Characterization of 4,6-α-glucanotransferase enzymes and their functional role in \textit{Lactobacillus reuteri}

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Chapter 5

Structural basis of the role of starch and sucrose in homopolysaccharide formation in *Lactobacilli reuteri* 35-5

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*In preparation for submission*
Abstract

Lactic acid bacteria (LAB) are abundant producers of exopolysaccharides (EPS) which are important for biofilm formation in the oral cavity and gastrointestinal tract of mammals. Sucrose is a well-known substrate for homo-EPS formation by glucansucrases (GS) of LAB. Starch is the main fermentable carbohydrate in the human diet, and often consumed simultaneously with sucrose. Recently, 4,6-α-glucanotransferases (4,6-α-GTases) of Lactobacillus reuteri strains were characterized that act on starch and maltodextrins, cleaving α1→4 linkages and synthesizing α1→6 linkages, yielding isomalto-/malto-polysaccharides (IMMP) that are soluble dietary fibers. Lactobacillus strains (e.g. L. reuteri 35-5) may possess both a GS (e.g. GtfA) and a 4,6-α-GTases (GtfB), both cell-associated and located extracellularly. In this study, we characterized the EPS formed in vivo by L. reuteri 35-5 cells during growth with either sucrose or maltodextrins, or with both. Size exclusion chromatography and enzymatic hydrolysis revealed variations in EPS size and structure depending on the substrate(s) provided. In vitro incubation of the purified GtfA-ΔN plus GtfB-ΔN enzymes in the presence of both sucrose and starch resulted in formation of oligosaccharides with terminally and internally consecutive α1→6 linkages, which are structurally different from the oligosaccharides produced from either of the single substrates. In conclusion, the data show that both in vivo and in vitro the GtfA and GtfB enzymes of L. reuteri 35-5, incubated with sucrose plus starch, cross-react and contribute to the synthesis of the final products. This may have strong effects on the functional properties of the EPS formed by lactobacilli, and influence their ability for biofilm formation in the mammalian oral cavity and gastrointestinal tract. Also the relationship between dietary intake of sucrose and starch, dental caries formation and degradability of EPS dietary fibers, are likely to be affected.
Abstract Figure. Schematic model of the exopolysaccharides produced by GtfA and GtfB enzymes in the presence of sucrose and starch in *Lactobacillus reuteri* 35-5.

**Introduction**

Exopolysaccharides (EPS) constitute a major fraction of the extracellular polymeric substances that form the scaffold for the three-dimensional architecture of biofilms (1). In many bacteria, EPS are indispensable for biofilm formation: mutants that have lost ability to synthesize EPS are unable or severely compromised in forming mature biofilms (2-5). Lactic acid bacteria (LAB) use glucansucrase (GS) and fructansucrase enzymes to synthesize α-glucan and β-fructan homo-EPS from sucrose (6-10). GS are family 70 glycoside hydrolase (GH70) enzymes that are able to synthesize a diversity of α-glucans varying in linkage type and degree of branching (11). In the oral cavity and gastrointestinal tract of mammals, the α-glucan polysaccharides produced by GS facilitate biofilm formation and significantly enhance the adhesion of bacteria to the tooth enamel or epithelium (12, 13).
Starch is an important carbohydrate in the human diet and usually consumed simultaneously with sucrose. Simultaneous intake of starch and sucrose resulted in enhanced cariogenicity in human compared to use of either carbohydrate alone (14-17). The EPS matrix produced by *Streptococcus mutans* from sucrose plus starch contains a larger amount of highly branched α-glucan compared to the EPS derived from either of the single substrates (18). In addition to *S. mutans*, many other species of LAB that produce EPS from sucrose, using GS, are found in the oral cavity and gastrointestinal tract of mammals (9, 19, 20). No information is available on the EPS synthesized by LAB strains challenged with both sucrose and starch as carbon sources for growth.

