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Highly Hydrolytic Reuteransucrase from Probiotic *Lactobacillus reuteri* Strain ATCC 55730

S. Kralj,[1,4] E. Stripling,[1,4] P. Sanders,[2] G. H. van Geel-Schutten,[1,3] and L. Dijkhuizen[1,4]*

Centre for Carbohydrate Bioengineering, TNO-University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands[1]; Innovative Ingredients and Products, TNO Nutrition and Food Research, Rouaanstraat 27, 9723 CC Groningen, The Netherlands[2]; Innovative Ingredients and Products, TNO Nutrition and Food Research, Utrechtseweg 48, 3704 HE Zeist, The Netherlands[3]; and Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands[4]

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Lactobacillus reuteri **strain ATCC 55730 (LB BIO) was isolated as a pure culture from a Reuteri tablet purchased from the BioGaia company. This probiotic strain produces a soluble glucan (reuteran), in which the majority of the linkages are of the α-(1→4) glucosidic type (~70%). This reuteran also contains α-(1→6)-linked glucosyl units and 4,6-disubstituted α-glucosyl units at the branching points. The LB BIO glucansucrase gene (*gtfO*) was cloned and expressed in *Escherichia coli*, and the GTFO enzyme was purified. The recombinant GTFO enzyme and the LB BIO culture supernatants synthesized identical glucan polymers with respect to linkage type and size distribution. GTFO thus is a reuteransucrase, responsible for synthesis of this reuteran polymer in LB BIO. The preference of GTFO for synthesizing α-(1→4) linkages is also evident from the oligosaccharides produced from sucrose with different acceptor substrates, e.g., isopanose from isomaltose. GTFO has a relatively high hydrolysis/transferase activity ratio. Complete conversion of 100 mM sucrose by GTFO nevertheless yielded large amounts of reuteran, although more than 50% of sucrose was converted into glucose. This is only the second example of the isolation and characterization of a reuteransucrase and its reuteran product, both found in different *L. reuteri* strains. GTFO synthesizes a reuteran with the highest amount of α-(1→4) linkages reported to date.**

Lactic acid bacteria are gram-positive bacteria, represented by genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* (2). Members of these genera are used for several food (and feed) applications (e.g., silage, dairy products, vegetables, sourdough, fish, and meats) (14, 15, 27). Many LAB produce an abundant variety of exopolysaccharides, either as heteropolysaccharides or as homopolysaccharides (8, 19), which may find applications as a new generation of food-grade ingredients.

α-Glucans represent one example of homopolysaccharides; they are synthesized from sucrose by large extracellular enzymes, glucosyltransferases (EC 2.4.1.5, commonly named glucansucrases [GTFs]). Glucansucrase enzymes catalyze two different reactions, depending on the nature of the acceptor substrate: (i) hydrolysis of sucrose (acceptor substrate, water) and (ii) glucosyl transfer (transferase), which can be divided into polymerization (acceptor substrate, the growing glucan chain) and oligosaccharide synthesis (acceptor substrates, oligosaccharides, such as maltose and isomaltose).

Glucansucrase enzymes have been characterized from *Leuconostoc*, *Streptococcus*, and *Lactobacillus* spp. (10–12, 19, 27, 30), synthesizing glucans with α-(1→2), α-(1→3) (mutan), α-(1→4) (reuteran), or α-(1→6) (dextran) glucosidic linkages. Only a single glucansucrase enzyme synthesizing a branched glucan (reuteran) with α-(1→4), α-(1→6) and α-(1→4,6) glucosidic bonds, has been characterized, namely, GTFA of *Lactobacillus reuteri* 121 (11, 13).

Glucansucrases from *L. reuteri* strains and their products are of special interest for different reasons. (i) Some *L. reuteri* strains have probiotic properties (24), as has been demonstrated with various animals and humans (6, 29). (ii) The glucans (and α-glucooligosaccharides) produced by *L. reuteri* (10) may also act as prebiotics by stimulating the growth of probiotic strains or of beneficial strains of the gastrointestinal tract. Prebiotic effects of these oligosaccharides have been demonstrated for piglets, broilers, and calves (20). Glucans and glucooligosaccharides from lactobacilli are thus interesting and feasible ingredients for the production of foods (e.g., sourdough, yogurts, health foods).

Besides GTFA from *L. reuteri* 121 (11), two other glucosyltransferase enzymes from different *L. reuteri* strains have been characterized recently: GTF180 [α-(1→6); dextran] from *L. reuteri* 180 and GTFML1 [α-(1→3); mutan] from *L. reuteri* ML1. These three enzymes display high sequence similarity (~60% identity, ~75% similarity) but nevertheless synthesize different glucan products (10, 11). This paper describes the molecular and biochemical characterization of a novel reuteransucrase gene (*gtfO*), and the reuteransucrase enzyme (GTFO) encoded, from the probiotic *L. reuteri* strain ATCC 55730 used by the BioGaia company in different human health products. This novel reuteransucrase displays a high hydrolysis/transferase activity ratio and synthesizes a branched glucan (reuteran) containing the highest amount of α-(1→4) glucosidic linkages reported to date.

* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands. Phone: 31.50.3632150. Fax: 31.50.3632154. E-mail: l.dijkhuizen@rug.nl.

