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Characterization and Mapping of Regions Encoding Clindamycin Resistance, Tetracycline Resistance, and a Replication Function on the *Bacteroides* R Plasmid pCP1

BARBARA G. MATTHEWS* AND DONALD G. GUINEY

Department of Medicine H811F, UCSD Medical Center, University of California at San Diego, San Diego, California 92103

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The *Bacteroides* drug resistance plasmid pCP1 encodes clindamycin resistance (Cl^r) and a cryptic tetracycline resistance (Tc^r) determinant that is expressed in *Escherichia coli* cells grown aerobically, but not anaerobically, and is not expressed phenotypically in *Bacteroides* spp. Localization of genetic functions on pCP1 was facilitated by the construction of hybrid shuttle plasmids containing portions of pCP1 ligated to pDG5, a pBR322 derivative carrying the RK2 transfer origin. pDP1Δ4 is a *Bgl*II deletion derivative of pCP1 linked to pDG5 and can be maintained in both *E. coli* and *Bacteroides fragilis*. By using Tn*5* mutagenesis and subcloning, we localized the Cl^r and Tc^r regions on the *Eco*RI B fragment between the 1.2-kilobase direct repeats of pCP1. The Cl^r and Tc^r determinants are distinct and appear to be transcribed separately. Control of the Tc^r phenotype is unusual in that expression is constitutive and is enhanced by a region encompassing the adjacent direct repeat. In addition, a region of pCP1 required for replication in *Bacteroides* spp. has been identified in the neighboring *Eco*RI A fragment.

Bacteroides species are the predominant gram-negative anaerobes in the gastrointestinal tract. Plasmids are common in *Bacteroides* spp., and many strains also express traits, such as antibiotic resistance and bacteriocin production, that are commonly found to be plasmid-mediated in other bacterial genera, such as members of the family *Enterobacteriaceae* (11). Clindamycin resistance has been shown conclusively to be encoded by plasmids in *Bacteroides* spp. Four clindamycin resistance plasmids from *Bacteroides* spp. have been isolated and characterized; they are pBF4, pBFTM10, pCP1, and pBI136 (3, 13, 14, 16). pBF4 is a 41-kilobase (kb) transmissible plasmid which carries clindamycin resistance (Cl^r) and a cryptic tetracycline resistance (Tc^r) determinant bounded by 1.2-kb direct repeats (4, 6, 13a). Cl^r is expressed in *Bacteroides* spp. but not in *Escherichia coli*, whereas Tc^r is expressed in *E. coli* grown aerobically, but not anaerobically, and is not expressed in *Bacteroides* spp. (4, 5). Recent evidence indicates that this antibiotic resistance region with the direct repeats is transposable in *E. coli* and presumably in *Bacteroides* spp. (12). pBFTM10 and pCP1 are closely related 15-kb plasmids that contain Cl^r and aerobic Tc^r regions and direct repeats that are highly homologous to the pBF4 transposon (3, 4, 14). The remaining regions of pCP1 (pBFTM10) and pBF4 do not share detectable homology, suggesting that the drug resistance region is transposed onto different plasmids in *Bacteroides* spp. pBI136 is a larger Cl^r plasmid (81 kb) containing homologous direct repeats but separated by a longer region of DNA which lacks the aerobic Tc^r gene, and this plasmid has little similarity to pBF4 or pCP1 in restriction sites (13, 13a). Within the direct repeat region, the only areas of homology between pBI136 and pBF4 are the direct repeats themselves and a 0.85-kb sequence containing a cluster of *Hin*dIII, *Ava*II, and *Dde*I sites arranged in opposite orientation with respect to the direct repeats. Based on this data, Smith and Gonda (13a) propose that the common Cl^r gene maps in this cluster of restriction sites.

