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Two Extremely Thermostable Xylanases of the Hyperthermophilic Bacterium *Thermotoga maritima* MSB8

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During growth with xylose or xylan as the source of carbon, xylanase production by *Thermotoga maritima* MSB8 was enhanced about 10-fold compared with growth with glucose or starch. Two extremely thermostable endoxylanases (1,4-β-D-xylan-xylanohydrolase, EC 3.2.1.8), designated XynA and XynB, were identified and purified from cells of this organism. XynA and XynB occurred as proteins with apparent molecular masses of about 120 and 40 kDa, respectively, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Maximum activity at the optimal pH (pH 6.2 and pH 5.4 for XynA and XynB, respectively) was measured at about 92°C for XynA (10-min assay) and at about 105°C for XynB (5-min assay). XynB activity was stimulated twofold by the addition of 500 mM NaCl, while XynA displayed maximum activity without the addition of salt. Both xylanases were tolerant of relatively high salt concentrations. At 2 M (about 12% wt/vol) NaCl, XynA and XynB retained 49 and 65%, respectively, of their maximum activities. In contrast to XynB, XynA was able to adsorb to microcrystalline cellulose. Antibodies raised against a recombinant truncated XynA protein cross-reacted with XynB, indicating that the enzymes may have sequence or structural similarities. Part of the xylanase activity appeared to be associated with the outer membrane of *T. maritima* cells, since more than 40% of the total xylanase activity present in the crude cellular extract was found in the membrane fraction after high-speed centrifugation. Most of the membrane-bound activity appeared to be due to the 120-kDa xylanase XynA.

Thermotoga maritima, one of the few hyperthermophilic representatives of the domain of *Bacteria* described to date, is able to thrive at temperatures of up to 90°C and does so via a fermentative metabolism (10, 16). Since no organotrophic bacteria which have a higher temperature optimum of growth are known, it is of interest to investigate the carbohydrase outfit of this organism (7, 11, 12). One of the carbohydrate polymers utilized efficiently by *T. maritima* MSB8 (DSM 3109) is xylan.

Xylan, an abundant component of plant biomass, has a relatively complex structure based on a nonbranched β-1,4-glycosidically linked xylose backbone. Depending on the origin, the backbone structure is substituted to various degrees with acetyl, L-arabinofuranosyl, glucuronyl, or 4-*O*-methylglucuronyl side chain groups (4). Typically, backbone depolymerization is accomplished by the action of endoxylanases (1,4-β-D-xylan-xylanohydrolase, EC 3.2.1.8) and β-xylosidases (β-D-xyloside-xylohydrolase, EC 3.2.1.37). These two enzyme activities, which constitute the basic xylanolytic enzyme complement of microorganisms capable of xylan utilization, are also detectable in crude extracts of *T. maritima* MSB8 cells (2a) (unpublished results). In this communication, we report the occurrence of two distinct endo-β-1,4-xylanases, XynA and XynB, in crude extracts of *T. maritima* and report their purification and properties. Xylanases are believed to have considerable potential in biotechnological processes, such as the large-scale industrial manufacture of pulp and paper (4). Highly thermostable xylanases such as those described here may be particularly interesting for these applications.

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MATERIALS AND METHODS

Bacterial strains and crude-extract preparation. The type strain of *T. maritima* MSB8 (DSM 3109) was used throughout this study. *T. maritima* was grown anaerobically in Difco marine broth (MB) medium (catalog no. 2216; Difco, Detroit, Mich.) supplemented with 0.25% soluble starch or xylose (Merck, Darmstadt, Germany), 0.5% Na_2S, and 0.0001% resazurin, pH 7.0. For large batch cultures, cells from a fresh overnight culture were inoculated into MBR medium, a reduced medium consisting of 0.5% peptone, 0.1% yeast extract, 0.1% soluble starch, 1% (vol/vol) MB medium, 3% NaCl, 0.5% Na_2S, and 0.0001% resazurin dissolved in tap water and preheated to 80°C. Crude extracts of *T. maritima* cells grown with xylose as the source of carbon were prepared by passing suspensions of cells in 20 mM Bis-Tris buffer (pH 6.2) through a French pressure cell (American Instrument Company, Silver Spring, Md.) at 6.9 MPa.

