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Engineering the Xylan Utilization System in *Bacillus subtilis* for Production of Acidic Xylooligosaccharides

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Xylans are the predominant polysaccharides in hemicelluloses and an important potential source of biofuels and chemicals. The ability of *Bacillus subtilis* subsp. *subtilis* strain 168 to utilize xylans has been ascribed to secreted glycoside hydrolase family 11 (GH11) and GH30 endoxylanases, encoded by the *xynA* and *xynC* genes, respectively. Both of these enzymes have been defined with respect to structure and function. In this study, the effects of deletion of the *xynA* and *xynC* genes, individually and in combination, were evaluated for xylan utilization and formation of acidic xylooligosaccharides. Parent strain 168 depolymerizes methylglucuronoxylans ($MeGX_n$), releasing the xylobiose and xylotriose utilized for growth and accumulating the aldouronate methylglucuronoxylotriose ($MeGX_3$) with some methylglucuronoxylotetraose ($MeGX_4$). The combined GH11 and GH30 activities process the products generated by their respective actions on $MeGX_n$ to release a maximal amount of neutral xylooligosaccharides for assimilation and growth, at the same time forming $MeGX_3$ in which the internal xylose is substituted with methylglucuronate (MeG). Deletion of *xynA* results in the accumulation of β-1,4-xylooligosaccharides with degrees of polymerization ranging from 4 to 18 and an average degree of substitution of 1 in 7.2, each with a single MeG linked α-1,2 to the xylose penultimate to the xylose at the reducing terminus. Deletion of the *xynC* gene results in the accumulation of aldouronates comprised of 4 or more xylose residues in which the MeG may be linked α-1,2 to the xylose penultimate to the nonreducing xylose. These *B. subtilis* lines may be used for the production of acidic xylooligosaccharides with applications in human and veterinary medicine.

Xylooligosaccharides without (XOS) and with (AXOS) arabinofuranosyl substitutions are of interest as value-added products derived from the hemicellulose fractions of lignocellulose. Neutral forms of these β-1,4-linked xylooligosaccharides have been reported to serve as prebiotics (1–3) and anti-inflammatory agents (4). Aldouronates, acidic xylooligosaccharides (U-XOS and U-AXOS, where U represents uronate) in which some xylose (X) residues are substituted with α-1,2-linked 4-*O*-methylglucuronate (MeG), have been shown to exhibit anti-inflammatory and other immunomodulating activities (5). These acidic forms comprise a portion of the pentosans that are used for the preparation of pentosan polysulfates (PPSs), which have several medical applications, including the treatment of interstitial cystitis, mucopolysaccharidoses, and osteoarthritis (6–8). The generation of different forms of XOS and AXOS or U-XOS and U-AXOS results from the depolymerization of both methylglucuronoxylans ($MeGX_n$) and methylglucuronoarabinoxylans ($MeGAX_n$), the predominant polymers comprising the hemicellulose fractions of lignocellulosics derived from most species of dicots and monocots, respectively (9, 10).

The production of neutral and acidic forms can be achieved with endoxylanases of glycoside hydrolase family 10 (GH10), GH11, and GH30 (CAZy; http://www.cazy.org/), as depicted in Fig. 1. Members of each family have been defined with respect to structure and function (11–16). With $MeGX_n$ as the substrate, GH10 xylanases generate xylobiose (X_2) and xylotriose (X_3) as XOS and the aldotetrauronate 4-*O*-methylglucuronoxylotriose ($MeGX_3$) as U-XOS, in which a single MeG substitution occurs on the nonreducing terminal xylose (Fig. 1). The products of the GH10 enzymes may be assimilated and processed for the complete metabolism of the xylose and MeG components of the $MeGX_n$. This intracellular processing depends upon the presence of a GH67 α-glucuronidase that cleaves the α-1,2-linked MeG from the nonreducing terminal xylose on the $MeGX_3$ generated by the GH10 xylanase. Treatment of $MeGX_n$ with GH11 xylanase generates X_2 and X_3 as XOS and the aldopentauronate methylglucuronoxylotetraose ($MeGX_4$) with a single MeG substitution on the xylose penultimate to the nonreducing terminal xylose (11). This aldouronate is not a substrate for a GH67 α-glucuronidase, and $MeGX_4$ may accumulate as a limit product in media of bacterial cultures secreting only a GH11 endoxylanase. With $MeGX_n$ as the substrate, GH30 xylanases exclusively generate aldouronates in which a MeG substitution occurs on a xylose residue penultimate to the reducing terminal xylose, producing U-XOS (15–17). These aldouronates may contain a variable number of xylose residues, depending upon the distribution of MeG substitutions in the polymeric $MeGX_n$ (Fig. 1). As in the case of the $MeGX_4$ generated by GH11 endoxylanases, the position of the MeGA substitution does not allow processing by a GH67 α-glucuronidase.

Bacillus subtilis subsp. *subtilis* 168 and other *B. subtilis* strains secrete GH11 and GH30 endoxylanases (16, 18). On the basis of analysis of the sequenced genome of *B. subtilis* subsp. *subtilis* 168, these are the only endoxylanases for which structural genes have been identified in this strain. Both GH11 and GH30 endoxylanases produced by *B. subtilis* strains have been well characterized with respect to the products formed and structure-function relationships (15, 19, 20). With a fully sequenced genome, genetically malleable *B. subtilis* subsp. *subtilis* 168 lends itself to genetic modifications for the selective production of neutral and acidic forms

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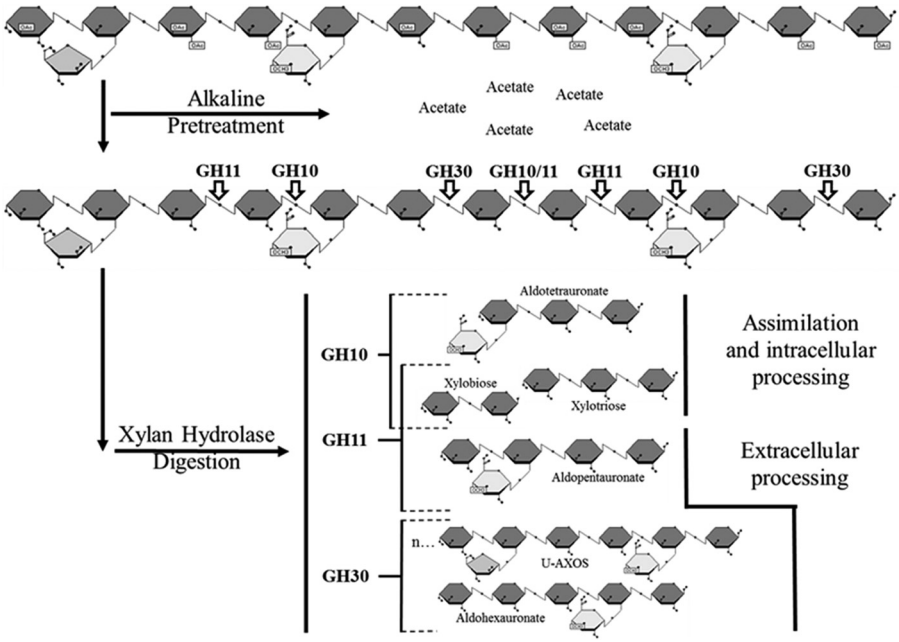

FIG 1 Scheme for the generation of XOS and U-XOS from $MeGX_n$ using GH10, GH11, and GH30 endoxylanases. OAc, acetate.

of XOS and AXOS from lignocellulosics. In this study, we have developed strains of *B. subtilis* that have defined the roles of the GH11 and GH30 endoxylanases for the cell-based conversion of $MeGX_n$ to release XOS for maximal metabolism without the need for a GH67 α-glucuronidase. The pathways for this conversion contribute to the efficiency with which *B. subtilis* subsp. *subtilis* 168 and other strains and species that have this GH11/GH30 system for xylan depolymerization are able to convert a lignocellulosic resource to targeted products. The deletion of the *xynA* gene results in a new strain that secretes only the GH30 XynC and accumulates aldouronates that have direct applications or serve as precursors for biologicals, e.g., pentosan polysulfate. With the generally regarded as safe status of several *B. subtilis* strains for production of enzymes and food additives (21, 22), these new strains may be developed as biocatalysts for the production of U-XOS from $MeGX_n$ for applications in human and veterinary medicine.

