APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 2958–2964 Vol. 61, No. 8
0099-2240/95/$04.00+0

A Xylan Hydrolase Gene Cluster in *Prevotella ruminicola* $B_1$4: Sequence Relationships, Synergistic Interactions, and Oxygen Sensitivity of a Novel Enzyme with Exoxylanase and β-(1,4)-Xylosidase Activities

ALES GASPARIC,[1] JENNIFER MARTIN,[2] ANNE S. DANIEL,[2] AND HARRY J. FLINT[2]*

Rowett Research Institute, Bucksburn, Aberdeen, United Kingdom,[2] and Biotechnical Faculty, University of Ljubljana, Slovenia[1]

Received 1 March 1995/Accepted 17 May 1995

Two genes concerned with xylan degradation were found to be closely linked in the ruminal anaerobe *Prevotella ruminicola* $B_1$4, being separated by an intergenic region of 75 nucleotides. *xynA* is shown to encode a family F endoxylanase of 369 amino acids, including a putative amino-terminal signal peptide. *xynB* encodes an enzyme of 319 amino acids, with no obvious signal peptide, that shows 68% amino acid identity with the *xsa* product of *Bacteroides ovatus* and 31% amino acid identity with a β-xylosidase from *Clostridium stercorarium*; together, these three enzymes define a new family of β-(1,4)-glycosidases. The activity of the cloned *P. ruminicola xynB* gene product, but not that of the *xynA* gene product, shows considerable sensitivity to oxygen. Studied under anaerobic conditions, the XynB enzyme was found to act as an exoxylanase, releasing xylose from substrates including xylobiose, xylopentaose, and birch wood xylan, but was relatively inactive against oat spelt xylan. A high degree of synergy (up to 10-fold stimulation) was found with respect to the release of reducing sugars from oat spelt xylan when XynB was combined with the XynA endoxylanase from *P. ruminicola* $B_1$4 or with endoxylanases from the cellulolytic rumen anaerobe *Ruminococcus flavefaciens* 17. Pretreatment with a fungal arabinofuranosidase also stimulated reducing-sugar release from xylans by XynB. In *P. ruminicola* the XynA and XynB enzymes may act sequentially in the breakdown of xylan.

Plant cell wall material is degraded in the rumens of mammals by a complex community of anaerobic microorganisms. Efficient degradation requires the breakdown of the matrix material, which is largely composed of hemicelluloses, in addition to cellulose breakdown. Cellulolytic rumen microorganisms are found to effectively degrade xylans, the major component of hemicellulose, but often utilize the breakdown products only poorly. On the other hand, many noncellulolytic bacteria utilize isolated xylans well and cause greatly increased utilization of hemicellulose from intact plant material when in coculture with cellulolytic species (5). The most abundant of these noncellulolytic, xylan-utilizing species appear to be *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* (28, 32). *P. ruminicola* $B_1$4 is an actively xylanolytic strain (2, 4) and is known to have a high-affinity system for xylose uptake (29).

Xylans are a highly diverse group of polysaccharides, in which the main chain of β-1,4-linked xylose residues can be substituted to various degrees with acetyl, α-2- or α-3-linked arabinose residues or 4-*O*-methyl glucuronic acid residues. Numerous endoxylanases that attack the main xylan chain have been described, together with β-xylosidases, which act on xylobiose or xylooligosaccharides, and a variety of debranching activities (3, 38). In common with cellulolytic rumen bacteria (8, 21) and nonrumen organisms, *P. ruminicola* $B_1$4 carries multiple xylanase genes. Four regions encoding xylanase activity have been identified in *P. ruminicola* $B_1$4 (14), one of which corresponds to a broad-specificity endoglucanase (24). Of the remaining regions, one encodes activities against *p*-nitrophenyl-β-D-xyloside (pNPX) and *p*-nitrophenyl-α-L-arabinofuranoside (pNPA), in addition to xylanase activity. In the present work, we show that this region carries at least two linked genes, one of which encodes an endoxylanase while the other encodes a novel oxygen-sensitive exoxylanase that has associated pNPX and pNPA activities. The exoxylanase exhibits a remarkable degree of synergy with endoxylanases, and its sequence relationships with β-xylosidases help to establish a new family of glycoside hydrolases.

* Corresponding author. Mailing address: Rowett Research Institute, Greenburn Rd., Bucksburn, Aberdeen AB2 9SB, United Kingdom. Phone: (+44) 1224 716651. Fax: (+44) 1224 716687. Electronic mail address: h.flint@rri.sari.ac.uk.

MATERIALS AND METHODS

Strains and isolated genes. *Escherichia coli* DH5α carrying various recombinant plasmids was used as a source of enzyme activities studied. The plasmids carried were pUC19/L3 (3.1-kb *P. ruminicola* $B_1$4 fragment carrying an intact *xynB* gene), pUC18/L30 (4.1-kb *P. ruminicola* $B_1$4 fragment carrying an intact *xynA* gene) (14), pUC13/X4723 (*Ruminococcus flavefaciens* 17 fragment encoding endoxylanase XynA domain C fused with a *lacZ* translational start), and pUC13/X4530 (*R. flavefaciens* 17 fragment encoding endoxylanase XynA domain A fused with a *lacZ* translational start) (11, 39). *R. flavefaciens* 17 was isolated from the rumen of a fistulated cow as described previously (8).

Growth conditions. Cultures of *E. coli* cells carrying pUC plasmids were grown in Luria broth (LB) medium containing 50 μg of ampicillin per ml. For enzyme assays, cells grown overnight were harvested by centrifugation, suspended in 1/40 of the culture volume of anaerobically prepared 0.05 M Na phosphate buffer–2 mM dithiothreitol (DTT) (pH 6.5) (see below), and subjected to sonication (9). Cell extracts were stored at −70°C under 100% CO_2. *R. flavefaciens* 17 cells were grown in a defined medium containing 0.5% freezer-milled oat straw as the sole source of energy. After 3 days of growth, cells were harvested by centrifugation under 100% CO_2, resuspended in 1/40 of the original culture volume of 0.05 M Na phosphate buffer–2 mM DTT (pH 6.5), and subjected to sonication. Culture supernatant was concentrated 12.5-fold by using an Amicon PM10 membrane (molecular weight cutoff, 10,000).

