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The levansucrase and inulosucrase enzymes of *Lactobacillus reuteri* 121 catalyse processive and non-processive transglycosylation reactions

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Bacterial fructosyltransferase (FTF) enzymes synthesize fructan polymers from sucrose. FTFs catalyse two different reactions, depending on the nature of the acceptor, resulting in: (i) transglycosylation, when the growing fructan chain (polymerization), or mono- and oligosaccharides (oligosaccharide synthesis), are used as the acceptor substrate; (ii) hydrolysis, when water is used as the acceptor. *Lactobacillus reuteri* 121 levansucrase (Lev) and inulosucrase (Inu) enzymes are closely related at the amino acid sequence level (86 % similarity). Also, the eight amino acid residues known to be involved in catalysis and/or sucrose binding are completely conserved. Nevertheless, these enzymes differ markedly in their reaction and product specificities, i.e. in β(2→6)- versus β(2→1)-glycosidic-bond specificity (resulting in levan and inulin synthesis, respectively), and in the ratio of hydrolysis versus transglycosylation activities [resulting in glucose and fructooligosaccharides (FOSs)/polymer synthesis, respectively]. The authors report a detailed characterization of the transglycosylation reaction products synthesized by the *Lb. reuteri* 121 Lev and Inu enzymes from sucrose and related oligosaccharide substrates. Lev mainly converted sucrose into a large levan polymer (processive reaction), whereas Inu synthesized mainly a broad range of FOSs of the inulin type (non-processive reaction). Interestingly, the two FTF enzymes were also able to utilize various inulin-type FOSs (1-kestose, 1,1-nystose and 1,1,1-kestopentaose) as substrates, catalysing a disproportionation reaction; to the best of our knowledge, this has not been reported for bacterial FTF enzymes. Based on these data, a model is proposed for the organization of the sugar-binding subsites in the two *Lb. reuteri* 121 FTF enzymes. This model also explains the catalytic mechanism of the enzymes, and differences in their product specificities.

INTRODUCTION

Bacterial fructosyltransferase (FTF) enzymes catalyse the transfer of the fructosyl residue from sucrose (and raffinose) to various acceptor substrates. FTF enzymes are known to catalyse two different reactions: (i) transglycosylation, using the growing fructan chain (polymerization), or sucrose, gluco- and fructosaccharides (oligosaccharide synthesis), as the acceptor substrate; (ii) hydrolysis of sucrose, when water is used as the acceptor. These FTF enzymes belong to glycoside hydrolase (GH) family 68 (GH68) (http://afmb.cnrs-mrs.fr/CAZY/) (Coutinho & Henrissat, 1999). They are β-retaining enzymes, employing a double-displacement mechanism that involves formation and subsequent hydrolysis of a covalent glycosyl–enzyme intermediate (a ping-pong type of mechanism) (Chambert *et al.*, 1974; Hernández *et al.*, 1995; Song & Jacques, 1999).

Most of the known bacterial FTFs are levansucrases (Lev; EC 2.4.1.10), synthesizing fructan polymers composed of β(2→6)-linked fructose units (levan) (Gross *et al.*, 1992; Steinmetz *et al.*, 1985; van Hijum *et al.*, 2004). Limited information is available about bacterial inulosucrases (Inu; EC 2.4.1.9), which produce β(2→1)-linked fructan polymers (inulin) (Baird *et al.*, 1973; Olivares-Illana *et al.*, 2002; Rosell & Birkhed, 1974; van Hijum *et al.*, 2002).

Abbreviations: DP, degree of polymerization; FOS, fructooligosaccharide; FTF, fructosyltransferase; GalGF, α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside; GH, glycoside hydrolase; HPAEC, high-performance anion-exchange chromatography; Inu, *Lb. reuteri* 121 inulosucrase; Lev, *Lb. reuteri* 121 levansucrase.

Only a few Lev enzymes have been characterized with respect to products synthesized from sucrose. The ratio between polymerization and oligosaccharide synthesis activities has been found to differ significantly, depending on the source of the enzyme. Hernandez *et al.* (1995) reported that SacB levansucrase of *Bacillus subtilis* catalyses the formation of a high-molecular-mass levan, without transient accumulation of oligofructan molecules. This suggests that the growing polymer chain remains bound to the enzyme, and that fructan-chain elongation proceeds via a processive type of reaction. In contrast, the enzymes of *Gluconacetobacter diazotrophicus*, *Zymomonas mobilis* and *Lactobacillus sanfranciscensis* have been found to synthesize mainly short fructooligosaccharides (FOSs; kestose and nystose) from sucrose (Doelle *et al.*, 1993; Hernández *et al.*, 1995; Korakli *et al.*, 2001, 2003); thus, these enzymes may employ a non-processive type of reaction, involving release of the fructan chain after (virtually) each fructosyl transfer. The available three-dimensional structures of the *B. subtilis* SacB levansucrase, which synthesizes mainly large polymers, and the FOS-synthesizing levansucrase from *G. diazotrophicus* (Martinez-Fleites *et al.*, 2005; Meng & Futterer, 2003), show that the active-site architecture of both levansucrase enzymes is identical (Martinez-Fleites *et al.*, 2005; Meng & Futterer, 2003). Therefore, it is unclear which structural features determine the polymerization versus oligosaccharide synthesis ratio, and the use of a processive or a non-processive mechanism for fructan-chain growth in FTF enzymes.

Besides sucrose, raffinose, but neither kestose (GF2) nor nystose (kestotetraose, GF3), is used as a substrate by the family GH68 FTF enzymes (Hernández *et al.*, 1995; Trujillo *et al.*, 2004). FTFs also use various saccharides (e.g. maltose, maltotriose, raffinose, arabinose, xylose and sucrose) as fructosyl-acceptor substrates in their transglycosylation reactions, with sucrose as the donor substrate (e.g. the levansucrase enzymes of *Aerobacter levanicum*, *B. subtilis* and *Lb. sanfranciscensis*) (Tanaka *et al.*, 1981; Tieking *et al.*, 2005; Hestrin *et al.*, 1956).

