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Cloning, Sequencing, and Expression of the Gene Encoding a Large S-Layer-Associated Endoxylanase from *Thermoanaerobacterium* sp. Strain JW/SL-YS 485 in *Escherichia coli*

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The gene (*xynA*) encoding a surface-exposed, S-layer-associated endoxylanase from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 was cloned and expressed in *Escherichia coli*. A 3.8-kb fragment was amplified from chromosomal DNA by using primers directed against conserved sequences of endoxylanases isolated from other thermophilic bacteria. This PCR product was used as a probe in Southern hybridizations to identify a 4.6-kb *Eco*RI fragment containing the complete *xynA* gene. This fragment was cloned into *E. coli*, and recombinant clones expressed significant levels of xylanase activity. The purified recombinant protein had an estimated molecular mass (150 kDa), temperature maximum (80°C), pH optimum (pH 6.3), and isoelectric point (pH 4.5) that were similar to those of the endoxylanase isolated from strain JW/SL-YS 485. The entire insert was sequenced and analysis revealed a 4,044-bp open reading frame encoding a protein containing 1,348 amino acid residues (estimated molecular mass of 148 kDa). *xynA* was preceded by a putative promoter at −35 (TTAAT) and −10 (TATATT) and a potential ribosome binding site (AGGGAG) and was expressed constitutively in *E. coli*. The deduced amino acid sequence showed 38 to 96% similarity to sequences of family F β-glycanases. A putative 32-amino-acid signal peptide was identified, and the C-terminal end of the protein contained three repeating sequences (59, 64, and 57 amino acids) that showed 46 to 68% similarity to repeating sequences at the N-terminal end of S-layer and S-layer-associated proteins from other gram-positive bacteria. These repeats could permit an interaction of the enzyme with the S-layer and tether it to the cell surface.

Xylans constitute the main polymeric component of the hemicellulose fraction of plant cell walls (9). They have been isolated from different sources and exhibit considerable variation in composition and structure, and so it is not surprising that in nature, the cooperative actions of a consortium of microbial enzymes are required for complete hydrolysis. Usually xylan is composed of a backbone structure of β-1,4-linked D-xylose residues with α-L-arabinofuranose and glucuronic acid side chains, most of which are acetylated. Biodegradation of these xylans involves the action of several hydrolytic enzymes, including two major xylanolytic enzymes, endoxylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). Endoxylanase randomly cleaves the internal xylosidic linkages of the xylan backbone, yielding xylooligosaccharides of various lengths, which are further hydrolyzed to xylose monomers by β-xylosidase after removal of the side chains (2). Xylanases from various microorganisms, including fungi and yeasts, have been isolated and characterized, and several of them, including some from thermophilic anaerobic bacteria, have been cloned and expressed in *Escherichia coli* (8, 19, 29, 42, 52, 60). However, comparatively little is known about the diversity of molecular and biochemical properties of the enzymes from anaerobic thermophiles.

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Recently, we isolated a highly xylanolytic anaerobic thermophile, *Thermoanaerobacterium* sp. strain JW/SL-YS 485 (DSM 8691). From this isolate, we have previously purified one glucuronidase (49), two acetyl xylan esterases (50), two xylosidases (unpublished results), and one unusually large, S-layer-associated endoxylanase (48). The apparent temperature optimum (80°C) of the endoxylanase is among the highest reported for endoxylanases from any microorganism. For example, the optimal temperature of endoxylanases from *Thermotoga* sp. strain Fjss3-B.1 was 105°C (52); the optimal temperature of endoxylanases from either *Caldicellulosiruptor saccharolyticus* (33) or *Thermoanaerobacterium saccharolyticum* B6A-RI (30) was 70°C. Originally, the purified endoxylanase was thought to be composed of two subunits (24 and 180 kDa). The 24-kDa subunit has an N-terminal sequence of S-Q-Y-A-A-F-E-Y-D-R-T-F-N-D and was thought to be necessary for maximum enzyme activity. The 180-kDa subunit contained 6% carbohydrate, suggesting that it was a glycoprotein. In addition, the endoxylanase was particularly interesting because greater than 80% of this enzyme activity localized to the S-layer of strain JW/SL-YS 485 (48). How this enzyme associates with the outermost layer of the bacterial cell is not known.

In this report, we describe the cloning, sequencing, and expression of the *xynA* gene from strain JW/SL-YS 485 in *E. coli*. From analysis of the deduced amino acid sequence, we now believe that the 24-kDa subunit is a breakdown product of the 180-kDa protein. In addition, several interesting features of the amino acid sequence suggest the nature of the interac-

