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Conjugal Transfer of a Shuttle Vector from the Human Colonic Anaerobe *Bacteroides uniformis* to the Ruminal Anaerobe *Prevotella* (*Bacteroides*) *ruminicola* B_14

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Prevotella ruminicola **(formerly *Bacteroides ruminicola*) is an anaerobic, gram-negative, polysaccharide-degrading bacterium which is found in the rumina of cattle. Since *P. ruminicola* is thought to make an important contribution to digestion of plant material in rumina, the ability to alter this strain genetically might help improve the efficiency of rumen fermentation. However, previously there has been no way to introduce foreign DNA into *P. ruminicola* strains. In this study we transferred a shuttle vector, pRDB5, from the colonic species *Bacteroides uniformis* to *P. ruminicola* B_14. The transfer frequency was 10^{-6} to 10^{-7} per recipient. pRDB5 contains sequences from pBR328, a cryptic colonic *Bacteroides* plasmid pB8-51, and a colonic *Bacteroides* tetracycline resistance (Tc^r) gene. pRDB5 was mobilized out of *B. uniformis* by a self-transmissible *Bacteroides* chromosomal element designated Tc^r Em^r 12256. pRDB5 replicated in *Escherichia coli* as well as in *Bacteroides* spp. and was also mobilized from *E. coli* to *B. uniformis* by using IncP plasmid R751. However, direct transfer from *E. coli* to *P. ruminicola* B_14 was not detected. Thus, to introduce cloned DNA into *P. ruminicola* B_14, it was necessary first to mobilize the plasmid from *E. coli* to *B. uniformis* and then to mobilize the plasmid from *B. uniformis* to *P. ruminicola* B_14.**

Prevotella ruminicola is one of the species of obligate anaerobes in the rumina of cattle which ferment polysaccharides. This species was originally classified as *Bacteroides ruminicola*, but recent taxonomic studies have shown that this and other species previously placed in the genus *Bacteroides* belong in a new genus, which has been named *Prevotella* (8). Since the growth of cattle is dependent on bacterial fermentation products, fermentation of polysaccharides by ruminal bacteria such as *P. ruminicola* is of considerable economic interest. Although there have been some biochemical studies of polysaccharide utilization by *P. ruminicola* and a xylanase gene has been cloned from this species (17), work in this area has been hampered by the lack of any way to introduce cloned DNA into *P. ruminicola*. Recently, Flint et al. (2) found a conjugal plasmid in *P. ruminicola* 223 which carried a gene for tetracycline resistance (Tc^r) (pRRI4) and showed that this plasmid could be transferred into another *P. ruminicola* strain, strain B_14, by conjugation. Subsequently, Thomson and Flint (15) demonstrated that plasmid DNA isolated from a *P. ruminicola* B_14 transconjugant could be introduced into wild-type strain B_14 by electroporation. Although these results were important because they showed that DNA could be introduced into *P. ruminicola* by conjugation and electroporation, plasmid pRRI4 could not be used as a shuttle vector because it was relatively large (19.5 kb) and did not replicate in *Escherichia coli*.

Since *P. ruminicola* is related to the human colonic *Bacteroides* species, a group which is now genetically manipulable, it seemed possible that some of the vectors developed for use with colonic *Bacteroides* species might work for rumen *Bacteroides* species. These vectors contain DNAs from cryptic *Bacteroides* plasmids which are able to replicate in a number of different colonic *Bacteroides* species and thus appear to have a broad host range (5, 7, 15). The vectors also contain sequences that enable them to replicate in *E. coli* and to be mobilized by IncP plasmids (R751, RK2). These shuttle vectors are transferred from *E. coli* into colonic *Bacteroides* species by conjugation. These same vectors can also be transferred by conjugation from one colonic *Bacteroides* species to another by using conjugal chromosomal *Bacteroides* elements (10).

Whereas shuttle vectors have been transferred successfully by conjugation from *E. coli* to *Bacteroides* species and from one *Bacteroides* species to another, transformation or electroporation appear to have a much narrower range of effectiveness. Smith (13) showed that plasmid DNA could be introduced into some colonic *Bacteroides* species by polyethylene glycol-facilitated transformation if the DNA was isolated from the same strain. However, if the plasmid DNA was isolated from *E. coli* or from another *Bacteroides* species, the number of transformants dropped at least 10^3-fold. Similarly, Thomson and Flint (15) found that *E. coli*-colonic *Bacteroides* shuttle vector pDP1, which was isolated from *Bacteroides uniformis*, could be electroporated into *B. uniformis* at a frequency of 10^6 transporants per μg of DNA, whereas the same plasmid isolated from *E. coli* EM24 gave only 10^3 transporants per μg of DNA. Accordingly, we decided to focus on transferring DNA into *P. ruminicola* by conjugation rather than by electroporation.

We used two different types of mobilizing elements, both of which were chosen because of their ability to transfer DNA among diverse genera. First, IncP plasmids R751 and RP4 have been shown to mobilize DNA from *E. coli* to a variety of other species, including colonic *Bacteroides* species (7). Second, the conjugal chromosomal Tc^r elements found in the colonic *Bacteroides* species have been shown to be capable of mobilizing coresident plasmids to a variety of colonic *Bacteroides* species and to *E. coli* (5, 7, 14). In this

