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Comparative Analyses of Two Thermophilic Enzymes Exhibiting both β-1,4 Mannosidic and β-1,4 Glucosidic Cleavage Activities from *Caldanaerobius polysaccharolyticus*▽†

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The hydrolysis of polysaccharides containing mannan requires endo-1,4-β-mannanase and 1,4-β-mannosidase activities. In the current report, the biochemical properties of two endo-β-1,4-mannanases (Man5A and Man5B) from *Caldanaerobius polysaccharolyticus* were studied. Man5A is composed of an N-terminal signal peptide (SP), a catalytic domain, two carbohydrate-binding modules (CBMs), and three surface layer homology (SLH) repeats, whereas Man5B lacks the SP, CBMs, and SLH repeats. To gain insights into how the two glycoside hydrolase family 5 (GH5) enzymes may aid the bacterium in energy acquisition and also the potential application of the two enzymes in the biofuel industry, two derivatives of Man5A (Man5A-TM1 [TM1 stands for truncational mutant 1], which lacks the SP and SLH repeats, and Man5A-TM2, which lacks the SP, CBMs, and SLH repeats) and the wild-type Man5B were biochemically analyzed. The Man5A derivatives displayed endo-1,4-β-mannanase and endo-1,4-β-glucanase activities and hydrolyzed oligosaccharides with a degree of polymerization (DP) of 4 or higher. Man5B exhibited endo-1,4-β-mannanase activity and little endo-1,4-β-glucanase activity; however, this enzyme also exhibited 1,4-β-mannosidase and cellodextrinase activities. Man5A-TM1, compared to either Man5A-TM2 or Man5B, had higher catalytic activity with soluble and insoluble polysaccharides, indicating that the CBMs enhance catalysis of Man5A. Furthermore, Man5A-TM1 acted synergistically with Man5B in the hydrolysis of β-mannan and carboxymethyl cellulose. The versatility of the two enzymes, therefore, makes them a resource for depolymerization of mannan-containing polysaccharides in the biofuel industry. Furthermore, on the basis of the biochemical and genomic data, a molecular mechanism for utilization of mannan-containing nutrients by *C. polysaccharolyticus* is proposed.

Bioenergy feedstocks consist primarily of the plant cell wall components cellulose and hemicellulose, and hydrolysis of these polysaccharides to their monomeric sugars involves a set of enzymes acting synergistically to cleave the different chemical linkages (9). Although cellulose consists of glucose units linked together in β-1,4-glycosidic linkages, the hemicellulosic component of feedstock may vary in chemical composition. Some feedstock hemicellulose molecules are mainly composed of β-1,4-linked xylose backbones with arabinose side chains, while others are comprised of a larger variety of sugars, including galactose and mannose existing as various forms of mannan (26). Mannans constitute a less significant portion of hemicellulose in bioenergy feedstocks, such as switchgrass. However, the presence of mannan may result in linkages that restrict release of fermentable sugars.

A variety of mannans are found in nature. These include linear mannan, glucomannan, galactomannan, and glucogalactomannan. In each case, the polysaccharide contains a β-1,4-linked backbone of mannose residues that may be substituted up to 33% (or up to 50% in hardwoods) with glucose residues (32). In galactomannans or glucogalactomannnans, galactose residues are linked in α-1,6-linkages to the mannan backbone (26). Therefore, hydrolysis of mannan to its component sugars requires endo-1,4-β-mannanases that hydrolyze the backbone linkages to generate short-chain manno-oligosaccharides that are further degraded to monosaccharides by 1,4-β-mannosidases.

Caldanaerobius polysaccharolyticus (21), originally named *Thermoanaerobacterium polysaccharolyticum* (5), is a thermophilic bacterium isolated from a waste pile from a canning factory in Illinois. It was demonstrated that the *C. polysaccharolyticus* genome encodes Man5A, a modular enzyme with mannanase/endoglucanase activities (4), and recently, the structures of the individual carbohydrate-binding modules (CBMs) that occur in tandem in this enzyme have been solved both in the apo form and also in complex with ligand (2). In the present report, we show that *C. polysaccharolyticus* contains a second mannanase gene, *man5B*. Interestingly, the gene product Man5B lacks the predicted signal peptide, CBMs, and surface layer homology (SLH) modules found in Man5A. The *C. polysaccharolyticus* Man5B hydrolyzes mannan-containing polysaccharides and both manno- and cello-oligosaccharides. Furthermore, we demonstrate that Man5B acts synergistically with Man5A-TM1 (TM1 for truncational mutant 1), a derivative of Man5A, to release products from substrates, such as β-mannan and car-

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boxymethyl cellulose (CMC). The catalytic efficiencies of Man5A-TM1, Man5A-TM2, and Man5B were determined on different substrates, and site-directed mutagenesis was used to identify the critical residues involved in catalysis in the two enzymes.

Reactions at high temperatures are critical for the enzymatic conversion of plant cell wall polysaccharides to fermentable sugars in the emerging biofuel industry. The two enzymes described in the present report represent examples of applicable enzymes necessary for enzymatic depolymerization of lignocellulose. In addition, the detailed description of the biochemistry of Man5A and Man5B aids us to propose a mechanism by which *Caldanaerobius polysaccharolyticus* derives energy from substrates, such as mannan-containing polysaccharides.

MATERIALS AND METHODS

Materials. *Thermoanaerobacterium polysaccharolyticum* (ATCC strain no. BAA-17) was originally isolated from the waste pile of a canning factory in Hoopeston, Illinois (5). Recently, the strain was reclassified and put into a new genus and given the name *Caldanaerobius polysaccharolyticus* (21). *Escherichia coli* JM109 and BL21-CodonPlus(DE3) RIL competent cells and the PicoMaxx high-fidelity PCR system were purchased from Stratagene (La Jolla, CA). The pGEM-T TA-cloning vector and GoTaq DNA polymerase were acquired from Promega (Madison, WI). The pET-28a vector and the pET-46b EK/LIC cloning kit were obtained from Novagen (San Diego, CA). The NdeI, XhoI, and DpnI restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). The DNeasy blood and tissue kit and the QIAprep spin miniprep kit were obtained from Qiagen, Inc. (Valencia, CA). Talon metal affinity resin was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Amicon Ultra-15 centrifugal filter units with molecular weight cutoffs (MWCOs) of 10,000 and 50,000 were obtained from Millipore (Billerica, MA).

Manno-oligosaccharides (mannobiose [M_2], mannotriose [M_3], mannotetraose [M_4], mannopentaose [M_5], and mannohexaose [M_6]), cello-oligosaccharides (cellobiose [G_2], cellotriose [G_3], cellotetraose [G_4], cellopentaose [G_5], and cellohexaose [G_6]), α-1,6-galactosyl-mannobiose (GM_2), α-1,6-galactosyl-mannotriose (GM_3), 1,4-β-D-mannan, and Konjac glucomannan were all obtained from Megazyme (Bray, Ireland). Sodium carboxymethyl cellulose (CMC) was purchased from Acros Organics (Geel, Belgium). Gel filtration standards were obtained from Bio-Rad (Hercules, CA). All other reagents were of the highest possible purity and purchased from Sigma-Aldrich (St. Louis, MO).

Cloning, expression, and purification of Man5A-TM1, Man5A-TM2, and Man5B. Genomic DNA from mid-log cultures of *C. polysaccharolyticus* was purified using the Qiagen DNeasy blood and tissue kit, and the genome was partially sequenced (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois) using the GSFLX Titanium from 454 Life Sciences (Branford, CT). The assembled contigs were uploaded onto the Rapid Annotation using Subsystem Technology (RAST) server for autoannotation (1). The open reading frame ORF0760 (*man5b*) was identified as a gene predicted to encode β-D-mannanase/endoglucanase activities. Man5A was previously identified in a recombinant phage library (4), and its full sequence was also identified in the partial genome sequence.

The DNA sequence corresponding to *man5B* (GenBank accession no. HM241690) was amplified with the PicoMaxx high-fidelity PCR kit and cloned into the pET-46b vector. Man5A-TM1, composed of the catalytic module and the two CBMs, was PCR amplified using genomic DNA as template with primers Man5A-TM1-F and Man5A-TM1-R. Thus, the forward primer and the reverse primer (indicated by F and R at the end of the primer designations, respectively) were designed to cleave the signal peptide and the three SLH repeats, respectively. Man5A-TM2 was amplified with primers Man5A-TM1-F and Man5A-TM2-R, designed to cleave the two C-terminal CBMs from Man5A-TM1. The forward and reverse primers for Man5A-TM1 and Man5A-TM2 were designed to include 5′-NdeI and 3′-XhoI restriction sites for directional cloning. The primers are shown in Table S1 in the supplemental material. The resulting amplicons were cloned in frame with the hexahistidine (His_6)-encoding sequence of a modified pET-28a expression vector via its NdeI-XhoI polylinker site.

