

4,6- α -Glucanotransferase activity occurs more widespread in *Lactobacillus* strains and constitutes a separate GH70 subfamily

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Abstract Family 70 glycoside hydrolase glucansucrase enzymes exclusively occur in lactic acid bacteria and synthesize a wide range of α -D-glucan (abbreviated as α -glucan) oligo- and polysaccharides. Of the 47 characterized GH70 enzymes, 46 use sucrose as glucose donor. A single GH70 enzyme was recently found to be inactive with sucrose and to utilize maltooligosaccharides [(1 \rightarrow 4)- α -D-glucopoligosaccharides] as glucose donor substrates for α -glucan synthesis, acting as a 4,6- α -glucanotransferase (4,6- α GT) enzyme. Here, we report the characterization of

two further GH70 4,6- α GT enzymes, i.e., from *Lactobacillus reuteri* strains DSM 20016 and ML1, which use maltooligosaccharides as glucose donor. Both enzymes cleave α 1 \rightarrow 4 glycosidic linkages and add the released glucose moieties one by one to the non-reducing end of growing linear α -glucan chains via α 1 \rightarrow 6 glycosidic linkages (α 1 \rightarrow 4 to α 1 \rightarrow 6 transfer activity). In this way, they convert pure maltooligosaccharide substrates into linear α -glucan product mixtures with about 50% α 1 \rightarrow 6 glycosidic bonds (isomalto/maltooligosaccharides). These new α -glucan products may provide an exciting type of carbohydrate for the food industry. The results show that 4,6- α GTs occur more widespread in family GH70 and can be considered as a GH70 subfamily. Sequence analysis allowed identification of amino acid residues in acceptor substrate binding subsites +1 and +2, differing between GH70 GTF and 4,6- α GT enzymes.

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Introduction

The glycoside hydrolase (GH) family 70 (Cantarel et al. 2009) is a group of extracellular bacterial glucansucrase (GTF) enzymes that synthesize α -D-glucan (abbreviated as α -glucan) polymers such as dextran, mutan, alternan, and reuteran (Côté and Robyt 1982; Monchois et al. 1999; van Hijum et al. 2006; Moulis et al. 2006; Schwab et al. 2007). These polymers form a protective layer around the cell (Kaditzky et al. 2008; Walter et al. 2008). In theory, four

types of α -glycosidic bonds can be formed between D-glucopyranose residues (at C-2, C-3, C-4, and C-6), and all four product specificities have been found among the different GH70 enzymes (Fabre et al. 2005; van Hijum et al. 2006). The GH70 enzymes from lactobacilli typically form products with two types of α -glycosidic linkages and synthesize branches, resulting in complex α -glucan structures. It was generally accepted that all GH70 enzymes use sucrose as glucose donor, but recently, we reported that *Lactobacillus reuteri* 121 encodes a GH70 enzyme, called 4,6- α -glucanotransferase (4,6- α GT-B), that uses maltooligosaccharides (α 1 \rightarrow 4-linked glucose residues), instead of sucrose, as glucose donor to synthesize linear isomalto/maltooligosaccharide product mixtures with up to 33% isomalto-sequences (α 1 \rightarrow 6-linked glucose residues) (Kralj et al. 2011; Dobruchowska et al. 2012). In addition, dextrin dextrinases (EC 2.4.1.2) form isomalto/maltooligosaccharides; however, these products contain branches (Naessens et al. 2005), whereas the 4,6- α GT-B products are linear (Dobruchowska et al. 2012). Although the physiological function of this 4,6- α GT-B enzyme is still unknown, it is interesting to note that this *L. reuteri* 121 also contains a standard type of GH70 enzyme (GTF-A), which synthesizes reuteran (a branched glucan with α 1 \rightarrow 4 and α 1 \rightarrow 6 linkages) from sucrose (Kralj et al. 2002; van Leeuwen et al. 2008a). The observation that the recombinant GTF-A enzyme makes an α -glucan polymer indistinguishable from the α -glucan polymer isolated from *L. reuteri* 121 supernatant implies that 4,6- α GT-B has no essential role in the synthesis of the extracellular α -glucan under the conditions tested, e.g., rich media with sucrose.

Today (November 2011), 135 GH70 sequences are reported in the CAZy database (Cantarel et al. 2009), of which 47 have been experimentally characterized (<http://www.cazy.org>). Three-dimensional structures have been reported for the N-terminally truncated GTF-180 enzyme of *L. reuteri* 180 (Vujičić-Žagar et al. 2010), which makes a branched glucan consisting of α 1 \rightarrow 6 and α 1 \rightarrow 3 glycosidic bonds (Kralj et al. 2004a; van Leeuwen et al. 2009), the N- and C-terminally truncated mutant [(1 \rightarrow 3)- α -glucan with about 10% α 1 \rightarrow 6 glycosidic bonds] synthesizer GTF-SI of *Streptococcus mutans* (Ito et al. 2011) and DSR-E of *Leuconostoc mesenteroides* NRRL B1299, which attaches glucose moieties on dextran via α 1 \rightarrow 2 glycosidic bonds (e.g., branches) (Brison et al. 2012). The catalytic domain of these enzymes has a (β / α)₈-fold, which is circularly permuted compared to the arrangement of the catalytic domain in the evolutionary related enzymes of the GH13 and GH77 families. Note that also the GH13 amylosucrase enzymes synthesize α -glucans from sucrose (Potocki de Montalk et al. 2000), though only linear (1 \rightarrow 4)- α -glucans, whereas all other GH13 enzymes either hydrolyze or disproportionate α -glycosidic bonds between two glucose

moieties (Stam et al. 2006; Kelly et al. 2009b). In the GH70 family, as well as in the GH13 and GH77 families, the catalytic nucleophile (Asp1025; numbered according to GTF-180), acid/base (Glu1063), and transition state stabilizer (D1136) are located at the C-terminal ends of the β -strands, as revealed by a sucrose bound structure of GTF-180 (Vujičić-Žagar et al. 2010). Despite the availability of crystal structures and the observation that mutations near the active site can alter the ratio of α -glycosidic bonds synthesized (Hellmuth et al. 2008), the reaction specificity of GH70 is not well understood, limiting the design of GH70 variants capable of synthesizing predefined α -glucan polymers.

Here, we describe the cloning, expression, and characterization of the second and third GH70 enzyme inactive with sucrose as donor substrate. The two GH70 genes of *L. reuteri* DSM20016 and *L. reuteri* ML1 encode enzymes that utilize maltooligosaccharides as substrate to synthesize linear α -glucans with α 1 \rightarrow 6-linked glucose segments at the non-reducing end of α 1 \rightarrow 4-linked glucose segments.

Materials and methods

Cloning of the *gtfW* gene

A truncated version of the 4,6- α GT-W encoding *gtfW* gene (GenBank accession number ABQ83597) was amplified from the genomic DNA of *L. reuteri* DSM 20016 using the primers For (*Nco*I) 5'-GAT GCA TCC ATG GGC ATA GAT GGT AAG AAC TAC CAC TTC GC-3' and Rev (*Bam*HI) 5'-ATA TCG ATG GAT CCT ATT AGT GAT GGT GAT GGT GAT GAA TAT TTT CTT GGT TTG CAT AGT AAT CTG C-3' and high fidelity DNA polymerase (Fermentas), and cloned in pET15b (Novagen), yielding pET15b_4,6- α GT-W. This N-terminally truncated version of 4,6- α GT-W (amino acids 458–1363) carries a fused (His)₆-tag at its C-terminal. The removal of the N-terminal variable region is based on the cloning and expression of other GTFs, as this improves protein expression without affecting the catalytic properties (Kralj et al. 2004b).

