Biochemical Characterization and Relative Expression Levels of Multiple Carbohydrate Esterases of the Xylanolytic Rumen Bacterium Prevotella ruminicola 23 Grown on an Ester-Enriched Substrate

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Received 30 April 2011/ Accepted 14 June 2011

We measured expression and used biochemical characterization of multiple carbohydrate esterases by the xylanolytic rumen bacterium Prevotella ruminicola 23 grown on an ester-enriched substrate to gain insight into the carbohydrate esterase activities of this hemicellulolytic rumen bacterium. The P. ruminicola 23 genome contains 16 genes predicted to encode carbohydrate esterase activity, and based on microarray data, four of these were upregulated >2-fold at the transcriptional level during growth on an ester-enriched oligosaccharide (XOSFA,Ac) from corn relative to a nonesterified fraction of corn oligosaccharides (AXOS). Four of the 16 esterases (Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A), including the two most highly induced esterases (Xyn10D-Fae1A and Axe1-6A), were heterologously expressed in Escherichia coli, purified, and biochemically characterized. All four enzymes showed the highest activity at physiologically relevant pH (6 to 7) and temperature (30 to 40°C) ranges. The P. ruminicola 23 Xyn10D-Fae1A (a carbohydrate esterase [CE] family 1 enzyme) released ferulic acid from methylferulate, wheat bran, corn fiber, and XOSFA,Ac, a corn fiber-derived substrate enriched in O-acetyl and ferulic acid esters, but exhibited negligible activity on sugar acetates. As expected, the P. ruminicola Axe1-6A enzyme, which was predicted to possess two distinct esterase family domains (CE1 and CE6), released ferulic acid from the same substrates as Xyn10D-Fae1 and was also able to cleave O-acetyl ester bonds from various acetylated oligosaccharides (AcXOS). The P. ruminicola 23 AxeA1, which is not assigned to a CE family, and Axe7A (CE7) were found to be acetyl esterases that had activity toward a broad range of mostly nonpolymeric acetylated substrates along with AcXOS. All enzymes were inhibited by the proximal location of other side groups like 4-O-acetyl and ferulic acid esters, but exhibited negligible activity on sugar acetates. As expected, the P. ruminicola Axe1-6A enzyme, which was predicted to possess two distinct esterase family domains (CE1 and CE6), released ferulic acid from the same substrates as Xyn10D-Fae1 and was also able to cleave O-acetyl ester bonds from various acetylated oligosaccharides (AcXOS). The P. ruminicola 23 AxeA1, which is not assigned to a CE family, and Axe7A (CE7) were found to be acetyl esterases that had activity toward a broad range of mostly nonpolymeric acetylated substrates along with AcXOS. All enzymes were inhibited by the proximal location of other side groups like 4-O-methylglucuronic acid, ferulic acid, or acetyl groups. The unique diversity of carbohydrate esterases in P. ruminicola 23 likely gives it the ability to hydrolyze substrates on the xylan backbone and enhances its capacity to efficiently degrade hemicellulose.

Hemicelluloses comprise a heterogeneous, highly branched mixture of complex polysaccharides. In plant cell walls, these polysaccharides form intimate associations with cellulose, pectin, and lignin and markedly reduce the degradability of plant fiber (20). In a microbial ecosystem such as the rumen, fiber-degrading microorganisms meet this challenge by operating as a consortium. Several hemicellulolytic rumen species, especially the ones belonging to Prevotella and Butyrivibrio, have been shown to synergistically enhance the rate and extent of plant cell wall hydrolysis during co- or sequential culturing with cellulolytic bacteria (12, 18, 31, 32).

The hemicelluloses present in the cell walls of grasses, cereals, and hardwoods largely comprise xylan, a polymer consisting of linear chains of D-xylopyranosyl units that are linked through β-(1→4) glycosidic linkages. For grasses and cereals, the xylan backbone is heavily substituted at the O-2 and/or O-3 position(s) with α-L-arabinofuranosyl and to a lesser extent with 4-O-methyl-α-D-glucuronic acids and O-acetyl esters (6, 9, 42, 46). Ferulic acid esters, linked to the O-5 of the arabinose moieties, may link with other esters, forming various types of di-ester bridges, resulting in the inter- and possibly even intralinking of polymers within plant cell walls (24). The xylan backbone from hardwoods is mainly decorated with O-acetyl esters and 4-O-methyl-α-D-glucuronic acids (17, 39, 44).

Hemicellulose degradation is largely affected by the presence of ferulic acid and O-acetyl esters. In nature, these esters can be hydrolyzed by microbial carbohydrate esterases. Acetyl xylan esterases catalyze the removal of acetyl ester groups from O-2 or O-3 positions of D-xylopyranosyl residues (5), while ferulic acid esterases release ferulic acid from the O-2 or O-5...
positions of α-1-arabinofuranosyl side chains (36, 37, 41). Consequently, acetyl and feruloyl esterases play important roles in hemicellulose degradation and, therefore, contribute to the complete hydrolysis of plant polysaccharides.

Various ruminal bacteria, including *Prevotella ruminicola* 23, have been shown to have esterase activities (21). However, knowledge regarding the diversity of esterases present in the genome, including the regulation, biochemical characteristics, and mechanistic action of these enzymes, is limited. Recently, the complete genome sequence of the ruminal bacterium *P. ruminicola* 23 was made available (38), therefore providing an opportunity to evaluate the repertoire of enzymes enabling this organism to function as a highly efficient hemicellulose-degrading bacterium.