Enzyme assays and analytical methods. Xylanase activity was determined by measuring the increase in reducing groups during the enzymatic hydrolysis of xylan by the dinitrosalicylic acid method of Bernfeld (2). Standard assay mixtures (500 μl) contained 0.8% oat spelts xylan (Roth, Karlsruhe, Germany), 250 mM NaCl, and 50 mM Bis-Tris buffer (pH 6.2) for XynA or 0.8% oat spelts xylan, 500 mM NaCl, 0.5% bovine serum albumin, and 50 mM sodium citrate buffer (pH 5.4) for XynB, plus appropriately diluted enzyme. Unless mentioned otherwise, incubation conditions were 10 min at 75°C (XynA) or 5 min at 90°C (XynB), after which the reaction was stopped by the addition of 0.75 ml of dinitrosalicylic acid solution (2). After color development for 20 min at 95°C and centrifugation of the samples (5 min, 15,000 × *g*), the A_{575} was determined. One unit of xylanase activity is the amount of enzyme which liberates 1 μmol of reducing groups (as xylose equivalents) per min. For the determination of enzyme activity versus pH (at 75°C for XynA or 90°C for XynB) or temperature (at pH 6.2 for XynA or pH 5.4 for XynB) profiles, the assay mixtures contained 250 μl of 0.2 M sodium phosphate-citrate (McIlvaine) buffer, 200 μl of 2% oat spelts xylan, 25 μl (XynA) or 50 μl (XynB) of 5 M NaCl, and 25 μl of enzyme solution. For XynB assays, 0.5% bovine serum albumin was added. Enzyme assays above the boiling point of water were done in high-pressure liquid chromatography sample vials made of glass with rubber seals. After preincubation of the reaction mixture without enzyme at the desired temperature, the reaction was started by injecting the enzyme with a syringe. Thermostability data were obtained by preincubating xylanase samples (20 μg/ml for XynA and 0.9 μg/ml for XynB) at elevated temperatures in 50 mM Bis-Tris (pH 6.2)–250 mM NaCl (for XynA) or 50 mM sodium citrate buffer (pH 5.4)–0.5% bovine serum albumin–250 mM NaCl (for XynB) and then measuring residual activity under the standard assay conditions described above. Previously published methods (11) were used for the determination of protein concentrations and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Thin-layer chromatographic analysis of oligosaccharides was done (11) with a solvent system consisting of chloroform, acetic acid, and H_2O (5:7:1, by volume).

TABLE 1. Xylanase activity in crude cellular extract of *T. maritima* MSB8[a]

Sugar or polysaccharide added	Xylanase activity		
	Total protein (mg)	Total activity (U)	Sp act (U/mg)
None	0.8	1.6	2.0
Glucose	1.8	2.3	1.3
Starch	4.0	2.4	0.55
Xylose	2.0	24	12.2
Xylan	3.1	27	8.8

[a] *T. maritima* was grown in MB broth supplemented with various carbon sources at 0.25% (wt/vol). Total and specific activities are expressed as units of oat spelts xylan-hydrolyzing activity and were determined in 100 mM sodium phosphate-citrate buffer (pH 6.2)–500 mM NaCl at 90°C.

Detection of xylanase bands after SDS-PAGE via activity staining and immunoblot. Xylanase bands resolved by SDS-PAGE were visualized by a zymogram technique. For this purpose, the gels were washed for 20 min at 40°C in McIlvaine buffer (pH 6.2)–25% isopropanol and subsequently in buffer without isopropanol. Then the gels were incubated at 75°C in a 1% oat spelts xylan suspension in the same buffer. After 30 min, the gels were transferred to a 0.1% Congo red solution to stain nonhydrolyzed xylan (20 min). Nonbound dye was washed out of the gels with 1 M NaCl (20 min, 20°C).

For the immunological detection of xylanase bands, proteins were electrophoretically transferred from SDS–10% PAGE gels to a nitrocellulose membrane (Schleicher and Schuell, Dassen, Germany). The membrane was washed at 20°C with blocking buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Tween 20, 0.02% sodium azide) three times for 10 min each and incubated for 16 h in 5 ml of the same buffer containing anti-XynA antiserum diluted 1:1,000. After three washing cycles (10 min each) with blocking buffer, 3 ml of a 1:3,000 dilution of anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate (Sigma, Deisenhofen, Germany) was added, and the incubation was continued for 2.5 h, followed by another series of three washing steps. Detection of specific signals took place by incubation in 15 ml of 100 mM Tris-HCl (pH 9.5)–100 mM NaCl–50 mM $MgCl_2$ supplemented with 53 μl of 50-mg/ml 5-bromo-4-chloro-3-indolyl phosphate in dimethyl formamide and 68 μl of 75-mg/ml nitro blue tetrazolium in 70% dimethyl formamide. Color development was stopped by washing with deionized water. For preparation of the anti-XynA antiserum, a rabbit was immunized with a recombinant truncated XynA derivative consisting of the catalytic domain of the enzyme (corresponds to the central one-third of the protein [20]).

