Intracellular mannanase was identified from the thermocacidophile *Alicyclobacillus acidocaldarius* Tc-12-31. This enzyme is particularly interesting, because it shows no significant sequence similarity to any known glycoside hydrolase. Gene cloning, biochemical characterization, and structural studies of this novel mannanase are reported in this paper. The gene consists of 963 bp and encodes a 320-amino acid protein, AaManA. Based on its substrate specificity and product profile, AaManA is classified as an endo-1,4-mannanase that is capable of transglycosylation. Kinetic analysis studies revealed that the enzyme required at least five subsites for efficient hydrolysis. The crystal structure at 1.9 Å resolution showed that AaManA adopted a (β/α)8-barrel fold. Two catalytic residues were identified: Glu282 at the C terminus of β4 and Glu231 at the C terminus of β7. Based on the structure of the enzyme and evidence of its transglycosylation activity, AaManA is placed in clan GH-A. Superpositioning of its structure with that of other clan GH-A enzymes revealed that six of the eight GH-A key residues were functionally conserved in AaManA, with the exceptions being residues Thr95 and Cys150. We propose a model of substrate binding in AaManA in which Glu282 interacts with the axial OH of C(2) in −2 subsites. Based on sequence comparisons, the enzyme was assigned to a new glycoside hydrolase family (GH113) that belongs to clan GH-A.

**Biochemical and Structural Characterization of the Intracellular Mannanase AaManA of *Alicyclobacillus acidocaldarius* Reveals a Novel Glycoside Hydrolase Family Belonging to Clan GH-A**

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Yueling Zhang, Jiansong Ju, Hao Peng, Feng Gao, Cheng Zhou, Yan Zeng, Yanfen Xue, Yin Li, Bernard Henisset, George F. Gao, and Yanhe Ma

From the 1State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China, the 6Graduate School, Chinese Academy of Sciences, Beijing 100049, China, and 5Architecture et Fonction des Macromolécules Biologiques, UMR6098, CNRS, and Universités d’Aix-Marseille I and II, 163 Avenue de Luminy, 13288 Marseille, France

An intracellular mannanase was identified from the thermocacidophile *Alicyclobacillus acidocaldarius* Tc-12-31. This enzyme is particularly interesting, because it shows no significant sequence similarity to any known glycoside hydrolase. Gene cloning, biochemical characterization, and structural studies of this novel mannanase are reported in this paper. The gene consists of 963 bp and encodes a 320-amino acid protein, AaManA. Based on its substrate specificity and product profile, AaManA is classified as an endo-1,4-mannanase that is capable of transglycosylation. Kinetic analysis studies revealed that the enzyme required at least five subsites for efficient hydrolysis. The crystal structure at 1.9 Å resolution showed that AaManA adopted a (β/α)8-barrel fold. Two catalytic residues were identified: Glu151 at the C terminus of β4 and Glu231 at the C terminus of β7. Based on the structure of the enzyme and evidence of its transglycosylation activity, AaManA is placed in clan GH-A. Superpositioning of its structure with that of other clan GH-A enzymes revealed that six of the eight GH-A key residues were functionally conserved in AaManA, with the exceptions being residues Thr95 and Cys150. We propose a model of substrate binding in AaManA in which Glu282 interacts with the axial OH of C(2) in −2 subsites. Based on sequence comparisons, the enzyme was assigned to a new glycoside hydrolase family (GH113) that belongs to clan GH-A.

Mannans are polysaccharides found in plants and consist of a backbone of β-1,4-linked mannose and glucose units. Mannose residues often carry an α-galactosyl substitute at O-6, and the degree of substitution depends on the plant of origin (Fig. 1A). Mannans are widely distributed in nature in parts of the hemicellulosic fraction in hardwoods and softwoods (1), legume seeds (2), and beans of carob trees (3).

