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Identification of GH10 xylanases in strains 2 and Mz5 of *Pseudobutyrivibrio xylanivorans*

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Abstract Genes encoding glycosyl hydrolase family 11 (GH11) xylanases and xylanases have been identified from *Pseudobutyrivibrio xylanivorans*. In contrast, little is known about the diversity and distribution of the GH10 xylanase in strains of *P. xylanivorans*. Xylanase and associated activities of *P. xylanivorans* have been characterized in detail in the type strain, Mz5. The aim of the present study was to identify GH10 xylanase genes in strains 2 and Mz5 of *P. xylanivorans*. In addition, we evaluated degradation and utilization of xylan by *P. xylanivorans* 2 isolated from rumen of Creole goats. After a 12-h culture, *P. xylanivorans* 2 was able to utilize up to 53 % of the total pentose content present in birchwood xylan (BWX) and to utilize up to 62 % of a ethanol-acetic acid-soluble fraction prepared from BWX. This is the first report describing the presence of GH10 xylanase-encoding genes in *P. xylanivorans*. Strain 2 and Mz5 contained xylanases which were related to GH10 xylanase of *Butyrivibrio* sp. Identifying xylanase-encoding genes and activity of these enzymes are a step toward understanding possible functional role of *P. xylanivorans* in the rumen ecosystem and contribute to providing an improved choice of enzymes for improving fiber digestion in ruminant animals, agricultural biomass utilization for biofuel production, and other industries.

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Introduction

Knowledge of diversity and organization of the enzymes involved in the breakdown of plant cell walls by rumen bacteria is essential for understanding important aspects of ruminant nutrition. The hydrolysis of the polysaccharides of plant cell walls mediated by microorganisms is highly efficient (Flint and Bayer 2008). Plant cell walls have a basic structure of cellulose surrounded by a matrix of hemicellulose, pectin, and proteins, whose composition varies among different plant species and maturation stages (Cosgrove 2005). Hemicellulose and pectin are branched polysaccharides with the capacity to cross-link the cellulose and form covalent bonds with lignin. The breakdown of plant cell walls requires the action of several enzymes capable of cleaving a broad range of different chemical bonds (Kelly et al. 2010). Hemicellulolytic rumen microorganisms are known to degrade and utilize xylans, the major component of hemicellulose, increasing the utilization of hemicellulose from intact plant material (Gasparic et al. 1995). The rumen microenvironment has a specific xylan-degrading microbial community and is an ideal environment to study the genetic diversity of functional xylanases. Xylanases reported from rumen bacteria belong to several families of glycosyl hydrolase enzymes (GH), according to the classification recorded in the Carbohydrate-active enzymes (CAZy) database. Brulc et al. (2009) have used genocentric analysis to examine randomly sampled pyrosequence data from fiber-adherent microbiomes

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from bovine rumen. These authors assessed the prevalence of enzymes involved in the degradation of polysaccharides and described a large number of glycosyl hydrolases, though only a few of them (3 GH11 and 18 GH10 xylanases) belong to families known to hydrolyze hemicellulose. Wang et al. (2011) reported a large number of xylanase-encoding genes belonging to GH10 and GH11 glycosyl hydrolase families from the rumen content of goat. These two families exhibit different kinetic, physicochemical, and catalytic properties, and their distribution could be related to different roles in the degradation of ruminal xylan (García Campayo et al. 1993). Biely et al. (1997) suggest that GH10 xylanases have substrate-binding sites smaller than GH11. This suggestion is in agreement with the finding that GH10 xylanases show higher affinity for shorter linear beta-1,4-xylooligosaccharides than GH11. Furthermore, GH10 predominantly attacks glycosidic linkages next to a substituted xylose residue, and it tends to exhibit broad catalytic versatility. In contrast, GH11 cannot cleave glycosidic linkages next to a branch and they are characterized by their exclusive activity on D-xylose-containing substrates (Hu et al. 2013). GH10 xylanase sequences related to *Bacteroides* spp. appear to be dominant in goat rumen, and half of them are closely related to xylanases from *Prevotella* (Wang et al. 2011), which is one of the most important xylan-degrading bacterial genera in the rumen (Gasparic et al. 1995). *Butyrivibrio* sp. also plays an important role in the degradation of xylan and hemicellulose from plant cell walls (Hespell and Cotta 1995), although xylanase gene sequences of this species have not yet been identified in goat rumen. *Butyrivibrio proteoclasticus* is present in high numbers in the rumen contents from cows consuming pasture or grass silage-based diets (Paillard et al. 2007). Kelly et al. (2010) determined the genome of *B. proteoclasticus* B316^{T}, and they found six CDSs encoding endo-1,4-beta-xylanases, belonging to family GH10 of glycosyl hydrolase enzymes involved in polysaccharide degradation.

