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Characterization of the glucansucrase GTF180 W1065 mutant enzymes producing polysaccharides and oligosaccharides with altered linkage composition

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ABSTRACT

Exopolysaccharides produced by lactic acid bacteria are extensively used for food applications. Glucansucrase enzymes of lactic acid bacteria use sucrose to catalyze the synthesis of α -glucans with different linkage compositions, size and physico-chemical properties. Crystallographic studies of GTF180- Δ N show that at the acceptor binding sites +1 and +2, residue W1065 provides stacking interactions to the glucosyl moiety. However, the detailed functional roles of W1065 have not been elucidated. We performed random mutagenesis targeting residue W1065 of GTF180- Δ N, resulting in the generation of 10 mutant enzymes that were characterized regarding activity and product specificity. Characterization of mutant enzymes showed that residue W1065 is critical for the activity of GTF180- Δ N. Using sucrose, and sucrose (donor) plus maltose (acceptor) as substrates, the mutant enzymes synthesized polysaccharides and oligosaccharides with changed linkage composition. The stacking interaction of an aromatic residue at position 1065 is essential for polysaccharide synthesis.

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1. Introduction

Microbial exopolysaccharides (EPS) vary in monosaccharide composition, structure and size, hence in physico-chemical properties, which facilitate the development of new innovations of commercial interest for food applications (Badel, Bernardi, & Michaud, 2011; Ryan, Ross, Fitzgerald, Caplice, & Stanton, 2015; Torino, Font de Valdez, & Mozzi, 2015). Lactic acid bacteria (LAB) have been traditionally used as starter cultures in dairy products and other fermented food products, to prolong their preservation and to improve their nutritional values and taste (Leroy & De Vuyst, 2004). Therefore, EPSs from LAB have been widely used in the development of functional food as texturizer, viscosifier and emulsifier (Ryan et al., 2015; Torino et al., 2015; Zannini, Waters, Coffey, & Arendt, 2016). Typically, α -glucan polysaccharides of LAB are

synthesized by the extracellular glucansucrase enzymes produced by the genera *Leuconostoc*, *Streptococcus*, *Lactobacillus*, *Weissella*, and *Oenococcus*, using sucrose as substrate (Leemhuis et al., 2013). Glucansucrase enzymes belong to glycoside hydrolase family 70 (GH70) (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014) and different glucansucrase enzymes synthesize α -glucan polysaccharides with different linkage compositions (i.e. dextran, mutan, alternan and reuteran) (Monsan et al., 2001; Naessens, Cerdobbel, Soetaert, & Vandamme, 2005). In addition, branching sucraes (a special subfamily of GH70 glucansucrase enzymes) are incapable of forming α -glucan polysaccharides in the presence of only sucrose; instead, in the presence of sucrose (donor substrate) and dextran (acceptor substrate), they form either (α 1 $\rightarrow$ 2) or (α 1 $\rightarrow$ 3) branches with a single glucosyl unit on a dextran acceptor substrate (Bozonnet et al., 2002; Vuillemin et al., 2016). All of these α -glucan polysaccharides find a broad application in industry, i.e. as blood plasma expander in medicine, as separation matrix in research, and as bio-thickening agent in food (Ryan et al., 2015; Zannini et al., 2016). In their biological environment, the sticky nature of α -glucan polysaccharides plays a key role in biofilm formation, enabling microorganisms to adhere to surfaces. Mutans produced by *Streptococcus* strains, especially *Streptococcus mutans*, facilitate microorganisms to adhere to teeth

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enamel; they have been recognized as the major pathogenic factor for dental caries (Bowen & Koo, 2011). When efficient acceptor substrates such as short chain oligosaccharides are available, glucansucrase enzymes shift their activity from polysaccharide synthesis to oligosaccharide synthesis (Koepsell et al., 1953). This unique feature of glucansucrase enzyme has been used to produce novel prebiotic oligosaccharides as functional food gradients (Barea-Alvarez, Benito, Olano, Jimeno, & Moreno, 2014; Côté, 2009; Díez-Municio, Herrero, Jimeno, Olano, & Moreno, 2012; Ruiz-Matute et al., 2011; Shi et al., 2016).

The GTF180 glucansucrase of *Lactobacillus reuteri* 180 produces an α -glucan polysaccharide with 69% (α 1 $\rightarrow$ 6) linkages and 31% (α 1 $\rightarrow$ 3) linkages (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008a). Truncation of the N-terminal variable region of GTF180 (GTF180- Δ N, residues 742–1772) had no significant effect on its activity and products from sucrose (Kralj, van Geel-Schutten, Dondorff, et al., 2004). Variations in product specificity are proposed to be determined by relatively small differences in acceptor substrate binding sites (Leemhuis, Pijning, Dobruchowska, Dijkstra, & Dijkhuizen, 2012). The crystal structure of GTF180- Δ N in complex with maltose revealed that several amino acid residues shape the acceptor substrate binding sites +1 and +2 (Vujicic-Žagar et al., 2010). Prominently, residue W1065 has hydrophobic stacking interactions with both the +1 and +2 glucosyl moiety of maltose, providing a binding platform for the acceptor substrate (Vujicic-Žagar et al., 2010). At subsite +1, the glucosyl moiety is sandwiched between W1065 on one side and L938 and L981 on the other side (Fig. 1a) (Vujicic-Žagar et al., 2010). In addition to this hydrophobic stacking interaction, W1065 is involved in a water-mediated hydrogen bond network involving the C4 hydroxyl group of the glucosyl moiety in subsite +1 and the C3 hydroxyl group of the glucosyl moiety in subsite +2 (Vujicic-Žagar et al., 2010). On the other hand, the structure of the inactive mutant GTF180- Δ N D1025N in complex with sucrose reveals that sucrose binds in the –1 and +1 site (Vujicic-Žagar et al., 2010); however, W1065 does not provide hydrophobic stacking interactions, but only is involved in two hydrogen bonds with the fructosyl moiety of the donor substrate. Sequence alignment shows that residue W1065 is located two residues downstream of the general acid/base catalyst in the conserved region III, and is conserved in most GH70 glucansucrase