Endo-β-1,4-mannanases (mannanases; EC 3.2.1.78) are glycoside hydrolases that randomly cleave the β-1,4-linkage in mannans (Figs. 1, A and B) (4); these enzymes have been isolated from bacteria, fungi, plants, and some mollusks (5–8). Interest in these enzymes has been increasing due to their importance in hemicellulose hydrolysis and various other applications (5, 9, 10). During the last 2 decades, more than 80 sequences of the catalytic domains of mannanase have been reported (see the CAZy site on the World Wide Web) and classified into glycoside hydrolase (GH) families 5 and 26, based on their sequence similarities (11). In recent years, crystallographic resolution of the structures of these enzymes has yielded information on their structure. At present, the tertiary structures of seven mannanases have been determined, of which five are from GH family 5 (12–16) and two are from family 26 (17, 18). All of these mannanases share a common (β/α)8 barrel fold, a retaining reaction mechanism (Fig. 1C), and two conserved catalytic residues (two glutamate residues at the C termini of β4 and β7, respectively). Therefore, all of these enzymes have been assigned to the same GH clan (i.e. GH-A) (19).

Mannanases of microbial origin are usually secreted extracellularly, but so far, only the mannanases from *Sporocytophaga myxococoides* and *Aerobacter mannoyticus* are reported to be intracellular (4, 20). However, there is a lack of sequence and structural information for both of these intracellular enzymes; therefore, knowledge of these is very limited. Extremophilic mannanases have obvious advantages in industrial applications (21, 22), and there have been many attempts to identify mannanases from extremophiles. Cloning, sequencing, and expres-

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1 To whom correspondence should be addressed: Institute of Microbiology, Chinese Academy of Sciences, A3 Datun Rd., Chaoyang District, Beijing 100101, China. Tel.: 86-10-64807590; Fax: 86-10-64807616; E-mail: mayanhe@im.ac.cn.

2 The abbreviations used are: GH, glycoside hydrolase; ORF, open reading frame; aa, amino acid(s); M1, mannoside; M2, mannobioside; M3, mannotriose; M4, mannotetraose; M5, mannopentose; M6, mannohexose; d.p., degree of polymerization.
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A general structure of the two major substrates of mannanase and scheme of the enzymatic action is shown. The nomenclature for sugar-binding subsites in glycoside hydrolase (57).

**EXPERIMENTAL PROCEDURES**

**Substrates and Chemicals**—Manno-oligosaccharides (mannobiose, mannotriose, mannotetraose, mannonpentose, and mannohexose), ivory nut mannann, yeast mannann, pecicto potato galactan, and potato galactan were purchased from Megazyme (Wicklow, Ireland). Other substrates were obtained from Sigma. All other chemicals were of the highest grade commercially available.

**Bacterial Strains, Plasmids, and Growth Conditions**—Strain Tc-12-31 was used to clone the mannanase gene and was obtained as follows. Selective agar plates (pH 4), containing basal salts, 0.5% konjac glucanmannan, and 1.5% agar (w/v), were prepared as described elsewhere (26). Mud and water samples from an acidic hot spring in Tengchong (Yunnan province, China) were spread on the selective agar plates at 100 μl/plate; this was followed by incubation for 48 h at 60 °C. The colonies thus obtained were replicated on selective agar plates and cultured. Mannanase-producing colonies were detected by flooding the plates with 0.1% (w/v) Congo Red solution (26, 27). Strain Tc-12-31, which gave the largest clear lysis zone, was selected. Phylogenetic analyses showed that strain Tc-12-31 belongs to the genus *Alicyclobacillus*, with the sequence of the 16S rRNA gene exhibiting the highest (99.5%) identity to that of *A. acidocaldarius* MIH 321 (GenBank™ accession number AB060166). Then the strain was designated *A. acidocaldarius* Tc-12-31. To prepare genomic DNA, *A. acidocaldarius* Tc-12-31 was grown under the conditions described elsewhere (26), but the medium was devoid of glycerol and soluble starch. Escherichia coli DH5α (Invitrogen), *E. coli* BL21(DE3) (Novagen), and pUC18 (TaKaRa) and pET28a (Novagen) plasmids were used. The *E. coli* strains were grown at 37 °C in Luria-Bertani medium, and 100 μg ml⁻¹ ampicillin or 50 μg ml⁻¹ kanamycin was added to select plasmid transformants.

