Engineering the Glucansucrase GTFR Enzyme Reaction and Glycosidic Bond Specificity: Toward Tailor-Made Polymer and Oligosaccharide Products†

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ABSTRACT: Two long-standing questions about glucansucrases (EC 2.4.1.5) are how they control oligosaccharide versus polysaccharide synthesis and how they direct their glycosidic linkage specificity. This information is required for the production of tailor-made saccharides. Mutagenesis promises to be an effective tool for enzyme engineering approaches for altering the regioselectivity and acceptor substrate specificity. Therefore, we chose the most conserved motif around the transition state stabilizer in glucansucrases for a random mutagenesis of the glucansucrase GTFR of *Streptococcus oralis*, yielding different variants with altered reaction specificity. Modifications at position S628 achieved by saturation mutagenesis guided the reaction toward the synthesis of short chain oligosaccharides with a drastically increased yield of isomaltose (47%) or leucrose (64%). Alternatively, GTFR variant R624G/V630I/D717A exhibited a drastic switch in regioselectivity from a dextran type with mainly α-1,6-glucosidic linkages to a mutan type polymer with predominantly α-1,3-glucosidic linkages. Targeted modifications demonstrated that both mutations near the transition state stabilizer, R624G and V630I, are contributing to this alteration. It is thus shown that mutagenesis can guide the transglycosylation reaction of glucansucrase enzymes toward the synthesis of (a) various short chain oligosaccharides or (b) novel polymers with completely altered linkages, without compromising their high transglycosylation activity and efficiency.

The synthesis of oligosaccharides and polysaccharides is a challenging goal for modern science. Despite the great importance and the enormous potential of oligosaccharides, their technical and pharmaceutical usage is still limited because of the high complexity of these molecules, causing enormous problems in classical chemical synthesis (*1*). The production of tailor-made oligosaccharides and polysaccharide structures is still a growing field of interest in modern biotechnology. An established industrial process is the fermentative production of polysaccharides (α-glucans) by lactic acid bacteria, e.g., dextran by *Leuconostoc mesenteroides*. Such polymers are used in the food industry, as additives for dyes, and in health care (*2, 3*). Differences in the glycosidic linkage type, the degree and type of branching, and the molecular mass of glucans available at present show promising variations in structural and functional properties, which needs to be elucidated further. Known are dextrans with different structural specificities [α-1,6-bound glucose (Glc) backbone, with α-1,2 and α-1,3 side chains] (*4, 5*), mutan (α-1,3-bound Glc units) (*6*), alternan (α-1,6- and α-1,3-bound Glc units) (*7*), and amylose/reuteran (α-1,4-bound Glc units) (*8*). In addition to defined polymers, oligosaccharides with specific structures are urgently needed. Oligosaccharides currently produced for commercial markets (*9*), including isomaltooligosaccharides (IMO)[1] (*10*), leucrose (*11*), and palatinose (*12*), are of interest in the fields of food, pharmacy, and cosmetics because of their ability to prevent and treat diseases from various biological origins. Isomaltose, for instance, enhances cytokine IL-12 production by macrophages stimulated with *Lactobacillus gasseri* in vitro, and dietary IMO significantly increased the number of lactobacilli in the intestinal microflora (*13*). For the synthesis of oligosaccharides, the concept of so-called glycosynthases is very promising, where the hydrolytic activity of glycosidases has been suppressed via random and rational site-directed mutagenesis, allowing transglycosylation reactions to occur instead (*14–18*). Here we report an alternative approach that switches the regioselectivity and acceptor specificity of GTFR of *Streptococcus oralis*, a dextran-producing enzyme, by random mutagenesis toward synthesis of (a) various short

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[1] Abbreviations: GTFR, glycosyltransferase R; IMO, isomaltooligosaccharide; HPAEC, high-performance anion exchange chromatography; PCR, polymerase chain reaction.

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chain oligosaccharides or (b) novel (mutan) polymers with completely altered linkages, without compromising its high transglycosylation activity.

EXPERIMENTAL PROCEDURES

Oligodeoxynucleotides. Oligodeoxynucleotides were obtained from MWG Biotech (Ebersberg, Germany). The mutagenic oligodeoxynucleotide was synthesized with 91% of the wild type (WT) and 3% of each non-WT nucleotide (NT) precursor at each degenerate position (for primer sequences, see the Supporting Information).

