

# Use of a Modified *Bacteroides-Prevotella* Shuttle Vector To Transfer a Reconstructed $\beta$ -1,4-D-Endoglucanase Gene into *Bacteroides uniformis* and *Prevotella ruminicola* B<sub>14</sub>

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A carboxymethyl cellulase (CMCase) gene from *Prevotella ruminicola* B<sub>14</sub> was reconstructed by adding a cellulose binding domain from a *Thermomonospora fusca* cellulase and was conjugally transferred from *Escherichia coli* to *Bacteroides uniformis* 0061 by using a chloramphenicol and tetracycline resistance shuttle vector (pTC-COW). pTC-COW was specifically constructed to facilitate conjugal transfer of vectors from *B. uniformis* donors to *P. ruminicola* recipients. *B. uniformis* transconjugants containing CMCase constructs cloned into pTC-COW expressed Cm<sup>r</sup>, but they did not produce the reconstructed CMCase until a xylanase promoter from *P. ruminicola* 23 was added upstream of the CMCase (pTC-XRCMC). The xylanase promoter allowed the *B. uniformis* transconjugants to produce large amounts of the reconstructed CMCase, which was present on the outside surface of the cells. Although the reconstructed CMCase alone did not allow *B. uniformis* to grow on acid-swollen cellulose, rapid growth was observed when two exocellulases were added to the culture supernatant. Under these conditions, the reconstructed CMCase permitted faster growth than the wild-type CMCase. The frequency of transfer of pTC-XRCMC from *B. uniformis* to *P. ruminicola* B<sub>14</sub> was increased 100-fold when strictly anaerobic conditions, nitrocellulose filters (cell immobilization), and more stringent selections were employed. Although the *P. ruminicola* B<sub>14</sub> (pTC-XRCMC) transconjugants expressed Tc<sup>r</sup> and had DNA that hybridized with a probe to the shuttle vector, these transconjugants did not produce detectable levels of the reconstructed CMCase even when xylan was the carbon source. On the basis of these results, it appears that not all of the promoters recognized by *B. uniformis* and *P. ruminicola* 23 are functional in *P. ruminicola* B<sub>14</sub>. However, the results with *B. uniformis* suggest that the introduction of a *P. ruminicola* B<sub>14</sub> promoter should allow expression of the reconstructed CMCase in *P. ruminicola* B<sub>14</sub>.

The relationship between ruminal microorganisms and ruminant animals is clearly symbiotic. The animal provides a habitat, the rumen, for microbial growth, and the microorganisms, in turn, provide the animal with fermentation acids and microbial protein (19). Because mammals do not produce cellulases, this interaction is particularly strong when the diet is rich in fiber. Modern methods of ruminant production which stress the use of cereal grains have placed new constraints on the rumen. When the rate of starch fermentation is rapid, declines in ruminal pH (36) inhibit the growth of cellulolytic bacteria (31, 40). Because the pH inhibition is closely linked to the method of intracellular pH (36) control (29, 42), simple schemes of genetic engineering will not solve this problem.

A variety of noncellulolytic ruminal bacteria produce carboxymethyl cellulases (CMCases) (1, 24, 44). These enzymes degrade carboxymethyl cellulose (CMC) and soluble glucans but cannot bind to or hydrolyze insoluble cellulose (6). Because the addition of a cellulose binding domain (CBD) to the C terminus of CMCase caused a greater than fivefold increase in cellulose hydrolysis, it appears that some CMCases can be converted into cellulases (22). Based on the observation that *Prevotella ruminicola* B<sub>14</sub>, an acid-resistant ruminal bacterium, produces a CMCase, gene reconstruction is a potential strategy for creating an acid-resistant, cellulolytic bacterium (22).

A variety of ruminal enzymes have been cloned into *Esche-*

*richia coli* (8, 11, 14, 37), but there have been few reports of gene transfer into ruminal bacteria. Gram-positive bacteria like *Ruminococcus flavefaciens* and *Streptococcus bovis* have been routinely transformed with a shuttle vector derived from *Streptococcus sanguis* (21, 47), but shuttle vectors for gram-negative ruminal bacteria are not as well developed. Antibiotic resistance plasmids have been isolated from *P. ruminicola* 223/M2/7 and 2202 (9, 10), but these plasmids are very large, with few useful cloning sites, and have not yet been transferred into other strains of *P. ruminicola* (41).

Shoemaker et al. (32) showed that an *E. coli*-*Bacteroides* shuttle vector could be transferred to *P. ruminicola* B<sub>14</sub>, but the efficiency of mating was low and the strain constructions were difficult. In this paper, we report the construction of a new shuttle vector (pTC-COW) that facilitates plasmid transfer to *P. ruminicola* B<sub>14</sub> by eliminating one mating step. A modification of the previous *Bacteroides-Prevotella* conjugation protocol results in a 100-fold increase in *Prevotella* transconjugants. This system was used to transfer a genetically reconstructed CMCase gene (RCMC) from *E. coli* via *Bacteroides uniformis* to *P. ruminicola* B<sub>14</sub>.