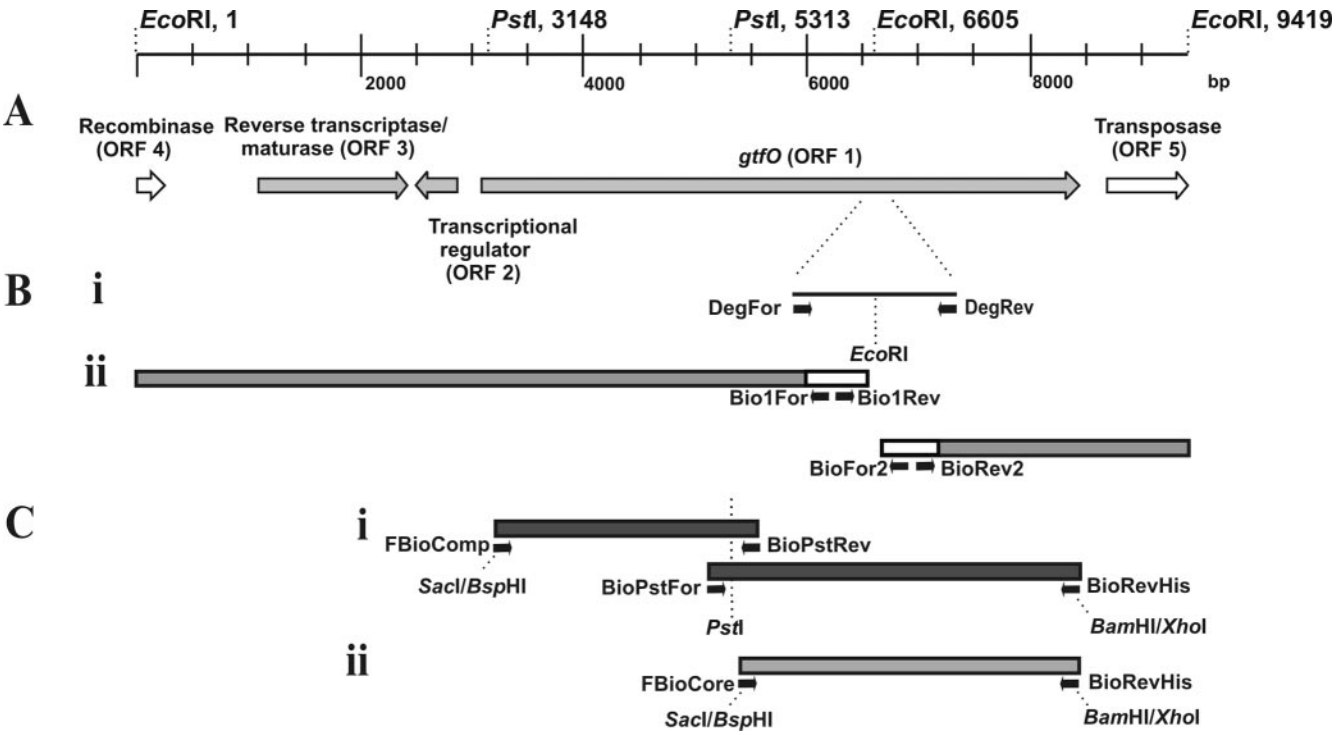

FIG. 1. (A) Schematic representation of the organization of *gtfO* and surrounding regions. (B) Strategy used for the isolation of the *gtfO* gene from *L. reuteri* strain ATCC 55730 chromosomal DNA. Primers are indicated with small, black arrows. (i) ~660-bp PCR product isolated with degenerate primers. (ii) Fragments amplified by inverse PCR of EcoRI-digested and ligated LB BIO chromosomal DNA (~6,000 bp and ~3,000 bp, respectively). Partial ORFs are shown as open arrows. (C) Primers and restriction sites used for the following. (i) Cloning of the complete *gtfO* gene. The two fragments were first cloned separately in pBluescript II SK(+). After construction of the complete *gtfO* gene in pBluescript II SK(+), the *gtfO* gene was cloned into the expression vector pET15b. (ii) Cloning of the *gtfO*-Δ*N* gene (N-terminally truncated variant) in the expression vector pET15b.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *L. reuteri* strain ATCC 55730 (LB BIO) was isolated as a pure culture from a "Reuteri tablet" (BioGaia AB, Stockholm, Sweden) and cultivated as described previously (12). The taxonomic position of *L. reuteri* ATCC 55730 was confirmed by 16S rRNA analysis (99% identity within 1,537 nucleotides with the *L. reuteri* type strain DSM 20016 T). *E. coli* TOP 10 (Invitrogen, Carlsbad, CA) and plasmid pCR-XL-TOPO (Invitrogen) were used for cloning of the *gtf* gene and for sequencing purposes. Plasmid pBluescript II SK$^+$ (Stratagene, La Jolla, CA) was used for cloning of the complete *gtf* gene. Plasmid pET15b (Novagen, Madison, WI) was used for expression of the *gtf* gene in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown as described previously (13) and when required were supplemented with the appropriate antibiotic (100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin). Agar plates were made by adding 1.5% agar to the LB medium; when appropriate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 μg ml^{-1}) was added.

Molecular techniques. General procedures for restriction, ligation, cloning, PCR, inverse PCR (iPCR), *E. coli* transformations, DNA isolation and manipulations, isolation of DNA fragments from gel, and agarose gel electrophoresis were as described previously (13). Primers were obtained from Eurogentec, Seraing, Belgium. Sequencing was performed by GATC (Konstanz, Germany).

Identification and nucleotide sequence analysis of the *gtf* gene. A first fragment of the glucansucrase gene was isolated by PCR amplification of chromosomal DNA of the *L. reuteri* strain ATCC 55730, using degenerate primers (DegFor and DegRev; Fig. 1B) (12). The ~660-bp amplified product was ligated into pCR-XL-TOPO and transformed to *E. coli* TOP 10. Ten random clones were subjected to restriction analysis, followed by sequencing of five of these clones (12), revealing identical nucleotide sequences and confirming *gtf* gene identity. The 660-bp amplified fragment was used to design primers for subsequent iPCR reactions (28) (Table 1; Fig. 1B).

Construction of plasmids for expression of (mutant) *gtfO* genes in *E. coli*. Appropriate primer pairs and template DNA were used to create two different expression constructs with a C-terminal His tag: for the complete GTFO (1,750 amino acids), constructed with two separate PCRs using the method previously described for GTFA from *L. reuteri* 121 (11), and an N-terminally truncated variant of GTFO (GTFO-ΔN; 1,042 amino acids) (Table 1; Fig. 1C).

