

Cloning and Characterization of the Glucooligosaccharide Catabolic Pathway β -Glucan Glucohydrolase and Cellobiose Phosphorylase in the Marine Hyperthermophile *Thermotoga neapolitana*[†]

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Characterization in *Thermotoga neapolitana* of a catabolic gene cluster encoding two glycosyl hydrolases, 1,4- β -D-glucan glucohydrolase (GghA) and cellobiose phosphorylase (CbpA), and the apparent absence of a cellobiohydrolase (Cbh) suggest a nonconventional pathway for glucan utilization in *Thermotogales*. GghA purified from *T. neapolitana* is a 52.5-kDa family 1 glycosyl hydrolase with optimal activity at pH 6.5 and 95°C. GghA releases glucose from soluble glucooligomers, with a preference for longer oligomers: k_{cat}/K_m values are 155.2, 76.0, and 9.9 mM⁻¹ s⁻¹ for cellotetraose, cellotriose, and cellobiose, respectively. GghA has broad substrate specificity, with specific activities of 236 U/mg towards cellobiose and 251 U/mg towards lactose. With *p*-nitrophenyl- β -glucoside as the substrate, GghA exhibits biphasic kinetic behavior, involving both substrate- and end product-directed activation. Its capacity for transglycosylation is a factor in this activation. Cloning of *gghA* revealed a contiguous upstream gene (*cbpA*) encoding a 93.5-kDa cellobiose phosphorylase. Recombinant CbpA has optimal activity at pH 5.0 and 85°C. It has specific activity of 11.8 U/mg and a K_m of 1.42 mM for cellobiose, but shows no activity towards other disaccharides or cellotriose. With its single substrate specificity and low K_m for cellobiose (compared to GghA's K_m of 28.6 mM), CbpA may be the primary enzyme for attacking cellobiose in *Thermotoga* spp. By phosphorolysis of cellobiose, CbpA releases one activated glucosyl molecule while conserving one ATP molecule per disaccharide. CbpA is the first hyperthermophilic cellobiose phosphorylase to be characterized.

To utilize polysaccharides such as glucans to meet carbon and energy requirements, heterotrophic organisms depend on a catabolic pathway involving the interaction of multiple hydrolytic enzymes, transporter complexes, and regulatory systems coordinating gene expression of pathway-specific proteins (41). During hydrolysis of the homopolymer cellulose, for example, the consortium of catalytic enzymes consists of endoglucanases (1,4- β -D-glucan 4-glucohydrolase [EC 3.2.1.4]), which randomly hydrolyze internal β -1,4 glycosidic bonds; cellobiohydrolases (β -D-glucan cellobiohydrolase [EC 3.2.1.91]), also referred to as exoglucanases, which remove cellobiose from either the nonreducing or reducing ends of cellooligomers; and β -glucosidases (β -D-glucoside glucohydrolase [EC 3.2.1.21]), which convert cellobiose to glucose. These classes of enzymes have been documented in a number of fungal and bacterial systems (4, 5, 39), while other glucan-catabolizing enzymes are less well understood.

Thermotoga neapolitana, a marine hyperthermophile isolated from geothermally heated biotopes, belongs to the order *Thermotogales*. *T. neapolitana* shares with other *Thermotogales*, specifically *Thermotoga maritima*, both the capacity to catabolize a wide variety of α - and β -linked glucans and a fermenta-

tive metabolism. While *Thermotogales* elaborate hydrolases such as amylases, cellulases, glucosidases, galactosidases, mannanases, and xylanases (6, 7, 10, 11, 23, 37), neither *T. neapolitana* (D. Y. Yernool, G. Swiatek, and J.-D. Bok, unpublished data) nor *T. maritima* (27) has been shown to have a gene for a cellobiohydrolase, a key enzyme in classical cellulolytic systems.

In this paper, we report the characterization of a catabolic gene cluster in *T. neapolitana* encoding two enzymes: a cellobiose phosphorylase (CbpA) and a β -glucan glucohydrolase (GghA). Cellobiose phosphorylases (EC 2.4.1.20) cleave cellobiose by phosphorolysis, yielding glucose-1-phosphate as one of the products, while β -glucan glucohydrolases or exoglucohydrolases (1,4- β -D-glucan glucohydrolase [EC 3.2.1.74]) preferentially act on cellooligomers, releasing glucose (17, 31, 37, 43). In addition to the functional and molecular characterization of GghA and CbpA, we propose a nonconventional, ATP-conserving, catabolic pathway for glucan utilization consisting of endoglucanases CelA and CelB (7), a β -glucan glucohydrolase (GghA), and a cellobiose phosphorylase (CbpA).

MATERIALS AND METHODS

Bacterial strains and culture media. *T. neapolitana* NS-E (kindly provided by K. O. Stetter and R. Huber, University of Regensburg, Regensburg, Germany) was grown anaerobically in MMS medium (9) at 77°C with cellobiose or glucose (1%, wt/vol) as a carbon source in static culture for 24 h. A cosmid DNA library in vector pLAFR3 (36) of *T. neapolitana* strain NS-E in *Escherichia coli* DH5 α (gift from Kenneth Knoll, University of Connecticut, Storrs, Conn.) was used. Commercially available *E. coli* strains were grown in Luria-Bertani (LB) medium or super broth (32) containing either ampicillin (50 μ g/ml) or tetracycline (25 μ g/ml). Some of the abbreviations used in this report are as follows: ORF, open reading frame; oNP, *o*-nitrophenol; PC, sodium phosphate-sodium citrate; pNPA, *p*NP- α -D-arabino-; pNPC, *p*NP- β -D-cellobio-; pNPG, *p*NP- β -D-gluco-; pNPL, *p*NP- β -D-lacto-; pNP, *p*-nitrophenol; pNPX, *p*NP- β -D-xylo-;

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pNP α G, *pNP*- α -D-glucoside; *oNPG*, *oNP*- β -D-glucoside; *oNPGal*, *oNP*- β -D-galactoside.