Cloning of the *gtfML4* gene

Previously, the 3'-end fragment of the 4,6- α GT-ML4 encoding *gtfML4* gene (GeneBank accession number AAU08003.1) has been identified in *L. reuteri* ML1, located upstream of a glucansucrase gene (*gtfML1*). The 5'-end of the *gtfML4* gene was obtained by inverse PCR. Briefly, genomic DNA of *L. reuteri* ML1 was digested by *Bcu*I or *Nsi*I, and the products were ligated at a concentration of ~10 ng/ μ l using T4 DNA ligase to obtain circular fragments. The ligation mixtures served as PCR template in an inverse PCR with the oligonucleotides HL115, 5'-TGA TCG TCC AGA TGT AGC-3' and

HL116, 5'-CCA GTTACT TTC ATA GAG G-3'. This yielded PCR fragments of 2 kbp (*BcuI*) and 8 kbp (*NsiI*), which were cloned in the pCR-XL-TOPO vector (Invitrogen). The obtained plasmids were used for DNA sequencing, yielding the 5'-end of the *gtfML4* gene.

Based on the DNA sequence obtained with the inverse PCR, the oligonucleotides HL117 (forward, *NcoI*), 5'-TTT TCC ATG GGG AAC CGC GTT GAT TAC TGG-3' and HL118 (reverse, *BglII*), 5'-AAA AGA TCT TAG TGA TGG TGA TGG TGA TGG TTG TTA AAA TTT AAT GAA ATT GC-3' were designed and used to amplify the gene from chromosomal DNA. The obtained fragment was cloned in pET15b (Novagen), yielding pET15b_4,6- α GT-ML4. This vector encodes an N-terminally truncated version of 4,6- α GT-ML4 (amino acids 714–1620) and carries a fused (His)₆-tag at its C terminus. Removal of the N-terminal variable region previously was shown to improve GTF protein expression without affecting catalytic properties (Kralj et al. 2004b).

Expression and purification of 4,6- α GT-W

The 4,6- α GT-W protein was produced in *Escherichia coli* BL21(DE3)/pET15b_4,6- α GT-W cultivated in Luria broth containing 100 mg/l ampicillin. Protein expression was induced at an OD₆₀₀ of 0.4–0.5 by adding isopropyl β -D-1-thiogalactopyranoside to 0.1 mM, and cultivation was continued for 4 h at 30°C. Cells were harvested by centrifugation, washed in phosphate buffer (50 mM, pH 8.0) and resuspended in phosphate buffer (50 mM, pH 8.0), containing NaCl (250 mM), imidazole (10 mM), and β -mercaptoethanol (5 mM). Cell-free extracts were made by sonication followed by centrifugation (10,000 \times g, 1 h). The 4,6- α GT-W protein was purified by nickel affinity chromatography using a 1-ml Hitrap IMAC HP column (GE Healthcare) and a gradient of 10–200 mM imidazole in phosphate buffer (50 mM, pH 8.0), containing NaCl (250 mM). Fractions containing 4,6- α GT-W were pooled. The imidazole and NaCl were removed using a 5-ml Hitrap desalting column (GE Healthcare) run with sodium phosphate buffer (50 mM, pH 8.0), and the protein was loaded on a 1-ml Resource-Q column (GE Healthcare). Protein was eluted with a gradient of 0–1 M NaCl in phosphate buffer (50 mM, pH 8.0). Fractions containing 4,6- α GT-W were transferred to sodium acetate buffer (50 mM, pH 5.5) using a 5-ml Hitrap desalting column. Protein purity was assessed on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration measured with Bradford reagent (BioRad) and bovine serum albumin as standard.

Expression and purification of 4,6- α GT-ML4

The 4,6- α GT-ML4 protein was produced in *E. coli* BL21 (DE3)/pET15b_4,6- α GT-ML4 cultivated in Luria broth

containing 100 mg/l ampicillin. Protein expression was induced at an OD₆₀₀ of 0.4–0.5 by adding isopropyl β -D-1-thiogalactopyranoside to 0.1 mM, and cultivation was continued for 4 h at 30°C or 24 h at 18°C. Cells were harvested by centrifugation, washed in Tris/HCl buffer (20 mM, pH 8.0), and resuspended in 10 ml BPer lysis reagent (Pierce), followed by centrifugation (10,000 \times g, 15 min). The 4,6- α GT-ML4 protein was purified from inclusion bodies by washing the pellet once with 5 ml BPer plus 2 mg lysozyme for 10 min at room temperature, followed by four washes with 50 ml 10 times diluted BPer. Finally, the inclusion bodies were resuspended in 10 ml 8 M urea, 20 mM Tris/HCl (pH 8.0), 1 mM CaCl₂, and 1 mM DTT to obtain unfolded protein. Refolding was done by dialysis against 20 mM Tris/HCl (pH 8.0), 1 mM CaCl₂, and 1 mM dithiothreitol. The 4,6- α GT-ML4 protein was then further purified by nickel affinity chromatography using a 1-ml Hitrap IMAC HP column (GE Healthcare). Following washing with 10 mM imidazole in 20 mM Tris/HCl (pH 8.0) with 1 mM CaCl₂, the protein was eluted with 200 mM imidazole in the same buffer. The imidazole was then removed using a 5-ml Hitrap desalting column (GE Healthcare) run with 20 mM Tris/HCl (pH 8.0) and 1 mM CaCl₂. Protein purity was assessed on 8% SDS-PAGE and protein concentration measured with Bradford reagent (BioRad) and bovine serum albumin as standard.

Enzyme assays

All reactions were performed in sodium acetate buffer (25 mM, pH 4.7) with CaCl₂ (1 mM) at 37°C, unless stated otherwise. The substrate spectra of the enzymes were investigated by incubating the various oligosaccharides (10 mg, 100 mM; Sigma-Aldrich) with 25 μ g/ml enzyme. The progress of the reactions was followed by high-pH anion-exchange chromatography (HPAEC) (described below) and/or thin-layer chromatography (TLC) using silica gel 60F254 plates (Merck) run with butanol/ethanol/water (5:3:3, v/v/v). TLC plates were developed with 10% H₂SO₄ in methanol/water (1:1, v/v), followed by heating at 100°C.

For 4,6- α GT-W activity, the optimal pH and temperature were determined over the pH range of 3.5–6.5 (in 25 mM sodium acetate buffer) and the temperature range of 20–60°C, using 100 mM maltose as substrate by following the release of glucose in time using the GOPOP kit (Megazyme) (Kaper et al. 2007). This assay could not be used with 4,6- α GT-ML4 as it is hardly active with maltose. The optimal pH and temperature of 4,6- α GT-ML4 were therefore determined using 50 mM maltotetraose as substrate and by following appearance of products on TLC plates.

The thermal inactivation rate of 4,6- α GT-W was determined by incubating the enzyme at a concentration of

0.26 mg/ml in sodium acetate buffer (50 mM, pH 5.5) with CaCl_2 (1 mM) at temperatures from 20°C to 55°C in a water bath for 10 min. Samples were then cooled in an ice bath, and residual activity was measured by following the release of glucose using maltose as substrate. The T_{50} is defined as the temperature at which 50% of the initial enzyme activity is retained after 10-min incubation.

