INTRODUCTION

Lactic acid bacteria (LAB) are known for their extracellular "coats", largely containing α-glucan-type polysaccharides (e.g., dextran, mutan, and reuteran), which are synthesized from sucrose by glucansucrase (GS) enzymes belonging to glycoside hydrolase family 70 (GH70) (http://www.cazy.org1). Recently however, within GH70, α-glucanotransferase (GT) enzyme subfamilies were discovered in LAB and non-LAB that are inactive on sucrose, synthesizing α-glucans from starch-like substrates. To date, only a few GT enzymes have been biochemically characterized;2-12 most of them display α-1,6 transglycosylation specificity, i.e., cleavage of the substrate α-1,4 glycosidic bond (donor half-reaction) followed by formation of an α-1,6 glycosidic bond (acceptor half-reaction). These subfamilies have been described, GtfB, GtfC, and GtfD, differing in bacterial origin, reaction and product specificity, and (predicted) domain organization and displaying variations of key amino acid residues in GH70 homology motifs I−IV.13 These subfamilies also provided important insights into the evolutionary relationships between GH13 α-amylases, which also act on starch substrates,14 and the GH70 glucansucrases (acting on sucrose only), placing the GtfB, -C, and -D subfamilies as structural and functional intermediates.5,7,15 Notably, starch-degrading GH13 enzymes feature an open-substrate binding groove, and we previously hypothesized that the evolution of reaction specificity from α-amylases toward GT and GS enzymes was accompanied by structural changes involving loop architecture around the active site.16

The GtfB-type α-glucanotransferases, exclusively found in LAB, share 45−50% sequence identity with GSs. Several GtfB enzymes have been characterized biochemically,3,5,7,9,12,17 revealing different substrate and product specificities. For example, the well-characterized GtfB from Limosilactobacillus reuteri NCC 2613 (Lr2613 GtfB) preferably converts linear starch-type substrates, e.g., from amyllose, it synthesizes linear isomalto-/malto-polysaccharides (IMMP),16-19 but is hardly active on amylopectin. On the other hand, for two other GtfB enzymes, identified on the basis of amino acid variations in homology motifs II and/or IV, initial characterization suggested that they act on linear (amylose) as well as branched (amylopectin) starch substrates. From amyllose, the GtfB from L. reuteri NCC 2613 (Lr2613 GtfB) synthesizes a branched reuteran-type α-glucan,20 while the GtfB from L. fermentum NCC 2970 (Lf2970 GtfB) remarkably displays a unique α-4,3 transglycosylation specificity.21 The activity of these enzymes on branched substrates has not been studied in detail, while such knowledge is highly relevant for the application of these enzymes for whole starch modification. For example, the GtfB from Streptococcus thermophilus was found to be suitable for converting wheat starch and potato starch.11,12 Together, the different reaction specificities of GTs offer great opportunities to convert starches to different α-glucans, in particular, because introduction of α-1,3 and α-1,6 linkages decreases their digestibility in the upper human gastrointestinal tract. This property makes these α-glucans attractive as low-glycemic ingredients in food applications.20−23
In order to exploit the varying specificities of GTs for α-glucan synthesis using different starch substrates, understanding the GT structure–function relationship is of great value. The accessibility of the substrate binding groove and the architecture of acceptor substrate binding subsites are key factors in determining substrate preference as well as the efficiency and specificity (e.g., α-4,6 vs α-4,3) of the transglycosylation reaction. In this regard, the so far only available experimental 3D structure of a GH70 GT enzyme, a truncated (ΔANV) GtB from \textit{L. reuteri} 121 (Lr121 GtB), provided important initial insights. In its groove, two long loops (loops A1 and B) cover donor subsites -2 and -3, leading to the hypothesis that the resulting tunnel-like feature is the main determinant of the enzyme’s limited ability to process branched starch substrates. Interestingly, sequence alignments showed that the lengths of the A1 and B loops differ in characterized GT enzymes. For example, both in Lr2613 GtB and Lf2970 GtB these loops are much shorter; homology models of these enzymes thus predicted a much more open binding groove, a feature that may determine higher enzyme activity.24,25 Partial deletion of positions containing gaps and missing data;31 the maximum likelihood method based on the JTT matrix model, with partial deletion of positions containing gaps and missing data;31 the bootstrap consensus tree was inferred from 1000 bootstrap replicates. Each entry was annotated with the combined number of residues in loops A1 and B. Deepening our understanding of GT specificity, we describe here the second crystal structure of a GH70 α-4,6-GT from \textit{L. reuteri} NCC 2613 (Lr2613 GtB), along with an improved homology model of the α-4,3-GT from \textit{L. fermentum} NCC 2970 (Lf2970 GtB). Both GtB enzymes feature shorter loops A1 and B and consequently, instead of having a tunnel, feature a much more open binding groove compared to Lr121 GtB; this allowed us to model the binding of branched starch-like substrates. Analysis of the products synthesized from starch substrates with various amounts of branching (amylose and amylopectin) revealed residues that interact with the donor substrate, acceptor substrate, or inhibitor, respectively.24,25 Mutational studies confirmed the contribution of these residues to reaction specificity.13,26 However, for GH70 GTs, no acceptor substrate complexes are available yet, and residues that may affect reaction specificity in these enzymes could only be identified by homology with GSs. Deepening our understanding of GT specificity, we describe here the second crystal structure of a GH70 α-4,6-GT from \textit{L. reuteri} NCC 2613 (Lr2613 GtB), along with an improved homology model of the α-4,3-GT from \textit{L. fermentum} NCC 2970 (Lf2970 GtB). Both GtB enzymes feature shorter loops A1 and B and consequently, instead of having a tunnel, feature a much more open binding groove compared to Lr121 GtB; this allowed us to model the binding of branched starch-like substrates. Analysis of the products synthesized from starch substrates with various amounts of branching (amylose and amylopectin) revealed the importance of difference in loop architecture, in particular of loops A1 and B, of GtB-type α-glucanotransferases for their substrate and product specificities. Sequence analysis of 287 putative GtB enzymes revealed an almost bimodal distribution of loop lengths, with the majority of GtBs (e.g., Lr121 GtB) having long A1 and B loops, while Lr2613 GtB represents a minority with much shorter loops, and Lf2970 GtB has intermediate loop lengths. The structure of Lr2613 GtB in complex with acarbose, occupying acceptor substrate subsites (+1 to +3), allowed the identification of residues that likely contribute to acceptor binding. Interestingly, the type of residue at these specific positions correlated with the length of loops A1 and B. Together, our results suggest that GH70 GtB enzymes with a more open architecture are evolutionary closer to starch-degrading GH13 α-amyloses, while those with a tunneled binding groove have evolved away. This extends our structural knowledge of GH70 GT enzymes regarding their reaction specificity and may accelerate the discovery and application of these enzymes for starch modification aimed at the synthesis of α-glucan food ingredients (with a low glycemic index).
nuclear magnetic resonance (1H NMR) spectroscopy, and methylation analysis as described in the Supporting Information.