In order to provide insight into the diversity and complexity of the multiple carbohydrate esterases present in *P. ruminicola* 23 cells, the two most upregulated esterases were biochemically characterized and their activities were compared with those of two other esterases that were constitutively expressed during growth on either an ester-enriched corn oligosaccharide, nonesterified corn oligosaccharide, or simple sugar substrates.

**MATERIALS AND METHODS**

Preparation of water-soluble acetylated and feruloylated XOS<sub>PAAC</sub>. Corn fibers were kindly provided by a corn processing plant. These fibers were milled (<1 mm) by using a Retsch ZM200 mill (Retsch Gmbh, Haan, Germany), and a suspension of milled fibers (231 g dry weight in total) in water (1,869 g) plus sulfuric acid (4.6 g) was heated at 140°C for 30 min, followed by cooling within 5 min to room temperature. A mixture of hemi celluloses with an endoxylanase (Humincola insolens GH10 [49]), β-xylanases (Trichoderma reesei GH3 [49]), and arabinofuranosidases (Meripilus giganteus GH51 and H. insolens GH43 [49] (Novozymes, Bagsvaerd, Denmark) and the commercially available amylglucosidase Spirizyme (Novozymes, Bagsvaerd, Denmark) were added to the suspension in order to degrade the most easily accessible starch and hemicellulose components (enzyme loading, 30 U/g of fiber; 30 U/g of fiber) in one fraction. Pure water was added to keep the oligomers in solution. After methanol evaporation, the XOS<sub>PAAC</sub> was lyophilized, yielding 19 g of dry material.

The nonbound C18 fraction, named AXOS, was collected as well (composition of the C18 fraction and AXOS has not been described by Dodd et al. (14). The cells were pelleted, resuspended in 25 ml of lysis buffer (1 ml of 0.1 M NaOH, 0.05 M NaCl, and 1 mM MgCl₂), and mixed with 0.5 ml isopropanol. The total RNA was mixed with 200 μl of concanavalin A-Sepharose beads (Sigma, St. Louis, MO). The beads were incubated in 1 ml of Trizol (Invitrogen, Carlsbad, CA) for 5 min at room temperature and then transferred to a sterile Eppendorf tube. The homogenate was mixed with 200 μl chloroform, and the mixture was centrifuged at 12,000 × g for 15 min at 4°C. The upper aqueous layer, containing total RNA, was collected, and RNA was precipitated using 0.5 ml isopropanol. The total RNA was subsequently pelleted, washed in 70% ethanol, and resuspended in sterile diethyl pyrocarbonate (DEPC)-treated double-distilled water (ddH₂O). The resuspended RNA was then further purified using an RNeasy kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. Purified RNAs were analyzed for quality using a Bioanalyzer RNA 6000 nano assay. All samples used gave an RNA index number (RIN) of 9 or greater. Total RNA was transcribed to cDNA, labeled, and purified using the Fairplay III system (Strategene, La Jolla, CA) and Cy3 and Cy5 dyes (GE Healthcare, Piscataway, NJ) per the manufacturer's instructions.

Genome analysis and sequence availability. The *P. ruminicola* 23 genome (accession number CP000206), along with the protein sequences of the three putative carbohydrate esterases designated Axe1-6A (YP_003575954), AxeA1 (YP_003575955), and Axe7A (YP_003575956) and the two most upregulated esterases that were constitutively expressed during growth on either a corn oligosaccharide or a simple sugar substrate, were deposited in GenBank database. Amino acid sequences were analyzed and classified into gene families using blastp (1) and Interpro (23). The cellular localization of proteins was predicted with NlocPloc (10, 45) and by the presence of transmembrane (30) or signal peptide (2) hidden Markov models (HMMs). HMMs were predicted using HMMER (15).

Microarray analysis. Transcriptional profiles were obtained using custom-designed 8- by 15-K microarrays supplied by Agilent (Santa Clara, CA). Each array carried five replicate probes for each of the 2,875 open reading frames (ORFs) and 12 structural RNAs identified in the *P. ruminicola* 23 genome. Microarray slides were scanned using a GenePix Professional 4200 scanner and GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA). Each condition (growth on glucose, xylose, XOS<sub>PAAC</sub>, or AXOS) was examined in triplicate, including a single dye swap. The resulting data were normalized and then analyzed in Bioconductor (19, 48) using a single-channel analysis. Genes with a transcriptional difference of 2-fold or greater and a false discovery rate (FDR) value of <0.05 were considered to be statistically significant. Since our interest was in carbohydrate esterase diversity, to validate the microarray analysis the transcriptional responses of 12 of the esterase genes were further examined by reverse transcription-quantitative PCR (RT-qPCR). All RT-qPCR assays were performed relative to three constitutively expressed genes, encoding the transduction factor IF2 (inB), the beta subunit of the ATP-synthase F1 (apfD), and the beta subunit of the RNA polymerase (rpoB). Unlabeled cDNAs, prepared as described above from cells grown on either XOS<sub>PAAC</sub> or glucose, were amplified using Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA) per the manufacturer's instructions. The 20-μl reactions were cycled in the following way in 384-well plates using an ABI 7000 thermal cycler (Applied Biosystems). Reaction mixtures were heated to 95°C for 10 min and then underwent 40 cycles of 95°C for 15 s followed by 62°C for 1 min. Reactions were performed in triplicate, and those with an amplification efficiency of ≥1.98 were analyzed using SDS software version 2.2.1 (Applied Biosystems). All primers used were designed using VectorNTI (Invitrogen, Carlsbad, CA) and are listed in Table 1.