Purification of GTFO(-ΔN) proteins. Proteins were purified using nickel-nitrilotriacetic acid affinity chromatography, followed by anion exchange chromatography as described previously (13). Protein concentrations were determined with the Bradford method using the Bio-Rad reagent and bovine serum albumin as the standard (Bio-Rad, Veenendaal, The Netherlands).

Enzyme activity assays. The various reuteransucrase activities were determined as initial rates by measuring glucose and fructose release (enzymatically) from sucrose conversion (six data points over a period of 6 min) (13, 30). Unless indicated otherwise, reactions were performed at 35°C in 25 mM NaAc buffer, pH 5.0, containing 1 mM $CaCl_2$ and 30 nM purified GTFO(-ΔN) enzymes. One unit of enzyme activity is defined as the release of 1 μmol of monosaccharide per min.

(i) pH and temperature optima. pH and temperature optima were determined by measuring the amounts of glucose and fructose released in 30 min from 50 mM sucrose (13).

(ii) Kinetic parameters. Kinetic assays were performed with 15 different sucrose concentrations, ranging from 0.25 to 100 mM (13).

(iii) GTFO activity with maltose as an acceptor substrate. The effect of maltose on GTFO(-ΔN) enzyme activity (initial rates) was determined, using 50 mM sucrose and 100 mM maltose, measuring fructose release.

Analysis of products synthesized from sucrose. After complete depletion of sucrose (100 mM, 60 h at 35°C) by 30 nM GTFO(-ΔN) enzymes, the concentrations of fructose, glucose, isomaltose, and leucrose in the reaction medium were determined using anion exchange chromatography (Dionex) (13).

Oligosaccharides synthesized from sucrose and (iso)maltose as acceptor substrates. (i) Synthesis and analysis. After complete depletion of sucrose (100 mM, 60 h at 35°C) by 30 nM GTFO(-ΔN) enzymes, incubated together with the acceptor substrates maltose or isomaltose (100 mM each), the oligosaccharides synthesized were analyzed by anion exchange chromatography (13).

TABLE 1. Primers and vectors used for inverse PCR reactions, amplification, cloning, and expression of *gtfO(-ΔN)* of *Lactobacillus reuteri* ATCC 55730

Gene or construct	Primer	Sequence of direct primer (5′→3′)[a]	Primer	Sequence of reverse primer (5′→3′)[a]	Vector
gtfO					
5′	Bio1For	GGAACGATTACCGGCAATAACGACCAGGCG	Bio1Rev	GTTCAGGAGGTAGTAGAGCCAGTTCAGCTG	pCR-XL-TOPO
3′	BioFor2	CATACGCAATCCTCCTAACCAACAAGGATACG	BioRev2	GGACGGAATATTGTACAGGTTGTACTTCTTAAC	pCR-XL-TOPO
Constructs					
gtfO[b]	FBiocomp	SacI BspHI GATGCATGAGCTCATGAATACCACCACGCC- GGCTGATAACCAGTCTG	BioPstRev	GTCAAACAGCCGGTCTAATGAAACGGTTCC	pBluescript pET15b
gtfO[b]	BioPstFor	GCAGTACATTCAGTCTTATTCACCAGTATTTG	BioRevHis	CTGATATTGGTCAGATGACTGATCAAAATAGA- ATT*CATCACCATCACCATCACTAATA*GGATCC- GCTCGAGATCGATAT BamHI XhoI	pBluescript pET15b
gtfO-ΔN[c]	FBioCore	SacI BspHI ATGCATGAGCTCATGAAGGATGGCAAGGA- TTACTACTATGACCC	BioRevHis	CTGATATTGGTCAGATGACTGATCAAAATAGA- ATT*CATCACCATCACCATCACTAATA*GGATCCG- CTCGAGATCGATAT BamHI XhoI	pET15b

[a] Relevant restriction sites are indicated; sequences encoding His tags are shown in italics

[b] The two PCR products were cloned separately in pBluescript using (SacI/PstIand PstI/XhoI, respectively). The resulting plasmids were used to construct the complete *gtfO* gene in pBluescript using PstI and XhoI restriction sites. Subsequently, the complete *gtfO* gene, using BspHI and NcoI, was cloned in the NcoI and BamHI sites of the expression vector pET15b (Fig. 1) (11).

[c] ΔN-GTFO PCR product was cloned directly in the NcoI and BamHI sites of the expression vector pET15b using BspHI and BamHI sites.

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

(iii) Characterization. The separate purified oligosaccharides were subjected to enzymatic degradation using dextranase from *Penicillium* sp. (EC 3.2.1.11; Sigma, St. Louis, MO), which hydrolyzes only α-(1→6)glucosidic bonds (3, 26), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3; Sigma), which was shown to hydrolyze α-(1→4), α-(1→3), and α-(1→6) linkages at decreasing rates, respectively, to produce glucose from the nonreducing end of linear oligosaccharides (21, 22) and α-glucosidase from *Bacillus stearothermophilus* (EC 3.1.2.20; Megazyme, Ireland), which hydrolyzes terminal, α-(1→4) linkages from the nonreducing end of oligosaccharides to produce glucose (17). Oligosaccharides (1 g · l^{-1}) were incubated with 0.1 U ml^{-1} amyloglucosidase, 66 U ml^{-1} dextranase, and 66 U ml^{-1} α-glucosidase. After 30 min, 2 h, and 18 h of incubation, samples were withdrawn and products formed in time were analyzed by anion exchange chromatography as described above. One endodextranase unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol isomaltose from dextran min^{-1} at 37°C and pH 6.0. One amyloglucosidase unit is defined as the amount of enzyme that hydrolyzes 1 mg of maltose per 3 min at 55°C and pH 4.5. One α-glucosidase unit is defined as the amount of enzyme that hydrolyzes 1 μmol ρ-nitrophenol-α-glucoside min^{-1} at 40°C and pH 6.5.

