4,6-alpha-Glucanotransferase activity occurs more widespread in Lactobacillus strains and constitutes a separate GH70 subfamily

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Structural characterization of linear isomalto-/malto-oligomer products synthesized by the novel GTFB 4,6-α-glucanotransferase enzyme from *Lactobacillus reuteri* 121

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Recently, a novel glucansucrase (GS)-like gene (*gtfB*) was isolated from the probiotic bacterium *Lactobacillus reuteri* 121 and expressed in *Escherichia coli*. The purified recombinant GTFB enzyme was characterized and turned out to be inactive with sucrose, the natural GS substrate. Instead, GTFB acted on malto-oligosaccharides (MOSs), thereby yielding elongated gluco-oligomers/polymers containing besides (α1 → 4) also (α1 → 6) glycosidic linkages, and it was classified as a 4,6-α-glucanotransferase. To gain more insight into its reaction specificity, incubations of the GTFB enzyme with a series of MOSs and their corresponding alditols [degree of polymerization, DP2(-ol)–DP7(-ol)] were carried out, and (purified) products were structurally analyzed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry and one-/two-dimensional ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. With each of the tested malto-oligomers, the GTFB enzyme yielded series of novel linear isomalto-/malto-oligomers, in the case of DP7 up to DP >35.

Keywords: α-D-glucans / glucansucrase / GTFB 4,6-α-glucanotransferase / *Lactobacillus reuteri* / structural analysis

Introduction

Lactic acid bacteria (LAB), including *Lactobacillus* species, produce exopolysaccharides (EPSs), which are used as ingredients in the food and dairy industry because of their beneficial physico-chemical properties (e.g. viscosifying, stabilizing, emulsifying, sweetening, gelling or water-binding agents) as well as their prebiotic properties. LAB employ extracellular glucansucrases (GSs)/glucosyltransferases (GTFs; EC 2.4.1.5) to convert their natural substrate sucrose for the synthesis of EPSs, being complex α-D-glucose polymers. Several LAB strains possess multiple GTF enzymes.

When searching for novel carbohydrate-modifying enzymes, which may be used in industrial applications, we have isolated several *gtf* genes (e.g. *gtfA*, *gtf180*, *gtfML1*, *gtfML4*, *gtfO*) from different *Lactobacillus reuteri* strains and investigated the corresponding enzymes for their reaction specificity and activity (Kralj, van Geel-Schutten, Dondorff, et al. 2004). Focusing on the *gtfA* gene from the probiotic bacterium *L. reuteri* 121, it has been reported that the corresponding GS GTFA (reuteransucrase) enzyme converts sucrose into oligosaccharides and polysaccharides consisting of D-glucose residues connected via (α1 → 4) glycosidic linkages, together with (α1 → 6) and (α1 → 4,6) linkages (Kralj, van Geel-Schutten, van der Maarel, et al. 2004; Van Leeuwen, Kralj, van Geel-Schutten, et al. 2008). Mutational experiments on GTFA residues located near the catalytic Asp1133 (putative transition-state-stabilizing residue) have shown that specific amino acid changes give rise to changes in the glycosidic linkage patterns of the formed products (Kralj et al. 2008). For instance, Asn1134, present in the N terminus of the catalytic domain, is a main determinant of the glycosidic-bond product specificity and the hydrolysis/transglycosylation activity ratio (Kralj et al. 2006).

Recently, we have shown that upstream of the *gtfA* gene of *L. reuteri* 121 another putative GS gene is located, designated *gtfB*, encoding the GTFB enzyme, having 45% identity and 65% amino acid similarity with GTFA. However, after cloning and expression of the *gtfB* gene in *Escherichia coli*, the purified recombinant GTFB enzyme turned out to be inactive with sucrose but displayed clear hydrolase/transglycosylase activity on malto-oligosaccharides (MOSs). Interestingly, the formed elongated linear gluco-oligomers contained besides (α1 → 4) also (α1 → 6) glycosidic linkages. This is the first example of such a 4,6-α-glucanotransferase enzyme activity in the GH70 family (Kralj et al. 2011).

To get more insights into the properties/activity of the novel recombinant GTFB enzyme, we report here the results of a structural analysis of the products of purified GTFB,

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incubated with a series of MOSs (degree of polymerization, DP2–DP7) and their corresponding alditols (DP, DP2-ol–DP7-ol), as deduced from high-pH anion-exchange chromatography (HPAEC), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and one-/two-dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (^{1}H-^{1}H TOCSY, total correlation spectroscopy; ^{1}H-^{13}C HSQC, ^{1}H-detected heteronuclear single-quantum coherence spectroscopy; ^{1}H-^{1}H ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy).

Results and discussion

General

Solutions of 100 mM MOSs(-alditols) [DP2(-ol)–DP7(-ol)], which are linear saccharides containing only (α1 → 4) linkages, were individually incubated with 500 nM recombinant GTFB for 72 h at 37°C and pH 4.7, and the product mixtures were analyzed by thin-layer chromatography (TLC; Supplementary data, Figure S1). The evaluation of both free oligosaccharides and their corresponding alditols will generate information about the substrate specificity in terms of elongation at the non-reducing end vs the reducing end and in terms of influence of the reduced form on the product outcome. The GTFB enzyme demonstrated both hydrolysis and transglycosylase activity by the appearance of lower- and higher-molecular-mass products than the substrate oligosaccharides(-alditols), including polysaccharide products. Activity was already observed with maltose (DP2), maltotriose (DP3) and maltotriitol (DP3-ol), but the highest activity, in terms of yield of newly formed products, was seen with the DP6(-ol) and DP7(-ol) substrates. It should be noted that recombinant GTFB was inactive on DP5 and DP6 isomalto-oligosaccharides (IMOs), which are linear saccharides containing only (α1 → 6) linkages (Kralj et al. 2011). In order to get detailed information about the structures of the formed products, preparative-scale incubations were performed with maltose, maltotriose, maltoheptaose and maltopentaitol. Furthermore, to get insight into the product formation in the progress of time, incubations were carried out with maltose and maltotriose.

With respect to the use of NMR spectroscopy in the structural analysis of the different carbohydrate products, it should be noted that the various NMR assignments were made on guidance of an earlier developed ^{1}H NMR structural-reporter-group concept for the analysis of α-D-glucans (Van Leeuwen, Kralj, van Geel-Schutten, et al. 2008; Van Leeuwen, Kralj, Gerwig, et al. 2008; Van Leeuwen, Leeflang, et al. 2008; Van Leeuwen et al. 2009, and references cited therein; see also Irague et al. 2011) and checked by the ^{1}H NMR CASPER database (http:www.casper.organ.su.se/casper).

Incubation of maltose and maltotriose with the recombinant GTFB enzyme

Maltose (DP2) and maltotriose (DP3) (50 mM) were incubated with 250 nM GTFB at 37°C and pH 4.7. The obtained mixtures of products were analyzed by MALDI-TOF MS, revealing a series of compounds ranging from DP2 to DP15 ($[M + Na]^{+}$, *m/z* 365–2471) for the maltose incubation and ranging from DP2 to DP20 ($[M + Na]^{+}$, *m/z* 365–3281) for the maltotriose incubation. According to the intensities of the observed sodiated molecular ions, in both cases the amounts of the compounds of DP > 10 were very low. By using HPAEC combined with pulsed amperometric detection (PAD) on CarboPac PA-1, 8 fractions for the maltose incubation (Figure 1A) and 12 fractions for the maltotriose incubation (Figure 2A) could be isolated. All fractions were analyzed by MALDI-TOF MS

