university of groningen

University Medical Center Groningen

University of Groningen

Characterization of the functional roles of amino acid residues in acceptor binding subsite +1 in the active site of the glucansucrase GTF180 enzyme of Lactobacillus reuteri 180

Meng, Xiangfeng; Pijning, Tjaard; Dobruchowska, Justyna M; Gerwig, Gerrit J; Dijkhuizen, Lubbert

Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M115.687558

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Meng, X., Pijning, T., Dobruchowska, J. M., Gerwig, G. J., & Dijkhuizen, L. (2015). Characterization of the functional roles of amino acid residues in acceptor binding subsite +1 in the active site of the glucansucrase GTF180 enzyme of Lactobacillus reuteri 180. The Journal of Biological Chemistry, 290(50), 30131-30141. https://doi.org/10.1074/jbc.M115.687558

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 22-05-2019

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 50, pp. 30131–30141, December 11, 2015
© 2015 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

Characterization of the Functional Roles of Amino Acid Residues in Acceptor-binding Subsite +1 in the Active Site of the Glucansucrase GTF180 from *Lactobacillus reuteri* 180*

Received for publication, August 24, 2015, and in revised form, October 17, 2015 Published, JBC Papers in Press, October 27, 2015, DOI 10.1074/jbc.M115.687558

Xiangfeng Meng[‡], Tjaard Pijning[§], Justyna M. Dobruchowska[‡], Gerrit J. Gerwig[‡], and Lubbert Dijkhuizen[‡1]

From the Departments of [‡]Microbial Physiology and [§]Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

α-Glucans produced by glucansucrase enzymes hold strong potential for industrial applications. The exact determinants of the linkage specificity of glucansucrase enzymes have remained largely unknown, even with the recent elucidation of glucansucrase crystal structures. Guided by the crystal structure of glucansucrase GTF180-ΔN from *Lactobacillus reuteri* 180 in complex with the acceptor substrate maltose, we identified several residues (Asp-1028 and Asn-1029 from domain A, as well as Leu-938, Ala-978, and Leu-981 from domain B) near subsite +1 that may be critical for linkage specificity determination, and we investigated these by random site-directed mutagenesis. First, mutants of Ala-978 (to Leu, Pro, Phe, or Tyr) and Asp-1028 (to Tyr or Trp) with larger side chains showed reduced degrees of branching, likely due to the steric hindrance by these bulky residues. Second, Leu-938 mutants (except L938F) and Asp-1028 mutants showed altered linkage specificity, mostly with increased (α1→6) linkage synthesis. Third, mutation of Leu-981 and Asn-1029 significantly affected the transglycosylation reaction, indicating their essential roles in acceptor substrate binding. In conclusion, glucansucrase product specificity is determined by an interplay of domain A and B residues surrounding the acceptor substrate binding groove. Residues surrounding the +1 subsite thus are critical for activity and specificity of the GTF180 enzyme and play different roles in the enzyme functions. This study provides novel insights into the structure-function relationships of glucansucrase enzymes and clearly shows the potential of enzyme engineering to produce tailor-made α-glucans.

The ability of lactic acid bacteria to produce large amounts of exopolysaccharides has drawn strong attention for industrial applications in recent years (1–4). Using sucrose as substrate, glucansucrase enzymes of lactic acid bacteria catalyze the synthesis of various α-glucans (5), which are used as biothickening agent in food industry, as plasma expander in medicine, and as separation matrix in research (1–4, 6). These enzymes are generally found in *Leuconostoc*, *Streptococcus*, *Lactobacillus,* and *Weissella* (5, 7) and belong to glycoside hydrolase family 70 (GH70) (8). Together with enzymes from GH13 and GH77, they form clan GH-H, sharing mechanistic, structural, and evolutionary characteristics (5, 9–11). Glucansucrases catalyze reactions via an α-retaining double-displacement mechanism (5, 7, 11, 12). First, the (α1↔2β) glycosidic linkage of the donor substrate sucrose is cleaved, resulting in the formation of a β-glucosyl-enzyme intermediate. Second, an acceptor substrate attacks the β-glucosyl-enzyme intermediate, after which the glucosyl moiety is transferred to the acceptor with retention of the α-anomeric configuration. Depending on the nature of available acceptor substrates, glucansucrases catalyze three different reactions (5, 7). In the polymerization reaction, α-glucan polysaccharide is synthesized using a growing glucan chain as acceptor. The hydrolysis reaction uses water as an acceptor substrate, and sucrose is hydrolyzed into glucose and fructose. In the acceptor reaction, the glucosyl moiety is transferred to either an oligosaccharide (resulting in oligosaccharide synthesis) (13, 14) or a hydroxyl group containing organic molecule (resulting in its glycosylation) (5, 15, 16).

Glucansucrases have a fully conserved catalytic center, but they produce α-glucans with different linkages, *i.e.* dextran with a majority of (α1→6) linkages, mutan with a majority of (α1→3) linkages, alternan with alternating (α1→6) and (α1→3) linkages, and reuteran with (α1→4) and (α1→6) linkages (17). In addition, DSR-E from *Leuconostoc mesenteroides* NRRL B-1299 forms single (α1→2) glucosyl branches on dextran (18–21). Thus, all four possible linkage types between D-glucopyranosyl residues have been found in glucansucrase products. It has been proposed that their linkage specificity is determined by the orientation in which the acceptor substrate binds to the enzyme (7, 9, 17, 22). Thus, residues forming acceptor-binding subsites are expected to be critical in determining the linkage specificity. Before the availability of structural information of glucansucrase proteins, the identification of such residues was difficult and mostly based on the sequence similarity between glucansucrases and closely related GH13 enzymes and crystal structures of the latter. The four homology regions (I to IV) of the GH13 family enzymes, with the three catalytic residues and other residues interacting with donor and acceptor substrate, are also found to be present in GH70 family enzymes (5, 7, 11). Mutation studies are thus mainly targeted residues in the four glucansucrase homology regions I–IV (7, 9, 11, 22, 23), of which some are strictly conserved and others are only moderately conserved. Indeed, mutations in regions I–IV were shown to

* This work was supported by the China Scholarship Council (to X. M.) and by European Union Project NOVOSIDES FP7-KBBE-2010-4-265854 (to L. D.). The authors declare that they have no conflicts of interest with the contents of this article.

[1] To whom correspondence should be addressed: Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands. Tel.: 31-503632150; Fax: 31-503632154; E-mail: l.dijkhuizen@rug.nl.

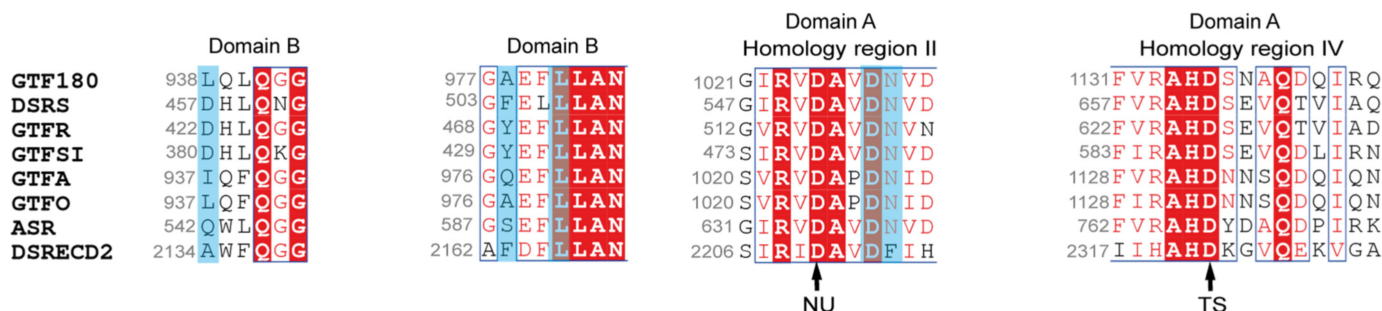

FIGURE 1. **Partial alignment of the amino acid sequences of GH70 glucansucrase enzymes.** Residues Leu-938, Ala-978, Leu-981, Asp-1028, and Asn-1029 of GTF180 and their corresponding residues in other glucansucrase enzymes are highlighted in *blue*. Two amino acid residues involved in enzyme catalysis, the nucleophile residue (*NU*) in homology region II and transition state stabilizer (*TS*) in homology region IV, are indicated.

affect acceptor substrate binding and linkage specificity, confirming the roles of these residues (9, 17, 22, 24–27). In particular, mutations in residues Ser-1137–Asp-1141 (GTF180 numbering) following the transition state stabilizer (Asp-1136) in homology region IV (Fig. 1) have been shown to change the linkage compositions of synthesized α-glucan products in several glucansucrase enzymes (9, 22, 24–28).

