Acetylxylan esterases hydrolyze the ester linkages of acetyl groups at positions 2 and/or 3 of the xylose moieties in xylan and play an important role in enhancing the accessibility of xylanases to the xylan backbone. The hemicellulolytic system and play an important role in enhancing the accessibility of lus acetate. The pH profiles for hydrolyzes the synthetic substrates 2-naphthyl acetate, 4-nitrophenyl acetate, 4-methylumbelliferyl acetate, and phenyl acetate. The pH profiles for $k_{cat}$ and $k_{cat}/K_m$ suggest the existence of two ionizable groups affecting the binding of the substrate to the enzyme. Using NMR spectroscopy, the reactivity of Axx2 was directly determined with the aid of one-dimensional selective total correlation spectroscopy. Methyl 2,3,4-tri-O-acetyl-β-D-xylopyranoside was rapidly acetylated at position 2 or at positions 3 and 4 to give either diacetyl or monoacetyl intermediates, respectively; methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside was initially acetylated at position 6. In both cases, the complete hydrolysis of the intermediates occurred at a much slower rate, suggesting that the preferred substrate is the peracetate sugar form. Site-directed mutagenesis of Ser-15, His-194, and Asp-201 resulted in complete inactivation of the enzyme, consistent with their role as the catalytic triad. Overall, our results show that Axx2 is a serine acetylxylan esterase representing a new carbohydrate esterase family.

Acetylxylan esterases take part in the degradation of xylan by microorganisms, which utilize plant biomass for growth (1). Many xylans are decorated with acetyl side groups attached at position 2 or 3 of the xylose backbone units. For example, in 4-O-methyl-β-D-glucuronoxylan, the main hardwood hemicellulose, 7 of 10 xylose units are acetylated (2). In general, the removal of these side chains improves the access for xylanases and facilitates the hydrolysis between the sugar backbone units (3). Acetylxylan esterases are classified in the CAZy Database and are found in 8 of 16 carbohydrate esterase (CE) families (4). Families CE3 (acetylxylan esterases) and CE12 (pectin acetyl esterases, rhamnogalacturonan acetyl esterases, and acetylxylan esterases) are also classified as lipase GDSL family proteins (Pfam accession number PF00657).

Geobacillus steathrothermophilus T-6 is a Gram-positive thermophilic bacterium that possesses an extensive hemicellulolytic system, with >40 genes involved in the utilization of hemicellulose (5, 6). The bacterium degrades xylan by initially secreting an extracellular xylanase (7–10), which partially degrades xylan to decorated xylo-oligomers that are transported into the cell via a specific ATP-binding cassette (ABC) transport system (11). Inside the cell, the decorated xylo-oligomers are hydrolyzed by side-chain-cleaving enzymes, arabino-furanosidases (12–14) and glucuronidase (15–17), and finally by intracellular xylanase (18) and xylosidases (19, 20).

The $axe2$ gene (GenBank™ accession number AB149953.1) was identified in G. steathrothermophilus as part of a three-gene operon, which also includes $xynB3$ (encoding $β$-xylosidase) (21, 22) and an uncharacterized gene, $xynX$ (GenBank™ accession number DQ868502.2). According to sequence similarities, the $axe2$ gene product, Axx2, is a serine hydrolase belonging to the lipase GDSL family (UniProtKB/TrEMBL accession number Q9QLX1) and is made up of 219 amino acids with a calculated molecular mass of 24,770 Da. The lipase GDSL family is one of four families that make up the SGNH hydrolase superfamily (Pfam clan accession number CL0264) (23, 24). The SGNH
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to four conserved sequence blocks identified as blocks I, II, III, and V of the five conserved blocks first used by Upton and Buckley to define a new family of lipolytic enzymes (25). The GDSX motif, which contains the nucleophile Ser residue, is part of block I and is equivalent to the classical GXSXG motif of lipases/esterases, whereas the consensus amino acids Gly, Asn, and His belong to blocks II, III, and V, respectively. The His residue acts as the base and activates the catalytic Ser residue, whereas the role of Asp, also from block V, is not clear and is thought to affect the catalytic His residue by increasing its basic character, stabilizing it during the formation of the tetrahedral intermediate, or ensuring its correct orientation (26, 27). The main chains of the conserved catalytic Ser and Gly residues in block II and the side chain of Asn in block III serve as the proton donors for the oxyanion hole, a positively charged pocket that activates the carbonyl and stabilizes the negatively charged oxyanion of the tetrahedral intermediates (26–31). In this work, we report the identification and characterization of Axe2, a new acetylxylan esterase belonging to the SGNH superfamily.

