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Molecular and Physiological Role of the Trehalose-Hydrolyzing α-Glucosidase from *Thermus thermophilus* HB27▽†

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Trehalose supports the growth of *Thermus thermophilus* strain HB27, but the absence of obvious genes for the hydrolysis of this disaccharide in the genome led us to search for enzymes for such a purpose. We expressed a putative α-glucosidase gene (TTC0107), characterized the recombinant enzyme, and found that the preferred substrate was α,α-1,1-trehalose, a new feature among α-glucosidases. The enzyme could also hydrolyze the disaccharides kojibiose and sucrose (α-1,2 linkage), nigerose and turanose (α-1,3), leucrose (α-1,5), isomaltose and palatinose (α-1,6), and maltose (α-1,4) to a lesser extent. Trehalose was not, however, a substrate for the highly homologous α-glucosidase from *T. thermophilus* strain GK24. The reciprocal replacement of a peptide containing eight amino acids in the α-glucosidases from strains HB27 (LGEHNLPP) and GK24 (EPTAYHTL) reduced the ability of the former to hydrolyze trehalose and provided trehalose-hydrolytic activity to the latter, showing that LGEHNLPP is necessary for trehalose recognition. Furthermore, disruption of the α-glucosidase gene significantly affected the growth of *T. thermophilus* HB27 in minimal medium supplemented with trehalose, isomaltose, sucrose, or palatinose, to a lesser extent with maltose, but not with cellobiose (not a substrate for the α-glucosidase), indicating that the α-glucosidase is important for the assimilation of those four disaccharides but that it is also implicated in maltose catabolism.

α-Glucosidases (EC 3.2.1.20) are a widespread group of enzymes that catalyze the hydrolysis of the α-glucosidic bond from the nonreducing end of a chain as well as the α-glucosidic bond of free disaccharides (21, 23). Many known α-glucosidases seem to prefer the α-1,4 bonds of maltose or maltooligosaccharides (21). However, the α-glucosidases from *Thermus thermophilus* strain GK24 and from *Bacillus* sp. strain SAM1606 preferentially hydrolyze the α-1,6 bond of isomaltose over other α linkages (20, 21, 23). Trehalose is a natural disaccharide that is widespread in nature and that can serve multiple roles, namely, as a general stress protectant, a source of carbon and energy, a sensing and regulatory compound, and a structural element of the bacterial cell wall (11). The assimilation of trehalose as a carbon and energy source requires the activity of enzymes that hydrolyze the α-1,1 bond of this disaccharide, including trehalase (EC 3.2.1.28), trehalose phosphorylase (EC 2.4.1.64 and EC 2.4.1.231), and other α-glucosidases (EC 3.2.1.20) with broad specificity (14, 16). These enzymes may also play important roles in the regulation of the level of trehalose in a cell. Trehalose can also be converted into maltose by trehalose synthase (TreS; EC 5.4.99.16) and then metabolized via amylomaltase, maltose/maltodextrin phosphorylase, or other α-glucosidases with α-1,4-linkage hydrolytic activity (7, 14, 18). Although *T. thermophilus* HB27 lacks the genetic machinery for trehalose biosynthesis, this disaccharide is taken up from the medium (2, 24) but does not serve as a compatible solute for strain HB27, unlike the majority of the strains of this species. In this organism, mannosylglycerate is the sole osmolyte under salt stress (2), indicating that the disaccharide is used only as a carbon source.

To understand the mechanisms involved in trehalose metabolism in *T. thermophilus* HB27, we attempted to identify the enzyme responsible for its hydrolysis. Unlike the HB8 and GK24 strains, the HB27 strain lacks a TreS gene and should not be able to convert trehalose into maltose. Additionally, no obvious trehalase or trehalose phosphorylase genes could be found in the HB27 genome (12). Hence, we hypothesized that gene TTC0107 could encode an enzyme required for trehalose utilization, although the highly homologous enzyme from strain GK24 was shown to lack trehalose-hydrolyzing activity (21). In fact, trehalose is the preferred substrate for the HB27 enzyme, which is, to our knowledge, the first report of an α-glucosidase with this property. To examine the importance of the affinity of a distinctive peptide containing eight amino acids from the HB27 α-glucosidase that is not found in the homologous GK24 enzyme for trehalose, we created site-specific mutant α-glucosidases from both strains. We also constructed an α-glucosidase disruption mutant to investigate the specific role of this enzyme in strain HB27 and studied the organism's adaptation to specific nutritional conditions.

MATERIALS AND METHODS

Strains and plasmids. *Thermus thermophilus* strains HB27 (DSM 7039) and HB8 (DSM 579) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, and *T. thermophilus* strain GK24 (27) was a gift from Richard Sharp of PHLS, Salisbury, United Kingdom. *Escherichia coli* DH5α and expression vector pTRC99A were used for the cloning and expression of α-glucosidase genes from *T. thermophilus* strains HB27 ($aglH_{HB27}$), GK24 ($aglH_{GK24}$), and HB8 ($aglH_{HB8}$). Plasmid pGEM-T Easy was used to accommodate the disrupted HB27 *aglH* gene and for the disruption of

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TABLE 1. Substrate specificity of recombinant α-glucosidases (AglH) from *Thermus thermophilus* strains HB27, HB8, and GK24