MATERIALS AND METHODS

***B. subtilis* strains and media.** *Bacillus subtilis* strains used in this study are listed in Table 1. *B. subtilis* subsp. *subtilis* strain 168 was obtained from the Bacillus Genetic Stock Center (http://www.bgsc.org). *B. subtilis* strains were cultured in LB broth (Lennox L broth), low-salt formula (RPI Corp.), at 37°C, and Spizizen's medium (23) was used for cultivation on different carbohydrate substrates. Spizizen's medium contained the following composition per liter: K_2HPO_4 (14 g), KH_2PO_4 (6 g), trisodium citrate dihydrate (1 g), $(NH_4)_2SO_4$ (2 g), and $MgSO_4{\cdot}7H_2O$ (0.2 g). Spizizen's medium was supplemented with tryptophan at 25 μg ml^{-1}. Unless otherwise noted, 0.1% yeast extract (Difco) was included along with a methylglucuronoxylan ($MeGX_n$) substrate at various concentrations.

Construction of *B. subtilis* xylanase mutants MR42, MR44, and MR45. For construction of a *B. subtilis xynC* GH30 xylanase mutant strain (MR42 [168 Δ*xynC*-Km]), the 4,270-bp DNA fragment containing *xynD-xynC-bglC′* genes was amplified using *B. subtilis* subsp. *subtilis* 168 genomic DNA as the template and bg-BS0104F (GCATACCTCGAGCG TCTGGCAATGGCGGTGTA) and bg-BS0104R (AGCAGCAGCAATCT ACAACCT) as the primers. The amplified product was ligated into plasmid vector pUC19 hydrolyzed by HinCII (pMSR450). The kanamycin resistance gene (Km) fragment (1,486 bp) was prepared from plasmid pMSP3535VA after hydrolysis by ClaI and filling in using the Klenow fragment (Klenow) of DNA polymerase I. A 1,235-bp fragment of *xynC* was removed from plasmid pMSR450 after hydrolysis by AflII and filling in the ends with Klenow, and the Km fragment was inserted at this location (pMSR451). A 4,527-bp fragment of *xynD-km-bglC′* was amplified by PCR and introduced into *B. subtilis* subsp. *subtilis* 168 according to the procedure described by Rhee et al. (24). Transformants were selected using LB agar medium with 5 μg ml^{-1} kanamycin. Disruption of the *xynC* gene in strain MR42 was confirmed by PCR amplification.

In order to construct the *B. subtilis xynA* GH11 xylanase mutant (strain MR44 [168 Δ*xynA*-Spc]), the 1,935-bp DNA fragment containing the *xynA* gene of *B. subtilis* subsp. *subtilis* 168 was amplified from genomic DNA using the primers xA-BS0204F (GGAGTGCTCGAGAGGAGGAAG

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Bacillus subtilis strains		
168	*trpC2*	
MR42	168 Δ*xynC*-Km	This study
MR44	168 Δ*xynA*-Spc	This study
MR45	168 Δ*xynA*-Spc Δ*xynC*-Km	This study
Plasmids		
pUC19		
pMSP3535VA	pVA380-1 and ColE1 replicons, *nisRK* P_{nisA} Km^r	44
pAW016	Mini-Tn*10*-delivering plasmid	45
pMSR450	pUC19 *xynD-xynC-bglC′*	This study
pMSR451	pMSR450 Km^r	This study
pMSR452	pUC19 *xynA*	This study
pMSR453	pMSR452 Spc^r	This study
pLSW3	pET15b *xynA*	This study

TCATGGTAAGC) and xA-BS0204R (GCGTTGTCTAGATCGTAGAGT CCCCATTCATAAAT). The PCR product was ligated into plasmid vector pUC19 hydrolyzed by HincII (pMSR452). A 519-bp fragment was removed from the middle of the *xynA* gene in plasmid pMSR452 after hydrolysis by NheI and EcoRV, and the NheI end was filled in after the Klenow treatment. The spectinomycin resistance gene (Spc) fragment (1,411 bp) from pAW016 was ligated into this region to yield plasmid pMSR453. A PCR product of 2,831 bp containing *xynA* interrupted with the Spc fragment was introduced into *B. subtilis* subsp. *subtilis* 168. Transformants were selected using LB agar medium containing spectinomycin (100 $\mu g\ ml^{-1}$). A double mutant lacking *xynA* and *xynC* (strain MR45 [168 Δ*xynA*-Spc Δ*xynC*-Km]) was constructed by transforming the 2,831-bp *xynA*-Spc fragment into strain MR42. Disruption of the *xynA* gene in the MR44 and MR45 mutants was confirmed by PCR amplification.

Preparation of GH11 and GH30 endoxylanases of *B. subtilis*. For purification of GH11 endoxylanase XynA, the *xynA* gene was amplified by PCR with *B. subtilis* subsp. *subtilis* 168 genomic DNA as the template and xynAF (ATGTCCCTCGAGAGCACAGACTACTGGCAAAATT) and xynAR (CGATAAGGATCCCCTACCTCCAGCAATTCCAA) as the primers. The amplified product (721 bp) hydrolyzed by XhoI and BamHI was ligated into plasmid pET15b, also hydrolyzed by XhoI and BamHI, yielding plasmid pLSW3. *Escherichia coli* Rosetta 2 cells were transformed with the ligation product, and transformants were selected on LB medium containing ampicillin and chloramphenicol. The Rosetta 2 strain containing pLSW3 was cultured in 500 ml of LB medium containing ampicillin and chloramphenicol in a 2.8-liter Fernbach flask at 37°C with shaking at 250 rpm. When the optical density at 600 nm (Beckman DU640 spectrophotometer) reached 0.8, isopropyl-β-D-1-thiogalactopyranoside (IPTG; 0.1 mM) was added to the culture to induce the T7 RNA polymerase. After 4 h of incubation at room temperature with shaking, cells were harvested by centrifugation (10,000 × *g*, 10 min, 4°C), washed twice with 25 ml of 20 mM sodium phosphate (pH 7.4), and resuspended in 20 ml of the same buffer. Cells were passed through a French pressure cell at 16,000 lb/in^2. The crude extract was clarified by centrifugation (30,000 × *g*, 45 min, 4°C), and the supernatant was filtered through a 0.22-μm-pore-size filter and loaded onto a HiTrap HP chelating column (5 ml; GE Life Sciences) preconditioned with 0.1 M $NiSO_4$. Unbound material was removed by washing with 10 column volumes of elution buffer (20 mM sodium phosphate [pH 7.4] containing 0.5 M NaCl), followed by 10 column volumes of elution buffer containing 50 mM imidazole. His-tagged XynA protein was eluted with 0.5 M imidazole in elution buffer. Imidazole was removed from the sample using a PD-10 column (GE Life Sciences), and protein was eluted with 50 mM sodium acetate, pH 6.0. The activity of this XynA enzyme was 37 U mg^{-1}. The GH30 endoxylanase XynC enzyme was prepared as a pure recombinant enzyme (44 U mg^{-1}), as previously described (15, 16). One unit was the activity that generated 1 μmol reducing terminus per min at 30°C.

Preparation of substrates and analyses of enzymes. $MeGX_n$ was purified from sweet gum wood as previously described (14, 25). The preparations were analyzed for total carbohydrate (26), total uronic acid (27), and total reducing sugar (28). The average degree of polymerization (DP; ratio of total carbohydrate to total reducing sugar) of these preparations was estimated to be 330. Xylanase assays were routinely performed using the reducing sugar assay with $MeGX_n$ as the substrate (14). In some cases, the multiwell plate bicinchoninic acid assay was used as described previously (29). Products generated from the enzyme assay were identified following resolution by thin-layer chromatography (TLC).

