Characterization of XynC from *Bacillus subtilis* subsp. *subtilis* Strain 168 and Analysis of Its Role in Depolymerization of Glucuronoxylan[▽]

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Secretion of xylanase activities by *Bacillus subtilis* 168 supports the development of this well-defined genetic system for conversion of methylglucuronoxylan (MeGAX_n [where n represents the number of xylose residues]) in the hemicellulose component of lignocellulosics to biobased products. In addition to the characterized glycosyl hydrolase family 11 (GH 11) endoxylanase designated XynA, *B. subtilis* 168 secretes a second endoxylanase as the translated product of the *ynfF* gene. This sequence shows remarkable homology to the GH 5 endoxylanase secreted by strains of *Erwinia chrysanthemi*. To determine its properties and potential role in the depolymerization of MeGAX_n, the *ynfF* gene was cloned and overexpressed to provide an endoxylanase, designated XynC, which was characterized with respect to substrate preference, kinetic properties, and product formation. With different sources of MeGAX_n as the substrate, the specific activity increased with increasing methylglucuronosyl substitutions on the β -1,4-xylan chain. With MeGAX_n from sweetgum as a preferred substrate, XynC exhibited a $V_{\max}$ of 59.9 units/mg XynC, a K_m of 1.63 mg MeGAX_n/ml, and a k_{cat} of 2,635/minute at pH 6.0 and 37°C. Matrix-assisted laser desorption ionization—time of flight mass spectrometry and ¹H nuclear magnetic resonance data revealed that each hydrolysis product has a single glucuronosyl substitution penultimate to the reducing terminal xylose. This detailed analysis of XynC from *B. subtilis* 168 defines the unique depolymerization process catalyzed by the GH 5 endoxylanases. Based upon product analysis, *B. subtilis* 168 secretes both XynA and XynC. Expression of *xynA* was subject to MeGAX_n induction; *xynC* expression was constitutive with growth on different substrates. Translation and secretion of both GH 11 and GH 5 endoxylanases by the fully sequenced and genetically malleable *B. subtilis* 168 recommends this bacterium for the introduction of genes required for the complete utilization of products of the enzyme-catalyzed depolymerization of MeGAX_n. *B. subtilis* may serve as a model platform for development of gram-positive biocatalysts for conversion of lignocellulosic materials to renewable fuels and chemicals.

Biocatalyst production of value-added products and fuel ethanol from lignocellulosics is being developed as an alternative to traditional chemical synthesis methods (24, 28, 39, 54, 59, 65). Hardwood and crop residues are attractive underutilized lignocellulosic resources which could be used for the generation of fermentable hexoses and pentoses, the former resulting from the cellulose fraction as glucose and the latter from the hemicellulose fraction as xylose with minor amounts of arabinose. The primary component of hemicellulose in hardwood and crop residue is 4-*O*-methyl- β -D-glucuronoxylan (MeGAX_n [where n represents the number of xylose residues]), in which xylose residues in the β -D-1,4-xylan polymer are substituted periodically with α -1,2-linked 4-*O*-methyl- β -D-glucuronopyranosyl (MeGA) residues (27, 49, 61). Significant limitations with current lignocellulose bioconversion lie in the pretreatment process required to liberate utilizable sugars from the complex hemicellulose fraction, and there is increasing interest in developing endoxylanases to enhance the release of fermentable pentoses (49). Enzyme systems in gram-positive spore-forming bacteria, e.g., *Geobacillus stearothermophilus* (55) and *Paenibacillus* sp. strain JDR-2 (58; G. Nong, V. Chow, J. D. Rice, F. St. John, and J. F. Preston, Abstr. 105th Gen. Meet.

Am. Soc. Microbiol. 2005, abstr. O-055, 2005), that allow efficient depolymerization and assimilation of MeGAX_n and complete catabolism of xylose and MeGA have been identified. The expression of these MeGAX_n utilization systems in microorganisms is needed to develop biocatalysts for the efficient conversion of the hemicellulose fraction to biobased products and alternative fuels.

Genomic review of *Bacillus subtilis* 168 reveals the presence of genes encoding enzymes which participate in the degradation of plant cell wall polysaccharides (<http://afmb.cnrs-mrs.fr/CAZY/>). To characterize the ability of *B. subtilis* 168 to utilize MeGAX_n and determine a starting point for engineering of MeGAX_n utilization enzyme systems, we assessed the genomic data available for possible MeGAX_n hydrolytic enzymes. We found no protein homolog for a glycosyl hydrolase family 10 (GH 10) β -xylanase or a GH 67 α -glucuronidase, both of which are presumed to be required for complete utilization of MeGAX_n (49, 55, 58). The *xynA* gene in *B. subtilis* 168 has been shown to encode a GH 11 xylanase, XynA (19, 36). Members of this family are known to generate xylobiose and xylotriose, along with the aldopentaurionate 4-*O*-methylglucuronosyl- α -1,2-xylotetraose (MeGAX₄), in which the MeGA is linked to a xylose residue penultimate to the nonreducing terminus (2). MeGAX₄ is not known to be assimilated and metabolized without the removal of the xylose residue at the nonreducing terminus first (41, 42, 45). Further review also identified the *ynfF* gene in the *B. subtilis* 168 genome. The translated protein product of this gene has 40% identity and

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60% similarity to XynA of *Erwinia chrysanthemi* D1, a GH 5 endoxylanase that has been well characterized (23, 35, 49).