**Inhibitory Effect of Acarbose on the Enzyme Activity of Lr2613 GtfB.** The initial total activity of Lr2613 GtfB was assayed by the amylose-iodine staining method as described before.3,4 The reaction mixture contained 0.125% (w/v) amylose V (AVEBE, Foxhol, The Netherlands), 2–100 µg mL⁻¹ (0.02–1.0 nM) of enzyme in 25 mM sodium acetate (pH 5.0) and 1 mM CaCl₂. The decrease in absorbance of the α-glucan-iodine complex resulting from transglycosylation and/or hydrolytic activity was monitored at 660 nm for 8 min at 40 °C. One unit of activity was defined as the amount of enzyme converting 1 mg of substrate per minute. The inhibitory effect of acarbose (Serva Electrophoresis GmbH, Heidelberg, Germany) on the initial total enzyme activity was measured by adding 1.0 mM acarbose to the reaction mixture (the acarbose concentration used did not significantly affect the amylose-iodine staining assay). The initial activity of Lr2613 GtfB on amylose was decreased by 62% in the presence of acarbose. Incubation of the Lr2613 GtfB with acarbose revealed that this enzyme is not able to use acarbose as a substrate (data not shown).

**HPSEC Analysis of Products.** The molecular mass distribution of the product mixtures was determined by high-performance size-exclusion chromatography (HPSEC) with multi-detection as described previously.5,7 The HPSEC system (Agilent Technologies 1260 Infinity) was equipped with a multi-angle laser light scattering detector (SLD 7000 PSS, Mainz), a viscometer (ETA-2010 PSS, Mainz), and a differential refractive index detector (G1362A 1260 RID Agilent Technologies). Separation was performed by using three PFG-SEC columns with porosities of 100, 300, and 4000 Å, coupled with a PFG guard column. DMSO-LiBr (0.05 M) was used as eluent at a flow rate of 0.5 mL min⁻¹. The system was calibrated and validated using a standard pullulan kit (PSS, Mainz, Germany) with Mn ranging from 342 to 708,000 Da. The specific RI increment value dn/dc was also measured by PSS and was 0.072 mL g⁻¹ (private communication with PSS). The multi-angle laser light scattering signal was used to determine the molecular mass of the high molecular mass products (>1 × 10⁵ Da). The specific RI increment value, dn/dc, for these polysaccharides in this system was taken as the same as for pullulan. The molecular mass of the low molecular mass products (<1 × 10⁵ Da) was determined by a universal calibration method. WinGPC Unity software (PSS, Mainz, Germany) was used for data processing.

