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High-Frequency Transfer of a Naturally Occurring Chromosomal Tetracycline Resistance Element in the Ruminal Anaerobe *Butyrivibrio fibrisolvens*

KAREN P. SCOTT,[1] TERESA M. BARBOSA,[1] KENNETH J. FORBES,[2] AND HARRY J. FLINT[1]*

Rowett Research Institute[1] and Department of Medical Microbiology, University of Aberdeen,[2] Aberdeen, United Kingdom

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Butyrivibrio fibrisolvens **strains resistant to tetracycline were isolated from the bovine rumen. Two of three Tc^r *B. fibrisolvens* tested were able to donate tetracycline resistance at frequencies ranging from 10^{-7} to 10^{-1} per donor cell in anaerobic filter matings to a rifampin-resistant mutant of the type strain of *B. fibrisolvens*, 2221^R. The recipient strain 2221^R exhibited rapid autoaggregation, which might be a factor in the high transfer rates observed. Tc^r transconjugants of *B. fibrisolvens* 2221^R were also capable of further transferring tetracycline resistance to a fusidic acid-resistant mutant, 2221^F. Comparison of genomic DNAs by pulsed-field gel electrophoresis demonstrated altered band profiles in transconjugants, consistent with the acquisition of a large mobile chromosomal element. The transferable elements from the two *B. fibrisolvens* donors 1.23 and 1.230 (Tn*B123* and Tn*B1230*, respectively) showed the same preferred insertion site in the *B. fibrisolvens* 2221^R chromosome and are likely to be similar, or identical, elements. Hybridization experiments showed no close relationship between Tn*B1230* and *int-xis* regions from Tn*916* or Tn*5253*. Although DNA from the *B. fibrisolvens* donor strains hybridized with probes carrying *tet*(M) or *tet*(O) sequences, transconjugants were found to have acquired a distinct band that hybridized only weakly with these probes, suggesting that a second, distantly related Tc^r determinant had been transferred.**

Butyrivibrio fibrisolvens is one of the most abundant bacteria isolated from the rumen and has also been reported to exist in other gut habitats (5, 38). Recent 16S rRNA sequencing has shown that this species encompasses a wide range of genetic diversity, but the different strain clusters appear to fall within the *Clostridium* subdivision of the gram-positive bacteria (12, 43). In addition to its natural role in rumen metabolism, *B. fibrisolvens* has attracted interest as a target for genetic manipulation aimed at enhancing rumen function (16). Plasmid-based vector systems have been developed (14, 20), and a manipulated strain that is capable of degrading the plant-derived toxin fluoroacetate and which survives without selection in the sheep rumen for at least 5 months has been produced (15). The development and proposed use of such manipulated strains contrasts with an almost complete lack of information on the natural capacity of rumen anaerobes such as *B. fibrisolvens* to transfer genetic material between strains or to other species. The conjugative transposon Tn*916*, carrying tetracycline resistance, has been shown to be capable of transfer under laboratory conditions from *Enterococcus faecalis* to *B. fibrisolvens* but did not show onward transfer to other *B. fibrisolvens* strains (17).

Tetracycline resistance has been detected in several of the predominant obligately anaerobic species of the rumens of cows and sheep (9), and a Tc^r determinant from *Prevotella ruminicola*, later shown to be of the Tet Q group, was found to be plasmid encoded and capable of transfer in vitro to other ruminal strains of *P. ruminicola* and to human hind gut *Bacteroides* spp. (10, 27, 32). In this paper we show that a tetracycline resistance determinant present in natural isolates of *B. fibrisolvens* can be transferred between strains under anaerobic conditions in vitro at rates comparable to the highest reported for conjugal transfers in gram-positive bacteria. Our evidence indicates that this transfer is chromosomally mediated.

* Corresponding author. Mailing address: Rowett Research Institute, Greenburn Rd., Bucksburn, Aberdeen, United Kingdom. Phone: 44 (0) 1224 716651. Fax: 44 (0) 1224 716687. E-mail: h.flint@rri.sari.ac.uk.

