Residues Leu\(^{940}\) Has a Crucial Role in the Linkage and Reaction Specificity of the Glucansucrase GTF180 of the Probiotic Bacterium \textit{Lactobacillus reuteri} 180*}

Received for publication, August 9, 2014, and in revised form, September 29, 2014. Published, JBC Papers in Press, October 6, 2014, DOI 10.1074/jbc.M114.602524

Xiangfeng Meng\(^1\), Justyna M. Dobruchowska\(^1\), Tjaard Pijning\(^3\), Cesar A. Lápez\(^2,3\), Johannis P. Kamerling\(^4\), and Lubbert Dijkhuizen\(^1,2\)

From the Departments of \(^4\)Microbial Physiology and \(^6\)Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

**Background:** Glucansucrases of lactic acid bacteria catalyze the synthesis of a variety of \(\alpha\)-glucans from sucrose. The structural determinants of glucansucrase specificity have remained unclear. Residue Leu\(^{940}\) in domain B of GTF180, the glucansucrase of the probiotic bacterium \textit{Lactobacillus reuteri} 180, was shown to vary in different glucansucrases and is close to the +1 glucosyl unit in the crystal structure of GT180-\(\Delta\)N in complex with maltose. Herein, we show that mutations in Leu\(^{940}\) of wild-type GT180-\(\Delta\)N all caused an increased percentage of \((\alpha1\rightarrow6)\) linkages and a decreased percentage of \((\alpha1\rightarrow3)\) linkages in the products. \(\alpha\)-Glucans with potential different physicochemical properties (containing 67–100% of \((\alpha1\rightarrow6)\) linkages) were produced by GTF180 and its Leu\(^{940}\) mutants. Mutant L940W was unable to form \((\alpha1\rightarrow3)\) linkages and synthesized a smaller and linear glucan polysaccharide with only \((\alpha1\rightarrow6)\) linkages. Docking studies revealed that the introduction of the large aromatic amino acid residue tryptophan at position 940 partially blocked the binding groove, preventing the isomaltos-oligosaccharide acceptor to bind in an favorable orientation for the formation of \((\alpha1\rightarrow3)\) linkages. Our data showed that the reaction specificity of GTF180 mutant was shifted either to increased polysaccharide synthesis (L940A, L940S, L940E, and L940F) or increased oligosaccharide synthesis (L940W). The L940W mutant is capable of producing a large amount of isomaltos-oligosaccharides using released glucose from sucrose as acceptors. Thus, residue Leu\(^{940}\) in domain B is crucial for linkage and reaction specificity of GTF180. This study provides clear and novel insights into the structure-function relationships of glucansucrase enzymes.

**Lactobacillus reuteri**, a bacterium of human origin, has been widely used as probiotic supplement in human nutrition (1–3). \textit{L. reuteri} strains possess glucansucrase enzymes and synthesize various \(\alpha\)-glucans (4–8), which have potential prebiotic activities and therefore can be used to stimulate growth of beneficial intestinal bacteria such as \textit{Bifidobacterium} and \textit{Lactobacillus} (9). Glucansucrases are \(\alpha\)-glucan-synthesizing enzymes only detected in lactic acid bacteria. Depending on the particular enzyme, \(\alpha\)-glucans with different types of glycosidic linkages are produced from sucrose (10, 11): dextran with mainly \((\alpha1\rightarrow6)\) linkages, mutan with predominantly \((\alpha1\rightarrow3)\) linkages, reuteran containing \((\alpha1\rightarrow4)\) and \((\alpha1\rightarrow6)\) linkages, and alternan with alternating \((\alpha1\rightarrow6)\) and \((\alpha1\rightarrow3)\) linkages. A notable case is that of DSRE CD2 from \textit{Leuconostoc mesenteroides} NRRL B-1299, forming \((\alpha1\rightarrow2)\) single glucose branches on dextran (12). Glucansucrases catalyze three different reactions to produce glucose, polysaccharides, and oligosaccharides, using water, growing glucan chains, and low molecular mass oligosaccharides as acceptors, respectively. The structural determinants of linkage and reaction specificity (the relative balance of three reactions catalyzed) of glucansucrase are still not fully understood.

Because of their ability to produce a diverse range of \(\alpha\)-glucans with different types of linkage, size, branching, and physicochemical properties, glucansucrases have attracted interest for industrial application such as food, medicine, cosmetics, etc. (13). Dextran produced by the glucansucrase DSRS\(^3\) from \textit{L. mesenteroides} NRRL B-512F was designated as a novel food ingredient by the European Union in 2001 (14). Bakery products with dextran have improved softness and increased volume (15). In addition to its use as a prebiotic food additive, \(\alpha\)-glucan is also applied as size exclusion chromatography material in research and as a plasma expander in medicine. A more detailed understanding of the linkage and reaction specificity of glucansucrases may allow the production of tailor-made \(\alpha\)-glucans with desired properties.

---

*This work was financially supported by the Chinese Scholarship Council (to X.M.), and by European Union project NOVOSIDES FP7-KBBE-2010-4-265854 (to L.D.).

1 Present address: Theoretical Division, Los Alamos National Laboratory, NM 87545.

2 To whom correspondence should be addressed: Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Inst., University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands. Tel: 31-503632153; Fax: 31-503632154; E-mail: l.dijkhuizen@rug.nl.

3 The abbreviations used are: DSR, dextran sucrase; DSRS, DSR from \textit{Leuconostoc mesenteroides} NRRL B-512F; HPAEC, high pH anion exchange chromatography.
Glucansucrases are classified in glycoside hydrolase family 70 with circularly permuted (B/α) barrel (16, 17). Three catalytically crucial residues of glucansucrases have been identified in previous studies (6, 17, 18). In glycoside hydrolase family 70 enzymes, Asp\(^{1025}\) (catalytic nucleophile, GTF180 numbering) is involved in the formation of a covalent glucosyl-enzyme intermediate, Glu\(^{1063}\) is the acid/base catalyst, and Asp\(^{1136}\) is the transition state stabilizer (19). Glucansucrases produce α-glucans with different structures especially regarding the glycosidic linkages. The linkage specificities of glucansucrases appear to be determined by only a small number of amino acids. In several glucansucrases, residues located C-terminally to the catalytic transition state stabilizer have been identified as important residues for linkage specificity (20–23). For example, mutations in the tripeptide (Ser\(^{1137}\)-Asn\(^{1138}\)-Ala\(^{1139}\)) following the transition state stabilizing residue (Asp\(^{1136}\)) in GTF180 of \(L.\) reuteri 180 altered its linkage specificity (22). Similarly, mutations in the corresponding tripeptide in GTFA-\(\Delta N\) of \(L.\) reuteri 121 (Asp\(^{1134}\)-Asn\(^{1135}\)-Ser\(^{1136}\)) increased the amount of (α1→6) linkages and decreased the amount of (α1→4) linkages (23). Mutations P1026V and I1029V (C-terminal to the nucleophile Asp\(^{1024}\)) in GTFA-\(\Delta N\) also altered linkage composition in the α-glucan produced (23). Random mutagenesis of Asp\(^{569}\) in GTF-I from *Streptococcus downei* showed that mutations at this position affected the structure of the α-glucan and the size of the synthesized oligosaccharides (24). Residues that are far away from the catalytic residues have also been found to influence the specificity of glucansucrases. For example, mutations T350K and S455K in DSRS of *L. mesenteroides* NRRL B-512F increased the amount of (α1→6) linkages in the glucan (25). Moreover, the double mutant T350K/S455K was able to produce (α1→2) branches on dextran similar to DSRE (25).