Bacterial Strains, Plasmids, and Culture Conditions. Escherichia coli XL10-Gold cells (Stratagene) were used for library construction. The recombinant plasmids were pTH275, harboring the full-length *gtfR* gene (*19*), and pAER100, containing a 5′-terminally truncated version (*20*). *E. coli* XL10-Gold, harboring pTH275 or its derivatives, was routinely grown at 37 °C in LB medium (*21*), supplemented with 100 mg/L ampicillin.

General DNA Techniques. In vitro DNA modifications, agarose gel electrophoresis, and transformations were carried out according to standard protocols (*21*), unless described in detail.

DNA Sequencing. Approximately 1 μg of plasmid DNA was subjected to *Taq* DNA polymerase-catalyzed cycle sequencing, as described previously (*22*).

Generation of a Mutant gtfR Library. A mixture of mutant *gtfR* fragments was generated by standard PCR (*23*), using pAER100 as the template. The primers were pETR3+5179, annealing to the multiple cloning site of the vector, and the mutagenic oligodeoxynucleotide gtfR-1908M3. An overlapping WT DNA helper fragment was synthesized by PCR with the same template, using primers gtfR+1895 and gtfR-2783. Both PCR products were purified by agarose gel electrophoresis and isolated with a gel purification kit (Qiagen, Hilden, Germany). They were fused in an overlap extension PCR (*24*). The product was isolated as described above. After cleavage by *Aar*I and purification with a PCR purification kit (Qiagen), the fragment library was ligated with identically cleaved and dephosphorylated pTH275. Transformants of *E. coli* strain XL10-Gold were selected on LB/ampicillin (100 mg/L). The quality of the library of mutants was assessed by screening for enzymatic activity (see below) and by sequencing of 28 randomly selected clones. This yielded the following results: percentage of active clones, 59%; percentage of clones that had obtained stop codons, 16%; number of mutations per clone, theoretically expected, 1.35; and number of mutations per clone, experimentally determined, 1.36.

Generation of Targeted Mutations. Targeted mutations were constructed via overlap extension PCR. pTH275 was used as the template. The mutagenic primers R624G, V630I, and R624G/V630I were used together with gtfR+1566 to synthesize and introduce mutations. Fragments were purified as stated above. After fusion with the gtfR+OE−gtfR-2192 overlapping helper fragment in an overlap extension PCR, isolation, and cleavage by *Hin*dIII and *Bgl*II, the fragments were ligated with identically cleaved and dephosphorylated pTH275. Transformants of *E. coli* strain XL10-Gold were selected on LB/ampicillin agar plates.

GTFR Library Screen for Enzymatic Activity. Cultures were grown in 96-well microtiter plates overnight at 37 °C in 300 μL of 2×YT medium (*21*), supplemented with 100 mg/L ampicillin. Cells were pelleted for 30 min at 2000*g* and resuspended in 50 μL of 50 mM sodium phosphate (pH 6.5), 0.1% (w/v) SDS, 1% (w/v) Triton X-100, and 1% (w/v) sucrose. After incubation for 4 h at 37 °C, 50 μL of 0.01% (w/v) triphenyltetrazolium chloride in 1 M NaOH was added (*25*). Reducing sugars (fructose and glucose) which are released in the reaction result in formation of a red color. Between 5 and 15 min later, the occurrence of absorption at 490 nm, indicating activity, was monitored with a microtiter plate reader (GENios, Tecan) and compared with that of the wild type reaction for a first selection of active candidates.

GTFR Library Screen for Altered Transglycosylation and Polymerization Products. Ninety-six-well microtiter plates with 200 μl of LB medium containing ampicillin (100 mg/L) were inoculated with the GTFR mutant library stored as a cryoculture and were shaken for 2 days at 30 °C. Cells were centrifuged down; medium was removed, and the pellets were resuspended in 40 μL of bacterial protein extraction reagent (BPER; Pierce). Ten microliters of the extracts was mixed with 10 μL of 584 mM sucrose in reaction buffer and the mixture incubated for 4 days at 30 °C. Samples were diluted 1/10 with MilliQ H_2O and analyzed via thin-layer chromatography (TLC) for changes in transglycosylation (different product pattern) and polymer solubility (frayed spots of polymer) (Supporting Information).