Reports on the occurrence of GH 5 xylanases are not as prevalent as those on GH 10 and GH 11 xylanases, which have been extensively studied (2, 11, 22, 47). Just like GH 10 and GH 11 xylanases, they are presumed to function through a pair of glutamate residues catalyzing hydrolysis by a double-displacement mechanism with retention of anomeric configuration (17, 35). Although their general protein fold is the same as those found for other GH 5-categorized glycosyl hydrolases (20–22), GH 5 xylanases are more similar in primary sequence to GH 30 hydrolases (12). Recently, the first crystal structure of a GH 5 xylanase was published (35). The catalytic domain (CD) contains an α/β_8 barrel similar to that in the GH 10 xylanases, but closely associated with this domain is a putative carbohydrate binding module (CBM). The β -sheet structure of the CBM is formed with N-terminal and C-terminal regions, suggesting that the CD and CBM may function synchronously together, rather than having a simple spatial relationship that has been suggested for many other CD and CBM associations (4).

Unlike GH 10 and GH 11 xylanases, GH 5 xylanases have not been linked to the process of MeGAX_n degradation and utilization in microbial ecosystems. XynA from the well-characterized pectinolytic phytopathogen *Erwinia chrysanthemi* D1 was suggested to facilitate access to the pectin component of biomass (7, 23). This XynA showed activity that correlated directly to the degree of substitution (DS) of the glucuronosyl moiety, and that reduction of the carboxyl carbon of MeGA greatly reduced XynA activity, suggesting a role for the MeGA substitution in directing the recognition of the substrate by the enzyme (23). Products resulting from sweetgum wood MeGAX_n (SG MeGAX_n) hydrolysis by XynA contained a single MeGA moiety as determined by ¹³C nuclear magnetic resonance (NMR) and biochemical analysis, but early matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) results were difficult to reconcile and interpretation predicted two MeGA substitutions per hydrolysis product (23). Xylanases with sequences homologous to the defined sequence of GH 5 in *Erwinia chrysanthemi* have been reported, although characterization has been limited with respect to substrate specificities and product formation (25, 46, 60). An earlier report (44) described a 42-kDa xylanase (XynC is 43.9 kDa) purified from a commercial *B. subtilis* enzyme preparation (Novo Ban L-120). Hydrolysis product analysis comparisons to our observations here suggest that it may have been a GH 5 xylanase with properties similar to those of XynC of *B. subtilis* 168. In the absence of sequence data and the identity of the *B. subtilis* strain that served as its source, we are unable to conclude that it was homologous to XynC.

In this study, we expressed and characterized the *ynfF* gene product (XynC) from *B. subtilis* 168. This is the second characterization of a true GH 5 xylanase showing no activity on carboxymethyl cellulose (12), and the first complete characterization of an endoxylanase classified as a member of glycosyl hydrolase family 5 from a gram-positive bacterium. The secretion of both XynA and XynC and the utilization of xylooligosaccharides for growth make *B. subtilis* an attractive candidate to genetically transform and define the requirements for the

complete utilization of MeGAX_n for conversion to biobased products.

MATERIALS AND METHODS

***B. subtilis* 168 *ynfF* cloning and XynC purification.** *Bacillus subtilis* subsp. *subtilis* strain 168 was obtained from the Bacillus Genetic Stock Center (<http://www.bgsc.org>). Genomic DNA from overnight cultures grown in Luria-Bertani (LB) broth at 37°C with shaking was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA). The *ynfF* gene was amplified using the ProofStart DNA polymerase PCR kit (QIAGEN) for directional cloning into the NcoI and XhoI restriction sites (highlighted by underlined regions in primer sequences, respectively) of the pET 41 expression vector (EMD Biosciences). The 5' primer (TTCCATGGCAGCAAGTGATGTAAACAGTTAATG) was designed to truncate the XynC secretion signal sequence as predicted using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (1) and create an in-frame fusion behind the affinity purification tags of pET 41. The 3' primer (GTTCTCGAGTTAACGATTTACAACAAATGTTGT) contained the last few nucleotides of *ynfF*, including the stop codon. The expression construct (pET41ynfF#1) contains a glutathione S-transferase (GST) tag, a His tag, a thrombin cleavage site, an S tag, an enterokinase cleavage site, and the *ynfF* gene which was verified by sequencing. The pET41ynfF#1 construct was introduced into chemically competent *Escherichia coli* Rosetta (DE3) (EMD Biosciences) and grown overnight at 37°C on LBKC medium (LB agar plates containing 30 µg kanamycin/ml and 34 µg chloramphenicol/ml). Transformants grown on LBKC medium at 35°C were induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) when the optical density at 600 nm reached 0.6 to 0.7 and were processed for XynC purification as recommended in the pET system manual, 10th ed. (EMD Biosciences). Expressed fusion protein was isolated using a HiTrap HP metal-chelating column in the nickel form as per the method outlined in the HiTrap Chelating HP instruction manual (GE Healthcare Bio-Sciences). Xylanase activity was localized in the elution buffer fraction containing 500 mM sodium chloride, 500 mM imidazole, and 20 mM sodium phosphate, pH 7.4. To minimize protein precipitation associated with rapid desalting of this particular protein, the fraction was dialyzed in 10,000 molecular weight cutoff tubing (Pierce Biotechnology) in a five-step series in which the elution buffer was successively diluted twofold with 50 mM Tris-HCl, pH 7.4, at 45-min intervals. The resulting fraction was further dialyzed with two twofold-volume dilutions from the 50 mM Tris-HCl (pH 7.4) and enterokinase cleavage buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl₂) and finally with undiluted enterokinase cleavage buffer. Following overnight digestion with enterokinase at room temperature and filtration through a 0.45-µm filter, the preparation was dialyzed against GST-Bind buffer (4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl [pH 7.3]), the GST tag removed with GST-Bind agarose beads, and site-specific protease enterokinase removed with EKapture agarose (EMD Biosciences) equilibrated in GST-Bind buffer. At each step, protein concentrations were determined (6) by using bovine serum albumin (fraction V) as the standard, purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (34), and xylanase activity was determined as described below. The final product was stored at 4°C on ice and exhibited no loss of activity after 11 months.

