Crystal structure of a 117 kDa glucansucrase fragment provides insight into evolution and product specificity of GH70 enzymes

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Supporting Information

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SI Text

Biochemical Assays. Enzymatic activities of GTF180-ΔN and derived catalytic mutants were determined enzymatically by measuring glucose and fructose release from sucrose (1). The amount of released fructose corresponds to the total enzyme activity. The amount of free glucose represents the hydrolytic activity; the transferase activity is represented by the amount of released fructose minus the amount of free glucose.

To study the effects of calcium ions on enzyme activity, the experiments were initially conducted with buffer without $CaCl_2$. The effect of $CaCl_2$ was investigated by adding 1 mM $CaCl_2$ or 1 mM EDTA to the reaction mixture. Reactions were performed at 50 °C in 25 mM sodium acetate buffer, pH 4.5 which are the pH and temperature optima for this enzyme (2), containing 1 mM $CaCl_2$, 50 mM sucrose and 5 nM purified GTF180-ΔN enzyme. Data points were taken every 5 min over a period of 30 min. In case of very low activity of mutant proteins, assay conditions were modified as follows: reactions were performed with 25-fold more protein, and data points were taken at 30 min intervals for at least 2 h. One unit of enzyme activity is defined as the release of 1 μmol of monosaccharide per min. Enzyme concentrations were determined using the Bradford reagent (BioRad, Germany) with bovine serum albumin as standard.

Sample Preparation for Thin Layer Chromatography (TLC Analysis). 1 μL of purified protein (~2 mg/mL) was added to 9 μL 25 mM sodium acetate, pH 4.5, 1 mM $CaCl_2$ and 50 mM sucrose followed by overnight incubation at 37 °C. TLC was performed on 20 × 20 cm TLC sheets (Silica gel 60 F_{254}, Merck, Darmstadt, Germany). A total volume of 2 μL of sample was spotted on TLC, after drying the plate was run in butanol:ethanol:water (vol/vol/vol = 5:5:3). The plate was developed by staining with a solution of methanol containing 10% H_2SO_4, followed by incubation at ~110 °C for 20 min.

Distant Maltose Binding Sites. While maltoses M1 and M2 bind in the active site (Fig. 2), maltoses M3 and M4 are bound approximately 25 Å from the catalytic center (Fig. S3). Maltose M3 is bound to domain B. The molecule has hydrophobic stacking interactions with W1531, is at hydrogen bonding distance to D1525, T1530, E1532, and V1533, and makes a water-mediated hydrogen bond to D934. M4 is at direct hydrogen bonding distance to N1115, T1110, S1112, L1113, and R1086 in domain A. Because a GTF180-ΔN W1531S mutation has no effect on product size compared to the wild type, this binding site does not appear important for catalysis and product specificity (Fig. S4).

Oligosaccharide Synthesis and Product Analysis. ***Synthesis.*** 30 nM GTF180-ΔN was incubated at 50 °C for 80 h with 100 mM sucrose in the presence of 100 mM maltose as acceptor substrate.

Analysis. After complete consumption of sucrose (80 h at 50 °C), the sample was diluted 1,000 times in a 90% DMSO solution. Maltose, isomaltose, maltotriose, panose (Sigma, St. Louis, Missouri), isomaltotriose (TNO Nutrition and Food Research, Groningen, The Netherlands), sucrose (Acros Organics, Geel, Belgium), fructose, glucose (Merck, Darmstadt, Germany), and leucrose (Pfeiffer and Langen, Köln, Germany) were used as standards.

Separation of oligosaccharides was achieved with a CarboPac PA1 anion exchange column (250 × 4 mm; Dionex, Sunnyvale, California, USA) coupled to a CarboPac1 guard column (Dionex). The following gradient was used: eluent A (0 min, 100%); (5 min, 100%); (50 min, 92%); (55–58 min, 0%); (60 min, 100%); and (75 min, 100%). Eluent A was sodium hydroxide (0.1 M) and eluent B was NaAc (0.6 M) in sodium hydroxide (0.1 M). Detection was performed with an ED40 Electrochemical detector (Dionex) with an Au working electrode and an Ag/AgCl reference electrode with a sensitivity of 300 nanoCoulomb. The pulse program used was: +1.0 Volt (0–0.40 s); +0.7 Volt (0.41–0.60 s); and −0.1 Volt (0.61–1.00 s). Data were integrated using a Turbochrom (Applied Biosystems) data integration system.