Characterization of the glucans produced. (i) Polymer production. Purified GTFO(-ΔN) enzyme preparations (30 nM) were incubated for 7 days with 146 mM sucrose, using the conditions described above under enzyme assays. Glucans produced by *L. reuteri* strain ATCC 55730 and by purified recombinant GTFO(-ΔN) enzymes were isolated by precipitation with ethanol (30).

(ii) Methylation analysis. Polysaccharides were permethylated using methyl iodide and dimsyl sodium ($CH_3SOCH_2^-$-Na^+) in dimethyl sulfoxide at room temperature (10).

(iii) NMR spectroscopy. Prior to nuclear magnetic resonance (NMR) spectroscopy, samples were dissolved in 99.96 atom % D_2O (Isotec). One-dimensional ^{1}H-NMR spectra were recorded on a 600 MHz Bruker AVANCE NMR spectrometer at a probe temperature of 353 K. The HOD signal was suppressed by applying a presaturation sequence. Chemical shifts are expressed in ppm by reference to external acetone (δ = 2.225). Proton spectra were recorded in 64K data sets, with a spectral width of 8,000 Hz. Resolution enhancement of the spectra was performed with a Lorentzian-to-Gaussian transformation; when necessary, a fifth-order polynomial baseline correction was performed.

(iv) Molecular masses of the glucans. Molecular mass analysis was performed as described previously, using high-performance size exclusion chromatography coupled on-line with a multiangle laser light-scattering (MALLS) and differential refractive index detection (11).

Nucleotide sequence accession number. The nucleotide sequence of *gtfO* has been assigned accession number AY911856 by GenBank.

RESULTS AND DISCUSSION

Isolation and nucleotide and amino acid sequence analysis of the putative *L. reuteri* strain ATCC 55730 glucosyltransferase gene/enzyme. Degenerate primers were designed and used for PCR with chromosomal DNA of *L. reuteri* strain ATCC 55730 as a template (Fig. 1B) (12). The method used resulted in identification of a single *gtf* gene in LB BIO (*gtfO*).

Using iPCR, a total of 9,419 bp of LB BIO DNA was cloned and sequenced, revealing the presence of three complete open reading frames (ORFs) and two partial ORFs (including a putative transposase) on the compiled sequence (Fig. 1A and B; Table 2). The presence of transposases near (putative) *gtf* genes has been previously observed in different lactobacilli (10). The *gtfO* gene encodes a putative protein of 1,781 amino acids, with a deduced molecular mass of 197,170 Da and a pI of 5.49.

Amino acid sequence alignments of *L. reuteri* ATCC 55730 GTFO with other glucosyltransferases from *Streptococcus*, *Leuconostoc*, and *Lactobacillus* subsp. using BLAST (1) revealed clear similarities. Highest similarity at the amino acid

TABLE 2. Overview of the highest identity and similarity scores of GTFO and surrounding ORFs of *Lactobacillus reuteri* ATCC 55730

(Putative) protein	Highest similarity to:	Genbank no.	Identity (%)	Similarity (%)	No. of amino acids[b]
GTFO	GTFA from *L. reuteri* 121 (reuteran)[c]	AY697435	68	80	1,799
	GTF180 from *L. reuteri* 180 (dextran)[c]	AY697430	59	75	1,801
	GTFML1 from *L. reuteri* ML1 (mutan)[c]	AY697431	57	73	1,789
ORF2	Putative transcriptional regulator, *Bacillus cereus* ATCC 14579	Q81CU4	34	51	110
ORF3	Putative reverse transcriptase/maturase family protein from *Clostridium acetobutylicum* ATCC 824	CAC3514	48	70	424
ORF4[a]	Putative site-specific recombinase from *Streptococcus agalactiae* 2603V/R	SAG1247	44	64	56
ORF5[a]	Putative transposase from *Lactobacillus fermentum* plasmid pLEM3	AF449484	83	91	231

[a] Partial open reading frames.
[b] No. of amino acids used to determine the identity and similarity percentages.
[c] Type of glucan synthesized.

level was found with reuteransucrase of *L. reuteri* 121 (GTFA), which synthesizes a reuteran with α-(1→4) and α-(1→6) linkages (11) (Table 2). The putative protein structure of GTFO was very similar to that of GTFA, GTF180, and GTFML1, containing (i) an N-terminal signal sequence of 38 amino acids, (ii) a variable N-terminal domain of 705 amino acids, (iii) a catalytic domain of 771 amino acids, and (iv) a C-terminal domain of 267 amino acids (10).

The deduced N-terminal amino acid sequence of GTFO contains a putative secretion peptide with a predicted signal peptidase cleavage site (SPase) between amino acid 38 and 39 (http://www.cbs.dtu.dk/services/SignalP/). Within the deduced N-terminal variable region of GTFO, a series of five RDV repeats, *R*(P/N)*DV*-x_{12}-SGF-x_{19-22}-R(Y/F)S (x, nonconserved amino acid residue) were found, as previously observed only in GTFA, GTF180, and GTFML1 from other *L. reuteri* strains (10, 11).

The C-terminal domain (glucan binding domain) of GTFO contains four YG-repeating units, NDGYYFxxxGxxH°x(G/N)H°H°H° (x, nonconserved amino acid residue; H°, hydrophobic amino acid residue) according to the definition in reference 9, and seven YG-repeating units which are less conserved. In the deduced amino acid sequence of the catalytic domain of GTFO, three (putative) catalytic residues were identified: Asp^{1024}, Glu^{1061}, and Asp^{1133}.