**NMR Spectroscopy.** One-dimensional 1H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 500 spectrometer (NMR Center, University of Groningen), using D₂O as solvent and at a probe temperature of 298 K. Before the analysis, samples were exchanged twice in D₂O (99.9 atom % D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization and then dissolved in 0.6 mL of D₂O. One-dimensional 500-MHz 1H NMR spectra were recorded at a 4000 Hz spectral width and 16 k complex points, using a WET1D pulse to suppress the HOD signal. All NMR spectra were processed with MestReNova 10.0.2 (Mestrelabs Research SL, Santiago de Compostella, Spain). Manual phase correction was performed and a Whittaker smoother baseline correction was applied. Chemical shifts (δ) were expressed in ppm and calibrated with the internal standard acetone (δ, 2.225 ppm). The percentage of different linkages was estimated by integration of the respective signal peak areas.5,6,33

**Methylation Analysis.** Methylation analysis was performed as described before. Briefly, the carbohydrate samples (~5 mg) were per-methylated using CH₃I and solid NaOH in DMSO and subsequently hydrolyzed with trifluoroacetic acid (2 h, 120 °C). The partially methylated monosaccharides generated were reduced with NaBD₄ (2 h, room temperature, aqueous solution). The solution was neutralized with acetic acid and boric acid was removed by co-evaporation with methanol. The resulting partially methylated alditois were per-acetylated using pyridine:acetic anhydride (1:1 v/v) at 120 °C yielding mixtures of partially methylated alditois acetates (PMAAs). PMAAs were analyzed by gas–liquid chromatography (GLC) coupled to electron impact mass spectrometry (EI-MS) and GLC coupled to flame ionization detection (FID) as previously described.34

**Crystallization and Data Collection.** Crystals of Lr2613 GtfB were obtained in hanging drop vapor diffusion experiments at 293 K, using either streakseeding or macroseeding methods. Drops for streakseeding experiments consisted of equal volumes of protein solution (7 mg mL⁻¹ in 25 mM HAc/NaCl, pH 5.0, 150 mM NaCl, and 1 mM CaCl₂) and reservoir solution (1.4–1.7 M (NH₄)₂SO₄ and 0.1 M Bis-Tris–HCl, pH 5.5) and were equilibrated against reservoir solution. The drops were streakseeded with a cat whisker 22 h after set up, from previously grown crystals. Drops for macroseeding experiments consisted of equal volumes of the same protein solution and 2.7–3.1 M (NH₄)₂SO₄ 75 mM Bis-Tris–HCl, pH 5.5, 25 mM HAc/NaCl, and 150 mM NaCl; they were equilibrated against reservoir solution and macroseeded immediately after set up. Crystals using either method appeared as thin plates and were often intergrown; for data collection, they were stabilized in 1.8 M (NH₄)₂SO₄, 20 mM HAc/NaCl, pH 5.0, and 75 mM NaCl and cryoprotected by including 25% (v/v) glycerol. Acarbose-complexed crystals were obtained by including 20 or 15 mM acarbose (Serva Electrophoresis GmbH, Heidelberg, Germany) in the stabilization and cryoprotectant solutions, respectively. Diffraction data were collected at beamlines P14 (native) and P11 (acarbose complex) of DESY (Hamburg, Germany) and indexed, integrated, and scaled using XDS.35 statistics are given in Table 1.

| Table 1. Crystallographic Data Collection and Refinement Statistics |
|-----------------------------|-----------------------------|
| **parameters** | **native** | **acarbose complex** |
| **PDB entry** | 7P38 | 7P39 |
| **data collection** | | |
| **space group** | P2₁,2₁,2₁ | P2₁,2₁,2₁ |
| **cell dimensions a, b, c (Å)** | 107.4, 134.5, 147.9 | 107.2, 133.8, 147.9 |
| **resolution (Å)** | 47.24–2.70 (2.77–2.70) | 99.21–2.90 (2.98–2.90) |
| **Rmerge** | 0.130 (0.060) | 0.141 (0.551) |
| **I/σ(I)** | 6.1 (1.7) | 3.6 (1.1) |
| **completeness (%)** | 99.9 (99.0) | 98.3 (98.9) |
| **redundancy** | 8.2 (8.3) | 9.1 (9.0) |
| **refinement** | | |
| **resolution (Å)** | 47.24–2.70 (2.77–2.70) | 99.21–2.90 (2.98–2.90) |
| **unique observations** | 56,749 (4107) | 44,476 (3254) |
| **R-factor** | 0.260 (0.299) | 0.293 (0.315) |
| **number of atoms** | | |
| **protein** | 13,001 | 13,022 |
| **Ca²⁺/waters** | 2/96 | 2/19 |
| **carbohydrate ligands** | acarbose | | |
| **other ligand molecules** | | |
| **glycerol (1)** | | |
| **sulfate ion (1)** | | |
| **B-factors** | | |
| **protein (Å²)** | 39.4 (molecule A), 36.9 (molecule B) | 44.5 (molecule A), 42.3 (molecule B) |
| **carbohydrate (Å²)** | 49.1, 46.1 (acarbose) | | |
| **root-mean-square deviations** | | |
| **bond lengths (Å)** | 0.007 | 0.007 |
| **bond angles (°)** | 1.16 | 1.14 |
| **Ramachandran** | 93.5 | 95.3 |
| **allowed (%)** | 5.6 | 3.9 |
| **outliers (%)** | 0.9 | 0.7 |