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s)[a]	Description and/or source	Reference(s)
Plasmids			
pVAL1	(Ap^r Tc^r) Em^r	Shuttle vector[b]	16
pRDB3	(Ap^r Tc^r) Tc^r Em^r	7-kb *Hin*cII fragment from Tc^r Em^r DOT cloned into pVAL1[b]	This study
pRDB5	(Ap^r Tc^r) Tc^r	Deletion of a 3-kb *Cla*I fragment from pRDB3[b]	This study
pFD160	(Ap^r)	C. J. Smith	13
pNFD13-2	(Ap^r) Tc^r	2.5-kb segment of Tc^r Em^r DOT element cloned into pFD160[b]	14
pEG920	(Ap^r Tc^r) Em^r	Shuttle vector which hybridizes with pVAL1 and pRDB5	11
pEG920::XBU4422	(Ap^r) Em^r Tra$^+$	pEG920 containing an insertion of a cryptic conjugal *Bacteroides* element which hybridizes with the Tc^r and Tc^r Em^r elements	11
pRRI4	Tc^r Tra$^+$	Transmissible 19.5-kb plasmid isolated from a *P. ruminicola* strain	2
R751	(Tp^r Tra$^+$)	Plasmid used to mobilize pRDB5, pVAL1, pNFD13-2, and pRDB3 from *E. coli* to *B. uniformis*	10
E. coli strains			
HB101	(RecA$^-$ Str^r)	H. Boyer	1
EM24	(RecA$^-$ Str^r)	RecA$^-$ derivative of LE392	4, 9
S17-1	(RecA$^-$ Tp^r Str^r ΩRP4-Tc::Mu-Kn::Tn*7*)	IncP RP4 inserted into chromosome, R. Simon	12
Bacteroides chromosomal elements			
Tc^r Em^r DOT	Tc^r Em^r Tra$^+$ Mob$^+$, Tc-inducible Tra and Mob functions	Chromosomal conjugal element from which the Tc^r region in pRDB5, pNFD13-2, and pRDB3 was cloned	9
Tc^r Em^r 12256	Tc^r Em^r Tra$^+$ Mob$^+$, constitutive Tra and Mob functions	Chromosomal conjugal element used to mobilize pRDB5, pNFD13-2, and pVAL1	7, 16
B. uniformis strains			
1001	Rif^r	N. B. Shoemaker et al.	10
1100	Tp^r Thy$^-$ Rif^s	N. B. Shoemaker et al.	10
1008	Tc^r Em^r Rif^r	*B. uniformis* 1001 carrying the Tc^r Em^r 12256 element	16
1108	Tc^r Em^r Tp^r, Thy$^-$ Rif^s	*B. uniformis* 1100 carrying the Tc^r Em^r 12256 element	16
1108(pRDB5)	Same as *B. uniformis* 1108	*B. uniformis* 1108 carrying pRDB5	This study
1108(pVAL1)	Same as *B. uniformis* 1108	*B. uniformis* 1108 carrying pVAL1	This study
1108(pNFD 13-2)	Same as *B. uniformis* 1108	*B. uniformis* 1108 carrying pNFD13-2	This study
P. ruminicola strains			
$B_1$4	Tc^s Em^s Rif^s Thy$^+$	M. Bryant[c]	
$B_1$4R	Tc^s Em^s Rif^r Thy$^+$	Rif^r derivative of *P. ruminicola* $B_1$4, recipient in matings with *B. uniformis* strains	This study
223	Tc^r	Strain from which pRR14 was originally isolated	14

[a] Phenotypes in parentheses are expressed only in *E. coli* and not in *Bacteroides* species. Phenotypes not in parentheses are expressed in *Bacteroides* species. Abbreviations: Ap, ampicillin; Tc, tetracycline; Tp, trimethoprim; Str, streptomycin; Em, erythromycin; Rif, rifampin; Thy, thymidine; Mob, ability to mobilize coresident plasmids; Tra, ability to self-transfer.

[b] See Fig. 1.

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paper we describe the first transfer of a shuttle vector from a strain of *B. uniformis* to rumen strain *P. ruminicola* $B_1$4.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are described in Table 1. *P. ruminicola* $B_1$4 was obtained from Marvin Bryant (Department of Animal Sciences, University of Illinois, Urbana). *B. uniformis* 0061 was originally obtained from the culture collection of the Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg.

Three types of media were used to cultivate *P. ruminicola*. In all cases the medium was made in glass tubes sealed with rubber stoppers. The atmosphere was 80% nitrogen–20% carbon dioxide. The broth medium used to grow *P. ruminicola* prior to mating, modified M10 medium (MM10), was similar to the M10 medium described in the *Anaerobe*

Laboratory Manual (3), except that the concentrations of Trypticase and yeast extract were increased 10-fold and amylopectin was used instead of glucose as the carbohydrate source. Also, titanium citrate (0.15 M) was added dropwise until the resazurin became colorless (usually 0.2 to 0.3 ml per liter of medium) prior to the addition of cysteine. The pH of this medium was 6.5 to 6.6.

The medium used for the matings, modified E agar medium, was similar to the Sweet E medium described in the *Anaerobe Laboratory Manual* (3), except that glucose was the only carbohydrate used and thymidine was added to a final concentration of 100 μg/ml. Agar was added to a final concentration of 2%. The final pH of this medium was 6.8. Modified E medium was used for mating, and the thymidine was needed to support the growth of the donor, which was a thymidine-requiring (Thy^-) strain of *B. uniformis*.

The third medium was MM10 to which agar (final concentration, 2%) and rifampin (final concentration, 40 μg/ml) were added (MM10-Rif medium). The pH of this medium was 6.2. MM10-Rif medium was used in roll tubes to enumerate the *P. ruminicola* recipients after mating. For selection of transconjugants, either tetracycline (final concentration, 5 μg/ml) or erythromycin (final concentration, 5 μg/ml) was added to MM10-Rif medium; these selective media were designated MM10-Rif-Tc and MM10-Rif-Em, respectively.