DNA Library Construction and Positive Clone Screening—Standard molecular methods were used for DNA isolation, genomic library construction, and restriction analyses (28). Mannanase-positive clones were identified on replica plates with 0.5% konjac glucanmannan using the substrate overlay method (27).

**Cell Fractionation, PAGE, and Zymogram**—Cell fractions of *A. acidocaldarius* Tc-12-31 were performed as described by Matzke et al. (29). Laemmli gels (10%) (30) were prepared in the presence of 0.2% konjac glucanmannan. After electrophoresis, the gels were washed once with buffer A (50 mM phosphate sodium citrate, pH 5.5) and incubated for 20 min at 60 °C in the same buffer. The remaining konjac glucanmannan in the gel was visualized by incubating the gel in 0.1% Congo Red solution. The mannanase activity was detected in situ by the formation of a clear lysis band on the gel.

**Sequence and Structural Analysis**—Open reading frames (ORFs) were predicted using an ORF finder on the NCBI Web site, and homologues of these ORFs were searched in the GenBank™ data base using BLAST (Basic Local Alignment Search Tool) (31, 32). Signal peptide prediction was performed using SignalP 3.0 (33). Sequences were aligned with ClustalX (34). The Pfam data base (35) and the Conserved Domain Data Base (36) were searched online for family and domain information. Fold recognition analysis was carried out using the BioInfoBank metaserver and its associated 3D-jury consensus method (37). Structure neighbors were searched against the Protein Data Bank™ using the secondary structure matching service (38). Three-dimensional molecular visualization and figure preparation were performed with PyMOL (39).

**Enzymatic Assays**—The AamanA gene was inserted into plasmid pET28a to produce the expression plasmid pETManA (40). Translation from the resulting ORF resulted in a recombinant protein with a 23-aa peptide (MGSSSHHHHHHHSSGLVPRGSHMAS) fused to Met1 of the AamanA gene product. The recombinant protein was expressed in *E. coli* BL21(DE3) as described elsewhere (26) and purified by a protamine sulfate precipitation method (41). The purified enzyme was used in all enzymatic assays. The enzyme activity was measured by measuring the absorbance at 300 nm after hydrolysis of congo red-bound mannan with 0.1% (w/v) Congo Red solution (26). The mannanase activity was determined as the sum of the induced activities of Tc-12-31 and the recombinant AamanA.
and purified for further characterization. The effects of temperature, pH, ions, EDTA, and SDS on AaManA activity were studied as described in the supplemental materials. In subsequent enzymatic assays, pH and temperature optima of 5.5 and 65 °C, respectively, were used (see Fig. S1). Specific activities on konjac glucomannan, locust bean gum, guar gum, and ivory nut mannan were determined. The substrate concentration was 0.5% (w/v) in buffer A, and incubation was carried out at 65 °C for 10 min with 0.05, 0.1, 5, or 10 µM enzyme. Bovine serum albumin (0.1 mg ml⁻¹) was added to all of the incubation reactions. Enzymatic activity was quantified by measuring the release of reducing sugars, as described earlier (41). One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of mannose-reducing sugar equivalents/min. To screen for other endoglycanase activity, 1 µg of enzyme (in a volume of 5 µl) was spotted onto a 1.2% (w/v) agar plate containing 0.5% (w/v) of the appropriate polysaccharides in buffer A. The plates were incubated at 60 °C for 180 min, and the enzyme activity, which was detected after flooding with 0.1% Congo Red solution, manifested as a clear halo surrounding the “enzyme spot.” To detect the exoglycosidase activity, the p-nitrophenylglycoside substrate (5 mM) was incubated with 1 µM enzyme for 30 min. The release of p-nitrophenol was monitored by measuring the absorbance at 405 nm.