Enzyme Production by Fermentation. One microliter of *E. coli* XL10-Gold cryocultures (encoding specific GTFR mutant proteins) was used to inoculate 12.5 mL of LB medium that contained 100 mg/L ampicillin (LB-Amp). Precultures were incubated with shaking for 24 h at 37 °C. Subsequently, these precultures were transferred to 250 mL of LB-Amp, and the culture was incubated with shaking (120 rpm) for 12 h at 30 °C. The cells were collected by centrifugation, washed with Sorensen buffer (pH 7), and resuspended in 6 mL of the same buffer. Cells were disrupted by sonication (4 min pulsed operation at 120 W). After sonication, cells were centrifuged at 2000*g*, and the supernatants were used as a source of enzyme. Incubation of supernatants from *E. coli* XL10-Gold cultures without the *gtfR* gene showed no activity with sucrose, showing that no background activity is present in the crude extract.

Thin-Layer Chromatography (TLC). The ethyl acetate/2-propanol/water (e/i/w) solvent system in a ratio of 6:3:1 (v/v/v) or an acetonitrile/water (a/w) solvent system (8:2, v/v) was used as the mobile phase.

The reaction samples were applied on silica thin-layer plates (20 cm × 20 cm TLC aluminum sheets, silica gel 60 F254 with a concentrating zone with dimensions of 20 cm × 2.5 cm; MERCK) after appropriate dilution (final concentration between 0.05 and 10 g/L). Standard solutions were prepared in the range of 0.05−5 g/L. Monosaccharides D-fructose and D-glucose, disaccharides sucrose (substrate) (Nordzucker) and leucrose, and isomaltose (possible products) (Sigma) were used as external standards. Carbohydrates were separated by using three ascents (60 min) for e/i/w or one to three ascents (45 min) for a/w. Spots were detected by dipping the plates into the detecting reagent [0.3% (w/v) *N*-(1-naphthyl)ethylenediamine (Fluka) and 5% (v/v) concentrated sulfuric acid in methanol] using a CAMAG

Chromatogram Immersion Device III (speed 2, time 4) (MERCK), followed by heating in an oven at 120 °C for 15 min. The sugars were visualized as colored spots.

High-Performance Anion Exchange Chromatography (HPAEC). HPAEC was performed with a Dionex BioLC chromatography system with 0.1 M NaOH, a Carbopac PA1 column, and a pulsed amperometric detector. Elution was carried out with a gradient of sodium acetate from 0 to 0.5 M. Glucose (acceptor substrate), fructose (acceptor substrate), sucrose (donor substrate), and product formation of leucrose [*O*-α-D-glucopyranosyl-(1 → 5)-D-fructopyranoside], palatinose [isomaltulose, *O*-α-D-glucopyranosyl-(1 → 6)-D-fructopyranoside], isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-α,β-D-glucopyranose], and 1,6-linked oligoglucosides were identified by comparison with standards.

Enzyme Activity Assays. Sucrose (292 mM) was incubated with 0.5% enzyme extract for 1 h in reaction buffer [Sorensen buffer (pH 7) containing 0.5 mM $CaCl_2$] at 30 °C. Samples were taken at five different time points (0, 5, 20, 40, and 60 min) and inactivated by being boiled for 10 min. Samples were diluted in an appropriate way and analyzed by HPAEC. One unit of enzyme activity was defined as the release of 1 μmol of fructose/min.

Kinetic Parameters. Enzyme activity assays were performed by measuring the sucrose consumption (two to five time points) up to a conversion of approximately 10% at five to nine different initial concentrations of sucrose (0.2−50 g/L) and quantification of sucrose via HPAEC. One unit of enzyme was defined as the consumption of 1 μmol of sucrose/min.

Sucrose consumption data were fitted to the Michaelis−Menten equation by using Sigma-plot, and apparent K_m and V_{max} values were determined.

k_{cat} Values. Enzyme samples were analyzed with a Coomassie-stained SDS−polyacrylamide gel and compared to a standard of BSA protein for quantification of the GTFR (mutant) protein concentrations. Quantitative determinations were performed by scanning densitometry using a Bio-Rad imaging densitometer utilizing Quantity One (version 4.2). k_{cat} values were calculated using the enzyme concentrations obtained and the calculated V_{max} values (GTFR is a monomeric protein).