The localization of genetic functions on plasmids from *Bacteroides* spp. has been hampered by the lack of a system for genetic manipulation of these anaerobic bacteria. We have recently described methods for cloning and manipulating *Bacteroides* plasmids in *E. coli* and transferring these plasmids back to *Bacteroides* by using the conjugation system of the IncP plasmid RK2 (5). The shuttle plasmid pDP1Δ4 (Fig. 1) contains the replication origin (*oriV*) and β-lactamase gene of pBR322, the transfer origin (*oriT*) of RK2, and a *Bgl*II deletion derivative of the *Bacteroides* plasmid pCP1. The shuttle plasmid pDP1Δ4 retains the Cl^r and aerobic Tc^r regions from pCP1 (the *Eco*RI B fragment) and a portion of the *Eco*RI A fragment required for replication in *Bacteroides* spp. We used the shuttle vector properties of pDP1Δ4 together with Tn*5* mutagenesis and subcloning to localize the Cl^r, aerobic Tc^r, and replication regions of the *Bacteroides* pCP1 plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JA221, HB101, and JM83 and *B. fragilis* 638 have been described previously (5, 6, 15). The source of Tn*5* was HB101::Tn*5* (2). The shuttle plasmid pDP1Δ4 is a *Bgl*II deletion derivative of pDP1 and expresses Cl^r in *Bacteroides* spp. and ampicillin resistance (Am^r) and aerobic Tc^r in *E. coli* (5). Plasmids pRK212.1 (*tra*$^+$ Am^r Tc^r) and pRK231 (*tra*$^+$ Km^r Tc^r) are deletion derivatives of RK2 (7). The small cloning vector pDGV1 is a minimal pBR322 replicon (7). Plasmids pUC8 and pUC9 have been described (15).

Bacterial matings. The standard filter mating procedure was used to transfer plasmids between *E. coli* strains (7). Triparental matings between *E. coli* and *B. fragilis* were performed by a modification of a previously described procedure (5), which was based on the method of Privitera et al. (10). The *E. coli* donors were grown overnight in brain heart infusion broth supplemented with cysteine and hemin

* Corresponding author.

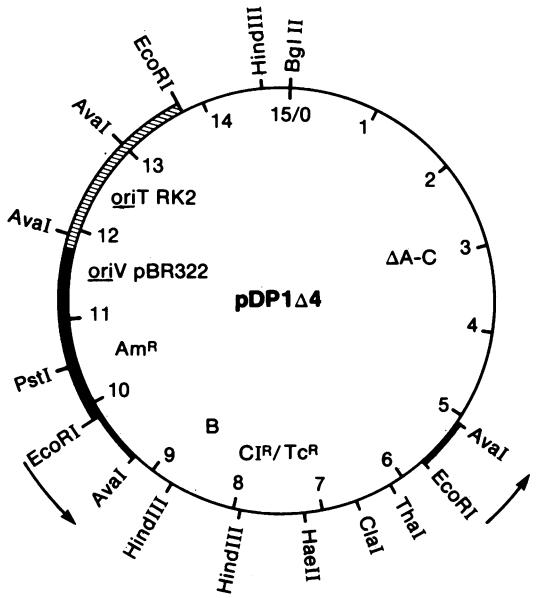

FIG. 1. Map of pDP1Δ4, showing regions derived from the *Bacteroides* plasmid pCP1 (——), pBR322 (▬), and RK2 (▨). The directly repeated DNA sequences between *Eco*RI and *Ava*I sites in pCP1 are indicated by arrows. Recent evidence shows that these repeats are about 1.2 kb and extend through the *Ava*I sites. ΔA-C indicates the fusion of the *Eco*RI A and C fragments of pCP1 as a result of the *Bgl*II deletion which generated pDP1Δ4. Am^R, Cl^R, and Tc^R indicate the approximate locations of the ampicillin, clindamycin, and tetracycline resistance determinants. The locations of *Hae*II and *Tha*I sites are shown only for the *Eco*RI B fragment; other sites for these enzymes in pDP1Δ4 are not indicated in this figure.

(BCH [6]) and with selective antibiotics. Immediately before mating, the donor strains were diluted to a cell density of 80 Klett units. The recipient was not diluted. The donors and recipient were mixed in a ratio of 1:1:2 by volume, and a 0.20-ml portion was plated on BCH and incubated anaerobically overnight. The cells were scraped from the plate, suspended in saline, diluted, and plated on BCH with 5 μg of clindamycin per ml and 50 μg of gentamicin per ml. Colony counts or absence of bacterial growth was recorded after 48 h of anaerobic growth.