Mutants with altered linkage distribution specificity have been the main focus in previous studies. Relatively little attention has been paid to the effects of mutations on reaction specificity. Several mutant glucansucrases have been reported to produce different amounts of polysaccharides, oligosaccharides, and glucose from sucrose (20, 21, 23). For instance, S628D and S628R mutations in GTF-I from *Streptococcus oralis* abolished polysaccharide synthesis and only produced short chain oligosaccharides (20). Moulis *et al.* (21) also reported that mutations in residues C-terminal to the transition state stabilizer in DSRS of *L. mesenteroides* NRRL B-512F and alternan-sucrase of *L. mesenteroides* NRRL B-1355 abolished or reduced polysaccharide synthesis. On the contrary, the H355V mutation in GTF-Ic of *S. downei* Mfe 28 increased the production of mutan from 62 to 75% (26). Engineering of reaction specificity of glucansucrases may allow efficient production of either polysaccharides or oligosaccharides to meet the demand for different applications.

Even though various mutagenesis studies have been performed on glucansucrases, the structural features determining linkage and reaction specificity of glucansucrases are still not understood. Further studies are required to explore regions that are critical for specificity and to expand the diversity of α-glucan products for different applications. In our previous studies, GTF180 of *L. reuteri* 180 was found to produce dextran with 69% (α1→6) linkages and 31% (α1→3) linkages (5, 27). Truncation of the N-terminal variable domain had no significant effect on the product spectrum of GTF180 (5). A careful inspection of the sequence alignment of GTF180 with other glucansucrases revealed that residue Leu\(^{940}\) is highly conserved in dextran- and mutan-producing glucansucrases and in alternan-sucrase (Fig. 1). Instead, a phenylalanine is located at this position in reuteran-producing glucansucrases (GTFA of *S. oralis* N123) and DSRE of *L. reuteri* ATCC 55730 and in the (α1→2)-branch forming DSRE (Fig. 1). The crystal structures of N-terminally truncated GTF180 (GTF180-\(\Delta N\)) in complex with the donor substrate sucrose and the acceptor substrate maltose show that the active site is delineated by residues from domain A and B (19). Residue Leu\(^{940}\) is located in a loop of domain B, which shapes the binding groove near the acceptor binding site (19); it is close to the +1 glucosyl unit of maltose (19). The crystal structures of GTFA-\(\Delta N\) (28) and DSRE\(\Delta N\) (29) is critical for glucansucrase linkage specificity, reaction specificity, and activity.

**EXPERIMENTAL PROCEDURES**

Sequence Alignment—Clustal Omega (29) was used to align the amino acid sequences of the dextran-producing glucansucrases GTF180 (Q5SN3) of *L. reuteri* 180, GTF-I (Q9LCH3) of *S. oralis* ATCC10557, DSRE (Q9LCH3) of *L. mesenteroides* B-1299CB4, DSRS (Q92AR4) of *L. mesenteroides* NRRL...
B-512F, and DSRWC (B9UNL6) of Weissella cibaria CMU; the manut-producing glucansucrases GTFML1 (Q55BN0) of L. reuteri ML1, GTF1smGS5 (P08987) of Streptococcus mutans GS 5, GTF1smUA159 (AAN58705.1) of S. mutans UA159, and GTF1ssoBOMZ176 (Q55263) of Streptococcus sobrinus ATCC 33478/OMZ176; the reuteran-producing glucansucrases GTFA (Q55BL9) of L. reuteri 121 and GTFO (Q4JLC7) of L. reuteri ATCC 55730; alternansucrase ASR (Q9RE05) of L. mesenteroides NRRL B-1355; and the (α1→2)-forming glucansucrase DSRE CD2 (Q8G9Q2) of L. mesenteroides NRRL B-1299. Then the aligned sequences were submitted to EsPrfit (30) for alignment based on the GTF180-ΔN crystal structure (Protein Data Bank code 3KLK).

Site-directed Random Mutagenesis of Leu940 in GTF180-ΔN—The plasmid p15GTF180-ΔN-SX, constructed in a previous study, was used as the template for site-directed random mutagenesis (22). Random mutations of Leu940 were introduced by PCR with primer L940X-For (5′-CGGTCTTGGGC-AANNSCAAGTGATATT-3′) and L940X-Rev (5′-GACCTTGCAACCACCG-3′) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers were synthesized by Sigma-Aldrich. The PCR product was cleaned up with the PCR cleaning up kit (Sigma-Aldrich) and transformed into BL21 star (DE3) and transformed into Escherichia coli BL21 star (DE3) (Invitrogen). After growth on LB agar plates, the colonies were inoculated in a 96-well plate with LB medium containing 100 μg/ml ampicillin. The overnight cultures were inoculated again in a new 96-well plate with fresh LB medium containing 100 μg/ml ampicillin and 0.1 mM isopropyl β-d-thiogalactopyranoside and were incubated overnight at 18 °C. Then the cells were lysed with B-PER protein extraction reagents (Thermo Scientific, Pierce) and used as crude extracts. The crude extract from each colony was incubated with 0.1 mM sucrose sodium acetate buffer, pH 4.5, at 37 °C for 15 min. Glucansucrase activities were measured with 3,5-dinitrosalicylic acid reagent (31). All mutations were identified by nucleotide sequencing (LGC Genomics, Berlin, Germany).

Enzyme Production and Purification—E. coli BL21 star (DE3) (Invitrogen) was used as a host for expression of wild-type GTF180-ΔN and all GTF180-ΔN Leu940 mutants. Precultures of E. coli BL21 star (DE3) harboring p15GTF180-ΔN-SX and different mutant plasmids were cultured overnight at 37 °C. Then fresh LB medium containing 100 μg/ml ampicillin was inoculated with 1% preculture. Expression of glucansucrases was induced with 0.1 mM isopropyl β-d-thiogalactopyranoside when the culture reached A590 of 0.4–0.6. Cultivation was continued at 18 °C overnight. The cells were collected by centrifugation (10 min, 4 °C, 10,000 × g) and washed with 50 mM Tris-HCl buffer, pH 8.0. The (mutant) glucansucrase enzymes were purified as previously described (6).

Production of α-Glucans by Wild-type GTF180-ΔN and GTF180-ΔN Leu940 Mutants from Sucrose—GTF180-ΔN and mutants (1.0 unit/ml) were incubated with 0.1 mM sucrose for 24 h at 37 °C in 25 mM sodium acetate buffer, 1 mM CaCl2, pH 4.5. The depletion of sucrose was analyzed by TLC. The reaction was stopped by incubation at 100 °C for 10 min. The polysaccharide and oligosaccharide fractions were isolated by chromatographic separation on a Bio-Gel P-6 column (2.5 × 50 cm; Bio-Rad) using 10 mM NH4HCO3 as eluent at a flow rate of 48 ml/h. Free fructose was not collected.