Chromatographic (TLC) analysis of xylan utilization. Samples were spotted onto silica gel 60 TLC plates (20 cm by 20 cm; Millipore). Reaction products were separated by ascension with 150 ml of developing solvent (chloroform-acetic acid-water; 6:7:1 [vol/vol/vol]) (30), allowing the solvent to migrate to within 1 cm of the top of the plate. Plates were allowed to dry prior to a second ascension. Plates were allowed to dry at ambient temperature overnight in a fume hood, sprayed with a solution containing 100 ml of methanol with 0.1685 g of *N*-(1-naphthyl)ethylenediamine dihydrochloride and 3 ml of H_2SO_4, and heated at 100°C to reveal the resolved components.

Preparation and analyses of oligosaccharides. Digestions and analyses of products of $MeGX_n$ depolymerization with XynA and XynC were carried out as previously described for XynC from *B. subtilis* subsp. *subtilis* 168 (16). Cultures with 0.2%, 0.5%, or 1.0% $MeGX_n$ as the carbon source in modified Spizizen's medium containing 0.1% yeast extract were incubated at 37°C with gyratory shaking (200 rpm) for 25 h. Samples of the cultures were directly spotted onto TLC plates for identification of the accumulated oligosaccharides as described above. Cells were removed by centrifugation (10,000 × *g*, 10 min, 4°C), and the supernatants were analyzed by matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry (MS) and ^1H nuclear magnetic resonance (NMR) as described in detail below.

MALDI-TOF MS analysis of $MeGX_n$ hydrolysis products. Products generated from the digestion of 0.2% $MeGX_n$ by recombinant XynA and/or recombinant XynC and 0.5% $MeGX_n$ by *B. subtilis* strains 168, MR42, and MR44 were analyzed without further concentration by MALDI-TOF MS. Analysis of the samples was performed on an Applied Biosystems Inc. Voyager-STR-DE MALDI-TOF MS operating in the positive-ion reflector mode with a delayed extraction time of 800 ns and a 20-kV accelerating voltage. Sufficient laser energy was employed to allow ionization, and 300 to 500 spectra were accumulated and averaged for each run. A stock matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in 1 ml of 30% acetonitrile containing 0.1% trifluoroacetic acid (MeCN-TFA). A working matrix solution was prepared by mixing 29 μl of the stock matrix solution with 1 μl of 2 mg ml^{-1} α-cyclodextrin (molecular mass = 972.86 g mol^{-1}) in MeCN-TFA. For analysis, 3.33 μl of sample was added to 30 μl of MeCN-TFA. This was added to a microcentrifuge tube containing 5 to 10 mg of Poros HS-20 strong cation exchanger that had previously been washed by suspension in MeCN-TFA and centrifugation. Samples were thoroughly mixed and centrifuged for 2 min to pellet the Poros resin. The resulting desalted supernatant aliquots of 1 μl were applied to the MALDI plate, followed by the addition of 1 μl of working matrix solution containing the internal standard, α-cyclodextrin (molecular mass = 972.86 g mol^{-1}). The drops were mixed with a pipette and allowed to dry at room temperature prior to loading into the instrument.

NMR analysis of $MeGX_n$ hydrolysis products. Samples for ^1H NMR were prepared as previously described (16). This involved three successive dissolutions in 3 ml 99.9 atom% D_2O (Sigma-Aldrich), each followed by lyophilization. Exchanged samples were dissolved to a concentration of 15 mg ml^{-1} total carbohydrate in 99.99% D_2O. To 1.0 ml of these preparations, 2.3 μl (31.3 μmol) of acetone was added as a reference (2.225 ppm), and the final samples were transferred to Wilmad 505-PS NMR tubes (Wilmad, Buena, NJ). ^1H NMR data collection was performed using a Mercury 300-MHz spectrometer with a 5 mm pulsed-field gradient broadband probe at the Department of Chemistry, University of Florida (acquisition time = 1.5 s, relaxation delay = 2 s, number of scans = 32). NMR data were analyzed and images were prepared using MestReNova software (Mestrelab Research, Chemistry Software Solutions). Assignment of shift positions for specific atoms was based upon the studies of U-XOS-derived methylglucuronoxylans of *Rudbeckia fulgida* by partial acid (TFA) hydrolysis (31).

RESULTS

Products generated by XynA, XynC, and their combination. *B. subtilis* subsp. *subtilis* 168 accumulates $MeGX_3$ when grown on polymeric $MeGX_n$, which may be the result of the combined actions of GH11 XynA and GH30 XynC. To test this possibility, the products generated from $MeGX_n$ by equivalent activity units of recombinant XynA, XynC, and a combination of the XynA and XynC enzymes were resolved by TLC (Fig. 2). As expected from previous studies, XynA generates X_2, X_3, and the aldouronate $MeGX_4$ as the predominant products. Aldouronates of larger size, presumably $MeGX_5$ and $MeGX_6$, are also present in lower concen-

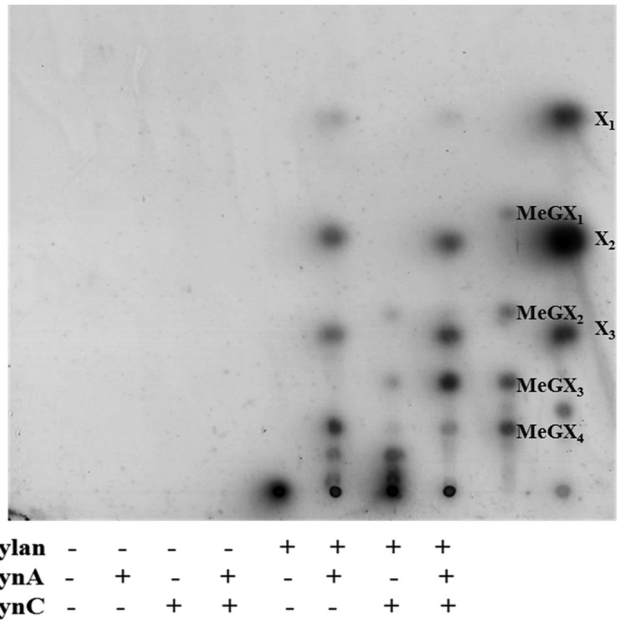

FIG 2 Products generated from $MeGX_n$ by recombinant XynA, XynC, and both enzymes together. Purified recombinant XynA and XynC, 0.1 unit of each in a 100-μl reaction mixture, were incubated with 0.2% sweet gum $MeGX_n$ in 0.05 M sodium acetate buffer, pH 6.0, for 18 h. Samples (10 μl) were resolved on silica gel TLC plates and detected as described in Materials and Methods. Standards of aldouronates (U-XOS) included 10 nmol each of $MeGX_1$, $MeGX_2$, $MeGX_3$, and $MeGX_4$. Standards of xylose and XOS included X_1 (10 nmol), X_2 (20 nmol), X_3 (10 nmol), and a trace amount of X_4 (xylotetraose) in the X_3 preparation.

trations and would likely be processed further to release more X_2 and X_1 (free xylose). XynC generates a mixture of large oligosaccharides, as well as components that correspond to $MeGX_4$, $MeGX_3$, and $MeGX_2$, by TLC, with no detectable X_1, X_2, or X_3. The combination of XynA and XynC generates predominantly X_2 and X_3 for rapid assimilation and growth by *B. subtilis* cultures, with $MeGX_3$ being a predominant limit product. As shown in Fig. 2, XynC generated small amounts of products that corresponded to $MeGX_4$, $MeGX_3$, and $MeGX_2$ with respect to mobility determined by TLC, with most products (estimated to be more than 95% of the products) being larger than $MeGX_4$. MALDI-TOF MS analysis identified a range of U-XOSs from $MeGX_4$ to $MeGX_{18}$ for the products generated from sweet gum $MeGX_n$ in this study (see Fig. 6A).

Evaluation of products by ^{1}H NMR. The products generated from sweet gum $MeGX_n$ by recombinant XynA, XynC, and the combination of both enzymes were analyzed by ^{1}H NMR. The products generated by XynA provided a ^{1}H NMR spectrum (Fig. 3A) that included limit product aldouronates X_3 and X_2 and a small amount of xylose (Fig. 2). Xylose ^{1}H-linked carbons in the aldouronate could not be quantitatively assigned. The ^{1}H linked to uronate C-1 showed a single doublet at 5.27 to 5.33 ppm, characteristic for ^{1}H on C-1 of MeG residues linked α-1,2 to xylose residues in oligosaccharides generated from methylglucuronoxylans by acid hydrolysis (31).