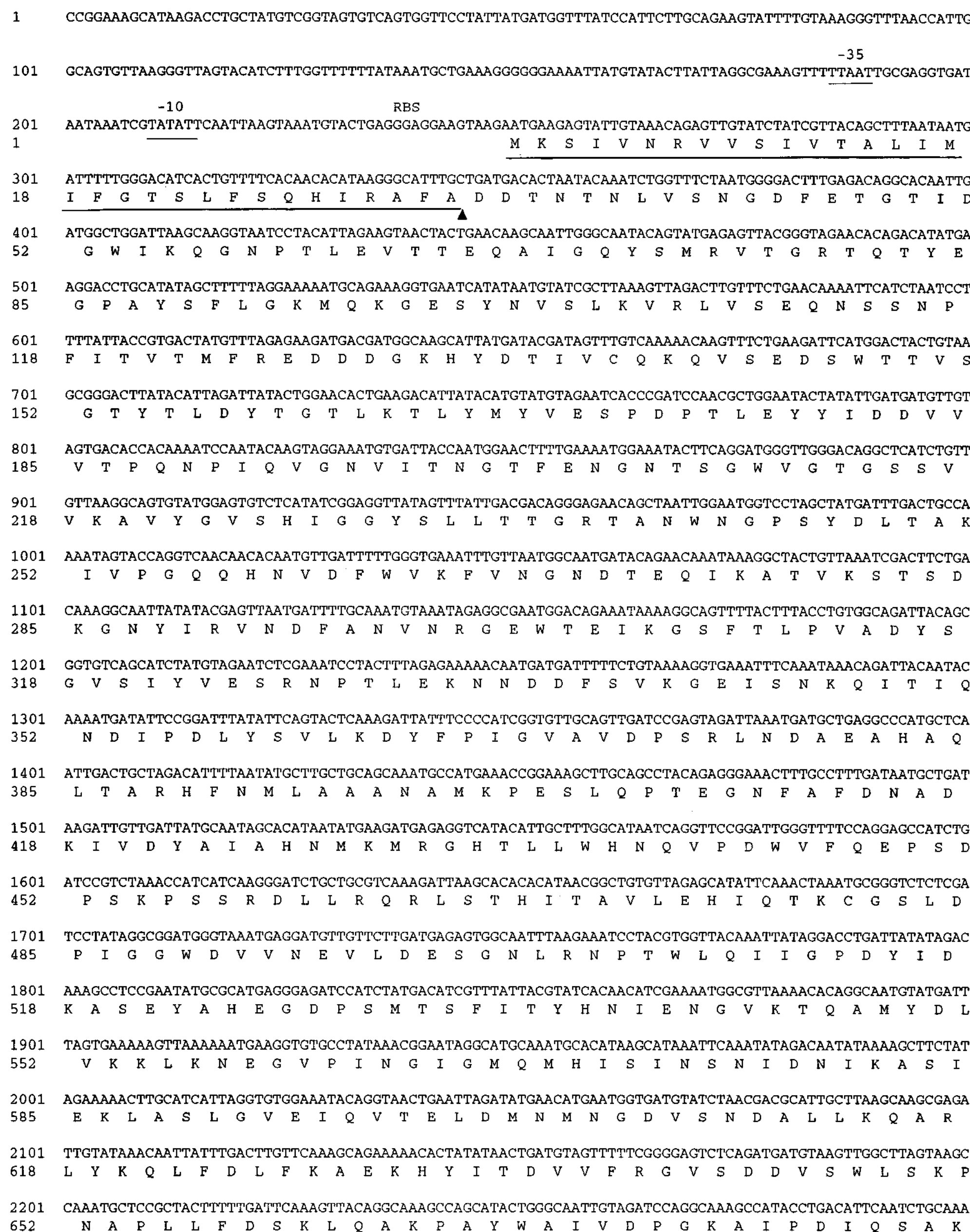

FIG. 1. Nucleotide and amino acid sequences of *xynA*, the xylanase gene of *Thermoanaerobacterium* sp. strain JW/SL-YS 485. The locations of the putative −35 and −10 sites and ribosome binding site (RBS) are shown above the sequence. The putative signal peptide for the *xynA* gene product is underlined, and a possible signal peptidase I cleavage site (Ala-32) is indicated by the triangle. The region of XynA with homology to the N-terminal sequence of the 24-kDa subunit is indicated by the solid line. Locations of SLR1, SLR2, and SLR3 are shown. Arrows indicate a putative terminator sequence.

tion of the enzyme with the S-layer. We also report the biochemical and molecular properties of the purified recombinant enzyme.

MATERIALS AND METHODS

Bacterial strains and growth condition. *Thermoanaerobacterium* sp. strain JW/SL-YS 485 (DSM 8691) was recently isolated by Liu and Wiegel (32a) from Yellowstone National Park, Wyoming, during a survey of anaerobic thermophiles able to grow with xylose or xylan as a sole carbon source at pH values below 4.5. Identification was based on physiological properties including the formation of elemental sulfur from thiosulfate (28) and 16S rRNA sequence analysis (45a). *Thermoanaerobacterium* sp. strain JW/SL-YS 485 was grown under anaerobic conditions at 60°C in modified M5 medium containing 3.7 mM KH_2PO_4, 11.6 mM Na_2HPO_4, 9.3 mM NH_4Cl, 3.8 mM $(NH_4)_2SO_4$, 17 mM NaCl, 0.2 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.5 mM each Na_2S and cysteic acid, 5 ml of trace element solution and 0.5 ml of vitamin solution per liter (16), 0.1% (wt/vol) yeast extract, and 0.5% (wt/vol) xylose as a carbon source and inducer of the enzyme. *E. coli* JM109 was grown in Luria broth as described by Maniatis et al. (36). Ampicillin (125 μg/ml) was added for selection.

