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Plasmid transfer from *Escherichia coli* to *Bacteroides fragilis*: Differential expression of antibiotic resistance phenotypes

(bacterial conjugation/broad host range plasmid/RK2/anaerobic bacteria/shuttle vector)

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ABSTRACT **A unique shuttle plasmid, pDP1, has been constructed to mediate gene transfer between *Escherichia coli* and the Gram-negative anaerobe *Bacteroides fragilis*. pDP1 contains the pBR322 replicon and the *Bacteroides* clindamycin resistance plasmid pCP1 linked to the transfer origin of the broad host range plasmid RK2. pDP1 can be transferred from *E. coli* to *B. fragilis* by the RK2 conjugation system even though RK2 itself is not maintained in the *Bacteroides* recipients. The antibiotic resistance and replication functions of pDP1 have been mapped by deletion analysis, and a 5-kilobase portion of the plasmid has been identified as the essential region for maintenance in *Bacteroides*. Comparison of the resistance conferred by pDP1 on *E. coli* and *B. fragilis* shows that antibiotic resistance genes are expressed differently in aerobic and anaerobic bacteria. These results document the feasibility of gene transfer from *E. coli* to *B. fragilis* and demonstrate the usefulness of this conjugation system to study genetic structure and expression in *Bacteroides*.**

Studies of genetic exchange between aerobic and anaerobic bacteria have produced inconclusive results. Systematic efforts to transfer well-characterized antibiotic resistance plasmids from *Escherichia coli* to *Bacteroides fragilis* have been unsuccessful, despite the fact that the *B. fragilis* group of Gram-negative anaerobes contains the most numerous organisms in the human colon, where they are in close contact with *E. coli* (1–3). Transfer of drug resistance from *Bacteroides* to *E. coli* has been reported, but the plasmids involved have not been characterized and no further work with these strains has been published (4–6). In an earlier study, we identified a large conjugative plasmid in *Bacteroides ochraceus* (subsequently renamed *Capnocytophaga ochraceus*) capable of transferring multiple antibiotic resistance to *E. coli* (7). However, this R plasmid could not be transferred into intestinal *Bacteroides*, a finding consistent with the subsequent classification of *C. ochraceus* as an oral microaerophilic organism, distinct from the strict anaerobes (2). Only one report has described transfer of antibiotic resistance from *E. coli* to *Bacteroides*, and the plasmid involved was not identified or characterized (8). We have tested plasmids from 14 different incompatibility groups in *E. coli* (*Inc*A-C, B, FII, FIV, H, I, K, L, N, P, U, W, Y, and 9) for transfer to *B. fragilis*, but none could be detected in the *Bacteroides* recipients (3). We have concluded that there is a substantial barrier to plasmid transfer between *E. coli* and *B. fragilis*. This barrier could operate at the level of plasmid DNA transfer during conjugation, plasmid DNA replication in the new host, or the expression of antibiotic resistance genes in the *Bacteroides* recipient.

To study this problem, we have constructed a unique shuttle vector to mediate gene transfer between *E. coli* and *B. fragilis*. This transfer system utilizes the conjugation functions of the broad host range plasmid RK2, a member of the P incompatibility group. We chose the conjugation system of RK2 because it is particularly well adapted to overcome species barriers and can mediate gene transfer between a wide variety of Gram-negative bacteria (3). In earlier studies, we mapped, cloned, and sequenced the origin of conjugal DNA transfer (*ori*T) of RK2 (9), which is contained within a 760-base-pair *Hae* II restriction fragment. Small vector plasmids containing this *oriT* sequence are transferred at high frequency by the RK2 conjugation system provided by an RK2 "helper" plasmid present in the same cell (9). Accordingly, we constructed a hybrid plasmid containing replicons from *E. coli* and *B. fragilis* linked to the *ori*T fragment from RK2. This shuttle vector can be transferred from *E. coli* to *B. fragilis* by the RK2 conjugation system. Furthermore, experiments with this hybrid plasmid show that differences in plasmid replication and the expression of drug resistance genes are both substantial barriers to genetic exchange between aerobic and anaerobic bacteria.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* strain used as a donor for the matings was JA221, *leu*B, Δ *trp*E5, *lac*Y, *rec*A, *hsd*R$^-$, *hsd*M$^+$. Recipients were *E. coli* HB101 *nal*^{R} (9), *B. fragilis* 638 *rif*^{R} (10), and *B. fragilis* TM4000 *rif*^{R} (11). *E. coli* K802, used for antibiotic sensitivity testing, is *gal*, *met*, *sup*E, *hsd*R$^-$, *hsd*M$^+$. pRK231 is a derivative of RK2 with a *Pst* I-generated deletion in the ampicillin resistance gene; its phenotype is Km^R, Tc^R, Tra$^+$ (9). pDG5 is a derivative of pBR322 lacking tetracycline resistance and containing the 760-base-pair *Hae* II *ori*T fragment from RK2 (9). pCP1 is a 15-kilobase (kb) clindamycin resistance plasmid originally isolated from *Bacteroides thetaiotaomicron* (13).