Recently, Grilli et al. (2013) isolated and identified functional groups of bacteria in the rumen of Creole goats involved in ruminal fermentation of native forage shrubs. A native forage diet allowed the isolation of strains related to *Butyrivibrio fibrisolvens*, such as *Pseudobutyrivibrio ruminis* 153 and *Pseudobutyrivibrio xylanivorans* 2. Based on 16 s rRNA sequential analysis, many strains of *B. fibrisolvens* have been reclassified as closely related to *P. ruminis* (van Gylswyk et al. 1996), *B. proteoclasticus* (Moon et al. 2008), *Butyrivibrio hungatei*, and *P. xylanivorans* (Kopečný et al. 2003). *B. fibrisolvens* and some closely related species constitute a significant proportion of rumen bacteria in domestic and wild ruminants fed with poor-quality forage diets (Orpin et al. 1985; Forster et al. 1996; Ichimura et al. 2004; Mrázek et al. 2006; Paillard et al. 2007; Sundset et al. 2007).

Xylanase-encoding genes have been identified in different strains of *B. fibrisolvens* and *P. ruminis* (Mannarelli et al. 1990; Lin and Thomson 1991; Dalrymple et al. 1999), although these rumen microorganisms produce only GH10 xylanases (Dalrymple et al. 1999). In turn, genes encoding GH11 xylanases were identified from *P. xylanivorans* Mz5 (Čepeljnik et al. 2004, 2006), but genes encoding GH10 xylanase have not yet been identified in this species. Therefore, our aim was to identify GH10 xylanase genes in strains 2 and Mz5 of *P. xylanivorans* and to analyze the distribution of GH10 xylanase from these strains.

P. xylanivorans Mz5 produces multiple xylanases (Zorec et al. 2000, 2001) that account for the highest xylanolytic activity among the rumen bacteria so far tested (Marinšek-Logar et al. 2000). However, little information is available on the abilities of other *P. xylanivorans* strains to degrade xylans and to utilize the products to grow. In the present study, birchwood xylan (BWX) was used as a substrate for growth of *P. xylanivorans* 2 isolated from rumen of Creole goats. Xylan degradation and utilization of breakdown products over time were determined to characterize the xylanolytic activity in this strain. Identifying xylanase-encoding genes and activity of these enzymes would help understand the possible functional role of *P. xylanivorans* in the rumen ecosystem.

Materials and methods

Bacterial culture

P. xylanivorans Mz5 (DSM 14809) was grown on M10 medium with 15 % of rumen fluid and cellobiose as carbon source in the Laboratory of Anaerobic Microbiology, Academy of Sciences of the Czech Republic (Kopečný et al. 2003). *P. xylanivorans* 2 was obtained from rumen content of Creole goats as previously described (Grilli et al. 2013). Strain 2 was maintained in RGM medium (Hespell et al. 1987) containing 40 % (*v*/*v*) ruminal fluid, 2 % (*w*/*v*) agar, and 0.2 % (*w*/*v*) cellobiose as energy sources. To determine xylanolytic activity, the cellobiose was promptly replaced with 0.4 % (*w*/*v*) birchwood xylan (BWX medium) (Cotta and Zeltwanger 1995). Bacterial growth was monitored by measuring cell protein concentrations (Lowry et al. 1951) after cellular lysis (0.1 mol/L NaOH, 70 C, 20 min), using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, US) as standard. Time-course experiments examining the degradation and utilization of BWX were carried out by inoculating cultures (5 %*v*/*v*) from late-logarithmic phase (18 h) in 0.5 % (*w*/*v*) cellobiose broth, which was identical in all other constituents to the culture medium. Samples were taken at 0, 2, 6, 12, 24, 48, and 72 h.

