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# Identification of Tn*4451* and Tn*4452*, Chloramphenicol Resistance Transposons from *Clostridium perfringens*

LAWRENCE J. ABRAHAM AND JULIAN I. ROOD*

*Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia*

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**The recombinant plasmids pJIR45 and pJIR97 contain the chloramphenicol resistance determinants derived from the *Clostridium perfringens* R plasmids pIP401 and pJIR27, respectively. *Escherichia coli* cultures which harbored these recombinant plasmids rapidly became chloramphenicol sensitive when grown in the absence of chloramphenicol. The loss of resistance was associated with the loss of 6.2-kilobase (kb) segments from both plasmids. Detailed restriction analysis of *E. coli*- and *C. perfringens*-derived deletion plasmids indicated that deletion of these segments was essentially precise. Transposition of the 6.2-kb segments was demonstrated by cloning the determinants into a temperature-sensitive plasmid, curing the recombinant plasmids, and selecting chloramphenicol-resistant, plasmid-free clones. Southern hybridization analysis of chromosomal DNA isolated from these *recA E. coli* clones indicated that the 6.2-kb segments had transposed to different sites on the chromosome. Heteroduplex analysis and restriction mapping indicated that the transposons, Tn*4451* (pIP401) and Tn*4452* (pJIR27), were closely related and did not contain large inverted or directly repeated sequences. These transposons represent the first transposable elements from the clostridia to be identified and characterized.**

Three conjugative chloramphenicol resistance plasmids, pIP401 (53 kilobases [kb]), pJIR25 (52 kb), and pJIR27 (50 kb), have been identified in the anaerobic pathogen *Clostridium perfringens* (3, 6, 24). Each plasmid carries an identical tetracycline resistance determinant and has at least 23 kb of DNA homology which includes the regions coding for chloramphenicol resistance (3).

The chloramphenicol resistance determinants are stable in their host strains, but when the plasmids are transferred to an antibiotic-sensitive recipient strain, two types of transconjugants are obtained. Most of the transconjugants are resistant to both chloramphenicol and tetracycline and carry the same plasmid as the original donor. However, the remaining transconjugants are tetracycline resistant but chloramphenicol sensitive. These strains carry plasmids that have a single deletion of approximately 6 kb (3, 6). Restriction analysis of the pIP401-derived deletion plasmid pJIR23 showed that the deletion event involved the reduction in size of an 8.7-kb *Xba*I fragment to 2.6 kb (3). We cloned this 8.7-kb fragment into the *Xba*I site of pUC18 and showed that the recombinant plasmid, pJIR45, confers chloramphenicol resistance in *Escherichia coli*. The 2.6-kb *Xba*I fragment from pJIR23 also was cloned in *E. coli*. The recombinant plasmid, pJIR47, does not confer chloramphenicol resistance (3).

In this paper, we show that the chloramphenicol resistance determinants from pIP401 and pJIR27 are unstable in *E. coli* and are located on the transposable genetic elements Tn*4451* and Tn*4452*, respectively.

(These results have been reported in part in abstract form [L. J. Abraham and J. I. Rood, Australian Microbiologist 7:204, 1986].)

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids are listed in Table 1. *C. perfringens* strains were cultured in Trypticase (BBL Microbiology Systems) peptone glucose broth or on nutrient agar (23). *E. coli* JM83 and its derivatives were cultured in 2× YT (26); all other *E. coli* strains were grown in R broth (2). Media were supplemented with the following antibiotics as required: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), rifampin (100 μg/ml), or tetracycline (10 μg/ml). JIR862, a spontaneous rifampin-resistant mutant, was isolated after plating MM294 *recA* on medium supplemented with rifampin (500 μg/ml).