Plasmid DNA Procedures. Plasmid DNA was purified from *E. coli* and *B. fragilis* by CsCl/ethidium bromide density gradient centrifugation as described (9, 11). Standard methods for restriction enzyme digestion, DNA ligation, agarose gel electrophoresis, and bacterial transformation were followed (12). The hybrid plasmid pDP1 was constructed by ligating a partial *Eco*RI digestion of pCP1 with a complete *Eco*RI digestion of pDG5. The ligation mix was transformed into *E. coli* JA221 selecting for tetracycline resistance at 10 μg/ml. To delete the *Eco*RI C and A fragments from pDP1, the plasmid was partially digested with *Eco*RI, religated, then digested to completion with *Hpa* I, and transformed into *E. coli* JA221. To generate the *Bgl* II and *Hin*dIII deletions, pDP1 was digested separately to completion with *Bgl* II or *Hin*dIII, then religated, and transformed into *E. coli* JA221.

Bacterial Matings. Matings between *E. coli* strains were done by a standard filter mating procedure for 3 hours with the transfer frequency calculated as described (9). Matings between *E. coli* and *B. fragilis* were performed according to a modification of the plate mating procedure of Privitera *et*

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Abbreviation: kb, kilobase(s).

al. (10). The *E. coli* donor strain was grown overnight in brain heart infusion broth, supplemented with hemin and cysteine (BHC, described in ref. 11), and containing selective antibiotics (penicillin, 200 μg/ml for pDP1; kanamycin, 50 μg/ml for pRK231). The *B. fragilis* recipient was grown overnight in BHC without antibiotics. The overnight donor culture was washed, diluted, and grown to early logarithmic phase on an aerobic shaker. The recipient was diluted and grown to stationary phase in an anaerobic jar (7). The donor and recipient cultures were mixed in a 1:2 ratio; 0.15 ml of this mating mix was spread on the surface of a BHC plate and incubated anaerobically overnight. The entire confluent growth was scraped from the plate, suspended in saline, diluted, and plated on BHC containing 5 μg of clindamycin per ml and 20 μg of nalidixic acid per ml (selection for pDP1 transfer) or 1 μg of tetracycline per ml and 20 μg of nalidixic acid per ml (selection for pRK231 transfer). Transfer frequency is the number of transconjugants containing the indicated plasmid divided by the total number of *B. fragilis* recipients recovered at the end of mating. The *B. fragilis* transconjugants were checked for lack of aerobic growth and purified by several subcultures prior to the isolation of plasmid DNA. Later mating experiments with pDP1 and its deletion derivatives were performed by a triparental mating procedure: the pRK231 helper plasmid in *E. coli* HB101 and pDP1 (or a derivative) in *E. coli* JA221 were mixed with the *B. fragilis* recipient for mating on plates as described above.

RESULTS

Construction and Properties of pDP1. To construct a hybrid plasmid capable of replication in both *E. coli* and *B. fragilis*, we ligated the *Bacteroides* R plasmid pCP1 to the *E. coli* pBR322 derivative, pDG5. pCP1 encodes high-level resistance to erythromycin and clindamycin by means of determinants located in the *Eco*RI B fragment between direct repeats (13). This *Eco*RI B fragment also contains a cryptic tetracycline resistance determinant that is expressed when cloned in *E. coli* but is not expressed by pCP1 in *Bacteroides* (14). We have used this tetracycline resistance phenotype to clone the entire pCP1 plasmid in *E. coli* linked to the vector pDG5, a pBR322 derivative that has been deleted for the pBR322 tetracycline resistance region and contains the transfer origin (*oriT*) of the broad host range plasmid RK2 (9). Fig. 1 shows the hybrid plasmid pDP1 with pDG5 inserted into the *Eco*RI site between fragments B and C of pCP1. We also obtained another plasmid, designated pDP1.1, with pDG5 inserted between *Eco*RI fragments A and C. pDP1 is dependent on the pBR322 replicon in *E. coli*, since pDP1 could not be transformed into the *pol*A mutant of *E. coli*, C2110. In addition, we were unable to transform the native pCP1 plasmid into *E. coli*, selecting for tetracycline resistance. These results show that the *Bacteroides* plasmid pCP1 cannot replicate in *E. coli*.

