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# Synthesis of New Hyperbranched α-Glucans from Sucrose by *Lactobacillus reuteri* 180 Glucansucrase Mutants

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Supporting Information

**ABSTRACT:** α-Glucans produced by glucansucrase enzymes of lactic acid bacteria attract strong attention as novel ingredients and functional biopolymers in the food industry. In the present study, α-helix 4 amino acid residues D1085, R1088, and N1089 of glucansucrase GTF180 of *Lactobacillus reuteri* 180 were targeted for mutagenesis both jointly and separately. Analysis of the mutational effects on enzyme function revealed that all D1085 and R1088 mutants catalyzed the synthesis of hyperbranched α-glucans with 15−22% branching (α1→3,6) linkages, compared to 13% in the wild-type GTF180. In addition, besides native (α1→6) and (α1→3) linkages, all of the mutations introduced a small amount of (α1→4) linkages (5% at most) in the polysaccharides produced. We conclude that α-helix 4 residues, especially D1085 and R1088, constituting part of the +2 acceptor binding subsite, are important determinants for the linkage specificity. The new hyperbranched α-glucans provide very interesting structural diversities and may find applications in the food industry.

**KEYWORDS:** *enzyme mutation, glucansucrase, hyperbranched α-glucan, product specificity, GTF180, Lactobacillus reuteri*



## INTRODUCTION

Sucrose is the second largest commercially available carbohydrate in terms of amount with an annual production of about 130 million tons.[1] An avenue for obtaining high-value products from sucrose is the synthesis of α-glucan polysaccharides and oligosaccharides for the food industry.[2,3] Lactic acid bacteria synthesize α-glucan polysaccharides from sucrose, using glucansucrase enzymes.[4−6] Depending on the linkage composition, different α-glucans are defined: dextran, containing mainly (α1→6) linkages; mutan, consisting predominantly of (α1→3) linkages; alternan, comprising alternating (α1→6) and (α1→3) linkages; and reuteran, containing (α1→4) and (α1→6) linkages.

The structural variability of α-glucans offers a large set of physicochemical properties and biological functionalities and, therefore, a wide range of applications in food, medicine, and research.[2,3,7] In the food industry, the generally recognized as safe (GRAS) status of lactic acid bacteria facilitates the use of their exopolysaccharides such as α-glucans as food additives,[2,3,8] where they are mainly used as thickening, gelling, and emulsifying agents to improve the texture and quality of food products such as yogurt and breads. It has been reported that bakery products with dextran have improved softness and increased volume.[9] Moreover, with the availability of efficient acceptor substrates such as maltose and isomaltose, the glucansucrase activity can be shifted from polysaccharide synthesis to oligosaccharide synthesis.[10−12] The oligosaccharides produced from sucrose by glucansucrases hold great potential as novel food ingredients with prebiotic activity to stimulate the growth/activity of intestinal beneficial bacteria.[7,13−15] For example, isomalto-oligosaccharides possessing prebiotic properties can be produced using a dextransucrase and maltose or glucose as acceptor substrate.[16,17] The oligosaccharides synthesized by glucansucrase enzymes using maltose, lactose, and cellobiose as acceptor substrates have been characterized and reported to have prebiotic activities.[11,13,14,18] Similarly, oligosaccharides with (α1→2) branching produced by DSRE from *Leuconostoc mesenteroides* NRRL B-1299 were shown to stimulate the growth of *Bifidobacterium* and increase the production of short-chain fatty acids (SCFA).[19] In the medical industry, dextrans with a molecular size between 40 and 100 kDa are used as blood plasma expander and recognized as clinical dextran.[7,20] In research, cross-linked dextrans (Sephadex) produced by Pharmacia (GE Healthcare) are used for purification and separation of biomolecules such as proteins and carbohydrates.[7]

Altering the linkage specificity of glucansucrases potentially allows synthesis of an even larger diversity of α-glucans with different properties and applications. Glucansucrases are classified in glycoside hydrolase family 70 (GH70) in the CAZy database.[21] The catalytic machinery of glucansucrases is highly conserved and employs a conserved catalytic triad (D1025 as nucleophile, E1063 as acid/base catalyst, and D1136 as transition state stabilizer; GTF180 numbering).[22] Linkage specificity has been proposed to be determined by only a small number of amino acids shaping the acceptor substrate binding site.[23,24] In this regard, previous mutagenesis studies based on sequence alignments and targeting mainly homology regions I−IV of glucansucrases have identified several residues that are assumed to be involved in defining acceptor binding sites of glucansucrases.[4,6,25,26] For instance, the amino acids (S1137, N1138, A1139, Q1140, and D1141) following the transition

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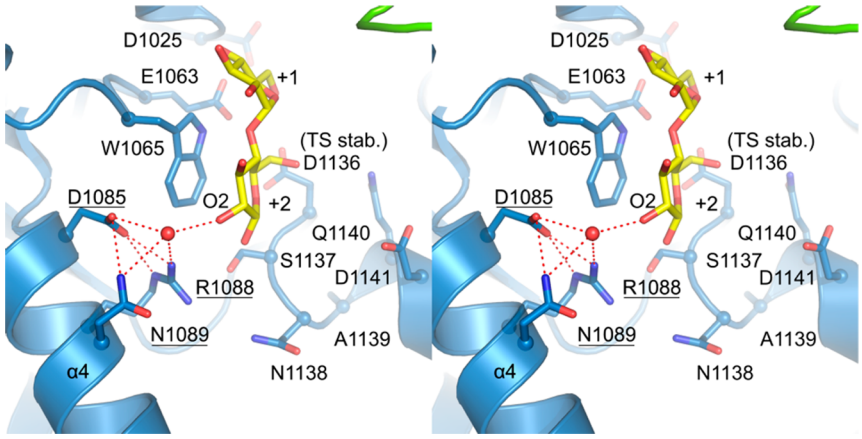


**Figure 1.** Stereoview of the GTF180-ΔN protein (PDB 3KLL) with the acceptor maltose (yellow carbon atoms) bound in subsites +1 and +2. Residues D1085, R1088, and N1089 (underlined) from α-helix 4 in domain A all form an indirect hydrogen bond with the C2-hydroxyl group of the subsite +2 bound glucosyl unit of maltose through the same water molecule. On the other (O6/O5/O1) side of this glucosyl unit, residues S1137–D1141 immediately downstream of D1136 (transition state stabilizing residue), are indicated as well.

state stabilizer D1136 (GTF180 numbering) in homology region IV were found to be important for linkage specificity of several glucansucrase enzymes.[23,24,26−29] Crystal structures of N-terminally truncated GTF180 (GTF180-ΔN, retaining amino acids 742−1772 and having the same product specificity as GTF180) with bound maltose confirmed that these amino acid residues are involved in shaping the acceptor binding sites and provided a structural explanation for the effects of different mutations observed previously.[22] At acceptor binding site +2, residues S1137, N1138, A1139, Q1140, and D1141 are located at one side of the +2 glucosyl unit (Figure 1).[22] Notably, S1137 has a direct hydrogen bond with the +2 C1 hydroxyl group.[22] The crystal structures also revealed that at the other side of the +2 glucosyl unit, the nonconserved residues D1085, R1088, and N1089 of α-helix 4 form a hydrogen bond network with the reducing end glucosyl unit of maltose (Figure 1).[30] The roles of these three residues in linkage specificity have not been studied in detail yet. Therefore, in our current study, residues D1085, R1088, and N1089 in GTF180-ΔN were mutated together to the corresponding residues in other glucansucrases or to some rationally conceived residues; in addition, each residue was mutated separately and randomly to evaluate its effects on enzyme function.

## MATERIALS AND METHODS

**Bacterial Strains, Plasmid, and Growth Conditions.** *Escherichia coli* DH5α (Phabagen, Utrecht, The Netherlands) was used as the host for DNA manipulation; *E. coli* BL21 DE3 star (Invitrogen, Carlsbad, CA, USA) was used to express various mutant enzymes. *E. coli* strains were routinely grown in LB medium with the appropriate antibiotic at 37 °C and 200 rpm, unless specified elsewhere. The plasmid p15GTF180-ΔN-SX, containing N-terminally truncated GTF180 (residues 742−1772), was used for mutagenesis and recombinant protein expression.[28]

**Sequence Alignments.** ClustalW2 was used to align the amino acid sequences of GTF180 (Uniprot: Q5SBN3) from *Lactobacillus reuteri* 180, DSRS (Q9ZAR4) from *L. mesenteroides* NRRL B-512F, DSRBCB4 (D2CFL0) from *L. mesenteroides* B-1299CB4, DSRWC (B9UNL6) from *Weissella cibaria* CMU, GTFR (Q9LCH3) from *Streptococcus oralis* ATCC10557, smGTFB (P08987) from *Streptococcus mutans* GS 5, smGTFC (P13470) from *S. mutans* GS 5, smGTFD (P49331) from *S. mutans* GS 5, 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[31] for alignment based on the GTF180-ΔN crystal structure (PDB 3KLK).[22]

**Site-Directed Mutagenesis.** The Quikchange site-directed mutagenesis protocol (Stratagene, La Jolla, CA, USA) was used to introduce various specific mutations using p15GTF180-ΔN-SX as the template. The appropriate primers used are summarized in Table 1. The PCR products were purified with a GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) and transformed into *E. coli* DH5α. The mutations were verified by isolation of the plasmids and by nucleotide sequencing (LGC Genomics, Berlin, Germany). In addition, site-directed random mutagenesis was performed targeting amino acid residues D1085, R1088, and N1089 separately, and 20 colonies were selected to isolate the plasmids for each targeted residue (residues D1085, R1088, and N1089). The mutations were identified by nucleotide sequencing.