High pH Anion Exchange Chromatography with Pulsed Amperometric Detection Analysis—The oligosaccharides produced by GTF180-ΔN (mutants) were analyzed by high pH anion exchange chromatography (HPAEC) on a Dionex DX500 work station, equipped with an ED40 pulsed amperometric detection system. The oligosaccharides were separated on a CarboPac PA-1 column (250 × 5 mm; Dionex) by using a linear gradient of 10–240 mM sodium acetate in 100 mM NaOH (1 ml/min).

1H NMR Spectroscopy—1H NMR spectra of polysaccharides and oligosaccharides produced by different mutants were recorded on a Varian Inova 500 Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. Samples were exchanged twice with 99.9% D2O, D2O (Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization and dissolved in 600 μl of D2O. Chemical shifts were expressed in ppm and calibrated by internal standard acetone (δ 2.225). The percentage of different linkages was estimated by integration of the respective signal peak areas.

Determination of the Reaction Specificity—The reaction specificity was defined as the relative balance of the three reactions (hydrolysis, oligosaccharide synthesis, and polysaccharide synthesis) catalyzed by glucansucrases. Changes in reaction specificity were monitored by determining the percentage of sucrose used for polysaccharide synthesis, oligosaccharide synthesis, and hydrolysis. The percentages of sucrose used for hydrolysis and polysaccharide synthesis were determined by detecting the released glucose in the incubation mixture and the amount of glucose in the polysaccharide, respectively. The percentage of sucrose used for oligosaccharide synthesis was calculated by subtracting the amount of sucrose for hydrolysis and polysaccharide synthesis from the total amount of sucrose added to the incubation mixture. The amount of glucose released in the incubation mixture was measured by converting the glucose into gluconate-6-phosphate with hexokinase and glucose-6-phosphate dehydrogenase and measuring the NADH release at A340 (32). The amount of glucose in the isolated polysaccharide was determined by measuring the weight of polysaccharides after lyophilization. The amounts of polysaccharide synthesis, oligosaccharide synthesis, and hydrolysis were expressed as the percentages of sucrose used for each reaction.

Enzymatic Activity Assays—The enzymatic activities of GTF180-ΔN (mutants) were measured as previously described (6). Briefly, enzyme assays were performed with 30 mM enzyme in 25 mM sodium acetate buffer, pH 4.5, at 50 °C. Samples of 25 μl of incubation mixture were withdrawn every 1 min for 5 min and inactivated with 2.5 μl of 1 M NaOH. One unit of enzyme activity was defined as the release of 1 μmol of fructose per min. The kinetic parameters (Km and kcat) were determined with 12 different sucrose concentration ranging from 0.5 to 200 mM. The kinetic parameters were calculated using SigmaPlot version 12.5.

Oligosaccharide Synthesis with 0.1 mM Malto as Acceptor by GTF180-ΔN Leu940 Mutants from Sucrose—GTF180-ΔN and mutants (1.0 unit/ml) were incubated with 0.1 mM sucrose and 0.1

The Crucial Role of Leu940 in the Specificity of GTF180

Bio-Rad) using 10 mM NH4HCO3 as eluent at a flow rate of 48 ml/h. Free fructose was not collected.

High pH Anion Exchange Chromatography with Pulsed Amperometric Detection Analysis—The oligosaccharides produced by GTF180-ΔN (mutants) were analyzed by high pH anion exchange chromatography (HPAEC) on a Dionex DX500 work station, equipped with an ED40 pulsed amperometric detection system. The oligosaccharides were separated on a CarboPac PA-1 column (250 × 5 mm; Dionex) by using a linear gradient of 10–240 mM sodium acetate in 100 mM NaOH (1 ml/min).

1H NMR Spectroscopy—1H NMR spectra of polysaccharides and oligosaccharides produced by different mutants were recorded on a Varian Inova 500 Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. Samples were exchanged twice with 99.9% D2O, D2O (Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization and dissolved in 600 μl of D2O. Chemical shifts were expressed in ppm and calibrated by internal standard acetone (δ 2.225). The percentage of different linkages was estimated by integration of the respective signal peak areas.

Determination of the Reaction Specificity—The reaction specificity was defined as the relative balance of the three reactions (hydrolysis, oligosaccharide synthesis, and polysaccharide synthesis) catalyzed by glucansucrases. Changes in reaction specificity were monitored by determining the percentage of sucrose used for polysaccharide synthesis, oligosaccharide synthesis, and hydrolysis. The percentages of sucrose used for hydrolysis and polysaccharide synthesis were determined by detecting the released glucose in the incubation mixture and the amount of glucose in the polysaccharide, respectively. The percentage of sucrose used for oligosaccharide synthesis was calculated by subtracting the amount of sucrose for hydrolysis and polysaccharide synthesis from the total amount of sucrose added to the incubation mixture. The amount of glucose released in the incubation mixture was measured by converting the glucose into gluconate-6-phosphate with hexokinase and glucose-6-phosphate dehydrogenase and measuring the NADH release at A340 (32). The amount of glucose in the isolated polysaccharide was determined by measuring the weight of polysaccharides after lyophilization. The amounts of polysaccharide synthesis, oligosaccharide synthesis, and hydrolysis were expressed as the percentages of sucrose used for each reaction.

Enzymatic Activity Assays—The enzymatic activities of GTF180-ΔN (mutants) were measured as previously described (6). Briefly, enzyme assays were performed with 30 mM enzyme in 25 mM sodium acetate buffer, pH 4.5, at 50 °C. Samples of 25 μl of incubation mixture were withdrawn every 1 min for 5 min and inactivated with 2.5 μl of 1 M NaOH. One unit of enzyme activity was defined as the release of 1 μmol of fructose per min. The kinetic parameters (Km and kcat) were determined with 12 different sucrose concentration ranging from 0.5 to 200 mM. The kinetic parameters were calculated using SigmaPlot version 12.5.

Oligosaccharide Synthesis with 0.1 mM Malto as Acceptor by GTF180-ΔN Leu940 Mutants from Sucrose—GTF180-ΔN and mutants (1.0 unit/ml) were incubated with 0.1 mM sucrose and 0.1
structural properties of polysaccharides and oligosaccharides produced by wild-type GTF180-ΔN and Leu⁹⁴⁰ mutants

| Enzymes | Polysaccharide chemical shift³ | Molecular mass⁴ | Relative molecular mass | Oligosaccharide chemical shift³ | Relative molecular mass |
|---------|-------------------------------|----------------|------------------------|--------------------------------|------------------------|
| GTF180-ΔN | 67% (α1→6) 33% (α1→3) | 22.6 × 10⁶ Da | 100.0% | 78% | 22% |
| L940G | 85% | 16.8 | 74.1 | 92% | 8% |
| L940C | 74% | 17.3 | 76.3 | 85% | 15% |
| L940A | 84% | 19.3 | 85.2 | 91% | 9% |
| L940S | 84% | 19.7 | 87.0 | 91% | 9% |
| L940M | 72% | 19.2 | 84.7 | 80% | 20% |
| L940E | 73% | 18.7 | 82.5 | 83% | 17% |
| L940F | 93% | 19.5 | 86.3 | 95% | 5% |
| L940YY | 100% | 6.3 | 27.9 | 100% | 0% |

³The data represent the ratios of integration of the surface areas of the (α1→6) linkage signal at 4.96 ppm and the (α1→3) linkage signal at 5.33 ppm in the 1H NMR spectra of the polysaccharides and oligosaccharides produced.