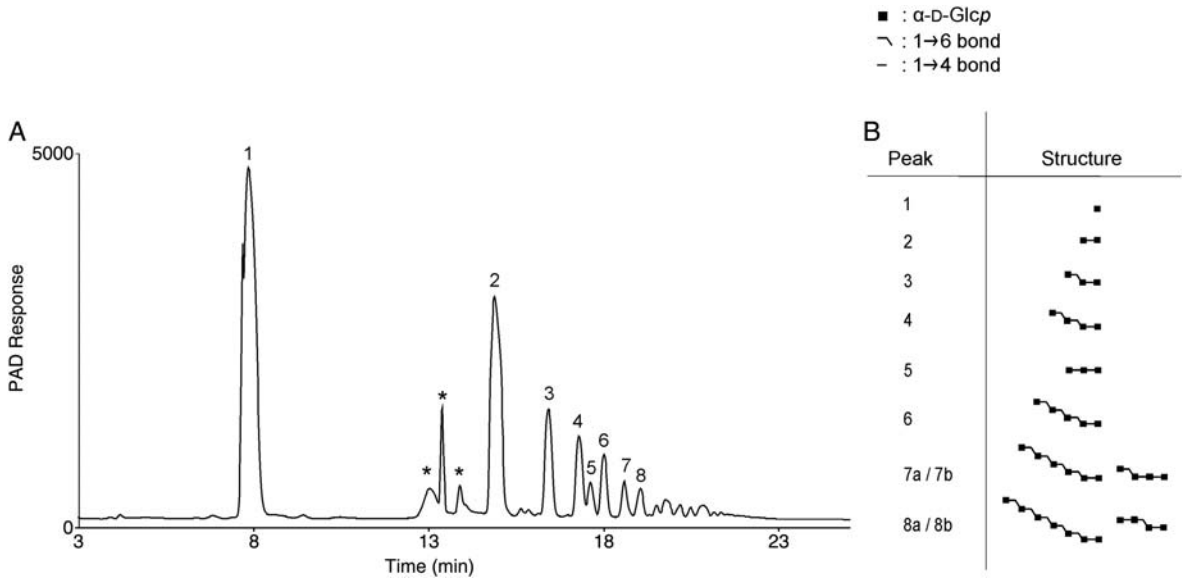

Fig. 1. The HPAEC-PAD profile (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 (250 × 9 mm) of the product mixture obtained from the incubation of maltose (**A**) with GTFB after 72 h at 37°C and pH 4.7. Asterisks denote the non-carbohydrate contamination. Established oligosaccharide structures for isolated fractions are included (**B**). Note that the reducing Glc units occur as the α/β mixture.

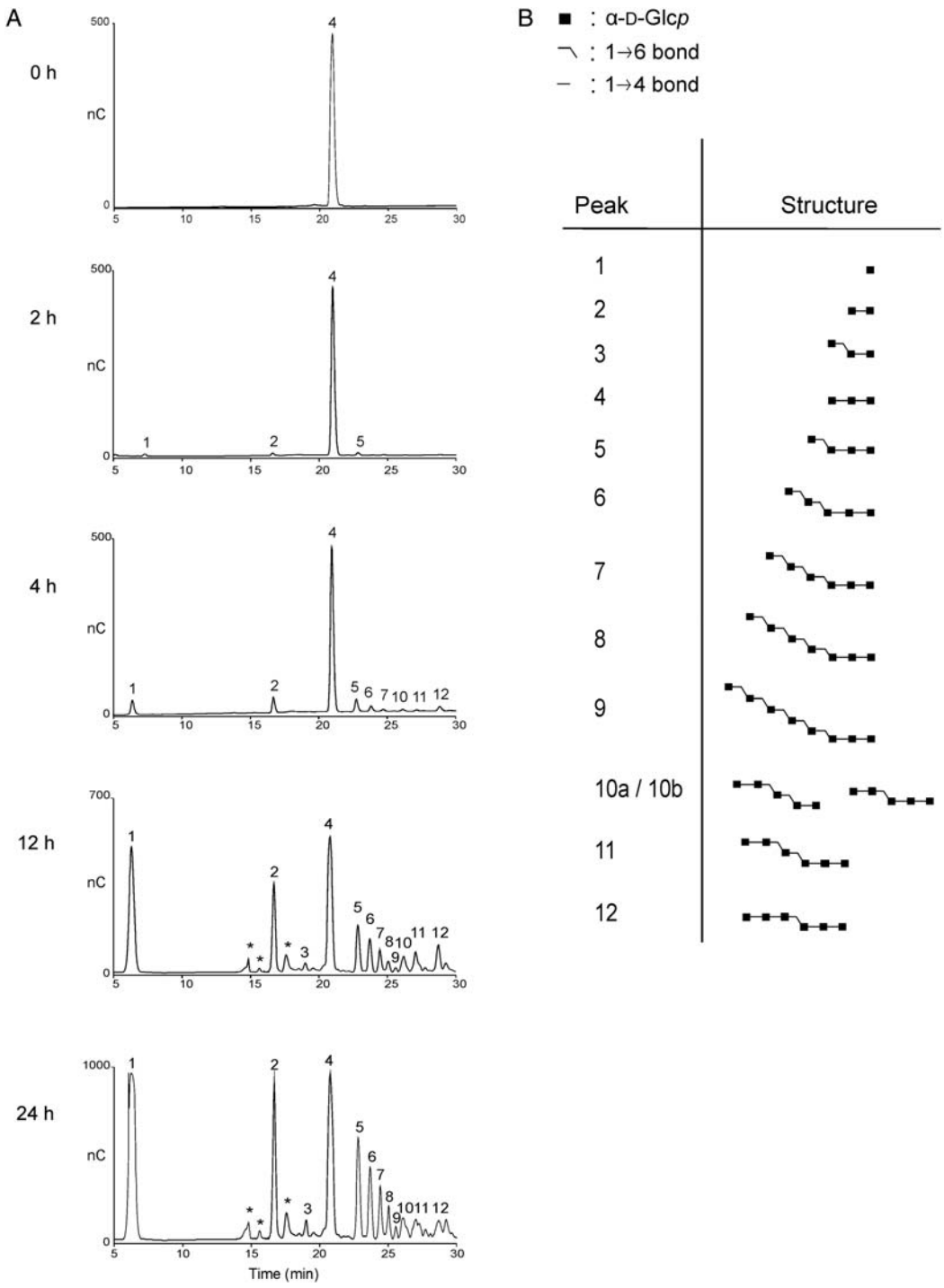

Fig. 2. HPAEC-PAD profiles (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 (250 × 4 mm) of the product mixtures obtained from the incubation of maltotriose (**A**) with GTFB after 0–24 h at 37°C and pH 4.7. Established oligosaccharide structures for isolated fractions are included (**B**). Note that the reducing Glc units occur as the α/β mixture.

and one-dimensional ^{1}H NMR spectroscopy, and the major fractions also by two-dimensional NMR spectroscopy (TOCSY, HSQC and ROESY). The established structures are included in Figures 1B and 2B, and most of their one-dimensional ^{1}H NMR spectra are depicted in Supplementary data, Figure S2. According to the ^{1}H chemical shifts of the anomeric signals around δ 5.39 and 4.96, the presence of (α1 → 4) and (α1 → 6) linkages, respectively, is indicated. More details in terms of -(1 → 4)-α-D-Glc*p*-(1 → 4)- (**A**), α-D-Glc*p*-(1 → 4)- (**B**), -(1 → 6)-α-D-Glc*p*-(1 → 4)- (**C**),

-(1 → 6)-α-D-Glc*p*-(1 → 6)- (**D**), -(1 → 4)-α-D-Glc*p*-(1 → 6)- (**E**), α-D-Glc*p*-(1 → 6)- (**F**) and -(1 → 4)-D-Glc*p* (**R**) units, revealing that only linear glucose sequences were present, were collected from the two-dimensional NMR measurements. Table I summarizes the NMR data of the differently substituted glucose residues. The rationalization behind the various assignments will be worked out in more detail for a DP8 product oligosaccharide of the maltoheptaose incubation (see below). Comparison of the structures found for the maltose and maltotriose incubations (Figures 1B and 2B) showed that both substrates produced similar kinds of products. The larger the structures, the more (α1 → 6) linkages were present. However, also a minor amount of (α1 → 4) elongations was observed. Some fractions contained more than one compound, having different structures. It should be noted that for isomeric compounds, an increase in (α1 → 6) linkages instead of (α1 → 4) linkages reduces the HPAEC retention time on CarboPac PA-1. This can lead to separation problems with complicated mixtures, giving an overlap of products with different/same molecular masses and different structures.