**Enzyme Production and Purification.** Recombinant wild-type GTF180-ΔN and mutant enzymes were expressed in *E. coli* BL21 DE3 star (Invitrogen). Fresh LB medium with 100 μg/mL ampicillin was inoculated with overnight cultures of the *E. coli* BL21 DE3 star strains harboring the appropriate plasmids. The cultures were grown at 37 °C to an absorbance of 0.4−0.6 at 600 nm. Subsequently, the enzyme expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration of 0.1 mM), and the cultures were incubated at 18 °C overnight. The cells were collected by centrifugation (10000*g*, 10 min) and washed with 50 mM Tris/HCl buffer, pH 8.0. The enzymes were purified by $Ni^{2+}$-nitrilotriacetic acid (NTA) affinity chromatography (Sigma-Aldrich) as previously described.[32] Enzyme concentrations were determined by measuring absorbance at 280 nm using a NanoDrop 2000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands).

**Enzyme Activity Assays.** Glucansucrase enzyme activity assays were performed as previously described.[33] Briefly, activity was measured by following the release of fructose from sucrose. One unit of enzyme activity was defined as the release of 1 μmol/min of fructose. Activity assays were routinely performed in 25 mM sodium acetate buffer, 1 mM $CaCl_2$, pH 4.5, at 50 °C with 30−100 nM enzyme. Samples of 25 μL were withdrawn per minute (over a 5 min period 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) and calculated with SigmaPlot using Michaelis−Menten kinetic equations.

**Production of α-Glucans by (Mutant) GTF180-ΔN Enzymes from 100 mM Sucrose.** α-Glucans were produced by incubation of 100 mM sucrose with wild-type and mutant GTF180-ΔN enzymes (1.0 U/mL) in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.5, at 37 °C. Depletion of sucrose was verified by thin-layer chromatography (TLC) analysis. Samples (1 μL) were spotted on silica gel 60 F254,


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**Table 1. Primer Pairs Used for Site-Directed Mutagenesis of *gtf180*-ΔN**

| mutant or targeted amino acid | primer pair (5′→3′) |
|---|---|
| DHT[a] | DHT-For: 5′-GATGATCGTTTACACACTGCAATTATGGATAC-3′<br>DHT-Rev: 5′-GTATCCATAATTGCAGTGTGTAAACGATCATC-3′ |
| NRL | NRL-For: 5′-CAATTAACAATGGATAATCGTTTACGATTGGCAATTATGG-3′<br>NRL-Rev: 5′-CCATAATTGCCAATCGTAAACGATTATCCATTGTTAATTG-3′ |
| DKN | DHT-For: 5′-GATGATCGTTTAAAGAATGCAATTATGGATAC-3′<br>DHT-Rev: 5′-GTATCCATAATTGCATTCTTTAAACGATCATC-3′ |
| VKG | VKG-For: 5′-CAATGGATGTGCGTTTAAAGGGCGCAATTATGGATAC-3′<br>VKG-Rev: 5′-GTATCCATAATTGCGCCCTTTAAACGCACATCCATTG-3′ |
| YTS | YTS-For: 5′-CAATGGATTACCGTTTAACGAGTGCAATTATGGATAC-3′<br>YTS-Rev: 5′-GTATCCATAATTGCACTCGTTAAACGGTAATCCATTG-3′ |
| ETL | ETL-For: 5′-CAATGGATGAGCGTTTAACGCTGGCAATTATGGATAC-3′<br>ETL-Rev: 5′-GTATCCATAATTGCCAGCGTTAAACGCTCATCCATTG-3′ |
| AAA | AAA-For: 5′-CAATGGATGCGCGTTTAGCAGCTGCAATTATGGATAC-3′<br>AAA-Rev: 5′-GTATCCATAATTGCAGCTGCTAAACGCGCATCCATTG-3′ |
| LLL | LLL-For: 5′-CAATTAACAATGGATCTGCGTTTACTGCTCGCAATTATGGATAC-3′<br>LLL-Rev: 5′-GTATCCATAATTGCGAGCAGTAAACGCAGATCCATTGTTAATTG-3′ |
| FFF | FFF-For: 5′-CAATTAACAATGGATTTCCGTTTATTCTTCGCAATTATGGATAC-3′<br>FFF-Rev: 5′-GTATCCATAATTGCGAAGAATAAACGGAAATCCATTGTTAATTG-3′ |
| DED | DED-For: 5′-CAATGGATGATCGTTTAGAGGACGCAATTATGGATAC-3′<br>DED-Rev: 5′-GTATCCATAATTGCGTCCTCTAAACGATCATCCATTG-3′ |
| D1085 | D1085X-For: 5′-CAATTAACAATGGATNNS[b]CGTTTACGAAATG-3′<br>D1085X-Rev: 5′-CATTTCGTAAACGSNNATCCATTGTTAATTG-3′ |
| R1088 | R1088X-For: 5′-GGATGATCGTTTANNSAATGCAATTATGG-3′<br>R1088X-Rev: 5′-CCATAATTGCATTSNNTAAACGATCATCC-3′ |
| N1089 | N1089X-For: 5′-GATGATCGTTTACGANNSGCAATTATGGATAC-3′<br>N1089X-Rev: 5′-GTATCCATAATTGCSNNTCGTAAACGATCATC-3′ |

[a]DHT represents mutating amino acid residues D1085, R1088, and N1089 to D, H, and T, respectively. The same is valid for other mutants. [b]NNS, randomized codon; N, A/G/C/T; S, G/C.

20 × 20 cm TLC sheets (Merck, Darmstadt, Germany). A mixture of glucose and malto-oligosaccharides (DP2−DP7) was used as standard. TLC plates were developed with 2-butanol/acetic acid/water (2:1:1, v/v/v) and stained with orcinol/sulfuric acid. Reactions were stopped by heating for 10 min at 100 °C. The percentages of sucrose used for hydrolysis were determined by measuring the amounts of glucose in the reaction media.[34] The amount of sucrose used for hydrolysis was expressed as the percentage of sucrose being hydrolyzed relative to the amount of sucrose initially added to the incubation mixture. Polysaccharides were then precipitated by adding 2 volumes of cold ethanol.[33]

**Linkage Composition Analysis of Polysaccharides Produced by (Mutant) GTF180-ΔN Enzymes.** The linkage composition of polysaccharide products was determined by NMR spectroscopic analysis and methylation analysis. One-dimensional $^{1}$H NMR, 2D $^{1}$H−$^{1}$H, and 2D $^{13}$C−$^{1}$H correlation spectra were recorded on a Varian Inova spectrometer (Varian, Palo Alto, CA, USA) at a probe temperature of 300 K. Prior to NMR analysis, samples were exchanged twice with $D_2O$ (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) with intermediate lyophilization and dissolved in 600 μL of $D_2O$. Chemical shifts were expressed in parts per million by reference to internal standard acetone ($\delta$ $^{1}$H 2.225; $^{13}$C 31.08). Percentages of different linkages were estimated by integration of the respective signal peak areas. 2D $^{1}$H−$^{1}$H TOCSY spectra were recorded with MLEV17 mixing sequences with 30, 60, and 150 ms spin-lock times. 2D $^{13}$C−$^{1}$H HSQC spectra were recorded with a spectral width of 5000 Hz in t2 and 10000 Hz in t1 direction. All spectra were processed using MestReNova 5.3 (Mestrelabs Research SL, Santiago de Compostela, Spain), using Whittaker Smoother baseline correction.