Isolation of Transglycosylation and Polymer Products. All reactions were carried out with 146 mM sucrose in Sorensen buffer, at the optimal pH of 7, and 200 units/L enzyme, at 30 °C, and incubated until all the sucrose was consumed. For isolation of transglycosylation products, an acceptor substrate (292 mM) was added. These reaction products were analyzed via HPAEC. Polymers were isolated after the reaction was finished. For total polymer isolation, 2 volumes of ethanol was added and incubated overnight at 4 °C prior to centrifugation. The supernatants were removed, and the pellets were washed five times with 1 volume of 66% ethanol. To distinguish between soluble and insoluble polymer fractions, insoluble material was spun down by centrifugation prior to ethanol precipitation and polymers in the supernatant were precipitated with ethanol as stated above. Both pellet fractions were washed separately. The polymers were partly dissolved in water and lyophilized. Amounts were measured by weighing.

Methylation Analysis of Polymers. Only the total polymer was analyzed. Methylation analysis was carried out as previously described (*6, 26*) with the following modification: prior to methylation samples were dissolved in DMSO and solubilized for 8 h by sonication.

Size Distribution. The polymers were dissolved in 0.9 M NaOH and characterized by multiangle laser light diffraction (MALLD; Dawn DSP, Wyatt Technologies) and RI analysis (RI-101, Shodex).

RESULTS AND DISCUSSION

Glycosyltransferase R (GTFR) from *S. oralis* is a dextransucrase type of enzyme (EC 2.4.1.5, this paper), belonging to glycoside hydrolase family 70 (*27, 28*). Polymer and transglycosylation products are not hydrolyzed by these so-called glucansucrases, thus displaying a main characteristic of glycosynthases. Besides glucan production, GTFR catalyzes transglycosylation of different acceptor substrates, including alcohols and amino acids (*29*). With a temperature optimum of 37 °C and no significant loss of activity at 30 °C for weeks, GTFR is a comparatively stable enzyme, making it an interesting catalyst for further investigations into elucidating and controlling its reaction and glycosidic bond specificity. A variety of approaches, involving both "rational" and "irrational" design, have been used successfully to alter protein function (*30, 31*). Examples of expanding substrate specificity and the control of the regioselectivity of a given enzyme, especially glycosyl transfer enzymes, are rare (*32, 33*). However, the three catalytic residues crucial for glucansucrase activity have been identified previously (*34*). In enzymes of GH70, D516 (putative nucleophile, GTFR numbering) is involved in formation of the covalent glucosyl−enzyme complexes (*35, 36*). The crucial roles of this Asp residue and the other two catalytic residues, E554 (putative acid/base catalyst) and D627 (putative transition state stabilizer), have been identified by site-directed mutagenesis experiments (*8, 37–39*). Using sequence alignments, the corresponding catalytic amino acids in GTFR were identified as D516 (putative catalytic nucleophile), E554 (putative acid/base catalyst), and D627 (putative transition state stabilizer) (*29*). On the basis of structural modeling, further amino acid residues putatively forming the enzyme active center could be identified (*40*). As changes in the transglycosylation activity have been reported for mutations in a highly conserved motif around the transition state stabilizer (*41–45*), this "RAHDSEV" (Table 1) segment was randomized by PCR mutagenesis to generate variants with novel (acceptor substrate) properties (Table 1 and the Supporting Information).

Glucansucrase Mutants Exhibiting Strong Variation in Oligosaccharide Product Synthesis. As a first approach, the resulting GTFR mutant library was screened for clones exhibiting high activity with the donor substrate sucrose. Subsequently, the positive clones were screened for altered product spectra using thin-layer chromatography (TLC). Of 2000 clones, we identified several variants exhibiting clear changes in oligosaccharide (OS) versus polymer (PS) formation.