In order to get information about the formation of products in the progress of time, both maltose and maltotriose were incubated with GTFB, and samples for HPAEC analysis were taken after 2, 4, 12 and 24 h of incubation. The formed products were identified by MALDI-TOF MS and, after isolation, by ^{1}H NMR spectroscopy. For maltose (DP2), the reaction products detected after 2 h were glucose and panose (DP3). Later in time, larger linear oligosaccharides (DP4–DP7), wherein maltose is mainly elongated with (α1 → 6) and less with (α1 → 4) linkages, were formed. When maltotriose (DP3) was used as a substrate (Figure 2), the reaction products detected after 2 h were glucose, maltose (DP2) and maltotriose elongated with one (α1 → 6)-linked glucose residue at the non-reducing site (DP4). After 4 h, also larger oligosaccharides (DP5 and DP6), elongated with (α1 → 6) but also with (α1 → 4) linkages, were seen. Like in the case of maltose, the amount of (α1 → 6) elongation increased after longer incubation times. This could indicate that in both cases initial products with (α1 → 4) elongations were used again as donor substrates.

It is evident that the GTFB enzyme transfers terminal (α1 → 4)-linked Glc residues from MOS donors to MOS acceptors attaching them as (α1 → 6)-linked Glc residues [(α1 → 4) to (α1 → 6) transfer activity]. The initial finding of transferred maltosyl/maltotriosyl units means either that maltose/maltotriose units are released from MOS donors via a slight additional endo-(α1 → 4)-glycosidase activity of GTFB and transferred via an (α1 → 6) linkage to MOS acceptors or that besides the (α1 → 4) to (α1 → 6) transfer activity also a slight additional (α1 → 4) to (α1 → 4) transfer activity of GTFB exists.

Incubation of maltoheptaose with the recombinant GTFB enzyme

Maltoheptaose (DP7) was incubated with GTFB for 120 h at 37°C and pH 4.7. One-dimensional ^{1}H NMR analysis of the generated product mixture (Figure 3) showed, besides the presence of (α1 → 4) linkages (H-1, ∼δ 5.39), also the presence of newly formed (α1 → 6) linkages (H-1, $\delta \sim 4.96$; broad signal). The (α1 → 4):(α1 → 6) linkage ratio is 64:36. Furthermore, also free glucose is present. MALDI-TOF-MS analysis of the generated product mixture revealed the presence of a series of compounds ranging from DP2 to DP35 ($[M + Na]^{+}$, *m*/*z* 365–5711). However, according to the *m*/*z* peak intensities of the sodiated molecular ions, the amounts of the compounds of DP > 10 were very low.

For further analysis, the generated product mixture was subjected to size-exclusion chromatography on Bio-Gel P-2, and the products-containing eluate was collected into 10 fractions, denoted **F1**–**F10**. MALDI-TOF-MS analysis showed that fraction **F1** contained oligosaccharides with DP > 12, **F2** mainly DP10, **F3** mainly DP10, DP9 and DP8, **F4** mainly DP9 and DP8, **F5** mainly DP7 and DP6, **F6** mainly DP6 and DP5, **F7** mainly DP5, **F8** mainly DP4, **F9** mainly DP3 and **F10** mainly DP2. The ^{1}H NMR analysis of the Bio-Gel P-2 fractions demonstrated that the amount of (α1 → 6) linkages increased with increasing DP. In a parallel Bio-Gel P-2 fractionation, the fraction containing DP12–DP19 revealed already a higher percentage of (α1 → 6) linkages than (α1 → 4) linkages; the fraction containing DP > 35 showed an (α1 → 4):(α1 → 6) linkage ratio of 15:85 (see ^{1}H NMR spectra in Supplementary data, Figure S3). The fractions **F2**–**F10** were subfractionated by HPAEC on CarboPac PA-1 (Figure 4) to yield fractions containing compounds of a single DP (MALDI-TOF-MS analysis; DP2–DP10), which were analyzed by one- and two-

Table I. ^{1}H and ^{13}C chemical shifts[a] (ppm, D_2O, 300 K) of Glc residues present in linear isomalto-/malto-oligomers (oligosaccharides and corresponding alditols) formed by the incubation of malto-oligomers (oligosaccharides and corresponding alditols) with GTFB

Residue	H-1a, C-1	H-1b	H-2, C-2	H-3, C-3	H-4, C-4	H-5, C-5	H-6a, C-6	H-6b
A, -(1 → 4)-α-D-Glc*p*-(1 → 4)-	5.395, 100.7	—	3.62, 72.5	3.96, 74.3	3.66, 77.8	3.84, 72.2	3.88, 61.5	3.81
A′, -(1 → 4)-α-D-Glc*p*-**R-ol**	5.129, 101.3	—	3.61, 72.4	4.01, 71.9	3.67, 77.7	3.84, 72.2	3.84, 61.5	3.76
B, α-D-Glc*p*-(1 → 4)-	5.399, 100.7	—	3.59, 72.7	3.67, 73.8	3.42, 70.3	3.72, 73.7	3.84, 61.5	3.75
C, -(1 → 6)-α-D-Glc*p*-(1 → 4)-	5.389, 100.7	—	3.60, 72.4	3.70, 74.3	3.49, 70.6	3.93, 71.2	3.74, 66.7	3.98
C′, -(1 → 6)-α-D-Glc*p*-**R-ol**	5.127, 101.6	—	3.60, 72.3	3.74, 74.0	3.51, 70.5	4.10, 72.1	3.76, 67.1	3.99
D, -(1 → 6)-α-D-Glc*p*-(1 → 6)-	4.970, 98.8	—	3.58, 72.3	3.70, 74.3	3.51, 70.6	3.91, 71.2	3.76, 66.7	3.98
E, -(1 → 4)-α-D-Glc*p*-(1 → 6)-	4.970, 98.9	—	3.61, 72.5	4.03, 74.4	3.65, 78.1	3.85, 71.3	3.87, 61.5	3.84
F, α-D-Glc*p*-(1 → 6)-	4.960, 98.8	—	3.55, 72.4	3.70, 72.8	3.43, 70.5	3.73, 72.3	3.85, 61.5	3.76
Rα, -(1 → 4)-D-Glc*p*α	5.225, 92.9	—	3.56, 72.3	3.98, 74.3	3.66, 77.9	3.94, 72.3	3.83, 61.5	3.79
Rβ, -(1 → 4)-D-Glc*p*β	4.650, 96.8	—	3.27, 75.1	3.77, 77.2	3.65, 77.9	3.59, 75.6	3.90, 61.5	3.77
R-ol, -(1 → 4)-D-Glc-ol	3.81, 63.2	3.70	4.02, 73.6	3.87, 72.5	3.89, 83.2	3.92, 71.4	3.81, 63.2	3.70

[a]In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

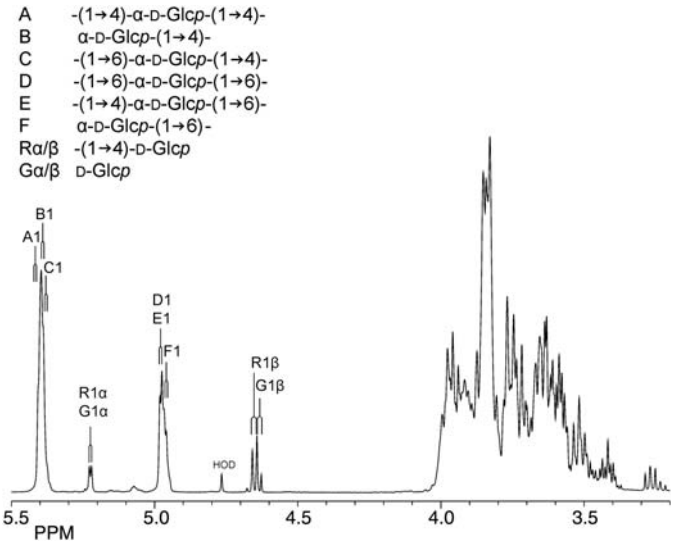

Fig. 3. The ^{1}H NMR spectrum of the generated oligosaccharide mixture after the incubation of maltoheptaose (DP7) with GTFB for 120 h at 37°C and pH 4.7.

dimensional NMR spectroscopy. Fractions **F1** and **F2** were not further investigated due to the complexity of the mixture.