*In the highest resolution shell.*
Structure Determination and Refinement of Lr2613 GtfB. The crystal structure of the Lr2613 GtfB construct was determined by molecular replacement using PHASER,\textsuperscript{41} a previously obtained homology model\textsuperscript{16} based on the crystal structure of \textit{L. reuteri} 121 GtfB(\textit{∆N\text{AV}})\textsuperscript{30} was used as the search model. The asymmetric unit of the \(P2_1\times 2_1\) cell contains two molecules. Models of the native and acarbose-soaked structures were refined with Refmac\textsuperscript{8} using non-crystallographic symmetry restraints, alternated with inspection of the electron density and manual rebuilding with Coot.\textsuperscript{43} The PDB-REDO server\textsuperscript{39} was used in the final refinement stages. The final refinement statistics and model quality are listed in Table 1. Structural figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) or UCSF Chimera.\textsuperscript{40} Atomic coordinates and structure factors have been deposited at the Protein Data Bank with entries 7F3B (native) and 7F3P (acarbose-bound).

Homology Modeling of Lf2970 GtfB. The one-to-one Phyre2 protocol\textsuperscript{41} was used to construct a homology model of Lf2970 GtfB, with the crystal structure of Lr2613 GtfB as the template. Residues 898–901 (in loop B) and 1114–1116 (in loop A1) were added based on the result from a normal Phyre2 modeling protocol.

Modeling Substrate Binding. To map the substrate binding groove of all three enzymes, first the crystal structure of Lr2613 GtfB in complex with acarbose (subsites +3 to −1) was superimposed with that of the Lr121 GtfB–maltoolentaose complex (subsites −1 to −5) (PDB: 5JBF).\textsuperscript{16} Guided by this superposition, a maltodetraose (G8) was then placed in subites +3 to −5 of Lr2613 GtfB, optimizing the substrate by adjusting glycosidic torsion angles while avoiding clashes with the protein. By superposition, this procedure was repeated for the crystal structure of Lr121 GtfB and the homology model of Lf2970 GtfB. To model a branched substrate, an \(\alpha\,1,6\)-linked glucosyl moiety was added at the sugar unit in subite +1.

RESULTS AND DISCUSSION

Lr2613 GtfB, Lf2970 GtfB, and Lr121 GtfB Have Different Substrate Specificities. To study the substrate specificity of Lr2613 GtfB and Lf2970 GtfB, the product mixtures synthesized from amylase or amylopectin were analyzed regarding linkage and molecular mass distribution and compared with that of the Lr121 GtfB (Table S1). As reported before,\textsuperscript{18} Lr121 GtfB synthesizes a linear IMMP (isomalto/maltopolsaccharides). While the IMMP derived from amylase contained 80% of \(\alpha\,1,6\)-linkages, only 14% were introduced in the amylopectin-derived products, indicating that the presence of \(\alpha\,1,6\) branching points in this substrate limits \(\alpha\,1,6\) transglycosylating activity. In contrast, Lr2613 GtfB synthesized branched reuteran-like products with similar linkage distributions for \(\alpha\,1,4\) and \(\alpha\,1,6\) (79 and 21%, respectively) regardless of the substrate used. Finally, for Lf2970 GtfB, we analyzed the products by methylation analysis, as the anomeric signals of the \(\alpha\,1,3\) and \(\alpha\,1,4\) linked \(\alpha\)-D-Glcp residues partially overlap. In the product mixture synthesized from amylase, terminal, 3-substituted, 4-substituted, 3,4-disubstituted, and 4,6-disubstituted glucopyranose residues are present in molar percentages of 18, 12, 59, 9, and 2%, respectively. In contrast, the relative amounts for the amylopectin-derived products were 22, 2, 69, 1, and 6%, respectively.

HPSEC analysis (Figure S1) of the products synthesized from amylase or amylopectin by the three enzymes supports the linkage distribution analyses. In the case of Lr121 GtfB, the amylose-derived products have a different molecular mass distribution than the amylopectin-derived ones, eluting at a narrow peak at \(\sim26\) mL (corresponding to a low molecular polymer with an average \(M_w\) of \(\sim15 \times 10^3\) Da), or with a broader molecular mass distribution around the same \(M_w\), respectively. This HPSEC profile, together with the low amount of \(\alpha\,1,6\) linkages detected by \(^1\text{H}\) NMR in the amylopectin-derived products (Table S1), fits with previous results reported by Bai et al.\textsuperscript{18} showing that this enzyme was only capable of modifying the linear side chains of amylopectin in wheat starches. In contrast, Lr2613 GtfB products from amylose or amylopectin showed a rather similar molecular mass distribution. The main peak at an elution volume of \(\sim29\) mL corresponds to a low molecular mass polymer with an average \(M_w\) of \(\sim5.6 \times 10^3\) Da, together with a small shoulder peak corresponding to maltose. Finally, for Lf2970 GtfB, the molecular mass distribution of synthesized products clearly depends on the substrate given. From amylase, Lf2970 GtfB gave a bimodal molecular mass distribution containing two main peaks corresponding to a high molecular mass polymer with an average \(M_w\) of \(26 \times 10^3\) Da, and oligosaccharides. From amylopectin, however, the synthesized \(\alpha\)-glucans showed more complex elution patterns because of their high polydispersity indices. This suggests that Lf2970 GtfB efficiently cleaves the \(\alpha\,1,4\) linkages present in amylopectin, while the presence of branching points limits the synthesis of new \(\alpha\,1,3\) linkages leading to polymer formation. As a result, a non-uniform poly-/oligosaccharide mixture is produced from amylopectin.