Enzymes synthesizing inulin polymers have been identified in three Gram-positive bacterial species: *Streptococcus mutans* JC2 (Baird *et al.*, 1973; Rosell & Birkhed, 1974), *Leuconostoc citreum* CW28 (Olivares-Illana *et al.*, 2002) and *Lactobacillus reuteri* 121 (van Hijum *et al.*, 2002). TLC analysis of the reaction products of the inulosucrase of *S. mutans* JC2 reveals that, beside traces of sucrose, kestose and nystose, a fructose-containing compound with a degree of polymerization (DP) >7 (FOS or/and inulin) is synthesized (Heyer *et al.*, 1998). Further information about products synthesized from sucrose by inulosucrase enzymes is lacking at present.

Previously, we have isolated and characterized two FTF enzymes from *Lb. reuteri* 121. One of these enzymes is capable of synthesizing a levan (levansucrase; Lev) (van Hijum *et al.*, 2004), and the other synthesizes an inulin (inulosucrase; Inu) (van Hijum *et al.*, 2002). These two FTF enzymes are very similar at the amino acid level (86 % similarity and 56 % identity, within 768 aa), and both depend on Ca^{2+} ions for activity (to a different extent), and display similar high temperature optima (Ozimek *et al.*, 2005). Characterization of the substrate and product specificities of the *Lb. reuteri* 121 Inu and Lev enzymes is yet to be carried out. The (eco)physiological functions of the Lev and Inu enzymes, and their products, in *Lb. reuteri* 121 are unknown. It is most likely that the fructans function in adhesion of the cells to surfaces, e.g. in the oral cavity (Rozen *et al.*, 2001). The fructan/FOS products synthesized by *Lb. reuteri* 121 are of special interest because they may contribute to human health as prebiotics (Menne *et al.*, 2000). Moreover, several *Lb. reuteri* strains have been designated probiotics (Casas *et al.*, 1998; Valeur *et al.*, 2004).

Here we report a detailed characterization and comparison of the products formed by the *Lb. reuteri* 121 Inu and Lev enzymes from sucrose and inulin-type FOS as substrates. The data clearly showed that Lev and Inu catalysed processive and non-processive reactions, respectively. Also, to the best of our knowledge, this was the first time that Lev and Inu were observed to catalyse a disproportionation reaction. The data allow us to propose the first known model of the organization of the FTF active site, and its acceptor substrate sugar-binding subsites.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* strain Top10 (Invitrogen) was used for expression of the *Lb. reuteri* 121 inulosucrase (*inu*; GenBank accession number AF459437) and levansucrase (*lev*; GenBank accession number AF465251) genes. Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning and expression purposes. Plasmid-carrying *E. coli* strains were grown at 37 °C on Luria–Bertani medium (Sambrook *et al.*, 1989), supplemented with 100 μg ampicillin ml^{-1}, and 0·02 % (w/v) arabinose for *ftf* gene induction. The proteins were expressed in *E. coli* as constructs with a C-terminal truncation of 100 (Inu) or 32 (Lev) aa residues, resulting in improved expression and a C-terminal polyhistidine tag (van Hijum *et al.*, 2002, 2004).

Purification of FTFs, and enzyme activity assay. Inu and Lev of *Lb. reuteri* 121 were produced and purified by Ni-NTA affinity chromatography, as described previously (van Hijum *et al.*, 2002). Purity was monitored by SDS-PAGE analysis. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad), with BSA as the standard. Unless indicated otherwise, all enzymic incubations were performed in the following reaction mixture: 50 mM sodium acetate buffer, pH 5·4, supplemented with 840 mM sucrose and 1 mM $CaCl_2$. Activities of purified Inu (6·9 μg ml^{-1}) and Lev (9·6 μg ml^{-1}) were determined at 22, 37 and 50 °C. After preincubation of the assay mixture at the appropriate assay temperature for 5 min, reactions were started by enzyme addition. Samples were taken every 3 min, and used to determine the amount of glucose and fructose released from sucrose (van Hijum *et al.*, 2001). The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). The amount of fructose (V_F) formed is a measure of the hydrolytic activity. The tranglycosylation activity was calculated by subtracting the amount of free fructose from glucose ($V_G - V_F$). One unit of enzyme activity is defined as the release of 1 μmol of monosaccharide per min. Curve fitting of the data was performed as described previously, using either the Michaelis–Menten formula, or the three-parameter Hill formula

(van Hijum *et al.*, 2002, 2004). Experiments were performed in duplicate, and the data presented are mean values, showing less than 5 % variation.

Analysis of reaction products

TLC analysis. To investigate the product specificity of Inu and Lev with sucrose and various acceptor substrates, reactions were performed at 22 and 37 °C with 2 U FTF ml^{-1} (measured at 37 °C) (Figs 2 and 4). Samples were taken at different time points (from 5 min to 24 h), diluted threefold in water, and spotted (1 µl) on TLC plates (Silica gel 60 F_{254}; Merck). Solutions (100 mM) of fructose (Merck), sucrose (Acros Organics), 1-kestose (GF2) (Fluka Chemie), 1,1-nystose (GF3) (Fluka Chemie AG), 1,1,1-kestopentaose (GF4) (Megazyme International Ireland) were used as standards (0·5 µl spots). The plates were run once in a butanol : ethanol : water (5 : 5 : 3, v/v/v) mixture. Specific staining of fructose-containing sugars was obtained using a urea spray (Trujillo *et al.*, 2004).

Lev and Inu enzyme activities with 1-kestose, 1,1-nystose, 1,1,1-kestopentaose and raffinose [α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside; GalGF] (Sigma Aldrich), and the products synthesized, were analysed by adding 840 mM of the sugar, instead of sucrose, to the reaction mixture.

High-performance anion-exchange chromatography (HPAEC). HPAEC (Dionex) was used to separate and/or determine the concentrations of fructose, glucose, sucrose, 1-kestose, 6-kestose, 1,1-nystose and 1,1,1-kestopentaose after complete consumption of sucrose (end-point conversion, incubation at 37 °C for 24 h, with 840 mM sucrose and 2 U FTF ml^{-1}) (Table 1). No standards were available for 'unknown-1' and bifructose (Fig. 3, Table 1); therefore, the calibration curve for 1-kestose, representing the oligosaccharide most closely related to these compounds, was used to estimate the approximate concentrations of unknown-1 and bifructose. The Dionex analysis protocol allowed us to calculate the concentration of most of the oligosaccharide products smaller than kestopentaose. The concentrations of all the individual oligosaccharides were added up, and, by subtracting this value from the sucrose concentration used, the amounts of products larger than kestopentaose were calculated (Table 1). Individual oligosaccharides, and products larger than kestopentaose, were expressed as a percentage of the total amount of sucrose initially present in the incubation mixture. There were unavoidable discrepancies because of the use of the 1-kestose calibration curve to determine concentrations of unknown-1 and bifructose (see above), and the presence of other unknown oligosaccharides (especially for Lev) smaller than kestopentaose, which were not taken into account in our calculations (Fig. 3, Table 1).