EXPERIMENTAL PROCEDURES

Enzyme Source—The axe2 gene was amplified via PCR from G. stearothermophilus T-6 genomic DNA using primers 5′-GGAGGAAAGAGGACCATGGAATCGGCTCTGGCCG-3′ (with Ncol restriction site, N-terminal) and 5′-GGATGTATGTATGCTAGCTTCATCTAGACCTAACCAC-3′ (with BplI restriction site, C-terminal), cloned into the pET9d expression vector (Novagen), expressed in Escherichia coli BL21(DE3) cells (Novagen), and purified by gel filtration as described previously (15). Xylanase XT6 was prepared as described previously (32).

Real-time RT-PCR Analysis—Growth conditions for G. stearothermophilus were as described by Shulami et al. (6). Total RNA was isolated with an RNeasy kit (Qiagen) according to the manufacturer’s protocol. Reverse transcription of RNA was performed with a Verso cDNA kit (Thermo Fisher Scientific) following the manufacturer’s protocol with 1 μg of total RNA and random hexamers as primers. Control reactions were carried out in the absence of reverse transcriptase. Gene relative quantification was performed with the Applied Biosystems 7300 real-time PCR system according to the manufacturer’s instructions. The reaction mixture (20 μl) included template cDNA, 300 nM each primer, and Power SYBR Green PCR Master Mix (Applied Biosystems). The amplification conditions for all reactions were one cycle at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s. Data analysis was carried out using the 7300 system software (Applied Biosystems) using the housekeeping gene citC for normalization.

Substrates—p-Nitrophenyl acetate, 2-naphthyl acetate, phenyl acetate, 4-methylumbelliferyl acetate, and N-acetylglucosamine were purchased from Sigma. Acetylxylan was prepared as described previously (33) using dimethyl sulfoxide, potassium borate, and acetic anhydride. Methyl-β−D−xylopyranoside and methyl-β−D−glucopyranoside (Sigma) were acetylated using pyridine and acetic anhydride to give the peracetate sugars (34). Xylobiose peracetate was prepared following enzymatic digestion of birch wood xylan by xylanase XT6, followed by acetylation (34) and purification using silica gel chromatography (230–400 mesh) with 1:1 (v/v) hexane/ethyl acetate. Acetylation of the sugars was confirmed, where possible, by proton NMR spectroscopy.