Enzyme assay conditions. Assays measuring the release of reducing sugar from xylan (final concentration, 1% unless otherwise stated) (20) and assays measuring the release of *para*-nitrophenol from pNPA and pNPX were as described previously (7, 14) except that they were routinely performed under anaerobic

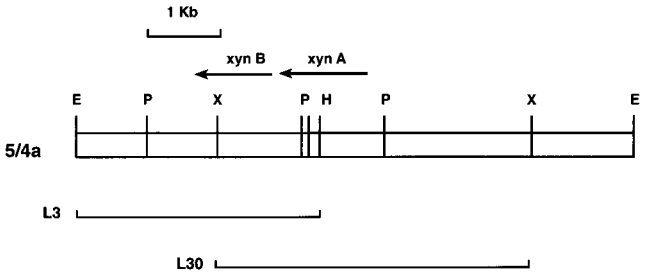

FIG. 1. Linked genes concerned with xylan utilization in *P. ruminicola* B_14. The positions and orientations of the *xynA* and *xynB* ORFs are indicated and are based on sequencing of the 3,689 bp between the leftmost *Eco*RI site and the rightmost *Pst*I site. A 2,500-bp stretch of this sequence, including the two identified genes, is in the database (EMBL accession no. Z49241). An incomplete ORF (414 amino acids) extending from the leftmost *Eco*RI site, in the opposite orientation relative to *xynA* and *xynB*, is also present but lacks its amino terminus and is not expected to be expressed in the clones described here. L3 and L30 are subclones made in pUC13 and were derived from the larger pUC13 clone 5/4a (14). E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I.

conditions in the present study. Anaerobic 50 mM Na phosphate buffer was prepared by boiling and bubbling with 100% CO_2, DTT was added to a final concentration of 2 mM, and the pH was adjusted to 6.5 with NaOH. The buffer was maintained under a 100% CO_2 atmosphere, and assays were performed in this atmosphere. Anaerobic growth of *E. coli* carrying plasmid L3 in LB medium with glucose did not result in any detectable activity. The best conditions for recovering XynB activity were, therefore, aerobic growth followed by anaerobic assay.

In order to examine the effects of preincubation, extracts were diluted 1:9 with aerobically or anaerobically prepared 50 mM Na phosphate buffer (pH 6.5) with or without 100 μg of bovine serum albumin per ml or 2 mM DTT. After a 30-min incubation at 30°C, the preincubation mix was diluted a further 10-fold by addition of the appropriate assay buffer containing the substrate pNPA or pNPX at 3 mM, and incubation was continued at 37°C for 15 min before termination of the reaction. For the experiment shown in Table 3, xylans (5% [wt/vol]) were pretreated with *Aspergillus niger* arabinofuranosidase (Megazyme Pty., Sydney, New South Wales, Australia) at a final concentration of 0.8 U/ml for 60 min at 37°C in 0.1 M Na acetate buffer, pH 4, under aerobic conditions. Unless otherwise stated, enzyme activities reported here refer to the means for duplicate assays; individual values differed from the mean by no more than 10%.

Molecular biology procedures. Subcloning and preparation of plasmid DNA were done by standard procedures (27). DNA sequencing was performed by the dideoxy-chain termination method with an Applied Biosystems automated sequencer. *Taq* polymerase was used according to the manufacturer's recommendations. M13 forward and reverse primers, and internal primers synthesized on a Cruachem synthesizer, were used to obtain the sequence shown in Fig. 2. Both strands of the region shown were sequenced. Sequence alignments and searches were performed by using the Seqnet facility, Daresbury, United Kingdom.

Carbohydrate analyses. Hydrolysis products were analyzed by thin-layer chromatography as described previously (9) following incubation of xylan (10 mg/ml), xylopentaose (2.5 mg/ml), or xylobiose (1 mg/ml) with enzyme preparations, under anaerobic assay conditions. Xylopentaose and aldotetraouronic acid were obtained from Megazyme Pty. Xylans were obtained from Sigma, Poole, United Kingdom (birch wood [BW] xylan X-0502 and oat spelt [OS] xylan X-0627), and from Roth, Karlsruhe, Germany, via Techmate, Milton Keynes, United Kingdom (OS xylan 9289 and BW xylan 7500). Xylan suspensions (1% in 50 mM sodium phosphate buffer–2 mM DTT [pH 6.5]) were fractionated by two cycles of centrifugation (2,000 × *g*, 20 min, ambient temperature) and resuspension. The final pellet was taken as the insoluble fraction, and the pooled supernatants were taken as the soluble fraction. Total carbohydrate in each fraction was determined by the phenol-sulfuric acid method (17). The monosaccharide composition of xylans was determined by gas-liquid chromatography (6).

RESULTS

Nucleotide sequences of two linked genes concerned with xylan utilization. The nucleotide sequence of a region of *P. ruminicola* B_14 DNA that specifies xylanase, pNP-xylosidase, and pNP-arabinofuranosidase activities (14) was found to contain two open reading frames (ORFs) (Fig. 1 and 2). The upstream ORF (designated *xynA*) gives rise to a predicted translation product of 369 amino acids, starting with a region resembling a typical bacterial signal peptide sequence (34), that shows close similarity with xylanases of family F, in particular XylI from the hind gut anaerobe *Bacteroides ovatus* (37) (Table 1). The second ORF (designated *xynB*), separated by 75 nucleotides from the *xynA* gene, encodes a polypeptide whose predicted translation product of 319 amino acids is similar in sequence to the predicted product of the *xsa* gene of *B. ovatus*, which encodes an enzyme with β-(1,4)-xylosidase and α-L-arabinofuranosidase activities (Fig. 3; Table 1). The *B. ovatus* gene (37) is also situated immediately downstream of a gene encoding a family F xylanase, so that the two clusters appear closely homologous. Neither the *P. ruminicola xynB* product nor the *B. ovatus xsa* product shows an obvious signal peptide sequence.

No sequence resembling a typical *E. coli* ribosome binding site was found upstream of either ORF. This appears to be a common feature of genes isolated from *Bacteroides* and *Prevotella* spp. (33, 36). Sequences showing a four- of five-base complementarity with the 3′ end of *E. coli* 16S rRNA are present within the 10 bases upstream of the proposed initiation codons for *xynA* and *xynB*, which might account for their expression in *E. coli*, although the possibility of alternative internal start points cannot be excluded. Sequences capable of forming stems and hairpin loops are present in the noncoding region between the *xynA* and *xynB* genes (ΔG, −8.4 kcal [−35.1 kJ]/mol), and also downstream from the *xynB* gene (ΔG, −23.4 kcal [−97.9 kJ]/mol according to reference 30) (Fig. 2). Codon usages are similar for the two genes and show considerable bias, in particular for glycine (GGT accounting for 73% of glycine codons in *xynA* and 82% in *xynB*), arginine (CGT accounting for 73% of arginine codons in *xynA* and 83% in *xynB*), and proline (CCA/CCT accounting for 94% of proline codons in *xynA* and 100% in *xynB*). AGG, CGG (arginine), CCG (proline), and TCC (serine) are not used in either gene. This pattern of usage resembles that reported for the endoglucanase gene of the same strain (24) but differs from that reported for *P. ruminicola* 23 (36).