Products generated by XynC (Fig. 3B) included aldouronate limit products with no detectable xylose, X_2, or X_3 (Fig. 2). This provides a defining ^{1}H NMR spectrum with signals from 4.32 to 4.34 ppm for ^{1}H atoms linked to the C-5 of MeG (U-5), from 4.08 to 4.14 ppm for ^{1}H atoms linked to the C-5 (X-5) of internal β-1,4-linked (including the reducing terminal) xylose, and from 3.95 to 3.98 ppm for ^{1}H atoms linked to the C-5 (X-5) of the nonreducing (nr) terminal xylose. The ratio of the ^{1}H integrals (int–X-1 + nr–X-1 + U–X-1 + αr–X-1 + βr–X-1)/U_1 (where int refers to xylose within the β-1,4 xylan chain, αr refers to xylose in the alpha configuration at the reducing terminus, and βr refers to xylose in the beta configuration at the reducing terminus) was 6.9, representing the average degree of substitution of xylose residues with MeG in the polymeric $MeGX_n$. The ratio of ^{1}H integrals (int–X-1 + nr–X-1 + U–X-1 + βr–X-1 + αr–X-1)/(βr–X-1+ αr–X_1) was 6.7. Together, these values confirm that that each U-XOS bears a single MeG substitution. The ^{1}H atoms in the common molecular environment of the C-4-linked —OCH_3 of the MeG residue showed a prominent signal at 3.46 ppm that was readily detectable in polymeric $MeGX_n$ as well as oligosaccharides (31). For all of the digests, the integration of ^{1}H–U-5 was assigned a value of 1 for comparison with other hydrogens. The ratio of ^{1}H-U-OCH_3/^{1}H–U-5 was 3.66:1, or 1.22:1 on a single-hydrogen basis. These results support previous ^{13}C NMR studies of sweet gum $MeGX_n$ that found that the U-1, U-4, and U-OCH_3 carbons are equivalent by integration, indicating that all of the C-4 carbons on the glucuronate residues contain —OCH_3 groups (32). The signal for the ^{1}H on C-1 of the MeG showed a split doublet at 5.27 to 5.32 ppm; in comparison, a single doublet at 5.27 to 5.30 ppm was found for the products generated by XynA. The splitting of this doublet is characteristic for the ^{1}H of a MeGA residue linked to the xylose penultimate to the reducing terminal xylose in the oligosaccharide, whereby the 60:40 anomeric equilibrium of the α and β forms of the reducing terminal xylose influences the environment of the ^{1}H on C-1 of the MeG that is α-1,2 linked to xylose adjacent to the reducing terminal residue (16, 33).

Products generated by the combination of the XynA and XynC enzymes showed a complex spectrum (Fig. 3C) that reflected, as in the case of the spectrum for the XynA digest (Fig. 3A), the presence of X_3, X_2, and xylose, as well as the aldouronate $MeGX_3$ (Fig. 2). The ^{1}H–U-1 signal showed a split doublet at 5.27 to 5.32 ppm characteristic of substitution at a xylose penultimate to the reducing terminal xylose. The 60:40 ratio for this split supports a structure for the $MeGX_3$ generated by the action of XynC on the $MeGX_4$ generated by XynA, as seen with the processing of birch wood xylan (34). The combination of XynA processing of the products generated by XynC and of XynC processing of the products generated by XynA is then responsible for the conversion of $MeGX_n$ to X_3, X_2, and xylose, as well as $MeGX_3$, in which a xylose flanked by xylose residues is substituted with an α-1,2-linked 4-*O*-methylglucuronate.

Effects of deletion of genes encoding XynA and/or XynC on the utilization of $MeGX_n$ by *B. subtilis*. To investigate the role that the XynA and XynC xylanases play in $MeGX_n$ utilization, the genes encoding these enzymes were deleted individually to provide mutants MR42 Δ*xynC* and MR44 Δ*xynA* or in combination to provide mutant MR45 Δ*xynA* Δ*xynC*. The growth of these strains (Table 1) was compared to that of parent strain 168 with 0.5% sweet gum $MeGX_n$ in a medium supplemented with yeast extract (Fig. 4).

The growth (apparent rate and yield based upon turbidity) of strain MR45, which is unable to produce either XynA or XynC, was markedly lower than that of parent strain 168, reflecting the inability to generate xylotriose and xylobiose for growth. Strain

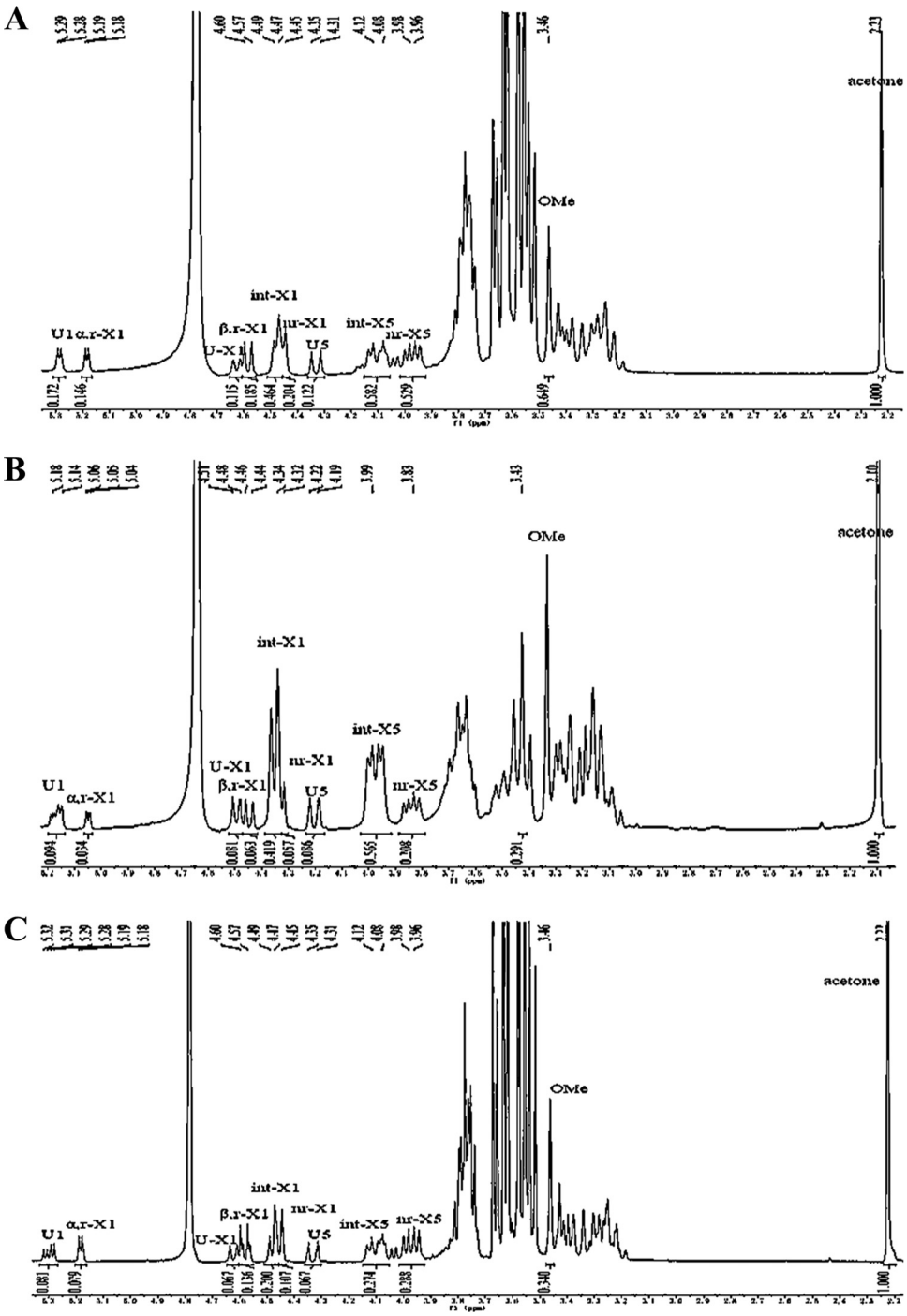

FIG 3 Comparison by ^{1}H NMR of products generated by recombinant XynA, XynC, and the combination of both enzymes. Reaction mixtures containing 0.5% sweet gum MeGX$_n$ in 0.05 M sodium acetate buffer, pH 6.5, and enzyme were incubated for 18 h at 37°C. Samples representing a 3.0-ml reaction mixture containing 15 mg MeGX$_n$ were exchanged with D_2O through successive lyophilization steps, dissolved in 99.99% D_2O to a final volume of 1.0 ml, and analyzed on a Mercury 300 spectrometer as described in Materials and Methods. The XynA digest contained 13.6 μmol of acetone as an internal standard. The XynC and the combination of XynA and XynC digests contained 31.3 μmol acetone. (A) Recombinant XynA at 0.1 unit; (B) recombinant XynC at 0.1 unit; (C) recombinant XynA at 0.1 unit and recombinant XynC at 0.1 unit. OMe, methoxy.