The percentage of oligosaccharide synthesis from sucrose and maltose acceptor was determined by subtracting the amount of unused acceptor from the initial acceptor concentration.

No standard was available for α-(1 → 6)-panose; therefore, the calibration curve for panose, representing the most closely related oligosaccharide available to us, was used to estimate the approximate concentrations of this compound. Total and individual oligosaccharide yields were calculated from the amount of acceptor substrate converted into total and individual oligosaccharides, expressed as a percentage of the total amount of acceptor substrate initially present in the incubation.

Purification. Oligosaccharides synthesized were purified on the basis of their degree of polymerization (DP) using a BC-200 Ca^{+2} column (at 85 °C; 300 by 7.8 mm; Benson Polymeric, Reno, USA) eluted with water (0.2 ml min^{-1}), using a model 830-RI refractive index detector at 40 °C (Jasco, Tokyo, Japan). The system was calibrated using linear maltooligosaccharides (G1–G7).

Characterization. The two separated purified oligosaccharides of DP3 and DP4 were subjected to enzymatic degradation using dextranase from *Penicillium sp*. (EC 3.2.1.11, Sigma, St. Louis, MO), which endo-hydrolyzes α(1 → 6) glucosidic bonds (3, 4), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, Sigma), which was shown to hydrolyze α(1 → 4), α(1 → 3), and α(1 → 6) linkages at decreasing rates, respectively, to produce glucose from the nonreducing end of linear oligosaccharides (5, 6), and α-glucosidase from *Bacillus stearothermophilus* (EC 3.1.2.20 Megazyme, Ireland), which hydrolyzes terminal, α(1 → 4) linkages from the nonreducing end of oligosaccharides to produce glucose (7). Oligosaccharides (1 $g{\cdot}l^{-1}$) were incubated with 0.1 U mL^{-1} amyloglucosidase, 66 U mL^{-1} dextranase, and 66 U mL^{-1} α-glucosidase. After 30 min, 2 h, and 18 h of incubation, respectively samples were withdrawn and products formed in time were analyzed by anion exchange chromatography as described above. One dextranase unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmole isomaltose from dextran min^{-1} at 37 °C and pH 6.0. One amyloglucosidase unit is defined as the amount of enzyme that hydrolyzes 1 mg of maltose per 3 min at 55 °C and pH 4.5. One α-glucosidase unit is defined as the amount of enzyme that hydrolyzes 1 μmole ρ-nitrophenol-α-glucoside min^{-1} at 40 °C and pH 6.5.

Analysis of Products Synthesized by GTF180-ΔN. GTF180-ΔN synthesized an oligosaccharide of DP3 and DP4 from sucrose and maltose as acceptor reaction substrate (Fig. S5). The structure of the DP3 oligosaccharide was identified by (*i*) spiking of a commercial sample of panose and (*ii*) preparative isolation of the DP3 oligosaccharide followed by enzymatic degradation of the purified DP3 product synthesized. The major oligosaccharide product (DP3) eluted at the same position (41 min; Dionex analysis;

Fig. S5) as panose [α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-D-glucose]. This oligosaccharide was also purified from the incubation mixture and analysis of products formed upon its enzymatic degradation in time, by amyloglucosidase and α-glucosidase, confirmed its identity as panose (Table S5). In contrast, isopanose [α-D-glucopyranosyl-(1 → 4)-[α-D-glucopyranosyl-(1 → 6)]-D-glucose] was degraded very fast by the latter two enzymes indicative of an α-(1 → 4) linkage at this saccharide's nonreducing end (8).

The structure of the DP4 product synthesized was identified by enzymatic degradation as α(1 → 6)-panose [α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-D-glucose]. Dextranase degraded the DP4 oligosaccharide into maltose and isomaltose (indicative of an endo α(1 → 6) linkage in this oligosaccharide). In contrast, α(1 → 6)-isopanose [α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-[α-D-glucopyranosyl-(1 → 6)-D-glucose]], containing an endo α(1 → 4) linkage, was not degraded by dextranase (8). Amyloglucosidase cleaved the DP4 oligosaccharide slowly to glucose and panose [α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl(1 → 4)-D-glucose], indicative of an α(1 → 6) linkage at its nonreducing end (see above), whereas for α-glucosidase no degradation was observed.