Methylation analysis was performed as previously described.[35,36] Samples of isolated polysaccharide (~5 mg) were permethylated using $CH_3I$ and solid NaOH in dimethyl sulfoxide. After hydrolysis with 2 M trifluoroacetic acid (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ for 2 h at room temperature in aqueous solution. Conventional workup, comprising neutralization by adding 4 M acetic acid and removal of boric acid by coevaporation with methanol, followed by acetylation with pyridine/acetic anhydride (1:1, v/v) for 30 min at 120 °C, yielded mixtures of partially methylated alditol acetates. Samples were analyzed by GLC-EI-MS on a GCMS-QP2010 plus instrument (Shimadzu, Kyoto, Japan) using an EC-1 column (30 m × 0.25 mm, Alltech) and a temperature program of 140−250 °C at 8 °C/min.


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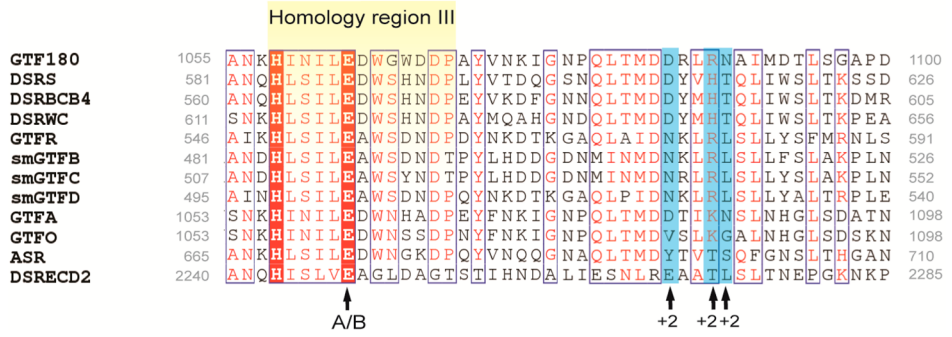


**Figure 2.** Sequence alignment of selected glucansucrases from family GH70 with homology region III highlighted in yellow. Residues D1085, R1088, and N1089, which were the subject of mutation in this study, and their corresponding residues in other glucansucrases aligned, are highlighted in blue.

**Table 2. Characteristics of (Mutant) GTF180-ΔN Enzymes and Their Polysaccharide Products**

| | methylation analysis[a] (%) | | | | | chemical shifts[b] (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| enzyme | Glc*p*(1→ | →3)Glc*p*(1→ | →6)Glc*p*(1→ | →3,6)Glc*p*(1→ | →4)Glc*p*(1→ | (*α*1→6) | (*α*1→3) | (*α*1→4) | MM[c] ($10^6$ Da) | relative total activities[d] | hydrolysis[e] |
| GTF180-ΔN (DRN) | 11 | 21 | 55 | 13 | <1 | 67 | 33 | <1 | 31 | 100 | 22.2 ± 1.3 |
| DHT | 13 | 20 | 50 | 15 | 2 | 64 | 34 | 2 | 22 | 70.0 ± 3.8 | 24.6 ± 0.4 |
| NRL | 16 | 14 | 53 | 16 | 1 | 67 | 32 | 1 | 22 | 48.2 ± 1.7 | 21.4 ± 0.4 |
| DKN | 13 | 22 | 49 | 15 | 1 | 65 | 34 | 1 | 23 | 42.4 ± 1.3 | 29.3 ± 0.8 |
| VKG | 17 | 13 | 52 | 17 | 1 | 68 | 30 | 2 | 19 | 56.7 ± 2.5 | 19.1 ± 1.1 |
| YTS | 16 | 13 | 53 | 17 | 1 | 66 | 33 | 1 | 20 | 44.3 ± 2.4 | 20.2 ± 1.4 |
| ETL | 17 | 17 | 46 | 18 | 2 | 61 | 35 | 4 | 17 | 39.6 ± 1.7 | 29.3 ± 1.4 |
| AAA | 17 | 10 | 54 | 17 | 2 | 69 | 29 | 2 | 17 | 112.8 ± 5.2 | 20.1 ± 1.0 |
| LLL | 17 | 13 | 49 | 19 | 2 | 65 | 33 | 2 | 14 | 102.4 ± 6.3 | 34.0 ± 1.8 |
| FFF | 21 | 13 | 40 | 22 | 4 | 58 | 37 | 5 | 8 | 18.1 ± 2.6 | 36.5 ± 1.9 |
| DED | 14 | 16 | 53 | 15 | 2 | 67 | 31 | 2 | 23 | 39.0 ± 1.2 | 22.9 ± 1.0 |
| D1085A | 16 | 13 | 52 | 18 | 1 | 69 | 29 | 2 | 20 | 47.0 ± 1.8 | 19.2 ± 1.0 |
| D1085V | 15 | 11 | 57 | 16 | 1 | 73 | 25 | 2 | 23 | 60.2 ± 2.5 | 18.3 ± 1.3 |
| D1085L | 17 | 13 | 50 | 18 | 2 | 69 | 29 | 2 | 20 | 48.5 ± 1.9 | 18.2 ± 1.0 |
| D1085E | 15 | 21 | 46 | 17 | 1 | 64 | 35 | 2 | 22 | 31.8 ± 1.0 | 23.3 ± 0.5 |
| D1085Q | 16 | 16 | 49 | 17 | 2 | 65 | 33 | 2 | 19 | 27.5 ± 2.4 | 20.6 ± 1.0 |
| D1085H | 16 | 14 | 51 | 17 | 2 | 69 | 29 | 2 | 23 | 27.6 ± 1.5 | 20.0 ± 0.7 |
| D1085Y | 17 | 12 | 51 | 18 | 2 | 68 | 30 | 2 | 20 | 15.1 ± 1.1 | 22.3 ± 1.4 |
| R1088G | 14 | 15 | 54 | 16 | 1 | 66 | 32 | 2 | 18 | 54.2 ± 2.0 | 27.9 ± 1.2 |
| R1088T | 14 | 18 | 51 | 15 | 2 | 66 | 32 | 2 | 22 | 63.3 ± 4.6 | 26.1 ± 1.1 |
| R1088N | 14 | 19 | 50 | 15 | 2 | 64 | 34 | 2 | 22 | 51.5 ± 2.5 | 27.5 ± 1.4 |
| R1088E | 15 | 15 | 51 | 17 | 2 | 64 | 33 | 3 | 20 | 50.8 ± 1.5 | 26.5 ± 1.1 |
| R1088H | 14 | 20 | 48 | 16 | 2 | 64 | 34 | 2 | 23 | 57.4 ± 2.6 | 22.9 ± 0.5 |
| R1088W | 18 | 11 | 49 | 19 | 3 | 66 | 30 | 4 | 16 | 53.5 ± 2.6 | 21.1 ± 0.5 |
| N1089G | 11 | 21 | 56 | 12 | <1 | 67 | 32 | 1 | 27 | 77.1 ± 4.4 | 21.3 ± 1.7 |
| N1089S | 12 | 20 | 56 | 12 | <1 | 67 | 32 | 1 | 29 | 95.2 ± 3.6 | 19.6 ± 1.6 |
| N1089L | 11 | 23 | 53 | 12 | 1 | 65 | 34 | 1 | 29 | 77.1 ± 2.9 | 22.3 ± 0.9 |
| N1089D | 12 | 18 | 58 | 11 | 1 | 65 | 34 | 1 | 27 | 72.3 ± 3.2 | 21.4 ± 0.9 |
| N1089R | 13 | 17 | 56 | 14 | <1 | 69 | 30 | 1 | 27 | 77.3 ± 3.7 | 18.5 ± 1.2 |
| N1089P | 14 | 17 | 54 | 14 | 1 | 66 | 33 | 1 | 24 | 35.9 ± 3.4 | 21.2 ± 0.7 |
| N1089Y | 13 | 16 | 56 | 14 | 1 | 67 | 32 | 1 | 26 | 55.8 ± 2.3 | 24.7 ± 1.9 |

[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 surface areas of the (*α*1→6) linkage signal at 4.99 ppm, the (*α*1→3) linkage signal at 5.34 ppm, and the (*α*1→4) linkage signal at 5.41 ppm in the $^1$H NMR spectra of the polysaccharides produced. [c]Average molecular mass values from duplicate analysis. [d]Total activities measured as fructose released using 100 mM sucrose at 50 °C. [e]The percentages of sucrose used for hydrolysis of the total amount of sucrose present initially in the incubations are presented.