MeGAX_n substrate preparation and carbohydrate analysis. MeGAX_n was isolated from sweetgum wood as previously described (29) and characterized by ¹³C NMR (30). Birch and beech wood xylans were obtained from Sigma-Aldrich. MeGAX_n was prepared from these xylans by solubilization of 20 mg (dry weight) xylan/ml deionized water for 5 h at 50°C to 60°C and centrifuged at 30,000 × g for 25 min at room temperature. Supernatants were decanted for carbohydrate analysis and use as reaction substrates. Xylose standards were used for total carbohydrate measurements (13) and determination of reducing termini (43). D-Glucuronic acid standards were used for total uronic acid determination (3). The degree of polymerization (DP) and the degree of uronic acid substitution for each substrate were determined. Xylanolytic activities of XynC toward these substrates were compared using the optimized reaction conditions for SG MeGAX_n described below.

Optimization of activity and kinetic evaluation of XynC. XynC activity was determined by measuring the increase in reducing termini (43) resulting from depolymerization of SG MeGAX_n substrate (10 mg substrate/ml). Reaction mixtures containing 250 µl substrate in 50 mM potassium phosphate or 50 mM sodium acetate with 200 ng XynC were run from pH 5.0 through pH 6.5 at 37°C. The temperature optimum under the conditions used was determined with reactions in 50 mM sodium acetate, pH 6.0, over the range from 50°C through 70°C. Specific activities are given as units/mg XynC, where one unit equals one micromole of reducing termini generated per minute. The kinetic analyses were determined from 250-µl reaction mixes containing 200 ng XynC in 50 mM

FIG. 1. Optimization of XynC was performed using standard reaction conditions described in Materials and Methods. Buffer and pH conditions were optimized and were applied for determination of the optimal reaction temperature and half-life analyses. These results were used to define the reaction conditions for kinetic analysis. (A) Buffer and pH optimization using 50 mM buffers with a pH between 5.0 and 6.5. Diamonds, potassium phosphate; squares, sodium acetate. (B) Temperature optimization using 50 mM sodium acetate, pH 6.0. (C) Half-life analysis determined by preincubating XynC at the specified temperatures and measuring remaining activity over time. Data are presented as the half-lives obtained from the linear regression of inactivation at each temperature. (D) Lineweaver-Burk kinetic analysis was based on a reaction velocity versus substrate concentration data set which was fit to a logarithmic equation.

sodium acetate, pH 6.0, with SG MeGAX_n as substrate at 37°C. Kinetic analysis was performed by the method of Lineweaver and Burk (37). Thermal stability assays were conducted with 250- μ l reaction mixes containing 2.5 mg SG MeGAX_n and 150 ng XynC in 50 mM sodium acetate, pH 6.0, between 30°C and 60°C. Aliquots were sampled four times in the first hour with decreasing sampling frequencies through 60 h of incubation.

XynC catalyzed depolymerization of MeGAX_n for product analysis. A batch reaction mixture of 20 ml consisting of 50 mg SG MeGAX_n/ml in 50 mM sodium acetate, pH 6.0, was prepared. The reaction was initiated by the addition of 1 unit of XynC in a volume of 100 μ l and maintained for 18 h at 30°C with slow vertical rotation on a roller drum-type rotator. The digest was processed by collecting consecutive filtrates from a Centriprep YM-3 centrifugal filter device (Millipore, Billerica, MA) by centrifugation at 2,000 $\times$ g at 20°C in one-hour intervals. After every other centrifugation, the volume in the filter unit was refilled to 15 ml with deionized water and filtrates combined to a total volume of 15 ml. Four 15-ml filtrate fractions, as well as a final 6-ml filtrate fraction, were collected. The resulting retentate was heated to 70°C for 15 min to inactivate XynC and was further processed twice by diluting to 50 ml with deionized water and concentrating in a 50-ml stir cell (Millipore, Billerica, MA) with a YM-3 membrane. The first recovered 15-ml filtrate from the Centriprep YM-3 concentrator (YM-3 filtrate) and the final washed retentate (YM-3 retentate) are the primary foci of the studies reported here. To verify compatibility of XynC with YM-3 membranes, which are made of regenerated cellulose acetate, XynC was checked for endoglucanase activity by using carboxymethyl cellulose as the substrate. No activity was detected.

MALDI-TOF MS analysis of XynC-generated MeGAX_n hydrolysis products. Samples for MALDI-TOF MS analysis were prepared as described previously (53), using a solution of DHBA (2,5-dihydroxybenzoic acid) dissolved to 2.5 mg/ml in 50% acetonitrile. Carbohydrate solutions, 1 to 2 mg/ml containing 0.1% trifluoroacetic acid, were prepared just prior to analysis, mixed with an equal volume of DHBA solution, and spotted (1 μ l) onto the MALDI-TOF MS plate. Mass spectra were collected using Voyager-DE Pro (Applied Biosystems, Foster City, CA) at the University of Florida Protein Core facility. The mass spectrometer was set for positive polarity in the reflector mode and the acceleration voltage set to 18,000 V with a laser intensity of 2,500. Data were converted into ASCII format and analyzed in Excel.

