JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5436–5441 Vol. 179, No. 17
0021-9193/97/$04.00+0

Isolation, Analysis, and Expression of Two Genes from *Thermoanaerobacterium* sp. Strain JW/SL YS485: a β-Xylosidase and a Novel Acetyl Xylan Esterase with Cephalosporin C Deacetylase Activity

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The genes encoding acetyl xylan esterase 1 (*axe1*) and a β-xylosidase (*xylB*) have been cloned and sequenced from *Thermoanaerobacterium* sp. strain JW/SL YS485. *axe1* is located 22 nucleotides 3′ of the *xylB* sequence. The identity of *axe1* was confirmed by comparison of the deduced amino acid sequence to peptide sequence analysis data from purified acetyl xylan esterase 1. The *xylB* gene was identified by expression cloning and by sequence homology to known β-xylosidases. Plasmids which independently expressed either acetyl xylan esterase 1 (pAct1BK) or β-xylosidase (pXylo-1.1) were constructed in *Escherichia coli*. Plasmid pXylAct-1 contained both genes joined at a unique *Eco*RI site and expressed both activities. Substrate specificity, pH, and temperature optima were determined for partially purified recombinant acetyl xylan esterase 1 and for crude recombinant β-xylosidase. Similarity searches showed that the *axe1* and *xylB* genes were homologs of the ORF-1 and *xynB* genes, respectively, isolated from *Thermoanaerobacterium saccharolyticum*. Although the deduced sequence of the *axe1* product had no significant amino acid sequence similarity to any reported acetyl xylan esterase sequence, it did have strong similarity to cephalosporin C deacetylase from *Bacillus subtilis*. Recombinant acetyl xylan esterase 1 was found to have thermostable deacetylase activity towards a number of acetylated substrates, including cephalosporin C and 7-aminocephalosporanic acid.

Efficient degradation of hemicellulose by microorganisms is achieved by the concerted action of several enzymes. Xylan (β-1,4-linked D-xylopyranosyl) is the predominant form of hemicellulosic polysaccharide in plant cell walls (10). Hydrolysis of xylan to xylose oligomers and ultimately to xylobiose and xylose is mediated by endoxylanases (β-D-xylanases) and exoxylanases (β-D-xylosidases), respectively (3). Xylan frequently contains multiple side groups, the most common being acetyl, arabinofuranosyl, and glucuronosyl residues (17). Acetyl xylan esterases are one of the "accessory" enzymes in xylan degradation required for the removal of acetyl groups esterified to the 2′ and/or 3′ position of xylan. In hardwoods, as much as 60 to 70% of the xylosyl residues are acetylated (21), whereas softwood xylan generally is not acetylated. The action of β-xylanases and β-xylosidases is often precluded by the presence of these side groups whose removal is necessary prior to, or concomitantly with, the action of xylanolytic enzymes (9, 35).

While a number of β-xylosidase genes have been isolated from a variety of fungal and mesophilic bacterial sources, few have been isolated from thermophilic bacteria (2, 20, 23). In contrast to the situation for xylanases and xylosidases, for which microbiological and biochemical information is available, very few genes encoding acetyl xylan esterases have been verified (12, 23, 24, 32), the *xynD* gene from *Pseudomonas fluorescens* being the only bacterial representative which has been expressed in *Escherichia coli* and characterized (12).

Thermoanaerobacterium sp. strain JW/SL YS485 is a thermophilic anaerobic bacterium which is capable of using xylan as its sole carbon source. We have recently described the purification and characterization of several enzymes involved in hemicellulose degradation by this organism, including a cell-associated xylanase (28), two acetyl xylan esterases (30), an α-glucuronidase (29), and two xylosidases (27a). To better understand the regulation of these enzymes and their relationship to similar proteins isolated from other sources, and to characterize at both the biochemical and genetic levels the components involved in xylan degradation, we have sought to isolate and express the genes encoding them. The gene encoding a cell-associated xylanase was recently isolated and expressed in *E. coli* (22). Here we report the isolation, expression, and analysis of the gene encoding acetyl xylan esterase 1 (*axe1*) and an adjacent gene (*xylB*) encoding a β-xylosidase. Recombinant acetyl xylan esterase 1 (rAXE1) had activity towards a number of acetylated substrates, including the antibiotic cephalosporin C. This is the first report of an acetyl xylan esterase gene isolated from an anaerobic thermophilic bacterium and expressed in *E. coli* whose recombinant product has cephalosporin C deacetylase activity.