A spontaneous rifampin-resistant (Rif^r) mutant of *P. ruminicola* B_14 was isolated by inoculating the bacteria into MM10 broth media containing progressively higher concentrations of rifampin. This mutant, *P. ruminicola* B_14R, could grow in the presence of rifampin concentrations as high as 60 μg/ml. To ascertain that the spontaneous Rif^r mutant was actually a derivative of *P. ruminicola* B_14, we compared its *Not*I digest pattern with that of the original strain B_14 (see below) and found that the patterns were identical.

B. uniformis 1108 (Thy^-) was grown in Trypticase-yeast extract-glucose broth (3) or agar medium to which thymidine (final concentration, 100 μg/ml) was added. This medium also contained vitamin K_3 (final concentration, 1 μg/ml). Since the absence of vitamin K_3 and thymidine was an important part of the selection process, we designated this medium TYG-Thy-K to emphasize these two components. The final pH of this medium was 7.0 to 7.3. The gas phase was 80% nitrogen–20% carbon dioxide. *E. coli* strains were grown in Luria broth or on Luria broth agar plates.

Antibiotic susceptibility of *P. ruminicola* B_14. MICs for various antibiotics were determined by inoculating MM10 containing different concentrations of antibiotics and incubating the preparations for 48 h. The antibiotic concentrations tested were 5, 10, 20, 50, 100, and 200 μg/ml. In the case of tetracycline and erythromycin, resistance levels on MM10 agar were also determined.

Construction of *B. uniformis* and *E. coli* donor strains. pRDB5, pVAL1, and pNFD13-2 (Fig. 1) were introduced into *E. coli* DH5αMCR (Bethesda Research Laboratories) or S17-1 (11) by transformation. When strain DH5αMCR was the donor, IncP mobilizing plasmid R751 was introduced by conjugation (8, 14). Strain S17-1 has a copy of IncP plasmid RP4 inserted in its chromosome. Both R751 and RP4 mobilize pRDB5, pVAL1, and pNFD13-2 from *E. coli* to *B. uniformis* at frequencies of 10^{-4} per recipient.

To construct the *B. uniformis* donor strains containing conjugal Tc^r Em^r element 12256 and plasmid pRDB5 (Tc^r) or pNFD13-2 (Tc^r), the plasmid was first introduced into *B. uniformis* 1100 as described previously (9, 14), with selection for Tc^r. The resulting transconjugant, which carried the Tc^r plasmid, was used as a recipient in a mating with *B. uniformis* 1008 (Tc^r Em^r), with selection for trimethoprim resistance (Tp^r) and erythromycin resistance (Em^r). To construct *B. uniformis* carrying Tc^r Em^r element 12256 and pVAL1 (Em^r), pVAL1 was first transferred from *E. coli* to *B. uniformis* 1100 by conjugation, with selection for Em^r. Then the Tc^r Em^r 12256 element was introduced by conjugation from *B. uniformis* 1008 into *B. uniformis* 1100(pVAL1), with selection for Tp^r and Tc^r.

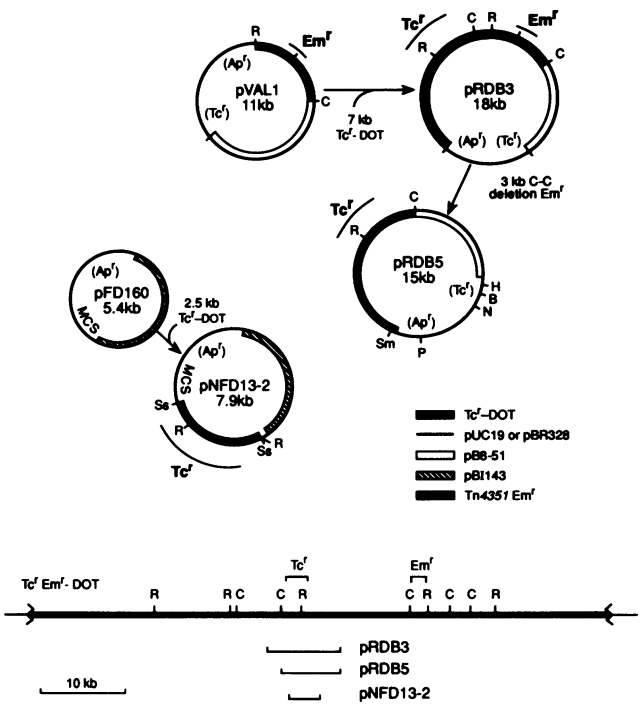

FIG. 1. Construction of pRDB3, pRDB5, and pNFD13-2. A 7-kb *Hin*cII fragment from a cosmid clone of the Tc^r Em^r DOT element was cloned into the unique *Pvu*II site of pVAL1 (16), resulting in pRDB3. A *Cla*I deletion of pRDB3 removed the Tn*4351* Em^r gene and formed pRDB5. With the aid of a Tn*1000* insertion into pRDB3, a 2.5-kb *Sst*I fragment containing the Tc^r gene from Tc^r Em^r DOT was isolated and cloned into the *Sst*I site of pFD160 (13), creating pNFD13-2 (14). All of these vectors can be mobilized both by the IncP plasmids and by the *Bacteroides* conjugal Tc^r elements. A partial map of the Tc^r Em^r DOT element is shown at the bottom, and the regions of this element which were cloned into the vectors are indicated by brackets under the map. Abbreviations for restriction sites: B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; N, *Nru*I; P, *Pst*I; Sm, *Sma*I; Ss, *Sst*I; C, *Cla*I. Only the unique restriction sites on pRDB5 are shown. On pNFD13-2, in addition to the sites shown, the following sites in the multiple cloning site are still unique: *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Bam*HI, *Sma*I, *Xma*I, and *Kpn*I. Antibiotic abbreviations are given in Table 1, footnote *a*.