enzymes (Fig. 1b). However, in GH70 branching sucraes, an aromatic residue is lacking at this position (Brison et al., 2012; Vuillemin et al., 2016). In (α 1 $\rightarrow$ 2) branching sucraes, a glycine is present at the corresponding position (Fig. 1b). DSRE from *L. mesenteroides* NRRL B-1299 is a novel enzyme that synthesizes dextran with a large amount of (α 1 $\rightarrow$ 2) branched linkages. Molecular and biochemical studies showed that this enzyme contains two catalytic domains, CD1 and CD2, catalyzing the synthesis of the glucan main chain with predominantly (α 1 $\rightarrow$ 6) linkages and the formation of (α 1 $\rightarrow$ 2) branched linkages on the (α 1 $\rightarrow$ 6) main chain, respectively (Bozonnet et al., 2002). Crystallographic studies of the DSRE-CD2 of *Leuconostoc citreum* NRRL B-1299 [(α 1 $\rightarrow$ 2) branching sucraes] showed that the space of W1065 is replaced by A2249-G2250 (DSRE numbering) (Brison et al., 2012). In a recent study, BRS-B of *L. citreum* NRRL B-742 and BRS-D of *Leuconostoc fallax* KCTC 3537 were reported to introduce (α 1 $\rightarrow$ 3) branching linkages on the (α 1 $\rightarrow$ 6) main chain (dextran) (Vuillemin et al., 2016). In BRS-B and BRS-D, the W1065 residue is replaced by a proline or a serine (Fig. 1b), respectively (Vuillemin et al., 2016). In order to elucidate the functional role of W1065 in glucansucrase GTF180, we set out to perform random mutagenesis targeting W1065 in GTF180- Δ N, and to characterize the activity and product specificity of mutant enzymes in detail.

2. Materials and methods

2.1. Sequence alignments

Clustal Omega was used to align the amino acid sequences of glucansucrase enzymes: GTF180 (AAU08001.1) of *L. reuteri* 180, DSRS (AAD10952.1) of *Leuconostoc mesenteroides* NRRL B-512F, DSRBCB4 (ABF5832.1) of *L. mesenteroides* B-1299 CB4, DSRWC (ACK38203.1) of *Weissella cibaria* CMU, GTFA (AAU08015.1) of *L. reuteri* 121, GTFO (AAY86923.1) of *L. reuteri* ATCC 55730, GTFML1 (AAU08004.1) of *L. reuteri* ML1; GTFR (BAA95201.1) of *Streptococcus oralis* ATCC10557, smGTFB (AAA88588.1) of *S. mutans* GS 5, smGTFC (BAA26114.1) of *S. mutans* GS 5, smGTFD (AAA26895.1) of *S. mutans* GS 5 and alternansucrase ASR (CAB65910.2) of *L. mesenteroides* NRRL B-1355; branching sucraes: DSRE CD2

Glucansucrase		Conserved region III
GTF180	1058	HINILEDWGWDDP
DSRS	584	HLSELEDWSHNDP
DSRBCB4	563	HLSELEDWSHNDP
DSRWC	614	HLSELEDWSHNDP
GTFA	1056	HINILEDWNHADP
GTFO	1056	HINILEDWNSSDP
GTFR	549	HLSELEAWSNDNDP
smGTFB	484	HLSELEAWSNDNDT
smGTFC	510	HLSELEAWSYNDT
smGTFD	498	HLSELEAWSNDNDP
ASR	668	HLSELEDWNGKDP
Branching sucraes		
DSRE CD2	2243	HISLVEAGLDAGT
BRS-A	705	HLSELEAGLDAGT
BRS-B	704	HISIVEAPKGETP
BRS-C	771	HVSIVEASADQTP

Fig. 1. (a) View of GTF180- Δ N with the acceptor substrate maltose (yellow carbon atoms) bound in subsites +1 and +2 (PDB: 3KLL). Residue W1065 stacks with both glucosyl residues of maltose, and is also involved in a water-mediated hydrogen bond network involving the C4 hydroxyl group of the glucosyl moiety in subsite +1 and the C3 hydroxyl group of the glucosyl moiety in subsite +2. (b) Sequence alignment of conserved region III of GH70 glucansucrase enzymes and GH70 branching sucraes enzymes. The acid/base catalyst is highlighted in blue; residue W1065 and the corresponding residues in different glucansucrase and branching sucraes enzymes are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(CDX66820.1) of *Leuconostoc citreum* NRRL B-1299, BRS-A (CDX66896.1) of *L. citreum* NRRL B-1299, BRS-B (WP_040190490.1) of *L. citreum* NRRL B-742 and BRS-C (WP_010006776.1) of *L. fallax* KCTC3537.

2.2. Thin-layer chromatography (TLC)

TLC analysis was performed on 20 × 20 cm TLC sheets (Merck, Silica Gel 60, F254). Product mixtures (1 µL) from incubations of donor and/or acceptor substrates with glucansucrase enzymes were analyzed by spotting on TLC sheets. The TLC plates were run in 2-butanol:acetic acid:water = 2:1:1 and stained with orcinol/sulfuric acid.

2.3. Construction and selection of GTF180 W1065 variants

2.3.1. Site-directed mutagenesis

The random site-directed mutations were introduced according to previous studies (van Leeuwen et al., 2009). The plasmid pB-GTF180-ΔN (pBluescript SKII with *gtf180*-ΔN insert) constructed in a previous study was used as the template for mutagenesis (van Leeuwen et al., 2009). For random mutagenesis targeting W1065 residue, primers W1065X-FW: ATTTTGGAGATNNSG-GATGGGATGATCCT and W1065X-RV: AGGATCATCCCATCCSN-NATCTTCCAAAAT were used for PCR reactions. The PCR conditions were as follows: 95 °C for 5 min, followed by 19 cycles of denaturing step at 95 °C for 30 s, annealing at 55 °C for 60 s and elongation at 68 °C for 12 min. PCR products were first digested with *DpnI*, followed by transformation into *E. coli* TOP 10 cells. After transformation, all colonies from agar plates were dissolved in 2 mL of sterile Milli-Q water and plasmids were isolated from this pool (GenElute plasmid miniprep kit, Sigma Aldrich). Plasmids were then digested with *NcoI* and *XhoI* and the fragments containing mutations were extracted from the gel. These mutated fragments were inserted into pET15-GTF180-ΔN (also digested with *NcoI* and *XhoI*) constructed in previous studies (van Leeuwen et al., 2009) and then transformed into *E. coli* BL21 DE3 Star.