MATERIALS AND METHODS

Reagents. All chemicals used in protein analysis and assays were purchased from Sigma or Boehringer Mannheim. DNA-modifying enzymes were obtained from New England Biolabs and Promega.

Bacterial strains and culture conditions. *Escherichia coli* TG-1 cells (*supE hsdΔ5 thi*Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) were used for all cloning and subcloning manipulations. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth containing the following supplements as necessary: ampicillin (100 μg/ml), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.008% 5-

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bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and 4-methylumbelliferyl-β-D-xyloside (4-MU xyloside) (20 μg/ml).

Peptide sequence analysis. Purified acetyl xylan esterase 1 (AXE1) was supplied by Weilan Shao and is described elsewhere (30). Internal peptide fragments were generated by trypsin digestion as described previously (34). Peptides were separated first by acid reverse-phase high-performance liquid chromatography (HPLC) (C_{18} column model HRLC 2700; Bio-Rad) with a linear gradient of 0.1% trifluoroacetic acid and 0.08% trifluoroacetic acid–80% acetonitrile. Peak fractions were collected, dried in a Speed Vac dehydrator (Savant), and resuspended in 15 mM ammonium acetate. Fractions were passed over a second neutral reverse-phase HPLC (C_{18} column model RP300; and Applied Biosystems) eluted with a linear gradient between 15 mM ammonium acetate and 120 mM ammonium acetate–80% acetonitrile. Peptide elution was monitored by noting absorbance at 220 nm. Peaks from the neutral HPLC separations were sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Library construction. Genomic DNA was isolated as described previously (1). Plasmid libraries were constructed by ligation of genomic DNA, which had been partially digested with either *Hin*dIII or *Nsi*I, into the *Hin*dIII or *Pst*I site of pUC18, respectively.

Xylosidase expression screening. Recombinant colonies were replica plated onto LB plates containing either ampicillin–IPTG–X-Gal or ampicillin–IPTG–4-MU xyloside. Recombinant clones expressing xylosidase activity were detected by illuminating the 4-MU xyloside plates with a handheld UV light (wavelength, 350 nm).

DNA manipulations. Plasmid pUC18/19 or plasmid pkk223-3 (Pharmacia) was used for all subcloning and expression constructs. Routine manipulations of DNA fragments and plasmids were carried out essentially as described elsewhere (27). PCRs were performed in a Perkin-Elmer Cetus 480 DNA thermocycler as described previously (26). DNA fragments were purified from low-melting-point agarose (Gibco BRL) (38). Competent-cell preparation and transformations were performed as described previously (13).

DNA analysis and synthesis. Southern transfer (33) and hybridization analyses using oligonucleotide probes ACT5PR and ACT3PR were performed as described previously (40). Probes were end labeled with [γ-^{32}P]ATP by using T-4 kinase (27). Oligonucleotide primer synthesis and DNA sequence analyses were done at the Molecular Genetics Instrumentation Facility at the University of Georgia. Computer analyses of DNA and amino acid sequences were done by using the Wisconsin package, version 8 (Genetics Computer Group). The GenBank sequence accession number for *xylB* and *axe1* is AF001926.

Isolation of *axe1*. Isolation of the *axe1* 3′ region was done by digesting YS485 DNA with *Nsi*I and purifying sized fractions from agarose. Each DNA fraction was used as a template for PCR using two 21-mer oligonucleotide primers, ACT5PR (AGG GAT TGA TAT GGG ACT TTT) and ACT3PR (TGT AAA TAC CTC ATT CTC CCT), synthesized to be specific to the 5′ and 3′ regions of the partial AXE1 open reading frame (ORF) identified to be present in pXylo-1. DNA fractions which yielded a PCR product of the appropriate size were ligated into *Pst*I-digested pUC19. Ligation of *Nsi*I-cut DNA into the *Pst*I site led to the abolition of both sites. Transformed *E. coli* TG-1 cells were selected on LB plates containing ampicillin, IPTG, and X-Gal. Recombinants were screened by PCR using ACT5PR and ACT3PR as primers and by alkaline miniprep analysis (5).