Crystal Structure of Lr2613 GtfB. The native crystal structure of Lr2613 GtfB was determined at a resolution of 2.9 Å. The asymmetric unit of the Lr2613 GtfB crystals contains two molecules (A and B), comprising residues 446–1277 and 445–1277, which can be superimposed with a root-mean-square difference (rmsd) of 0.11 Å on Ca atoms. Dynamic light scattering analysis clearly indicated a monodisperse solution containing a monomeric protein (apparent molecular mass 101 kDa). Additional weak electron density, likely representing extra N-terminal residues of Lr2613 GtfB, was not sufficient to include these in the model; also, the N-terminal His-tag was not visible in electron density. In the native structure, residues 805–807 of molecule B were omitted because of weak electron density. Superposition with the structure of Lr121 GtfB\textsuperscript{16} gave an rmsd of 0.42 Å (for 574 Ca atoms), reflecting the high sequence identity (79.4% for the segments visible in the structures). In fact, these two GtfB structures are very similar, except for some loop regions (as will be discussed below). Lr2613 GtfB has the GH70-like domain arrangement;\textsuperscript{24,28} the crystal structure comprises the three core domains A, B, and C, and the auxiliary domain IV can thus be considered a \(\Delta\text{N\text{AV}}\)-structure (Figure 1).

Residues preceding the first visible residue (V446), constituting domain V and N-terminal variable domain (N), likely extend away from domain IV. Like in other GH70 enzymes, the catalytic domain A is circularly permuted, such that the order of the four homology motifs is II-III-IV-I, differing from the GH13 I-II-III-IV homology motif order.\textsuperscript{24,28} The catalytic site of Lr2613 GtfB is located at the interface of domains A and B with the three catalytic residues D679 (nucleophile), E717 (acid/base), and D788 (transition-state stabilizing residue) lining a poxet that lies halfway a groove running along the domain interface; four other residues (R677, H787, D1135, and Q1140) that are conserved in GH70 enzymes also suround the conserved subsite −1 (Figure S2).

Of these seven residues, six are also conserved in GH13 \(\alpha\)-amylasses, while the glutamine Q1140 replaces a histidine that is present at the corresponding position in \(\alpha\)-amylasses. Below subite −1 is the side chain of Y1095, known to provide an
aromatic stacking interaction with sugar moieties in this subsite.24 Close to the active site is a conserved Ca²⁺-binding site also found in other GH70 enzyme structures.

Most notably, the Lr2613 GtfB structure shows an open and fully accessible binding groove. In agreement with earlier modeling,6 the short loops A1 (residues 802–811) and B (residues 590–593) do not form a tunnel; details and comparison with other GtfB structures are discussed below (section “GtfB Enzymes Display Varying A1 and B Loop Lengths and Binding Groove Architectures”).

**The Lr2613 GtfB−Acarbose Complex Reveals Accept-or Binding Subsites.** Acarbose is a known inhibitor of GH13 and GH70 enzymes and the inhibitory effect on Lr2613 GtfB activity was confirmed (Supporting Information). Soaking the crystals with acarbose resulted in binding of the pseudotetrasaccharide in subsites −1 to +3 (Figure 2). The valeinamine moiety at the non-reducing end of the inhibitor is bound in subsite −1 where, in case of a natural substrate (e.g., a maltooligosaccharide fragment), the covalent enzyme−glucosyl intermediate is formed in the first half-reaction. The valeinamine moiety has a distorted 2H3 conformation, mimicking the transition state,42 with the non-cleavable N-glycosidic linkage oriented toward the catalytic acid/base residue E717. A total of seven hydrogen bonds to the valeinamine moiety and its N-glycosidic linkage are provided by two of the three catalytic residues (acid/base E717 and D788) as well as the strictly conserved H787. The orientation of this sugar moiety is further stabilized by an aromatic stacking interaction with a

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**Figure 1.** Crystal structure of Lr2613 GtfB-ΔΝΔΥ with the four domains A, B, C, and IV indicated. The active site is at the interface of domains A and B, with the three catalytic residues (D679, E717, and D788) shown in stick representation. The figure is prepared with PyMOL.