NMR analysis of XynC-generated MeGAX_n hydrolysis products. Samples for ¹H NMR were prepared by three successive dissolutions in 4 ml 99.9 atom% D₂O (Sigma-Aldrich), each with subsequent lyophilization. Each time the lyophilized MeGAX_n XynC hydrolysate residue was dissolved in D₂O, it was warmed to 50°C for 15 min to enhance ¹H displacement with deuterium. Final carbohy-

drate samples were prepared by dissolving the lyophilized carbohydrate powder to a concentration of 15 mg/ml in D₂O. To 750 μ l of these preparations, 2.5 μ l of acetone was added as the reference, and the final samples were transferred to 505-PS NMR tubes (Wilmad, Buena, NJ). ¹H NMR data collection was performed using a Bruker Avance 500 MHz spectrometer with a 5-mm TXI probe at the Advanced Magnetic Resonance Imaging and Spectroscopy facility at the McKnight Brain Institute, University of Florida. NMR data were analyzed and images prepared using Nuts Lite (Acorn NMR Inc., Livermore, CA).

Fractionation and analysis of xylanase activities secreted by *B. subtilis* 168. A single colony of *B. subtilis* 168 from an overnight LB agar plate was inoculated into 25 ml of 1% yeast extract (YE), Spizizen salts (56) in a 250-ml baffled flask and grown for 15 h at 37°C with rotation on a New Brunswick G-2 gyratory shaker at 200 rpm. Twenty milliliters of this culture was inoculated into a Fernbach flask containing 1 liter of prewarmed medium and incubated as described above until an optical density of 600 nm of approximately 1.5 absorbance units was obtained. Cells were removed by centrifugation at 13,200 $\times$ g for 20 min at 4°C. The supernatant was filtered through a 0.45- μ m filter and 1 ml of protease inhibitor cocktail for bacterial cell extracts (Sigma, St. Louis, MO) added. This preparation was concentrated/dialyzed using a 350-ml Amicon stir cell with a YM-3 membrane (Millipore, Billerica, MA) with 20 mM sodium phosphate, pH 6.0, containing 150 mM NaCl. The YM-3 retentate (*B. subtilis* spent medium concentrate [BSC]) was concentrated to less than 5 ml and loaded onto a calibrated BioGel P-60 chromatography column (60 cm by 0.75 cm) in the buffer system described above. Fractions comprising two peaks of xylanase activity (fraction A and fraction B) were concentrated/dialyzed separately to less than 1 ml against 20 mM sodium phosphate, pH 6.0, with Amicon YM-3 centrifugal filtration devices (Millipore, Billerica, MA). Equal volumes of each fraction were reacted with 20 mg SG MeGAX_n/ml in 50 mM sodium acetate, pH 6.0, and incubated overnight at 37°C. Thin-layer chromatography (TLC) was performed as previously described (5, 58). Controls for TLC included undigested SG MeGAX_n, SG MeGAX_n digested with recombinantly expressed XynC (330-nmol xylose equivalents loaded for samples and controls), MeGAX₁ to MeGAX₄ oligomers, and xylooligomers with one to four xyloses (25-nmol xylose equivalents for each oligomer).

Quantification of levels of xylanase transcripts by Q-RT-PCR. Primers for quantitative reverse transcriptase PCR (Q-RT-PCR) were designed using Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 WWW.cgi>) (52) with simultaneous secondary structure analysis using mfold (<http://www.bioinfo.rpi.edu/applications/mfold/>) to avoid DNA secondary structure in priming sites (66). Primers of approximately 20 nucleotides were designed to have an annealing temperature of 60°C, and mfold secondary structure modeling was performed at

TABLE 1. Relationship of XynC activity to the degree of MeGA substitution on MeGAX_n

MeGAX _n source ^a	DP ^b	DS ^c	Sp act ^d
Sweetgum wood	231	6.8	43.7 ± 1.4
Beech wood	156	7.2	36.5 ± 1.5
Birch wood	229	10	21.9 ± 0.4

^a Sweetgum wood xylan was prepared as described in Materials and Methods; all other sources were purchased from Sigma-Aldrich.
^b DP equals the molar ratio of total anhydroxylose to total reducing terminal xylose.
^c The degree of uronic acid substitution (DS) equals the molar ratio of total anhydroxylose to total uronic acid.
^d Specific activity is given as units/mg XynC, for which 1 unit is equal to 1 μmol of reducing terminal xylose equivalents generated per minute.

59°C under simulated conditions normal for PCR (50 mM Na⁺ and 3 mM Mg²⁺). All primer pairs yielded efficiencies between 85% and 95%, and all amplicons were less than 200 bp. High-quality genomic DNA of *B. subtilis* 168 was obtained for use in making primer set standard curves (63). Standard curves were prepared from 10² to 10⁶ gene copies for all genes. The reference genes were extended to 10⁸ copies based on their possible relative level to the genes of interest. All RT data collections were performed using an iCycler (Bio-Rad Laboratories, Hercules, CA). For primer set optimization and standard curve analysis, iQ SYBR green supermix was used, and for Q-RT-PCR, the iScript one-step RT-PCR kit (Bio-Rad Laboratories) was used. Reaction volumes were 16 μl for both kits. Cultures for RNA extraction (25 ml) contained glucose, arabinose, arabinose and xylose, SG MeGAX_n, or Birch MeGAX_n at 0.5% (xylose was at 0.28%) supplemented with 0.1% YE and 0.005% tryptophan in

FIG. 2. MALDI-TOF MS analysis of the YM-3 filtrate (A) and YM-3 retentate (B) resulting from 3-kDa ultrafiltration of an SG MeGAX_n XynC digest. Peak *m/z* values were tabulated and show that each cluster of peaks is composed of various single- and double-salt adducts which differ from the previous cluster by a single xylose residue. Each chemical species is composed of the designated number of xylose residues containing a single MeGA residue. Complements of different sodium and/or potassium adducts comprise designated clusters.