Cellulose binding studies. Microcrystalline (approx. 0.019 mm) cellulose (Avicel PH105; Serva, Heidelberg, Germany) was boiled twice for 30 min each in deionized H_2O before use. This material was added to columns (2.5 by 10 cm) by gravity flow. The columns were washed with deionized H_2O and equilibrated with 10 mM Bis-Tris (pH 6.2)–0.5 M NaCl. *T. maritima* cells grown on xylose were disrupted by French press lysis in 10 mM Bis-Tris (pH 6.2)–0.5 M NaCl supplemented with 0.75% Triton X-100 to solubilize membrane-bound proteins. Ten milliliters of the lysate, which contained 6 mg of total protein per ml, was applied to the column at a flow rate of 1 ml/min. After 16 h at 4°C, the column was washed extensively with 10 mM Bis-Tris (pH 6.2)–0.5 M NaCl. Bound protein was eluted with 0.1 M cellobiose or 0.2 M glucose. The washing fluid and the eluate were concentrated and analyzed by SDS-PAGE and activity staining.

RESULTS

Induction of xylanase activity during growth on xylan and xylose. *T. maritima* MSB8 from a fresh culture grown in MB broth without carbohydrate supplementation was inoculated at 1% into MB medium containing various carbon sources at concentrations of 0.25%. After incubation at 80°C for 24 h, the cells were harvested, washed with 20 mM Bis-Tris buffer (pH 7.0), suspended in the same buffer supplemented with 1% (vol/vol) Triton X-100, and disrupted by passage through a French press cell. Since part of the xylanase was cell bound (see below), the enzyme activity of the crude extracts was measured without prior centrifugational clearing. Compared with *T. maritima* MSB8 cells grown on glucose or starch, the specific xylanase activity of cells cultivated in the presence of xylose or xylan was increased by a factor of 7 to 22, and total xylanase production was increased about 10-fold (Table 1).

Separation and purification of the xylanases from crude cell extracts of *T. maritima*. SDS-PAGE and zymogram staining specific for thermostable xylanolytic enzymes of crude extracts of *T. maritima* MSB8 cells grown on xylose revealed two distinct bands with xylanase activity at about 40 and 120 kDa (Fig. 1A, lane 7; also see Fig. 4, lane 1). The purification of both enzymes, designated XynA and XynB, is summarized in Table 2. If not stated otherwise, all solutions contained 20 mM Bis-Tris (pH 6.2).