In recent years, we have identified a new GH70 subfamily with 4,6-α-glucanotransferase (4,6-α-GTase) enzymes in *Lactobacillus* strains. These 4,6-α-GTases do not act on sucrose but on α-glucans (starch, maltodextrins), cleaving α1→4 linkages and forming α1→6 linkages, resulting in isomalto-/malto-polysaccharide (IMMP) synthesis that act as soluble dietary fibers (21-23). According to current database searches (http://www.ncbi.nlm.nih.gov/), around 25% of the LAB strains carrying a 4,6-α-GTase gene also possess a GS gene. In our previous study, *L. reuteri* 121 cultures with the extracellular and cell-associated 4,6-α-GTase GtfB were shown to synthesize IMMP-like EPS from starch (Bai et al., submitted). Here we study whether co-feeding of sucrose and starch influences EPS formation by *L. reuteri* 121 strain 35-5. This mutant only has GS (GtfA) and 4,6-α-GTase (GtfB) enzymes but no fructansucrase or other homo-EPS producing enzymes (24). GtfA converts sucrose into reuteran with alternating α1→4 and α1→6 glycosidic linkages; GtfB converts starches and maltodextrins into IMMP with consecutive α1→6 linkages (Abstract Figure) (25-28). Here, we analyzed the different *L. reuteri* 35-5 EPS synthesized during growth with sucrose, maltodextrins, and sucrose plus maltodextrins. We also studied the EPS synthesized by GtfA-ΔN and GtfB-ΔN enzymes *in vitro*, by analyzing initial oligosaccharide formation from these substrates. The data shows that homo-EPS formation by *L. reuteri* 35-5 cells results from the combined activity of GtfA plus GtfB with sucrose plus maltodextrins, in addition to the conventional single-enzyme reactions of GtfA and GtfB with either sucrose or maltodextrins, respectively. Synthesis of such hybrid EPS from sucrose and maltodextrins/starches may widely occur in LAB strains that have similar gene
combinations. This study provides new insights into homo-EPS formation by LAB, potentially generating new EPS functionalities.

Materials and Methods

Growth conditions
*Lactobacillus reuteri* 35-5 (29) was subcultured from stocks stored at -80 °C in 10 ml of sugar-free MRS medium (liquid culture or agar plate) supplemented with 1% (w/v) glucose. The fresh cultures were inoculated anaerobically in modified sugar-free MRS medium with sucrose (5%, w/v), or maltodextrins (dextrose equivalent (DE) = 13-17, Sigma-Aldrich, US, 5%, w/v), or sucrose (2.5%, w/v) plus maltodextrins (2.5%, w/v). One liter medium contained 10 g bactopeptone, 4 g yeast extract, 5 g sodium acetate, 2 g tri-ammonium citrate, 0.2 g MgSO$_4$·7H$_2$O, 0.05 g MnSO$_4$·7H$_2$O; and 1 ml Tween 80.

Extraction of *L. reuteri* 35-5 whole cell proteins
The cells from different *L. reuteri* 35-5 cultures (10 ml) grown with various carbon sources were collected by centrifugation (10,000 ×g) and then suspended in 200 µl B-PER protein extraction buffer (Thermo, Rockford, US). The suspensions were frozen in liquid nitrogen and thawed in a 37 °C water bath for three times. Then, 2 µl lysozyme (150,000 U) was added to the suspension. After 1 h incubation at room temperature, 10 µl of each suspension was boiled with 5 µl SDS loading buffer for 10 min. After centrifugation (12,000 ×g, 5 min), the supernatants were analyzed by SDS-PAGE.

Activity staining of EPS synthesizing enzymes
After electrophoresis, the gels with *L. reuteri* 35-5 proteins were washed 3 times with dd H$_2$O (30 min each), allowing protein renaturation. Then, the gels were incubated overnight at 37°C in sodium acetate buffer (25 mM Tris/HCl, pH 5.0, 1 mM CaCl$_2$) with 5% (w/v) sucrose, 5% (w/v) maltodextrins or 2.5% (w/v) sucrose plus 2.5% (w/v) maltodextrins. The activities of EPS synthesizing enzymes were detected by staining of the polysaccharides by a periodic acid-Schiff (PAS) procedure (50). The full-length GtfB expressed and purified from *E. coli* was loaded as a reference.
**Extraction of exopolysaccharides (EPS)**

EPS were produced by *L. reuteri* 35-5 in the sugar-free MRS medium supplemented with sucrose (5%, w/v), or maltodextrins (DE = 13-17, 5%, w/v), or sucrose (2.5%, w/v) plus maltodextrins (2.5 %, w/v). Strains were grown anaerobically for 72 h at 37 °C. EPS were extracted from supernatants obtained by centrifugation of liquid MRS cultures (30). Two volumes of cold ethanol (-20 °C) were added to the supernatants and held at 4 °C overnight. The precipitates were harvested by centrifugation (12,000 ×g, 30 min, 4 °C) and re-dissolved in 1 volume of dd H₂O. The EPS were re-precipitated with 2 volumes of ethanol at 4 °C overnight. After centrifugation, the harvested precipitates were dissolved in water, and then dialyzed (10 kDa MWCO, Thermo Scientific) against water for 48 h with changes of dd H₂O every 12 h followed by freeze drying (31).