Spizizen minimal salt base (56). RNA was extracted from early- to mid-log-phase cultures by using an RNeasy RNA extraction kit (QIAGEN, Valencia, CA), treated with RQ1 DNase (Promega, Madison, WI) and repurified using an RNeasy column. RNA (10 ng) without reverse transcriptase treatment was used to amplify the *rpoA* gene to check for DNA contamination. All data obtained from Q-RT-PCR were based on a 10-ng RNA starting quantity as determined by absorbance at 260 nm. The housekeeping genes *rpoA* and *atpA* were used to provide a reference for relative expression in response to glucose and xylan, using triplicate measurements of each growth condition averaged together. Threshold values for *atpA* under all conditions averaged 17.37 (cycle threshold [C_T]) with a standard deviation of 0.31. For the *rpoA* gene, the C_T average was 15.53 with a standard deviation of 0.53. Transcript data were analyzed with Gene Expression Macro V1.1 software available through Bio-Rad (<http://www.Bio-Rad.com/LifeScience/jobs/2004/04-0684/genex.xls>) (38, 64).

RESULTS

Cloning, overexpression, and characterization of XynC. Following cloning and expression in pET 41, protein was produced in quantities sufficient for enzyme purification. The N-terminal His tag was used for affinity purification and resulted in a protein which was relatively insoluble at low ionic strengths. Qualitatively, solubility was maintained in tertiary amine buffers, such as 50 mM Tris-HCl or imidazole, containing 20 mM NaCl. After enterokinase protease release of the affinity tag fusion product from XynC, there were no further apparent problems with solubility.

Optimization of reaction conditions and kinetic evaluation of XynC. The relationship between activity and pH showed similarly broad optima of pH 6 with 50 mM potassium phosphate or 50 mM sodium acetate buffers (Fig. 1A). The relationship between temperature and activity (Fig. 1B) showed that the maximum amount of reducing terminus formation for a 15-min digestion was obtained at 65°C. The relationship between stability and temperature was evaluated by half-life stability analysis (Fig. 1C), with half the activity retained for 25 h at 40°C and for 5 h at 50°C. All further studies, including kinetic analyses, were performed at 37°C in 50 mM sodium acetate, pH 6.0, for 15 min with 0.012 units (200 ng) XynC.

Kinetic analysis (Fig. 1D) of XynC using SG MeGAX_n as the substrate showed it to have a K_m of 1.63 mg/ml and a V_{max} of 59.5 units/mg XynC, which correspond to a k_{cat} of 2,635/minute. As seen in Table 1, measurements of XynC activity on different MeGAX_n sources indicate that activity is directly correlated to the degree of substitution of the glucuronosyl moiety on the xylan chain.

Mass analysis of SG MeGAX_n XynC hydrolysis products. YM-3 filtrate and retentate fractions of the SG MeGAX_n XynC hydrolysate were analyzed by MALDI-TOF MS. As shown in Fig. 2, clusters of peaks were observed, with each cluster being a defined aldouronate with the individual peaks being some salt adduct form of the specific aldouronate. Table 2 lists the mass-to-charge ratios and designated products along with the ion adducts. In each case, the species with a single Na adduct was the most prominent. Based upon these assignments, the YM-3 filtrate and YM-3 retentate fractions contain detectable xylooligosaccharides ranging in DP from 4 to 12 and from 4 to 20, respectively, each with a single MeGA substitution. There was no evidence for the presence of unsubstituted xylooligosaccharides in these fractions.

NMR analysis of SG MeGAX_n XynC hydrolysis products. Analysis of the YM-3 filtrate fraction by ¹H NMR (Fig. 3A)

TABLE 2. MALDI-TOF peak assignments

Xylooligomer	Peak no.	Adduct	Mol wt
MeGAX ₄	1	Na	759.23
MeGAX ₅	2	Na	891.18
	3	Na + Na	913.18
	4	Na + K	929.08
	5	K + K	945.11
MeGAX ₆	6	Na	1,023.22
	7	Na + Na	1,045.07
	8	Na + K	1,061.08
	9	K + K	1,076.97
MeGAX ₇	10	Na	1,155.08
	11	Na + Na	1,177.11
	12	Na + K	1,192.92
	13	K + K	1,208.97
MeGAX ₈	14	Na	1,287.00
	15	Na + Na	1,308.89
	16	Na + K	1,324.87
	17	K + K	1,340.81
MeGAX ₉	18	Na	1,419.06
	19	Na + K	1,456.77
MeGAX ₁₀	20	Na	1,550.83
	21	Na + Na	1,572.73
	22	Na + K	1,588.73
	23	K + K	1,604.65
MeGAX ₁₁	24	Na	1,682.83
	25	Na + K	1,721.83
MeGAX ₁₂	26	Na	1,814.73
	27	Na + Na	1,836.63
	28	Na + K	1,852.63
MeGAX ₁₃	29	Na	1,947.75
MeGAX ₁₄	30	Na	2,078.54
MeGAX ₁₆	31	Na	2,342.39
MeGAX ₁₈	32	Na	2,607.07
MeGAX ₂₀	33	Na	2,871.04