The GTF180-ΔN glucansucrase from *Lactobacillus reuteri* 180 produces an α-glucan with 69% (α1→6) and 31% (α1→3) linkages, the latter being present both in the linear sections as well as forming branch points (29). The elucidation of the GTF180-ΔN three-dimensional structure provided new insights and details of donor and acceptor substrate binding in glucansucrases (12), and it allowed us to expand the set of residues contributing to acceptor-binding subsites, including residues outside homology regions I–IV. GTF180-ΔN has five domains (A, B, C, IV, and V) with the active site lying at the interface of the catalytic domains A and B, as revealed by the protein complexes with the donor substrate sucrose (PDB[2] code 3HZ3) and with the acceptor substrate maltose (PDB code 3KLL) (12). First, the crystal structure of the inactive mutant GTF180-ΔN D1025N bound with sucrose revealed that the seven strictly conserved residues (Arg-1023, Asp-1025, His-1135, Asp-1136, Glu-1063, Tyr-1465, and Gln-1509), six of them also employed by GH13 enzymes, make similar interactions with the glucosyl moiety of sucrose (12) bound in subsite −1 (nomenclature according to Ref. 30). Residues Arg-1023, Asp-1025, His-1135, Asp-1136, and Gln-1509 make direct H-bonds to glucosyl hydroxyl groups (12). Residue Asp-1025 acts as the nucleophile residue that attacks the anomeric C1 carbon of the glucosyl unit of sucrose to form a β-glucosyl-enzyme covalent intermediate, stabilized by the transition state stabilizing residue (Asp-1136). Residue Glu-1063 is the acid/base catalyst donating a proton to facilitate the release of fructose and depronating the acceptor molecule to activate it (12). Second, the crystal structure of GTF180-ΔN in complex with maltose revealed this acceptor substrate bound in subsites +1 and +2. At subsite +2, the residues following the transition state stabilizer (Asp-1136) in homology region IV, which have been shown to be important for linkage specificity in previous mutagenesis studies (9, 22, 25–28), are located close to the reducing end moiety of maltose, especially Ser-1137, which has a direct hydrogen bond with the +2 C1 hydroxyl group (12). These observations confirmed the involvement of these residues in forming acceptor binding sites as predicted in previous studies and explain the altered linkage specificity caused by mutating these residues. At subsite +1, residues from homology regions II (Asp-1028 and Asn-1029) in domain A enclose the non-reducing end glucosyl moiety of maltose, providing direct and indirect hydrogen bonds with the C4 and C3 hydroxyl groups (Asn-1029), and a water-mediated hydrogen bond with the C4 hydroxyl group (Asp-1028) (Fig. 2) (12). Notably, in addition to these residues from the homology regions in domain A, residues from two loops in domain B (Leu-938, Leu-940, Ala-978, and Leu-981) are also close to the non-reducing end glucosyl moiety in subsite +1 (Fig. 2). Because of the hydrophobic nature of their side chains, they do not provide hydrogen bond interactions to the acceptor substrate molecule, but they do contribute to shaping the active site near subsite +1 (12, 17). Indeed, in a recent study of dextransucrase DSRS from *L. mesenteroides* NRRL B-512F, guided by the homologous GTF180-ΔN crystal structure, several residues (including residues corresponding to residues Leu-938, Ala-978, and Asn-1029 of GTF180) were targeted for combinatorial mutagenesis (31). Several of these mutants were found to display an altered product linkage distribution. Moreover, in one of our recent studies, we showed that residue Leu-940 from domain B contributes to determining linkage and reaction specificity (32); one of the mutations (L940W) even abolished synthesis of (α1→3) linkages in the products. Finally, residue Trp-1065 has a hydrophobic stacking interaction with both the +1 and +2 glucosyl moiety of maltose. In the complex of GTF180-ΔN D1025N with sucrose, it also has a direct hydrogen bond with the C1 hydroxyl group of the fructosyl moiety. It has been shown that mutating Trp-491 of GTFI from *Streptococcus mutans* (equivalent to Trp-1065 of GTF180-ΔN) to either glycine or alanine resulted in an enzyme devoid of detectable activity (17, 33). Mutation of Trp-1065 in GTF180-ΔN indeed heavily impaired the enzyme activity (preliminary results). Therefore, we did not include Trp-1065 mutations in this study. Instead, residues (Leu-938, Ala-978, and Leu-981) of domain B and domain A (Asp-1028 and Asn-1029) in GTF180-ΔN were individually subjected to random mutagenesis. A large number of mutants were characterized, and their α-glucan polysaccharide products were structurally analyzed, determining the linkage type distributions and their substitution pattern by NMR

[2] The abbreviation used is: PDB, Protein Data Bank.

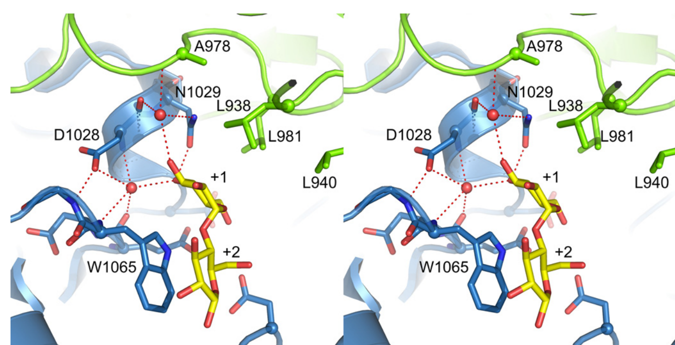

FIGURE 2. **Stereo view of GTF180-ΔN with the acceptor maltose (*yellow* carbon atoms) bound in subsites +1 and +2 (PDB code 3KLL) (12).** Residues Asn-1029 and Asp-1028 from domain A (*blue*) provide direct and indirect (water-mediated) hydrogen bonds to the non-reducing end glucosyl unit bound at subsite +1, respectively. Residues Leu-938, Leu-940, Ala-978, and Leu-981 from domain B (*green*) are also near subsite +1.

spectroscopy and methylation analysis, respectively. Combining the experimental results with the three-dimensional structures of the GTF180-ΔN sucrose and maltose complexes showed that in GTF180-ΔN (*a*) Ala-978 is important for the degree of branching, (*b*) Asp-1028 and Leu-938 are critical for linkage specificity, and (*c*) Leu-981 and Asn-1029 are essential for the transglycosylation reaction. This study provides further insights into the structure-function relationships of GH70 glucansucrase enzymes and offers tools to expand and control the diversity of their α-glucan products. These results are discussed in combination with previous mutagenesis studies and residues present in other glucansucrase enzymes.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions—*Escherichia coli* DH5α (Phabagen, Utrecht, The Netherlands) was used for DNA manipulations. *E. coli* BL21 Star DE3 (Invitrogen) was used for recombinant protein expression. *E. coli* strains were routinely grown in LB medium with the appropriate antibiotic at 37 °C. LB agar plates were made by adding 1.5% agar to the LB medium. The plasmid p15GTF180-ΔN-SX, containing N-terminally truncated GTF180 (residue 742–1772), was used for mutagenesis and protein production (27).

Sequence Alignments—Clustal Omega was used to align the amino acid sequences of GTF180 (Q5SBN3) from *L. reuteri* 180, DSRS (Q9ZAR4) from *L. mesenteroides* NRRL B-512F, GTFR (Q9LCH3) from *Streptococcus oralis* ATCC10557, GTFSI (P13470) from *Streptococcus mutans*, GTFA (Q5SBL9) from *L. reuteri* 121, GTFO (Q4JLC7) from *L. reuteri* ATCC 55730, alteransucrase Asr (Q9RE05) from *L. mesenteroides* NRRL B-1355, and DSRE CD2 (Q8G9Q2) from *L. mesenteroides* NRRL B-1299. Then the aligned sequences were submitted to ESPript for alignment with the GTF180-ΔN crystal structure (PDB code 3KLK) (34).

Site-directed Random Mutagenesis—Site-directed random mutagenesis was performed as described previously (32). Briefly, the QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) was used to introduce mutations using primer pairs for each targeted residue (Table 1) and p15GTF180-ΔN-SX as a template. The PCR product was cleaned up with the PCR clean-up kit (Sigma) and was then transformed into *E. coli*

TABLE 1
Primer pairs used for site-directed mutagenesis of *gtf180*-ΔN
NNS indicates the randomized codon; N, A/G/C/T; S, G/C.