Substrate	Glycosidic linkage	Glucose released (μmol/min · mg protein)[a]		
		$AglH_{HB27}$	$AglH_{HB8}$	$AglH_{GK24}$
Disaccharides				
α,α-Trehalose	*O*-α-D-Glucosyl-(1→1)-D-glucose	126 ± 5.3	0.0	0.0
α,β-Trehalose	*O*-α-D-Glucosyl-(1→1)-β-D-glucose	49 ± 3.6	0.0	0.0
β,β-Trehalose	*O*-β-D-Glucosyl-(1→1)-β-D-glucose	0.0	0.0	0.0
Kojibiose	*O*-α-D-Glucosyl-(1→2)-D-glucose	26 ± 3.1	31 ± 3.3	38 ± 4.7
Sucrose	*O*-α-D-Glucosyl-(1→2)-β-D-fructose	10 ± 1.1	9 ± 4.1	67 ± 3.4
Nigerose	*O*-α-D-Glucosyl-(1→3)-D-glucose	20 ± 1.1	36 ± 8.1	56 ± 2.2
Turanose	*O*-α-D-Glucosyl-(1→3)-D-fructose	18 ± 3.6	28 ± 3.5	3 ± 3.4
Maltose	*O*-α-D-Glucosyl-(1→4)-D-glucose	12 ± 0.2[b]	3.1 ± 0.2[b]	1.8 ± 0.1[b]
Cellobiose	*O*-β-D-Glucosyl-(1→4)-D-glucose	0.0	0.0	0.0
Leucrose	*O*-α-D-Glucosyl-(1→5)-D-fructose	9.3 ± 0.4	14 ± 2.1	14 ± 0.8
Isomaltose	*O*-α-D-Glucosyl-(1→6)-D-glucose	80 ± 3.6	108 ± 5.9	113 ± 7.7
Palatinose	*O*-α-D-Glucosyl-(1→6)-D-fructose	41 ± 1.6	45 ± 6.7	40 ± 1.4
Gentiobiose	*O*-β-D-Glucosyl-(1→6)-D-glucose	0.0	0.0	0.0
Melibiose	*O*-α-D-Galactosyl-(1→6)-D-glucose	0.0	0.0	0.0
Trisaccharides				
Melizitose	*O*-α-D-Glucosyl-(1→3)-β-D-fructosyl-(2→1)-D-glucose	0.0	0.0	0.0
Maltotriose	*O*-α-D-Glucosyl-(1→4)-α-D-glucosyl-(1→4)-D-glucose	18 ± 3.7[b]	8.3 ± 1.2[b]	5.8 ± 0.8[b]
Raffinose	*O*-α-D-Galactosyl-(1→6)-α-D-glucosyl-(1→2)-β-D-fructose	0.0	0.0	0.0
Panose	*O*-α-D-Glucosyl-(1→6)-α-D-glucosyl-(1→4)-D-glucose	45 ± 0.2	45 ± 5.8	50 ± 2.7
Isomaltotriose	*O*-α-D-Glucosyl(1→6)-α-D-glucosyl-(1→6)-D-glucose	38 ± 4.5	49 ± 8.2	5 ± 4.5

[a] Activity was determined with 0.5 μg of pure enzymes and 10 mM of each substrate at 70°C for 20 min, except where indicated otherwise.
[b] Activity was determined with 4 μg of pure enzymes and 100 mM of each substrate at 70°C for 1 h.

this gene in the strain HB27 chromosome as described below. Plasmid pMK18 was used as the source for the kanamycin (Kan) resistance cassette (9).

Amplification and cloning of the α-glucosidase genes. All PCRs were performed with the GC-Rich PCR system kit (Roche) according to the manufacturer's instructions. DNA from strains HB27, GK24, and HB8 was isolated and used as a template for the amplification of α-glucosidase genes (22). The primer sequences are listed in Table S1 in the supplemental material. Primers AG1 and AG2 were designed with additional EcoRI and HindIII sites, respectively, and used for the amplification of the HB27 *aglH* gene (TTC0107). Primer AG3 was designed based on the 5′ end of the α-glucosidase gene from strain GK24 ($aglH_{GK24}$) (GenBank accession number AF096282) with an EcoRI site and used with primer AG2 for the amplification of the $aglH_{GK24}$ and $aglH_{HB8}$ genes. The products were cloned into pTrc99A and sequenced (AGOWA GmbH, Berlin, Germany).

Expression and purification of recombinant enzymes. The constructs were transformed into *E. coli* DH5α, and the recombinant bacteria were grown and induced as previously described (26). Cells were harvested and suspended in 25 mM Bis-Tris propane buffer (BTP), pH 7.5, containing a protease inhibitor cocktail (Roche), DNase I (1 μg ml^{-1}), and 10 mM $MgCl_2$, followed by disruption on a French press. Cell debris was removed, and cell extracts were heat denatured to remove the majority of the host proteins.

Recombinant α-glucosidases were purified with two sequential Q-Sepharose columns (Hi-Load 16/10 Q FF) equilibrated with 25 mM BTP, pH 7.5, at a constant flow rate of 3 ml/min. Elution was carried out with linear NaCl gradients (0.0 to 1.0 M), and activity was determined as described below. Active fractions were concentrated and loaded onto a Superdex 200 column equilibrated with 50 mM BTP and 0.2 M NaCl, pH 7.5, at a constant flow rate of 1 ml/min. The purest fractions were pooled, concentrated, equilibrated with 25 mM BTP (pH 7.5), and stored at −20°C. The molecular mass of the HB27 recombinant α-glucosidase was estimated by gel filtration as previously described (8).

