Characteristics of adjacent family 6 acetylxylan esterases from *Fibrobacter succinogenes* and the interaction with the Xyn10E xylanase in hydrolysis of acetylated xylan

Dong Keun Kam, Hyun-Sik Jun, Jong K. Ha, G. Douglas Inglis, and Cecil W. Forsberg

Abstract: Acetylxylan esterase genes *axe6A* and *axe6B* located adjacent to one another on a *Fibrobacter succinogenes* chromosome have been separately cloned and their properties characterized. The corresponding esterases contained an N-terminal carbohydrate esterase family 6 catalytic domain (CD) and a C-terminal family 6 carbohydrate-binding module (CBM). The amino acid sequences of the CDs and CBMs were found to exhibit 52% and 40% amino acid similarity, respectively. The CDs of the two esterases exhibited the highest similarity to CDs of acetylxylan esterases: AxeA from the ruminal fungi *Orpinomyces* sp. and BnaA from *Neocallimastix patriciarum*. Axe6A and Axe6B were optimally active at neutral pH and had low K_m values of 0.084 and 0.056 mmol·L^{-1}, respectively. Axe6A and Axe6B were shown to bind to insoluble cellulose and xylan and to soluble arabinoxylan. Axe6A deacetylated acetylated xylan at the same initial rate in the presence and absence of added Xyn10E xylanase from *F. succinogenes*, but the action of the xylanase on acetylated xylan was dependent upon the initial activity of Axe6A. The capacity of acetylxylan esterases to bind to plant cell wall polymers and to independently deacetylate xylan enabling xylanase to release xylooligosaccharides, documents the central role these enzymes have to improve access of *F. succinogenes* to cellulose.

Key words: Fibrobacter succinogenes S85, acetylxylan esterase, xylanase, synergy.

Résumé : Les acétylxylane estérases *axe6A* et *axe6B* placées l'une à côté de l'autre sur le chromosome de *Fibrobacter succinogenes* ont été clonées séparément et leurs propriétés furent caractérisées. Les deux estérases contenaient un domaine catalytique (DC) N-terminal de la famille 6 des estérases de glucides et un module de liaison aux glucides (MLG) C-terminal de la famille 6. Les séquences d'acides aminés des DC et MLG ont présenté 52 % et 40 % de similarité au niveau des acides aminés, respectivement. Les DC des deux estérases ont présenté un plus haut le degré de similarité aux CD des acétylxylane estérases AxeA des champignons ruminaux *Orpinomyces* sp. et BnA de *Neocallimastix patriciarum*. Axe6A et Axe6B démontraient une activité optimale à un pH neutre et avaient des valeurs de K_m basses de 0,084 et 0,056 mmol·L^{-1}, respectivement. Il fut démontré que Axe6A et Axe6B se liaient à du cellulose et du xylane insolubles ainsi qu'à de l'arabinoxylane soluble. Axe6A a désacétylé le xylane acétylé au même taux initial en présence ou absence de la xylanase Xyn10E de *F. succinogenes*, mais l'action de la xylanase sur le xylane acétylé dépendait de l'activité initiale de Axe6A. La capacité des acétylxylane estérases à se lier aux polymères de parois cellulaires végétales et de désacétyler indépendamment le xylane, permettant ainsi à la xylanase de libérer les xylooligosaccharides, documente le rôle central de ses enzymes pour faciliter l'accès de *F. succinogenes* au cellulose.

Mots clés : Fibrobacter succinogenes S85, acétylxylane estérases, xylanase, synergie.

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Introduction

Ruminant animals digest plant materials to satisfy their primary nutritional requirements. Much of the plant material, and in particular, the cell walls, which may constitute more than 90% of the plant biomass, are composed of recalcitrant cellulose, hemicellulose, pectin, and variable amounts of lignin (Brett and Waldren 1996). Digestion of plant cell

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Table 1. Oligonucleotide primers used for amplifying *Fibrobacter succinogenes* S85 genes *axe6A* and *axe6B*.

Gene	Primer set	Sequences (5′→3′)
axe6A	3724-F; *Nde*I	AAGGGAGATA**CATATG**AGTGTGG
	3724-R; *Xho*I	TTTG**CTCGAG**TTCGAAAACGACAACC
	3724-N-F; *Bgl*II	TTAATA**AGATCT**AGGCCTCTCTATGTTTGCG
	3724-N-R; *Xho*I	TATATA**CTCGAG**TTCGAAAACGACAACCTT
axe6B	3723-F2; *Nde*I	TAACTAATT**CATATG**AGCGTGGAAAGAAAC
	3723-R2; *Xho*I	TATCTA**CTCGAG**TTCATGTATCACCACC
	3723-G-F; *Sma*I	TATTAA**CCCGGG**AGCTGGGCTTTCTATGTTT
	3723-G-R; *Xho*I	TATATA**CTCGAG**ATGTATCACCACCTTTTTT

Note: Bold sequences are recognition sites of restriction enzyme.

walls requires the presence of microorganisms because the animal lacks the unique complement of enzymes necessary for hydrolysis of the diversity of linkages in this intertwined mesh of polymers (Selinger et al. 1996). Xylan, the major component of hemicellulose in cell walls of grasses and other forages is composed of a backbone of β-1,4-linked xylopyranosyl residues substituted by arabinofuranose, glucuronic acid, methylglucuronic acid, and acetyl side groups (Selinger et al. 1996). Acetyl substituents on the O-2 and O-3 positions of xylose residues of xylan, which account for 50% to 70% of the substituents of grasses and white clover (*Trifolium pretense*), respectively (Chesson et al. 1983), limit hydrolysis of xylan by xylanases (EC 3.2.1.8) (Biely et al. 1986; Wood and McCrae 1986). Acetylxylan esterases (EC 3.1.1.72) hydrolyze acetyl side chains from the xylan backbone, enabling the action of xylanases (Biely et al. 1986). To improve understanding of the role of acetylxylan esterases present in ruminal bacteria, Hespell and O'Bryan-Shah (1988) tested *Butyrivibrio fibrisolvens* strains for the presence of acetylxylan esterase and found the enzyme to be present in three strains. Shortly thereafter McDermid et al. (1990*a*, 1990*b*) reported the presence of acetylxylan esterase activity in *Fibrobacter succinogenes* and purified and characterized catalytic properties of one of the enzymes detected in the extracellular culture fluid of the bacterium. They documented that the purified esterase could cleave acetyl residues from xylan without the need for xylanase activity. Since these initial studies, acetylxylan esterase genes of the ruminal fungi *Orpinomyces* sp. and *Neocallimastix patriciarum* have been cloned and the catalytic properties of the purified enzymes characterized (Blum et al. 1999; Cybinski et al. 1999). As well, a multidomain enzyme containing an acetylxylan esterase from *Ruminococcus flavefaciens* was also characterized by Aurilia et al. (2000).

Because of the dominant role *F. succinogenes* plays in the biodegradation of acetylated plant cell walls (Béra-Maillet et al. 2004), visually documented by the capability of individual cells to create digestion craters (Lynd et al. 2002), the role of acetylxylan esterase in this genus was targeted in the current study. An acetylxylan esterase gene (*axeA*) was cloned from *F. succinogenes* S85 and sequenced (AF180369; Ha et al. 1999). In addition to this gene, the cloned DNA sequence appeared to contain the partial sequence of a second acetylxylan esterase gene at the 3′ end.

The objectives of this research were to complete the sequence of the second acetylxylan esterase, to characterize the catalytic properties of the two esterase proteins, and to examine the interaction of acetylxylan esterase with a *F. succinogenes* xylanase in the hydrolysis of hemicellulose.