**Isolation and characterization of DNA.** Plasmid DNA from *C. perfringens* was isolated by a sodium dodecyl sulfate lysis procedure, followed by isopycnic gradient centrifugation (1). *E. coli* plasmid DNA was isolated from chloramphenicol- or spectinomycin-amplified cells by the cleared-lysate method (28). Multiple small-scale plasmid preparations were made from 5-ml R-broth cultures (12). Chromosomal DNA was isolated as follows. Stationary-phase cells were lysed as described previously (1). Lysates then were treated with RNase A (Sigma Chemical Co.) (15 μg/ml). After incubation at 37°C for 30 min, pronase (8 mg/ml, predigested; Boehringer-Mannheim Biochemicals) was added, and the lysates were incubated for a further 60 min at the same temperature. Preparations were then phenol-chloroform extracted and ethanol precipitated.

Restriction endonucleases were purchased from either Boehringer-Mannheim, Bethesda Research Laboratories, Inc., or Pharmacia, Inc. Restriction enzyme digestion and agarose gel electrophoresis were carried out as described previously (1). Restriction fragments were isolated from low-melting-temperature agarose (SeaPlaque; FMC Corp., Marine Colloids Div.) by a cetyltrimethylammonium bromide extraction procedure (15). Polyacrylamide gel electrophoresis of restriction fragments was performed as described previously (17).

**Cloning methodologies.** The DNA to be cloned was digested with the appropriate restriction endonuclease, mixed with the vector DNA in a 5:1 ratio, and ligated with T4 DNA ligase as outlined by the supplier (New England BioLabs,

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| Strains | | |
| *C. perfringens* JIR204 | CW504(pJIR27) | 1329 × CW504 (3) |
| *E. coli* | | |
| HB101 | *recA13 hsdS20 rpsL20 supE44* | 5 |
| JM83 | *ara* Δ(*lac-pro*), *strA thi* φ80d*lacZ*Δ*M15* | 27 |
| JIR862 | *endA thi hsdR hsdM*$^+$ *recA* Rif$^r$ | Rif$^r$ mutant of MM294 *recA* (4) |
| JIR863 | JIR862(pJIR94) | Recombinant |
| JIR888 to JIR892 | JIR862::Tn*4451* | Cured derivatives of JIR863 |
| Plasmids | | |
| pJIR27 | Tc$^r$ Cm$^r$ Tra$^+$ | 3 |
| pUC18 and pUC19 | Ap$^r$ Lac$^+$ | 19 |
| pSC301 | Tc$^r$ Rep(Ts) | 14 |
| pJIR45 | pUC18Ω(*Xba*I:pIP401, 8.7 kb) | 3 |
| pJIR46 | pUC18Ω(*Xba*I:pIP401, 8.7 kb) | Opposite orientation to pJIR45 |
| pJIR47 | pUC18Ω(*Xba*I:pJIR23, 2.6 kb) | 3 |
| pJIR51 | pUC18Ω(*Hin*dIII:pJIR45, 5 kb) | Recombinant |
| pJIR84 | pUC18Ω(*Xba*I-*Sma*I:pJIR45, 3.6 kb) | Recombinant (*Xba*I-*Eco*RV pJIR45 fragment) |
| pJIR85 | pUC18Ω(*Xba*I-*Sma*I:pJIR45, 5.1 kb) | Recombinant (*Xba*I-*Eco*RV pJIR45 fragment) |
| pJIR86 | pJIR45ΔTn*4451* | Spontaneous Cm$^s$ deletion |
| pJIR94 | pSC301Ω(*Eco*RI:pJIR45, 5.1 kb, + pJIR46, 3.6 kb) | Recombinant (*Eco*RI-*Eco*RV pJIR45 and pJIR46) |
| pJIR95 | pUC19Ω(*Eco*RI-*Xba*I:pJIR85, 5.1 kb) | Recombinant |
| pJIR97 | pUC18Ω(*Xba*I:pJIR27, 8.5 kb) | Recombinant |
| pJIR102 | pJIR94ΔTn*4451* | Spontaneous Cm$^s$ deletion |

[a] Rif$^r$, rifampin resistant; Tc$^r$, tetracycline resistant; Tra$^+$, transferable; Cm$^r$, chloramphenicol resistant; Cm$^s$, chloramphenicol sensitive; and Ap$^r$, ampicillin resistant.