Enzyme assays and substrate specificity. The activities of the recombinant α-glucosidases were determined by measuring the release of glucose using the glucose oxidase assay kit (Sigma), according to the manufacturer's instructions. Standard reaction conditions were 25 mM of BTP at pH 7 with 20 mM $CaCl_2$ and incubation at 70°C. Mixtures containing 10 mM of substrate were incubated with 0.5 μg of pure recombinant α-glucosidase for 20 min, unless stated otherwise. Alternative reaction conditions used for the determination of activities with maltose and maltotriose were 25 mM of BTP at pH 7 with 20 mM $CaCl_2$, 100 mM of each substrate, and 4 μg of pure enzyme incubated for 1 h at 70°C. Several di-, tri-, and polysaccharides—namely, starch, amylopectin, glycogen, and pullulan—were tested as possible substrates (Table 1). Trehalose-6-phosphate was also tested as a possible substrate for the HB27 α-glucosidase ($AglH_{HB27}$). The reverse reaction was tested with different combinations of 5 mM (each) ADP-glucose, GDP-glucose, UDP-glucose, glucose, glucose-6-phosphate, or glucose-1,6-bisphosphate. To test transglucosylation, standard reaction mixtures containing trehalose, isomaltose, maltose, or glucose (5 to 500 mM) were incubated with 2 μg of pure recombinant $AglH_{HB27}$ at 90°C for 24 h and cooled on ice. Products were visualized by thin-layer chromatography as previously described (5).

Characterization of the HB27 recombinant α-glucosidase. The effect of pH on enzyme activity was tested with 25 mM of each buffer, i.e., acetate (pH 4.0 to 5.5), MES (morpholineethanesulfonic acid; pH 5.5 to 6.5), BTP (pH 6.0 to 9.5), Tris-HCl (7.0 to 9.0), and TAPS [*N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; pH 8.0 to 9.0], at 70°C and calculated as previously described (8). The standard mixtures containing trehalose were preheated for 2 min; the reaction was initiated by the addition of enzyme and stopped at different times by cooling on ethanol-ice. Prior to glucose quantification, the enzyme was inactivated by the addition of 0.5 M HCl (required for irreversible inhibition), incubated at room temperature for 20 min, and neutralized with 0.5 M NaOH. The temperature profile was determined by the incubation of reaction mixtures with trehalose at different temperatures (30 to 100°C). The thermal inactivation was determined by incubating α-glucosidase aliquots (30 μl of a solution of 0.5 mg ml^{-1} in 25 mM BTP buffer at pH 7.0) at 70, 80, and 90°C. Aliquots were withdrawn at appropriate times (up to 48 h) and examined for residual activity under standard conditions. The effect of the cations Ca^{2+}, Fe^{2+}, Zn^{2+}, Sr^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, and Ba^{2+} was tested using 25 mM BTP buffer, pH 7, containing cations at concentrations of 2 or 20 mM or no cations. The effect of Ca^{2+} was also determined at 10, 30, 40, or 80 mM. The effects of NaCl (20, 50, 100, and 500 mM) and KCl (50, 100, 500, and 1,000 mM) were also tested. To investigate the possibility of a phosphorylase reaction, the α-glucosidase activity was tested in the presence of 1 to 10 mM of ATP, ADP, and AMP or 10 mM phosphate buffer.

Kinetic parameters. The kinetic parameters for the recombinant α-glucosidases from strains HB27 and GK24 and for the mutant enzymes created as described below were determined under standard reaction conditions, with trehalose or isomaltose (0.25 to 50 mM) as the substrate. Reactions were initiated by the addition of 0.5 μg of the enzyme and stopped at different times (up to 6 min) by cooling on ethanol-ice. The kinetic parameters for the recombinant α-glucosidases with maltose as the substrate were calculated with maltose concentrations ranging from 5 to 250 mM, 4 μg of enzyme, and incubation times up to 1.5 h. The protein content of each sample was determined (6). All experiments were performed in triplicate.

Site-directed mutagenesis and expression of mutant α-glucosidases. The replacement of the $AglH_{HB27}$ peptide (LGEHNLPP) with the $AglH_{GK24}$ peptide (EPTAYHTL) was carried out by two rounds of PCR. First, we separately amplified a 1,176-bp fragment with primers AG1 and SV24Rv and a 435-bp fragment with primers SV24Fw and AG2 (see Table S1 in the supplemental material), using the DNA from strain HB27 as a template. Primer SV24Fw was designed to introduce the sequence coding for EPTAYHTL (Fig. 1), and primer SV24Rv was designed to introduce the complementary sequence for EPTAY HTL. The PCR products (1,176 bp and 435 bp), which had 24 overlapping bp (corresponding to the target EPTAYHTL sequence), were purified and used as templates in a second PCR with additional AG1 and AG2 primers. The identity of the final 1,587-bp product coding for a mutant HB27 α-glucosidase, where the LGEHNLPP peptide was replaced with the EPTAYHTL peptide, was confirmed by sequencing (AGOWA GmbH, Berlin, Germany). The substitution of the $AglH_{GK24}$ peptide EPTAYHTL with the peptide LGEHNLPP of $AglH_{HB27}$ was achieved by a similar strategy. Mutant α-glucosidase genes were expressed in *E. coli* DH5α, and the recombinant enzymes were purified as described above for the unmodified enzymes.

Construction of *T. thermophilus* HB27 α-glucosidase disruption mutant. $aglH_{HB27}$ was cloned into pGEM-T Easy to obtain pGEM-*aglH*. A 1,217-bp fragment containing the kanamycin resistance cassette was removed from pMK18 by digestion with BamHI and subcloned into the BglII site of pGEM-*aglH* to disrupt the α-glucosidase gene (pGEM-*aglH*-Kan). The orientation of the Kan insert was confirmed by restriction analysis as previously described (1). *Escherichia coli* DH5α carrying pGEM-*aglH*-Kan was grown overnight at 37°C in medium containing 30 μg ml^{-1} of kanamycin, and plasmids were isolated and sequenced (AGOWA GmbH, Berlin, Germany). Pure pGEM-*aglH*-Kan was used to transform strain HB27 and to disrupt the α-glucosidase gene as previously described (1, 9). The mutant's DNA was isolated, and successful disruption was confirmed by PCR with the following primer combinations: AG1 plus AG2; kan1 plus kan2, both designed based on the Kan cassette of pMK18; 27vegFw plus kan2; and kan1 plus 27mtRv (see Table S1 in the supplemental material and Fig. 2). Primer 27vegFw was designed based on the putative ATP pyrophosphatase gene adjacent to the α-glucosidase gene on the HB27 genome (12), and primer 27mtRv was designed based on the 3′ end of the putative methyltransferase gene located immediately downstream of the α-glucosidase gene (Fig. 2).