Cloning and Mutagenesis of *gtf180*. To facilitate manipulation the N terminally truncated GTF180 gene was amplified using PCR with primers 180expCF 5′-GATGCAT*GAGCTC***CCATGG**G-CATTAACGGCCAACAATATTATATTGACCC-3′, containing *Sac*I (Italics) and *Nco*I (bold) restriction sites, and 180expCR 5′-ATATCGAT*GGGCCCC***GGATCC**TATTAGTGATGGTGAT-GGTGATGTTTTTGGCCGTTTAAATCACCAGGTTTTAATG-G-3′, containing *Apa*I (italics) and *Bam*HI (bold) restriction sites and a C-terminal His-tag (underlined) (9). The product was digested with *Sac*I/*Apa*I and cloned in the corresponding sites of pBluescript II SK+, yielding plasmid pBGTF180-ΔN. Furthermore, two unique restriction sites (*Sal*I, 2802 bp; *Xho*I, 3606 bp) were introduced using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used in the PCR: 5′-GGAATAAGGATAGTGAAAATGTCGAC-TACGGTGGTTTGC-3′ and 5′-ATAAAGATTCAGTTCCTCGA-GTTTACTATGGAGACC-3′, and the complementary reverse primers, containing an introduced *Sal*I and *Xho*I site, respectively (underlined, silent mutation by change of base shown in bold face), yielding the plasmid pBGTF180-ΔNSX. This plasmid was digested with *Nco*I and *Bam*HI and ligated in the corresponding sites of pET15b yielding p15GTF180-ΔNSX. pBGTF180-ΔNSX was used for site-directed mutagenesis, sequencing, and rapid exchange (using the *Nco*I and *Xho*I restriction sites) with p15GTF180-ΔNSX. The single mutants were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) and pBGTF180SX as template using forward primers 5′-GATGG-TATTCGAGTGAATGCTGTTGATAATGTAG-3′ (D1025N, *Bsm*I restriction site underlined), 5′- GCACATTAATATTTTG-CAAGATTGGGGATGGGATGATCC-3′ (E1063Q), 5′-GCTA-TAATTTTGTTCGGGCCCATAATAGTAATGC-3′, (D1136N, *Apa*I restriction site underlined), and 5′-CACGGTACTACATCG-GAAGTTTCGCCAATA-3′ (W1531S) and complementary reverse primers. After successful mutagenesis (confirmed by restriction analysis and/or nucleotide sequencing), pBGTF180SX was digested with *Nco*I and *Xho*I and ligated in the corresponding sites of p15GTF180-ΔN.

Docking Studies. A model of a covalent glucosyl-GTF180-ΔN intermediate (in subsite −1) was made based on the structure of the glucosyl-intermediate of *Neisseria polysaccharea* amylosucrase [PDB code 1S46; (10)]. Isomaltose (Glc-α(1 → 6)-Glc), nigerose (Glc-α(1 → 3)-Glc), and isomaltotriose (Glc-α(1 → 6)-Glc-α(1 → 6)-Glc) were docked into this model. The automatic docking calculations were done with the program AutoDock 4 (11). For every carbohydrate a total of 256 docked structures were obtained; posterior selection was done by evaluating: (*i*) the binding free energy, (*ii*) the proximity of the docked carbohydrates to the active site, (*iii*) the proper direction of the C1-O1 bond and sufficient space to extend the docked saccharide with additional glucose residues, and (*iv*) the correct dihedral angle around the glycosidic bonds (ϕ, ψ and ω).