Kinetic properties of 4,6- α GT-W were determined with maltose (0–500 mM) as substrate and by following the release of glucose. Reactions were initiated by the addition of 4,6- α GT-W enzyme at a concentration of 64 nM. The rates were fitted to the Michaelis–Menten equation.

Isolation of individual products made by 4,6- α GT-W via HPAEC

Twenty milligrams of maltose was incubated with 16 μg 4,6- α GT-W in 1.17 ml sodium acetate buffer (50 mM, pH 4.7) at 37°C for 4 days. The reaction mixture was freeze-dried, dissolved in 200 μl Milli-Q water, and 20- μl aliquots were loaded on a 9 \times 250 mm CarboPac PA-1 column (Dionex), which was run with a gradient of 0–600 mM sodium acetate in 100 mM NaOH (3 ml/min). Eluting peaks were collected manually and neutralized immediately with 4 M acetic acid. The collected fractions were desalted on 150 mg CarboGraph SPE columns (DiscoverySciences) using acetonitrile/water=1:3 (v/v) as eluent, followed by lyophilization. The desalted fractions were analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and ^1H nuclear magnetic resonance (NMR) spectroscopy. Fractions containing multiple oligosaccharides were separated again by HPAEC using a different elution gradient.

4,6- α GT-W and 4,6- α GT-ML4 product analysis with hydrolytic enzymes

Reaction products (250 μg) of the enzyme incubations with maltose up to maltoheptaose were dissolved in 10 μl sodium acetate buffer (50 mM) and incubated for 1 day at 37°C with α -amylase (porcine pancreas type I-A, pH 6.5; SigmaAldrich), amyloglucosidase (*Rhizopus*, pH 5.0; Megazyme), or pullulanase M1 (*Klebsiella planticola*, pH 5.0; Megazyme). α -Amylase cleaves α 1 \rightarrow 4 glycosidic bonds in longer α 1 \rightarrow 4 fragments, amyloglucosidase is an exo-acting enzyme that cleaves terminal α 1 \rightarrow 4 and α 1 \rightarrow 6 glycosidic bonds at the non-reducing end to form glucose, and pullulanase M1 cleaves α 1 \rightarrow 6 glycosidic bonds at the reducing end of an α 1 \rightarrow 4 glycosidic bond [in (-) α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp-(1 \rightarrow 6)- sequences] (Domań-Pytka and Bardowski 2004). The degree of degradation was assessed by TLC and/or HPAEC analysis.

High-pH anion-exchange chromatography

Carbohydrate samples were analyzed on a 4 \times 250 mm CarboPac PA-1 column using a Dionex DX500 workstation (Dionex), run with a gradient of 30–600 mM sodium acetate in 100 mM NaOH (1 ml/min), and detected with an ED40 pulsed amperometric detector. Calibration was done by running samples with known concentrations of glucose and maltotetraose.

Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry

MALDI-TOF-MS experiments were performed on an AximaTM mass spectrometer (Shimadzu), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 FWHM and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2%; the mirror voltage ratio was 1.12, and the acquisition mass range was 200–3,000 Da. Samples (1 μl) were mixed in 1:1 ratio with 10 mg/ml 2,5-dihydroxybenzoic acid in acetonitrile/water=1:1 (v/v). The compounds fly as sodium ion adduct.

Linkage analysis

Samples (~2 mg) were permethylated using CH_3I and solid NaOH in Me_2SO as described previously (Ciucanu and Kerek 1984). After hydrolysis with 2 M trifluoroacetic acid (2 h, 120°C), the partially methylated monosaccharides were reduced with NaBD_4 (2 h at room temperature, aqueous solution). Conventional work-up, comprising neutralization (by adding 4 M acetic acid) and removal of boric acid by co-evaporation with methanol, followed by acetylation with pyridine/acetic anhydride (1:1, v/v) (30 min, 120°C), yielded mixtures of partially methylated alditol acetates, which were analyzed by gas-liquid chromatography electron impact mass spectrometry.

NMR spectroscopy

Resolution-enhanced 1D 500-MHz ^1H NMR spectra were recorded in D_2O on a Varian Inova Spectrometer (NMR Center, University of Groningen) or a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at probe temperatures of 300 K. Prior to analysis, samples were exchanged twice in D_2O (99.9 atm% D, Cambridge Isotope Laboratories, Inc.) with intermediate lyophilization and then dissolved in 0.6 ml D_2O . Suppression of the HOD signal (on the Bruker DRX-500 spectrometer only) was achieved by applying a WEFT pulse sequence. Chemical shifts (δ)

are expressed in parts per million by reference to internal acetone (δ 2.225 for ^1H).

Sequence alignments

The amino acid sequences of GH70 proteins were compared using ClustalW2 run at EMBL-EBI (<http://www.ebi.ac.uk>) (Larkin et al. 2007; Goujon et al. 2010).

Results

Cloning of the *gtfW* and *gtfML4* genes

L. reuteri DSM 20016 possesses a *gtfW* gene (4092 bps; GeneBank accession ABQ83597), which encodes a 154-kDa protein, designated 4,6- α GT-W. Sequence comparison indicates that, in analogy with other GH70 enzymes, 4,6- α GT-W possesses a variable N-terminal region (amino acids 1–427), a catalytic domain [amino acids 428–1223; e.g., domains A, B, and C in the crystal structure of GTFs (Vujičić-Žagar et al. 2010; Ito et al. 2011)] and a C-terminal region (amino acids 1224–1363). The signal sequence predictor SignalP 3.0 does not predict an N-terminal signal sequence for 4,6- α GT-W. The enzyme shares 47% sequence identity with 4,6- α GT-B of *L. reuteri* 121; the similarity of domains A, B, and C is much higher with 70% sequence identity. A protein BLAST search identifies three hypothetical GH70 enzymes in the genomes of *L. reuteri* JCM 1112 (Morita et al. 2008), *L. reuteri* MM2-3 (UniProt accession C0YXW9) and *L. reuteri* MM4-1A (UniProt accession C2F8B9) that are virtually identical to 4,6- α GT-W, the only difference being that the three hypothetical proteins have an extra 125 amino acids at their N termini. The next hit in the BLAST search is a hypothetical GH70 protein (UniProt accession C0X0D3) from *Lactobacillus fermentum* ATCC 14931 with 71% sequence identity to 4,6- α GT-W.

Previously, it was shown that *L. reuteri* ML1 possesses a *gtfML1* gene, which encodes a glucansucrase and that the direct upstream DNA sequence shows similarity to GH70 genes (Kralj et al. 2004a), though a full gene sequence was not obtained. This gene fragment was designated as (part of) *gtfML4*, and the encoded protein fragment showed most sequence similarity to 4,6- α GT-B of *L. reuteri* 121. Therefore, we isolated the full *gtfML4* gene by inverse PCR. The gene is 4863 nucleotides and encodes a protein of 180 kDa, which has the typical arrangement of a GH70 family enzyme, with an N-terminal signal sequence of 39 amino acids as predicted by the SignalP 3.0 (Bendtsen et al. 2004), a large variable N-terminal region (amino acids 40–738), a catalytic domain (amino acids 739–1486), and a C-terminal region (amino acids 1487–1620). 4,6- α GT-ML4 shares 96%

sequence identity with GTF-106B of *L. reuteri* TMW1.106, which has been reported to slowly hydrolyze sucrose but does not synthesize a polymer from sucrose (Kaditzky et al. 2008) and 86% and 46% sequence identity with 4,6- α GT-B and 4,6- α GT-W, respectively.