Purification of glucan glucohydrolase. *T. neapolitana* was grown on cellobiose, collected by centrifugation, washed twice in Tris-HCl (0.1 M [pH 7.5] at 4°C), resuspended in the same buffer, and lysed by sonication. Cell debris was removed, the proteins in the supernatant were precipitated using ammonium sulfate (final concentration, 80%), collected, and dissolved in 50 mM Tris-HCl (pH 7.5) (20 ml). The ammonium sulfate precipitation was repeated twice, and the resultant cell extract was dissolved in 20 mM piperazine-HCl buffer (pH 5.1) (20 ml) and desalted by ultrafiltration using a PM 10 membrane (Amicon, Cambridge, Mass.). The pH of this cell-free extract was adjusted to 4.3 by addition of sodium citrate (0.15 M, pH 4.1); precipitated proteins were removed by centrifugation. The pH of the supernatant containing aryl β -glucosidase activity was raised to 5.1 using 0.2 M sodium citrate. Purification of the aryl-glycosidase was achieved by anion-exchange chromatography using an FFQ-Sepharose column (XK26; 60-ml bed volume; Pharmacia, Piscataway, N.J.) equilibrated with 20 mM piperazine buffer (pH 5.1). Bound proteins were eluted with a linear gradient of 0 to 0.3 M NaCl. Fractions from a single peak with activity towards aryl-glycosidase were pooled, desalted by ultrafiltration, and subjected to gel filtration (BioGel P-60 column, 1.5 by 97 cm; 50 mM MOPS [pH 7.0]). Active fractions were pooled, the concentration of ammonium sulfate was adjusted to 0.2 M, and the proteins were then fractionated by hydrophobic-interaction chromatography (Phenyl-Sepharose FF; XK26 column; 20-ml bed volume; Pharmacia). Proteins bound to the column were eluted using a reverse gradient (0.2 M ammonium sulfate in 20 mM Tris-HCl [pH 7.5] to distilled water). Active fractions were pooled, desalted, and further purified using a galactose-agarose column (*p*-aminobenzyl-1-thio- β -D-galactopyranoside; Sigma, St. Louis, Mo.) equilibrated with binding buffer (0.1 M NaPO₄ [pH 7.0], 0.15 M NaCl). Bound proteins were eluted with elution buffer (binding buffer with 0.3 M glucose). After buffer exchange to 20 mM BisTris (pH 6.5), pooled active fractions were applied to a Mono-Q column (HR 10/10; Pharmacia) and eluted with a linear gradient of 0.05 to 0.5 M NaCl. Final purification was achieved by discontinuous preparative polyacrylamide gel electrophoresis (PAGE) using a PrepCell (Bio-Rad, Richmond, Calif.) following the manufacturer's instructions.

Purification of recombinant cellobiose phosphorylase. *E. coli* clone 13CBPFL was grown in 5 liters of LB medium containing ampicillin (50 μ g/ml) to an optical density at 600 nm of 0.5. Expression of the *cbpA* gene was induced by addition of arabinose (final concentration, 0.015%). Four hours postinduction, cells were collected by centrifugation (4,000 \times g, 15 min, 4°C). Cell pellets were resuspended in buffer (50 mM Tris-HCl buffer [pH 7.9], 50 mM dextrose, 1 mM EDTA) containing lysozyme (4 mg/ml) (Sigma) and sonicated. Heat-labile *E. coli* proteins were removed from the cell extract by heat treatment (75°C for 15 min) and precipitated by centrifugation (15,000 \times g, 30 min, 4°C). Supernatant was equilibrated with ammonium sulfate (final concentration, 1.0 M) and loaded onto a phenyl-Sepharose column (XK26; Pharmacia; 100-ml bed volume). Bound proteins were eluted with a reverse gradient, ammonium sulfate (1.0 M) in Tris-HCl buffer (20 mM, pH 7.8) to distilled H₂O and distilled H₂O to 50% acetonitrile. Active fractions from a single peak were pooled, desalted, and further purified using immobilized metal-ion chromatography (Ni-nitrilotriacetic acid resin; Qiagen, Valencia, Calif.; 10-ml bed volume) with a linear gradient of 0.01 to 0.3 M imidazole, following the manufacturer's instructions. Active fractions were pooled, buffer exchanged by ultrafiltration (Amicon YM10; Millipore, Bedford, Mass.), and further purified by anion-exchange chromatography (Mono-Q HR 5/5; Pharmacia). The pool was equilibrated with loading buffer (20 mM piperazine [pH 6.0], 10 mM NaCl), and bound protein was eluted with a linear gradient of 0.01 to 0.5 M NaCl. Active fractions from the single peak were pooled and further purified by ultrafiltration (Microcon YM50; Millipore).

Glucan glucohydrolase assays. Aryl-glycosidase activity was determined using either *pNP*- or *oNP*-glycoconjugates at 5 mM final concentration in a 15-min assay (42). The glycoconjugates used included *pNPA*, *pNPC*, *pNPG*, *pNPL*, *pNPX*, *pNP* α G, *oNPG*, and *oNPGal*. Released *pNP* and *oNP* were measured at 405 and 420 nm, respectively; activity was calculated using a standard curve developed under assay conditions. Hydrolysis of cellobiose and lactose was analyzed using the glucose hexokinase kit following the manufacturer's instructions (Sigma). One unit of enzyme activity corresponds to release of 1 μ mol of *pNP*, *oNP*, or glucose/min (for cellobiose as the substrate, one unit corresponds to the release of 2 μ mol/min). All assays were carried out in 100 mM (final concentration) PC buffer (pH 6.4) at 85°C unless otherwise cited.

Cellobiose phosphorylase assay. Cellobiose phosphorylase activity was determined by measuring the amount of glucose 1-phosphate formed from phosphorylation of cellobiose (Sigma). The enzyme was incubated at 85°C for 15 min with 10 mM cellobiose in PC buffer (pH 5.5). The reaction was stopped by boiling for 10 min, and the amount of glucose 1-phosphate produced was determined by a coupled enzyme assay measuring the appearance of NADPH at 340 nm (30). The reaction mixture contained phosphoglucomutase (4 U ml⁻¹), glucose-6-phosphate dehydrogenase (2.0 U ml⁻¹) (Boehringer Mannheim, Indianapolis, Ind.), 3 mM NADP, and 5 μ M glucose 1,6-bisphosphate (Sigma) in 80 mM triethanolamine buffer (with 4.4 mM MgCl₂ [pH 7.5]). One unit of activity corresponds to the release of 1 μ mol of glucose 1-phosphate/min.

