Sigma) staining bath (0.5 μg/ml) for 15 min and destained in distilled water for 15 min. The gel was subsequently visualized with a UV transilluminator (Ultra-Violet Products Inc.) and photographed with type 57 Polaroid high-speed film. The size of each fragment was determined by reference to a 123-base-pair DNA ladder (Bethesda Research Laboratories). Plasmid pHB101 and the chloramphenicol resistance (*cat*) gene from plasmid pHR106 were isolated from low-melting-temperature agarose (NuSieve Agarose; FMC Corp., Marine Colloids Div., Rockland, Maine) by phenol-chloroform extraction of heated (65°C) DNA-containing agarose gel bands. Insertion of the *cat* gene into plasmid pAK101 was performed by using an in-gel method (7) with slight modifications. The ligation mixture consisted of 8 μl (ca. 1 μg of DNA) of digested vector, 10 μl of melted gel containing the *cat* gene (ca. 250 ng of DNA), 2 μl of reaction buffer (5×), and 1 μl (1 U) of T4 DNA ligase (Bethesda Research Laboratories). The ligation mixture (4:1 ratio, vector/insert) was incubated overnight at room temperature.

Plasmid transformation. Two different methods were developed for electroporation-induced transformation of intact cells of *E. coli* and *C. perfringens*.

(i) *E. coli* protocol. A mid-log-phase *E. coli* cell culture (200 ml) was harvested by centrifugation at 5,000 × *g* for 10 min. The cell pellet was washed with electroporation buffer A (270 mM sucrose, 1 mM $MgCl_2$, 7 mM $NaHPO_4$, pH 7.4, containing 15% [vol/vol] glycerol). The washed cell pellet was suspended in 1/20 of a volume of electroporation buffer A to give ca. 10^9 cells per ml. A volume (0.8 ml) of cell suspension was mixed with 10 to 20 μl of DNA (0.1 to 1 μg) in an electroporation cuvette (Bio-Rad Laboratories, Richmond, Calif.), and the mixture was incubated for 30 min on ice to allow for DNA-cell saturation. The chilled cell mixture was shocked, using a Gene Pulser (Bio-Rad) set at 2,500 V and 25 μF, and incubated for 30 min on ice. The cell culture was diluted and plated onto antibiotic-containing selective plates (LB-Amp/Tet, LB-Amp, LB-Tet, and LB-Chl), which were incubated overnight at 37°C.

(ii) *C. perfringens* protocol. A 6-h-old, late-stationary-phase *C. perfringens* cell culture was harvested by centrifugation as described above. The cell pellet was washed with electroporation buffer B (15% [vol/vol] glycerol and distilled water). The washed cell pellet was suspended in 1/20 of a volume of electroporation buffer B to give ca. 10^{12} to 10^{14} cells per ml. Cell suspension (0.8 ml) was mixed with 10 to 20 μl of DNA (ca. 1 μg) in the electroporation cuvette and incubated for 10 min on ice. The chilled cell mixture was shocked as described previously. Following postelectroporative incubation (10 min on ice), the cells were diluted into 9 volumes of TGY medium (1:10 dilution) and incubated for 1 h at 37°C. The culture was centrifuged and suspended in

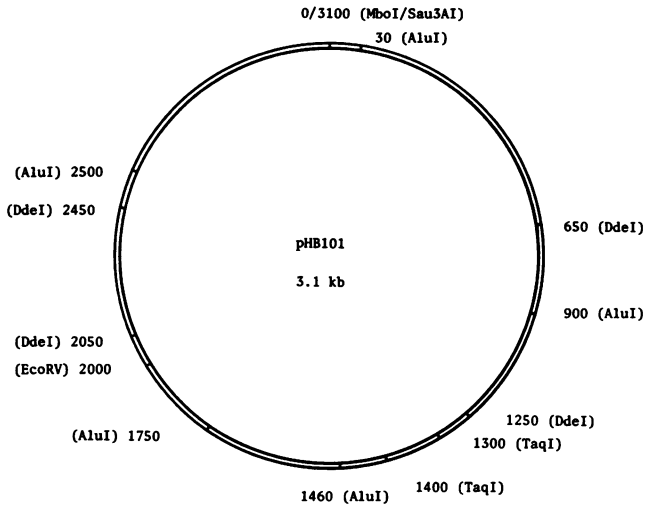

FIG. 1. Plasmid pHB101 restriction map. Single-cut enzymes: *Mbo*I (*Sau*3AI) and *Eco*RV; two-cut enzyme: *Taq*I; four-cut enzyme: *Dde*I; and five-cut enzyme: *Alu*I; no cut enzymes: *Bam*HI, *Eco*RI, *Hae*III, *Hin*dIII, *Pvu*II, *Ava*I, and *Pst*I.