Saccharide analysis

Saccharides in culture samples were separated into saccharide soluble in ethanol-acetic acid solution (xylooligosaccharides)

and saccharide precipitable in ethanol-acetic acid solution (undegraded xylan fractions) on the basis of solubility in an acidic alcohol solution (Cotta and Zeltwanger 1995). To precipitate high molecular weight xylan, three volumes of cold ethanol-acetic acid solution (95 %*v*/*v* ethanol, 5 %*v*/*v* glacial acetic acid) were added to the culture, and the mixture was kept on ice for 30 min. This mixture was then centrifuged at 12,000*g* at 4 °C for 15 min. Centrifugation yielded a xylooligosaccharide-containing supernatant and a pellet containing undegraded xylan. The insoluble residue was washed once with acidified alcohol solution and then solubilized with 5 %*w*/*v* NaOH prior to pentose analysis. These fractions, as well as the whole untreated culture samples, were analyzed for total pentose by using the orcinol reagent, with xylose as a standard (Schneider 1957). Xylan utilization was estimated from the loss of pentose, and degradation was calculated on the basis of the amount of xylan converted from an acidified alcohol insoluble form to a soluble form (Dehority 1967). Percent of utilization and degradation of xylan were calculated based on the results of total and converted pentose in an uninoculated BWX medium.

Enzyme assays

Cells were grown in BWX medium for 48 h and then centrifuged at 12,500*g* for 30 min at 4 °C. Twenty microliters of the supernatant were mixed with 230 μL of 1 % (*w*/*v*) BWX in 50 mmol/L sodium phosphate buffer and incubated for 2.5 h at 37 °C. Xylan-degrading activity was determined by measuring the release of reducing sugars and expressed as released reducing sugars per second per milligram of protein (nkat/mg). Content of reducing sugars was measured by the Somogyi's modified method (Nelson 1944), using xylose as the standard.

Identification of xylanase genes

The Mz5 and 2 strains of *P. xylanivorans* were cultured in M10 medium (Caldwell and Bryant 1966) containing 10 % rumen fluid and 4 g/L cellobiose (as carbon source) for 14 h. A 0.5-mL volume of bacterial culture was used for total DNA extraction by DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions for Gram-positive bacteria. DNA concentrations were measured at 260 nm with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Xylanase gene homologues were amplified with the PCR primers described by Dalrymple et al. (1999). Purified DNA was used as a template following the reaction system and PCR conditions reported by Dalrymple et al. (1999). First, generic primers for PCR amplification of genes encoding GH10 enzymes were used. The primers Xyn10f (5′-ATGA GRGGNCAYACNCTNGTNTGG-3′) and Xyn10r (5′-AGRTGNGAYTGCATNCCYTYNCC-3′) were designed with the DNA sequence encoding two regions of a highly conserved amino acid sequence in GH10 enzymes, one of the major families of xylanases (Henrissat 1991). PCR was performed with the Taq DNA Polymerase kit (Invitrogen, Brazil) according to the manufacturer's directions. The PCR conditions were as follows: initial denaturing at 94 °C for 3 min, followed by 35 cycles of 45 s of denaturing at 94 °C, 30 s of annealing at 55 °C, and 1.5 min of elongation at 72 °C with a final extension at 72 °C for 10 min. Additionally, two primer sets (xynAf 5′-ACTATGGACGAACGTCTTGA-3′ and xynAr 5′-CCTT TTTACCCTGGTTGAT-3′; xynBf 5′-AAGTGGTTTTTC TGCGA-3′ and xynBr 5′-TAATGAAGTCTCTTTTCC-3′) specific for GH10 xylanases were used to amplify xylanase gene fragments. PCR was performed with the RedTaq Readymix (Sigma-Aldrich, USA) using the following program: initial denaturing at 95 °C for 5 min, followed by 35 cycles of 30 s of denaturing at 95 °C, 30 s of annealing at 46 °C, and 30 s of elongation at 72 °C with a final extension at 72 °C for 5 min. PCR products of strain 2 were visualized on an agarose gel, and bands of the expected size were excised and purified with the NucleoTraP®CR Agarose Gel DNA Purification kit (Macherey-Nagel, Germany). The PCR product of Mz5 was purified by Qiaquick PCR Purification kit (Qiagen, Valencia, CA, USA). Purified DNA was sequenced with BigDye v3.1 Terminator Kit (Life, USA). For each gene, PCR product was completely sequenced on both strands. After editing, DNA sequences were translated to protein and compared to the previous reported xylanase sequences in GenBank by BlastP search. Sequences were aligned with the MUSCLE software (v3.7) configured for highest accuracy (Edgar 2004). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT) (Guindon and Gascuel 2003). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.066) and four gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma= 1.826). Reliability for internal branch was assessed using the aLRT test (SH-Like) (Anisimova and Gascuel 2006). Branches with a bootstrap support under 70 % were collapsed. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) (Chevenet et al. 2006).