yielded the expected distribution of peaks based on previous proton shift assignments for similar xylooligomers substituted with methylglucuronosyl residues (8, 15, 30). The integration ratio of the signals for the proton on carbon five of the MeGA, the proton on carbon five of the nonreducing xylose, and the proton on carbon one of the reducing xylose (α and β configurations) was 1.0:1.1:0.9, establishing that the products of XynC depolymerization contained a single MeGA moiety α -1,2 linked to β -1,4-xylooligosaccharides. The signals ascribed to the proton of carbon one for the MeGA residue (α/β -U1) appear as two doublets, the first at 5.31 and 5.30 ppm and the second at 5.29 and 5.28 ppm. The integrated ratio for these two doublets is 0.23:0.77. This is nearly identical to the α/β intensity split of 0.26:0.74 for the integrated signals from the proton on the α anomer of carbon one (doublet at 5.18 and 5.17 ppm) and the proton on the β anomer of carbon one (doublet at 4.58 ppm and 4.63 ppm) for the reducing terminal xylose. Thus, the doublet shift values for α/β -U1 that is α -1,2 linked to a xylose residue are split to a ratio that reflects the equilibrium of the α and β anomers of the xylose residue at the reducing terminus. As stated above, this interpretation is consistent with other published interpretations of ¹H NMR spectra of xylooligomers substituted with MeGA and indicates that the substitution is penultimate to the reducing terminal xylose (8, 15). Figure 3B represents the predicted limit products based on the ¹H NMR observations described above and shows the expected positioning of the MeGA moiety with respect to the

FIG. 3. ^1H NMR of SG MeGAX_n 3-kDa filtrate revealing the general action of XynC hydrolysis of MeGAX_n and the predicted limit product of XynC MeGAX_n digestion. Integrated intensity values for specific shift positions have been used to determine the product of an XynC digestion, establishing that there is a single MeGA substitution for every reducing terminal xylose and every nonreducing terminal xylose and that this substitution is penultimate to the reducing terminal xylose. (A) Shift assignments are labeled as follows: α , β -U1, 4-*O*-methylglucuronic acid carbon one hydrogen; U5, 4-*O*-methylglucuronic acid carbon five hydrogen; α , β -r-X1, reducing terminal xylose carbon one hydrogen; nr-X5, nonreducing terminal xylose carbon five hydrogen; int-X5, internal xylose carbon five hydrogen. (B) Limit product generated by XynC-catalyzed hydrolysis of MeGAX_n. X, xylose; MeGA, 4-*O*-methylglucuronic acid; n, some number of β -1,4-linked xylose residues.

reducing end xylose and some number of β -1,4-linked xylose residues.

Fractionation of xylanases secreted by *B. subtilis* 168. The resolution of extracellular xylanase activities was achieved following concentration of medium by YM-3 filtration, and chromatographic separation of 2.9 units of the BSC with a BioGel P-60 column as described in Materials and Methods. Two xylanase-positive peaks, fraction A and fraction B, were collected, concentrated as described above, and evaluated by TLC for products generated by their respective actions on SG MeGAX_n (Fig. 4). Fraction A eluted at a position expected for a globular protein with a molecular mass of approximately 31 kDa. The activity in this fraction catalyzed the depolymerization of SG MeGAX_n to form aldouronate products resolved by TLC that were identical to those generated by the recombinantly expressed XynC protein, with the notable absence of xylobiose or xylotriose (Fig. 4). Fraction B eluted at a position that corresponded to a molecular mass, extrapolated from the relationship of $\log M_r$ to elution position for standards, of approximately 3 kDa, indicating adsorption to the acrylamide matrix. This activity catalyzed the generation of products from

SG MeGAX_n that would be expected for a typical GH 11 xylanase, releasing xylobiose, xylotriose, and aldopentauronate (MeGAX₄) as primary limit products (2). SG MeGAX_n digestion with unfractionated BSC generated products that were primarily a result of XynA hydrolysis but also included aldopentauronate (MeGAX₃) (Fig. 4).

Relative levels of xylanase transcripts determined by Q-RT-PCR. To study xylanase gene expression in *B. subtilis* 168, the genes *xynA*, *xynB*, *xynC*, *abnA*, and *gapA* were analyzed. The *abnA* gene codes for an extracellular arabinofuranosidase and is well studied, being highly expressed while *B. subtilis* is growing on arabinose and repressed by glucose (50). The *gapA* gene, which codes for the glycolytic glyceraldehyde-3-phosphate dehydrogenase in *B. subtilis*, was reported to be upregulated while *B. subtilis* was growing on glucose (16, 40). The *abnA* and *gapA* genes thus served as internal controls. This was confirmed in our studies in which *abnA* showed the greatest dynamic range of all genes analyzed (Table 3). Our results also support previous findings for the *xynB* gene, showing that expression was greatest during growth on xylose. However, the same effect was not observed for *xynA* (36) and *xynC*. Changes

FIG. 4. Identification of products generated by XynA (GH 11) and XynC (GH 5) secreted by *B. subtilis* 168. Spent medium from a mid-log-phase culture of *B. subtilis* 168 was concentrated by YM-3 filtration to provide the BSC fraction. This was fractionated using a BioGel P-60 column to provide fractions A and B, which were used to digest SG MeGAX_n and identify the xylanase hydrolysis pattern by TLC. SG MeGAX_n and an SG MeGAX_n digested with recombinantly expressed XynC were used as controls. Lanes: 1, SG MeGAX_n; 2, SG MeGAX_n digestion with fraction A; 3, SG MeGAX_n digestion with recombinant XynC; 4, MeGAX₁ to MeGAX₄ aldouronate standards; 5, xylooligomer standards with one to four xyloses; 6, SG MeGAX_n digestion with fraction B; 7, SG MeGAX_n digestion with BSC.