Sequence alignments of the GH70 enzymes revealed differences in the amino acids forming the acceptor subsites +1/+2 between 4,6- α GTs and GTFs (Table 1). In addition, it showed that the loop connecting domains IV and B (residues 932–943 in GTF-180), containing the subsite +1 residue 938, is eight residues longer in 4,6- α GTs compared to GTFs (Fig. 1). Phylogenetic analysis of the GH70 protein sequences revealed that the 4,6- α GT proteins form a small but clearly separate cluster (Suppl. Info. Fig. S1).

Table 1 Comparison of the amino acid residues at the acceptor subsites +1 and +2 of GH70 enzymes

	GTFs	4,6- α GTs	Function in GTFs
Subsite +1			
938 ^a	D,L,I ^{b,c}	G	4,6- α GTs have a longer loop here
981	<i>L</i>	L	–
1028	<i>D</i>	D	–
1029	<i>N</i>	N	–
1065	<i>W,G</i>	Y	–
1140	<i>Q</i>	N	Glycosidic bond specificity ^d
Subsite +2			
978	Y,F,A,Q,S	P	Loop region with variation in length
1028	<i>D</i>	D	–
1065	<i>W,G</i>	Y	–
1088	R,H,K,T,S	F	–
1137	<i>S,N</i>	Q	Glycosidic bond specificity ^{d,e}
1141	T,D,S,A,G	L,V	Glycosidic bond specificity ^f

The information is based on the crystal structure of GTF-180 of *L. reuteri* 180 [protein databank files 3HZ3 and 3KLL (Vujičić-Žagar et al. 2010)], and the amino acids are numbered according to GTF-180. Eighty-eight GH70 sequences were used for the alignment, including the three 4,6- α GTs (B, W, and ML4) and the two putative 4,6- α GTs of *Lactobacillus reuteri* JCM 1112 (UniProt accession number B2G8K2) and *Lactobacillus reuteri* TMW1.106 (UniProt accession number A9Q0J0). The equivalent residues in the 4,6- α GTs are based on sequence alignments. Catalytic residues are excluded from the comparison as they are conserved in both GTFs and 4,6- α GTs

^a Amino acids are numbered according to GTF-180 of *L. reuteri* 180 of which the three dimensional structure is known (Vujičić-Žagar et al. 2010)

^b The amino acids are given in a descending order of occurrence

^c The residues in GTF-180 are indicated in italics

^d van Leeuwen et al. (2009)

^e Irague et al. (2011)

^f Shimamura et al. (1994)

GTF-A	929-SENVN-----FSGIQFQGGFLKYEN-948
GTF-ML1	930-SENEDE-----FGGLQLQGGFLKYVN-949
GTF-180	930-SENVDE-----YGGLQLQGGFLKYVN-949
GTF-O	929-TENVN-----YSGIQFQGGFLKYEN-948
GTF-SI	374-SEKPF-----DDHLQKGALLYSN-391
GTF-DSRE	429-SES-----KGNDDLQGGALLYVN-446
4,6- α GT-ML4	904-SELSLQSMPNYRPDESQTVDSQVIFVN-931
4,6- α GT-B	903-SELSLQSMPNYRPDKSGTIDSDQVIFVN-930
4,6- α GT-W	640-SELPVEYSNGYVPDVSGSIDNNQLIFIN-667

Fig. 1 The loop connecting the domains IV and B is longer in 4,6- α GTs than in GTFs. The sequence alignment shows the region around the subsite +1 residue L938 (indicated by the triangle) of GTF-180. The sequences used are as follows: GTF-A (Q5SBL9) of *Lactobacillus reuteri* 121; GTF-180 (Q5SBN3) of *L. reuteri* 180; GTF-O (Q4JLC7)

of *L. reuteri* ATCC 55730; GTF-ML1 (Q5SBN0) of *L. reuteri* ML1; GTF-DSRE (Q8G9Q2) of *Leuconostoc mesenteroides* NRRL B-1299; GTF-SI (P13470) of *Streptococcus mutans* GS-5; 4,6- α GT-B (Q5SBM0) of *L. reuteri* 121; 4,6- α GT-W (A5VL73) of *L. reuteri* DSM20016; 4,6- α GT-ML4 (Q5SBN1) of *L. reuteri* ML1

Expression and basic properties of 4,6- α GT-W and 4,6- α GT-ML4

N-terminally truncated versions of 4,6- α GT-W (amino acids 458–1363) and 4,6- α GT-ML4 (amino acids 714–1620) were expressed in *E. coli*. 4,6- α GT-W was purified by His-tag affinity and anion-exchange chromatography, yielding 1.0 mg of pure protein per liter of culture. The enzyme displays optimal activity around pH 4.5 and 40–45°C and is resistant to thermal inactivation up to 45°C in sodium acetate buffer (pH 5.5), but at 51°C, it loses half of its activity in 10 min. 4,6- α GT-ML4 was purified from inclusion bodies using urea unfolding, refolding by dialysis and subsequent His-tag affinity chromatography, yielding 1.2 mg of soluble and pure 4,6- α GT-ML4 protein per liter of culture. The enzyme displays optimal activity around pH 4.5 and 40°C.

4,6- α GT-W and 4,6- α GT-ML4 disproportionate maltooligosaccharides

The substrate preference of the 4,6- α GT-W and 4,6- α GT-ML4 enzymes was explored by incubating them with various oligosaccharides. As shown by TLC, both enzymes use linear maltooligosaccharides [(1→4)- α -D-glucooligosaccharides] as substrate, forming a range of shorter and longer products (Fig. 2), but not sucrose (the typical substrate of GH70 enzymes), trehalose, raffinose, 1-kestose, nystose, isomaltose, and isomaltopentaose. The difference between the two enzymes is that 4,6- α GT-W already efficiently disproportionates the disaccharide maltose, whereas 4,6- α GT-ML4 requires maltotetraose or longer maltooligosaccharides (Fig. 2). Besides oligomeric material, TLC analysis showed that both enzymes also produce polymeric material [degree of polymerisation (DP) >10] (Fig. 2). Note that the polymeric material is not of very high molecular weight. For the homolog enzyme 4,6- α GT-B, we showed that the larger