**Size exclusion chromatography (SEC)**

DMSO-LiBr (0.05M) was prepared by stirring for 3 h at room temperature followed by degassing for 15 min using an ultrasonic cleaner (Branson 1510, Branson, Danbury, CT). Samples were dissolved at a concentration of 4 mg/ml in DMSO-LiBr by overnight rotation at room temperature, followed by 30 min heating in an oven at 80 °C, obtaining clear sample solutions. The samples, cooled to room temperature, were filtered through a 0.45-µm Millex PTFE membrane (Millipore Corporation, Billerica, MA). The SEC system set-up (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) consisted of an isocratic pump, auto sampler, an online degasser, an inline 0.2 µm filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz) and MALLS (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS, Mainz) was used for data processing. Samples (100 µl) were injected into a PFG guard column using an autosampler at a flow rate of 0.5 ml/min and DMSO-LiBr as eluent. Three separate PFG-SEC columns with porosities of 100, 300 and 4000 Å were used. The columns were held at 80°C, the refractive index detector at 45 °C and the viscometer was thermostatted at 60 °C. A standard pullulan kit (PSS, Mainz, Germany) with molecular masses from 342 to 805000 Da was used. The specific RI increment value dn/dc was measured by PSS and is 0.072 (obtained from PSS company).
Enzymatic hydrolysis of EPS

The EPS samples (5 mg) were hydrolyzed by pullulanase M1 (Megazyme, Ireland) based on the protocol established previously for the *L. reuteri* 35-5 EPS derived from sucrose (25). The hydrolyzed samples were subjected to HPAEC analysis.

Monosaccharide analysis

EPS samples (0.5 mg) were dissolved in 200 µl Milli-Q water in a glass reaction tube. An equal volume of 4 M trifluoroacetic acid (TFA) was added to the samples. The samples were hydrolyzed for 4 h at 100 °C. Samples were then dried under a flow of dry nitrogen. The dry samples were dissolved in 100 µl isopropanol and dried again by evaporation under dry nitrogen. Samples were dissolved in 1 ml dimethyl sulfoxide (DMSO) and were subjected to HPAEC analysis.

Expression and purification of GtfA-ΔN, GtfB-ΔN and full-length GtfB

The N-terminally truncated GtfA-ΔN and GtfB-ΔN enzymes were produced as described before (32, 33). The full-length GtfB protein was produced in *E. coli* BL21 Star (DE3) carrying the plasmids pRSF-GtfB and pBAD22-GroELS as described previously (21). All three enzymes were first purified by His-tag affinity chromatography using a 1-ml Hitrap IMAC HP column (GE Healthcare) followed by anion exchange purification using Hitrap Q FF (GE Healthcare). Purity was checked on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by Bradford reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Enzymatic reactions

All reactions were performed in sodium acetate buffer (25 mM, pH 4.7, 1 mM CaCl₂) at 37 °C for 4 h. GtfA-ΔN (750 nM) and/or GtfB-ΔN (150 nM) were used in the reactions with 0.1 M sucrose and/or 0.25% amylose V. After 4 h incubation, the reactions were terminated by heating the samples in boiling water for 10 min. Then the products were subjected to TLC and high-pH anion-exchange chromatography (HPAEC) analyses and further separation.
**Amylose-iodine assay**

The iodine staining method was used to quantify (remaining) amylose in the reaction mixture in order to determine the degradation rate of amylose V in the absence or presence of different acceptor substrates (34). It was also used for characterization of the GtfB enzyme (33). I$_2$ (0.26 g) and KI (2.6 g) were dissolved in 10 ml distilled water to prepare the 260× concentrated stock. Prior to use, the stock was diluted 260× with distilled water. For each measurement, 150 µl iodine reagent was pipetted into microtiter plate wells, followed by addition of 15 µl enzyme reaction sample solution. The microtiter plate was shaken slowly for 5 min to allow color formation and the optical density was measured at 660 nm. Amylose V solutions (0-0.25%, w/v) were used to prepare a calibration curve. The acceptor reactions were performed using 0.125 % (w/v) amylose V as donor substrate and the activity was measured as described previously (33).

**Separation of oligosaccharides**

The individual oligosaccharides produced by GtfA-ΔN (and GtfB-ΔN) enzymes from sucrose or sucrose plus amylose V were isolated by a combination of Bio-Gel P2 size exclusion chromatography followed by preparative HPAEC. The reaction product mixtures were loaded onto the Bio-Gel P2 size exclusion column (100 x 0.9 cm) (Bio-Rad) using ammonium bicarbonate (10 mM) as eluent at a rate of ~10 ml/h. The oligosaccharide fractions were pooled and each pool was further fractionated by HPAEC-PAD on a Dionex DX500 workstation (Dionex, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (250×9mm; Dionex) and an ED40 pulsed amperometric detector, using a linear gradient of 0-500mM sodium acetate in 100 mM NaOH (3ml/min). 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 eluent and lyophilized (35). The isolated oligosaccharides were subjected to MALDI-TOF-MS and NMR analysis as described below.

**Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)**

The degree of polymerization of isolated oligosaccharides was determined 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 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 200-7000 Da. Samples (1 µl) were mixed in 1:1 ratio with a solution of 10 mg/ml 2,5-dihydroxybenzoic acid in 1:1 acetonitrile:H₂O.

**Nuclear Magnetic Resonance (NMR) spectroscopy**

The oligosaccharides produced by GtfA-ΔN plus GtfB-ΔN were desalted and exchanged twice with D₂O (99.9 atm% D, Cambridge Isotope Laboratories, Inc.) with intermediate lyophilization. The dried samples were dissolved in 0.6 ml D₂O and were filled in NMR tube for analysis. Resolution-enhanced 1D/2D 500-MHz ¹H NMR spectra were recorded with a spectral width of 4500 Hz in 16k complex data sets and zero filled to 32k in D₂O on a Varian Inova Spectrometer (NMR Center, University of Groningen) or a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 300/310 K. Suppression of the HOD signal was achieved by applying a WET1D pulse sequence. The 2D TOCSY spectra were recorded with spin-lock times of 20–200 ms using an MLEV-17 (composite pulse devised by (36)) mixing sequence. The 2D ROESY spectra were recorded with a mixing time of 200 ms using the standard Bruker XWINNMR software. The carrier frequency was set at the downfield edge of the spectrum to minimize TOCSY transfer during spin-locking. 2D 13C–¹H HSQC experiments (1H frequency 500.0821 MHz, 13C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the 1H free induction decay. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multiplication with a squared-bell function phase shifted by π/(2.3) for 2D spectra. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for 1H and δ 31.07 for 13C). MestReNova 5.3 was used for spectra annotation.

**High-pH anion-exchange chromatography (HPAEC)**

The reaction products of GtfA-ΔN, GtfB-ΔN and GtfA-ΔN plus GtfB-ΔN, and the hydrolysis products of EPS, were analyzed on a Dionex DX500 workstation (Dionex) with a 4×250 nm CarboPac PA-1 column. Samples were run with a
gradient of 30-600 mM NaAc in 100 mM NaOH (1 ml/min) and detected by an ED40 pulsed amperometric detector.

**Results and Discussion**

*4,6-α-Glucanotransferase encoding genes occur concomitantly with glucansucrase encoding genes in lactic acid bacteria*

The purified 4,6-α-GTase GtfB enzyme from *L. reuteri* 121 (strain 35-5) has been shown to produce IMMP *in vitro*. *L. reuteri* 121 (strain 35-5) cells grown on starch *in vivo* produce EPS that are structurally similar to IMMP; the extracellular and cell-associated GtfB is essential for IMMP-like EPS formation (Bai et al. submitted). Similar 4,6-α-GTase enzymes have been identified and characterized (GtfW and GtfML4) in other *L. reuteri* strains (22, 33, 37). To date, 28 gene sequences encoding characterized or putative 4,6-α-GTase enzymes (>70% similarity with GtfB enzyme) from *Lactobacillus* species are present in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). In 8 cases, the same strain also carries a gene encoding a characterized or putative glucansucrase (GS) (Table 1) (38, 39); GS and 4,6-α-GTase genes are located next to each other in 3 *L. reuteri* strains. These GS are known to convert sucrose into homo-EPS (α-glucan), e.g. the GS GtfA enzyme from *L. reuteri* 121 (strain 35-5) synthesizes reuteran with alternating α1→4 and α1→6 linkages (25, 40, 41). With larger numbers of 4,6-α-GTase encoding genes rapidly accumulating, more combinations of GS and 4,6-α-GTase enzymes are likely to be discovered in time.

**Table 1.** Combinations of 4,6-GTase and GS encoding genes occurring in LAB strains. The sequences were retrieved from NCBI database and are labelled by Genebank number.