Inc.). *E. coli* strains were transformed as previously described (16). HB101- and JIR862-derived transformants were selected on R broth agar supplemented with tetracycline, ampicillin, or chloramphenicol. JM83-derived recombinants were identified as described previously (19).

**Heteroduplex analysis.** Heteroduplexes were prepared and analyzed by the method of Davis et al. (8). Heteroduplexes were allowed to form at room temperature for 45 min in the presence of 50% formamide. The DNA was mounted, shadowed with platinum-palladium, and viewed and photographed in a Phillips EM300 electron microscope at magnifications of 27,000 to 33,000×.

**Southern hybridization analysis.** DNA was transferred bidirectionally from agarose gels to nitrocellulose filters (16). Hybridization of denatured DNA present on the filters to labeled probe DNA was done as described previously (25). Stringency washes were normally carried out in 0.16× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C. When required, low-stringency washes were carried out at the same temperature in 2× SSC. Probe DNA was labeled with photobiotin (BRESA Inc.) (9). Hybridized probe DNA was detected by an avidin-alkaline phosphatase detection system (BRESA).

## RESULTS

**Instability of the chloramphenicol resistance region from pIP401.** Unexpected plasmid profiles were observed when plasmid DNA was prepared from HB101 cells carrying the 11.4-kb recombinant plasmid pJIR45. Agarose gel electrophoresis revealed the presence of an additional 5.3-kb plasmid (pJIR86) that was indistinguishable from the cloned deletion derivative pJIR47 (3).

When these heterogeneous pJIR45 preparations were used to transform HB101 to ampicillin resistance, only 30 to 60% of the transformants were chloramphenicol resistant and carried pJIR45. The remaining transformants were sensitive to chloramphenicol and carried 5.3-kb deletion plasmids. Further experiments showed that even after the chloramphenicol-resistant transformants were cultured for ca. 120 generations in the presence of chloramphenicol, chloramphenicol-sensitive segregants could still be isolated at a high frequency.

Six independently derived deletion plasmids (including pJIR86) were then compared with pJIR47, which was derived from a conjugative deletion event that occurred in *C. perfringens*. The plasmids were digested with a variety of restriction enzymes, each of which digested the plasmids into numerous small fragments. Polyacrylamide gel electrophoresis showed that these plasmids had identical restriction profiles (limit of resolution, 10 to 20 base pairs). We concluded that the deletion events which occurred independently in both *C. perfringens* and *E. coli* were essentially identical and precise.

The *E. coli* deletion phenomenon was investigated further by determining whether both ends of the 6.2-kb segment were required for excision of the chloramphenicol resistance region. The left and right ends of this region were located separately on 3.6- and 5.1-kb *Xba*I-*Eco*RV fragments (3). These fragments, which together make up the 6.2-kb region and the surrounding sequences of pIP401, were cloned into pUC18 to form the recombinant plasmids pJIR84 and pJIR85, respectively. As expected from previous results (3), strains carrying these plasmids were sensitive to chloramphenicol. Both of these plasmids were stable in *E. coli*, indicating that neither end of the chloramphenicol resistance region by itself promotes the excision event characteristic of the intact segment.

Owing to the location of the *Eco*RV site within the chloramphenicol resistance gene, these results could be interpreted as evidence for the involvement of that gene in the deletion process. However, this interpretation could be

ruled out because pJIR51 was stable in *E. coli*. This plasmid confers chloramphenicol resistance but only contains the left end of the 6.2-kb segment.

**Transposition of the chloramphenicol resistance segment in *E. coli*.** The pIP401-derived chloramphenicol resistance determinant was recloned into the tetracycline resistance, temperature-sensitive plasmid pSC301 (14) to form the recombinant plasmid pJIR94. The strain carrying this plasmid (JIR863) exhibited the same spontaneous deletion phenomenon, since tetracycline-resistant, chloramphenicol-sensitive derivatives could be isolated at a high frequency. Restriction analysis of a deletion plasmid (pJIR102) that was isolated from one of these derivatives showed that it was identical to pJIR94, except that it had lost the 6.2-kb segment.

