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Rational Transformation of *Lactobacillus reuteri* 121 Reuteransucrase into a Dextransucrase

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ABSTRACT: Glucansucrase or glucosyltransferase (GTF) enzymes of lactic acid bacteria display high sequence similarity but catalyze synthesis of different α-glucans (e.g., dextran, mutan, alternan, and reuteran) from sucrose. The variations in glucosidic linkage specificity observed in products of different glucansucrase enzymes appear to be based on relatively small differences in amino acid sequences in their sugar-binding acceptor subsites. This notion was derived from mutagenesis of amino acids of GTFA (reuteransucrase) from *Lactobacillus reuteri* strain 121 putatively involved in acceptor substrate binding. A triple amino acid mutation (N1134S:N1135E:S1136V) in a region immediately next to the catalytic Asp1133 (putative transition state stabilizing residue) converted GTFA from a mainly α-(1→4) (∼45%, reuteran) to a mainly α-(1→6) (∼80%, dextran) synthesizing enzyme. The subsequent introduction of mutation P1026V:I1029V, involving two residues located in a region next to the catalytic Asp1024 (nucleophile), resulted in synthesis of an α-glucan containing only a very small percentage of α-(1→4) glucosidic linkages (∼5%) and a further increased percentage of α-(1→6) glucosidic linkages (∼85%). This changed glucosidic linkage specificity was also observed in the oligosaccharide products synthesized by the different mutant GTFA enzymes from (iso)maltose and sucrose. Amino acids crucial for glucosidic linkage type specificity of reuteransucrase have been identified in this report. The data show that a combination of mutations in different regions of GTF enzymes influences the nature of both the glucan and oligosaccharide products. The amino acids involved most likely contribute to sugar-binding acceptor subsites in glucansucrase enzymes.

Glucansucrase or glucosyltransferase (GTF)[1] enzymes (EC 2.4.1.5) of lactic acid bacteria (LAB) are able to synthesize a diversity of α-glucans with α-(1→6) [dextran by dextransucrases (DSR), mainly found in *Leuconostoc*], α-(1→3) [mutan by mutansucrases, mainly found in *Streptococcus*], and alternating α-(1→3) and α-(1→6) [alternan by alternansucrase (ASR), only reported in *Leuconostoc mesenteroides*] glucosidic bonds. Two novel types of α-glucans, and the glucansucrase enzymes involved in their synthesis, have been characterized only recently. A unique glucan with α-(1→2) glucosidic branch linkages is synthesized by DSRE of *Ln. mesenteroides* NRRL B-1299 (*1*), and glucans with α-(1→4) glucosidic linkages (reuteran) are synthesized by GTFA from *Lactobacillus reuteri* 121 [∼45% α-(1→4) linkages] and GTFO from *Lb. reuteri* ATCC 55730 [∼70% α-(1→4) linkages] (*2*, *3*). Furthermore, two other *Lb. reuteri* GTF enzymes have been recently characterized [GTF180 of *Lb. reuteri* 180 synthesizing a branched dextran and GTFML1 of *Lb. reuteri* ML1 synthesizing a highly branched mutan (*4*)]. The four *Lb. reuteri* GTF enzymes are highly similar (∼65% identity, ∼70% similarity) yet synthesize different glucan products.

Glucansucrase enzymes catalyze two different reactions, depending on the nature of the acceptor substrate: (i) hydrolysis, when water is used as acceptor; (ii) glucosyl transfer (transferase), which can be divided in (a) polymerization, when the growing glucan chain is used as acceptor, and (b) oligosaccharide synthesis, when oligosaccharides (e.g., maltose, isomaltose) are used as acceptor.

Virtually all glucansucrases possess a common pattern of structural organization: their N-terminal end starts with (i)

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[1] Abbreviations: GTF, glucosyltransferase; GTFA, reuteransucrase from *Lactobacillus reuteri* 121; ASR, alternansucrase from from *Leuconostoc mesenteroides* NRRL B-1355; DSR, dextransucrase; GH, glycoside hydrolase, CGTase, cyclodextrin glycosyltransferase; PCR, polymerase chain reaction; DP, degree of polymerization; DMSO, dimethyl sulfoixde; NMR, nuclear magnetic resonance; ppm, parts per million; MHz, megahertz, G*n*, maltooligosaccharide with a degree of polymerization *n*; IG*n*, isomaltooligosaccharide with a degree of polymerization *n*.

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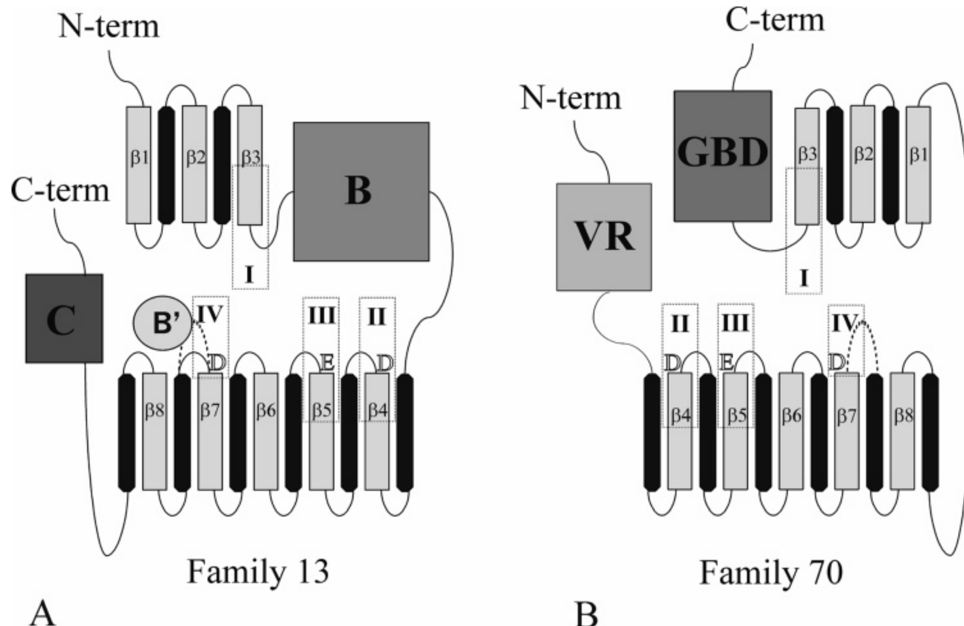

FIGURE 1: Topology diagrams of a family GH13 enzyme (α-amylase; A) and of glucansucrases of family GH70 (B). The catalytic domain of α-amylases has a $(\beta/\alpha)_8$ barrel structure, starting with *β*-strand 1 and ending with α-helix 8. The B domain is located between *β*-strand 3 and α-helix 3. Glucansucrases have a circularly permuted $(\beta/\alpha)_8$ barrel structure (*9*), which starts with α-helix 3 (α-amylase order) and ends with *β*-strand 3. Between α-helix 8 and *β*-strand 1, a large stretch with unknown function is located. The locations of the four conserved regions (I−IV) in family GH13 (and family GH70) are indicated with dashed boxes. Amylosucrase GH13 has a domain loop (B′-domain; important for polymerizing activity; indicated with a dashed line and circle) consisting of approximately 60 amino acid residues which is located after *β*-strand 7 (*8*, *34*), immediately after the two catalytically important H392 and D393 residues in conserved region IV (*33*). Glucansucrases also contain an additional loop (∼45 amino acids; indicated with a dashed line) compared to α-amylase enzymes, which is located between *β*-strand 7 and α-helix 7. This loop is also important for polymerizing activity in glucansucrases (this study). The approximate sites of the three catalytic residues (D, E, and D) are indicated. B = B domain, C = C domain, GBD = glucan binding domain, VR = variable region. Panel A was adapted from ref *35*.