MATERIALS AND METHODS

Strains and growth conditions. Tetracycline-resistant *B. fibrisolvens* strains were isolated from rumen fluid obtained from a cannulated cow housed at the Rowett Research Institute (Aberdeen, United Kingdom) which received a mixed diet of hay (50%), barley (30%), molasses (10%), fish meal, and a vitamin and mineral supplement. Samples were taken 2 h after feeding and maintained anaerobically under 100% CO_2. Anaerobic dilutions (4) were spread directly onto M2GSC agar plates (medium 2 of Hobson [18] containing 0.2% glucose, 0.2% cellobiose, and 0.2% soluble starch as energy sources) with and without tetracycline (10 μg/ml) in a Coy anaerobic cabinet in an atmosphere of 55% CO_2, 2.5% H_2, and 42.5% N_2 and grown for 2 days at 38°C. Hybridization to the *tet*(M) probe was assayed, and single hybridizing colonies were picked from the plates and repurified as described below. Routine anaerobic growth and storage were as described previously (9). The relevant characteristics and origins of the *B. fibrisolvens* strains used here are listed in Table 1. *E. faecalis* CG110::Tn*916* (Tc^r [10 μg/ml], Rif^r [25 μg/ml], and Fus^r [25 μg/ml]) and *Escherichia coli* CG120 harboring plasmid pAM120::Tn*916* (Tc^r [4 μg/ml]) were kind gifts from A. Salyers. *Prevotella bryantii* F115 (previously *P. ruminicola* [1a]) is a transconjugant of strain $B_1$4 that carries plasmid pRRI4 containing the *tet*(Q) gene (10). Plasmid pVJ444 was kindly supplied by M. N. Vijayakumar.

The antibiotic concentrations used were 10 μg/ml in solution unless otherwise stated.

DNA purification. Chromosomal DNA was purified from cells grown overnight in M2GSC medium by using a small-scale version of a method described previously (11). Intact chromosomal DNA for pulsed-field gel electrophoresis (PFGE) was purified by using a modification of the method of Smith et al. (35). The optical density at 650 nm (OD_{650}) of overnight cultures of *B. fibrisolvens* was measured, and, typically, 7×10^8 to 1.5×10^9 cells were chilled on ice, harvested at 4°C, and washed three times in SB (10 mM Tris-HCl, 1 mM NaCl [pH 7.5]). Final pellets were resuspended in 1 ml of SB at 37°C, thoroughly mixed with an equal volume of 1% insert agarose (Sigma; agarose for PFGE sample preparation), poured into molds, and allowed to solidify at 4°C. The inserts were then injected into 5 ml of LB (10 mM Tris-HCl [pH 7.5], 1 M NaCl, 100 mM EDTA, 0.5% 20 Cetyl ether, 0.2% deoxycholate, 0.5% Sarkosyl, 1 mg of lysozyme per ml, 20 μg of RNase per ml) and incubated for 24 h at 37°C to lyse the cells. The LB solution was then replaced with an equal volume of PB (0.5 M EDTA [pH 9], 1% Sarkosyl, 1 mg of proteinase K per ml), and the incubation was continued for a

TABLE 1. Characteristics of *B. fibrisolvens* strains studied here

Strain(s)	Antibiotic resistance[a]	α-Fucosidase activity[b]	Aggregating phenotype	No. of plasmids (size [kb])	Source and/or reference
1.230	TC (10)	−	No	0	Rumen (this study)
1.23	TC (10)	−	No	0	Rumen (this study)
1.210	TC (10)	−	No	2 (10–25)	Rumen (this study)
2221		+	Yes	1 (>200)	Type strain ATCC 19171
2221^R	RIF (10)	+	Yes	1 (>200)	Spontaneous Rif^r mutant
2221^F	FUS (150)	+	Yes	1 (>200)	Spontaneous Fus^r mutant
D6/1		−	No	0	C. S. Stewart, Rowett Research Institute
$D6/1^R$	RIF (10)	−	No	0	Spontaneous Rif^r mutant
Tc3 and Tc2	TC (10), RIF (10)	+	Yes	1 (>200)	Transconjugants (this study)

[a] The antibiotic concentrations (micrograms per milliliter) routinely used are given in parentheses. TC, tetracycline; RIF, rifampin; FUS, fusidic acid. All strains were resistant to streptomycin and kanamycin (5, 10, and 30 μg/ml) but sensitive to chloramphenicol and erythromycin (5, 10, and 30 μg/ml).

[b] Enzyme activity was tested by using API rapid ID32A enzymatic test strips (Biomérieux). All strains were positive for α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and α-arabinofuranosidase and negative for urease, glutamate decarboxylase, β-glucuronidase, and β-*N*-acetylglucosaminidase.

further 48 h at 50°C. Finally, the blocks were washed for three 1/2-h periods in 10 mM Tris-HCl–1 mM EDTA (TE) and stored until required in TE at 4°C.

Plasmid DNA was purified by the alkaline lysis method (3). Larger plasmids were screened for by using methods described previously (1, 19).