Two GTFR variants that had lost the ability to synthesize polymer are mutated at position 628: S628D and S628R (GTFR numbering). This residue is part of the ^{628}SEV^{630} sequence motif (Table 1) which is known to affect glucansucrase glycosidic linkage specificity. Recent findings by

Table 1: Polymer Linkage Type and Alignment of Amino Acid Sequences of Various (mutant) Glucansucrase Enzymes with Wild Type GTFR and Mutant Variants Derived[a]

Wild-type and Mutant Enzymes	Main α-linkage in glucan polymer products	Amino acid sequence around transition state stabilizer D627 in GTFR
DSRS	(1-6)	655 YSFVRAHDSEVQTVI
GTFI	(1-3)	557 YSFIRAHDSEVQDLI
GTFA	(1-4)	1126 YSFVRAHDNNSQDQI
GTFA'	(1-6)	1126 YSFVRAHDSEVQDQI
GTFR	(1-6)	620 YIFVRAHDSEVQTVI 713 SPYHDAIDA
S628D		620 YIFVRAHDDEVQTVI
S628R		620 YIFVRAHDREVQTVI
R624G/V630I/D717A		620 YIFVGAHDSEIQTVI 713 SPYHAAIDA
R624G		620 YIFVGAHDSEVQTVI
V630I		620 YIFVRAHDSEIQTVI
R624G/V630I		620 YIFVGAHDSEIQTVI
D717A		713 SPYHAAIDA

[a] Abbreviations: DSRS, *L. mesenteroides* NRRL B-1355 (*38*); GTFI, *Streptococcus downei* Mfe28 (*49*); GTFA, *Lactobacillus reuteri* 121 (*50*); GTFA′, mutant GTFA (*44*); GTFR, *S. oralis* (*19*); GTFR variants, mutants constructed in this study. Mutations are shown with a black background, and the putative transition state stabilizer is shown with a grey background. The mutation at position 717 was probably the result of a PCR error.

Table 2: Kinetic Parameters of Wild Type GTFR and Mutant Variants (determining sucrose consumption as a measure of total activity)[a]

	apparent K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
wild type	2.0 (0.6)	1344 (101)	672
Variants with Altered OS Synthesis			
S628D	3.1 (0.2)	1788 (29)	577
S628R	5.0 (0.5)	96 (3)	19
Variants with Altered PS Synthesis			
R624G/V630I/D717A	9.8 (0.3)	1536 (18)	157
R624G	1.4 (0.3)	312 (12)	223
V630I	1.6 (0.4)	1956 (74)	1223
R624G/V630I	3.5 (0.3)	870 (23)	249

[a] Errors (standard errors of regression) are given in parentheses.

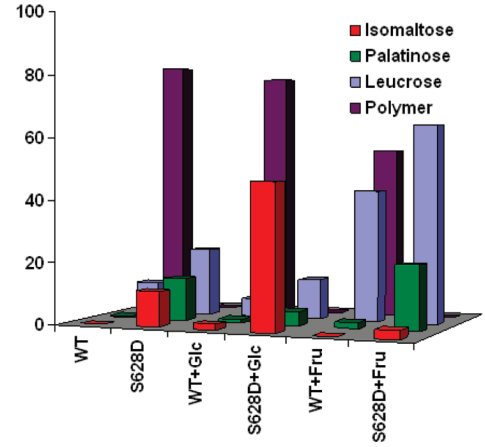

FIGURE 1: Product spectra of WT GTFR and mutant S628D enzymes (200 units/L) incubated (7 days at 30 °C) with sucrose (146 mM) and different acceptor substrates (292 mM; Glc, glucose; Fru, fructose). Yields are given in % (moles per mole of Glc). Yields of higher oligosaccharides (DP > 5) and hydrolysis products are not shown.

Kralj et al. (*44*) suggested that amino acids C-terminal to the invariant His626-Asp627 motif of glucansucrases (GTFR numbering) influence the structure of the oligo- and polysaccharides formed. Reduction or the absence of polymer formation by rational mutations at this position is consistent with the findings of Kralj (*44, 45*) and Moulis (*46*), but in the latter studies, with other glucansucrases, polymer formation was slightly inhibited (*44, 45*) or associated with a great loss of enzyme activity (*46*). The third amino acid residue next to the transition state stabilizer was also mutated in GTFA of *Lb. reuteri* 121 S1136V (*45*), but in this particular enzyme, virtually no shift in glucosidic linkages was observed as we perceive here for V630I.