Enzyme assays. Endoxylanase activity was assayed by measuring the increase in reducing sugar, using a modification of the method of Lever (31) with oat xylan as a substrate. A 500-μl volume of a xylan solution (0.25% xylan in 100 mM citrate in 100 mM sodium phosphate buffer [pH 6.0]) was mixed with 25 μl of suitably diluted enzyme extract and incubated at 75°C. After 5 min, the reaction was terminated by adding 1.5 ml of ice-cold PAHBAH reagent (0.1% *para*-hydroxybenzoic acid hydrazide in 0.4 M NaOH prepared as described by Lever [31]). The reaction mixture was boiled for 10 min, and the A_{410} was measured. Xylose was used as a standard for the reducing sugar assay. One unit of endoxy-

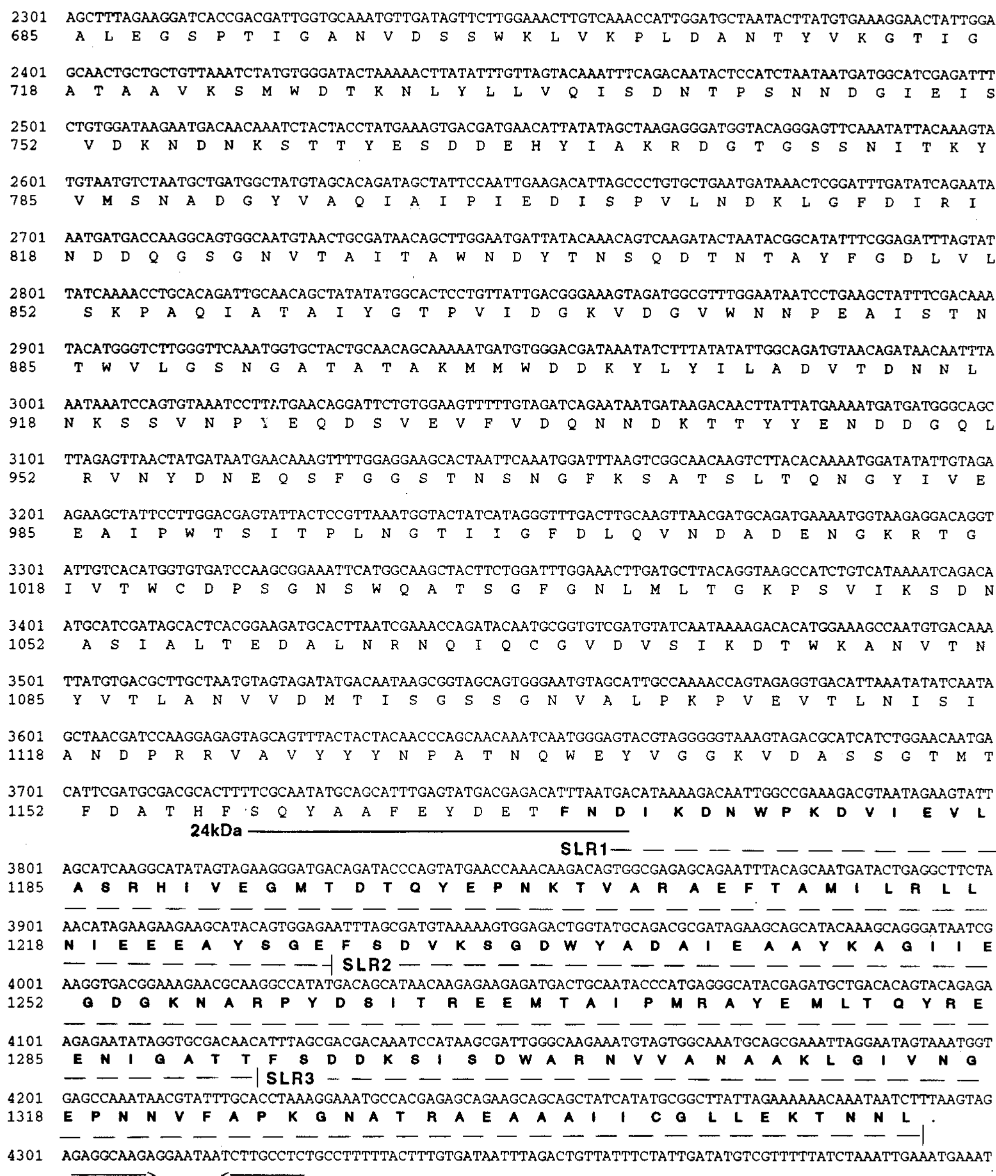

FIG. 1—*Continued.*

lanase activity was defined as the amount of enzyme producing 1 μmol of reducing equivalents per min under the assay conditions. Endoxylanase activity in recombinant clones was measured by using a modification of the plate assay method described by Teather and Wood (55). Replica plates were incubated overnight at 37°C, overlaid with 0.8% soft agar containing xylan coupled to Remazol brilliant blue or oat xylan, and incubated at 70°C for 2.5 h. A positive clone had a pale blue zone of clearing (xylan-Remazol brilliant blue) or a clear zone (oat xylan) surrounding the bacterial colony. In some cases, these plates were subsequently stained with Congo red to enhance the clear zones.

DNA manipulations. Genomic DNA was isolated from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 cells by a modification of the Marmur method (38). Cells were harvested in mid-exponential growth phase by centrifugation (8,000 × *g* for 10 min at 4°C) and suspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). Cells were treated with lysozyme (1 mg/ml) for 20 min and then treated with 0.5% sodium dodecyl sulfate (SDS) and proteinase K (100 μg/ml) at 37°C for 1 h. The lysate was extracted with chloroform-isoamyl alcohol (24:1) twice; DNA was precipitated with 0.6 volume of isopropanol and harvested by centrifugation (10,000 × *g* for 15 min). DNA was suspended in TE buffer and further purified by cesium chloride-ethidium bromide density gradient centrifugation (36).