## MATERIALS AND METHODS

**Strains, plasmids, and enzymes.** The strains used in this study are described in Table 1. The restriction enzymes were obtained from New England Biolabs (Beverly, Mass.) and Gibco-BRL (Gaithersburg, Md.). *P. ruminicola* B<sub>14</sub> strains show phenotypic variation (30), and we used a B<sub>14</sub> strain that was originally obtained from M. P. Bryant (University of Illinois, Urbana) and maintained in our laboratory for approximately 15 years (B<sub>14</sub>R).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) <sup>a</sup>	Description and/or source	Reference
<b>Plasmids</b>			
pC43	(Ap <sup>r</sup> )	pUC 19 with the B <sub>1</sub> 4 CMCase gene	25
pGF7	(Ap <sup>r</sup> )	pUC 18 with E2 CBD fused to the 40.5-kDa CMCase	22
pBCMC	(Ap <sup>r</sup> )	pBluescript KS+ with the B <sub>1</sub> 4 CMCase gene	This study
pBRCMC	(Ap <sup>r</sup> )	pBCMC with E2 CBD fused to the B <sub>1</sub> 4 CMCase gene	This study
pFD342	(Sp <sup>r</sup> )	Vector with the <i>P. ruminicola</i> 23 xylanase promoter	45
pXYL	(Ap <sup>r</sup> )	pBluescript KS+ with PCR-amplified xylanase promoter-CMCase fragment	This study
pBXCRCMC	(Ap <sup>r</sup> )	pBluescript KS+ xylanase promoter-CMCase gene	This study
pBXRRCMC	(Ap <sup>r</sup> )	pBluescript KS+ xylanase promoter-CMCase-E2 CBD	This study
pRDB5	(Ap <sup>r</sup> Tc <sup>r</sup> )Tc <sup>r</sup>	Shuttle vector	32
pFD308	(Ap <sup>r</sup> Cm <sup>r</sup> )Er <sup>r</sup> Cm <sup>r</sup>	<i>cat</i>	38
pTC-COW	(Ap <sup>r</sup> Cm <sup>r</sup> )Tc <sup>r</sup> Cm <sup>r</sup>	pVAL1 derivative with <i>cat</i> and <i>tetQ</i>	43, This study
pTC-RCMC	(Ap <sup>r</sup> Cm <sup>r</sup> )Tc <sup>r</sup> Cm <sup>r</sup>	pTC-COW with the CMCase-E2 CBD gene	This study
pTC-XCMC	(Ap <sup>r</sup> Cm <sup>r</sup> )Tc <sup>r</sup> Cm <sup>r</sup>	pTC-COW with the xylanase promoter-CMCase gene	This study
pTC-XRCMC	(Ap <sup>r</sup> Cm <sup>r</sup> )Tc <sup>r</sup> Cm <sup>r</sup>	pTC-COW with the xylanase-CMCase-E2 CBD gene	This study
<b><i>E. coli</i></b>			
DH5α	(RecA <sup>-</sup> )		Gibco BRL
S17-1	(RecA <sup>-</sup> ΩRP4-Tc::Mu-Kn::Tn7)	IncP RP4 inserted into the chromosome	35
<b><i>B. uniformis</i> 0061</b>			
1008	Rif <sup>r</sup> Tc <sup>r</sup> Em <sup>r</sup>	<i>B. uniformis</i> with the conjugal transposon Tc <sup>r</sup> Em <sup>r</sup> 12256	32
1100	Tp <sup>r</sup> Thy <sup>-</sup> Rif <sup>r</sup>		32
1108	Tc <sup>r</sup> Em <sup>r</sup> Tp <sup>r</sup> Thy <sup>-</sup> Rif <sup>r</sup>	1100 with the conjugal transposon Tc <sup>r</sup> Em <sup>r</sup> 12256	32
pTC-XCMC	Same as 1108, Cm <sup>r</sup>	1108 carrying pTC-XCMC	This study
pTC-RCMC	Same as 1108, Cm <sup>r</sup>	1108 carrying pTC-RCMC	This study
pTC-XRCMC	Same as 1108, Cm <sup>r</sup>	1108 carrying pTC-XRCMC	This study
<b><i>P. ruminicola</i></b>			
B <sub>1</sub> 4R	Tc <sup>s</sup> Em <sup>s</sup> Thy <sup>+</sup> Rif <sup>r</sup>	Rif <sup>r</sup> derivative of B <sub>1</sub> 4	32
pTC-RCMC	Same as B <sub>1</sub> 4R, Tc <sup>r</sup>	B <sub>1</sub> 4R carrying pTC-RCMC	This study
pTC-XRCMC	Same as B <sub>1</sub> 4R, Tc <sup>r</sup>	B <sub>1</sub> 4R carrying pTC-XRCMC	This study

<sup>a</sup> *B. uniformis* and *P. ruminicola* resistances, with *E. coli* resistances in parentheses.