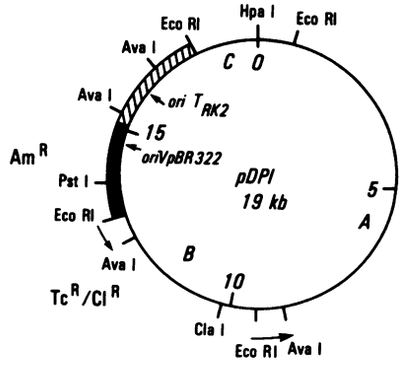

FIG. 1. Map of pDP1. The *Bacteroides* plasmid pCP1 is shown by the thin line; A, B, and C refer to the three *Eco*RI fragments of pCP1. The arrows show the locations of the direct repeats of pCP1 (13). The *E. coli* plasmid pDG5 is shown by the thick line: the solid portion represents the region of pBR322 containing the origin of replication (*oriV*) and the TEM β-lactamase gene (Am^R), and the hatched line shows the RK2 DNA with its origin of transfer (*oriT*, ref. 9). Fragment B encodes clindamycin resistance (Cl^R) in *Bacteroides* and tetracycline resistance (Tc^R) in *E. coli* (refs. 13 and 14 and Table 3). In pDP1.1, the pDG5 DNA is inserted in the *Eco*RI site between fragments A and C of pCP1.

Transfer of pDP1 to *Bacteroides*. The data in Table 1 show that pDP1 can be transferred at a high frequency in *E. coli* by the RK2 helper plasmid pRK231, which is also co-transferred at about the same frequency. Both pDP1 and pDP1.1 could be transferred into *B. fragilis* with a frequency of approximately 10^{-6}. pDP1 was transferable into two different *B. fragilis* recipients, 638 and TM4000, at the same frequency. pDP1 transfer into both the *E. coli* and *B. fragilis* recipients was completely dependent on the helper plasmid, pRK231. However, no transfer of pRK231 into *B. fragilis* could be detected by selecting for the tetracycline resistance on pRK231. Plasmid DNA extracted from the clindamycin-resistant *Bacteroides* transconjugants revealed that pDP1 was transferred and maintained intact in *B. fragilis*. Fig. 2 shows that the pDP1 DNA extracted from *B. fragilis* 638 after transfer from *E. coli* JA221 is identical to the pDP1 DNA present in the *E. coli* donor. This pDP1 DNA from *Bacteroides* was transformed back into *E. coli* and expressed both ampicillin and tetracycline resistance. However, none of 10 *B. fragilis* transconjugants (selected for pDP1 transfer) contained pRK231 DNA. Since pRK231 is nearly 100% co-transferred with pDG5 in *E. coli* matings (9), these results suggest that pRK231 cannot be maintained in *B. fragilis*.

Properties of pDP1 Deletions. To reduce the size of pDP1 and locate the region essential for replication in *Bacteroides*, we isolated the deletions shown in Fig. 3. In pDP1 Δ1, the *Eco*RI C fragment is deleted, whereas in pDP1 Δ2, both the A and C fragments have been removed. pDP1 Δ3 contains a 3.3-kb *Hin*dIII-generated deletion from fragment C extending into the adjacent A fragment and also a small internal deletion of 0.7 kb in the B fragment. pDP1 Δ4 has a large 4.6-kb *Bgl* II deletion extending further into the A fragment. All four deletion plasmids contain pDG5 with *oriT* as well as an intact *Eco*RI B fragment expressing tetracycline resistance in *E. coli*. Table 2 shows the transfer data for these plasmids into *B. fragilis*. pDP1 Δ1 and pDP1 Δ4 can still be transferred to *Bacteroides*, whereas transfer of pDP1 Δ2 and pDP1 Δ3 could not be detected. Both pDP1 Δ1 and pDP1 Δ4 could be recovered intact from the *Bacteroides* transconjugants and transformed back into *E. coli*. These results show that the C fragment and an adjacent 3-kb portion of the A fragment are not required for replication in *Bacteroides*. Since pDP1 Δ2 cannot be transferred to *B. fragilis*, the essential region for