Targeted amino acid	Primer pairs (5′ →3′)
Leu-938	GATTACGGTGGTNNSCAATTACAAGG
	CCTTGTAATTGSNNACCACCGTAATC
Ala-978	AGAACTATGGTGGTNNSGAATTCTTATTAGC
	GCTAATAAGAATTCSNNACCACCATAGTTCT
Leu-981	GGTGCGGAATTCNNSTTAGCTAATGAT
	ATCATTAGCTAASNNGAATTCCGCACC
Asp-1028	CGAGTGGATGCTGTTNNSAATGTAGATGTTGAC
	GTCAACATCTACATTSNNAACAGCATCCACTCG
Asn-1029	GATGCTGTTGATNNSGTAGATGTTGAC
	GTCAACATCTACSNNATCAACAGCATC

BL21 Star (DE3). After selection on LB agar plates containing 100 μg/ml ampicillin, colonies were inoculated in 96-well plates with LB medium containing 100 μg/ml ampicillin. The overnight cultures were inoculated and propagated into new 96-well plates with fresh LB medium containing 100 μg/ml ampicillin and 0.1 mM isopropyl β-D-thiogalactopyranoside at 18 °C for 24 h. The cells were lysed with B-PER protein extraction reagent (Thermo Scientific, Bleiswijk, The Netherlands). The supernatants obtained were used as crude enzyme extracts and were incubated with 0.1 M sucrose in 50 mM sodium acetate buffer, pH 4.5, 1 mM $CaCl_2$, at 37 °C for 15 min. The activity of each putative GTF180-ΔN mutant was monitored by measuring the release of reducing sugar with dinitrosalicylic acid reagent (35) and normalized with $A_{600\ nm}$ of the corresponding culture. To reduce the probability of mutation redundancy, putative GTF180-ΔN mutants, displaying different level of activities, were selected to isolate plasmid DNA and sequenced to verify the mutations. DNA sequencing was performed by LGC Genomics (Berlin, Germany).

Expression and Purification of GTF180-ΔN Mutant Enzymes—Wild-type GTF180-ΔN and selected mutants were expressed using *E. coli* BL21 Star (DE3) as host. Fresh LB medium was inoculated with 1% (v/v) of the overnight culture of *E. coli* BL21 Star (DE3) containing the relevant plasmids and propagated to $A_{600\ nm}$ 0.4–0.6. Then, the expression of enzymes was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside, and cultivation was continued for 20 h at 18 °C. Cells were harvested by centrifugation (10,000 × *g*, 10 min). Mutant enzymes were purified as described previously (36). Protein concentrations were determined by reading the absorbance at 280 nm, using a NanoDrop 2000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands).

Enzyme Activity Assays—The enzymatic activities of the different mutants were assayed as described previously (37). Briefly, the total activity with sucrose was measured by the release of fructose. One unit of enzyme activity was defined as the release of 1 μmol of fructose/min. The enzymatic assay was routinely performed in 25 mM sodium acetate buffer, 1 mM $CaCl_2$, pH 4.5, at 50 °C with 30–100 nM enzymes. Samples of 25 μl were withdrawn per min (5 min in total) and inactivated with 2.5 μl of 1 M NaOH. Kinetic parameters (K_m and k_{cat}) were determined using 12 different sucrose concentrations (ranging from 0.5 to 200 mM) using Michaelis-Menten kinetic equations in SigmaPlot.

Production of α-Glucans by GTF180-ΔN Mutants Incubated with 0.1 M Sucrose—Sucrose (0.1 M) was incubated with mutant enzymes (1.0 unit/ml) in 25 mM sodium acetate buffer, 1 mM $CaCl_2$, pH 4.5, at 37 °C. The depletion of sucrose was verified by thin layer chromatography (TLC). Samples (1 μl) were spotted on TLC sheets (Merck Silica Gel 60 F254, 20 × 20 cm). A mixture of glucose and malto-oligosaccharides (DP2 to DP7) was used as standard. The TLC plates were developed with 2-butanol/acetic acid/water = 2:1:1 and stained with orcinol/sulfuric acid. Reactions were stopped by heating at 100 °C for 10 min. Polysaccharides were isolated by adding 2 volumes of cold ethanol as described previously (37). The mixtures were incubated overnight at 4 °C, and the polysaccharides were collected by centrifugation (4500 × *g*, 20 min). The precipitated polysaccharides were washed with 2 volumes of cold ethanol. At the end of the reaction and before ethanol precipitation, the amount of glucose released was measured as described previously (37) and used to calculate the percentage of sucrose used for the hydrolysis reaction.

Linkage Composition Analysis of Polysaccharides Produced by Mutant Enzymes—The linkage composition of the polysaccharide produced by each mutant enzyme was analyzed by 500-MHz ^{1}H NMR spectroscopy and methylation analysis. ^{1}H NMR spectra were recorded on a Varian Inova Spectrometer (NMR Center, University of Groningen) at a probe temperature of 300 K. Prior to NMR analysis, samples were exchanged twice with D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization and dissolved in 600 μl of D_2O. Chemical shifts were expressed in parts/million by reference to internal standard acetone (δ 2.225). The percentage of different linkages was estimated by integration of the respective signal peak areas.

Methylation analysis was performed as described previously (29, 38). Briefly, samples of isolated polysaccharides (~5 mg) were permethylated using CH_3I and solid NaOH in Me_2SO. 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) and neutralized by adding 4 M acetic acid. Then, boric acid was removed by co-evaporation with methanol. The mixture was acetylated with pyridine/acetic anhydride (1:1, v/v) for 30 min at 120 °C, yielding mixtures of partially methylated alditol acetates, which were analyzed by GLC-EI-MS on a GCMS-QP2010 plus instrument (Shimadzu) using an EC-1 column (30 m × 0.25 mm, Alltech) and a temperature program of 140–250 °C at 8 °C/min.

Size Exclusion Chromatography Analysis of Product Mixtures—The product mixtures obtained by incubating enzymes (1.0 unit/ml) with 0.1 M sucrose were first freeze-dried and dissolved in the eluent (DMSO with 0.05 M LiBr). Samples were incubated overnight and then heated for 30 min at 100 °C. The samples were filtered through a 0.45-μm Millex PTFE membrane (Millipore Corp., Billerica, MA). Size exclusion chromatography analysis was performed using a size exclusion chromatographic system (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany). The setup consisted of an isocratic pump, an auto-sampler without temperature regulation, an online degasser, an inline 0.2-μm filter, a refractive index detector (G1362A 1260 refractive index detector, Agilent Technologies), and multi-angle laser light scattering signal (SLD 7000 PSS). As eluent, DMSO with 0.05 M LiBr was used at a flow rate of 0.5 ml/min. The samples (100 μl) were injected into a PFG guard column and three PFG size exclusion chromatography columns of 100, 300, and 4000 Å. Columns were thermostated at 80 °C, and the refractive index detector was kept at 45 °C. WinGPC Unity software (PSS) was used for data processing.

Results

Mutagenesis of GTF180-ΔN and Identification of Mutant Enzymes—Guided by three-dimensional protein structural information, residues Leu-938, Ala-978, Leu-981, Asp-1028, and Asn-1029 near the acceptor subsite +1 of GTF180 were selected for mutagenesis to characterize their roles in enzyme function. Random mutagenesis was performed targeting these residues separately. For each targeted residue (except for Leu-981), GTF180-ΔN inserts of 18 active colonies were isolated for DNA sequencing to identify their mutations. DNA sequencing of the various selected clones resulted in identification of 24 mutants in total (Table 2). Except for Leu-981 (only two mutants), a proper number of mutants for each residue (Leu-938, five; Ala-978, six; Asp-1028, six; and Asn-1029, five) was obtained. However, most Leu-981 mutations resulted in severely impaired activity, and only eight active colonies were selected for DNA sequencing resulting in the identification of only two Leu-981 mutants. When eight inactive colonies of Leu-981 mutants were selected for further sequencing, four additional mutants (L981G, L981K, L981N, and L981W) were found. To exclude the influence of protein expression on their activities, all mutant enzymes were expressed in *E. coli* BL21 Star DE3 and purified to homogeneity as described previously (36). These mutations had no significant effect on protein expression levels. Compared with wild-type enzyme, four Leu-981 mutants were inactive or showed very low activities (at 100 mM sucrose) (0% for L981K and L981W; <1% for L981G; <3% for L981N). Because of their low activities, these four mutants were not included in the further analysis.

Ala-978 Has an Important Role in Branched Linkage Formation—^{1}H NMR analysis of the polysaccharides produced by Ala-978 mutants (A978G, A978S, A978L, A978P, A978F, and A978Y) showed that the ratio of (α1→6) and (α1→3) linkages showed no significant change compared with that of wild-type GTF180-ΔN (Table 2). In our previous study, it has been shown that the polysaccharide produced by wild-type GTF180-ΔN is built up from five different building blocks, with different lengths of isomalto-oligosaccharides interconnected by (α1→3) linkages (Fig. 3*a*) (29). The (α1→3) linkages are present in both linear and branched orientation {→3)Glc*p*(1→ and →3,6)Glc*p*(1→, respectively} (Fig. 3*a*) (29). The relative intensity of the H-4 signal (t4, between ~δ 3.40 and 3.45) stemming from terminal residues of α-glucan polysaccharides is an indicator for the amount of branching (Fig. 3*b*) (27, 29, 39). Examination of NMR spectra of polysaccharides produced by Ala-978 mutants with a larger side chain (A978L, A978P, A978F, and A978Y) revealed that the intensity of the t4 signal was reduced, indicating a decreased amount of branched linkages; those of Ala-978 mutations with a small amino acid resi-

TABLE 2
Linkage composition of polysaccharides produced and activities of GTF180-ΔN and mutants derived
ND means not determined.