In the region downstream of the (putative) catalytic nucleophile Asp^{1024}, ^{1024}DAPDNI, GTFO differs in two out of five amino acids conserved in virtually all studied GTF enzymes from *Streptococcus*, *Leuconostoc*, and *Lactobacillus* (12, 19). In GTFO, Pro^{1026} is found in a position where a conserved Val is present in all other glucansucrases (except GTFA of *L. reuteri* 121, which also possesses a Pro residue at this position). Another conserved amino acid substitution in this region of GTFO, Ile^{1029} (instead of Val), was also found in amylosucrase, a glucosyltransferase from *Neisseria polysaccharea* synthesizing an α-(1→4) glucan (7), CD2 of DSRE of *L. mesenteroides* NRRLB-1299, responsible for the synthesis of α-(1→2) linkages (4), and GTFA of *L. reuteri* 121 (11). This region in GTFO is identical to that in GTFA of *L. reuteri* 121, which also synthesizes a reuteran; this region thus may be responsible for or contribute to the α-(1→4) bond specificity in both enzymes.

The region following the putative acid base catalyst E1061 (putative acceptor substrate-binding region) is not highly conserved in glucansucrases (19). Immediately following the motif ^{1061}E(A/D)W(S/N), two serine residues are found in GTFO, whereas GTFA of *L. reuteri* 121 possesses a His and an Ala residue at the corresponding positions, respectively. We speculate that differences in this (putative) acceptor substrate binding region between GTFO and GTFA may at least partly explain the larger amount of α-(1→4) bonds synthesized by GTFO in its products (see below) and/or its higher hydrolytic activity (see below).

The region following D1133 (transition state stabilizer) in GTFO differs from the sequence ^{1133}DSEVQTVI, conserved in many glucansucrases from *Streptococcus*, *Leuconostoc*, and *Lactobacillus* (12, 19). In GTFO as well as in GTFA, an original tripeptide, NNS is found immediately downstream of this catalytic Asp. Also, GTF180 and GTFML1 both contain an original tripeptide at this position, SNA and NGS, respectively.

TABLE 3. Comparison of the kinetic properties and product spectra of GTFO of *Lactobacillus reuteri* ATCC 55730 and GTFA of *Lactobacillus reuteri* strain 121

Parameter	Value for enzyme	
	GTFA[a]	GTFO[b]
Total activity		
V_{max}^{F}	25.5 ± 0.4	13.5 ± 0.4
K_m^{F}	0.9 ± 0.1	1.2 ± 0.1
Transferase activity		
$V_{max}^{F\text{-}G}$ ($\mu \cdot mg^{-1}$)	11.3 ± 1.2	—[d]
$K_m^{F\text{-}G}$ (mM)	4.6 ± 0.8	—
Hydrolysis activity		
V_{max}^{G} ($\mu \cdot mg^{-1}$)[e]	20.3 ± 0.5	13.9 ± 0.3
K_m^{G} (mM)	2.1 ± 0.1	1.5 ± 0.1
Reuteran synthesis (%)	73.7 ± 3.6	35.9 ± 4.3
Isomaltose synthesis (%)	1.6 ± 0.2	4.0 ± 0.2
Leucrose synthesis (%)	2.1 ± 0.1	5.7 ± 0.2
Hydrolysis (%)	22.7 ± 3.2	54.5 ± 3.9
Acceptor reaction oligosaccharide yield[c] (%) in the presence of:		
Maltose	59.6 ± 3.9	63.4 ± 0.8
Isomaltose	20.3 ± 1.1	43.8 ± 0.9

[a] Data from reference 13.
[b] GTFO-ΔN and full-length GTFO displayed similar kinetic properties and product spectra (data not shown).
[c] Oligosaccharide yield is expressed as a percentage of the total amount of acceptor substrate used in the incubation.
[d] No transferase activity could be determined for GTFO: only hydrolysis occurred at sucrose concentrations below 10 mM.
[e] Hydrolysis reaction of GTFA was fitted with the Michaelis-Menten equation with substrate inhibition (Ki^{G} = 111.0 ± 12.0 mM). No substrate inhibition was observed with GTFO.