Growth of α-glucosidase mutant with single carbon sources. The wild-type HB27 strain and the *aglH*::Kan α-glucosidase disruption mutant were grown in complex *Thermus* medium or in a minimal medium (MM) containing 1.0 g $liter^{-1}$ NH_4Cl, 0.01 g $liter^{-1}$ of yeast extract, and a vitamin solution (25). Filter-sterilized glutamate, proline, glucose, cellobiose, trehalose, isomaltose, palatinose, sucrose, or maltose (1.0 g $liter^{-1}$) was independently added to the medium. The *aglH*::Kan mutant was grown with and without kanamycin (30 μg ml^{-1}). Cultures were grown at 65°C as previously described (25).

RESULTS

Sequence analysis, genome environment, and functional expression of *Thermus thermophilus* α-glucosidases in *E. coli*. The 1,587-bp gene (TTC0107) from strain HB27, coding for a polypeptide with 528 amino acids with a calculated molecular mass of 61.8 kDa, was annotated as a putative α-glucosidase/glycoside hydrolase (12). This protein exhibits high sequence homology (90% amino acid identity) with the putative oligo-1,6-glucosidase from *T. thermophilus* HB8 (GenPept accession number YP143747), the α-glucosidase from *T. thermophilus* GK24 (GenBank accession number AF096282), and the oligo-1,6-glucosidase from "*Bacillus flavocaldarius*" (EMBL accession number BAB18518). Lower sequence identity (43%) was detected with an α-glucosidase from *Bacillus* sp. strain SAM1606 (EMBL accession number CAA54266). Amplification of the α-glucosidase genes (*aglH*) from strains HB27, GK24, and HB8 yielded products with the expected gene sizes and sequences, and their expression in *E. coli* resulted in high yields of protein, which were purified to homogeneity (data not shown).

We identified in the *T. thermophilus* α-glucosidases the seven conserved regions (CRs) and five invariable residues common to family 13 glycoside hydrolases (GH13) (http://www.cazy.org/fam/GH13.html) that belong to the α-amylase superfamily (19). Based on a comparison with the sequences from homologous enzymes, the three catalytic residues in the α-glucosidase of strain HB27 ($AglH_{HB27}$) are Asp197, Glu264, and Asp326, and the substrate-binding histidines are His100 and His325 (Fig. 1) (17, 19, 28).

In the region immediately upstream from the α-glucosidase genes of strains GK24 and HB8, we detected an incomplete operon-like structure containing genes implicated in the synthesis of trehalose, namely, a partial *otsA* gene and complete *otsB* and *treS* genes (2). This structure is absent from the HB27 genome, and no trehalose biosynthetic genes were found (2, 12). All genes surrounding the α-glucosidase gene, with the exception of the trehalose cluster that is absent from the HB27 chromosome, are similar in strains HB27 and HB8 (Fig. 2A). The regions downstream of *treS* and downstream of α-glucosidase in GK24 have not been identified.

Substrate specificity of the α-glucosidases from strains HB27, HB8, and GK24. $AglH_{HB27}$ hydrolyzed the glucosidic bonds, with declining relative activity, of the disaccharides α,α-trehalose (100%), isomaltose (63.5%), α,β-trehalose (39.3%), palatinose (32.8%), kojibiose (20.7%), nigerose (16.3%), turanose (14.3%), sucrose (8.3%), and leucrose (7.4%) and of the trisaccharides panose (35.6%) and isomaltotriose (30.1%). Maltose and maltotriose were poor substrates for this enzyme (Table 1). Trehalose-6-phosphate was not a substrate. $AglH_{HB27}$ could also catalyze transglucosylation reactions only in the presence of higher levels of trehalose or isomaltose and with incubation times up to 24 h. Glucose and oligosaccharides with different molecular weights (not identified in this study) were detected by thin-layer chromatography (data not shown). Our results indicate that the recombinant enzyme exists as a dimeric protein with a molecular mass of 132 ± 5.3 kDa.

The preferred substrate of the α-glucosidases from strains GK24 and HB8 was isomaltose. Both enzymes could, however, also hydrolyze sucrose, nigerose, palatinose, kojibiose, turanose, leucrose, and the trisaccharides panose and isomaltotriose. The disaccharides α,α-trehalose or α,β-trehalose were not hydrolyzed by these enzymes under any conditions tested (Table 1). The α-glucosidases from the GK24 and HB8 strains could also hydrolyze maltose and maltotriose but only at higher substrate and enzyme concentrations as described above (Table 1). None of the enzymes were able to hydrolyze the disaccharides β,β-trehalose, cellobiose, gentiobiose, and melibiose or the trisaccharides raffinose and melizitose. None of the polysaccharides tested, namely, starch, amylopectin, glycogen, and pullulan, served as substrates for the enzymes.