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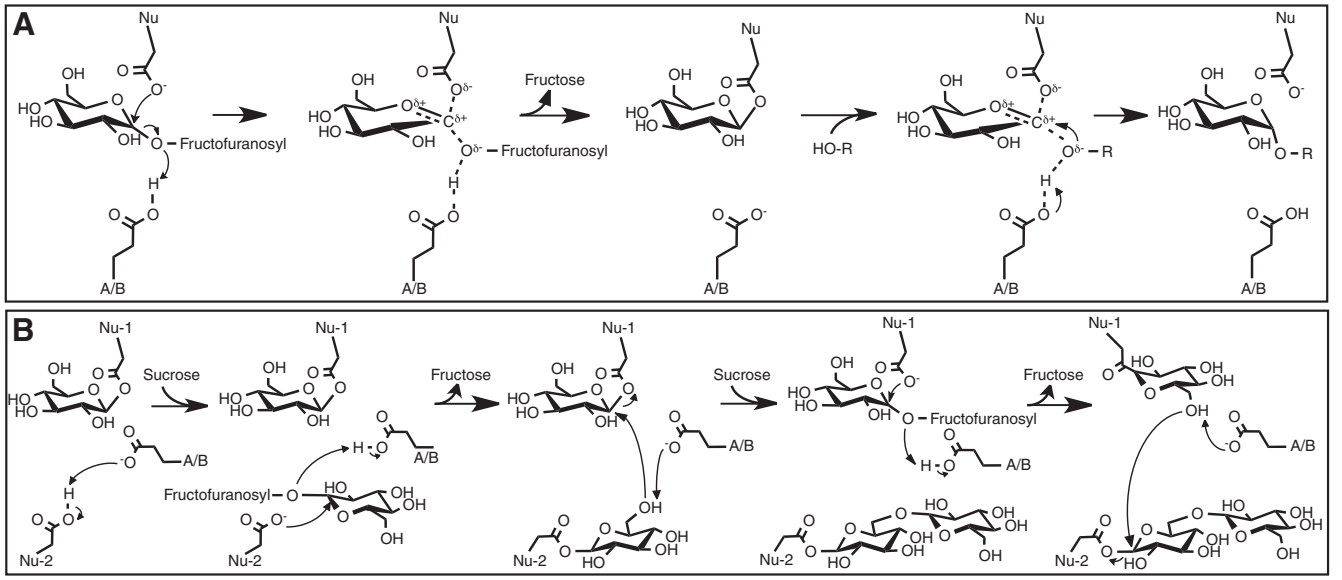

Fig. S1. (*A*) Reaction scheme of the single-catalytic site reaction mechanism of α-amylase superfamily enzymes (GH13/70/77 families) (1, 2) and (*B*) the two catalytic site/double-nucleophile insertion mechanism of GH70 enzymes proposed by Robyt et al. (3). The formation of the first glucosyl-enzyme intermediate in reaction mechanism (B) is the same as for the mechanism (A) and was therefore omitted. The second nucleophilic residue (Nu-2) of (*B*) corresponds to the transition state stabilizer of (*A*). Nu, nucleophile; A/B, acid-base; R, glucan chain, acceptor or water.

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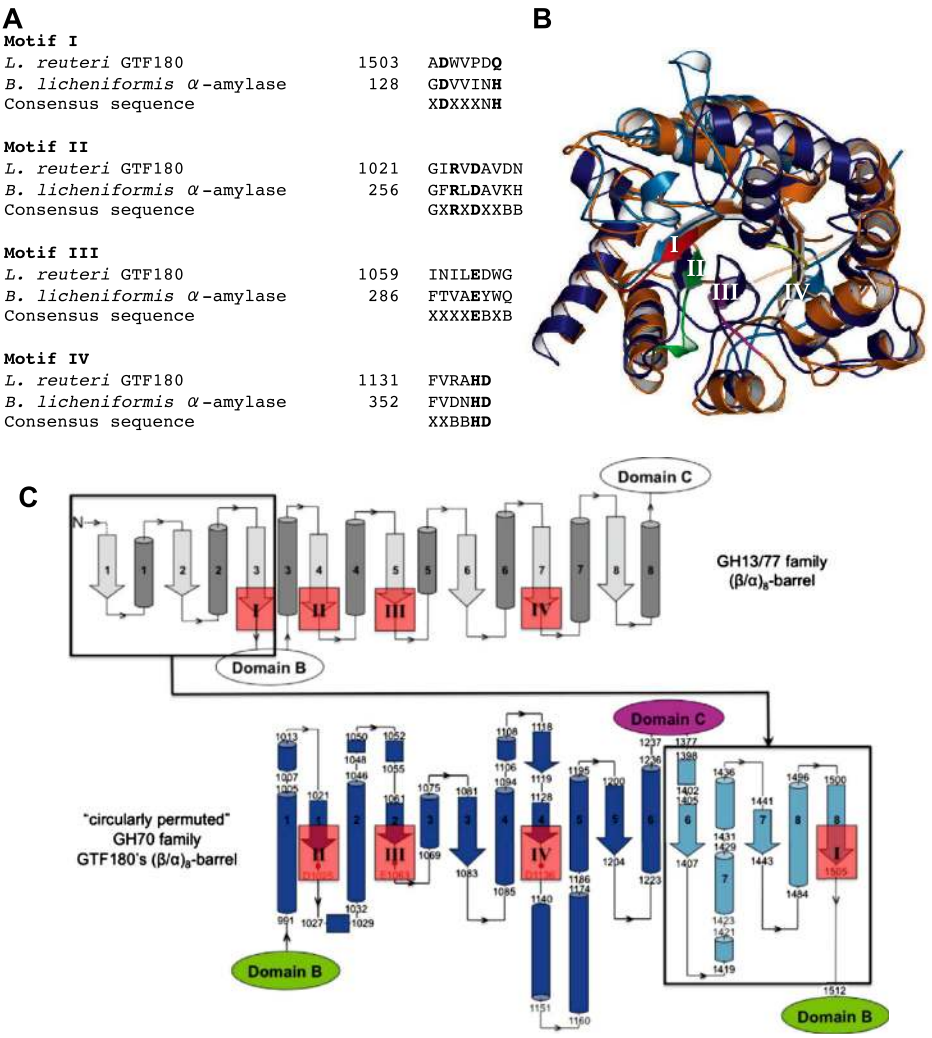