**Media and culture conditions.** The media and growth conditions for *E. coli*, *P. ruminicola*, and *B. uniformis* 0061 were as previously described (32).

**Joining of the *P. ruminicola* 23 xylanase promoter to the *P. ruminicola* B<sub>1</sub>4 CMCase gene.** The xylanase promoter from the *P. ruminicola* 23 xylanase gene was previously cloned into pFD325 to make pFD342, which was provided by T. Whitehead (38, 45). The xylanase promoter was introduced upstream of the *P. ruminicola* B<sub>1</sub>4 CMCase gene by using the PCR overlap extension method (18). All PCRs were performed with Vent DNA polymerase (New England Biolabs) in 1× Vent polymerase buffer with 200 μM each deoxynucleoside triphosphate, 0.5 μM each PCR primer, and 1 ng of the template. The conditions for the PCR were 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min. Primer 1 (5′ CGGT GACGATCCTATTGTAGCCGACCTTG 3′) and primer 2 (5′ AATCAAGTA AAATGTGGCGTATTAAGATTGATGTCGTT 3′) were used to amplify the xylanase promoter with pFD342 as template. Primer 3 (5′ AACGACATCAA CAATCTTAATACGCCACATTTACTTGATT 3′) and primer 4 (5′ CAGC CCATTCAGTAACCGGA 3′) were used to amplify 1 kb of the N-terminal portion of the CMCase gene with pBCMC as template. Approximately 50-μl samples of PCR products were combined in a Microcon 100 filter device (Amicon, Beverly, Mass.), washed three times with 300 μl of distilled water, and concentrated to 10 μl according to the manufacturer's instructions. Overlap extension PCR was performed with 1 μl of the previous concentrate as template, 0.5 μM primer 1, and 0.5 μM primer 4 in a PCR similar to the one described above. The subsequent PCR product was digested with *Eco*RI at an internally located *Eco*RI site (480 bp from the 3′ end) and ligated into *Eco*RI- and *Sma*I-digested pBluescript KS+ (Stratagene, La Jolla, Calif.) to yield pXYL. pXYL was digested with *Bam*HI and *Eco*RI, and the xylanase promoter-CMCase fragment was ligated into *Bam*HI- and *Eco*RI-digested pBCMC. The resulting vector was designated pBXCRCMC. All vectors were transformed into *E. coli* DH5α by electroporation as previously described (24).

**Construction of RCMCase and XRCMCCase.** The CBD from the *Thermomonomospora fusca* endocellulase E2 was previously ligated in frame with the 40.5-kDa CMCase portion of the *P. ruminicola* B<sub>1</sub>4 CMCase (designated plasmid pGF7) and expressed in *E. coli* (22). pGF7 was digested with *Nco*I and *Sac*I, and the CMCase-E2 CBD fragment was ligated into *Nco*I- and *Sac*I-digested pB-CMC or pBXCRCMC. The subsequent vectors were designated pBRCMC and pBXRRCMC, respectively, and transformed into *E. coli* DH5α by electroporation.

**Construction of *E. coli*-*Bacteroides*-*Prevotella* shuttle vectors.** The construction of pTC-COW was based on the *E. coli*-*Bacteroides* shuttle vector pVAL1 (43). pVAL1 was digested with *Eco*RI and *Cla*I to remove the IS4351′ and *ermF*, filled in, and ligated to the 2.7-kb blunted *Sac*I fragment containing *tetQ* from pNFD13-2 (26). The resulting tetracycline-resistant shuttle vector, pTC-COW, was partially digested with *Bsa*I and ligated to the blunted 2.5-kbp *Pst*I-*Sac*I fragment isolated from pFD308 (38), which contained IS4351′ and the *cat* gene (chloramphenicol resistance) from Tn9. The *Bam*HI site between the IS4351′ and the *cat* gene was eliminated. The resulting chimeric shuttle vector, pTC-COW, contains pB8-51 for replication and mobilization in *E. coli* and *Bacteroides* recipients, the ColE1 replicon from pBR328 for replication in *E. coli*, a chloramphenicol resistance that is expressed in both *E. coli* and *Bacteroides* spp., a tetracycline resistance (TetQ) that is expressed in *Bacteroides* and *Prevotella* strains, and the tetracycline and ampicillin resistances from pBR328 that are expressed in *E. coli*. pTC-XCMC, pTC-RCMC, and pTC-XRCMC were made by digesting pBXCRCMC, pBRCMC, and pBXRRCMC, respectively, with *Sal*I and *Bam*HI and ligating the CMCase fragments into *Sal*I- and *Bam*HI-digested pTC-COW. The subsequent plasmids were electroporated into *E. coli* S17-1 (35).