Plasmid pJIR94 was cured from its host strain (JIR863) by daily subculturing for 6 days at the nonpermissive temperature (42°C). Chloramphenicol-resistant (20 μg/ml) colonies were isolated at a frequency of ca. $10^{-6}$. The eight clones that were purified and examined were all tetracycline sensitive and did not have any detectable plasmid DNA.

Chromosomal DNA was purified from five of these clones (JIR888 to JIR892) and subjected to Southern hybridization analysis with either the entire pJIR45 molecule or the 4.2-kb *Cfo*I fragment as a probe. This fragment is contained wholly within the 6.2-kb deletion region of pJIR45. Three of the clones had single hybridizable 11.7-kb *Eco*RI bands. The remaining clones had hybridizable *Eco*RI bands of 23 and 11.6 kb. No hybridizable bands were detected in chromosomal DNA prepared from the parent strain (Fig. 1). In all five clones, a homologous 4.2-kb *Cfo*I fragment was observed after hybridization of chromosomal *Cfo*I digests with the 6.2-kb region-specific probe (Fig. 1). When the chromosomal preparations were cleaved with *Hind*III, which has a single recognition site within the chloramphenicol resistance region, two hybridizable bands were observed (data not shown). When, under conditions of reduced stringency, the chromosomal digests were probed with pJIR102 (the deletion derivative of pJIR94), no homology was observed (data not shown). On the basis of these results, we concluded that the 6.2-kb segment had undergone a precise *recA*-independent transposition to the *E. coli* chromosome and accordingly designated the segment as Tn*4451*.

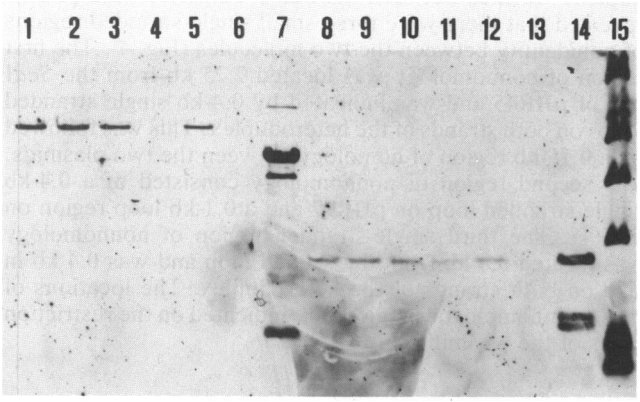


FIG. 1. Southern hybridization analysis of the chloramphenicol-resistant, tetracycline-sensitive derivatives of JIR863. Hybridization analysis was carried out as described in the text. Plasmid pJIR45 was photobiotin labeled and used to probe *Eco*RI- or *Cfo*I-digested chromosomal DNA. Lanes: 1 to 5, *Eco*RI-digested JIR888 to JIR892, respectively; 6, *Eco*RI-digested JIR862; 7, *Eco*RI-digested pJIR94; 8 to 12, *Cfo*I-digested JIR888 to JIR892, respectively; 13, *Cfo*I-digested JIR862; 14, *Cfo*I-digested pJIR94; 15, photobiotin-labeled, *Hind*III-digested lambda DNA.