Molecular biology procedures and DNA-DNA hybridizations. Agarose gel electrophoresis, restriction enzyme cleavage, Southern transfers, random-primed ^{32}P labelling, and hybridizations were all by standard procedures (29). Boehringer positive nylon membranes were used for transfers, and hybridization was at 65°C (unless otherwise stated) according to the manufacturer's guidelines. After hybridization, filters were washed for 15-min intervals at 65°C, four times in 2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice in 0.1× SSC, both containing 0.1% sodium dodecyl sulfate (SDS). Hybridizing bands were then visualized by exposure to X-ray film. For screening colonies of rumen bacteria resistant to 10 μg of tetracycline per ml, these were either transferred directly onto sterile membranes or picked, gridded, and regrown before transfer. Colonies on membranes were denatured and neutralized by placing the filters, colony side up, on filter paper soaked in 0.5 M NaOH–1.5 M NaCl (7 min), 1 M Tris-HCl (pH 7.5) (5 min), and 3 M NaCl–1 M Tris-HCl (pH 7.5) (5 min), blotting the filters dry briefly between each stage (29). The DNA was then fixed on the membranes by UV cross-linking with a Biorad GS Gene Linker.

Conjugation experiments. Matings between *B. fibrisolvens* strains were based on the method of Hespell and Whitehead (17). Cells were grown anaerobically in M2GSC medium containing appropriate antibiotics to the desired cell density and pelleted at 6,000 × *g* for 15 min at 15°C. The pellet was washed twice, anaerobically, in half the original volume of RGM buffer lacking a carbohydrate source (RGM-C) and finally resuspended in 1/10 of the original volume of RGM-C buffer. The donor and recipient cultures were then mixed (1:1; 200 to 300 μl each) and centrifuged gently. Most of the fluid was decanted, and the cells were resuspended in the remainder and placed on the center of a sterile 0.2-μm-pore-size Millipore filter disc on an M2GSC agar plate. After incubation on the filter discs for 16 h, cells were washed into RGM-C buffer and dilutions were plated onto selective M2GSC agar plates containing the appropriate antibiotics. Potential transconjugants grew in 2 days, and their resistance phenotypes were confirmed by replating selected colonies on antibiotic plates.

Tetracycline uptake assays. Tetracycline uptake assays were done by the method described by McMurry et al. (24), where the accumulation of tritiated tetracycline is measured in cells preincubated for 10 min at 31°C with or without the addition of 1 mM dinitrophenol (DNP). *B. fibrisolvens* and *P. bryantii* cultures were maintained anaerobically for the duration of the experiment.

Aggregation experiments. Autoaggregation of cultures was tested by using methods described previously (21, 28). Cell mixing experiments were done by the method of Kolenbrander and Andersen (21), using washed cells. The OD_{600} of anaerobically grown overnight cultures was measured, the cells were pelleted and washed, and the ODs were measured over a 4-h period for pure cultures and for mixtures containing equal numbers of washed cells.

PFGE. PFGE of the digested DNA was performed on a CHEF-DR II system (Bio-Rad). The electrophoresis was carried out in 1% agarose gels at 14°C and 200 V in 0.5× Tris-borate-EDTA buffer. Pulse times and running times varied with the enzymes used (for *Apa*I, 15 to 20 s and 24 to 36 h, respectively, for *Not*I, 80 s and 22 h, respectively). Slight differences in pulse times and running times account for the differences in resolution between the gels shown in Fig. 3 and 4. Gels were stained for 30 min in ethidium bromide, and the sizes of the DNA fragments were estimated by comparison with either *Saccharomyces cerevisiae* or phage lambda concatenated DNA, according to the sizes of the fragments.

PCR amplification. 16S rDNA sequences of selected *Butyrivibrio* strains were amplified by using a forward PCR primer based on *Butyrivibrio* 16S rDNA sequences in the database and the general eubacterial T primer (42). Amplification was done with a Techne PHC-3 thermal cycler with an annealing temperature of 55°C.

Protein profiles. Protein profiles of the isolates were obtained by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (22) with 12.5% acrylamide gels. Cells were pelleted from overnight cultures (10 ml) and resuspended in 500 μl of 1× SDS sample loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). Samples were boiled for 5 min before being loaded. The running buffer was 1× Tris-glycine, and gels were run at a constant voltage of 130 V.

RESULTS

Transfer of tetracycline resistance between strains of *B. fibrisolvens*. Bacteria resistant to 10 μg of tetracycline per ml were isolated under anaerobic conditions from the rumen of a cow. Approximately 10% of Tc^r colonies were found to hybridize with a ^{32}P-labelled probe carrying a 1.6-kb *Hin*cII/*Kpn*I fragment of the *tet*(M) gene from *E. faecalis*, corresponding to bases 582 to 2345 in the sequence published by Su et al. (39). Among these, the largest single group (5 of 48 isolates) were curved, gram-negative-staining, butyrate-producing, obligately anaerobic rods, and three of these isolates were identified as strains of *B. fibrisolvens* from their motility, API 32A enzyme profiles, and total protein SDS-PAGE profiles (Table 1). In their SDS-PAGE profiles the three newly isolated strains resembled *B. fibrisolvens* D6/1 rather than the type strain 2221 (see Fig. 1), but 16S rRNA sequencing has demonstrated at least two major groups of *B. fibrisolvens* isolates that diverge significantly (12, 43). PCR amplification of these three rumen isolates with specific primers showed that they, and strain D6/1, fall into the second group of strains as defined by Willems et al. (43) (rRNA types 8 to 11), which includes NCDO 2223.