**Mating between *E. coli* and *B. uniformis*.** The procedure used for the matings between *E. coli* donors and *B. uniformis* recipients was as previously described (33, 39). Because of the presence of the chloramphenicol resistance gene on pTC-COW, pTC-COW derivatives were directly transferred to *B. uniformis* 1108 or 1008 strains that already contained the conjugal tetracycline-erythromycin resistance transposon Tc<sup>r</sup>Em<sup>r</sup> 12256. *B. uniformis* transconjugants were selected for gentamicin (200 μg/ml) and chloramphenicol (30 μg/ml) resistance.

**Mating between *B. uniformis* and *P. ruminicola* B<sub>1</sub>4R.** The procedure used for matings between *B. uniformis* 1108 and *P. ruminicola* B<sub>1</sub>4R was changed from the original protocol (32). All manipulations were done in an anaerobic glove box with an atmosphere of 95% CO<sub>2</sub> and 5% H<sub>2</sub>. The matings (2 × 10<sup>9</sup> B<sub>1</sub>4 cells and 1 × 10<sup>9</sup> *B. uniformis* cells were concentrated into a 100-μl volume of plating medium) were performed on Millipore HAWP 0.45-μm-pore-size filters (Millipore, Marlborough, Mass.) for 16 h. The concentration of rifampin in the selective media to select for the transconjugants in B<sub>1</sub>4R was increased from 40 to 60 μg/ml. Plates, rather than roll tubes, were used to enumerate the bacteria and isolate transconjugants. Southern blot analysis was used to determine if the Tc<sup>r</sup>

transconjugants were due to transfer of the pTC-COW derivatives or to transfer of Tc<sup>r</sup>Em<sup>r</sup> 12256.

**SDS-PAGE and Western and Southern blots.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots (immunoblots) were performed as previously described (24). In addition to the *P. ruminicola* B<sub>1</sub>4 CMCase antiserum, *T. fusca* E2 antiserum (5) was also used to detect reconstructed CMCases on Western blots.

Probes for Southern blot analysis were made by following the manufacturer's instructions for the Genius system nonradioactive DNA labelling kit (Boehringer Mannheim, Indianapolis, Ind.) and by using *Eco*RI-digested pTC-RCMC as template. Total DNA from *P. ruminicola* was isolated as previously described (49). Southern blotting was performed as previously described (24).

**Assays and agglutination experiments.** CMC and swollen cellulose assays were performed as previously described (12). Agglutination experiments were performed as previously described (13).

**Growth of *B. uniformis* transconjugants on acid-swollen cellulose.** *B. uniformis* pTC-XCMC and *B. uniformis* pTC-XRCMC were grown in basal media (12) supplemented with 4 g of cellobiose per liter to an optical density (600 nm) of 1.5. Basal medium (10 ml) was supplemented with swollen cellulose (3.5 mg/ml) and inoculated with 150  $\mu$ l of the previous cultures (approximately 20  $\mu$ g of cell protein per ml). In some cases the cultures were also supplemented with 120  $\mu$ g of purified exocellulases per ml (either *T. fusca* E3 [48], *Trichoderma reesei* CBHI [20], or both). Growth was monitored by increases in cell protein. Samples (100  $\mu$ l) were centrifuged (12,000  $\times$  g, 4°C, 10 min) to harvest the cells. Cell pellets were washed with 50 mM phosphate buffer (pH 6.5) and resuspended in 50  $\mu$ l of phosphate buffer (50 mM, pH 6.5). Cells were sonicated (Branson model 200 sonifier, micro tip, maximum output, 0°C, 5 min), and protein was measured by the Bradford assay (4) using bovine serum albumin as a standard.

## RESULTS

**Selection of transconjugants and mating.** The *Bacteroides-Prevotella* shuttle vector pRDB5 was transferred from *E. coli* S17-1 to *B. uniformis* 1100 by conjugation, and transconjugants were selected for tetracycline resistance (Tc<sup>r</sup>). These *B. uniformis* 1100 transconjugants, however, lacked the *Bacteroides* conjugal transposon (Tc<sup>r</sup>Em<sup>r</sup> 12256) that is necessary for plasmid mobilization from *Bacteroides* to *P. ruminicola* B<sub>1</sub>4 (34). pRDB5 could not be transferred directly from *E. coli* to *B. uniformis* 1108, which contains the conjugal element, because it is already Tc<sup>r</sup>. The necessary *B. uniformis* 1108(pRDB5) donor strain could be constructed by doing a second mating between *B. uniformis* 1100 (pRDB5) and *B. uniformis* 1008 with selection for erythromycin resistance. By using pTC-COW (Fig. 1), a shuttle vector containing the chloramphenicol resistance gene (*cat*), direct transfer from *E. coli* S17-1 to *B. uniformis* 1108 could be accomplished by using chloramphenicol resistance (Cm<sup>r</sup>) for selection of transconjugants and for retention of the plasmid.