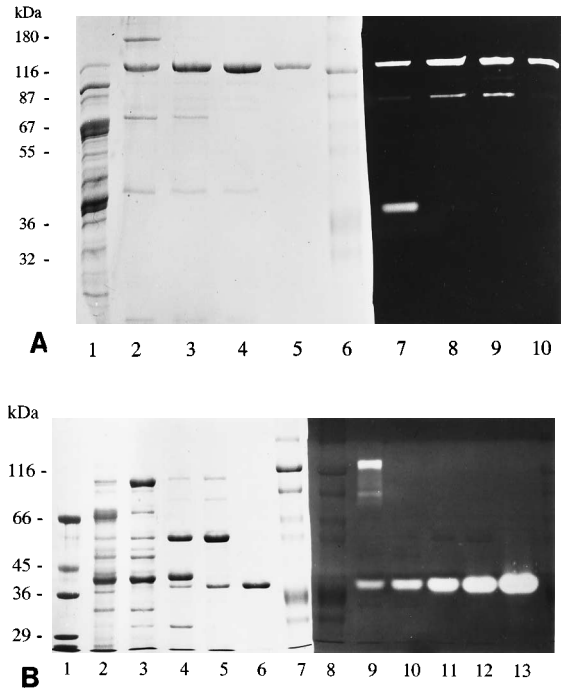

FIG. 1. SDS-PAGE of protein samples after various steps of purification of XynA (A) and XynB (B). The lanes correspond to the purification steps listed in Table 2. (A) Protein samples: lanes 1 and 7, crude extract; lanes 2 and 8, membrane fraction; lanes 3 and 9, after Q-Sepharose chromatography; lane 4, after phenyl-Sepharose chromatography; lanes 5 and 10, after second phenyl-Sepharose chromatography and ultrafiltration. (B) Protein samples: lanes 2 and 9, crude extract; lanes 3 and 10, after Q-Sepharose chromatography; lanes 4 and 11, after phenyl-Sepharose chromatography; lanes 5 and 12, after Mono P chromatography; lanes 6 and 13, after gel permeation chromatography. Lane 6 of panel A and lanes 1 and 7 of panel B contained protein molecular mass standards. The right part of the gel was stained for xylanolytic activity (white halos). Additional activity bands are visible at about 80 and 100 kDa in panel A, lanes 7 to 9. These proteins were also detected with anti-XynA antibodies (see Fig. 3, lanes 1 and 2) and therefore presumably represent truncated XynA derivatives which may have been formed by proteolytic processing.

XynA was purified from 12 g (wet mass) of cells grown with 0.25% xylose as the source of carbon. High-speed centrifugation of the crude extract for 120 min at 140,000 × *g* led to a fivefold increase in xylanase specific activity in the membrane pellet. SDS-PAGE analysis of the crude extract and the pellet fraction (Fig. 1A, lanes 7 and 8) indicated that the sedimentation of the high-molecular-mass xylanase XynA was responsible for this effect. Therefore, this method was used as the first purification step in the XynA isolation protocol (Table 2). The xylanase activity in the membrane fraction was solubilized in 50 ml of Bis-Tris (pH 6.2)–2% Triton X-100, loaded onto a Q-Sepharose Fast Flow (Pharmacia, Freiburg, Germany) 26/30 column equilibrated with 20 mM Bis-Tris (pH 6.2), and eluted with a linear 0 to 1,000 mM NaCl gradient. XynA was further purified with two hydrophobic interaction chromatography steps on a Phenyl-Sepharose 16/10 column (Pharmacia), using linear NaCl elution gradients of 3,000 to 0 mM and 500 to 0

TABLE 2. Purification of XynA and XynB from crude cellular extracts of *T. maritima* MSB8[a]

Xylanase and purification step	Protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
XynA					
Crude extract	768	7,716	10.0	1.0	100
Membrane fraction	68	3,360	49	4.9	44
Q-Sepharose	22	1,800	81	8.1	23
Phenyl-Sepharose (3–0 M NaCl)	2.0	370	185	18.5	4.8
Phenyl-Sepharose (0.5–0 M NaCl) and ultrafiltration	1.1	337	306	31	4.4
XynB					
Crude extract	428	6,848	16	1.0	100
Q-Sepharose	27	1,072	40	2.5	16.0
Phenyl-Sepharose	3.0	690	230	14.3	10.0
Mono P	0.56	460	821	51	6.7
Gel filtration	0.052	239	4,600	288	3.5

[a] Total and specific activities are expressed as units of oat spelts xylan-hydrolyzing activity and were determined in 100 mM sodium phosphate-citrate buffer (pH 6.2) at 90°C and in 50 mM sodium citrate buffer (pH 5.4)–1% bovine serum albumin at 90°C for XynA and XynB, respectively. Please note that the starting material for XynA and XynB isolation, i.e., *T. maritima* crude extract, contains two different xylanases. Therefore, the values for purification factor and yield given are underestimates of the real situation.

mM, respectively. The final purification step was ultrafiltration (membrane cutoff, 100,000 Da).