Biochemical Characterization and Kinetic Studies—The activity of Axe2 on synthetic substrates was determined by measuring the release of the leaving groups using an Ultraspec 2100 pro UV/visible spectrophotometer (GE Healthcare) equipped with a temperature-stabilized water-circulating bath. The extinction coefficients (ε) and wavelengths (λ) that were used at pH 6 are as follows: 4-nitrophenyl, ε = 1.17 mm−1 cm−1 and λ = 420 nm; 2-naphthyl, ε = 1.15 mm−1 cm−1 and λ = 330 nm; phenyl, ε = 0.91 mm−1 cm−1 and λ = 277 nm; and 4-methylumbelliferyl, ε = 0.94 mm−1 cm−1 and λ = 356 nm. The extinction coefficients for 2-naphthyl at different pH values were also determined. The reactions contained 450 μl of either citrate phosphate buffer (for pH 5–8) or Clark and Lubs buffer (for pH 8–10) with the enzyme at the appropriate concentration (1.5 × 10−4 to 3.75 × 10−5 mM final concentrations in the reactions) and 350 μl of substrate dissolved in either isopropyl alcohol or Me2SO. Blank samples contained either isopropyl alcohol or Me2SO and the appropriate buffer. Control mixtures containing all of the reactants except the enzyme were used to correct for spontaneous hydrolysis of the substrates. The standard reaction was carried out with 43 mM p-nitrophenyl acetate in citrate phosphate buffer (pH 6) at 30 °C. Michaelis–Menten constants were obtained from initial rates at different substrate concentrations and following analysis using GRAFIT 5.0.1 (Erithacus Software, Surrey, United Kingdom). Thermal stability was determined by incubating the enzyme at different temperatures for 5 min and measuring the residual activity under the standard assay conditions. Protein melting temperature was determined using differential scanning calorimetry over a temperature range of 40–90 °C as described previously (15). The effect of temperature on the reaction rate was determined by performing the standard reaction at different temperatures ranging from 20 to 70 °C. The pH dependence at a range of 5–10 was measured using 2-naphthyl acetate as a substrate at different concentrations (1–100 mM stock concentrations) depending on the pH. The activity of Axe2 on acetylated xylan was determined by following the release of acetic acid (see below). The activities of Axe2 on N-acetylglucosamine and on acetylated xylobiose, methyl-β−D−xylopyranoside, and methyl-β−D−glucopyranoside were determined by TLC and proton NMR spectroscopy (see below). For enzyme kinetics using proton NMR spectroscopy, 1% substrate was dissolved in D2O and centrifuged to ensure a homogeneous solution. The reaction mixture contained 350 μl of substrate solution, 450 μl of deuterated citrate phosphate buffer (pH 6.9), and 35 μl of 2.4 mg/ml Axe2.
Analytical Methods—The release of acetic acid was determined with a K-ACETRM acetic acid kit (Megazyme) according to the manufacturer’s instructions. Axe2 activity on acetylated sugars was followed by TLC using precoated plates (Silica Gel 60 F254, 0.25 mm; Merck) with 1:2:7 (v/v) water/methanol/ethyl acetate as the running solvent. Sugars were visualized by charring with solution containing 120 g of (NH4)Mo7O24 and 50 g of (NH4)2Ce(NO3)6 in 800 ml of 10% H2SO4. Xylanase XT6 activity was determined using the BCA assay for reducing sugars (35) with xylose as a standard.

Structural Analysis of Substrates and Products—Proton NMR spectroscopy was performed in D2O or deuterated buffers on Bruker Avance AV-III 400 and AV-III 600 spectrometers operating at 400.40- and 600.55-MHz resonance frequencies, respectively. Five-mm outer diameter glass tubes were used with broadband (BBO) probe heads with automatic tuning equipped with z-gradients and 2H lock. Typical data on the Bruker Avance AV-III 600 spectrometer were collected with eight shots, a 69.3-s dwell time, 65,536 real points (in the time domain), and a relaxation time of 4 s to allow quantification of the spectra. Total correlation spectroscopy (TOCSY) was performed with the Bruker AV-III 600 spectrometer irradiating at anomeric protons (homonuclear Hartman-Hahn transfer using the MLEV-17 sequence for mixing) (36). Typical data were collected with 32 shots, a 83.2-s dwell time, 65,536 real points, a relaxation time of 4 s, and mixing times of 20–80 ms.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene) with the following primers (with mutated nucleotides in boldface): 5’-CTC-TTT-ATT-GGT-GAT-GCT-ATC-ATT-GAT-TGG-GCG-GAT-CAT-GAT-AGC-ACC-AAT-AAC-AAA-GAG-3’ for the S15A mutant, 5’-GTC-CGG-CTC-GCT-TGG-GCC-CGG-GTT-CAC-CGG-TCT-GCT-3’ and 5’-AC-GGA-CGG-GTG-AAC-CCG-GGC-AGC-GAG-CCG-AG-C-3’ for the D191A mutant, and 5’-GTC-GTG-ACT-GCC-GTG-CAC-CTC-3’ for the H194A mutant. DNA production and purification were performed with E. coli XL1-Blue (Stratagen) and an SV Miniprep DNA purification kit (Promega). All mutations were verified by DNA sequencing. The DNA was transformed into E. coli BL21(DE3) cells, and the mutant enzymes were purified as described previously (15).