The original protocol for mating *B. uniformis* to *P. ruminicola* was difficult and not always reliable. *P. ruminicola* B<sub>1</sub>4R and *B. uniformis* pTC-XCMC or *B. uniformis* pTC-XRCMC could be grown and washed in tubes outside an anaerobic chamber, but oxygen was often introduced during the washing step, subsequently inhibiting the growth of *P. ruminicola* B<sub>1</sub>4R. When the growth and washing steps were performed in an anaerobic chamber, anaerobiosis was more easily maintained and *P. ruminicola* and *B. uniformis* both grew reliably ( $2 \times 10^{10}$  and  $1 \times 10^{10}$  viable cells, respectively). Because *B. uniformis* donors were sometimes detected at frequencies of  $10^{-8}$  on the selection plates, the rifampin concentration was increased from 40 to 60  $\mu$ g/ml, which reduced the parental background to  $\leq 10^{-9}$ . The frequency of *P. ruminicola* B<sub>1</sub>4R transconjugants was approximately  $10^{-7}$  when the matings were done on an agar slant. By allowing the cells to mate on nitrocellulose filters, the conjugal frequency could be increased to approximately  $10^{-6}$  transconjugants per recipient.

**Transfer of the reconstructed CMCase gene from *E. coli* to *B. uniformis*.** *E. coli* DH5 $\alpha$  carrying plasmid pC43 (25), which contains the entire CMCase gene from *P. ruminicola* B<sub>1</sub>4, pro-

duces 88-, 82-, and 40.5-kDa CMCases, as well as other fortuitous proteins. None of these proteins can degrade cellulose at a rapid rate, but a reconstructed CMCase having a cellulose binding site did (24). When *E. coli* S17-1 was transformed with a plasmid carrying the reconstructed CMCase gene (pTC-RCMC), the dominant CMCases (approximately 90% of the total activity) were approximately 14 kDa larger, and all of these reconstructed enzymes could degrade cellulose (Table 2). When pTC-RCMC was transferred by conjugation from *E. coli* S17-1 to *B. uniformis* 1108, the *B. uniformis* transconjugants did not express any CMCase activity. However, when a xylanase promoter from *P. ruminicola* 23 (45) was placed upstream of the reconstructed CMCase gene (pTC-XRCMC), the *B. uniformis* transconjugants, *B. uniformis* pTC-XRCMC, produced 100- and 96-kDa cellulases (Fig. 2) that could degrade cellulose (Table 2). *B. uniformis* transconjugants lacking the binding domain from *T. fusca*, *B. uniformis* pTC-XCMC, produced 88- and 82-kDa CMCases, but these enzymes could not degrade cellulose at a significant rate.

*B. uniformis* pTC-XRCMC and *B. uniformis* pTC-XCMC both had CMCase-specific activities of approximately 230 nmol/mg of protein per min (Table 2). Both the CMCase and the reconstructed CMCase appeared to be on the surface of the cells. This is supported by the observation that whole cells had essentially the same activity as cell extracts (data not shown). In addition, antisera to the 40.5-kDa CMCase agglutinated *B. uniformis* cells containing either the CMCase or the reconstructed CMCase but did not agglutinate untransformed *B. uniformis* cells (data not shown). Finally, antisera to the *T. fusca* cellulase, E2, agglutinated only *B. uniformis* pTC-XRCMC cells.

*B. uniformis* 1108 could not grow on acid-swollen cellulose, but growth was observed if an excess of exocellulase (E3 from *T. fusca* or CBHI from *T. reesei*) was added to the culture supernatant (Table 3). E3 and CBHI together caused a more than additive increase in the growth rate than either of the two exocellulases alone. Transconjugants carrying the unmodified CMCase gene (pTC-XCMC) showed no improvement in growth over *B. uniformis* 1108, but transconjugants carrying the reconstructed CMCase (pTC-XRCMC) had a higher growth rate when both exocellulases were present.

**Transfer of the reconstructed CMCase gene from *B. uniformis* to *P. ruminicola* B<sub>1</sub>4.** When *B. uniformis* pTC-RCMC was mated with *P. ruminicola* B<sub>1</sub>4R, the frequency of transfer was  $10^{-6}$ . The *P. ruminicola* transconjugants had the same SDS-PAGE profile as *P. ruminicola* B<sub>1</sub>4R (Fig. 3), and Southern blots indicated that the *P. ruminicola* transconjugants contained pTC-RCMC (Fig. 4). These transconjugants, however, did not produce detectable amounts of reconstructed CMCase (Fig. 2). When pTC-XRCMC which provided a *P. ruminicola* 23 xylanase promoter was conjugally transferred to *P. ruminicola* B<sub>1</sub>4R, there was still no detectable amount of reconstructed CMCase even when xylan was used as the carbohydrate source.