Analytical methods and enzymatic characterization. The molecular mass of glucan glucohydrolase and cellobiose phosphorylase was determined by sodium dodecyl sulfate (SDS)-PAGE according to Laemmli (24) and by gel filtration

using an analytical Suparose 12 HR column (Pharmacia). Protein bands in SDS-PAGE gels were visualized with either silver stain for GghA or Coomassie blue for CbpA. To determine the N-terminal sequence, the protein band was transferred to a polyvinylidene difluoride membrane, and the sequence was determined using an Applied Biosystems 475A gas phase sequencer (Applied Biosystems, Foster City, Calif.). Protein concentrations were estimated by the Bradford dye-binding method (Bio-Rad) with bovine serum albumin as the standard and using A_{280} and the molar extinction coefficient (12). Isoelectric focusing was used to determine the isoelectric point (pI) (Phast System; Pharmacia). pH optima were determined using PC buffer (0.1 M, pH 3.6 to 7.0) and phosphate buffer (pH 6.0 to 7.5). To determine the optimum temperature for activity for GghA, a mixture of GghA in PC buffer (0.1 M, pH 6.4) and *pNPG* (5 mM) were sealed in 2-ml gas chromatography vials (Wheaton, Vineland, N.J.). Activity was measured after heating in an oil bath (70 to 120°C) for 20 min. Released *pNP* was quantified as described for the enzyme assays. The temperature optimum for CbpA was determined by the cellobiose phosphorylase assay run for 15 min at various temperatures prior to the coupled enzyme assay as described above. The glucan glucohydrolase's thermal stability was evaluated by heating the enzyme (0.19 μ g) in sealed vials in an oil bath at 90 and 95°C for up to 9 h in MOPS buffer (20 mM, pH 7.0). After cooling on ice, residual activity was estimated using *pNPG* as the substrate. The effect of end product on hydrolytic activity was assessed by measuring the rate of release of *pNP* from *pNPG* in the presence of increasing concentrations of glucose (0 to 1.0 M).

Purified glucan glucohydrolase (37 to 185 ng) was mixed with each substrate (2 to 40 mM) in a total volume of 120 μ l and incubated at 75°C in 5 mM HEPES buffer (pH 7.2). The celooligosaccharide substrates used included cellobiose, cellotriose, and cellotetraose (V-Labs, Covington, La.). The products of hydrolysis of celooligosaccharides and transglycosylation were fractionated on an Aminex HPX42A column (Bio-Rad) attached to a high-pressure liquid chromatograph (HPLC; model LC600; Shimadzu, Kyoto, Japan) and detected using a refractometer (Waters, Milford, Mass.) linked to a Hewlett-Packard 3390A integrating recorder. The released sugars were eluted isocratically with distilled water (0.6 ml/min). The data were compared to commercially available standards analyzed under identical conditions. One unit is defined as the amount of enzyme required to hydrolyze 1 μ mol of cellobiose to glucose (2 μ mol) or to release 1 μ mol of glucose from cellotriose and cellotetraose in 1 min under assay conditions.

Determination of anomeric carbon configuration of the hydrolysis products.

The method described by Baker and Himmel (3) was used with minor modifications. GghA (0.9 μ g) was mixed with *oNPG* (10 mM final concentration) in 5 mM HEPES buffer (pH 7.2), incubated at 22°C for 10 min, and immediately placed on ice. A 20- μ l aliquot of the reaction mixture was fractionated on an Aminex HPX-87C column run at 25°C at 0.5 ml/min. The retention times and the extent of mutarotation were compared to those of freshly prepared controls (α -glucose and β -glucose). Under these conditions, mutarotation was negligible ($\beta/\alpha = 4.26$; at equilibrium, $\beta/\alpha = 1.6$).

Cloning, sequencing, and analysis of *gghA* and *cbpA* from *T. neapolitana*. All DNA manipulations were carried out using standard methods (32). A genomic DNA library of *T. neapolitana* was constructed and screened for thermostable glycosidases as described earlier (7). The DNA sequence (partial) of the inserts contained in selected clones was determined using fluorescent dye terminator sequencing chemistry with an ABI 373 automated sequencer (Applied Biosystems). The sequence was assembled and analyzed using BLAST (2). Initially, clone p46B1 containing part of the sequence of the *gghA* gene was identified. The sequence at the other end of the p46B1 insert revealed similarities to known cellobiose phosphorylases. Based on this sequence, two PCR primers, OT305PA1 (5'TCCAGTC CGCCCTTCCTATGAG3') and OT317PA1 (5'AAGGCATT TCA CAGGAGAG GTC 3'), were designed. A PCR screen was conducted using these primers on a *T. neapolitana* cosmid library. By analyzing PCR amplicons by agarose gel electrophoresis, two cosmid clones, RUC 270 and RUC 278, containing the *gghA* and *cbpA* genes were identified; both *gghA* and *cbpA* genes were cloned, and the DNA sequence was determined.

Comparison of amino acid sequences of cellobiose phosphorylases and β -(1-2) glucan synthetases. Percent similarity was calculated using the GAP program of GCG version 9.1 (Genetics Computer Group, Madison, Wis.). Complete amino acid sequences were used except for domains from *Clostridium thermocellum* Cbp (amino acids 564 to 975), *Rhizobium meliloti* NdvB (amino acids 2090 to 2843), and *Brucella abortus* Cgs (amino acids 2084 to 2817).

Expression of the cellobiose phosphorylase *cbpA* gene in *E. coli*. The *cbpA* gene was amplified using primers CBP.509 (5'AGGATCCATAGCATGAAGTTTCG GCTACTT 3') and CBP.R510 (5'CAAGCTTTCGCTCTTTGCTGTAGTTT 3'), designed with *Bam*HI and *Hind*III restriction sites, respectively (underlined sequence). The amplicon was cloned into the pBAD-TOPO-TA vector (Invitrogen, Carlsbad, Calif.) under the control of an inducible arabinose promoter, resulting in the addition of a C-terminal (His)₆ tag.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank and EMBL Data Bank under accession number AF039487.

TABLE 1. Purification of glucan glucohydrolase from *T. neapolitana*

Purification step	Total protein (mg)	Total activity ^a (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract	2,425	4,994	2.06	— ^b	— ^b
Acidification to pH 4.3 (supernatant)	628	2,747	4.37	1	100
Anion-exchange chromatography (Q-Sepharose)	152	2,200	14.47	3	80
Gel filtration (Biogel P-60)	82.8	2,025	24.45	6	74
Hydrophobic-interaction chromatography (phenyl-Sepharose)	24.4	1,735	71.1	16	63
Affinity chromatography (galactose-agarose)	3	1,460.9	374.3	86	53
Anion-exchange chromatography (Mono-Q)	3.2	1,413	441.5	97	51
Preparative PAGE (native)	1.1	888	807.27	184	32

^a The substrate used in enzyme assays was *o*NPG.