Mating between *B. uniformis* and *E. coli*. To ascertain that the *B. uniformis* recipients carrying a conjugal Tc^r or Tc^r Em^r element and a plasmid were capable of mobilizing the plasmid at a high frequency, *B. uniformis* 1108(pRDB5), 1108(pVAL1), or 1108(pNFD13-2) was mated with *E. coli* HB101 or EM24. The procedure used for the *B. uniformis-E. coli* mating has been described previously (9, 14).

Mating between *B. uniformis* and *P. ruminicola*. To test for transfer of the plasmids from *B. uniformis* to *P. ruminicola*, it was necessary to devise a selective medium on which *P. ruminicola* grew but *B. uniformis* did not grow. To obtain a

background low enough to allow us to detect transfer levels as low as 10^{-9} per recipient, a combination of selection procedures was required. First, we used a thymidine-requiring strain of *B. uniformis* (strain 1100) so that the lack of thymidine could be used to select against the strain. However, spontaneous reversion to the wild type occurred at relatively high frequencies (10^{-6}). *B. uniformis* grows best in medium containing vitamin K, whereas *P. ruminicola* has no vitamin K requirement. Thus, we also omitted vitamin K from the selective medium. Finally, we took advantage of the fact that *P. ruminicola* B_14 grows well at pH 6.2, whereas *B. uniformis* does not grow well at pH values lower than 6.8. The combination of selection for Rif^r, lack of thymidine and vitamin K, and low pH provided a reproducibly clean background.

For matings between *B. uniformis* and *P. ruminicola*, the *B. uniformis* donor was grown in TYG-Thy-K broth to an optical density at 650 nm of 0.15 to 0.20. *P. ruminicola* B_14R was grown in MM10 broth to an optical density at 650 nm of 0.25 to 0.30. Optical densities were measured in 18-mm-diameter culture tubes with a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, N.Y.). The donor (30 ml) was centrifuged by using a Sorvall model GLC28 bench top centrifuge (SP/X rotor; Dupont Instruments, Wilmington, Del.) at 3,000 rpm for 15 min at room temperature to pellet the bacteria. The bacteria were washed in 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) and resuspended in 1 ml of TYG-Thy-K. Manipulations of *B. uniformis* were done under aerobic conditions, whereas all manipulations of *P. ruminicola* were done under anaerobic conditions.

P. ruminicola B_14R (10 ml) was centrifuged in a sealed culture tube at 3,000 rpm for 15 min at room temperature as described above. The supernatant was removed with a sterile syringe. The resuspended donor (1 ml) and 5 ml of anaerobic 0.1 M potassium phosphate buffer (pH 7.0) were injected into the tube. Anaerobic phosphate buffer was made by boiling phosphate buffer and cooling it under a stream of oxygen-free carbon dioxide. After vortexing to dislodge the pelleted recipient, the bacterial mixture was centrifuged again in the sealed tube, and the wash solution was withdrawn with a syringe. TYG-Thy-K (1.5 ml) and MM10 (1.5 ml) were injected into the tube, and the tube was vortexed to resuspend the bacteria. The resuspended mixture was injected into a sealed anaerobic tube containing a slant of modified E medium. The tube was centrifuged as described above to pellet the bacteria on the slant. The tube was inverted gently, and the supernatant was removed with a syringe. The tube was then incubated upside down at 37°C for 15 to 18 h.

After incubation, 1 ml of MM10 (pH 6.6) containing no thymidine or vitamin K was added to the slant tube, and the tube was vortexed to remove the bacteria. Resuspended bacteria were removed with a sterile syringe which had been gassed out with nitrogen-carbon dioxide. To select for transconjugants, 0.1 to 0.2 ml of resuspended cells or 0.1 to 0.2 ml of a 1:10 dilution was inoculated into a roll tube containing MM10-Rif-Tc or MM10-Rif-Em selection medium. To determine the total number of *B. uniformis* donors, 0.1-ml portions of a 10^{-6} dilution of the resuspended cells were plated onto TYG-Thy-K agar plates, and the preparations were incubated in a GasPak jar. To determine the number of *P. ruminicola* recipients, 0.1-ml portions of 10^{-6} and 10^{-8} dilutions were inoculated into an MM10-Rif roll tube. All preparations were incubated at 37°C for 3 to 4 days.

Matings between *E. coli* and *P. ruminicola*. The conditions used for matings between *E. coli* and *P. ruminicola* were the same as those described above for matings between *B. uniformis* and *P. ruminicola*, except that the *E. coli* donor was grown in Luria broth rather than TYG-Thy-K broth.

Identity of transconjugants. To ascertain that the putative *P. ruminicola* transconjugants contained pRDB5, total DNA (plasmid DNA plus chromosome DNA) was digested with *Eco*RI, and the fragments were separated on a 1.0% agarose gel. After blotting onto Optibind (Schleicher and Schuell), the digests were probed with ^{32}P-labeled pFD160. This plasmid hybridizes with the pBR328 sequences in pRDB5 but not with the Tc^r gene. To check that putative transconjugants were not Thy$^+$ Rif^r mutants of *B. uniformis* 1108 (pRDB5), total DNAs from the donor, the recipient, and transconjugants were digested with *Hin*dIII and *Eco*RI, and the digests were blotted onto Optibind. The blot was hybridized with labeled pEG920::XBU4422 (10); this probe detects not only RDB5 but also the Tc^r Em^r 12256 element in the *B. uniformis* 1108 chromosome.

To ascertain that the transconjugants were *P. ruminicola* B_14 derivatives, total DNA was digested with *Not*I, and the fragments were separated by pulsed-field gel electrophoresis on a Beckman Geneline instrument according to the manufacturer's directions. In stage 1, the pulses were 4 s long, the voltage was 170 V, and the duration was 30 min. In stage 2, the pulses were 45 s long, the voltage was 150 V, and the duration was 18 h. The gels were stained with ethidium bromide.