2.3.2. Enzyme production & activity screening

The colonies from agar plates (~440) were cultured in 96 well plates with 250 µL LB medium at 37 °C. The cells (100 µL) were lysed with 35 µL B-PER Bacterial Protein Extraction Reagent (Thermo Scientific, Bleiswijk, The Netherlands) and the suspensions were used as crude enzyme preparations. Afterwards, 15 µL (1 M NaAC pH 4.5 containing 1 M sucrose and 10 mM CaCl₂) was added and the complete solution (150 µL) was incubated for 2 h at 37 °C. The activity of putative mutant enzymes was measured by determining the amount of reducing sugar formed at OD540 nm with 3,5-dinitrosalicylic acid (DNS) reagent as follows: 50 µL of the enzyme sucrose solution and 50 µL DNS [10 g/L-dinitrosalicylic acid 16 g/L NaOH, 300 g/L potassium-sodium-tartrate] were incubated for 7 min at 95 °C. The OD600 nm of the corresponding culture was used for normalization. In addition, the product profiles of selected mutant enzymes were determined by analyzing 1–3 µL of the “complete solution” on TLC. On the basis of activity and the product spectrum compared to wild-type GTF180-ΔN, different clones were selected for sequencing (GATC Biotech, Konstanz, Germany) and mutations were identified.

2.4. Enzyme expression and purification

Fresh LB media with 100 µg/mL Ampicillin were inoculated with overnight culture of *E. coli* BL21 DE3 Star strains, containing the appropriate expression plasmid with *gtf180*-ΔN and derived mutant genes. Cultures were grown at 37 °C and 200 rpm until

OD600 nm 0.4–0.6 and the expression of recombinant proteins was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Afterwards, cultures were continued overnight at 18 °C and 180 rpm. Cells were collected by centrifugation (10,000×g, 10 min) and washed with 50 mM Tris/HCl buffer, pH 8.0. Proteins were purified as previously described (Kralj, van Geel-Schutten, van der Maarel, & Dijkhuizen, 2004). The concentration of each purified enzyme was measured by absorbance at 280 nm, using a NanoDrop 2000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands).

2.5. Enzyme activity assay

The enzyme assays were routinely performed in 25 mM sodium acetate/1 mM CaCl₂ buffer, pH 4.5. The activity of glucansucrase enzymes was measured with 30–100 nM enzymes and 100 mM sucrose. Samples of 25 µL were withdrawn every min over 5 min from the incubation mixture and inactivated with 2.5 µL 1 M NaOH. Glucansucrase enzyme activity was determined by measuring the amount of released fructose (enzymatically) from sucrose as previously described (Kralj, van Geel-Schutten, van der Maarel, et al., 2004). Briefly, the amount of glucose (Glu) released in the incubation mixture was first determined by measuring the release of NADH at OD340 nm during the conversion of glucose into gluconate-6-phosphate with hexokinase (Roche, Mannheim, Germany) and glucose-6-phosphate dehydrogenase (Roche, Mannheim, Germany). Then the phosphoglucose isomerase (Roche, Mannheim, Germany) was added to convert fructose released in the incubation mixture into glucose, which was further converted to gluconate-6-phosphate with hexokinase and glucose-6-phosphate dehydrogenase. The total amount of NADH released (Total) was measured. The amount of fructose in the incubation mixture was determined by subtracting Glu from Total. One unit (U) of glucansucrase enzyme activity was defined as the release of 1 µmol of fructose per min. *K_m* of mutant enzyme were determined using 12 different sucrose concentrations (ranging from 0.5 to 200 mM) using Michaelis-Menten kinetic equations in Sigma Plot.

2.6. High performance anion-exchange chromatography (HPAEC)

Samples of incubation mixtures were analyzed by HPAEC on a Dionex DX500 workstation (Dionex, Amsterdam, The Netherlands), equipped with an ED40 pulsed amperometric detection (PAD) system. The separation of oligosaccharides was carried out on a Carbo-Pac PA-1 column (250 × 4 mm for analytical runs, 250 × 9 mm for preparative runs; Dionex).

2.7. Product distribution in incubations of wild-type and mutant enzymes with 0.1 M sucrose

Wild-type GTF180-ΔN and derived mutant enzymes (1.0 U/mL) were incubated with 100 mM sucrose at 37 °C in 25 mM sodium acetate/1 mM CaCl₂ buffer, pH 4.5. The depletion of sucrose was determined by TLC analysis. Afterwards, these mixtures were incubated at 100 °C for 10 min to inactivate the enzyme. The reaction mixtures were diluted 500 fold and analyzed by HPAEC using a linear gradient of 25–300 mM sodium acetate in 100 mM NaOH (1 mL/min). The amount of glucose, leucrose and isomaltose was quantified using respective standards. The amount of glucose produced represented the amount of sucrose that had been hydrolyzed. Polysaccharides produced by wild-type GTF180-ΔN and W1065F mutant enzymes were precipitated by adding two volumes of cold ethanol as previously described (Meng, Dobruchowska, Pijning, Gerwig, & Dijkhuizen, 2016). The amount of polysaccharides produced was determined by measuring the

weight of polysaccharides. The percentage of sucrose used for hydrolysis (glucose production) and for the synthesis of leucrose, isomaltose and polysaccharide was calculated in relation to the total amount of sucrose added to the incubation mixture. The percentage of sucrose used for glucan oligosaccharide synthesis was determined by subtracting the summed amount of sucrose for hydrolysis, leucrose, isomaltose and polysaccharide synthesis from 1.

2.8. Oligosaccharide synthesis by incubating sucrose (donor substrate) and maltose (acceptor substrate) with wild-type and mutant enzymes

The wild-type GTF180-ΔN and mutant enzymes (1.0 U/mL) were incubated with 100 mM sucrose and 100 mM maltose at 37 °C in 25 mM sodium acetate/1 mM CaCl₂ buffer, pH 4.5. After the depletion of sucrose, the enzymes were inactivated by incubation at 100 °C for 10 min. Product mixtures were diluted 100-fold and analyzed by HPAEC-PAD using a linear gradient of 25–240 mM sodium acetate in 100 mM NaOH (1 mL/min). The oligosaccharides synthesized by incubating GTF180-ΔN, mutant enzymes W1065K and W1065L with sucrose and maltose were first fractionated on a Bio-Gel P-2 column (50 × 2.5 cm), eluted with 10 mM NH₄HCO₃ at a flow rate of 48 mL/h. Subsequently, the pooled Bio-Gel P-2 fractions were separated by HPAEC-PAD using a linear gradient of 0–300 mM sodium acetate in 100 mM NaOH (3 mL/min) or isocratic conditions of 100 mM sodium acetate in 100 mM NaOH (3 mL/min). Fractions containing products with a single DP were collected and neutralized immediately by adding 4 M acetic acid, and desalted on CarboGraph SPE columns (Alltech, Breda, The Netherlands) using acetonitrile:water (2:3, v/v) as eluent. Isolated oligosaccharide samples were lyophilized and subjected to further structural analysis.