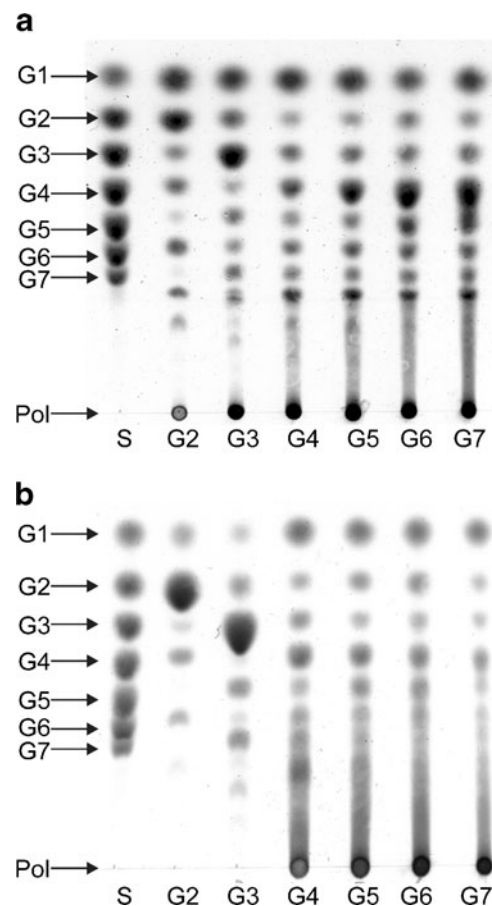


Fig. 2 4,6- α GT-W and 4,6- α GT-ML4 disproportionate maltooligosaccharides [(1→4)- α -D-glucooligosaccharides]. TLC analysis of product mixtures from maltooligosaccharide incubations with **a** 4,6- α GT-W and **b** 4,6- α GT-ML4. Conditions: pH 4.7 and 37°C, 40 h, 25 μ g/ml enzyme. S standard: glucose (G1) to maltoheptaose (G7); Pol polymer; lanes 2–7 are the product mixtures obtained from maltose (G2) to maltoheptaose (G7)

molecules synthesized from maltoheptaose had DPs below 50 (Dobrurowska et al. 2012).

The initial rate of 4,6- α GT-W with maltose was determined by measuring the release of glucose (as maltose + maltose \rightarrow a DP3 compound + glucose) using glucose oxidase, displaying a k_{cat} of $37 \pm 4 \text{ s}^{-1}$ and a K_M of $150 \pm 40 \text{ mM}$. The enzyme also processes maltopentaose, yielding maltotetraose + glucose (hydrolysis) or maltotetraose + a DP6 compound (transglycosylation). With 43 mM of maltopentaose as substrate, the initial rate of glucose formation was 56% of that of maltotetraose formation, showing that the enzyme is rather hydrolytic at the start of the reaction. However, when reaction products start to accumulate, transglycosylation becomes more efficient, as the reaction products are better acceptor substrates than maltopentaose.

4,6- α GT-W and 4,6- α GT-ML4 form $\alpha 1 \rightarrow 6$ glycosidic bonds

HPAEC analysis of the reaction products of the incubations of 4,6- α GT-W and 4,6- α GT-ML4 with maltooligosaccharides [(1 \rightarrow 4)- α -D-glucooligosaccharides] of DP2 to DP7 (for DP7, see Suppl. Info. Fig. S2) revealed the formation of products with retention times different from those of maltooligosaccharides. The elution profiles of both enzyme incubations per DP also showed that the product ensembles are not identical, despite the fact that both reaction mixtures contain the same type of glycosidic linkages, at nearly identical ratios, as discussed below.

^1H NMR analysis of the various product mixtures obtained with 4,6- α GT-W and 4,6- α GT-ML4 showed the presence of $\alpha 1 \rightarrow 4$ linkages (H-1, $\delta \sim 5.40$) and newly formed $\alpha 1 \rightarrow 6$ linkages (H-1, $\delta \sim 4.97$; broad signal). As typical examples, Fig. 3 presents the very similar ^1H NMR spectra for the product mixtures generated from maltohexaose, showing a linkage ratio $\alpha 1 \rightarrow 4 : \alpha 1 \rightarrow 6 = 50 : 50$ for 4,6- α GT-W (Fig. 3a) and a linkage ratio $\alpha 1 \rightarrow 4 : \alpha 1 \rightarrow 6 = 53 : 47$ for 4,6- α GT-ML4 (Fig. 3b). The slightly different built-up of the broad H-1 signal at $\delta \sim 4.97$, comparing both cases, supports the HPAEC observation that different product ensembles are formed. The reducing-end glucose residues are 4-substituted [(1 \rightarrow 4)-D-Glcp; R α H-1, δ 5.225; R β H-1, δ 4.652]. The spectra also revealed the presence of free glucose (G α H-1, δ 5.225; G β H-1, δ 4.637). Furthermore, small amounts of six-substituted reducing-end glucose residues [(1 \rightarrow 6)-D-Glcp; R α H-1, δ 5.241; R β H-1, δ 4.670] were detected, demonstrating that both enzymes can use glucose as acceptor to make isomaltose, which can then be further elongated by the enzyme. The ^1H NMR spectra (Fig. 3) reveal that both 4,6- α GT-W and 4,6- α GT-ML4 create linear structures only, as no signal typical for -(1 \rightarrow 4,6)- α -D-Glcp-(1 \rightarrow 4)- branches is seen [H-1, $\delta \sim 5.36$] (van Leeuwen et al. 2008a).

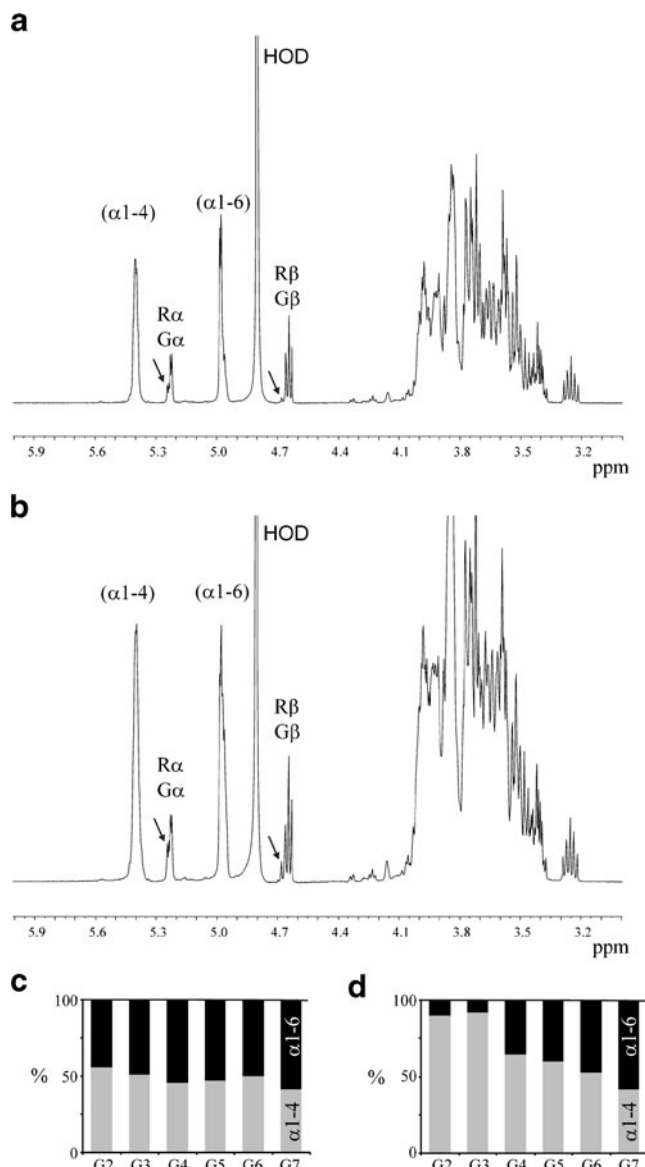


Fig. 3 4,6- α GT-W and 4,6- α GT-ML4 form $\alpha 1 \rightarrow 6$ glycosidic linkages. 500-MHz ^1H NMR spectra (without water suppression) of product mixtures obtained from maltohexaose (DP6) incubations with **a** 4,6- α GT-W or **b** 4,6- α GT-ML4. The bar diagrams give the percentage of $\alpha 1 \rightarrow 4$ (light gray) and $\alpha 1 \rightarrow 6$ glycosidic linkages (black) in the product mixtures obtained from maltose (G2) to maltoheptaose (G7) incubated with **c** 4,6- α GT-W or **d** 4,6- α GT-ML4. The anomeric signals reflecting the $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glycosidic linkages are indicated by ($\alpha 1-4$) and ($\alpha 1-6$), respectively. The anomeric signals assigned as G α/β and R α/β indicate the presence of free glucose and the presence of reducing -(1 \rightarrow 4)-D-Glcp units, respectively. The arrows in the spectra of the product mixtures indicate the presence of small amounts of reducing -(1 \rightarrow 6)-D-Glcp units