The resulting plasmid constructs pET28a-Man5A-TM1, pET28a-Man5A-TM2, and pET46-Man5B were sequenced to confirm the integrity of the cloned genes (W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign). The plasmid constructs were introduced into *E. coli* BL21-CodonPlus(DE3) RIL competent cells that were then cultured at 37°C in LB (1 liter) supplemented with ampicillin and chloramphenicol with vigorous shaking (225 rpm/min). At an optical density of 0.3 at 600 nm, the temperature was shifted to 16°C and gene expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) (0.1 mM). After 16 h of growth, the cells were harvested by centrifugation, and the cell pellets were resuspended in 30 ml lysis buffer (50 mM Tris-HCl, 300 mM NaCl [pH 7.0]) and ruptured by passage through an EmulsiFlex C-3 cell homogenizer from Avestin (Ottawa, Canada). The cell lysate was heated at 65°C for 30 min to denature the heat-labile *E. coli* proteins. The recombinant proteins were then purified by Talon metal affinity resin with a Tris-based binding buffer (50 mM Tris-HCl, 300 mM NaCl [pH 7.5]) and elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole [pH 7.5]). The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) and staining with Coomassie brilliant blue G-250. After the proteins were transferred into storage buffer (50 mM Tris-HCl, 150 mM NaCl [pH 7.5]), protein concentrations were calculated using the following extinction coefficients: Man5A-TM1, $\varepsilon_{280} = 189\ mM^{-1}\ cm^{-1}$; Man5A-TM2, $\varepsilon_{280} = 113\ mM^{-1}\ cm^{-1}$; Man5B, $\varepsilon_{280} = 103\ mM^{-1}\ cm^{-1}$.

Hydrolysis of *p*NP-linked sugars. The hydrolytic activities of Man5A-TM1, Man5A-TM2, and Man5B with *para*-nitrophenyl (*p*NP)-linked substrates were assayed using a thermostated Cary 300 UV-visible light spectrophotometer from Varian Inc. (Palo Alto, CA). Fifteen different substrates were screened. The 15 substrates were *p*NP-α-L-arabinopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-β-D-fucopyranoside, *p*NP-α-L-fucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-maltopyranoside, *p*NP-α-D-maltopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-α-L-rhamnopyranoside, *p*NP-β-D-xylopyranoside, and *p*NP-β-D-cellobioside. The different *p*NP substrates (final concentration of 1.0 mM) were incubated at 65°C in the presence or absence of Man5A-TM1, Man5A-TM2, or Man5B (final concentration of 100 nM) in citrate buffer (50 mM sodium citrate [pH 5.5]) for 30 min, and the rate of *p*NP release was determined by monitoring the absorbance at 400 nm continuously. The extinction coefficient of *p*NP at pH 5.5 was determined as $1{,}636\ M^{-1}\ cm^{-1}$.

Optimal temperature and pH for Man5B. The optimum temperature for Man5B was determined by incubating the protein (final concentration of 5 nM) with *p*NP-β-D-mannopyranoside or *p*NP-β-D-cellobioside in citrate buffer at temperatures ranging from 45 to 80°C in increments of 5°C. The optimum pH for Man5B was determined with *p*NP-β-D-mannopyranoside and *p*NP-β-D-cellobioside at 65°C. Buffers with different pH values ranging from 3.0 to 10.0 in increments of 0.5 pH values (pH 3.0 to 6.0 for citrate buffer [50 mM], pH 6.5 for phosphate-citrate buffer [50 mM], pH 7.0 to 8.0 for Tris-HCl buffer [50 mM], and pH 9.0 to 10.0 for glycine buffer [50 mM]) were employed. For both the pH and optimum temperature experiments, the release of *p*NP was monitored by continuously monitoring the absorbance at 400 nm. Extinction coefficients were determined for *para*-nitrophenol in each of the different buffers, and the resulting values were used to calculate the initial velocities from the slopes of the changes in absorbance at 400 nm as a function of time.

Hydrolysis of oligosaccharides by Man5A-TM1, Man5A-TM2, and Man5B. The capacity of Man5A-TM1, Man5A-TM2, and Man5B to hydrolyze oligosaccharides was assessed by incubating the enzymes with cello-oligosaccharides (cellobiose [G_2], cellotriose [G_3], cellotetraose [G_4], cellopentaose [G_5], and cellohexaose [$G_{6]}$), manno-oligosaccharides (mannobiose [M_2], mannotriose [M_3], mannotetraose [M_4], mannopentaose [M_5], and mannohexaose [M_6]), or galacto-manno-oligosaccharides (α-1,6-galactosyl-mannobiose [GM_2] and α-1,6-galactosyl-mannotriose [GM_3]) and measuring the products released by either high-performance anion-exchange chromatography (HPAEC) or thin-layer chromatography (TLC).

For endpoint assays, the substrates (10 mg/ml) were independently incubated with Man5A-TM1, Man5A-TM2, or Man5B (final concentration of 0.5 μM) in citrate buffer (final volume of 10 μl) at 65°C. After 12 h of incubation, 2 volumes of ethanol was added to the hydrolysates, and then the mixtures were evaporated using a Savant DNA120 SpeedVac concentrator (Savant, Ramsey, MN), and the end products were resuspended in 2.5 μl of double-distilled water (ddH_2O). For TLC analysis, 0.5 μl of sample was spotted on Silica Gel 60 F_{254} TLC plates from Merck (Whitehouse Station, NJ). Sugar standards, including mannose (M_1), glucose (G_1), manno-oligosaccharides (M_2 to M_4), or cello-oligosaccharides (G_2 to G_4) (2.5 μg each) were spotted onto TLC plates where appropriate. The products of enzyme reactions with cello-oligosaccharides as substrates were resolved by one ascent with a mobile phase consisting of 1-butanol–acetic acid–H_2O (10:5:1 [vol/vol/vol]) (18). The products of enzyme reactions with manno-

oligosaccharides or galactosyl-manno-oligosaccharides as substrates were resolved by one ascent with a mobile phase consisting of *n*-propanol–ethanol–water (7:1:2 [vol/vol/vol]) according to Megazyme's instructions. The plates were then visualized by spraying with a 1:1 (vol/vol) mixture of methanolic orcinol (0.1% [wt/vol]) and sulfuric acid (10% [vol/vol]), followed by heating at 75°C for 10 min (10). For high-performance anion-exchange chromatography (HPAEC-PAD) analysis, a System Gold HPLC instrument from Beckman Coulter (Fullerton, CA) equipped with a CarboPac PA1 guard column (4 by 50 mm) and a CarboPac PA1 analytical column (4 by 250 mm) from Dionex Corporation (Sunnyvale, CA) and a Coulochem III electrochemical detector from ESA Biosciences (Chelmsford, MA) was employed. The resuspended hydrolysate was diluted 100-fold in ddH_2O, and 30 μl was injected onto the column. The elution conditions for HPAEC were as follows: 0 to 25 min, 0 to 0.3 M sodium acetate gradient in 100 mM NaOH; 25 to 30 min, 0.3 to 1 M sodium acetate gradient in 100 mM NaOH; 30 to 50 min, 100 mM NaOH (20, 31). Monomeric mannose (M_1) and α-1,6-galactosyl-mannobiose (GM_2) were used as standards.

Determination of catalytic efficiencies for Man5A-TM1, Man5A-TM2, and Man5B with oligosaccharides. The catalytic efficiencies of Man5A-TM1, Man5A-TM2, and Man5B for manno-oligosaccharides (M_2 to M_6), gluco-oligosaccharides (G_2 to G_6), and galactosyl-manno-oligosaccharides (GM_2 and GM_3) were determined by HPAEC by the method of Matsui et al. (24). Reaction mixtures were prepared with oligosaccharide substrates (final concentration of 30 μM) in citrate buffer, and the reaction was initiated by the addition of Man5A-TM1, Man5A-TM2, or Man5B. For oligosaccharides M_2, M_3, G_2, G_3, GM_2, and GM_3, the Man5A-TM1 or Man5A-TM2 enzyme was added to a final concentration of 5 μM, and for M_4 to M_6 and G_4 to G_6, the two enzymes were added to a final concentration of 3 nM. The Man5B enzyme was added to the reaction mixture to a final concentration of 5 μM for G_2 and to a final concentration of 3 nM for the other oligosaccharides. The relationship between hydrolysis rate and oligosaccharide substrate concentration (0, 30, 60, and 90 μM) was linear; therefore, the substrate concentration of 30 μM should be well below K_m. A linear relationship was also observed between substrate depletion and hydrolysis time (0, 5, 10, and 15 min), so the hydrolytic reactions were terminated at 5 min. The substrate concentrations at the beginning of the reaction ($[S_0]$) and at specified times ($[S_t]$) (5 min) during the reaction were fitted to equation 1 to determine the catalytic efficiencies (6, 7) as follows:

$$k = \ln[S_0]/[S_t] \quad (1)$$

where $k = (k_{cat}/K_m)([\text{enzyme}])(\text{time})$ and [enzyme] is the concentration of enzyme.