As a typical example, the one- and two-dimensional (TOCSY, ROESY and HSQC) NMR spectra of one of the four isomeric DP8 product oligosaccharides are depicted in Figure 5. The one-dimensional ^{1}H NMR spectrum showed two low-intensity anomeric signals at δ 5.225 (α) and δ 4.650 (β), in agreement with a reducing -(1 → 4)-D-Glc*p* **R** unit. As mentioned already for the maltose/maltotriose incubations, the groups of anomeric signals around δ 5.39 and 4.96 reflect the presence of the (α1 → 4) and (α1 → 6) linkages, respectively. For a further fine-tuning of the precise environment of the various Glc units, two-dimensional NMR measurements were carried out, and the use was made of the earlier developed structural-reporter-group concept for the analysis of α-D-glucans.

Starting from the anomeric signals of the residues **A**, **C**, **D** and **F** in the two-dimensional TOCSY spectrum (Figure 5), all chemical shifts of the non-anomeric protons of the differently substituted Glc residues could be determined (Table I). Although the anomeric signals of **A** (H-1, δ 5.395) and **C** (H-1, δ 5.389) strongly overlap, the differences in chemical shift of their H-3, H-4 and H-5 signals could be deduced from the TOCSY built-up series of mixing times [20, 40, 100 (data not shown) and 200 ms]. The set of chemical shifts of **A** H-2, H-3, H-4 and H-5 at δ 3.62, 3.96, 3.66 and 3.84, respectively, corresponds to that of an internal -(1 → 4)-α-D-Glc*p*-(1 → 4)- unit, whereas the set of chemical shifts of **C** H-2, H-3, H-4 and H-5 at δ 3.60, 3.70, 3.49 and 3.93, respectively, corresponds to that of an internal -(1 → 6)-α-D-Glc*p*-(1 → 4)- unit. The 4- and 6-substitution of the residues **A** and **C**, respectively, are further supported by their ^{13}C chemical shifts (deduced from HSQC measurements; Figure 5): **A** C-4 at δ 77.8 and **C** C-6 at δ 66.7 (for comparison: maltotriose C-4′, δ 77.8; C-6′, δ 61.4; C-4″, δ 70.1; C-6″, δ 61.4; and isomaltotriose C-4′, δ 70.4; C-6′, δ 66.5;

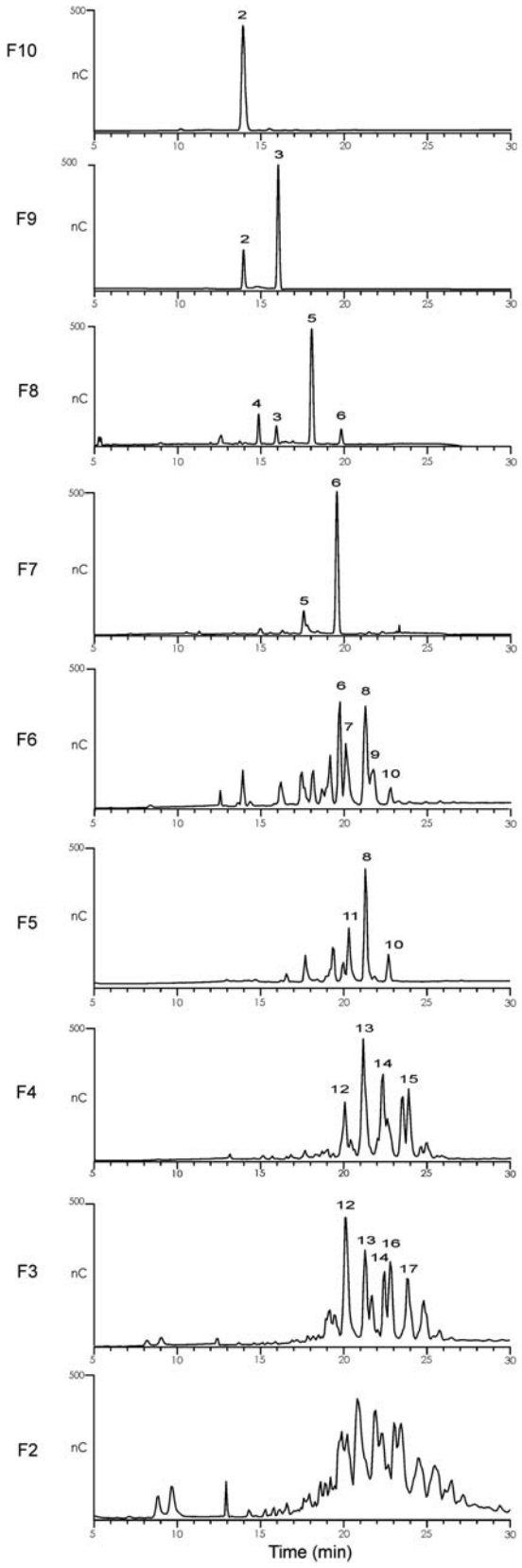

Fig. 4. The HPAEC-PAD subfractionation (0–500 mM NaOAc gradient in 100 mM NaOH) of Bio-Gel P-2 fractions **F2**–**F10** on CarboPac PA-1 [incubation of maltoheptaose (DP7) with GTFB].