RESULTS

Axe2 Represents a New Carbohydrate Esterase Family—The axe2 gene in G. stearothermophilus T-6 is located within a large gene cluster for xylan utilization. The gene is part of a three-gene operon, which includes the xynB3 gene (encoding an intracellular GH43 β-xylanase) (21, 22) and a putative regulatory gene with unknown function, xynX. The axe2 gene product does not contain any recognizable Gram-positive leader sequence and is therefore most likely an intracellular enzyme. Real-time RT-PCR analysis indicated that the expression level of axe2 was ~8-fold higher in cultures grown on xylose than on arabinose as the carbon source. These results suggest that the gene is induced by xylose, in agreement with its physical arrangement on the chromosome. Amino acid sequence analysis of Axe2 using the ConSurf server (37) provided nine
homologs, which were further subjected to multiple sequence alignment using MUSCLE 3.7. The homologs showed >50% identity and included the four conserved blocks of the SGNH hydrolase superfamily (25). None of the homologs are assigned to a known family in the CAZY Database, and no published experimental data are available for these enzymes. Further analysis using the Pfam Database (24), which is based on domain identification, indicated that Axe2, together with families CE12 and CE3, can be assigned to the lipase GDSL family (Pfam accession number PF00657). Using sequences from families CE3 and CE12 (obtained from the CAZY Database) (4), a phylogenetic tree yielded three distinct clusters: the CE3 sequences, the CE12 sequences, and Axe2 with its homolog sequences (Fig. 1). Close inspection of the conserved blocks of the three groups revealed some differences in the amino acid composition specific to each group. In addition, family CE3 does not possess block II, in contrast to the other two groups (38). Taken together, the data suggest that Axe2 and its homologs constitute a new family of carbohydrate esterases.

**Biochemical Characterization of Axe2**—The **axe2** gene was overexpressed efficiently in *E. coli* using the T7 polymerase expression system. The purification procedure included heat treatment and gel filtration, providing >700 mg of purified enzyme/liter of culture. Based on SDS-PAGE, the protein was >90% pure. Thermal stability was determined after incubating the enzyme at different temperatures for 5 min. The residual activity was measured under the standard assay conditions. The enzyme was stable at 60 °C and lost most of its activity at 70 °C. This result is in good agreement with the melting temperature of the protein (72 °C), measured by differential scanning calorimetry. The effect of temperature on the reaction rate was determined using 4-nitrophenyl acetate as the substrate at temperatures ranging from 20 to 70 °C. The highest activity in a 5-min reaction was between 50 and 60 °C, and the activation energy calculated from an Arrhenius plot was 40 kJ/mol. The pH dependence of the kinetic constants at a range of 5–10 was determined using 4-p-nitrophenyl acetate as the substrate at substrate concentrations of 0.1–100 mM. The catalytic constant (*k*_cat) and the specificity constant (*K*_m) were determined using 4-nitrophenyl acetate as the substrate at substrate concentrations of 0.1–100 mM. The catalytic constant (*k*_cat) versus pH gave a flat bell-shaped curve, with a pH optimum range of 7.1–9.2 (Fig. 2A). The pH dependence of the specificity constant (*k*_cat/*K*_m) gave a symmetrical sharper curve with a pH optimum of 8.5 (Fig. 2B). The differences between the curves are mainly due to changes in the *K*_m values with pH. For example, the *K*_m value at pH 7.1 was 2.3 mM compared with 0.62 mM at pH 8.5. Axe2 was capable of hydrolyzing synthetic substrates bearing leaving groups with different *pK*_a values (Table 1). There were no significant changes in activity (*k*_cat) toward the different substrates.
strates, suggesting that the rate-limiting step is the second deacetylation step.