Table 1. HPAEC of the reaction products of Inu and Lev

The values for reaction products of Inu and Lev are percentages of the total amount of sucrose converted. The reaction was carried out using 840 mM sucrose and 2 U FTF ml^{-1}, at 37 °C for 24 h.

Reaction product	Enzyme	
	Inu	Lev
Hydrolysis	18·3	51·6
Transglycosylation	81·7	48·4
1-Kestose	6·7	5·4
Unknown-1	3·3	7·4
Bifructose	0·0	6·0
1,1-Nystose	9·2	1·7
1,1,1-Kestopentaose	5·5	0·0
> Kestopentaose	57	28*

*Lev synthesized additional unknown small-size oligosaccharides (Fig. 3) that were not taken into account; therefore, this value is an overestimate. Experiments were performed in duplicate. Data shown are representative values.

Separation of oligosaccharides was achieved by using a CarboPac PA1 anion-exchange column (250 mm × 4 mm; Dionex) coupled to a CarboPac1 Guard column (Dionex). The following gradient was used: eluent A at 100 % (0 min), 70 % (10 min), 60 % (25 min), 10 % (80 min), 0 % (83 min), 100 % (91 min). Eluent A was 0·1 M sodium hydroxide, and eluent B was 0·1 M sodium hydroxide in 0·6 M sodium acetate. Detection was performed with an ED40 electrochemical detector (Dionex), with an Au working electrode, and an Ag/AgCl reference electrode. The amount of sucrose utilized during the reaction reflected the total enzyme activity. The amount of fructose synthesized reflected the hydrolytic enzyme activity. The total activity minus the hydrolytic activity reflected the transglycosylation enzyme activity (polymer and FOS formation). Based on these data, the hydrolysis versus transglycosylation ratio (end-point conversion) was calculated.

The reaction products synthesized by Inu and Lev (2 U ml^{-1} at 37 °C; activity measured with sucrose), with sucrose and kestopentaose (840 mM) as substrates, were analysed in the same way (after 24 h incubation), using Raftilose L85 as the FOS standard, and a 1 : 1 mixture of Raftiline ST-Gel and Raftiline HP (Orafti), representing chicory inulin standards. The Raftilose L85 standard is based on a partially hydrolysed (fructanase-treated) chicory inulin, and contains a range of GF*n* and F*n* molecules (GF represents glucose linked to fructose, and F represents fructose) (Figs 3 and 5). Identification of the FOS in the standard was performed by preparative separation (HPAEC), followed by determination of the monosugar composition of the different fractions. Pure 6-kestose (obtained from Professor K. Buchholz, Technical University of Braunschweig, and Dr Thielecke, Innosweet), 1-kestose and 1,1-nystose confirmed the retention times of the short-fructan components. Oligofructose molecules were identified as fractions containing pure fructose: F2 was assigned as the first pure-fructose peak after fructose itself, F3 as the second pure-fructose peak after fructose, and so on. Retention times and order were in agreement with van Loo *et al.* (1995).

RESULTS

FTF activity

Kinetic studies at 22, 37 and 50 °C revealed that the transglycosylation activity of *Lb. reuteri* 121 Inu increased gradually with increasing sucrose concentration, and that the enzyme could not be saturated by its substrate (Fig. 1, 37 °C) (see also van Hijum *et al.*, 2003). At sucrose concentrations lower than 200 mM (at 37 °C), hydrolysis was the main enzyme activity (not shown). At higher sucrose concentrations, transglycosylation increased gradually, reaching 90 % or more of total enzyme activity at 1·7 M sucrose.

Lb. reuteri 121 Lev mainly hydrolysed sucrose at concentrations below 85 mM (at 37 °C). Above 85 mM sucrose, the transglycosylation activity of Lev gradually became significant (Fig. 1); both the Lev activities displayed Michaelis–Menten type kinetics. Lev transglycosylation activity,

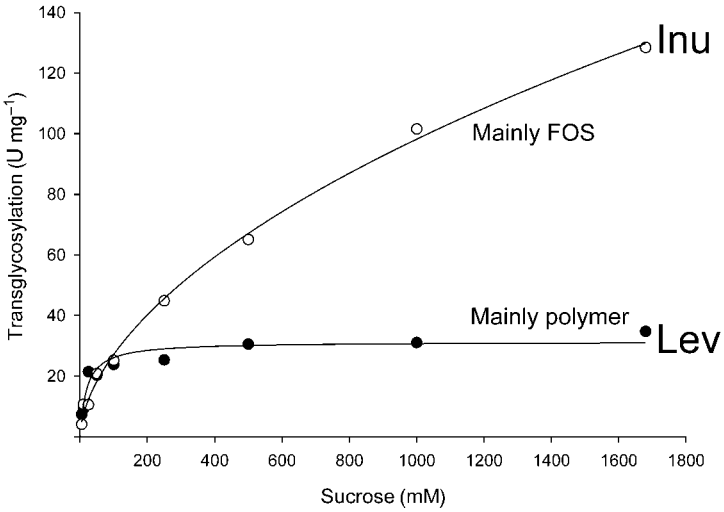

Fig. 1. Comparison of the transglycosylation enzyme activities (initial rates at 37 °C) at different sucrose concentrations, using purified Inu (6·9 μg ml^{-1}) and Lev (9·6 μg ml^{-1}) proteins. Experiments were performed in duplicate. Product analysis showed that Inu synthesized mostly FOS, whereas Lev synthesized mostly polymeric material.

however, never exceeded 50 % of total enzyme activity (not shown). Compared with Inu, the transglycosylation activity of Lev varied with sucrose concentration in a relatively small range of concentrations (Fig. 1). These marked differences in enzyme kinetics prompted us to study the substrate and reaction product specificities of the *Lb. reuteri* 121 Lev and Inu enzymes in more detail.