Enzymes	Methylation (%)[a]				Chemical shift (%)[b]		Relative activities[c]	Hydrolysis[d]
	Glc*p*(1→	→3)Glc*p*(1→	→6)Glc*p*(1→	→3,6)Glc*p*(1→	(α1→6)	(α1→3)		
GTF180-ΔN	11	21	55	13	67	33	100	22.2 ± 1.3
L938A	9	12	69	10	78	22	73.7 ± 4.2	36.6 ± 1.6
L938S	8	15	67	10	76	24	65.2 ± 2.0	40.9 ± 0.8
L938F	10	32	46	12	58	42	57.5 ± 2.6	68.2 ± 1.2
L938K	5	7	82	6	90	10	46.3 ± 1.7	33.6 ± 1.5
L938M	13	23	50	14	64	36	67.4 ± 1.5	39.3 ± 0.9
A978F	6	27	60	7	67	33	36.9 ± 1.3	19.7 ± 0.9
A978S	12	20	56	12	68	32	86.9 ± 3.0	19.8 ± 1.0
A978G	9	18	63	10	71	29	92.9 ± 3.2	20.2 ± 1.8
A978L	5	30	59	6	64	36	23.5 ± 0.5	22.5 ± 0.8
A978P	5	28	60	7	68	32	92.3 ± 3.5	16.7 ± 1.5
A978Y	5	27	62	6	67	33	32.8 ± 1.2	24.1 ± 1.0
L981A	15	19	53	13	64	36	7.4 ± 1.2	82.8 ± 4.7
L981E	ND	ND	ND	ND	ND	ND	8.4 ± 2.2	96.2 ± 3.2
D1028Y	8	12	73	7	84	16	8.1 ± 0.5[e]	10.0 ± 1.3
D1028W	7	12	75	6	84	16	13.1 ± 0.4[e]	11.8 ± 1.4
D1028L	12	11	65	12	78	22	30.4 ± 1.9	15.6 ± 1.6
D1028K	13	5	70	12	82	18	7.4 ± 0.4[e]	17.4 ± 0.8
D1028G	13	4	71	12	85	15	31.1 ± 1.3	12.1 ± 0.9
D1028N	11	10	67	12	80	20	36.7 ± 2.6	17.1 ± 0.8
N1029Y	12	21	55	12	68	32	47.7 ± 1.0	59.4 ± 1.5
N1029G	ND	ND	ND	ND	42	58	16.9 ± 1.9	74.2 ± 3.4
N1029T	18	53	10	19	25	75	9.3 ± 0.9	72.2 ± 2.4
N1029M	17	39	24	20	38	62	23.5 ± 2.1	75.7 ± 2.6
N1029R	ND	ND	ND	ND	ND	ND	25.5 ± 1.1	100.5 ± 4.0

[a] The average linkage distribution data are shown in molar percentage based on GLC intensities from duplicate analysis.
[b] The data represent the ratios of integration of the peak areas of the (α1→6) linkage signal at 4.99 ppm and the (α1→3) linkage signal at 5.34 ppm in the ^{1}H NMR spectra of the polysaccharides produced.
[c] Average activities (mean ±S.D.) of triplicate experiments measured with 100 mM sucrose at 50 °C. The activities of all mutant enzymes were relative to that of wild-type GTF180-ΔN (100%).
[d] Values (mean ±S.D.) of triplicate experiments show the percentages of sucrose used for hydrolysis of the total amount of sucrose present initially in the incubations.
[e] The displayed activities were measured at 37 °C with 100 mM sucrose due to reduced stability at 50 °C.

due (A978G and A978S) did not show such a change (Fig. 3*b*). This change was further confirmed by methylation analysis (Table 2, chromatograms in Fig. 4 for methylation analysis). Mutation of Ala-978 to a larger residue (Leu, Pro, Phe, or Tyr) reduced the amount of branched units {→3,6)Glc*p*(1→)} to approximately half of the wild-type value. However, the (α1→3) linkages in the linear section {→3)Glc*p*(1→)} increased correspondingly, explaining the similar overall ratio of (α1→6) and (α1→3) linkages as shown by ^{1}H NMR analysis. In contrast, mutation of Ala-978 to a smaller amino acid (Gly or Ser) had less effect on the degree of branching (Table 2 and Fig. 3). All Ala-978 mutants showed a reduced activity compared with the wild-type enzyme. Only relatively small decreases in activity were observed for mutants A978G, A978S, and A978P, whereas larger decreases were observed for mutants A978L, A978F, and A978Y (Table 2). Kinetic studies revealed that the K_m values of A978G and A978F for sucrose were similar to that of GTF180-ΔN (Table 3), although their k_{cat} decreased, causing an impaired activity. The hydrolysis reactions of all Ala-978 mutants were hardly affected, with some of them showing a slight decrease (Table 2).

Leu-938 and Asp-1028 Are Involved in Linkage Specificity Determination—Analysis of Leu-938 and Asp-1028 mutants revealed a clear shift in linkage type specificity. ^{1}H NMR analysis of mutants L938A/L938S/L938K showed that the percentages of (α1→6) linkages in the α-glucan polysaccharide products increased from 67% in the wild-type to 78, 76, and 90%, respectively (Table 2). Methylation analysis confirmed the increase of (α1→6) linkages in the polysaccharide produced by these mutants (Table 2, L938A, L938S, and L938K). Methylation analysis also revealed that the polysaccharide produced by L938K contained only 6% of branched glucopyranose units {→3,6)Glc*p*(1→)} (as compared with 13% for the wild-type GTF180-ΔN polysaccharide products). The structure of α-glucan produced by mutant L938M was essentially unaffected, while α-glucan polysaccharide synthesized by mutant L938F showed a slight increase in the percentage of (α1→3) linkages.

Substitution of Asp-1028 with different amino acid residues (Gly, Leu, Asn, Lys, Tyr, and Trp) all caused a clear increase in the percentage of (α1→6) linkages at the expense of (α1→3) linkages (Table 2). As shown by methylation analysis of the D1028G, D1028L, D1028N, and D1028K mutant polysaccharides, the relative amount of 6-substituted glucopyranyl units {→6)Glc*p*(1→)} increased from 55 to 65–71% at the expense of the 3-substituted glucopyranyl unit (→3)Glc*p*(1→)}, whereas the percentage of branched glucopyranyl units {→3,6)Glc*p*(1→)} remained almost unaffected. However, the mutations of Asp-1028 to Tyr or Trp resulted in a significant decrease of the percentage of branched units (from 13% to 7 and 6%, respectively). The decrease of branched linkages in the polysaccharides produced by mutants D1028Y and D1028W was also reflected by a reduced t4 signal intensity in their NMR spectra (data not shown), as observed for Ala-978 mutants.

Besides product specificity, the relative activity was also affected. At 100 mM sucrose, mutations of Asp-1028 had more severe effects on relative activity (down to 7–37% of wild-type