## DISCUSSION

The modification of ruminal cellulose degradation by the introduction of genetically constructed cellulolytic organisms is complicated by many factors. In the rumen, free enzymes become too diffuse to be nutritionally advantageous to an organism (28); consequently, ruminal cellulases are usually cell associated (16, 27). Most of the well-characterized cellulases available for cloning, however, are from nonruminal organisms and are cell-free enzymes (15, 48). In addition, cloning foreign genes into ruminal bacteria may be unsuccessful because little

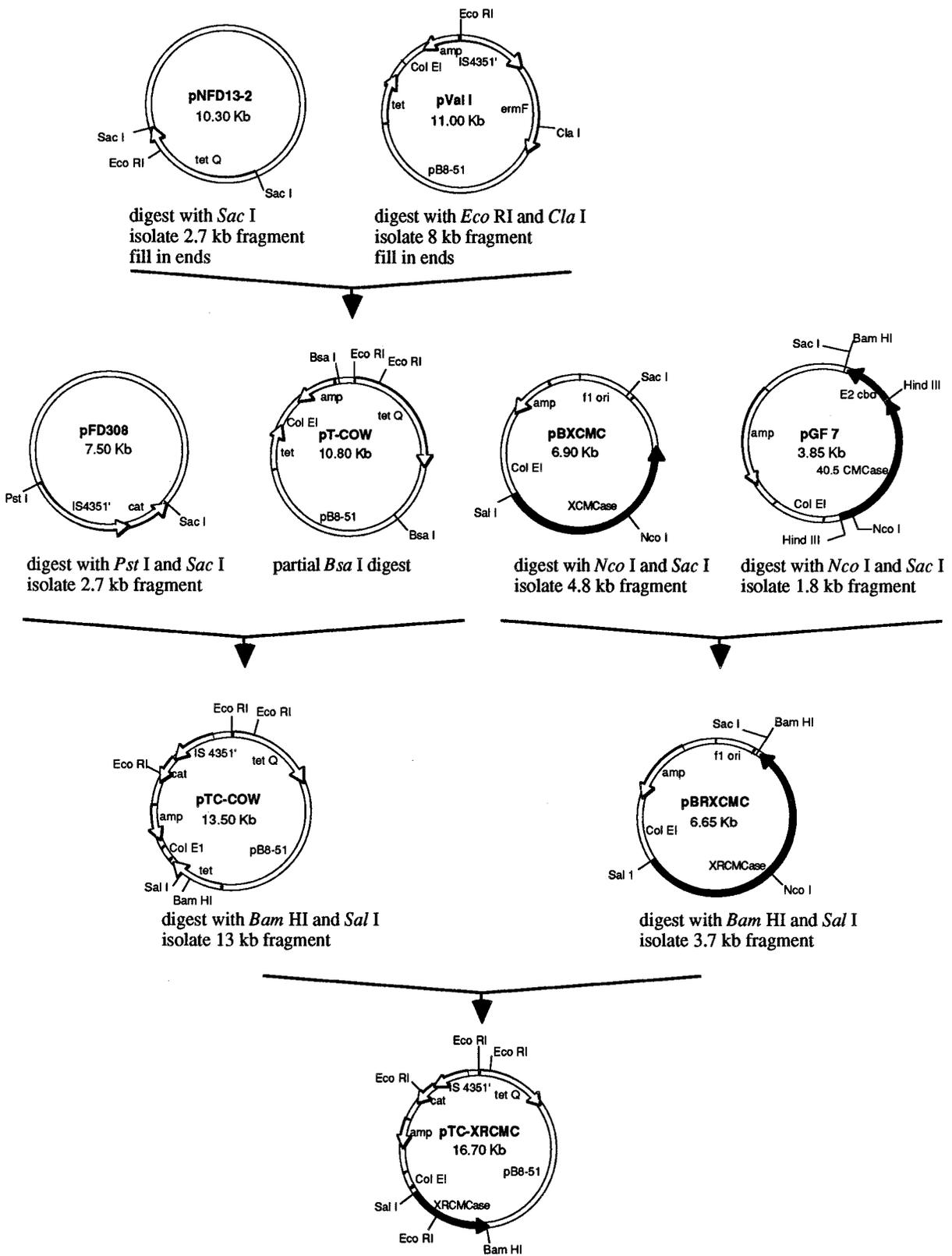


FIG. 1. Construction of shuttle vectors pTC-COW and pTC-XRCMC. □, *E. coli* sequences; ■, *Prevotella* sequences.