Cloning, expression, and purification of Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A. The genes encoding the esterases, designated the Axe1-6A, Axe1A, Axe7A, and Axe7A, were amplified using the primers listed in Table 1. The amplified PCR products were cloned into the TA cloning vector pGEM-T (Promega, Madison, WI), transformed into *E. coli* DH5α (Invitrogen, Carlsbad, CA), and sequenced to confirm the integrity of the coding sequence. The correct inserts were subcloned into a modified pET28a vector (Novagen, San Diego, CA). The modified expression vector contained a replacement of the kanamycin resistance gene present in the plasmid with the antibiotic resistance gene. The modified pET28a vector was described in our earlier publication (14). Each resulting pET28a-gene construct was introduced into *E. coli* BL21-21C-Porton (DE3) RIL competent cells (Strategene, La Jolla, CA), and protein was expressed as described by Dodd et al. (14). The cells were pelleted, resuspended in 25 ml of lysis
other carbohydrates were released under N2 in alkali methanol (2% [vol/vol] methanol in 0.5 M HCl). The analysis was performed on an Acella ultra performance liquid chromatography (UPLC) system (Thermo Scientific, Rockford, IL). Separation was performed on a Hypersil GOLD column (1.9 by 150 mm; 1.9 μm; Thermo Scientific, Rockford, IL) at a flow rate of 0.4 ml per minute at 30°C. The mobile phase was composed of H2O plus 1% (vol/vol) acetonitrile plus 0.2% (vol/vol) acetic acid (phase A) and acetonitrile plus 0.2% (vol/vol) acetic acid (phase B). The elution profile was as follows: the first 5 min isocratic on 50% phase B; 5 to 23 min, phase B (linearly 0% to 50%); 23 to 24 min, phase B (linearly 50% to 100%); followed by cleaning and reconditioning of the column. Spectral data were collected from 200 to 600 nm, and quantification was performed at 320 nm. Mass spectrometry (MS) data were collected in the negative mode with an ion spray voltage of 3.5 kV, a capillary voltage of –20 V, and a capillary temperature of 350°C. Full MS scans were made within the range m/z 150 to 1,500, and MS2 data of the most intense ions were recorded.

MALDI-TOF MS. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was performed using an Ultraflex instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm. The sample preparation and choice of matrix were described previously (26). Oligosaccharides were detected using the reflector mode. Proteins were detected in the linear mode.

Capillary electrophoresis (CE) of 9-aminopropyl-1,6,6-trisulfonate (APTS)-derivatized oligosaccharides was performed as described previously (27). Both CE coupled to a laser-induced fluorescence (LIF) detector and CE coupled to an electrospray ion-trap mass spectrometry (ESI-MS) detector were used for the identification and characterization of the oligosaccharides (28).

**Enzyme assays.** Feruloyl esterase activity was assayed by evaluating the release of ferulic acid from methylferulate (Sigma-Aldrich, St. Louis, MO), wheat bran (25), and corn fiber isolated in the current study. Ferulic acid released was analyzed as described above under “Ester content.” Various acetylated substrates were used to evaluate the acetyl esterase activity. The 4-O-methylumbelliferylacetate, xyllose-tetraacetate, β-N-glucose-pentaacetate, cephalosporin C were purchased from Merck (Whitehouse Station, NJ), and the xanthan-acetylated CX911 was obtained from Megazyme (Bray, Ireland).

**Temperature and pH optima.** The pH and temperature ranges for AxsA1, AxsA7, and AxsA6 were determined by analyzing the release of acetic acid and released from 1.2 mM glucose-pentaacetate in 30 min using 0.05 μM the appropriate enzyme. Buffers used for pHs in the range 3.0 to 6.5 were 50 mM citrate plus 150 mM NaCl, and those used in the range pH 7.0 to 8.0 were 50 mM HEPES plus 150 mM NaCl.

**Microarray data accession number.** Microarray results have been deposited in the Gene Expression Omnibus (GEO accession number GSE19088) in accordance with Minimum Information about a Microarray Experiment (MIAME) standards.

**RESULTS**

**Characteristics of XOSFAC.** The main compositional characteristics of fraction XOSFAC are described in Table 2. The
fraction XOSFA,Ac was found to be soluble and also to contain large amounts of esterified O-acetyl and ferulic acid groups. The mass spectrum of fraction XOSFA,Ac (Fig. 1A) revealed a range of oligosaccharide structures that either contain a ferulic acid, an O-acetyl group, or both. The addition of NaOH caused a shift in the mass spectra (Fig. 1B). Mass decreases of 42, 176, or 218 Da were observed for oligosaccharides, consistent with the expected mass decreases resulting from the loss of an O-acetyl or ferulic acid moiety or both, respectively, confirming the presence of alkali-labile ester groups. The mass spectra also revealed the oligosaccharides present to be mostly pentose-based oligomers (Fig. 1), and this was confirmed by the sugar composition (Table 2), which suggested that the main pentose present in XOSFA,Ac was xylose. Based on 100 xylosyl residues (molar ratio), the relative amount of side chains was calculated to be 32 arabinosyl, 10 galactosyl, 6 glucuronic acid, 24 (bound) ferulic acid, 5 (bound) coumaric acid, and 50 (bound) O-acetyl residues. Based on 100 arabinosyl residues (molar ratio), the relative amounts of ferulic acid and coumaric acid were calculated to be 75 and 17, respectively. Collectively, these results show that the corn fraction used as a substrate was composed of an arabinobioxylo-oligosaccharide rich in O-acetyl and ferulic acid ester linkages.