Digestion of DNA with restriction endonucleases, separation of fragments by agarose gel electrophoresis, ligation of DNA fragments, and transformation of *E. coli* with plasmid DNA were performed as described by Maniatis et al. (36). DNA fragments were recovered from agarose gels by using GeneClean (Bio 101, Inc., La Jolla, Calif.).

PCR amplification of *xynA* probe. Several homologous regions of *xyn* genes exist in *Thermoanaerobacterium thermosulfurigenes* EM1 (38a), *T. saccharolyticum* B6A-RI (29), and *Clostridium thermocellum* (43a). On the basis of these sequence homologies, two PCR primers were designed. The forward oligonucleotide primer, *xynA*1 (5′-TAT-CTA-TTG-TTA-CAG-CTT-3′), was synthesized on the basis of the homologous regions which are near the N termini of the endoxylanases, and the reverse oligonucleotide primer, *xynA*2 (5′-YGT-CAT-CTC-TTC-TCT-TGT-3′, Y = C + T), was designed according to the homologous regions which are near the C termini of the endoxylanases (sequence data were from the GenBank/EMBL data bank). Synthetic oligonucleotide primers *xynA*1 and *xynA*2 were prepared in the Molecular Genetics Instrumentation Facility, Department of Genetics, University of Georgia. The chromosomal DNA from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 was used as a template.

Template DNA (500 ng) and primers *xynA*1 and *xynA*2 (50 pmol of each) were incubated in a 100-μl reaction mixture containing 10 mM Tris (pH 8.5), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase (Promega Corp., Madison, Wis.) in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass.) at 94°C (1 min), 45°C (45 s), and 72°C (2 min). Forty-five cycles were performed. The PCR product (PCR-231) was used to probe Southern blot (36)

containing *Thermoanaerobacterium* sp. strain JW/SL-YS 485 and *E. coli* chromosomal DNA digested with *Bgl*II, *Eco*RI, and *Pst*I.

Cell fractionation. To determine the location of the recombinant endoxylanase in *E. coli*, cells were fractionated by a modification of the method described by Ames et al. (1). A culture of 10 ml was grown to stationary phase, and cells were harvested by centrifugation (6,000 × *g* for 10 min). Periplasmic proteins were released by incubation with 20 μl of chloroform at room temperature for 20 min. After centrifugation (6,000 × *g* for 20 min), the supernatant containing the periplasmic proteins was withdrawn and stored at −20°C. The cytoplasmic proteins were obtained by sonic disruption of the pellet (50% pulse for 1 min at 4°C). Unlysed cells and cell debris were removed by centrifugation (16,000 × *g* for 20 min at 4°C), and the supernatant fractions containing the cytoplasmic proteins were stored at −20°C. To ensure that fractionation had occurred properly, enzyme activities of the marker proteins, β-galactosidase (41) for the cytoplasmic fractions and alkaline phosphatase (7) for the periplasmic fractions, were measured.

Purification of the recombinant endoxylanase. Five hundred milliliters of *E. coli* cells harboring pSYL2 was grown at 37°C in LB broth plus ampicillin overnight and harvested by centrifugation (6,000 × *g* for 10 min at 4°C). Cells were suspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride and disrupted by sonication (or in a French press), and cell debris were removed by centrifugation (12,000 × *g* for 20 min). The supernatant was heated (70°C for 10 min) and cooled to 4°C, and denatured proteins were removed by centrifugation (12,000 × *g* for 20 min). Proteins from the enzyme preparation were separated by nondenaturing polyacrylamide gel electrophoresis, (PAGE), and the xylanase activity was localized by the activity stain method of Biely et al. (3). The reactive band was further purified by electroelution using a model 422 Electro-Eluter (Bio-Rad, Hercules, Calif.). Protein concentrations were determined by the method of Bradford (6), with bovine serum albumin (Bio-Rad) as a standard.

Electrophoresis. Electrophoretic analysis was performed with the Phast system (Pharmacia, Piscataway, N.J.) (27). A high-molecular-weight electrophoresis calibration kit (Pharmacia) was used as a standard in SDS-PAGE (4 to 15% gel) and nondenaturing gradient PAGE (8 to 25% gel). Protein bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad). Isoelectric focusing was carried out with two different pH ranges, 3 to 10 and 4 to 6.5.

DNA sequencing. Insert DNA was sequenced by the dideoxy-chain termination method (46, 63) in the Molecular Genetics Instrumentation Facility, Department of Genetics, University of Georgia. The sequence information was analyzed using the software package from the Genetics Computer Group Inc., Madison, Wis. (11).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the GenBank/EMBL data bank and assigned accession number U27183.

RESULTS

Cloning and DNA sequence analysis of *xynA*. PCR probe PCR-231 (3.8 kbp) was obtained by amplification of total *Thermoanaerobacterium* sp. strain JW/SL-YS 485 chromosomal DNA, using primers *xynA*1 and *xynA*2 (see Materials and Methods). PCR-231 was used to probe Southern blots containing JW/SL-YS 485 DNA that had been digested with several restriction endonucleases. A 4.6-kb DNA fragment from the *Eco*RI-digested DNA was identified, isolated from the agarose gel, and ligated to pUC19, and recombinant plasmids were transformed into *E. coli* JM1094. Recombinant clones were replica plated and assayed for xylanase activity. Positive clones were detected with xylan-Remazol brilliant blue (RBB xylan) or as a clearing zone surrounding the colonies on plates containing oat xylan. Plasmid DNA was isolated from colonies with high endoxylanase activity and analyzed by using restriction endonucleases. One recombinant plasmid, pSYL2, containing a 4.6-kb *Eco*RI fragment was selected for further characterization.