⁴The average molecular mass of polysaccharides was determined in duplicate.

m maltose for 24 h at 37 °C. The reaction was stopped by incubation at 100 °C for 10 min. The amounts of remaining maltose and panose in the incubation mixture were determined by HPAEC pulsed amperometric detection with the respective standards. Because of the lack of a standard for glucosyl-(α1→6)-panose (α-D-Glc p-(1→6)α-D-Glc p-(1→6)α-D-Glc p-(1→4)α-D-Glc p) and [glucosyl-(α1→6)]2-panose (α-D-Glc p-(1→6)α-D-Glc p-(1→6)α-D-Glc p-(1→6)α-D-Glc p-(1→4)α-D-Glc p), their concentrations were estimated using panose as the standard. Different oligosaccharide yields were calculated as the percentages of the amount of maltose converted to the respective oligosaccharides. Approximately 90–100% of maltose initially present in the incubation was recovered from the analysis. The lower percentages may be due to the use of the D-panose calibration curve to determine the amount of glucosyl-(α1→6)-panose and [glucosyl-(α1→6)]2-panose.

Size Determination of Polysaccharide Produced by GTF180-ΔN Leu⁹⁴⁰ Mutants—The size of polysaccharides was determined using a SEC system (Agilent Technologies 1260 Infinity) from Polymer Standards Service (Mainz, Germany). The setup consisted of an isocratic pump, an auto sampler without temperature regulation, an online degasser, an inline 0.1-μm filter, a refractive index detector (G1362A 1260 RID; Agilent Technologies), and multiangle laser light scattering (SLD 7000; Polymer Standards Service). As eluent, 0.1 M Na₂SO₄ with 0.02% NaN₃ was used at a flow rate of 0.8 ml/min. Polysaccharide samples were also dissolved in 0.1 M Na₂SO₄ with 0.02% NaN₃. The samples (100 μl) were injected into a Suprema guard column and three Suprema SEC columns: 100, 3000, and 30000. Columns and detectors were kept at 50 °C. A standard pullulan kit (Polymer Standards Service) with molecular masses from 342 to 805,000 Da was used for making a calibration curve.

RESULTS AND DISCUSSION

Construction and Expression of GTF180-ΔN Leu⁹⁴⁰ Mutants—The mutations were introduced by PCR using appropriate primers. The PCR library containing random mutations was transformed into E. coli BL21 DE3 star. Twenty clones were selected based on activity as described under "Experimental Procedures." Sequencing of 20 clones revealed five of L940G, one of L940C, two of L940A, two of L940S, two of L940M, one of L940E, four of L940F, and one of L940W. Thus, eight different GTF180-ΔN Leu⁹⁴⁰ mutants were found in total. Wild-type GTF180-ΔN and the eight Leu⁹⁴⁰ mutants were expressed and purified. Compared with GTF180-ΔN, no significant difference in expression levels of the mutants was observed. The eight GTF180-ΔN Leu⁹⁴⁰ mutants covered various classes of amino acids with the exception of amino acids with a positive charge.

Effects of Mutation on Linkage Specificity and Size of α-Glucan Polysaccharides Produced from Sucrose—To explore the effects of mutations at position 940 on the linkage specificity of GTF180-ΔN, the α-glucans produced were analyzed by 1H NMR spectroscopy. The results showed that all eight mutants synthesized α-glucans with a higher percentage of (α1→6) linkages than that of GTF180-ΔN (Table 1). Based on the extent of the shift to (α1→3) linkages, three different groups of mutants were observed. First, mutations of Leu⁹⁴⁰ to similar size amino acids (cysteine, methionine, and glutamate) showed a relatively small shift with ~73% of (α1→6) linkages in the polysaccharide produced (Table 1). A larger shift was observed when the mutant residue (L940G, L940A, and L940S) has a smaller side chain than the wild-type leucine. Mutants L940G, L940A, and L940S produced α-glucans with similar percentages of (α1→6) linkages, which shifted from 67% in wild-type GTF180-ΔN to 85% (Table 1). The largest shift was observed when a large aromatic side chain was introduced (L940F and L940W).
of Leu$_{940}$ to the bulky residue phenylalanine, which is present in reuteransucrase GTFA and GTFO, creating different ratios of ($\alpha_1 \rightarrow 4$) and ($\alpha_1 \rightarrow 6$) linkages (4, 6), and DSRE CD2, creating ($\alpha_1 \rightarrow 2$) branch linkages on dextran (12), caused a shift in the percentage of ($\alpha_1 \rightarrow 6$) linkages to 93% (Table 1). However, the L940F mutation did not enable the synthesis of ($\alpha_1 \rightarrow 4$) or ($\alpha_1 \rightarrow 2$) linkages. Notably, mutation of Leu$_{940}$ to tryptophan abolished ($\alpha_1 \rightarrow 3$) linkage synthesis completely, resulting in synthesis of 52% 6-monosubstituted, and 12% 3,6-disubstituted polysaccharides. The L940W mutant is deficient in the linkage specificity of GTF180-\(\Delta N\) (27). The 1H NMR spectrum of L. reuteransucrase GTFA and GTFO, creating different ratios of ($\alpha_1 \rightarrow 1$) and ($\alpha_1 \rightarrow 2$) linkages. Typically, glucansucrases catalyze the synthesis of $\alpha$-glucans with ($\alpha_1 \rightarrow 6$) linkages exclusively (Fig. 2). Typically, glucansucrases catalyze the synthesis of $\alpha$-glucans with two types of linkage (10). To our knowledge, our present study is the first to report that a single mutation completely abolished one type of linkage in the synthesis of $\alpha$-glucan by a glucansucrase. The polysaccharide produced by wild-type GTF180-\(\Delta N\) is built up from different lengths of isomalto-oligosaccharides, intercon- nected by single ($\alpha_1 \rightarrow 3$) glycosidic linkages in linear and branched orientations (12%) terminal, 24% 3-monosubstituted, 52% 6-monosubstituted, and 12% 3,6-disubstituted $\alpha$-$\delta$-gluco-pyanose residues (27). The L940W mutant is deficient in the synthesis of ($\alpha_1 \rightarrow 3$) branch linkages, resulting in the synthesis of linear $\alpha$-glucans. Until now, it has not been understood how product specificity (including linkage specificity) is determined in glucansucrases; it appears to involve an interplay of residues surrounding the active site that affect the binding mode and orientation of acceptor sugars. In particular, residues from the catalytic domain (domain A), especially those in the region following the transition state stabilizing residue, seem to play an important role (20–24). In our present study, the linkage distribution of the $\alpha$-glucans produced by Leu$_{940}$ mutants clearly suggests that Leu$_{940}$ of domain B plays an important role in linkage specificity of GTF180-\(\Delta N\). Although residue Leu$_{940}$ in GTF180-\(\Delta N\) is not close to the catalytic residues (the distance to Asp$^{1225}$/Glu$^{1063}$ is $\sim$10–11 Å), its side chain points toward the substrate/acceptor binding groove and is located at 6–8 Å distance from the +1 glucosyl moiety of the bound maltose (Protein Data Bank code 3KLL (19)). The clear influence of the size of the (mutant) side chain at position 940 on linkage specificity suggests that steric effects may affect acceptor binding and thus determine which linkage type is favored. In a recent combinatorial engineering study of DSRS from L. mesen- teroides NRRL B-512F, residue Phe$^{553}$ (DSRS numbering, corre- sponding to Ala$^{978}$ in GTF180) of domain B was shown to slightly alter linkage specificity (36). The present study indicates that not only residues from domain A but also from domain B of glucansucrase are critical for linkage specificity determination.