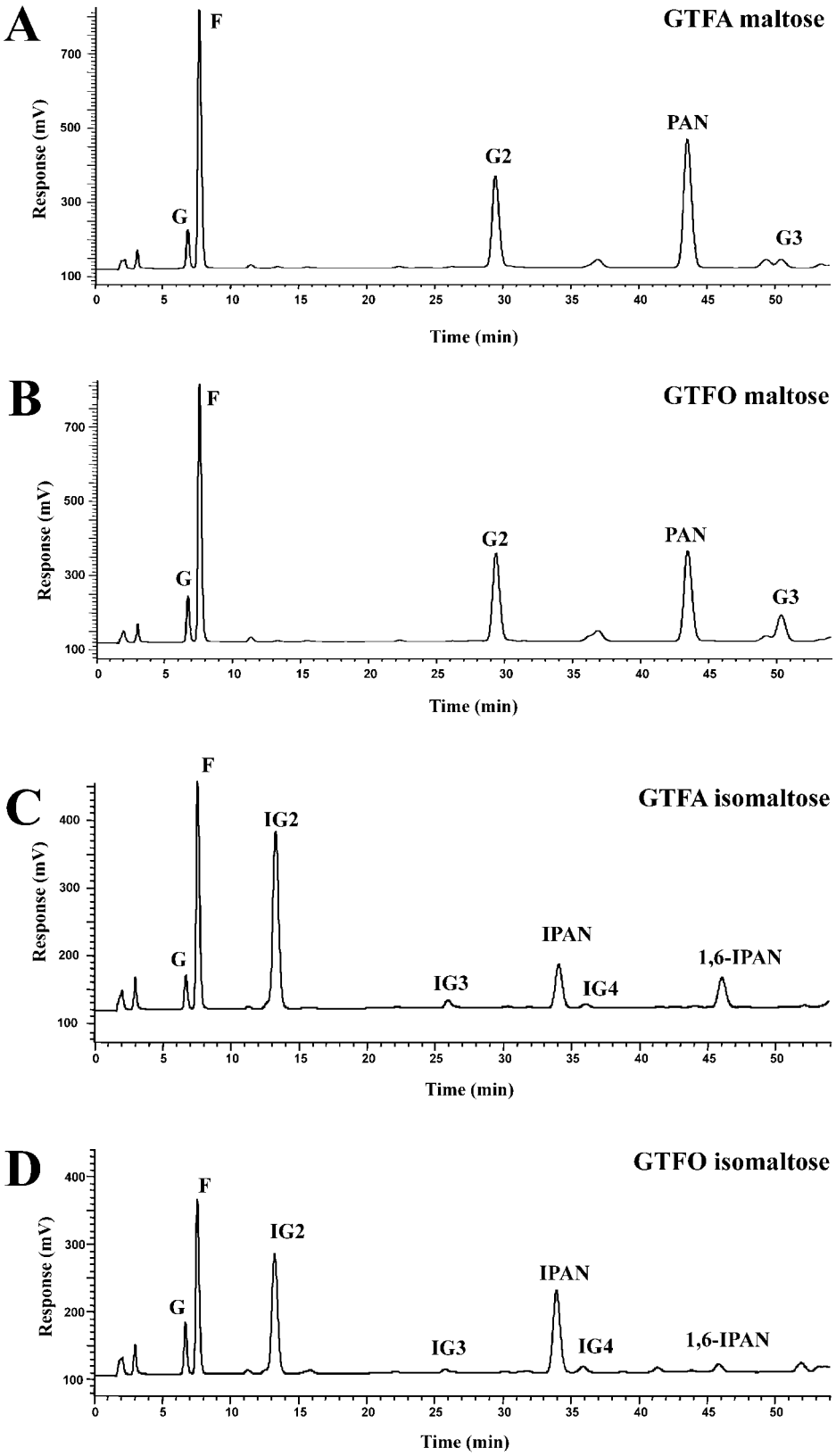

FIG. 2. Anion exchange chromatography of GTFO and GTFA (data from reference 13) acceptor reaction products. Products formed upon incubation of 30 nM GTFA (A) and 30 nM GTFO (B) enzyme with 100 mM sucrose and 100 mM maltose for 60 h. Products formed upon incubation of 30 nM GTFA (C) and 30 nM GTFO (D) enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h. F, fructose; G, glucose; G2, maltose; G3, maltotriose; IG2, isomaltose; IG3, isomaltotriose; IG4, isomaltotetraose; PAN, panose; IPAN, isopanose; 1,6-IPAN, α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose.

TABLE 4. Identification of major oligosaccharides formed by GTFO from sucrose and different acceptor substrates, deduced from enzymatic degradation products[e]

Degree of polymerization of oligosaccharide (acceptor used)	Degradation product(s) of[e]:			ID, oligonucleotide structure[e]
	Dextranase	Amyloglucosidase	Alpha-glucosidase	
3 (Maltose)	—[c]	G + G2[b]	G[b]	PAN
3 (Isomaltose)	—	G + IG2[a]	G + IG2[a]	IPAN
4 (Isomaltose)[d]	—	G + IG2 + IPAN[b]	G[b]	α-(1→6)-IPAN

[a] Relatively fast degradation.
[b] Relatively slow degradation.
[c] —, no degradation observed.
[d] The DP4 product formed by GTFA was analyzed, because GTFO synthesized only relatively small amounts.
[e] G, glucose; G2, maltose; IG2, isomaltose; PAN, panose; IPAN, isopanose; ID, identification.

Finally, also alternansucrase and CD2 of DSRE of *L. mesenteroides* NRRLB-1299 also both contain an original tripeptide at this position, YDA and KGV, respectively (4). Mutational evidence is available that the region following this catalytic Asp^{1133} is important in glucan structure determination in GTF enzymes (18, 23, 25). The presence of an original tripeptide, NNS, in both GTFA and GTFO may at least partly explain the unique structure of the glucans synthesized by both enzymes containing high amounts of α-(1→4) linkages.

Purification, pH, and temperature optima. *L. reuteri* ATCC 55730 GTFO(-ΔN) enzymes expressed in *E. coli* were purified to homogeneity. The predicted M_r's of the N-terminal deletion mutant (117 kDa, without signal sequence and N-terminal variable region) and full-length protein (194 kDa, without signal sequence) were in agreement with the results obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). In order to define the best conditions for subsequent kinetic studies, the pH and temperature optima of the full-length GTFO enzyme were examined. The pH optima for both the hydrolyzing and transferase activities were at pH 5.0 (data not shown). The temperature optima for both reactions were 35°C (data not shown). The N-terminal deletion mutant (GTFO-ΔN) showed the same pH and temperature optima (data not shown). The pH optimum of GTFO(-ΔN) was similar to that of GTFA of *L. reuteri* 121 (pH 4.7). However, the temperature optimum of GTFO(-ΔN) was 15°C lower than that of GTFA (50°C) (13).

Kinetic studies with GTFO(-ΔN). (i) Kinetic parameters. In the presence of sucrose, GTFO displayed a Michaelis-Menten type of kinetics for hydrolysis (V_G) and for total enzyme activity (V_F) (Table 3). Transferase activity (initial rates; $V_F - V_G$) was observed only at sucrose concentrations above 10 mM. GTFO thus favors hydrolysis at low sucrose concentrations and polymerization at higher sucrose concentrations. GTFA of *L. reuteri* 121 clearly displayed transferase activity (initial rates) at sucrose concentrations below 10 mM (13). GTFO-ΔN showed similar kinetics as full-length GTFO (data not shown). Affinity for the substrate sucrose in the hydrolysis reaction was similar for both GTFO(-ΔN) enzymes. Deletion of the N-terminal variable domain in GTFA resulted in drastic changes in enzyme activity, especially resulting in a strongly increased transferase activity and an approximately two times lower turnover rate for the hydrolysis reaction (13). Deletion of the N-terminal variable domain in GTFO did not affect its transferase activity. However, the GTFO-ΔN hydrolysis reaction turnover rates (k_{cat} s^{-1}) were about 1.5 times higher than for full-length GTFO (data not shown).