TABLE 2. CMC and swollen cellulose specific activities of *E. coli* and *B. uniformis*

Substrate	Sp act (nmol of reducing sugar/min/mg of protein)			
	<i>E. coli</i> (pTC-XCMC)	<i>E. coli</i> (pTC-XRCMC)	<i>B. uniformis</i> (pTC-XCMC)	<i>B. uniformis</i> (pTC-XRCMC)
CMC	377	386	221	239
Swollen cellulose	23	110	18	75

TABLE 3. Effects of exocellulases on the growth of *B. uniformis* strains

Exocellulase(s)	Growth rate (h <sup>-1</sup> ) <sup>a</sup>		
	BU1108	BU1108 (pTC-XCMC)	BU1108 (pTC-XRCMC)
E3	0.013	0.014	0.014
CBHI	0.021	0.021	0.022
E3 + CBHI	0.082	0.082	0.137

<sup>a</sup> Values are the averages of three determinations. Standard deviations were all less than 10%. No growth was observed without exocellulases.

is known about their transcriptional and translational machinery. Previous work indicates that the *P. ruminicola* B<sub>14</sub> CMCase is a cell-associated enzyme (13) and can be made active against cellulose by the addition of a CBD (22). On the basis of these observations, we decided to modify the *P. ruminicola* B<sub>14</sub> CMCase gene before attempting to clone foreign cellulase genes into *P. ruminicola* B<sub>14</sub>.

To facilitate cloning genes into *P. ruminicola* B<sub>14</sub>, the protocol developed by Shoemaker et al. (32) was modified in this study. A new *Bacteroides-Prevotella* shuttle vector, pTC-COW, was constructed, eliminating one mating step. More stringent mating conditions and selection procedures were also developed, increasing the yield of transconjugants in *P. ruminicola* B<sub>14</sub> about 100-fold. These improvements made it easier to transfer the reconstructed CMCase vectors from *E. coli* to *B. uniformis* recipients and then to *P. ruminicola* B<sub>14</sub>. This new protocol should inspire future genetic manipulations of *P. ruminicola* B<sub>14</sub>.

When the vector pTC-RCMC was transferred to *B. uniformis* 1108 and then to *P. ruminicola* B<sub>14</sub>R, neither strain produced any detectable amounts of reconstructed CMCase. Whitehead (45) cloned the promoter region of a xylanase gene from *P. ruminicola* 23 upstream of a promoterless chloramphenicol gene, and it allowed transcription of the gene in both *E. coli* and *B. uniformis*. This promoter also allowed expression of the reconstructed CMCase in *B. uniformis*, but no expression of the reconstructed CMCase was detected in *P. ruminicola* B<sub>14</sub> (carrying pTC-XRCMC) even when xylan was the carbon source. This is not altogether unexpected since *P. ruminicola* 23 and B<sub>14</sub> are genetically distinct strains that have less than 20% DNA homology (2, 23), and it would appear that they do not have identical promoters. The fact that *B. uniformis* pTC-XRCMC can express the reconstructed gene with the aid of the xylanase promoter region could indicate that either *B. uniformis* can recognize the *P. ruminicola* 23 xylanase pro-

motor or that there is a fortuitous *Bacteroides* promoter sequence contained within the cloned region. In either case, these results show that promoter recognition cannot be assumed between *Bacteroides* and *Prevotella* spp. or even among *Prevotella* spp. However, *Bacteroides* spp. and *P. ruminicola* B<sub>14</sub> both express the tetracycline resistance gene (*tetQ*), and the vectors containing the *Bacteroides* plasmid pB8-51 can replicate in both strains, indicating that they can sometimes recognize the same promoters. Because its xylanase promoter was placed upstream of the CMCase gene, *P. ruminicola* 23 might have expressed the reconstructed CMCase, but no transconjugants were detected from matings between *B. uniformis* pTC-XRCMC and *P. ruminicola* 23. This could be due to the possibility that the system of gene transfer between *B. uniformis* 1108 and *P. ruminicola* B<sub>14</sub> does not work with *P. ruminicola* 23, the shuttle vector does not replicate in *P. ruminicola* 23, or the *tetQ* gene is not expressed in *P. ruminicola* 23.

Unfortunately, the effect on cellulose degradation could not be assessed in *P. ruminicola* B<sub>14</sub> transformed with pTC-XRCMC or pTC-RCMC because the reconstructed CMCase gene was not expressed. However, since the reconstructed CMCase was expressed in *B. uniformis*, the benefit of this gene to a noncellulolytic organism could still be determined. Whitehead et al. (46) transformed *Bacteroides thetaiotamicron* with a *P. ruminicola* 23 xylanase gene and demonstrated that the *B. thetaiotamicron* transconjugant could degrade xylan but it could not use the digestion products for growth. This could cause potential problems when the organism is introduced into the rumen to increase xylan degradation because there is no selection pressure to keep the xylanase from accumulating mutations and being rendered inactive. Because *B. uniformis* can grow on cellobiose, it would be able to utilize the products of cellulose hydrolysis and could maintain selection pressure