Axe2 Is an Acetylxylan Esterase—To test whether Axe2 can act on natural sugars, the specificity of the enzyme on acetylated carbohydrates was determined. Axe2 hydrolyzed 20–30% of the available acetyl groups on fully acetylated birch wood xylan. Interestingly, the resulting partially deacetylated xylan could not be degraded further by the GH10 family extracellular xylanase XT6. On the other hand, Axe2 completely deacetylated xylobiose peracetate (fully acetylated) at pH 7 (Fig. 3). Following the reaction by proton NMR spectroscopy showed that the enzyme was active on both the α- and β-forms of the sugar (immediate disappearance of both anomeric protons of the substrate). TLC and proton NMR analysis showed that the enzyme completely deacetylated methyl 2,3,4-tri-O-acetyl-β-D-xylpyranoside and methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside but failed to deacetylate N-acetylglucosamine. Taken together, the results suggest that the enzyme is an intracellular O-linked deacetylase, active on short xylo-oligomers.

FIGURE 4. Identification of partially acetylated methyl-β-D-xylopyranoside by one-dimensional selective TOCSY. A, methyl 2-mono-O-acetyl-β-D-xylopyranoside; B, methyl 3,4-di-O-acetyl-β-D-xylopyranoside. These acetylated sugars were formed during the deacetylation of methyl 2,3,4-tri-O-acetyl-β-D-xylpyranoside by Axe2 at 44 °C in deuterated citrate phosphate buffer at pH 6.8 (spectrum obtained during the reaction is shown at the top). The irradiated anomeric protons are marked by asterisks. Mixing times and assignments of the peaks are indicated.

TABLE 2
1H NMR data of acetylated and non-acetylated methyl-β-D-xylopyranoside
Data are as viewed after partial deacetylation of methyl 2,3,4-tri-O-acetyl-β-D-xylopyranoside by Axe2 in citrate phosphate buffer (pH 6.8) at room temperature. The chemical shifts of the fully acetylated and deacetylated forms of the sugar are also given in D2O, calibrated with DSS. The chemical shifts were determined by one-dimensional selective TOCSY using a Bruker Avance AV-III 600 spectrometer (one-dimensional homonuclear Hartman-Hahn transfer using the MLEV-17 sequence for mixing). Data were collected with 32 shots, a 83.2-μs dwell time, 65,536 real points, a 4-s relaxation time, and 20–80-ms mixing times.

|                   | H1   | H2   | H3   | H4   | H5a  | H5b  |
|-------------------|------|------|------|------|------|------|
| Methyl 2,3,4-tri-O-acetyl-β-D-xylopyranoside | 4.64 | 4.83 | 5.16 | 4.95 | 4.12 | 3.56 |
| Methyl 3,4-di-O-acetyl-β-D-xylopyranoside     | 4.42 | 3.41 | 5.03 | 4.89 | 4.04 | 3.47 |
| Methyl 2-mono-O-acetyl-β-D-xylopyranoside     | 4.46 | 4.61 | 3.58 | 3.65 | 3.95 | 3.30 |
| Deacetylmethyl-β-D-xylopyranoside             | 4.26 | 3.18 | 3.37 | 3.55 | 3.90 | 3.26 |
| Methyl 2,3,4-tri-O-acetyl-β-D-xylopyranoside* | 4.7  | 4.88 | 5.22 | 5.01 | 4.16 | 3.60 |
| Methyl-β-D-xylopyranoside*                    | 4.31 | 3.23 | 3.42 | 3.60 | 3.96 | 3.31 |