^b Purification and recovery were not calculated because the crude extract contains at least two enzymes with aryl-glycosidase activity.

RESULTS

Purification of glucan glucohydrolase (GghA). A seven-step protocol was used to purify GghA, giving 184-fold purification and a total recovery of 32% (Table 1). The specific activity of the purified enzyme is 807 U/mg toward *o*NPG. SDS-PAGE analysis showed that the purified enzyme had an apparent molecular mass of 52.5 kDa (Fig. 1). A few faint bands were visible on the silver-stained gel as purified sample (610 ng) was loaded in excess (sensitivity of silver staining is 2 to 10 ng of protein/band [13]). The N-terminal sequence of GghA was MKKFPEGFLWGVATASYQIEG, which agrees with the DNA sequence data (see below).

Characterization of GghA. GghA exhibited optimal activity over the pH range 6.4 to 7.0 and at 95°C. The enzyme was highly thermostable, retaining 85% activity after incubation for 9 h at 90°C and 88% activity after 1 h at 95°C. Active GghA was a monomer (determined by gel filtration) with an apparent molecular mass of 58.9 kDa. GghA showed broad-spectrum glycosidase activity, releasing *o*NP from both *o*NPG and *o*NPGal with high specific activities, 807 and 778 U/mg, respectively (Table 2). GghA showed higher activity towards aryl-glucosides in which the nitrophenol group was attached to the *ortho* rather than the *para* position. In addition, GghA hydrolyzed *p*NPC and *p*NPL, and the lower rate of *p*NP release compared to the release of reducing sugars suggests that the attack on the substrate takes place from the nonreducing end of the glycoside. To determine the true substrate specificity of the enzyme,

GghA was tested for its ability to hydrolyze naturally occurring disaccharides, cellobiose and lactose. The specific activity towards lactose (251 U/mg) was marginally higher than that towards cellobiose (236 U/mg). In view of the organism's ability to grow on glucans, and to establish the precise role for GghA in the glucan catabolic pathway, the kinetic constants for hydrolysis of glucooligosaccharides were determined. Comparable k_{cat} values of 345.7 and 333.7 s⁻¹ were found for cello-triose and cellotetraose, respectively (Table 3). The K_m for cellotetraose (2.15 mM), however, is significantly lower than that for cello-triose (4.55 mM), resulting in a twofold increase in the k_{cat}/K_m value with cellotetraose as the substrate. Interestingly, the K_m for cellobiose is 13-fold higher than the K_m for cellotetraose, indicating a weak interaction between the enzyme and cellobiose (Table 3).

The kinetics of the hydrolysis of *p*NPG by GghA is described in Fig. 2A. The double reciprocal plot of v versus $[S]$ shows a biphasic pattern. The inflection point on the curve, at a substrate concentration of 2 mM, suggests the binding of an additional substrate molecule. The Michaelis-Menten equation was used to calculate the kinetic constants for both low (0 to 2 mM) and high (2 to 40 mM) substrate concentrations by fitting the data at the extremes of v and $[S]$. At lower substrate concentrations (0.2 to 2.0 mM *p*NPG), the K_m and k_{cat} are 0.28 mM and 512.55 s⁻¹, respectively, while at higher concentrations of the substrate (2 to 40 mM), both K_m and k_{cat} increase, to 1.93 mM and 883.9 s⁻¹, respectively (Table 3). The biochemical basis for such biphasic kinetic behavior was investigated by analyzing the effect of the end product (glucose, 0 to 200 mM) on GghA's hydrolytic activity towards *p*NPG. Surprisingly, glucose acts as an activator even up to a concentration of 200 mM, and there was a direct relationship between glucose concentration and the rate of activation (Fig. 2B). End product inhibition was observed only at glucose concentrations

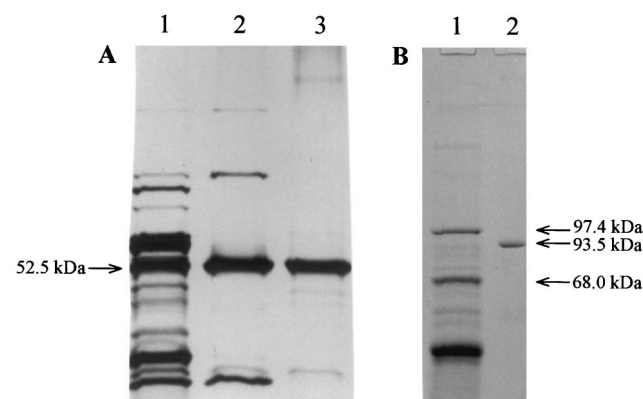


FIG. 1. SDS-PAGE analysis of purified native glucan glucohydrolase (GghA) and recombinant cellobiose phosphorylase (CbpA). (A) Glucan glucohydrolase. Lane 1, fraction after hydrophobic interaction chromatography (2 μ g); lane 2, fraction after affinity chromatography (1 μ g); lane 3, purified GghA after preparative PAGE (0.61 μ g). (B) Cellobiose phosphorylase. Lane 1, high-range protein molecular size markers; lane 2, purified CbpA after Mono-Q chromatography (6.7 μ g).

TABLE 2. Substrate specificity of *T. neapolitana* glucan glucohydrolase towards aryl-glycosides

Substrate	Sp act ^a (U/mg)	Relative activity (%)
<i>o</i> NPG	807	100
<i>o</i> NPGal	778	96.4
<i>p</i> NPG	664	82.3
<i>p</i> NPL	260	32.2
<i>p</i> NPC	225	27.9
<i>p</i> NPX	117	14.5
<i>p</i> NPA	86	10.6
<i>p</i> NP α G	0	0

^a Determined with 5 mM (final concentration) of aryl-glycosides as the substrate.

TABLE 3. Kinetic parameters of *T. neapolitana* GghA towards glucooligosaccharides and pNPG

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
Sugars^a			
Cellobiose	28.6	285.04	9.96
Cellotriose	4.55	345.71	75.98
Cellotetraose	2.15	333.68	155.2
pNP^b			
Hydrolysis (low [S] = 0 to 2 mM)	0.28	512.55	1,830.54
Transglycosylation (high [S] = 2 to 40 mM)	1.93	883.9	457.97

^a Activity determined by analysis of products of hydrolysis using HPLC (see text).