**Size Determination of Polysaccharides Produced by (Mutant) GTF180-ΔN Enzymes.** The size of polysaccharides was determined using a 1260 Infinity SEC system from PSS (Agilent Technologies, Mainz, Germany). The setup consisted of an isocratic pump, an autosampler without temperature regulation, an online degasser, an inline 0.2 *μ*m filter, a G1362A 1260 RID refractive index



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(RI) detector (Agilent Technologies), and a SLD 7000 multiangle laser light scattering signal (MALLS) detector (PSS, Mainz). As eluent, DMSO with 0.05 M LiBr was used at a flow rate of 0.5 mL/min. Polysaccharide samples were also dissolved in the eluent solvent and incubated overnight. Then, samples were heated for 30 min at 100 °C. The samples (100 μL) were injected into a PFG guard column and three PFG SEC columns 100, 300, and 4000 Å. Columns were thermostated at 80 °C, and the RI detector was kept at 45 °C. The MALLS signal was used to assess the molar masses with an RI increment value (d*n*/d*c*) of 0.072 mL/g. WinGPC Unity software (PSS, Mainz) was used for data processing.

## RESULTS AND DISCUSSION

**Construction and Production of Mutant GTF180-ΔN Enzymes.** GTF180-ΔN of *Lactobacillus reuteri* 180 produces α-glucan polysaccharides with 69% (α1→6) linkages and 31% (α1→3) linkages.[36] Sequence alignments (Figure 2) revealed that D1085, R1088, and N1089 of GTF180 are located downstream of homology region III of glucansucrase proteins and that amino acid residues at these positions vary among different glucansucrases. Dextran-producing glucansucrases of *Leuconostoc* (DSRS, DSRBCB4) and *Weissella* (DSRWC) have residues D, H, and T at these positions, whereas N, R, and L residues are present in glucansucrases of *Streptococcus* (GTFR, smGTFB, smGTFC, and smGTFD). In the reuteran-producing glucansucrases GTFA and GTFO of *L. reuteri*, D, K, and N and V, K, and G are present, respectively. ASR, producing alternan with alternating (α1→6) and (α1→3) linkages, contains Y, T, and S at these positions, whereas DSRE CD2, introducing (α1→2) branches in dextran, possesses E, T, and L. Therefore, residues D1085, R1088, and N1089 in GTF180-ΔN were mutated to the above-described variations to evaluate their roles in enzyme function. To investigate the effects of residue size at these positions, the AAA, LLL, and FFF mutants were constructed. The DED mutant was constructed to study the effects of charge. Furthermore, at each position, site-directed random mutagenesis was performed to investigate the roles of individual residues. All mutant proteins (Table 2) showed a similar expression level in *E. coli* BL21 DE3 star as wild-type GTF180-ΔN and were purified by NTA affinity chromatography.

**Effects of Mutations in GTF180-ΔN on Enzyme Activity.** The effects of mutations on enzyme activity were evaluated first at 100 mM sucrose, showing that relative activities varied between 15 and 113%, compared to wild-type GTF180-ΔN (Table 2). Triple mutants AAA and LLL had a relatively high activity (112.8 and 102.4%, respectively). However, mutant FFF displayed a much lower activity (18.1%), possibly due to the fact that the presence of three bulky side chains close to acceptor binding site +2 may cause a larger structural rearrangement compared to AAA and LLL. All other constructed triple mutants had varying degrees of lower activity compared to that of wild-type GTF180-ΔN. In the single mutants, the relative activities of D1085 mutants were on average lower than those of R1088 or N1089 mutants. Mutant D1085Y retained the lowest activity (15.1%) among all single mutants, whereas the highest relative activity was observed for mutant N1089S (95.2%). In general, mutations of D1085 had more detrimental effects on the activity, followed by R1088 and then by N1089 (Table 2).

Determination of the kinetic parameters of selected triple and single mutants showed that all characterized mutant GTF180-ΔN enzymes displayed Michaelis−Menten type of kinetics with the substrate sucrose, similar to the wild type.[34,37]

**Table 3. Kinetic Parameters[a] of Selected GTF180-ΔN Mutant Enzymes**

| enzyme | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| GTF180-ΔN[b] DRN | 5.0 ± 0.3 | 303.0 ± 3.6 | 60.6 |
| VKG | 4.5 ± 0.3 | 165.9 ± 5.7 | 36.9 |
| ETL | 5.3 ± 0.3 | 116.3 ± 3.9 | 21.9 |
| AAA | 10.9 ± 0.9 | 336.3 ± 11.8 | 30.9 |
| LLL | 7.6 ± 0.5 | 304.3 ± 11.2 | 40.0 |
| FFF | 4.5 ± 0.3 | 64.8 ± 2.8 | 14.4 |
| DED | 2.5 ± 0.2 | 102.0 ± 4.8 | 40.8 |
| D1085A | 6.1 ± 0.5 | 147.7 ± 7.2 | 24.2 |
| D1085E | 1.8 ± 0.2 | 87.0 ± 2.6 | 48.3 |
| R1088T | 3.2 ± 0.2 | 204.5 ± 6.3 | 63.9 |
| R1088E | 4.1 ± 0.3 | 157.0 ± 4.5 | 38.3 |
| N1089L | 3.9 ± 0.2 | 228.5 ± 5.4 | 58.6 |
| N1089D | 5.3 ± 0.5 | 240.2 ± 9.6 | 45.3 |

[a]The kinetic parameters ($K_m$ and $k_{cat}$) were determined with 12 different sucrose concentrations ranging from 0.5 to 200 mM. [b]Data from previous study.[37]

All characterized mutants had a lower catalytic efficiency ($k_{cat}/K_m$) than wild-type GTF180-ΔN, the only exception being R1088T having both a lower $k_{cat}$ and a lower $K_m$ (Table 3). The AAA and LLL mutants, which retained high activity at 100 mM sucrose, showed higher $k_{cat}$ values but an increased $K_m$ (10.9 and 7.6 mM, respectively). All other characterized mutants showed similar or slightly lower $K_m$ values compared to wild-type GTF180-ΔN. The decreased catalytic efficiencies thus were mainly due to the decreased $k_{cat}$ values. The relative amounts of sucrose used for hydrolysis were not significantly affected (Table 2). Only mutants LLL and FFF showed a clear increase in hydrolysis, to 34.0 and 36.5%, respectively.

**Effects of Mutations in GTF180-ΔN on the Structures of Polysaccharides Produced.** The linkage type distributions of the polysaccharides synthesized by the different GTF180-ΔN mutant enzymes, determined by $^1$H NMR spectroscopic and methylation analysis, are summarized in Table 2.

The first striking observation is that for every mutant, the mutation(s) resulted in the introduction of a new linkage type, namely, (α1→4) (Figure 3A). The triple mutant FFF produced the highest amount of (α1→4) linkages (5%) in its polysaccharide, whereas the single mutant R1088W products had 4% (α1→4) linkages (Table 2; Figure 3A). The size of the amino acid at position 1088 may contribute to the introduction of this new linkage type. N1089 mutations introduced fewer (α1→4) linkages (~1%), suggesting that this position is less critical in this regard. Together, the results indicate that the triad D1085, R1088, and N1089 affects the orientation of acceptor substrates in GTF180-ΔN and that it is possible to change the linkage type distribution by mutagenesis and even introduce (α1→4) linkages in addition to the (α1→6) and (α1→3) linkages present in wild-type GTF180-ΔN polysaccharides. The introduction of (α1→4) linkage types in GTF180-ΔN products was also reported in previous mutagenesis studies,[28,38] by targeting a different region near subsite +2 (S1137, N1138, A1139, and Q1140). Notably, the polysaccharide (**mEPS-PNNS**) produced by the triple mutant V1027P:S1137N:A1139S contained 12% (α1→4) linkages.[38] Due to the presence of (α1→4) and more branched (α1→3) linkages, **mEPS-PNNS** is less soluble and more resistant to acid hydrolysis,[38] indicating that it may form