MR44, which secretes XynC but lacks XynA, initially grew to a higher turbidity than MR45, and then the turbidity dropped to the level seen for MR45. This result, which was repeated, was surprising, as the XynC enzyme does not generate detectable quantities of xylotriose, xylobiose, or even xylose from MeGX$_n$ (Fig. 2). Strain MR42, which secretes XynA but lacks XynC, grew to a greater extent than MR44, as expected, as it does generate xylotriose and xylobiose from MeGX$_n$.

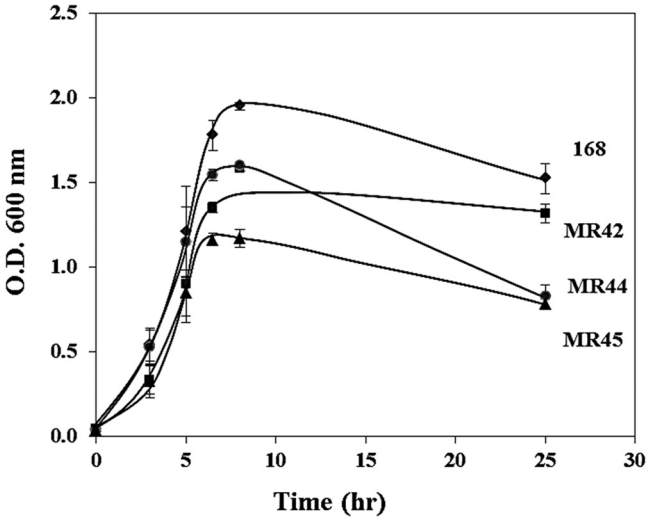

FIG 4 Growth of *B. subtilis* strains 168, MR42, MR44, and MR45 on $MeGX_n$. For preparing inocula for growth comparisons, 18-h standing cultures (1.0 ml of LB with kanamycin at 5 μg ml^{-1} for MR42, spectinomycin at 100 μg ml^{-1} for MR44, and kanamycin at 5 μg ml^{-1} and spectinomycin at 100 μg ml^{-1} for MR45) of 0.03 ml were inoculated into 1.0 ml of the same medium without antibiotics and incubated for 3 h at 37°C with shaking. Cultures of these strains (Table 1) grown in LB medium to late log phase (optical density [O.D.] at 600 nm, 0.6 to 0.7) were inoculated (0.4 ml) into 20 ml of Spizizen's minimal medium with 0.5% $MeGX_n$ and 0.1% yeast extract without antibiotics to give an optical density at 600 nm of 0.03. Cells were cultured at 37°C with gyratory shaking (200 rpm). The experiment was repeated, and the results are given as the means of the 2 sets of values with error bars (SEM).

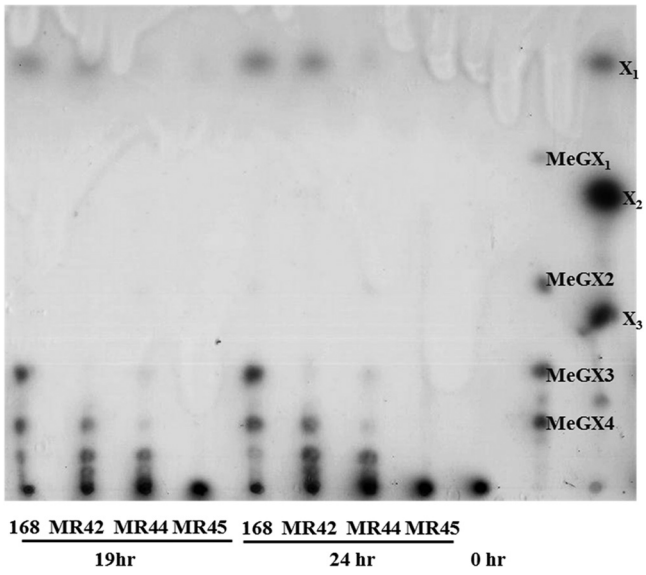

FIG 5 Accumulation of U-XOS by *B. subtilis* strains. Media (10 μl) from cultures of *B. subtilis* strains described in the legend for Fig. 4 were resolved and detected by TLC as described in Materials and Methods. Standards of aldouronates (U-XOS) included 10 nmol each of $MeGX_1$, $MeGX_2$, $MeGX_3$, and $MeGX_4$. Standards of xylose and XOS included X_1 (10 nmol), X_2 (20 nmol), X_3 (10 nmol), and a trace amount of X_4 (xylotetraose) in the X_3 preparation.

The utilization of carbohydrate (Table 2) was, as expected, the greatest for strain 168 but incomplete, with 28% remaining at 25 h, 17 h past the time of maximal growth, at which time 53% of the total carbohydrate had been consumed. During 8 h of exponential growth, strain MR45 lacking the XynA and XynC xylanases utilized 30% of the $MeGX_n$. The absence of either xylose or XOS detectable by TLC (Fig. 5) suggested the possibility that other sugars may provide some carbohydrate that does not depend upon xylanolytic depolymerization. On the basis of analysis of the sugar composition in hydrolysates of sweet gum lignocelluloses, glucans may comprise a small amount of the hemicellulose (xylan) fraction of sweet gum, although these were not detected as significant components upon NMR analyses of the polymeric $MeGX_n$. Strain MR42, which secretes XynA, consumed 57% of the total carbohydrate, as expected, with the generation of X_2 and X_3, which were readily consumed. It is surprising that MR44, which secretes the GH30 (XynC) enzyme, showed 54% consumption of the $MeGX_n$ substrate, as the aldouronate products of XynC digestion were not directly utilized. There may be exoxylanolytic activities that can process XynC products to release xylose and/or XOS that support some growth. However, the ^1H NMR spectra of MR44 medium (see Fig. 7C and Table 3) indicate that xylose and MeG were present in a ratio similar to that found in the $MeGX_n$, which indicates nearly complete conversion of the $MeGX_n$ substrate to U-XOS products and their accumulation, a result which fits the established model of $MeGX_n$ processing by this enzyme.

TABLE 2 Utilization of carbohydrates[a] during growth

Time (h)	Total carbohydrate (mM xylose equivalents)			
	168	MR42	MR44	MR45
0	30.0 ± 2.6	30.0 ± 2.6	30.0 ± 2.6	30.0 ± 2.6
5	15.6 ± 1.4	22.1 ± 1.2	19.2 ± 0.9	24.7 ± 2.2
8	14.2 ± 0.8	19.0 ± 0.3	16.4 ± 0.9	21.2 ± 1.2
25	8.4 ± 1.1	13.3 ± 0.3	14.5 ± 0.5	21.1 ± 1.8

[a] Cultures were cultivated on Spizizen's medium containing 0.1% yeast extract and 0.5% sweet gum $MeGX_n$, as described in the Materials and Methods section.