XynB was isolated from a different batch (about 6.5 g, wet mass) of *T. maritima* cells grown in MBR broth containing xylose. In this case, the crude extract obtained by French press disruption was cleared by centrifugation (10,000 × *g*, 20 min, 4°C), and then the supernatant was loaded onto a Q-Sepharose Fast Flow 26/30 column and eluted as described above for XynA. The fractions with xylanase activity were applied to a phenyl-Sepharose 16/10 column after adjusting the salt concentration to 3 M. Elution was achieved with a linear 3,000 to 0 mM NaCl gradient. Next, the enzyme was loaded onto a Mono-P 5/5 column (Pharmacia) preequilibrated with 25 mM Bis-Tris (pH 6.2). The XynB fractions eluted with 1:20-diluted Polybuffer (Pharmacia), pH 4.0, at a flow rate of 0.5 ml/min were pooled and concentrated by ultrafiltration (membrane cutoff, 30,000 Da), and the buffer was changed to 0.2 M potassium phosphate (pH 6.2). XynB was separated from remaining contaminant proteins by gel permeation chromatography on a HiLoad 16/60 Superdex 200 column (Pharmacia) equilibrated with 0.2 M potassium phosphate buffer (pH 6.2) at a flow rate of 0.5 ml/min. The amino-terminal sequence of XynB was Phe-Gln-Asn-Val. This string is not present in the amino acid sequence of XynA (20).

Biochemical properties of the purified enzymes. The sizes of XynA and XynB as determined by SDS-PAGE were 120 and 40 kDa, respectively. During SDS-PAGE, XynA comigrated with the recombinant enzyme purified from *Escherichia coli* WCM105(p76) (20). Determination of the sizes of the native enzymes via analytical gel filtration on a HiLoad 16/60 Superdex 200 column (Pharmacia) was hampered by the fact that both enzymes had greater elution volumes than expected on the basis of the SDS-PAGE results (apparent size by gel filtration was 28 kDa for both XynA and XynB). This retardation phenomenon was presumably due to interaction of the enzymes with the column matrix.

Kinetic analysis of the reaction products released during xylan hydrolysis via thin-layer chromatography indicated that XynA and XynB act via an endo-type mechanism, releasing a variety of substituted and nonsubstituted xylooligosaccharides (data not shown). The major xylooligomers found after extensive hydrolysis of oat spelts xylan were xylotriose and xylobiose, but some monomeric xylose was also formed. The effects of pH, temperature, and sodium chloride concentration on the activity of XynA and XynB are summarized in Fig. 2. While XynA did not require the addition of NaCl for maximum activity, XynB activity was stimulated by NaCl addition.

XynA and XynB behaved similarly with respect to the hydrolysis of various polysaccharides (Table 3). Xylans were hydrolyzed most efficiently, followed by the mixed-linkage (β-1,3/β-1,4) glucans barley β-glucan and lichenan. XynB also displayed significant hydrolysis of laminarin, a β-1,3-linked glucan. Various other polysaccharides, including microcrystalline cellulose and carboxymethyl cellulose, were not hydrolyzed.

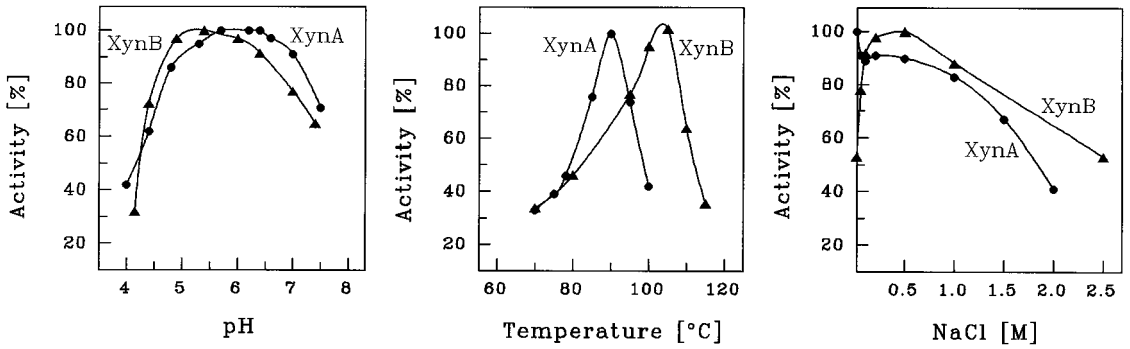

FIG. 2. Effects of pH, temperature, and NaCl concentration on the activities of XynA (●) and XynB (▲). The maximal activity in each curve was defined as 100%.