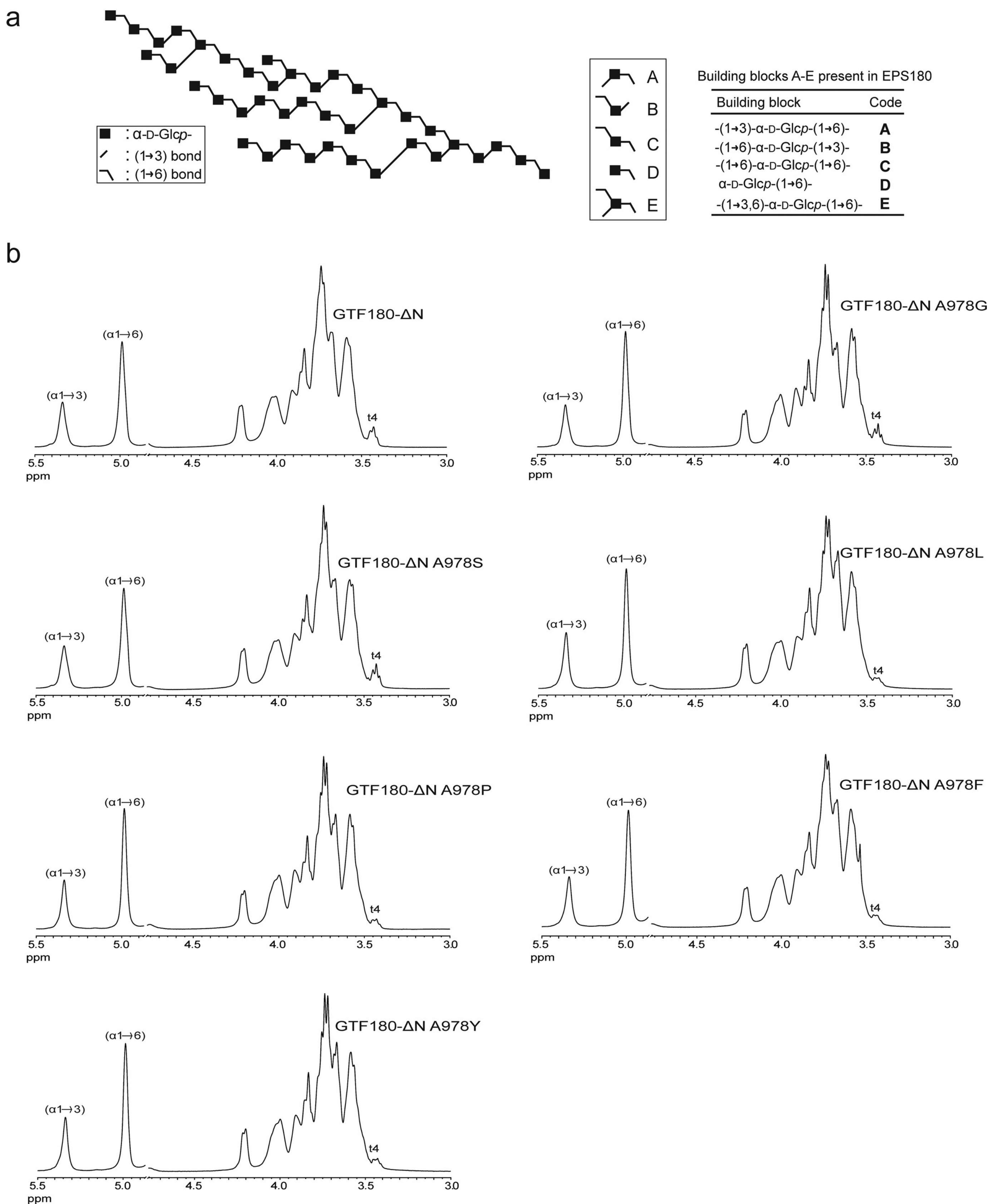

FIGURE 3. *a*, composite model structure of EPS180 as reported in our previous study (29). The building blocks of EPS180 are presented on the *right*. *b*, 500 MHz one-dimensional ^{1}H NMR spectra of the α-glucans produced by GTF180-ΔN and mutants A978G, A978S, A978L, A978P, A978F, and A978Y as indicated in the figure. The H-4 signal of the terminal residue (t4, between ~ δ 3.40 and 3.45), which is an indicator of branched linkages, is indicated.

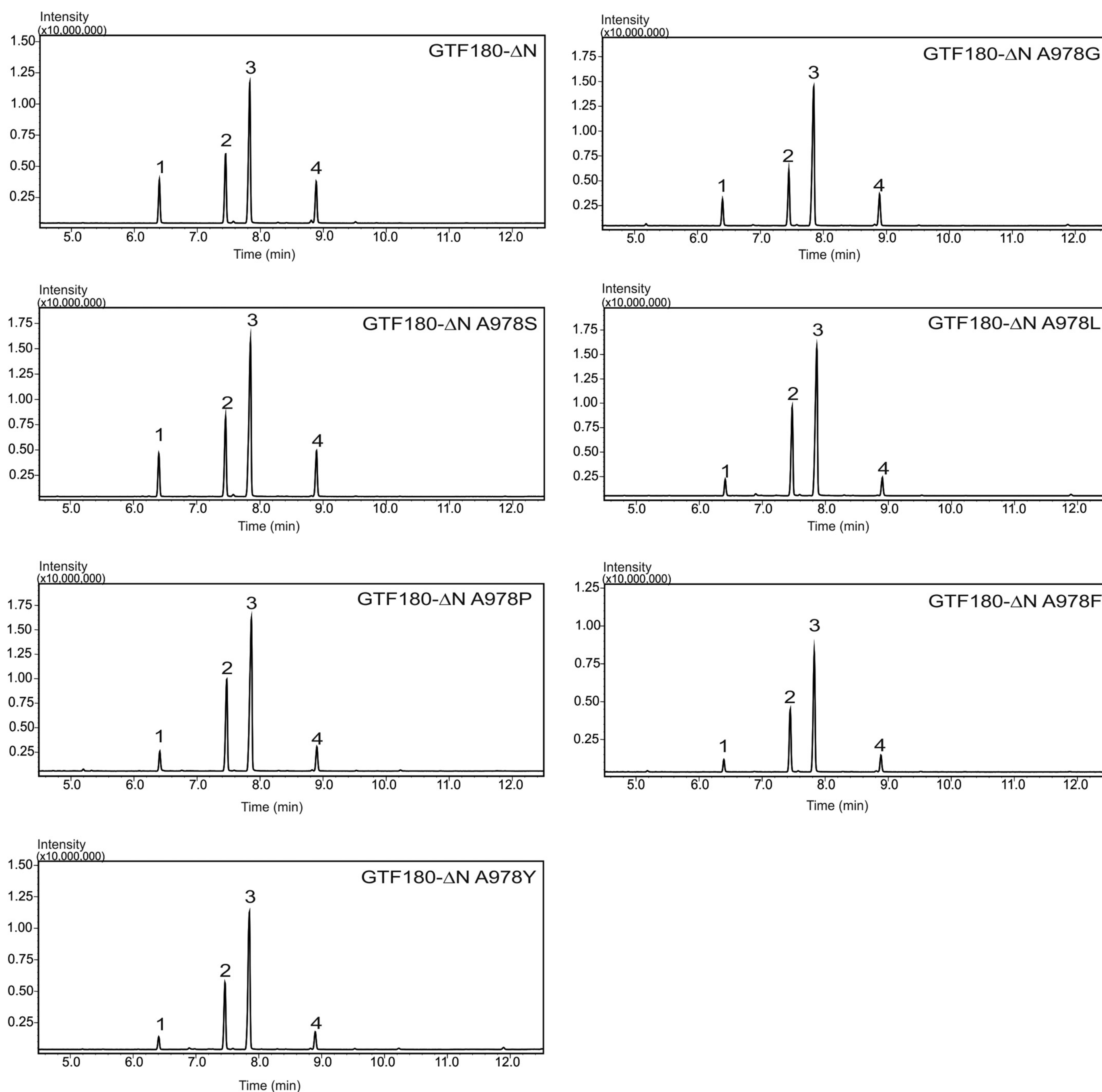

FIGURE 4. **Methylation analysis chromatogram of polysaccharides produced by GTF180-ΔN, A978G, A978S, A978L, A978P, A978F, and A978Y as indicated in the figure by GLC-EI-MS.** *1,* {Glc*p*(1→} (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol); *2,* {→3)Glc*p*(1→)} (1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol); *3,* {→6)Glc*p*(1→)} (1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol); *4,* {→3,6)Glc*p*(1→)} (1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol).

activity) than mutations of Leu-938 (down to 46–74%) (Table 2). Kinetic analysis of L938A, L938F, and D1028L revealed that the major cause of their decrease in activity was a reduced k_{cat} (Table 3), because their K_m values for sucrose were hardly affected (L938A and D1028L) or showed a slight decrease (L938F) (Table 3). In addition, all Leu-938 mutants showed an increased level of hydrolysis, up to 68% for L938F (Table 2). In contrast, Asp-1028 mutants were less hydrolytic, to a varying extent (Table 2).

Leu-981 and Asn-1029 Are Essential for Transglycosylation—Mutants L981A and L981E retained only 7.4 and 8.4% activity, respectively, at 100 mM sucrose. Kinetic analysis of L981A showed that both its K_m and k_{cat} values were affected significantly, resulting in a very low catalytic efficiency (Table 3). Product analysis of L981A and L981E showed that their relative amount of hydrolysis increased significantly to 83 and 96%, respectively (Table 2), indicating that they prefer to use water as acceptor substrate. L981E hardly produced any polysaccharides, while L981A produced minor amounts of polysaccharide. As shown by ^{1}H NMR and methylation analysis, the structure of the α-glucan polysaccharide produced by mutant L981A was similar to that of wild-type GTF180-ΔN (Table 2). To conclude, Leu-981 mutations significantly impaired the enzyme activity

TABLE 3
Kinetic properties of wild-type GTF180-ΔN and mutants derived

Enzymes	K_m[a]	k_{cat}[a]	k_{cat}/K_m
	mM	s^{-1}	$s^{-1}\ mM^{-1}$
GTF180-ΔN[b]	5.0 ± 0.3	303.0 ± 3.6	60.6
L938A	5.7 ± 0.4	212.7 ± 4.4	37.3
L938F	2.8 ± 0.2	165.1 ± 5.7	59.0
A978G	4.8 ± 0.2	259.5 ± 3.7	54.1
A978F	5.4 ± 1.1	88.9 ± 2.9	16.5
L981A	38.4 ± 4.2	31.1 ± 2.6	0.8
D1028L	4.4 ± 0.3	85.2 ± 3.8	19.4
N1029Y	2.9 ± 0.2	136.5 ± 3.2	47.1

[a] The kinetic parameters (K_m for sucrose and k_{cat}) were determined with 12 different sucrose concentrations ranging from 0.5 to 200 mM and presented as means ±S.D. of triplicate experiments.
[b] Data are from a previous study (21).

and affected the reaction specificity of the enzyme, shifting the reaction balance to hydrolysis (L981A and L981E).