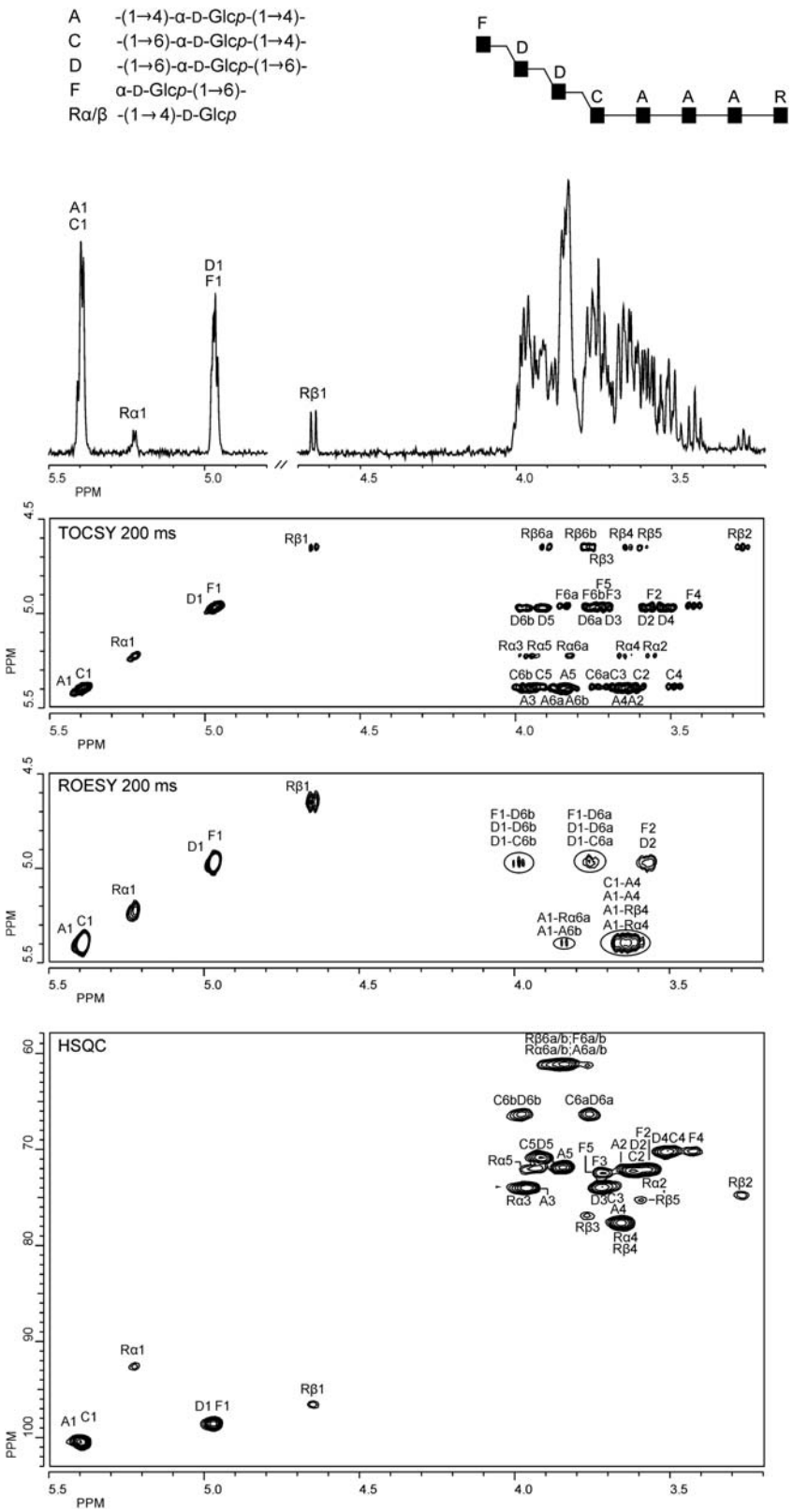

Fig. 5. One-dimensional ^{1}H NMR, TOCSY (200 ms), ROESY (200 ms) and HSQC spectra of a DP8 product oligosaccharide [α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-D-Glc*p*].

C-4″, δ 70.5; C-6″, 61.4). The anomeric signals of **D** (H-1, δ 4.970) and **F** (H-1, δ 4.960) also overlap, but the chemical shifts of their H-4, H-5 and H-6a signals clearly assigned **D** as an internal -(1 → 6)-α-D-Glc*p*-(1 → 6)- unit (H-4, H-5 and H-6a at δ 3.51, 3.91 and 3.98, respectively) and **F** as a terminal α-D-Glc*p*-(1 → 6)- unit (H-4, H-5 and H-6a at δ 3.43, 3.73 and 3.85). The 6- and non-substitution of **D** and **F**, respectively, are further supported by their ^{13}C chemical shifts (deduced from HSQC measurements): **D** C-6 at δ 66.7 and **F** C-6 at δ 61.5. In the ROESY spectrum (Figure 5), inter-residual cross-peaks were observed indicating the linkages **A**(1 → 4)**R**α/β, **A**(1 → 4)**A**, **C**(1 → 4)**A**, **D**(1 → 6)**C**, **D**(1 → 6)**D** and **F**(1 → 6)**D**. In conclusion, the NMR chemical shift data, together with the peak areas of the anomeric signals and the molecular mass (1314 Da, octasaccharide) revealed the complete structure of the oligosaccharide, being a linear octasaccharide containing from the non-reducing to the reducing end three successive (α1 → 6) linkages, followed by four successive (α1 → 4) linkages (Figure 5). The one-dimensional ^{1}H and TOCSY spectra of the other three isomeric DP8 product oligosaccharides are included in Supplementary data, Figures S4–S6.

The results of the analysis of the compounds ranging from DP1 to DP10, obtained from the incubation of maltoheptaose (DP7) with recombinant GTFB, are summarized in Figure 6, indicating the elucidation of 17 different structures. It should be noted that the elucidated structures are only a part of the total amount of compounds that were formed during the incubation. The characterization of the higher-molecular-mass products is currently under investigation. In principle, the five products of DP < 7 having (α1 → 4) linkages only (maltose to maltohexaose), obtained from maltoheptaose (DP7), can also act as new substrates for elongation with (α1 → 6) linkages, and four structures of DP < 8, reflecting these possibilities, have been elucidated. In a similar way as discussed for maltotriose, the finding of a DP8 structure in which two maltotetraosyl units are connected via an (α1 → 6) linkage suggests

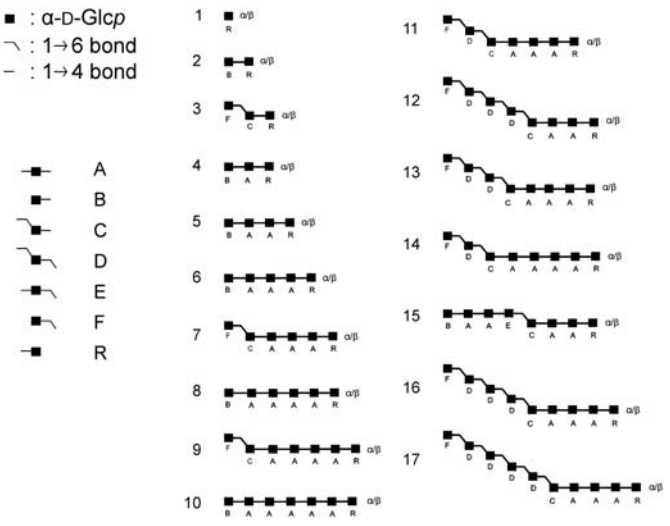

Fig. 6. Elucidated structures of oligosaccharides from the incubation of maltoheptaose (DP7) with GTFB for 120 h at 37°C and pH 4.7.

that either a slight additional endo-(α1 → 4)-glycosidase activity combined with the (α1 → 6) transfer activity or a slight additional (α1 → 4) to (α1 → 4) transfer activity of GTFB exists. Although among the identified oligosaccharides no structures were found with a 6-substituted reducing-end Glc residue, the ^{1}H NMR spectrum of the total product mixture (Figure 3) shows trace amounts of such a unit [-(1 → 6)-D-Glc*p*, H-1α at δ 5.240 and H-1β at δ 4.669). This could indicate that a (1 → 4) linkage in the -α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)- sequence is poorly susceptible to hydrolysis via the endo-(α1 → 4)-glycosidase activity of the GTFB enzyme.

Incubation of maltopentaitol with the recombinant GTFB enzyme

As far as known, the linkage specificity of GSs is conserved in gluco-oligosaccharide synthesis, whereby oligosaccharides are elongated at their non-reducing end (Monchois et al. 1999; Moulis et al. 2006). To test whether GTFB specificity differs between free oligosaccharides and oligosaccharide-alditols, additional incubations were performed with MOS-alditols ranging from triitol to heptaitol (DP3-ol to DP7-ol). It should be noted that (product) oligosaccharide-alditols elute faster on HPAEC and give a better separation. Furthermore, the interpretation of NMR spectra of oligosaccharide-alditols is in general less complicated, due to the absence of the mixed α/β configuration of the reducing-end Glc residue.