* In D2O calibrated with DSS (DSS = 0 ppm).
To reveal further the enzyme mode of action and regioselectivity, partially acetylated intermediates were followed with time using proton NMR. Preliminary data indicated that acetylation of the sugars resulted in deshielding of the proton in the acetylated position, and as a result, there was a significant difference in the chemical shift (0.4–1.5 ppm). One-dimensional selective TOCSY was used to identify partially acetylated sugars by stopping the reaction at different time points and irradiating the anomeric protons of the forming sugar intermediates. During the deacetylation of methyl 2,3,4-tri-O-acetyl-β-D-xylopyranoside, two partially acetylated products were observed, which were later identified as methyl 2-mono-O-acetyl-β-D-xylopyranoside (Fig. 4A) and methyl 3,4-di-O-acetyl-β-D-xylopyranoside (Fig. 4B). The most shielded anomeric proton that was observed at 4.26 ppm was confirmed to be the deacetylated form of the sugar: methyl-β-D-xylopyranoside. The chemical shifts of the acetylated and non-acetylated methyl-β-D-xylopyranosides are summarized in Table 2. According to the results, the enzyme initially deacetylates either position 2 or positions 3 and 4, with a preference for the later (Fig. 5). Deacetylation of methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside also resulted in specific partially acetylated intermediates, which eventually became completely deacetylated (Fig. 6). At the start of the reaction, there was a slight movement of the peaks corresponding to positions 2, 3, and 4 and the formation of a peak at 3.8 ppm corresponding to position 6, which suggested that the sugar was not deacetylated at positions 2, 3, and 4 but only at position 6. Deacetylation progressed through two observable intermediates (designated as 1 and 2 in Fig. 6), which could not be resolved using one-dimensional selective TOCSY. Eventually, those intermediates were deacetylated completely to give methyl-β-D-glucopyranoside (designated as 3 in Fig. 6). The chemical shifts of methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside and the deacetylated form of the sugar in D$_2$O, calibrated with sodium 3-(trimethylsilyl)propanesulfonate (DSS), commonly called sodium 2,2-dimethyl-2-silapentane-5-sulfonate (39), are given in Table 3. Following the reaction on-line...
# Acetylxylan Esterase from G. stearothermophilus

The classification of carbohydrate active enzymes in the CAZy Database is based on sequence similarities and reflects structural features and evolutionary relationships, which have implications in catalysis (4). Based on bioinformatics analysis, Axe2 and its homologs do not belong to any known family in the CAZy Database and thus represent a new family of carbohydrate esterases. The axe2 gene is part of the hemicellulolytic system of *G. stearothermophilus*; the gene is induced by xylose; and the gene product acts on different O-acetylated substrates, such as acetylated methyl-β-D-xylanopyranoside, methyl-β-D-glucopyranoside, and xylobiose. Taken together, the results show that Axe2 is an acetylxylan esterase.

Axe2 Regioselectivity—The complete deacetylation of methyl-β-D-xylanopyranoside peracetate was shown to occur through two intermediates: methyl 3,4-di-O-acetyl-β-D-xylanopyranoside and methyl 2-mono-O-acetyl-β-D-xylanopyranoside. The ability of Axe2 to work through two different partially acetylated intermediates may be explained by the formation of three productive complexes (including the fully acetylated sugar) of the enzyme to deacetylate positions 3 and 4 rather than position 2 on methyl-β-D-xylanopyranoside peracetate. Axe2 completely deacetylates methyl-β-D-glucopyranoside peracetate, initially by removing the acetate group from position 6. The appearance of partially acetylated sugars during deacetylation of either methyl-β-D-xylanopyranoside or methyl-β-D-glucopyranoside peracetate indicates the preference of the enzyme for the fully acetylated forms of the sugars rather than the partially acetylated ones. Enzymes from the CE3 family were shown to deacetylate acetylxylan and xylo-oligosaccharides and, in some cases, to enhance xylanase activity on these substrates (38, 43). Biely *et al.* (3) showed that the CE1, CE4, and CE5 families exhibit acetylxylan esterase activities with strong preference for position 2 on acetylated xylodinoyranosides. Enzymes in the CE2 family, which were shown to be 6-O-deacetylases (44) with a preference for glucosaminan over xylan, also showed activity for positions 3 and 4 on xylodinoyranosyl residues. This suggests that they could also function as acetylxylan esterases (3).

**Catalytic Mechanism of Axe2**—All CE families in the CAZy Database use Ser as the nucleophile during catalysis, except for the CE4 family, which comprises metalloenzymes and is deprived of a catalytic Ser in the active site. To date, Axe2 and its homologs were not assigned a CE family, and thus, their catalytic activity cannot be inferred from the CAZy Database. However, the Axe2 structural model, which is based on an SGH hydrolase (*E. coli* thioesterase I/protease I/lysophospholipase L1), suggests an orientation of the catalytic residues similar to that of the CE3 family structure (Protein Data Bank code 2VPT) from *Clostridium thermocellum*. For this structure, the acetate ion was modeled into the catalytic site and was shown to be able to have favorable interactions with the catalytic residues (43). Mutating the three putative catalytic residues of Axe2, Ser-15, Asp-191, and His-194, abolished the catalytic activity of the enzyme, suggesting that this new group comprises serine esterases and thus operates via a double-displacement mechanism. The catalytic cycle occurs in two steps: acetylation, in which the enzyme is acetylated by the substrate and the leaving group is released, followed by a deacetylation step, in which the enzyme is deacetylated by introducing a nucleophilic water molecule (Fig. 8). There is no available information on the residues involved in the formation of the oxyan-