Plasmid DNA procedures. Plasmid DNA was extracted from *E. coli* by the cleared lysate method and purified by CsCl-ethidium bromide density gradient centrifugation (8). Restriction fragments of plasmid DNA were purified by agarose gel electrophoresis by standard techniques (9). Routine methods for restriction enzyme digestion, ligation, agarose and polyacrylamide gel electrophoresis, and bacterial transformations were used (8).

Tn*5* insertions in pDP1Δ4. To obtain random insertions of Tn*5* into plasmid pDP1Δ4, HB101::Tn*5* was transformed with pDP1Δ4 with selection for kanamycin and tetracycline. One transformant was selected for use in a triparental mating with HB101 Nal^r and HB101(pRK212.1) with selection for resistance to kanamycin and nalidixic acid. The resultant clones were HB101 Nal^r cells containing pDP1Δ4::Tn*5* transpositions. The location of Tn*5* on these various pDP1Δ4::Tn*5* molecules was mapped by restriction digestion with *Eco*RI and double digests with *Eco*RI and *Cla*I, *Bgl*II, and *Pst*I. The distance of the Tn*5* insert from the *Cla*I site was determined by digestion with *Cla*I and *Hpa*I.

Assay of antibiotic resistance. Relative resistance to tetracycline was assayed by recording the extent of growth on L broth plates containing either 5 or 20 μg of tetracycline per ml. A score of +3/+3 was used to indicate good growth out to the tertiary streak, whereas +1/+3 indicated growth only on the primary streak. The tetracycline resistance was quantitated by determining the MIC in L broth by a tube dilution assay with a heavy inoculum of 10^7 cells per ml. The transfer of the pDP1Δ4::Tn*5* plasmids to *B. fragilis* and the assay of Cl^r were performed by doing triparental matings with HB101(pRK231), HB101(pDP1Δ4::Tn*5*), and *B. fragilis* 638; the mating mix was plated on BCH agar containing clindamycin (5 μg/ml) and gentamicin (50 μg/ml). Plasmid pDP1Δ4 was included in each set of matings as a positive control, and the number of viable *Bacteroides* recipients was determined by plating the mating mix on BCH agar containing gentamicin (50 μg/ml) alone.

RESULTS

Localization of the tetracycline resistance gene on pDP1Δ4. Insertions of Tn*5* into plasmid pDP1Δ4 were isolated and mapped as described in Materials and Methods. To locate the Tc^r determinant, the pDP1Δ4::Tn*5* derivatives were screened for expression of Tc^r. Because the tra^+ helper plasmid pRK212.1 (Am^r Tc^r) was found in a number of the initial pDP1Δ4::Tn*5* transconjugants, the pDP1Δ4::Tn*5* plasmids to be studied were extracted and transformed individually into HB101 Nal^r. Subsequent analysis showed these transformants to be free of the larger pRK212.1 helper. The *Cla*I site on the *Eco*RI B fragment of pDP1Δ4 was used as the reference point in mapping the Tn*5* insertions (Fig. 2). Tn*5* insertions between insert 11, at 315 base pairs (bp) from *Cla*I in the direction of the *Eco*RI A fragment, and insert 6, at 435 bp in the opposite direction (clockwise) toward *Hin*dIII, interrupted expression of the tetracycline resistance gene (Fig. 2, Table 1). In the direction of the *Hin*dIII site, the next Tn*5* insertion (insert 75) occurred 1.14 kb from *Cla*I. The tetracycline resistance gene was completely expressed by this plasmid and by those in which Tn*5* was inserted further from *Cla*I. Insert 31, at 815 bp from *Cla*I at the other end of the tetracycline gene, partially interfered with the expression of resistance. The *E. coli* strain with this plasmid grew well on plates containing 5 μg of tetracycline per ml (+3/+3 growth) but only on the primary streak at a concentration of 20 μg/ml (+1/+3 growth). This decreased resistance to a higher concentration of tetracycline occurred whenever Tn*5* was inserted in this region up to 1.8 kb from the *Cla*I site. Insertion more distant in the *Eco*RI A fragment had no effect on expression of antibiotic resistance.