(ii) Effect of maltose on initial GTFO activity. The presence of maltose strongly stimulated GTFO total activity (initial rates) (sevenfold increase to V_F of 95.4 ± 2.2 U · mg^{-1}). A similar observation was made for GTFA (3.5-fold increase to V_F of 97.7 ± 0 · 9 U · mg^{-1}) (13).

Product analysis. (i) Product spectrum from sucrose. Compared to GTFA, GTFO converted less sucrose into glucan but produced larger amounts of isomaltose and leucrose. GTFO action on sucrose resulted in considerably more glucose release from sucrose (>54% of sucrose; Table 3) than that for GTFA (22.7% of sucrose; Table 3). Two unknown GTFO products eluted after 36 and 54 min, respectively (data not shown). GTFO-ΔN showed a product spectrum similar to that of GTFO (data not shown).

(ii) Enzymatic analysis of the structures of oligosaccharides synthesized from maltose and isomaltose as acceptor substrates. The major oligosaccharide products (DP3) synthesized by both GTFA and GTFO from sucrose in the presence of maltose as an acceptor reaction substrate eluted at the same position (43.5 min; Dionex analysis) (Fig. 2A and B) as panose (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose) (13). This oligosaccharide was purified, and analysis of products formed upon its enzymatic degradation in time, by amyloglucosidase and alpha-glucosidase, confirmed its identity as panose (Table 4) (13).

The major oligosaccharide products (DP3) synthesized by both GTFA and GTFO from sucrose in the presence of isomaltose as an acceptor reaction substrate eluted after 34 min (Fig. 2C and D) (13). Its identity (α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose; isopanose) was deduced from the products formed upon its degradation to isomaltose and glucose by amyloglucosidase and alpha-glucosidase (Table 4). Besides isopanose, GTFO synthesized small amounts of a DP4 oligosaccharide, eluting after 46 min. GTFA synthesized larger amounts of this DP4 oligosaccharide (Fig. 2C and D) (13). Therefore, the DP4 oligosaccharide synthesized by GTFA was used for enzymatic analysis of its structure. It was degraded slowly by amyloglucosidase, whereas degradation by alpha-glucosidase was poor (Table 4). Isopanose was one of the degradation products from amyloglucosidase; this is taken to suggest that isopanose formed by GTFA and GTFO is used as an acceptor reaction substrate to form α-(1→6)-isopanose (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose).

TABLE 5. Methylation analysis, NMR analysis (see also Fig. 3), and masses of the glucans produced by culture supernatants of *Lactobacillus reuteri* strain ATCC 55730 under the conditions tested and the purified recombinant GTFO(-ΔN) and GTFA enzymes

Parameter	Type of glucosyl units	Glucan produced by:			
		L. reuteri strain ATCC 55730	GTFO	GTFO-ΔN	GTFA
Methylation analysis (%)	Glc*p*-(1→	9	5	5	7
	→4)-Glc*p*-(1→	69	67	69	47
	→6)-Glc*p*-(1→	11	13	14	35
	→4,6)-Glc*p*-(1→	13	15	13	11
NMR analysis (%)	α-(1→6) (~5.0 ppm)	19	ND[a]	21	43
	α-(1→4) (~5.3 ppm)	81		79	57
Molecular mass (10^6 Da)		28	45	42	45

[a] ND, not determined.

Degradation of this DP4 oligosaccharide by dextranase was not possible, whereas another DP4 oligosaccharide with an α-(1→6) linkage in the middle was cleaved by dextranase (data not shown).

(iii) Comparison of the amounts of oligosaccharides synthesized by GTFA and GTFO. Maltose is an equally good acceptor reaction substrate for oligosaccharide synthesis by both GTFO and GTFA (Table 3). In the presence of sucrose and maltose, GTFA and GTFO formed panose as the most abundant acceptor reaction product (see above, approximately 44 mM and 33 mM, respectively) (Fig. 2B). Compared to GTFA, GTFO synthesized larger amounts of maltotriose (5 mM and 17 mM, respectively) (Fig. 2A and B).

Isomaltose is a two-times-better acceptor reaction substrate for oligosaccharide synthesis by GTFO than for synthesis by GTFA (Table 3). In the presence of sucrose and isomaltose, GTFO formed small amounts of isomaltotriose and isomaltotetraose, plus isopanose (Fig. 2D; see above). GTFO synthesized approximately 2.5 times more isopanose than GTFA (Fig. 2C and D). Besides isopanose, GTFO also synthesized minor amounts of α-(1→6)-isopanose. GTFA synthesized significantly larger amounts of α-(1→6)-isopanose (Fig. 2C and D) (13).

GTFO-ΔN displayed a similar product distribution with maltose and isomaltose as acceptor reaction substrates (data not shown).

Compared to GTFA, GTFO thus introduced more α-(1→4) linkages in its acceptor substrates, yielding larger amounts of maltotriose from maltose and larger amounts of isopanose from isomaltose and only minor amounts of α-(1→6)-isopanose (Fig. 2). The ability of GTFO to synthesize an α-glucan with large amounts of α-(1→4) linkages (see below) thus also is reflected in its oligosaccharide product spectrum.