Maltopentaitol (DP5-ol) was incubated with GTFB for 72 h at 37°C and pH 4.7. MALDI-TOF-MS analysis of the obtained mixture of products revealed a series of oligosaccharide-alditols ranging from DP2-ol to DP20-ol ($[M + Na]^+$, *m/z* 367–3283), although the peak intensities of their sodiated molecular ions showed that products of DP-ol > 12 are present in very low amounts. Additionally, the sodiated molecular ions of a series of free oligosaccharides ranging from DP2 to DP6 ($[M + Na]^+$, *m/z* 365–1013) were observed, showing hydrolysis [endo-(α1 → 4)-glycosidase] and transfer activities of GTFB. Besides the anomeric signals around δ 5.39, in accordance with the presence of the (α1 → 4) linkages, the one-dimensional ^{1}H NMR spectrum of the generated product mixture (Figure 7) demonstrated also the presence of newly formed (α1 → 6) linkages by a broad anomeric signal around δ 4.96. The (α1 → 4):(α1 → 6) linkage ratio is 80:20. The anomeric signals at δ 5.225 (H-1α) and δ 4.650 (H-1β) indicate the presence of reducing -(1 → 4)-α-D-Glc*p* units, stemming from free oligosaccharides; also free Glc was shown to be present.

The generated product mixture was subfractionated according to size by gel-filtration chromatography on Bio-Gel P-2, and the products-containing eluate was collected into five fractions, denoted **F′1**–**F′5**. MALDI-TOF-MS analysis showed that fraction **F′1** contained product oligosaccharide-alditols with DP-ol > 12, but due to the complexity of the mixture, this fraction was not further investigated. The other fractions were fractionated by HPAEC on CarboPac PA-1 (Supplementary data, Figure S7), and in this way, a number of subfractions could be collected containing compounds of a single DP(-ol), as deduced from MALDI-TOF-MS (data not

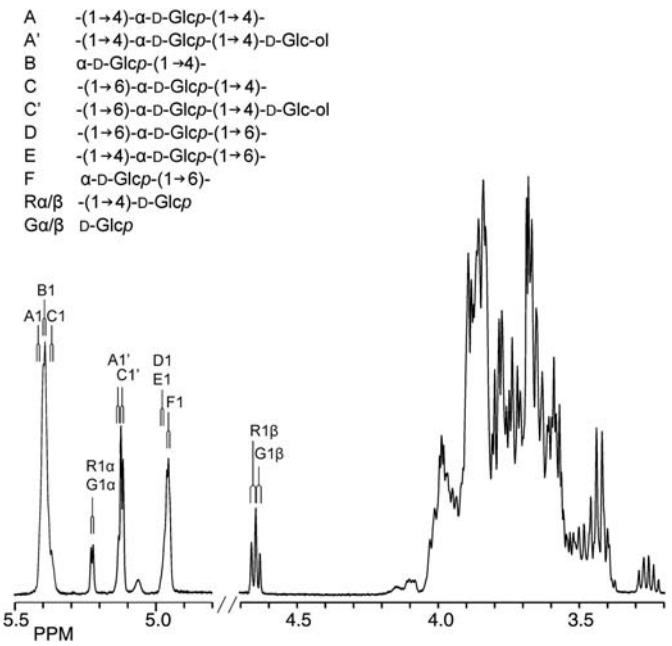

Fig. 7. The ^{1}H NMR spectrum of the generated oligosaccharide mixture after the incubation of maltopentaitol (DP5-ol) with GTFB for 72 h at 37°C and pH 4.7.

shown). Subsequently, these subfractions were subjected to NMR analysis.

As typical examples, the NMR analysis of three isomeric DP5-ol product structures (MALDI-TOF-MS: $[M+Na]^{+}$, *m/z* 853) will be worked out (Figure 8A–C). Because of the alditol character of the three products (Glc **R-ol**), no α,β-anomeric signals of a reducing-end Glc **R** residue are seen. Starting from the anomeric signals in the two-dimensional TOCSY spectra, run at mixing times of 20, 40, 100 (data not shown) and 200 ms, together with the information from the HSQC spectra, all chemical shifts of the non-anomeric protons of the differently substituted Glc residues could be determined; also all Glc-ol protons could be assigned (Table I). Although the anomeric signals of **A** and **B** (Figure 8A), **D** and **E** (Figure 8B) and **D** and **F** (Figure 8C) strongly overlap, the set of chemical shifts of the non-anomeric protons can be used for the discrimination between these residues. Following the earlier developed structural-reporter-group concept rules, the set of chemical shifts of residue **A** (H-1 track, δ 5.395) was in agreement with an internal -(1 → 4)-α-D-Glc*p*-(1 → 4)- unit; that of residue **B** (H-1 track, δ 5.399) with a terminal α-D-Glc*p*-(1 → 4)- unit; that of residue **C′** (H-1 track, δ 5.127) with an internal -(1 → 6)-α-D-Glc*p*-(1 → 4)- unit specifically coupled to D-Glc-ol [a -(1 → 6)-α-D-Glc*p*-(1 → 4)- unit in a Glc sequence is reflected by a **C** H-1 track at δ 5.389]; that of residue **D** (H-1 track, δ 4.970) with an internal -(1 → 6)-α-D-Glc*p*-(1 → 6)- unit; that of residue **E** (H-1 track, δ 4.970) with an internal -(1 → 4)-α-D-Glc*p*-(1 → 6)- unit and that of residue **F** (H-1 track, δ 4.960) with a terminal α-D-Glc*p*-(1 → 6)- unit. Comparison of the NMR data of the three isomeric DP5-ol products, including the peak areas of the various anomeric signals, makes clear that each structure has a -(1 → 6)**C′**(1 → 4)**R-ol** end. One structure has a terminal **F** (1 → 6)- and two internal -(1 → 6)**D**(1 → 6)- units, resulting in the structure **F**(1 → 6)**D**(1 → 6)**D**(1 → 6)**C′**(1 → 4)**R-ol** (Figure 8C). The two other structures have a terminal **B**(1 → 4)- unit. One of them has an internal -(1 → 4)**A**(1 → 4)- and an internal -(1 → 4)**E**(1 → 6)- unit, resulting in the structure **B** (1 → 4)**A**(1 → 4)**E**(1 → 6)**C′**(1 → 4)**R-ol** (Figure 8A). The other one has an internal -(1 → 4)**E**(1 → 6)- unit and an internal -(1 → 6)**D**(1 → 6)- unit, resulting in the structure **B**(1 → 4)**E** (1 → 6)**D**(1 → 6)**C′**(1 → 4)**R-ol** (Figure 8B).

In a similar way, 12 other product oligosaccharide-alditols could be elucidated by NMR analysis of the HPAEC fractions (Figure 9). Some HPAEC fractions contained free oligosaccharides, and NMR analysis of these fractions revealed structures (Figure 9) identical to those depicted in Figures 1B, 2B and 6, thereby demonstrating that these products are stemming from hydrolysis alone [endo-(α1 → 4)-glycosidase activity] or combined with the transfer activity of GTFB. The sequence of the Glc residues in most of the elucidated oligosaccharide-alditol product structures (Figure 9) is identical to that in the non-reduced forms depicted in Figure 6, indicating that elongation indeed takes place at the non-reducing-end Glc residue. Typically, all oligosaccharide-alditol products have an α-D-Glc*p*-(1 → 4)-D-Glc-ol unit. Furthermore, it seems that compared with the results with free oligosaccharides, the transfer specificity (α1 → 6) vs (α1 → 4) has changed slightly in favor of more (α1 → 4) linkages and shorter (α1 → 6) sequences in the final product mixtures.