Enzyme	Main α-linkages in glucan polymer	Residue	Region II	Residue	Region III	Residue	Region IV
			∀ □□ ‡		↓□□□		◆ ‡ ‡
GTFA	1→4 / 1→6	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNHADPEY	1126	YSFVRAHDNNSQDQIQNA
GTFO	1→4	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNSSDPEY	1126	YSFIRAHDNNSQDQIQNA
GTF180	1→6	1017	ANFDGIRVDAVDNVDVDLLSI	1058	HINILEDWGWDDPAY	1129	YNFVRAHDSNAQDQIRQA
GTFML1	1→3	1017	ANFDSIRVDAVDNVDADLLDI	1058	HINILEDWGGQDPYY	1125	YSFIRAHDNGSQDDIKRA
GTFB	1→3	443	ANFDSIRVDAVDNVDADLLQI	484	HLSILEAWSDNDTPY	555	YSFIRAHDSEVQDLIADI
GTFD	1→6	457	ANFDGVRVDAVDNVNADLLQI	498	HLSILEAWSDNDPQY	577	YIFIRAHDSEVQTVIAKI
GTFI	1→3	445	ANFDSIRVDAVDNVDADLLQI	486	HVSIVEAWSDNDTPY	557	YSFARAHDSEVQDLIRDI
DSRS	1→6	543	ANFDGIRVDAVDNVDADLLQI	584	HLSILEDWSHNDPLY	655	YSFVRAHDSEVQTVIAQI
ASR	1→6 / 1→3	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKDPQY	759	YSFVRAHDYDAQDPIRKA
DSRE CD1	1→6	519	ANFDGYRVDAVDNVDADLLQI	560	HISILEDWDNNDSAY	631	YAFIRAHDSEVQTVIAQI
DSRE CD2	1→2	2202	ANFDSIRIDAVDFIHNDTIQR	2243	HISLVEAGLDAGTST	2315	YSIIHAHDKGVQEKVGAA
			*:**. *:** * : * :.		::.::* .		* : :*** * :

FIGURE 2: Amino acid sequence alignment of (highly) conserved regions (II, III, IV) in the catalytic domains of dextran-, mutan-, alternan- and reuteransucrase enzymes of lactic acid bacteria (also see refs *5* and *16*) and amino acid residues with important functional roles, previously identified by mutagenesis or targeted in this study. Key: GTFA, *Lb. reuteri* 121 (*2*); GTFO, *Lb. reuteri* ATCC 55730 (*3*); GTF180, *Lb. reuteri* 180 (*4*); GTFML1, *Lb. reuteri* ML1 (*4*); GTFB, *S. mutans* GS5 (*36*); GTFD, *S. mutans* GS5 (*37*); GTFI, *S. downei* MFe28 (*38*); DSRS, *Ln. mesenteroides* NRRL B-512F (*30*); ASR, *Ln. mesenteroides* NRRL B-1355 (*17*); DSRE CD1 and CD2, *Ln. mesenteroides* NRRL B-1299 (*1*, *39*). Symbols: ∗, identical residue; :, highly conserved residue; ., conserved residue; ∀, putative catalytic nucleophile (*9*, *10*); ↓, putative acid/base catalyst (*9*, *10*); ◆, putative residue stabilizing the transition state intermediate (*9*, *10*); □, putative sugar-binding/glycosyl transfer sites (*9*); ‡, residues involved in glucan solubility and structure determination [α-(1→3)/α-(1→6)] as shown separately for different glucansucrases; mutated amino acids shown in bold face and underlined (*13*, *14*, *40*). The GTFB tryptophan residue shown underlined is important for activity; its mutagenesis resulted in complete loss of enzyme activity (*41*). Catalytic amino acids mutated in different studies (*5*, *10*, *18*) are shown in bold italic type. GTFA amino acid residues targeted in this study are shown in bold type.

a signal peptide, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain, and (iv) a C-terminal domain composed of a series of tandem repeats (*5*).

More than 30 different glucansucrases have been (at least partly) (http://afmb.cnrs-mrs.fr/∼cazy/CAZY/index.html) characterized, but no detailed structural information is yet available. The 3D structures have been elucidated for several enzymes of glycoside hydrolase family 13 [GH13; mainly containing enzymes acting on starch, e.g., cyclodextrin glycosyltransferase (CGTase) and α-amylase] (*6*), including that of amylosucrase, the only enzyme of family GH13 that

uses sucrose as substrate to synthesize α-glucan polymers containing α-(1→4) glucosidic linkages (*7*, *8*).

Secondary structure predictions revealed that the catalytic domains of GTF enzymes possess a $(\beta/\alpha)_8$ barrel structure similar to members of family GH13. The core of the proteins belonging to family GH13 constitutes eight β-sheets alternated with eight α-helices (Figure 1). In GTFs, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted (*9*, *10*) (Figure 1). Therefore, GTF enzymes are classified in family GH70 (*11*). Also, the four conserved regions (I−IV) identified in members of the α-amylase family GH13 (*12*) can be found in glucansucrases. However, as a consequence of the circular permutation, region I occurs C-terminal of region II−IV in glucansucrase enzymes (Figure 1).

On the basis of a comparison with sugar-binding acceptor subsites in family GH13 enzymes (*9*), the locations of two regions putatively involved in acceptor substrate binding in GTF enzymes were identified, C-terminal of the catalytic residues D1024 (GTFA numbering, region II, Figure 2) and E1061 (region III, Figure 2). A third (putative) acceptor substrate binding region was identified on the basis of earlier mutagenesis studies with different GTF enzymes, involving amino acid residues 1138 and 1142 (GTFA *Lb. reuteri* 121 numbering, used throughout the paper), located C-terminal of the catalytic residue D1133 (region IV, Figure 2), determining the solubility of the glucan products and the ratio of [α-(1→3) versus α-(1→6)] glucosidic linkages present (*13*−*15*).

The region directly C-terminal of the catalytic Asp1024 (region II) contains the conserved amino acids Asp1024-Ala-Val(Pro)-Asp-Asn-Val(Ile)1029. In the second catalytic domain (CD2) of DSRE of *Ln. mesenteroides* NRRL B-1299 [responsible for α-(1→2,6) linkage synthesis] the second Val residue is replaced by an Ile residue (Ile2215) (Figure 2). The Pro1026 and Ile1029 combination is present only in GTFA and GTFO, strikingly the only glucansucrases that synthesize reuterans [α-(1→4) polymers] (*2*, *3*). Therefore, these two GTFA residues were mutated (P1026V and I1029V, Figure 2).

The region following the putative acid/base catalyst E1061 (region III) is less conserved. GTFA possesses an Ala residue at amino acid position 1066, while an Asn residue is located here in most other glucansucrases (Figure 2). Therefore, this GTFA residue was mutated (A1066N) (*5*, *16*). Both reuteransucrase (GTFA and GTFO) enzymes are highly similar in the three (putative) acceptor substrate binding regions and only differ in two amino acid residues in the region following the acid/base catalyst: Glu1061-Asp-Trp-Asn-His(Ser)-Ala-(Ser)-Asp1067 (residues shown in parentheses are present in GTFO). Thus, residues 1065 and 1066 of GTFA were exchanged by site-directed mutagenesis for the residues present in GTFO (H1065S:A1066S, Figure 2).

The region following D1133 (transition state stabilizer) in both reuteransucrases (GTFA and GTFO) differs from the sequence Asp1133-Ser-Glu-Val-Gln-Thr-Val-Ile1140, conserved in many glucansucrases from *Streptococcus*, *Leuconostoc*, and *Lactobacillus* species (Figure 2) (*4*, *5*, *16*). In both GTFA (reuteran) and GTFO (reuteran), an original tripeptide is found immediately downstream of this catalytic Asp, Asn-Asn-Ser. Also, GTF180 (dextran) and GTFML1 (mutan) both contain an original tripeptide at this position, Ser-Asn-Ala and Asn-Gly-Ser, respectively (*4*, *16*). Finally, also alternansucrase (ASR) of *Ln. mesenteroides* NRRL B-1355 (alternan) and CD2 of DSRE of *Ln. mesenteroides* NRRL-B1299 both contain an original tripeptide at this position, Tyr-Asp-Ala and Lys-Gly-Val, respectively (Figure 2) (*1*, *17*).