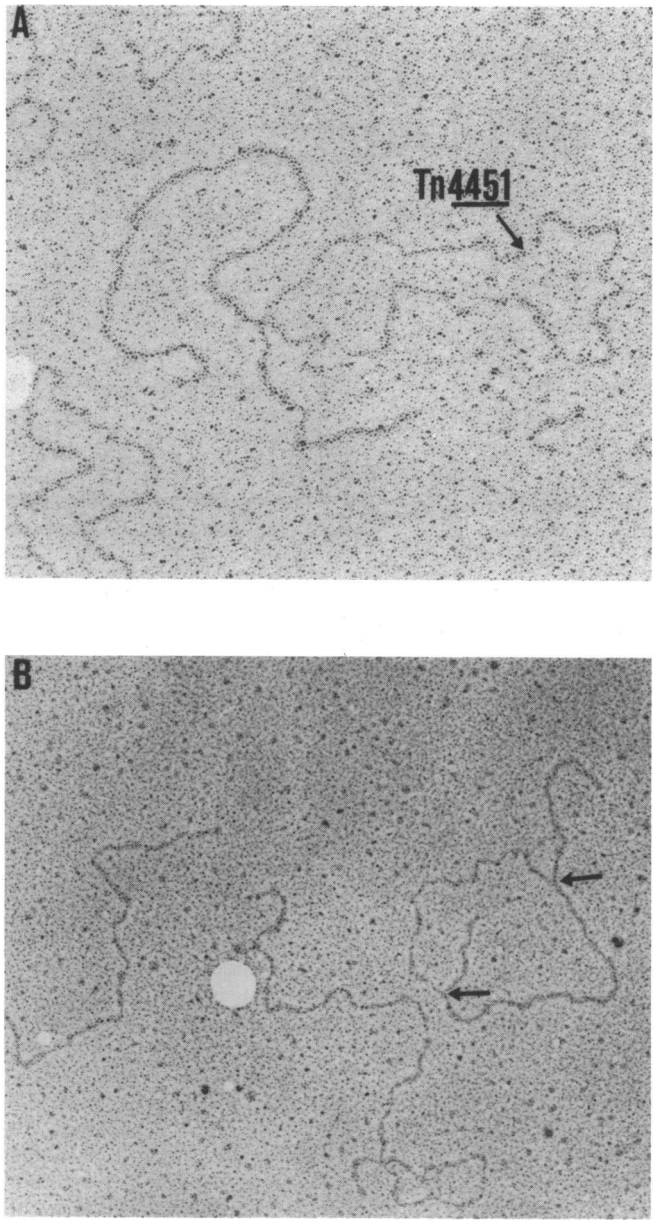


FIG. 2. Heteroduplex analysis of Tn*4451*. (A) Heteroduplex formed between pJIR45 and pJIR47; the single-stranded region corresponding to Tn*4451* is indicated. (B) Heteroduplex formed between pJIR84 and pJIR95; the junctions between the single- and double-stranded regions of the heteroduplex are indicated by the arrows. Plasmid DNA was digested with either *Eco*RI (A) or *Sca*I (B), alkali denatured, reannealed, and mounted for electron microscopy (8). Plasmid pUC18 (19) and bacteriophage φX174 were included as double- and single-stranded standards, respectively. Molecules were photographed, and contour lengths were determined from an average of at least 10 measurements with a Carl Zeiss MOP-1 digital analyzer.

**Hybridization analysis of Tn*4451*.** Heteroduplex analysis was carried out to determine the exact location of the Tn*4451* excision site and also to determine whether inverted repeat sequences were present at the ends of the transposon. Plasmids pJIR45 and pJIR47 were digested with *Eco*RI

