# Cloning and Expression in *Escherichia coli* of a Xylanase Gene from *Bacteroides ruminicola* 23

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A gene coding for xylanase activity in the ruminal bacterial strain 23, the type strain of *Bacteroides ruminicola*, was cloned into *Escherichia coli* JM83 by using plasmid pUC18. A *B. ruminicola* 23 genomic library was prepared in *E. coli* by using *Bam*HI-digested DNA, and transformants were screened for xylanase activity on the basis of clearing areas around colonies grown on Remazol brilliant blue R-xylan plates. Six clones were identified as being xylanase positive, and all six contained the same 5.7-kilobase genomic insert. The gene was reduced to a 2.7-kilobase DNA fragment. Xylanase activity produced by the *E. coli* clone was found to be greater than that produced by the original *B. ruminicola* strain. Southern hybridization analysis of genomic DNA from the related *B. ruminicola* strains, D31d and H15a, by using the strain 23 xylanase gene demonstrated one hybridizing band in each DNA.

Xylan is a major component of hemicellulose in plants and is used as a source of carbon and energy by the microflora of the rumen. However, compared with other plant polysaccharides, such as starch and cellulose, hemicellulose is degraded to a lesser extent in the rumen (19). In order to understand the limiting factors in hemicellulose degradation, we are studying the enzymes involved in the breakdown of complex polysaccharides, such as xylan, by ruminal microorganisms. *Bacteroides ruminicola* strains are the predominant microorganisms in the rumen, and a number of strains have been shown to be xylanolytic (5).

*B. ruminicola* is a member of the phylogenetic true *Bacteroides* species cluster and is related to the colonic *Bacteroides* species, such as *B. fragilis* and *B. thetaiotaomicron* (15). Relatively little is known about the activity, structure, and genetic regulation of enzymes produced by ruminal microorganisms. By means of molecular biology, we are beginning to study the individual genes and proteins involved in degradation of polysaccharides. In this regard, we now report the cloning of a xylanase gene from *B. ruminicola* 23. This is the first report of the cloning and expression in *Escherichia coli* of a gene from a *B. ruminicola* strain.

## MATERIALS AND METHODS

**Bacterial strains and media.** *B. ruminicola* 23 (3), the type strain of the species; strain D31d (6), a pectinolytic isolate; and strain H15a (5), a hemicellulolytic isolate, were grown anaerobically on RGM medium (7) containing 0.2% carbon source at 37°C. *E. coli* strains were grown on LB medium (10 g of tryptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter) or on LB medium supplemented with ampicillin (75 µg/ml) (LBA) to select for transformants. LBA agar plates containing 3.5 mg of 4-*O*-methyl-D-glucurono-D-xylan-Remazol brilliant blue R per ml (RBB-xylan) were used for screening transformants for the cloned xylanase gene.

**Cloning procedures.** Plasmid pUC18 (20) was used as the cloning vector; *E. coli* JM83 and JM105 (20) were used as the cloning hosts. *B. ruminicola* genomic DNA was a gift from Bruno Mannarelli (Agricultural Research Service, Peoria, Ill.) and was isolated by methods described previously (2,

10). Genomic DNA was partially digested with *Bam*HI; conditions were made optimal for obtaining fragments from 5 to 10 kilobases (kb) in length. The digested DNA was ligated into *Bam*HI-digested and dephosphorylated pUC18 by using T4 DNA ligase, and the ligated plasmid DNA was transformed into competent strain JM83. Transformed cells were screened on LBA–RBB-xylan plates, and xylanase-positive clones were identified on the basis of clearing zones around the colonies. Xylanase-positive clones were picked and restreaked to confirm formation of clearing zones around the colonies on the RBB-xylan.

**DNA analysis.** DNA was digested with restriction endonucleases, electrophoresed through agarose gels in 89 mM Tris–68 mM phosphoric acid–2 mM EDTA, and stained with ethidium bromide. For hybridization with radiolabeled probes, the DNA was transferred to nitrocellulose by the method of Southern (17). Gel-purified DNA fragments were radiolabeled by means of a randomly primed DNA labeling kit and $[\alpha\text{-}^{32}P]dATP$ to a specific activity of $>10^9$ cpm/µg as recommended by the manufacturer. DNA was hybridized by the method of Maniatis et al. (9). For heterologous hybridizations, stringency was increased by raising the 6× SSC (0.9 M NaCl–0.09 M sodium citrate, pH 7.0) wash temperatures from the hybridization temperature (55°C) to 69°C.