in transcript levels for *xynA* and especially for *xynC* were modest with respect to *abnA* (Fig. 5). The *xynA* transcript was in greatest quantity while cells were growing on MeGAX_n as the substrate and lowest while cells were growing on glucose, sug-

FIG. 5. Regulation of expression of *xynA* and *xynC* genes in early- to mid-exponential-phase growth cultures of *B. subtilis* 168 with different sugars as the substrate, measured by using Q-RT-PCR. G, glucose; A, arabinose; B, birch wood MeGAX_n; S, sweetgum wood MeGAX_n; 1, *xynA*; 2, *xynC*.

gesting that *xynA* expression may be activated by growth on MeGAX_n. In contrast, the *xynC* transcript was constitutively expressed without significant changes in regulation observed while growing on the different sugars.

DISCUSSION

XynC from *B. subtilis* 168 is the first GH 5 xylanase from a gram-positive bacterium to be fully characterized with respect to substrate requirements and resulting hydrolysis products. The turnover rate of 2,635/minute is close to the rate observed for the GH 5 xylanase (XynA) from the gram-negative bacterium *Erwinia chrysanthemi* strain PI (data not shown). A review of publications concerning GH 10 and GH 11 xylanases in which a V_{max} or k_{cat} is given reveals a very broad range of catalytic rates (9, 10, 14, 18, 31, 47, 49). In general, the GH 5 xylanases have a catalytic rate which is at the lower range of those reported for GH 10 and GH 11 xylanases.

Both the MALDI-TOF MS data and the 1H NMR data indicate that XynC catalyzes the depolymerization of MeGAX_n to release products in which β -1,4-linked xylooligosaccharides are substituted with a single α -1,2-linked 4-O-methylglucuronate. MALDI-TOF MS analysis of products generated by XynC revealed an array of peak clusters, each differing by a single xylose residue. Tabulation of mass data showed that single-salt adducts differed from double-salt adducts by a single mass unit, indicating that formation of adducts results from proton displacement. Studies in which this technique for carbohydrate analysis was developed (57) reported single-salt adducts for neutral polysaccharides but no double-salt adducts. The occurrence of double-salt adducts may result from the presence of the carboxylic acid. The generation of xylooligosaccharides with a single MeGA substitution calls into question the interpretation of the MALDI-TOF MS data for products generated from the action of the GH 5 endoxylanase from *Erwinia chrysanthemi* strain D1 (23). Recent studies with the GH 5 endoxylanase from *Erwinia chrysanthemi* strain PI have applied MALDI-TOF MS to identify the products generated from the depolymerization of SG MeGAX_n along with potassium ion supplements (J. Rice, G. Nong, A. Ragunathan, F. St. John, and J. P. Preston, Abstr. 105th Gen. Meet. Am. Soc. Microbiol. 2005, abstr. B-138, 2005). These results support the assignments made in Table 2 and the interpretation provided here. Further support for this interpretation comes from the

TABLE 3. Relative transcript quantity measured by Q-RT-PCR for *gapA*, *abnA*, *xynA*, and *xynC* genes

Growth substrate ^a	Relative transcript quantity ^b				
	<i>gapA</i>	<i>abnA</i>	<i>xynB</i>	<i>xynC</i>	<i>xynA</i>
Glucose	32.6 $\pm$ 5.0	1.0 $\pm$ 0.3	1.2 $\pm$ 0.3	1.1 $\pm$ 0.2	1.0 $\pm$ 0.3
Arabinose	1.3 $\pm$ 0.2	611.9 $\pm$ 105.9	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1	1.1 $\pm$ 0.1
Arabinose-xylose	1.0 $\pm$ 0.1	621.3 $\pm$ 68.0	132.3 $\pm$ 13.4	1.4 $\pm$ 0.1	1.6 $\pm$ 0.2
SG MeGAX _n	3.6 $\pm$ 0.4	40.7 $\pm$ 4.7	1.4 $\pm$ 0.4	1.3 $\pm$ 0.2	3.1 $\pm$ 0.3
Birch MeGAX _n ^c	2.8 $\pm$ 0.3	57.1 $\pm$ 11.8	2.2 $\pm$ 0.6	1.2 $\pm$ 0.1	3.7 $\pm$ 0.3

^a Media for growth consisted of 0.1% YE in Spizizen salts with the specified sugar added to a final concentration of 0.5% except for xylose, which was added to a final concentration of 0.25%.

^b C_T values were used to calculate transcript copy number, which was normalized to the lowest transcript level in each conditional gene set by using Bio-Rad Gene Expression Macro V1.1.

^c Birch wood xylan was used as received from Sigma-Aldrich.

ratio 1.0:1.1:0.9 of the integrated signals for the proton on carbon five of the MeGA, the proton on carbon five of the nonreducing terminal xylose, and the proton on carbon one of the reducing terminal xylose.