Nucleotide sequence accession numbers

XynAPx and *xynBMz5* sequences have been deposited under GenBank ID JQ948036 and GenBank ID JX136954, respectively.

Results

Utilization of xylan and xylooligosaccharides by *P. xylanivorans*

Utilization of pentose sugars was monitored by assaying total orcinol reactive material, during growth of *P. xylanivorans* 2 in a medium containing BWX as the sole energy source (Fig. 1). After 24 h of culture, approximately 53 % of BWX had been utilized. There was a small accumulation of acidified alcohol-soluble pentosans (xylooligosaccharides) during the growth of *P. xylanivorans* 2 in the medium containing xylan (Fig. 1). This suggests that the degradation products were metabolized as quickly as they were generated and that this bacterial strain was able to utilize the majority of xylooligosaccharides generated by the hydrolysis of xylan. Degradation of xylan by *P. xylanivorans* 2 occurred primarily within 6 h and approached maximal degradation (62±1.5 %) by 24 h. No considerable utilization was observed in *P. xylanivorans* 2 until 6 h, and maximal utilization (53±0.5 %) was not reached until approximately 12 h. The xylanolytic activity of secreted xylanases in the culture supernatant was 1.04±0.30 nkat/mg. This value represents only 5 % of the xylanolytic activity reported by Čepeljnik et al. (2004) for XynA11 of *P. xylanivorans* Mz5 using the same source of xylan and the cultivation conditions.

Identification of xylanase genes

The generic primers (Xyn10f and Xyn10r) used did not clearly identify GH10 xylanase genes from the DNA of *P. xylanivorans* 2 and Mz5. Using primers GH10 xylanase-specific, gene fragments of about 315 bp for GH10 xylanases were amplified directly from genomic DNA of *P. xylanivorans* 2 and Mz5. PCR products from strains 2 (*xynAPx*) and Mz5 (*xynBMz5*) were sequenced. The nucleotide sequences were translated to deduced amino acid sequences and compared to the previously reported xylanases in the GenBank database by BLAST search. After phylogenetic tree construction of the amino acid sequences of *xynAPx* and *xynBMz5* with the previously reported xylanases from cultured bacteria (Fig. 2), the results showed that the xylanases were divided into two clusters. Putative amino acid sequences of *xynBMz5* (XynBMz5) were designated to the cluster I and were clearly found to form a separate cluster together with xylanases obtained from *B. fibrisolvens* (Lin and Thomson 1991; Dalrymple et al. 1999), *B. proteoclasticus* (Kelly et al. 2010), and *Ruminococcus* sp. (Moon et al. 2011; Suen et al. 2011). XynBMz5 showed similarity score of 90–99 % to xylanases of strains *B. fibrisolvens* isolated from cow rumen. The amino acid sequence of *xynBMz5* was 99 % identical to amino acid sequences of *xynY* from *B. fibrisolvens* D1 (Dalrymple et al. 1999).

On the other hand, putative amino acid sequences of *xynAPx* (XynAPx) were designated to the cluster II. In this cluster are sequences of xylanases reported in *Clostridium* sp. and sequences of xylanases reported in digestive tract of ruminants (*Lachnobacterium bovis*, *Eubacterium cellulosolvens*, *Cellulosilyticum ruminicola*, *B. fibrisolvens*, *B. proteoclasticus*, and *P. ruminis*) and humans (*B. fibrisolvens*