**Protein and enzyme analyses.** *B. ruminicola* strains were grown to mid-log phase ($A_{660}$, approximately 0.6; 1-cm light path), centrifuged at 10,000 × g at 20°C for 10 min, and washed twice with 50 mM potassium phosphate (pH 6.8)–10 mM dithiothreitol. Cells were resuspended at 5% of the original culture volume in the same buffer and broken in French pressure cell at 12,000 lb/in². The broken-cell suspension was centrifuged at 15,000 × g for 20 min at 20°C, and the supernatant fluid (crude extract) was recovered and used for enzyme assays and protein analyses. *E. coli* strains carrying the vector or hybrid plasmids were grown in LBA to an $A_{660}$ of 1.0 and processed as described above.

Xylanase activity was determined aerobically by monitoring release of sugars from larchwood xylan (50 µg/ml) with orcinol as described previously (7), with xylose as the standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol of xylose equivalents in 1 h at 37°C. Xylosidase activity was
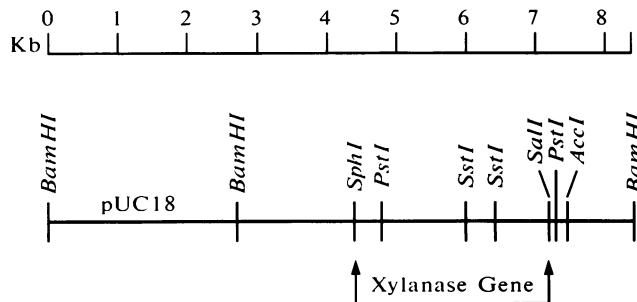
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FIG. 1. Restriction endonuclease map of plasmid pRX1 carrying the xylanase gene. The bracket indicates the location of the xylanase gene.

also assayed as described previously (7), and one unit was defined as the amount of enzyme that catalyzed the release of 1 μmol of p-nitrophenol in 1 h at 37°C. Protein concentrations were estimated by the dye-binding assay of Bradford (4) with the commercial reagent (Bio-Rad Laboratories, Richmond, Calif.), and fraction V of bovine serum albumin was used as the standard. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (8). The stacking gel was 4% acrylamide, and the resolving gel was 10% acrylamide. Proteins were stained with Coomassie brilliant blue R-250.

**Materials.** Restriction enzymes, T4 DNA ligase, isopropyl-thio-β-galactoside (IPTG), and plasmid pUC18 were purchased from Bethesda Research Laboratories, Gaithersburg, Md. Larchwood xylan, RBB-xylan, and p-nitrophenyl-β-D-xylopyranoside were obtained from Sigma Chemical Co., St. Louis, Mo. [α-$^{32}$P]dATP was purchased from New England Nuclear Corp., Boston, Mass. Calf intestinal alkaline phosphatase and a randomly primed DNA labeling kit were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Nitrocellulose BA-85 (0.45 μM) was obtained from Schleicher & Schuell, Keene, N.H. All other reagents were of reagent grade or higher grade.

## RESULTS

**Cloning of *B. ruminicola* 23 xylanase gene.** A genomic library of *B. ruminicola* 23 DNA was prepared in *E. coli* JM83 by using pUC18. The transformed bacteria were screened on LBA–RBB-xylan plates for xylanase activity. Initially, 10 clones out of approximately 8,000 colonies screened were identified as being xylanase positive on the basis of formation of clear halos around the colonies. After secondary screening, six clones were found to be xylanase positive. Colonies of JM83 cells carrying pUC18 did not produce clear zones on the RBB-xylan plates.