Electroporation. pRRI4, which was isolated from *P. ruminicola* B_14 or 223, and pRDB5, which was isolated from *P. ruminicola* B_14 or *B. uniformis* 1108, were used in an attempt to introduce DNA into *P. ruminicola* B_14 by electroporation. The plasmid isolation procedures used have been described previously (6, 9). For electroporation, *P. ruminicola* B_14 was grown in six sealed tubes (total volume, 75 ml) containing MM10 broth to an optical density at 650 nm of 0.4 to 0.5, and the tubes were centrifuged to pellet the bacteria. After the supernatant was removed by using a syringe, the bacterial pellets were washed three times in distilled water which had been boiled and cooled under a stream of oxygen-free nitrogen. The pellets were then pooled, resuspended in 0.2 ml of 10% glycerol (pH 7.0), and stored on ice for 30 min. Electroporation was performed with a Bio-Rad Gene Pulser (Bio-Rad, Richmond, Calif.). The resuspended bacteria (50 μl) were placed in a prechilled 0.1-cm electrode-gap cuvette which had been flushed with nitrogen. A field strength of 16 kV/cm (capacitance, 25 μF; resistance, 200 Ω) was used. The resulting time constant was 4.2 to 4.3 ms. After electroporation, the cells were injected into a sealed tube containing 1 ml of modified E medium and allowed to stand for 2 to 3 h at 37°C. A portion of this mixture was then introduced into a roll tube made with MM10 agar containing tetracycline (5 μg/ml) and incubated at 37°C for 48 h.

DNA manipulations. Plasmids were isolated as described by Maniatis et al. (4). Chromosomal DNA was obtained from *B. uniformis* 1108 and from the various *P. ruminicola* strains as described previously (6, 8, 9). Southern blotting was done as described by Maniatis et al. (4).

RESULTS

Antibiotic susceptibility pattern of *P. ruminicola* B_14. To determine what antibiotic resistance genes might be detected in *P. ruminicola* B_14, we tested the susceptibility of *P. ruminicola* to a variety of antibiotics. *P. ruminicola* B_14 was susceptible to rifampin (10 μg/ml), tetracycline (2 μg/ml), erythromycin (1 μg/ml), gentamicin (20 μg/ml), and ampicil-

lin (5 μg/ml). It was resistant to chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), trimethoprim (200 μg/ml), and nalidixic acid (100 μg/ml).

P. ruminicola B_14 was susceptible to all of the antibiotics which inhibited the growth of *B. uniformis* except chloramphenicol. *E. coli* HB101 and EM24 were also susceptible to chloramphenicol. Accordingly, we first tried using chloramphenicol to select for *P. ruminicola* and against the donor. However, when mixtures of *E. coli* (or *B. uniformis*) and *P. ruminicola* B_14 were plated onto MM10 agar containing 10 μg of chloramphenicol per ml, donor colonies were still able to grow enough to obscure the true transconjugants. Thus, we isolated a spontaneous Rif^r derivative of *P. ruminicola* B_14 to use as a recipient in the matings. The combination of rifampin and chloramphenicol produced a background low enough to enable us to detect transfer frequencies below 10^{-9} per recipient.

Attempts to transfer plasmids from *E. coli* to *P. ruminicola*. Initially, since we had previously found that IncP plasmids could mobilize shuttle vectors such as pVAL1 (Fig. 1) from *E. coli* to the colonic *Bacteroides* species, we attempted to demonstrate mobilization of these shuttle vectors from *E. coli* to *P. ruminicola* B_14R. Since we did not know what antibiotic resistance genes would be expressed in *P. ruminicola* B_14, we tried the two antibiotic resistance genes that have been cloned from colonic *Bacteroides* species, a Tc^r gene from conjugal element Tc^r Em^r DOT and an Em^r gene from *Bacteroides* transposon Tn*4351*. pVAL1 carries the Em^r gene, whereas pRDB5 and pNFD13-2 (Fig. 1) carry the Tc^r gene. Moreover, the cryptic *Bacteroides* plasmid cloned in pRDB5 and pVAL1 (pB8-51) is different from and compatible with the cryptic plasmid cloned in pNFD13-2 (pBI143). Thus, we had two different *Bacteroides* replication origins represented on our vectors. However, no Tc^r or Em^r *P. ruminicola* transconjugants were detected.

Conjugal transfer between *B. uniformis* and *P. ruminicola*. Since we could not demonstrate transfer from *E. coli* to *P. ruminicola*, we tried using *B. uniformis* as a donor in the matings. Colonic *Bacteroides* strains have been found to harbor large conjugal chromosomal elements which carry a Tc^r gene and sometimes also carry an Em^r gene. These elements can mobilize coresident plasmids not only from *Bacteroides* species to *Bacteroides* species but also from *Bacteroides* species to *E. coli*. Thus, the Tc^r and Tc^r Em^r conjugal elements found in the colonic *Bacteroides* strains appear to be able to mediate mating pair formation between members of diverse genera of bacteria. We chose the Tc^r Em^r 12256 element for most experiments because it mobilizes coresident plasmids at high frequencies (15). Also, the Tc^r Em^r 12256 element exhibits constitutive transfer, whereas it is necessary to preexpose other Tc^r and Tc^r Em^r elements to tetracycline to obtain maximum transfer frequencies.

B. uniformis donors containing the Tc^r Em^r 12256 element and pVAL1, pNFD13-2, or pRDB5 were constructed and tested for mobilization of the plasmid from *B. uniformis* to *E. coli*. Mobilization of these plasmids from *B. uniformis* to *E. coli* occurred at frequencies of 10^{-4} to 10^{-5} per recipient (Table 2).