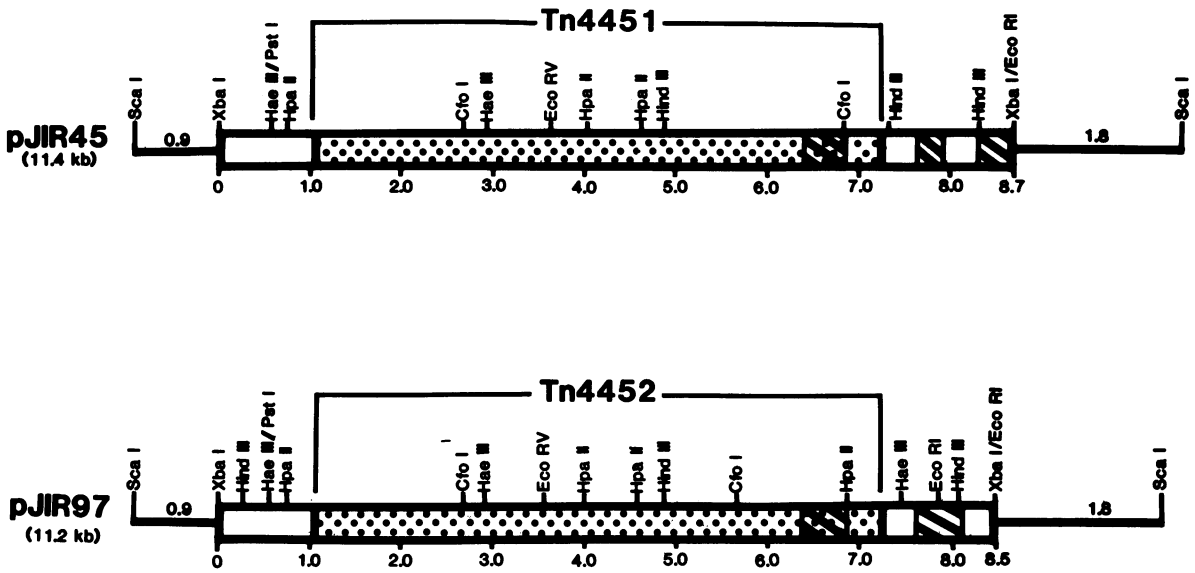


FIG. 3. Linear restriction maps of pJIR45 and pJIR97. Both plasmids are linearized at the single *Sca*I sites in the pUC18 portions of the plasmids. The pUC18 portions of the plasmids are indicated by the single lines. The boxed regions show the cloned *Xba*I fragments of the plasmids. The extents of Tn*4451* and Tn*4452* are indicated by the dotted regions. The hatched areas show the regions of nonhomology between pJIR45 and pJIR97, as determined from heteroduplex analysis of the two plasmids. Sizes indicated are in kilobases. Restriction sites present in pUC18 are not shown.

(which has a single cleavage site within the pUC18 multiple cloning region) and denatured. Heteroduplexes formed between the two plasmids consisted of double-stranded structures with a single-stranded loop (Fig. 2A). Physical analysis showed that the 6.2-kb single-stranded loop was located 1.4 and 3.9 kb from the ends of the pJIR47 strand of the heteroduplex. We did not observe any double-stranded stem structures which would indicate that the transposon was bound by large inverted repeat sequences.

Heteroduplex studies also were used to determine if directly repeated sequences were present at the ends of Tn*4451*. The 5.1-kb *Xba*I-*Eco*RI fragment of pJIR85 was recloned into pUC19 to form pJIR95. The aim of this manipulation was to place any putative direct repeats located within pJIR84 and pJIR95 in the same orientation with respect to the pUC portion of these plasmids. Both plasmids were cut with *Sca*I (which has a single cleavage site within the pUC portion of the molecules), denatured, and allowed to anneal together. Heteroduplexes showed homologous 1.8- and 0.9-kb regions which corresponded to the pUC portions of pJIR84 and pJIR95. These double-stranded regions were bound by 3.6-kb (pJIR84) or 5.1-kb (pJIR95) single-stranded regions (Fig. 2B). We did not observe any regions of homology between the cloned inserts, suggesting that there were no substantial directly repeated sequences located within Tn*4451*.

These conclusions were confirmed by Southern hybridization analysis. The 3.6-kb *Eco*RI-*Xba*I fragment of pJIR84 and the 2.4-kb *Hin*dIII fragment of pJIR85 (each fragment containing one end of Tn*4451*) were used to probe *Eco*RI-*Hin*dIII-digested pJIR84 and pJIR85 DNAs. The pJIR84-derived probe hybridized to the 3.6-kb fragment of pJIR84 but not to any fragments of pJIR85. Conversely, the pJIR85-derived probe hybridized to the 2.4-kb fragment of pJIR85 but not to any fragments of pJIR84.