**Figure 2.** (a) Stereo view of the Lr2613 GtfB−acarbose complex, with the pseudotetrasaccharide inhibitor occupying donor subsite −1 and acceptor subsites +1 to +3. Residues close to acarbose are shown with side chains (catalytic residues D679, E717, and D788 are indicated with bold labels); hydrogen bond interactions are shown as dotted lines. The figure is prepared with PyMOL. (b) Chemical structure of acarbose.

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conserved tyrosine residue (Y1095). The remaining three sugar moieties occupy subsites +1 to +3 and, for the first time, map these three acceptor substrate subsites in a GH70 GtfB-type enzyme. Surrounding these subsites, there are six residues (apart from the catalytic residues) closer than 4 Å from the bound inhibitor, shaping the binding site and providing a hydrogen bond or aromatic stacking interactions at subsites +1 and +2; they will be discussed below. No interactions were observed at subsite +3, although we cannot exclude that water-mediated hydrogen bonds are present, which are unresolved at 2.9 Å resolution.

**Improved Homology Model of Lf2970 GtfB.** The previously reported homology model of Lf2970 GtfB was based on the crystal structure of glucansucrase Gtf180-ΔN from *L. reuteri* 180 (sequence identity: 38.6% for segments visible in the structure). Because of the much higher sequence identity with Lr2613 GtfB (93.7%), the homology model of Lf2970 GtfB presented here is more accurate. The new model comprises residues 752–1589 of Lf2970 GtfB; with a virtually identical chain trace, superposition with molecule A of the Lr2613 GtfB crystal structure resulted in an rmsd of only 0.13 Å (for 750 Cα atoms). The most notable differences occur in loops A1 and B; how the Lf2970 GtfB binding groove compares to that of the two other GtfB enzymes will be reviewed below.

**GtfB Enzymes Display Varying A1 and B Loop Lengths and Binding Groove Architectures.** Comparing the two crystal structures (Lr2613 GtfB and Lr121 GtfB) and an improved homology model (Lf2970 GtfB) highlights the differences in the architecture of the binding groove and the importance of loops A1 and B; how the Lf2970 GtfB binding groove compares to that of the two other GtfB enzymes will be reviewed below.

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**Figure 3.** (a–c) Comparison of loops A1 (purple), A2 (red), and B (brown) in the crystal structure of Lr2613 GtfB (this study), the homology model of Lf2970 GtfB (this study), and the crystal structure of Lr121 GtfB. The three loops are labeled with their lengths; in the first two GtfBs, amino acid residues at the tip of loops A1 and B are indicated. The catalytic residues (D679, E717, and D788) are shown in stick representation (lower left). In Lr2613 GtfB and Lf2970 GtfB, loops A1 and B are relatively short, leading to a (half-)open binding groove architecture; in contrast, in Lr121 GtfB the corresponding segments are long and cover donor subsites. The figure is prepared with PyMOL.

**Figure 4.** Sequence alignment of regions in Lr2613 GtfB, Lf2970 GtfB, and Lr121 GtfB in domains A (blue header) and B (green header) in which residues closer than 4 Å to a modeled substrate are shown below the alignment with the corresponding subsite. The six acceptor subsite residues discussed in the text are highlighted in yellow. The three catalytic residues (D679, E717, and D788) are indicated with a black triangle (NU = nucleophile, A/B = general acid/base, TS = transition-state stabilizing residue). Color coding of the loops B, A1, and A2 corresponds to the scheme used in Figure 3. The figure is prepared using the ESPript 3.0 server (https://espirpt.ibcp.fr).
residues, respectively (Figures 3a and 4). As a result, and in stark contrast to Lr121 GtfB (Figure 3c), loop B hardly protrudes from the surface; in fact, only a non-conserved residue (Y592) does so. Also loop A1 (residues H802–Y810) protrudes considerably less from the surface than in Lr121 GtfB.

As a result, and as was previously predicted from sequence alignment and homology modeling, the crystal structure of Lr2613 GtfB now clearly shows that the shorter loops A1 and B do not form a tunnel (the hydroxyl groups of residues Y806 and Y592 at their tips are about 9 Å apart); this creates a much more open architecture. It has to be noted that the electron density for residues K800–A811 is weak and/or ambiguous in both molecules, suggesting possible flexibility of loop A1. Nevertheless, the active site groove of Lr2613 GtfB is fully accessible, reminiscent of the situation in GH13 starch-degrading α-amylases. In Lf2970 GtfB, loops A1 and B are somewhat longer than in Lr2613 GtfB (by three and four residues, respectively) and loop B protrudes more from the surface (Figure 3b). Still, the residues at the tips (S900 and G1115) are at such a distance that Lf2970 GtfB displays a (half-)open architecture.