Substitution of Asn-1029 by various other residues (Gly, Thr, Met, Arg, and Tyr) strongly increased the hydrolysis, especially for N1029R (Table 2). N1029Y still catalyzed the synthesis of polysaccharide from sucrose (Fig. 5). NMR and methylation analysis showed that the polysaccharide produced by N1029Y was not significantly different compared with that of wild-type GTF180-ΔN. All the other Asn-1029 mutants produced virtually no polysaccharides (Fig. 5). Using sucrose as substrate, N1029G, N1029T, and N1029M catalyzed the synthesis of a small amount of short oligosaccharides (mainly leucrose). NMR analysis of the very low amounts of ethanol-precipitable polymeric material obtained with these mutant enzymes showed an increased amount of (α1→3) linkages (from 58 to 75%), indicating that Asn-1029 is also involved in determination of linkage specificity. Most Asn-1029 mutants showed a relatively low activity (Table 2). Kinetic analysis of mutant N1029Y indicated a slightly decreased K_m (2.9 mM) and a clearly reduced k_{cat} (136.5 s^{-1}) (Table 3). Thus, this mutant enzyme can still efficiently bind the substrate sucrose, and its relatively low activity is mainly caused by a reduced k_{cat}.

Discussion

Although the linkage specificity determinants of glucansucrase enzymes have been the focus of research in several studies, they are still not fully understood. Initially, amino acid residues that were important for linkage specificity determination were identified by primary sequence alignment with GH13 family enzymes, which have been studied extensively, and their crystal structures are available (9, 22). For example, the tripeptide following the transition state stabilizer (Asp-1136) varies among different glucansucrases (Fig. 1) (9, 22, 25, 27). GTF180 contains SNA at this position; mutations targeting these residues altered linkage specificity of GTF180 and even introduced a third type of linkage ((α1→4) linkage) not present in the α-glucan produced by wild-type enzyme (27, 29, 40). The corresponding tripeptide in the dextransucrase DSRS (SEV) has been the subject of mutation studies (22, 31). GTFR from *S. oralis* producing mainly (α1→6)-linked α-glucan and GTFSI from *S. mutans* producing mainly (α1→3) linkages have the same tripeptide as DSRS. Reuteransucrases GTFA and GTFO (synthesizing (α1→4) and (α1→6) linkages) contain an NNS tripeptide, while alternansucrase ASR producing an α-glucan with alternating (α1→6) and (α1→3) linkages has YDA. In all cases, the tripeptide has been shown to be important for linkage specificity determination in the respective glucansucrases (9, 22, 25, 27, 28, 31). Likewise, the fourth and fifth residues following the transition state stabilizer have been targeted for mutagenesis studies and were demonstrated to be critical for linkage specificity (24, 26, 27). The available crystal structures of glucansucrases showed that residues following the transition state stabilizer are close to the +2 acceptor subsite, thus explaining their effects on linkage specificity. However, whereas the above described residues are in domain A, the crystal structure of GTF180-ΔN in complex with maltose revealed that the acceptor-binding site is shaped by residues not only from domain A but also from domain B (12). Thus, the latter may also contribute to acceptor binding and linkage specificity. For example, our previous study, targeting residue Leu-940 from domain B of GTF180-ΔN, showed that it is critical for linkage specificity (32). Surprisingly, mutation L940W completely abolished (α1→3) linkage synthesis and only synthesized (α1→6) linkages (32). This residue shows limited variations among glucansucrases; in reuteransucrase GTFA and GTFO, and DSRE CD2 producing (α1→2) linkages, it is replaced by phenylalanine.

In this study, we show that other residues from domain B (Leu-938, Leu-981, and Ala-978) and domain A (Asp-1028 and Asn-1029) of GTF180-ΔN are also critical for linkage specificity and activity. First, the polysaccharides produced by Ala-978 mutant enzymes showed no significant changes in overall ratio of (α1→6) and (α1→3) linkages. Nevertheless, our detailed structural analysis showed that this residue is important for branched linkage formation, reflected by the reduced amount of branched linkages in the polysaccharides produced by Ala-978 mutant enzymes with larger side chains (Leu, Pro, Phe, and Tyr). These results may be explained by inspection of the GTF180-ΔN-isomaltotriose complex model (Fig. 6) (12). In this model, residue Ala-978 is located near subsite +II′ with its main chain nitrogen hydrogen-bonded to the C3 hydroxyl group of the +II′ glucosyl moiety. Mutating Ala-978 to a large residue may partially block the +II′ subsite and disfavor formation of branched linkages. Such mutations of Ala-978 may also invoke local conformational changes, possibly involving the protein main chain. The observed adverse effects on the synthesis of branched linkage may thus be a result of steric effects near subsite +II′ as well as the loss of hydrogen bond interactions with acceptor substrates. Thus, residue Ala-978 of GTF180 is the first glucansucrase residue identified to be clearly involved in branch formation. Amino acid sequence alignment analysis of different glucansucrase enzymes showed that in DSRS, which produces less branched linkages in its polysaccharide compared with GTF180, the corresponding residue is a phenylalanine. However, Ala-978 is replaced by Tyr, Tyr, and Gln in GTFR, GTFSI, and GTFA, respectively, which still produce relatively high amounts of branches (25, 36, 41); this indicates that the residue at this position may play different roles in different glucansucrase enzymes. Mutant A978G and A978F showed no significant change in their K_m values for sucrose, which can be explained by the fact that in the GTF180-ΔN sucrose complex (PDB code 3HZ3), residue Ala-978 is far (≈11 Å) from the +1 fructosyl moiety of sucrose. The

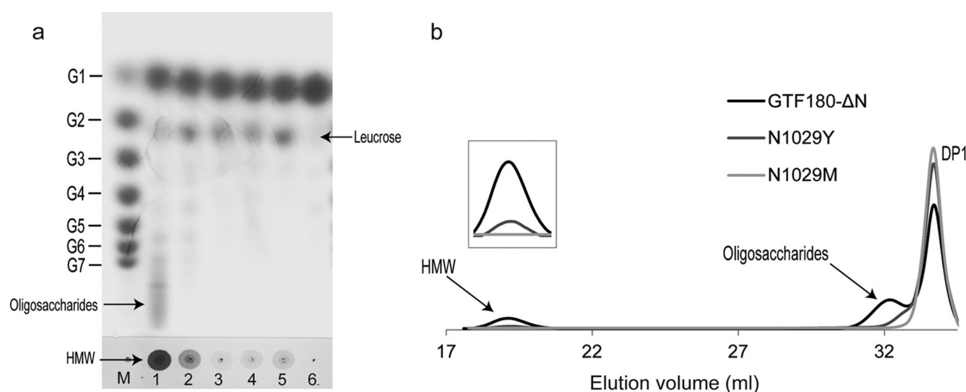

FIGURE 5. *a,* TLC analysis of products formed from incubation of GTF180-ΔN and Asn-1029 mutants (1.0 unit/ml) with 0.1 M sucrose in 25 mM NaAC, 1 mM $CaCl_2$ buffer, pH 4.5, at 37 °C. A mixture of glucose (*G1*) to maltoheptaose (*G7*) was used as standard (*M*). *Lane 1,* GTF180-ΔN; *lane 2,* N1029Y; *lane 3,* N1029G; *lane 4,* N1029T; *lane 5,* N1029M; and *lane 6,* N1029R. *b,* size exclusion chromatography analysis of product mixtures obtained by incubating 1.0 unit/ml GTF180-ΔN, N1029Y, and N1029M with 0.1 M sucrose in 25 mM NaAC, 1 mM $CaCl_2$ buffer, pH 4.5, at 37 °C. *HMW*, high molecular weight polysaccharides (*inset* enlarged for better view).

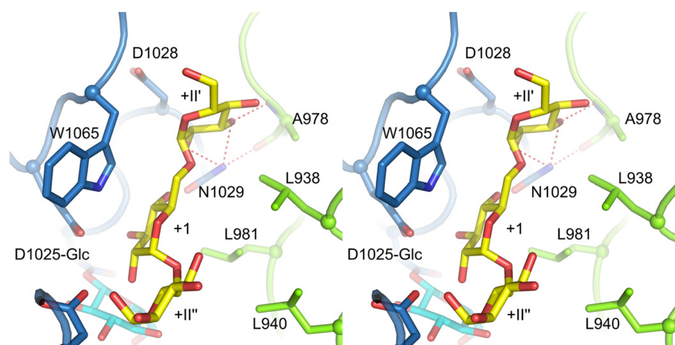

FIGURE 6. **Stereo view of docked isomaltotriose in the active site of modeled GTF180-ΔN glucosyl-enzyme intermediate (7).** Residues from domain A (*blue*) and domain B (*green*) surrounding the +1 and +I′ subsites are indicated, including those (Asp-1028, Asn-1029, Leu-938, Ala-978, and Leu-981) mutated in this study.

impaired activity of mutants can thus be explained by the observed decrease in k_{cat}. Mutations of Ala-978 had no effect on sucrose binding; however, it did affect acceptor substrate binding, as shown above.