Materials and methods

Bacterial strains, growth conditions, and plasmids

Bacterial strains used in this study included *Escherichia coli* DH5α and BL21 (DE3) and *F. succinogenes* subsp. *succinogenes* S85 (ATCC 19169; Montgomery et al. 1988). The vectors used included pBluescript (Strategene, La Jolla, California), pET-30a (Novagen Inc., Madison, Wisconsin), and pGEX4T1 (Pharmacia (Canada) Ltd., Dorval, Quebec). *Escherichia coli* DH5α and BL21 (DE3) were grown in Luria–Bertani (LB) medium containing either 100 μg of ampicillin·mL^{-1} or 30 μg of kanamycin·mL^{-1} at 37 or 25 °C with rotary shaking at 150 r·min^{-1}, depending upon which vector the host contained. *Fibrobacter succinogenes* S85 was grown in a chemically defined medium (lacking casamino acids) with glucose at 0.5% (*w*/*v*) as the carbohydrate source and carbon dioxide as the gas phase (Scott and Dehority 1965) at 37 °C. Cultures were harvested after 18 h of growth with shaking, and DNA were isolated using a modification of the method described by Stanley (1990). To express the gene *axe6A*, two different gene constructs were prepared. (*i*) The *axe6A* gene was amplified from *F. succinogenes* genomic DNA using the primer pair 3724-F and 3724-R (Table 1), with an annealing temperature of 52 °C for 30 s, followed by digestion with *Nde*I and *Xho*I. The digested polymerase chain reaction (PCR) product was inserted into pET-30a (Novagen) producing a recombinant plasmid with a C-terminal His-tag. (*ii*) The *axe6A* gene was amplified using primers 3724-N-F and 3724-N-R (Table 1), followed by digestion by *Bgl*II and *Xho*I and insertion into the corresponding sites in pET-30a to construct a recombinant plasmid with both N-terminal and C-terminal His-tags. To overexpress the gene *axe6B*, two different gene constructs were prepared using pET-30a and pGEX4T1: (*i*) *axe6B* was amplified by PCR using the primer pair 3723-F2 and 3723-R2, followed by digestion with *Nde*I and *Xho*I and insertion into pET-30a to conserve the C-terminal His-tag; and (*ii*) *axe6B* was also amplified by using the primer pair 3723-G-F and 3723-G-R, subjected to digestion by *Sma*I and *Xho*I. The digested PCR product was subcloned into the corresponding sites in the glutathione *S*-transferase (GST) expression vector pGEX4T1. Each construct was sequenced to ensure that there were no mutations in the insert. Constructs were tested for expression of acetylxylan esterase by streaking the ex-

pression host containing the construct onto LB agar containing either 50 μg of ampicillin·mL^{-1} or 30 μg of kanamycin·mL^{-1} and 0.1 mmol isopropyl-β-D-galactopyranoside·L^{-1}. Once colonies had formed, a solution containing α-naphthyl acetate (α-NA) coupler solution (α-NA – Diazo Blue B; Rosenberg et al. 1975) was poured over the colonies, and the plates were incubated at room temperature for color to develop around colonies expressing esterase activity.

Characterization of the nucleotide sequences

The gene *axeA* encoding acetylxylan esterase was previously cloned from *F. succinogenes* S85 and submitted to GenBank with the accession No. AF180369 (Ha et al. 1999). Part of an open reading frame (ORF) downstream of *axeA* was cloned as part of a restriction fragment. The amino acid sequence of the truncated ORF exhibited a high similarity to the catalytic domain (CD) of AxeA. To determine the unknown 3′ sequence of the truncated gene, the 810 bp immediately upstream was used as the query sequence in a BLASTn search of the *F. succinogenes* S85 genome sequence database available at the TIGR website (http://www.tigr.org). The BLAST result showed an extra 1000 bp upstream and 1000 bp downstream of the input query sequence. The combined downstream sequence from *axe6A*, (1801 bp) was submitted to the ORF finder program provided by NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to identify the complete gene. The ORF was determined to be a 1659-bp nucleotide sequence that we designated *axe6B*.

The amino acid sequence, molecular weight, and pI were obtained using http://ca.expasy.org/. The amino acid sequences of Axe6A and Axe6B were further analyzed by using the Pfam search tool (http://www.sanger.ac.uk/Software/Pfam/) and the Prodom program (http://www.protein.toulouse.inra.fr) to identify protein domains and families, including the CD and the carbohydrate binding module (CBM). The signal peptide was predicted using http://www.cbs.dtu.dk/services/SignalP-2.0/.

Analytical methods

Acetylxylan esterase activity was determined using α-NA as the substrate at a final concentration of 1.0 mmol·L^{-1} (McDermid et al. 1990*b*). The assay mixture contained 20 μL of 0.5 mol sodium phosphate buffer·L^{-1} (pH 6.5) and diluted enzyme made to a volume of 180 μL and preincubated at 37 °C. The reaction was initiated by the addition of 20 μL of freshly prepared 10 mmol·L^{-1} α-NA solution in methanol. After 15 min of incubation, the reaction was stopped by the addition of 50 μL of 5 mg Fast Garnet GBC salt·mL^{-1} (Sigma Chemical Co., St. Louis, Missouri) in 10% (*w*/*v*) SDS. After color development for 10 min, the absorbance was measured at 560 nm using a microtitre plate reader (V_{max}; Kinetic Microplate Reader, Molecular Devices Corporation, Sunnyvale, California). A reagent blank and a series of α-naphthol standards ranging from 5 to 25 nmol were included for each set of measurements. Other substrates, including α-naphthyl butyrate, α-naphthyl caprylate, or α-naphthyl laurate, were used at the same concentrations in place of α-NA. The K_m and V_{max} were determined using α-NA as the substrate at concentrations ranging from 0.1 to 4.0 mmol·L^{-1} and the kinetic parameters calculated by a Lineweaver–Burke plot.

Acetylxylan esterase activity was determined by using 1% (*w*/*w*) chemically acetylated birch-wood xylan as the substrate. Birch-wood xylan containing 8% (*w*/*w*) of acetyl groups was prepared as described by Johnson et al. (1988). The degree of acetylation of xylan was determined by high performance liquid chromatography (HPLC) after extraction with NaOH, as described by Khan et al. (1990). The enzyme reaction was performed in a final volume of 500 μL of 50 mmol sodium phosphate buffer·L^{-1} (pH 6.5). After incubation at 37 °C, the assay was stopped by heating at 100 °C for 5 min. The mixture was centrifuged at 8000*g* for 5 min, and the released acetic acid was quantified by HPLC using an Aminex HPX-87H column with 5 mmol H_2SO_4·L^{-1} as the solvent at a flow rate of 0.6 mL·min^{-1} and detected at 210 nm. A standard curve with a range of 0–0.4 μmol per 20 μL injection was prepared with each set of samples analyzed. No free acetic acid was released during the heat treatment to stop the reaction by inactivation of the esterase.

Xylanase activity was measured with 1% (*w*/*v*) oat spelt xylan (Sigma-Aldrich Canada, Oakville, Ontario) as the substrate in a final volume of 0.1 mL of 50 mmol sodium phosphate buffer·L^{-1} (pH 6.5) at 37 °C for 15 min. Reducing sugars generated were quantified with *p*-hydroxybenzoic acid hydrazide reagent (Lever 1972) using xylose as the standard over a range of 0–0.267 μmol (0–40 μg). The unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of product per min, which could be α-naphthol, acetic acid, or reducing sugar per min, depending upon the assay. Specific activity was defined as units per minute per milligram of protein.

Xylan hydrolysis products, including xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose, were separated by HPLC with a CarboPac PA1 ion-exchange column (Dionex, Sunnyvale, California) and the products quantified with a pulsed amperometric detector, as described by Jun et al. (2003), by reference to standard xylooligosaccharides at concentrations of 1 and 2 nmol per 20-μL injection analyzed under the same conditions.

All assays were conducted in triplicate and repeated twice. The data reported are the means of one set of data.