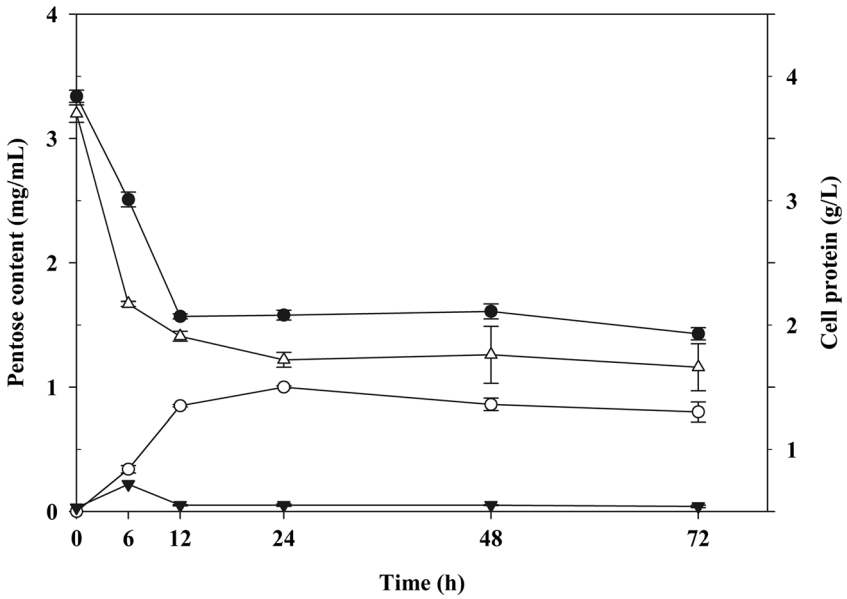

Fig. 1 Utilization of BWX by cultures of *Pseudobutyrivibrio xylanivorans* 2. Total cell protein (*white circles*), total pentose sugar (*black circles*), and ethanol-soluble (*black triangles*) and insoluble (*white triangles*) pentose sugar are shown for cultures grown in a medium containing BWX as energy source (0.4 %*w/v*, final concentration). *Bars* indicate standard error of two experiments