The complete nucleotide sequence of the insert in pSYL2 was determined, and one open reading frame of 4,044 bp (*xynA*) encoding a protein composed of 1,348 amino acid residues (estimated molecular mass of 148 kDa) was identified (Fig. 1). There was a region similar to an *E. coli*-like promoter sequence with putative −35 (TTAAT) and −10 (TATATT) sites followed by a potential Shine-Dalgarno sequence (AGG GAG) nine nucleotides from the putative start codon (58). When the insert DNA from pSYL2 was flipped into the reverse orientation by using *Eco*RI, no decrease in enzyme activity was observed. This suggested that *xynA* was being transcribed and translated from its own promoter and not from sequences in pUC19. A hairpin loop structure corresponding to a putative transcriptional terminator was identified six nucleotides downstream of the termination codon (TAA; arrows in Fig. 1). At the DNA level, the *xynA* gene had 38 to 96% similarity to other genes encoding for β-glycanases of family F (data not shown).

TABLE 1. Similarity among amino acid sequences of endoxylanase from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 and family F β-glycanases from other microorganisms

Strain (protein)	Similarity (%)	Identity (%)
Thermoanaerobacterium saccharolyticum B6A-RI (XynA)	92	87
Clostridium stercorarium (Xyn)	64	43
Bacillus sp. strain C-125 (XynA)	61	40
Caldicellulosiruptor saccharolyticus (XynA)	60	37
Aspergillus kawachii (XynA)	56	35
Thermoascus aurantiacus (XynA)	54	30
Pseudomonas fluorescens (XynA)	53	30
Butyrivibrio fibrisolvens (XynB)	52	30
Streptomyces lividans (XlnA)	52	29
Clostridium thermocellum (XynZ)	51	28
Cellulomonas fimi (Cex)	51	28
Cryptococcus albidus (XynA)	50	30
Caldicellulosiruptor saccharolyticus (CelB)	49	27
Butyrivibrio fibrisolvens (XynA)	47	24
Pseudomonas fluorescens (XynB)	45	23
Ruminococcus flavefaciens (XynA)	42	18
Bacillus polymyxa (XynD)	39	18

Amino acid sequence analysis. Analysis of the deduced amino acid sequence revealed several interesting features. First, the deduced amino acid sequence of XynA revealed that the N-terminal end of the 24-kDa protein (S-Q-Y-A-A-F-E-Y-D-R-T-F-N-D) which copurified with the 180-kDa protein from JW/SL-YS 485 is contained within the coding region (Fig. 1, amino acids 1158 to 1171). In fact, the region from nucleotide 3721 (amino acid residue 1158) to the end of the open reading frame encodes a 22-kDa protein. This finding strongly suggests that the 24-kDa protein is a proteolytic breakdown product of 148-kDa protein. Second, comparisons of amino acid sequences of family F β-glycanases from several thermophilic anaerobic bacteria (18, 21, 29, 32, 34, 37, 47, 64), fungi (24, 42, 51, 54), and aerobic bacteria (8, 20, 22, 24, 25) with the deduced amino acid sequence (Fig. 1) of XynA from strain JW/SL-YS 485 showed highest similarity (92%) with the *T. saccharolyticum* XynA (Table 1). XynA from strain JW/SL-YS 485 showed 39 to 64% similarity to the other Xyn proteins (Table 1). Third, 10 highly conserved motifs were observed in multiple sequence alignments of the catalytic domains of these enzymes (Fig. 2) (29, 39). Fourth, a 32-amino-acid signal peptide (underlined in Fig. 1) was identified and contained a typical signal peptidase I Ala-X-Ala processing site (▲ in Fig. 1). A signal peptide of this length is typical in gram-type positive bacteria and would be expected to be present in secreted proteins.

Finally, the deduced amino sequence revealed very interesting features at the C-terminal end of XynA. There were three repeats of 59 (S-layer repeat 1 [SLR1]), 64 (SLR2), and 57 (SLR3) amino acids which showed 52% similarity and 32% identity to themselves (dashed lines in Fig. 1). In addition, these repeats were homologous to repeats in the C-terminal ends of other xylanases, such as the endoxylanases from *T. saccharolyticum* B6A-RI (29), *Butyrivibrio fibrisolvens* (32), and

FIG. 2. Multiple sequence alignment between the catalytic domains of *Thermoanaerobacterium* sp. strain JW/SL-YS 485 XynA and seven family F β-glycanases. TsXynA, *T. saccharolyticum* B6A-RI XynA (29); 485-xynA, *Thermoanaerobacterium* sp. strain JW/SL-YS 485 XynA (this study); BaXynA, *Bacillus* sp. strain C-125 XynA (23); CstXyn, *Clostridium stercorarium* Xyn (18); CsaXynA, *Caldicellulosiruptor saccharolyticus* XynA (34); CsaCelB, *Caldocellosiruptor saccharolyticus* CelB (47); CfCex, *Cellulomonas fimi* Cex (8); CtXynZ, *C. thermocellum* XynZ (21). Alignment was performed using the PILEUP program (software package from the Genetics Computer Group Inc.). The consensus residues are shown in boldface; highly conserved regions are indicated by boxes.