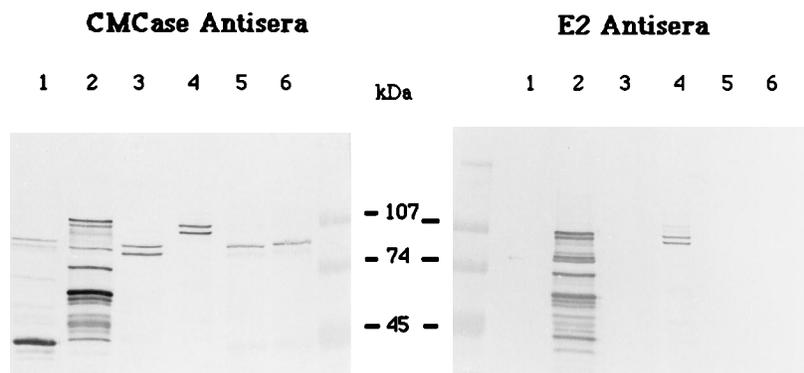


FIG. 2. Western blot of XRCMC strains carrying pTC-XRCMC or pTC-XCMC. Lanes: 1, *E. coli*(pTC-XCMC); 2, *E. coli*(pTC-XRCMC); 3, *B. uniformis*(pTC-XCMC); 4, *B. uniformis*(pTC-XRCMC); 5, *P. ruminicola* B<sub>14</sub>; 6, *P. ruminicola*(pTC-XRCMC).

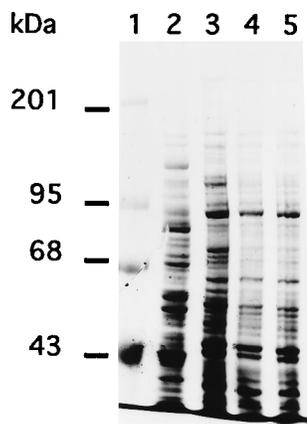


FIG. 3. SDS-PAGE gel of *P. ruminicola* transconjugants. Lanes: 1, molecular mass standards; 2, *B. uniformis* 1108; 3, *P. ruminicola* B<sub>14</sub>R; 4, *P. ruminicola* (pTC-RCMC); 5, *P. ruminicola* (pTC-XRCMC).

against mutations that would inactivate the reconstructed CM-Case gene. It would also mean that *B. uniformis* could become truly cellulolytic because cellulose would be its carbon source.

*B. uniformis* pTC-XCMC or pTC-XRCMC could not grow on acid-swollen cellulose, so it appears that the addition of an endocellulase is not enough to create a cellulolytic organism. It has long been recognized that cellulose is degraded via synergistic interactions of more than one enzyme (17, 50). Irwin et al. (20) noted a pronounced synergistic effect when two exocellulases and an endocellulase were used together to hydrolyze cellulose. Endocellulases provide reducing and nonreducing ends which can then be attacked by the exocellulases which cleave cellobiose from the ends (48). Exocellulase CHBI (from *T. reesei*) cleaves cellobiose from the reducing end, while exocellulase E3 (from *T. fusca*) cleaves cellobiose from the non-reducing end (3, 7). Because significant growth was observed with the *B. uniformis* strains only when two different exocellu-

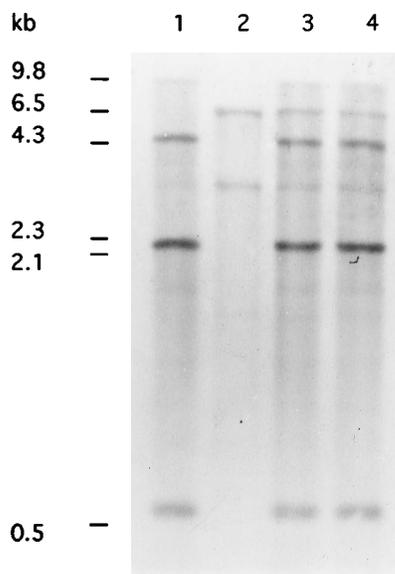


FIG. 4. Southern blot of *P. ruminicola* transconjugants. Total cellular DNAs were isolated and digested with *Eco*RI. The probe was made to pTC-RCMC. Lanes: 1, *E. coli*(pTC-RCMC); 2, *P. ruminicola* B<sub>14</sub>; 3, *P. ruminicola*(pTC-RCMC); 4, *P. ruminicola*(pTC-XRCMC).

lases were added to the culture supernatant, it would appear that the addition of exocellulases is a vital step in creating a cellulolytic organism. However, *B. uniformis* pTC-XRCMC did grow significantly faster than *B. uniformis* 1108 or *B. uniformis* pTC-XCMC in the presence of E3 and CBHI, so the production of the reconstructed CMCase by *B. uniformis* did indeed improve cellulose hydrolysis. Thus, it may require the introduction of both the exocellulases and the reconstructed CMCase to create an efficient cellulolytic *P. ruminicola* strain.

Future work will concentrate on isolating a *P. ruminicola* B<sub>14</sub> promoter that will allow expression of the reconstructed CMCase. Recent work indicates that the CMCase has virtually the same pattern of expression as mannanase activity in *P. ruminicola* B<sub>14</sub>, and it appears that the mannanase and CMCase genes may be part of an operon (12). We are currently attempting to clone a 16-kb *Sac*I fragment which appears to contain both the mannanase and CMCase and to identify the DNA sequence that serves as a promoter in *P. ruminicola* B<sub>14</sub>.

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