GTF proteins mutated in the tripeptide immediately following the putative transition state stabilizing residue have not been reported yet. This GTFA region therefore was mutated, substituting the unique triplet Asn-Asn-Ser for the conserved Ser-Glu-Val residues found in many other glucansucrases (N1134S:N1135E:S1136V). Finally, various combinations of the mutations made in the separate acceptor substrate binding regions were constructed and analyzed as well.

Aims of our studies are to elucidate structure/function relationships of these intriguing enzymes. The largely similar structural features of members of families GH13 and GH70, together with the new glucansucrase (and reuteransucrase) sequence information, provided a perfect starting point for such investigations. GTFA amino acid residues putatively responsible for glucosidic linkage specificity located close to the three catalytic residues and putative acceptor substrate binding regions were identified as targets for site-directed mutagenesis experiments (Figure 2).

The effect of the amino acid substitutions on GTFA enzyme activity, glucan and oligosaccharide synthesis, were determined. Regions and specific amino acid residues crucial for glucosidic linkage type specificity, in glucan and oligosaccharide synthesis, of reuteransucrase of *Lb. reuteri* 121 (GTFA) have been identified in this report.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Growth Conditions. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA) was used as host for cloning purposes. Plasmid pBPE1500, containing the 3′-part of the catalytic core of the *gtfA* gene of *Lb. reuteri* 121, was used as template for mutagenesis, and plasmid pBGTF2, with the full-length *gtfA* gene including a C-terminal His tag, was used for cloning purposes (*18*). Plasmid pET15b (Novagen, Madison, WI) was used for expression of the different *gtf* genes in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (*19*). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with 100 μg mL^{-1} ampicillin. Agar plates were made by adding 1.5% agar to the LB medium.

Molecular Techniques. General procedures for restriction, ligation, cloning, PCR, *E. coli* transformations, DNA isolation and manipulations, isolating DNA fragments from gel, and agarose gel electrophoresis were as described (*18*). Primers were obtained from Eurogentec (Seraing, Belgium). Sequencing was performed by GATC (Konstanz, Germany).

Construction of Plasmids for Site-Directed Mutagenesis Experiments. The QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce mutations in pBPE1500. Constructs with multiple mutations were made using pBPE1500 containing mutation(s) as template and different primer pairs (Table 1). Successful mutagenesis resulted in appearance of new restriction sites, allowing rapid screening of potential mutants (Table 1). After successful mutagenesis (confirmed by DNA nucleotide sequencing), the

Table 1: Templates and Oligonucleotides Used for Site-Directed Mutagenesis of *gtfA*[a]

Mutation in GTFA protein	Template	Primer pairs (5'→3')	Restriction site
P1026V	pBPE 1500	GTAGATGCA***GTC***GACAATATTGATGCCG CGGCATCAATATTGTC***GAC***TGCATCTAC	*Sal*I
I1029V	pBPE 1500	GTAGATGCACCGGATAAT***GTC***GACGCC GGCGTC***GAC***ATTATCCGGTGCATCTAC	*Sal*I
P1026V:I1029V	pBPE 1500 P1026V	GTAGATGCACCGGATAAT***GTC***GACGCC GGCGTC***GAC***ATTATCCGGTGCATCTAC	*Sal*I
A1066N	pBPE 1500	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I
H1065S:A1066S	pBPE 1500	GAAGACTGGAAT***TC***T***TC***G***G***ATCCGGAATACTTT AAAGTATTCCGGATC***C***G***A***A***GA***ATTCCAGTCTTC	*Bam*HI
P1026V:I1029V:A1066N	pBPE 1500 P1026V:I1029V	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I
N1134S:N1135E:S1136V	pBPE 1500	CGTTCGGGC***C***CACGATA***GT***G***A***A***GT***TCAAGATCAAATTCAAAATGC GCATTTTGAATTTGATCTTGA***ACT***T***C***A***C***TATCGTG***G***GCCCGAACG	*Apa*I
P1026V:I1029V: N1134S:N1135E:S1136V	pBPE 1500 P1026V:I1029V	CGTTCGGGC***C***CACGATA***GT***G***A***A***GT***TCAAGATCAAATTCAAAATGC GCATTTTGAATTTGATCTTGA***ACT***T***C***A***C***TATCGTG***G***GCCCGAACG	*Apa*I
A1066:N:N1134S:N1135E :S1136V	pBPE 1500 N1134S:N1135E: S1136V	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I
P1026V:I1029V: A1066N:N1134S:N1135E: S1136V	pBPE 1500 P1026V:I1029V: N1134S:N1135E: S1136V	GAAGACTGGAATCAT***AA***TGA***C***CCGGAATAC GTATTCCGG***G***TCA***TT***ATGATTCCAGTCTTC	*Nci*I

[a] Nucleotides in bold type/italics represent mismatches with the sequence of *gtfA*. Underlined nucleotides represent introduced restriction sites.

pBPE1500 derivative was digested with *Pst*I and *Eco*RV and exchanged for the corresponding fragment of pBGTF2 (*18*). The resulting 5.3 kb fragment was introduced, using *Nco*I/*Bam*HI restriction sites, in the expression vector pET15b (Novagen, Madison, WI) (*18*). Plasmid pBGTF2 containing mutation H1065S:A1066S was digested with *Nco*I/*Xho*I, and the resulting 4.5 kb fragment was ligated into the corresponding sites of the vector p15GTF2.

Enzyme Assays and Enzyme Purification. GTFA proteins were produced and purified as described previously (*18*). All reactions were performed at 50 °C in 25 mM NaAc buffer, pH 4.7, containing 1 mM $CaCl_2$ and 30 nM (12−55 units) purified (mutant) GTFA enzyme.

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, using 15 different sucrose concentrations ranging from 0.25 to 100 mM). One unit of enzyme activity is defined as the release of 1 μmol of monosaccharide per minute (*18*, *20*).

The effect of maltose on GTFA enzyme activity (initial rates) was determined using 50 mM sucrose and 100 mM maltose, measuring fructose release.

Characterization of the Glucans Produced. Polymers were produced by incubation of (mutant) enzyme preparations with 146 mM sucrose for 7 days, using the conditions described above under enzyme activity assays. Glucans produced were isolated by precipitation with ethanol as described previously (*20*).

Methylation analysis was performed as described by permethylation of the polysaccharides using methyl iodide and dimsylsodium ($CH_3SOCH_2^-{-}Na^+$) in DMSO at room temperature (*4*).

One-dimensional ^1H NMR spectra were recorded on a 600 MHz Bruker AVANCE NMR spectrometer at a probe temperature of 353 K. Prior to NMR spectroscopy samples were dissolved in 99.96 atom % D_2O (Isotec). The HOD signal was suppressed by applying a pressat sequence. Chemical shifts are expressed in parts per million by reference to external acetone (δ = 2.225). Proton spectra were recorded in 64K data sets, with a spectral width of 8000 Hz. Resolution enhancement of the spectra was performed with a Lorentzian-to-Gaussian transformation; when necessary, a fifth-order polynomial baseline correction was performed.

Analysis of Products Synthesized from Sucrose. After complete depletion of sucrose (100 mM, 60 h at 50 °C) by 30 nM GTFA enzymes, the concentrations of fructose, glucose, isomaltose, and leucrose in the reaction medium were determined using anion-exchange chromatography (Dionex) (*18*).