These Tn*5* insertion mutations indicated that the tetracycline resistance gene was on the 2.3-kb *Eco*RI-*Hin*dIII piece of the *Eco*RI B fragment of plasmid pDP1Δ4. To further delineate the length of this gene, the plasmid pOA0 (Fig. 3) was constructed by the ligation of the gel-purified *Eco*RI-*Hin*dIII fragments of plasmids pDGV1 and pDP1Δ4 and transformation into strain HB101 with selection for Am^r and Tc^r. The 2.4-kb fragment from pDGV1 contains the β-lactamase gene and the replication region of pBR322. The deletion plasmids pOA1 and pOA2 were constructed from pOA0 (Fig. 3). The plasmid pOA1 was generated by a complete *Hae*II digestion of pOA0, ligation of the fragments, and transformation of *E. coli* HB101 Nal^r with selection for penicillin resistance. Plasmid pOA1 lacks the 0.95-kb *Hae*II fragment extending from the single *Hae*II site on the *Hin*dIII-*Eco*RI region from pDP1Δ4 to the adjacent *Hae*II site in pDGV1. Cells containing pOA1 showed no increment

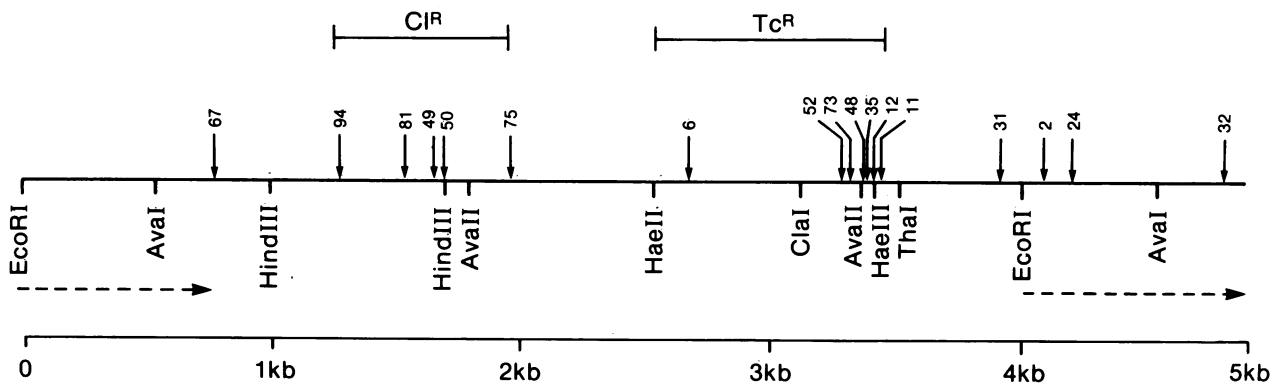

FIG. 2. Map of Tn*5* insertions in the region of the *Eco*RI B fragment of pDP1Δ4. The arrows indicate the approximate positions of the direct repeats but not the extent of these regions. The locations of the Tn*5* inserts are indicated by the numbers. The bars labeled Cl^R and Tc^R show the minimal extent of these regions as determined by insertion and deletion analyses (see the text and Tables 1 through 3).

in Tc^r over the plasmid-free parent strain, whereas cells with pOA0 expressed a moderate level of Tc^r. This result indicates that the Tc^r region extends through the adjacent *Hae*II site (Fig. 2, Table 2). Plasmid pOA2 was constructed by a partial *Tha*I digest of pOA0 followed by blunt-end ligation, digestion with *Eco*RI, and transformation of HB101 Nal^r with selection for penicillin resistance. This procedure selected for plasmids lacking the *Tha*I fragment containing the *Eco*RI site, and one of these, designated pOA2, was tested for Tc^r expression. This 580-bp *Tha*I deletion still left Tc^r expression intact and localized one end of the Tc^r region in the sequence between the *Tha*I site and Tn*5* insert 11 (Fig. 2, Table 2).