**TABLE 2. Compositional data of the corn fiber fraction XOSFA,Ac**

<table>
<thead>
<tr>
<th>Component</th>
<th>Content (% [w/w]) XOSFA,Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>13</td>
</tr>
<tr>
<td>Carbohydrates (total)</td>
<td>45</td>
</tr>
<tr>
<td>Monomeric sugars</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Arabinose</td>
<td>8</td>
</tr>
<tr>
<td>Xylose</td>
<td>25</td>
</tr>
<tr>
<td>Galactose</td>
<td>3</td>
</tr>
<tr>
<td>Glucose</td>
<td>6</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.3</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>2</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>17 (9, 8)</td>
</tr>
<tr>
<td>Coumaric acid</td>
<td>2.5 (1, 1.5)</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>4 (0, 4)</td>
</tr>
</tbody>
</table>

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Growth characteristics on corn fiber fractions. The growth dynamics of _P. ruminicola_ 23 cells on XOSFA,Ac were determined. The growth rate of _P. ruminicola_ was 0.12 h⁻¹, and the bacterium attained a maximum cell density (OD₆₀₀) for growth of 0.997 on this substrate (Fig. 2). Measures of sugar consumption across the growth curve showed that as much as 50% of the available sugars were consumed upon entering stationary phase (Fig. 2).

Bioinformatic analysis of the esterases of _P. ruminicola_ 23. Using BlastP alignments and hidden Markov model searches, a total of 16 putative carbohydrate esterases (Table 3; Fig. 3) were identified. Eight of these carbohydrate esterases (PRU_1592, PRU_1811, PRU_2656, PRU_2678, PRU_2694, PRU_2707, PRU_2728, and PRU_2746) were clustered with one or more glycoside hydrolase-encoding genes. Additionally, PRU_1726 was found proximal to a predicted GPS family sugar transporter, and PRU_1396 was contiguous with a group 2 glycosyl transferase. Three of the putative esterases (PRU_2678, PRU_2707, and PRU_2728), in particular, were contiguous with a large number of genes likely to be involved
in polysaccharide hydrolysis. For example, PRU_2678 was surrounded by six putative glycoside hydrolase (2 endo-1,5-α-L-arabinosidases, a β-1,4-xyllosidase, an α-amylase, an endo-1,4-β-galactanase, and a GH 43 family protein)-encoding genes, as well as two glycosyl transferases (GTs) (GT families 1 and 2).

Nine of the putative esterases were predicted to possess signal peptides (Fig. 3). Using GnegPloc (45), three of the signal peptide-possessing esterases (PRU_2630, PRU_2656, and PRU_2678) and two esterases devoid of signal peptides (PRU_1726 and PRU_2728) were predicted to be targeted to the periplasm (Table 3). Six esterases (PRU_0012, PRU_1101, PRU_1396, PRU_2212, PRU_2694, and PRU_2707) were predicted to be secreted out of the cell, and all other esterases were predicted to be located in the cytoplasm (Table 3).

Microarray analysis of P. ruminicola 23 grown on XOSFA,Ac.

The results of the transcriptional analyses are summarized in Table 3. The data from the microarray measurements revealed that 4 of 16 putative esterases in P. ruminicola 23 were significantly upregulated (>2 fold) during growth on XOSFA,Ac compared to growth on the nonesterified corn arabininoxyloligosaccharide fraction (AXOS). The upregulated esterase-encoding genes included xyn10D-fae1A (PRU_2728) and genes predicted to encode an acetyl-xylan esterase, the Axel-6A gene (PRU_2707), and two esterases (PRU_2033 and PRU_2746), whose specificities were unclear from bioinformatic evidence. Comparing this transcriptional response to that obtained during growth on XOSFA,Ac relative to that on xylose, revealed much of the transcriptional induction observed in the comparison between XOSFA,Ac and AXOS was influenced by the presence of the corn xlyo-oligomers rather than the ester groups themselves, which caused a much smaller, albeit significant transcriptional induction. Two additional esterases were upregulated when glucose was used as the comparator to XOSFA,Ac, suggesting that their induction was purely attributable to the presence of xylose, or perhaps a pentameric sugar as opposed to glucose, and their oligomserized derivatives (PRU_1592, PRU_1726). Reverse transcription-quantitative PCR (RT qPCR) largely supported the microarray results, although the data also suggested that the true induction of xyn10D-fae1A exceeded the measurable dynamic range of the microarray analysis. The RT qPCR data also suggested that two esterase-encoding genes, including a pectin esterase, pecE2 (PRU_1101), and PRU_2584, were also induced greater than 2-fold. In both the microarray analysis and RT qPCR analysis, the most significantly upregulated esterase gene was xyn10D-fae1A, which was determined by RT qPCR to be induced 120-fold during growth on XOSFA,Ac relative to that on glucose. All other esterases were constitutively expressed under each of the conditions examined. Despite its constitutive expression, the Axe1 gene (PRU_2212) was found to be highly expressed, exceeding all others during growth on glucose and having just a 7.5-fold-lower growth rate than xyn10D-fae1A.