Hydrolysis of plant polysaccharides by Man5A-TM1, Man5A-TM2, and Man5B. To evaluate the enzymatic activities of Man5A-TM1, Man5A-TM2, and Man5B on polysaccharide substrates (guar gum, locust bean gum, glucomannan, β-mannan, CMC, xylan, or lichenan), the specific activities were determined in citrate buffer (50 mM sodium citrate, pH 5.5) at 65°C. For locust bean gum, glucomannan, β-mannan, and lichenan, the enzymes were added to a final concentration of 0.5 nM, and for CMC, xylan, and guar gum, the three enzymes were added to a final concentration of 5 nM. At regular time intervals (3, 6, and 9 min), 100-μl aliquots were removed from the reaction mixtures, and the enzymes were inactivated by heating at 100°C for 10 min. The concentration of reducing ends in the supernatant was then determined using the *para*-hydroxybenzoic acid hydrazide (PHBAH) method with glucose as a standard (23).

To assess the synergistic activities of Man5A-TM1, Man5A-TM2, and Man5B in the hydrolysis of polysaccharide substrates (locust bean gum, guar gum, glucomannan, β-mannan, and CMC), the substrates (0.5% [wt/vol]) were incubated with each enzyme in citrate buffer at 65°C in a final reaction mixture volume of 2.5 ml. The enzymes and enzyme mixtures analyzed for hydrolysis were as follows: (i) Man5A-TM1 (50 nM), (ii) Man5A-TM2 (50 nM), (iii) Man5B (50 nM), (iv) Man5A-TM1 (25 nM) and Man5A-TM2 (25 nM), (v) Man5A-TM1 (25 nM) and Man5B (25 nM), and (vi) Man5A-TM2 (25 nM) and Man5B (25 nM). At regular time intervals (5, 10, 20, 30, and 60 min), 100-μl aliquots were removed from reaction mixtures, and the enzymes were inactivated by heating at 100°C for 10 min. The concentration of reducing ends was then determined by the PHBAH method.

Site-directed mutagenesis. Multiple amino acid sequence alignments were constructed using ClustalW (http://align.genome.jp). Mutagenesis was carried out using the PCR-based QuikChange XL site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The mutagenic primers are listed in Table S1 in the supplemental material. The inserts in the mutated plasmids were sequenced to confirm that only the mutations introduced through the primers were present. The mutant recombinant proteins were expressed and purified as described above for the nonmutated proteins.

RESULTS

Cloning and expression of Man5A derivatives and Man5B. The analysis of the genome sequence of *Caldanaerobius polysaccharolyticus* revealed the presence of two glycoside hydrolase family 5 (GH5) genes that were predicted to encode enzymes with β-mannanase activity. Consequently, they were designated Man5A and Man5B. The modular architecture of Man5A was described in an earlier report (4). Briefly, Man5A is composed of an N-terminal GH5 catalytic domain, flanked at the C terminus by a region of unknown function, followed by two family 16 carbohydrate-binding modules (CBMs) in tandem and then three surface layer homology (SLH) repeats. Ahead of the catalytic domain is a predicted signal peptide, which was removed during PCR amplification of each Man5A derivative. The polypeptide sequence of Man5A was deposited in GenBank with accession number AAD09354. In the current report, two truncational mutants of Man5A were constructed to probe the importance of the SLH and CBMs for the hydrolytic activity of Man5A on different mannan-containing substrates (Fig. 1A). Man5A-TM1 (TM1 stands for truncational mutant 1) was constructed by deleting the signal peptide and the three SLH repeats. An additional truncational mutant (Man5A-TM2) was constructed by deleting the two CBMs from Man5A-TM1 to facilitate comparison of the GH5 catalytic modules of Man5A and Man5B (Fig. 1A). In addition, the gene coding for the single-module Man5B was cloned. The gene product of Man5B lacks a signal peptide, which suggests an intracellular function. The three proteins were expressed in *E. coli* cells as N-terminal hexahistidine fusion proteins to facilitate protein purification. Following a two-step purification procedure involving heat denaturation of mesophilic host proteins and metal affinity chromatography, the three proteins were highly purified as determined by SDS-PAGE analysis (Fig. 1B). The molecular masses were calculated from the primary amino acid sequences, and the values for Man5A-TM1 (98.3 kDa), Man5A-TM2 (66.5 kDa), and Man5B (39.2 kDa) were in good agreement with the molecular masses estimated by SDS-PAGE.

Screening for activity with *p*NP-linked sugars. Man5B exhibited hydrolytic activity with *p*NP-β-D-mannopyranoside and *p*NP-β-D-cellobioside as substrates, the specific activities of which were 14.2 IU/mg and 11.3 IU/mg, respectively. Compared to Man5B, Man5A-TM1 and Man5A-TM2 exhibited very little detectable activity with the two *p*NP-linked substrates. The temperature and pH optima for Man5B were determined with both *p*NP-β-D-mannopyranoside and *p*NP-β-D-cellobioside as substrates (data not shown). The enzyme was active at temperatures ranging from 45°C to 80°C. Interestingly, Man5B has different temperature optima with the two *p*NP substrates. For *p*NP-β-D-cellobioside, the optimal temperature of the enzyme was around 65 to 70°C. About 50% activity was retained by incubation for 30 min at 80°C. The optimal temperature of Man5B against *p*NP-β-D-mannopyranoside was around 60 to 65°C. Man5B was active at a pH range of 4.5 to 7.0, and the optimal pH was around 5.5 with both of the *p*NP substrates. The optimum temperature and pH of Man5A were 65 to 75°C and 5.8, respectively, as previously reported (4). The catalytic efficiencies of Man5A-TM1 and Man5A-TM2, using

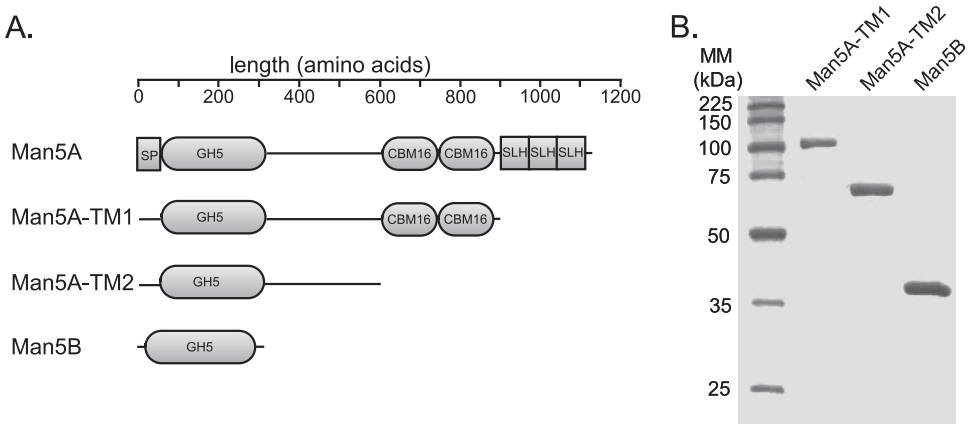


FIG. 1. (A) Domain architecture for Man5A, Man5A-TM1, Man5A-TM2, and Man5B. The functional domains were assigned by using the Conserved Domains Database (CDD) search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Abbreviations: SP, signal peptide; GH5, glycoside hydrolase family 5 catalytic module; CBM16, carbohydrate-binding module family 16; SLH, surface layer homology module. (B) Purification of Man5A-TM1, Man5A-TM2, and Man5B. The elution fractions from cobalt affinity chromatography were pooled for each protein and analyzed by 12% SDS-PAGE. The positions of molecular mass markers (MM) (in kilodaltons) are indicated to the left of the gel.

the two pNP-linked substrates, were 2 orders of magnitude less than that of Man5B (Table 1).

Man5A and Man5B exhibit different specificities with oligosaccharide substrates. To compare the substrate specificities of Man5A-TM1, Man5A-TM2, and Man5B, the three purified enzymes were screened for hydrolytic activity with manno- and cello-oligosaccharides. The products of hydrolysis were resolved by thin-layer chromatography. Man5A-TM1 and Man5A-TM2 were unable to hydrolyze mannobiose, mannotriose, and mannotetraose (Fig. 2A and C) and likewise cellobiose, cellotriose, and cellotetraose (Fig. 2B and D). However, as the degree of polymerization of the oligosaccharide increased to 5 and 6, both Man5A-TM1 and Man5A-TM2 hydrolyzed cello- and manno-oligosaccharides, releasing end products that ranged from trisaccharides to monosaccharides (Fig. 2A to D). In contrast to the Man5A constructs, Man5b exhibited hydrolytic activity with all of the oligosaccharides tested (Fig. 2E and F) with the exception of cellobiose. All of the manno-oligosaccharides were converted to a mixture of mannobiose and mannose, while the cello-oligosaccharides were converted to shorter oligosaccharides. The products of hydrolysis of cello-oligosaccharides were not completely converted to cellobiose and glucose during the course of the reaction (Fig. 2E and F).

To provide a more quantitative assessment of the substrate specificities of Man5A-TM1, Man5A-TM2, and Man5B, the catalytic efficiencies for the enzymes were determined with manno- and cello-oligosaccharides. There was no detectable activity for Man5A-TM1 and also Man5A-TM2 with oligosaccharides with a degree of polymerization (DP) of less than 4. However, for these Man5A derivatives, in general, the catalytic efficiency (k_{cat}/K_m) increased with substrates with a higher DP (Table 1). Furthermore, the k_{cat}/K_m values for the two Man5A derivatives with manno-oligosaccharides were higher than those with cello-oligosaccharides (Table 1).