Using the conditions described in Materials and Methods and ratios of donors to recipients ranging from 1.5:1.0 to 3.0:1.0, we detected Tc^r transconjugants in a mating between *B. uniformis* 1108(pRDB5) and *P. ruminicola* B_14R at frequencies of 10^{-6} to 10^{-7} per recipient (Table 2). However, no transconjugants were detected in matings in which the donor was *B. uniformis* 1108(pNFD13-2) or 1108 (pVAL1).

TABLE 2. Transfer frequencies of various shuttle vectors from *B. uniformis* 1108 to either *P. ruminicola* B_14 or *E. coli*

Donor strain	Frequency[a] of transfer from *B. uniformis* to:	
	E. coli	*P. ruminicola*
B. uniformis 1108(pRDB5)	3×10^{-4}	10^{-7}–10^{-6}
B. uniformis 1108(pRDB5)[b]	2×10^{-4}	10^{-7}–10^{-6}
B. uniformis 1108(pNFD 13-2)	1×10^{-4}	$<10^{-9}$
B. uniformis 1108(pVAL1)	1×10^{-4}	$<10^{-9}$

[a] Frequencies are expressed as the number of transconjugants per recipient. The ranges are the ranges of values obtained from at least three separate experiments.

[b] The plasmid used in this experiment was pRDB5, which was isolated from a *P. ruminicola* B_14 transconjugant and was reintroduced into the *B. uniformis* 1108 background.

Analysis of *P. ruminicola* transconjugants. Plasmid preparations made from *P. ruminicola* B_14R had a background staining material that made it difficult to see plasmid DNA unambiguously. However, when a plasmid preparation was used to transform *E. coli*, pRDB5 was recovered in *E. coli*. The restriction profile of this plasmid was identical to that of the original pRDB5 (data not shown). Additionally, a Southern blot containing *Eco*RI-digested total DNA from several putative transconjugants was probed with ^{32}P-labeled pFD160, a probe which hybridizes with the pBR328 sequences in pRDB5 (Fig. 2A). *Eco*RI cuts once in pRDB5 to produce a 15-kb linear segment. All of the putative transconjugants contained a single band of the correct size which hybridized with the probe.

Several tests were used to ascertain that the putative transconjugants were, in fact, *P. ruminicola* B_14 rather than a contaminant or a revertant of the donor. Since the transconjugants were not able to grow in Trypticase-yeast extract-glucose broth, on Trypticase-yeast extract-glucose medium containing thymidine, or in MM10 containing gentamicin, they were not revertants of the *B. uniformis* donor. Also, the transconjugants were able to grow in MM10 containing xylan instead of glucose, a medium that does not support growth of *B. uniformis*. A Southern blot of *Hin*dIII-*Eco*RI-digested total DNAs from the donor, the recipient, and a transconjugant was probed with pEG920::XBU4422 (Fig. 2B). This probe hybridized not only with pRDB5 but also with the Tc^r Em^r 12256 element. A revertant of the donor would still contain the Tc^r Em^r 12256 element as well as pRDB5. As Fig. 2B shows, a mixture of bands due to pRDB5 and the Tc^r Em^r 12256 element was observed in the *B. uniformis* donor, whereas only the bands associated with pRDB5 were observed in the transconjugant.

The results described above indicated that the transconjugants were not revertants of the donor. However, they did not rule out the possibility that we had isolated a Tc^r contaminant which had properties similar to those of *P. ruminicola*. To test this, we compared the *Not*I digestion patterns of DNAs from *B. uniformis*, *P. ruminicola*, and a transconjugant on pulsed-field gels. The *Not*I digestion pattern of *P. ruminicola* B_14 differed not only from the pattern of *B. uniformis* 1108, but also from the patterns of other *P. ruminicola* strains (data not shown). The *Not*I restriction patterns of the Rif^r *P. ruminicola* B_14R recipient and the Tc^r transconjugant were identical to each other and to that of *P. ruminicola* B_14 (Fig. 3).

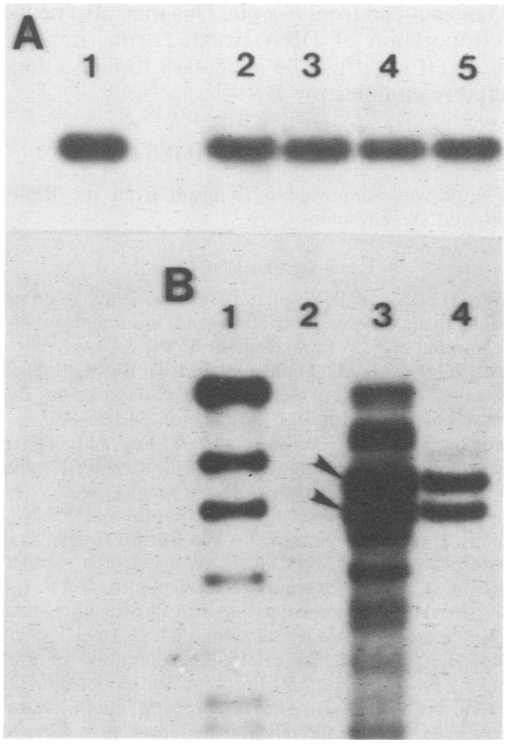

FIG. 2. (A) Total DNAs from the *B. uniformis* 1108 donor containing pRDB5 (lane 1) and four *P. ruminicola* B_14 Tc^r transconjugants (lanes 2 through 5) digested with *Eco*RI. The Southern blot was probed with pFD160, which cross-hybridized with pRDB5 but not with *P. ruminicola* B_14 DNA. (B) Total DNAs from the *P. ruminicola* B_14R recipient (lane 2), the *B. uniformis* 1108 donor (lane 3), and one of the *P. ruminicola* B_14 transconjugants (lane 4) digested with *Eco*RI and *Hin*dIII. The Southern blot was probed with pEG920::XBU4422 (10). This probe hybridized not only with pBR328 sequences on pRDB5 but also with the Tc^r Em^r 12256 element in the donor. The two bands corresponding to pRDB5 are indicated in lane 3 by arrowheads. Lane 1 contained DNA size standards. The four largest standards were 23.1, 9.4, 6.7, and 4.4 kb.