<table>
<thead>
<tr>
<th>Strains</th>
<th>4,6-α-GTase</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus reuteri</em> 121</td>
<td>AAU08014.2</td>
<td>AAU08015.1</td>
</tr>
<tr>
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<td>ABP88725.1</td>
<td>ABP88726.1</td>
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<tr>
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<td>KEK16955.2</td>
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<td>ADQ61508.1</td>
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<tr>
<td><em>Lactobacillus plantarum</em> 16</td>
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<td>AGO09550.1</td>
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<td><em>Lactobacillus salivarius</em> GJ-24</td>
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<tr>
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<td>ACJ54706.1</td>
<td>ACJ64929.1</td>
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</table>
Activity staining of GtfA and GtfB enzymes of Lactobacillus reuteri 35-5

Periodic acid-Schiff (PAS) staining was used to visualize enzymes producing polysaccharides present in cells of L. reuteri 35-5. The PAS method has been used successfully to screen for GS positive Lactobacillus strains as denatured GS can be refolded and regain activity in SDS-PAGE gels under mild conditions (42). Following SDS-PAGE of L. reuteri 35-5 whole cell proteins the gels were washed with dd H2O and reaction buffer, and incubated with either maltodextrins (DE = 13.0-17.0), sucrose, or both, to show EPS formation by different enzymatic reactions. Cells of L. reuteri 35-5 grown on different substrates constitutively expressed an active GtfA protein (199 kDa; Fig. 1, upper panel) (24). The other gel band (~120 kDa) most likely is due to (proteolytic) degradation of GtfA (43). It has been shown that removal of the N-terminal region, involved in cell wall anchoring, does neither affect the overall activity of the GtfA enzyme nor the linkage types and size of the polymer synthesized (26, 32, 44). Previously, we already reported that also the denatured GtfB enzyme can be easily renatured in dd H2O (37). After incubation of the gels with maltodextrins, a band at ~180 kDa became visible (Fig. 1, bottom panel), which reflects active GtfB protein (179 kDa). This band was most clearly visible in extracts of L. reuteri 35-5 cells grown on glucose and maltodextrins. As expected, the 199 kDa GtfA band was not visible: maltodextrins do not act as (donor) substrates for EPS formation by GtfA (Fig. 1, bottom panel) (28). The L. reuteri 121 genome sequence (Gangoiti et al., in preparation) analysis showed that GtfA and GtfB are the only two carbohydrate acting enzymes with molecular masses larger than 150 kDa. The combined results thus show that the visible band at ~180 kDa represents the GtfB enzyme (179 kDa). To better distinguish the bands representing GtfA and GtfB activities, gels also were incubated with both sucrose and maltodextrins. This resulted in appearance of two bands at the expected and clearly different positions, demonstrating that these two enzymes are independently active on their own preferred substrate for polysaccharide formation (Fig. 1, middle panel). The combined data thus shows that GtfA and GtfB enzymes are the only two enzymes contributing to homo-EPS formation in the presence of sucrose or maltodextrins/starch.
Fig. 1. Periodic Acid-Schiff (PAS) staining of α-glucans synthesized in SDS-PAGE gels of whole-cell-extracted proteins of *L. reuteri* 35-5, visualizing the position of the renatured and active GS GtfA and 4,6-α-GTase GtfB enzymes. *L. reuteri* 35-5 was grown in MRS medium with glucose, sucrose, maltodextrins, or sucrose plus maltodextrins. Gels were incubated with sucrose (5%, w/v), maltodextrins (5%, w/v) or sucrose (2.5%, w/v) plus maltodextrins (2.5%, w/v) overnight at 37 °C, pH 4.7. Heterologously expressed and purified GtfB from *E. coli* was loaded as reference protein.
SEC analysis of the products formed by *L. reuteri* 35-5 from sucrose, maltodextrins, and sucrose plus maltodextrins

The GS GtfA mainly acts on sucrose as donor/acceptor substrate, resulting in chain length elongation and oligo-/polysaccharide synthesis. To some extent, GtfA also is able to use maltose/maltodextrins as acceptor substrate (with sucrose as donor substrate) (32). 4,6-α-GTase GtfB mainly acts on maltodextrins/starches, cleaving α1→4 linkages and making α1→6 linkages. Here, we report that, to some extent, GtfB also use sucrose as acceptor substrate (with amylose V as donor substrate) (Fig. 5). It is therefore entirely likely that the final EPS formed by *L. reuteri* 35-5 cells in the presence of both substrates is not just a mixture, but may have different structures and thus characteristics. The EPS produced by *L. reuteri* 35-5 from sucrose (5%, w/v), maltodextrins (5%, w/v), and sucrose (2.5%, w/v) plus maltodextrins (2.5%, w/v) were isolated from liquid cultures by ethanol precipitation and subjected to SEC analysis (Fig. 2). The α-glucan EPS derived from sucrose is of high molecular mass (MM) up to 34.7 MDa (24), whereas the majority (97.5%) of the EPS derived from maltodextrins is of low MM (Bai et al., submitted). Growth of *L. reuteri* 35-5 on sucrose plus maltodextrins resulted in synthesis of an EPS mixture with both high and low MM (Fig. 2) In view of the size distribution obtained, the EPS35-5-sucrose+maltodextrins appears to be composed of a mixture of the EPS35-5-sucrose and EPS35-5-maltodextrins. A slight difference was observed in the low MM region (at approx. 3.5 kDa) between the SEC chromatograms of EPS35-5-maltodextrins and EPS35-5-sucrose+maltodextrins (Fig. 2). The question remained whether the EPS structures at high and low MM changed when *L. reuteri* 35-5 was grown in the presence of both sucrose and maltodextrins.
Enzymatic hydrolysis of the products formed by \textit{L. reuteri} 35-5 from sucrose, maltodextrins, and sucrose plus maltodextrins