^b Activity determined by measuring the rate of release of pNP.

of >200 mM. The activation of GghA both at higher substrate concentrations (2 to 40 mM pNPG) and in the presence of end product glucose (0 to 200 mM) may be due to transglycosylation activity of GghA, where the substrate or end product can act as the preferential glycosyl acceptor compared to water. To test the possibility of simultaneous occurrence of hydrolysis and transglycosylation, GghA was reacted with 40 mM cellobiose and 16 mM cellotriose, and the products were fractionated by HPLC. Figure 3A and B show the production of cellotriose from cellobiose and cellotetraose from cellotriose, respectively. Moreover, cellopentaose was produced when GghA was reacted with 16 mM cellotetraose (data not shown). The stereochemistry of the newly formed chiral center was assayed using purified GghA enzyme under conditions in which the mutarotation is negligible (see Materials and Methods). The results show that GghA retains the anomeric configuration at the C-1 atom of the glucoside (Fig. 3C).

Purification of recombinant cellobiose phosphorylase (CbpA). Recombinant CbpA was purified by a three-step protocol, resulting in 17-fold purification. The specific activity of the purified enzyme is 11.8 U/mg towards cellobiose. SDS-PAGE analysis showed a homogeneous enzyme preparation with an apparent molecular mass of 93 kDa (Fig. 1B), which is in agreement with sequence-derived data.

Characterization of CbpA. CbpA showed optimal activity at 85°C over a pH range of 4.5 to 6.0. Its pI was 4.8. The thermostability of CbpA was affected by the presence or absence of substrate: when incubated with substrate at 85°C, CbpA remained active for 2 h; when incubated without substrate, there was little activity after 15 min. Without phosphate in the reaction mixture, CbpA failed to hydrolyze the substrate. CbpA shows activity only towards cellobiose; it was not active towards cellotriose or the disaccharides lactose, chitobiose, and xylobiose. The kinetic constants were determined for the phosphorylase of cellobiose by fitting data to the Michaelis-Menten equation using linear regression: the K_m for cellobiose was 1.42 mM, the k_{cat} was 26.3 s^{-1} , and k_{cat}/K_m was 18.3 $s^{-1} mM^{-1}$.

Cloning, sequencing, and analysis of the glucan catabolic cluster. Preliminary DNA sequencing and analysis were conducted on the inserts from eight clones identified in a prior screening protocol (7). Of these clones, p46B1 contained the partial sequence of the *gghA* gene and an incomplete upstream ORF with similarities to cellobiose phosphorylases. Together, the two genes constitute a cluster involved, in all likelihood, in the catabolism of glucooligosaccharides. To define this cluster, two cosmid clones, RUC 270 and RUC 278, with similar restriction maps were identified by screening the *T. neapolitana*

cosmid library. An insert within clone RUC 270 was subcloned to obtain the full-length *gghA* gene, and primer walking was used to completely sequence the cellobiose phosphorylase (*cbpA*) gene. *cbpA* and *gghA*, encoding 812 and 444 amino acid proteins, respectively, are separated by a 103-bp intergenic region (Fig. 4). The structural organization of the cluster consisting of *cbpA* and *gghA* was confirmed by Southern blotting. Further analysis of the sequence 3' to the *gghA* gene revealed the presence of a partial ORF encoding a putative α -amylase (*amyB*); and 5' to the *cbpA* gene, several short AT-rich sequence motifs are present, one of which has 95% identity over its 23-bp length (Fig. 4) to motifs present 5' of endoglucanase clusters from *T. maritima* and *T. neapolitana* (10, 25). Such regions may be involved in the regulation of glucan catabolic gene clusters.

Based on amino acid sequence comparisons, cellobiose and cellodextrin phosphorylases form a homologous group of proteins. Interestingly, CbpA is 84.7, 82.5, and 76.9% similar to the cellobiose phosphorylases of the cellulolytic organisms *C.*

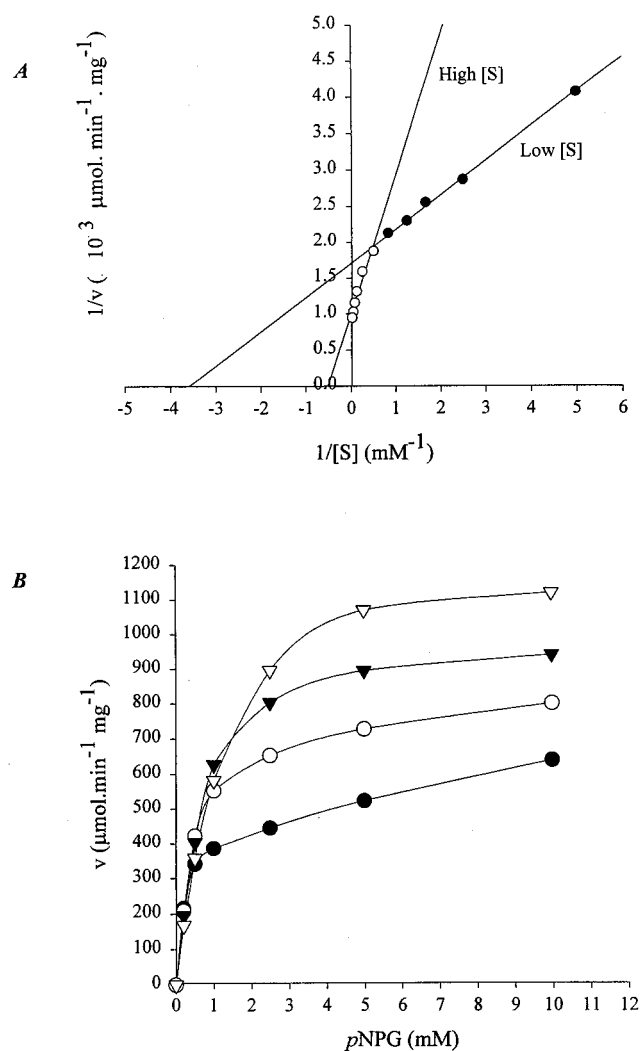


FIG. 2. Effect of substrate (pNPG) and end product (glucose) on activity of GghA. (A) Lineweaver-Burke plot of rate of pNP release as a function of pNPG concentration. (B) Effect of end product (glucose) on rate of pNP release from pNPG. ●, $v(U/mg)@[I] = 0$; ○, $v(U/mg)@[I] = 50$; ▼, $v(U/mg)@[I] = 100$; ▽, $v(U/mg)@[I] = 200$. $v(U/mg)@[I]$ = specific activity at various concentrations of inhibitor.