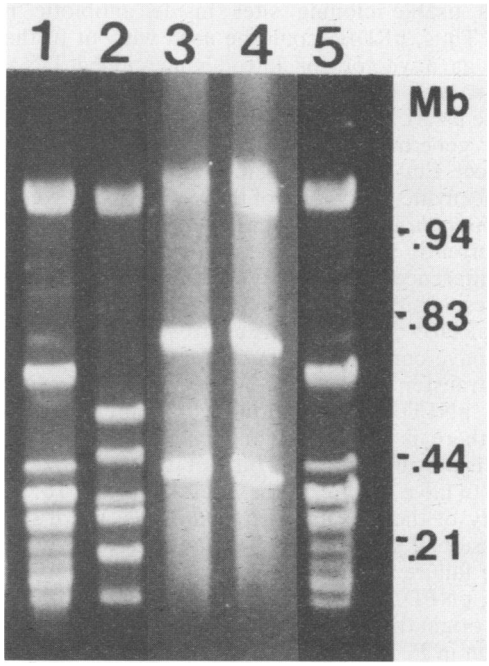

FIG. 3. Pulsed-field electrophoresis to verify the identity of *P. ruminicola* B_14 Tc^r transconjugants. *Not*I digests of DNA from the donor, *B. uniformis* 1108 carrying pRDB5, were placed in lanes 1 and 5. *Not*I-digested DNAs from *P. ruminicola* GA33 (lane 2) and B_14R (lane 3) and one of the strain B_14(pRDB5) transconjugants (lane 4) were also examined. The *Not*I pattern of *P. ruminicola* B_14 is identical to that of strain B_14R (data not shown). The migration distances of some of yeast chromosome size standards are shown in megabases (Mb) on the right.

Retransfer of pRDB5. Since the transfer frequency of pRDB5 from *B. uniformis* 1108 to *P. ruminicola* B_14 was relatively low, we tested the possibility that the plasmids found in the transconjugants might have sustained mutations that improved their survival in the *P. ruminicola* B_14 recipient. To do this, we reconstructed the *B. uniformis* donor by using pRDB5 reisolated from a *P. ruminicola* B_14 transconjugant. The transfer frequency from this donor was the same as the transfer frequency from the original donor strain.

Attempt to introduce pRDB5 into *P. ruminicola* by electroporation. Since we showed that pRDB5 could replicate in *P. ruminicola* B_14, we attempted to introduce this plasmid into *P. ruminicola* B_14 by electroporation. First, we used pRRI4, which was isolated from *P. ruminicola* 223, to establish the conditions for electroporation. Using this plasmid and the procedure described in Materials and Methods, we got 8×10^{-5} transporant per recipient. However, when pRDB5, which was isolated from *P. ruminicola* B_14R, was used instead of pRRI4, no transporants were obtained. We noted that plasmid preparations made from *P. ruminicola* B_14 containing pRDB5 often contained a substantial amount of contaminating material, which made it difficult to see the plasmid on stained gels. This could have been due to the low copy number of pRDB5 in *P. ruminicola* B_14 or to some physiological effect due to the plasmid. Since much cleaner plasmid preparations were obtainable from *B. uniformis* 1100, we also tried to electroporate a plasmid preparation from this strain into *P. ruminicola* B_14. However, no transporants were obtained ($<10^{-9}$ transporant per recipient).

DISCUSSION

We found that plasmid pRDB5 can be transferred by conjugation from one of the colonic *Bacteroides* species, *B. uniformis*, to *P. ruminicola* B_14. This is the first report of a successful transfer of cloned DNA into *Prevotella* species. The Tc^r colonies obtained after the mating were true transconjugants because they contained pRDB5, did not contain the Tc^r Em^r 12256 element, had the same phenotypic characteristics as *P. ruminicola* B_14, and had a *Not*I digestion pattern that was identical to that of *P. ruminicola* B_14. The fact that pRDB5 was present in *P. ruminicola* B_14R as a plasmid and conferred tetracycline resistance on the strain indicates that both the replication machinery and the tetracycline resistance gene of pRDB5 appeared to function in *P. ruminicola* B_14. Given the genetic distance that separates strains classified as *P. ruminicola*, we cannot conclude that pRDB5 will replicate in all *P. ruminicola* strains, but the fact that pRDB5 replicates in many colonic *Bacteroides* species as well as in *P. ruminicola* B_14 indicates that its host range is very wide. Although pRDB5 is relatively large, there are

several usable cloning sites inside antibiotic resistance genes. Thus, pRDB5 could be used without further modification as a vector for introducing cloned DNA into *P. ruminicola*.

Since pVAL1 was virtually identical to pRDB5 except that the Tc^r gene had been replaced by an Em^r gene, the failure to detect Em^r transconjugants of *P. ruminicola* B_14 may have been due to a failure of the Em^r gene to be expressed in *P. ruminicola* B_14. However, given the low transfer frequencies currently attainable, we cannot rule out the possibility that differences in stability between pRDB5 and pVAL1 were responsible for the failure to detect transfer of pVAL1. Also, pRDB5 contains DNA upstream from the Tc^r gene that might have contributed to transfer or stability.