Consistent with the results for the two Man5A derivatives, Man5B showed higher hydrolytic activity with manno-oligosac-

TABLE 1. Catalytic efficiencies for Man5A-TM1, Man5A-TM2, and Man5B with various substrates

Substrate	Catalytic efficiency (k_{cat}/K_m)[a] for the following enzyme:		
	Man5A-TM1	Man5A-TM2	Man5B
*p*NP-cellobioside[b]	$(1.8 \pm 0.3) \times 10^{-2}$	$(2.5 \pm 0.3) \times 10^{-2}$	25 ± 2
*p*NP-β-D-mannopyranoside[b]	$(1.4 \pm 0.3) \times 10^{-2}$	$(1.1 \pm 0.2) \times 10^{-2}$	1.6 ± 0.1
Mannobiose	ND	ND	23 ± 3
Mannotriose	ND	ND	27 ± 5
Mannotetraose	17 ± 4	11 ± 1	63 ± 6
Mannopentaose	33 ± 3	12 ± 2	$(2.2 \pm 0.3) \times 10^2$
Mannohexaose	$(1.2 \pm 0.2) \times 10^2$	28 ± 8	$(2.7 \pm 0.7) \times 10^2$
6-Galactosyl-mannobiose	ND	ND	1.5 ± 0.2
6-Galactosyl-mannotriose	ND	ND	30 ± 7
Cellobiose	ND	ND	ND
Cellotriose	ND	ND	1.0 ± 0.07
Cellotetraose	11 ± 2	1.8 ± 0.5	1.4 ± 0.2
Cellopentaose	9.3 ± 2	2.2 ± 0.3	17 ± 3
Cellohexaose	20 ± 2	25 ± 8	43 ± 3

[a] The catalytic efficiencies (k_{cat}/K_m) for *p*NP-cellobioside, *p*NP-β-D-mannopyranoside, and oligosaccharides are reported as $mM^{-1}\ s^{-1}$. The experiments were performed in triplicate, and data are reported as means ± standard deviations from the means. ND, not detectable (activity was below the detection limit for the assay).
[b] The catalytic efficiencies (k_{cat}/K_m) for *p*NP-cellobioside and *p*NP-β-D-mannopyranoside were determined by the method of Dodd et al. (10).

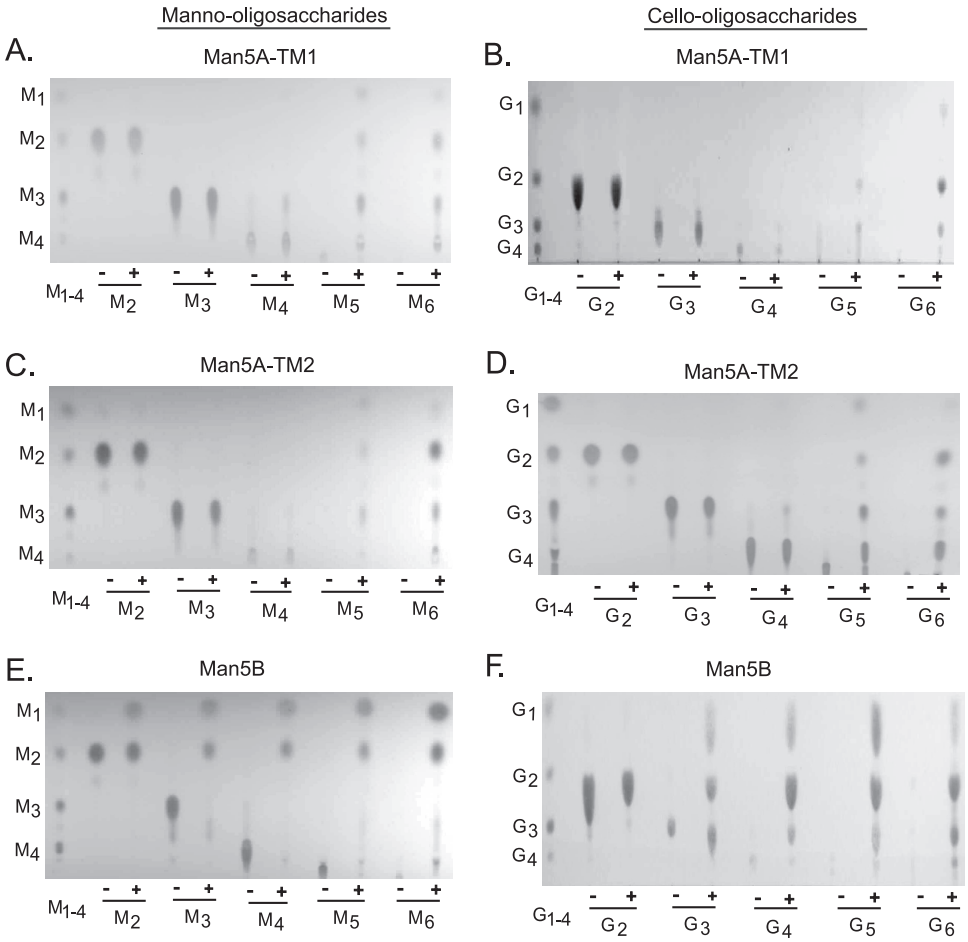


FIG. 2. Hydrolysis of β-1,4-linked manno-oligosaccharides and cello-oligosaccharides by Man5A-TM1, Man5A-TM2, and Man5B. Each protein (final concentration of 0.5 μM) was incubated with either manno-oligosaccharides (M_2 to M_6) or cello-oligosaccharides (G_2 to G_6) (final concentration of 10 mg/ml) at 65°C for 12 h, and the products were resolved by thin-layer chromatography followed by staining with methanolic orcinol. (A) Man5A-TM1 on manno-oligosaccharides; (B) Man5A-TM1 on cello-oligosaccharides; (C) Man5A-TM2 on manno-oligosaccharides; (D) Man5A-TM2 on cello-oligosaccharides; (E) Man5B on manno-oligosaccharides, and (F) Man5B on cello-oligosaccharides.

charides than with cello-oligosaccharides, and the k_{cat}/K_m also increased with substrates with a higher DP. Compared to mannobiose, there was a substantial increase (>10-fold) in k_{cat}/K_m of Man5B with mannohexaose, and similar results were also observed for cello-oligosaccharides.

Hydrolysis of substrates of increasing complexity. The capacity of the three enzymes to hydrolyze two substrates of medium complexity, galactosyl mannobiose (GM_2) and galactosyl mannotriose (GM_3), were investigated. Man5A-TM1 and Man5A-TM2 were incapable of releasing products from both GM_2 and GM_3 substrates (Fig. 3A, B, D, and E). Man5B released GM and mannose products from GM_2; however, this was detected only by high-performance anion-exchange chromatography (HPAEC) analysis (Fig. 3D). Man5B was able to effectively release mannose and GM_2 from GM_3 (Fig. 3C and E). The catalytic efficiencies of Man5B for GM_2 and GM_3 were also determined, and the value for GM_3 was 20-fold higher than that for GM_2 (Table 1). For the hydrolysis of both GM_3 and GM_2 with Man5B, no galactose was detected in the products, indicating that Man5B cleaves only the β-1,4-bond between mannose residues and does not cleave the α-1,6-bond between galactose and mannose (Fig. 3E).

Analyses of the hydrolytic activities of the enzymes with polymeric substrates were performed by determining the specific activity of each enzyme on locust bean gum, guar gum, β-mannan, glucomannan, CMC, lichenan, or xylan. Man5A-TM1 exhibited hydrolytic activity with each of the polymeric substrates, and the highest activity was found with glucomannan (Fig. 4). The specific activity of Man5A-TM2 was highly reduced with each of the substrates, suggesting that the carbohydrate-binding modules that are present in Man5A-TM1 but absent in Man5A-TM2 are generally important for the hydrolytic activity of Man5A with polysaccharide substrates. Man5B exhibited differences in substrate specificity compared to the two Man5A derivatives, with almost no activity when incubated with CMC, lichenan, or xylan from oat spelt (Fig. 4). Although Man5B lacks CBMs, its specific activities on locust bean gum, guar gum, β-mannan, and glucomannan were very high; however, in each case, the specific activity was less than that of Man5A-TM1. As observed for Man5A-TM1, the highest spe-