Cryptococcus albidus (42) and the exoxylanase from *C. thermocellum* (43a) (Fig. 3A [SLR1-like sequences], B [SLR2-like sequences], and C [SLR3-like sequences]). It should be noted that SLR1-, SLR2-, and SLR3-like sequences appear in the same order in these proteins except for repeats R1 and R2 of the cellulase from *Bacillus* sp. strain KSM-635, which are in reverse order (Fig. 3A and B).

Sequence homology, although not as significant, was observed in other extracellular enzymes, such as the pullulanase from *T. thermosulfurigenes* EM1 (39), the cellulase from *Bacillus* sp. strain KSM-635 (43), and the putative cellulosome-anchoring protein from *C. thermocellum* (17) (Fig. 3). The S-layer repeats also showed some sequence homology to the N-terminal segments of the S-layer proteins from *Thermotoga maritima* (13), *Thermus thermophilus* (15), *Thermoanaerobacter kivui* (45), *Bacillus stearothermophilus* (26), and *Bacillus sphaericus* (5), *Bacillus brevis* (57), and *Bacillus anthracis* (14). However, only one or two repeats were identified from the S-layer proteins. It is possible that these S-layer repeats provide a region of the protein that is capable of direct interaction with S-layer proteins on the cell surface.

Cellular localization of XynA in *E. coli*. Preliminary localization experiments showed that >95% of endoxylanase activity in *E. coli* was associated with the soluble fraction. This finding raised the question of whether *E. coli* was accumulating the enzyme in the cytoplasm or recognizing the export signal and translocating the endoxylanase to the periplasmic space. Therefore, subcellular fractions from *E. coli* cells harboring pSYL2 were isolated by spheroplasting and assayed for β-galactosidase (a soluble marker), alkaline phosphatase (a periplasmic marker), and endoxylanase activities. Eighty-six percent of β-galactosidase activity was found in the cytoplasmic fraction, and the remaining 14% was found in the periplasmic fraction. On the other hand, 85% of the alkaline phosphatase activity was present in the periplasmic fraction, while 15% was in the cytoplasmic fraction. The majority of the endoxylanase activity (65%) was found in the periplasm, while 35% of the activity was cytoplasmic, suggesting that *E. coli* was

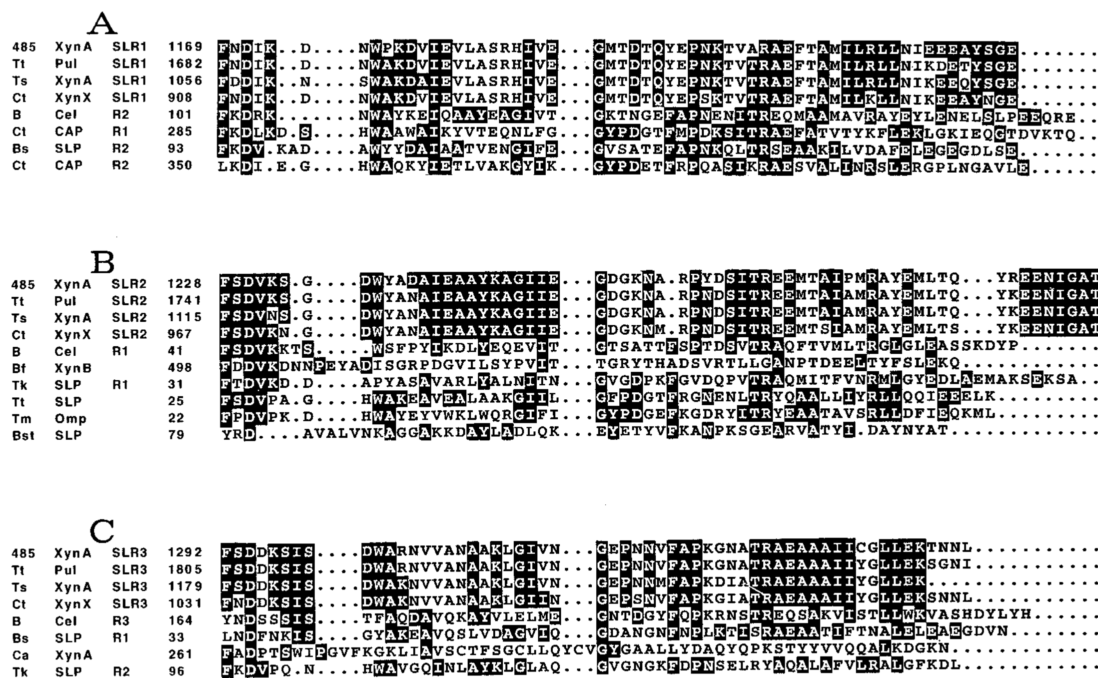

FIG. 3. Alignment of the C-terminal repeats from eight extracellular enzymes and the N-terminal repeats from five S-layer proteins. Identical residues are shown in black boxes. (A) Comparison of SLR1 from strain JW/SL-YS 485 with other SLR1-like sequences from other enzymes or S-layer proteins; (B) comparison of SLR2 with SLR2-like sequences; (C) comparison of SLR3 with SLR3-like sequences. Organisms and proteins are abbreviated as follows: 485 XynA, *Thermoanaerobacterium* sp. strain JW/SL-YS 485 endoxylanase (this study); Tt Pul, *T. thermosulfurigenes* EM1 pullulanase (39); Ts XynA, *T. saccharolyticum* B6A-RI endoxylanase (29); Ct XynX, *C. thermocellum* exoxylanase (38); B Cel, *Bacillus* sp. strain KSM-635 alkaline cellulase (43); Ct CAP, *C. thermocellum* cellulosome-anchoring protein (17); Bs SLP, *Bacillus sphaericus* S-layer protein (5); Bf XynB, *Butyrivibrio fibrisolvens* endoxylanase (32); Tk SLP, *Thermoanaerobacterium* (*Acetobacterium*) *kivui* S-layer protein (45); Tt SLP, *Thermus thermophilus* S-layer protein (15); Tm Omp, *Thermotoga maritima* outer membrane protein α precursor (13); Bst SLP, *Bacillus stearothermophilus* S-layer protein (26); Ca XynA, *Cryptococcus albidus* endoxylanase (42).