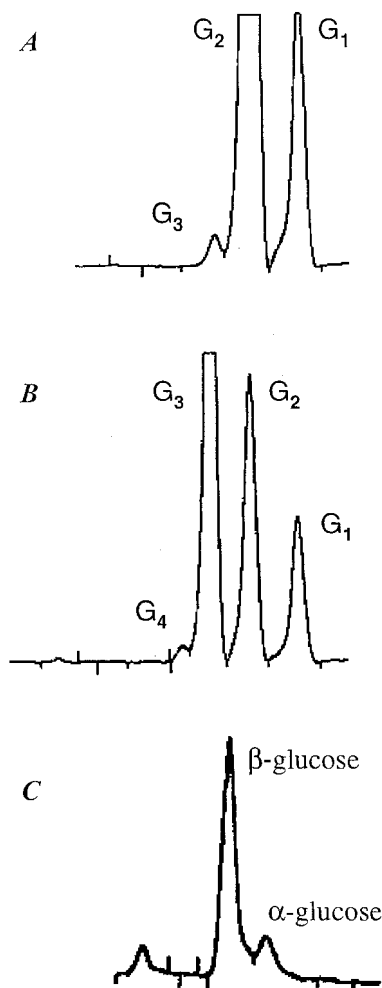


FIG. 3. HPLC analysis of products of hydrolysis and transglycosylation of GghA. (A) Production of cellotriose (G₃) and glucose (G₁) from cellobiose (G₂). (B) Production of cellotetraose (G₄), cellobiose (G₂), and glucose (G₁) from cellotriose (G₃). (C) Determination of the configuration at the newly formed anomeric center.

thermocellum (GenBank accession no. AB013109), *Clostridium stercorarium* (30), and *Cellvibrio gilvus* (26), respectively. It is also noted that the cellobiose phosphorylases, including *T. neapolitana* CbpA, have >50% similarity to the C-terminal domains of *R. meliloti* NdvB (21) and *B. abortus* Cgs proteins, which are responsible for β-(1,2) glucan synthetase activity (22). The similarity in primary sequence between glucan synthetic enzymes and phosphorolytic enzymes with significant transglycosylation activity indicates common biochemical mechanisms in these functionally diverse groups of enzymes.

The GghA protein belongs to family 1 of glycosyl hydrolases, containing mainly β-glucosidases, phospho-β-glucosidases, and phospho-β-galactosidases (16). Sequence alignment of *T. neapolitana* gghA and the *Bacillus polymyxa* β-glucosidase gene (*bglA*) (33), shown in Fig. 5, reveals 68% sequence similarity and 46% identity over their entire length. The crystal structure of *B. polymyxa* BglA has been determined, and those structural data have been used to delineate its secondary structural elements. Conserved active-site glutamates, E166 and E342, are located at the ends of β-sheets 7 and 13, respectively. Of particular interest are Gln-20, conserved in all members of

family 1, and Glu-405, conserved among glycosidases but not phosphoglycosidases, both of which are capable of forming bidentate hydrogen bonds and also loop C (amino acids 296 to 327), which is involved in substrate binding, including gluco-oligomers above G₂. The sequences of *B. polymyxa* BglA loop C and the homologous region in *T. neapolitana* GghA exhibit 52% similarity.

DISCUSSION

We report the purification, characterization, and cloning of a cellobiose phosphorylase and a β-glucan glucohydrolase cluster from *T. neapolitana*. Few cellobiose phosphorylases or glucan glucohydrolases have been defined at the biochemical and molecular level; this may be due in part to the lack of methods for direct screening and selection of these enzymes. For instance, characterization of an enzyme as a glucan glucohydrolase requires HPLC analysis of the products of hydrolysis of gluco-oligomers. Also, no direct colorimetric or fluorometric methods are available for analysis of cellobiose phosphorylase activity.

GghA was purified from *T. neapolitana* extracts using a multistep purification protocol, as *Thermotoga* spp. have the potential to produce a number of glycosyl hydrolases (7, 8, 10, 11, 23, 37). Purified GghA has broad substrate specificity, with similar specific activities towards the disaccharides lactose and cellobiose, reflecting the polyspecific nature of family 1 glycosyl hydrolases to which it belongs (15). GghA, like other family 1 enzymes, hydrolyzes various aryl-β-glycosides, but it also exhibits biphasic kinetic behavior towards pNPG: its activity increases at higher substrate concentrations (Fig. 2A). This apparent activation may be due to higher concentrations of substrate or the de novo end product or both. Our data show the involvement of both substrate-directed and end product-directed activation, suggesting that GghA's greater kinetic activity at higher substrate or end product concentrations is due to its transglycosylating capability. For example, the production of oligomers of $N + 1$ length by GghA ($N =$ length of substrate) is indicative of both substrate-induced and end product-activated (glucose concentrations up to 200 mM) transglycosylation (Fig. 3A and B). This effect is similar to that reported for a cytosolic β-glucosidase from guinea pig liver (14). Although similar end product activation of glucoside hydrolases has been reported for a β-glucosidase from a *Streptomyces* sp. (29), and a family 1 glycosyl hydrolase from *Microbispora bispora* (44), the biochemical basis was not defined. While transglycosylation clearly plays a role in activation of GghA, our data do not rule out other mechanisms of activation operating in concert with transglycosylation.

T. neapolitana GghA was identified initially as an aryl-β-glycosidase based on its activity towards pNPG, an activity it shares with other glucan glucohydrolases (17, 18, 28, 31). The basis for designating *T. neapolitana* GghA a glucan glucohydrolase (EC 3.2.1.74) instead of a β-glucosidase (EC 3.2.1.21) is its greater catalytic efficiency towards cellotetraose: the k_{cat}/K_m for GghA's hydrolysis of cellotetraose is 16-fold greater than that for cellobiose, while its overall substrate preference is cellotetraose > cellotriose > cellobiose. GghA's k_{cat} values for cellotetraose (333.6 s⁻¹) and cellotriose (345.7 s⁻¹) are comparable, indicating that the rate of formation of the covalent glycosyl-enzyme intermediate and subsequent release of glucose from the complex are essentially the same for both cello-oligomers. The K_m for cellotetraose, however, is half that of cellotriose, implying a better fit for the enzyme with the longer substrate. Indeed, it can be suggested that GghA has at least four binding subsites, all of which, in the case of cellotetraose,