Regarding product size, the polysaccharides produced by mutants L940A, L940S, L940M, L940E, and L940F were similar to that of wild-type GTF180-\(\Delta N\) (Table 1). L940G and L940C synthesized $\sim$25% smaller polysaccharides than that of GTF180-\(\Delta N\) (Table 1). Notably, the $\alpha$-glucan polysaccharide synthesized by mutant L940W showed a size reduction of 70%. Previous studies have reported glucansucrase mutations that resulted in synthesis of polysaccharides with reduced molecular masses (22, 37, 38), but a clear picture of the mechanism of size determination has not emerged. Mutations at position 940, lining the binding groove, may affect the affinity for acceptor molecules during product elongation and thus the final size of the polysaccharides.

Effects of Mutations on Oligosaccharide Synthesis from Sucrose Only and from Sucrose and Maltose—The altered linkage specificity of GTF180-\(\Delta N\) Leu$_{940}$ enzyme variants was also observed in the oligosaccharides produced from sucrose (Table 1). For each enzyme, the percentage of ($\alpha_1 \rightarrow 6$) linkages in the oligosaccharides is higher than that in the polysaccharides produced (Table 1). There is a significant positive correlation between the number of ($\alpha_1 \rightarrow 6$) linkages in the polysaccharide and the amount of ($\alpha_1 \rightarrow 6$) linkages in the oligosaccharides. It is noteworthy that the HPAEC (CarboPac PA-1) profile of the oligosaccharides produced by the L940W mutant showed a relatively simple pattern of peaks with increasing degrees of polymerization (according to MALDI-TOF-MS) (Fig. 3A). The peaks were assigned by combining NMR data of the oligosaccharide pool and by comparison of the retention times with those of reference compounds (Fig. 3). The 1H NMR spectrum of the oligosaccharide pool produced by the L940W mutant
The Crucial Role of Leu940 in the Specificity of GTF180

enzyme with sucrose as substrate (Fig. 3B) showed one major signal and four minor signals in the anomeric region (δ 4.5–5.5). The predominant signal at δ 4.96 belongs to successive (α1→6) linkages (39). The 1H chemical shifts of the minor anomeric signals at δ 5.242 (R1α) and 4.675 (R1β) indicate the presence of 6-substituted reducing Glc residues (39). Typical chemical shift values for sucrose fragments (Glc, g; Fru, f) are δ 5.431, stemming from Glc g H-1, and δ 4.223, stemming from Fru f H-3 (39). The anomeric signal at δ 5.110 (Glc, g) indicates the presence of leucose (α-D-Glc-(1→5)-β-D-Fru) (40). The NMR data showed that released glucose and sucrose were used as acceptors by the L940W mutant and that they are further elongated with successive (α1→6) linkages. Fructose was also used as acceptor to produce leucrose. The majority of the oligosaccharide products produced by the L940W mutant from sucrose are linear isomalt-oligosaccharides with increasing degrees of polymerization, whereas sucrose-containing oligosaccharides and leucrose are present in lesser amounts (Fig. 3B). Therefore, the L940W mutant is efficient in the synthesis of isomalt-oligosaccharides from sucrose and holds great potential for the production of isomalt-oligosaccharides. However, the HPAEC profiles of oligosaccharides produced by the wild-type GTF180-ΔN enzyme (Fig. 3A) and other mutants (data not shown) showed very complex oligosaccharide mixtures. The wild-type GTF180-ΔN and other mutants produced oligosaccharides containing both (α1→6) and (α1→3) linkages using glucose and sucrose as acceptors (Table 1). Here, fructose was also used as acceptor to produce leucrose.

With the availability of maltose as acceptor, GTF180-ΔN catalyzes the synthesis of panose, glucosyl-(α1→6)-panose (α- D-GlcP-(1→6)-α- D-GlcP), and [glucosyl-(α1→6)]2-panose (α- D-GlcP-(1→6)-α- D-GlcP-(1→4)-α- D-GlcP), and [glucosyl-(α1→6)]3-panose (α- D-GlcP-(1→6)-α- D-GlcP-(1→4)-α- D-GlcP-(1→3)-α- D-GlcP), etc. (19). Table 2 shows that there are no significant differences in the amounts of panose and glucosyl-(α1→6)-panose produced by wild-type GTF180-ΔN and Leu940 mutants in the presence of 100 mM sucrose (donor) and 100 mM maltose (acceptor). This result may be explained by the fact that maltose (as an acceptor) binds “away” from the position of Leu940 (19). In other words, the mutations at position Leu940 are not close enough to the maltose binding site to affect the linkage specificity of oligosaccharides produced. However, a positive correlation was found between the percentage of (α1→6) linkages in the polysaccharides and the amount of [glucosyl-(α1→6)]2-panose produced by GTF180-ΔN and Leu940 mutants in the presence of 100 mM sucrose and 100 mM maltose (Table 2). Thus, the altered linkage specificity was also reflected in the oligosaccharide synthesis from sucrose and maltose. Monchois et al. (24) also reported that no additional new oligosaccharides were produced by Asp569 mutants of GTF-I from S. downei in the presence of maltose and sucrose. However, the distribution of different sizes of oligosaccharides was affected by using different concentrations of maltose and sucrose (24). Effects of Mutation on Reaction Specificity—Several of the mutations at residue 940 in GTF180-ΔN affected the reaction specificity, reflecting in the relative amounts of sucrose used for hydrolysis, oligosaccharide synthesis, and polysaccharide synthesis, respectively (Table 3). Wild-type GTF180-ΔN used 23.9,