**TABLE 3. Predicted location and expression of the carbohydrate esterases of P. ruminicola 23**

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Product</th>
<th>family&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CE family&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cellular location&lt;sup&gt;c&lt;/sup&gt;</th>
<th>RT qPCR&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Microarray&lt;sup&gt;e&lt;/sup&gt; (Glu; Xyl; AXOS)</th>
<th>RE&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRU_0012</td>
<td>Pectin esterase, PecE1</td>
<td></td>
<td>8</td>
<td>Extracellular</td>
<td>0.93</td>
<td>0.61; 0.83; 0.79</td>
<td>8.7; 0.07</td>
</tr>
<tr>
<td>PRU_1101</td>
<td>Pectin esterase, PecE2</td>
<td></td>
<td>8</td>
<td>Extracellular</td>
<td>2.18</td>
<td>1.37; 1.14; 1.35</td>
<td>6.1; 0.12</td>
</tr>
<tr>
<td>PRU_1396</td>
<td>Pectin acetyl esterase, PacE1</td>
<td></td>
<td>8</td>
<td>Extracellular</td>
<td>0.94</td>
<td>0.26; 0.37; 0.96</td>
<td>1.4; 0.01</td>
</tr>
<tr>
<td>PRU_1592</td>
<td>Conserved hypothetical protein</td>
<td></td>
<td>12</td>
<td>Cytoplasm</td>
<td>ND</td>
<td>2.25; 1.77; 0.72</td>
<td>ND</td>
</tr>
<tr>
<td>PRU_1726</td>
<td>Carbohydrate esterase</td>
<td></td>
<td>7</td>
<td>Periplasm</td>
<td>11.5</td>
<td>2.36; 0.6; 1.26</td>
<td>2.7; 0.27</td>
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<tr>
<td>PRU_1811</td>
<td>Polysaccharide deacetylase</td>
<td></td>
<td>4</td>
<td>Cytoplasm</td>
<td>ND</td>
<td>0.61; 0.86; 0.87</td>
<td>ND</td>
</tr>
<tr>
<td>PRU_2033</td>
<td>Carbohydrate esterase</td>
<td></td>
<td>7</td>
<td>Carbohydrate esterase</td>
<td>ND</td>
<td>7.19</td>
<td>6.16; 3.60; 3.14</td>
</tr>
<tr>
<td>PRU_2212</td>
<td>Carbohydrate acetyl esterase, AxeA1</td>
<td></td>
<td>Extracellular</td>
<td>Carbohydrate esterase</td>
<td>1.15</td>
<td>0.66; 0.58; 1.40</td>
<td>13.2; 0.13</td>
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<tr>
<td>PRU_2584</td>
<td>Carbohydrate esterase</td>
<td></td>
<td>Cytoplasm</td>
<td>Carbohydrate esterase</td>
<td>3.17</td>
<td>1.02; 1.26; 1.61</td>
<td>0.9; 0.02</td>
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<tr>
<td>PRU_2630</td>
<td>Pectin acetyl esterase, PacE2</td>
<td></td>
<td>1</td>
<td>Periplasm</td>
<td>1.86</td>
<td>1.58; 1.65; 1.25</td>
<td>5.4; 0.09</td>
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<tr>
<td>PRU_2656</td>
<td>Isoamylase/esterase</td>
<td></td>
<td>7</td>
<td>Periplasm</td>
<td>ND</td>
<td>1.68; 1.11; 0.90</td>
<td>ND</td>
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<tr>
<td>PRU_2678</td>
<td>Carbohydrate esterase, Axe7A</td>
<td></td>
<td>1</td>
<td>Extracellular</td>
<td>1.71</td>
<td>1.81; 1.26; 1.05</td>
<td>1.1; 0.02</td>
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<tr>
<td>PRU_2694</td>
<td>Isoamylase/esterase</td>
<td></td>
<td>1</td>
<td>Extracellular</td>
<td>ND</td>
<td>1.62; 1.26; 0.85</td>
<td>ND</td>
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<tr>
<td>PRU_2707</td>
<td>Acetyl-xylan esterase, Axe1-6A</td>
<td>1/6</td>
<td>Extracellular</td>
<td>48.77</td>
<td>41.72; 36.01; 46.7</td>
<td>1.0; 0.38</td>
<td></td>
</tr>
<tr>
<td>PRU_2728</td>
<td>Xylanase/ferulic acid esterase, Xyn10D-Fae1A</td>
<td></td>
<td>1</td>
<td>Periplasm</td>
<td>119.54</td>
<td>41.73; 38.10; 4.35</td>
<td>1.0; 1.00</td>
</tr>
<tr>
<td>PRU_2746</td>
<td>Carbohydrate esterase</td>
<td></td>
<td>1</td>
<td>Cytoplasm</td>
<td>ND</td>
<td>20.36</td>
<td>24.97; 18.00; 2.45</td>
</tr>
</tbody>
</table>

<sup>a</sup> Biochemically characterized proteins are distinguished in boldface type. ND, not determined.