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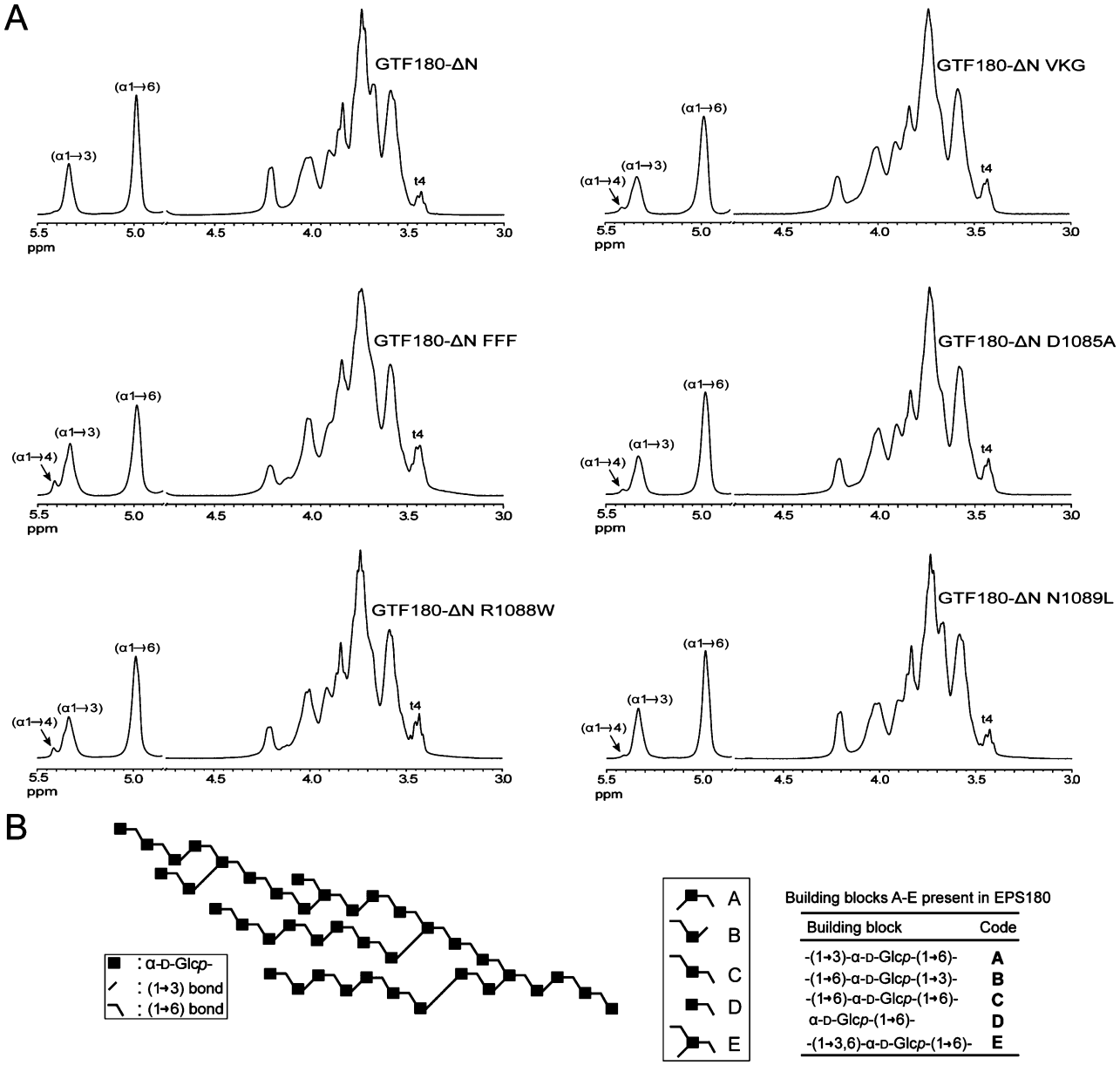


**Figure 3.** (A) 500 MHz 1D $^1$H NMR spectra of the $\alpha$-glucans produced by GTF180-ΔN and mutants VKG, FFF, D1085A, R1088W, and N1089L as indicated. The H-4 signal of terminal residue (t4, between ~$\delta$ 3.40 and 3.45), which is an indicator of branched linkages, was assigned. (B) Composite model structure of EPS180 as reported in our previous study.[36] The building blocks of EPS180 are presented.

a more rigid structure and possess different physicochemical properties.

Second, $^1$H NMR spectroscopic and methylation analysis of the polysaccharides produced revealed that mutant enzymes shifted from synthesis of ($\alpha$1→3) linkages [→3)Glc*p*(1→)] in linear sections to ($\alpha$1→3,6) branched points [→3,6)-Glc*p*(1→)], with the overall ratio of ($\alpha$1→6) and ($\alpha$1→3) linkages virtually unaffected (Table 2). As shown in our previous study,[36] the polysaccharide produced by wild-type GTF180-ΔN is built from five different building blocks, with different lengths of isomalto-oligosaccharides interconnected by ($\alpha$1→3) linkages (Figure 3B), which are present in both linear sections [→3)Glc*p*(1→)] and branched points [→3,6)-Glc*p*(1→)]. Examination of NMR spectra of polysaccharides produced by mutant enzymes revealed that in most mutant products, except for those of N1089, the relative intensity of the H-4 signal (t4 between $\delta$ ~3.40 and 3.45) stemming from the terminal residue of polysaccharides was higher than that in wild-type GTF180-ΔN products (Figure 3A). This H-4 signal (t4) is an indicator for the amount of branching;[28,39] its increase thus indicates an increased amount of branched linkages.[28,36,39] Methylation analysis confirmed the increased percentage of branched →3,6)Glc*p*(1→glucosyl units (14−22%) for all of these mutants (Table 2; Figure 4). In most cases, their products simultaneously showed a reduced amount of →3)Glc*p*(1→ glucosyl units, resulting in a virtually unchanged overall ratio of ($\alpha$1→6) and ($\alpha$1→3) linkages as analyzed by NMR spectroscopy. In some mutants (e.g., FFF and R1088W), both the amount of →3)Glc*p*(1→ glucosyl units and →6)Glc*p*(1→ glucosyl units decreased; instead, they synthesized higher amounts of branched linkages [→3,6)Glc*p*(1→, 22 and 19%, respectively]. As shown by methylation analysis, the triple mutants AAA, LLL, and FFF, containing different sizes of amino acid residues, synthesized polysaccharides with a gradual increase


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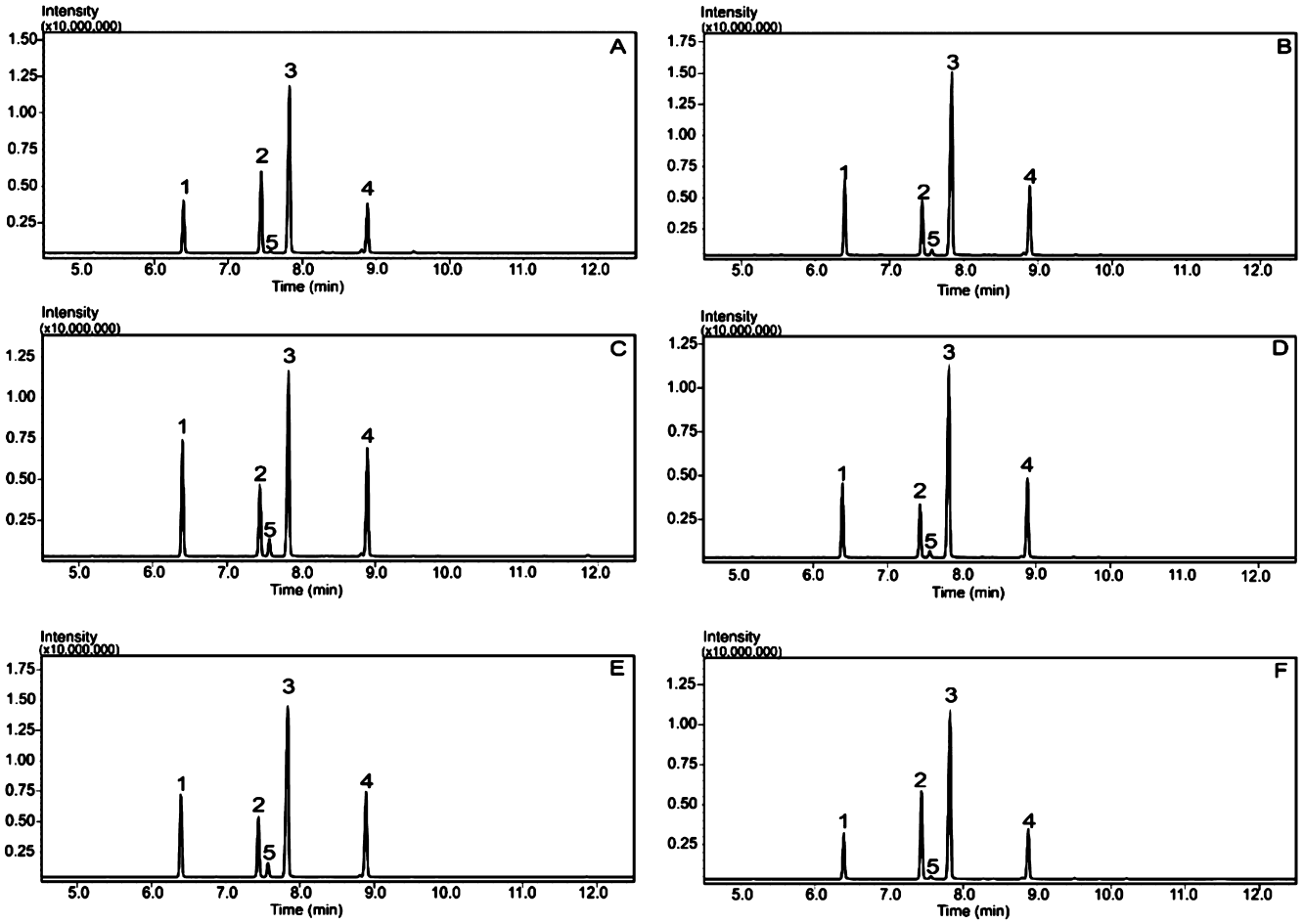