Fig. S2. Conserved sequence motifs of the catalytic domain of α-amylase superfamily members. (*A*) Motifs I-IV of GH70 representative *Lactobacillus reuteri* GTF180 and GH13 representative *Bacillus licheniformis* α-amylase (which shows highest structural similarity to domains A, B, and C of *L. reuteri* GTF180; rmsd of 3.2 Å for 297 equivalent Cα atoms and 23% sequence identity). The consensus sequence of the GH13 family enzymes is indicated at the bottom (legend: X, usually hydrophobic; B, usually hydrophilic, modified from MacGregor et al. (1). The strictly conserved GH13 residues are depicted in bold. Residues of the catalytic triad are enlarged. (*B*) Superimposed $(\beta/\alpha)_8$-barrels of *B. licheniformis* α-amylase (orange) and *L. reuteri* GTF180 (dark and light blue show the N- and the C-terminal portions of the polypeptide chain, respectively). Conserved sequence motifs I-IV in the structure of *B. licheniformis* α-amylase are colored red, green, magenta, and yellow, respectively. (*C*) Order of the $(\beta/\alpha)_8$-barrel elements in the GH13/77 and GH70 family enzymes. Locations of the conserved sequence motifs I-IV are shown with red boxes.

1 MacGregor EA, Janecek S, Svensson B (2001) Relationship of sequence and structure to specificity in the alpha-amylase family of enzymes. *Biochim Biophys Acta* 1546:1–20.

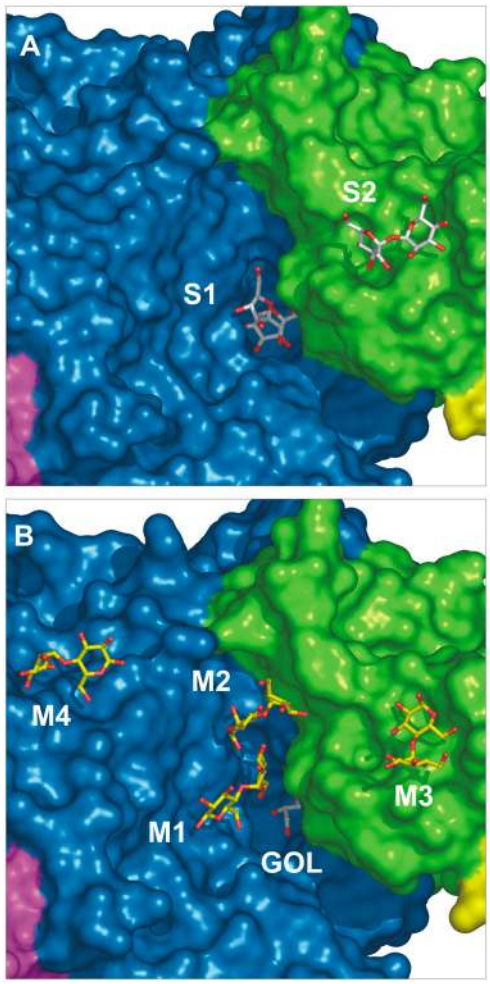


Fig. S3. Surface presentation of the GTF180-ΔN active site gorge. (*A*) Sucrose binding in GTF180(D1025N)ΔN-sucrose complex; (*B*) Maltose binding in GTF180ΔN-maltose complex. Sucrose and maltose molecules are depicted as white and yellow sticks. Domains A and B are colored in blue and green, respectively; GOL = glycerol.