The *xynB* product belongs to a new family of β-(1,4)-glycoside hydrolases. No significant sequence relationships between the *P. ruminicola xynB* product and β-xylosidases or an arabinofuranosidase-xylosidase from the rumen bacterium *B. fibrisolvens* were found (31) (Table 1). On the other hand, there is significant similarity with a recently described arabinofuranosidase-xylosidase from *Clostridium stercorarium* (26). Together with the *B. ovatus* enzyme, these enzymes define a new enzyme family (Fig. 3), distinct from the families 39 and 43 described by Henrissat and Bairoch (16), which include β-xylosidases. More than 25% of the residues that are conserved among the three sequences are aromatic amino acids, including 15 tyrosines (Fig. 3).

Activity and stability of the *P. ruminicola xynA* and *xynB* products. A 3.1-kb fragment (L3) that contained an intact *xynB* coding sequence and an inactive, truncated *xynA* gene was subcloned previously. Activities against pNPX, pNPA, BW xylan, and OS xylan that were detected in *E. coli* cells carrying this fragment (14) must all be attributed to the XynB enzyme. Another fragment (L30), which contains the complete *xynA* gene, encoding the family F xylanase, but an incomplete *xynB* coding sequence, was previously shown to encode activity against BW xylan and OS xylan but not against pNPX or pNPA.

The activities associated with XynB were highly thermolabile and decreased exponentially at 50°C in 0.05 M Na phosphate buffer–2 mM DTT at pH 6.5; half-lives for pNPX and pNPA hydrolytic activities at 50°C were approximately 4 min (results not shown). It was found that activity at 37°C was enhanced by performing assays under a 100% CO_2 atmosphere

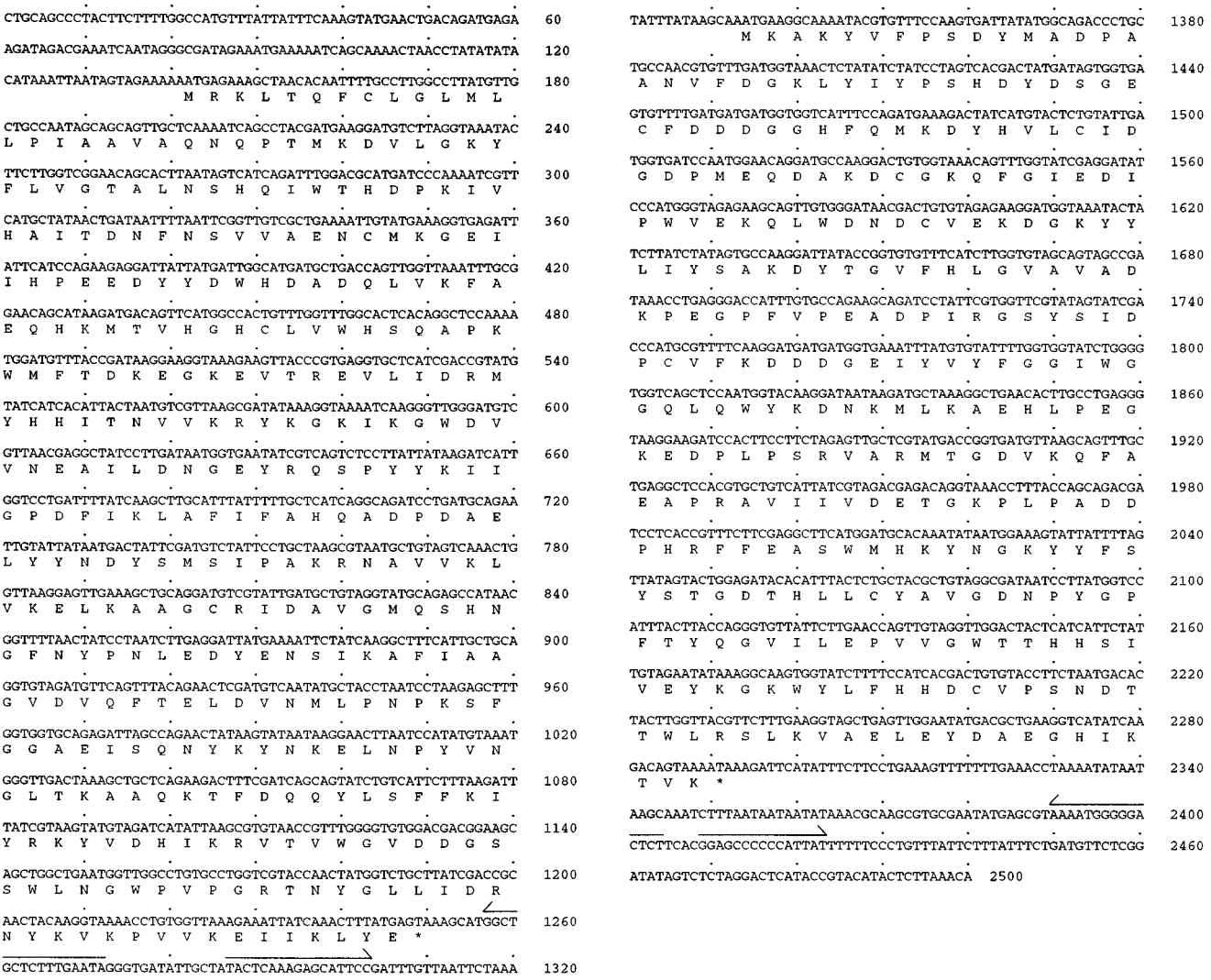

FIG. 2. Nucleotide sequence of a xylan utilization gene cluster from *P. ruminicola* B_14. The sequence of both DNA strands was determined as described in Materials and Methods. The first ORF (residues 142 to 1258, encoding 369 amino acids) corresponds to *xynA*, and the second (residues 1334 to 2290, 319 amino acids) corresponds to *xynB*. Arrows indicate sequences capable of forming stem-loop structures. Encoded amino acids are indicated below the nucleotide sequence. Asterisks represent proposed termination codons.

in anaerobically prepared buffers. On exposure to air without DTT, pNPX hydrolysis ceased to be linear after 5 min, whereas with DTT and anaerobiosis a linear reaction rate was maintained for at least 60 min (Fig. 4). Hydrolysis of pNPA was also sensitive to air. When enzyme preparations were incubated aerobically in the presence of 2 mM DTT for 30 min at 30°C and then assayed anaerobically, pNPX and pNPA activities were the same as they were without preincubation, indicating that inactivation was reversible, although aerobic preincubation without DTT gave only partial restoration of activity. Addition of bovine serum albumin to a concentration of 100 μg/ml did not increase the stability of the enzyme under aerobic or anaerobic conditions (results not shown).