**Figure 4.** Methylation analysis GLC-EI-MS chromatograms of polysaccharides produced by (A) GTF180-ΔN, (B) VKG, (C) FFF, (D) D1085A, (E) R1088W, and (F) N1089L. Peaks: 1, [Glc*p*(1→] (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol); 2, [→3)Glc*p*(1→)] (1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol); 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); 5, [→4)Glc*p*(1→)] (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol).

of branched linkages, suggesting that the size of amino acid residues plays a role in the formation of these branching linkages, although one has to consider the fact that multiple mutations involving large side chains may cause rearrangements of adjacent amino acid residues. Thus, by detailed analysis of the polysaccharides produced by GTF180-ΔN mutant enzymes obtained from combined site-directed mutagenesis and individual site-directed mutagenesis, we showed that hyperbranched α-glucans [containing more (α1→3,6) branched linkages, up to 22%] were produced by α-helix 4 mutant enzymes. They catalyzed the synthesis of higher amounts of branched linkages by switching the synthesis of (α1→3) linkages in the linear sections to that of (α1→3) linkages in branched points. These results indicate that the triad D1085, R1088, and N1089 may affect the orientation of acceptor substrates in GTF180-ΔN and that it is possible to fine-tune the linkage-type distribution by mutation.

The third general observation is that positions 1085 and 1088 are most critical regarding linkage specificity, whereas position 1089 is less so. All D1085 mutants produced polysaccharides with increased relative amounts of →3,6)Glc*p*(1→ glucosyl units; the same is valid for all R1088 mutants, although to a slightly lesser extent. On the other hand, all N1089 mutants showed a relatively small change in linkage specificity (Table 2).

Fourth, we observed that mutations of D1085:R1088:N1089 to the corresponding amino acid residues in other glucansucrases, producing different glycosidic linkages, did not change the linkage specificity accordingly. This observation further supports the idea that linkage specificity is determined not only by these residues from α-helix 4 but by an interplay of residues defining the acceptor binding sites, including the residues that have been identified in previous mutagenesis studies and from structural characterization of glucansucrases in complex with acceptor substrates.[4,22] It remains unknown why different amino acid substitutions cause similar changes in linkage distribution.

Finally, the polysaccharides synthesized by some mutant enzymes showed clearly reduced molecular sizes as compared to those produced by wild-type GTF180-ΔN (Table 2). The polysaccharide size of triple mutant FFF was only 8 MDa, the most significant reduction in size. The polysaccharide sizes produced by the D1085 and R1088 single mutants were reduced to about 20 MDa, whereas the polysaccharides produced by N1089 mutants with similar degrees of branching to that of the wild type showed no significant change in size (about 27 MDa).

Glucansucrase mutagenesis studies so far have targeted different regions around acceptor subsites +1 and +2 and were based on glucansucrase sequence alignment as well as the


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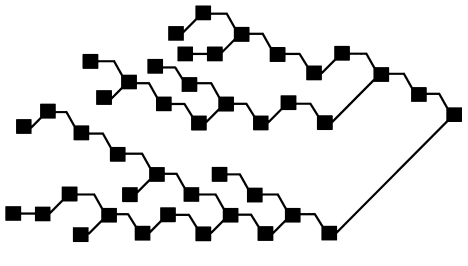


Building blocks A-H observed in mPS-FFF

| Building block | Code | Abundance (%) |
|---|---|---|
| -(1→3)-α-D-Glc*p*-(1→6)- | **A** | 13 |
| -(1→6)-α-D-Glc*p*-(1→3)- | **B** | 22 |
| -(1→6)-α-D-Glc*p*-(1→6)- | **C** | 18 |
| α-D-Glc*p*-(1→6)- | **D** | 6 |
| -(1→3,6)-α-D-Glc*p*-(1→6)- | **E** | 22 |
| α-D-Glc*p*-(1→3)- | **F** | 11 |
| α-D-Glc*p*-(1→4)- | **G** | 4 |
| -(1→4)-α-D-Glc*p*-(1→3)- | **H** | 4 |

**Figure 5.** Composite model for **mPS-FFF** that includes all of the structural features identified. The structural building blocks (residues A−H) observed in **mPS-FFF** using 2D NMR spectroscopy and their abundance are shown.

maltose-bound structures of glucansucrase GTF180-ΔN[22] and GTFSI[40] when these became available. Mutations in residues S1137−Q1140 in GTF180-ΔN immediately downstream of the transition state stabilizing residue (D1136), near the O1/O5/O6 side of the reducing end glucosyl moiety of maltose in subsite +2 (Figure 1), were shown to affect linkage type specificity and even introduced a third linkage type.[28,38] Our current study shows that residues in α-helix 4 (D1085, R1088, and N1089), adjacent to the other (O2/O3) side of the maltose in the +2 subsite (Figure 1), contribute to linkage specificity as well. By mutating residues D1085, R1088, and N1089 at subsite +2, the branching degree of α-glucans produced increased to 22%, and (α1→4) linkages were introduced as a third linkage type, resulting in the production of novel, hyperbranched α-glucans. It appears possible to tune the degree of branching in the α-glucan polysaccharides by glucansucrase enzyme engineering, depending on the desired functional properties and applications. A recent combinatorial mutagenesis study with DSRS of *L. mesenteroides* NRRL B-512F showed that combinatorial mutations involving the corresponding α-helix 4 residues D460, H463, and T464 affected linkage type distribution, yielding products with increased percentages of (α1→3) linkages.[41] The involvement of residues from α-helix 4 in linkage specificity determination thus may be a general feature in glucansucrases. Together these studies show that, in addition to residues downstream of the transition state stabilizing residue, residues in α-helix 4 also contribute to determining the orientation of the acceptor sugar unit in subsite +2 and help define the linkage type synthesized during transglycosylation. The structures of GTF180-ΔN in complex with maltose so far allowed the identification of residues close to subsites +1 and +2. Most likely, these are the strongest determinants of linkage specificity, but it is possible that residues at further subsites, yet unidentified, also contribute.

**Structural Analysis of Selected Polysaccharides Produced by Mutant Enzymes Using 2D NMR Spectroscopy.** Polysaccharide produced by mutant FFF (**mPS-FFF**) contained the highest amount of branching and (α1→4) linkages and its structural analysis by 2D NMR spectroscopy is presented here as an example (Figure S1). Integration of the anomeric signals in the $^{1}$H NMR spectrum revealed 5% (α1→4) linkages ($\delta_{H\text{-}1} \sim 5.41$), 37% (α1→3) linkages ($\delta_{H\text{-}1} \sim 5.34$), and 58% (α1→6) linkages ($\delta_{H\text{-}1} \sim 4.99$), which is in agreement with the methylation analysis [21% Glc*p*(1→ , 13% →3)Glc*p*(1→ , 4% →4)Glc*p*(1→, 40% →6)Glc*p*(1→ and 22% →3,6)Glc*p*(1→]. To unravel the structural elements present in **mPS-FFF**, 2D $^{1}$H−$^{1}$H TOCSY experiments with increasing mixing times of 30, 60, and 150 ms and a $^{13}$C−$^{1}$H HSQC experiment (Figure S1) were performed and interpreted on the basis of previously established structural reporter-group signals for α-glucans.[28,36,38,39] Despite the strong partial overlap of signals, careful examination of the spectra provided sufficient structural information. The detailed interpretations of the NMR signals of different structural units are presented in the Supporting Information. In total, **mPS-FFF** contains eight different building blocks (Figure 5), namely, -(1→3)-α-D-Glc*p*-(1→6)- (**A**), -(1→6)-α-D-Glc*p*-(1→3)- (**B**), -(1→6)-α-D-Glc*p*-(1→6)- (**C**), α-D-Glc*p*-(1→6)- (**D**), -(1→3,6)-α-D-Glc*p*-(1→6)- (**E**), D-Glc*p*-(1→3)- (**F**), α-D-Glc*p*-(1→4) (**G**), and -(1→4)-α-D-Glc*p*-(1→3)- units (**H**). The relative quantitation of different building blocks in **mPS-FFF** was achieved by combining the data from methylation analysis and integration of $^{1}$H NMR analysis as described previously[28,36,39] and presented in Figure 5. The detailed quantitation of different building blocks in **mPS-FFF** is described in the Supporting Information. Making use of the quantitation of the different building blocks, a composite model including all identified structural units was built for **mPS-FFF** (Figure 5).