For the conversion of maltotetraose by 4,6- α GT-W (Suppl. Info. Fig. S3), methylation analysis supports the conclusion that only 4-O- and 6-O-substituted glucose residues are present, but no branched residues, thereby demonstrating that indeed the products are linear. Interestingly, the ^1H NMR analysis revealed that in case of

4,6- α GT-W the size of the substrate had only little influence on the α 1 \rightarrow 4: α 1 \rightarrow 6 glycosidic linkage ratio in the product mixtures (Fig. 3c), whereas for 4,6- α GT-ML4 the percentage of α 1 \rightarrow 6 glycosidic linkages introduced increased with increasing size of the substrate (Fig. 3d). In conclusion, both 4,6- α GT-W and 4,6- α GT-ML4 convert (1 \rightarrow 4)- α -D-glucooligosaccharides in linear α -glucans with α 1 \rightarrow 4 and α 1 \rightarrow 6 glycosidic linkages.

Purification and characterization of 4,6- α GT-W products

To gain more insight in the carbohydrate structures made by 4,6- α GT-W, the reaction mixture obtained from maltose was separated by HPAEC (Fig. 4). The isolated compounds were analyzed by MALDI-TOF-MS for their molecular mass and for their purity in terms of DP. Fractions containing multiple oligosaccharides were separated again by HPAEC using a different elution gradient. The structures of the isolated compounds were established by 1D 1 H NMR spectroscopy making use of a 1 H NMR library data base of α -glucans (van Leeuwen et al. 2008b; Dobruchowska et al. 2012). Besides glucose, the starting substrate maltose and panose [α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 4)-D-Glcp], four compounds could be identified, i.e., α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp (maltotriose), α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp, α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp, and α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp (Fig. 4). For 1 H NMR spectra, see (Suppl. Info. Fig. S4).

Enzymatic hydrolysis of the 4,6- α GT-W and 4,6- α GT-ML4 products

To investigate to what extent the α -glucan product mixtures made from maltose up to maltoheptaose using 4,6- α GT-W and 4,6- α GT-ML4 were resistant to α -amylase digestion, they were incubated with a high dose of pig pancreatic α -amylase. Subsequent TLC analysis (Fig. 5) revealed small oligosaccharides but also larger oligosaccharides and polymeric material. These data thus shows that both 4,6- α GTs synthesize α -glucans partly resistant to the endo- α 1 \rightarrow 4-hydrolase action of α -amylase. The product mixtures are, however, completely degraded to glucose by amyloglucosidase (data not shown), demonstrating that the products are composed of α 1 \rightarrow 4 and α 1 \rightarrow 6 glycosidic bonds only, which is in agreement with the 1 H NMR analysis.

4,6- α GT-W mainly synthesizes α 1 \rightarrow 6 glycosidic linkages, but also occasionally α 1 \rightarrow 4 glycosidic bonds, as maltotriose is one of the products made from maltose (Fig. 4). To explore whether 4,6- α GT-W also elongates via an α 1 \rightarrow 4 glycosidic bond onto oligosaccharides of which the non-reducing glucose moiety is attached via an α 1 \rightarrow 6 linkage, thereby creating - α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp-(1 \rightarrow 6)- elements, the product mixtures obtained from maltose up to maltoheptaose were treated with pullulanase type M1. This enzyme hydrolyzes α 1 \rightarrow 6 glycosidic linkages in (-) α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp-(1 \rightarrow 6)- sequences only. Comparison of the HPAEC elution profiles before and after pullulanase treatment showed only a few differences in the larger oligosaccharide and polymeric fractions (Suppl. Info. Fig. S5), demonstrating that 4,6- α GT-W occasionally synthesizes such a sequence.

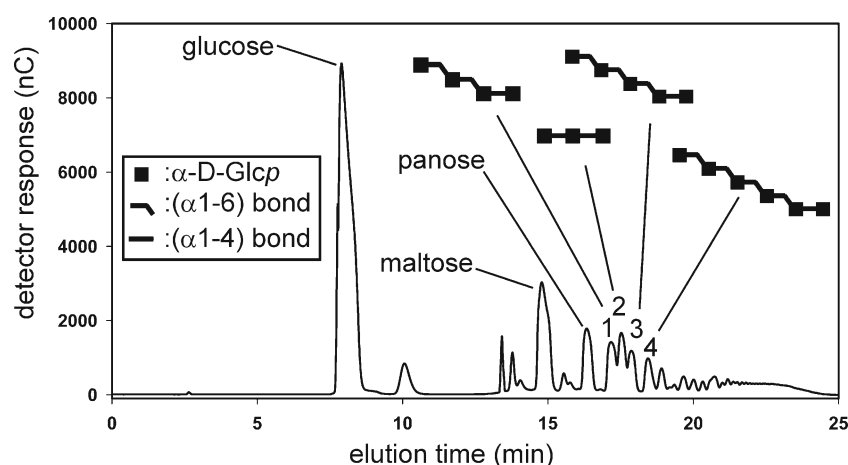


Fig. 4 HPAEC elution profile of the reaction mixture obtained from maltose, incubated with 4,6- α GT-W at pH 4.7 and 37°C for 4 days. Fractions 1–4 were collected, and after desalting, the products were characterized by 1 H NMR spectroscopy (Suppl. Info. Fig. 4a–d),

making use of a 1 H NMR library data base of α -glucans (van Leeuwen et al. 2008b; Dobruchowska et al. 2012) (and references cited therein). Identified structures are indicated in *symbol notation above the peaks*; for the complete names, see text

Discussion

It was generally accepted that all GH70 enzymes synthesize α -glucans from sucrose, until we recently demonstrated that *L. reuteri* 121 encodes a GH70 enzyme (called 4,6- α GT-B) that uses (1 \rightarrow 4)- α -glucans, but not sucrose, as substrate (Kralj et al. 2011; Dobruchowska et al. 2012). Even though it is known that some GH70 enzymes possess a low disproportionating activity with α -glucans in addition to their main activity (Binder et al. 1983), it was surprising to find a GH70 enzyme to be so effective in utilizing (1 \rightarrow 4)- α -glucans as glucose donor because the glycosidic linkage of sucrose is labile compared with the α 1 \rightarrow 4 glycosidic bond. Phylogenetic tree analysis of the GH70 protein sequences

revealed that 4,6- α GT-B clusters with a few hypothetical proteins (Kralj et al. 2011) (Suppl. Info. Fig. S1). Here, we show that the hypothetical GH70 proteins of *L. reuteri* strains DSM 20016 and ML1 encode enzymes that convert maltooligosaccharides [(1 \rightarrow 4)- α -D-glucooligosaccharides] into linear α -glucans (in fact isomalto/maltooligosaccharides) with about 50% α 1 \rightarrow 6 glycosidic bonds. It thus appears that 4,6- α GT activity is a common activity within the GH70 family.