B. fibrisolvens 2221 was chosen as a recipient strain to test for transfer of tetracycline resistance from the newly isolated Tc^r strains. A spontaneous rifampin-resistant mutant, 2221^R, was isolated to allow selection of transconjugants (Table 1). Strain 2221 differed from the other four strains examined here in being α-fucosidase positive, thus aiding the identification of transconjugants. Matings were carried out under anaerobic conditions as described in Materials and Methods. With *B. fibrisolvens* 1.230 and 1.23 as donors, putative transconjugants grew on selective plates containing 10 μg of rifampin per ml and 10 μg of tetracycline per ml after 2 days. All putative

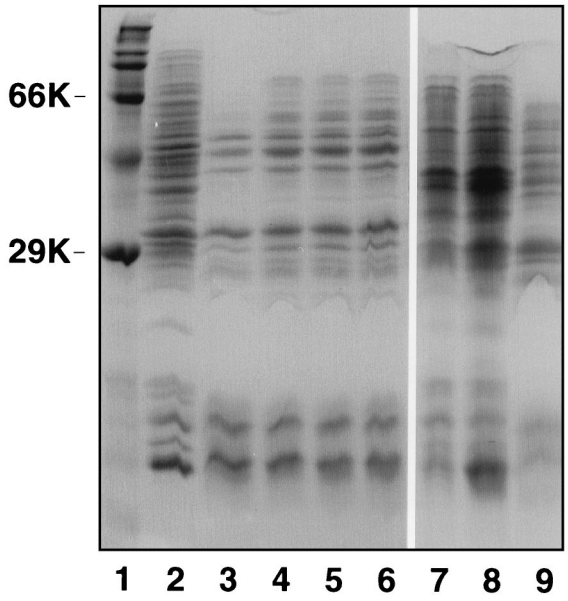

FIG. 1. SDS-PAGE comparison of total cell proteins from the *B. fibrisolvens* strains studied here. Lanes 2 to 6, strains 2221^R, D6/1, 1.230, 1.23, and 1.210, respectively. Lane 7 contains a typical Tc^r Rif^r transconjugant from a mating between 2221^R (lane 8) and 1.230 (lane 9). Lane 1, molecular size markers (in kilodaltons) (Sigma SDS-6H).

transconjugants were α-fucosidase positive and gave SDS-PAGE profiles resembling that of the recipient, showing that they could not be rifampin-resistant mutants of the donor strain (Fig. 1). The possibility that the putative transconjugants arose as spontaneous tetracycline-resistant mutants of 2221^R was eliminated, since no Tc^r colonies were obtained from plating 2221^R on the doubly selective media. The frequency of resistance transfer was highly variable between experiments (Table 2). Transfer frequencies were highest in matings involving donor and recipient cells grown to an OD_{650} of 0.10 to 0.15, but it is not clear whether this is a function primarily of cell density or of growth stage, since transfer was also detectable when stationary-phase cells were used (Table 2). The presence of tetracycline in the initial growth medium, which had been shown to enhance transfer of Tn*916* (33), did not have any dramatic effect on the rate of resistance transfer observed here (Table 2), but parent cells were pregrown in the presence of tetracycline for the crosses involving young cells. No transfer of Tc^r from the third potential donor strain tested, 1.210, to 2221^R was detected either with or without pregrowth in the presence of tetracycline.

Onward transfer of the tetracycline resistance determinant from a 2221^R transconjugant, Tc3, was also demonstrated, using a spontaneous fusidic acid-resistant mutant, 2221^F, as the recipient. Transconjugants were selected on 10 μg of tetracycline per ml and 150 μg of fusidic acid per ml and were detected at a frequency of greater than 10^{-6} per recipient in experiments involving stationary-phase parent strains.