Properties of the recombinant α-glucosidase from strain HB27. The α-glucosidase from strain HB27 had maximal activity at 90°C and about 50% of maximal activity at 70°C but was inactive below 30°C. The enzyme also retained 44% of maximal activity at 100°C. The optimum pH was 6.2, and 80% of maximal activity was retained between pH 5.2 and 6.8. The enzyme had a half-life of 44 ± 9.8 h (mean ± standard deviation) at 70°C (inactivation constant [K_d] = 0.0237 ± 0.0059 min^{-1}) and 23 ± 3.4 h at 80°C (K_d = 0.0431 ± 0.0073 min^{-1}). At 90°C, the residual activity progressively decreased during the initial 2 h, but after this period, it remained constant, with about 35% of maximum activity for 48 h. The enzyme was

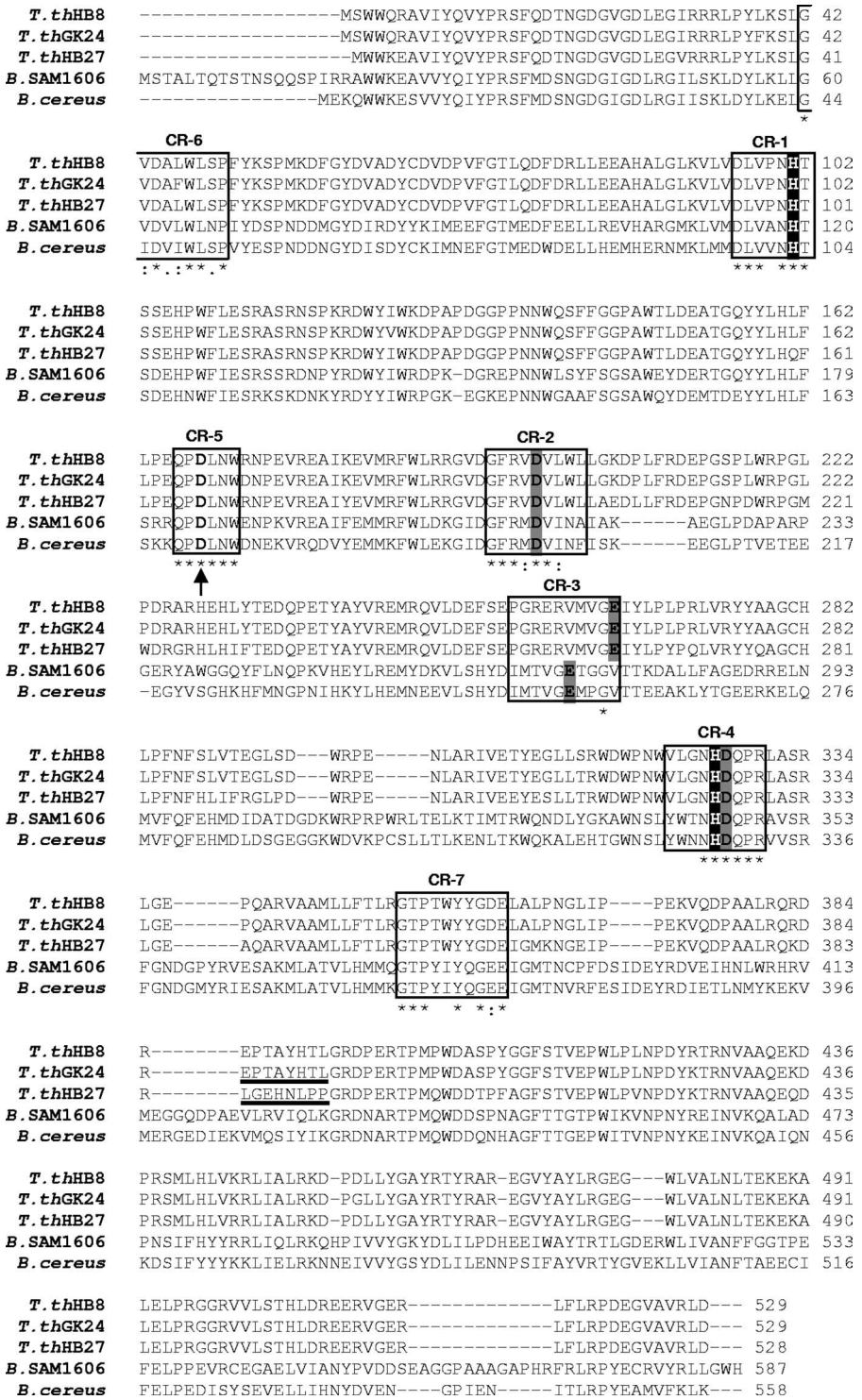

FIG. 1. ClustalX alignment (29) of α-glucosidases from *T. thermophilus* strains HB27, HB8, and GK24 with homologous proteins from *Bacillus* species. The boxes represent the seven CRs of the α-amylase family. The boldface amino acids indicate putative catalytic and substrate-binding residues, and the underlined portions indicate an eight-amino-acid sequence, which is the target sequence for mutagenesis, that is identical in the α-glucosidases of strains GK24 and HB8 but different in the HB27 α-glucosidase. Gray shading, putative catalytic aspartates and glutamate; black shading, putative functional histidines (substrate-binding sites); arrow, Ca^{2+}-binding aspartate; star, conserved identical amino acids; colon, conservative substitutions; period, semiconservative substitutions; *T. th*HB27, *T. thermophilus* HB27 (NCBI accession number YP_004082); *T. th*HB8, *T. thermophilus* HB8 (NCBI accession number YP_143747); *T. th*GK24, *T. thermophilus* GK24 (EMBL accession number AAD50603); *B.* SAM1606, *Bacillus* sp. strain SAM1606 (EMBL accession number CAA54266); *B. cereus*, *Bacillus cereus* ATCC 7064 (EMBL accession number CAA37583).