Enzyme expression and purification

To express the proteins in *E. coli* strains harboring recombinant plasmids, single colonies were transferred into 100 mL of LB medium containing either 30 μg kanamycin·mL^{-1} or 100 μg ampicillin·mL^{-1} and incubated at 37 °C for 12 h. The inoculum was added to 2 L of the same medium and grown at 25 °C for 12 h with rotary shaking until the cells reached an absorbance of 0.7–0.8 at 675 nm. Expression was induced after cooling the culture to 18 °C with the addition of 1 mmol·L^{-1} IPTG (isopropyl-β-D-thiogalactopyranoside), and incubation at 18 °C with continued rotary shaking for a further 24 h. To purify Axe6A, the culture was harvested and resuspended in 40 mL of lysis buffer composed of 50 mmol·L^{-1} sodium phosphate buffer (pH 8.0), 300 mmol·L^{-1} NaCl, and 10 mmol·L^{-1} imidazole containing 0.5 mmol·L^{-1} phenylmethylsulfonyl fluoride (PMSF), protease inhibition cocktail (Sigma-Aldrich Canada), and 0.02% of sodium azide. Cells were disrupted by passage though a French

press (Thermo Electron Corporation, Burlington, Ontario) at a pressure of 8300 kPa. After centrifugation of the extract at 15 000*g* for 20 min and 4 °C, the cytoplasmic fraction was filtered through a 0.45-μm pore sized membrane, and the extract was incubated with 3 mL of Ni – nitrilotriacetic acid (NTA) resin at 4 °C for 1 h with gentle mixing. The suspension was then applied to a small column (0.9 cm by 12.5 cm) and the packed gel washed with the same buffer and then eluted with 5 mL of imidazole buffer (50 mmol sodium phosphate buffer·L^{-1} (pH 8.0), 300 mmol NaCl·L^{-1}, and 250 mmol imidazole·L^{-1}). The enzyme was concentrated and the buffer exchanged to 0.02 mol potassium phosphate buffer·L^{-1} (pH 6.5) with 0.1 mol NaCl·L^{-1} by ultrafiltration using a membrane with a 10 000 molecular weight cutoff (PM10, Amicon; Millipore (Canada) Ltd., Nepean, Ontario). When using the Axe6A construct with the N-terminal His-tag, the concentrated protein collected after binding to the Ni–NTA resin was digested with 10 U of thrombin at room temperature for 16 h to remove the N-terminal His-tag, as recommended in the instruction manual (Sigma). At this stage enzyme from either tag system was applied to a Sephadex G-75 chromatography column (2.6 cm by 100 cm) in the same buffer, and the eluted fractions were analyzed for acetylxylan esterase activity and protein concentration, and active fractions were analyzed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE). Selected fractions containing activity were concentrated by ultrafiltration, diluted in 0.02 mol potassium phosphate buffer·L^{-1} (pH 6.5) to reduce the ionic strength, and then loaded onto DEAE–Sepharose CL-6B (2.5 cm by 77 cm) pre-equilibrated with 0.02 mol potassium phosphate buffer·L^{-1} (pH 6.5). After the sample was applied to the column, it was washed with 1 bed volume (400 mL) of starting buffer and eluted with a linear 500-mL gradient of 0–1 mol NaCl·L^{-1} in 0.02 mol potassium phosphate buffer·L^{-1} (pH 6.5). Axe6A eluted from DEAE–Sepharose was again concentrated by ultrafiltration with a PM10 membrane.

For purification of Axe6B fused to GST, the cells containing the pGEX construct were grown as described above, and the harvested cells were resuspended in PBS buffer (50 mmol sodium-potassium phosphate·L^{-1} (pH 7.2), 150 mmol NaCl·L^{-1}) containing 0.5 mmol PMSF·L^{-1}, protease inhibition cocktail (Sigma-Aldrich Canada) and 0.01% (*w*/*v*) sodium azide. The cells were disrupted by using a French press, followed by centrifugation at 15 000*g* at 4 °C for 20 min. The cytoplasmic fraction was incubated with 3 mL of glutathione agarose at 4 °C for 1 h with gentle mixing. The mixture was loaded onto a column and the effluent collected. The agarose was washed with 40 mL of PBS containing 1% (*v*/*v*) Triton X-100 and eluted with buffer containing 10 mmol reduced glutathione·L^{-1} in 50 mmol Tris-HCl·L^{-1} (pH 8.0) (Frangioni and Neel 1993). The eluted enzyme was exchanged with 50 mmol sodium phosphate buffer·L^{-1} (pH 6.5) by ultrafiltration. The enzyme was treated with thrombin and reapplied to a glutathione column to remove the GST. The proteins were loaded onto a G-75 Sephadex column pre-equilibrated with 50 mmol sodium phosphate buffer·L^{-1} (pH 6.5) with 100 mmol NaCl·L^{-1} containing 1 mmol PMSF·L^{-1} and 0.01% (*w*/*v*) sodium azide. Fractions containing esterase activity that were free from contaminating proteins (shown by SDS–PAGE analysis of the fractions) were pooled and concentrated by ultrafiltration. Homogeneity of the purified proteins was confirmed by SDS–PAGE using the Page Ruler™ Protein Ladder (Fermentas International Inc., Burlington, Ontario) molecular weight markers.

Xyn10E protein was purified by using the cloned gene in pET-30a, as described by Jun et al. (2003).

Mass spectrometry analysis of proteins

The purified proteins (10–15 μg) were separated by one-dimensional SDS–PAGE and detected by staining with colloidal Coomassie brilliant blue G-250 (Neuhoff et al. 1988). The excised gel pieces were destained in 50% (*v*/*v*) acetonitrile in 50 mmol ammonium bicarbonate·L^{-1} and dried in 100% acetonitrile. The proteins were subjected to reduction in 10 mmol dithiothreitol·L^{-1} at 50 °C for 30 min and alkylation in 100 mmol iodoacetamide·L^{-1} at 22 °C for 1 h. After several washes in 100 mmol ammonium bicarbonate·L^{-1} and dehydration in acetonitrile, the gel pieces were completely dried using a Speedvac (Thermo Electron Corporation), and 20 μL of acid treated trypsin (20 ng·μL^{-1}, Sigma) was suspended in 50 mmol ammonium bicarbonate·L^{-1} was added to each gel piece, followed by rehydration for 1 h at room temperature after which 50 μL of 50 mmol ammonium bicarbonate·L^{-1} was added, and digestion continued for 18 h at 37 °C. The digested fragments were then extracted with a solution containing 50% (*v*/*v*) acetonitrile and 5% (*v*/*v*) formic acid in water. The extracted peptides were dried in a Speedvac and dissolved in approximately 200 μL of 0.1% (*w*/*v*) trifluoroacetic acid and applied to a C18 ZipTip (Millipore (Canada) Ltd.), as described in the instruction manual. The purified peptides were eluted from the ZipTip with 4 μL of a saturated solution of α-cyano-4-hydroxycinamic acid containing 0.1% trifluoroacetic acid in 50% acetonitrile. A 0.5-μL volume of this solution was spotted in a well of a 96-well MALDI plate (Hellman et al. 1995; Rosenfeld et al. 1992). Peptide mass analyses were performed using a Bruker Reflex III (Bruker Datonics, Breman, Germany) matrix-assisted laser desorption ionization – time-of-flight (MALDI–TOF) mass spectrometer (Biological Mass Spectrometry Facility, University of Guelph).

Peptide mass profiles were analyzed by using the MS-FIT Protein Prospector program (UCSF Mass Spectrometry, prospector.ucsf.edu/) against the *F. succinogenes* nonredundant protein database.

Substrates binding assay

Binding of Axe6A to each insoluble polysaccharide was qualitatively assessed by mixing purified enzyme (~1.5 μg), in triplicate with 25 mg of a water-washed insoluble polysaccharide, which was one of microcrystalline cellulose (Avicel PH105), insoluble fraction of oat spelt xylan or beech-wood xylan suspended in a final volume of 500 μL of 0.05 mol MOPS buffer·L^{-1} (pH 7.0) in a 1.5-mL microcentrifuge tube. Each suspension was incubated at 37 °C for 15 min with rotary mixing and then centrifuged at 14 000*g* for 2 min. The supernatant containing unbound enzyme was assayed for esterase activity using α-NA as the substrate. The comparative binding of Axe6A and Axe6B to insoluble polysaccharides was performed with cell extracts as a source of enzyme due to a lack of purified Axe6B.