**DNA and enzyme analysis.** Plasmid DNA was isolated from the six independent clones and subjected to restriction endonuclease mapping. All six plasmids were found to contain an identical 5.7-kb *Bam*HI insert in the same orientation. One plasmid was designated pRX1 and used for further restriction analysis and characterization. The results of subcloning of various restriction fragments indicated that the xylanase gene was located on the 2.7-kb *Sph*I-*Sal*I fragment of pRX1 (bracketed segment, Fig. 1). Further attempts to reduce the insert size with the few enzymes that would digest the insert DNA resulted in loss of xylanase activity. The 2.7-kb fragment was subcloned into pUC18 in both orientations, and the resultant plasmids were termed pRX26 and pRX27. Both plasmids coded for active xylanase
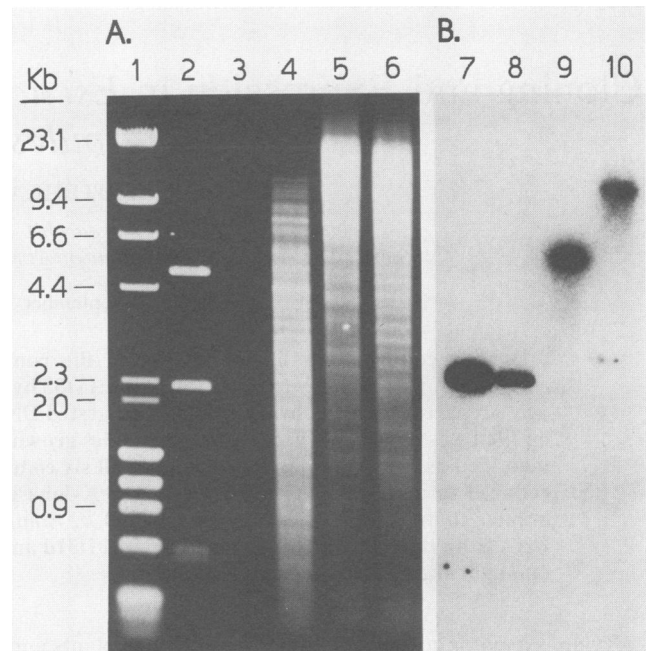


FIG. 2. Southern hybridization analysis of *B. ruminicola* 23 genomic DNA digests with a nick-translated 2.2-kb *Pst*I fragment of pRX1. (A) Photograph of a 0.8% agarose gel stained with ethidium bromide. (B) Autoradiogram of DNA transferred to nitrocellulose and hybridized with the $^{32}$P-labeled *Pst*I fragment. Lanes: 1, molecular weight markers (lambda-*Hind*III digest); 2, 0.2 μg of *Pst*I-digested pRX1; 3 and 7, 0.002 μg of *Pst*I-digested pRX1. Lanes 4 through 6 and lanes 8 through 10 contain 5 μg of strain 23 DNA: lanes 4 and 8, *Pst*I digest; lanes 5 and 9, *Bam*HI digest; lanes 6 and 10, *Eco*RI digest.

in *E. coli* JM83 as determined by formation of clear zones on RBB-xylan. In addition, when pRX26 and pRX27 were transformed into *E. coli* JM105, equal clearing was observed in the presence and absence of IPTG. These data indicate that the xylanase gene is being transcribed from an endogenous promoter and that it is not under the control of the pUC18 β-galactosidase promoter.

Xylanase specific activity in crude extracts of JM83(pRX26) was found to be 1.1 U/mg of protein, an activity which is approximately 37-fold and 1.2-fold higher than that found in *B. ruminicola* 23 grown on glucose and oat spelt xylan, respectively. No xylosidase activity was found in crude extracts of JM83 carrying pRX1 or pRX26. When proteins from crude extracts of JM83(pRX26) and JM83(pUC18) were subjected to comparative analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, no additional proteins could readily be identified (data not shown).

**Southern analysis.** The cloned xylanase gene was used as a probe to carry out Southern hybridization analysis of genomic DNA from *B. ruminicola* 23. The DNA was digested with *Pst*I, *Bam*HI, or *Eco*RI and hybridized with the nick-translated 2.2-kb *Pst*I fragment of pRX1. The results are shown in Fig. 2. An identical 2.2-kb band is visible in the genomic *Pst*I digest (lane 8), and the *Bam*HI digest demonstrates a 5.7-kb hybridizing band, as is visible in the original clone (lane 9). The *Eco*RI digest reveals a 15-kb hybridizing band (lane 10). These results indicate that the same 5.7-kb *Bam*HI fragment is present in the strain 23 chromosome and that it did not rearrange during the cloning procedures.
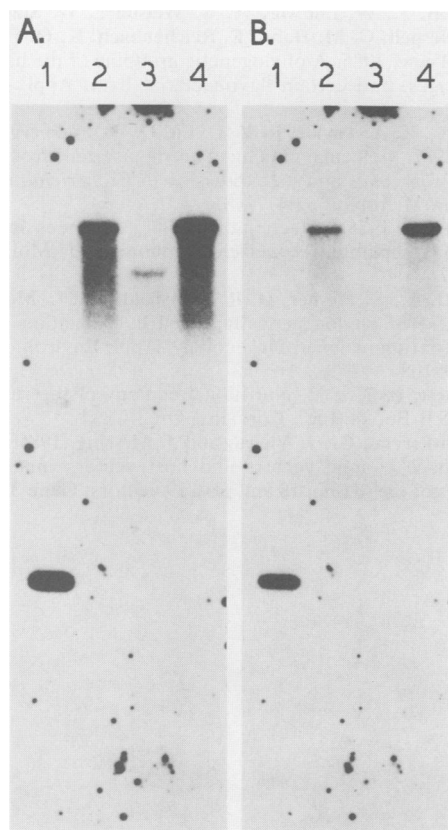
FIG. 3. Southern hybridization analysis of genomic DNA from *B. ruminicola* 23, D31d, and H15a with the nick-translated *Pst*I fragment of pRX1 carrying the strain 23 xylanase gene. (A) Analysis after 55°C washes. Lanes: 1, 0.002 μg of *Pst*I-digested pRX1; 2, 5 μg of *Eco*RI-digested strain 23 DNA; 3, 5 μg of *Eco*RI-digested D31d DNA; 4, 5 μg of *Eco*RI-digested H15a DNA. (B) Analysis after 69°C washes. The lanes contain the same DNAs as in panel A.