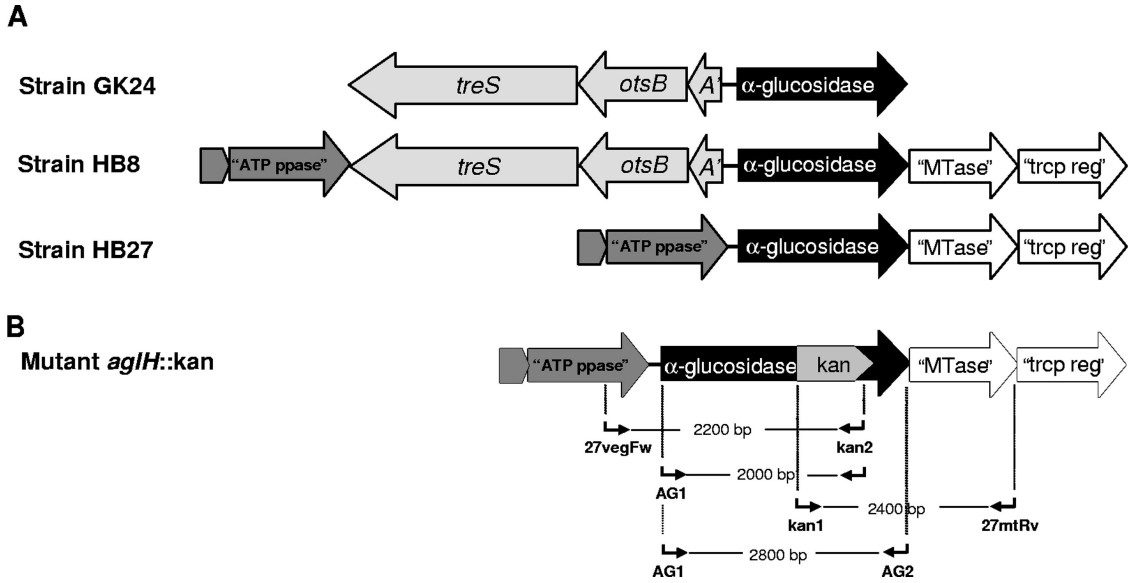

FIG. 2. (A) Schematic representation of the α-glucosidase gene environment in *T. thermophilus* strains HB7 and HB8 (http://www.genome.ad.jp/kegg/catalog/org_list.html) and in GK24 (2); (B) strain HB27 α-glucosidase gene disruption with the kanamycin resistance cassette and representation of primers 27vegFw/kan2, AG1/kan2, AG1/AG2, and kan1/27mtRv used to confirm the expected mutation. *treS*, trehalose synthase gene; *otsB*, trehalose-6-phosphate phosphatase gene; *A′*, partial trehalose-phosphate synthase; "ATP ppase," putative ATP pyrophosphatase gene; "MTase," putative methyltransferase gene; "trcp reg," putative transcriptional regulator gene.

completely inactivated after incubation at 100°C for 30 min. Cations were not required for enzyme activity, but 20 mM Ca^{2+} (153%) followed by 20 mM Sr^{2+} (123%) and Mn^{2+} (114%) stimulated activity. Mg^{2+} did not affect the activity of the enzyme, but Fe^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, and Ba^{2+} were inhibitory. Increasing NaCl and KCl concentrations up to 0.5 M and 1 M, respectively, gradually inhibited the enzyme activity to about 50% (data not shown). To examine trehalose phosphorylase activity, we also determined the activity of the enzyme using trehalose and ATP, ADP, AMP, or phosphate as the substrates, with negative results (data not shown).

Kinetic studies of wild-type and mutant α-glucosidases. Lineweaver-Burk plots allowed the calculation of apparent K_m values for the substrates trehalose, isomaltose, and maltose as well as the rate constants (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) of each of the wild-type and mutant α-glucosidases with each substrate (Table 2). Our data confirm previous results reporting that trehalose was not a substrate for $AglH_{GK24}$ and that isomaltose was the preferred substrate (21). However, we now show that $AglH_{GK24}$ is also able to hydrolyze maltose at a low rate (Tables 1 and 2).

Amino acid substitution comprised residues 385 to 392 (LG EHNLPP) in $AglH_{HB27}$ and residues 386 to 393 (EPTAY HTL) in $AglH_{GK24}$ (Fig. 1). Upon the replacement of the HB27 α-glucosidase peptide by the GK24 peptide, the affinity of the HB27 enzyme for trehalose was significantly affected (the K_m increased from 4.9 mM to 17 mM and the k_{cat} decreased 7-fold), resulting in a 25-fold decrease in the catalytic efficiency (Table 2). The K_m values for isomaltose and maltose between the native enzyme and the mutant $AglH_{HB27}$ were not significantly altered, although isomaltose replaced trehalose as the preferred substrate of this mutant enzyme (Table 2).

The replacement of the GK24 α-glucosidase peptide (EPT AYHTL) by the peptide LGEHNLPP from the HB27 enzyme

TABLE 2. Effect of the reciprocal replacement of specific peptides containing eight amino acids of the α-glucosidases from *T. thermophilus* HB27 and GK24 on kinetic parameters determined with trehalose, isomaltose, and maltose as substrates[a]