Plasmid constructs. Plasmid pXylo-1.1 was made by ligating the pXylo-1 *Hin*dIII insert into plasmid pkk223-3, thereby placing it under the control of the *tac* promoter. Plasmid pXylAct-1 was created by ligation of a *Hin*dIII-*Eco*RI pXylo-1 fragment to an *Eco*RI-*Sac*I fragment from pAct1-2. The *Sac*I site on pAct1-2 was donated from the pUC19 multiple cloning site. The resulting 2.7-kbp *Hin*dIII-*Sac*I fragment was ligated into pUC19 digested with *Hin*dIII/*Sac*I. Plasmid pAct1BK was derived from pXylAct-1 by isolation of a *Bsi*HKAI-*Kpn*I fragment and ligation into *Pst*I-*Kpn*I-digested pUC19.

Enzyme assays. In both xylosidase and acetyl xylan esterase assays, 1 U of activity was defined as the amount of enzyme required to produce 1 μmol of product/min. Specific activity was expressed as units of activity per milligram of protein. Recombinant xylosidase activity was measured by using crude *E. coli* lysates which had been clarified by centrifugation. Xylosidase activity was determined in a 4-min assay performed at 70°C, pH 6.0, with a *p*-nitrophenyl-β-D-xylopyranoside (pNPX) substrate as described elsewhere (31). Other *p*-nitrophenyl (pNP) conjugates, including pNP-β-D-arabinoside, pNP-β-D-arabinofuranoside, pNP-β-D-glucoside, and pNP-β-D-mannoside, were tested under the same conditions. Optimum-pH determinations were made at 70°C in either 50 mM sodium phosphate buffer (pH 5 to 8) or 50 mM sodium phthalate buffer (pH 5 to 6.5). The temperature optimum was determined in 50 mM sodium phosphate, pH 6. In both cases, determinations were made with pNPX in a 3-min assay with 97 μg of crude lysate.

rAXE1 activity was measured by using a partially purified preparation (see below). Routine assays were performed for 2 min at 75°C, pH 7.0, with 4-MU acetate as described elsewhere (30). Acetylated xylan was made according to the method of Johnson et al. by using birch xylan (Sigma) (16). Assays with acetylated xylan and all other acetylated substrates tested were performed by using 60 μg of partially purified protein in a 2-min reaction at 75°C, pH 7.0, as described elsewhere (30). Liberated acetic acid was quantified by using a kit from Boehringer Mannheim (catalog no. 148261). Acetylesterase activity on nitrocellulose filters after Western blotting (8), or in situ after native gel electrophoresis, was determined by incubation with α-naphthyl acetate in the presence of Fast Red TR at 25°C (11, 36). Optimum-pH determinations were made at 70°C in either 50 mM sodium phthalate buffer (pH 5 to 6.5) or 50 mM sodium phosphate buffer (pH 6 to 8). The temperature optimum was determined in 50 mM sodium phosphate at pH 7. In both cases, determinations were made with 4-MU acetate in a 2-min assay with 64 μg of purified protein from an S-200 column pool. Buffers were titrated at 25°C.

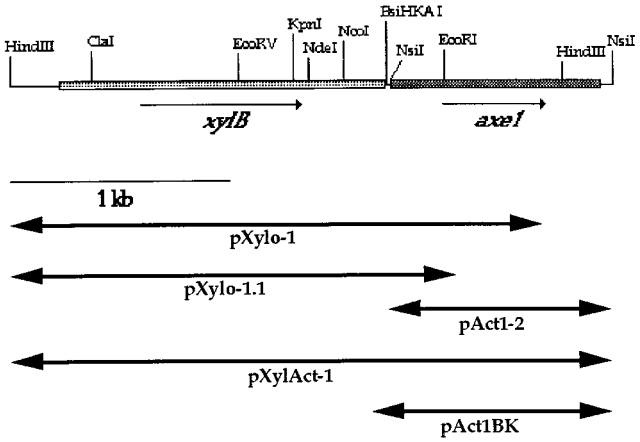

FIG. 1. Restriction map of the clone pXylAct-1. Arrows indicate the extents of genomic clones and subclones which were isolated for the reconstruction of pXylAct-1 as well as for expression of β-xylosidase and AXE1 activities.