Because Tn*5* insertion in the region of the right direct repeat reduced the level of tetracycline resistance expressed in plasmid pDP1Δ4 (Fig. 2), we investigated the possibility that an adjacent promoter function was being interrupted. The 2.3-kb *Eco*RI-*Hin*dIII fragment from plasmid pOA0 was cloned in opposite orientations downstream from the *lac* promoter in plasmids pUC8 and pUC9 to yield plasmids pOA8 and pOA9, respectively. These were constructed by complete *Hin*dIII-*Eco*RI digestion of pOA0, pUC8, and pUC9 with subsequent ligation. Strain JM83 was transformed with the ligation products, selected for penicillin resistance, and screened for lack of β-galactosidase production on plates coated with the *lac*$^+$ color indicator X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside; Bethesda Research Laboratories). There was no difference in the MICs of tetracycline for *E. coli* JA221, HB101 Nal^r or JM83 with either pOA8, or pOA9. The MICs for all strains were higher than with pOA0, and they grew well in 24 h on plates with 20 μg of tetracycline per ml. Both HB101(pOA8) and HB101(pOA9) were sensitive to tetracycline when grown anaerobically.

Clindamycin resistance gene. The effect of Tn*5* insertion on the expression of clindamycin resistance in *Bacteroides* spp.

TABLE 1. Location and antibiotic sensitivity testing of Tn*5* inserts[a]

pDP1Δ::Tn*5* insert	Distance from *Cla*I (bp)[b]	Sensitivity to antibiotic (μg/ml):[c]		
		Tc (5)	Tc (20)	Cl (5)
67	−2,340	R	R	R
94	−1,840	R	R	S
81	−1,590	R	R	S
68	−1,490	R	R	S
50	−1,440	R	R	S
75	−1,140	R	R	S
6	−435	S	S	R
52	+185	S	S	R
73	+215	S	S	R
48	+265	S	S	R
35	+275	S	S	R
12	+300	S	S	R
11	+315	S	S	R
31	+815	R	WR	R
2	+985	R	WR	R
24	+1,085	R	WR	R
32	+1,800	R	WR	R

[a] As shown in Fig. 2.
[b] − indicates distance to the left, and + indicates distance to the right in Fig. 2.
[c] Abbreviations: R, resistant (+3/+3 growth); S, sensitive; WR, weakly resistant (+1/+3 growth). See the text for details of sensitivity testing.

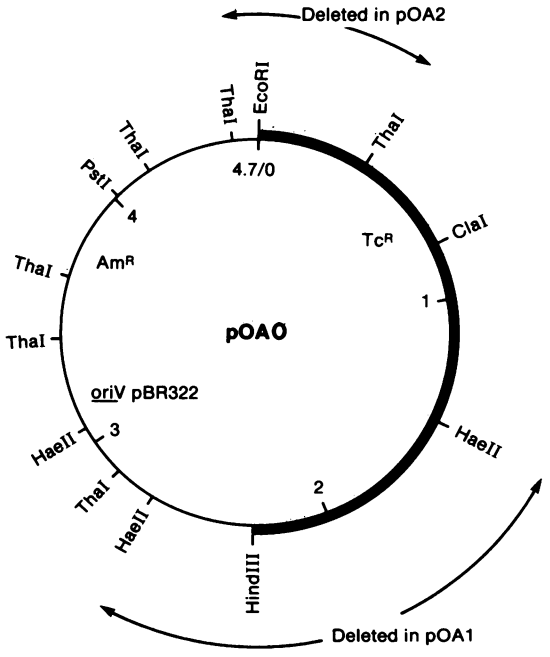

FIG. 3. Map of the *Eco*RI-*Hin*dIII fragment (▬) containing the Tc^r region (Tc^R) cloned onto pDGV1 (——) to form pOA0. The regions deleted to form pOA1 and pOA2 are indicated.