**Kinetic Analysis**—The Michaelis-Menten constants of the hydrolysis of locust bean gum were determined by using 0.1 µM enzyme and varying concentrations of the substrate (from 0.3 to 5 mg ml⁻¹). The kinetic parameters of manno-oligosaccharide hydrolysis were determined using different substrates: 0.25–6 mM mannotetraose (M4), 0.125–3 mM mannopentose (M5), and 0.125–3 mM mannohexose (M6). The enzyme concentrations used for M4, M5, and M6 were 0.6, 0.3, and 0.2 µM, respectively. All hydrolysis experiments were performed in buffer A to which bovine serum albumin (0.1 mg ml⁻¹) was added. The hydrolysis products were detected using high pH anion exchange chromatography-pulsed amperometric detection, as described earlier (15). Manno-oligosaccharides in the concentration range of 0.5–100 µM were used as standards. \( K_m \) and \( k_{cat} \) were determined by nonlinear regression using the GraphPad Prism 5.0 software (available on the World Wide Web).

**Time Course of Hydrolysis**—The time course of hydrolysis was established using 10 µM enzyme in buffer A with 2% (w/v) substrate supplemented with 0.1 mg/ml bovine serum albumin at 60 °C. At 0, 5, 10, 20, 40, 80, and 180 min, 10-µl aliquots were taken out and boiled for 2 min to stop enzymatic hydrolysis. The hydrolysates were measured by TLC (42).

**Phasing, Model Building, and Structure Determination**—Crystallization and data collection of AaManA and its selenomethionyl derivative have been described elsewhere (40). The SOLVE program (43) was used to determine and refine the positions of the selenium atoms. The RESOLVE program (43) was then used to perform solvent flattening and initial phase calculations. Automatic model building was performed with the ARP/wARP program (44). Subsequent manual rebuilding and refinement cycles were carried out with COOT (45) and REFMAC5 (46). Finally, stereochemical assessments of the structure were performed with PROCHECK (47). The statistics of structure refinement are summarized in Table 1.

**Site-directed Mutagenesis**—The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce amino acid substitutions. The pETManA expression plasmid was used as the mutation template. After confirmation of the desired mutation by DNA sequencing, the mutant AaManA enzymes were expressed and purified by the same procedure as that used for the wild-type enzyme. The global structural integrity of the mutant enzyme was evaluated by CD spectroscopy (48).

### RESULTS AND DISCUSSION

**Characterization of the Gene Cluster Containing AamanA**—The genomic library of *A. acidocaldarius* Tc-12-31 was constructed and screened for clones exhibiting mannanase activity on plates. One positive clone was found in ~10,000 transformants analyzed. The plasmid (pUCM1) purified from this clone contained a 4180-bp insert. Five ORFs were found along its entire length; these are referred to as glo (459 bp), AamanA (963 bp), pitA (984 bp), pfr (486 bp), and unk (1536 bp), as shown in Fig. 2A. Data base searches using BLAST revealed that none of the genes in the pUCM1 insert showed any similarity to the established mannanases. *AamanA* was the only gene that encoded a protein that was similar to glycoside hydrolases. The highly sensitive iterative PSI-BLAST search (e value threshold was 0.01) was conducted with AaManA and led to the identification of two galactanases of the GH53 family (i.e., EAN09439 from *Enterococcus faecium* (EfaGAL) and EAN12989 from *Frankia* sp. (FraGAL)) (see Fig. S2), after two iterations. Both galactanases exhibited 17% sequence identity with AaManA.