The observed effects of Leu-938 mutations in GTF180-ΔN for linkage specificity may be explained by the fact that this residue is located in the N-terminal part of domain B and contributes to shaping the groove near the catalytic site (5, 12), with the +1 glucosyl moiety of maltose bound between Trp-1065 on one side and Leu-938 and Leu-981 on the other side (Fig. 2). Mutating Leu-938 to methionine (similar in size and properties to leucine) hardly affected the linkage composition. However, mutation to alanine or serine, both smaller than the wild-type leucine, increased the amount of (α1→6) linkages. The long and positively charged side chain of lysine in mutant L938K may disfavor the acceptor binding mode necessary for the formation of (α1→3) branches. In contrast, the bulky and neutral aromatic side chain of phenylalanine in mutant L938F does not show such an effect, indicating that not only size but also charge may play a role. Together, the results show that mutations in Leu-938 affect the linkage specificity of GTF180-ΔN, depending on the size and physicochemical properties of the amino acid side chain introduced.

Residue Leu-981 is located in the same N-terminal loop of domain B as Ala-978, delineating the sucrose binding pocket. However, it is much closer (≈4 Å) to the sucrose-binding site. Moreover, Leu-981 is strictly conserved in all the glucansucrase enzymes, suggesting an important role for this residue. Our mutational results with Leu-981 in GTF180-ΔN confirmed this; all mutants showed heavily impaired activities. Mutations in Leu-981 probably impaired the substrate binding causing low activity (L981A, L981E, L981G, and L981N) or even inactivation of the enzyme (L981K and L981W). In addition, the ratio of hydrolysis of mutants L981A and L981E was significantly increased, suggesting that Leu-981 is also essential for transglycosylation.

Our mutation studies show that Asp-1028 affects (α1→6) and (α1→3) linkage synthesis and is also involved in branched linkage formation. Residue Asp-1028 is part of homology region II and is highly conserved in glucansucrases. In the GTF180-ΔN maltose complex (PDB code 3KLL), it is involved in a hydrogen bond network around subsite +1 formed by residues Asp-1028, Asn-1029, and Trp-1065, and it makes a water-mediated hydrogen bond to the C4 hydroxyl group of the non-reducing end glucosyl unit, which is in a productive orientation to form an (α1→6) linkage (Fig. 2) (12). Thus, the observed effects of Asp-1028 mutations on (α1→6) and (α1→3) linkage synthesis are likely due to changes in the interactions possible with acceptor substrates at subsite +1, disfavoring (α1→3) linkage synthesis. In docking studies with wild-type GTF180-ΔN, isomaltotriose was observed to bind such that its middle glucosyl unit is oriented in favor of (α1→3) branch formation (Fig. 6) (12). In this model, residue Asp-1028 is close (within 5 Å) to the non-reducing end glucosyl moiety at the +II′ subsite (Fig. 6). For mutations involving a large aromatic side chain, the decrease of branched (α1→3) linkage synthesis indicates that steric effects play a role, as these bulky residues may partially block the +II′ subsite (Fig. 6). All Asn-1029 mutants significantly increased the ratio of hydrolysis and (α1→3) linkage synthesis in their α-glucan products (except N1029Y). In the GTF180-ΔN complexes with sucrose and maltose, residue Asn-1029 is involved in a hydrogen bond network, making indirect or direct hydrogen bonds with the sugar moiety in subsite +1 (fructosyl or glucosyl, respectively; Fig. 2), explaining its involvement in the determination of reaction and linkage spec-

ificity. Asn-1029 is part of homology region II and is highly conserved in glucansucrases (Fig. 1), confirming its importance in glucansucrase function. Because glucansucrases producing different linkages contain the same residue in positions 1028–1029 (except for DSRECD2), these two residues are probably not specific residues that determine the difference of linkage specificity in these glucansucrases.

Together, these mutagenesis studies showed that the glucansucrase linkage specificity is not determined by one or a few amino acid residues. Instead, it is determined by an interplay of different amino acid residues from both domain A and domain B. Together, these residues shape the acceptor-binding sites and create the specific micro-physicochemical environment, thus determining which hydroxyl group of the non-reducing end glucosyl moiety of an acceptor substrate is capable of attacking the glucosyl-enzyme intermediate to form the corresponding linkage type. This interplay explains why, even with different amino acid residues at a certain position, different glucansucrases still may have similar linkage specificities, and the glucansucrases containing identical amino acid residues in the specific positions described above synthesize different products. Although the contribution of different residues complicates the rationalization of acceptor specificity in glucansucrases, this study shows that three-dimensional structure-guided mutagenesis is an effective approach for changing the linkage specificity of glucansucrase enzymes and producing novel α-glucans. Combinatorial mutagenesis (involving several residues at the same time) represents an alternative outstanding approach to diversify the linkage composition of α-glucans produced. Recently, such a combinatorial mutagenesis approach, covering several residues from both domain A and domain B, was applied to DSRS from *L. mesenteroides* NRRL B-512F making use of the NMR-based high throughput screening carbohydrate-active enzyme specificity method (31, 42). Indeed, the obtained mutants produced a range of novel α-glucans with an altered relative amount of (α1→3) linkage (3–20%) (43). The ultimate goal of glucansucrase mutation studies would be to have glucansucrase mutants produce tailor-made α-glucans, with desired linkage-type distribution, by creating the acceptor substrate-binding site with the appropriate physicochemical microenvironment. With current insights, this goal is still beyond reach. This study contributes to a better understanding toward the linkage specificity, especially for branched linkage formation, in relation to the three-dimensional structure of the acceptor-binding sites.

Conclusions—This paper reports the three-dimensional structure-guided identification and mutation of amino acid residues around subsite +1 in GTF180-ΔN, some of which are outside the homology regions I–IV that previously have been targeted. Using mutagenesis, we changed the relative amounts of linkage types (increase of (α1→6) linkages in Leu-938 and Asp-1028 mutants), changed the balance between (α1→3) linkages in the linear section and branched (α1→3) linkages (several Ala-978 mutants), or shifted the reaction balance to hydrolysis (Leu-981 and Asn-1029 mutants). Analysis of these results combining those of previous mutagenesis studies makes it evident that the interplay of residues from both domain A and domain B, forming the acceptor-binding site, determines the catalytic activity, reaction, and linkage specificity of the glucansucrase enzymes. This study provides novel insights into the structure-function relationship of glucansucrases and clearly shows the potential of enzyme engineering for the synthesis of tailor-made α-glucans.

Author Contributions—X. M., T. P., and L. D. designed the study. X. M. performed and analyzed most of experiments and wrote the paper. T. P. analyzed the data in combination with enzyme structural information in Figs. 2 and 6. J. M. D. conducted the NMR analysis of polysaccharides in Fig. 3 and Table 2. G. J. G. contributed to the methylation analysis in Table 2. All the authors reviewed the results, revised the manuscript and approved the final version of the manuscript.

Acknowledgment—We acknowledge the strong support of Prof. Johannis P. Kamerling in writing the manuscript. We thank Albert Woortman (Macromolecular Chemistry and New Polysaccharide Materials, University of Groningen) for assistance in size exclusion chromatography analysis of α-glucan products.

References

1. Badel, S., Bernardi, T., and Michaud, P. (2011) New perspectives for lactobacilli exopolysaccharides. *Biotechnol. Adv.* **29,** 54–66
2. Jolly, L., Vincent, S. J., Duboc, P., and Neeser, J. R. (2002) Exploiting expolysaccharides from lactic acid bacteria. *Antonie Van Leeuwenhoek* **82,** 367–374
3. Monsan, P., Bozonnet, S., Albenne, C., Joucla, G., Willemot, R.-M., and Remaud-Siméon, M. (2001) Homopolysaccharides from lactic acid bacteria. *Int. Dairy J.* **11,** 675–685
4. Welman, A. D., and Maddox, I. S. (2003) Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends Biotechnol.* **21,** 269–274
5. Leemhuis, H., Pijning, T., Dobruchowska, J. M., van Leeuwen, S. S., Kralj, S., Dijkstra, B. W., and Dijkhuizen, L. (2013) Glucansucrases: three-dimensional structures, reactions, mechanism, α-glucan analysis and their implications in biotechnology and food applications. *J. Biotechnol.* **163,** 250–272
6. Naessens, M., Cerdobbel, A., Soetaert, W., and Vandamme, E. J. (2005) *Leuconostoc* dextransucrase and dextran: production, properties and applications. *J. Chem. Technol. Biotechnol.* **80,** 845–860
7. Monchois, V., Willemot, R.-M., and Monsan, P. (1999) Glucansucrases: mechanism of action and structure-function relationships. *FEMS Microbiol. Rev.* **23,** 131–151
8. Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., and Henrissat, B. (2014) The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **42,** D490–495
9. Kralj, S., van Geel-Schutten, I. G., Faber, E. J., van der Maarel, M. J., and Dijkhuizen, L. (2005) Rational transformation of *Lactobacillus reuteri* 121 reuteransucrase into a dextransucrase. *Biochemistry* **44,** 9206–9216
10. Svensson, B. (1994) Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity, and stability. *Plant Mol. Biol.* **25,** 141–157
11. MacGregor, E. A., Jespersen, H. M., and Svensson, B. (1996) A circularly permuted α-amylase-type α/β-barrel structure in glucan-synthesizing glucosyltransferases. *FEBS Lett.* **378,** 263–266
12. Vujičić-Žagar, A., Pijning, T., Kralj, S., López, C. A., Eeuwema, W., Dijkhuizen, L., and Dijkstra, B. W. (2010) Crystal structure of a 117-kDa glucansucrase fragment provides insight into evolution and product specificity of GH70 enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 21406–21411
13. Côté, G. L., and Sheng, S. (2006) Penta-, hexa-, and heptasaccharide acceptor products of alternansucrase. *Carbohydr. Res.* **341,** 2066–2072
14. Côté, G. L. (2009) Acceptor products of alternansucrase with gentiobiose. Production of novel oligosaccharides for food and feed and elimination of bitterness. *Carbohydr. Res.* **344,** 187–190