0.8 ml (cuvette volume) of TGY medium. The cell suspension was plated onto TGY-chloramphenicol (20 μg/ml) selective plates and incubated overnight to determine the transformation frequency.

Determination of plasmid stability. The stability of plasmids pAK201 and pHR106 was examined. Colonies of *C. perfringens* transformants containing plasmid pAK201 or pHR106 were isolated and inoculated into fresh TGY containing chloramphenicol. Cultures were inoculated twice daily into fresh TGY media without chloramphenicol pressure over a 3-day period and plated onto both nonselective and selective TGY agar plates. The colonies on selective and nonselective plates in a given 24-h period were counted to determine plasmid stability (percent: [Chl^r colonies on selective plates/total colonies on nonselective plates] × 100). Randomly picked colonies from selective plates were examined for the presence of plasmid DNA.

RESULTS

Plasmid pHB101 restriction mapping. A pHB101 plasmid restriction map was constructed (Fig. 1) by using restriction enzymes that cleave plasmid pBR322 (i.e., *Eco*RV, *Bam*HI, *Taq*I, *Hae*III, *Hin*dIII, *Pst*I, *Mbo*I [*Sau*3AI], *Dde*I, *Pvu*II, *Ava*I, and *Alu*I). Although cutting plasmid pHB101 at the *Eco*RV site did not disrupt the clostridial origin of replication, caseinase-encoded activity (4) was apparently inactivated, since transformants of *C. perfringens* 3624A containing *Eco*RV-linearized pHB101 within plasmid pAK201 did not demonstrate caseinase-encoded activity. It was found in our laboratory that transconjugants of *C. perfringens* 3624A containing intact plasmid pHB101 have been shown to produce caseinase enzyme activity (B. C. Hampson, personal communication).

Shuttle vector construction in *E. coli* and *C. perfringens*. The construction of shuttle vector pAK201 is summarized in Fig. 2. Plasmids pHB101 and pBR322 were digested with restriction enzyme *Eco*RV to generate blunt ends. The linearized plasmids were ligated with T4 DNA ligase following overnight incubation at room temperature. The resultant ligated plasmid, designated pAK101, was transformed into *E. coli* HB101 and isolated from *E. coli* transformants by

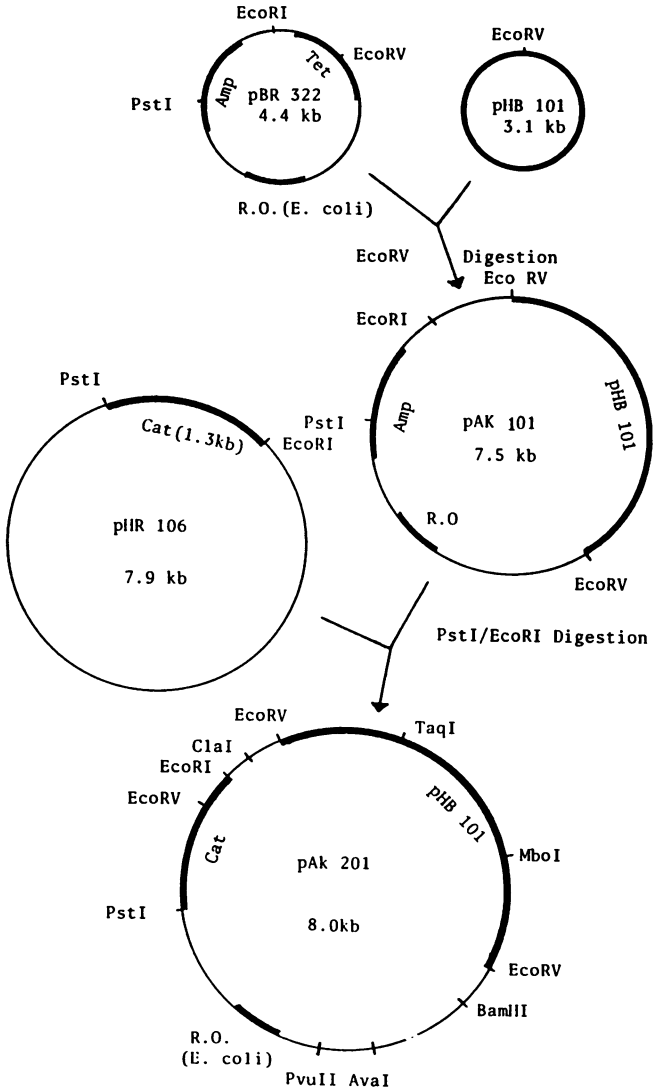

FIG. 2. Protocol for construction of shuttle vector pAK201. R.O., Replication origin.