translocating a significant amount of recombinant enzyme across the cytoplasmic membrane.

Purification and characterization of XynA. Recombinant endoxylanase was purified from *E. coli* JM109 harboring plasmid pSYL2. Heat treatment increased the specific activity of the enzyme approximately 4.5-fold and drastically reduced the amount of host cell protein. A homogeneous recombinant endoxylanase was obtained after electroelution of the heat-treated enzyme preparation. An approximate molecular mass of 150 kDa was estimated from SDS-PAGE and nondenaturing PAGE, indicating that the recombinant enzyme is a monomer. Also, the purified recombinant enzyme contained no detectable carbohydrate, suggesting that *E. coli*, unlike strain JW/SL-YS 485, was not glycosylating the endoxylanase. The isoelectric point of the enzyme was pH 4.5. The apparent pH optimum for enzyme activity at 75°C was pH 6.3 (Fig. 4a). The apparent temperature optimum for enzyme activity at pH 6.4 was 80°C (Fig. 4b). At pH 6.4 in the absence of substrates, the enzyme was stable at 65°C for more than 2 h (Fig. 5) and had a half-life of 1 h at 77°C. Neither the recombinant nor the native enzyme had any detectable activity against carboxylmethyl cellulose.

DISCUSSION

Previously, we have reported on the isolation and characterization of the endoxylanase from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 (48). The enzyme appeared to be composed of two heterosubunits of 180 and 24 kDa, it had a pI of 4.37 and a half-life of 1 h at 70°C, and maximal activity was observed at pH 6.2 at 80°C. These physical and biochemical properties are slightly different from those of recombinant enzyme reported here. One obvious difference that is resolved by analysis of the recombinant XynA and its sequence is that the 24-kDa subunit is most likely a proteolytic breakdown product of the larger subunit that does not separate during purification. This conclusion makes sense in light of the fact that this region of the 24-kDa protein contains the S-layer repeats that are thought to be involved with self-assembly of the endoxylanase to the S-layer.

There are two possible explanations for the other observed differences. First, the recombinant enzyme may be an endoxylanase different from that of strain JW/SL-YS 485. Multiplicity of endoxylanases is common in microorganisms (10). For example, five different xylanases have been purified from *Aspergillus niger* 11 (60), while at least three xylanases each have been isolated from *Clostridium stercorarium*, *Streptomyces* sp. strain 3137, *Streptomyces exfoliatus* MC1, *Trichoderma harzianum* E58, *Trichoderma reesei* QM9414, *Aeromonas* sp. strain 212, *Penicillium janthinellum*, and *Talaromyces byssochlamydoided* YH-50 (60). This phenomenon appears to be due to inaccessibility of linkages and the heterogeneity of the xylan polysaccharide. Microbes deal with this variation in substrate by secreting several specialized xylanases. However, this may not be the case for strain JW/SL-YS 485. Shao et al. (48) reported that only one thermostable endoxylanase activity was observed in this strain during enzyme purification and after different growth conditions using native gel electrophoresis and activity staining of crude extracts.

Second, the observed differences between the recombinant and native endoxylanases may arise from posttranslational modification, such as glycosylation. Many xylanases are glyco-

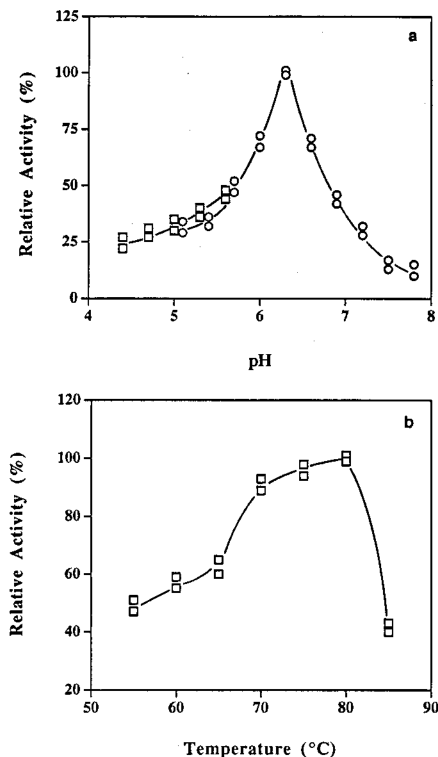

FIG. 4. Effects of pH and temperature optima of the recombinant endoxylanase. (a) Enzyme was assayed in 100 mM sodium acetate buffer at pH 4.4 to 5.6 (□) and 100 mM potassium phosphate buffer at pH 5.1 to 7.8 (○) at 70°C for 10 min (pH was measured at 70°C). The 100% activity corresponds to 15.7 U/mg of protein; each assay was performed with 17 μg of protein per ml. (b) Enzyme activity was assayed in 100 mM potassium phosphate buffer at pH 6.4 at 55 to 85°C for 5 min. The 100% activity corresponds to 12.6 U/mg of protein; each assay was performed with 17 μg of protein per ml.