The XynB enzyme showed a pH optimum between 6.3 and 7 for the hydrolysis of Sigma BW xylan when assayed in anaerobic 50 mM Na phosphate buffer–2 mM DTT. Activities determined anaerobically at pH 6.5 (Table 2) were substantially higher than those reported previously, which were based on aerobic assays (14). Once again, a strong preference for BW xylan over OS xylan was found, and this was confirmed with two different sources of each xylan (Table 2). This variation may be partly explained by differences in the content of soluble material, but the two types of xylan also differ markedly in their patterns of substitution with arabinose and 4-*O*-methyl glucuronic acid (Table 2). The XynB enzyme was shown to be active against both the insoluble and soluble fractions of Sigma BW xylan; after the concentrations of the two fractions were adjusted to 6 mg/ml, the activity against the soluble material (304 nmol/min/mg of protein) was approximately three times that against the insoluble material (105 nmol/min/mg of protein).

The xylanase activity of the XynA enzyme was also thermolabile (60% activity loss in 5 min at 50°C), but no evidence for oxygen sensitivity was found. The XynA enzyme shows approximately twice the activity with BW xylan as a substrate as it does with OS xylan (14). The pH optimum was approximately 6.5 in aerobically prepared 50 mM Na phosphate buffer (results not shown).

Modes of action of the XynA and XynB enzymes. The XynB

TABLE 1. Comparison of sequence of *P. ruminicola* $B_1$4 XynA with those of xylanases of family F[a] and comparison of sequence of *P. ruminicola* $B_1$4 XynB with those of bacterial enzymes showing β-xylosidase or β-xylosidase/α-L-arabinofuranosidase activities

P. ruminicola enzyme and organism and gene used for comparison[b]	% Identical amino acids
XynA	
Bacteroides ovatus xylI (BOU04957)	49.3
Bacillus sp. strain C125 *xynA* (XYNA_BACS5)	39.3
Caldocellum saccharolyticum xynA (XYNA_CALSA)	36.5
Clostridium thermocellum xynZ (XYNZ_CLOTM)	35.6
Ruminococcus flavefaciens xynA (XYNA_RUMFL)	33.8
Butyrivibrio fibrisolvens xynA (XYNA_BUTFI)	32.1
Butyrivibrio fibrisolvens xynB (XYNB_BUTFI)	31.4
XynB	
Bacteroides ovatus xsa (BOU04957)	67.8
Clostridium stercorarium xylA (JS0770)	30.7
Bacillus pumilus xynB (XYNB_BACPU)	22.0
Butryivibrio fibrisolvens xsa (A49776)	20.7
Caldocellum saccharolyticum xynB (XYNB_CALSA)	18.0
Thermoanaerobacter xylB (TEOENDXYLB)	14.4

[a] According to the nomenclature of Gilkes et al. (15) or family 10 according to the nomenclature of Henrissat and Bairoch (16).

[b] Database code names or accession numbers are given in parentheses. Comparisons were made by using the Bestfit program.

enzyme was found to convert xylopentaose and xylobiose to xylose (Fig. 5), and xylose was the only low-molecular-weight degradation product of BW xylan detected by thin-layer chromatography (Fig. 5) or gas chromatography (data not shown). This suggests that the XynB enzyme degrades xylan chains by removing terminal xylose residues, presumably from the nonreducing end of the chain. The activities of the XynB enzyme are therefore consistent with its being an exoxylanase capable of acting on certain xylans and xylooligosaccharides. XynB did not degrade aldotetraouronic acid (xylotriose in which the xylose residue at the nonreducing end is substituted with 4-*O*-methyl glucuronic acid) and is therefore unable to cleave 4-*O*-methyl glucuronic acid from xylose, or to attack bonds between xylose residues from the reducing end of this molecule (result not shown).

In contrast, the XynA enzyme produced mainly xylobiose and xylotriose from xylan and from xylopentaose (Fig. 5), suggesting an action similar to that of previously studied endoxylanases from rumen bacteria (11). XynA did not hydrolyze xylobiose (Fig. 5). There was no evidence for release of arabinose from OS xylan by XynA or XynB.

Synergistic interactions between endo- and exoxylanases in xylan breakdown. The proposed action of XynB suggested that it might display synergy with other enzymes with respect to xylan degradation. Endoxylanases should generate free chain ends upon which the XynB enzyme could act, while debranching activities could remove substituents that may otherwise block the enzyme's progress. L3 extracts gave dramatic synergy with respect to release of reducing sugar from OS xylan when added to diluted extracts of *R. flavefaciens* 17 cells grown on oat straw (Table 3). Similar synergy was observed with each of two endoxylanase domains expressed from cloned *R. flavefaciens* 17 genes and with the XynA xylanase from *P. ruminicola* $B_1$4, and synergy was also observed with respect to the degradation of BW xylan (Fig. 6; Table 3). Thus, a large part of the synergistic effect with *R. flavefaciens* extracts can be attributed to the formation by endoxylanases of xylooligosaccharides that can be utilized by the XynB enzyme.

Pretreatment with a fungal arabinofuranosidase from *A. niger* (10) resulted in increases of twofold and fourfold in the release of reducing sugar (not including the arabinose released in the preincubation) from BW and OS xylans, respectively, during subsequent incubation with the XynB enzyme (Table 3). The yield of reducing sugar in incubations with XynB and a cloned endoxylanase from *R. flavefaciens* 17 was enhanced approximately 50% and twofold for BW and OS xylans, respectively, following preincubation with the arabinofuranosidase. These effects indicate that the removal of arabinose substituents from both types of xylan opens up larger tracts of xylose residues to hydrolysis by the XynB enzyme. For OS xylan, the action of the *R. flavefaciens* endoxylanase was also enhanced following pretreatment (Table 3).