15. Desmet, T., Soetaert, W., Bojarová, P., Křen, V., Dijkhuizen, L., Eastwick-Field, V., and Schiller, A. (2012) Enzymatic glycosylation of small molecules: challenging substrates require tailored catalysts. *Chem. Eur. J.* **18,** 10786–107801
16. Seo, E.-S., Lee, J.-H., Park, J.-Y., Kim, D., Han, H.-J., and Robyt, J. F. (2005) Enzymatic synthesis and anti-coagulant effect of salicin analogs by using the *Leuconostoc mesenteroides* glucansucrase acceptor reaction. *J. Biotechnol.* **117,** 31–38
17. Leemhuis, H., Pijning, T., Dobruchowska, J. M., Dijkstra, B. W., and Dijkhuizen, L. (2012) Glycosidic bond specificity of glucansucrases: on the role of acceptor substrate binding residues. *Biocatal. Biotransform.* **30,** 366–376
18. Brison, Y., Pijning, T., Malbert, Y., Fabre, É., Mourey, L., Morel, S., Potocki-Véronèse, G., Monsan, P., Tranier, S., Remaud-Siméon, M., and Dijkstra, B. W. (2012) Functional and structural characterization of α-(1→2) branching sucrase derived from DSR-E glucansucrase. *J. Biol. Chem.* **287,** 7915–7924
19. Bozonnet, S., Dols-Laffargue, M., Fabre, E., Pizzut, S., Remaud-Simeon, M., Monsan, P., and Willemot, R.-M. (2002) Molecular characterization of DSR-E, an α-1,2 linkage-synthesizing dextransucrase with two catalytic domains. *J. Bacteriol.* **184,** 5753–5761
20. Brison, Y., Fabre, E., Moulis, C., Portais, J. C., Monsan, P., and Remaud-Siméon, M. (2010) Synthesis of dextrans with controlled amounts of α-1,2 linkages using the transglucosidase GBD-CD2. *Appl. Microbiol. Biotechnol.* **86,** 545–554
21. Brison, Y., Laguerre, S., Lefoulon, F., Morel, S., Monties, N., Potocki-Véronèse, G., Monsan, P., and Remaud-Simeon, M. (2013) Branching pattern of gluco-oligosaccharides and 1.5-kDa dextran grafted by the α-1,2 branching sucrase GBD-CD2. *Carbohydr. Polym.* **94,** 567–576
22. Moulis, C., Joucla, G., Harrison, D., Fabre, E., Potocki-Veronese, G., Monsan, P., and Remaud-Simeon, M. (2006) Understanding the polymerization mechanism of glycoside-hydrolase family 70 glucansucrases. *J. Biol. Chem.* **281,** 31254–31267
23. van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L., and van Geel-Schutten, I. G. (2006) Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. Rev.* **70,** 157–176
24. Côté, G. L., and Skory, C. D. (2014) Effects of mutations at threonine-654 on the insoluble glucan synthesized by *Leuconostoc mesenteroides* NRRL B-1118 glucansucrase. *Appl. Microbiol. Biotechnol.* **98,** 6651–6658
25. Hellmuth, H., Wittrock, S., Kralj, S., Dijkhuizen, L., Hofer, B., and Seibel, J. (2008) Engineering the glucansucrase GTFR enzyme reaction and glycosidic bond specificity: toward tailor-made polymer and oligosaccharide products. *Biochemistry* **47,** 6678–6684
26. Monchois, V., Vignon, M., and Russell, R. R. (2000) Mutagenesis of Asp-569 of glucosyltransferase I glucansucrase modulates glucan and oligosaccharide synthesis. *Appl. Environ. Microbiol.* **66,** 1923–1927
27. van Leeuwen, S. S., Kralj, S., Eeuwema, W., Gerwig, G. J., Dijkhuizen, L., and Kamerling, J. P. (2009) Structural characterization of bioengineered α-D-glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180. *Biomacromolecules* **10,** 580–588
28. Kralj, S., Eeuwema, W., Eckhardt, T. H., and Dijkhuizen, L. (2006) Role of asparagine 1134 in glucosidic bond and transglycosylation specificity of reuteransucrase from *Lactobacillus reuteri* 121. *FEBS J.* **273,** 3735–3742
29. van Leeuwen, S. S., Kralj, S., van Geel-Schutten, I. H., Gerwig, G. J., Dijkhuizen, L., and Kamerling, J. P. (2008) Structural analysis of the α-D-glucan (EPS180) produced by the *Lactobacillus reuteri* strain 180 glucansucrase GTF180 enzyme. *Carbohydr. Res.* **343,** 1237–1250
30. Davies, G. J., Wilson, K. S., and Henrissat, B. (1997) Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J.* **321,** 557–559
31. Iraque, R., Tarquis, L., André, I., Moulis, C., Morel, S., Monsan, P., Potocki-Véronèse, G., and Remaud-Siméon, M. (2013) Combinatorial engineering of dextransucrase specificity. *PloS one* **8,** e77837
32. Meng, X., Dobruchowska, J. M., Pijning, T., López, C. A., Kamerling, J. P., and Dijkhuizen, L. (2014) Residue Leu^{940} has a crucial role in the linkage and reaction specificity of the glucansucrase GTF180 of the probiotic bacterium *Lactobacillus reuteri* 180. *J. Biol. Chem.* **289,** 32773–32782
33. Tsumori, H., Minami, T., and Kuramitsu, H. K. (1997) Identification of essential amino acids in the *Streptococcus mutans* glucosyltransferases. *J. Bacteriol.* **179,** 3391–3396
34. Gouet, P., Courcelle, E., Stuart, D. I., and Metoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* **15,** 305–308
35. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31,** 426–428
36. Kralj, S., van Geel-Schutten, G. H., van der Maarel, M. J., and Dijkhuizen, L. (2004) Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase. *Microbiology* **150,** 2099–2112
37. Van Geel-Schutten, G. H., Faber, E. J., Smit, E., Bonting, K., Smith, M. R., Ten Brink, B., Kamerling, J. P., Vliegenthart, J. F., and Dijkhuizen, L. (1999) Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. *Appl. Environ. Microbiol.* **65,** 3008–3014
38. Ciucanu, I., and Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **131,** 209–217
39. van Leeuwen, S. S., Leeflang, B. R., Gerwig, G. J., and Kamerling, J. P. (2008) Development of a ^{1}H NMR structural-reporter-group concept for the primary structural characterisation of α-D-glucans. *Carbohydr. Res.* **343,** 1114–1119
40. van Leeuwen, S. S., Kralj, S., Gerwig, G. J., Dijkhuizen, L., and Kamerling, J. P. (2008) Structural analysis of bioengineered α-D-glucan produced by a triple mutant of the glucansucrase GTF180 enzyme from *Lactobacillus reuteri* strain 180: generation of (α1→4) linkages in a native (1→3)/(1→6)-α-D-glucan. *Biomacromolecules* **9,** 2251–2258
41. Ito, K., Ito, S., Shimamura, T., Weyand, S., Kawarasaki, Y., Misaka, T., Abe, K., Kobayashi, T., Cameron, A. D., and Iwata, S. (2011) Crystal structure of glucansucrase from the dental caries pathogen *Streptococcus mutans*. *J. Mol. Biol.* **408,** 177–186
42. Iraque, R., Massou, S., Moulis, C., Saurel, O., Milon, A., Monsan, P., Remaud-Siméon, M., Portais, J. C., and Potocki-Véronèse, G. (2011) NMR-based structural glycomics for high-throughput screening of carbohydrate-active enzyme specificity. *Anal. Chem.* **83,** 1202–1206
43. Iraque, R., Rolland-Sabaté, A., Tarquis, L., Doublier, J. L., Moulis, C., Monsan, P., Remaud-Siméon, M., Potocki-Véronèse, G., and Buléon, A. (2012) Structure and property engineering of α-D-glucans synthesized by dextransucrase mutants. *Biomacromolecules* **13,** 187–195