Protein analysis. Cells were grown at 37°C in 50 ml of LB to an optical density at 600 nm of 0.5 to 0.6. Cultures were split, IPTG was added to one sample, and cells were grown for 5 to 18 h before harvesting. Cells were lysed by resuspending pellets in one-sixth of the culture volume with 0.1 mg of lysozyme per ml in TE buffer (10 mM Tris, 1 mM EDTA [pH 7.6]) and freeze-thawing twice at −70°C. Following brief sonication (Branson Cell Disruptor; power setting at 7 for 20 s), the lysate was centrifuged at 17,000 × *g* for 20 min. Protein concentration was determined by the method of Bradford by using bovine serum albumin as a reference standard (6). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described elsewhere (18). Native PAGE was performed in the same gel system but in the absence of β-mercaptoethanol in the sample buffer and without SDS. Molecular mass markers were purchased from Pharmacia. Masses, in kilodaltons, are as follows: phosphorylase *b*, 94; albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; trypsin inhibitor, 20.1; and α-lactalbumin, 14.4.

Partial purification of rAXE1 was performed in four steps as follows. A 500-ml culture was grown to an optical density at 600 nm of 0.6, and IPTG was added to give a final concentration of 0.5 mM. After a further 6 h of growth, the cells were harvested by centrifugation and resuspended in 40 ml of TE buffer. The cells were lysed by two cycles of freezing at −20°C and thawing. Lysozyme was added to a final concentration of 2 mg/ml. After being incubated for 1 h at 25°C, the cells were sonicated with 20 0.5-s bursts by using a Branson Cell Disruptor. The lysate was centrifuged at 20,000 × *g* for 20 min and the supernatant was heated at 55°C for 10 min followed by a second, identical centrifugation. Chromatography was performed by using a Pharmacia FPLC system equipped with prepacked columns. The clarified lysate was first passed over a Q-Sepharose column equilibrated in 25 mM Tris–0.1% β-mercaptoethanol (pH 8.0) and eluted with a linear gradient (0 to 1 M NaCl) at a rate of 0.5 ml/min. Pooled fractions were then chromatographed on an S-200 column equilibrated in 10 mM Tris–50 mM NaCl (pH 7.8).

RESULTS

Gene isolation and sequence analysis. (i) *xylB*. The *xylB* gene encoding a β-xylosidase was isolated by expression screening of a plasmid library on 4-MU xyloside plates. Several clones expressing xylosidase activity were isolated. The clone pXylo-1 was analyzed by restriction endonuclease digestion and found to contain a 2.5-kbp insert (Fig. 1). DNA sequence analysis verified that pXylo-1 contained the complete coding region of a β-xylosidase gene, *xylB*. *xylB* encodes a polypeptide of 500 residues with a calculated molecular mass of 58.5 kDa. Putative prokaryotic-sequence-like −10 (TATAGA) and −35 (TATGTG) promoter sequences as well as a Shine-Dalgarno (GGGGG) site were noted.

Screening of protein and nucleic acid data banks revealed that the *xylB* gene is a homolog of the previously described *xynB* gene isolated from *Thermoanaerobacterium sacharrolyticum* (20), the products having 98% amino acid similarity and 91% identity by Bestfit analysis. The differences between the peptide sequences of these two genes are, for the most part, conservative. Similarly, the *xylB* gene isolated from *Caldicellosiruptor saccharolyticus* (23) exhibited 59% similarity and 35% identity to the *xylB* gene from *Thermoanaerobacterium* sp. strain JW/SL YS485. The consensus pattern for glycosyl hydrolase family 39 (14), WXFEXWNEP(D/N) was located in the deduced sequence between residues 153 and 163. The deduced N-terminal sequence of *xylB* did not, however, agree with either of two N-terminal sequences from xylosidases purified from *Thermoanaerobacterium* sp. strain JW/SL YS485 (27a).

(ii) *axe1*. Three peptide sequences were determined for the trypsin-digested, HPLC-purified AXE1 peptides. The amino acid sequences of these peptides were as follows: Acet3-2, VYPDYG; Acet3-3, TEGKHPALIR; and Acet15-2, FQVSFAECYDLY. These sequence data, along with the previously reported N-terminal sequence GLFXMMXWLQKLREY TGT (30), were used to confirm that the partial ORF found 3′ of *xylB* was the gene, *axe1*, encoding AXE1. All internal peptide sequences matched the deduced sequence; however, the N-terminal sequence data for the native protein were found to differ slightly from the predicted sequence MGLFDMPLQK LREYTGT. This difference may have been due to a partially blocked or cleaved methionine which led to aberrant N-terminal sequencing.