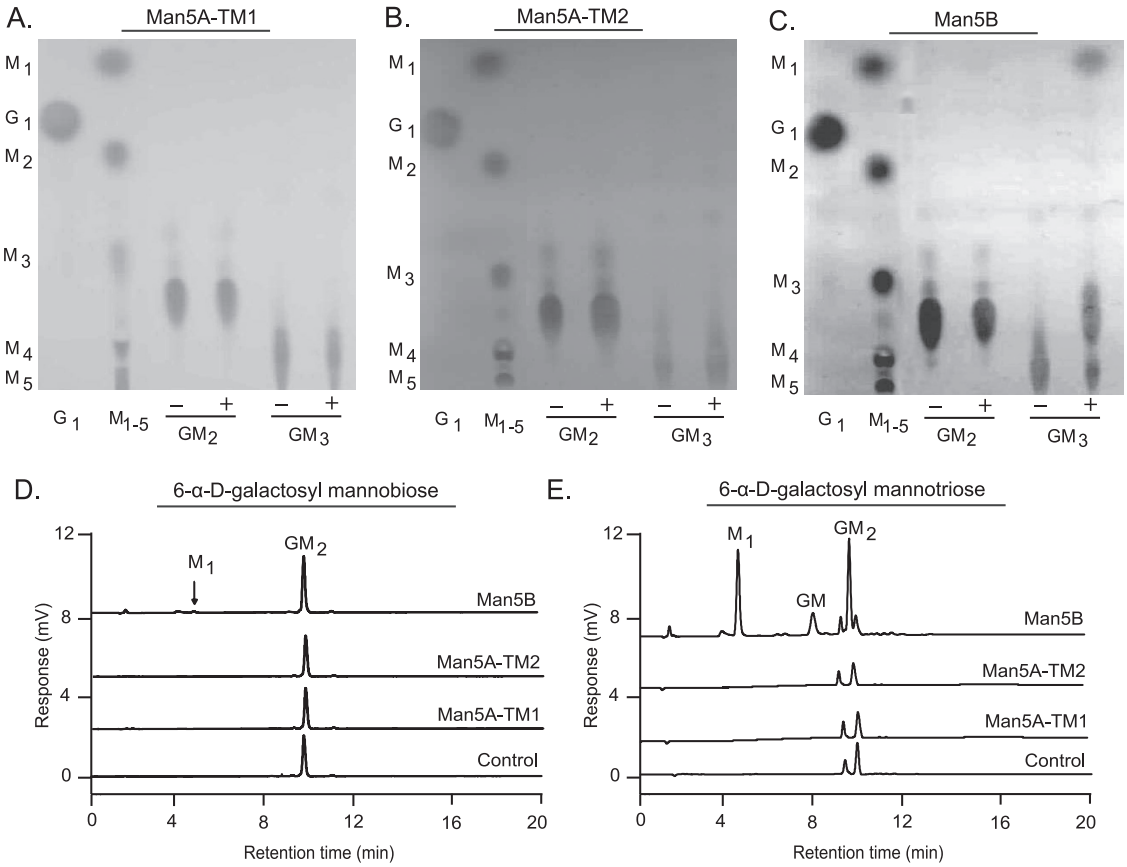


FIG. 3. Hydrolysis of galactomanno-oligosaccharides by Man5A-TM1, Man5A-TM2, and Man5B. (A to C) Man5A-TM1, Man5A-TM2, and Man5B (each enzyme at a final concentration of 0.5 μM) were incubated with 6-α-D-galactosyl mannobiose (GM_2) or 6-α-D-galactosyl mannotriose (GM_3) (final concentrations of 10 mg/ml) at 65°C for 12 h, and the products of hydrolysis were resolved by TLC followed by staining with methanolic orcinol. Galactose (G_1) and manno-oligosaccharides (M_1 to M_5 [M_{1-5}]) were used as standards. The minus sign indicates reactions in which the indicated enzyme was omitted, and the plus sign indicates reactions in which the indicated enzyme was added. (D and E) Man5A-TM1, Man5A-TM2, and Man5B (each enzyme at a final concentration of 0.5 μM) were incubated with 6-α-D-galactosyl mannobiose (GM_2) or 6-α-D-galactosyl mannotriose (GM_3) (final concentration of 10 mg/ml) at 65°C for 12 h, and the products of hydrolysis were analyzed by HPAEC. The peaks corresponding to mannose (M_1), 6-α-D-galactosyl mannose (GM), and 6-α-D-galactosyl mannobiose (GM_2) were determined by comparison of retention times to calibration standards.

cific activity for ManB was also recorded with glucomannan as the substrate. The results suggest that although Man5A-TM2 and Man5B are similar in architecture, in that they lack carbohydrate-binding modules, there may be other differences in the catalytic modules that confer some differences in substrate specificities and enzymatic activities in the two proteins.

To evaluate the product distribution following hydrolysis of polysaccharide substrates with Man5A-TM1, Man5A-TM2, or Man5B, the enzymes were incubated with guar gum, locust bean gum, glucomannan, β-mannan, or carboxymethyl cellulose, and the products of hydrolysis were analyzed by HPAEC. The results from these studies indicated that only relatively long oligosaccharides (DP greater than 3) were released from polysaccharides incubated with Man5A-TM1 (locust bean gum, guar gum, β-mannan, glucomannan, and carboxymethyl cellulose) and Man5A-TM2 (glucomannan and β-mannan) (see Fig. S1 to S5 in the supplemental material). Conversely, when Man5B was incubated with various polysaccharides, the product distribution was highly enriched for shorter oligosaccharides, with mannose and mannobiose being the predominant products (see Fig. S1 to S5 in the supplemental material). These results suggest that Man5A and Man5B have distinct differences in the products that they release from polysaccharides.

Synergistic activities of Man5A derivatives and Man5B. The *C. polysaccharolyticus* enzymes were tested for synergistic activity. The enzymes were incubated independently or in binary combinations with guar gum, locust bean gum, glucomannan, β-mannan, or CMC, and the amount of reducing ends released was monitored over time (Fig. 5). In addition, an aliquot of each reaction mixture from all substrate-enzyme combinations was removed at the 1-h time point, and the product composition was analyzed by HPAEC (see Fig. S1 to S5 in the supplemental material). The total concentration of enzyme in each of the reaction mixtures was maintained at 50 nM.

In the case of locust bean gum, glucomannan, and CMC, the activity of Man5A-TM1 was not influenced by the addition of Man5A-TM2 to the reaction mixture. With guar gum as the substrate, the release of reducing ends in the presence of the two enzymes was additive. Interestingly, the release of reduc-

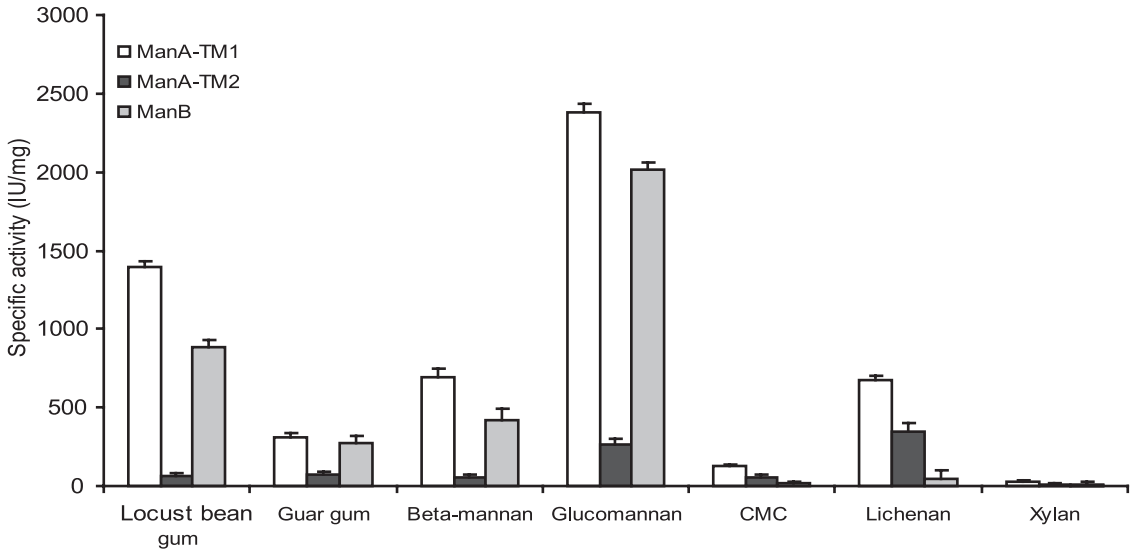


FIG. 4. Hydrolysis of polysaccharides by Man5A-TM1, Man5A-TM2, and Man5B. The specific activities of the three enzymes on the indicated polysaccharides were determined by the rate of release of reducing ends in reaction buffer (50 mM citrate buffer [pH 5.5]) at 65°C. For locust bean gum, glucomannan, β-mannan, or lichenan (0.5% [wt/vol]), the enzymes were at a final concentration of 0.5 nM, and for CMC, xylan, and guar gum, the enzymes were at a final concentration of 5 nM. For each experiment, the reactions were performed three independent times, and the data were reported as means plus standard deviations from the means.