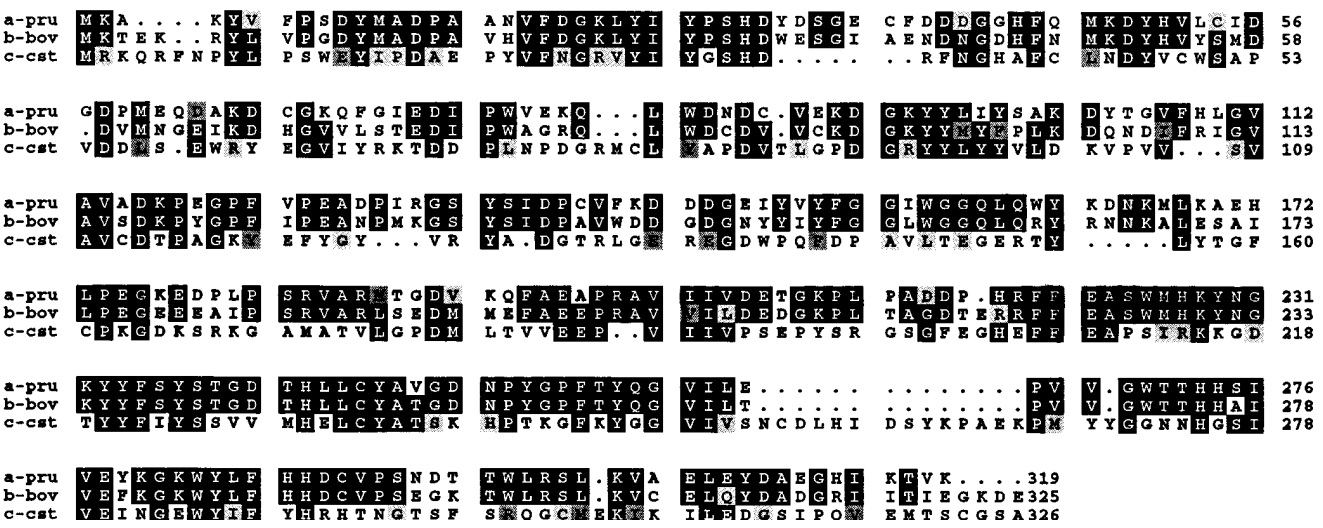

FIG. 3. A new family of β-(1,4)-glycoside hydrolases. A multiple alignment was performed by using the ClustalV program. pru refers to XynB of *P. ruminicola* $B_1$4 (this paper), bov refers to the Xsa product of *B. ovatus* (accession no. BOU04957), and cst refers to the Xsa product of *C. stercorarium* (accession no. JS0770). Dots indicate gaps in the alignment.

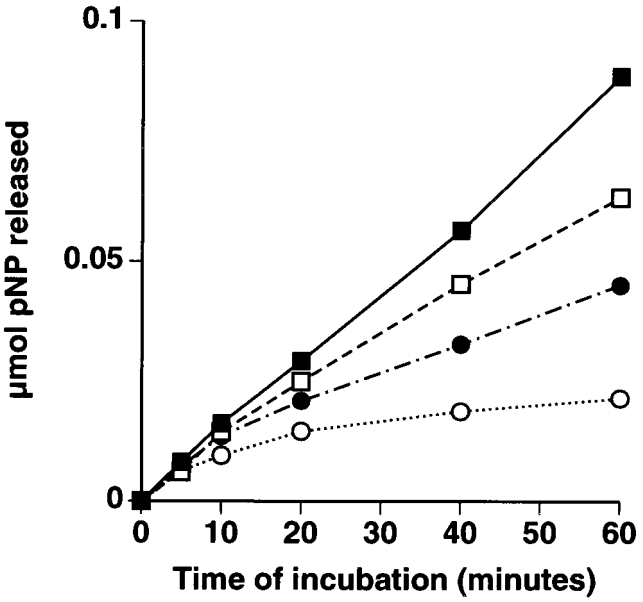

FIG. 4. Sensitivity of the *xynB* exoxylanase to aerobic conditions. Anaerobically prepared extracts of *E. coli* carrying the L3 clone, expressing *xynB*, were incubated with pNPX either in anaerobically prepared 0.05 M Na phosphate buffer, pH 6.5, with (■) or without (□) 2 mM DTT or in the same buffer prepared aerobically, with (●) or without (○) 2 mM DTT.

DISCUSSION

This work shows that the rumen bacterium *P. ruminicola* B_14 carries linked genes concerned with xylan utilization, consisting of a gene encoding a family F endoxylanase (*xynA*) situated upstream from a gene (*xynB*) encoding a putative exoxylanase. This is the first report of the characterization of specific xylanolytic genes from *P. ruminicola* and the first demonstration that family F xylanases occur in this group, although a broad-specificity xylanase-endoglucanase enzyme from *P. ruminicola* 23 belonging to family A has been described previously (36). Family F xylanases from other rumen bacteria have been reported (22, 39), but the *P. ruminicola* B_14 enzyme was found to be less closely related to these than to a family F xylanase from the human hind gut organism *B. ovatus*. This is also the first report of a gene cluster from a rumen anaerobe, although a similar cluster is present in *B. ovatus* (35, 37). Xylanase activity is regulated in *P. ruminicola* B_14 (13, 14), and it is likely, although not yet established, that these two genes are regulated. It is also possible that further functionally related genes will be found in this gene cluster.

In its amino acid sequence, the *P. ruminicola* XynB enzyme resembles two enzymes reported previously, from *B. ovatus* and *C. stercorarium*, that are described as processing β-xylosidase and α-L-arabinofuranosidase activities and with them establishes a new family of glycoside hydrolases. Although the *P. ruminicola* XynB enzyme resembles the other two in showing activity against pNPX and pNPA, it also has some highly significant properties that either are absent from the two related enzymes or were not identified. First, the enzyme expressed in *E. coli* is highly temperature labile and oxygen sensitive. It is not yet established whether this applies to the native enzyme. If the oxygen sensitivity is due to disulfide bond formation, then the greater number of cysteine residues in the *P. ruminicola* enzyme than in the *B. ovatus* enzyme (seven compared with four) may be relevant and could explain why oxygen sensitivity was not reported for the *B. ovatus* enzyme. Since the rumen environment contains less dissolved oxygen than the hind gut does (18), there may be less selection for oxygen

TABLE 2. Activities associated with the *P. ruminicola* XynB enzyme, expressed from the L3 clone in *E. coli*, assayed under anaerobic conditions

Substrate (source)	Enzyme activity (nmol/min/mg of protein)	Substrate composition (ratio)[a]		
		Soluble/insoluble xylan	Arabinose/xylose	Uronic acid/xylose
BW xylan (Sigma)	540[b]	3.3[c]	0.009	0.16
BW xylan (Roth)	210	1.5	0.007	0.14
OS xylan (Sigma)	22[b]	0.75	0.091	0.02
OS xylan (Roth)	39	0.37	0.072	0.02
pNPX	101[b]			
pNPA	89[b]			

[a] Xylans were fractionated as described in Materials and Methods. Monosaccharide composition was determined as described elsewhere (6).