Also, the N1134S mutation in GTFA resulted in a drastically changed specificity but no major changes in polymer versus oligosaccharide formation as observed in the S628X mutants. In S628, now instead of these mutants, synthesized were formed.

Kinetic studies (measuring sucrose consumption) revealed that the apparent K_m values for sucrose of the S628D,R variants were comparable to those of wild type GTFR. While substitution of serine for arginine in S628R resulted in a 14-fold reduction in activity, the aspartate variant S628D was not significantly affected in terms of either its K_m or k_{cat} value (Table 2).

Thus, transglycosylation experiments were conducted for the wild type GTFR enzyme and its variants (Table 2)

Table 3: Linkage Analysis of α-Glucan Polymers Produced by WT GTFR and Its Mutants[a]

	WT	V630I/R624G/D717A	R624G	V630I	R624G/V630I	D717A
Glcp-(1−	11 ± 1	9 ± 2	11 ± 0	10 ± 1	11 ± 1	10 ± 1
−3)-Glcp-(1−	14 ± 1	46 ± 1	23 ± 1	20 ± 1	39 ± 7	7 ± 0
−6)-Glcp-(1−	62 ± 2	28 ± 1	53 ± 1	57 ± 1	38 ± 9	70 ± 0
−3,6)-Glcp-(1−	14 ± 1	17 ± 1	15 ± 1	14 ± 0	14 ± 1	14 ± 1

[a] Analysis was done via methylation experiments.

(Supporting Information). The wild type enzyme synthesized predominantly glucan and higher oligosaccharides (together amounting to 85% of the total products) from sucrose. Side products are leucrose [*O*-α-D-glucopyranosyl-(1 → 5)-D-fructopyranoside, 10%] and palatinose [isomaltulose, *O*-α-D-glucopyranosyl-(1 → 6)-D-fructopyranoside, 1%].

The latter two products are synthesized from sucrose (donor substrate) and fructose (acceptor substrate). Fructose is derived from sucrose, accumulating in increasing amounts during the reaction. Isomaltose formation [*O*-α-D-glucopyranosyl-(1 → 6)-α,β-D-glucopyranose] was not detected. However, HPAEC analysis (Supporting Information) showed that wild type GTFR synthesized acceptor reaction products of the isomaltooligosaccharide series in low yields, which was confirmed by comparing it with a dextran hydrolysate (yielding 1,6-linked glucooligosaccharides), with yields increasing with a higher degree of polymerization (DP) of products. Addition of glucose as the acceptor substrate did not result in a major increase in the level of isomaltose formation (2%) by wild type GTFR. Polymer formation remained the dominant reaction. On the other hand, addition of fructose as the acceptor substrate increased the leucrose and palatinose yields to 43 and 2%, respectively, while the dextran yield was reduced to 55% (Figure 1).

Compared to the wild type enzyme, S628X variants exhibited tremendous changes in their transglycosylation product spectra. Polymer formation was virtually not detected, and increased amounts of sucrose hydrolysis products were found (23%), but without the loss of transglycosylation capacity. With sucrose, the S628D variant synthesized isomaltose in 12% yield; also, the extent of leucrose synthesis (22%) increased. Additional products, such as palatinose (14%) and several unknown compounds, were formed as well. With glucose as the acceptor substrate, the isomaltose yield (47%) was increased 25-fold compared to that of the wild type enzyme. Addition of fructose as the acceptor substrate enhanced the leucrose yield (64%) and palatinose yield (21%) impressively. These data show that (mutant) glucansucrase enzymes have a tremendous potential for technical applications, particularly for production of isomaltose, leucrose, or palatinose oligosaccharides.