α-Glucosidase source	Trehalose				Isomaltose				Maltose			
	V_{max}	K_m	k_{cat}	k_{cat}/K_m	V_{max}	K_m	k_{cat}	k_{cat}/K_m	V_{max}	K_m	k_{cat}	k_{cat}/K_m
HB27												
Wild type	333 ± 24	4.9 ± 1.4	343 ± 39	71 ± 11	250 ± 15	8.7 ± 1.8	257 ± 20	31 ± 5.9	21 ± 0.9	50 ± 3.3	22 ± 1.8	0.44 ± 0.02
Mutant[b]	46 ± 2.5	17 ± 0.8	47 ± 4.5	2.8 ± 0.4	286 ± 19	8.5 ± 0.7	295 ± 20	35 ± 2.5	1.9 ± 0.4	55 ± 6.3	2.0 ± 0.6	0.04 ± 0.02
GK24												
Wild type	0	0	0	0	357 ± 42	5.2 ± 0.4	361 ± 44	68 ± 16	11 ± 0.6	69 ± 7.7	11 ± 0.9	0.17 ± 0.04
Mutant[c]	20 ± 0.1	6.9 ± 0.1	20 ± 1.3	2.9 ± 0.1	162 ± 8.0	4.1 ± 0.2	164 ± 12	39 ± 0.9	3.4 ± 0.1	72 ± 6.2	3.5 ± 0.1	0.05 ± 0.01

[a] V_{max} values expressed as μmol/min · mg protein, K_m values expressed as mM, k_{cat} values expressed as s^{-1}, and k_{cat}/K_m values expressed as $mM^{-1}s^{-1}$. All values are means ± standard deviations.
[b] HB27 α-glucosidase with the EPTAYHTL peptide of the GK24 enzyme (see Fig. 1).
[c] GK24 α-glucosidase with the LGEHNLPP peptide of the HB27 enzyme (see Fig. 1).

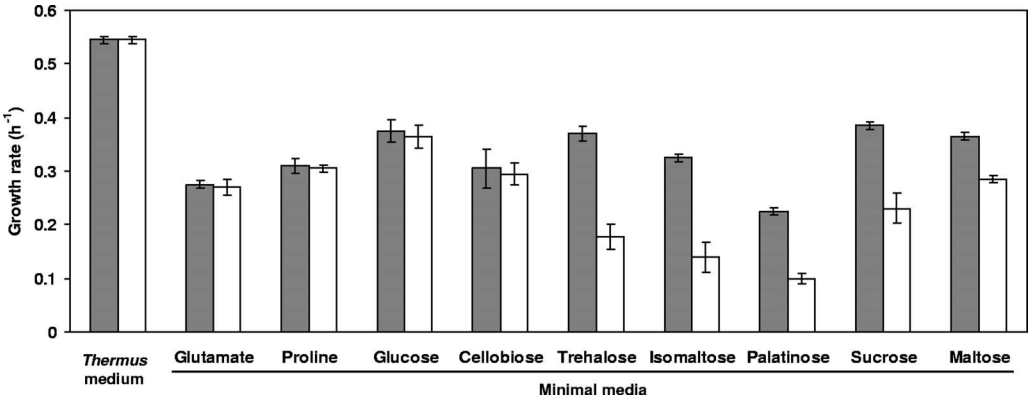

FIG. 3. Growth rates of the wild-type *T. thermophilus* strain HB27 (gray bars) and the *aglH*::Kan α-glucosidase mutant (white bars) in *Thermus* medium (complex) and in MM with glutamate, proline, glucose, cellobiose, trehalose, isomaltose, palatinose, sucrose, or maltose as the sole carbon source. Data are the mean values from three independent experiments.

had a marked effect on substrate utilization, since the mutant $AglH_{GK24}$ acquired the ability to hydrolyze trehalose (K_m = 6.9 mM and k_{cat} = 20 s^{-1}). The K_m values of isomaltose and maltose were not significantly affected for the mutant $AglH_{GK24}$; however, the rate decreased more than twofold in comparison with that of the GK24 native enzyme (Table 2). Nevertheless, isomaltose was still the preferred substrate for the mutant $AglH_{GK24}$.

Phenotypic analysis of the wild type and the HB27 α-glucosidase disruption mutant. Growth rates and final cell yields of wild-type HB27 and the *aglH*::Kan α-glucosidase disruption mutant were similar in complex *Thermus* medium and in MM with glutamate, proline, glucose, or cellobiose as sources of carbon and energy. However, the growth rate of this mutant in MM containing trehalose, isomaltose, sucrose, and palatinose was about 50% lower than the growth rate of the wild type, while the growth rate of the mutant in MM with maltose was about 20% lower than that of the wild-type organism (Fig. 3). Despite the lower growth rates with these substrates, the mutant was always able to reach the final cell yields of the wild type.