The capacity of Axe6A and Axe6B to bind to soluble polysaccharides was evaluated by affinity gel electrophoresis, a method in which migration of a test protein through nondenaturing polyacrylamide gel containing a polysaccharide is assessed relative to that in a gel lacking the polysaccharide. (Freelove et al. 2001; Tomme et al. 2000). The migration of a nonbinding protein (bovine serum albumin; BSA) was electrophoresed at the same time to serve as a reference. Axe6A, Axe6B, and BSA, each at approx. 2 μg, were loaded onto 7.5% native polyacrylamide gels containing 0.1% (*w*/*v*) of hydroxyethyl cellulose, rye arabinoxylan, barley β-glucan, or arabinogalactan, and a reference, a gel not containing a polysaccharide. The gels were prepared with 25 mmol Tris·L^{-1} and 250 mmol glycine buffer·L^{-1} (pH 8.3) and electrophoresed at 50 V for 3 h at 22 °C. The proteins were stained with Coomassie brilliant blue G-250, and the relative migration distances of Axe6A, Axe6B, and BSA were compared.

Results

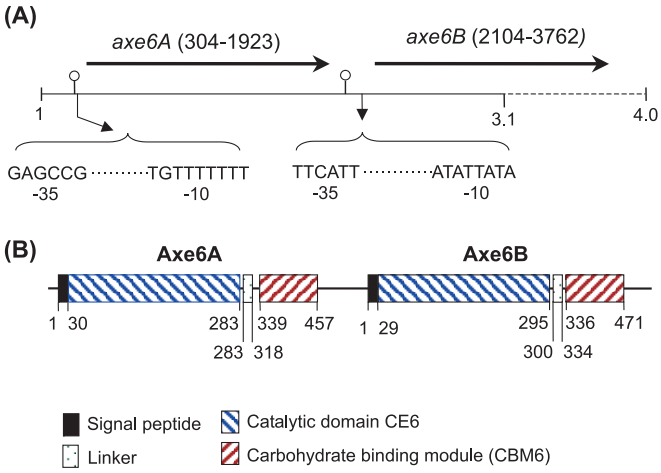
Characteristics of genes *axe6A* and *axe6B*

The genes *axe6A* and *axe6B* are located adjacent to one another in the *F. succinogenes* chromosome separated by a 183-bp intervening sequence. The putative –10 and –35 promoter sequences of *axe6A* and *axe6B* shown in Fig. 1 were predicted by using the program BPROM (http://www.softberry.com/berry.phtml?topic = bprom&group = programs&subgroup = gfindb). At the 3′ terminus of the *axe6A* gene after the stop translation codon TAA (bases 1921–1923 bp) there is an inverted repeat region between nucleotides 1939 and 1964 that may serve as a rho-independent transcriptional termination (Brendel and Trifonov 1984).

Both Axe6A and Axe6B are modular enzymes composed of an N-terminal carbohydrate esterase family 6 CD and a C-terminal family 6 CBM. The molecular masses and pIs of Axe6A and Axe6B derived from the amino acid sequences were 58.6 and 60.5 kDa and 5.52 and 5.43, respectively. Axe6A and Axe6B were predicted to contain signal peptides of 30 and 29 residues, respectively, with putative cleavage sites between pairs of alanine residues. The CD of Axe6A exhibited 61% similarity to that of Axe6B (Fig. 2). The CDs of Axe6A and Axe6B also exhibited 61% and 57% similarity to the CD of the AxeA from *Orpinomyces* sp. (Blum et al. 1999), and 59% and 54% similarity to the CD of BnaA of *N. patriciarum* (Dalrymple et al. 1997), respectively, with large segments of homology.

The C-terminal CBMs of Axe6A and Axe6B showed 40% similarity to each other. The CBM of Axe6A exhibited 25% and 23% similarity to the *Cellvibrio mixtus* endoglucanase 5a CBMs, CBM6-1 and CBM6-2, respectively (Henshaw et al. 2004), and 12% similarity to the *Clostridium thermocellum* CBM of xylanase 11A, Xyn11A_Ct (Czjzek et al. 2001), while Axe6B exhibited 17% and 20% similarity, respectively, to the CBMs of *Cellvibrio mixtus* endoglucanase 5a and 6% similarity to the CBM *Clostridium thermocellum* of xylanase 11A (Fig. 3).

Fig. 1. Organization of the acetylxylan esterases *axe6A* and *axe6B* in genomic DNA of *Fibrobacter succinogenes*. (A) Gene structures and (B) domain organization of the enzymes. The vertical bar with a circle in (A) indicates an inverted repeat region.



Expression and purification of Axe6A and Axe6B

Escherichia coli BL21 (DE3) harboring the recombinant plasmid pET-30a-Axe6A overexpressed Axe6A; however, the C-terminal His-tagged protein did not efficiently bind to a Ni–NTA column. Therefore a construct designed with both N- and C-terminal His-tags was used for purification of Axe6A. Following Ni–NTA affinity chromatography and removal of the N-terminal His-tag by thrombin treatment, chromatography on Sephadex G-75 and on DEAE–Sepharose resulted in purification of Axe6A to practical homogeneity (Table 2; Fig. 4).

For the purification of Axe6B, *E. coli* DH5α harboring the GST recombinant plasmid pGEX4T1–Axe6B was used, since *E. coli* BL21 (DE3) harboring the pET-30a–Axe6B plasmid did not overexpress Axe6B efficiently and the enzyme produced did not bind efficiently to Ni–NTA resin. The protein Axe6B fused to GST was purified by glutathione agarose affinity chromatography, followed by Sephadex G-75 chromatography (Table 2; Fig. 4). The xylanase Xyn10E was purified as described previously (Jun et al. 2003). The purified enzymes (Axe6A, Axe6B, and Xyn10E) were electrophoresed in a single dimension, subjected to in-gel trypsin digestion, and the peptide products were subjected to MALDI–TOF mass spectroscopy, as described in the Materials and Methods section, to confirm that the purified proteins matched the corresponding gene. The tryptic peptides from Axe6A, Axe6B, and Xyn10E matched the respective translated ORFs with 70%, 57%, and 70% amino acid coverage, respectively.

General characterization of Axe6A and Axe6B

Axe6A and Axe6B had the highest activities with α-NA as the substrate, had sevenfold lower activity on α-naphthyl propionate, and showed almost no activity on α-naphthyl butyrate and naphthyl substrates with longer alky chain lengths (Table 3). Although Axe6A and Axe6B exhibited lower activities on xylose tetraacetate, glucose peptaacetate, and chemically acetylated xylan than on naphthyl acetate, the activities of these substrates demonstrated the capacity to de-