A site for the enzyme *Nsi*I was located in the intergenic region of the *xylB* and *axe1* genes (Fig. 1). The full-length *axe1* gene was isolated from a "shotgun" library constructed in pUC18 with agarose-purified, *Nsi*I-digested DNA (size ranges, 2.4 to 4.0 and 1.3 to 2.0 kbp). These size ranges were selected based on Southern data (not shown) for *Nsi*I-digested DNA hybridized with two oligonucleotide primers, ACT5PR and ACT3PR. PCR analysis of the size-fractionated DNA, done by using the two primers, amplified the expected 760-bp fragment (data not shown).

Fifty recombinant clones were analyzed. One clone, pAct1-2, contained a 1.9-kbp insert having the entire *axe1* gene on a single *Nsi*I fragment as confirmed by restriction enzyme analysis (Fig. 1). The *axe1* gene spanned 963 bp encoding 320 residues. The calculated molecular mass of 36 kDa approximated the subunit molecular mass of 32 kDa reported for the native AXE1 (30). Putative −10 (TAAATT) and −35 (TGGATA) promoter elements were found in the 3′ coding region of *xylB*. A Shine-Dalgarno site (AGGGA) was also noted.

axe1 is a homolog of the unidentified ORF-1 located 3′ of the *xynB* gene from *T. saccharolyticum* (20). The predicted amino acid sequences were 98% similar and 96% identical by Bestfit analysis. No significant amino acid sequence similarity between *axe1* and any published acetyl xylan esterase sequence was found by Genetics Computer Group Blast and FastA analyses; however, strong similarity between the deduced *axe1* sequence and the cephalosporin C deacetylase (CAH) gene isolated from *Bacillus subtilis* (25) was found. Bestfit analysis scores showed 59% similarity and 37% identity between the CAH and AXE1 protein sequences (Fig. 2). Comparison of the C-terminal halves of these two sequences increased these values to 65% similarity and 43% identity. In a region containing a number of these identities (AXE1 residues 170 to 194), an active-site motif, Gly-X-Ser-X-Gly, found in many serine proteases, esterases, and lipases (7) was located.

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  1 MGLFDMPLQKLREYTGTNPCPEDFDEYWNRALDEMRSVDPKIELKESSFQ 50
    |.|||:||:.|..|......|.||.|:|. .|:|: .|::. :|.. .:.
  1 MQLFDLPLDQLQTYKPEKTAPKDFSEFWKLSLEELAKVQAEPDLQPVDYP 50

 51 VSFAECYDLYFTGVRGARIHAKYIKPKTEGKHPALIRFHGY.SSNSGDWN 99
    .. ...| | :.:. .||| :.|  |..:|.|||:::::||| .| .|: :
 51 ADGVKVYRLTYKSFGNARITGWYAVPDKQGPHPAIVKYHGYNASYDGEIH 100

100 DKLNYVAAGFTVVAMDVRGQGGQSQDVGGVTGNTLNGHIIRGLDDDADNM 149
    :.:|:.  |:...:| ||||.: .:.  :: |:.| | :.:|: |. |.
101 EMVNWALHGYAAFGMLVRGQQSSEDTSISLHGHAL.GWMTKGILDK.DTY 148

150 LFRHIFLDTAQLAGIVMNMPEVDEDRVGVMGPSQGGGLSLACAALEPRVR 199
    .:| ::||...  ::: .:.||||.|:|| |.||||||.:|.|||.. .:
149 YYRGVYLDAVRALEVISSFDEVDETRIGVTGGSQGGGLTIAAAALSDIPK 198

200 KVVSEYPFLSDYKRVWDLDLAKNAYQEITDYFRLFDPRHERENEVFTKLG 249
     .|.:||:||::.|. |:.| ..:| ||..:||  :...| | :.:..|:
199 AAVADYPYLSNFERAIDVAL.EQPYLEINSFFRR.NGSPETEVQAMKTLS 246

250 YIDVKNLAKRIKGDVLMCVGLMDQVCPPSTVFAAYNNIQSKKDIKVYPDY 299
    |:|:.|||.|:|..|||::||:|.|.|||||||||:::..|::|||. :
247 YFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETEKELKVYRYF 296

300 GHEPMRGFGDLAMQFMLELYS 320
    ||| :.:|..  :.|: : ..
297 GHEYIPAFQTEKLAFFKQHLK 317
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FIG. 2. Bestfit amino acid sequence alignment of the deduced AXE1 sequence (top) and the deduced CAH sequence (bottom) from *B. subtilis*. Identities are indicated by vertical lines.