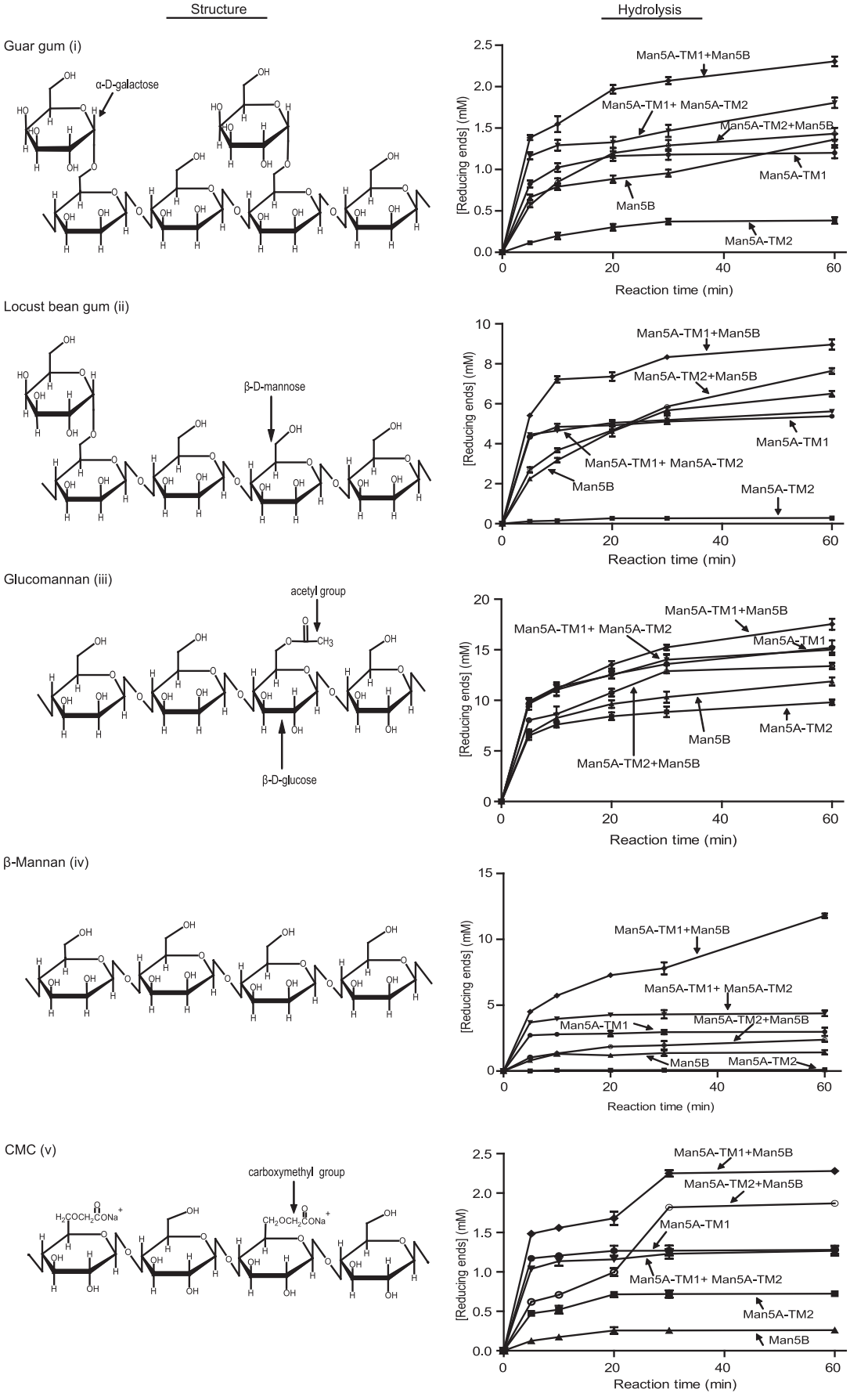
ing ends or products by Man5A-TM1 was stimulated in the presence of Man5A-TM2 with β-mannan as the substrate. The activity of Man5A-TM1 was also studied in the presence of Man5B. The release of reducing ends from guar gum and locust bean gum by Man5A-TM1 in the presence of Man5B was additive. However, Man5A-TM1 and Man5B acted synergistically to release reducing ends from CMC and β-mannan (Fig. 5iv and v). This synergistic activity was confirmed by results from HPAEC analysis for hydrolysis of β-mannan (see Fig. S4 in the supplemental material [Man5A-TM1, Man5B, and Man5A-TM1/Man5B]) and CMC (see Fig. S5 in the supplemental material [Man5A-TM1, Man5B, and Man5A-TM1/Man5B]).

Mutational analysis to identify catalytic residues in Man5A and Man5B. To identify active site residues for Man5A, we constructed amino acid sequence alignments and generated a three-dimensional homology model based on the available crystal structure of a β-mannanase from *Thermobifida fusca* (Protein Databank accession no. 1BQC) (13). The *T. fusca* protein shares 23% identity with the catalytic domain of Man5A. The sequence alignments and the homology model suggested that E177 and E285 may be catalytic residues in Man5A (results not shown). Each residue was replaced with either glutamine or alanine, and circular dichroism scan was used to confirm that the mutations did not severely impact the secondary structural elements of the protein (results not shown). The hydrolytic activities of the E177A, E1771Q, E285A, and E285Q mutants on locust bean gum, guar gum, β-mannan, or glucomannan were all markedly decreased than the hydrolytic activity of Man5A-TM1 (results not shown). Similar experiments were carried out on Man5B through amino acid sequence alignments and a three-dimensional homology model based on the crystal structure of the *Clostridium thermocellum* endoglucanase CelC (Protein Databank accession no. 1CEC) (11, 27). The likely catalytic residues identified were E137 and E258, and replacing each residue with either glutamine or alanine, although not severely impacting the secondary structural elements, led to severe decreases in the hydrolysis of *p*NP-β-D-cellobioside and *p*NP-β-D-mannopyranoside (results not shown). These data suggested that E177 and E285 in Man5A and E137 and E258 in Man5B, which based on amino acid sequence alignment correspond to catalytic residues in other GH5 enzymes (3, 29), are important for catalysis.

DISCUSSION

The complete hydrolysis of (hetero)mannans to their component monosaccharides requires the synergistic action of endo-acting β-mannanases, exo-acting β-mannosidases, exo-acting β-glucosidases, α-galactosidases, and acetylmannan esterases (26). Galactose and acetyl decorations are removed by the side chain cleaving activities of α-galactosidases and acetylmannan esterases. The internal glycosidic linkages within the backbone of mannans are hydrolyzed into manno-oligosaccharides or gluco-manno-oligosaccharides by endo-β-1,4-mannanases, and then β-mannosidases and β-glucosidases release mannose or glucose, respectively, from the nonreducing end. Previously, our laboratory cloned and characterized a multidomain β-mannanase (Man5A) from *C. polysaccharolyticus* (4). In the present report, we identified an additional gene encoding β-mannanase (*man5B*) from the partial genome sequence of this bacterium. To gain insight into how *C. polysaccharolyticus* may utilize these enzymes in nutrient acquisition, especially from mannan-containing polysaccharides, we studied the properties of recombinant Man5B and two truncational mutants of Man5A.

Deletion of the two family 16 carbohydrate-binding modules (CBMs) from Man5A resulted in a significant loss of both



mannanase and carboxymethyl cellulase (CMCase) activities (4). In the current study, we studied the influence of the CBM domains on Man5A catalytic activity with a variety of oligosaccharide and polysaccharide substrates. When the two CBM domains were deleted from Man5A, catalytic activity with polysaccharides was drastically reduced. This result suggests that the CBMs influence the catalytic activity of Man5A, which is consistent with previous results that reported that CBMs increase the catalytic activities of associated catalytic modules (28). Although CBMs are generally thought to influence catalytic activities of associated catalytic domains mainly on insoluble substrates, our results indicated that the catalytic activities with soluble substrates (locust bean gum, guar gum, carboxymethyl cellulose, and glucomannan) could also be increased by the presence of CBMs. This finding is in agreement with an observation made by others (17), although the mechanism by which the CBMs increase activity with soluble substrates is currently unknown. We postulate that these CBMs target the enzyme to regions lacking decorations in the polysaccharide and therefore reduce formation of unproductive complexes. We look forward to investigating this hypothesis.

Both truncational mutants of Man5A had no detectable hydrolytic activity with manno- or cello-oligosaccharides with a degree of polymerization (DP) of less than 4 but cleaved cello- and manno-oligosaccharides with a DP from 4 to 6. Conversely, Man5B cleaved shorter manno- and cello-oligosaccharides, in addition to the longer oligosaccharides. Man5A and Man5B both have catalytic domains that group within the glycoside hydrolase family 5 (GH5) (http://www.cazy.org/), and by mutational analysis, the two glutamic acid residues, important for catalysis, were shown as conserved in the two enzymes (3, 29). Despite this similar domain grouping, the two proteins share relatively low amino acid sequence similarity (23% identity over 115 amino acids aligned). The differences in amino acid sequence similarity between these two proteins may have significant consequences for the catalytic activities of the enzymes and may explain in part, the differences in substrate specificities and end product distributions of Man5A and Man5B. The observation that Man5A-TM1 and Man5A-TM2 released similar products from polysaccharides suggests that the two CBM domains do not influence the product distribution for Man5A and further supports the idea that the product distribution is largely determined by the GH5 catalytic module. The catalytic activity of Man5A-TM1 on glucomannan was the highest among the five polysaccharides. Also, the specific activity of Man5A-TM1 for locust bean gum was considerably higher than that of β-mannan and guar gum. These results suggest that a lower degree of α-linked galactose substitution (mannose/galactose ratio of 4:1) found in locust bean gum (26) might promote the hydrolysis of the mannan backbone, whereas the higher galactose substitution in guar gum (mannose/galactose ratio of 2:1) might decrease the accessibility of substrates to the CBMs or active site of Man5A-TM1. This hypothesis can be tested by including an α-galactosidase in the reaction mixture. In fact, *C. polysaccharolyticus* has been demonstrated to elaborate an α-galactosidase activity (16). In regards to β-mannan, it is likely that its lower solubility might have contributed to lower accessibility to the enzyme. The pattern of enzymatic activity of Man5B on the mannan-containing substrates followed a trend similar to that of Man5A-TM1 (glucomannan > locust bean gum > β-mannan > guar gum), which suggests that these two enzymes have evolved in this bacterium to work on similar substrates as described below.