Fig. 2. Alignment of the amino acid sequences of the catalytic domains of *Fibrobacter succinogenes* Axe6A and Axe6B with carbohydrate esterase family 6 enzymes. Axe6A_Fs and Axe6B_Fs, *F. succinogenes* S85 (accession No. AF180369); AxeA_Orp, *Orpinomyces* sp. strain PC-2 (accession No. AF001178); BanA_Npat, *Neocallimastix patriciarum* (accession No. U66251). Asterisks indicate conserved amino acids among sequences. Putative signal peptide sequences are underlined.

```
Axe6A_Fs        MSVEMSFKKLMGIAGVAAGLSMFAVMGANAAPDPNFHIYIAYGQSNMEGNARNFTDVDKK 60
Axe6B_Fs        MSVERNLKKFMALAGVAAGLSMFAVG-ANAAPNPNFHIYIAYGQSNMAGNGDIVPSEDQA 59
AxeA_Orp        ------MRTSVVITFLAAALTVMAKP--HAKPDPNFHIYLALGQSNMEGQG-NVEAQDRV 51
BnaA_Npat       ------MRTFAIAAFVATTLSAVSQT--FAAPDPNFHIYLAFGQSNMEGQG-PIGSQDR  T 51
                      ::.    : :*: *: .:     * *:******:* ***** *:.  .   *:

Axe6A_Fs        EHPR-VKMFATTSCPSLGRP-------TVGEMYPAVPPMFKCGEGLSVADWFGRHMADSL 112
Axe6B_Fs        EAPKNFIMLASHNANASQRSGKTNQSIKTGEWYPAIPPMFHPFENLSPADYFGRAMADSL 119
AxeA_Orp        EDKR-FKLISTADE-CMGRE--------LGEWYPALPPIVNCYGNLGPVDYFGRTLTKKL 101
BnaA_Npat       VDKR-FQMISTVSG-CNGRQ--------MGNWYDAVPPLANCDGKLGPVDYFGRTLVKKL 101
                   : . :::: .  .  *          *: * *:**: :    *. .*:*** :...*

Axe6A_Fs        P-NVTIGIIPVAQGGTSIRLFDPDDYKNYLNSAESWLKNGAKAYGDDGNAMGRIIEVAKK 171
Axe6B_Fs        P-GVTVGIIPVAIGAVSIRAFDKDQYEAYFRGDGKDIMNWGWPKDYDNNPPGRILELAKK 178
AxeA_Orp        PKEVKVGVCAVAVAGCDIQLFEEENYKSYEIPD----WMQGRIDHYGGNPFRRLVNIAKK 157
BnaA_Npat       PQEIKVGVAVVAVAGCDIQLFEKNNYRNYRLES----YMQGRVNAYGGNPYGRLIEVAKK 157
                *  :.:*:  ** .. .*: *: ::*. *           .     ..*.  *::::***

Axe6A_Fs        AQEKGVIKGIIFHQGETDGGMSNWEQIVKKTYEYMLKQLGLNAEETPFVAGEMVDGGS-- 229
Axe6B_Fs        AKEVGVIKGFIFHQGESDGTDANWRKTVYKTYKDVIDALGLDENEVPFVAGELLQEGQN- 237
AxeA_Orp        AQKAGVIKGILLHQGETNNGQEDWPKRIKVVYERLLKELNLKAEEVPLLAGEVVREEYEG 217
BnaA_Npat       AQQVGVIKGILLHQGETNTGQQNWPNRVKAVYEDMLKDLGLNAKDVPLLAGEVVQSNQGG 217
                *:: *****:::****::    :* : :  .*: ::. *.*. ::.*::***::

Axe6A_Fs        -CAGFSSRVRGLSKYIANFGVASSKGYGSKG-DGLHFTVEGYRGMGLRYAQQMLKLINVA 287
Axe6B_Fs        CCSSKNGGIAQLKQNFKKFGLASSKGLQGNGKDPYHFGRAGVIELGKRYCSEMLKLID-- 295
AxeA_Orp        MCSLHNTVIKKLPEVIPTAHVISAEGLDDGG-DDLHFSSASYRILGERYADKMLELL--- 273
BnaA_Npat       QCGSMNSIIQKLPSVIPTAHVISSQGLGQQG-DGLHFSSQAYRTFGERYADEMLKIL--- 273
                 *.  .  :  * . : .  : *::*    * *  **   .   :* **..:**:::
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Table 2. Axe6A and Axe6B purified from the cytoplasmic fraction of *Escherichia coli*.

Purification	Total protein (mg)	Total units (U)	Specific activity ($U \cdot mg^{-1}$)	Yield (%)
Axe6A				
Cell extract	1679	14 554	8.7	100
Ni–NTA binding	13.5	4155	264	29
Sephadex G-75	ND	1258	ND	8.6
DEAE Sepharose	0.8	85.9	113	0.6
Axe6B				
Cell extract	1195	3464	2.9	100
GST elution	16.6	284	17.1	8.2
Sephadex G-75	0.77	45.1	58.6	1.3

Note: ND, not determined; Ni–NTA, Ni – nitrilotriacetic acid; GST, glutathione *S*-transferase.

grade acetylxylans in plant cell wall polymers (Table 3). It is worth noting that Khan et al. (1990) tested the activity of different acetylxylan esterases on naturally acetylated and chemically acetylated xylans. They found that some enzymes showed up to 44% lower activity on chemically acetylated xylan than on naturally acetylated xylan, while others exhibited up to 61% higher activity on acetylated xylan, and yet other enzymes exhibited similar activities on both forms of xylan. The lack of hydrolytic activity of the enzymes on *p*-nitrophenyl glycosides further affirms the specificity of the enzymes.

The properties of the enzymes are shown in Table 4. The low K_m values for Axe6A and Axe6B at 0.084 and 0.056 $mmol \cdot L^{-1}$, respectively, indicates the capacity of the enzymes to function at very low substrate concentrations. The neutral pH values for maximum enzyme activity fit well with the generally neutral pH of the rumen. The enzyme activity of Axe6A was decreased by greater than 50% by Fe, Cu, and Zn at a concentration of 1.0 $mmol \cdot L^{-1}$ but was unaffected by Mn, Co, Ca, Mg, and EDTA at a concentration of 10 $mmol \cdot L^{-1}$, whereas the activity of Axe6B was inhibited by all minerals but Ca at this latter concentration.

Binding to polysaccharides

Axe6A bound to Avicel cellulose and beech-wood xylan with greater than 90% efficiency, whereas only 51% of the

Fig. 3. Alignment of the amino acid sequences of the carbohydrate-binding modules (CBMs) of *Fibrobacter succinogenes* Axe6A and Axe6B with CBM 6 enzymes Axe6A_Fs and Axe6B_Fs, *F. succinogenes* S85 (accession No. AF180369); Xyn10D_Fs, Xyn10E_Fs, and Xyn10B_Fs (accession No. AF180368); *Cm*CBM6-1 and *Cm*CBM6-2 *Cellvibrio mixtus* Cel5a; Xyn11A_Ct, *Clostridium thermocellum* xylanase 11A. The bold letters indicate the conserved residues that have been shown to be involved in ligand binding. Asterisks indicate conserved amino acids among sequences.

```
Axe6A_Fs        ---------DSD---YRKDTG----------VDLYKAGDGVALGYTQTGEWLEYT---VD 374
Axe6B_Fs        VEAENYNKGGADKAYYDLSKGNEGGKLRKNDVDIYQPNMGIVVGHCQKSEWLKYT---VN 392
Xyn10D_Fs       --------GDSD---YRKDTG----------VDLYKKATGVIVGYNSEGDWLEWT---VN 455
Xyn10E_Fs       --------GDSD---YRKDTG----------ADLYKKATGVALGYNTTGDWYEYT---IN 446
Xyn10B_Fs       --------GDSD---YRKSDAS--------DVDIYKKATGNIVGYNTTGDWLEYS---VD 433
CmCBM6-1        ---------QAES--WCQMSG-------VKTETTSDAGDGLNVGYIDGGDWMTYS---VN 39
CmCBM6-2        ----VIATIQAED--HSQQSG-------TQQETTTDTGGGKNVGYIDAGDWLSYAGTPVN 48
Xyn11A_Ct       --RSAFSKIESEE--YNSLKSS-------TIQTIGTSDGGSGIGYIESGDYLVFN--KIN 47
                          ::        .                  *  :*:   .::  :    ::

Axe6A_Fs        VKADGEYNIDASVAAGNSTSAFKLYIDEKA--ITDDVSVPQT--ADNSWDTYKTISVKEK 430
Axe6B_Fs        VKADGDYGITANVAGDNATGSIVLYMDDKR--IGDEMVNE-----GKGFDTFS-IVDGGK 444
Xyn10D_Fs       VKEAGDYTMFAAVAAAGSTSSFQLSLDGKA--LTEKITVPAAKEGEENYDHYN--KVKGN 511
Xyn10E_Fs       IAEAGDYTAIASVATEG-TGAFTLSLDGKS--LAE-FEVT-----GTSYDDFS--DVKKK 495
Xyn10B_Fs       IAEAGDYTATASVAADG-SGSFKLSIDGKS--VGE-FDVTWT---GSSWDNFI--DVKKK 484
CmCBM6-1        IPTTGTYKVSYRVAAQAGGGQLQLEKAGGSP-VYSNINVPAT----GGWQNWQ--TISHN 92
CmCBM6-2        IPSSGSYLIEYRVASQNGGGSLTFEEAGGAP-VHGTIAIPAT----GGWQTWT--TIQHT 100
Xyn11A_Ct       FGNGANSFKARVASGADTPTNIQLRLGSPTGTLIGTLTVAST----GGWNNYE--EKSCS 101
                .   .       .:       : :        :   .          .:: :       .