In order to extend our analysis of loop length beyond these three enzymes to the entire GtfB subfamily, we performed a BLAST search with the core domains (A, B, and C) of Lr121 GtfB. This yielded 287 putative GtfB enzyme sequences (Table S2), including the three enzymes described in our study. The sequence identity of the hits ranged from 99.9 to 44.2%. Given the importance of loops A1 and B in determining the accessibility of the substrate binding groove (especially near donor subsites) and knowing that in homology motif IV, the fourth residue after the transition-state stabilizing aspartate (D788), and almost fully conserved in homology motif V, the third residue after the pseudo-nonasaccharide (PDB: 6BS644), we modeled a branch unit in the crystal structure of Lr2613 GtfB di-1,4-linked maltooligosaccharide segment showing that the branch unit may interact with residue N792 in homology motif IV, the fourth residue after the transition-state stabilizing aspartate (D788), and almost fully conserved in the GtfB subfamily (Figure 7). In SusG, such an interaction is indeed experimentally observed with the corresponding residue K541. The orientation of the branch unit is such that the branch can be extended via α-1,4 linkages without sterical hindrance. In Lf2970 GtfB, the situation is very similar since

The most common length for loop A1 is 17 residues (78.4% of cases), while most of the other sequences feature a much shorter loop A1. The length of loop B varies between 4 and 20 residues, again with the majority (80.1% of cases) having the long version. The distribution of total loop length (A1 + B) is similar, with two distinct groups: 78.4% (225 sequences) of the enzymes feature long loops (37 or 40 residues combined), while most of the remaining ones have shorter loops (17.8% have a combined length of 12–14 residues). The three enzymes that we compared structurally represent GtfBs with (almost) the shortest (Lr2613 GtfB: 14 residues), intermediate (Lf2970 GtfB: 21 residues), and (almost) the longest combined loop lengths (Lr121 GtfB: 37 residues). The small subset with intermediate loop lengths includes S. thermophilus GtfB (combined loop length of 21 residues). A phylogenetic tree (Figure S3) constructed from the 287 putative GtfB sequences, annotated with the total loop length (A1 + B), revealed that those with short loops cluster together in two opposite segments, one mostly containing Lactiplantibacillus plantarum or Fructilactobacillus sanfranciscensis species, and the other mainly containing Limosilactobacillus reuteri and other species (e.g., Limosilactobacillus fermentum).

**Modelling Substrate Binding.** The first step of the reaction catalyzed by GTs is donor substrate binding; we combined structural data from two GtfB enzymes to gain more insights into the molecular details. Superposition of the Lr2613 GtfB–α-acarbose complex with the Lr121 GtfB–maltpentaose complex (PDB: 5JBF46) showed that the (pseudo)sugar units in subsite −1 have a similar orientation and almost all hydrogen bond interactions are conserved. This allowed us to construct a model of maltoctaose (G8) occupying subsites −5 to +3 in Lr2613 GtfB (Figure 6b); only minor adjustments of glycosidic torsion angles were required to fit the oligosaccharide substrate in the binding groove. Similarly, we modeled maltoctaose in the homology model of Lf2970 GtfB (Figure 6c) and in the crystal structure of Lr121 GtfB (Figure 6d), visualizing how linear α-1,4-linked maltooligosaccharide segments of starch substrates can be bound. Comparing our models with the crystal structure of the α-amylase from Bacillus halmapalus complexed with a pseudo-nonasaccharide (PDB: 1W9X)43 (Figure 6a) showed that the global binding mode of a maltooligosaccharide substrate in GH70 GtfBs is similar to that observed in this α-amylase.

After modeling a bound linear donor substrate in GtfB-type proteins, we investigated the possibility to model α-1,4,6 branched ones. As described above, the open architecture of the binding groove of Lr2613 GtfB differs considerably from that of the tunneled one observed in Lr121 GtfB. The (linear) G8 models show that the positions of the O6 atoms of the glucosyl moieties in several subsites are such that a branched structure would fit without sterical hindrance in Lr2613 GtfB but not in Lr121 GtfB. Therefore, analogous to the observed binding mode of a branched oligosaccharide in the GH13 pullulanase SusG (PDB: 6BS644), we modeled a branched glucosyl unit linked to the O6 at subsite +1 (Figure S4), showing that the branch unit may interact with residue N792 in homology motif IV, the fourth residue after the transition-state stabilizing aspartate (D788), and almost fully conserved in the GtfB subfamily (Figure 7). In SusG, such an interaction is indeed experimentally observed with the corresponding residue K541. The orientation of the branch unit is such that the branch can be extended via α-1,4 linkages without sterical hindrance. In Lf2970 GtfB, the situation is very similar since
the only main differences (somewhat longer loops A1 and B) are far from subsite +1. In contrast, as is clear from Figure S4, in Lr2613 GtfB, residues in the region S918−D922 of loop B would clash with branch sugar units in subsite +1′, hindering the binding of branched starch substrates.