**AaManA Is an Intracellular Mannanase**—Upstream sequence analysis showed that the first ATG codon of *AamanA* was preceded (9–15 bp) by a typical bacterial ribosome-binding sequence analysis showed that the first ATG codon of *AamanA* was preceded (9–15 bp) by a typical bacterial ribosome-binding sequence.
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sequence and a −35 promoter region (Fig. 2C). The 320-aa protein encoded by AaManA had no signal peptide, suggesting that the AaManA protein was an intracellular enzyme. This was proved by examining the zymogram corresponding to that of the subclone E. coli DH5α/pUCM1 in different cell fractions of A. acidocaldarius Tc-12-31 (Fig. 2B). The mannanase activity was only observed in the fraction of cellular soluble proteins (Fig. 2B, lane Aa-SP), not in the fractions of the culture fluid (Fig. 2B, lane Aa-CF), cell debris, and membrane proteins (Fig. 2B, lane Aa-CD). Therefore, native AaManA was an intracellular enzyme in A. acidocaldarius Tc-12-31.

AaManA Hydrolyzes β-1,4-Mannosidic Substrates—Purified AaManA hydrolyzed polysaccharides containing β-1,4-mannosidic linkages (konjac glucomannan, locust bean gum, guar gum, and ivory nut mannan). The highest activity (1056 units mg⁻¹) was observed when unsubstituted konjac glucomannan was used as the substrate. This activity was much higher than that against the galactosyl-substituted locust bean gum (724 units mg⁻¹) (Table 2). An activity of only 79 units mg⁻¹ was observed toward the more highly substituted galactomannan from guar gum. Therefore, enzymatic activity seems to be limited by the galactose side groups, which is the case in most other mannanases, such as those from Bacillus sp. N16-5 and Cellvibrio japonicus (5, 18). The very low activity (69 units mg⁻¹) toward ivory nut mannan could be attributed to the limited access to the glycosidic linkages in this crystalline substrate. This low activity would be expected on the basis of the intracellular location of AaManA, which is unlikely to encounter crystalline mannan as a substrate. AaManA did not hydrolize crystalline cellulose, chitin, pectin, soluble starch, oat spelled xylan, or α-1,6-linked yeast mannan. No activity was found on p-nitrophenyl β- and α-glucosides, β- and α-galactosides, and β- and α-mannosides; the lack of activity in this case indicates that the enzyme was unable to cleave terminal mannosides. Although the closest relatives of AaManA were β-1,4-galactanases, it is interesting to note that the enzyme did not exhibit any activity against pectic galactan and galactan.

AaManA Is an Endo-β-1,4-mannanase with Transglycosylation Activity—The hydrolysis products released from konjac glucomannan and locust bean gum were examined by TLC. The products were mainly oligosaccharides with a higher degree of polymerization (d.p.) than biose (Fig. 3A). The enzyme showed no activity on M2; however, cleaved manno-oligosaccharides with d.p. of >3 and manno-oligosaccharides with d.p. of >2 were the main reaction products (Fig. 3, B–E). These results indicate that AaManA hydrolyzed substrates by endo-type cleavage. In addition, during the hydrolysis of manno-oligosaccharides, products with higher d.p. than the substrates were formed, such as M4, M5, and M6 from mannotriose (M3) (Fig. 3B). M5 and M6 from M4 (Fig. 3C), and M6 from M5 (Fig. 3D). These are obvious signs of transglycosylation. Another piece of evidence of transglycosylation was the formation of unequal amounts of end products. For example, among the products of

### Table 2

| Enzyme | Substrate | Units mg⁻¹ |
|--------|-----------|------------|
| Wild type | Locust bean gum | 724.4 ± 20.0 |
| E151A | Konjac glucomannan | 0.091 ± 0.002 |
| E151Q | Guar gum | 0.847 ± 0.021 |
| E231A | Ivory nut mannan | 0.102 ± 0.003 |
| E231Q | | 0.027 ± 0.001 |
| C150A | | 310.3 ± 14.3 |
| Wild type | Locust bean gum | 79.4 ± 1.2 |
| E151A | Konjac glucomannan | 69.3 ± 5.0 |
| E151Q | Guar gum | NA* |
| E231A | Ivory nut mannan | NA |
| E231Q | | NA |
| C150A | | 23.9 ± 0.69 |
| E151A | Locust bean gum | 25 ± 0.68 |