subsequent insertional inactivation of the tetracycline resistance gene (Amp^r Tet^s). Ten percent of the ampicillin-resistant *E. coli* transformants were Amp^r Tet^s. These transformants contained plasmids that ranged in size from 3 to 16 kb. All of these plasmids contained the *E. coli* origin of replication and the ampicillin resistance gene of plasmid pBR322. Restriction enzyme digestion analysis showed that the deletions occurred at the *Eco*RV sites on plasmid pBR322 (data not shown). Only 33% of Amp^r Tet^s transformants contained a chimera which corresponded in size and restriction pattern to plasmid pAK101. Plasmid pAK101 was linearized by *Pst*I and *Eco*RI digestion, and the *cat* gene, a 1.3-kb fragment, was cut out of plasmid pHR106 by *Pst*I and *Eco*RI digestion. The ligation mixture was used to transform *E. coli* HB101, and transformants were isolated by subsequent insertional inactivation of the ampicillin resistance gene (Chl^r Amp^s). The plasmid DNA content of *E. coli* transformants was analyzed by the miniprep plasmid isolation method, followed by *Eco*RV digestion. The resultant 8-kb presumptive pAK201 shuttle vector was subsequently transformed into *C. perfringens*. The fragments generated

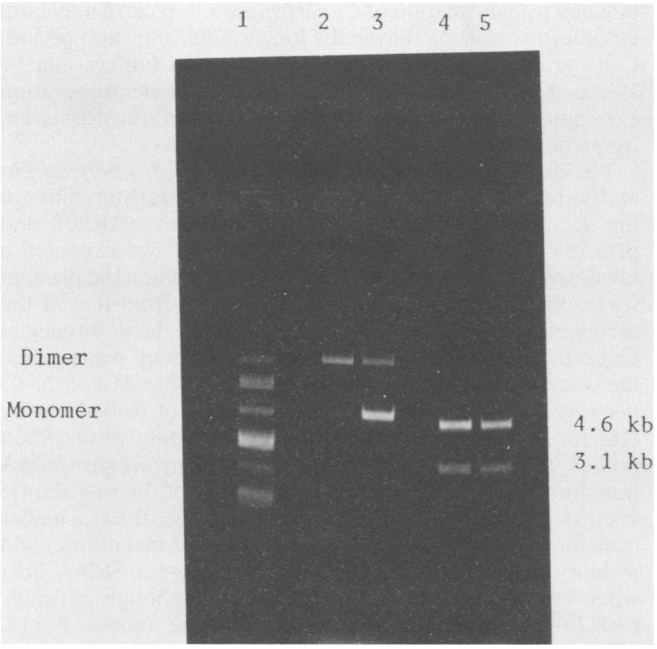

FIG. 3. Agarose gel (0.7%) electrophoresis of intact and *Eco*RV-digested plasmid pAK201. Lane 1, *E. coli* V-517 DNA molecular weight marker; lane 2, plasmid pAK201 isolated from *C. perfringens* 3624A; lane 3, plasmid pAK201 isolated from *E. coli*; lane 4, *Eco*RV-digested plasmid pAK201 isolated from *C. perfringens* 3624A; lane 5, *Eco*RV-digested plasmid pAK201 isolated from *E. coli*. (*Eco*RV digestion of pAK201 also resulted in a 300-base fragment which is not shown.)

following *Eco*RV, *Mbo*I, *Taq*I, *Ava*I, or *Pvu*II digestion of pAK201 isolated from either *E. coli* or *C. perfringens* were identical. The results of *Eco*RV digestion of pAK201 can be seen in Fig. 3. The orientation of the pHB101 insert was determined by *Mbo*I and *Bam*HI double digestion (Fig. 2). Shuttle vector pAK201 expresses chloramphenicol resistance (20 μg/ml) in both *E. coli* and *C. perfringens*.