Axe6A_Fs        VTLKAGKHVLKLEITANYVNIDWIQFSEPKK-- 461
Axe6B_Fs        VSLKAGEHELKIEIANDWIDIDYIEFK------ 471
Xyn10D_Fs       VTLPAGKHVLRMDVTGAWFDVDYFTFVK----- 539
Xyn10E_Fs       VTLPAGKHVLRLDVTQQYFDIDYINFVK----- 523
Xyn10B_Fs       VTLPAGKHTLRMDVTAQYFDIDYINFTK----- 512
CmCBM6-1        VVLPAGEQLIALSAITGGFNINWLKVES----- 120
CmCBM6-2        VNLSAGSHQFGIKANAGGWNLNWIRINKTH--- 130
Xyn11A_Ct       ITNTTGQHDLYLVFSG-PVNIDYFIFDSNGVNP 133
                :   :*.: : :       ::::: .
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Table 3. Substrate specificities of Axe6A and Axe6B.

	Axe6A		Axe6B	
Substrate	Activity	%	Activity	%
α-Naphthyl acetate	110.3±7.3	100.0	26.0±2.5	100.0
α-Naphthyl propionate	16.8±1.9	15.2	3.8±0.1	14.6
α-Naphthyl butyrate	0.6±0.1	0.6	0.5±0.1	1.9
α-Naphthyl caprylate	1.4±0.3	1.3	0.4±0.2	0.2
α-Naphthyl laurate	0.1±0.1	0.1	0.02±0.1	0.1
β-Naphthyl acetate	45.9±5.2	41.6	14.9±0.0	57.3
Acetylated birch-wood xylan	6.2±0.1	5.6	1.5±0.1	5.8
Xylose tetraacetate	11.4±0.2	10.3	1.3±0.03	5.0
Glucose pentaacetate	11.4±0.3	10.3	0.6±0.1	10.0
p-Nitrophenyl xylopyranoside	1.4±0.4	1.3	0.04±0.04	0.2
p-Nitrophenyl arabinofuranoside	0.3±0.02	0.3	0.0±0.01	0.0

Note: Activity is expressed in units per milligram of protein ± standard error (n = 3).

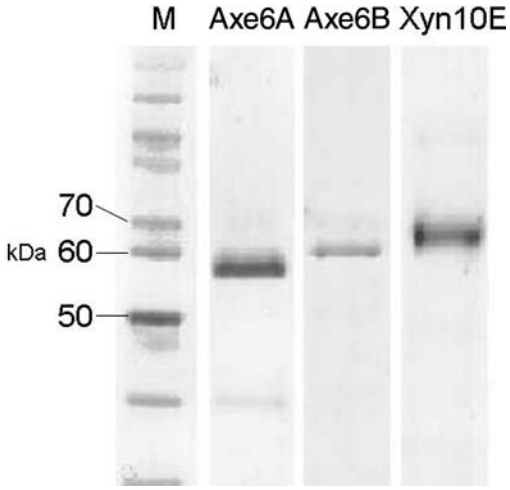
enzyme bound to the insoluble fraction of oat spelt xylan (Table 5). An experiment using cell extracts was also conducted to compare the binding of Axe6A and Axe6B to insoluble polysaccharides. Under these conditions Axe6A bound to Avicel cellulose and beech-wood xylan with 69% and 67% efficiency, respectively, compared with 57% and 55% efficiency, respectively, for Axe6B. Using cell extracts added the complication that Axe6B was substituted at the N terminus with a bulky domain. However, the enzyme still retained binding capacity. To assess the binding of Axe6A and Axe6B to soluble polysaccharides, nondenaturing electrophoresis in the presence of the polysaccharide (affinity gel electrophoresis) was adopted with BSA serving as the reference, since it does not bind to polysaccharides (Fig. 5; Freelove et al. 2001). Axe6A bound to the soluble substrates barley β-glucan and rye arabinoxylan, as shown by retardation of the enzyme mobility relative to that of BSA during migration in the presence of these substrates. No retardation of migration was observed with hydroxyethyl cellulose and arabinogalactan and hence no binding. Because of reduced

Table 4. Characteristics of Axe6A and Axe6B.

Enzyme	Mol. mass* (kDa)	pH of max. activity	Temperature (°C) of max. activity	pI*	K_m (mmol·L^{-1})	V_{max} (U·mg^{-1})
Axe6A	58.6	6.5–8.5	40	5.52	0.084	105.3
Axe6B	60.5	7.5	40	5.43	0.056	31.2

Note: α-Naphthyl acetate was used as substrate. Assay results are the mean values of triplicate determinations.
*Molecular masses and pI values were computed from the amino acid sequences of the mature enzymes.

Fig. 4. SDS–PAGE analysis of purified Axe6A, Axe6B, and Xyn10E. Molecular mass marker (M); Axe6A (~58.6 kDa); Axe6B (~60.5 kDa); Xyn10E (~66.7 kDa).



concentrations of Axe6B, due to the lack of enzyme, the result was less definitive. However, it appeared that migration was reduced in the presence of rye arabinoxylan but not in the presence of β-glucan or other polysaccharides tested, thereby indicating binding to arabinoxylan but not to glucan.

Synergy of Axe6A with xylanase

Axe6A was able to cleave acetyl residues from acetylated xylan at a similar initial rate in either the absence of Xyn10E or after pretreatment with Xyn10E (Fig. 6). However, the final level of acetate released without xylanase action was lower than that in the presence of xylan, suggesting that xylan hydrolysis was, at least initially, not necessary for deacetylation of xylan. No xylooligosaccharides were released from the acetylated birch-wood xylan by the acetylxylan esterase.

In the absence of Axe6A, the amount of xylose released from acetylated xylan by Xyn10E increased slowly for up to 4 h of incubation but did not increase further (Fig. 7). However, after pretreatment of acetylated xylan with Axe6A there was a continued increase in reducing sugar release during 12 h of incubation (Fig. 7). Analysis of the products of hydrolysis by HPLC confirmed and expanded the results observed, by following reducing sugar production. Xyn10E action alone on acetylated xylan released xylooligosaccharides at a very slow rate, and these consisted mainly of longer chain oligosaccharides with little xylose produced (Fig. 8). With either the simultaneous presence of Axe6A and Xyn10E or with pretreatment with Axe6A before addition of Xyn10E, there were transient increases in xylohexaose, xylopentaose, and xylotetraose followed by large increases in xylotriose, xylobiose, and xylose (Fig. 8).

Table 5. Test for binding of Axe6A to insoluble polysaccharides.

Polysaccharide substrate	Unbound activity (nmol/min·mL^{-1})	Bound activity (%)
None	23.1±0.9	—
Avicel	1.5±0.2	93.5
IF-OSX	11.4±0.9	49.4
Beech-wood xylan	1.3±0.2	94.4

Note: IF-OSX, insoluble fraction of oat spelt xylan.

Discussion

In a previously unpublished study, we cloned the *axeA* (*axe6A*) acetylxylan esterase gene (accession No. AF180369) and a segment of a second downstream gene exhibiting high homology to Axe6A (Ha et al. 1999). Now using the genome database of *F. succinogenes* S85, the complete sequence of *axe6B* was located downstream of *axe6A*, and the two acetylxylan esterases were cloned, expressed, and characterized. Both proteins exhibited modular structures composed of an N-terminal CD belonging to carbohydrate esterase family 6 and a C-terminal CBM also belonging to family 6. They have catalytic and biochemical characteristics different from the acetylxylan esterase purified from the culture fluid of *F. succinogenes* S85 by McDermid et al. (1990*a*). The K_m and V_{max} values of Axe6A and Axe6B were substantially lower and the masses of the proteins were slightly greater than values for the esterase from the culture fluid reported by McDermid et al. (1990*a*). In addition, the pI of the previous purified acetylxylan esterase was 4.0, while the computed pIs of Axe6A and Axe6B were 5.52 and 5.43, respectively, suggesting that they are different from the enzyme purified by McDermid et al. (1990*a*). They also reported a number of active acetylxylan esterase activity bands from zymogram analysis of the extracellular culture fluid by SDS–PAGE renaturing gels. Therefore, it is not surprising that we have isolated genes that code for proteins with different properties.