Expression and analysis of recombinant proteins. (i) β-Xylosidase. Low levels of β-xylosidase activity against pNPX were detected in crude extracts from *E. coli* harboring pXylo-1 (Table 1). Specific activities determined for pXylo-1.1 were 10-fold higher than those for pXylo-1, while activity produced from pXylAct-1 (Fig. 1), a clone which had the full-length *xylB* and *axe1* genes joined in their natural orientation, was 2.5-fold higher than that from pXylo-1.1 (Table 1). No xylosidase activity was detected when pNP derivatives arabinose, mannose, and glucose were used as substrates.

Temperature and pH optima for the β-xylosidase activity were determined with clarified lysates from pXylo-1.1. The pH optimum for xylosidase was between 5.5 and 6.0, depending upon the buffer used. The highest activity was seen in sodium phosphate buffer at pH 6.0. The optimum temperature was 65°C. Activity declined rapidly at higher temperatures. SDS-PAGE analysis of the recombinant xylosidase (Fig. 3) revealed a band migrating at approximately 57 kDa (lane C) which was not seen in the control lane (lane B) and indicated that the *xylB* gene product is not β-xylosidase 1 (lane D) or β-xylosidase 2 (lane E), which were purified from *Thermoanaerobacterium* sp. strain JW/SL YS485 (27a), as these migrate at approximately 86 and 78 kDa, respectively.

(ii) AXE1. rAXE1 activity against 4-MU acetate was determined with clarified lysates from pXylAct-1 and pAct1BK (Ta-

TABLE 1. β-Xylosidase and AXE1 activities in transformed *E. coli* lysates

Plasmid	Sp act (U/mg of protein)	
	pNPX[a]	4-MU acetate[b]
pXylo-1	0.024	ND[c]
pXylo-1.1	0.22	ND
pXylAct-1	0.53	0.063
pAct1BK	ND	4.5

[a] Cultures were induced with 1 mM IPTG at mid-log phase and harvested after 12 h of incubation at 30°C. Activity was determined with pNPX as a substrate.

[b] Cultures were induced with 1 mM IPTG at mid-log phase and harvested after 5 h of incubation at 37°C. Activity was determined with 4-MU acetate as a substrate.

[c] ND, not determined.

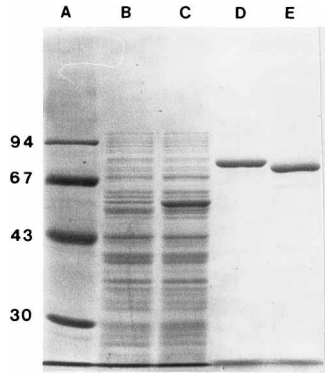

FIG. 3. SDS–7% PAGE analysis of recombinant xylosidase expression in *E. coli*. Lanes: A, molecular mass markers (in kilodaltons); B, pUC18-transformed TG-1 cell lysate (15 μg); C, pXylo-1.1-transformed TG-1 cell lysate (15 μg); D, purified native xylosidase 1 (5 μg); E, purified native xylosidase 2 (5 μg).

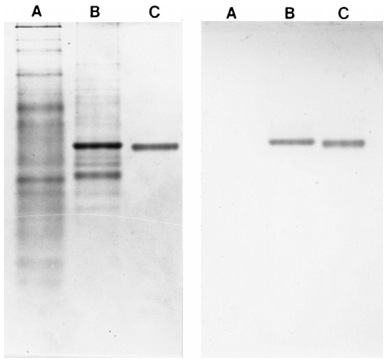

FIG. 5. (Left) Nondenaturing 8% PAGE analysis of rAXE1 and native AXE1. (Right) In situ acetylesterase activity against α-naphthyl acetate after nondenaturing 8% PAGE. Lanes (both panels): A, pUC18-transformed TG-1 cell lysate (5 μg); B, rAXE1 after S-200 chromatography (5 μg); C, native AXE1 (2 μg).

ble 1). While significant activity was seen in pXylAct-1 extracts, the activity from pAct1BK was sevenfold higher. rAXE1 expression from pXylAct-1 was optimum after a 5-h induction with IPTG at 37°C, while a 16-h induction at 30°C produced very little rAXE1 activity. The reverse was true for xylosidase expression from the same clone; however, both activities were detectable under either growth condition. Although transcription of *axe1* may be independent from that of *xylB*, translation from a dicistronic transcript remains a possibility.

rAXE1 was partially purified from extracts of pAct1BK as described in Materials and Methods. The pH optimum of the partially purified rAXE1 was 7.0, and the temperature optimum was 75°C; both of these values are in agreement with values determined for the native enzyme. SDS-PAGE analysis of a pAct1BK cell lysate (Fig. 4) showed a prominent band migrating at approximately 31 kDa in the heat-treated sample (lane B) and in the sample which had been partially purified by chromatography in Q-Sepharose and S-200 resins (lane C). Purified native AXE1 (lane D) was slightly larger than rAXE1.