TABLE 3. Comparison of XynA and XynB

Characteristic	XynA	XynB
Molecular mass	120 kDa	40 kDa
Isoelectric point	ND[a]	5.6
Optimum pH	6.2	5.4
Optimum temp (5-min assay)	92°C	105°C
Thermal stability (half-life) at:		
75°C	Stable	ND
90°C	45 min[b]	>180 min
95°C	ND	125 min
Optimum NaCl concn	0–250 mM	500 mM
Kinetic parameters (substrate: oat spelts xylan, Roth)		
Assay at 75°C		
K_m	0.11%	ND
V_{max}	109 U/mg	ND
Assay at 90°C		
K_m	0.11%	0.029%
V_{max}	374 U/mg	4,600 U/mg
Cellulose binding	+	−
Substrate specificity		
Polymeric substrates[c] (relative activity, %)		
Xylan (oat spelts, Roth)	100	100
Xylan (oat spelts, Sigma)	120	110
Xylan (birch wood, Sigma)	87	85
4-*O*-Methylglucuronoxylan (Sigma)	118	84
Xylan (larch wood)	96	82
β-Glucan	53	40
Lichenan	15	37
Laminarin	ND	8
Microcrystalline cellulose (Avicel, Serva) (24-h assay)	0	0
Carboxymethyl cellulose (Sigma)	0	0
Mannan	0	0
Arabinogalactan	0	0
Pullulan	0	0
Amylose	ND	0
Starch	0	ND
Nitrophenyl glycosides[d] (U/mg)		
pNP-β-D-Cellobioside	4.7	200
oNP-β-D-Cellobioside	9.9	166
pNP-β-D-Xylopyranoside	0.1	17
oNP-β-D-Xylopyranoside	7.5	216
pNP-β-D-Fucoside	2.0	26
pNP-α-D-Arabinofuranoside	0.2	0
pNP-β-D-Glucopyranoside	0	1.2

[a] ND, not determined.
[b] This value was estimated from the initial rate of thermal denaturation observed during the first 45 min. During further incubation at 90°C, the residual activity remained relatively constant at 50 to 60% for at least 90 min (repeated experiments). The reason for this phenomenon is presently unknown.
[c] Activity on Roth oat spelts was defined as 100%.
[d] pNP, *p*-nitrophenyl; oNP, *o*-nitrophenyl.

Both enzymes hydrolyzed various nitrophenyl glycosides. However, XynB was much more active towards these low-molecular-mass substrates than XynA (Table 3). The enzymes differed significantly in their catalytic efficiencies during the hydrolysis of oat spelts xylan. The apparent substrate affinities and the maximum initial velocities were much higher for XynB than for XynA, i.e., XynB had an approximately 4-fold-lower apparent K_m than XynA and an approximately 12-fold-higher V_{max} (Table 3).

Immunological comparison of the xylanases. Western (immunoblot) experiments were carried out to see if both xylanases were structurally related. Rabbit antibodies raised against the catalytic domain of recombinant XynA (corresponds to the central one-third of the enzyme [20]) displayed significant cross-reactivity with XynB protein (Fig. 3). Despite the immunological similarity, XynB is probably encoded by a different gene than XynA because the enzymatic properties of the enzymes differ widely (see above).

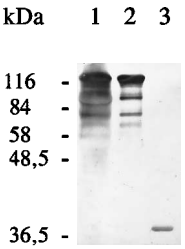

FIG. 3. Immunoblot analysis of protein fractions containing XynA and/or XynB with anti-XynA antibodies. Lane 1, *T. maritima* crude extract (100 μg of protein); lane 2, 2.7 μg of partially purified XynA enzyme preparation containing approximately 2 μg of XynA (the same sample as in lane 4 of Fig. 1A); lane 3, 7-μg protein sample containing about 1.25 μg of XynB (the same sample as in lane 5 of Fig. 1B). The migration positions of protein molecular mass standards are indicated. The additional bands appearing at about 80 and 100 kDa in lanes 1 and 2 are presumably truncated XynA derivatives. These proteins also have xylanase activity (see Fig. 1A, lanes 7 to 9).