Transformation of plasmid DNA by electroporation. The preliminary electroporation experiments were carried out with plasmids pBR322 (*E. coli*) and pHR106 (*C. perfringens*). *E. coli* transformants were screened for resistance to ampicillin (50 μg/ml), while *C. perfringens* transformants were screened for resistance to chloramphenicol (20 μg/ml). Glycerol (15%, vol/vol) added to the electroporation buffers increased the transformation frequencies of both *E. coli* and *C. perfringens* (data not shown). This compound increases the electrical resistance, and consequently the pulse duration time, while limiting cytoplasmic leakage during transient pore formation. During the electroporation-induced transformation of *C. perfringens*, all salts were removed from the electroporation buffer to increase the pulse duration time. The pulse duration time was 7.0 to 7.8 ms with electroporation buffer A and 36.0 to 45.0 ms with electroporation buffer B. Electroporation-induced transformation of *E. coli* HB101 and *C. perfringens* 3624A with plasmids derived from either *E. coli* or *C. perfringens* can be seen in Table 2. The transformation frequency of *E. coli* (10^6 transformants per μg of DNA) with plasmid pBR322, pAK201, or pHR106 is equivalent to that obtained by $CaCl_2$-mediated transformation methods (13). The transformation frequency of *C. perfringens* (ca. 10^4 transformants per μg of DNA) with plasmid pAK201, derived from *E. coli* or *C. perfringens*, is

TABLE 2. Electroporation-induced transformation of *E. coli* HB101 and *C. perfringens* 3624A with various plasmids

Recipient	Plasmid[a]	Source of plasmid	Transformation frequency (transformants per μg of plasmid DNA)
E. coli HB101	pBR322	*E. coli* HB101	1.3×10^6
	pAK201	*C. perfringens* 3624A	2.0×10^6
	pAK201	*E. coli* HB101	2.0×10^6
	pHR106	*E. coli* HB101	3.2×10^6
C. perfringens 3624A	pAK201	*C. perfringens* 3624A	1.0×10^4
	pAK201	*E. coli* HB101	6.9×10^3
	pHR106	*E. coli* HB101	1.2×10^3

[a] Selection for individual plasmids was carried out as follows: pBR322, 50 μg of ampicillin per ml; pAK201 and pHR106, 20 μg of chloramphenicol per ml.

sufficiently high to allow for screening of the transformants without the long posttransformation incubation period shown to be required in *C. perfringens* L-phase variant transformations (9, 12). Polyethylene glycol-induced transformation experiments require incubation for 4 h (12) to overnight (9) for recovery of transformants. The electroporation-induced transformation method described herein requires only a 1-h incubation period.

Restriction enzyme digestion analysis of plasmid pAK201. The restriction map of plasmid pAK201 is shown in Fig. 2. Cutting at any of the six single-cut enzyme sites (i.e., *Pst*I, *Eco*RI, *Cla*I, *Bam*HI, *Pvu*II, or *Ava*I) did not adversely affect the transferability, the stability, or the *cat* gene of plasmid pAK201. Consequently, the pBR322 segment within plasmid pAK201 can be used for cloning experiments at this time.

Shuttle vector stability. The stability of shuttle vectors pAK201 and pHR106 in *C. perfringens* was examined. Some 77% of the initial pAK201-containing *C. perfringens* 3624A transformants continued to be resistant to chloramphenicol following repeated culture transfer over a 3-day period in the absence of antibiotic pressure, while only 8% of the initial pHR106-containing *C. perfringens* 3624A transformants were resistant following similar treatment (Fig. 4). The

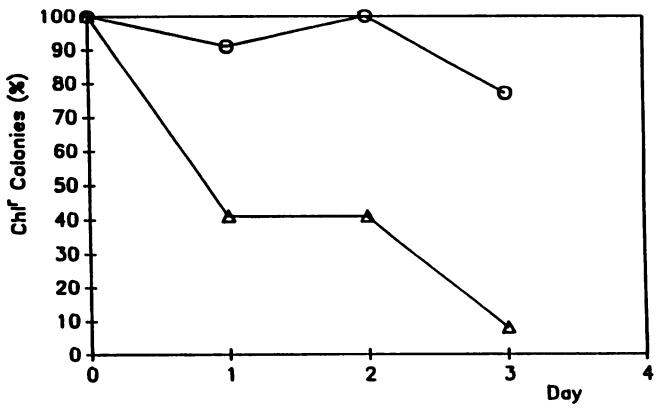

FIG. 4. Stability of plasmids pAK201 (○) and pHR106 (△) in *C. perfringens* 3624A following repeated transfer in nonseletive media over a 3-day period.

presence of plasmid DNA isolated from randomly selected colonies was confirmed by agarose gel electrophoresis (data not shown).