Accumulation of U-XOS by *B. subtilis* strains. The culture media from each strain were evaluated for the accumulation of oligosaccharides by thin-layer chromatography (Fig. 5). Strain 168 accumulated $MeGX_4$, which is an expected product of the recombinant XynA, and also $MeGX_3$, which is an expected product of the combination of recombinant XynA and XynC (Fig. 2). The appearance of some xylose was observed following digestion of $MeGX_n$ by XynA or a combination of XynA and XynC. Strain MR42 Δ*xynC* showed the accumulation of $MeGX_4$, the expected product of XynA, as well as larger oligosaccharides with mobilities expected for $MeGX_5$ and $MeGX_6$. A similar mixture was noted in the XynA-generated digest of $MeGX_n$ (Fig. 2). The much lower levels of these larger aldouronates in the medium from strain 168 cultures indicate the synergistic role that XynA and XynC play in maximizing the production of xylose and XOS for assimilation and growth. Strain MR44 Δ*xynA* accumulated $MeGX_4$ to $MeGX_{18}$ (Fig. 6B) and also traces of aldouronates with mobilities corresponding to those of $MeGX_4$, $MeGX_3$, and $MeGX_2$. The absence of detectable xylose indicates that this strain and other strains lack an extracellular β-xylosidase that significantly participates in the further processing of generated XOS. Strain MR45 Δ*xynA* Δ*xynC* accumulated no detectable XOS, indicating that XynA and XynC are the only endoxylanases secreted by *B. subtilis*.

The size of the oligosaccharides generated by the recombinant XynC provides a basis for the analysis of the medium of the MR44 culture by MALDI-TOF MS to determine the role of XynC in the

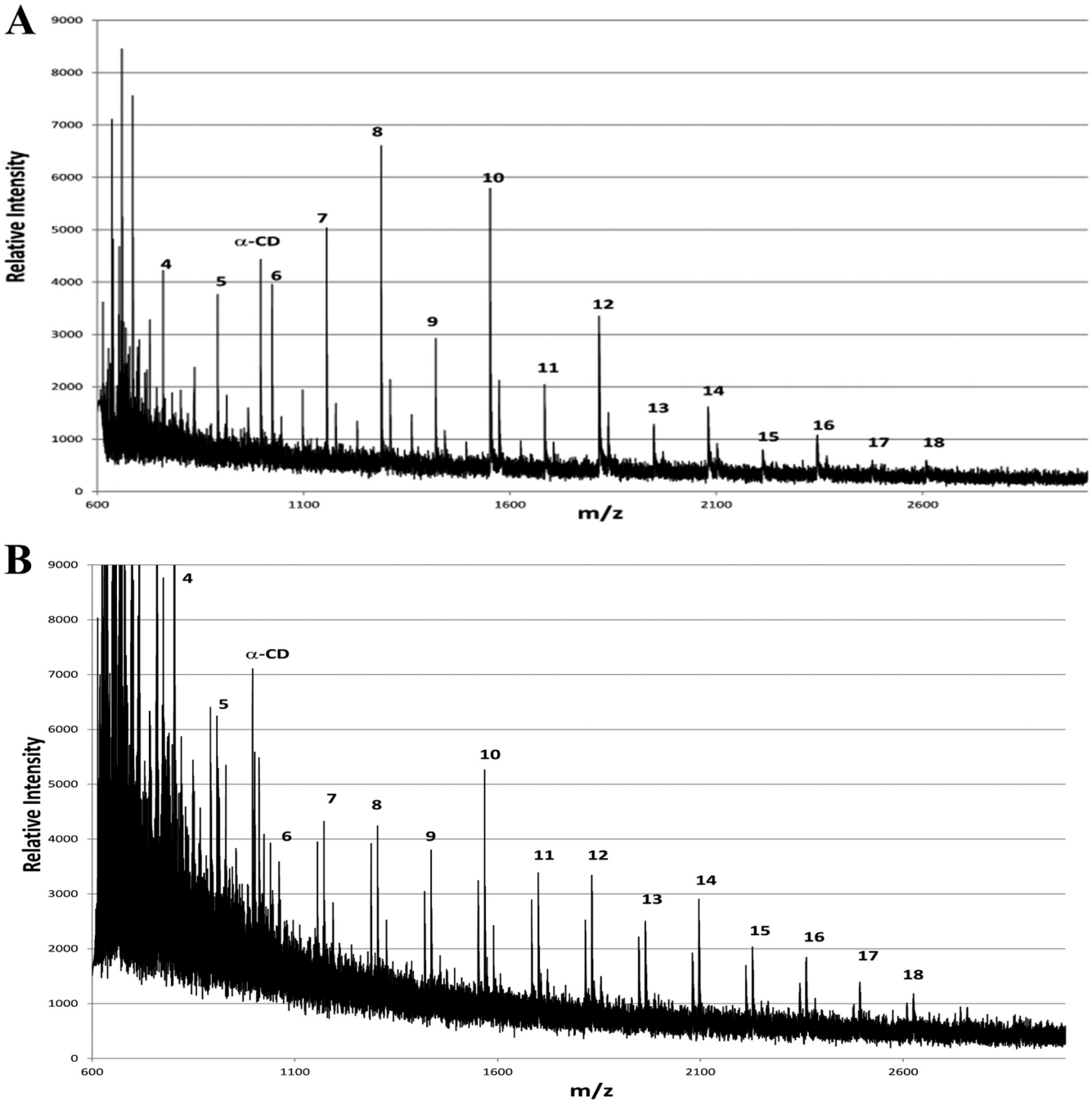

FIG 6 MALDI-TOF MS analysis of products generated by recombinant GH30 XynC and cultures of strain MR44 from $MeGX_n$. Numbers are assigned to species on the basis of the number of xylose units appended to an aldouronate containing a single MeG and Na^+ and/or K^+. (A) Recombinant XynC (0.1 unit) from *B. subtilis* subsp. *subtilis* 168 was incubated at 37°C in 0.1 ml of 0.5% sweet gum $MeGX_n$ in 0.05 M sodium acetate buffer, pH 6.0, for 18 h. Samples were removed and processed for MS as described in Materials and Methods. As Na^+ was the predominant cation in the reaction medium, the Na^+ adduct was the prominent species detected. (B) MR44 was cultured for 24 h as described in the legend for Fig. 4. Samples were removed and processed for MS as described in Materials and Methods. With K^+ as the predominant cation in the medium, the K^+ adduct was the prominent species detected. Numbers above the predominant adduct species represent the number of xylose residues in the U-XOS. Alpha-cyclodextrin (α-CD) was the internal standard used in all analyses.

accumulation of aldouronates that are not assimilated and metabolized. Figure 6A shows the products generated by *in vitro* reaction with the recombinant XynC on the $MeGX_n$ used in the medium for the MR44 culture. XynC generated aldouronate products with *m/z* values that corresponded to those for the sodium salts of $MeGX_4$ to $MeGX_{18}$ and that were similar to those previously documented (16). Figure 6B shows the products accumulated by MR44 in the medium, with an *m/z* profile qualitatively similar to that observed for products generated by recombinant XynC *in vitro*.

^{1}H NMR analysis of U-XOS products accumulated in cultures. To identify the products accumulating in the media of parent strain 168 as well as mutant strains MR42 Δ*xynC* and MR44