Oligosaccharides were synthesized from sucrose and (iso)-maltose as acceptor substrates. After complete depletion of sucrose (100 mM, 60 h at 50 °C) by 30 nM GTFA enzymes, incubated together with the acceptor substrates maltose or isomaltose (100 mM each), the oligosaccharides synthesized were analyzed by anion-exchange chromatography (*18*). No standards were available for α-(1→6)-panose, isopanose, and α-(1→6)-isopanose. Therefore, the calibration curve for

Table 2: Kinetic Parameters of GTFA Wild Type and Site-Directed Mutants Derived

	total activity		transferase activity		hydrolysis activity			
(A) enzyme[a]	V_{max}^{F} (units·mg^{-1})	K_m^{F} (mM)	V_{max}^{F-G} (units·mg^{-1})	K_m^{F-G} (mM)	V_{max}^{G} (units·mg^{-1})	K_m^{G} (mM)	K_i (mM)	(B) acceptor reaction,[b] V_{max}^{F} (units·mg^{-1})
wild type	28.4 ± 1.0	0.9 ± 0.2	11.9 ± 0.8	4.8 ± 1.1	24.3 ± 1.0	1.0 ± 0.1	106.9 ± 16.8	97.7 ± 0.9
P1026V	55.2 ± 2.2	1.8 ± 0.3	24.4 ± 1.2	8.3 ± 1.4	46.0 ± 4.3	1.6 ± 0.4	144.9 ± 54.6	130.0 ± 5.5
I1029V	38.1 ± 1.2	1.4 ± 0.2	19.5 ± 1.9	8.5 ± 1.4	29.9 ± 2.0	1.3 ± 0.2	105.6 ± 25.0	122.0 ± 8.5
P1026V:I1029V	45.0 ± 3.2	1.2 ± 0.3	17.0 ± 1.2	9.0 ± 1.8	44.1 ± 5.2	1.4 ± 0.4	76.2 ± 25.8	110.5 ± 3.4
A1066N	29.9 ± 1.3	1.1 ± 0.2	14.5 ± 0.9	3.3 ± 0.7	22.6 ± 1.0	1.1 ± 0.1	103.4 ± 16.6	114.6 ± 0.2
H1065S:A1066S	12.1 ± 1.1[c]	1.2 ± 0.3	ND	ND	10.6 ± 0.6	1.1 ± 0.2	56.6 ± 9.9	33.4 ± 2.1
P1026V:I1029V:A1066N	39.0 ± 2.0	1.1 ± 0.2	16.1 ± 1.6	5.1 ± 1.7	34.5 ± 2.3	1.2 ± 0.2	95.7 ± 22.1	97.3 ± 1.1
N1134S:N1135E:S1136V	27.6 ± 1.6	8.5 ± 1.6	102.6 ± 71.6[d]	540.9 ± 432.8[d]	13.9 ± 0.2	2.6 ± 0.1	–[e]	110.2 ± 1.7
P1026V:I1029V:N1134S:N1135E:S1136V	45.7 ± 1.4	4.8 ± 0.5	29.4 ± 2.4	36.7 ± 7.2	26.1 ± 0.5	2.3 ± 0.2	–[e]	120.1 ± 2.1
A1066N:N1134S:N1135E:S1136V	19.8 ± 1.0	7.9 ± 1.3	35.8 ± 13.9[d]	224.9 ± 116.8[d]	10.2 ± 0.3	2.8 ± 0.3	–[e]	125.2 ± 2.5
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	31.2 ± 0.9	4.1 ± 0.4	17.2 ± 1.4	33.1 ± 6.5	19.6 ± 0.2	2.2 ± 0.1	–[e]	102.5 ± 1.4

[a] Kinetic parameters determined using 15 different concentrations of sucrose (0.25–100 mM). [b] Total activity determined using 50 mM sucrose and 100 mM maltose. [c] Total activity for mutant H1065A:A1066S was fitted using the Michaelis–Menten equation with substrate inhibition: K_i 64.1 ± 16.9 mM. [d] The absence of transferase activity at low substrate concentrations resulted in a high standard error with curve fitting. ND = no transferase activity (initial rate) could be detected. [e] Data obtained were fitted using the Michaelis–Menten equation (with or without substrate inhibition).

panose, representing the most closely related oligosaccharide available to us, was used to estimate the approximate concentrations of these three compounds. Total and individual oligosaccharide yields were calculated from the amount of acceptor substrate converted into total and individual oligosaccharides, expressed as a percentage of the total amount of acceptor substrate initially present in the incubation. The Dionex analysis protocol used allowed recovery of 85–110% of the (iso)maltose initially present in the incubation, as remaining (iso)maltose and/or as oligosaccharide products formed. The lower percentages may be due to synthesis of unknown products from (iso)maltose. Both the lower and higher percentages may be due to use of the panose calibration curve to determine concentrations of the three other compounds (see above).

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, NV) eluted with water (0.2 mL min^{-1}), using a model 830-RI refractive index detector at 40 °C (Jasco, Tokyo, Japan). The system was calibrated using linear maltooligosaccharides (G1–G7).

The separate purified oligosaccharides were characterized by enzymatic degradation using dextranase from *Penicillium* sp. (EC 3.2.1.11; Sigma, St. Louis, MO), which hydrolyzes only α-(1→6) glucosidic bonds (*21*, *22*), 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 (*23*, *24*), 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 (*25*). Oligosaccharides (1 g·L^{-1}) were incubated with 0.1 unit mL^{-1} amyloglucosidase, 66 units mL^{-1} dextranase, and 66 units 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 of isomaltose from dextran per minute 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 of *p*-nitrophenol-α-glucoside per minute at 40 °C and pH 6.5.

RESULTS

Kinetic Parameters of Wild-Type and Mutant GTFs. Mutants in conserved region I, near the catalytic D1024 residue (P1026V, I1029V, and P1026V:I1029V), displayed 1.5–2-fold higher activities than GTFA wild type in all three reactions catalyzed. Affinities for sucrose in the hydrolysis and transferase reactions were slightly lower (Table 2A). Mutant A1066N, in the vicinity of the catalytic E1061 residue, displayed similar kinetic parameters as wild type GTFA (Table 2A). The double mutant H1065S:A1066S lacked transferase activity (initial rates; Table 2A). The hydrolytic activity of this mutant was 2-fold lower than in wild type, whereas the affinity for sucrose in the hydrolysis reaction was 2-fold higher compared to wild type. The combination of the P1026V:I1029V and A1066N mutations resulted in higher activities in all reactions, whereas affinities for the substrate sucrose were comparable to wild type levels in all reactions (Table 2A). Both triple N1134S:N1135E:S1136V and quadruple A1066N:N1134S:N1135E:S1136V mutants showed no transferase activity at low sucrose concentrations (initial rates); the affinity of both mutants for the substrate sucrose in the transferase reaction was extremely low. The affinity for sucrose for total activity was also strongly decreased (Table 2A). The same phenomena were observed for the other two mutants containing the N1134S:N1135E:S1136V mutation in combination with mutations in the other (putative) acceptor substrate binding region(s).

Table 3: Product Spectra of GTFA Wild Type and Derived Site-Directed Mutants Incubated with Sucrose for 60 h

enzyme	reuteran (%)[c]	leucrose (%)	isomaltose (%)	glucose (%)
wild type[a]	73.7 ± 3.6	2.1 ± 0.1	1.6 ± 0.2	22.7 ± 3.2
P1026V	66.1 ± 2.9	3.8 ± 0.4	6.2 ± 0.5	23.9 ± 2.0
I1029V	74.1 ± 3.2	2.3 ± 0.3	1.7 ± 0.3	21.9 ± 2.6
P1026V:I1029V	65.0 ± 4.0	4.0 ± 0.1	6.2 ± 0.7	24.8 ± 3.2
A1066N	76.3 ± 2.7	1.9 ± 0.1	1.4 ± 0.2	20.4 ± 2.5
H1065S:A1066S	73.4 ± 0.4	1.7 ± 0.1	1.4 ± 0.1	23.5 ± 0.2
P1026V:I1029V:A1066N	67.3 ± 2.9	3.6 ± 0.1	5.5 ± 0.3	23.5 ± 2.5
N1134S:N1135E:S1136V	74.6 ± 1.2[b]	1.9 ± 0.3	0.6 ± 0.1	23.0 ± 1.3
P1026V:I1029V:N1134S:N1135E:S1136V	76.5 ± 0.1[b]	3.0 ± 0.6	1.0 ± 0.1	19.5 ± 0.6
A1066N:N1134S:N1135E:S1136V	69.1 ± 1.7[b]	0.7 ± 0.2	0.5 ± 0.2	29.7 ± 2.4
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	72.9 ± 1.0[b]	2.1 ± 0.4	1.1 ± 0.1	23.9 ± 0.5

[a] Data from ref *18*. [b] Sucrose consumed for 20−70% after 60 h of incubation. [c] Percentages indicate the relative conversion of sucrose into reuteran, oligosaccharides (leucrose and isomaltose), and glucose (hydrolysis). The 100% value is equivalent to the total amount of sucrose consumed after 60 h of incubation, in most cases resulting in sucrose depletion.