Concluding remarks

GTFB from *L. reuteri* 121 has recently been identified as a 4,6-α-glucanotransferase, a novel enzyme of the GH70 family, that is inactive with sucrose, but active on MOSs. The reported structural data of the obtained product mixtures indicated that GTFB converted specific MOS into elongated non-branched gluco-oligosaccharides with (α1 → 4) and a growing percentage of (α1 → 6) glycosidic linkages in the higher DPs (Kralj et al. 2011). Here, we present a detailed structural analysis of isolated products obtained from the incubation of GTFB with a series of MOS and their corresponding alditols, which has deeply broadened our insights into the hydrolase/transglycosylase activities of this enzyme. Inspection of the structures of the various isolated oligosaccharides (Figures 1, 2, 6 and 9) revealed that besides residual malto-oligomers, only linear isomalto-/malto-oligomers with a high DP (in the case of DP7 up to DP > 35) are formed. The GTFB enzyme showed a clear (α1 → 4) to (α1 → 6) transfer activity, but besides that also a slight endo-(α1 → 4)-glycosidase activity combined with the (α1 → 6) transfer activity or a slight (α1 → 4) to (α1 → 4) transfer activity exists. As GTFB is inactive with IMO (Kralj et al. 2011), products with terminal (α1 → 6)-linked Glc residues do not act as donor substrates, they can only act as acceptor substrates. However, products with terminal (α1 → 4)-linked Glc residues, initially formed during the incubations (e.g. Figure 2), can act as donor substrates, so that the amount of (α1 → 6) linkages increases with the DP of the products (Binder et al. 1983). This results in product mixtures wherein long IMO segments are seen,

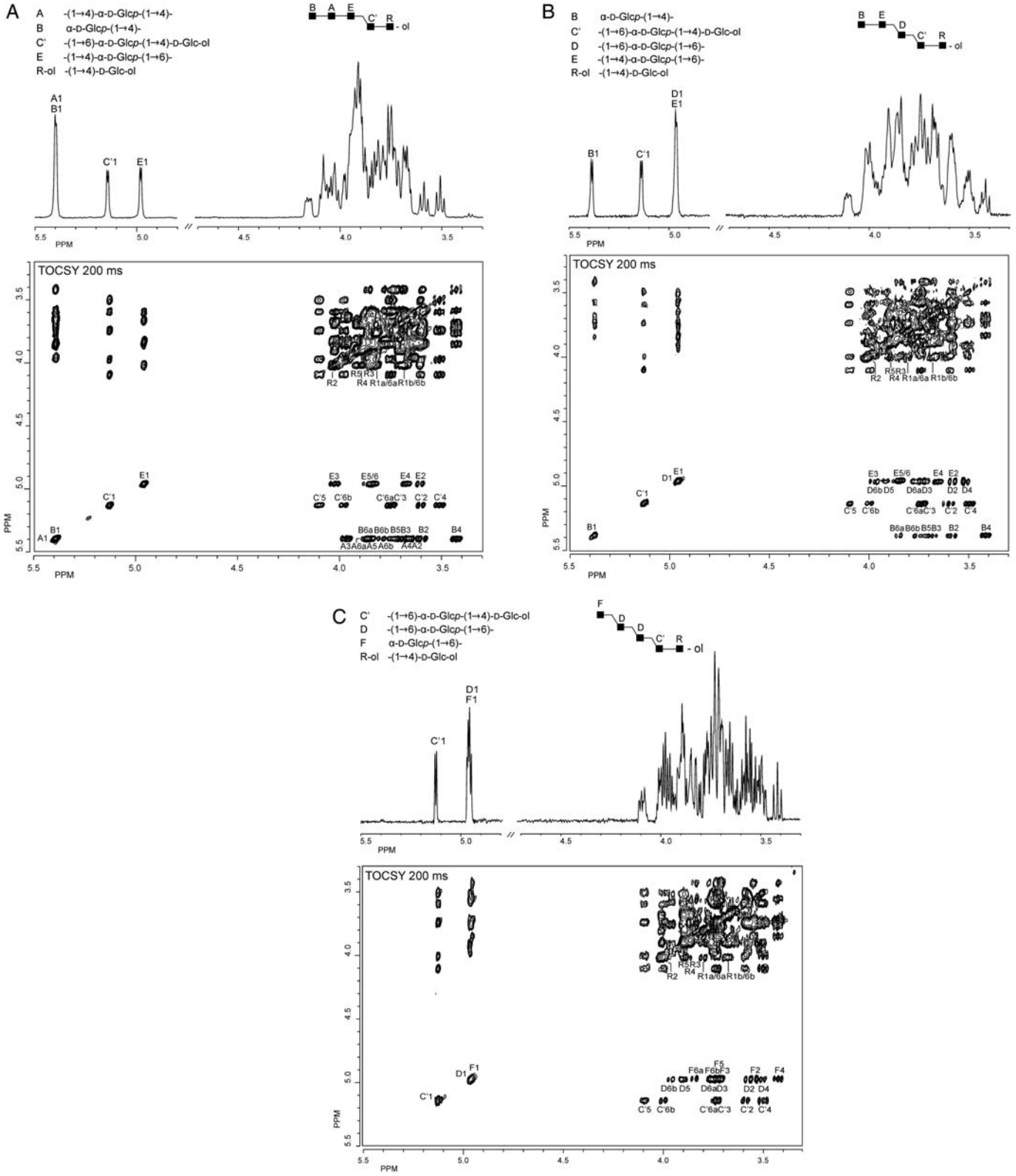

Fig. 8. One-dimensional ^{1}H NMR and TOCSY (200 ms) spectra of three isomeric DP5-ol product oligosaccharides. (**A**) α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc-ol; (**B**) α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc-ol; (**C**) α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc-ol.

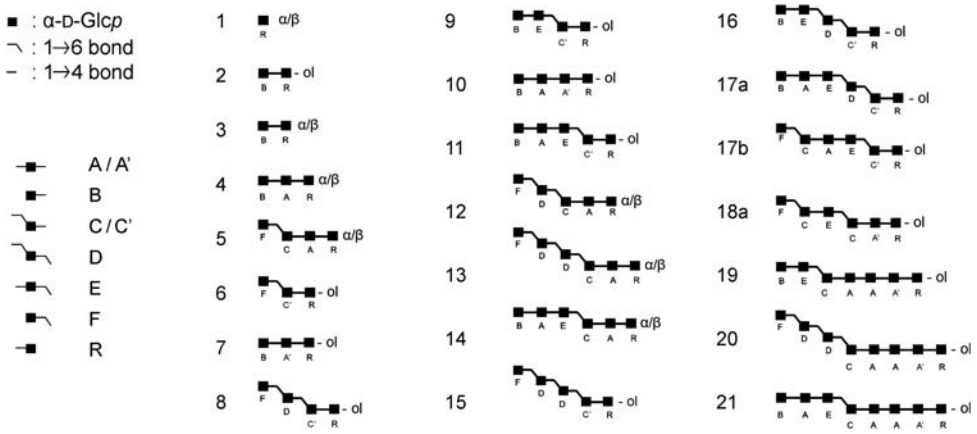

Fig. 9. Elucidated structures of oligosaccharides/oligosaccharide-alditols from the incubation of maltopentaitol (DP5-ol) with GTFB for 72 h at 37°C and pH 4.7.

e.g. in the case of DP7 in a decasaccharide with five (α1 → 6) linkages and four (α1 → 4) linkages (Figure 6), and the fraction containing DP > 35 represented an (α1 → 4) to (α1 → 6) linkage ratio of 15:85 (Supplementary data, Figure S3). The incubation with the MOS alditols supported that elongation takes place at the non-reducing-end Glc residues. It should be stressed that the type of IMO–MOS structures, presented in this study, has never been synthesized before, neither by organic chemical synthesis nor by enzymatic synthesis, and represents a completely unique array of IMO–MOS compounds.

Nowadays, functional food gets great attention from the food industry. Prebiotics are non-digestible food ingredients, which beneficially affect the host's health by selective stimulation of the growth of one or a limited number of bacteria in the colon. MOS are either degraded completely in the small intestine to glucose, which is taken up in the blood, or those parts that escape digestion, end up in the large intestine, where they serve as a general substrate for the colonic microflora. IMO sequences have been claimed to be more suitable to reach the distal part of the colon, thereby reducing the blood glucose levels after food consumption. In this context, it will be of interest to investigate the prebiotic properties of the newly formed linear IMO–MOS oligosaccharides and their possible industrial application. In such an approach, it will be possible to test the incubation mixtures of defined DP MOS donors and their formed (IMO-)MOS products. But also MOS donors built up from a range of DPs, simply prepared from starch, can be used as starting materials. The final product can then be defined as a MOS mixture, partially decorated with IMO segments. In fact, such materials compare the commercially available and widely used galacto-oligosaccharide (GOS) ingredients, prepared from lactose and some specific β-galactosidase enzyme and built up from a broad array of GOSs (in general DP2–DP8; different isomeric forms per DP), starting lactose and produced galactose and glucose (Playne and Crittenden 2009).