2.9. Structural analysis of polysaccharides and oligosaccharides synthesized by wild-type and mutant enzymes

The linkage distribution present in the polysaccharides produced by GTF180-ΔN and the W1065F mutant enzyme was determined by methylation analysis as previously described (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008a). The molecular mass of isolated oligosaccharides was determined by MALDI-TOF-MS using an Axima™ Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, UK) as previously described (Dobrichowska et al., 2013). One-dimensional ¹H NMR, 2D ¹H–¹H and 2D ¹³C–¹H correlation spectra were recorded on a Varian Inova 500 Spectrometer (NMR Center, University of Groningen) at a probe temperature of 300 K or 310 K. Prior to NMR analysis, samples were prepared by exchanging twice with D₂O (Cambridge Isotope Laboratories, Inc.; Andover, MA) with intermediate lyophilization. ¹H and ¹³C chemical shifts are expressed as ppm with acetone (δ ¹H 2.225; δ ¹³C 31.07) as an internal standard. Suppression of the deuterated water signal (HOD) was achieved by applying a WET1D pulse. The ratio of different linkages was determined by integration of the respective anomeric signal peak areas in ¹H NMR spectra of polysaccharides. 2D ¹H–¹H TOCSY spectra were recorded with MLEV17 mixing sequences with 30, 60, and 150 ms spin-lock times. 2D ¹H–¹H ROESY spectra with a mixing time of 300 ms were recorded in 256 increments of 2000 complex data points with a spectral width of 5000 Hz. 2D ¹³C–¹H HSQC spectra were recorded without decoupling during acquisition of the ¹H free induction decay and with a spectral width of 5000 Hz in the t₂ and 10,000 Hz in the t₁ direction. MestReNova5.3 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process all the record spectra using Whittaker Smoother baseline correction.

3. Results and discussion

3.1. Site-directed mutagenesis of W1065 in GTF180-ΔN, identification and production of mutant enzymes

Mutations targeting W1065 of GTF180-ΔN were introduced by site-directed random mutagenesis as described in Material and Methods. Based on the level of activity and the product spectrum, 22 clones were selected for sequencing to identify the mutations. Among these, all enzymes with wild-type like activity (4 out of 22 clones) had a Trp at position 1065. Sequencing of the other clones resulted in the identification of 10 mutant enzymes (W1065F, W1065K, W1065R, W1065L, W1605N, W1065Q, W1065M, W1065P, W1065E, and W1065G, Table 1). These mutant enzymes covered various classes of amino acid residues; we characterized their activities and product spectra in detail (see below). The wild-type and mutant enzymes were expressed in *E. coli* BL21 DE3 Star and showed comparable expression levels. They were purified to homogeneity as previously described (Kralj, van Geel-Schutten, van der Maarel, et al., 2004).

3.2. Activity and product specificity of mutant enzymes in the presence of 100 mM sucrose

Compared to wild-type GTF180-ΔN, mutant enzymes with a non-aromatic amino acid residue at position 1065 showed a heavily reduced activity (below 6.1 U/mg) when incubated with sucrose, whereas mutant W1065F retained approximately 50% of wild-type activity (Table 1). Mutating W1065 to glycine resulted in an enzyme devoid of any detectable activity. Similar results have been reported for GTFI from *S. mutans* (Tsumori, Minami, & Kuramitsu, 1997), in which W491 (corresponding to W1065 in GTF180) was mutated to either glycine or alanine. Both mutations resulted in an inactive enzyme, and it was concluded that this residue is essential for activity. Our results demonstrate that in GTF180 residue W1065 is important but not essential for the activity of glucansucrase enzymes. The W1065F mutant enzyme retained a higher activity compared to non-aromatic mutant enzymes; this may be explained by the fact that a phenylalanine residue at this position may still provide an aromatic stacking interaction during transglycosylation. It is worth to note that mutant enzymes with either a positively or a negatively charged amino acid residue (K, R or E) at position 1065 retained the lowest activity among the mutant enzymes that were still active. Kinetic analysis of mutants W1065F and W1065L showed increased K_m values (24.9 mM and 16.6 mM, respectively), compared to that of wild-type GTF180-ΔN (5.0 mM). The impaired activity of W1065 mutants may be due to altered or even disabled hydrogen-bond capabilities to the fructosyl moiety, as seen in the GTF180-ΔN D1025N-sucrose complex (Vujicic-Zagar et al., 2010).

The product spectra of wild-type and all mutant enzymes from the sucrose incubations (except W1065G) were first evaluated by thin-layer chromatography (TLC) analysis (Fig. 2a). Wild-type GTF180-ΔN enzyme produced polysaccharides and a range of oligosaccharides from sucrose. Among the mutant enzymes, only W1065F still produced polysaccharide albeit in greatly reduced amounts (2.2%, Table 1) compared to wild-type GTF180-ΔN. All other mutations involving a non-aromatic substitution completely abrogated polysaccharide synthesis and only produced short chain oligosaccharides (DP < 5, mainly leucrose and isomaltose). These results indicate that the stacking interaction of W1065 with the growing acceptor glucan chain is critical for polysaccharide synthesis. The sticky α-glucan polysaccharides produced by oral bacteria (i.e. *S. mutans*) are considered as an important pathogenic factor for dental caries. Thus, specific inhibition of glucansucrase

Table 1

Specific activity and product spectra of GTF180-ΔN and W1065 mutant enzymes incubated with 100 mM sucrose.