Cellulose binding properties of XynA and XynB. A crude extract of *T. maritima* MSB8 cells grown with xylose as the source of carbon was prepared and loaded onto a microcrystalline cellulose column as described in Materials and Methods. Nonbound proteins were washed from the column and collected. Proteins which had specifically adsorbed to the cellulose matrix could be eluted with 0.2 M glucose (or, alternatively, with 0.1 M cellobiose). The nonbound proteins and the eluted proteins were concentrated via ultrafiltration (10,000-Da cutoff) and subjected to SDS-PAGE followed by

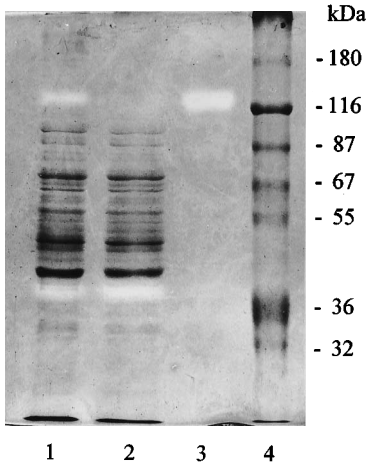

FIG. 4. Cellulose binding properties of XynA and XynB. A crude extract of *T. maritima* cells grown with xylose as the source of carbon was prepared and applied to a cellulose column as described in Materials and Methods. The fractions obtained were analyzed by SDS-PAGE and xylanase-specific activity staining. Subsequently, the gel was stained for proteins with Coomassie brilliant blue. White halos indicate xylanase activity, and dark bands represent proteins. Lane 1, *T. maritima* crude extract (15 μg); lane 2, proteins which passed through the column without binding (15 μg); lane 3, proteins eluted with 0.2 M glucose (1 μg); lane 4, protein molecular mass standards.

xylanase activity staining of the gel (Fig. 4). The XynA activity present in the crude extract appeared to bind to microcrystalline cellulose quantitatively, while most of XynB passed through the column without being retained.

DISCUSSION

T. maritima MSB8 regulates the synthesis of xylanolytic enzymes in response to the presence of xylan or xylose as a carbon source in the culture medium (Table 1). Two distinct endoxylanases (1,4-β-D-xylan-xylanohydrolase, EC 3.2.1.8) contribute to the total xylanolytic activity produced during growth of this organism on xylan or xylose. We were able to separate the two activities by physical (high-speed centrifugation) and protein chromatographic means and have obtained both as highly purified enzyme preparations. XynA (120 kDa) and XynB (40 kDa) are distinguished by various biochemical characteristics (Fig. 2; Table 3). Also, immunoblot experiments (Fig. 3) revealed a merely weak cross-reactivity of XynB with rabbit antibodies raised against the central catalytic part of XynA. Also, the N-terminal amino acid sequence determined for XynB was not present in the primary structure of XynA. Therefore, there is no doubt that *T. maritima* MSB8 xylanases XynA and XynB are encoded by two different genes and that the 40-kDa enzyme cannot be a proteolytic processing product derived from the 120-kDa enzyme. On the other hand, the cross-reactivity of XynB with the anti-XynA antiserum was significant and therefore implies some degree of structural relatedness between the two enzymes. Since the primary structure of XynA (20) proves that this enzyme is a member of family 10 of glycosyl hydrolases (9) (corresponds to β-glycanase family F as defined by Gilkes et al. [8]), it seems likely that XynB belongs to the same enzyme family. It is interesting that most of the highly thermostable xylanases investigated so far belong to this enzyme family. To our knowledge, the list of members of the other xylanase family (family G according to the classification used by Gilkes et al. [8], which corresponds to glycosyl hydrolase family 11 of Henrissat [9]) contains only one enzyme produced by a thermophilic bacterium, i.e., xylanase A of *Clostridium stercorarium* (15).

The 40-kDa xylanase was most active in the slightly acidic pH range between pH 5 and 6, with an optimum at pH 5.4, whereas the pH profile of the 120-kDa enzyme was shifted towards neutral incubation conditions (more than 85% activity between pH 5 and pH 7, with an optimum at pH 6.2). Thus, the pH optima of the *T. maritima* MSB8 enzymes are within the range reported for most xylanases and β-xylosidases (pH 4.0 to 6.7 [3]). One of the most outstanding properties of the xylanases of *T. maritima* is their thermostability. In our assay, XynB displayed maximum activity at 105°C, while maximum XynA activity was measured at about 15°C lower (Fig. 2). Consequently, XynB also had greater long-term thermostability than XynA (Table 2). In terms of thermophilicity, the xylanases described here are matched only by the xylanolytic enzymes of *Thermotoga* sp. strain FjSS3-B.1 (18) and the aerobic thermophile *Rhodothermus marinus* (6). The approximately 40-kDa enzyme XynB of *T. maritima* MSB8 is similar in some biochemical features to the xylanase of *Thermotoga* sp. strain FjSS3-B.1, which, however, was reported to be significantly smaller (31 to 35 kDa).