* NA, no activity was detected.
M3, the amount of mannobiose (M2) was much higher than that of mannose (M1) (Fig. 3B). Intuitively, it is expected that M3 cleavage should result in equal amounts of M1 and M2 or in an excess of M1 if the enzyme can further hydrolyze M2 into M1. Similar product inequalities were also observed during the hydrolysis of M4, M5, and M6. In summary, AaManA is an endo-β-1,4-mannanase that is capable of transglycosylation. Since transglycosylation is restricted to retaining hydrolases (49), hydrolysis by AaManA probably operates via a mechanism in which the stereocchemistry is retained at the site of cleavage (Fig. 1C).

Enzyme Kinetics of AaManA—The $k_{cat}$ and $K_m$ for the hydrolysis of locust bean gum by AaManA are 340 s$^{-1}$ and 2.4 mg ml$^{-1}$, respectively. This $K_m$ value is similar to that of CfMan26A from Cellulomonas fimi (2.3 mg ml$^{-1}$) (18) and approximately 4 times lower than that of CjMan26A from C. japonicus (8.5 mg ml$^{-1}$) (17). The Michaelis-Menten parameters for manno-oligosaccharides are summarized in Table 3. The results show that M5 and M6 were hydrolyzed with similar efficiencies ($k_{cat}/K_m$), whereas a 3.5-fold decrease in the $k_{cat}/K_m$ values occurred when the degree of substrate polymerization was decreased from M5 to M4. The M3 hydrolysis rate was too low to permit accurate kinetic analysis; therefore, it was not analyzed further. These results indicate that AaManA requires at least five subsites to achieve efficient hydrolysis, which is similar to the case of CfMan26A (18). In contrast, CjMan26A requires only four sites, and to date, it is the most efficient mannotetraose-degrading mannanase (17). Other mannanases that require more sites have also been reported. CjMan5A, CjMan6B from C. japonicus, and MeMan5A from Mytilus edulis require at least six sites for efficient hydrolysis (15, 50).

The $k_{cat}/K_m$ for M6 was much lower than the values of other mannanases, such as MeMan5A (198 s$^{-1}$ mm$^{-1}$) and CfMan26A (111 s$^{-1}$ mm$^{-1}$). It is possible that AaManA hydrolysis is compromised by its transglycosylation activity, because evidence for transglycosylation, such as inequality in the amounts of products formed and the occurrence of products with d.p. higher than that of the original substrates, was also detected by high pH anion exchange chromatography-pulsed amperometric detection.

**Overall Structure of AaManA**—The final model of AaManA was refined at a resolution of 1.9 Å using the data obtained with a native crystal. The final R-factor and Rfree are 17.2 and 21.2%, respectively (Table 1). The refined model consists of 316 residues, corresponding to residues Glu5–Arg320. The 27 aa at the C-terminus (23-residue histidine tag and first four N-terminal residues of AaManA) are not visible in the electron density map. The AaManA structure is composed of seven α-helices, eight β-strands, and six 310-helices (Fig. 4). As shown in Fig. 5A, AaManA folds into a typical (β/α)$_g$ barrel. On the top of the barrel, AaManA features a groove (indicated by a red arrow in Fig. 5A (right)) that binds to mannan; this is also observed in the structures of other mannanases.