Enzyme	Activity (U/mg)	Glucose (%) ^a	Leucrose (%)	Isomaltose (%)	Glucan oligosaccharides (%)	Polysaccharides (%)
GTF180-ΔN	47.7 ± 1.5	24.4 ± 1.7	6.2 ± 0.4	2.9 ± 0.2	50.0 ± 2.5	16.5 ± 1.6
W1065F	22.1 ± 1.2	30.9 ± 1.6	7.1 ± 0.4	5.9 ± 0.3	53.8 ± 1.5	2.2 ± 0.7
W1065K	1.8 ± 0.3	84.0 ± 2.2	2.4 ± 0.4	6.4 ± 0.4	7.3 ± 2.8	–
W1065R	2.1 ± 0.1	65.2 ± 2.7	1.9 ± 0.2	13.3 ± 0.6	19.7 ± 3.2	–
W1065L	6.0 ± 0.2	55.1 ± 3.7	2.6 ± 0.1	12.3 ± 0.3	30.0 ± 3.8	–
W1065N	5.9 ± 0.2	64.3 ± 2.5	2.1 ± 0.2	11.0 ± 0.4	22.6 ± 3.0	–
W1065Q	3.4 ± 0.4	67.0 ± 1.4	4.4 ± 0.3	9.3 ± 0.1	19.3 ± 1.6	–
W1065M	6.1 ± 0.2	54.0 ± 3.5	3.6 ± 0.3	13.4 ± 0.6	29.1 ± 3.2	–
W1065P	3.0 ± 0.1	69.3 ± 1.3	3.2 ± 0.3	9.7 ± 0.5	17.8 ± 1.7	–
W1065E	1.7 ± 0.1	67.0 ± 3.5	3.9 ± 0.4	11.6 ± 0.4	17.5 ± 4.2	–
W1065G	–	–	–	–	–	–

–: No activity or no products detected.

^a The amount of glucose in the product mixtures represents the amount of sucrose being hydrolyzed.

Fig. 2. (a) TLC analysis of products formed by incubation of 100 mM sucrose with GTF180-ΔN and W1065 mutant enzymes. G1–G7 depict the malto-oligosaccharide mixture (glucose to maltoheptaose) used as a standard. m: standard; W, GTF180-ΔN; F, W1065F; K: W1065K; L: W1065L; Q: W1065Q; E: W1065E; M: W1065M; P: W1065P; R: W1065R; N: W1065N. (b) 500-MHz 1D ¹H NMR spectra of the α-glucan polysaccharides formed by GTF180-ΔN and the GTF180-ΔN W1065F mutant enzyme, recorded in D₂O at 300 K. The H-4 signal of terminal residue (t4, between ~δ 3.40 and 3.45), which is an indicator of branched linkages, is indicated.

activity has been proposed as a strategy to prevent dental caries. Importantly, such inhibitors should not affect the activity of human GH13 α-amylases essential for the digestion of our food. In the light of the structural similarities between the GH13 and GH70 family active sites, the search for specific inhibitors is challenging. In view of the important role of W1065 for polysaccharide synthesis, this aromatic residue may be a target for inhibitors aiming to block polysaccharide synthesis of glucansucrases.

High performance anion-exchange chromatography (HPAEC) analysis showed that the level of hydrolysis increased dramatically (to more than 55%) by mutating W1065 to non-aromatic residues (Table 1). The strongest increase was observed with W1065K (84%) while hydrolysis by the W1065F mutant enzyme (30.9%) was only slightly increased compared to that of wild-type GTF180-ΔN (24.4%). Thus, the presence of an aromatic residue at position 1065 is essential for the transglycosylation reaction in GTF180. An aromatic residue at position 1065 facilitates carbohydrate acceptor substrate binding by providing stacking interactions (Vujicic-Žagar et al., 2010), which activates the transglycosylation reaction (induced-fit mechanism) (Leemhuis, Uitdehaag, Rozeboom, Dijkstra, & Dijkhuizen, 2002). The replacement of W1065 with non-aromatic amino acid residues resulted in the loss of stacking interaction with carbohydrate acceptor substrates, explaining the increased hydrolysis seen with the non-aromatic

mutant enzymes. When glucose is available as acceptor substrate, GTF180 catalyzes the synthesis of isomaltose, while leucrose is synthesized when fructose is used as acceptor substrate. The production of leucrose by mutant GTF180-ΔN enzymes (except W1065F) was reduced (Table 1); in contrast, the synthesis of isomaltose was significantly increased in all mutant enzyme incubations (Table 1). These results are explained by a dramatic increase in hydrolysis reaction, resulting in high amounts of glucose in the reaction mixtures, serving as acceptor substrates for isomaltose synthesis. The increased isomaltose synthesis and the decreased leucrose synthesis indicate that GTF180 prefers to use glucose as acceptor substrate compared to fructose. This characteristic of mutant enzymes can be explored for the production of isomalto-oligosaccharides as food ingredients.

3.3. Structural analysis of α-glucan polysaccharide produced wild-type GTF180-ΔN and W1065F mutant enzymes

Integration of the ¹H resonance signals in the NMR spectrum of polysaccharide produced by W1065 mutant enzymes, showed that (α1→6) linkages in the polysaccharide produced by W1065F decreased to 49%, compared to 67% in the wild-type polysaccharides, while the ratio of (α1→3) linkages increased from 33% to 45% (Fig. 2b). It is worth to note that the W1065F mutant