Table 1. Conjugal transfer of pDP1 and pDP1.1

Plasmids in *E. coli* JA221 donor	Recipient	Transfer frequency pDP1	Transfer frequency pRK231
pDP1	*E. coli* HB101 nal^R	$<10^{-8}$	—
+ pRK231	*E. coli* HB101 nal^R	0.7	1
pDP1	*B. fragilis* 638	$<10^{-8}$	—
+ pRK231	*B. fragilis* 638	3×10^{-6}	$<10^{-8}$
+ pRK231	*B. fragilis* TM4000	3×10^{-6}	—
pDP1.1 + pRK231	*B. fragilis* TM4000	0.6×10^{-6}	—

Transfer frequency is the number of transconjugants containing the indicated plasmid divided by the total number of *E. coli* or *B. fragilis* recipients recovered at the end of the mating. pDP1 transfer was selected by penicillin resistance in *E. coli* and clindamycin resistance in *B. fragilis*. pRK231 transfer was selected by kanamycin resistance in *E. coli* and tetracycline resistance in *B. fragilis*.

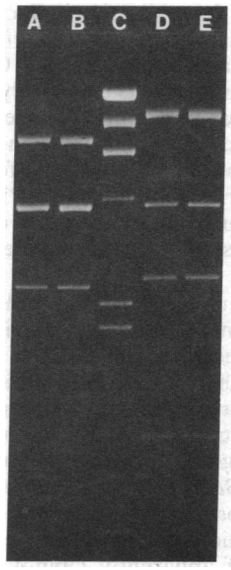

FIG. 2. Restriction digestion of pDP1 extracted from *B. fragilis* 638 and *E. coli* JA221. Lanes A and B are *Eco*RI digests, and lanes D and E show *Ava* I digests of pDP1 extracted from *B. fragilis* (lanes A and D) or *E. coli* (lanes B and E). The middle band in the *Eco*RI digests is a doublet containing fragment B and pDG5 (see Fig. 1). Lane C is a *Hin*dIII digest of λ DNA.

maintenance of pDP1 in *Bacteroides* is most likely located in a 5-kb region of fragment A between coordinates 4 and 9 kb on the map.

Expression of pDP1 Resistance Genes in *E. coli* and *B. fragilis*. Since pDP1 can replicate in both *E. coli* and *B. fragilis*, expression of the drug resistance genes on the plasmid was tested in these hosts. Table 3 shows that the TEM β-lactamase gene from pBR322 confers high-level ampicillin resistance on *E. coli* but gives no resistance to *Bacteroides*. The erythromycin resistance determinant from the *Bacteroides* plasmid pCP1 is not expressed in *E. coli* although it gives high-level resistance to *B. fragilis*. The cryptic tetracycline resistance determinant, encoded by *Eco*RI fragment B of pCP1 (see Fig. 1), is expressed in *E. coli* but not in *Bacteroides*. A particularly interesting feature of this tetracycline

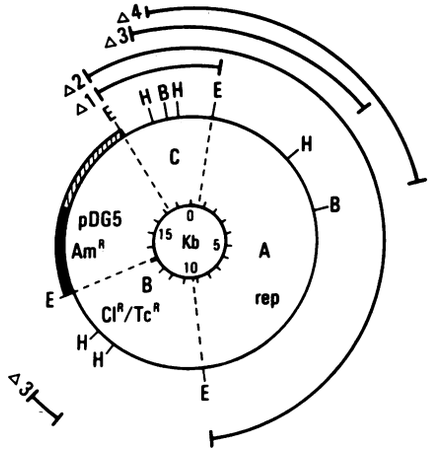

FIG. 3. Deletions of pDP1. The *Eco*RI fragments A, B, C, and pDG5 are shown as in Fig. 1. Restriction sites are indicated by the following letters: E, *Eco*RI; B, *Bgl* II; and H, *Hin*dIII. The DNA deleted in Δ1, Δ2, Δ3, and Δ4 is represented by the bars. Two additional *Bgl* II sites in the A fragment deleted in Δ4 are not shown. *rep* refers to the region of fragment A required for replication in *Bacteroides* (see text).