[b] Mean for three separate experiments (unless indicated otherwise, values represent means for duplicate assays from single experiments). Assays were performed as described in Materials and Methods, with incubation times at 37°C of 15 min for pNPA and pNPX and 45 min for xylanase activities.

[c] Activity against the soluble fraction of Sigma BW xylan was approximately three times that against the insoluble fraction, assayed at equal concentrations (wt/vol) (see text).

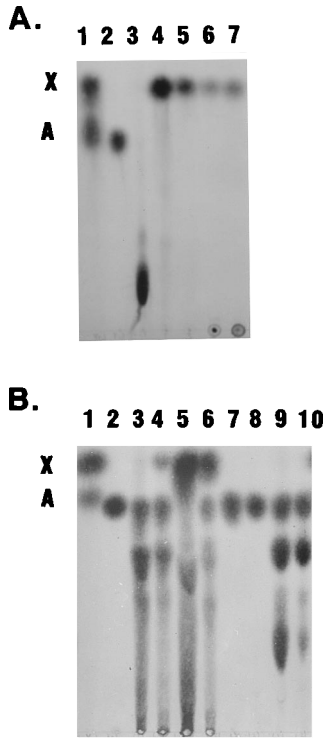

FIG. 5. Products of hydrolysis of xylan, xylopentaose, and xylobiose by extracts containing XynA or XynB enzymes. Products of hydrolysis were separated by thin-layer chromatography. (A) Lane 1, xylose plus arabinose; lane 2, xylobiose; lane 3, xylopentaose; lanes 4, 5, and 6, xylobiose, xylopentaose, and BW xylan, respectively, following anaerobic incubation for 2 h with XynB. Lane 7 shows hydrolysis of BW xylan for 6 h with XynB. (B) Lanes 1 and 2, as in panel A; lanes 3 and 4, BW and OS xylans, respectively, incubated with XynA (6 h); lanes 5 and 6, BW and OS xylans, respectively, incubated with both XynA and XynB (6 h); lanes 7 and 8, xylobiose incubated for 2 and 6 h, respectively, with XynA; lanes 9 and 10, xylopentaose incubated for 2 and 6 h, respectively, with XynA. The sources of XynA and XynB were cell extracts of clones L30 and L3. X, xylose; A, arabinose.

TABLE 3. Synergy between *P. ruminicola* B_14 XynB exoxylanase, endoxylanases from *R. flavefaciens* 17 and *P. ruminicola*, and arabinofuranosidase from *A. niger*

Enzyme addition(s)	Xylose equivalents (μmol) released from xylan with or without addition of second enzyme[a]:				
	P. ruminicola B_14 XynB (clone L30)	*R. flavefaciens* cell extract	*R. flavefaciens* supernatant	*R. flavefaciens* XynA domain C (clone X4723)	
				OS xylan	BW xylan
B_14 exoxylanase only[b] (clone L3)	0.034	0.023	0.023	0.071	0.66
B_14 exoxylanase + second enzyme	0.42	1.10	0.45	1.09	1.39
Second enzyme alone	0.088	0.25	0.042	0.041	0.043
B_14 exoxylanase + arabinofuranosidase				0.32[c]	1.29[c]
B_14 exoxylanase + second enzyme + arabinofuranosidase				1.94[c]	2.18[c]
Second enzyme + arabinofuranosidase				0.25[c]	0.037[c]
Arabinofuranosidase alone				0.02[c]	0.00[c]

[a] OS xylan was the substrate for *P. ruminicola* B_14 XynB and *R. flavefaciens* cell extract and supernatant. For *R. flavefaciens* XynA, the first value is for OS xylan and the second is for BW xylan. Incubations were for 90 min (OS xylan) or 45 min (BW xylan) under anaerobic conditions at 37°C. *R. flavefaciens* 17 cell extract and supernatant were from a culture grown for 72 h in medium containing 0.5% oat straw (see Materials and Methods). Material was preincubated with *A. niger* arabinofuranosidase as described in Materials and Methods. Other enzymes were derived from sonicated cell extracts of *E. coli* strains carrying cloned genes. Incubation mixtures contained added extracts at the following protein concentrations: L3, 180 μg/ml; L30, 150 μg/ml; X4723, 0.2 μg/ml; *R. flavefaciens* sonicate, 65 μg/ml; *R. flavefaciens* supernatant, 18 μg/ml. OS xylan and BW xylan were both from Sigma.

[b] The L3 extract used for *R. flavefaciens* XynA was from a batch different from that used for the first three columns. This largely accounts for the differences in the low level of xylose release from OS xylan seen with L3 extract alone. Within each experiment (i.e., column), duplicate assay values differed from the mean by less than 10%.

[c] Not including reducing sugar released as arabinose during the preincubation (0.29 μmol/ml for OS xylan and 0.072 μmol/ml for BW xylan).

tolerance of individual proteins in microbes from the rumen than in those from the hind gut.