Finally, in view of the foregoing, it is not surprising that the direct action of GTFB on starch produces starch derivatives, which are highly decorated with IMO chains (Dijkhuizen et al. 2010). This decoration may result in an increased resistance to human α-amylase degradation and thus in a slower digestibility of these starch derivatives in the human gastrointestinal tract, serving to reduce the blood glucose levels after food consumption. Details of this research will be published elsewhere (Leemhuis et al., in preparation).

Materials and methods

Preparation and isolation of GTFB enzyme

The recombinant GTFB enzyme was prepared and isolated as described previously (Kralj, van Geel-Schutten, Dondorff, et al. 2004; Kralj et al. 2011). Briefly, plasmid pET15b-gtfB (Novagen, Madison, WI) was used for expression of the *gtfB* gene in *E. coli* BL21 DE3 star (Invitrogen, Carlsbad, CA). After cultivation aerobically at 37°C in Luria–Bertani medium, containing ampicillin (100 μg/mL), until optical density $OD_{600} = 0.5$ (incubation time, ~18 h), the culture was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside and grown for another 4 h. Then, the cells were harvested by centrifugation (10,000 × *g*, 20 min, 4°C), washed twice with 50 mM sodium phosphate buffer, pH 8.0, and subsequently disrupted by sonication (4 × 15 s, 0°C). After centrifugation (10,000 × *g*, 40 min, 4°C), a mixture of proteins, including the recombinant GTFB protein, was isolated from the supernatant by His-tag affinity chromatography (Mw GTFB + His-tag = 176,193 Da) using Ni^{2+}-nitrilotriacetate (Ni-NTA) as column material (Sigma-Aldrich, St Louis, MO). To this end, the supernatant was added to Ni-NTA, and after gently incubation for 2 h at 4°C, the mixture was poured into a column (10 × 2 cm). The column was connected to an fast protein liquid chromatography system (AKTA workstation, GE Healthcare, Roosendaal, The Netherlands; detection, UV 214 nm), washed with 20 mM Tris–HCl buffer, pH 8.0, containing 1 mM $CaCl_2$, for 2 min, and eluted with an imidazole gradient (5–200 mM) in 20 mM Tris–HCl buffer, pH 8.0, containing 1 mM $CaCl_2$, at a flow rate of 1 mL/min. Fractions (1 mL) were collected and checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The GTFB-containing fractions were pooled, and imidazole was removed on a HiTrap column (5 mL; GE Healthcare), eluted with 20 mM Tris–HCl buffer, pH 8.0. Activity assays of GTFB were carried out by incubation with maltopentaose, followed by TLC analysis. Further purification was performed

by anion-exchange chromatography (AKTA workstation) on a Resource Q column (6 mL; GE Healthcare) using a gradient elution of 0–1 M NaCl in 20 mM Tris–HCl buffer, pH 8.0, at a flow rate of 1 mL/min and detection at 214 nm, yielding purified recombinant GTFB as the first eluting peak. Again the presence of activity was checked by incubation of maltopentaose, followed by TLC analysis. Fractions were also monitored by SDS–PAGE and the GTFB-containing fraction revealed a band at 176 kDa. The additional fractions, containing proteins with a molecular mass <176 kDa, showed no enzymatic activity.

Incubation of MOSs(-alditols)

Before incubation, commercially available MOSs (DP2–DP7) were purified via size-exclusion chromatography on Bio-Gel P-2 (DP2–DP4) or via HPAEC on CarboPac PA-1 (DP5–DP7). MOS-alditol samples (DP2-ol–DP7-ol) were prepared by a reduction of the corresponding MOS samples with sodium borohydride and purification on Bio-Gel P-2 or CarboPac PA-1. The purified samples (100 mM solutions) were individually incubated in sterile Greiner tubes with 500 nM GTFB in 250 mM sodium acetate, pH 4.7, containing 10 mM $CaCl_2$, at 37°C for 2–120 h. The progress of the reactions was followed by analyzing aliquots of the incubation mixtures with TLC, MALDI-TOF MS and HPAEC-PAD. Comparable incubations without the addition of GTFB showed no product formation.

Isolation and purification of product oligosaccharides (-alditols)

Product mixtures of oligosaccharides(-alditols) obtained after incubation of DP2 and DP3 (50 mM solutions) with 250 nM GTFB were directly fractionated by HPAEC on a Dionex DX500 workstation (Dionex, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (Dionex; 250 × 4 mm for analytical runs, 250 × 9 mm for preparative runs) and an ED40 pulsed amperometric detector. Product mixtures obtained from DP7 and DP5-ol were first prefractionated on a Bio-Gel P-2 column (90 × 1 cm), eluted with 10 mM NH_4HCO_3 at a flow rate of 12 mL/h. Subsequently, pooled Bio-Gel P-2 fractions were fractionated by HPAEC-PAD, using a linear gradient of 0–500 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), and products having a single DP were isolated. Collected fractions were immediately neutralized with 4 M acetic acid, desalted on CarboGraph SPE columns (Alltech, Breda, The Netherlands) using acetonitrile:water = 1:3 as the eluent and lyophilized.

Thin-layer chromatography

Samples were spotted in 1-cm lines on TLC sheets (Merck Kieselgel 60 F254, 20 × 20 cm), which were developed with *n*-butanol:acetic acid:water = 2:1:1. Bands were visualized by orcinol/sulfuric acid staining and compared with a simultaneous run of the standard oligosaccharides.

MALDI-TOF mass spectrometry

MALDI-TOF-MS experiments were performed on an Axima™ mass spectrometer (Shimadzu Kratos Inc., Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 Full Width at Half Maximum (FWHM) and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2%; the mirror voltage ratio was 1.12, and the acquisition mass range was 200–6000 Da. Samples were prepared by mixing on the target 0.5 μL sample solutions with 0.5 μL aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

NMR spectroscopy

Resolution-enhanced one-/two-dimensional 500-MHz ^{1}H NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or a Varian Inova Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. Prior to analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL of D_2O. Suppression of the deuterated water signal (HOD) was achieved by applying a WEFT (water eliminated Fourier transform) pulse sequence for one-dimensional experiments and by a pre-saturation of 1 s during the relaxation delay in two-dimensional experiments. The TOCSY spectra were recorded using an MLEV-17 (composite pulse devised by M. Levitt) mixing sequence with spin-lock times of 20–200 ms. The ROESY spectra were recorded using standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance ^{1}H-^{13}C HSQC experiments (^{1}H frequency 500.0821 MHz, ^{13}C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the ^{1}H Free Induction Decay (FID). Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for one-dimensional spectra or by multiplication with a squared-bell function phase shifted by $\pi/(2.3)$ for two-dimensional spectra, and when necessary, a fifth-order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and δ 31.08 for ^{13}C).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

DP, degree of polymerization; EPS, exopolysaccharide; GOS, galacto-oligosaccharide; GS, glucansucrase; GTFB, glucosyltransferase B from *L. reuteri* 121; HPAEC, high-pH anion-exchange chromatography; HSQC, ^{1}H-detected heteronuclear single-quantum coherence spectroscopy; IMO, isomalto-oligosaccharide; LAB, lactic acid bacteria; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MOS, malto-oligosaccharide; Ni-NTA, Ni^{2+}-nitrilotriacetate; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy.

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