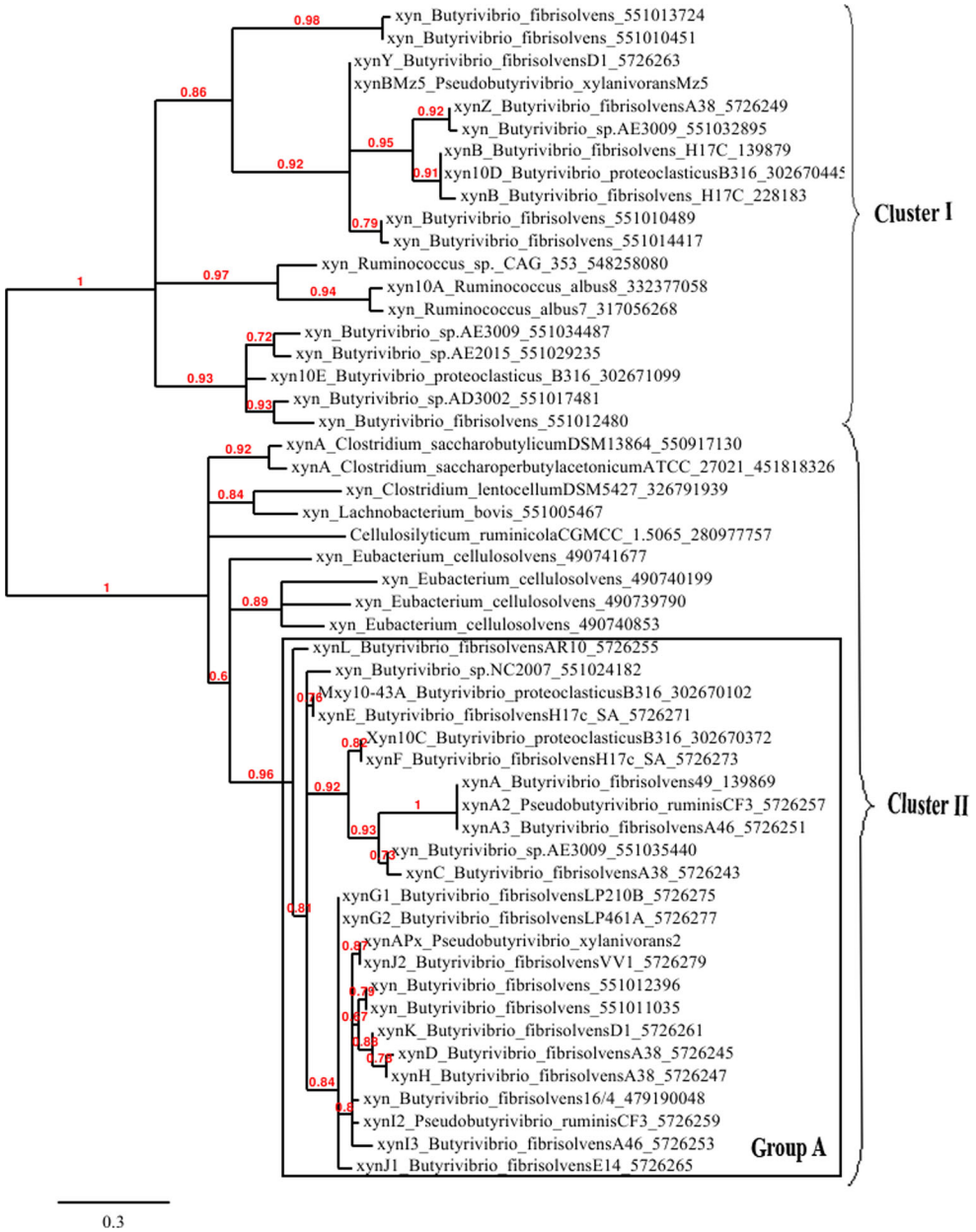

Fig. 2 Phylogenetic tree of GH10 xylanase from cultured bacteria. Phylogenetic placement of amino acid sequences of GH10 xylanase obtained from database and amino acid sequences of GH10 xylanase homologues deduced from DNA sequences of xylanase genes detected in *Pseudobutyrivibrio xylanivorans* Mz5 and 2. The xylanases were divided into two clusters: cluster I, xylanases obtained from *Pseudobutyrivibrio xylanivorans* Mz5, *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*, and *Ruminococcus* sp., and cluster II, xylanases reported in *P. xylanivorans* 2, *Clostridium* sp., *Lachnobacterium bovis*, *Eubacterium cellulosolvens*, *Cellulosilyticum ruminicola*, *B. fibrisolvens*, *B. proteoclasticus*, and *Pseudobutyrivibrio ruminis*. The group A into cluster II contained XynAPx and several sequences of GH10 xylanases belonging to *B. fibrisolvens* and some closely related species (*B. proteoclasticus* and *P. ruminis*) isolated from cow rumen and human gut. *Bar*, 30 substitutions per 100 amino acids. The tree was constructed using the maximum likelihood analysis of a distance matrix obtained from a multiple-sequence alignment. Branches with a bootstrap support under 70 % were collapsed. Numbers above each node are confidence levels (proportion) generated from 1,000 bootstrap trees. The sequences have the name of the enzyme, genus, species and strain of bacteria, and the accession numbers

16/4). The group A (Fig. 2) into cluster II contained XynAPx and several sequences of GH10 xylanases belonging to *B. fibrisolvens* (Mannarelli et al. 1990; Dalrymple et al. 1999), two sequences of GH10 xylanases (Mxy10-43A and XynC10) obtained from *B. proteoclasticus* B136^{T} (Kelly et al. 2010), two GH10 xylanases (xynA2 and xynI2) from *P. ruminis* CF3 (Moon et al. 2008), and one GH10 xylanase obtained from *B. fibrisolvens* isolated from human gut. However, XynAPx showed similarity of 93–99 % to GH10 xylanases of strains *B. fibrisolvens* isolated from cow rumen.

XynAPx was 99 % identical to amino acid sequences of *xynJ2* from *B. fibrisolvens* VV1 (Dalrymple et al. 1999).