The CDs of Axe6A and Axe6B from *F. succinogenes* S85 had greater than 50% similarity to those of the acetylxylan esterases from the ruminal fungi *Orpinomyces* sp. (strain PC-2) and *N. patriciarum*, but they did not show significant homology to esterases of the same carbohydrate esterase family, 6, in other organisms found in different environments (e.g., *Arabidopsis*, *Clostridium*, *Oryza*, and *Phodopirellula*). This would suggest that the catalytic domains from the ruminal microorganisms may have either diverged from a common ancestor in a different environment or perhaps converged in the stable environment of the rumen.

Fig. 5. Nondenaturing electrophoresis of bovine serum albumin (BSA), Axe6B, and Axe6A in polyacrylamide gel containing 0.1% (*w*/*v*) of soluble polysaccharide. Gel panels include: no polysaccharide, hydroxyethyl cellulose (HEC), rye arabinoxylan, starch, arabinogalactan, barley β-glucan. Electrophoresis was at 50 V for 3.5 h at 22 °C and staining was with Coomassie brilliant blue G-250.

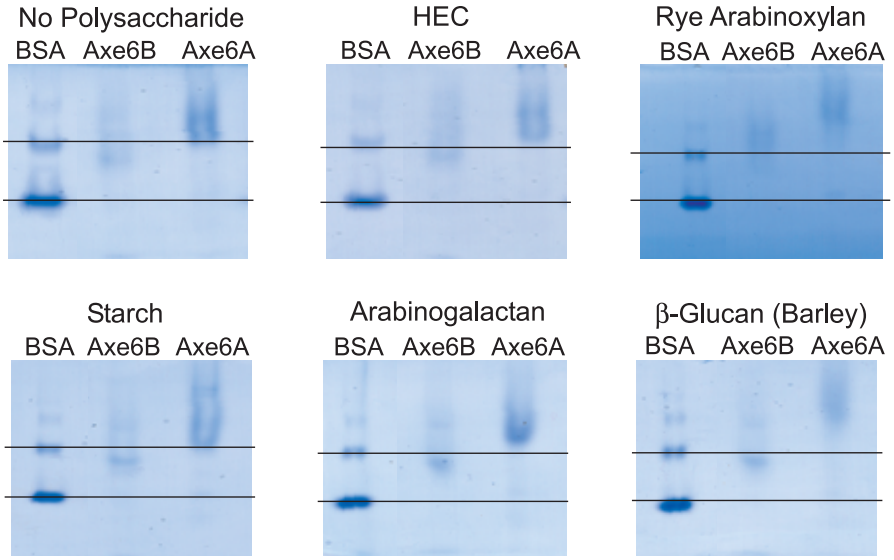


Fig. 6. Effect of pretreatment of acetylated xylan with xylanase on the release of acetate by acetylxylan esterase. Treatments: pretreatment with Xyn10E for 10 h followed by the addition of Axe6A (●); Axe6A alone (○); Xyn10E alone (▼). The vertical bars indicate the standard error of the mean (n = 3).

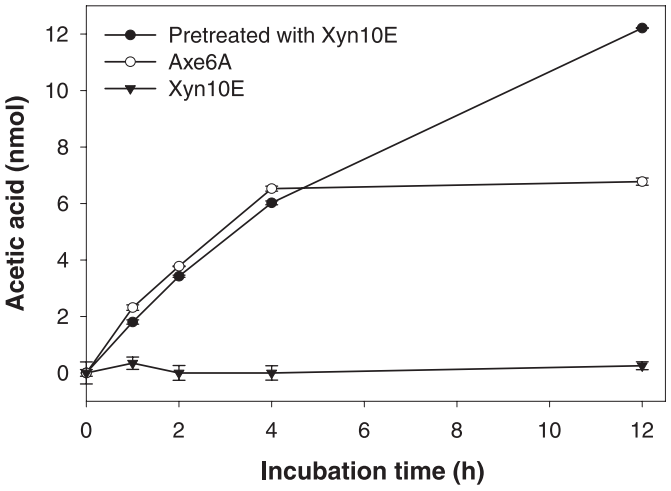
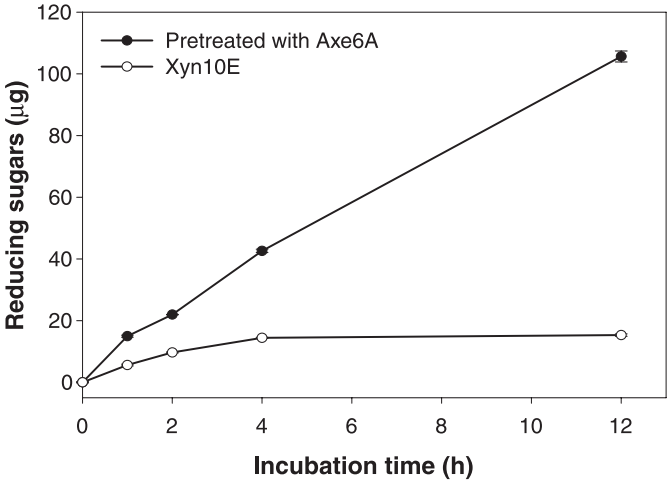


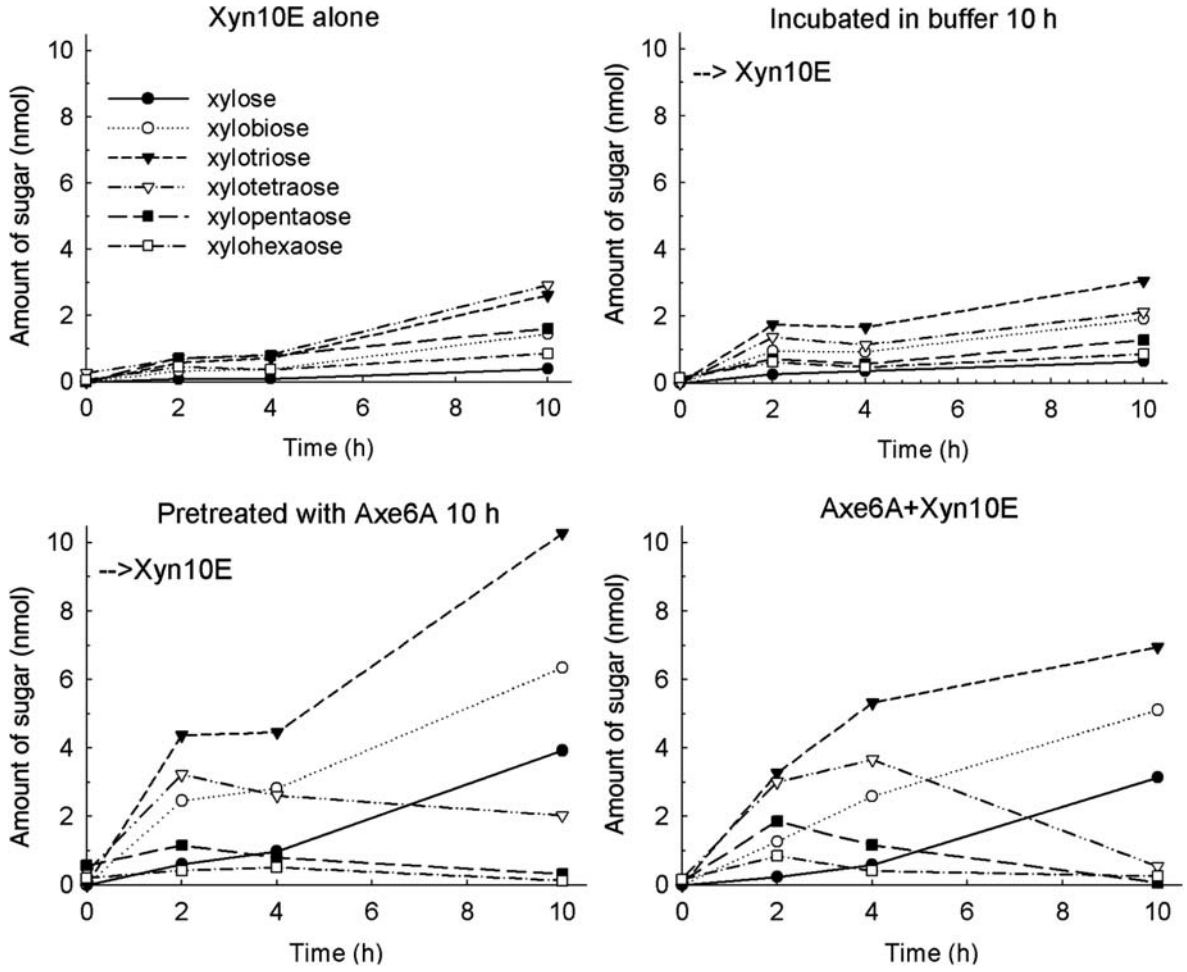
Fig. 7. The effect of pretreatment of acetylated birch-wood xylan with Axe6A on hydrolysis by Xyn10E. Treatments: pretreatment with AxeA for 10 h prior to the addition of Xyn10E (●); Xyn10E alone (○).The vertical bars indicate the standard error of the mean (n = 3).