DISCUSSION

This report describes the development of a stable shuttle vector, designated pAK201, as well as an intact-cell electroporation-induced transformation system for introducing it into either *E. coli* or *C. perfringens*. Since electroporation of shuttle vector pAK201 into *E. coli* results in high numbers of transformants, gene structure analysis of *C. perfringens* can be carried out. *C. perfringens* genes can be inserted into plasmid pAK201 and efficiently transformed into the *E. coli* host system, while gene regulation can be examined in *C. perfringens*. The transformation frequency of pAK201 (10^4 transformants per μg of DNA) into *C. perfringens* is good enough to allow for the rapid screening of transformants.

When voltages below 2,000 V were applied during electroporation, transformation with plasmid pBR322 was not detected in *E. coli* (data not shown). DNA rearrangement of the pAK101 ligation mixture was observed following electroporation of *E. coli* HB101. DNA transferred into the *recA* *E. coli* HB101 host may be broken by the high-voltage pulse, and the breaks may be repaired by an unknown host repair mechanism which produces either small- or large-sized plasmids in *E. coli*. An examination of the effect of electroporation and the *E. coli* host repair mechanisms is required to understand these DNA rearrangements. DNA rearrangement of the closed circular form of plasmid pAK201 was also observed in *E. coli* following electroporation. The plasmids isolated from Chl^r transformants ranged in size from 8 to 24 kb. Restriction enzyme digestion analysis indicated that the presence of plasmids larger than 8 kb was due to multimer formation rather than concatemer formation by DNA recombination (data not shown). DNA rearrangement in the *C. perfringens* host was not observed following electroporation-induced transformation, although plasmid pAK201 is recovered predominantly in the dimer form from this microorganism while the monomer form predominates in *E. coli* (Fig. 3). It is unclear at this time why DNA rearrangement was observed in *E. coli* and not in *C. perfringens*.

When comparing electroporation-induced transformation in *C. perfringens* and *E. coli*, it may be that the gram-positive cell wall represents an additional barrier to DNA uptake in *C. perfringens*. Although the cell membrane is permeable to macromolecules such as plasmid DNA, the cell wall must be removed or compromised in some way to allow for DNA passage without affecting cellular viability. When electroporating *C. perfringens*, we routinely used cells in the late stationary phase. Some strains of *C. perfringens* undergo autolysis at late stationary phase (9, 17), which may destabilize the cell wall. We found that *C. perfringens* 3624A also underwent autolysis during late stationary phase (data not shown). Because of the autolysin activity, the cell wall of late-stationary-phase *C. perfringens* cells may be more vulnerable to electroporation-induced pore formation and thereby allow for plasmid DNA penetration. We were unable to transform *C. perfringens* 3624A when mid-log-phase cells were used.

The presence of 15% (vol/vol) glycerol in the electroporation buffers was found to increase the pulse duration time (ca. 5 to 7 ms). At the same time, a reduction in the ionic strength of the electroporation buffer drastically increased the pulse duration time (ca. 7 to 40 ms). The combination of glycerol and distilled water allowed for electroporation-induced transformation of *C. perfringens*. Glycerol may limit cytoplasmic leakage during the longer pulse duration period. Cell cultures suspended in electroporation buffers can be stored at −70°C for a few weeks and used for electroporation experiments without a significant decrease in transformation frequency.

We examined the effect of *E. coli* and *C. perfringens* restriction and modification systems on the transferability of the *E. coli-C. perfringens* shuttle vectors pAK201 and pHR106. On the basis of earlier work (19), we expected a lower frequency of plasmid transformation when the plasmid was isolated from a host which is different from that of the recipient. However, in the case of pAK201, the difference in *C. perfringens* 3624A transformation frequency was negligible when the source of the plasmid was either *E. coli* or *C. perfringens*, in agreement with the results of Roberts et al. (16) for plasmid pHR106. Another interesting observation relates to the difference between *C. perfringens* 3624A transformation frequency and the stability of the two shuttle vectors, pAK201 and pHR106. Plasmid pAK201 has a higher transformation frequency (nearly 1 order of magnitude) and stability (77 versus 8%) in the *C. perfringens* 3624A host when compared with plasmid pHR106. Although plasmids pAK201 and pHR106 are similar in size (about 8 kb), differences exist with respect to the source of the clostridial DNA. Plasmid pAK201 may have functional sequences derived from plasmid pHB101 which stabilize it in *C. perfringens*. These results suggest that, in addition to the origin of replication, the construction of a stable shuttle vector requires a DNA sequence specifically related to plasmid stability.

The relatively small size (8 kb) of shuttle vector pAK201 together with six unique restriction cut sites will allow for the cloning of various clostridial gene segments. Functional mapping of plasmid pAK201 is currently under way in our laboratory to identify the *C. perfringens* origin of replication, the pHB101-encoded caseinase structural gene, and a specific sequence(s) related to plasmid stability.

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