NMR spectroscopic analysis (data not shown) of polysaccharides produced by the selected mutant enzymes (VKG, D1085A, and R1088W) revealed the presence of the same structural elements in different relative amounts (Table 2). Although the linkage distribution of polysaccharide produced by N1089L mutant enzyme was similar to that of wild-type GTF180-ΔN, 2D NMR analysis (data not shown) revealed the presence of terminal α-D-Glc*p*-(1→3)- (**F**) units, which was not observed in the polysaccharide produced by wild-type GTF180-ΔN (Figure 3B),[36] indicating structural differences between these polysaccharides.

The composite model for the polysaccharide produced by the FFF mutant enzyme (Figure 5) showed significant differences compared with that of wild-type EPS180 (Figure 3).[36] The physicochemical properties of the novel α-glucan polysaccharides produced by the GTF180-ΔN mutant enzymes in the current study remain to be determined to explore their potential food applications. Dextrans with 3−20% of (α1→3) linkages have been produced by DSRS of *L. mesenteroides* B-512F and derived mutants and were shown to have different properties regarding molecular mass and rheological behavior.[42] Several studies showed that hyperbranched α-glucans have different properties, such as a denser structure and a higher solubility.[42−45] Biopolymers, extracted from plants (e.g., starch and cellulose) and animals (e.g., chitin) or produced by microorganisms or their enzymes (exopolysaccharides, e.g., gellan, pullulan, fructan and α-glucan), are already widely used in the food, medicine, cosmetic, and bioplastic industries.[2,7] Lactic acid bacteria are widely used in fermented food as starter cultures. In recent years, the biopolymers produced by lactic acid bacteria have also been explored for novel applications in the food industry.[2,3] The α-glucans (dextran) produced in situ


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by *Weissella* strains were shown to improve the texture of sourdoughs and breads in the baking industry.[8,9] As biothickening agents in the fermented milks and beverages, and in the production of low-fat cheeses, the exopolysaccharides produced by lactic acid bacteria were shown to improve their viscosity, mouthfeel, and yield.[2,3] Some branched α-glucans and their derivatives (e.g., amylopectin and glycogen) have interesting biomedical applications, for example, as nanovehicles for controlled release of drug molecules and as artificial materials for coating of implantable devices.[46−48] Although several in vitro systems, involving a combination of different enzymes, have been developed for the enzymatic synthesis of hyperbranched α-glucans,[43−45,49] the use of engineered glucansucrases, such as *L. reuteri* 180 glucansucrase GTF180-ΔN and its derived mutant enzymes, to synthesize specific α-glucans from a cheap substrate (sucrose) represents a very attractive alternative for producing biopolymers. The additional advantage of glucansucrases is the synthesis of various linkage types in their polysaccharide products. α-Helix 4 (D1085, R1088, and N1089) mutant enzymes constructed in this study catalyzed the synthesis of hyperbranched α-glucans, opening new perspectives to expand their range of products and applications.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05161.

1D and 2D NMR analysis and the identification and quantitation of structural units of polysaccharides produced by mutant enzyme FFF (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

GRAS, generally recognized as safe; DP, degree of polymerization; EPS, exopolysaccharide; TOCSY, total correlation spectroscopy; HSQC, $^{1}$H detected heteronuclear single-quantum coherence spectroscopy; MLEV, composite pulse devised by M. Levitt; MALLS, multiangle laser light scattering signal

## ■ REFERENCES


(1) Godshall, M. A. Future directions for the sugar industry. *Int. Sugar J.* **2001**, *103*, 378−384.

(2) Badel, S.; Bernardi, T.; Michaud, P. New perspectives for Lactobacilli exopolysaccharides. *Biotechnol. Adv.* **2011**, *29*, 54−66.

(3) Torino, M. I.; Font de Valdez, G.; Mozzi, F. Biopolymers from lactic acid bacteria. Novel applications in foods and beverages. *Front. Microbiol.* **2015**, *6*, 834.

(4) Leemhuis, H.; Pijning, T.; Dobruchowska, J. M.; Dijkstra, B. W.; Dijkhuizen, L. Glycosidic bond specificity of glucansucrases: on the role of acceptor substrate binding residues. *Biocatal. Biotransform.* **2012**, *30*, 366−376.

(5) Leemhuis, H.; Pijning, T.; Dobruchowska, J. M.; van Leeuwen, S. S.; Kralj, S.; Dijkstra, B. W.; Dijkhuizen, L. Glucansucrases: three-dimensional structures, reactions, mechanism, α-glucan analysis and their implications in biotechnology and food applications. *J. Biotechnol.* **2013**, *163*, 250−272.

(6) Monchois, V.; Willemot, R.-M.; Monsan, P. Glucansucrases: mechanism of action and structure-function relationships. *FEMS Microbiol. Rev.* **1999**, *23*, 131−151.

(7) Naessens, M.; Cerdobbel, A.; Soetaert, W.; Vandamme, E. J. *Leuconostoc* dextransucrase and dextran: production, properties and applications. *J. Chem. Technol. Biotechnol.* **2005**, *80*, 845−860.

(8) Galle, S.; Schwab, C.; Arendt, E.; Gänzle, M. Exopolysaccharide-forming *Weissella* strains as starter cultures for sorghum and wheat sourdoughs. *J. Agric. Food Chem.* **2010**, *58*, 5834−5841.

(9) Galle, S.; Schwab, C.; Dal Bello, F.; Coffey, A.; Gänzle, M. G.; Arendt, E. K. Influence of *in-situ* synthesized exopolysaccharides on the quality of gluten-free sorghum sourdough bread. *Int. J. Food Microbiol.* **2012**, *155*, 105−112.

(10) Côté, G. L.; Sheng, S. Penta-, hexa-, and heptasaccharide acceptor products of alternansucrase. *Carbohydr. Res.* **2006**, *341*, 2066−2072.

(11) Diez-Municio, M.; Montilla, A.; Jimeno, M. L.; Corzo, N.; Olano, A.; Moreno, F. J. Synthesis and characterization of a potential prebiotic trisaccharide from cheese whey permeate and sucrose by *Leuconostoc mesenteroides* dextransucrase. *J. Agric. Food Chem.* **2012**, *60*, 1945−1953.

(12) Koepsell, H. J.; Tsuchiya, H. M.; Hellman, N. N.; Kazenko, A.; Hoffman, C. A.; Sharpe, E. S.; Jackson, R. W. Enzymatic synthesis of dextran: acceptor specificity and chain initiation. *J. Biol. Chem.* **1953**, *200*, 793−801.

(13) Ruiz-Matute, A. I.; Brokl, M.; Sanz, M. L.; Soria, A. C.; Côté, G. L.; Collins, M. E.; Rasta, R. A. Effect of dextransucrase cellobiose acceptor products on the growth of human gut bacteria. *J. Agric. Food Chem.* **2011**, *59*, 3693−3700.

(14) Sanz, M. L.; Côté, G. L.; Gibson, G. R.; Rastall, R. A. Prebiotic properties of alternansucrase maltose-acceptor oligosaccharides. *J. Agric. Food Chem.* **2005**, *53*, 5911−5916.

(15) Sanz, M. L.; Côté, G. L.; Gibson, G. R.; Rastall, R. A. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-based oligosaccharides by human gut bacteria. *J. Agric. Food Chem.* **2006**, *54*, 9779−9784.

(16) Goffin, D.; Delzenne, N.; Blecker, C.; Hanon, E.; Deroanne, C.; Paquot, M. Will isomalto-oligosaccharides, a well-established functional food in Asia, break through the European and American market? The status of knowledge on these prebiotics. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 394−409.

(17) Moulis, C.; Medina, G. V.; Suwannarangsee, S.; Monsan, P.; Remaud-Siméon, M.; Potocki-Vèronése, G. One-step synthesis of isomalto-oligosaccharide syrups and dextrans of controlled size using engineered dextransucrase. *Biocatal. Biotransform.* **2008**, *26*, 141−151.

(18) Shi, Q.; Juvonen, M.; Hou, Y.; Kajala, I.; Nyyssölä, A.; Maina, N. H.; Maaheimo, H.; Virkki, L.; Tenkanen, M. Lactose- and cellobiose-derived branched trisaccharides and a sucrose-containing trisaccharide produced by acceptor reactions of *Weissella confusa* dextransucrase. *Food Chem.* **2016**, *190*, 226−236.