TLC analysis of FTF products from sucrose

TLC analysis of the reaction products formed after incubation with sucrose showed that at 22 °C Inu initially synthesized a broad range of FOSs (Fig. 2). Compared with Inu, Lev produced a relatively limited range of FOSs, but synthesized much more levan polymer (see also Trujillo *et al.*, 2004), which was detected at very early incubation times. Both Inu and Lev synthesized similar amounts of kestose (confirmed by anion-exchange chromatography, see below; Fig. 3, Table 1). Inu was able to synthesize nystose from the very early stage of the reaction: after only 5 min incubation, traces of nystose were visible. The nystose concentration remained constant once it reached a similar level to kestose (after 1 h), and synthesis of FOS of a larger size started. This scheme applied to all sizes of FOS synthesized during Inu incubations with sucrose; the presence of FOSs of DP $(n+1)$ was detected only when DP n reached a certain threshold level.

Both Inu and Lev of *Lb. reuteri* 121 have the highest specific activity towards sucrose utilization at 50 °C (Ozimek *et al.*, 2005). During incubation at higher temperatures (37 and 50 °C, data not shown), the increase in Inu and Lev enzyme activities resulted in a much earlier depletion of sucrose. Inu subsequently started to degrade its larger-size FOSs, and Lev started to degrade its larger-size FOSs and the levan polymer (data not shown). Chemical degradation of inulin, levan and FOS substrates was not observed at 50 °C for 24 h, in the absence of enzymes. Furthermore, when Inu and Lev were incubated with purified inulin oligosaccharides and levan, respectively, they also showed fructose release (data not shown).

HPAEC analysis of FTF products from sucrose

HPAEC allowed the identification and quantification of the oligosaccharide products formed by the two FTF enzymes,

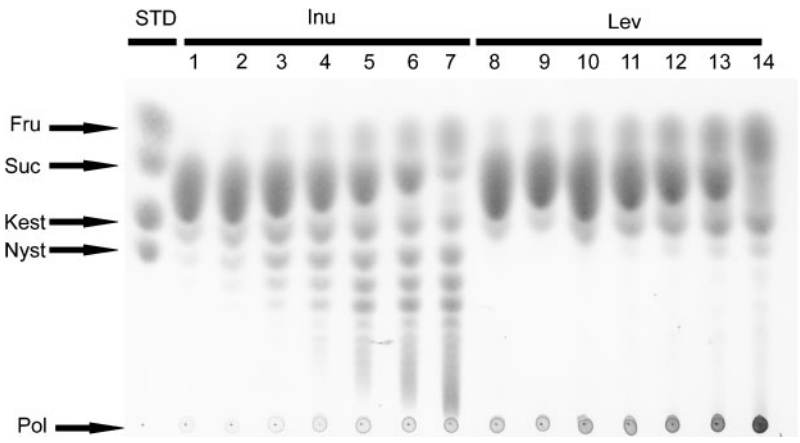

Fig. 2. TLC analysis of the Inu and Lev products from sucrose (840 mM) at 22 °C (using FTF enzymes at 2 U ml^{-1}, measured at 37 °C). Samples were taken at different time points. Lanes: 1 and 8, 5 min; 2 and 9, 20 min; 3 and 10, 1 h; 4 and 11, 2 h; 5 and 12, 4 h; 6 and 13, 7 h; 7 and 14, 24 h. The STD lane contained the following standards (from the top): Fru, fructose; Suc, sucrose; Kest, 1-kestose (GF2); Nyst, 1,1-nystose (GF3). Pol, polymer.

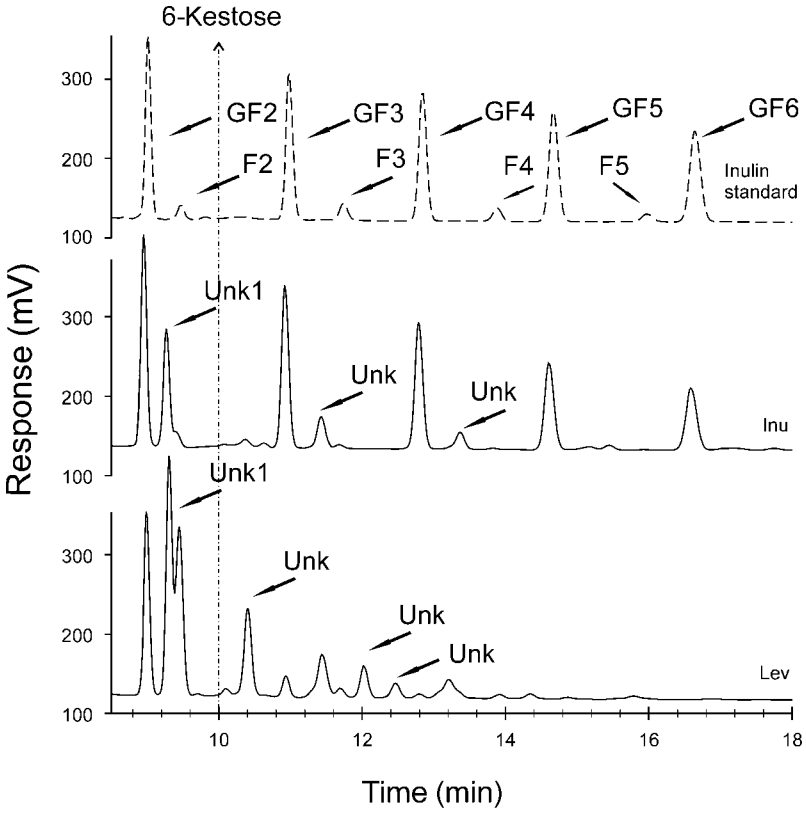

Fig. 3. HPAEC of the Inu and Lev products from sucrose (840 mM) at 37 °C after 24 h incubation (using FTF enzymes at 2 U ml^{-1}). Raftilose L85 was used as an FOS standard, and a 1 : 1 mixture of Raftiline ST-Gel and Raftiline HP was used as an inulin standard. Known oligosaccharides [GF*n* and F*n* with $\beta(2\rightarrow1)$ linkages, and 6-kestose] are indicated. Unk, unknown oligosaccharides.