sylated, and in this case, posttranslational glycosylation could be the reason for the observed differences. The purified enzyme from strain JW/SL-YS 485 contains 6% carbohydrate (48), which is not observed with the recombinant enzyme because *E. coli* does not glycosylate proteins. Therefore, the difference in apparent molecular mass (180 kDa for the native enzyme, 150 kDa for the recombinant enzyme) as well as the slight differences in biochemical properties could result from the lack of glycosyl residues as well as a slight overestimation of the molecular mass by SDS-PAGE and gel filtration. Since these methods are less accurate than obtaining the molecular mass from the deduced sequence, we assume that despite the above-mentioned differences, the enzyme isolated by Shao et al. (48) and the recombinant enzyme are the same.

Previously characterized microbial xylanases vary in molecular mass (either determined by SDS-PAGE or calculated from the deduced amino acid sequences) from 29 to 130 kDa, with the majority of family F xylanases having molecular masses of 40 and 50 kDa, and all are monomers (8, 18, 20–22, 24, 25, 30, 32, 34, 37, 42, 51, 54, 64). Among them, only xylanases from *Ruminococcus flavefaciens* (90 kDa) (64), *C. thermocellum* (92 kDa) (21), and *T. saccharolyticum* B6A-RI (130 kDa) (30) are larger than 90 kDa. From the results of denaturing and nondenaturing PAGE, as well as the deduced amino acid sequence, the recombinant endoxylanase from strain JW/SL-YS 485 was determined to be a monomer with an approximate molecular mass of 150 kDa. This is the largest microbial xylanase yet described.

Endoxylanases are thought to be secreted enzymes, and as expected, the first 32 amino acid residues of the endoxylanase from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 carried typical characteristics of a signal peptide (59). There is a positively charged amino acid (Lys-2) within the first four amino acids, a hydrophobic core sequence, and a possible signal peptidase I cleavage site (Ala-32). Since the recombinant endoxylanase was located mainly in the periplasmic fraction of *E. coli*, it seems that the signal peptide of endoxylanase from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 was being recognized and processed by *E. coli*. Some xylanases cloned in *E. coli* have also been found to be transported to the periplasm (30, 53, 61), while others were mainly located in the cytoplasm (33, 44, 62). It is, however, unusual for *E. coli* to translocate such a large recombinant protein (148 kDa).

The xylanase isolated from strain JW/SL-YS 485 was characterized as a cell-associated enzyme which was found in the S-layer fraction of cells (48). Crystalline bacterial cell surface layers have been identified as an outermost cell envelope component of numerous eubacteria and represent an almost universal feature of archaebacteria (40). The oblique, trimeric, square, and hexagonal lattices of mostly monolayered arrays are formed through the self-assembly of identical protein or glycoprotein subunits with molecular masses ranging from 30 to 200 kDa. S-layers can form the only cell wall layer, or they can be part of complex cell walls. The localization of extracellular enzymes to this outermost cell structure would be advantageous because the enzyme could function to mediate a close association between the living cell and a high-molecular-weight substrate.

Fujino et al. (17) and Matuschek et al. (39) have proposed a hypothetical model for the attachment of extracellular enzymes to the cell surface. Because of the presence of structurally homologous sequences, the C-terminal domain of the extracellular enzymes might be able to integrate into the S-layer lattice. Domains structurally homologous to S-layer proteins which were suggested to be involved in the recognition mechanism were described for the pullulanase of *T. thermosulfurigenes* EM1 (39), the cellulosome of *C. thermocellum* (17), and the endoxylanase of *T. saccharolyticum* (35). The XynA from strain JW/SL-YS 485 contains three S-layer sequences, SLR1, SLR2, and SLR3, that show significant homology to these other S-layer-like sequences. Because of the localization of this enzyme to that structure, it is possible that these repeats are

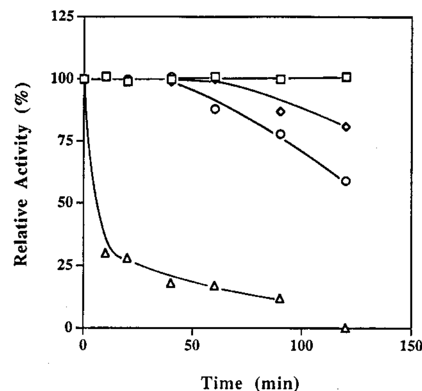

FIG. 5. Thermostability of purified recombinant endoxylanase. Residual activities were assayed in 100 mM potassium phosphate buffer (pH 6.4) at 75°C for 5 min after preincubation of the enzyme in 100 mM potassium phosphate buffer (pH 6.4) at 65°C (□), 70°C (◇), 75°C (○), and 80°C (△). The 100% activity corresponds to 13.9 U/mg of protein.

involved in that association. However, the specific interactions between conserved S-layer-like domains of these proteins or XynA from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 and the S-layer proteins from the same strains remain to be determined (56). It should be noted that the repeated S-layer-like sequences are found mainly in enzymes from thermophiles (12). It is not known, however, whether this is an adaptation to an environment (such as hot springs) in which free extracellular enzyme can quickly diffuse away from the organism producing it without benefiting the organism.

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