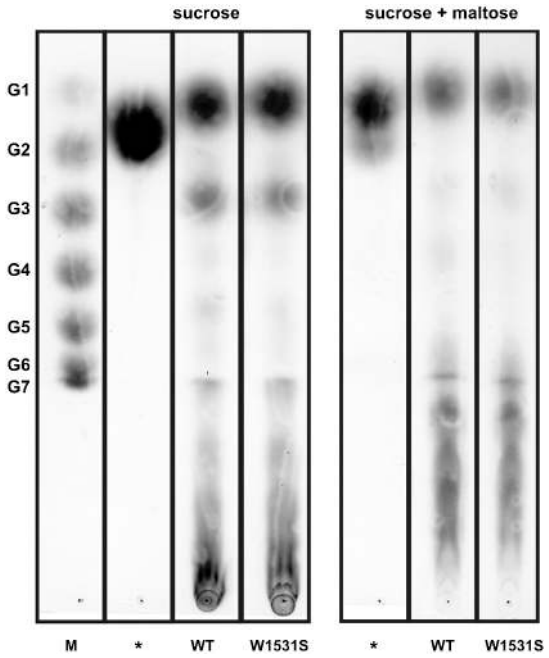


Fig. S4. TLC analysis of products synthesized by wild type GTF180-ΔN and its W1531S mutant in presence of either sucrose or sucrose and maltose. Nomenclature is as follows: "M," glucose G1–G7 marker; "*," substrate (and acceptor substrate); "WT," wild type GTF180-ΔN; "W1531S," GTF180-ΔN-W1531S mutant.

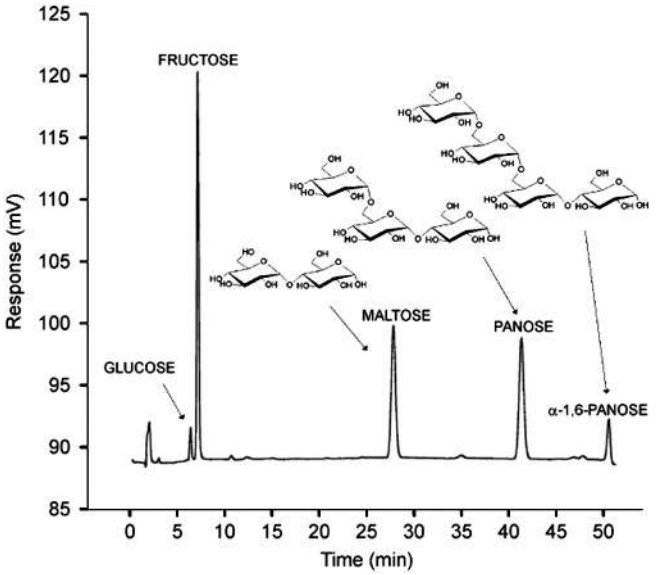


Fig. S5. Anion exchange chromatography of *L. reuteri* GTF180-ΔN products synthesized in the presence of both sucrose and maltose. From left to right are shown peaks for glucose, derived from hydrolysis of sucrose; fructose, total amount released in the medium; maltose, not used during reaction; and panose and $\alpha(1 \rightarrow 6)$-panose, which are the products of glucosyl transfer to maltose. For details see *SI text*.