and determination of the transglycosylation versus hydrolysis product ratio. At the final stage of the reaction (end-point conversion, 24 h incubation), Inu and Lev had utilized (almost) all available (840 mM) sucrose (94 and 98 %, respectively). Inu converted 81·7 % of the sucrose into transglycosylation products, and 18·3 % of the sucrose was hydrolysed; for Lev, the values were 48·4 and 51·6 %, respectively (Table 1). In the case of Inu, 57 % of the sucrose was converted into FOS material larger than GF4, and 25 % was converted into FOS that was the same size as GF4 or smaller (i.e. 1-kestose, 1,1-nystose, and an unidentified product eluting after 9·5 min) (Table 1, Fig. 3). The amount of

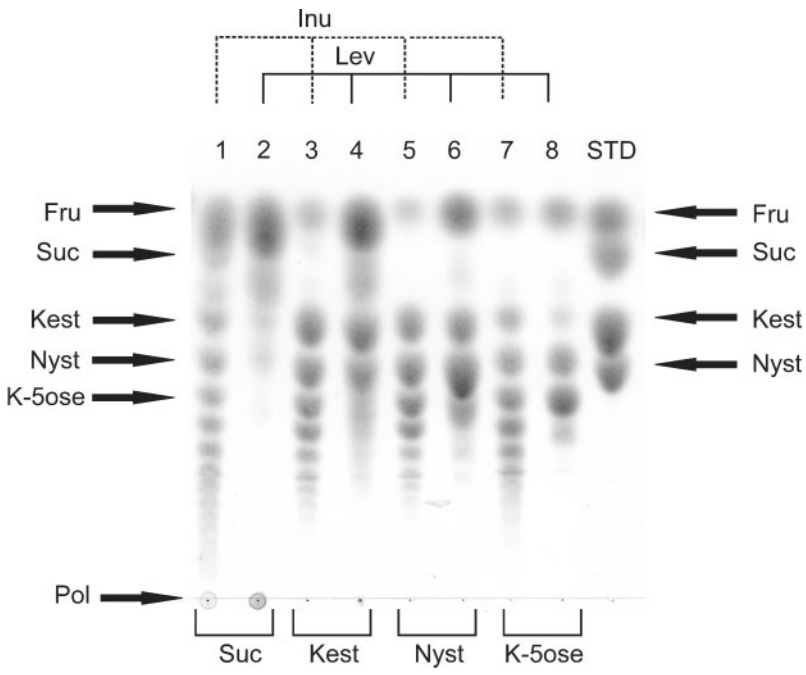

Fig. 4. TLC analysis of the Inu and Lev products from different substrates (840 mM) at 22 °C after 16 h incubation (using FTF enzymes at 2 U ml^{-1}, measured at 37 °C). Odd and even numbers represent Inu and Lev samples, respectively. Enzymes were incubated with the following substrates: sucrose (lanes 1 and 2), 1-kestose (lanes 3 and 4), 1,1-nystose (lanes 5 and 6), and 1,1,1-kestopentaose (K-5ose, lanes 7 and 8). The STD lane contains the standards indicated. See legend to Fig. 2 for abbreviations.

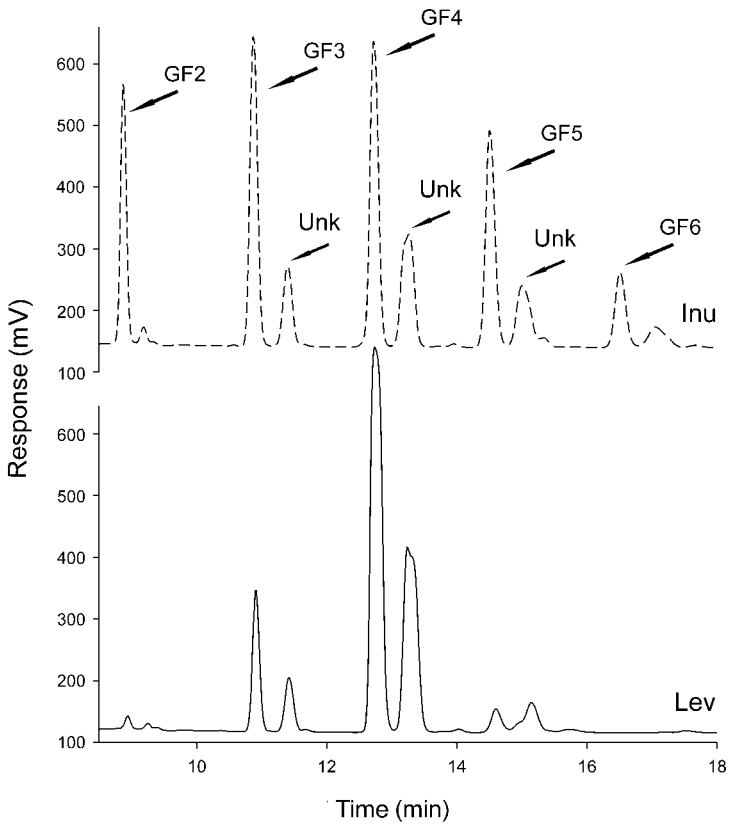

Fig. 5. HPAEC of the Inu (dashed line) and Lev (continuous line) products from 1,1,1-kestopentaose (840 mM) at 37 °C after 24 h incubation (using FTF enzymes at 2 U ml^{-1}; activity measured with sucrose). Known oligosaccharides [GF*n* with $\beta(2\rightarrow1)$ linkages] are indicated. Unk, unknown oligosaccharides.

FOS decreased gradually with increasing DP, with DP 15 representing the largest-size FOS detected with HPAEC (not shown in Fig. 3). In the case of Lev, only 28 % of the transglycosylation products were larger than GF4 (virtually all levan polymeric material), and 20 % of the products were FOS smaller than GF5 (i.e. 1-kestose, 1,1-nystose and bifructose) (Table 1, Fig. 3). Lev synthesized several additional products that could not be identified with the inulin standards used [GF*n* and F*n* molecules containing $\beta(2\rightarrow1)$ linkages]. The largest FOS product made by Lev had a DP of about 4–5 (Figs 2 and 3). The main FOS synthesized from sucrose by Lev (7·4 % of total; Table 1), eluted after 9·5 min, was not an inulin-type oligosaccharide, and remained unidentified. 6-Kestose is a very likely intermediate in levan [with $\beta(2\rightarrow6)$ glycosidic bonds] synthesis, but it did not accumulate at levels that were detected (Fig. 3).