A second significant feature of the XynB enzyme is its mode of action on xylan molecules. The evidence presented here is consistent with the *P. ruminicola* XynB enzyme's being an exoxylanase capable of releasing xylose progressively from the nonreducing ends of substrates ranging in size from xylan polysaccharides down to xylobiose. Very few exoxylanases have been reported previously (25, 38), and this appears to be the first case in which a putative exoxylanase gene has been isolated and its sequence determined. This mode of action may account for the dramatic synergy (up to 10-fold enhancement of reducing-sugar release) in xylan degradation that was observed when the enzyme was incubated along with endoxylanases, since the concentration of termini available to the exoxylanase will be increased by random cleavage of the chain by endoxylanases. Addition of β-(1,4)-xylosidases to endoxylanases has also been reported to enhance the release of reducing sugar from xylans, but by much smaller factors of around twofold (12, 19). Since the activity of the XynB enzyme against xylan is expected to depend on the concentration of available termini, the greater content of soluble material found in preparations of BW xylan may largely account for the strong preference for this substrate over OS xylan. On the other hand, the patterns of substitution are also quite different between the two types of xylan (Table 2) and may also play an important role. The enhancement of XynB action following pretreatment of xylans with fungal arabinofuranosidase indicates that enzyme hydrolysis is not able to progress past xylose residues that carry arabinose substituents. Therefore, there is no evidence that the activity against the artificial substrate pNPA reflects an ability to cleave arabinose residues from xylan, although the possibility that oligosaccharides might be attacked in this manner is not ruled out. Since XynB did not hydrolyze aldotetraouronic acid, 4-*O*-methyl glucuronic acid substituents are also likely to form a barrier to hydrolysis by the enzyme. Resolution of the precise specificity of action of the XynB exoxylanase is clearly of considerable interest but must await more detailed kinetic studies with better-defined substrates.

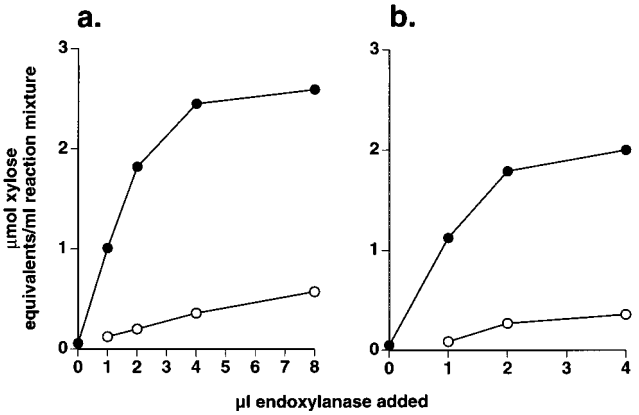

FIG. 6. Synergistic interactions between the *P. ruminicola* XynB exoxylanase and endoxylanases from *R. flavefaciens*. Reaction mixtures containing a constant amount of *P. ruminicola* crude exoxylanase (L3 cell extract, 45 μg of crude protein) were incubated under anaerobic conditions for 90 min at 37°C with various amounts of a cloned endoxylanase activity from *R. flavefaciens* 17 expressed in *E. coli* (●). Reducing-sugar release from OS xylan was stimulated five- to eightfold compared with the amount obtained with the endoxylanase acting alone (○). (a) Endoxylanase from clone X4723 (XynA domain C) (extract containing 0.54 mg of protein per ml). (b) Endoxylanase from clone X4530 (XynA domain A) (extract containing 2 mg of protein per ml). Specific activities with respect to OS xylan were 1.87 μmol/min/mg of protein for X4723 and 0.28 μmol/min/mg of protein for X4530 (assayed aerobically).

While these studies show that the XynB enzyme is active against large xylan molecules and can act synergistically with endoxylanases in the degradation of these molecules, it is not clear whether this is its major function in vivo. The lack of a signal peptide suggests that this enzyme is located within the *P. ruminicola* cell and could therefore be concerned with hydrolysis of transported oligosaccharides, while the XynA endoxylanase, which carries a signal peptide, is likely to be either periplasmic or extracellular. This suggests that the XynA and XynB enzymes could act successively, rather than simultaneously, in vivo. Although *P. ruminicola* transports xylose

efficiently (29), there is little evidence to indicate whether xylan-derived material enters the cell mainly as xylose, xylooligosaccharides, or polysaccharide. There is evidence that in related *Bacteroides* spp., starch molecules can be transported across the cell membrane and are hydrolyzed in the periplasm (1). Recent evidence shows that the endoglucanase activity of *P. ruminicola* $B_1$4 is largely cell associated (13). We have found that this also applies to xylanase activity and, furthermore, that most of the xylanase and endoglucanase activity of *P. ruminicola* $B_1$4 is present either in the periplasm or inside the cell (23). Further studies of the localization of individual gene products are now required to elucidate the roles of the different components of the xylan-utilizing system of this species. The work reported here provides a first step towards understanding the organization and interactions of xylan-utilizing enzymes in this important group of strict anaerobes.

ACKNOWLEDGMENTS

This work was supported by the Scottish Office Agriculture and Fisheries Department.

We are grateful for permission to use the Daresbury Seqnet facility and thank the British Council/Alis Link scheme for supporting visits between Aberdeen and Slovenia. We are also grateful to Terry R. Whitehead for making available sequences for the genes from *B. ovatus*, to Peter Dewey for carbohydrate analyses, and to Tom M. Wood and Peter Hotten for valuable discussions.