<sup>b</sup> Classified according to carbohydrate esterase (CE) families described in the CAZy database (8) (www.cazy.org).

<sup>c</sup> Cellular location of enzyme as predicted by GnegPloc (10, 45).

<sup>d</sup> Expression of each esterase relative to that of xyn10D-fae1A during growth on XOSFA,Ac relative to growth on glucose.

<sup>e</sup> Expression of each esterase during growth on XOSFA,Ac relative to that during growth on glucose, xylose, or AXOS.

<sup>f</sup> Expression of each esterase relative to that of xyn10D-fae1A during growth on xylose or XOSFA,Ac.
during growth on XOSF$_{Ac}$. This finding suggested, despite its constitutive expression, the AxeA1 gene could make significant contributions to the esterase activities of P. ruminicola 23.

Purification and general characteristics of esterases Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A. To gain further insights into the diverse esterase activities present in P. ruminicola 23, the two most upregulated esterases (Xyn10D-fae1A and Axe1-6A), the esterase (axeA1) with the highest basal expression on xylose relative to xyn10D-fae1A, and a fourth esterase (axe7A; PRU_2678) that appeared to be neither upregulated nor strongly expressed were selected for biochemical studies. The esterase genes were cloned, expressed heterologously in E. coli, and purified to homogeneity. Protein concentrations of purified Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A were 2.08, 0.24, 1.82 and 0.11 g/liter, respectively. The molecular masses of Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A were determined by MALDI-TOF mass spectrometry to be 83.0, 74.1, 54.5, and 49.2 kDa, respectively, which were in agreement with the predicted masses (84, 74, 53, and 48 kDa, respectively) based on their polypeptide sequences.

Enzymatic activities of Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A. The most highly induced gene, xyn10D-fae1A, was biochemically characterized previously by Dodd et al. (14). These authors demonstrated that Xyn10D-Fae1A possesses both endo-β-1,4-xylanase and ferulic acid esterase activities. In the current study, the esterase activity of Xyn10D-Fae1A was evaluated in more detail. The reported ferulic acid esterase activity was confirmed by the release of ferulic acid from methylferulate (Fig. 4A), and from the more natural substrates wheat bran and corn fiber. Only mono-ferulic acids were released, as di- and tri-ferulic acids were not identified in the enzyme digests. The enzymatic release of acetic acid from glucose pentaacetate (Table 4), acetylated xylo-oligosaccharides (Fig. 5), and acetylated xylan was negligible. The xylanase activity resulted in a decreased size of acetylated xylo-oligosaccharides (Fig. 5), and acetylated xylan (not shown).

The esterase Axe1-6A released all of the acetic acid esters present in 1.2 mM glucose-pentaacetate in 2 h (Table 4). Axe1-6A also released part of the acetyl esters from the acetylated xylo-oligosaccharides, resulting in a completely deacetylated series of oligosaccharides, including xylobiose and xylotriose up to xylohexaose (Fig. 5). The protein possesses both

![FIG. 3. Putative esterase genes from the genome of P. ruminicola 23, illustrating the size and domain structure variability as predicted from their amino acid sequence. HMM identifiers are given, with the exceptions of CBM (carbohydrate-binding domain) and SP (signal peptide).](http://aem.asm.org/)

![FIG. 4. UV chromatograms of methylferulate with XynD-Fae1A digest (A), methylferulate with Axe1-6A digest (B), and methylferulate without enzymes (C). Methylferulate in 50 mM citrate buffer, pH 6 (4 mg/ml), was digested overnight at 37°C, with 0.12% (wt/wt) (enzyme protein/substrate) for Axe1-6A or 1.5% (wt/wt) for XynD-Fae1A.](http://aem.asm.org/)
acetyl esterase activity and ferulic acid esterase activity, as evidenced by the release of ferulic acid from methyl ferulate (Fig. 4), and from the more natural substrates wheat bran and corn fiber (not shown). The Axe1-6A enzyme is, therefore, a bifunctional esterase.

Analysis of enzymatic activity revealed AxeA1 to be an acetyl esterase with broad substrate specificity, releasing acetic acid from acetylated xylo-oligosaccharides and acetylated xylan as well as xylose-tetra-acetate, 4-O-methylumbelliferyl acetate, glucose-pentaacetate, and cephalosporin C (Table 4). AxeA1 released 100% and 88%, respectively, of the acetic acid esters present in 1.2 mM glucose-pentaacetate and 1.7 mM cephalosporin C (Table 4). AxeA1 appeared to have greater activity on xylo-oligosaccharides than on the polymeric substrates, releasing 58% of the acetic acid esters present in 0.4% (wt/vol) acetylated xylan. AxeA1 and Axe7A were able to release some acetic acid esters present in 0.4% (wt/vol) acetylated xylan in 20 h, compared to just 2% of the acetic acid esters present in 0.4% (wt/vol) acetylated xylo-oligosaccharides with two or fewer O-acetyl groups (see Fig. S1 in the supplemental material). At the conclusion of the incubation, only xylo-oligosaccharides with two O-acetyl esters or fewer remained (Fig. 6B). The residual xylo-oligosaccharides from the 24 h of incubation were labeled with APTS and studied in more detail using capillary electrophoresis coupled to a fluorescence detector and an electrospray ion-trap mass spectrometer (CE-LIF-ESI-MSn). CE-LIF-ESI-MSn results revealed that the remaining esters were located mostly on the second xylosyl residue from the reducing end of the residual xylo-oligosaccharides containing just one O-acetyl group. However, it could not be determined whether these residual O-acetyl groups were located at the O-2 or O-3 position of the xylosyl residue. The position of the O-acetyl groups for xylo-oligosaccharides containing two O-acetyl esters could not be determined, although it was revealed that at least two xylosyl residues from the nonreducing end were not O-acetylated. The corresponding analysis revealed that the acetyl esterase activities of Axe1-6A and Axe7A were limited to acetylated xylo-oligosaccharides smaller than xylo-heptaose (Fig. 5D and 6C); however, the remaining mixture of acetylated oligosaccharides was still too heterogeneous following 24 h of incubation to extract detailed information regarding the decactylation mechanism of Axe1-6A and Axe7A.