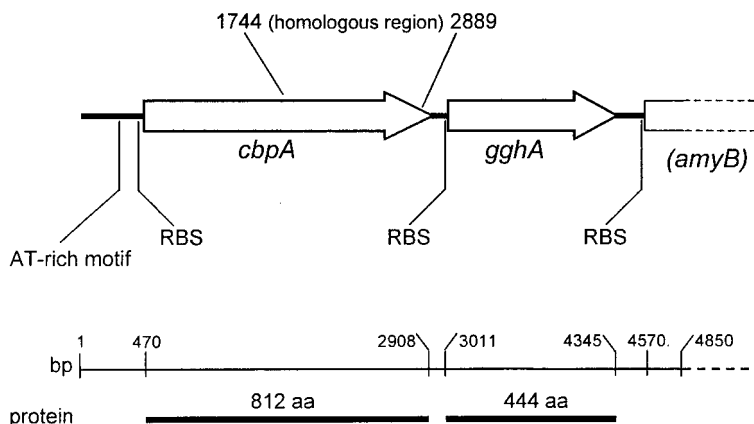


FIG. 4. Molecular organization of oligosaccharide catabolic cluster. Arrows indicate the direction of gene transcription. *cbpA*, cellobiose phosphorylase; *gghA*, glucan glucohydrolase; *amyB*, partial ORF of putative α -amylase; AT-rich region, conserved-sequence motif present upstream of other endoglucanase genes in *Thermotoga* spp.; RBS, ribosome-binding site; bases 1744 to 2889, domain common to *C. thermocellum* cellobiohydrolase (1, 38); aa, amino acids.

interact with the substrate and result in the strongest enzyme-substrate interaction.

Such tandem multisugar binding sites and a tendency towards greater catalytic efficiency (k_{cat}/K_m) with increasing degrees of substrate polymerization have been reported for a family 1 glucan glucohydrolase (BII) from barley (19), an exo-glucosidase from the archaeon *Sulfolobus solfataricus* (28), and a β -glucosidase (BglA) from *B. polymyxa* (33). Loop C in *B. polymyxa* BglA (amino acids 297 to 328) (Fig. 5), for example, has been specifically identified both with substrate binding and with providing additional subsites for the binding of larger glucooligomers. Given the high level of primary sequence conservation between *T. neapolitana* GghA and *B. polymyxa* BglA (68% sequence similarity and 46% identity over the entire

length), it is not surprising that GghA shows greater catalytic efficiency towards glucooligomers longer than cellobiose. In light of GghA's activity towards cellobiose and lactose, it is interesting that conserved amino acids Gln-20 and Glu-405, which enable the enzyme to form bidentate hydrogen bonds with oxygen atoms of glycosides, may account for the dual glycosidase and galactosidase activity of family 1 members, including GghA from *T. neapolitana* (33, 40).

From a bioenergetic perspective, cellobiose phosphorylases are interesting enzymes, for the energy of the β -1,4 glycosidic bond is conserved during phosphorolysis in the form of glucose 1-phosphate. One ATP molecule is needed to activate the other glycosyl product of the reaction. Glucose 1-phosphate is then converted to glucose 6-phosphate by phosphoglu-

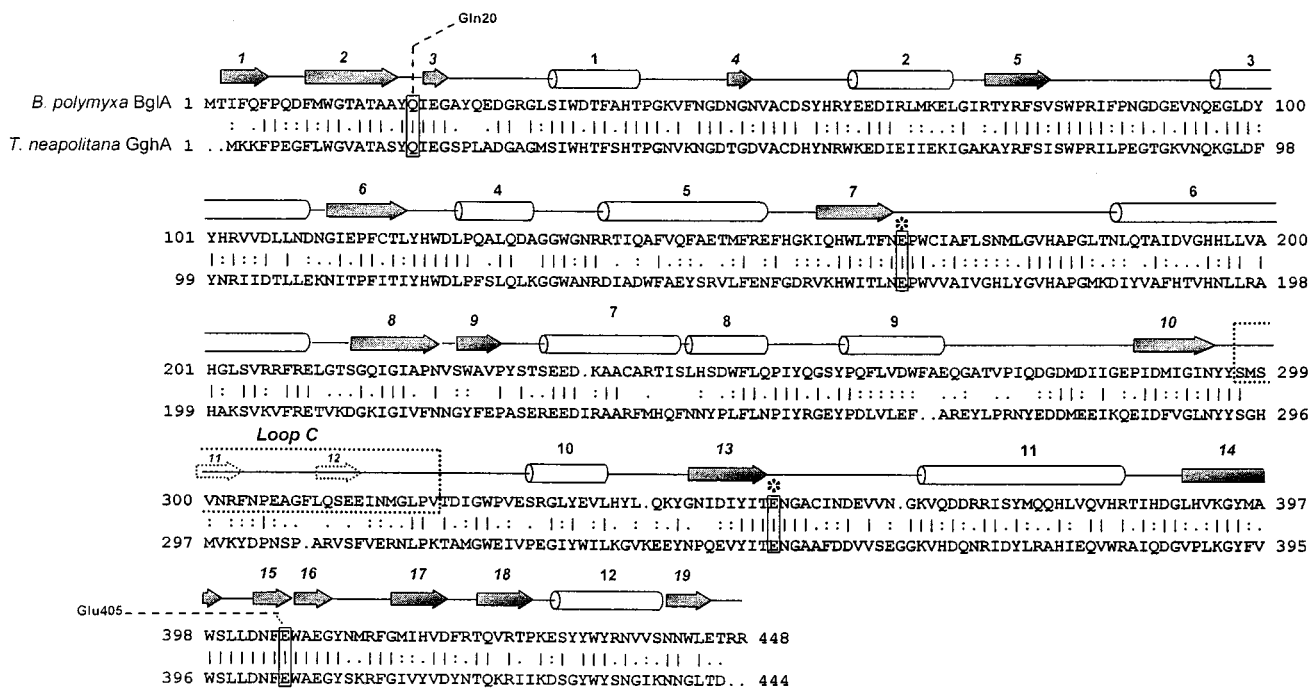


FIG. 5. Comparison of primary sequence of GghA with that of β -glucosidase from *B. polymyxa*. The secondary-structure elements were defined using the crystal structure of *B. polymyxa* β -glucosidase (33). Barrel, helix; arrow, β -strand; dashed arrow, secondary structure within loop C; *, conserved active-site amino acids; Gln20 and Glu405, amino acids capable of bidentate hydrogen bonding.