DISCUSSION

The α-glucosidase (AglH) from *T. thermophilus* strain HB27 is unique among α-glucosidases (EC 3.2.1.20), since it preferentially hydrolyzes the α-1,1-glucosidic linkage of trehalose, with lower activity for disaccharides containing α-1,6, α-1,2, α-1,3, and α-1,5 linkages, such as isomaltose, palatinose, kojibiose, sucrose, nigerose, turanose, and leucrose. Moreover, this enzyme possesses much lower activity for the α-1,4 bond of maltose. This broad substrate specificity is common among α-glucosidases, but trehalose has never been shown to be the preferred substrate and is not used at all by several of these enzymes (15, 19). Surprisingly, the HB27 α-glucosidase ($AglH_{HB27}$) shares 90% identical amino acids with the homologous enzymes from *T. thermophilus* strains HB8 and GK24 that are able to hydrolyze the α-1,2, α-1,3, α-1,5, and α-1,6 linkages of several disaccharides but cannot hydrolyze α-1,1-trehalose (21); the α-1,4 linkage of maltose was also hydrolyzed but at a lower rate. It has been suggested that enzymes with high amino acid identity but different substrate specificity might have "evolved," through a limited number of amino acid substitutions, to give rise to enzymes with extended substrate specificity (23). Several amino acids within the CRs of the *Bacillus* sp. strain SAM1606 α-glucosidase, which is able to hydrolyze trehalose, are different from those of the homologous enzyme from *Bacillus cereus*, which is not able to hydrolyze this disaccharide. Site-specific mutagenesis demonstrated the involvement of these residues in the affinity for trehalose and confirmed that slight differences within the CRs dictate significant differences in α-glucosidase substrate specificity (13, 21). However, the α-glucosidases from *T. thermophilus* strains HB27, GK24, and HB8 have identical CRs, implying that other residues outside the CRs must be involved in substrate affinity. Our results clearly show that a unique peptide with eight amino acids (LGEHNLPP) in $AglH_{HB27}$, located outside the CRs, was crucial for the specificity of the enzyme, since its substitution affected the preference for trehalose. The implication of this sequence in trehalose utilization was strongly supported by the acquisition of trehalose-hydrolyzing activity by the GK24 enzyme, when the specific EPTAYHTL sequence was replaced by the HB27 sequence (LGEHNLPP). Indeed, the peptide substitution significantly decreased the affinity of $AglH_{HB27}$ for trehalose and the k_{cat} value. The kinetic values of isomaltose and maltose hydrolysis were not significantly altered, indicating that the interaction with these substrates has not been affected by the substitution. The GK24 mutant enzyme acquired the ability to hydrolyze trehalose, and the K_m for this substrate was similar to that of $AglH_{HB27}$; however, the k_{cat} of the GK24 mutant enzyme was much lower, which resulted in a 25-fold decrease in the k_{cat}/K_m, indicating that trehalose hydrolysis was not as efficient with the GK24 mutant enzyme as with $AglH_{HB27}$ and that additional residues in the sequence were important for an enhanced catalytic rate.

The results obtained with the HB27 α-glucosidase mutant indicate that the α-glucosidase has an important role in the assimilation of trehalose, isomaltose, sucrose, and palatinose, since the growth rates of the α-glucosidase mutant were considerably affected when these disaccharides were the sole carbon sources. Surprisingly, the growth rate of the α-glucosidase mutant on maltose was also slightly affected, suggesting that

the enzyme has a role in maltose degradation, despite the low affinity for this substrate in vitro. Different mechanisms exist for maltose degradation, and the one operating in *T. thermophilus* HB27 is unclear. In *E. coli*, for example, maltose is taken up and metabolized by the combined action of 4-α-glucanotransferase (MalQ), maltodextrin phosphorylase (MalP), and maltodextrin glucosidase (MalZ), leading to glucose and glucose-1-phosphate that can be utilized in glycolysis (4). Many of the *mal* genes from *E. coli*, including the global regulator *malT* and those encoding MalQ, MalP, and MalZ, have homologues in the genome of strain HB27 (4, 12). In strain HB8, the 4-α-glucanotransferase (MalQ) (TTC0897 in HB27) had very low activity with maltose, the maltodextrin glucosidase (MalZ) (TTC1283 in HB27) has been found to hydrolyze sucrose and maltose, and the maltodextrin phosphorylase (MalP) (TTC0808 in HB27) hydrolyzed maltooligosaccharides and glycogen but not maltose (3, 17, 30). In *Bacillus* species, maltose can be taken up and hydrolyzed to glucose and glucose-1-phosphate by a maltose phosphorylase, which is absent from strain HB27 (14). The difference between the growth rates of the wild-type HB27 and the α-glucosidase mutant with maltose allowed us to conclude that AglH, as deduced from the enzyme assays, is not the primary hydrolase for maltose and oligosaccharides with α-1,4 bonds but that a minor role in their hydrolysis cannot be excluded. The hypothesis that trehalose, isomaltose, palatinose, and sucrose are, to a large extent, hydrolyzed by AglH in vivo is consistent with the decrease in the growth rate of the α-glucosidase mutant with these disaccharides. Nevertheless, the organism has other enzymes to degrade the disaccharides with enough efficiency to support a lower but steady growth rate for the mutant. We searched the genome of strain HB27 for the corresponding enzymes that could possibly hydrolyze disaccharides and found a hypothetical protein (TTC0614), with a conserved domain common to trehalases, which we experimentally confirmed to have very low activity toward trehalose and isomaltose but which was not further characterized. The HB27 genome also contains other genes coding for putative disaccharide-hydrolyzing enzymes, namely, a plasmid-borne α-glucosidase gene (TTP0221) and a putative pullulanase gene (TTC1198). However, the function of the corresponding enzymes in strain HB27 can be unequivocally established only after biochemical characterization. Cellobiose with a β-1,4 linkage is not a substrate for the α-glucosidase in vitro: the lack of involvement of the α-glucosidase in cellobiose assimilation in vivo was also confirmed by the similar growth behaviors of the wild type and the α-glucosidase mutant. Other studies indicate that this disaccharide is probably hydrolyzed by a plasmid-encoded β-glycosidase (TTP0042) (10).

Previous results have shown that among the *T. thermophilus* strains studied, HB27 was the only strain that did not accumulate trehalose under salt stress (2, 24). The present data suggest that the trehalose taken up cannot be accumulated in HB27 to serve as a compatible solute due to the trehalose-hydrolyzing ability of the α-glucosidase from this strain. On the other hand, strains HB8 and GK24 can grow with trehalose as a carbon and energy source and concomitantly accumulate this disaccharide under salt stress, possibly because the homologous α-glucosidases are unable to hydrolyze this disaccharide.

While the mechanisms involved in the regulation of the assimilation/accumulation of trehalose in *T. thermophilus* strains remain elusive, our data from comparative site-specific and disruption mutagenesis revealed unique determinants for the substrate specificity and the physiological role of the HB27 α-glucosidase, allowing us to propose a central role for this enzyme in the assimilation of trehalose and other disaccharides by this particular strain of *T. thermophilus*.

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