enzyme also introduced a small amount of ($\alpha 1 \rightarrow 4$) linkages (6%), absent in the product of the wild-type enzyme. 2D ^1H - ^1H TOCSY analysis (Fig. S1) revealed that all these ($\alpha 1 \rightarrow 4$) linkages were present in the form of terminal α -D-Glcp(1 $\rightarrow$ 4)-units. The H-4 signal of the terminal residue (Fig. 2b, t4, between $\sim\delta$ 3.40 and 3.45), also showed a significant increase, implying an increased degree of branching in the polysaccharide products (Meng et al., 2016; van Leeuwen, Leeflang, Gerwig, & Kamerling, 2008). Methylation analysis revealed that the polysaccharide produced by wild-type GTF180- ΔN contained 11% Glcp(1 $\rightarrow$, 23% $\rightarrow$ 3)Glcp(1 $\rightarrow$, 54% $\rightarrow$ 6)Glcp(1 $\rightarrow$, and 13% $\rightarrow$ 3,6)Glcp(1 $\rightarrow$ units, while polysaccharides produced by the W1065F mutant enzyme composed of 17% Glcp(1 $\rightarrow$, 30% $\rightarrow$ 3)Glcp(1 $\rightarrow$, 31% $\rightarrow$ 6)Glcp(1 $\rightarrow$, 18% $\rightarrow$ 3,6)Glcp(1 $\rightarrow$ and 4% $\rightarrow$ 4)Glcp(1 $\rightarrow$ units. The higher amount of $\rightarrow$ 3,6)Glcp(1 $\rightarrow$ units confirmed a higher branching degree in the polysaccharides produced by the W1065F mutant enzyme. The size (13 MDa) of the polysaccharide produced by the W1065F mutant was reduced approximately 60% compared to the polysaccharide (30 MDa) produced by the wild-type enzyme. These results show that there are clear structural differences between the polysaccharides synthesized by W1065F mutant and wild-type GTF180- ΔN , prominently illustrated by introduction of ($\alpha 1 \rightarrow 4$) linkages, the increased degree of branching and the reduced size of the mutant product. Thus, the amino acid at position 1065 in GTF180- ΔN not only affects the transglycosylation efficiency, but also is involved in determining the linkage specificity of glucansucrase enzymes. Given the conservation of the tryptophan at this position, its functional role most likely is similar in almost all other glucansucrases, except for branching sucrases. Indeed, branching sucrases, which are mainly hydrolytic in the presence of only sucrose, have a glycine or serine or proline at this position (Fig. 1b) (Brison et al., 2012; Vuillemin et al., 2016). Notably, mutating W1065 in GTF180- ΔN to non-aromatic residues (including proline and glycine) also resulted in a high level of hydrolysis when incubated with only sucrose (except in the W1065G mutant enzyme for which we could not detect any activity) (Table 1). We attempted to detect branching sucrose activity in non-aromatic GTF180- ΔN mutants by incubation with sucrose as donor substrate and dextran (64–76 kDa) as acceptor substrate. However, no branching linkages were observed in the final product mixtures. In a previous study (Brison et al., 2012), the 'reverse' mutation was performed in the ($\alpha 1 \rightarrow 2$)-branching sucrose DSRE CD2; the two residues (A2249-G2250) at the position of W1065 in GTF180- ΔN were mutated individually to tryptophan. None of these mutants was capable of forming polysaccharides in the presence of sucrose, but when dextran was added as acceptor substrate, mutant enzymes were still able to catalyze the formation of ($\alpha 1 \rightarrow 2$) branching linkages (Brison et al., 2012). Together, these results suggest that not only the presence of an aromatic or a non-aromatic residue at position 1065 (GTF180- ΔN numbering) determines whether the enzyme is a 'classical' glucansucrase able to synthesize polysaccharides from sucrose only, or a branching sucrose that needs an additional acceptor substrate (dextran); other amino acid residues must contribute to this specificity as well. Indeed, there are other differences between the acceptor substrate binding sites of DSRE CD2 and GTF180. For example, residues following the transition state stabilizer are located close to the +2 acceptor substrate binding site and have been shown to be important for linkage specificity determination (Côté & Skory, 2014; Hellmuth et al., 2008; Moulis et al., 2006). In GTF180 this region contains the residues SNAQ (1137–1140) while in DSRE CD2, it is KGVQ (2323–2326) (Brison et al., 2012; Vujicic-Zagar et al., 2010). Further studies are needed to pinpoint the structural differences that are responsible for the different specificities of glucansucrase and branching sucrose enzymes.

3.4. Product specificity of mutant enzymes in the presence of 100 mM sucrose (donor substrate) and 100 mM maltose (acceptor substrate)

None of the mutant enzymes (except W1065F) produced a polysaccharide product (Table 1). To further explore the linkage specificity of mutant enzymes, we structurally characterized the oligosaccharides synthesized by incubation of sucrose (donor substrate) and maltose (acceptor substrate) with wild-type GTF180- ΔN and mutant enzymes. The HPAEC profiles of wild-type GTF180- ΔN and different mutant enzymes (except for the inactive W1065G mutant) were classified in 3 groups (Figs. 3 and S2). Group 1 includes the wild-type GTF180- ΔN and mutant W1065F; they produced similar oligosaccharides, only differing slightly in synthesized amounts. Group 2 contains W1065K and W1065R (introducing positively charged residues). The third group is formed by W1065L, W1065N, W1065Q, W1065M, W1065P and W1065E, showing similar product profiles. As a representative for each group, the oligosaccharides synthesized by wild-type GTF180- ΔN , mutant enzymes W1065K and W1065L were isolated and structurally studied by MALDI-TOF-MS and NMR spectroscopy (^1H , COSY, TOCSY, ROESY and ^1H - ^{13}C HSQC). Our earlier developed structural-reporter-group concept for α -D-glucans was used for the assignment of the various NMR signals (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008b). The established structures for the obtained oligosaccharides are depicted in Fig. 3 and the 1D ^1H NMR spectra of compounds 4–13, including some exact chemical shift values, are presented in Fig. 4. The identification of glucose (G), fructose (F), leucrose (1), isomaltose (2) and maltose (3) was achieved by comparing them with respective standards. The ^1H NMR spectra of compounds 4, 5 and 6 were identical to the oligosaccharides characterized in our previous study (Dobrurowska et al., 2013). The NMR data, including ^1H and ^{13}C chemical shifts, of compounds 7–13 can be found in Table 2.

As an example of the detailed interpretation of the ^1H and ^{13}C NMR signals, 2D NMR spectra (TOCSY 150 ms, ROESY 300 ms and HSQC) of compound 12 (Fig. S3) are interpreted in detail. MALDI-TOF-MS analysis of compound 12 gave rise to one $[\text{M}+\text{Na}]^+$ pseudo-molecular ion at m/z 689.07, corresponding with a tetrasaccharide. The ^1H NMR spectrum shows five anomeric proton signals at δ 5.405 (A1), 5.332 (C1), 5.227 (R α 1), 4.955 (B1) and 4.656 (R β 1). The anomeric signals at 5.227 (R α 1) and 4.656 (R β 1) are in agreement with a reducing $-(1\rightarrow 4)\text{-D-Glcp R}$ units (van Leeuwen, Leeflang, et al., 2008). The anomeric signals at δ 5.405 (A1), 5.332 (C1) and 4.955 (B1) reflect the presence of ($\alpha 1 \rightarrow 4$), ($\alpha 1 \rightarrow 3$) and ($\alpha 1 \rightarrow 6$) linkages, respectively (van Leeuwen, Leeflang, et al., 2008). Starting from the anomeric signals in the TOCSY spectra (Fig. S3), all chemical shifts of the non-anomeric protons of the differently substituted glucose residues could be assigned (Table 2). The presence of B H-4 and C H-4 at δ 3.43 reflects the presence of two terminal α -D-Glcp-(1-x)- units, according to the previously developed NMR library data (van Leeuwen, Leeflang, et al., 2008), indicating that a branching point is present in this tetrasaccharide. The chemical shifts of the set of B H-2, H-3, H-4, H-5, H-6a and H-6b at δ 3.56, 3.74, 3.43, 3.71, 3.85 and 3.77, respectively, correspond to that of a terminal α -D-Glcp-(1-6)- unit, while the set of chemical shifts of C H-2, H-3, H-4, H-5, H-6a and H-6b at δ 3.58, 3.74, 3.43, 4.02, 3.84 and 3.78, respectively, are in agreement with a terminal α -D-Glcp-(1-3)-unit (van Leeuwen, Leeflang, et al., 2008). The non-substitution of B and C is further supported by their ^{13}C chemical shifts without downfield shifts (Table 2, deduced from HSQC experiments) as compared to those of α -D-glucose (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008a; van Leeuwen, Leeflang, et al., 2008). The set of chemical shifts of A H-2, H-3, H-4, H-5, H-6a and H-6b are found at δ 3.70, 3.83, 3.75, 3.93, 3.72 and 3.98, respectively. Interpretation of the HSQC spectrum (Fig. S3) revealed that the ^{13}C chemical shifts of A-3 and A-