(19) Sarbini, S. R.; Kolida, S.; Naeye, T.; Einerhand, A.; Brison, Y.; Remaud-Siméon, M.; Monsan, P.; Gibson, G. R.; Rastall, R. A. In vitro




DOI: 10.1021/acs.jafc.5b05161
*J. Agric. Food Chem.* 2016, 64, 433−442

fermentation of linear and α-1,2-branched dextrans by the human fecal microbiota. *Appl. Environ. Microbiol.* **2011**, *77*, 5307−5315.
(20) Spence, J. H.; Higgins, A. R.; Kimmel, J. R. The clinical effects of dextran as a plasma volume extender. *U.S. Armed Forces Med. J.* **1952**, *3*, 515−518.
(21) Lombard, V.; Golaconda Ramulu, H.; Drula, E.; Coutinho, P. M.; Henrissat, B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **2014**, *42*, D490−D495.
(22) Vujičić-Žagar, A.; Pijning, T.; Kralj, S.; López, C. A.; Eeuwema, W.; Dijkhuizen, L.; Dijkstra, B. W. 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.* **2010**, *107*, 21406−21411.
(23) Kralj, S.; van Geel-Schutten, I. G. H.; Faber, E. J.; van der Maarel, M. J. E. C.; Dijkhuizen, L. Rational transformation of *Lactobacillus reuteri* 121 reuteransucrase into a dextransucrase. *Biochemistry* **2005**, *44*, 9206−9216.
(24) Moulis, C.; Joucla, G.; Harrison, D.; Fabre, E.; Potocki-Vèronése, G.; Monsan, P.; Remaud-Siméon, M. Understanding the polymerization mechanism of glycoside-hydrolase family 70 glucansucrases. *J. Biol. Chem.* **2006**, *281*, 31254−31267.
(25) van Hijum, S. A.; Kralj, S.; Ozimek, L. K.; Dijkhuizen, L.; van Geel-Schutten, I. G. H. Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 157−176.
(26) Kang, H. K.; Kimura, A.; Kim, D. Bioengineering of *Leuconostoc mesenteroides* glucansucrases that gives selected bond formation for glucan synthesis and/or acceptor-product synthesis. *J. Agric. Food Chem.* **2011**, *59*, 4148−4155.
(27) Hellmuth, H.; Wittrock, S.; Kralj, S.; Dijkhuizen, L.; Hofer, B.; Seibel, J. Engineering the glucansucrase GTFR enzyme reaction and glycosidic bond specificity: toward tailor-made polymer and oligosaccharide products. *Biochemistry* **2008**, *47*, 6678−6684.
(28) van Leeuwen, S. S.; Kralj, S.; Eeuwema, W.; Gerwig, G. J.; Dijkhuizen, L.; Kamerling, J. P. Structural characterization of bioengineered α-D-glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180. *Biomacromolecules* **2009**, *10*, 580−588.
(29) Côté, G. L.; Skory, C. D. Effects of mutations at threonine-654 on the insoluble glucan synthesized by *Leuconostoc mesenteroides* NRRL B-1118 glucansucrase. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6651−6658.
(30) Davies, G. J.; Wilson, K. S.; Henrissat, B. Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J.* **1997**, *321* (2), 557−559.
(31) Gouet, P.; Courcelle, E.; Stuart, D. I.; Metoz, F. ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* **1999**, *15*, 305−308.
(32) Kralj, S.; van Geel-Schutten, I. G. H.; van der Maarel, M. J. E. C.; Dijkhuizen, L. Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase. *Microbiology* **2004**, *150*, 2099−2112.
(33) Van Geel-Schutten, I. G. H.; Faber, E. J.; Smit, E.; Bonting, K.; Smith, M. R.; Ten Brink, B.; Kamerling, J. P.; Vliegenthart, J. F. G.; Dijkhuizen, L. 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.* **1999**, *65*, 3008−3014.
(34) Meng, X.; Dobruchowska, J. M.; Pijning, T.; López, C. A.; Kamerling, J. P.; Dijkhuizen, L. 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.* **2014**, *289*, 32773−32782.
(35) Ciucanu, I.; Kerek, F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **1984**, *131*, 209−217.
(36) van Leeuwen, S. S.; Kralj, S.; van Geel-Schutten, I. G. H.; Gerwig, G. J.; Dijkhuizen, L.; Kamerling, J. P. Structural analysis of the α-D-glucan (EPS180) produced by the *Lactobacillus reuteri* strain 180 glucansucrase GTF180 enzyme. *Carbohydr. Res.* **2008**, *343*, 1237−1250.
(37) Meng, X.; Dobruchowska, J. M.; Pijning, T.; Gerwig, G. J.; Kamerling, J. P.; Dijkhuizen, L. Truncation of domain V of the multidomain glucansucrase GTF180 of *Lactobacillus reuteri* 180 heavily impairs its polysaccharide-synthesizing ability. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 5885−5894.
(38) van Leeuwen, S. S.; Kralj, S.; Gerwig, G. J.; Dijkhuizen, L.; Kamerling, J. P. 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* **2008**, *9*, 2251−2258.
(39) van Leeuwen, S. S.; Leeflang, B. R.; Gerwig, G. J.; Kamerling, J. P. Development of a $^{1}$H NMR structural-reporter-group concept for the primary structural characterisation of α-D-glucans. *Carbohydr. Res.* **2008**, *343*, 1114−1119.
(40) Ito, K.; Ito, S.; Shimamura, T.; Weyand, S.; Kawarasaki, Y.; Misaka, T.; Abe, K.; Kobayashi, T.; Cameron, A. D.; Iwata, S. Crystal structure of glucansucrase from the dental caries pathogen *Streptococcus mutans*. *J. Mol. Biol.* **2011**, *408*, 177−186.
(41) Irague, R.; Tarquis, L.; André, I.; Moulis, C.; Morel, S.; Monsan, P.; Potocki-Vèronése, G.; Remaud-Siméon, M. Combinatorial engineering of dextransucrase specificity. *PLoS One* **2013**, *8*, e77837.
(42) Irague, R.; Rolland-Sabaté, A.; Tarquis, L.; Doublier, J. L.; Moulis, C.; Monsan, P.; Remaud-Siméon, M.; Potocki-Vèronése, G.; Buléon, A. Structure and property engineering of α-D-glucans synthesized by dextransucrase mutants. *Biomacromolecules* **2012**, *13*, 187−195.
(43) Ciric, J.; Woortman, A. J. J.; Gordiichuk, P.; Stuart, M. C. A.; Loos, K. Physical properties and structure of enzymatically synthesized amylopectin analogs. *Starch/Staerke* **2013**, *65*, 1061−1068.
(44) Grimaud, F.; Lancelon-Pin, C.; Rolland-Sabaté, A.; Roussel, X.; Laguerre, S.; Viksø-Nielsen, A.; Putaux, J. L.; Guilois, S.; Buléon, A.; D'Hulst, C.; Potocki-Vèronése, G. In vitro synthesis of hyperbranched α-glucans using a biomimetic enzymatic toolbox. *Biomacromolecules* **2013**, *14*, 438−447.
(45) Rolland-Sabaté, A.; Guilois, S.; Grimaud, F.; Lancelon-Pin, C.; Roussel, X.; Laguerre, S.; Vikso-Nielsen, A.; Putaux, J. L.; D'Hulst, C.; Potocki-Véronèse, G.; Buléon, A. Characterization of hyperbranched glycopolymers produced in vitro using enzymes. *Anal. Bioanal. Chem.* **2014**, *406*, 1607−1618.
(46) van der Vlist, J.; Schonen, I.; Loos, K. Utilization of glycosyltransferases for the synthesis of a densely packed hyperbranched polysaccharide brush coating as artificial glycocalyx. *Biomacromolecules* **2011**, *12*, 3728−3732.
(47) Zeng, D.; Fan, J.; Deng, Z.; Tan, J.; Deng, Z.; Zhang, L. M.; Yang, L. One-step synthesis of amphiphilic hyperbranched amylopectin derivatives, characterization and use as functional nanovehicles. *Carbohydr. Polym.* **2013**, *98*, 905−913.
(48) Semyonov, D.; Ramon, O.; Shoham, Y.; Shimoni, E. Enzymatically synthesized dextran nanoparticles and their use as carriers for nutraceuticals. *Food Funct.* **2014**, *5*, 2463−2474.
(49) van der Vlist, J.; Reixach, M. P.; van der Maarel, M.; Dijkhuizen, L.; Schouten, A. J.; Loos, K. Synthesis of branched polyglucans by the tandem action of potato phosphorylase and *Deinococcus geothermalis* glycogen branching enzyme. *Macromol. Rapid Commun.* **2008**, *29*, 1293−1297.


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