Table 4: Product Spectra of GTFA and Derived Site-Directed Mutants after 60 h of Incubation with 100 mM Sucrose and 100 mM Maltose

enzyme	oligosaccharide yield (%)[a]	panose (%)	maltotriose (%)	α-(1→6)-panose (%)[b]
wild type[c]	59.6 ± 3.9	54.5 ± 4.2	5.1 ± 0.4	−[d]
P1026V	55.1 ± 7.8	51.7 ± 9.6	3.3 ± 1.7	−[d]
I1029V	60.8 ± 4.1	56.9 ± 1.7	3.9 ± 0.3	−[d]
P1026V:I1029V	59.5 ± 3.0	55.9 ± 5.1	3.5 ± 0.5	−[d]
A1066N	62.6 ± 8.1	55.8 ± 8.1	3.9 ± 0.3	−[d]
H1065S:A1066S	63.2 ± 1.1	57.8 ± 1.0	5.3 ± 0.1	−[d]
P1026V:I1029V:A1066N	62.3 ± 2.5	60.4 ± 2.6	1.9 ± 0.1	−[d]
N1134S:N1135E:S1136V	64.8 ± 4.7	40.5 ± 1.3	2.1 ± 0.1	22.1 ± 3.3
P1026V:I1029V:N1134S:N1135E:S1136V	60.7 ± 1.3	34.8 ± 0.2	1.3 ± 0.1	24.7 ± 1.0
A1066N:N1134S:N1135E:S1136V	68.9 ± 4.7	45.1 ± 8.6	2.0 ± 0.2	21.8 ± 2.2
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	63.4 ± 0.1	37.2 ± 0.1	1.3 ± 0.1	24.9 ± 0.9

[a] The total and individual oligosaccharide yields indicate the amount of maltose consumed as a percentage of the total amount of maltose initially present in the incubation. [b] The calibration curve of panose was used to calculate α-(1→6)-panose concentrations. [c] Data from ref *18*. [d] Not detectable.

Table 5: Product Spectra of GTFA and Derived Site-Directed Mutants after 60 h of Incubation with 100 mM Sucrose and 100 mM Isomaltose

enzyme	oligosaccharide yield (%)[a]	isopanose (%)[b]	α-(1→6)-isopanose (%)[b]	isomalto-triose (%)	isomalto-tetraose (%)	isomalto-pentaose (%)	isomalto-hexaose (%)
wild type	28.3 ± 2.1	12.2 ± 3.2	11.6 ± 0.8	2.8 ± 0.5	1.6 ± 0.1	−[c]	−[c]
P1026V	29.9 ± 1.8	5.6 ± 0.5	8.9 ± 0.2	12.8 ± 0.5	2.6 ± 0.2	−[c]	−[c]
I1029V	27.3 ± 2.3	8.5 ± 1.7	13.2 ± 0.5	3.9 ± 0.3	1.6 ± 0.1	−[c]	−[c]
P1026V:I1029V	32.0 ± 3.6	6.2 ± 1.3	8.6 ± 0.3	14.2 ± 3.3	2.9 ± 0.6	−[c]	−[c]
A1066N	28.7 ± 0.9	11.6 ± 0.8	11.9 ± 0.1	3.5 ± 0.1	1.7 ± 0.1	−[c]	−[c]
H1065S:A1066S	24.9 ± 2.9	8.6 ± 0.8	11.6 ± 1.6	3.1 ± 0.4	1.6 ± 0.2	−[c]	−[c]
P1026V:I1029V:A1066N	32.6 ± 7.7	6.0 ± 1.4	9.6 ± 2.2	14.1 ± 3.4	2.9 ± 0.7	−[c]	−[c]
N1134S:N1135E:S1136V	61.0 ± 3.4	8.9 ± 0.5	−[c]	27.1 ± 1.4	20.4 ± 1.2	4.6 ± 0.3	−[c]
P1026V:I1029V:N1134S:N1135E:S1136V	61.3 ± 1.2	1.5 ± 0.1	−[c]	26.3 ± 0.4	23.5 ± 0.6	7.2 ± 0.2	2.8 ± 0.1
A1066N:N1134S:N1135E:S1136V	57.3 ± 2.8	7.7 ± 0.3	−[c]	26.5 ± 0.5	19.1 ± 1.4	4.0 ± 0.7	−[c]
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	59.6 ± 0.5	1.4 ± 0.1	−[c]	26.3 ± 0.3	22.9 ± 0.2	6.7 ± 0.1	2.2 ± 0.1

[a] The total and individual oligosaccharide yields indicate the amount of isomaltose consumed as a percentage of the total amount of isomaltose initially present in the incubation. [b] The calibration curve of panose was used to calculate isopanose and α-(1→6)-isopanose [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose] concentrations. [c] Not detectable.

Normal Michaelis−Menten kinetics was observed for the hydrolysis reaction of the four mutants containing mutation N1134S:N1135E:S1136V, whereas wild type and the other mutants showed substrate inhibition for the hydrolysis reaction (Table 2A). The V_{max} for total activity (V_F) in the reaction with sucrose and maltose as acceptor reaction substrate had increased for most mutants. Mutant P1026V:I1029V:A1066N showed a similar activity as wild type; mutant H1065S:A1066S displayed a 3-fold reduced activity (Table 2B).

Product Spectra of Mutants Incubated with 100 mM Sucrose. The single mutant P1026V, the double mutant P1026V:I1029V, and the triple mutant P1026V:I1029V:A1066N showed 3−4-fold increased levels of isomaltose production (and a slight decrease in reuteran synthesis) with sucrose alone or with sucrose and fructose as acceptor reaction substrate (Table 3 and data not shown) compared to wild type. Compared to wild-type GTFA, these three mutants showed up to 2-fold higher leucrose yields when incubated with sucrose alone, or with sucrose and fructose as acceptor reaction substrate (∼10 mM leucrose synthesized) (Table 3 and data not shown). All other mutants showed similar product spectra as wild-type GTFA upon incubation with sucrose (Table 3). The triple mutant N1134S:N1135E:

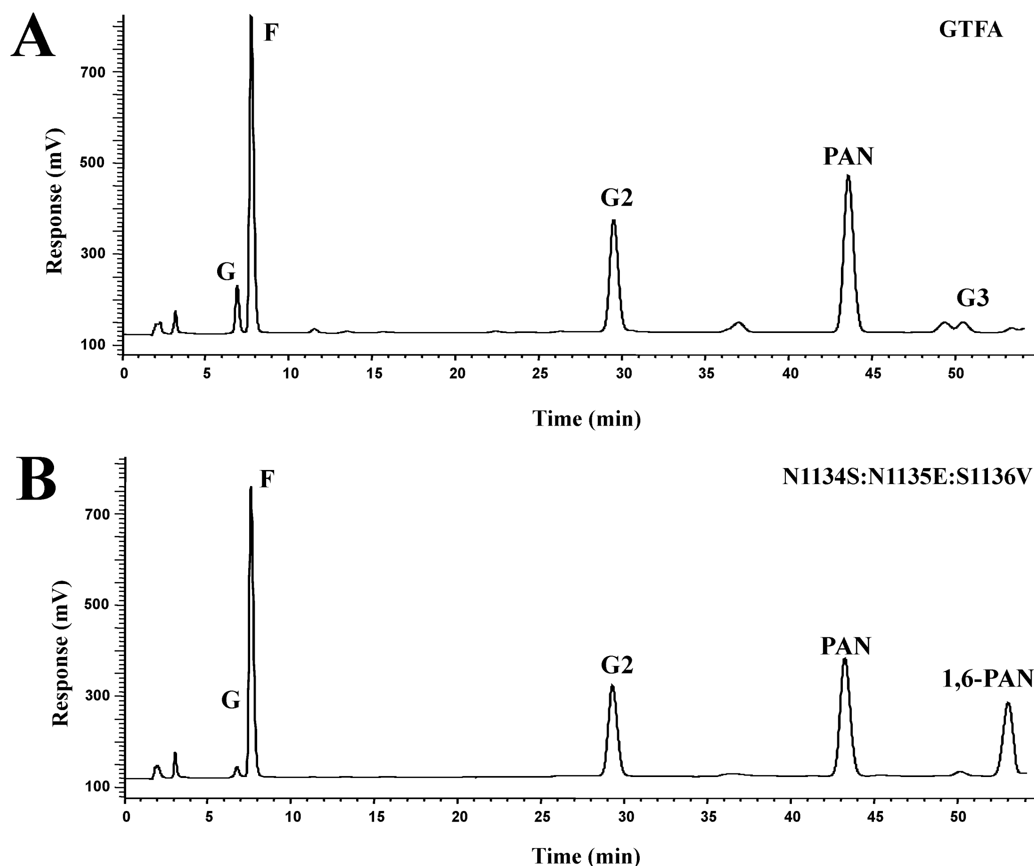

FIGURE 3: Anion-exchange (Dionex) analysis of (mutant) GTFA acceptor reaction products produced from sucrose plus maltose. (A) Products formed upon incubation of 30 nM GTFA enzyme with 100 mM sucrose and 100 mM maltose for 60 h. (B) Products formed upon incubation of 30 nM GTFA mutant N1134S:N1135E:S1136V enzyme with 100 mM sucrose and 100 mM maltose for 60 h. F = fructose, G = glucose, G2 = maltose, G3 = maltotriose, PAN = panose, and 1,6-PAN = α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-[α-D-glucopyranosyl-(1→4)]-D-glucose.

Table 6: Methylation (A) and 600 MHz ¹H NMR (B) Analysis of the Glucans Produced by Purified GTFA Wild Type and Derived Site-Directed Mutants

	(A) methylation (%) for glycosyl unit type				(B) chemical shift (%)[a]	
	Glc*p*-(1→	→4)-Glc*p*-(1→	→6)-Glc*p*-(1→	→4,6)-Glc*p*-(1→	α-(1→4)	α-(1→6)
wild type	9	46	34	12	57	43
P1026V	10	42	40	8	53	47
I1029V	7	47	35	11	58	42
P1026V:I1029V	7	41	40	12	53	47
A1066N	7	46	36	16	ND[b]	ND
H1065S:A1066S	10	45	29	16	ND	ND
P1026V:I1029V:A1066N	6	44	39	10	ND	ND
N1134S:N1135E:S1136V	7	11	76	6	16	84
P1026V:I1029V:N1134S:N1135E:S1136V	6	2	82	9	8	92
A1066N:N1134S:N1135E:S1136V	6	13	74	7	ND	ND
P1026V:I1029V:A1066N:N1134S:N1135E:S1136V	6	4	88	3	7	93

[a] The resolution with NMR was too low to trace the terminal and (1→4,6) linked residues as detected by methylation analysis. Displayed are the anomeric signals at 5.0 ppm [α-(1→6) linkages] and 5.3 ppm [α-(1→4) linkages]. [b] ND, not determined.

S1136V, incubated with 100 mM sucrose for 60 h, did not consume all sucrose. Only prolonged incubation resulted in complete consumption of sucrose. The other three mutants containing this triple amino acid mutation displayed the same properties.

Product Spectra of Mutants Incubated with both 100 mM Sucrose and 100 mM Maltose. When sucrose and maltose were both present in the assay mixtures, GTFA synthesized mainly panose and some maltotriose (Table 4). All GTFA-derived enzyme variants (including the four enzymes containing the triple amino acid mutation N1134S:N1135E:S1136V) were able to completely consume sucrose within 60 h. Wild-type GTFA and all mutants derived produced similar percentages of oligosaccharides (∼60%) from sucrose and maltose as acceptor reaction substrate (Table 5). Interestingly, the four enzymes with the N1134S:N1135E:S1136V mutations synthesized an oligosaccharide of DP4 from sucrose and maltose as acceptor reaction substrate (Figure 3). The structure of this oligosaccharide was identified by enzymatic degradation as α-D-glucopyranosyl-(1→6)-α-