The 4,6- α GT products are synthesized by cleaving off the non-reducing glucose moiety of a (1 \rightarrow 4)- α -glucan and transferring the glucose moiety to the HO-6 function of the non-reducing end of an acceptor α -glucan chain [a (1 \rightarrow 4)- α -glucan or a (1 \rightarrow 4)- α -glucan already elongated with a number of (α 1 \rightarrow 6)-linked glucose residues] (Fig. 6). The enzymes possess, in addition, minor hydrolytic and α 1 \rightarrow 4 transglycosylase activities. All three disproportionating GH70 4,6- α GT enzymes characterized so far synthesize α 1 \rightarrow 6 glycosidic bonds and occasionally an α 1 \rightarrow 4 glycosidic bond. In contrast, the common GH70 GTF enzymes synthesize all four types of α -glycosidic linkages possible. It appears unlikely that this is an intrinsic property of the non-sucrose utilizing type of GH70 enzymes. Therefore in the future α 1 \rightarrow 2 and α 1 \rightarrow 3 synthesizing non-sucrose utilizing type of GH70 enzymes may be found encoded by the ever increasing number of bacterial genome sequences, unless of course such synthetic capacities are not beneficial for the bacteria.

The distinct reaction specificities of 4,6- α GTs and GTFs

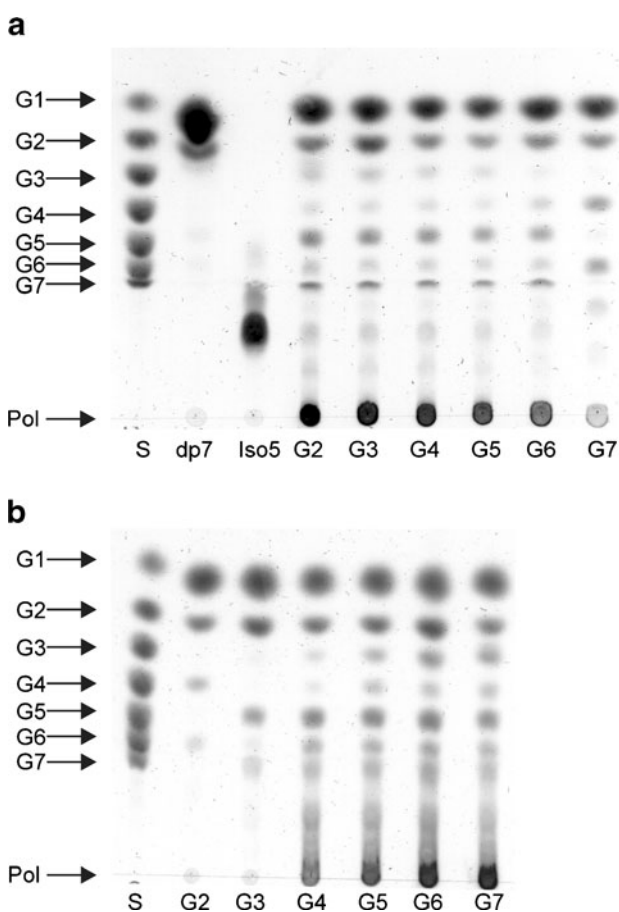
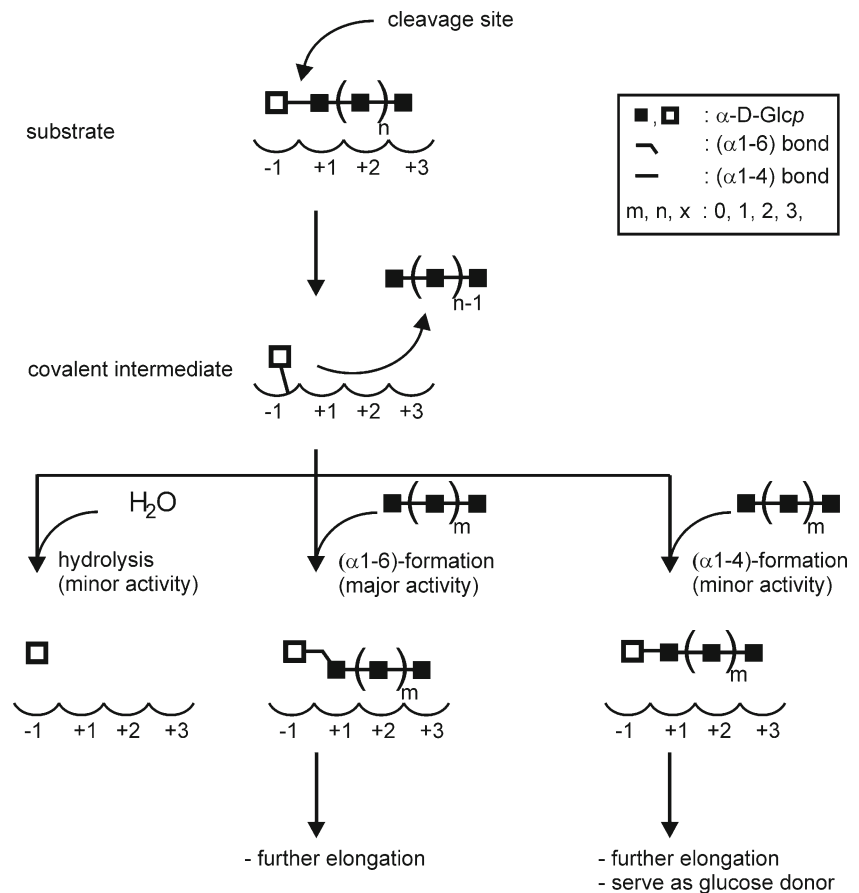


Fig. 5 4,6- α GT-W and 4,6- α GT-ML4 generate α -amylase resistant α -glucans. TLC analysis of the product mixtures from maltooligosaccharide (G2-G7) incubations with 4,6- α GT-W (a) and 4,6- α GT-ML4 (b) (see Fig. 2), after treatment with a high-dose of pig pancreatic α -amylase. *S* standard: glucose (G1) to maltoheptaose (G7); *Pol* polymer. Lanes 2–7 are the product mixtures from G2 to G7, generated by the sequential 4,6- α GT/ α -amylase incubations. The upper panel shows as controls isomaltopentaose (Iso5; not degraded by α -amylase) and maltoheptaose (dp7; degraded to maltose and glucose by α -amylase). Note that Iso5 is not entirely pure

Currently it is puzzling why 4,6- α GTs use maltooligosaccharides, but not sucrose, as glucose donor. Because GTFs cleave the glycosidic linkage between the glucosyl and fructosyl moieties of sucrose, and 4,6- α GTs the α 1 \rightarrow 4 glycosidic linkage between two glucosyl moieties, structural differences are expected at substrate binding subsite +1, which holds either a glucosyl or fructosyl unit (see Fig. 6 for subsite nomenclature). Whether the differences at subsite +1 (Table 1) are the only requirements to interchange the reaction specificities of GH70 enzymes or that additional mutations more remote from the catalytic center are required currently is unknown. In addition, acceptor subsite +2 has a few different amino acids (Table 1), which might contribute to the 4,6- α GT reaction specificity. For GTFs, it is known that amino acid substitutions at subsite +1 (in position 1140) and subsite +2 (in positions 1137 and 1141) drastically alter the ratio of α -glycosidic linkages in the α -glucan polymers made by the enzymes (Shimamura et al. 1994; van Leeuwen et al. 2009). Further mutational analysis of the 4,6- α GT acceptor subsites thus may allow elucidation and modification of their glycosidic linkage specificity.