Detection of Tc^r transfer by hybridization. Plasmid DNA was not detected in the Tc^r *B. fibrisolvens* strains 1.230 and 1.23 although plasmids were detected in the third Tc^r *B. fibrisolvens* strain, 1.210 (Table 1). Additionally a very large (200- to 300-kb) plasmid was detected in the recipient strain 2221^R, as reported previously (40). Chromosomal DNAs from all three Tc^r *B. fibrisolvens* strains hybridized under high-stringency conditions with the previously mentioned probe carrying *tet*(M) sequences from Tn*916* and with plasmid pAT121 (36) containing the *Campylobacter coli tet*(O) gene (results not shown). No bands of comparable intensity were detected in the transconjugants under these conditions, although two hybridizing bands were detectable at the same position both in transconjugants and in the recipient strain under conditions of reduced stringency (Fig. 2). Under these conditions, an additional 3.5-kb band was detected in the transconjugants that hybridized weakly to the *tet*(M) probe and was absent from the recipient strain (Fig. 2A). This band hybridized at an intensity comparable to that of a similarly sized band in the donor strains, 1.23

TABLE 2. Frequency of tetracycline resistance transfer in anaerobic matings between *B. fibrisolvens* strains

Donor		Recipient		TC[a]	Transfer frequency[f] per:	
Strain	Growth stage (OD_{650})	Strain	Growth stage (OD_{650})		Donor	Recipient[c]
1.210	Stat[b]	2221^R	Stat	+	ND[e]	0
				−	ND	0
1.23	Stat	2221^R	Stat	+	ND	3.7×10^{-5}, 4×10^{-7}
				−	ND	7.1×10^{-8}
1.230	Stat	2221^R	Stat	+	ND	1.6×10^{-5}, 1.2×10^{-4}, 2.3×10^{-7}
				−	ND	1.4×10^{-6}, 1.7×10^{-7}
1.230	0.11	2221^R	0.24	+	1.2×10^{-1}	1.6×10^{-5}[d]
	0.29		0.45	+	1.9×10^{-5}	2.0×10^{-5}
	0.02		0.13	+	5.0×10^{-2}[d]	2.1×10^{-4}
	0.11		0.15	+	5.0×10^{-2}[d]	6.6×10^{-3}
	0.15		0.08	+	1.7×10^{-4}[d]	5.1×10^{-3}
	0.23		0.16	+	2.9×10^{-7}[d]	1.6×10^{-4}
	0.21		0.24	+	1.5×10^{-5}	3.5×10^{-4}
	0.18		0.14	+	5.8×10^{-6}	7.1×10^{-3}
	0.22	D6/1^R	0.19	+	1.0×10^{-6}	3.3×10^{-7}
	0.22		0.11	+	4.2×10^{-7}	1.0×10^{-6}

[a] Pregrowth with (+) or without (−) 10 μg of tetracycline per ml.
[b] Stat, Stationary phase ($OD_{650} > 1.0$).
[c] In some cases, results of repeat experiments are shown.
[d] Cell numbers estimated from known OD values.
[e] ND, not determined.
[f] Transfer frequencies represent the number of transconjugants per number of parent cells in the mating mixture.

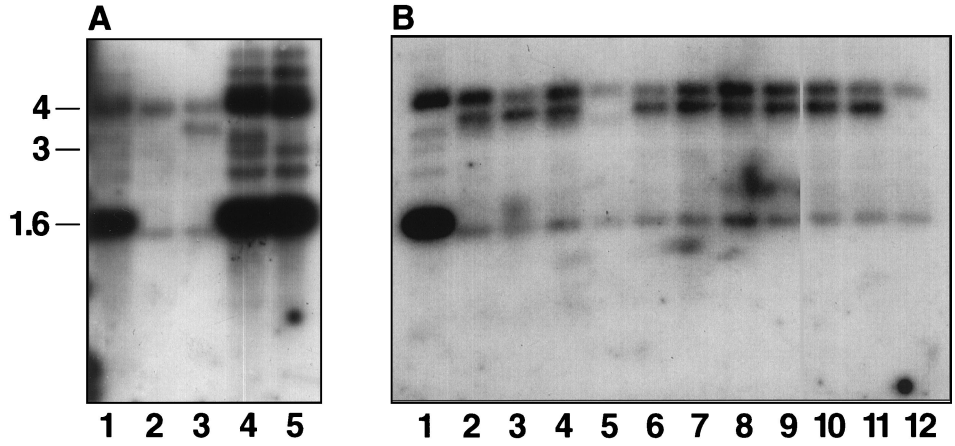

FIG. 2. Hybridization of a ^{32}P-labelled *tet*(M) probe to *Hin*dIII-restricted chromosomal DNA from *B. fibrisolvens* strains under conditions of reduced stringency. (A) Hybridization to DNA from Tc^r strains 1.230 (lane 1), 1.23 (lane 4), and 1.210 (lane 5). A weakly hybridizing band that is lacking in the recipient strain 2221^R (lane 2) is seen in a Tc^r transconjugant (Tc5) (lane 3). (B) Hybridization to 10 different Tc^r transconjugants (lanes 2 to 11) from a mating between 1.230 (lane 1) and 2221^R (lane 12). Both filters were hybridized and washed at 60°C (see text). Molecular size markers (in kilobases) are indicated on the left.

and 1.230, which was absent from strain 1.210 (Fig. 2A, lane 5). This suggests that there are two tetracycline resistance determinants present in *B. fibrisolvens* 1.23 and 1.230, one of which is homologous to Tet M and Tet O and is not transferable and the other of which is only distantly related to Tet M and is transferable. Strain 1.210 appears to carry only the nontransferable Tet M Tet O homolog.