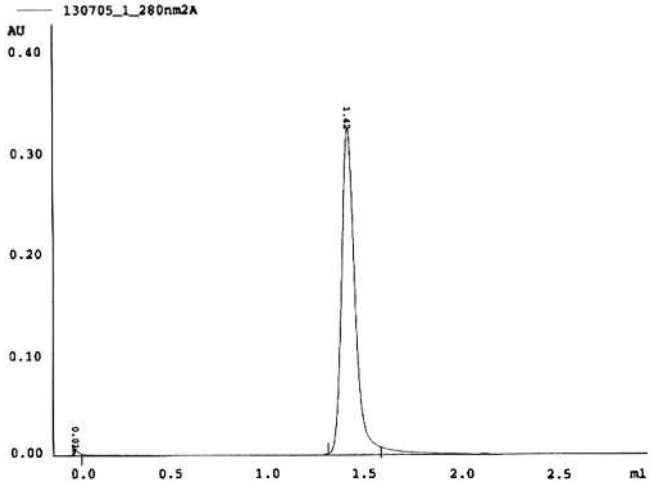
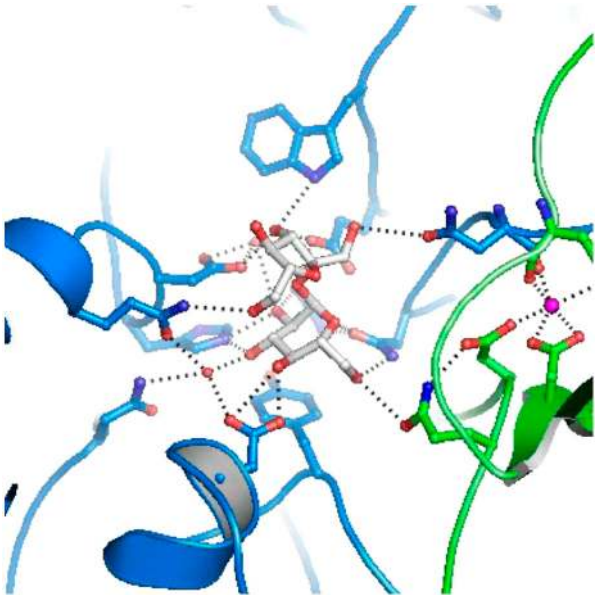
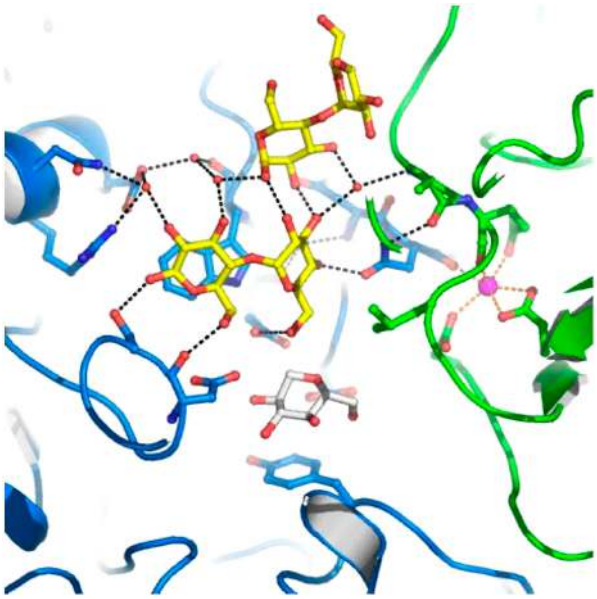


Fig. S6. Size exclusion chromatogram of GTF180-ΔN in 25 mM sodium acetate buffer, pH 5.0, 100 mM NaCl, 1 mM $CaCl_2$ using Superdex200 PC 3.2/30 column (Pharmacia SmartSystem). The protein elution volume is 1.42 mL, which corresponds to molecular mass of 94 kDa based on the BioRad gel filtration markers run in the same conditions.



Movie S1. Sucrose binding to the active site of *L. reuteri* GTF180-ΔN.

Movie S1 (AVI)



Movie S2. Maltose binding to the active site of *L. reuteri* GTF180-ΔN.

Movie S2 (AVI)

Table S1. Data processing statistics for GTF180-ΔN "MAD," "sucrose," and "maltose" datasets