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**TABLE 3**

| H | H2 | H3 | H4 | H5 | H6a | H6b |
|---|----|----|----|----|-----|-----|
| Methyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside | 4.75 | 4.95 | 5.35 | 5.12 | 4.05 | 4.40 | 4.23 |
| Methyl-β-D-glucopyranoside | 4.37 | 3.25 | 3.48 | 3.36 | 3.44 | 3.92 | 3.71 |

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**FIGURE 7.** Catalytic triad of Axe2. The model is based on *E. coli* thioesterase I/protease I/lysophospholipase L1 (Protein Data Bank code 1IVN) (29) obtained from the HHpred server (41) with 26% identity.
Acetylxylan Esterase from *G. stearothermophilus*

![Diagram of Acetylxylan Esterase from G. stearothermophilus](image)

**FIGURE 8. Proposed double-displacement catalytic mechanism for Axe2.** The first stage of catalysis involves the acetylation of the enzyme. His acts as a general base and increases Ser nucleophilicity. Ser attacks the ester bond, and a tetrahedral intermediate is formed, which is stabilized by the hydrogen bonds of the oxanion hole (formed by backbone amino acids). His then acts as a general acid and donates a proton to the sugar. The sugar is released, and the enzyme is deacetylated. His, again, acts as a general base and makes a water molecule a nucleophile. The water molecule attacks the ester bond of the acetyl group, creating a second tetrahedral intermediate. The acetyl group is released from the enzyme. His acts as a general acid, donates a proton to Ser, and restores the state of the catalytic site.

ion hole in acetylxylan esterases, but based on information on the SGNH hydrolase family, the conserved catalytic Ser-15 residue in block I, Gly-63 in block II, and Asn-92 in block III of Axe2 are the likely candidates (23, 30, 42). In the double-displacement mechanism, $k_{cat} = k_3/k_2(k_3 + k_2)$ and is made up of the two first-order rate constants, $k_2$ (acylation step) and $k_3$ (deacetylation step). Because the mechanism involves two steps, $K_m$ is in the form of $K_m = K_m/k_2/k_3$. The pH dependence profile of Axe2 shows that $k_{cat}$ remains constant over the wide pH range of 7.1–9.2, but $k_{cat}/K_m$ gives a sharp symmetrical curve at pH $\sim$8.5. This type of behavior indicates that the two ionizable groups observed in the pH profile of $k_{cat}/K_m$ with $pK_a$ values of 7.6 and 8.5 affect $K_m$. It is likely that the changes in the $K_m$ values reflect changes in the dissociation constant ($K_d$) rather than changes in the rate constants. Thus, the ionizable groups are actually affecting the binding of the substrate to the enzyme ($E \cdot ROAc$). This behavior is somewhat different from what was found for other serine proteases. For example, the pH profile of chymotrypsin suggests that one of the ionizable group is affecting $k_{cat}$ whereas the other one is affecting $K_m$ (45).

**Conclusion**—In this study, we have reported the existence of a new family of carbohydrate esterases represented by Axe2 from *G. stearothermophilus* T-6, a hemicellulose-degrading bacterium. The location of the axe2 gene, its induction by xylose, and the ability of the gene product to remove acetyl groups from acetylated xylo-oligosaccharides indicate that Axe2 is an acetylxylan esterase. Together with the CE3 and CE12 families, Axe2 belongs to the SGNH hydrolase superfamily and uses Ser-15, Asp-191, and His-194 for catalysis. Kinetic studies suggest that the observed ionizable groups are related to substrate binding rather than to the catalytic rate constants.

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