TABLE 2. Level of tetracycline resistance expressed by plasmids containing the cloned Tc^r region from pDP1Δ4

E. coli strain (plasmid)	Tetracycline MIC (μg/ml)[a]
HB101 Nal^r	3.6
HB101 Nal^r(pDP1Δ4::Tn*5*)[b]	40
HB101 Nal^r(pOA1)	3.6
HB101 Nal^r(pOA2)	10
HB101 Nal^r(pOA0)	12.5
JM83	<5
JM83(pOA8)	50
JM83(pOA9)	50

[a] MIC was determined by tube dilution in broth with an inoculum of 10^7 cells.

[b] Plasmid pDP1Δ4::Tn*5* contains a random insertion of Tn*5* distant from the Tc^r gene on the *Eco*RI B fragment.

was studied by transferring the various pDP1Δ4::Tn*5* plasmids via conjugation into *B. fragilis* 638 (Table 3). Triparental matings were performed with pRK231 to mobilize the pDP1Δ4::Tn*5* plasmids into *B. fragilis*. Cl^r recipients from HB101(pDP1Δ::Tn5) had an average frequency of transfer of 10^{-7} to 10^{-6}, compared with 10^{-6} to 10^{-5} for HB101(pDP1Δ4). The inability to obtain Cl^r transconjugants of *B. fragilis* 638 in at least three separate mating attempts with appropriate positive controls indicated a disruption either in the Cl^r gene or in the region required for replication in *B. fragilis*. Insertion of Tn*5* in the *Eco*RI B fragment between insert 75 at 1.14 kb and insert 94 at 1.84 kb from the *Cla*I site in the direction of the *Hin*dIII sites (Fig. 2) interrupted expression of the clindamycin resistance gene (Table 1). Flanking this region, the closest Tn*5* inserts occurred at 0.44 kb (insert 6) and 2.34 kb (insert 67) from the *Cla*I site and did not interfere with gene expression (Tables 1 and 3). These inserts define the Cl^r region on the B fragment.

In addition, clindamycin-resistant *Bacteroides* transconjugants could not be recovered when Tn*5* was inserted in the *Eco*RI A fragment of two plasmids, inserts 69 and 88, at 3.45 and 3.90 kb, respectively, from the *Cla*I site (Table 3). The *Eco*RI A fragment is required for replication in *Bacteroides* spp. but has not been implicated in Cl^r (5). Adjacent Tn*5* inserts at 3.01 and 5.42 kb from *Cla*I and all other inserts in the A fragment did not affect the ability of the plasmid to replicate and express Cl^r in *B. fragilis*.

DISCUSSION

The effects on expression of tetracycline resistance in aerobic *E. coli* cells by random Tn*5* insertion into pDP1Δ4 locate the Tc^r gene in a 1-kb region extending to either side of the *Cla*I site on the *Eco*RI B fragment (Fig. 2). On the basis of the deletion analysis with plasmids pOA1, pOA2 and pOA0, one end of the gene probably lies in the 560-bp area between the *Hae*II site and the adjacent Tn*5* insert 75. The opposite end is between the *Tha*I site and the adjacent Tn*5* insert 11 (Fig. 2). Using heteroduplex and restriction enzyme analysis, Smith and Gonda (13a) found that pBFTM10 (a plasmid similar to pCP1) differs from pBF4 in this region by a 100-bp deletion between the *Hae*III and *Eco*RI sites. Because both pCP1 and pBF4 express Tc^r, the deletion must be in a nonessential region; this is in agreement with the map in Fig. 2, which places one end of the gene close to the *Tha*I site. The Tn*5* insertions to the right of the *Tha*I site (inserts 31, 2, 24, and 32) in the region of the direct repeat decrease but do not abolish the expression of Tc^r. In addition, this region is deleted when the *Eco*RI-*Hin*dIII fragment containing Tc^r is cloned in pDGV1 to form pOA0, which expresses only low-level Tc^r. These results indicate that the region of the right-hand direct repeat is needed for full expression of Tc^r. The region may contain one or more promoters whose function strongly influences transcription of the Tc^r gene. The Tc^r gene probably has a secondary promoter located close to the gene, because pOA2 expresses low-level resistance, and cloning the Tc^r gene in either orientation with respect to the *lac* promoter of pUC9 (pOA8 and pOA9) resulted in equal expression of Tc^r (Table 2). The increased but constant level of Tc^r seen in both pOA8 and pOA9 could be the result of the high copy number of these plasmids. Alternatively, the direct repeat may enhance Tc^r expression by a mechanism other than transcription. The expression of Tc^r by pCP1 is constitutive (4, 5) and represents an unusual control system for Tc^r gene expression, since most Tc^r in both gram-positive and gram-negative organisms is inducible and regulated by a repressor protein (1). The results for pCP1 indicate that the region of the right-hand direct repeat has a positive effect on Tc^r expression, although the mechanism is still unknown.