axe6A and *axe6B* are contiguous genes on the *F. succinogenes* S85 chromosome and have similar overall structures. Clustered genes encoding fibrolytic enzymes have been reported previously for *F. succinogenes*, and these include two endoglucanase genes, *celD* and *celE* (Malburg et al. 1996), and three xylanase genes, *xyn10D*, *xyn10E*, and *xyn10B* (Jun et al. 2003). Previous researchers suggested the contiguous genes may result from gene duplication and subsequent evolution or from horizontal gene transfer (Jordan et al. 2002; Kondrashov et al. 2002). Based on high sequence identity and related overall organization of the genes, the acetylxylan esterases may also have resulted from gene duplication and subsequent evolutionary events, a hypothesis proposed by Jun et al. (2003) to explain the origin of the xylanases B, D, and E cluster.

Recently, the crystal structures were determined for two family-6 CBMs, *Cm*CBM6-2 at the C terminus of a *Cellvibrio mixtus* GH5 endoglucanase (Henshaw et al. 2004) and a CBM from the *Clostridium thermocellum* xylanase 11A (Czjzek et al. 2001; Henshaw et al. 2004). Both CBMs exhibited a classic lectin-like β-jelly-roll structure. Using the structure profiling program 3D PSSM developed by Kelley et al. (2000), we have shown that the crystal structure of *Cm*CBM6-2 is a suitable model for predicting the CBM tertiary structure of the Axe6A CBM. From the comparison, it appears that the CBM of Axe6A has a similar folding pat-

Fig. 8. Xylooligosaccharide products of hydrolysis of acetylated birch-wood xylan by Xyn10E without and with pretreatment by Axe6A. Treatments: Xyn10E alone; after incubation in buffer for 10 h; after preincubation in buffer for 10 h with Axe6A; or Xyn 10E and Axe6A added simultaneously. Acetylated xylan was included at a final concentration of 1% (*w*/*v*). The hydrolysis products were xylose (●), xylobiose (○), xylotriose (▼), xylotetraose (▽), xylopentaose (■), and xylohexaose (□).



tern to *Cm*CBM6-2 and xylanase 11A_CBM6. Using a combination of site-directed mutagenesis, high resolution X-ray crystallography, and NMR, Czjzek et al. (2001) and Henshaw et al. (2004) have shown that the three solvent-exposed residues (tyrosine, tryptophan, and asparagine) of cleft A of *Cm*CBM6-2 and xylanase 11A_CBM6 play a key role in ligand binding. These residues correspond to Tyr-362, Trp-419, and Asn-450, respectively, of Axe6A. Although the CBM of Axe6B exhibits comparatively high similarity to that of Axe6A and *Cm*CBM6-2 (Fig. 3), modeling of Axe6B did not reveal similar key residues, and instead His-380, Phe-434, and Asp-464 replaced the key solvent-exposed aromatic residues. In addition, 3D PSSM did not show any suitable target structure in Axe6B, leading to the suggestion that the binding mechanism, the ligand specificity, or the binding constant was different from that of CBM of Axe6A. Trp-39 in cleft B of *Cm*CBM6-2 that also plays a pivotal role in ligand binding was shown to be highly conserved in CBMs of both Axe6A (W-368) and Axe6B (W-386). In this respect, Axe6B seems to belong to a different subgroup within family 6 CBMs because of the difference in putative key amino acid residues involved in binding. The observation that Axe6B binds to the soluble substrate arabinoxylan but not to β-glucan supports the contention that Axe6A and Axe6B belong to different subgroups within the family. The Axe6A protein was shown to bind to both Avicel cellulose and insoluble xylan, as reported for the xylanase 11A_CBM6 (Czjzek et al. 2001), in contrast to *Cm*CBM6-2, which did not bind to xylan (Czjzek et al. 2001; Henshaw et al. 2004). The basis for the difference in binding characteristics among these CBM6 modules remains to be resolved. It has been shown that CBMs potentiate catalytic activity by mediating prolonged and intimate association between the enzyme and its target substrate (Bolam et al. 1998; Gill et al. 1999), thereby providing an important opportunity for synergistic hydrolysis of plant cell walls. This similar attribute of the *F. succinogenes* acetylxylan esterases helps illuminate the mechanism of plant cell wall biodegradation in this bacterium.

Studies have shown that the action of xylanase is essential for complete deacetylation of xylan by some acetylxylan esterases (Biely et al. 1986). Other acetylxylan esterases deacetylated acetylated xylan as efficiently in the absence as in the presence of xylanase (Halgasova et al. 1994; Kormelink et al. 1993; Sundberg and Poutanen 1991). The rate of deacetylation by different acetylxylan esterases may

also be influenced by whether the xylan is naturally acetylated or chemically acetylated, some exhibit increased activity on chemically acetylated xylan while others have decreased activity (Khan et al. 1990). Because Axe6A cleaved acetate residues from acetylated xylan in the absence of xylanase action, it obviously does not require cleavage of the xylan backbone of the polymer for removal of acetate groups, as has been demonstrated for other esterases. The observation that after extensive deacetylation of acetylated xylan, xylanase action is beneficial may stem from the fact that the xylan was not initially solubilized, a situation in which all acetylxylan esterases are probably stimulated by xylanase activity. Resolving this issue will require further research.

Acetylxylan-esterase-enhanced digestion of acetylated xylan by xylanase has been documented for the ruminal fungus *N. patriciarum* by measuring the increase in xylose-reducing equivalents when BnaA acted in consort with the *N. patriciarum* XynA on spear grass holocellulose (Cybinski et al. 1999). Our data with *F. succinogenes* extends these observations to the ruminal bacteria and adds detail by showing that a xylanase will cleave acetylated xylan, releasing long chain oligosaccharides, presumably by cleavage at sites not blocked by acetylation. The action of acetylxylan esterase cleaves acetyl residues from the xylooligosaccharides allowing their further cleavage to smaller products by xylanase. In a static system this gives rise to a transient appearance of longer chain oligosaccharides that are gradually hydrolyzed to xylotriose, xylobiose, and xylose. However, in the dynamic environment of the rumen, one can visualize the action of these enzymes allowing individual cells of *F. succinogenes* to produce digestion craters in acetylated plant cell walls as individual cells access cellulose to meet essential carbon and energy needs, since the bacterium is unable to metabolize xylose (Matte et al. 1992). It has been observed by microscopy that there are always a number of organisms in close association with *F. succinogenes* (Cheng et al. 1983). One explanation for this phenomenon is that the associated organisms are accessing cellodextrins released by *F. succinogenes* during growth (Wells et al. 1995); however, in addition to the release of cellodextrins, the continual purging of xylooligosaccharides at the *F. succinogenes* – plant cell wall interface provides another major energy source for growth of a broad range of saccharolytic rumen microorganisms.

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