PFGE analysis of transconjugants. In order to detect large-scale genetic changes that might accompany Tc^r transfer, chromosomal DNAs from the donor, recipient, and transconjugants were examined by PFGE following cleavage by *Apa*I (Fig. 3 and 4) or *Not*I (results not shown). This provided firm evidence for the acquisition of genetic material in all Tc^r transconjugants, all of which showed the loss of a 240-kb *Apa*I band, suggesting that there is a common preferred insertion site. Twelve transconjugants from three independent crosses involving the donor strain 1.230 were tested, of which 10 are shown in Fig. 3. The most common profile (4 of 12 transconjugants) (Fig. 3, lanes 4, 7, 10, and 13) showed the appearance of a single 290-kb band, consistent with an insertion of approximately 50 kb of DNA. Two further transconjugants appeared to have gained a 280-kb band (Fig. 3, lanes 8 and 11), which might indicate loss of part of the element upon insertion. Several transconjugants exhibited multiple changes also involving acquisition of a large 390-kb band (Fig. 3, lanes 3, 9, and 12) or of smaller bands of 140 kb (lane 2), and in some of these cases it was evident that bands additional to the 240-kb band had been lost (see, e.g., lane 3). These complex patterns pre-

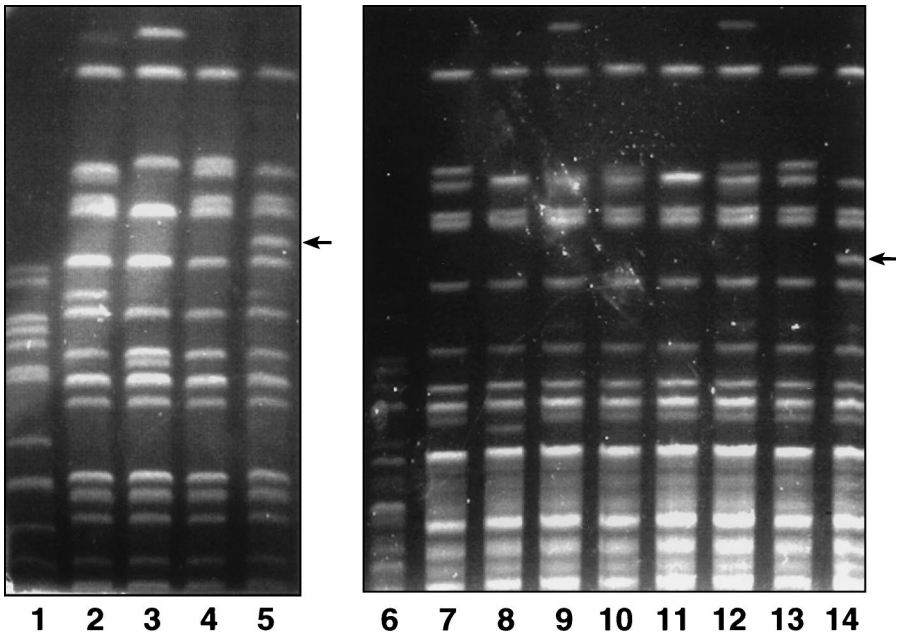

FIG. 3. PFGE of *Apa*I-digested DNAs from *B. fibrisolvens* strains. DNAs from the donor (1.230) (lanes 1 and 6) and recipient (2221^R) (lanes 5 and 14) are compared to those from Tc^r Rif^r transconjugants (lanes 2 to 4 and 7 to 13) from matings of 1.230 and 2221^R. The arrows indicate the position of the 240-kb band, which is present in the recipient strain but has disappeared in all transconjugants studied (see text).

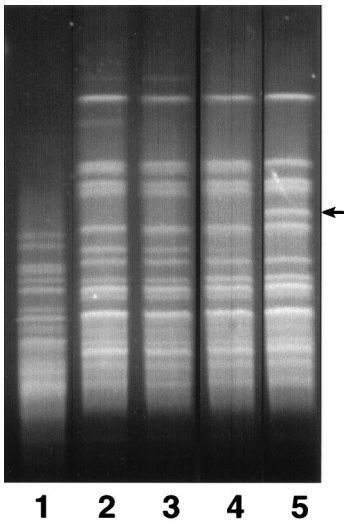

FIG. 4. PFGE of *Apa*I-digested DNAs from *B. fibrisolvens* 1.23 (lane 1) and 2221^R (lane 5) and three representative transconjugants from a mating between 1.23 and 2221^R (lanes 2 to 4). The arrow indicates the 240-kb band, which is present only in the recipient strain. The significance of the other bands is explained in the text.