TABLE 4. Activity of P. ruminicola 23 esterases tested on various acetylated substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction conditions</th>
<th>Protein concn (µM)</th>
<th>Activity (nmol HAc/µmol protein) at 20 min; 2 h; 20 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Substrate (g/liter)</td>
<td>AxeA1</td>
<td>Axe7A</td>
</tr>
<tr>
<td>Glucose-pentaacetate</td>
<td>0.47 (1.2 mM)</td>
<td>0.05; 0.05; 0.08; 0.25</td>
<td>27; 63; ND</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>0.68 (1.9 mM)</td>
<td>0.05; 0.05</td>
<td>11; 13; ND</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>0.64 (2.9 mM)</td>
<td>0.33; 0.33</td>
<td>&lt;0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-Acetyl-neuraminic acid (type IV-S)</td>
<td>0.60 (1.9 mM)</td>
<td>0.33; 0.33</td>
<td>ND</td>
</tr>
<tr>
<td>Acetylated xanthan</td>
<td>2.0</td>
<td>0.04; 0.37</td>
<td>&lt;0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sugar beet pectin (acetylated homogalacturonan)</td>
<td>4.0</td>
<td>0.04; 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>Acetylated xylo-oligosaccharides (DP 2-20; wood)</td>
<td>4.0</td>
<td>0.04; 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>Acetylated xylo-oligosaccharides (DP 4-12)</td>
<td>4.0</td>
<td>0.04; 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>X&lt;sub&gt;2&lt;/sub&gt;GlCAc + X&lt;sub&gt;4&lt;/sub&gt;GlCmeAc (FA-20)</td>
<td>1.0</td>
<td>0.04; 0.37</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not determined.
<sup>b</sup> Amount of acetic acid released below the level of detection (0.5 µM) in 20 h.
673, 919, 1,081 Da) remained, suggesting that the proximal location of ferulic acid groups and the O-acetyl group hindered the activities of these two enzymes.

**Temperature and pH optima for enzymatic activities of Xyn10D-Fae1A, Axe1-6A, AxeA1, and Axe7A.**

The pH and temperature range in which Axe1-6A, AxeA1, and Axe7A were active was determined by the release of acetic acid on glucose-pentaacetate (Fig. 7). Xyn10D-Fae1A had low activity on glucose-pentaacetate. The latter protein was characterized previously (14) and exhibited measurable activity for both the endo-xylanase and the esterase activity at pH 6.0 and 30 to 40°C.

Axe1-6A, AxeA1, and Axe7A were active in a pH range of 5 to 8. Whereas Axe1-6A displayed maximum activity at pH 6.5, both AxeA1 and Axe7A showed a maximum at pH 6.0. Axe1-6A and AxeA1 were active between 20 and 50°C and still exhibited 40 to 70% of the maximum activity after 20 h of incubation at 50°C. Axe7A was active at temperatures between 30 and 50°C, but at temperatures of 30°C and lower, less than 10% of the maximum activity was left. At 50°C Axe7A retained 60% of its maximum activity. The optimum temperatures for enzymatic activity of Axe1-6A, AxeA1, and Axe7A were 35°C, 30 to 35°C, and 40°C, respectively.

**DISCUSSION**

The genus *Prevotella* is one of the more numerically dominant and metabolically versatile bacteria in the rumen (16, 18, 19).
Corn, a common ingredient in the diet of U.S. cattle, contains the major hemicellulose xylan and is known to contain a relatively high content of ferulic and coumaric acid esters (22, 40, 41). This observation was further confirmed by our analysis of the corn-derived substrates used in the present study. Microbial capture of nutrients from polysaccharides, including hemicelluloses, followed by their fermentation in the rumen results in the production of short-chain fatty acids that serve as the major energy source for the host ruminant. The enzymatic hydrolysis of ester linkages in hemicelluloses by rumen microbes is, therefore, an important step in the complete degradation and efficient utilization of corn hemicellulose.

Previously, it was shown that Xyn10D-Fae1A possessed both endo-xylanase activity on oat spelt xylan and esterase activity on methylferulate (14). In the present study, Xyn10D-Fae1A was also tested on other ester-containing substrates, including wheat bran, corn fiber, and the ester-rich arabinoxylo-oligosaccharide fraction from corn (XOSFA,Ac). For all substrates the release of ferulic acid was observed, although the activity was clearly hindered by the presence of neighboring ester groups such as acetic acid esters. Negligible activity was observed when Xyn10D-Fae1A was incubated with sugar acetates, indicating that the enzyme behaved as a true ferulic acid esterase belonging to the carbohydrate esterase family 1 (8) (http://www.cazy.org).