**Overall Structure Comparison with Other Glycoside Hydrolases**—Comparison between the AaManA structure and that of other proteins in the Protein Data Bank showed that the overall folding of AaManA was most similar to that of endoglucanase Cel5G from Pseudomaro- monas haloplanktis (51). There was less similarity to the structures of endoglucanase Cel5 from Thermosascus aurantiacus (52), mannanases TfMan5A from T. fusca (14), HjMan5A from Hypocrea jecorina (formerly known as Trichoderma reesei) (16), and galactanase AacGAL from Aspergillus aculeatus (53). All of these closely matched structures were from the GH-A clan. Pairwise comparison showed that the structures of AaManA and Cel5G were similar (Fig. 5B), although the sequence identity was extremely low (9%). The root mean square distance value was 2.66 Å based on 228 Ca atoms.
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Comparison of the Active Pocket—The above results revealed that the structure of AaManA was most similar to those of the enzymes from clan GH-A, indicating that AaManA was a possible member of this clan. The enzymes from this clan, as described by Durand et al. (54), possess an active pocket consisting of seven to eight functionally conserved residues. To determine whether AaManA has the steric counterparts of these conserved residues, the structure around the active pocket of Cel5G (Fig. 5C) was investigated. The results revealed that AaManA possesses eight equivalent residues, namely Lys93, Thr95, Cys150, Glu151, Ser201, Tyr203, Glu231, and Trp281, that AaManA possesses eight equivalent residues, namely the structure around the active pocket of Cel5G (Fig. 5C) was investigated. The results revealed that AaManA possesses eight equivalent residues, namely Lys93, Thr95, Cys150, Glu151, Ser201, Tyr203, Glu231, and Trp281, which correspond to the GH-A functionally conserved residues Arg57, His100, Asn134, Glu135, His194, Tyr196, Glu222, and Trp256 in Cel5G, respectively.

Catalytic Residues—Primary sequence alignment of AaManA with GH53 enzymes showed that Glu151 and Glu231 aligned well with the acid/base catalyst and the nucleophile catalyst of GH53 enzymes, respectively (Fig. S2). This suggested that Glu151 and Glu231 probably had catalytic roles in AaManA. This was supported by structural comparisons between AaManA and Cel5A, which showed that Glu151 and Glu231 were sterically identical to the acid/base catalyst Glu135 and the nucleophile catalyst Glu222, respectively, in Cel5A (Fig. 5, B and C). To investigate the proposed roles of the two glutamates, the mutations E151A, E151Q, E231A, and E231Q were created.

Other Residues in the Active Pocket—A schematic representation of the interactions among residues in the active pocket of AaManA is shown in Fig. 6. The interactions among Lys93, Ser201, Tyr203, and the two catalytic glutamic acids were conserved in the GH-A enzymes and contribute to both the position and ionization state of the two catalytic residues. However, Thr95 and Cys150 were too far apart to form hydrogen bonds with the catalytic residues or the sugar unit in subsite −1. The most striking and interesting residue is Cys150, which precedes the acid/base catalyst Glu151. This position is strictly occupied by an asparagine in GH-A enzymes, with the exception of GH26 mannanases in which the corresponding residue is histidine (17). Both asparagine and histidine at this position play important roles in maintaining the position of the catalytic nucleophile through hydrogen bonding (17, 53). Mutations of the asparagine and histidine to alanine resulted in a >100-fold decrease in the activities (17, 48). However, cysteine (Cys150) was located at this position in AaManA, and no hydrogen bond was detected between Cys150 and Glu231. The C150A mutation resulted in only a 3-fold reduction in the activities (Table 3). This suggests that the role of the residue immediately preceding the acid/base catalyst in AaManA may differ from that in other

FIGURE 5. Overall structure of AaManA and stereoview of the structure superpositioned with endoglucanase Cel5G from P. haloplanktis. A, overall structure of AaManA. Left, overall structure of AaManA viewed from the top of the (β/α)8-barrel. The α-helices and β-strands belonging to the (β/α)8-barrel motif are numbered. The catalytic residues Glu151 and Glu231 are shown in orange in the stick model. Right, the view perpendicular to the (β/α)8-barrel axis. The surface model is shown in pink. A red arrow indicates the mannan-binding cleft. B and C, stereoview of the superpositioning of the structure of AaManA with those of other clan GH-A members. B, superpositioning of the overall structure of AaManA (purple) with that of Cel5G (cyan; Protein Data Bank code 1TVN). The catalytic residues (i.e. Glu135 and Glu222 in Cel5G and Glu151 and Glu231 in AaManA) are shown as ball models. C, comparison of the active site pockets of AaManA (purple) and Cel5G (cyan). The schematic diagram is drawn in white. The figures were prepared using PyMOL (39).