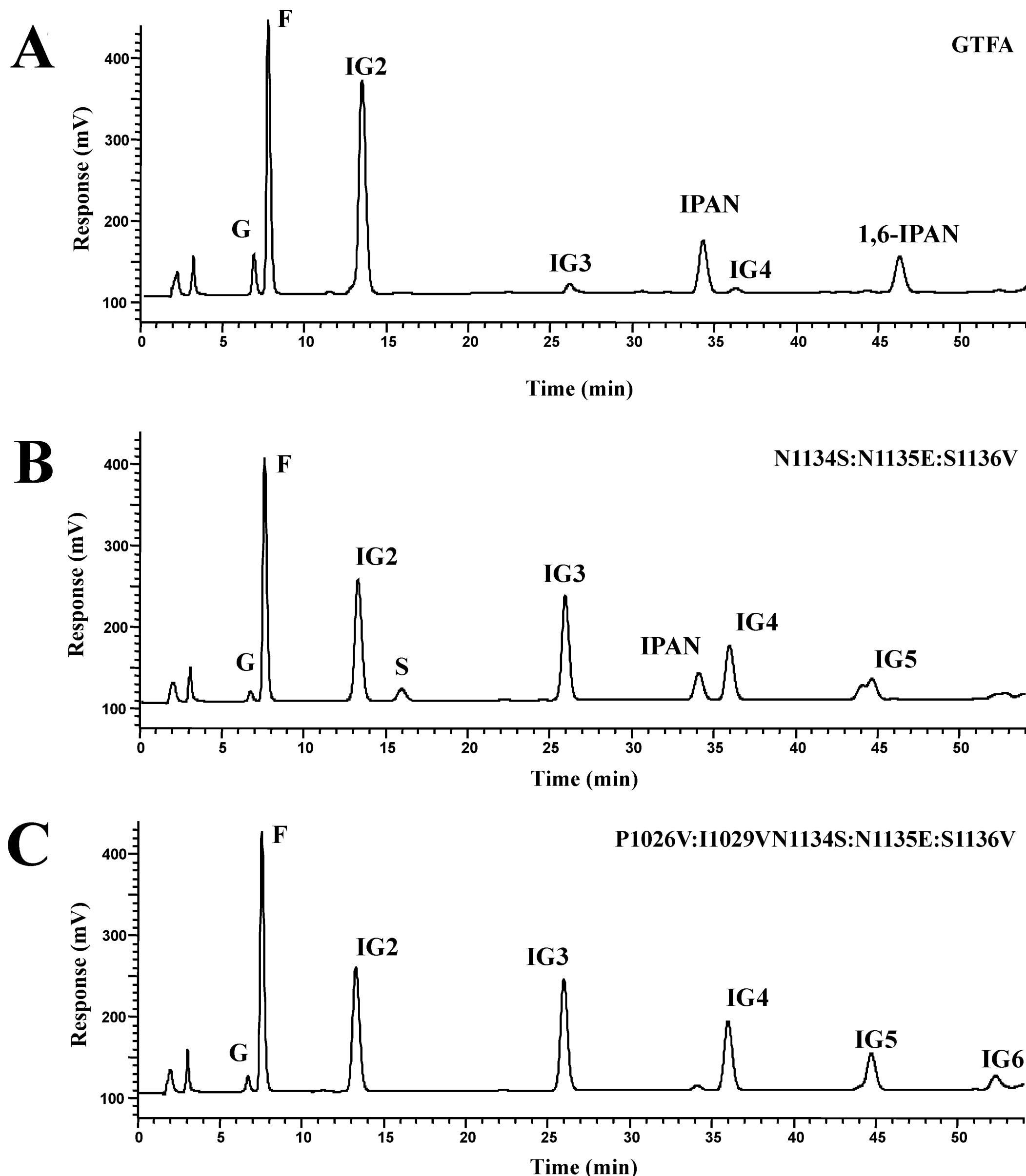

FIGURE 4: Anion-exchange (Dionex) analysis of (mutant) GTFA acceptor reaction products produced from sucrose plus isomaltose. (A) Products formed upon incubation of 30 nM GTFA enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h. (B) Products formed upon incubation of 30 nM GTFA mutant N1134S:N1135E:S1136V enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h. (C) Products formed upon incubation of 30 nM GTFA mutant P1026V:I1029V:N1134S:N1135E:S1136V enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h. F = fructose, G = glucose, S = sucrose, IG2 = isomaltose, IG3 = isomaltotriose, IG4 = isomaltotetraose, IG5 = isomaltopentaose, IG6 = isomaltohexaose, IPAN = isopanose, and 1,6-IPAN = α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose.

D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose; α-(1→6)-panose (data not shown). Dextranase degraded the DP4 oligosaccharide into maltose and isomaltose. Amyloglucosidase and α-glucosidase cleaved the oligosaccharide slowly to glucose and panose [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose]. In contrast, isopanose [α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose] was degraded very fast by the latter two enzymes (*3*). Wild-type GTFA and the other mutants did not produce α-(1→6)-panose at all. The four enzymes containing the triple amino acid mutation N1134S:N1135E:S1136V showed up to a 1.5-fold decreased level of panose synthesis and synthesized also lower amounts of maltotriose compared to wild type and the other mutants.

Product Spectra of Mutants Incubated with both 100 mM Sucrose and 100 mM Isomaltose. When sucrose and isomaltose were both present in the assay mixtures, GTFA synthesized predominantly isopanose and α-(1→6)-isopanose (Table 5; Figure 4A) (*18*). The three GTFA enzyme variants with the P1026V mutation showed up to a 5-fold increased synthesis of isomaltotriose. These three mutants showed also slightly increased isomaltotetraose synthesis. Oligosaccharide yields from sucrose and isomaltose increased drastically (2-fold) for all four GTFA-derived enzyme variants with the N1134S:N1135E:S1136V mutation. These four mutant enzymes also showed a 10-fold increased production of isomaltotriose and isomaltotetraose compared to wild type (Table 5; Figure 4B,C) and no synthesis of α-(1→6)-isopanose. Instead, isomaltopentaose was produced in low amounts. Combination of N1134S:N1135E:S1136V with P1026V:I1029V or P1026V:I1029V:A1066N resulted in a slightly higher production of isomaltotetraose and isomal-

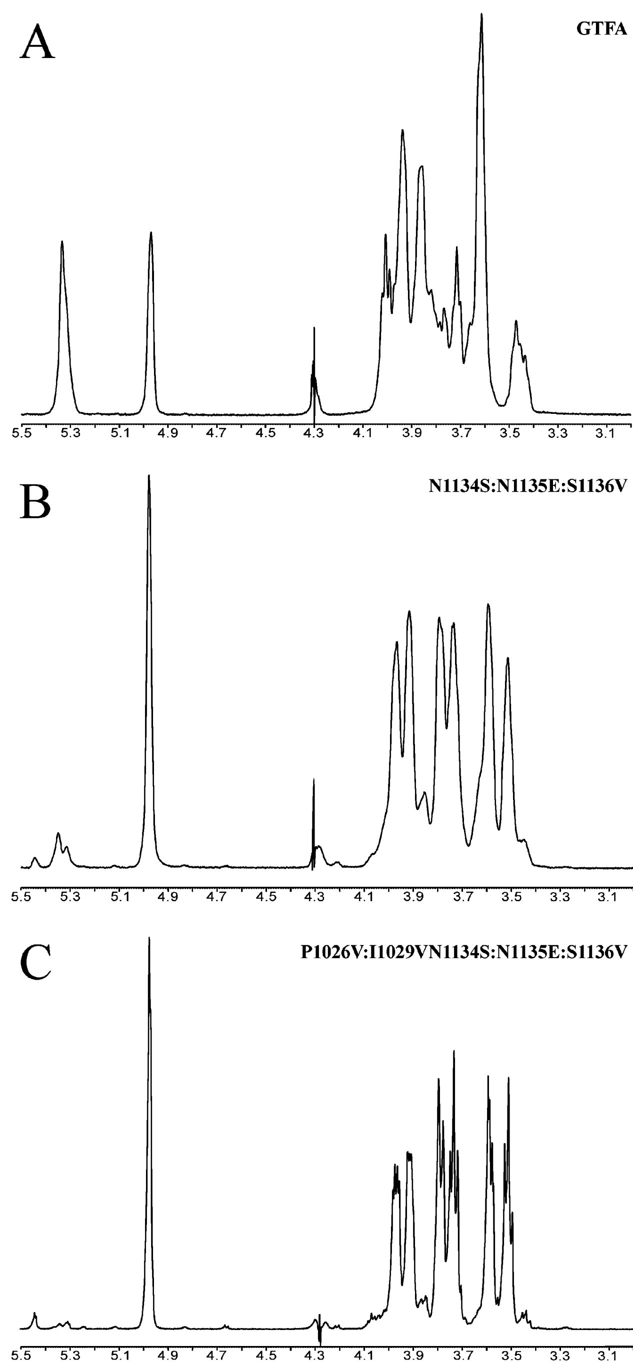

FIGURE 5: 600 MHz ^{1}H NMR spectra of the glucans produced by (A) purified recombinant GTFA protein, (B) GTFA mutant protein N1134S:N1135E:S1136V, and (C) GTFA mutant protein P1026V:I1029V:N1134S:N1135E:S1136V, recorded in D_2O at 80 °C. Chemical shifts are given in parts per million relative to the signal of internal acetone ($\delta = 2.225$). 5.0 ppm [α-(1→6) linkages and 5.3 ppm [α-(1→4) linkages. As also indicated in the legends to Table 6.

topentaose. Only these two mutant enzymes also synthesized isomaltohexaose. Interestingly, both mutants showed strongly reduced levels of isopanose synthesis, whereas α-(1→6)-isopanose was not produced at all (Table 5; Figure 4C).

Influence of Mutations on Glucan Synthesis and Glucosidic Bond Specificity. The mutations did not affect the ability of the mutants to convert sucrose into glucan polymer during 60 h incubation (Table 3). Methylation and ^{1}H NMR analysis (Table 6 and Figure 5) of the polymers produced by the mutant enzymes showed that mutation P1026V resulted in the production of a polymer with a slightly elevated level of α-(1→6) linkages and a decrease in the amount of α-(1→4) linkages. Mutations I1029V and A1066N did not change the nature of the glucan produced (Table 6). The glucan polymers synthesized by the double mutant P1026V:I1029V and the triple mutant P1026V:I1029V:A1066N showed similar distribution of glucosidic linkages inside their polymers as the glucan polymer synthesized by the single mutant P1026V. Both mutants N1134S:N1135E:S1136V and A1066N:N1134S:N1135E:S1136V showed a drastic increase of α-(1→6) linkages (∼40%) and a decrease of α-(1→4) linkages (∼40%) in their polymers compared to wild type (Table 6 and Figure 5). Combination of N1134S:N1135E:S1136V with P1026V:I1029V or P1026V:I1029V:A1066N resulted in an even further increased production of α-(1→6) linkages and a decrease of α-(1→4) linkage synthesis (as shown by methylation and ^{1}H NMR analysis), yielding a polymer containing very low amounts of α-(1→4) linkages (Table 6 and Figure 5).