Table 2. Transfer of pDP1 deletions to *B. fragilis*

Plasmid in *E. coli* JA221	Transfer frequency to *B. fragilis* 638
*Eco*RI deletion*	
pDP1	8×10^{-6}
pDP1 Δ1	4×10^{-6}
pDP1 Δ2	$<10^{-8}$
*Hin*dIII and *Bgl* II deletions†	
pDP1	0.6×10^{-6}
pDP1 Δ3	$<10^{-8}$
pDP1 Δ4	1.2×10^{-6}

*Matings were done with the helper plasmid pRK231 present in the same cell, as described in *Materials and Methods* and in Table 1.
†Matings were done by using the triparental cross procedure: pDP1 or the deletion derivative in *E. coli* JA221 was mated with *E. coli* HB101 containing pRK231 and *B. fragilis* 638. The transfer frequencies using this triparental technique are somewhat lower, but pDP1 and the deletions are more stable in *E. coli* in the absence of the helper plasmid pRK231.

resistance region of pCP1 is that it is not expressed in *E. coli* when the cells are grown anaerobically (14). Table 3 also shows the level of tetracycline resistance encoded by pDP1 under various growth conditions in *E. coli*. Even the addition of nitrate as an alternative electron acceptor did not restore tetracycline resistance expression under anaerobic conditions. These results suggest that this differential expression of tetracycline resistance is not due to differences in the availability of energy or electron transport during anaerobic growth. Furthermore, the failure of *Bacteroides* to express tetracycline resistance was not due to instability or deletion of this region, since pDP1 isolated from *B. fragilis* could be transformed back into *E. coli* with full expression of tetracycline resistance.

DISCUSSION

These results document the feasibility of plasmid transfer from *E. coli* to *Bacteroides*. Both *E. coli* and the *B. fragilis* groups of anaerobes are present in large numbers in the human colon and also frequently co-exist in infected tissues. Since plasmid-mediated transfer of antibiotic resistance is widespread in aerobic and facultative bacteria, we and others anticipated that R plasmids would transfer between these Gram-negative bacteria that share the same ecological niche. However, systematic studies with both narrow and broad

Table 3. Expression of antibiotic resistance by pDP1 in *E. coli* and *B. fragilis*

Bacterial strain	Growth condition*	Minimal inhibitory concentration, μg/ml		
		Am	Em	Tc
E. coli	$+O_2$	10	70	2
+ pDP1	$+O_2$	>2000	70	40
+ pDP1	$-O_2$	—	—	1
+ pDP1	$-O_2$, $+NO_3$	—	—	1
B. fragilis	$-O_2$	20	<1	<1
+ pDP1	$-O_2$	20	>320	<1

The *E. coli* strain was K802; the *B. fragilis* was strain 638. The minimal inhibitory concentration was determined by a standard tube dilution technique (11). Am, ampicillin; Em, erythromycin; Tc, tetracycline. Although exact minimal inhibitory concentration values of Am and Em were not determined for *E. coli* grown anaerobically, no substantial differences from the aerobic values were noted.

*$+O_2$ refers to growth in atmospheric oxygen, $-O_2$ is growth in anaerobic jars (7), and $+NO_3$ refers to supplementation with 0.2% sodium nitrate. The medium used throughout was brain heart infusion broth supplemented with hemin and cysteine (11).

host range conjugative plasmids of *E. coli* have failed to detect transfer to *Bacteroides* (1–3). The results described here with pDP1 clarify the genetic basis for this barrier to gene exchange. Although the broad host range conjugation system of RK2 can mediate transfer of plasmid DNA from *E. coli* to *B. fragilis*, differences in plasmid replication and gene expression prevent its maintenance in *Bacteroides*. Despite the broad host range property of RK2 replication in aerobic bacteria (15), pRK231 DNA was never found in the *B. fragilis* transconjugants selected for pDP1 transfer. Although it is possible that pRK231 DNA is more sensitive to restriction systems in the *Bacteroides* recipient, it seems unlikely that it would be selectively degraded, while the pDP1 DNA remained intact. Furthermore, our inability to detect pDP1 Δ2 in *Bacteroides* (Table 2) indicates that pBR322 cannot maintain this hybrid plasmid in *B. fragilis*. pDP1 Δ2 is missing the A and C fragments but still contains the B fragment required for clindamycin resistance expression in *Bacteroides*. Similarly, the *Bacteroides* plasmid pCP1 cannot replicate in *E. coli* and requires the pBR322 replicon on pDP1 for maintenance. Therefore, the inability of plasmids to replicate in the foreign host constitutes one substantial barrier to plasmid transfer between *E. coli* and *Bacteroides*.