Parameter	MAD (Multiple wavelength anomalous dispersion)			Sucrose soak	Maltose soak
Space group	*P*1			*P*1	P1
Cell dimensions					
a b c (Å)	58.3 65.9 82.5			57.8 66.2 82.7	58.2 65.9 82.3
α β γ (°)	73.3 78.5 85.8			72.9 78.6 85.4	73.5 78.6 86.4
	Peak	Inflection	Remote		
Wavelength (Å)	0.97911	0.97927	0.97564	0.9795	0.9760
Resolution range (Å)	50.00–2.00 (2.10–2.00)	50.00–2.20 (2.32–2.20)	50.00–2.30 (2.42–2.30)	50.00–2.22 (2.34–2.22)	38.75–2.00 (2.10–2.00)
R_{sym} (%)*	7.8 (27.4)	5.1 (15.7)	5.7 (17.5)	5.5 (25.3)	5.3 (17.1)
$I/\sigma(I)$	22.6 (7.8)	27.4 (9.6)	26.3 (9.8)	15.6 (4.4)	10.7 (4.3)
Completeness (%)	97.1 (95.9)	94.2 (72.9)	97.6 (95.6)	96.2 (89.3)	97.5 (94.0)
No. of unique observations	76,371	55,580	50,571	54,782 (7,438)	75,992 (10,726)
Redundancy	10.4 (10.6)	7.5 (6.9)	7.5 (7.2)	3.9 (3.5)	2.4 (2.4)
Phasing					
Resolution range in phasing (Å)	20.0–2.3	20.0–2.3	20.0–2.3		
Figure of merit	After SOLVE		0.65		
	After RESOLVE		0.78		

Values in parentheses are for the highest resolution shell.

*$R_{sym} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \overline{I(hkl)}|/\Sigma_{hkl}\Sigma_i\overline{I(hkl)}$

Table S2. Refinement statistics for "native," "sucrose," and "maltose" data

Parameter	Native	Sucrose soak	Maltose soak
Resolution range (Å)	20.00–1.65 (1.69–1.65)	20.00–2.22 (2.27–2.22)	20.00–2.00 (2.05–2.00)
R_{cryst}/R_{free} (%)*	16.5/19.5/(26.2/29.5)	19.5/24.3/(29.8/36.2)	16.3/19.9/(22.4/27.4)
No. of atoms			
Protein	7,977	7,960	7,983
Ligands: SUC/MAL/GOL†	-/-/48	46/-/-	-/92/24
Ca^{2+}/water	1/1,065	1/244	1/779
Average *B*-factors (Å^2)			
Protein	22.2	31.9	28.9
Ligands†	-		M1 42.4
		S2 61.9	M2 49.8
		S1 42.3	M3 47.8
			M4 29.1
Ca^{2+}/water/GOL	9.9/25.3/27.6	36.5/31.5/-	20.0/35.1/45.4
R.m.s. deviations from ideal			
Bond lengths (Å)/angles (°)	0.022/1.6	0.014/1.4	0.015/1.4
Ramachandran statistics (%)			
Most favored	97.0	96.3	97.8
Additionally allowed	3.0	3.7	2.1
Disallowed	-	-	0.1‡

Values in parenthesis are for the highest resolution shell.
*$R_{cryst} = \Sigma_{hkl}|F_{obs} - F_{calc}|/\Sigma_{hkl}|F_{obs}|$, R_{free} is R_{cryst} for 5% of the unique observations not included in the refinement;
†SUC(S) = sucrose, MAL(M) = maltose, GOL = glycerol;
‡Asn1692 (174.3, −30.4).

Table S3. The effect of EDTA and $CaCl_2$ on GTF180-ΔN hydrolytic and transferase activities

Compound (1 mM)	Hydrolytic activity (%)	Transferase activity (%)
none	100 ± 3	100 ± 9
EDTA	57 ± 11	59 ± 3
$CaCl_2$	244 ± 5	234 ± 4

Table S4. Activity of GTF180- ΔN active site mutants

Enzyme	Relative total activity (%)
GTF180-ΔN	100.00
GTF180-ΔN D1025N	2.67
GTF180-ΔN E1063Q	0.01
GTF180-ΔN D1136N	0.27

Table S5. Identification of the major oligosaccharides formed by GTF180-ΔN from sucrose and maltose, deduced from the enzymatic degradation products

Degree of polymerization	Dextranase	Amyloglucosidase	α-glucosidase	Identity ofoligosaccharide
3	-*	G + G2†	-*	PAN
4	G2 + IG2‡	G + PAN†	-*	1,6-PAN

G = glucose, G2 = maltose, IG2 = isomaltose, PAN = panose, 1,6-PAN = $\alpha(1 \rightarrow 6)$panose.
*no degradation observed
†relatively slow degradation in time
‡relatively fast degradation in time