Fig. 6 Schematic diagram of the reactions catalyzed by 4,6- α GT enzymes. The *open squares* indicate the glucose moiety transferred by the enzyme. The sugar binding subsite nomenclature is according to Davies et al. (1997), in which the glycosidic bond is cleaved between donor subsite -1 and acceptor subsite +1



Another remarkable difference is that 4,6- α GTs synthesize linear α -glucans only, whereas GTFs often produce branched polymers. Since linear α -glucans take up less space than branched ones, 4,6- α GTs may possess a narrower acceptor binding cleft. In this regard, it is noteworthy that the loop connecting domains IV and B (residues 932-943 in GTF-180) is eight residues longer in 4,6- α GTs compared to GTFs (Fig. 1). The crystal structures of GTF-180 and GTF-SI (Vujičić-Žagar et al. 2010; Ito et al. 2011) show that part of this loop delineates the active site; a longer loop at this position thus may result in a more restricted binding cleft in 4,6- α GTs. To further study this hypothesis, 3D structural information is needed, and currently, we are trying to obtain crystal structures of 4,6- α GT proteins.

Differences and similarities with other α -glucan acting enzymes

Furthermore, enzymes such as 4- α -glucanotransferase, cyclodextrin glucanotransferase, branching enzyme, and dextrin dextrinases disproportionate (1 \rightarrow 4)- α -glucans (MacGregor et al. 2001; Cantarel et al. 2009; Zona et al. 2004; Palomo et al. 2011; Kelly et al. 2009a; Naessens et al. 2005). However, these GH13, 57, and 77 enzymes

synthesize different products than 4,6- α GTs. Note that the sequences of dextrin dextrinases are unknown, as the genes have not been identified yet. Cyclodextrin glucanotransferases make cyclic (1 \rightarrow 4)- α -glucans (e.g., cyclodextrins), 4- α -glucanotransferases cleave and synthesize α 1 \rightarrow 4 glycosidic bonds thereby altering the (1 \rightarrow 4)- α -glucan chain distribution, and branching enzymes cleave α 1 \rightarrow 4 glycosidic bonds and transfer the cleaved-off chain to a HO-6 group within a (1 \rightarrow 4)- α -glucan, yielding an α 1 \rightarrow 6 branch. These enzymes cleave internal α 1 \rightarrow 4 glycosidic linkages (i.e., endo-acting), whereas 4,6- α GTs are exo-acting enzymes that cleave off the non-reducing glucose unit from an (1 \rightarrow 4)- α -glucan chain.

Moreover, cyclodextrin glucanotransferases and 4- α -glucanotransferases synthesize α 1 \rightarrow 4 glycosidic bonds, whereas 4,6- α GTs make α 1 \rightarrow 6 bonds. Although branching enzymes also form α 1 \rightarrow 6 bonds, they create α 1 \rightarrow 6 bonds in the middle of α -glucan chains, resulting in branch points, whereas 4,6- α GTs attach the glucose unit via an α 1 \rightarrow 6 glycosidic linkage to the non-reducing end of an α -glucan chain yielding linear products.

The reaction specificity of dextran dextrinases comes closest to that of 4,6- α GTs, both transferring single glucose moieties from the non-reducing end of maltooligosaccharides to synthesize isomalto/maltooligosaccharides (Yamamoto et al.

1993a; Kralj et al. 2011). The crucial difference between the two types of enzymes is that dextrin dextrinases form branched carbohydrates (Yamamoto et al. 1993b; Wang et al. 2011; Sims et al. 2001; Tsusaki et al. 2009). A second difference is that dextrin dextrinases disproportionate isomaltooligosaccharides (Yamamoto et al. 1993a), whereas isomaltooligosaccharides are not a substrate of 4,6- α GTs. This is relevant as this means that dextran dextrinases can disproportionate their own reaction products, whereas 4,6- α GTs cannot. A key question that remains to be answered is whether dextran dextrinases are evolutionary related to the GH70 enzymes. Based on their activity they combine the disproportionation activity of 4,6- α GTs and the branching activity of GH70 glucansucrases. The answer to this question requires the identification of the dextran dextrinase (gene) sequence.

A further difference is that GH70 enzymes, including 4,6- α GTs, are more hydrolytic than the GH13 and GH77 transglycosylases. For example, the $k_{\text{cat}}(\text{transglycosylation})/k_{\text{cat}}(\text{hydrolysis})$ ratio of the GH13 *Neisseria polysaccharea* amylosucrase is 20 (Albenne et al. 2004), that of GH13 *Bacillus circulans* CGTase is 90 (van der Veen et al. 2001), GH13 branching enzymes have ratios of $\gg 1,000$, and the GH77 4- α -glucanotransferases of *Thermus thermophilus* and *T. Brockianus* have ratios of 5,000 (Kaper et al. 2007; Jung et al. 2011). GH70 GTFs, in contrast, are predominantly hydrolytic in the early phase of sucrose conversion, and they become transglycosylases when better acceptor substrates become available as result of the transglycosylation activity or when good acceptor substrates are added at the start of a reaction (Kralj et al. 2004b). Here, we find that also 4,6- α GT-W has about 50% hydrolytic activity at the start of a reaction with low concentrations of linear (1 \rightarrow 4)- α -D-glucopoligosaccharides and becomes a better transglycosylase once products appear that again function as acceptor substrate. The acceptor subsites of GH70 enzymes seem thus to be optimized for recognizing and utilizing the products made by the enzyme as acceptor substrates in a polymerization reaction.

Applications and future perspectives of 4,6- α GTs

4,6- α GTs are an exciting type of novel enzymes that convert (1 \rightarrow 4)- α -glucan substrates into linear α -glucan products with a high percentage of α 1 \rightarrow 6 glycosidic linkages (i.e., isomaltooligosaccharides built on maltooligosaccharides). These enzymes are potentially very useful for the synthesis of carbohydrate displaying compounds. Indeed, GH13 and GH70 enzymes are being used for the glycosylation of a variety of compounds (Desmet and Soetaert 2011; Leemhuis et al. 2010; Homann and Seibel 2009). The fact that 4,6- α GTs synthesizes mainly α 1 \rightarrow 6 glycosidic bonds ensures that the products are water-soluble, which is beneficial for

increasing the solubility of poorly water-soluble compounds such as drug molecules.

Because 4,6- α GTs generate α -glucans with a high degree of α 1 \rightarrow 6 bonds, their products are water-soluble, which is advantageous for applications such as energy and soft drinks. The α 1 \rightarrow 6 linkages, in addition, make the products resistant to α -amylase digestion and the products as such are expected to pass the human stomach and small intestine and enter the colon where they can serve as carbon source for health promoting bacteria. Thus, 4,6- α GTs are expected to convert readily degradable maltooligosaccharides into a novel type of “resistant” α -glucans and are thus potentially of great interest to the food industry (Dijkhuizen et al. 2011).

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