Analysis of the glucans produced by *L. reuteri* ATCC 55730 culture supernatants and purified recombinant GTFO(-ΔN) enzymes. Purified recombinant GTFO(-ΔN) enzymes and supernatants of sucrose grown cultures of *L. reuteri* ATCC 55730, incubated with sucrose, produced high-molecular-weight glucans. Using high-performance size exclusion chromatography–multiangle laser light scattering, the average molecular weights of the glucans produced by LB BIO and by the purified GTFO(-ΔN) enzymes were determined (Table 5). The identical nature of these glucans was confirmed by methylation analysis (Table 5). The ^{1}H-NMR spectra of the glucans produced by LB BIO culture supernatants and by the purified recombinant GTFO-ΔN enzyme were virtually identical (Table 5; Fig. 3A and B). Comparison of both ^{1}H-NMR spectra with that of the reuteran produced by the *L. reuteri* 121 GTFA enzyme (Table 5; Fig. 3C) (11, 30) showed that both glucans consist of α-(1→4) (~5.3 ppm) and α-(1→6) (~5.0 ppm)-linked glucopyranosyl units. Due to poor resolution of the NMR spectra, it was not possible to trace the terminal and α-(1→4,6)-linked residues present (as indicated by the methylation analysis) (Table 5).

NMR (Table 5; Fig. 3) and methylation analysis (Table 5) clearly showed that GTFO and GTFA synthesize different reuteran products. GTFO reuteran contains approximately 20% more α-(1→4) glucosidic linkages [and 20% fewer α-(1→6) glucosidic linkages]. The degrees of branching of the GTFO- and GTFA-synthesized reuterans [amount of both terminal and α-(1→4,6) glucosyl units] are rather similar (see methylation analysis, Table 5). The precise structures of both reuterans remain to be elucidated.

Conclusions. LB BIO is able to colonize the human gastrointestinal tract (29), but a possible role for GTFO and its glucan product remains to be elucidated. The concentrations of sucrose needed to yield significant glucan polymer production may normally not be achieved in the gut. Glucans produced by streptococci play a key role in the development of human dental caries, enhancing the attachment and colonization of cariogenic bacteria to teeth (16). Recently, different lactobacilli have been identified in carious dentine (5). LB BIO may also be able to colonize the oral cavity, in which case GTFO may contribute to polymer formation and colonization on oral surfaces.

This paper reports the molecular and biochemical characterization of the novel *L. reuteri* ATCC 55730 reuteransucrase gene (*gtfO*) and the novel reuteransucrase enzyme (GTFO) encoded. The GTFO, GTFA, GTF180, and GTFML1 structures and amino acid sequences are highly similar (10) (~60% identity, ~75% similarity), but they synthesize glucans with different glucosidic linkages [mainly α-(1→4), α-(1→4)/α-(1→6), mainly α-(1→6), and mainly α-(1→3) linkages, respectively]. These enzymes thus are very interesting for structure/function studies aiming to identify amino acid residues responsible for glucosidic bond specificity. Furthermore,

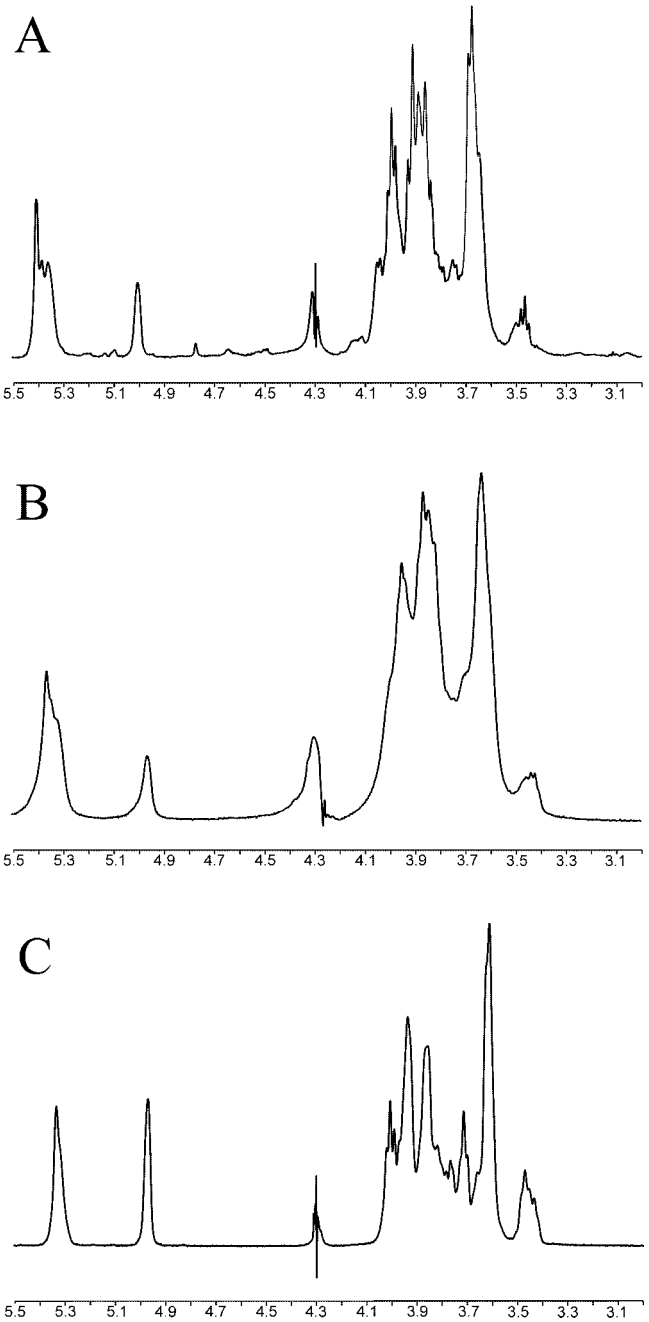

FIG. 3. 600-MHz ^{1}H-NMR spectra of glucans produced by *L. reuteri* strain ATCC 55730 culture supernatants (A), by purified recombinant GTFO-ΔN protein (B), and by purified recombinant GTFA protein of *L. reuteri* strain 121 (C) (13) recorded in D_2O at 80°C. Chemical shifts are given in parts per million relative to the signal of internal acetone (δ = 2.225); 5.0 ppm α-(1→6) linkages and 5.3 ppm α-(1→4) linkages.

GTFA and GTFO are interesting candidates for examination of structural differences responsible for hydrolysis/transferase activity ratios.

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