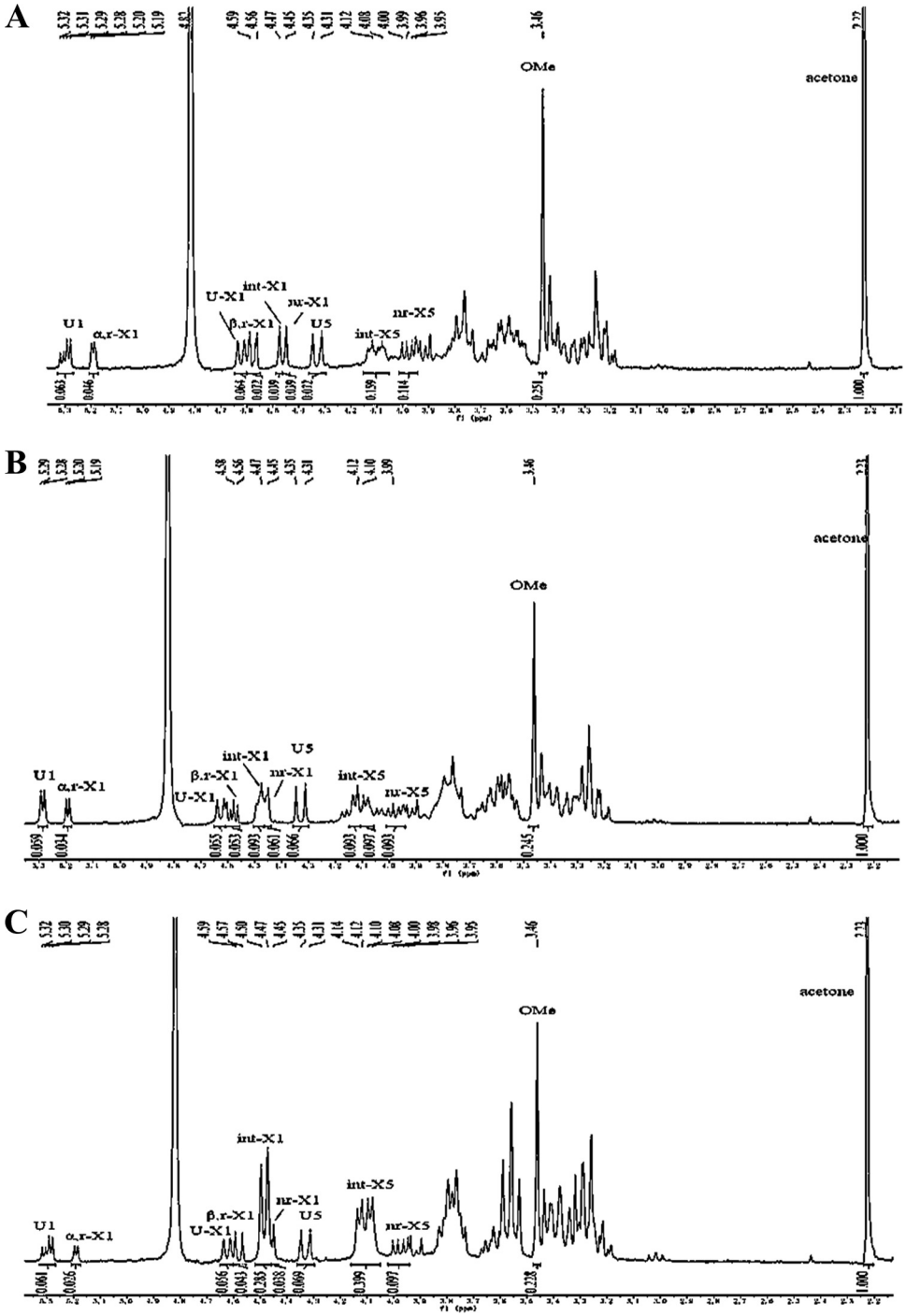

FIG 7 ^{1}H NMR analysis of U-XOS products accumulated in cultures. Samples from stationary-phase cultures (3.0 ml of a 20-ml culture at 25 h; Fig. 4) were centrifuged to remove cells. The cell-free medium was concentrated by lyophilization and exchanged with 99.9% D_2O with 3 successive treatments. After a final lyophilization, the sample was dissolved in 99.9% D_2O to a volume of 1.00 ml, to which was added 2.3 μl of 99.7% acetone (31.3 μmol), and the mixture was analyzed on a 300-MHz Mercury 300 spectrometer as described in Materials and Methods. (A) *B. subtilis* subsp. *subtilis* 168; (B) mutant MR42; (C) mutant MR44.

Δ*xynA*, cultures were grown to stationary phase and the media were analyzed for accumulated products by ^{1}H NMR. Strain 168 showed the accumulation of $MeGX_3$ as the most prominent aldouronate, along with products with TLC mobilities corresponding to those of $MeGX_4$ and $MeGX_5$, as well as small amounts of xylose (Fig. 5). Both X_2 and X_3 were prominent products in the digestion of $MeGX_n$ by a combination of recombinant XynA and XynC (Fig. 3). These products would have been formed and consumed by strain 168, which secretes both of these enzymes. The clean spectrum for the medium for strain 168 identified the aldouronate $MeGX_3$ as the only accumulated product. The integration of the ^{1}H signals for all strains (Fig. 7A to C) provides a semiquan-

TABLE 3 Quantitation of U and X residue accumulation in cultures by ^{1}H NMR

Strain	U-1 and X-1			U-5 and X-5			
	Concn (mM)			Concn (mM)			
	U-1[a]	X-1[b]	X-1/U-1	U-5[c]	X-5[d]	X-5/U-5	% $MeGX_n$[e]
168	11.8	48.9	4.1	13.5	51.3	3.8	88
MR42	11.1	55.6	5.0	12.4	53.2	4.3	83
MR44	11.5	84.2	7.3	13.0	93.2	7.1	86

[a] ^{1}H–U-1 determined as the integration ratio of ^{1}H atom equivalents of MeG ^{1}H–C-1 (5.28 to 5.32 ppm) to acetone [^{1}H-$(CH_3)_2$; 2.23 ppm] set to 1.00 for 188 mM ^{1}H atom equivalents.

[b] ^{1}H–X-1 determined as the ratio of the sum of ^{1}H integrations for α–X-1 (5.19 to 5.20 ppm), U–X-1 (4.60 to 4.68 ppm), β–X-1 (4.56 to 4.59 ppm), int–X-1 (4.47 to 4.5 ppm), and nr–X-1 (4.45 ppm) to 1.00 for acetone at 188 mM ^{1}H atom equivalents.

[c] ^{1}H–U-5 determined as the ratio of ^{1}H integration (4.31 to 4.35 ppm) to 1.00 for acetone at 188 mM ^{1}H atom equivalents.

[d] ^{1}H–X-5 (axial only) determined as the ratio of ^{1}H integration (4.08 to 4.12 ppm) to 1.00 for acetone at 188 mM ^{1}H atom equivalents.

[e] The percentage of the $MeGX_n$ substrate converted to accumulated aldouronate products by each culture was determined on the basis of the X/MeG ratio found in the products generated from 0.5% $MeGX_n$ substrate after complete digestion with pure XynC. XynC digestion generated exclusively U-XOS containing a single MeG with an X/MeG ratio of 6.9 for integration of X-1/U-1 by ^{1}H NMR. The concentration of $MeGX_n$ in the uninoculated medium was 5 mg ml^{-1}, and following the 3× concentration of 3.0 ml of culture medium during the process of D_2O exchange prior to NMR analysis (see Materials and Methods), the accumulated products would have been derived from 15 mg ml^{-1} $MeGX_n$. Using the molecular mass for the product $MeGX_{6.9}$ (191 + 5.9 × 132 + 150 = 1,120 mg mmol^{-1}), the concentration of $MeGX_{6.9}$ equivalents, equal to the concentration of MeG equivalents in the uninoculated medium, was 15 mg ml^{-1}/1,120 mg mmol^{-1}, or 13.3 mM. This value, divided by the concentration of U-1, provides an estimate of the fraction (in percent) of the $MeGX_n$ accumulated as products of $MeGX_n$ digestion.

titative estimate of the quantities of aldouronate products and the extent of conversion of $MeGX_n$ that led to these products (Table 3). The ^{1}H NMR spectra of MR44 medium (Fig. 7C; Table 3) indicate that MeG and xylose were present in a ratio similar to that found in the XynC digest of $MeGX_n$, indicating optimal conversion without further processing of the $MeGX_n$ substrate to U-XOS products by strain MR44.

DISCUSSION

Alternative strategies for efficient bioconversion of methylglucuronoxylans. An efficient process for depolymerization of $MeGX_n$, followed by assimilation and metabolism of all of the products of depolymerization, has been ascribed to bacteria that secrete GH10 endoxylanases and intracellularly process both the acidic U-XOS and the neutral XOS (12). Bacteria that secrete a GH10 endoxylanase generate X_2, X_3, and $MeGX_3$ in which the MeG is linked to the nonreducing terminal xylose and produce a GH67 α-glucuronidase to process the assimilated $MeGX_3$ and generate the greatest yields of fermentation products from $MeGX_n$ with this level of MeG substitution. *Paenibacillus* sp. strain JDR2 (Pjdr2) provides an example in which the efficient utilization of $MeGX_n$ involves extracellular depolymerization catalyzed by a cell-associated multimodular GH10 endoxylanase (35) coupled with assimilation of aldouronates and XOS by ABC transporters and intracellular processing of U-XOS and XOS to xylose. The intracellular processing is catalyzed by a combination of glycoside hydrolases, including a GH67 α-glucuronidase, a GH10 endoxylanase, and a GH43 β-xylosidase/α-L-arabinofuranosidase (14, 36). On the basis of genomic sequences, these systems may occur in a few other bacteria as well.