Discussion

In this paper, fragments of xylanase genes could not be amplified using generic xylanase primers (Xyn10f and Xyn10r). However, Dalrymple et al. (1999) used the same generic primers and amplified a 440-bp product in only 4 of the 20 strains of *B. fibrisolvens* evaluated. Because of the limitations of primers used and the impossibility to design a single set of universal PCR primers for all genes encoding GH10 xylanases, specific primers were used for amplification of GH10 xylanase gene. Amplified gene fragments were sequenced and translated to amino acid sequences. Putative amino acid sequence of *xynAPx* from *P. xylanivorans* 2 was very similar to amino acid sequence of *xynJ2* from *B. fibrisolvens* VV1 (Dalrymple et al. 1999). Xylanase activity of *B. fibrisolvens* VV1 has not been determined, and the purification and activity of enzyme coding *xynJ2* gene has not been reported. XynAPx was 78 % identical to previously characterized endo-1,4-beta-xylanase XynA of family GH10 (Xyn10A), isolated from *B. fibrisolvens* 49 (Mannarelli et al. 1990). The Xyn10A remained cell-associated, and it did not display arabinosidase, cellulase, α-glucosidase, or xylosidase activities (Mannarelli et al. 1990). In *B. fibrisolvens* 49, xylanase activity is constitutive, but higher levels are obtained when the cells are grown in the presence of xylan, and the enzyme is secreted into the medium (Hespell et al. 1987). Putative amino acid sequence of *xynBMz5* was very similar to amino acid sequence of *xynY* from *B. fibrisolvens* D1 (Dalrymple et al. 1999). The amino acid sequence of *xynBMz5* was 86 % identical to endo-1,4-beta-xylanase XynB of family GH10 (Xyn10B). The predicted XynB product does not appear to have a signal sequence and therefore may be an intracellular enzyme (Lin and Thomson 1991). Dalrymple et al. (1999) have proposed that XynA10 and XynB10 play quite different roles in the degradation of plant fiber. The secretion of a range of polysaccharide-degrading enzymes which initiate the breakdown of xylan and diverse intracellular enzymes to break down oligosaccharides constitute the degradative capability of several ruminal microorganisms (Berg Miller et al. 2009; Brulc et al. 2009; Kelly et al. 2010). XynAPx of *P. xylanivorans* 2 was phylogenetically distant from XynBMz5 of *P. xylanivorans* Mz5 and were classified in different clusters. This suggests that strains of *P. xylanivorans* produce different xylanases that would be a potential bioresource for xylanolytic enzymes, which may give an opportunity to obtain a potential enzyme for agricultural biomass utilization for biofuel production and other industries.

The degradation and utilization of BWX evaluated in strain *P. xylanivorans* 2 allowed us to understand the possible functional role of the xylanolytic enzyme encoded by the gene *xynAPx*, although this study cannot rule out the participation of other xylanase-encoding genes (belonging to other glycosyl hydrolase families), not found even in this strain. *P. xylanivorans* 2 showed maximal degradation of BWX after 24 h. These data were quite similar to those obtained with other sources of xylan, which indicated that, for instance, no marked differences occurred in the rate of degradation between the utilizing strains (H10b and H17c) of *B. fibrisolvens* (Dehority 1967). Maximum utilization of xylan (53±0.5 %) by *P. xylanivorans* 2 occurred at 12 h of incubation, coinciding with 48 % utilization of xylan by *B. fibrisolvens* H17c for the first 12 h of incubation (Hespell and Cotta 1995), while the maximum utilization of xylan (51 %) achieved by another hemicellulolytic strain (*Prevotella bryantii* $B_1$4) occurred after 24 h of incubation (Miyazaki et al. 1997). It should be noted that for all bacterial species, utilization lagged approximately 6 h behind degradation. The lower xylanolytic activity shown by *P. xylanivorans* 2 compared with the type strain Mz5 was evident under the same growth conditions. Xylanase activity was assessed from culture supernatants of strain *P. xylanivorans* 2, which suggests possible participation of extracellular xylanases in BWX digestion. One possible enzyme involved in this digestion may be the xylanase encoded by *xynAPx* identified in this study. Further studies on xylanase enzyme encoded by *xynAPx* are needed to confirm these findings.

This is the first description of the presence of GH10 xylanase-encoding genes in *P. xylanivorans* Mz5 and 2. These strains contained xylanases related to GH10 xylanase of *Butyrivibrio* sp. The secretion of a range of polysaccharide-degrading enzymes which initiate the breakdown of xylan, along with diverse intracellular enzymes to break down oligosaccharides, constitute the degradative capability of several ruminal microorganisms. Identification of enzymes that constitute the degradative capability of ruminal bacteria is a step toward understanding ruminant digestion and will contribute to providing an improved choice of enzymes for improving fiber digestion in ruminant animals. Information on the strategies used by rumen bacteria to break down plant cell walls is also likely to be relevant to initiatives seeking to develop enzyme-based processes for converting plant biomass into milk and meat for human consumption.

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