DISCUSSION

The different glucansucrase enzymes (GH70) are highly similar in their catalytic cores. Nevertheless, they synthesize a diverse range of linear and branched α-glucans and oligosaccharides from sucrose. Depending on the main glucosidic linkages present in their glucan products they can be divided into dextran [α-(1→6)], mutan [α-(1→3)], alternan [α-(1→3)/α-(1→6)], and reuteran [α-(1→4)] (*2*, *5*). The aim of this study was to identify amino acid residues that determine the linkage specificity in the poly- and oligosaccharide products synthesized by GTFA of *Lb. reuteri* 121.

Putative Sugar-Binding Acceptor Subsites in GTFs. For amylosucrase and CGTase (family GH13) the different sugar-binding acceptor subsites have been mapped out on the basis of 3D structural information, labeled −2, −1, +1, +2, etc., according to the definition of ref *26*. Since GTF enzymes of family GH70 and members of family GH13 have similar structural features (Figure 1) (*9*, *10*), we postulate that members of both enzyme families have a similar acceptor subsite organization.

Mutations in Conserved Region II. GTFA derivatives containing mutation P1026V showed a clear change in oligosaccharide and glucan products, with an increase of α-(1→6) and a decrease of α-(1→4) glucosidic linkages (Tables 3−6). Mutations in the corresponding region in GTFB (D457N) and GTFD of *Streptococcus mutans* GS5 (N471D) resulted in an interchange between soluble and insoluble glucan synthesis (*13*). In CGTase the corresponding region constitutes part of the sugar-binding acceptor subsites +1 and +2 [residues Asp^{229} (−1)-Ala- (+1)-Val-Lys-(+2)-His^{233} (+1), in *Bacillus circulans* 251 CGTase] (*27*), responsible for the stereospecific positioning of the molecule accepting the glucosyl unit (*28*). The structure of this acceptor site contributes to the determination of the type of glucosidic bond formed (*6*). In neopullulanase from *B. stearothermophilus* this region is also important in specificity toward α-(1→4) or α-(1→6) glucosidic linkages (*29*). The postulation made by ref *9*, that conserved region II in GH13 also is present at a similar position in GH70, is confirmed by our mutant data showing its importance in acceptor binding and glucosyl transfer (this paper).

Mutations in Conserved Region III. GTFA mutations A1066N and H1065S:A1066S (changes into residues present in GTFO) (*3*) did not result in changes in glucosidic linkages synthesized. However, the double mutant showed a drastic change in enzyme activity: overall activity was lower than in wild-type GTFA, and no transferase activity could be measured (initial rates) (Table 2) as was the case with GTFO (*3*). Residues H1065 and A1066 (corresponding to *B. circulans* 251 CGTase G261 and V262, close to subsites +2/+3) are located further away from the −1 and +1 subsites and might therefore have no direct effect on the nature of glucosidic linkages synthesized. Features responsible for the larger amounts of α-(1→4) glucosidic linkages in glucan and oligosaccharide products synthesized by GTFO compared to GTFA remain to be investigated.

Mutations in Conserved Region IV. Changing the tripeptide C-terminal of the catalytic D1133 in GTFA to N1134S:N1135E:S1136V resulted in a drastic reduction in affinity for sucrose and changes in the distribution of glucosidic linkages in the oligosaccharide and glucan products synthesized. This indicates that conserved region IV is important in sucrose binding/processing, consistent with the suggested role of D1133 as transition state stabilizer (*9*, *18*) (Figure 2). Where studied the linkage specificity of glucansucrases is conserved in oligosaccharide synthesis, and oligosaccharides are elongated at their nonreducing end (*18*). Dextransucrase (DSRS) from *Ln. mesenteroides* NRRL B-512F synthesizes panose and other members of the panose series from sucrose plus maltose (*30*, *31*). GTFA wild type only synthesized panose (*3*, *18*), whereas all four mutant enzymes containing the triple amino acid mutation N1134S:N1135E:S1136V synthesized α-(1→6)-panose (Figure 3). The data thus clearly indicate that, with respect to oligosaccharides synthesized from maltose, the GTFA mutants in conserved region IV have an increased α-(1→6) glucosidic bond specificity. DSRS from *Ln. mesenteroides* NRRL B-512F incubated with sucrose and isomaltose synthesizes isomaltotriose as the main product, followed by gradually lower amounts of isomaltooligosaccharides with increased DP (*32*). Whereas GTFA wild type incubated with sucrose and isomaltose synthesizes minor amounts of isomaltotriose and isomaltotetraose, GTFA derivatives containing mutation N1134S:N1135E:S1136V clearly produced isomaltotriose and isomaltotetraose (triple and quadruple mutants). The quintuple and sextuple mutants showed a product spectrum similar to that of the dextran synthesizing enzyme DSRS, with a series of isomaltooligosaccharides up to DP6 (Table 5; Figure 4). Also, the oligosaccharide yields with sucrose and isomaltose as acceptor reaction substrate increased drastically (2-fold) with these four GTFA derivatives (Table 5). A similar strong shift in glucosidic bond specificity also was observed in the glucan products synthesized by the four GTFA N1134S:N1135E:S1136V mutants, reaching levels of 85% of α-(1→6) linkages and very low amounts of α-(1→4) linkages, in the quintuple and sextuple mutants (Table 5; Figure 5). The data thus show that, with respect to both oligosaccharide and glucan synthesis, the combination of mutations in conserved regions II and IV of GTFA of *Lb. reuteri* 121 successfully transformed the enzyme from a reuteransucrase into a dextransucrase. In amylosucrase the D394 residue, next to the catalytically important H392 and D393 (transition state stabilizer) residues (*33*), is part of acceptor binding subsite +1 and is involved in the correct positioning of the glucosyl residue at this site. Mutant D394A displayed changes in the product spectrum (mono- and oligosaccharides) from sucrose (*7*). Mutant M329T in acarviosyltransferase (ATase) of *Actinoplanes* sp. strain SE50/110, immediately next to the catalytic Asp328 (CGTase *B. circulans* 251 numbering), also resulted in changed reaction specificity (10 times higher transferase activity on maltotetraose) (*27*). The drastic changes observed in the mutant GTFA enzymes with respect to affinity for sucrose, type of oligosaccharide, and glucan products synthesized, strongly support the hypothesis that residues in this conserved region IV are involved in acceptor substrate binding in GTF enzymes. However, most likely there are also other regions important for glucosidic linkage specificity. Many mutansucrases possess the same amino acid triplet "SEV" in region IV as GTFA mutant N1134S:N1135E:S1136V, and instead of synthesizing dextran they synthesize mutans. Furthermore, the higher amount of α-(1→4) glucosidic linkages synthesized by GTFO could also not be explained by the GTFA mutants in regions investigated in this study.

CONCLUSIONS

Amino acid residues and conserved enzyme regions determining the linkage specificity of glucan and oligosaccharide products formed by GTF enzymes have been identified. By rational design we were able to transform GTFA from a reuteransucrase into a dextransucrases type of enzyme. Furthermore, we show for the first time that two distantly located regions in a glucansucrase enzyme both affect linkage type distribution, in glucans as well as in oligosaccharide products. Although there are clear differences in glucosidic linkages in the glucan products synthesized by the different (mutant) GTFA enzymes, the precise glucan structures remain to be elucidated. Our data indicate that glucan and oligosaccharide acceptor substrates in GTFA are elongated at the same sugar-binding acceptor subsite. Further investigation of the precise roles of amino acid residues involved in acceptor substrate binding in glucansucrases that synthesize other types of glucosidic linkages may serve to expand the range of glucans and glucooligosaccharides that can conveniently be synthesized. Clear understanding of the structural features in glucansucrase enzymes that determine the nature and ratio of glucosidic linkages synthesized eventually may allow production of tailor-made glucan and oligosaccharide products for diverse applications.

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