No transfer of pNFD13-2 to *P. ruminicola* B_14 was detected. pNFD13-2 has the same Tc^r gene as pRDB5. However, there is a 4-kb region upstream from the Tc^r gene which is present in pRDB5 but not in pNFD13-2. This region seems to have no effect on expression of the Tc^r gene or the stability of the plasmid in *B. uniformis*, but it might affect expression or stability in *P. ruminicola*. Another explanation for the failure to detect *P. ruminicola* transconjugants containing pNFD13-2 was that pNFD13-2 has a different replication origin than pRDB5 and this replication origin does not function in *P. ruminicola* B_14. If so, pNFD13-2 could serve as a suicide vector for introducing DNA into the chromosome of *P. ruminicola* B_14. Work on this is currently under way in our laboratory.

The system which we used to transfer DNA into *P. ruminicola* B_14 was cumbersome. Construction of the *B. uniformis* donor required two steps. Another drawback to this transfer system is that the only selectable marker on pRDB5 (the Tc^r gene) was also found on the mobilizing Tc^r Em^r 12256 element. Thus, selection for Tc^r may not be sufficient to retain the plasmid if it is unstable. This was not a problem with pRDB5, which was stably maintained in the donor, but might become a problem if DNA cloned into this vector made it less stable than pRDB5. Nonetheless, the demonstration that pRDB5 replicates and expresses tetracycline resistance in *P. ruminicola* provides an important first step toward development of more tractable methods for introducing DNA into *P. ruminicola* because it provides a positive control for further tests. Also, some genetic experiments can be done even with the tools now available.

Clearly, genetic experiments would be much easier if *E. coli* were the donor or if DNA were introduced directly by electroporation. Failure to demonstrate transfer of pRDB5 from *E. coli* to *P. ruminicola* could have been due to the failure of IncP plasmids to mediate formation of mating pairs between *E. coli* and *P. ruminicola*. However, since IncP plasmids mediate mating between *E. coli* and the colonic *Bacteroides* strains, this seems unlikely. A more likely possibility is that the transfer frequency is lowered by the anaerobic mating conditions. We have found previously that transfer frequencies out of *E. coli* increase 10^3-fold when matings are done aerobically rather than anaerobically (10). Aerobic matings are feasible with the colonic *Bacteroides* species, which are quite aerotolerant, but not with the oxygen-sensitive *P. ruminicola* strains. Nonetheless, it may be possible to find conditions that raise the frequency of mating under conditions which allow *P. ruminicola* to survive. Work on this is currently under way. Finally, restriction enzymes in *P. ruminicola* may prevent survival of pRDB5 introduced from *E. coli*. This may also be the barrier to electroporation of DNA isolated from *E. coli* into *P. ruminicola*. If so, it will be necessary to find a donor strain that suitably modifies the DNA.

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REFERENCES

1. **Boyer, H. B., and D. Rouliand-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
2. **Flint, H. J., A. M. Thomson, and J. Bisset.** 1988. Plasmid-associated transfer of tetracycline resistance in *Bacteroides ruminicola*. Appl. Environ. Microbiol. **54**:855–860.
3. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
4. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Odelson, D. S., J. L. Rasmussen, C. J. Smith, and F. L. Macrina.** 1987. Review: extrachromosomal systems and gene transmission in anaerobic bacteria. Plasmid **17**:87–109.
6. **Saito, H., and K. I. Muira.** 1963. Preparation of transforming DNA by phenol treatment. Biochim. Biophys. Acta **72**:619–629.
7. **Salyers, A. A., N. B. Shoemaker, and E. P. Guthrie.** 1987. Recent advances in *Bacteroides* genetics. Crit. Rev. Microbiol. **14**:49–71.
8. **Shah, H. N., and M. D. Collins.** 1990. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int. J. Syst. Bacteriol. **40**:205–208.
9. **Shoemaker, N. B., R. D. Barber, and A. A. Salyers.** 1989. Cloning and characterization of a *Bacteroides* conjugal tetracycline-erythromycin resistance element by using a shuttle cosmid vector. J. Bacteriol. **171**:1294–1302.
10. **Shoemaker, N. B., C. Getty, E. P. Guthrie, and A. A. Salyers.** 1986. Two *Bacteroides* plasmids, pBFTM10 and pB8-51, contain transfer regions that are recognized by broad-host-range IncP plasmids and by a conjugative *Bacteroides* tetracycline resistance element. J. Bacteriol. **166**:959–965.
11. **Shoemaker, N. B., and A. A. Salyers.** 1990. A cryptic 65-kilobase-pair transposonlike element isolated from *Bacteroides uniformis* has homology with *Bacteroides* conjugal tetracycline resistance elements. J. Bacteriol. **172**:1694–1702.
12. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
13. **Smith, C. J.** 1985. Development and use of cloning systems for *Bacteroides fragilis*: cloning of a plasmid-encoded clindamycin resistance determinant. J. Bacteriol. **164**:294–301.
14. **Stevens, A. M., N. B. Shoemaker, and A. A. Salyers.** 1990. The region of a *Bacteroides* conjugal chromosomal tetracycline resistance element which is responsible for production of plasmidlike forms from unlinked chromosomal DNA might also be involved in transfer of the element. J. Bacteriol. **172**:4271–4279.
15. **Thomson, A. M., and H. J. Flint.** 1989. Electroporation induced transformation of *Bacteroides ruminicola* and *Bacteroides uniformis* by plasmid DNA. FEMS Microbiol. Lett. **61**:101–104.
16. **Valentine, P. J., N. B. Shoemaker, and A. A. Salyers.** 1988. Mobilization of *Bacteroides* plasmids by *Bacteroides* conjugal elements. J. Bacteriol. **170**:1319–1324.
17. **Whitehead, T. R., and R. B. Hespell.** 1989. Cloning and expression in *Escherichia coli* of a xylanase gene from *Bacteroides ruminicola* 23. Appl. Environ. Microbiol. **55**:893–896.