To explore structural differences among EPS derived from different substrates, these 3 EPS were hydrolyzed by an excess amount of pullulanase type I. This enzyme specifically attacks $\alpha_1 \rightarrow 6$ linkages in the backbone chains of pullulan and at branching points of starch molecules (45). As reported, this pullulanase degraded the EPS35-5-sucrose (reuteran) into mainly maltose, panose, maltotriose and maltooltriaose (25) (see Fig. 3). This pullulanase also degraded the EPS35-5-maltodextrins, resulting in a low yield of maltooligosaccharides and a trace amount of glucose. Interestingly, when the EPS35-5-sucrose+maltodextrins were hydrolyzed by pullulanase, glucose and some maltooligosaccharides were the main product. Interestingly also a series of new oligosaccharides were formed (eluting between 20 to 30 min) (Fig. 3). Obviously, the EPS35-5-sucrose+maltodextrins is not a simple mixture of EPS35-5-sucrose and EPS35-5-
maltodextrins. *L. reuteri* 35-5 cells grown on MRS medium with sucrose plus maltodextrins synthesized EPS that are structurally different from those derived from either sucrose or maltodextrins.

![HPAEC analysis](image)

**Fig. 3.** HPAEC analysis of the hydrolysis products of EPS (5 mg) digested by excess amounts of pullulanase M1 for 72 h. EPS were produced by *L. reuteri* 35-5 in MRS liquid cultures supplemented with sucrose (5%, w/v), maltodextrins (5%, w/v), and sucrose (2.5%, w/v) plus maltodextrins MD13-17 (2.5%, w/v) and isolated by ethanol precipitation.

**In vitro reactions of GtfA-ΔN or GtfB-ΔN with sucrose and amylose V**

As the *L. reuteri* 35-5 in vivo system for EPS formation is too complex to study directly, the purified GtfA-ΔN and GtfB-ΔN enzymes (26, 33) were used in vitro to study the initial formation of oligosaccharides from sucrose and/or maltodextrins. GtfA-ΔN or GtfB-ΔN were separately incubated with sucrose, amylose V and sucrose plus amylose V. Amylose V was used instead of maltodextrins to avoid interference between the substrate and the oligosaccharide products in the HPAEC analysis. As shown in Fig. 4, GtfA-ΔN is inactive with amylose V as no degradation products resulting from hydrolysis activity were visible in the HPAEC spectrum. We also failed to detect activity of GtfA-ΔN with amylose V when using the amylose-iodine assay (data not shown). Sucrose
is the preferred substrate for GtfA-ΔN and the initial products have been annotated previously (Fig. 4), representing linear oligosaccharides with alternating α1→4 and α1→6 linkages (28). Addition of amylose V to the sucrose/GtfA-ΔN incubation mixture also did not result in differences in the oligomer product profile. Unlike maltose, amylose V may be too large to act as acceptor substrate for sucrose/GtfA-ΔN reaction. Addition of amylose V, however, may influence the formation of polysaccharides, resulting in formation of new α1→6 and α1→4 linkages at the non-reducing ends of the amylose V substrate.

Fig. 4. HPAEC spectra of the products of GtfA-ΔN (750 nM) incubated with 0.1 M sucrose or/and 0.25% (w/v) amylose V for 2 h at 37 °C and pH 4.7. The oligosaccharide structures were annotated as in Dobruchowska et al (28).

GtfB is inactive on sucrose, but acts on amylose V, cleaving α1→4 linkages and making α1→6 linkages, which also results in the formation of glucose and maltose (Fig. 5), as reported previously (23). In the presence of both sucrose and amylose V, GtfB-ΔN uses sucrose as acceptor substrate and elongates it with one glucosyl unit, introducing either an α1→4 or α1→6 linkage (predominant), using amylose V as donor substrate. Almost no glucose was generated from both sucrose and amylose V, indicating that the (minor) hydrolysis activity of GtfB-ΔN was strongly suppressed.
Fig. 5. HPAEC analysis of the products of GtfB-ΔN (150 nM) incubated with 0.1 M sucrose or/and 0.25% (w/v) amylose V for 2 h at 37 °C and pH 4.7. Peaks are annotated on the basis of retention times of known controls (28).