Fig. 3. HPAEC-PAD (Carbopac PA-1) profiles of the incubation of 100 mM sucrose and 100 mM maltose with GTF180-ΔN, W1065K and W1065L. Established oligosaccharide structures of isolated fractions are shown on the right. G = glucose; F = fructose.

6a/6b both showed downfield shifts to 80.9 and 66.4, respectively, indicating the 3, 6-substitution of **A** (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008a; van Leeuwen, Leeflang, et al., 2008). Taking into account of **A** H-1 at δ 5.405 and the 4-substitution of **R**, **A** was assigned as a $-(1\rightarrow3, 6)-\alpha\text{-D-Glcp}-(1\rightarrow4)\text{-unit}$. In the ROESY spectrum (Fig. S3), inter-residual cross peaks were observed between **B** H-1 and **A** H-6a, between **C** H-1 and **A** H-3, and **A** H-1 and **R** α/β H-4, demonstrating the sequence of **B1** $\rightarrow$ **6**[**C1** $\rightarrow$ **3**]**A1** $\rightarrow$ **4R**. Taken together, compound 12 was characterized as $\alpha\text{-D-Glcp}-(1\rightarrow6)-[\alpha\text{-D-Glcp}-(1\rightarrow3)]\text{-}\alpha\text{-D-Glcp}-(1\rightarrow4)\text{-D-Glcp}$.

Inspecting the HPAEC profiles of the incubation mixtures of wild-type GTF180-ΔN and mutant enzymes revealed that the relative amount of sucrose hydrolyzed by mutant enzymes (except W1065F) was high, in view of the high intensity of the glucose peak (G) in HPAEC profiles (Figs. 3 and S1). Wild-type GTF180-ΔN and mutant W1065F (Group 1) produced relatively minor amounts of glucose from sucrose (Figs. 3 and S1). Maltose was elongated with glucosyl residues from sucrose to produce a range of oligosaccharide products. Minor amounts of leucrose (compound 1) and isomaltose (2) were also observed, suggesting that fructose and glucose were also used as acceptor, similar to the experiments involving only sucrose. Wild-type GTF180-ΔN mainly

catalyzed the successive elongation of maltose with $(\alpha1\rightarrow6)$ linkages at the non-reducing end, resulting in the production of compounds 5 (panose), 7 and 8. Minor amounts of oligosaccharides with $(\alpha1\rightarrow3)$ linkages (compounds 9, 10, 11 and 12) were also produced by wild-type GTF180-ΔN. These results fit with the linkage specificity of GTF180-ΔN regarding polysaccharide synthesis (Kralj, van Geel-Schutten, Dondorff, et al., 2004; van Leeuwen, Kralj, van Geel-Schutten, et al., 2008a). Interestingly, one of the tetrasaccharides was characterized as a branched oligosaccharide (compound 12), indicating that GTF180-ΔN is able to introduce branching linkages in relatively short $\alpha\text{-glucan}$ chains. In addition, two structures (compounds 4 and 6) with a $(1\rightarrow2)$ -linked $\alpha\text{-D-Glcp}$ residue were detected; in the polysaccharides produced by wild-type GTF180-ΔN such $(\alpha1\rightarrow2)$ linkages have not been observed. The same oligosaccharides (compounds 4 and 6) were also synthesized by incubation of sucrose and maltose with GTFA of *L. reuteri* 121 (Dobrichowska et al., 2013), which does not synthesize $(\alpha1\rightarrow2)$ linkage in its polysaccharide either (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008b).

In spite of its high glucose production, W1065K (Group 2) mainly catalyzed the synthesis of compound 9, which is the product of maltose elongation with a $(1\rightarrow3)$ -linked $\alpha\text{-D-Glcp}$ unit.

Fig. 4. ^1H NMR spectra of compounds 4–13 (Fig. 3) isolated from the incubation of 100 mM sucrose and 100 mM maltose with GTF180- ΔN , W1065K and W1065L. The spectra of compounds 4 and 6 were recorded at 310 K to shift the HOD peak up-field and those of the other compounds were recorded at 300 K.

Surprisingly, it was found that compound 9 can be further elongated with a (1 $\rightarrow$ 3)-linked α -D-Glcp unit (compound 13); successive (α 1 $\rightarrow$ 3) linkages are absent in the oligosaccharide and polysaccharide products of wild-type GTF180- ΔN (van Leeuwen, Kralj, van Geel-Schutten, et al., 2008a). In contrast, W1065K produced only very low amounts of compound 5 (panose) with a (α 1 $\rightarrow$ 6) linkage. These results demonstrate that mutant W1065K switched linkage specificity from mainly (α 1 $\rightarrow$ 6) to mainly (α 1 $\rightarrow$ 3) linkage synthesis. This changed linkage specificity of mutant enzymes may be used to produce novel oligosaccharides that hold the potential to have prebiotic effects. The fact that mutant W1065R showed similar profiles to W1065K suggest that the positive charge in these mutants is an important determinant

of the changed linkage specificity, favoring (α 1 $\rightarrow$ 3) linkage synthesis.

The representative of Group 3, mutant W1065L, was still capable of catalyzing the synthesis of large amounts of compound 5 (panose). However, the production of compound 7 was reduced severely and compound 8 was not detected. In contrast, the synthesis of (α 1 $\rightarrow$ 3) linkage-containing oligosaccharides (compounds 9, 10, 11 and 12) increased slightly. These features were observed for other Group 3 mutant enzymes as well (Figs. 3 and S1). These results indicate that Group 3 mutant enzymes retained the ability of elongating maltose with a (α 1 $\rightarrow$ 6) linkage, but could not elongate it further. These mutants therefore are unable to catalyze the synthesis of polysaccharides.