Glucansucrase Mutants with Changed Glycosidic Bond Specificity in Polysaccharides Produced. In a second approach, we focused on GTFR variants with altered polymer synthesis. The existing GTFR mutant library was screened visually for different polymer properties. One variant produced a kind of fluffy polymer with reduced solubility in the reaction medium, visible as frayed spots on TLC. This turned out to be a triple mutant variant GTFR (R624G/V630I/D717A), thus modified in its SEV motif (V630I). To characterize this variant in detail, we have generated three single mutants (R624G, V630I, and D717A) and one double mutant (R624G/V630I) by site-directed mutagenesis. Kinetic characterization of the variants (Table 2) revealed only slight changes compared to the wild type. R624G underwent a 4-fold reduction in k_{cat}. The triple mutant showed an increased apparent K_m value for sucrose, but it was still less than 10 mM. In reactions with sucrose and additional acceptor substrates, it was clearly visible that the D717A mutation had virtually no effect on specificity, displaying the same acceptor spectrum as the wild type (Supporting Information). The triple mutants and the double mutants exhibited a slightly increased amount of isomaltose synthesis (Supporting Information).

The amounts of polymer synthesized by the triple and double mutants were reduced to 50−60%, compared to that of the wild type. Polymer synthesis remained at a higher level in the single mutants. All mutants (except D717A) clearly produced enhanced amounts of insoluble polymer, especially the triple and double mutants (Supporting Information). However, not only the triple and double mutants but also both single mutants in the RAHDSEV motif (which lays around the transition state stabilizer) produced the same kind of fluffy polymer. Therefore, these polymers were characterized in more detail. Multiangle laser light diffraction (MALLD) experiments showed that all the polymers had molecular masses comparable to that of the soluble wild type polymer, ranging from 10^4 to 10^5 Da, without a significant change in the size distribution.[2] Because of their low solubility, NMR analysis of the polymers was not possible. GC analysis of methylated polymers revealed that the changes in solubility apparently are caused by a shift in the ratio of α-1,6- and α-1,3-linkages (Table 3).

The wild type polymer possessed predominantly α-1,6-glucosidic linkages (62%), together with approximately 14% α-1,3-glucosidic linkages and 14% α-1,3/6-branching points. The double and triple mutant polymers contained strongly increased amounts of α-1,3-linkages, which shifted from 14% in the wild type to 39 and 46%, respectively.

Also, the polymers synthesized by the single mutants showed increased amounts of α-1,3-linkages, except for the single mutant D717A. The latter exhibited an opposite shift; its polymer contained more α-1,6-glucosidic linkages and fewer α-1,3-glucosidic linkages compared to the wild type dextran polymer (Table 2). Glucans with predominantly α-1,3-linkages (mutans) are known to be less soluble than other types of α-glucans (*47*).

CONCLUSIONS

Our data suggest that the amino acids in the RAHDSEV motif participate in the positioning of acceptor substrates in the glucansucrase active site. The wild type appears to be very stringent in transglycosylation when glucose is present

[2] Sincere thanks are given to Steffen Harling (TU-Braunschweig, Technical Chemistry) for these measurements.

as the acceptor substrate, binding the formed isomaltose with high affinity and strict positioning, using it, as shown for DSRS (*46*), for further chain elongation and α-glucan synthesis. The mutational changes introduced into this motif apparently facilitate nucleophilic attack of the GTFR−Glc intermediate by water and other acceptor substrates, as well as release of products of the acceptor reaction. This is, for instance, the case for the two S628X variants and the R624G/V630I double mutant. The mutations R624G and V630I appear to affect the positioning of glucose polymer chains as acceptor substrates in the active center, explaining the shift in glucosidic linkages synthesized. Pijning et al. (*48*) reported very recently a successfull crystallization approach for the N-terminally truncated glucansucrase GTF180 from *Lactobacillus reuteri* 180; an upcoming structure may help to interpret the data obtained in the future.

Here we have demonstrated that the oligosaccharide product and the glycosidic linkage specificity of the glucansucrase GTFR enzyme can be modified by random mutagenesis, while maintaining a high transglucosylation efficiency, resulting in excellent product yields. Other glucansucrase and fructansucrase enzymes, known to efficiently produce homopolysaccharides with different monosaccharide and linkage compositions (*34*), might be subjected to similar enzyme engineering approaches. They provide very interesting enzyme engineering targets aiming at the development of new biocatalysts that produce tailor-made polymers and oligosaccharides.

SUPPORTING INFORMATION AVAILABLE

Two figures of TLC analysis, two figures of the library screen, one figure of HPAEC analysis and two tables, one of primers and one of the transglycosylation yields. This material is available free of charge via the Internet at http://pubs.acs.org.

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