Substrate and product specificity of Inu and Lev

Both Inu and Lev converted sucrose, 1-kestose, 1,1-nystose and 1,1,1-kestopentaose into various transglycosylation products. TLC analysis of the Inu reaction products revealed that all the substrates were converted into small amounts of fructose, and into a range of FOSs. Small amounts of polymer were synthesized from sucrose and 1-kestose (Fig. 4). Lev converted all the sucrose, but some 1-kestose, 1,1-nystose and 1,1,1-kestopentaose substrate remained after a 16 h incubation period (Fig. 4). The product specificity of Lev varied significantly depending on the substrate used. Lev synthesized polymer from sucrose only (Fig. 4). The amount of fructose released decreased with increasing size of the oligosaccharide substrate, suggesting that Lev has a lower hydrolytic activity with these larger inulin-type substrates. Lev converted these GF*n* substrates into mainly GF($n\pm1$), and small amounts of GF($n\pm2$). Thus, the data show that the two *Lb. reuteri* 121 FTF enzymes are able to catalyse a disproportionation type of reaction with 1-kestose, 1,1-nystose and 1,1,1-kestopentaose.

HPAEC of the products synthesized from kestopentaose (GF4) showed that Inu synthesized a broad range of FOSs (DP 3–7) from kestopentaose (Fig. 5). The main products synthesized were kestose, nystose and GF5, which were all apparently of the inulin type. Lev also synthesized inulin-type nystose in significant amounts, and some GF5 (Fig. 5). Again, more kestopentaose remained in incubations with Lev than in those with Inu (after 24 h). Both Inu and Lev also produced a range of products that could not be identified with the inulin-type FOS standards used; these products included the main product made by Lev (made in lower amounts by Inu as well, eluting after 13·5 min). It was unlikely that these products were unbranched F*n* products of the inulin type (compare with standards in Fig. 3). The exact identity of these oligosaccharides remains to be determined.

The two FTFs of *Lb. reuteri* 121 used raffinose as a fructosyl-donor and/or -acceptor substrate, synthesizing fructosyl-raffinose (most likely GalGF2). Only Inu synthesized a

whole range of larger oligomers (up to GalGF6), and some polymeric material (data not shown). The large amount of free fructose, and the small quantities of oligosaccharides, synthesized by Lev, indicate that Lev has relatively high hydrolysis and low transglycosylation activities with raffinose compared with sucrose (data not shown).

DISCUSSION

Although the *Lb. reuteri* 121 Inu and Lev enzymes are highly similar at the amino acid sequence level, marked differences were observed in their reaction kinetics with sucrose (Fig. 1). With the aim of understanding the molecular and structural basis for these differences, we analysed the transglycosylation products synthesized by these enzymes using sucrose and related short inulin-type FOSs as substrates.

The *Lb. reuteri* FTF enzymes catalyse processive and non-processive reactions

Van Hijum *et al.* previously reported synthesis of inulin-type FOSs from sucrose by Inu, but FOSs larger than nystose were not observed (van Hijum *et al.*, 2002). However, our present studies revealed that Inu was very efficient at synthesizing inulin-type FOSs of DP 15 and larger, when using relatively high sucrose and high Inu enzyme concentrations. Lev, in contrast, synthesized mostly polymer from sucrose (Fig. 2). The Inu and Lev enzymes of *Lb. reuteri* 121 thus employ a non-processive reaction and a processive reaction, respectively. With increasing sucrose concentrations, the range and concentration of synthesized FOSs increased, resulting in increased total reaction velocity, which also explained the non-Michaelis–Menten reaction kinetics observed for Inu (Fig. 1). Lev can be saturated by its substrate sucrose, resulting in a Michaelis–Menten type of kinetics (Fig. 1). The larger FOS molecules synthesized as intermediates in levan formation apparently remain bound to the Lev protein, and are directly used in further chain-elongation steps, resulting in their rapid further processing.

FTF synthesis of inulin- and levan-like FOSs

The FTF enzymes of *Lb. reuteri* 121 differed markedly in product spectrum from sucrose. Inu catalysed the synthesis of mostly $\beta(2\rightarrow1)$-linked FOSs, and a relatively low amount of inulin polymeric material (Fig. 3; see also van Hijum *et al.*, 2002). Lev synthesized relatively more of a large polymer of the levan type (this study), containing almost exclusively ($>95\,\%$) $\beta(2\rightarrow6)$ linkages (see van Hijum *et al.*, 2001), plus small amounts of $\beta(2\rightarrow1)$ FOS, and a range of unidentified molecules that may constitute $\beta(2\rightarrow6)$ FOSs (Table 1, Fig. 2).

The ability of levansucrase enzymes to synthesize $\beta(2\rightarrow1)$- as well as $\beta(2\rightarrow6)$-linked FOSs has been reported elsewhere (Euzenat *et al.*, 2005; Támbara *et al.*, 1999; Korakli *et al.*, 2003). *Lb. reuteri* 121 Lev also synthesized 1-kestose, and did not accumulate 6-kestose. Presumably, 1-kestose cannot be further elongated by Lev, resulting in its accumulation; 6-kestose may also be synthesized but does not accumulate, i.e. it is rapidly used as acceptor substrate for levan synthesis (Euzenat *et al.*, 2005). Besides bifructose and 1-kestose, the *Lb. reuteri* 121 Lev enzyme also synthesized a range of unidentified FOS molecules from sucrose (Table 1, Fig. 3).

Model for the organization of the FTF sugar-binding subsites

The disproportionation data indicate that FTF enzymes have one sugar-binding donor subsite (-1) only (see below). This correlates with the geometry of the active site of SacB from *B. subtilis*, which shows a salt bridge between E342 and R246, possibly disrupting further -2 and -3 subsites (Meng & Futterer, 2003; Ozimek *et al.*, 2004). A similar situation exists in the amylosucrase of *Neisseria polysaccharea* (Albenne *et al.*, 2002).