Table 2

^1H and ^{13}C chemical shifts of glucosyl residue in oligosaccharides (compounds 7–13, Fig. 3) synthesized from 100 mM sucrose and 100 mM maltose by incubation with wild-type GTF180-ΔN and the W1065 mutant enzyme.

	7		8		9		10		11		12		13	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
Rα-1	5.225	92.9	5.226	92.9	5.225	92.8	5.226	92.7	5.227	nd	5.227	92.7	5.225	92.8
Rα-2	3.57	72.3	3.57	72.3	3.56	72.5	3.57	72.4	3.57	nd	3.57	72.4	3.57	72.5
Rα-3	3.97	74.1	3.97	74.1	3.98	74.1	3.97	74.0	3.98	nd	3.98	74.0	3.98	74.0
Rα-4	3.64	78.1	3.64	78.1	3.65	77.9	3.66	77.8	3.65	nd	3.65	78.1	3.65	77.8
Rα-5	3.95	70.9	3.95	70.8	3.95	70.9	3.94	70.7	3.94	nd	3.95	70.8	3.95	70.9
Rα-6a	3.87	61.5	3.87	61.4	3.85	61.4	3.87	61.2	3.85	nd	3.86	61.3	3.86	61.3
Rα-6b	3.83		3.83		3.83		3.84		nd		3.83		3.83	
Rβ-1	4.652	96.7	4.652	96.8	4.656	96.7	4.652	96.6	4.653	nd	4.656	96.6	4.653	96.7
Rβ-2	3.27	74.8	3.27	74.8	3.27	74.9	3.27	74.7	3.27	nd	3.27	74.8	3.27	74.9
Rβ-3	3.77	77.1	3.77	77.0	3.78	77.1	3.77	77.0	3.77	nd	3.78	76.9	3.77	77.0
Rβ-4	3.64	78.1	3.64	78.1	3.65	77.9	3.66	77.8	3.64	nd	3.65	78.1	3.65	77.8
Rβ-5	3.61	75.5	3.61	75.5	3.60	75.5	3.61	75.3	3.61	nd	3.62	75.4	3.61	75.5
Rβ-6a	3.94	61.7	3.94	61.6	3.92	61.6	3.93	61.5	3.94	nd	3.93	61.5	3.91	61.5
Rβ-6b	3.79		3.79		3.79		3.77		3.80		3.79		3.79	
A-1	5.405	100.7	5.405	100.6	5.405	100.8	5.416	100.4	5.415	nd	5.405	100.8	5.408	100.9
A-2	3.59	72.5	3.59	72.4	3.68	71.3	3.59	72.4	3.61	nd	3.70	71.1	3.69	71.2
A-3	3.68	74.0	3.68	74.0	3.83	80.4	3.68	73.9	3.68	nd	3.83	80.9	3.84	80.4
A-4	3.49	70.4	3.49	70.4	3.65	70.7	3.51	70.1	3.51	nd	3.75	70.5	3.67	70.7
A-5	3.93	72.1	3.93	72.1	3.74	73.4	3.93	72.1	3.93	nd	3.93	71.9	3.74	73.4
A-6a	3.74	66.8	3.73	66.6	3.86	61.2	3.73	66.5	3.73	nd	3.72	66.4	3.86	61.2
A-6b	3.99		3.99		3.78		3.99		3.99		3.98		3.77	
B-1	4.970	98.9	4.969	98.7	5.358	100.1	4.960	98.9	4.965	nd	4.955	98.9	5.370	100.1
B-2	3.58	72.3	3.58	72.3	3.56	72.5	3.66	70.9	3.66	nd	3.56	72.2	3.67	71.2
B-3	3.71	74.0	3.70	74.0	3.75	73.8	3.87	80.6	3.85	nd	3.74	73.8	3.88	80.7
B-4	3.51	70.4	3.51	70.4	3.43	70.3	3.67	70.8	3.68	nd	3.43	70.2	3.66	70.7
B-5	3.91	71.1	3.91	71.1	4.02	72.7	3.74	72.4	3.74	nd	3.72	72.6	4.04	72.4
B-6a	3.75	66.4	3.77	66.4	3.84	61.3	3.85	61.1	3.85	nd	3.85	61.3	3.85	61.1
B-6b	3.97		3.98		3.78		3.76		3.76		3.77		3.78	
C-1	4.961	98.9	4.961	98.7			5.357	100.1	5.342	nd	5.332	100.0	5.359	100.1
C-2	3.55	72.4	3.55	72.3			3.56	72.4	3.57	nd	3.58	72.4	3.56	72.5
C-3	3.74	74.2	3.74	74.2			3.75	73.7	3.73	nd	3.74	73.8	3.75	73.8
C-4	3.42	70.4	3.42	70.3			3.45	70.1	3.55	nd	3.43	70.2	3.43	70.4
C-5	3.72	72.7	3.74	72.7			4.00	72.4	4.20	nd	4.02	72.6	4.01	72.7
C-6a	3.85	61.4	3.85	61.3			3.83	61.0	4.03	nd	3.84	61.2	3.86	61.2
C-6b	3.78		3.77				3.79		3.71		3.78		3.78	
D-1									4.965	nd				
D-2									3.56	nd				
D-3									3.74	nd				
D-4									3.42	nd				
D-5									3.73	nd				
D-6a									3.84	nd				
D-6b									3.77					

nd: not determined.

4. Conclusion

Residue W1065 of GTF180-ΔN was targeted for random mutagenesis in order to investigate its specific role in the reaction catalyzed by glucanase. Our results suggest that the aromatic stacking interaction provided by W1065 in GTF180 and other glucanases is important for the activity and transglycosylation reaction, but not an absolute requirement. Except for W1065G, all tested mutants at this position retained the ability to synthesize oligosaccharides. Regarding polysaccharide synthesis, the aromatic stacking interaction is an absolute requirement. The importance of residue W1065 was further demonstrated by the effects of its mutation on linkage specificity in polysaccharide and oligosaccharides synthesis, using sucrose, and sucrose (donor) plus maltose (acceptor) as substrates, respectively. Thus, residue W1065 is critical but not essential for the activity of GTF180-ΔN and is also important for the linkage specificity of GTF180-ΔN. The stacking interaction of an aromatic residue at position 1065 is essential for polysaccharide synthesis. The polysaccharides and oligosaccharides produced by mutant enzymes from sucrose is interesting to be explored for food applications.

Conflict of interest

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.08.087>.

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