**Cloning and analysis of the chloramphenicol resistance segment from pJIR27.** The homologous chloramphenicol resistance determinant from the *C. perfringens* R plasmid pJIR27 (3) was cloned into the *Xba*I site of pUC18 to form the recombinant plasmid pJIR97. Experiments showed that this plasmid carried the transposon Tn*4452*, which was very similar to Tn*4451* in that it was spontaneously deleted at a high frequency in *E. coli* and was able to transpose to different sites in the *E. coli* chromosome.

**Structural comparison of pJIR45 and pJIR97.** Comparative restriction analysis of pJIR45 and pJIR97 showed that Tn*4451* and Tn*4452* had considerable restriction identity which included the region containing the chloramphenicol resistance genes. However, the transposons did differ at their right-hand ends (Fig. 3).

Heteroduplex analysis was carried out to determine the precise relationship between the transposons. Heteroduplexes formed between *Sca*I-cleaved pJIR45 and pJIR97 revealed that there were three small single-stranded regions of nonidentity between the two molecules (Fig. 4). The first region of nonhomology was located 7.25 kb from the *Sca*I site of pJIR45 and was indicated by 0.4-kb single stranded loops on both strands of the heteroduplex. This was followed by a 0.75-kb region of homology between the two plasmids. The second region of nonhomology consisted of a 0.4-kb single-stranded loop on pJIR97 and a 0.1-kb loop region on pJIR45. The third single-stranded region of nonhomology was located 0.4 kb from the second loop and was 0.4 kb in size on both strands of the heteroduplex. The locations of these nonhomologous regions are indicated on the restriction maps of the plasmids (Fig. 3).

## DISCUSSION

In previous studies on the *C. perfringens* R plasmids pIP401 and pJIR27, we suggested that the homologous 6-kb chloramphenicol resistance segments may actually be transposable genetic elements (3). This hypothesis has been confirmed by the results presented in this paper. We demonstrated that each of the 6.2-kb segments could transpose to different sites in the chromosome of a *recA* strain of *E.*

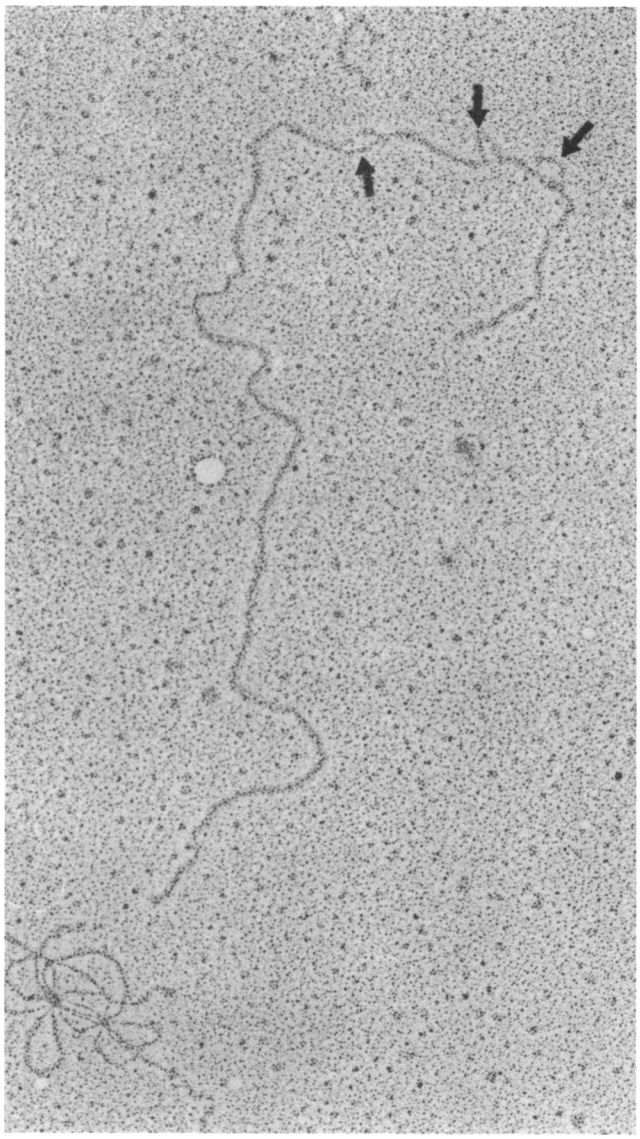

FIG. 4. Heteroduplex molecules formed between pJIR45 and pJIR97. Heteroduplex analysis was carried out as described in the legend to Fig. 2. The arrows indicate the three single-stranded regions of the heteroduplex.