![FIGURE 3. A, HPAEC (CarboPac PA-1) analysis of the oligosaccharides synthesized by the wild-type GTF180-ΔN and L940W mutant enzymes using 100 mM sucrose as substrate. The y axes of chromatograms were elevated to better visualize oligosaccharide chromatograms. G, glucose; F, fructose; L, leucrose; ι2, isomalto-oligosaccharides; ι3, isomaltotriose; ι5, isomaltopentaose; ι1, glucosyl-(α1→6)-sucrose. *, minor amounts of oligosaccharides that are expected to stem from glucosyl-(α1→6) elongations of ι1 and coelute with the isomalto-oligosaccharides at higher degrees of polymerization. B, the one-dimensional 1H NMR spectrum of the oligosaccharide fraction obtained after incubation of sucrose with the L940W mutant enzyme.](image-url)
16.3, and 59.7% of sucrose for hydrolysis, polysaccharide synthesis, and oligosaccharide synthesis, respectively (Table 3). The hydrolysis of L940G and L940C accounted for more than 30% of sucrose added, whereas the percentage for polysaccharide synthesis decreased to ~8% (Table 3). As seen for linkage specificity, mutants L940A and L940S also behaved similarly regarding reaction specificity with only a slight increase in hydrolysis and an increased sucrose consumption for polysaccharide synthesis (~22%; Table 3). The L940M mutation had no effect on reaction specificity (Table 3), which may be explained by the similar properties of leucine and methionine (both are hydrophobic and similar in size). The L940E and L940F mutants both showed a significant increase in polysaccharide synthesis (to ~30% of sucrose). Hydrolysis of L940E remained the same as in GTF180-ΔN, whereas in L940F, hydrolysis was reduced to 16%. The strongest reduction in hydrolysis was observed with L940W: it decreased 6-fold compared with wild-type GTF180-ΔN. A possible explanation is that the tryptophan side chain increases the hydrophobicity of the active site and/or partially shields off a bound sucrose for attack by water. Reducing hydrolysis is an important aspect in engineering of glucan-synthesizing enzymes; the L940W mutation thus provides a promising starting point for further engineering. In addition, the percentage of sucrose utilization for polysaccharide synthesis of L940W was reduced to ~4.3%, whereas oligosaccharide synthesis increased to 91.8%.

Overall, our results show that mutations of Leu<sup>940</sup> in GTF180-ΔN affect reaction specificity significantly. Most mutation studies involving glucansucrases have focused on changing linkage specificity (10, 11, 41), whereas only a few have investigated the effects of mutations on reaction specificity. For example, mutations S628D and S628R in GTFR from <i>S. oralis</i> were reported to have a heavily impaired polysaccharide synthesis and synthesize many more short chain oligosaccharides instead (20). Mutations at Leu<sup>940</sup> in GTF180-ΔN showed that the relative balance of the three reactions could be engineered toward either polysaccharide synthesis (L940A, L940S, L940E, and L940F) or oligosaccharide synthesis (L940W).

**Effects of Mutation on Kinetic Parameters of Enzymes**—The kinetic parameters (K<sub>m</sub> and k<sub>cat</sub>) determined for wild-type GTF180-ΔN and Leu<sup>940</sup> mutants are summarized in Table 4. In general, all Leu<sup>940</sup> mutants showed an increased K<sub>m</sub> for sucrose. Compared with wild-type GTF180-ΔN, the K<sub>m</sub> values of L940G, L940M, and L940F showed only a minor increase, whereas L940C, L940A, L940S, L940E, and L940W showed a 4–5-fold increase in K<sub>m</sub>. L940C and L940M showed only slightly decreased k<sub>cat</sub> values compared with that of wild-type GTF180-ΔN, whereas the k<sub>cat</sub> values of the other mutants were reduced approximately by 2–4-fold (Table 4). Again, L940A and L940S behaved similarly, as previously shown for linkage and reaction specificity (Tables 1, 3, and 4). Mutation to methionine had the smallest effects (only 50% reduction) on activity (k<sub>cat</sub>/K<sub>m</sub>), possibly because of its similar properties with leucine. Taken together, all mutants showed a decrease in catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub> Table 4), which may be due to the fact that mutations change the shape of the binding groove and thus may affect the affinity (19, 41).

**Docking Studies**—Linkage type distribution analysis showed that, using sucrose, mutant L940W is incapable of (α1→3) linkage formation, and only catalyzes the synthesis of (α1→6) linkages (Table 1). Another interesting feature of the L940W mutant is its 6-fold decreased hydrolysis. The available high resolution crystal structure of GTF180-ΔN and a model of its covalent glucosyl-enzyme intermediate (19) allowed us to investigate the structural effects of the L940W mutation, by performing docking experiments using isomaltotriose (α-D-Glc-p(1→6)-α-D-Glc-p(1→6)-α-D-Glc-p) as the ligand. The top-ranked mutants are shown in Fig. 4; the results are consistent with those from product analysis and kinetic analysis described above.

Previously, it was shown that in wild-type GTF180-ΔN, docked (α1→6)-linked gluco-oligosaccharides adopt conformations favorable for either (α1→3)- or (α1→3)-linkage formation, although the former is preferred (19). In our docking experiments with wild-type GTF180-ΔN, isomaltotriose indeed adopted a conformation where the O3 hydroxyl group of the nonreducing end glucosyl unit closely interacts with the Cl atom of the covalent glucosyl-enzyme intermediate to form an (α1→3) linkage (this conformation was similar to the previously reported one, except that the trisaccharide is shifted by one glucosyl unit because of the definition of the docking space box). The other two glucosyl units (subsites +2 and +3) were located near residue Leu<sup>940</sup> (Fig. 4A), in the groove lined on the one side by residues of domain B.

Docking experiments with the GTF180-ΔN L940W mutant revealed important differences regarding the configurational space for isomaltotriose. When applying the most energetically stable rotamer of L940W (the first rotamer; Fig. 4B), the tryptophan side chain was observed to block the

### Table 3

| Enzymes       | Hydrolysis | Polysaccharide synthesis | Oligosaccharide synthesis |
|---------------|------------|--------------------------|----------------------------|
| GTF180-ΔN     | 23.9 ± 0.7 | 16.3 ± 0.7               | 59.7 ± 1.2                 |
| L940G         | 30.1 ± 0.3 | 8.9 ± 0.2                | 61.0 ± 0.4                 |
| L940C         | 32.0 ± 0.7 | 8.1 ± 0.3                | 59.9 ± 0.9                 |
| L940A         | 26.3 ± 1.0 | 22.3 ± 0.4               | 51.4 ± 0.7                 |
| L940S         | 27.9 ± 0.3 | 21.4 ± 0.2               | 50.7 ± 0.2                 |
| L940M         | 24.4 ± 0.9 | 18.6 ± 0.1               | 57.0 ± 0.9                 |
| L940E         | 23.9 ± 0.8 | 29.9 ± 0.3               | 46.2 ± 0.9                 |
| L940F         | 15.8 ± 0.1 | 30.4 ± 0.5               | 53.8 ± 0.5                 |
| L940W         | 3.9 ± 0.4  | 4.3 ± 0.1                | 91.8 ± 0.5                 |

*The values show the percentages of sucrose used for hydrolysis, polysaccharide synthesis, and oligosaccharide synthesis of the total amount of sucrose present initially in the incubations.*