**In vitro reactions of GtfA-ΔN plus GtfB-ΔN with sucrose and/or amylose V**

The purified GtfA-ΔN plus GtfB-ΔN enzymes were incubated with sucrose, amylose V, or sucrose plus amylose V, to mimic the *in vivo* synthesis of EPS by *L. reuteri* 35-5 cells. When incubating both enzymes with the single substrates, similar product profiles were obtained as seen with the single enzymes (Fig. 6). Incubation of both enzymes with both substrates, however, resulted in clear differences. The amount of oligomers with an α1→6 linked non-reducing end (peaks 1, 4, and 5 in Fig. 6) increased compared to those derived from sucrose as sole substrate.

In addition, several new products were formed (e.g. peaks I and II) (Fig. 6). Both fractions were found to contain compounds of a single DP (MALDI-TOF-MS analysis; DP6, data not shown).
Fig. 6. HPAEC analysis of the products of GtfA-ΔN (750 nM) and GtfB-ΔN (150 nM) incubated with 0.1 M sucrose or/and 0.25% (w/v) amylose V for 2 h at 37 °C and pH 4.7. The oligosaccharide structures were annotated as in Dobruchowska et al (28). Two novel peaks labelled I and II were annotated based on 1D/2D NMR analysis (Fig. 7).

NMR analysis of novel products synthesized by GtfA-ΔN plus GtfB-ΔN from sucrose plus amylose V

The new products synthesized in vitro by GtfA-ΔN plus GtfB-ΔN from sucrose plus amylose V were analyzed by 1D and 2D NMR spectroscopy including TOCSY (150 ms). Starting from the anomeric signals of the residues g, f, A, B, E, J in the 2D TOCSY spectra (Fig. 7) all chemical shifts of the non-anomeric protons of differently substituted Glc residues could be assigned for two isomeric structures I and II (Table 2). Following the earlier developed structural-reporter-group concept rules (46), the set of chemical shifts of residue A (H-1 track, δ 5.380I/5.374II) was in agreement with an internal –(1→4)-α-D-Glc(1→4)- unit. Although the anomeric signals of B, E and J strongly overlap, the differences in chemical shifts of their non-anomeric protons could be deduced from the TOCSY built-up series of mixing times (20 ms, 60 ms, 80 ms, 150 ms). The TOCSY B H-1 track (δ 4.961) showed the set of chemical shifts corresponding to the values found for internal –(1→4)-α-D-Glc(1→6)- unit; that of residue E (δ 4.955I/4.947II) with a terminal α-D-Glc(1→6)- unit; that of residue J (δ 4.967I/II) with an internal –(1→6)-α-D-Glc(1→6)- unit (Table 2). Typical chemical shift
values for sucrose fragment (Glc, \(g\); Fru, \(f\)) are \(\delta 5.427\), stemming from Glc \(g\) H-1, and \(\delta 4.220\), stemming from Fru \(f\) H-3 (28). The small differences in chemical shift between residue A\(^I\) H-1 and A\(^{II}\) H-1, are due to proximity of A to the sucrose fragment and/or terminal residue E. Additional assignments and confirmation of the assignments were obtained from ROESY cross-peaks and by correlating the \(^1\)H resonances to the corresponding \(^13\)C resonances in the HSQC spectra (data not shown). These two novel oligosaccharides (DP = 6) with internally or terminally consecutive \(\alpha1\rightarrow6\) linkages are structurally different from all the reported oligosaccharides produced by the single GtfA or GtfB enzymes from their preferred substrates.

*In vitro* incubations of GtfA-\(\Delta N\) and GtfB-\(\Delta N\) with sucrose plus amylase V clearly affected initial oligosaccharide synthesis (Figs. 4-7). This reflects the cross-activity of the cell-associated GtfA and GtfB enzymes in EPS formation *in vivo*, also resulting in modified product structures (Fig. 3).