We decided to use the xylanase gene cloned from strain 23 as a probe for the presence of a homologous gene in two other strains of *B. ruminicola*, D31d and H15a. The nick-translated *Pst*I 2.2-kb fragment of pRX1 was used to probe *Eco*RI digests of genomic DNA from strains D31d and H15a. The results are shown in Fig. 3. When less stringent hybridization and washing conditions of 55°C were used, a hybridizing band of about 10 kb was seen with strain D13d (Fig. 3A, lane 3) and one of about 15 kb was seen with strain H15a (lane 4) that was almost identical to the 15-kb band seen with strain 23 (lane 2). When the stringency of the hybridization conditions was increased by using 69°C washes, the D31d band disappeared (Fig. 3B, lane 3), whereas a strong hybridization signal was still present with strain H15a DNA (lane 4).

## DISCUSSION

The xylanase gene from *B. ruminicola* 23, the type strain of the species, was cloned into *E. coli* by using pUC18. This is the first report of a gene from any true *Bacteroides* species of ruminal origin being cloned and expressed in *E. coli*. A xylanase gene from *Bacteroides succinogenes* has been reported to be expressed in *E. coli* (16); however, *B. succinogenes* has been shown not to be in the *Bacteroides* family by 16S RNA analysis (15, 18). The cloned strain 23

xylanase gene appears to be expressed from an endogenous promoter and is not under the control of the pUC18 β-galactosidase promoter. This conclusion is based on the fact that xylanase activity was observed when the genomic insert was cloned in both orientations. Second, no difference in formation of clear zones was noted when the insert in either orientation was cloned into *E. coli* JM105 in the presence and absence of IPTG, an inducer of the β-galactosidase gene of pUC18. It is interesting that the xylanase activity in the *E. coli* clone is greater than that of strain 23 grown on oat spelt xylan, which is the highest activity we have observed. Reports of other genes coding for xylanolytic enzymes and cloned into *E. coli* have shown that the activities have been much lower in *E. coli* than in the original organism (for example, see references 1, 12, and 13). The reason(s) for this higher activity is unclear. The enzyme produced in *E. coli* is functionally active and capable of degrading both larchwood and RBB-xylan. However, the structure and characteristics of this enzyme in either *B. ruminicola* or *E. coli* have yet to be determined. Further studies on the DNA sequence of the 2.7-kb fragment containing the xylanase gene should prove helpful in determining the regulatory sequences of the gene and the subunit structure of this enzyme. Assuming that most of the 2.7-kb insert consists of sequence coding for the enzyme, one could predict a protein with a molecular weight of 90,000. Bacterial xylanases tend to be monomers having molecular weights that range from 24,000 (in *Bacillus pumilus* [14]) to 145,000 (in an *Aeromonas* sp. [11]).

The cloned strain 23 xylanase gene was also used as a probe to determine whether homologous genes are present in *B. ruminicola* D31d and H15a. The results demonstrated only one hybridizing band in genomic digests of both organisms. Under more stringent conditions, only H15a hybridized to a fragment which was almost identical in size to that from strain 23 DNA. These data are in agreement with the total DNA-DNA hybridization results, as strains 23 and H15a are more closely related than are strains 23 and D31d (B. Mannarelli, personal communication). These results also suggest that the cloned strain 23 xylanase gene may be used as a probe to isolate xylanase genes from other *B. ruminicola* strains for comparative studies.

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