*coli*. Only the segments were transposed, since Southern hybridization experiments showed that the transposition derivatives did not contain any of the surrounding regions. These 6.2-kb transposons (Tn*4451* and Tn*4452*) represent the first transposons to be identified in *Clostridium* spp. However, we have been unable to demonstrate transposition in the original host species, probably because of the lack of a suitable system for the detection of transposition events in *C. perfringens*.

Restriction and heteroduplex analyses enabled the construction of detailed physical maps of Tn*4451* and Tn*4452* (Fig. 3). Comparison of the two transposons indicated that they were identical for most of their length and differed only at their right-hand ends. It appears most likely that one of the transposons probably arose from the other by an inversion or deletion-insertion event. Interestingly, the sequences surrounding Tn*4451* and Tn*4452* also were related. This relationship may indicate a similar target site for transposition or, alternatively, that a rearrangement of one of the transposons and its surrounding sequences led to the formation of the other transposon.

Both transposons were spontaneously excised when present on multipcopy plasmids in *E. coli*. Even after growth for many generations on chloramphenicol (50 μg/ml), chloramphenicol-sensitive deletion plasmids still could be isolated. Similar transposon excision occurred in *C. perfringens* but only upon conjugative transfer of pIP401 or pJIR27. Despite these differences in excision conditions, the results showed that the products of the deletion of Tn*4451* were essentially identical in *E. coli* and *C. perfringens*.

It is not known whether the excision mechanism of Tn*4451* (and Tn*4452*) is related to the mechanism of transposition. However, the stability of the subcloned derivatives pJIR84, pJIR85, and pJIR51 led us to conclude that both ends of Tn*4451* must be present for excision to occur. The exact structure of the transposon ends is not known, but heteroduplex and Southern hybridization experiments showed that no substantial direct or inverted repeat sequences were present.

Tn*4451* and Tn*4452* share a number of features with other transposons found in gram-positive bacteria. Transposons such as Tn*554* (22) from *Staphylococcus aureus* and the streptococcal transposons Tn*916* (10) and Tn*1545* (7) have been cloned in *E. coli* and have been shown to transpose to the *E. coli* chromosome (7, 10, 21). It appears that transposons from gram-positive bacteria, once allowed to cross the species barrier, can function in unrelated gram-negative bacteria such as *E. coli*.

All transposons are able to undergo precise excision, but most transposons (e.g., Tn*551* [20] and Tn*10* [11]) are excised at very low frequencies (13). In contrast, Tn*4451*, Tn*4452*, Tn*1545*, and Tn*916* are excised at much higher frequencies. These four transposons are unstable and are deleted at a very high frequency when grown without antibiotic selection in *recA E. coli* strains. Furthermore, the deletion events are all essentially precise in nature (7, 10, 18). Further studies are required to see if there is any molecular relationship among these transposons.

Gawron-Burke and Clewell (10) suggested that the precise deletion of Tn*916* in *E. coli* may be useful for the cloning of streptococcal genes. If transposition of Tn*4451* (or Tn*4452*) could be demonstrated in *C. perfringens*, then a similar cloning strategy could be feasible. The procedure would involve the isolation of Tn*4451* insertion mutants of the gene to be cloned, the cloning of the transposon and the surrounding gene sequences in *E. coli*, and the isolation of the regenerated gene after spontaneous deletion of the transposon. The development of this cloning strategy would enable major advances to be made in the analysis of clostridial genes and their industrially and clinically significant gene products.

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