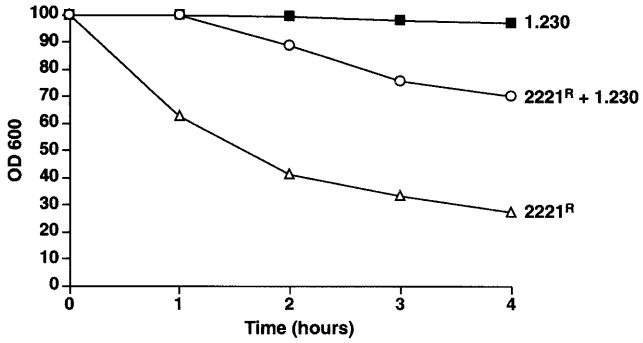

FIG. 5. Reduction in OD_{600} of the donor 1.230 (■), the recipient 2221^R (△), and a mixture of both (○) measured after set time periods. OD values for each sample were corrected to percentages of the starting OD value (time zero).

sumably result from insertion at secondary sites and possibly from multiple insertions at the same site. The patterns may be further complicated if transposition events occur during growth of the isolated transconjugants, since this could lead to hybrid patterns in which part of the cell population carries insertions at a particular site and part does not. The variable intensity of some of the bands appears consistent with this possibility.

Three representative transconjugants from the second mating involving donor strain 1.23 are shown in Fig. 4. These transconjugants seemed to have a more consistent profile, and six of eight tested had a banding pattern identical to that shown in Fig. 4, lane 3, again involving the acquisition of a 290-kb band and a large 390-kb band. The others either did not gain the 390-kb band (Fig. 4, lane 4) or gained an additional, slightly smaller band (lane 2). Thus, 1.23 carries an element similar, if not identical, to that in 1.230. We will refer to the 1.23 element as Tn*B123* and to the element in 1.230 as Tn*B1230*.

Relationship to known conjugative transposons. A 5.5-kb *Hin*cII fragment from Tn*916* that encodes regions required for conjugative transfer and a 3.6-kb *Hin*cII fragment containing the *xis-int* genes of Tn*916* (31) were tested for cross-hybridization with DNAs from *B. fibrisolvens* transconjugants. Although weak hybridization was detected for both probes, the same bands were present in transconjugants and in the recipient strain, and different bands were present in the donor. Therefore, the element involved in the transfer of tetracycline resistance is not closely related to Tn*916* (results not shown). No evidence was found either for similarity to the integrase gene of Tn*5252*, present in the plasmid pVJ444 (reference 41 and results not shown).

Mechanism of resistance to tetracycline in transconjugants. DNP is known to inhibit transport of tetracycline out of cells that carry active efflux-type resistance determinants, resulting in intracellular accumulation of labelled tetracycline (24), although the effects in anaerobes have been less studied. We found no evidence for increased accumulation of tetracycline in a representative *B. fibrisolvens* transconjugant (Tc2) following treatment with DNP, suggesting that an efflux mechanism was not responsible for the resistance (Table 3).

Aggregation phenotypes of *B. fibrisolvens* strains. During the course of this work, it was noted that the recipient strain 2221^R, but not the donor strains 1.230 and 1.23, showed rapid autoaggregation (Table 1). This was considered potentially significant, since aggregation has an important influence on the frequency of transfer of genetic elements in several gram-positive species (13, 28). However, we found no evidence that 1.230 coaggregated with 2221^R, since the OD of mixtures decreased at a rate consistent with clumping of 2221^R alone (Fig. 5). The nonaggregating strain D6/1 was able to act as a recipient in matings with 1.230, although frequencies were considerably lower than the maximal frequencies obtained with 2221^R as the recipient (Table 2).

TABLE 3. Uptake of tetracycline by a Tc^r transconjugant of *B. fibrisolvens*

Time (min)	[^{3}H]tetracycline uptake (cpm/mg of protein)[a] by:					
	E. coli HB101(pBR322)[b]		*P. bryantii* F115[c]		*B. fibrisolvens* Tc2[d]	
	Without DNP	With DNP (1 mM)	Without DNP	With DNP (1 mM)	Without DNP	With DNP (1 mM)
0	0	0	151	147	145	130
7.5	0	150	1,112	821	565	370
15	6	303	1,181	989	965	748

[a] Values are the means for two replicates. The two values differed from the mean by less than 12%. Counts were corrected for background by subtracting counts obtained from filters treated in the same way but without the addition of cells.
[b] *E. coli* HB101 containing plasmid pBR322, which contains *tetA*(C) encoding an efflux mechanism.
[c] *P. bryantii* F115 containing *tet*(Q), which encodes a ribosome protection mechanism.
[d] Tc2 is a representative transconjugant from a mating between 1.230 and 2221^R.