![Fig. 7. 1D \(^1\)H NMR and TOCSY analysis of the DP6 compounds in peaks I and II (Fig. 6).](image)

<table>
<thead>
<tr>
<th>Residue</th>
<th>H-1a</th>
<th>H-1b</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>H-6a</th>
<th>H-6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. \(^1\)H chemical shifts\(^a\) (D\(_2\)O, 300K) of linear oligomers (DP6) labelled as I and II in Figs. 6 and Fig. 7.
Specificity of GtfB-∆N using GtfA-∆N products as acceptor substrates

The possible use of GtfA-∆N products from sucrose as acceptor substrates for GtfB-∆N activity with amylose V was studied in more detail. Specific oligosaccharides produced by GtfA-∆N from sucrose were isolated as described by Dobruchowska et al. and incubated with GtfB-∆N in the presence or absence of amylose V as a donor substrate (28). As shown in Fig. 8a, a DP7 oligomer (product 1) with an α1→6 linked non-reducing end can serve as acceptor substrate for GtfB-∆N in the presence of amylose V as donor substrate, resulting in formation of larger products. When compared to another GtfA-∆N product (product 2, DP6) with an α1→4 linked non-reducing end, product 1 with α1→6 linked non-reducing end is clearly preferred as acceptor substrate by GtfB-∆N incubated with amylose V (Fig. 8b). Product 1 is completely converted into a series of elongated products, while most of product 2 remains in the reaction. During polymer synthesis by GtfA-∆N from sucrose, GtfB-∆N thus is able to use the intermediate products with α1→6 linked non-reducing ends as acceptor substrates.

$^a$In ppm relative to the signal of internal acetone (δ 2.225 for $^1$H)
Fig. 8. HPAEC spectra of products of GtfB-ΔN (150 nM) incubated with isolated GtfA-ΔN products in the presence or absence of amylose (0.25%, w/v) for 2 h at 37 °C and pH 4.7. The structures of products 1 and 2 were annotated according to Dobruchowska et al (28).

To further characterize the acceptor substrate specificity of GtfB-ΔN, the transglycosylation factors (TF) with amylose V in the presence of absence of small acceptor substrates were determined (47-49). The preferred substrate for GtfA-ΔN is sucrose, which serves as a strong acceptor substrate for GtfB-ΔN, compared to glucose, maltose, and maltotriose. This is in accordance with the observation in Fig. 5. The TF for panose, a trisaccharide with an α1→6 linked non-reducing end, is higher than for maltotriose with an α1→4 linked non-reducing end (see Table 3), proving that acceptor substrates with a α1→6 linked non-reducing ends are stronger acceptors than those with α1→4 linked non-reducing ends.
Table 3. Amylose V (0.25%, w/v) degradation activity (total activity, measured using iodine staining assay) (33) and transglycosylation factors of the *L. reuteri* 121 GTFB-ΔN (60 nM) enzyme incubated with amylose V in the presence of different acceptor substrates (10 mM). Experiments were carried out in duplicate and the average data is given.

<table>
<thead>
<tr>
<th>Amylose V degradation rate (U/mg)</th>
<th>Transglycosylation factors(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without acceptor substrate</td>
<td>2.8</td>
</tr>
<tr>
<td>Maltose(^b)</td>
<td>7.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.4</td>
</tr>
<tr>
<td>Maltotriose(^b)</td>
<td>7.5</td>
</tr>
<tr>
<td>Panose</td>
<td>24.7</td>
</tr>
</tbody>
</table>

\(^a\) The transglycosylation factor is defined as the ratio of amylose degradation rates in the presence/absence of an acceptor substrate.

\(^b\) The data refers to the paper published previously (33).

Conclusions

In this study we have demonstrated that *L. reuteri* 35-5 with both GS and 4,6-α-GTase enzymes synthesizes a new type of EPS when grown with sucrose plus maltodextrins, compared to the EPS derived from either of the single carbon sources. Subsequent *in vitro* enzymatic reactions of GtfA-ΔN plus GtfB-ΔN with sucrose plus amylose V resulted in synthesis of new initial oligosaccharides with terminally and internally consecutive α1→6 linkages. These initially formed GtfA-ΔN oligosaccharides are strong acceptor substrates for the GtfB-ΔN enzyme when incubated with amylose V. This underpins the above observation that *in vivo*, GtfB/maltodextrins affect EPS synthesis by GtfA from sucrose. In the presence of starch plus sucrose, EPS synthesized by *L. reuteri* strain 35-5 cells resulted not only from the action of the GtfA and GtfB enzymes with their preferred substrates sucrose and maltodextrins, but also yielded a new type of EPS reflecting the cross-reactivity of both enzymes. EPS formation in the presence of sucrose plus starch thus may influence biofilm formation by lactobacilli in the oral cavity or gastrointestinal tract of mammals. Also the relationship between dietary intake of sucrose and starch, dental caries formation and degradability of EPS dietary fibers, are likely to be affected.
Acknowledgements

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References