Differential gene expression (Table 3) represents a second major barrier to plasmid exchange between these bacteria. The TEM β-lactamase gene on pBR322 is widespread in aerobic bacteria and is expressed in diverse genera including many enterobacteriaceae, *Pseudomonas*, *Neisseria*, and *Hemophilus*. However, this gene confers no increment of resistance on *B. fragilis*. This result is similar to the lack of expression of the TEM β-lactamase gene in the Gram-positive organism *Bacillus subtilus* (16). The erythromycin/clindamycin resistance determinant encoded by fragment B of pDP1 has disseminated widely in *Bacteroides* but is not expressed in *E. coli*. Expression of phenotypically similar erythromycin resistance genes (encoding the macrolide-lincosamide-streptogramin B, or MLS, resistance pattern) from Gram-positive bacteria has been detected in *E. coli* (17, 18). However, the *Bacteroides* erythromycin resistance region does not share detectable DNA homology with the common MLS gene from streptococci, indicating that the *Bacteroides* gene is probably distinct from the Gram-positive MLS genes (11). Our earlier study had identified a cryptic tetracycline resistance determinant, encoded by the B fragment of pCP1 and expressed in *E. coli* but not *B. fragilis* (14). We are now able to show that the host-dependent expression of this tetracycline resistance region is not due to alteration or mutation of the fragment during cloning in *E. coli*. As shown in Table 3, pDP1 confers tetracycline resistance on *E. coli* but not *Bacteroides*. Furthermore, pDP1 isolated from *B. fragilis* transconjugants could be transformed back into *E. coli* with full expression of tetracycline resistance. The lack of expression in *B. fragilis* is due to the dependence of the resistance phenotype on aerobic growth of the host cell, since pDP1 does not express tetracycline resistance in *E. coli* under anaerobic growth conditions (ref. 14 and Table 3). Although nitrate can replace oxygen as the terminal electron acceptor in respiration, addition of nitrate to *E. coli* containing pDP1 did not restore expression of tetracycline resistance under anaerobic conditions. These results suggest that the defect in expression in anaerobic cells is related to the absence of oxygen rather than a block in the respiratory process.

Analysis of the deletions shown in Fig. 3 allows us to locate functional regions on the *Bacteroides* plasmid pCP1 contained in pDP1. Since fragment C and an adjacent 3.4-kb portion of fragment A (defined by the *Bgl* II deletion in pDP1 Δ4) are not required for replication in *Bacteroides*, the essential sequences for maintenance in *B. fragilis* lie in the remaining 5-kb region of fragment A. The small 0.7-kb *Hin*dIII deletion in fragment B of pDP1 Δ3 tentatively locates the clindamycin resistance coding region, since the larger 3.3-kb *Hin*dIII deletion occurs within the non-essential region of the A and C fragments and should not affect the maintenance of pDP1 Δ3 in *Bacteroides*. Since pDP1 Δ3 still expressed tetracycline resistance in *E. coli*, the cryptic tetracycline resistance gene must be distinct from the clindamycin resistance determinant.

These results demonstrate the usefulness of this unique gene transfer system for studying plasmid function and gene expression in anaerobic bacteria. The genetic analysis of *Bacteroides* and other anaerobes has been frustrated by the lack of a system for genetic manipulation. This obstacle can be overcome by the use of shuttle vectors like pDP1, which allow *Bacteroides* genes and plasmids to be cloned in *E. coli* and returned to *Bacteroides* for functional analysis. The broad host range properties of the RK2 conjugation system can mediate plasmid transfer from *E. coli* to *B. fragilis*, although RK2 itself apparently cannot replicate in *Bacteroides*. The transfer system of RK2 is well adapted to promote mating between unrelated types of aerobic Gram-negative bacteria (3). In addition, RK2-mediated plasmid transfer from *E. coli* to cyanobacteria has been reported (19). Our results further extend the host range of RK2 conjugation to include anaerobic bacteria. The use of a bifunctional vector plasmid containing two replicons and the RK2 transfer origin can facilitate gene transfer between bacteria that rarely, if ever, exchange plasmids under natural conditions.

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