By studying the effects of deletions on the function of the parent plasmid pDP1, we tentatively located the clindamycin resistance gene in the area of the *Hin*dIII sites on the *Eco*RI B fragment of pCP1 (5). The map of the Tn*5* inserts (Fig. 2) which interfered with expression of clindamycin resistance has located this gene more precisely, showing that it covers at least 700 bp in this region. The clindamycin resistance region of pCP1, homologous to those of pBF4 and pBI136, contains a *Hin*dIII and *Ava*II site (14). Plasmids pBF4 and pCP1 differ in this area by a 500-bp insert on pCP1, which gives pCP1 a second *Hin*dIII site close to the direct repeat (Smith and Gonda, in press). Because neither pBF4 nor pBI136 has this second *Hin*dIII site and still expresses clindamycin resistance, the end of this gene on pCP1 probably lies in the 0.26-kb area that extends from its additional *Hin*dIII site to the adjacent Tn*5* insert 94 at 1.84 kb from *Cla*I. The Tn5 inserts clearly show that the Cl^r and Tc^r determinants are separate genetic loci on the *Eco*RI B fragment and are probably transcribed from individual promoters, since none of the Tn*5* inserts in one gene shows polar effects on the other resistance loci.

Earlier work with plasmid pDP1 revealed that some of the

TABLE 3. Conjugal transfer frequencies of selected pDP1Δ4::Tn*5* plasmids into *B. fragilis* 638

PDP1Δ4::Tn*5* insert	Distance from *Cla*I (bp)[a]	Transfer frequency[b]
67	−2,340	1.0×10^{-7}
94	−1,840	$<1.0 \times 10^{-10}$
81	−1,590	$<1.0 \times 10^{-10}$
68	−1,490	$<1.0 \times 10^{-10}$
50	−1,440	$<1.0 \times 10^{-10}$
75	−1,140	$<1.0 \times 10^{-10}$
6	−435	2.3×10^{-6}
52	+185	4.2×10^{-7}
35	+275	2.0×10^{-6}
24	+1,085	2.7×10^{-7}
63	+2,400	1.0×10^{-7}
69	+3,450	$<1.0 \times 10^{-10}$
88	+3,900	$<1.0 \times 10^{-10}$

[a] −, Distance to the left of *Cla*I in Fig. 2; +, distance to the right of *Cla*I in Fig. 2.

[b] Number of Cl^r *B. fragilis* 638 transconjugants divided by the total number of *B. fragilis* 638 recipients recovered at the end of the mating.

difficulties with transfer of antibiotic resistance between *E. coli* and *Bacteroides* spp. were caused by differences in the replication functions of the plasmids (14). pDP1 could not be isolated in *Bacteroides* spp. after deletion of the whole *Eco*RI A fragment. It was suspected that an origin of replication on pDP1Δ4, required in *Bacteroides* spp., was located between its *Bgl*II site and the *Eco*RI site adjacent to the B fragment. The function of this replication region was interrupted in two plasmids where Tn*5* was inserted into the *Eco*RI A fragment of pDP1Δ4. We can tentatively locate an essential replication region in *Bacteroides* spp. at 2.0 to 2.5 kb clockwise from the *Bgl*II site (Fig. 1).

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