### Table 4

| Enzyme       | Km (mM) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (s<sup>-1</sup>·mM<sup>-1</sup>) |
|--------------|---------|---------------------------------|-------------------------------------------------|
| GTF180-ΔN    | 5.0 ± 0.3 | 303.0 ± 3.6 | 60.6                                              |
| L940G        | 9.0 ± 1.0 | 134.0 ± 3.1 | 14.9                                              |
| L940C        | 24.1 ± 1.5 | 253.8 ± 4.8 | 10.5                                              |
| L940A        | 27.9 ± 2.1 | 135.6 ± 4.9 | 4.9                                               |
| L940S        | 20.1 ± 2.6 | 84.0 ± 4.0  | 4.2                                               |
| L940M        | 7.9 ± 0.5  | 237.0 ± 32  | 30                                                |
| L940E        | 19.3 ± 2.6 | 117.9 ± 4.0 | 6.1                                               |
| L940F        | 7.1 ± 0.4  | 158.7 ± 1.8 | 22.4                                              |
| L940W        | 21.1 ± 2.2 | 190.7 ± 6.1 | 9.0                                               |

*The kinetic parameters (K<sub>m</sub> and k<sub>cat</sub>) were determined with 12 different sucrose concentrations ranging from 0.5 to 200 mM.*
The Crucial Role of Leu\textsuperscript{940} in the Specificity of GTF180

groove entrance, preventing the reducing end units of isomaltotriose to occupy the space observed in the wild-type enzyme. Instead, the ligand reoriented in such a way that the reducing end sugar moiety was now bound near residues Asn\textsuperscript{1138}, Asp\textsuperscript{1141}, and Gln\textsuperscript{1142}. Importantly, the C3 hydroxyl group of the glucosyl unit bound in subsite +1 is located too far from the C1 atom of the glucosyl-enzyme intermediate. Instead, the C6 hydroxyl group is now found to be well positioned for the formation of an (\(\alpha1\rightarrow6\)) linkage. In the case of the second rotamer of L940W, the tryptophan side chain aligned with the groove surface (Fig. 4C), modifying the shape of the groove to a lesser extent than that of the first

FIGURE 4. Molecular docking of isomaltotriose in the acceptor binding site of GTF180-\(\Delta N\). Residues of domain A are depicted in blue; residues of domain B are in green. A, wild-type GTF180-\(\Delta N\). The O3 of the glucosyl unit bound in subsite +1 is at close distance (3.1 Å, see arrow) from the C1 atom of the covalent glucosyl-enzyme intermediate. B, in mutant L940W rotamer 1, the tryptophan side chain blocks the groove and forces the reducing end of the trisaccharide toward residues from the loop following residue Asp\textsuperscript{1136}, the transition state stabilizing residue. Consequently, the glucosyl unit in subsite +1 cannot orient its C3-hydroxyl toward the glucosyl-enzyme intermediate; rather its C6-hydroxyl group is at closer distance to form an (\(\alpha1\rightarrow6\)) linkage. C, in rotamer 2 of Trp\textsuperscript{940}, the shape of the groove is less affected. Nevertheless, the trisaccharide is reoriented such that at subsite +1 neither of the hydroxyl groups is close enough to form a glycosidic linkage.
rotamer. Although isomaltotriose was still able to sample similar configurations as observed in the wild-type enzyme, the orientation of the middle glucosyl unit did not allow the C3 hydroxyl group of the middle glucosyl unit to approach the C1 atom of the glucosyl-enzyme intermediate close enough to favor glycosidic linkage formation.

In summary, the docking studies showed that the binding mode of isomaltotriose is constrained by the groove shape that is lined (among others) by residue Leu$^{940}$, which is positioned near subsites +2 and +3 of this trisaccharide (Fig. 4A). Introduction of the bulky tryptophan side chain forces the trisaccharide to adopt a different conformation, such that the subsite +1 glucosyl no longer favors ($\alpha_1 \rightarrow 3$) linkage formation, while ($\alpha_1 \rightarrow 6$) linkage formation is retained. Thus, these results explain the altered linkage specificity of the L940W mutant and indicate the importance of steric effects for acceptor binding. Indeed, a structural inspection of other mutations (Gly, Cys, Ala, Ser, Met, Glu, or Phe) at position 940 showed that they all change the shape of the binding groove (data not shown), apparently disfavoring ($\alpha_1 \rightarrow 3$) linkage formation.

**Conclusions**—Our data show that residue 940 of GTF180-$\Delta N$ is of critical importance for linkage specificity, reaction specificity, and activity. Mutation of Leu$^{940}$ to eight different residues (Gly, Cys, Ala, Ser, Met, Glu, Phe, and Trp) all caused a shift in linkage specificity to ($\alpha_1 \rightarrow 6$) linkage in the polysaccharides and oligosaccharides synthesized from sucrone only or from both sucrose and maltose. Interestingly, in mutant L940W, ($\alpha_1 \rightarrow 3$) linkage synthesis was completely abolished, and a smaller linear polysaccharide with only ($\alpha_1 \rightarrow 6$) linkages was synthesized. Docking studies provide molecular insight into how the introduction of the tryptophan side chain prevents ($\alpha_1 \rightarrow 3$) linkage-favoring acceptor orientation and point to the importance of steric effects for linkage specificity.

In addition to the altered linkage specificity, our data also showed that the reaction specificity can be engineered to increase either polysaccharide synthesis (L940A, L940S, L940E, and L940F) or oligosaccharide synthesis (L940W) or to reduce hydrolysis (L940W). The L940W mutant is capable of producing a large amount of isomalto-oligosaccharides from sucrone. All Leu$^{940}$ mutants retained a relatively high activity even though their $K_{m}$ was increased and $k_{cat}$ was reduced.

$\alpha$-Glucans containing 67–100% of ($\alpha_1 \rightarrow 6$) linkages are produced by GTF180-$\Delta N$ and Leu$^{940}$ mutants and may show different physicochemical properties. Given the conservation of residue Leu$^{940}$ and its equivalents among glucansucrases (only leucine or phenylalanine is observed), the importance of this residue for product specificity likely extends to other glucansucrases. This may also be true for other residues from domain B, such as Tyr$^{935}$, Leu$^{938}$ and Leu$^{981}$ in GTF180-$\Delta N$ (Fig. 1), shaping the binding groove at the same side. Supplemeting previously reported residues from domain A, these residues provide new targets for enzyme engineering, aiming at tailor-made products with novel properties. Our study provides novel insights into the structure-function relationships of glucansucrases regarding their linkage specificity, reaction specificity, and activity.

**Acknowledgment**—We thank Albert Woortman (Macromolecular Chemistry and New Polysaccharide Materials, University of Groningen) for assistance in size determination of $\alpha$-glucan oligosaccharides.