Amino acid residues located at subsites -1 and $+1$ in the active centre of FTF enzymes (nomenclature according to Davies *et al.*, 1997), and that directly interact with sucrose, have been identified based on the three-dimensional structure of *B. subtilis* levansucrase with bound sucrose (Meng & Futterer, 2003), and several mutagenesis studies with FTF enzymes (Chambert & Petit-Glatron, 1991; Yanase *et al.*, 2002). A total of eight residues, known to be involved in catalysis and/or interaction with the bound fructose and glucose at the -1 (W85, D86, R246, D247 and W163) and $+1$ subsites (R360, E340 and R246), are completely conserved in the Lev and Inu enzymes of *Lb. reuteri* 121.

The FTF -1 subsite is highly specific for accommodating fructose units (Chambert *et al.*, 1974; Hernández *et al.*, 1995; Song & Jacques, 1999), whereas the $+1$ subsite appears to be more flexible, exhibiting affinity for both glucose (binding of sucrose and raffinose) and fructose (binding sucrose as an acceptor substrate during transglycosylation) (this study) (Fig. 6). Upon complex formation with sucrose, the subsite $+1$ is occupied by a glucosyl moiety. As there is only one funnel-like channel leading towards the active site (Meng & Futterer, 2003), we propose the following reaction mechanism. Sucrose enters the active site and occupies the -1 and $+1$ subsites. Its glycosidic bond is cleaved, a covalent fructosyl–enzyme intermediate is formed at -1 (Chambert & Gonzy-Treboul, 1976), and glucose is released through the channel (Fig. 6A). Subsequently, water may enter the active site, react with the fructosyl–enzyme intermediate, resulting in hydrolysis and release of fructose (Fig. 6B). Alternatively, a second (sucrose) acceptor substrate enters the active site, binds to the $+1$ and $+2$ subsites, and reacts with the fructosyl–enzyme intermediate at -1, resulting in FOS formation (Fig. 6C). Further transglycosylation reactions may occur, resulting in chain elongation and polymer formation (Fig. 6C). A similar mechanism has been proposed for the amylosucrase (family GH13) of *N. polysaccharea* that catalyses transfer of a D-glucopyranosyl moiety from sucrose to an acceptor molecule (Mirza *et al.*, 2001).

Lb. reuteri Inu and Lev enzymes both use sucrose, kestose and raffinose as acceptor substrates (Figs 4 and 5, and data

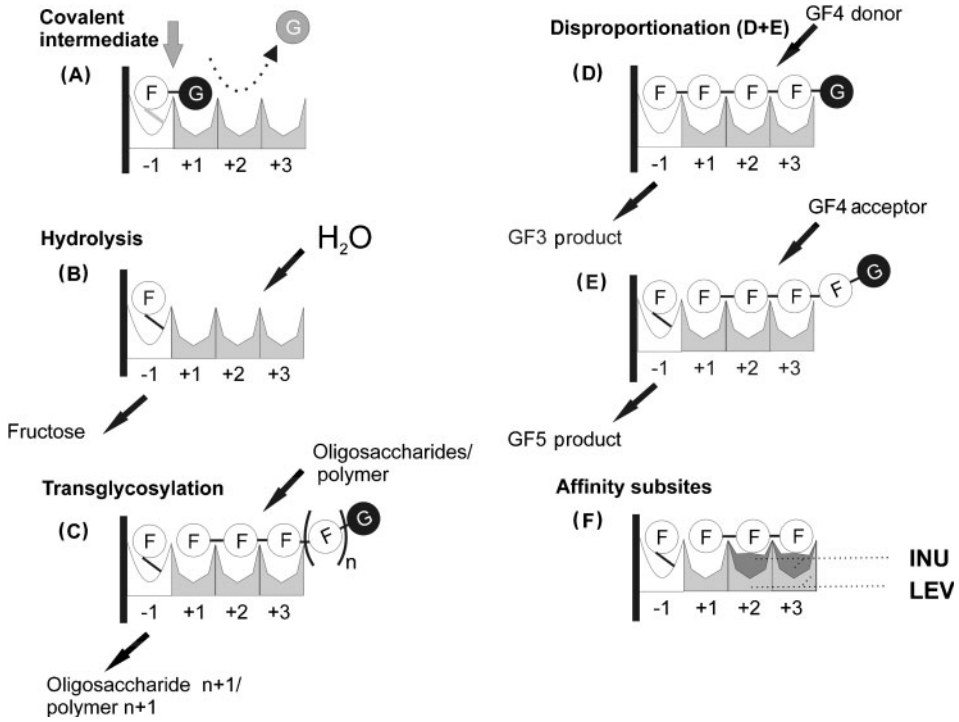

Fig. 6. Schematic representation of the reaction sequences occurring in the active site of FTF enzymes. The donor and acceptor subsites of FTF enzymes are mapped out based on the available three-dimensional structural information (Martinez-Fleites *et al.*, 2005; Meng & Futterer, 2003), and data obtained in the present study. (A) Binding of sucrose to subsites −1 and +1 results in cleavage of the glycosidic bond (glucose released, shown in grey), and formation of a (putative) covalent intermediate at subsite −1 (indicated by a grey line). Depending on the acceptor substrate used, hydrolysis (with water) (B) or transglycosylation (C) reactions may occur [with oligosaccharides or the growing polymer chain, resulting in FOS synthesis ($n+1$) or polymer synthesis ($n+1$), respectively]. *Lb. reuteri* 121 FTF enzymes also catalyse a disproportionation (D, E) reaction with inulin-type oligosaccharides. Kestopentaose (GF4), for instance, is converted into GF3 and GF5 (D, E). (F) The differences in affinity between Inu and Lev at the +2 and +3 subsites are shown by a shallow cleft (dark grey; low affinity), and a deep cleft (light grey; high affinity), respectively. Sugar-binding subsites are shown either in white (−1 subsite), reflecting specific and constant affinity for binding of fructosyl residues only, or in light/dark grey (+1, +2 and +3 subsites), reflecting their ability to bind fructosyl, glucosyl (with GF*n* substrate) or galactosyl (with raffinose) residues. The vertical grey arrow indicates the position where glycosidic bond cleavage/formation occurs. The vertical black bar indicates the salt bridge in FTF enzymes (E342 and R246 in SacB from *B. subtilis*) (Martinez-Fleites *et al.*, 2005; Meng & Futterer, 2003) that possibly blocks further donor sugar-binding subsites. F, fructose; G, glucose.

not shown), indicating further similarities in their acceptor substrate-binding sites. However, Inu and Lev clearly differ in their ratio of hydrolysis vs transglycosylation activities, and in their ratio of synthesis of FOS versus fructan polymer, inulin and levan, respectively. Since the residues constituting the −1 and +1 binding sites in Inu and Lev are virtually identical, unidentified structural differences in further acceptor substrate-binding subsites must exist, which are responsible for these differences in Lev and Inu product specificity.