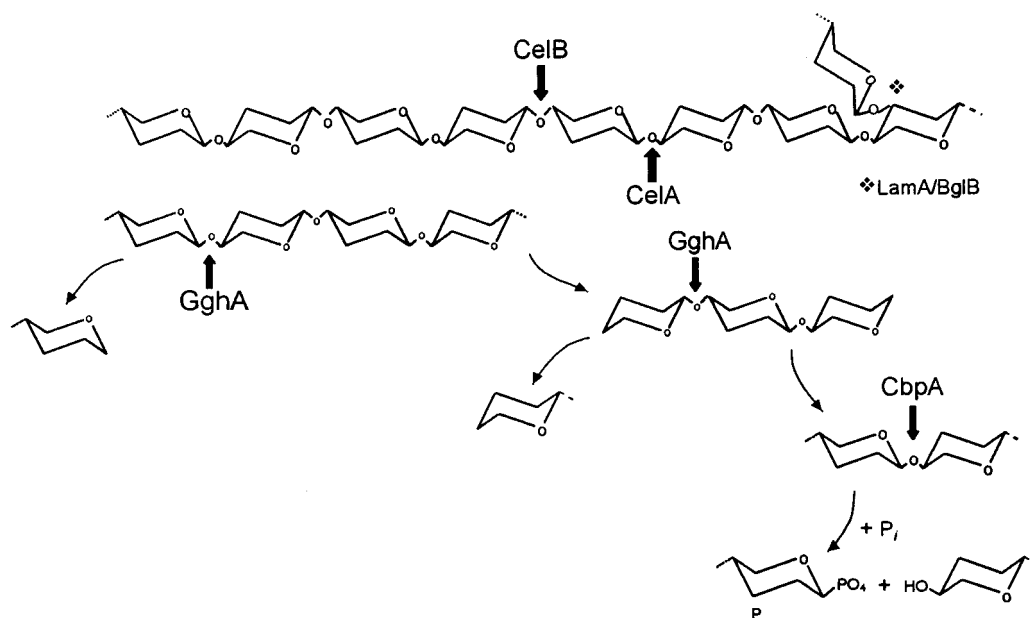


FIG. 6. Proposed glucon catabolic pathway in *T. neapolitana*. A schematic conversion of celooligomers to activated glucosyl molecules. Enzymes indicated are CelA and CelB, endoglucanases (7) active in or on the toga; GghA, glucon glucohydrolase, and CbpA, cellobiose phosphorylase, intracellular enzymes (this work); and LamA and BglB, laminarinase and β -glucosidase, respectively (45, 46).

comutase, a non-energy-consuming isomerization. Conversely, when a β -glucosidase hydrolyzes cellobiose, glycosidic bond energy is lost, and activation of the two glycosyl products requires two ATP molecules rather than one. To date, only cellobiose phosphorylases from *C. gilvus* (34), *C. thermocellum* (1, 38), and *C. stercorarium* (30) have been purified and characterized. The CbpA from *T. neapolitana* is the first report of a cellobiose phosphorylase from any hyperthermophile.

T. neapolitana and *T. maritima* are closely related species with minor differences in their physiological characteristics (20). A comparison of their glycosyl hydrolases, made possible by recent publication of the *T. maritima* genome (27), reveals that both gene sequences and the arrangement of catabolic gene clusters are highly conserved. Notably, both species apparently lack a gene encoding a cellobiohydrolase (27; D. Y. Yernool, G. Swiatek, and J.-D. Bok, unpublished data). The absence of the enzyme may explain the inability of *Thermotoga* spp. to hydrolyze crystalline substrates such as Avicel despite their capacity for hydrolysis of noncrystalline substrates such as acid-swollen cellulose and barley β -glucan. As cellobiohydrolases play a key role in classical cellulolysis and as sugar catabolism has been studied in detail in several hyperthermophilic genera, including *Thermotoga* (35), we propose a previously unreported catabolic pathway for glucon utilization. This non-conventional, ATP-conserving pathway consists of endoglucanases (CelA and CelB) (7), a β -glucon glucohydrolase (GghA), and a cellobiose phosphorylase (CbpA) (Fig. 6). Other potential components of this catabolic pathway might include a laminarinase (LamA) and a laminaribiase/ β -glucosidase (BglB), which act on both β -1,3 and β -1,4 linkages (45, 46).

Hydrolysis of internal glycosidic linkages of polymeric glucons by endoglucanases CelA and CelB begins the attack and results in the production of soluble glucooligomers. As CelB has an N-terminal signal peptide sequence, this initial processing probably occurs in or on the toga, the proteinaceous outer sack covering all *Thermotogales*. That neither CelB nor CelA is

secreted but is extracted from the pellet when purified from *T. neapolitana* grown on cellobiose (6) further suggests that initial activity involves the toga. The subcellular location of CelA, however, is not clear, as it lacks a secretory signal peptide sequence. The soluble products of the initial hydrolysis are then acted on by GghA, presumably an intracellular enzyme, which preferentially attacks the longer glucooligomers cellobiose to cellohexaose (the longer the glucooligomers, the lower their K_m s), releasing glucose from the nonreducing end and eventually producing the disaccharide intermediate cellobiose. At low concentrations of cellobiose (CbpA's K_m for cellobiose is 1.42 mM, compared to 28.6 mM for GghA), and given the enzyme's preference for a single substrate, CbpA, also presumably intracellular, then attacks cellobiose, producing the activated molecule glucose 1-phosphate and glucose.

Some cellobiose might be processed by other resident glycosyl hydrolases; however, the K_m of BglB (46) for cellobiose (50 mM) is 35-fold greater than that of CbpA, a strong indication that CbpA may be the primary enzyme for processing cellobiose in *T. neapolitana*. Further support for inclusion of CbpA in this pathway is the enzyme's energy-conserving mode of action: when cellobiose is converted to its glycosyl substituents by CbpA, one fewer ATP molecule is expended for every cellobiose molecule processed. For an organism with fermentative metabolism such as *T. neapolitana*, this represents a significant metabolic advantage. It is interesting that if cellobiohydrolase were present, the energy conserved would be greater, as there would be higher yields of cellobiose per molecule of glucooligosaccharide processed. Other important questions that bear on this issue include the type of glucons and polysaccharides present in the organism's ecological niche and what role, if any, other microbes play in complementing the degradative capability of *Thermotogales*. Finally, a number of genes encoding putative glycosyl hydrolases have been described in *T. maritima* (27). Functional analyses of these gene products and studies on regulation of their expression will undoubtedly further our understanding

of the process of acquisition of carbon and energy by *Thermotogales*.

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