Man5A-TM1 and Man5B acted synergistically to release end products. A possible explanation is that Man5A and Man5B release different products from polysaccharides, with Man5A cleaving polysaccharides into oligosaccharides that are converted to mono- or disaccharides by Man5B. Also, some CBMs have been found to have "disruptive" activity on the substrate, thus promoting catalysis by increasing substrate accessibility to the enzyme (8, 30). The hydrolytic activity of Man5A-TM1 was stimulated in the presence of Man5A-TM2 with β-mannan as the substrate, and this might be attributable to the CBMs of Man5A-TM1 making the substrate more accessible.

Man5A has a putative N-terminal Sec-dependent signal peptide, suggesting a protein secreted outside the cell. Man5A also contains surface layer homology (SLH) repeats which have predicted roles in anchoring proteins to the cell surface (25). These observations suggest that Man5A may be secreted and subsequently tethered to the cell surface. Our data suggest that the products of enzymatic hydrolysis of mannan polysaccharides by Man5A are relatively long manno-oligosaccharides. Therefore, we hypothesize that Man5A cleaves polysaccharides into oligosaccharides in close proximity to the cell surface, such that they are transferred into the cell by a membrane-associated transport system (Fig. 6A). In fact, clustering with *man5B* in the genome of *C. polysaccharolyticus* is an ABC transporter that may serve this function (Fig. 6B). We further postulate that Man5B remains in the cytoplasm and cleaves the transported oligosaccharides into mono- and disaccharides for subsequent metabolism. A similar strategy for capture of nutrients from water-insoluble xylan was recently reported for *Paenibacillus* sp. strain W-61 (12). We look forward to testing our hypothesis in the near future.

The biochemical characterization of the two thermophilic β-mannanases provides insight into the physiological role of these enzymes in mannan degradation, and the genomic context of Man5B has allowed us to propose a possible mechanism by which *C. polysaccharolyticus* transports the end products for utilization. Similar to other mannanases, such as *Bacillus agaradhaerens* Man5A (29), *Bacillus subtilis* Man26A (29), *Cellu-*

FIG. 5. Time course of polysaccharide hydrolysis by Man5A-TM1, Man5A-TM2, and Man5B. For each substrate, the chemical structure is shown on the left, and the reducing sugars released following incubation of the substrate with enzyme are shown on the right. For the hydrolysis reactions, the enzyme or combination of enzymes was incubated with the appropriate substrate (0.5% [wt/vol]) in citrate buffer (50 mM, pH 5.5) at 65°C. At regular time intervals (0, 5, 10, 20, 30, and 60 min), aliquots were removed for analysis of reducing ends released, as described in Materials and Methods. For reactions with a single enzyme, the final enzyme concentration was 50 nM, and for reactions with two enzymes, the final enzyme concentration was 25 nM (each) for the two enzymes.

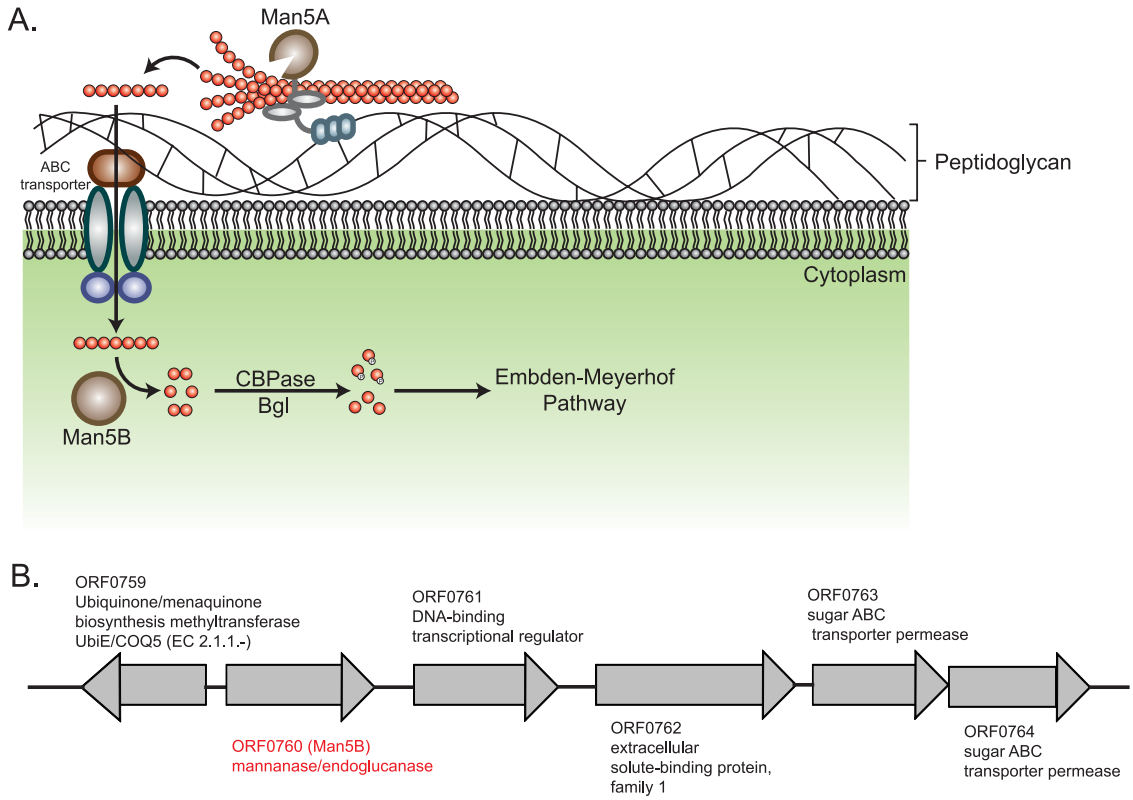


FIG. 6. Mannan utilization by *C. polysaccharolyticus*. (A) Schematic representation predicting the mechanism involved in nutrient acquisition from mannan-containing polysaccharides by *C. polysaccharolyticus.* Man5A has a signal peptide that allows its transport outside the cell after synthesis in the cell. The large polypeptide with its three surface layer homology (SLH) domains at the extreme C terminus is anchored to the peptidoglycan by the SLH repeats of the polypeptide. The enzyme is, therefore, in very close proximity to the cell. Upon coming into contact with mannan-containing polysaccharides, Man5A cleaves the substrate into oligosaccharides that are then transported into the cytoplasm where Man5B cleaves them into disaccharides and monosaccharides for metabolism by *C. polysaccharolyticus*. The genome of the bacterium harbors genes predicted to encode β-glucosidase (Bgl) and cellobiose phosphorylase (CBPase) enzymes, which may be involved in the fermentation of cellobiose. (B) Genomic context of *man5B.* ORF0760 encodes the polypeptide designated Man5B. Downstream of *man5B* are genes predicted to encode a DNA-binding transcriptional regulator (ORF0761), an extracellular solute-binding protein (ORF0762), and two permease components of an ABC transport system (ORF0763 and ORF0764). The proximity of these genes to *man5B* lends support to the model shown in panel A, wherein ORF0762, ORF0763, and ORF0764 may encode proteins involved in the transport of manno-oligosaccharides.

lomonas fimi Man26A (22), *Cellvibrio japonicus* Man26A (14, 15), and *Cellvibrio japonicus* Man5A (14), the catalytic efficiencies of both Man5A and Man5B of *C. polysaccharolyticus* increased with increasing length of manno-oligosaccharides. Furthermore, it has been reported that in GH5 mannanases with known three-dimensional structures, a relaxed specificity for glucose or mannose is observed; however, due to their absolute specificity for mannose at the −1 subsite, they cleave only mannosidic bonds, as observed for other mannanases (29). In the present report, Man5A and Man5B showed the highest specific activities with glucomannan as substrate. However, these two enzymes are quite unique in their capacity to cleave both β-1,4 mannosidic and β-1,4 glucosidic bonds. The synergistic effects of these two enzymes also suggest that they may be valuable in the saccharification of mannan-containing plant cell wall polysaccharides for subsequent biofuel production.