An extremely interesting facet of the biochemical characterization of the *T. maritima* xylanases was the observed differences in the abilities of XynA and XynB to hydrolyze aryl-β-glycosides (Table 3). In contrast to XynA, XynB displayed high activity with *o*-nitrophenyl and *p*-nitrophenyl β-D-xylosides, which are typical β-xylosidase substrates. With a few exceptions, e.g., the xylanases from *Caldocellum saccharolyticum* (13) and *Talaromyces emersonii* (19), most other xylanases investigated do not cleave aryl β-D-xylosides.

Both xylanases displayed near-optimal activity at about 500 mM NaCl (Fig. 2), which is similar to the salt content of seawater, the natural habitat of *T. maritima*. This observation corresponds well to the fact that *T. maritima* grows most rapidly at an NaCl concentration of about 2.7% (about 460 mM) (10). Both XynA and XynB tolerated relatively high salt concentrations (Fig. 2). The residual activities measured in the presence of 2.0 M (about 12% wt/vol) NaCl were 49 and 65%, respectively. While XynA was maximally active without salt addition, XynB may in fact be regarded as moderately halophilic, since the relative activity was stimulated more than twofold at an NaCl concentration of 500 mM over the basal activity without NaCl. To our knowledge, the effect of high salt concentrations on the activity of xylanases of marine bacteria was not investigated before. However, a thermostable cellobiohydrolase of *Thermotoga* sp. strain FjSS3-B.1 was reported to be stabilized by the presence of 0.8 M NaCl (14). Also, it was observed that the long-term thermostability of two cellulases purified from *T. maritima* MSB8 at 80°C was enhanced in the presence of 2 M NaCl (2a).

The biochemical properties of the xylanases described here, especially their extraordinary stability, and the fact that they do not hydrolyze microcrystalline cellulose or carboxymethyl cellulose (Table 3) indicate that these enzymes are promising candidates for biotechnological applications such as kraft pulp prebleaching or plant fiber processing. For these purposes, however, relatively large quantities of enzyme must be available. The molecular cloning of the 120-kDa xylanase XynA is described elsewhere (20). The enzymatic properties of recombinant XynA expressed in *E. coli* do not differ significantly from those of authentic XynA isolated from *T. maritima* itself, indicating that probably no *Thermotoga*-specific modification, i.e., glycosylation, contributes to the thermostability of the enzyme.

Although xylanases generally cannot hydrolyze insoluble cellulose, some xylanases curiously have cellulose binding domains which enable them to specifically adsorb to this polymer (1, 5, 15). It seems that cellulose-binding xylanases may play an important role in the synergistic cooperation of various endo- and exo-acting β-glycanases necessary for the complete decomposition of complex cellulosic material. XynA of *T. maritima* MSB8 displayed binding affinity to microcrystalline cellulose (Fig. 4). Interestingly, the bound enzyme could be released with 0.2 M glucose or 0.1 M cellobiose (even under high-salt conditions; unpublished results), which is very unusual for cellulose binding proteins. Recombinant XynA also bound to microcrystalline cellulose, and we were able to prove that this property resides within the carboxy-terminal part of the enzyme (20). XynB, on the other hand, apparently lacks a cellulose binding domain, as it did not bind to cellulose.

The xylanases described here were purified from crude extracts prepared by disintegration of *T. maritima* cells. Our cell fractionation data indicate that most of XynA, in contrast to XynB, seems to be membrane associated. Since polymeric xylan molecules probably cannot traverse the outer membrane without prior degradation and intact cells have high xylan-hydrolyzing activity (unpublished results), we conclude that the most probable localization of XynA is the outer membrane (the toga) rather than the cytoplasmic membrane. If the specific activities and purification factors listed in Table 2 are taken as a measure, XynA appears to be a rather abundant protein of the outer membrane, because an approximately sixfold purification was sufficient to obtain an essentially pure

enzyme from the membrane fraction as the starting material (see Table 2). In this context, it is interesting that a localization at the cell surface has also been proposed for the amylolytic activities of *T. maritima* (17). Finally, we have detected both XynA and XynB not only in crude extracts but also in the culture supernatant of *T. maritima* MSB8 (data not shown). The goal of future work will be to study the processing and the precise cellular localization of the xylanases in relation to the unique cell morphology of this microorganism.

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