Nondenatured rAXE1 was compared to native AXE1 in an 8% PAGE gel to determine if the multisubunit character of the native enzyme persisted in the recombinant protein (Fig. 5, left panel). The rAXE1 (Fig. 5, left panel, lane B) appeared as a slightly larger band relative to the native protein (lane C) on the Coomassie blue-stained gel. An identical gel was stained for in situ acetylesterase activity (Fig. 5, right panel). No activity was detected in the pUC18-transformed *E. coli* extract (Fig. 5, right panel, lane A), while both rAXE1 (lane B) and native AXE1 (lane C) exhibited activity against α-naphthyl acetate. The activity could also be demonstrated after Western blotting to nitrocellulose filters, although the native enzyme did not transfer as efficiently as did the recombinant form (data not shown). To see whether the apparent differences between the native and recombinant enzymes were due to glycosylation, both rAXE1 and AXE1 were fractionated in a native 8% PAGE gel and tested for the presence of carbohydrate staining by the Schiff method (19). No evidence for the presence of carbohydrate was obtained from either sample by this method.

rAXE1 was tested for its ability to hydrolyze acetate from a number of substrates (Table 2) which had been tested previously with the native enzyme (30). The highest specific activities were observed with xylose tetraacetate and triacetin substrates. Due to the amino acid sequence similarity between the *axe1* product and CAH, specific activities were determined for cephalosporin C and a derivative, 7-aminocephalosporanic acid. The specific activity against 7-aminocephalosporanic acid was almost one-half the value for xylose tetraacetate and was over twofold higher than that for cephalosporin C. Curiously, rAXE1 had no activity against acetylated birch xylan.

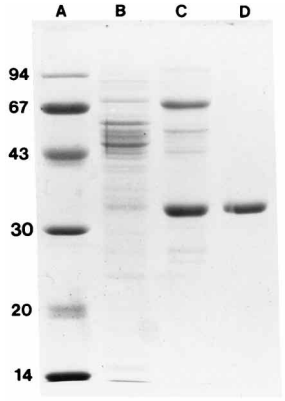

FIG. 4. SDS–12.5% PAGE analysis of rAXE1 expression in *E. coli*. Lanes: A, molecular mass markers (in kilodaltons); B, heat-treated crude lysate from pAct1BK cells (15 μg); C, rAXE1 after S-200 chromatography (15 μg); D, purified native AXE1 (5 μg).

DISCUSSION

The actions of β-xylosidase and acetyl xylan esterase are required for the complete degradation of many xylan-containing tissues. In this work, we have continued our efforts to understand the genetic organization and regulation of genes

TABLE 2. Substrate specificity of rAXE1

Substrate	Sp act[a] (U/mg)
4-MU acetate	47
Triacetin	165
Xylose tetraacetate	210
Acetylated xylan	0
Cephalosporin C	41
7-Aminocephalosporanic acid	91

[a] Each assay mixture contained 60 μg of partially purified protein taken from a S-200 column pool.

from *Thermoanaerobacterium* sp. strain JW/SL YS485 involved in xylan degradation. Here we report the isolation, expression, and analyses of a recombinant β-xylosidase and AXE1 encoded by the *xylB* and *axe1* genes, respectively.

The *xylB* gene is a homolog of the *xynB* gene isolated from *T. saccharolyticum* B6A1 (20). Not surprisingly, the substrate specificities of the *xylB* and *xynB* gene products (both active against pNPX only), their relative molecular masses on SDS-PAGE (57 versus 55 kDa), pH optima (6.0 versus 5.5), and temperature optima (65 versus 70°C) closely parallel one another. However, the apparent molecular size of the *xylB* gene product on SDS-PAGE does not correspond to the size of either xylosidase 1 (86 kDa) or xylosidase 2 (78 kDa), nor does the deduced N-terminal sequence of *xylB* corroborate N-terminal sequence data from purified β-xylosidases 1 and 2 (27a). These data, taken together, imply that *Thermoanaerobacterium* sp. strain JW/SL YS485 has at least three distinct β-xylosidases.