DISCUSSION

This is the first report of a naturally occurring transmissible chromosomal element in an obligately anaerobic rumen bacterium. The frequency of tetracycline resistance transfer seen here in in vitro experiments performed anaerobically is comparable to some of the highest transfer rates seen for matings between the more aerotolerant gram-positive lactic acid bacteria (28). While the frequencies observed for filter matings in vitro give little indication of the likelihood of transfer in vivo, there is evidence that a high proportion of rumen microorganisms exist in close association with biofilms and with the surfaces of food particles (8). Our evidence suggests that three of the Tc^r *B. fibrisolvens* strains isolated carry a gene closely related to Tet M and Tet O determinants but that tetracycline resistance transfer from 1.230 and 1.23 is due to a second gene that is more distantly related to Tet M. Hybridization results indicate that the third strain, 1.210, which did not transfer Tc^r, lacks this second gene. The presence of two tetracycline resistance genes in a single bacterial strain is not without precedent. Three species of staphylococci were found to contain more than one Tc^r gene (30), and some *Clostridium perfringens* also contain two genes conferring tetracycline resistance (34). In these cases the two genes code for different resistance mechanisms. Assuming that the occurrence of tetracycline resistance among ruminal bacteria is a result of antibiotic exposure, it is possible that intense selection pressure resulted in the acquisition of multiple Tc^r determinants which, in combination, confer increased levels of resistance. In fact, recent work has shown that strains 1.23 and 1.230 exhibit greater resistance to tetracycline and minocycline (a tetracycline analog) than either 1.210 or the 2221^R transconjugants (2a).

The nature of the transmissible tetracycline resistance determinant from *B. fibrisolvens* 1.230 remains unknown. Our evidence indicates that it is unlikely to encode an efflux type of resistance mechanism, and since the only case of resistance due to tetracycline modification involves an oxygen-dependent reaction (37), ribosome protection appears to be the likely resistance mechanism. This conclusion is also suggested by the weak hybridization obtained with *tet*(M) and *tet*(O) probe sequences, since *tet*(M) genes show significant homology with other tetracycline resistance genes involved in ribosome protection [75% homology to *tet*(O) (36), 40% to *tet*(Q) (26), and 79% to *tet*(S) (6)].

Conjugative transposons have been identified as one of the main mechanisms for the dissemination of antibiotic resistance, and the identification of such an element in a major rumen anaerobe is significant both in this context and in view of recent developments in genetic manipulation of *Butyrivibrio* spp. (16). Further work is needed to establish the nature of the elements involved in these transfers. The most likely size for the elements is in the range of 40 to 60 kb, based on the evidence from PFGE, and they have preferred insertion sites, although secondary insertions are also quite common. The fact that the preferred insertion sites for the Tn*B123* and Tn*B1230* elements are the same suggests that they are similar, if not identical, elements. Conjugative transposons are known to be prevalent in gram-positive bacteria to which *B. fibrisolvens* is related. Tn*916*-related elements carrying *tet*(M) genes are generally found in the size range of 18 to 25 kb and generally do not show a single preferred insertion site (7). Another group of elements unrelated to Tn*916* is exemplified by the Tn*5252* component of transposon Tn*5253* from *Streptococcus pneumoniae*, identified by Vijayakumar et al. (41), which carries a chloramphenicol resistance gene. Tn*5253* (2) and another conjugative transposon isolated from *Streptococcus pyogenes* (Tn*3701*) (23) have been shown to be composite elements capable of independent transposition, whose internal elements have homologies to Tn*916*. Conjugative transposons from *Clostridium difficile* that show partial homology with Tn*916* have also been reported (25). We have so far found no evidence for close sequence similarity between the Tn*B1230* element and conserved regions of Tn*916* or Tn*5253* and therefore must conclude that Tn*B1230* may represent a novel type of element. If this conjugative transposon was a composite element, size differences in the primary product after chromosomal insertion could be explained.

The possible role of cell aggregation in transfer of the *B. fibrisolvens* element has not been firmly established, since there was no clear evidence of coaggregation of donor and recipient. However, it seems very likely that autoaggregation of the recipient strain could still promote onward transfer of an acquired element from transconjugants to other recipients during crosses. This could be a factor in the high transfer frequency observed with 2221^R as the recipient.

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