Taken together, AaManA was folded into a (β/α)8-barrel, containing two GH-A featured catalysts, and performed hydrolysis by the retaining mechanism. Thus, we concluded that AaManA belongs to clan GH-A, similar to other mannanases characterized thus far (55).
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GH113, the New GH Family—As shown above, the BLAST results revealed that the primary sequence of AaManA had no significant similarity to any known glycoside hydrolase. Pfam (e value threshold was 1.0) and CDD (e value threshold was 0.01) analyses failed to reveal any conserved domain or any similarity to existing glycoside hydrolase families. Although 17% sequence similarity was observed between AaManA and two GH53 galactanases, sequence alignment revealed that four of the eight GH53 conserved residues (indicated by hollow stars in Fig. S2) were not present in AaManA; this excluded AaManA from being classified in GH53. In conclusion, AaManA could not be assigned to any of the 112 existing GH families. A BLAST search conducted with the AaManA sequence revealed significant similarity to dozens of hypothetical proteins. The most similar (with 37–46% identities in the entire sequence) were Pjdr2DRAFT_4355 from Paenibacillus sp. JDR-2, COPEUT_01901 from Coprococcus eutactus ATCC 27759, RUMGNA_02939 from Ruminococcus gnarus ATCC 29149, Cphy_0886 from Clostridium phytofermentans ISDg, LACR_1479 from Lactococcus lactis subsp. cremoris SK11, and FAEPARAM12_01134 from Faecalibacterium prausnitzii M21/2. Hence, a novel GH family (GH113) is proposed for AaManA and its relatives (Table S1).

AaManA adopts the GH-A (β/α)_8 barrel and belongs to clan GH-A. Additionally, the possible fold of each of the members assigned to the novel GH113 family was analyzed by the Bio-InfoBank metaserver. In each case, the GH-A (β/α)_8 barrels were predominant in the 3D-jury consensus results with the scores ranging from 74 to 198. The scores were well above the benchmark of 50, suggesting that all GH113 members folded into a GH-A (β/α)_8 barrel. Sequence alignment between AaManA and the other GH113 members showed that the catalytic residues (indicated by the diamond and triangle in Fig. 4) together with the other GH-A key residues were strictly conserved in this new family (indicated by the solid star in Fig. 4), with the exception of one residue at the position of Thr^95 (indicated by the hollow star in Fig. 4). Therefore, we concluded that all GH113 members fold in the GH-A (β/α)_8 barrel and possess the counterparts of the GH-A conserved residues. Taken together, the data strongly suggest that family GH113 belongs to clan GH-A.

CONCLUSION

To our knowledge, this is the first report on the presence of an endo-β-1,4-mannanase in the cytoplasm of the thermoacidophilic bacterium A. acidocaldarius. Data from sequence alignment, structural analysis, and mutagenesis studies show that the enzyme and its relatives formed a new GH family called GH113; this family was assigned to glycoside hydrolase clan GH-A. The three-dimensional structure of AaManA, which is the first reported structure for this new family, sheds new light on the evolution of glycoside hydrolases and provides useful information for investigating other GH113 members.

Hydrolysis of short soluble manno-oligosaccharides and the intracellular localization suggest a role for AaManA in the hydrolysis of short chain degradation products in the cytoplasm. Similar cytoplasmic functions have also been proposed for a cyclomaltodextrinase and an endoglucanase from A. aci-
docalarius (29, 56). We note that the intracellular location of AaManA and its preference for longer substrates requires a special manno-oligosaccharide uptake system, because such substrates are too large to passively cross the membrane. In addition, the significant transglycosylation activity and relatively low hydrolytic activity on manno-oligosaccharides suggest that this mannanase might produce longer manno-oligosaccharides in the cytoplasm of A. acidocaldarius Tc-12-31.

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