Acceptor Subsite Residues Correlate with Loop Lengths. In addition to the architecture of the binding groove, residues surrounding acceptor subsites are of importance for the specificity of the transglycosylation reaction, since they determine the orientation of bound acceptor molecules after formation of the covalent glycosyl−enzyme intermediate. The Lr2613 GtfB−acarbose complex as well as the modeled G8 substrate complex in all three enzymes show that at subsites +1 to +3, there are six residues (apart from the catalytic residues) that are at less than 4 Å distance from the sugar moieties (Figures 2 and 4). Four of these residues are from homology motifs II, III, and IV: residues H683, R720, and N792 provide hydrogen bond interactions with hydroxyl groups of (pseudo)sugar moieties in subsites +1, +1′ (in the case of a branched substrate), and +2, while Y719 in motif III provides an aromatic stacking interaction at subsite +2. Finally, residues from the 630’s loop in domain B, especially Y632 and L635, do not directly interact with the inhibitor but they help shape the binding site. Analysis of the conservation of these six residues in 287 putative GtfB sequences revealed that those corresponding to positions 635, 719, and 792 (Lr2613 GtfB numbering) are almost fully conserved, while the others show strong preference for a certain residue type, remarkably correlating with the loop length (Figure 7). Sequences with long loops A1 and B almost invariably have P, L, N, Y, H, and N (e.g., Lr121 GtfB is representative of this group), while in sequences with short or intermediate loop lengths, the first, third, and fifth residues are often Y, H, and R/N, respectively. The residues at these latter positions (632, 683, and 720 in Lr2613 GtfB) cluster together near subsites +1 and +2, and one may speculate that a different set of residues (with different hydrogen bond capabilities) is needed to process starch(-like) substrates in a more open

Figure 6. (b−d) Modeled maltooctaose shown as spheres in subsites +3 to −5 of Lr2613 GtfB, Lf2970 GtfB, and Lr121 GtfB, compared to (a) the structure of B. halmapalus α-amylase with a bound nonasaccharide.43 With shorter loops A1 and B, Lr2613 GtfB and Lf2970 GtfB feature a more open architecture like in the α-amylase, compared to the tunneled one in Lr121 GtfB. The figure is prepared with UCSF ChimeraX.40

Figure 7. Sequence conservation of six residues near acceptor subsites +1 and +2 in 287 putative GH70 GtfB enzymes and GH13 α-amylases. The GtfB enzymes are divided in 57 sequences with short or intermediate combined loop lengths (12−21 residues) and 230 sequences with long lengths (31−40 residues). In α-amylases, the six corresponding residues are fully conserved. Below the logos, the residues present in B. licheniformis α-amylase, Lr2613 GtfB, Lf2970 GtfB, and Lr121 GtfB are shown; the three residues in Lf2970 GtfB that are unique are indicated with an asterisk. The figure is based on use of the WebLogo server.29
binding groove. In fact, a superposition with bacterial α-amylase structures (not shown) revealed that the corresponding residues are invariably Y, H, and K. Together, these observations suggest that the GH70 GtfB subgroup with short loops are evolutionary closer to GH13 α-amylases, while the large subgroup with long loops have evolved further away. The GtfB subgroup with open architecture (short loops A1 and B) therefore provides an opportunity to study how they gained transglycosylation capabilities (most α-amylases are hydrolytic).

The six aforementioned residues likely also contribute to transglycosylation linkage specificity. Most of the GtfB enzymes characterized so far display α-4,6 specificity, but Lf2970 GtfB is the only known GT with α-4,3 transglycosylation specificity. Notably, within our set of 287 putative GtfB sequences, Lf2970 GtfB has unique substitutions at the second, third, and fifth positions (V943, D991, and G1028) (Figure 7), one may speculate that they play a key role in determining the orientation of acceptor substrates and thus the type of glycosidic linkage in the product. Preliminary modeling of acceptor substrates complexes in Lr2613 GtfB and Lf2970 GtfB so far did not give conclusive insight into this regard nor did single mutations targeting all six positions in Lr2613 GtfB affect the transglycosylation linkage specificity (personal communication). Possibly, simultaneous mutation of several residues is needed to achieve such changes.

In addition to transglycosylation linkage specificity, the acceptor subsite residues may also play a role in determining the branching characteristics of the products. An interesting comparison in this regard is the pair of GtfB-type enzymes, GtfY and GtfX from Ligilactobacillus aviarius DSM 20655 of which ΔNAC-constructs were characterized by Meng et al. While GtfY was shown to synthesize linear (IMMP) products from amylose V, the GtfX product is branched. The recent findings that Streptococcus thermophilus GtfB, with loop lengths similar to Lf2970 GtfB, can be used in potato starch and wheat starch conversion but single mutations targeting all six positions in Lr2613 GtfB (short loops) and Lr121 GtfB (long loops), the latter synthesizes higher molecular mass products from the same substrate (Figure S1). In any case, the quest for GH70 α-glucanotransferases with broad starch substrate specificity should focus on enzymes with shorter A1 and B loops. 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Evelien M. te Poele — Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Groningen 9747 AG, The Netherlands; CarbExplore Research B.V., Groningen 9747 AA, The Netherlands

Tim Börner — Nestlé Research, Société des Produits Nestlé SA, 1000 Lausanne, Switzerland; orcid.org/0000-0003-1120-225X

Lubboert Dijkhuizen — Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Groningen 9747 AG, The Netherlands; CarbExplore Research B.V., Groningen 9747 AA, The Netherlands

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c05657

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