**REFERENCES**

1. Shornikova, A.-V., Casas, I. A., Isolauri, E., Mykkänen, H., and Vesikari, T. (1997) *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. *J. Pediatr. Gastro. Nutr.* 24, 399–404

2. Rosenfeldt, V., Fleischer Michaelsen, K., Jakobsen, M., Nexmann Larsen, C., Möller, P. L., Pedersen, P., Tyved, M., Weyrehter, H., Valerius, N. H., and Paerregaard, A. (2002) Effect of probiotic *Lactobacillus* strains in young children hospitalized with acute diarrhea. *Pediatri. Infect. Dis. J.* 21, 411–416

3. Tubelius, P., Stan, V., and Zachrisson, A. (2005) Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomised, double-blind placebo-controlled study. *Environ. Health* 4, 25

4. Kralj, S., Stripling, E., Sanders, P., van Geel-Schutten, G. H., and Dijkhuizen, L. (2005) Highly hydrolytic reuteransucrase from probiotic *Lactobacillus reuteri* strain ATCC 55730. *Appl. Environ. Microbiol.* 71, 3942–3950

5. Kralj, S., van Geel-Schutten, G. H., Dondorff, M. M., Kirsanovs, S., van der Maarel, M. I., and Dijkhuizen, L. (2004) Glucan synthesis in the genus *Lactobacillus*: isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. *Microbiology* 150, 3681–3690

6. 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

7. van Geel-Schutten, G. H., Fleisch, F., ten Brink, B., Smith, M. R., and Dijkhuizen, L. (1998) Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides. *Appl. Microbiol. Biotechnol.* 50, 697–703

8. Kaditzky, S. B., Behr, J., Stocker, A., Kaden, P., Gänzle, M. G., and Vogel, R. F. (2008) Influence of pH on the formation of glucan by *Lactobacillus reuteri* TMW 1.106 exerting a protective function against extreme pH values. *Food Biotechnol.* 22, 398–416

9. Olano-Martín, E., Mountzouris, C. K., Gibson, G. R., and Rastall, R. A. (2000) *In vitro* fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. *Br. J. Nutr.* 83, 247–255

10. Leemhuis, H., Pijning, T., Dobruchowska, J. M., Dijkstra, B. W., and Paerregaard, A. (2002) Effect of probiotic *Bifidobacterium longum* subsp. *longum* BB12 on the small intestinal microflora of young children hospitalised with acute diarrhoea. *J. Pediatr. Gastr. Nutr.* 24, 287, 7915–7924

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

12. Brison, Y., Pijning, T., Malbert, Y., Fabre, É., Mourey, L., Morel, S., Potocki-Vérone, G., Monsan, P., Tranier, S., Remaud-Siméon, M., and Dijkstra, B. W. (2012) Functional and structural characterization of $\alpha$-1→2) branching sucrase derived from DSR-E glucansucrase. *J. Biol. Chem.* 287, 7915–7924

13. Badel, S., Bernardi, T., and Michaud, P. (2011) New perspectives for *Lactobacillus* exopolysaccharides. *Biotechnol. Adv.* 29, 54–66

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

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

16. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrisaat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* 37, D233–D238

17. MacGregor, E. A., Jespersen, H. M., and Svensson, B. (1996) A circularly permuted $\alpha$-amylose-type $\alpha/\beta$-barrel structure in glucan-synthesizing
The Crucial Role of Leu\textsuperscript{940} in the Specificity of GTF180

glucosyltransferases. FERS Lett. 378, 263–266
18. Moozer, G., Hefta, S. A., Paxton, R. J., Shively, J. E., and Lee, T. D. (1991) Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two Streptococcus sobrinus α-glucosyltransferases. J. Biol. Chem. 266, 8916–8922
19. Vujicic-Zagar, A., Pijning, T., Kralj, S., Lopez, C. A., Eeuwema, W., Dijkhuizen, L., and Dijkstra, B. W. (2008) Structural analysis of the coreregion of dextransucrase. Biochemistry 47, 6678–6684
20. Hellmuth, H., Wittrock, S., Kralj, S., Dijkhuizen, L., Hofer, B., and Seibel, J. (2008) Engineering the glucansucrase GTFR enzyme reaction mechanism and glycosidic bond specificity: toward tailor-made polymer and oligosaccharide products. Biochemistry 47, 6678–6684
21. 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
22. van Leeuwen, S. S., Kralj, S., Lopez, C. A., Eeuwema, W., Dijkhuizen, L., and Kamerling, J. P. (2006) Role of aspartic acid residues at the N-terminal end of the core region Streptococcus downei glucansucrase, GTF-I. Appl. Microbiol. Biotechnol. 52, 660–665
23. van Leeuwen, S. S., Kralj, S., van Geel-Schutten, I. H., Gerwig, G. J., Dijkhuizen, L., and Kamerling, J. P. (2008) Structural characterization of the asparagine 1134 in glucosidic bond and transglycosylation specificity of glucansucrase GTFR enzyme mutants of Lactobacillus reuteri strain 180. Biomacromolecules 10, 580–588
24. Kralj, S., van Geel-Schutten, I. G., Fabre, E., I., van der Maarel, M. J., and Dijkhuizen, L. (2005) Rational transformation of Lactobacillus reuteri 121 reuteransucrase into a dextransucrase. Biochemistry 44, 9206–9216
25. Monchois, V., Vignon, M., and Russell, R. R. (2000) Mutagenesis of Asp-569 of glucosyltransferase I glucansucrase modulates glucan and oligosaccharide synthesis. Appl. Environ. Microb. 66, 1923–1927
26. Funane, K., Ishii, T., Ono, H., and Kobayashi, M. (2005) Changes in linkage pattern of glucan products induced by substitution of Lys residues in the dextransucrase. FEBS Lett. 579, 4739–4745
27. Monchois, V., Vignon, M., and Russell, R. R. (1999) Isolation of key amino acid residues at the N-terminal end of the core region Streptococcus downei glucansucrase, GTF-I. Appl. Microbiol. Biotechnol. 52, 660–665
28. 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-1,6-glucan (EP5180) produced by the Lactobacillus reuteri strain 180 glucansucrase GTFR enzyme. Carbohydr. Res. 343, 1237–1250
29. Pijning, T., Vujicic-Zagar, A., Kralj, S., Dijkhuizen, L., and Dijkstra, B. W. (2012) Structure of the α-1,6/α-1,4-specific glucansucrase GTFR from Lactobacillus reuteri 121. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 68, 1448–1454
30. Goujon, M., McWilliam, H., Li, W., Valentín, F., Squizatto, S., Paern, J., and Lopez, R. (2010) A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res. 38, W695–W699
31. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428
32. 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. Microb. 65, 3008–3014
33. Wang, J., Wolf, R. M., Caldwell, J. W., Kollman, P. A., and Case, D. A. (2004) Development and testing of a general amber force field. J. Comput. Chem. 25, 1157–1174
34. Dunbrack, R. L., Jr. (2002) Rotamer libraries in the 21st century. Curr. Opin. Struct. Biol. 12, 431–440
35. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785–2791
36. Irague, 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
37. 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. FERS J. 273, 3735–3742
38. Irague, 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-1-glucansucrase glucansucrase mutants. Biomacromolecules 13, 187–195
39. Dobruchowska, J. M., Meng, X., Leemhuis, H., Gerwig, G. J., Dijkhuizen, L., and Kamerling, J. P. (2013) Gluco-oligomers initially formed by the glucansucrase enzyme of Lactobacillus reuteri 121 incubated with sucrose and malto-oligosaccharides. Glycobiology 23, 1084–1096
40. Thompson, J., Robrish, S. A., Pikis, A., Brust, A., and Lichtenhaler, F. W. (2001) Phosphorylation and metabolism of sucrose and its five linkage-isomeric α-1-glucosyl-α-fructoses by Klebsiella pneumoniae. Carbohydr. Res. 331, 149–161
41. 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