The close proximity of *axe1* 3′ of *xylB* suggests that AXE1 is involved in xylan degradation since genes involved in this process have been found to be clustered or adjacent to one another (2, 23, 32, 39). The fact that rAXE1 has significant levels of activity towards a number of low-molecular-weight acetylated substrates, including cephalosporin C and 7-aminocephalosporanic acid, but no activity towards acetylated xylan raises the question as to whether deacetylation of xylan is the primary function for this enzyme.

The ability of rAXE1 to utilize all acetylated substrates tested except acetylated xylan may be explained by one of several possibilities. Native AXE1 was shown by chromatography and SDS-PAGE data to be a hexamer of six identical subunits (30). Chromatography elution profiles of rAXE1, direct comparison to the native enzyme via nondenaturing PAGE, and in situ activity against α-naphthyl acetate together demonstrate that rAXE1 forms a multisubunit protein whose catalytic functions remain intact. Slight mobility differences between the native and recombinant proteins were seen in both SDS and native PAGE gels, and AXE1 did not efficiently transfer to nitrocellulose by Western blotting while rAXE1 did. These apparent differences are not likely due to glycosylation since carbohydrate was not detected on AXE1 by Schiff base analysis. The lack of activity of rAXE1 towards acetylated xylan could be due to its expression in a mesophilic background leading to perturbations in protein folding and/or quarternary structure formation that preclude the binding to xylan. Another, although unlikely, possibility is that the partially purified rAXE1 sample contains an inhibitor or compound that prevents the binding of xylan.

We suggest that the reason for the lack of rAXE1 activity towards acetylated xylan is that the primary substrate of AXE1 is acetylated xylo-oligomers. Biely et al., using the *Trichoderma reesei* system, attributed the cooperative effect of xylanases on the action of acetyl xylan esterases to a preference of the latter for shorter polymers (4). We reported previously that AXE1 activity towards acetylated xylan was 5.2 U/mg (the lowest activity for any substrate tested and 142-fold less than that for xylose tetraacetate, the best substrate tested) and that AXE1 was primarily cell associated (30). The lack of any apparent signal peptide sequence in *axe1* implies that it is not a secreted protein. Since an intracellular location for AXE1 is incongruous with a role in deacetylating xylan, an extracellular substrate, we hypothesize that the role of this enzyme in *Thermoanaerobacterium* sp. strain JW/SL YS485 is the deacetylation of transported oligosaccharides.

One of the most interesting aspects of this work is the lack of significant amino acid similarity between the *axe1* product and any published acetyl xylan esterase sequence and its high degree of similarity to CAH from *B. subtilis* (25). The amino acid identities found in the C-terminal halves of these two sequences, which contain the putative active site regions, suggest that *axe1* and the CAH gene divergently evolved from a common ancestral gene and belong to the same gene family. Furthermore, enzyme activity of rAXE1 towards cephalosporin C and 7-aminocephalosporanic acid substrates demonstrates that the sequence similarity seen between AXE1 and CAH has functional consequences.

Cephalosporin C is in the class of β-lactam antibiotics which includes penicillin. These antibiotics inhibit cell growth by inhibiting the enzyme that cross-links peptide glycans required for cell wall formation (37). It has been shown that deacetylation of cephalosporin C leads to an 80% loss in activity against *Staphylococcus aureus* and *Salmonella typhi* (15). *Thermoanaerobacterium* sp. strain JW/SL YS485 can grow in a wide temperature range (35 to 66°C). When grown at lower temperatures which would not lead to thermal degradation of cephalosporin C, *Thermoanaerobacterium* sp. strain JW/SL YS485 is sensitive to concentrations of cephalosporin C as low as 25 μg/ml (23a). Thus, deacetylation of cephalosporin C by AXE1 does not appear to be linked to the inactivation of cephalosporins in this organism. The importance of this activity for the survival or metabolic needs of *Thermoanaerobacterium* sp. strain JW/SL YS485 remains to be studied.

ACKNOWLEDGMENTS

We thank Weilan Shao for providing purified native AXE1 and β-xylosidases 1 and 2.

This research was supported by a grant from the U.S. Department of Energy (DE-FG05-95ER-20199).

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