FTF enzymes catalyse disproportionation reactions

The *Lb. reuteri* 121 Inu enzyme converted FOSs (DP 3–5 of the inulin type) into the same range of FOSs observed with sucrose (Fig. 4). Two mechanisms may explain these observations: (i) oligosaccharides are degraded by hydrolysis of the terminal fructose units until sucrose is formed, and sucrose is used as a substrate for transglycosylation; (ii) oligosaccharides are used directly as fructose donor and acceptor substrates, involving a disproportionation type of reaction, resulting in the first step in synthesis of GF($n\pm1$) from a GF*n* substrate. In a second step, multiple reactions will occur. Indeed, the relatively small amounts of free fructose released by Inu (Fig. 4), and the product range synthesized by Inu from kestopentaose (Figs 4 and 5), show that Inu catalysed a disproportionation reaction with these FOSs (Fig. 6D, E). Lev utilized FOSs of the inulin-type less efficiently, with more of the substrates remaining after 16 h incubation (Figs 4 and 5). The main products of the reaction of Lev with these inulin-type FOSs were FOS($n\pm1$) (plus fructose) (Figs 4 and 5), indicating that Lev also catalysed a disproportionation reaction (Fig. 6D, E). To the

best of our knowledge, this type of reaction has not been reported for bacterial FTF enzymes. Thus, both Inu and Lev cleave the $\beta(2\rightarrow1)$ linkage at the non-reducing end of an FOS donor, and transfer the fructosyl unit to the non-reducing end of another FOS acceptor. A similar scheme has been proposed for the amylosucrase (family GH13) of *N. polysaccharea* (Albenne *et al.*, 2002).

The ability of FTF enzymes to catalyse disproportionation reactions will undoubtedly be of interest for testing inulin hydrolysates as fructosyl donors for oligosaccharide and fructo-conjugate synthesis (see also Albenne *et al.*, 2002).

Fructan polymer synthesis requires the disaccharide sucrose as a fructosyl donor substrate

Both Inu and Lev were unable to synthesize large polymers when 1,1-nystose or 1,1,1-kestopentaose were used as substrates. Only Inu synthesized a small amount of polymer from 1-kestose, and this may depend on sucrose availability, generated by kestose hydrolysis (Fig. 4). A likely explanation for the lack of fructan polymer synthesis from FOSs is that polymerization, as a processive reaction, requires constant interaction of the enzyme with the growing polymer chain at subsites $+2$, $+3$ and further. Sucrose, because of its small size, apparently remains able to enter the active site, and binds to -1 and $+1$ subsites of the enzyme, even when fructan polymer is bound at subsites $+2$, $+3$ and further. However, an FOS larger than DP 2 can serve as a fructosyl donor substrate, but only if the subsite $+2$ (in case of DP 3), subsite $+3$ (in case of DP 4) and further subsites (longer FOSs) are accessible (Fig. 6). This competition between the growing polymer chain and fructosyl donors for the $+2$ and $+3$ subsites may result in a non-processive reaction (disproportionation, oligomerization), and loss of ability to synthesize (larger) polymer. Whereas Lev was efficient in polymer synthesis (processive reaction), Inu displayed a clearly non-processive reaction with sucrose (Fig. 2). Thus, the Inu subsites $+2$, $+3$ and/or further have a relatively low affinity for binding the growing fructan polymer chain, resulting in its release after virtually every chain elongation with a fructosyl unit from sucrose (Fig. 6F). The overall effect of this is synthesis of mainly FOS, as observed with Inu (Fig. 2).

Conclusions

Characterization of two FTF enzymes of *Lb. reuteri* 121 (Inu and Lev) revealed that, although very similar in amino acid sequence, they differ significantly not only in the type of polymers synthesized (inulin and levan), but also in the size distribution of FOS products synthesized from sucrose and related substrates. Inu synthesized a whole range of well-defined inulin-type FOSs of sizes that reached DP 15 and larger, and a small amount of inulin polymer, whereas Lev produced mainly a levan polymer, and small quantities of short oligosaccharides, probably containing [$\beta(2\rightarrow1)$ and/or $(2\rightarrow6)$ linkages]. Synthesis of FOS was the main activity of Inu, reflecting a much higher affinity of its acceptor-binding subsites for shorter FOSs than for longer FOSs. Lev synthesized mainly a large polymer, probably because its acceptor-binding subsites have a much higher affinity for large polymers (DP 5 and larger) than for short FOSs. With Inu, the result was a non-processive reaction with sucrose (after every fructosyl transfer, the growing polymer chain is released from the enzyme). Lev catalysed a processive type of reaction (the growing fructan chain remains bound to the additional acceptor binding subsites). The product specificities of Inu and Lev are also reflected in the products synthesized by *Lb. reuteri* 121 cell cultures: only levan polymer and inulin oligosaccharides have been detected (van Hijum *et al.*, 2002). The fructan products synthesized by *Lb. reuteri* cells and FTF enzymes may in future replace the prebiotic fructans isolated from plant material (Menne *et al.*, 2000). *Lb. reuteri* 121 and its fructan products may also be used as a combination of pro- and prebiotics in food and health products. The availability of high-resolution three-dimensional structures of FTF proteins with bound long-chain acceptor substrates, and site-directed mutagenesis studies of the residues involved, may help to understand and predict the outcome of the transglycosylation reactions catalysed by different FTF enzymes.

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