Axel-6A was found to release acetic acid from acetylated...
xylo-oligosaccharides, as well as from glucose-pentaacetate. This esterase was also able to release ferulic acid from methy-lylferulate, wheat bran, corn fiber, and XOS_{F_A,Ac}. The protein Axel-6A was predicted to have two esterase domains corre-sponding to distinct carbohydrate esterase families, CE1 and CE6 (8). It can be hypothesized that the release of acetic acid is related to the CE6 domain, an esterase family that exclu-sively contains acetyl xylan esterases (EC 3.1.1.72), while the release of ferulic acid may relate to the CE1 domain. Family I carbohydrate esterases include enzymes of numerous specific-ities, including both acetyl- and feruloyl-linked esterases. Ob-taining a clearer understanding of the functions of these two CE domains will be an important and interesting focus of future work.

In addition to Axel-6A, AxelA1 and Axel7A were also found to possess acetyl esterase activity, making these three esterases the first biochemically characterized acetyl esterases from a Prevotella sp. Axel7A was predicted to belong to CE family 7 (8). The CE-7 family enzymes have previously been suggested to comprise a single class of proteins with multifunctional deacetylase activity against a range of small substrates (11, 53). This is consistent with our findings, with Axel7A demonstrating deacetylase activity toward a variety of substrates, in-including xylose-tetraacetate, 4-O-methylumbelliferylacetate, glucose-pentaacetate, cephalosporin C, and acetylated xylo-oligosaccharides (DP 2-20), but not toward acetylated xylo-oligosaccharides larger than xylo-heptaose. AxelA1 was active toward a similar range of substrates but, in addition, displayed some activity toward polymeric acetyl-xylan. The high basal expression of AxelA1, relative to that of Xyn10D-Fae1A, sug-gests that this esterase is critical for _P. ruminicola_ 23, a bacte-

rrium with a hemicellulose-degrading lifestyle in the rumen. The preference of both AxelA1 and Axel7A for xylo-oligosaccharides over polymeric xylan illustrates the need for these enzymes to work in synergy with xylan-depolymerizing en-zymes, such as xylanases and β-xyllosidases. This has previously been reported for other acetyl-xylan esterases, which alone have shown low activities on acetylated xylan (3).

Insight into the mechanistic action of AxelA1 revealed that the enzyme preferentially targeted xylo-oligosaccharides pos-sessing three or more O-acetyl esters, but following their de-

pletion the enzyme was active on the less acetylated portion of the substrate. It also revealed several factors that limited the enzyme’s esterase activity. The residual xylo-oligosaccharides following incubation with AxelA1 were enriched for those possessing O-acetyl groups on the second xylosyl residue from the reducing end (X2), suggesting an inability to remove O-acetyl groups at this position. The deacetylase activities of various acetyl-xylan esterases have been described to possess posi-tional specificity (4, 35, 52). This limitation is likely conferred through their binding cleft or catalytic site, and this may be applicable to AxelA1. In addition to xylo-oligosaccharides with an O-acetyl group on X2, a xylo-tetraose having an O-acetyl ester on the nonreducing end xylosyl was observed following incubation with AxelA1. We cannot rule out the possibility that this O-acetyl is present on the O-4 position of the nonreducing end xylosyl residue and not on the O-2 or O-3 position typical of xylan (39, 51). The O-4 position could be the result of internal migration, as has been proposed previously (27). Biely et al. (4) reported that acetyl-xylan esterases from _Streptomyces lividans_, _Trichoderma reesei_, and _Schizophyllum commune_ were able to release O-acetyl esters from the O-2 and O-3 positions of methyl 2,3,4-tri-O-acetyl-β-D-xylopyranoside but not from the O-4 position.

The deacetylase activity of Axel-6A, AxelA1, and Axel7A also appeared to be hindered by the proximal localization of 4-O-methylglucuronic or ferulic acid esters. Likewise, the fer-

ulic acid esterase activity of Axel-6A and Xyn10D-Fae1A appeared to be hindered by the proximal localization of acetyl esters. As almost all previous investigations into the mechanis-tic action of other esterases have been performed on substrates possessing only ferulic acid or 4-O-methyl glucuronic or O-acetyl esters and not on substrates with combinations of these esters, it is unclear if such inhibition is a widespread feature of esterase enzymes. However, our results clearly provide signifi-cant insights into the biochemical activities of these four es-

terases from _P. ruminicola_ 23. In addition, this report begins to illuminate the importance of multiple or diverse esterase ac-

tivities that support the hemicellulose-degrading lifestyle of this important ruminal bacterium.

**ACKNOWLEDGMENTS**

This project was supported by National Research Initiative Com-

petitive Grant no. 2008-35206-18784 from the USDA National Insti-

tute of Food and Agriculture (to R.I.M., I.K.O.C., and M.M.) and by the Dutch Ministry of Economic Affairs via a grant supporting the short-term research of M.A.K. (EOS-KTO grant).

We thank David van Eylen, Femke van Dongen, and Maaike Ap-

peldoorn for their valuable contributions to this work.

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