REFERENCES

1. **Anderson, K. L., and A. A. Salyers.** 1989. Biochemical evidence that starch breakdown by *Bacteroides thetaiotaomicron* involves outer membrane starch binding sites and periplasmic starch-degrading enzymes. J. Bacteriol. **171:** 3192–3198.
2. **Avgustin, G., H. J. Flint, and T. R. Whitehead.** 1992. Distribution of xylanase genes and enzymes among strains of *Prevotella* (*Bacteroides*) *ruminicola* from the rumen. FEMS Microbiol. Lett. **99:**137–144.
3. **Biely, P.** 1985. Microbial xylanolytic systems. Trends Biotechnol. **3:**286–290.
4. **Bryant, M. P., N. Small, C. Bouma, and H. Chu.** 1958. *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica* the new genus and species. J. Bacteriol. **76:**15–23.
5. **Dehority, B. A.** 1993. Effects of microbial synergism on fibre digestion in the rumen. Proc. Nutr. Soc. **50:**149–159.
6. **Englyst, H. N., and J. H. Cummings.** 1984. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars by alditol acetates. Analyst **109:**937–942.
7. **Flint, H. J., J. Martin, C. A. McPherson, A. S. Daniel, and J.-X. Zhang.** 1993. A bifunctional enzyme, with separate xylanase and β(1,3-1,4)-glucanase domains, encoded by the *xynD* gene of *Ruminococcus flavefaciens*. J. Bacteriol. **175:**2943–2951.
8. **Flint, H. J., C. A. McPherson, and J. Bisset.** 1989. Molecular cloning of genes from *Ruminococcus flavefaciens* encoding xylanase and β(1-3,1-4)glucanase activities. Appl. Environ. Microbiol. **55:**1230–1233.
9. **Flint, H. J., C. A. McPherson, and J. Martin.** 1991. Expression of two xylanase genes from the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 cloned in pUC13. J. Gen. Microbiol. **137:**123–129.
10. **Flipphi, M. J. A., M. van Heuvel, P. van der Veen, J. Visser, and L. H. de Graaff.** 1993. Cloning and characterization of the *abfB* gene coding for the major α-L-arabinofuranosidase of *Aspergillus niger*. Curr. Genet. **24:**525–532.
11. **Garcia-Campayo, V., S. Macrae, J. Zhang, H. J. Flint, and T. M. Wood.** 1993. Mode of action, kinetic properties and physico-chemical characterization of two different domains of a bifunctional (1-4)-β-D-xylanase from *Ruminococcus flavefaciens* expressed separately in *Escherichia coli*. Biochem. J. **296:**235–243.
12. **Garcia-Campayo, V., and T. M. Wood.** 1993. Purification and properties of a β-xylosidase from the anaerobic rumen fungus *Neocallimastix frontalis*. Carbohydr. Res. **242:**235–243.
13. **Gardner, R. G., J. E. Wells, J. B. Russell, and D. B. Wilson.** 1995. The effect of carbohydrates on the expression of the *Prevotella ruminicola* 1,4-β-D-endoglucanase. FEMS Microbiol. Lett. **125:**305–310.
14. **Gasparic, A., R. Marinsek-Logar, J. Martin, R. J. Wallace, F. V. Nekrep, and H. J. Flint.** 1995. Isolation of genes encoding β-D-xylanase, β-D-xylosidase and α-L-arabinofuranosidase activities from the rumen bacterium *Prevotella ruminicola* $B_1$4. FEMS Microbiol. Lett. **125:**135–142.
15. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, and R. A. J. Warren.** 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function and enzyme families. Microbiol. Rev. **55:**303–315.
16. **Henrissat, B., and A. Bairoch.** 1993. New families in the classification of glycosyl hydrolases based on amino acid similarities. Biochem. J. **293:**781–788.
17. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 265–302. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, London.
18. **Hillman, K., A. L. White, and C. S. Stewart.** 1993. Dissolved oxygen in the porcine gastrointestinal tract. Lett. Appl. Microbiol. **16:**299–302.
19. **Kormelink, F. J. M., and A. G. J. Voragen.** 1992. Combined action of xylan-degrading and accessory enzymes on different {[glucurono]arabino}xylans, p. 415–418. *In* J. Visser, G. Beldman, M. A. Kusters-van Someren, and A. G. J. Voragen (ed.), Xylans and xylanases. Progress in biotechnology 7. Elsevier Science Publishers, Amsterdam.
20. **Lever, M.** 1977. Carbohydrate determination with 3-hydroxybenzoic acid hydrazide (PAHBAH): effect of bismuth on the reaction. Anal. Biochem. **81:**21–27.
21. **Mahlberg, L. M., Jr., D. C. Smith, H. E. Schellhorn, and C. F. Forsberg.** 1993. *Fibrobacter succinogenes* S85 has multiple xylanase genes. J. Appl. Bacteriol. **75:**564–573.
22. **Mannarelli, B. M., S. Evans, and D. Lee.** 1990. Cloning, sequencing, and expression of a xylanase gene from the anaerobic ruminal bacterium *Butyrivibrio fibrisolvens*. J. Bacteriol. **172:**4247–4254.
23. **Marinsek-Logar, R., J. Martin, A. Gasparic, and H. J. Flint.** Unpublished data.
24. **Matsushita, O., J. B. Russell, and D. B. Wilson.** 1990. Cloning and sequencing of a *Bacteroides ruminicola* $B_1$4 endoglucanase gene. J. Bacteriol. **172:** 3620–3630.
25. **Reilly, P. J.** 1981. Xylanases: structure and function. Basic Life Sci. **18:**111–129.
26. **Sakka, K., K. Yoshikawa, Y. Kojima, S. Karita, K. Ohmiya, and K. Shimada.** 1993. Nucleotide sequence of the *Clostridium stercorarium xylA* gene encoding a bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities, and properties of the translated product. Biosci. Biotechnol. Biochem. **57:**268–272.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Stewart, C. S., and M. P. Bryant.** 1988. The rumen bacteria, p. 21–75. *In* P. N. Hobson (ed.), The rumen anaerobic ecosystem. Elsevier Applied Science, London.
29. **Strobel, H. E.** 1993. Pentose utilization and transport by the ruminal bacterium *Prevotella ruminicola*. Arch. Microbiol. **159:**465–471.
30. **Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246:**40–41.
31. **Utt, E. A., C. K. Eddy, K. F. Keshav, and L. O. Ingram.** 1991. Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl. Environ. Microbiol. **57:**1227–1234.
32. **Van Gylswyk, N. O.** 1990. Enumeration and presumptive identification of some functional groups of bacteria in the rumen of dairy cows fed grass-silage based diets. FEMS Microbiol. Ecol. **73:**243–254.
33. **Vercoe, P. E., and K. Gregg.** 1992. DNA sequence and transcription of an endoglucanase gene from *Bacteroides* (*Prevotella*) *ruminicola* AR20. Mol. Gen. Genet. **233:**284–292.
34. **von Heijne, G.** 1988. Transcending the impenetrable: how proteins come to terms with membranes. Biochim. Biophys. Acta **947:**307–333.
35. **Weaver, J., T. R. Whitehead, M. A. Cotta, P. C. Valentine, and A. A. Salyers.** 1992. Genetic analysis of a locus on the *Bacteroides ovatus* chromosome which contains xylan utilization genes. Appl. Environ. Microbiol. **58:**2764–2770.
36. **Whitehead, T. R.** 1993. Analyses of the gene and amino acid sequence of the *Prevotella* (*Bacteroides*) *ruminicola* xylanase reveals unexpected homology with endoglucanases from other genera of bacteria. Curr. Microbiol. **27:**27–33.
37. **Whitehead, T. R.** Nucleotide sequences of xylan-inducible xylanase and xylosidase/arabinosidase genes from *Bacteroides ovatus* V975. Submitted for publication.
38. **Wong, K. K. Y., L. U. L. Tan, and J. N. Saddler.** 1988. Multiplicity of β-1,4-xylanase in microorganisms. Microbiol. Rev. **52:**305–317.
39. **Zhang, J.-X., and H. J. Flint.** 1992. A bifunctional xylanase encoded by the *xynA* gene of the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 comprises two dissimilar catalytic domains linked by an asparagine/glutamine rich sequence. Mol. Microbiol. **6:**1013–1023.