The evidence for the position of the xylose residue that is substituted with MeGA in XynC-generated products is circumstantial in that it is based upon the NMR shift assignment peak intensities ascribed to an induction of an α/β resonance split in the α -1,2-linked MeGA moiety. This induction can be rationalized by a direct interaction with the proton on carbon one of the reducing terminal xylose residue, which is in anomeric equilibrium between α and β configurations. Such an effect has been interpreted to explain the observations obtained from the ^1H NMR and two-dimensional $^1\text{H}/^{13}\text{C}$ NMR analyses of aldouronates in which the nonreducing terminal xylose in β -1,4-xylobiose and the internal xylose in β -1,4-xylotriose are substituted with α -1,2-linked 4-*O*-methylglucuronate (8, 15). The products generated by a xylanase purified from a commercial preparation of *Bacillus subtilis* were previously characterized by methylation analysis that identified the MeGA substitution on the xylose residue penultimate to xylose at the reducing terminus (44), and this activity may have been encoded by a gene homologous to *xynC*. These data identify a site of cleavage that is different from that indicated by previous studies of the GH 5 endoxylanase secreted by the D1 strain of *Erwinia chrysanthemi* (23), which was based upon ^{13}C NMR and limited digestion by β -xylosidase. Two-dimensional heteronuclear multiple-quantum coherence NMR spectra of the products generated by the GH 5 xylanase secreted by *Erwinia chrysanthemi* PI (J. Rice et al., Abstr. 105th Gen. Meet. Am. Soc. Microbiol. 2005) support the interpretation provided for the products generated by the XynC GH 5 endoxylanase secreted by *B. subtilis* 168. From this, it may be concluded that GH 5 endoxylanases from both *B. subtilis* 168 and the *Erwinia chrysanthemi* strains catalyze the exclusive cleavage of a β -1,4-xylosidic bond penultimate to that linking carbon one of the xylose residue that is substituted with α -1,2-linked MeGA as depicted in Fig. 3B.

Previous studies on expression of xylanase activity in *B. subtilis* have focused on the *xynA* gene, whose protein product (XynA) has long been thought to be the primary extracellular xylanase (36). The *xynA* gene is constitutively expressed even while cells are growing on glucose, suggesting that *xynA* regulation may not be subject to catabolite repression (36, 40). The *xynC* gene was originally identified by genome sequencing of *B. subtilis* 168 (33, 51). Analysis of the fractions derived from the BioGel P-60 chromatography of BSC indicates that *B. subtilis* 168 produces XynC as a xylanase activity separable from that of XynA. These results are supported by a recent proteomic evaluation of the *B. subtilis* secretome which identifies XynA and XynC (and XynD) as being present as secreted protein products (62).

Expression of *xynC* is unresponsive to metabolite-mediated induction or repression while *B. subtilis* 168 is growing on a variety of carbohydrates. In contrast, *xynA* expression appears to be activated by growth on MeGAX_n. Transcriptome analyses of *xynA* and *xynC* in the *B. subtilis* wild type (strain ST100) and a *ccpA* mutant (strain ST101) showed little change with and without glucose as the substrate (40) (<http://www.biology.ucsd.edu/~imsaier/regulation/>). Unlike the xylose utilization

FIG. 6. Limit aldouronates expected from an SG MeGAX_n digestion with a GH 11 xylanase and a GH 5 xylanase cosecreted in the growth medium of *B. subtilis* 168. (A) MeGAX₄ with a MeGA substitution penultimate to the nonreducing terminal xylose, the smallest aldouronate product resulting from a GH 11 hydrolysis of MeGAX_n (4). (B) The predicted hydrolysis limit product of a GH 5 xylanase as presented in this publication, having a single MeGA substitution penultimate to the reducing terminal xylose. (C) MeGAX₃ with a MeGA substitution on the second of three xylose residues positioned penultimate to the reducing and nonreducing ends.

operon of *B. subtilis* (19, 26, 32) and the Q-RT-PCR control genes mentioned above, neither of the two secreted xylanases of *B. subtilis* 168 seems to be repressed by glucose via the CcpA/cre-mediated mechanism. XynA may be upregulated threefold while growing on MeGAX_n by a process as yet undefined, and XynC is strictly constitutive.

GH 11 xylanases, e.g., XynA, are known to release xylobiose as a major limit product which may be utilized by *B. subtilis* (19). However, the products of XynC hydrolysis of MeGAX_n are too large for direct utilization. The hydrolysis of MeGAX_n by XynA and XynC together (in the BSC) releases a unique aldouronate smaller than those generated by the individual xylanases (Fig. 6). This aldotetrauronate (MeGAX₃), with a MeGA substitution on the second xylose of xylotriose, is distinct from the well-studied MeGAX₃ limit product of a GH 10 xylanase, which is substituted with MeGA directly on the non-reducing terminal xylose of β -1,4-xylotriose (2, 48). The MeGAX₃ generated by GH 10 xylanases may be readily assimilated and metabolized by some gram-positive bacteria (55, 58), while the assimilation and metabolism of MeGAX₃ generated by the combined action of a GH 11 and a GH 5 endoxylanase have yet to be demonstrated.

In summary, XynC of *B. subtilis* 168 is a GH 5 endoxylanase that, based on substrate preference and product analysis, cleaves at positions that are directed by the glucuronosyl substitution on the MeGAX_n chain. Hydrolysis products are decorated with a single MeGA moiety penultimate to the reducing end xylose as proposed by Nishitani and Nevins (44), and there are no detectable neutral xylooligosaccharides released. With its unique mode of action and moderate thermotolerance, XynC may complement GH 10 and GH 11 xylanases for pretreatment of lignocellulosic. *B. subtilis* 168 may be engineered for aldouronate utilization and help define genetic requirements for efficient enzymatic pretreatment of hemicellulose. Selection or engineering of ferment-

tative strains of *B. subtilis* 168 may then allow development of second-generation gram-positive biocatalysts for efficient conversion of MeGAX_n to "green" chemicals and fuel ethanol.

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