B. subtilis subsp. *subtilis* 168 has no gene encoding GH10 endoxylanases or GH67 α-glucuronidases yet efficiently depolymerizes $MeGX_n$ and assimilates and metabolizes the neutral XOS, X_2, and X_3 generated by the combined action of the secreted GH11 XynA and the GH30 XynC enzymes. The combined action of these two xylanases on $MeGX_n$ is depicted in Fig. 8, wherein xylose accumulates as $MeGX_3$ and xylobiose and xylotriose are assimilated by ABC transporters. Support for this scheme comes from the structural definition of the $MeGX_3$ with the MeG linked to the xylose penultimate to the reducing terminus, precluding potential processing by a GH67 α-glucuronidase.

With a ratio of X to MeG of 6 to 7:1, an approximate average range for $MeGX_n$ from sweet gum (*Liquidambar syraciflua*), the products of complete digestion would be X_1, X_2, X_3, and $MeGX_3$, with the neutral XOS representing approximately 50% of the total available xylose for assimilation and metabolism. Both X_3 and X_2 could be rapidly assimilated by ABC transporters, with xylose being assimilated more slowly. However, if the ratio of X to MeG reaches 20, as it may for the methylglucuronoarabinoxylans ($MeGAX_n$) in the hemicellulose fraction of grasses, the GH30-GH11 xylanase combination may achieve utilization of 85% of the xylose without processing the $MeGX_3$. In this case, *B. subtilis* and other bacteria that secrete GH11 and GH30 endoxylanases may be further developed as biocatalysts for the efficient fermentation of $MeGAX_n$ to targeted products.

U-XOS accumulation by *B. subtilis* strains with deletions in XynA or XynC. The generation of a series of aldouronates with an increasing number of xylose residues and a single MeG linked α-1,2 to a xylose penultimate to the reducing terminal xylose is a characteristic of GH30 endoxylanases, with XynC from *Bacillus subtilis* (15, 16, 37) and XynA from *Dickeya dadantii* (previously *Erwinia chrysanthemi*) (25, 38, 39) being examples of these enzymes. For *B. subtilis*, XynC generates few, if any, neutral XOS products for assimilation and metabolism from its action on the polymeric $MeGX_n$. In contrast GH11 endoxylanases generate aldouronates in which MeG is linked α-1,2 to a xylose penultimate to the nonreducing terminal xylose with $MeGX_4$ as the limit product, along with xylotriose, xylobiose, and some xylose (11). The studies presented here confirm the products expected for XynA and XynC from *B. subtilis* subsp. *subtilis* 168 with $MeGX_n$ from the dicotyledonous hardwood sweet gum. The path of carbon during growth on $MeGX_n$ may proceed sequentially either through XynA-mediated depolymerization followed by XynC-mediated depolymerization or first through XynC-mediated depolymerization followed by XynA-mediated depolymerization.

In strain MR42, XynA is the only xylanase secreted, resulting in the expected limit for a GH11 endoxylanase of $MeGX_4$. As seen in

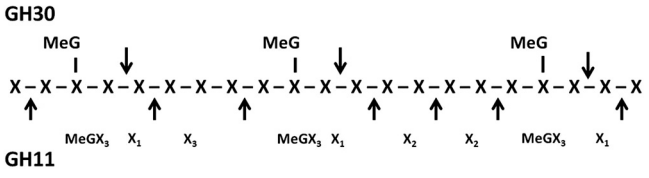

FIG 8 Schematic for the release of X_1, X_2, X_3, and $MeGX_3$ in *B. subtilis* subsp. *subtilis* 168. GH11 XynA (lower arrows) and GH30 XynC (upper arrows) hydrolyzed $MeGX_n$ to produced X_1, X_2, X_3, and $MeGX_3$. X_2 and X_3 were assimilated by *B. subtilis* subsp. *subtilis* 168, and X_1 was consumed slowly, while $MeGX_3$ was accumulated in the culture medium.

the TLC analysis (Fig. 2) of the products generated by recombinant XynA, XynC, and the combination of XynA and XynC, XynA generates $MeGX_4$ but also significant levels of aldouronates with the mobilities expected for $MeGX_5$ and $MeGX_6$. When XynC is present with XynA, $MeGX_4$ and the larger products are processed to $MeGX_3$.

MALDI-TOF MS provided profiles supporting the common identities of the products generated by XynC and strain MR44, in which the gene encoding the GH11 XynA was deleted, indicating that XynC is the only endoxylanase activity other than XynA that is secreted by *B. subtilis* subsp. *subtilis* 168. This was confirmed by the ^{1}H NMR spectra of the XynC digest (Fig. 3B) and the MR44 culture medium (Fig. 7C), which structurally defined the products and provided qualitative and quantitative information on the yields and average DP values of the accumulated aldouronates. The average DP values of the accumulated products determined by the ratios of ^{1}H on C-1 or axial C-5 on all xylose residues to ^{1}H on the xylose on the reducing terminus are similar to the average xylose-to-methylglucuronate ratios (Table 3). This supports the process for the accumulation of aldouronates of different compositions by strains secreting only XynA, XynC, or both enzymes. For parent strain 168, the recovery of MeG in the medium was 88% of the MeG provided in the substrate $MeGX_n$, estimated from the ratio of X to MeG in the products accumulated in strain MR44. The estimated recovery of MeG in strain MR44 was approximately the same at 86%. These estimated values are dependent on the accuracy of the integrations of different peaks from the ^{1}H NMR spectra and may be subject to some error derived from the contributions to a given peak by more than a single ^{1}H atom. However, the MeG recoveries in accumulated aldouronates for strains 168 and MR44 as well as strain MR42 showed essentially the same recovery of MeG, indicating that they can be used as biocatalysts for the production of defined aldouronates: $MeGX_3$ for strain 168, $MeGX_4$ for strain MR42, or mixtures of $MeGX_4$ to $MeGX_{18}$ for strain MR44.

Aldouronates, acidic xylooligosaccharides (U-XOS) containing one or more methylglucuronate residues linked α-1,2 to xylose residues in the β-1,4-xylan backbone in methylglucuronoxylans, have been shown to have a range of immunomodulating and antimicrobial activities (4, 5, 40, 41). These have received increasing attention for additional applications, including the production of pentosan polysulfate (PPS). PPS refers to products derived from U-XOS that are chemically sulfated to produce homologues of the naturally occurring glycosaminoglycan sulfates heparin and chondroitin sulfate (5). These have been applied to the treatment of interstitial cystitis in humans (6) and osteoarthritis in horses (8, 42). Novel properties of PPS that are expected to extend to the treatment of disease associated with mucopolysaccharidoses have been discovered (7).

The formation of PPS from pentosans involves the chemical sulfation of methylglucuronoxylans from hardwoods. A prominent source is wood from European beech, which is subjected to thermochemical pretreatment to release the soluble U-XOS generated from the partial hydrolysis of $MeGX_n$ (43). Chemical sulfation provides a mixture of sulfated U-XOSs that contain one or more uronic acids. The ability of *B. subtilis* to process glucuronoarabinoxylans and metabolize released arabinose, as well as metabolize α- and β-glucans, indicates that strain MR44 can be used to process impure preparations of hemicelluloses generated by the alkaline pretreatment of lignocellulosic biomass. Strain MR44 may then serve as a biocatalyst to process hemicellulose fractions from various resources, including energy crops and agricultural residues, to provide pentosans for the production of PPS with a defined composition for applications to human and veterinary medicine. *B. subtilis* strains MR42 and 168 may also serve as biocatalysts for the production of $MeGX_4$ and $MeGX_3$ to develop applications for these acidic xylooligosaccharides.

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