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REFERENCES

1. **Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal, F. Meyer, G. J. Olsen, R. Olson, A. L. Osterman, R. A. Overbeek, L. K. McNeil, D. Paarmann, T. Paczian, B. Parrell, G. D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke, and O. Zagnitko.** 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9:**75.
2. **Bae, B., S. Ohene-Adjei, S. Kocherginskaya, R. I. Mackie, M. A. Spies, I. K. O. Cann, and S. K. Nair.** 2008. Molecular basis for the selectivity and specificity of ligand recognition by the family 16 carbohydrate-binding modules from *Thermoanaerobacterium polysaccharolyticum* ManA. J. Biol. Chem. **283:**12415–12425.
3. **Bourgault, R., A. J. Oakley, J. D. Bewley, and M. C. J. Wilce.** 2005. Three-dimensional structure of (1,4)-β-D-mannan mannanohydrolase from tomato fruit. Protein Sci. **14:**1233–1241.
4. **Cann, I. K. O., S. Kocherginskaya, M. R. King, B. A. White, and R. I. Mackie.** 1999. Molecular cloning, sequencing, and expression of a novel multidomain mannanase gene from *Thermoanaerobacterium polysaccharolyticum*. J. Bacteriol. **181:**1643–1651.
5. **Cann, I. K. O., P. G. Stroot, K. R. Mackie, B. A. White, and R. I. Mackie.** 2001. Characterization of two novel saccharolytic, anaerobic thermophiles, *Thermoanaerobacterium polysaccharolyticum* sp. nov. and *Thermoanaerobac-*

terium zeae sp. nov., and emendation of the genus *Thermoanaerobacterium*. Int. J. Syst. Evol. Microbiol. **51:**293–302.
6. **Cartmell, A., E. Topakas, V. Ducros, M. D. L. Suits, G. J. Davies, and H. J. Gilbert.** 2008. The *Cellvibrio japonicus* mannanase *Cj*MAN26C displays a unique exo-mode of action that is conferred by subtle changes to the distal region of the active site. J. Biol. Chem. **283:**34403–34413.
7. **Charnock, S. J., T. D. Spurway, H. Xie, M. H. Beylot, R. Virden, R. A. J. Warren, G. P. Hazlewood, and H. J. Gilbert.** 1998. The topology of the substrate binding clefts of glycosyl hydrolase family 10 xylanases are not conserved. J. Biol. Chem. **273:**32187–32199.
8. **Davies, G. J., T. M. Gloster, and B. Henrissat.** 2005. Recent structural insights into the expanding world of carbohydrate-active enzymes. Curr. Opin. Struct. Biol. **15:**637–645.
9. **Dodd, D., and I. K. O. Cann.** 2009. Enzymatic deconstruction of xylan for biofuel production. Glob. Change Biol. Bioenergy **1:**2–17.
10. **Dodd, D., S. A. Kocherginskaya, M. A. Spies, K. E. Beery, C. A. Abbas, R. I. Mackie, and I. K. O. Cann.** 2009. Biochemical analysis of a β-D-xylosidase and a bifunctional xylanase-ferulic acid esterase from a xylanolytic gene cluster in *Prevotella ruminicola* 23. J. Bacteriol. **191:**3328–3338.
11. **Dominguez, R., H. Souchon, S. Spinelli, Z. Dauter, K. S. Wilson, S. Chauvaux, P. Béguin, and P. M. Alzari.** 1995. A common protein fold and similar active site in two distinct families of β-glycanases. Nat. Struct. Mol. Biol. **2:**569–576.
12. **Fukuda, M., S. Watanabe, S. Yoshida, H. Itoh, Y. Itoh, Y. Kamio, and J. Kaneko.** 2010. Cell surface xylanases of the glycoside hydrolase family 10 are essential for xylan utilization by *Paenibacillus* sp. W-61 as a generator of xylo-oligosaccharide inducers for the xylanase genes. J. Bacteriol. **192:**2210–2219.
13. **Hilge, M., S. M. Gloor, W. Rypniewski, O. Sauer, T. D. Heightman, W. Zimmermann, K. Winterhalter, and K. Piontek.** 1998. High-resolution native and complex structures of thermostable β-mannanase from *Thermomonospora fusca*–substrate specificity in glycosyl hydrolase family 5. Structure **6:**1433–1444.
14. **Hogg, D., G. Pell, P. Dupree, F. Goubet, S. M. Martín-Orúe, S. Armand, and H. J. Gilbert.** 2003. The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. Biochem. J. **371:**1027–1043.
15. **Hogg, D., E. J. Woo, D. N. Bolam, V. A. McKie, H. J. Gilbert, and R. W. Pickersgill.** 2001. Crystal structure of mannanase 26A from *Pseudomonas cellulosa* and analysis of residues involved in substrate binding. J. Biol. Chem. **276:**31186–31192.
16. **King, M. R., B. A. White, H. P. Blaschek, B. M. Chassy, R. I. Mackie, and I. K. O. Cann.** 2002. Purification and characterization of a thermostable α-galactosidase from *Thermoanaerobacterium polysaccharolyticum*. J. Agric. Food Chem. **50:**5676–5682.
17. **Kittur, F. S., S. L. Mangala, A. A. Rus'd, M. Kitaoka, H. Tsujibo, and K. Hayashi.** 2003. Fusion of family 2b carbohydrate-binding module increases the catalytic activity of a xylanase from *Thermotoga maritima* to soluble xylan. FEBS Lett. **549:**147–151.
18. **Kurokawa, J., E. Hemjinda, T. Arai, T. Kimura, K. Sakka, and K. Ohmiya.** 2002. *Clostridium thermocellum* cellulase CelT, a family 9 endoglucanase without an Ig-like domain or family 3c carbohydrate-binding module. Appl. Microbiol. Biotechnol. **59:**455–461.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
20. **Lee, Y. C.** 1996. Carbohydrate analyses with high-performance anion-exchange chromatography. J. Chromatogr. A **720:**137–149.
21. **Lee, Y. J., R. I. Mackie, I. K. O. Cann, and J. Wiegel.** 2008. Description of *Caldanaerobius fijiensis* gen. nov., sp. nov., an inulin-degrading, ethanol-producing, thermophilic bacterium from a Fijian hot spring sediment, and reclassification of *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* as *Caldanaerobius polysaccharolyticus* comb. nov. and *Caldanaerobius zeae* comb. nov. Int. J. Syst. Evol. Microbiol. **58:**666–670.
22. **Le Nours, J., L. Anderson, D. Stoll, H. Stalbrand, and L. L. Leggio.** 2005. The structure and characterization of a modular endo-β-1, 4-mannanase from *Cellulomonas fimi*. Biochemistry **44:**12700–12708.
23. **Lever, M.** 1972. A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. **47:**273–279.
24. **Matsui, I., K. Ishikawa, E. Matsui, S. Miyairi, S. Fukui, and K. Honda.** 1991. Subsite structure of *Saccharomycopsis* α-amylase secreted from *Saccharomyces cerevisiae*. J. Biochem. **109:**566–569.
25. **Matuschek, M., G. Burchhardt, K. Sahm, and H. Bahl.** 1994. Pullulanase of *Thermoanaerobacterium thermosulfurigenes* EM1 (*Clostridium thermosulfurogenes*): molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface. J. Bacteriol. **176:**3295–3302.
26. **Moreira, L. R. S., and E. X. F. Filho.** 2008. An overview of mannan structure and mannan-degrading enzyme systems. Appl. Microbiol. Biotechnol. **79:**165–178.
27. **Navas, J., and P. Béguin.** 1992. Site-directed mutagenesis of conserved residues of *Clostridium thermocellum* endoglucanase CelC. Biochem. Biophys. Res. Commun. **189:**807–812.
28. **Shoseyov, O., Z. Shani, and I. Levy.** 2006. Carbohydrate binding modules: biochemical properties and novel applications. Microbiol. Mol. Biol. Rev. **70:**283–295.
29. **Tailford, L. E., V. M. Ducros, J. E. Flint, S. M. Roberts, C. Morland, D. L. Zechel, N. Smith, M. E. Bjornvad, T. V. Borchert, and K. S. Wilson.** 2009. Understanding how diverse mannanases recognise heterogeneous substrates. Biochemistry **48:**7009–7018.
30. **Vaaje-Kolstad, G., D. R. Houston, A. H. K. Riemen, V. G. H. Eijsink, and D. M. F. van Aalten.** 2005. Crystal structure and binding properties of the *Serratia marcescens* chitin-binding protein CBP 21. J. Biol. Chem. **280:**11313–11319.
31. **Weitzhandler, M., R. Slingsby, J. Jagodzinski, C. Pohl, L. Narayanan, and N. Avdalovic.** 1996. Eliminating monosaccharide peak tailing in high pH anion-exchange chromatography with pulsed amperometric detection. Anal. Biochem. **241:**135–136.
32. **Yeoman, C. J., Y. J. Han, D. Dodd, C. M. Schroeder, R. I. Mackie, and I. K. O. Cann.** 2010. Thermostable enzymes as biocatalysts in the biofuel industry. Adv. Appl. Microbiol. **70:**1–55.