Inulin and levan synthesis by probiotic *Lactobacillus gasseri* strains: characterization of three novel fructansucrase enzymes and their fructan products

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Fructansucrase enzymes polymerize the fructose moiety of sucrose into levan or inulin fructans, with $\beta(2-6)$ and $\beta(2-1)$ linkages, respectively. Here, we report an evaluation of fructan synthesis in three *Lactobacillus gasseri* strains, identification of the fructansucrase-encoding genes and characterization of the recombinant proteins and fructan (oligosaccharide) products. High-performance anion-exchange chromatography and nuclear magnetic resonance analysis of the fructo-oligosaccharides (FOS) and polymers produced by the *L. gasseri* strains and the recombinant enzymes revealed that, *in situ*, *L. gasseri* strains DSM 20604 and 20077 synthesize inulin (and oligosaccharides) and levan products, respectively. *L. gasseri* DSM 20604 is only the second *Lactobacillus* strain shown to produce inulin polymer and FOS *in situ*, and is unique in its distribution of FOS synthesized, ranging from DP2 to DP13. The probiotic bacterium *L. gasseri* DSM 20243 did not produce any fructan, although we identified a fructansucrase-encoding gene in its genome sequence. Further studies showed that this *L. gasseri* DSM 20243 gene was prematurely terminated by a stop codon. Exchanging the stop codon for a glutamine codon resulted in a recombinant enzyme producing inulin and FOS. The three recombinant fructansucrase enzymes characterized from three different *L. gasseri* strains have very similar primary protein structures, yet synthesize different fructan products. An interesting feature of the *L. gasseri* strains is that they were unable to ferment raffinose, whereas their respective recombinant enzymes converted raffinose into fructan and FOS.

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INTRODUCTION

A diverse number of naturally occurring *Lactobacillus* species inhabit the gastrointestinal (GI) tract of humans and animals and are widely considered to exert a number of beneficial effects on human health. *Lactobacillus gasseri*, which is speculated to be an autochthonous human

intestinal organism (Azcarate-Peril *et al.*, 2008), constitutes a major part of the homofermentative *Lactobacillus* species occupying the human GI tract (Kullen *et al.*, 2000). *L. gasseri* is associated with a variety of probiotic functions including reduction of activities of faecal mutagenic enzymes (Pedrosa *et al.*, 1995), adherence to intestinal tissues (Conway *et al.*, 1987; Kirjavainen *et al.*, 1998), stimulation of macrophages (Kirjavainen *et al.*, 1999; Tejada-Simon & Pestka, 1999) and production of bacteriocins (Itoh *et al.*, 1995). The probiotic role of several lactobacilli has been attributed to the synthesis of prebiotic fructose-based polysaccharides (fructans) from sucrose involving fructansucrase or fructosyltransferase (FTF) enzymes (Armuzzi *et al.*, 2001; Korakli *et al.*, 2002; Oda *et al.*, 1983).

FTF enzymes possess either levan- or inulosucrase activity, polymerizing the fructose moiety of their substrate sucrose into fructans with either levan or inulin structures, with $\beta(2-6)$ and $\beta(2-1)$ linkages, respectively. Although all

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Abbreviations: FOS, fructo-oligosaccharides; FTF, fructosyltransferase; GI, gastrointestinal; HPAE, high-pressure anion-exchange [chromatography]; LB, Luria–Bertani; NMR, nuclear magnetic resonance.

The GenBank/EMBL/DDBJ accession numbers for the *Lactobacillus gasseri* *ftf* genes and their flanking regions are GU166814 (*inuGB*) and GU166815 (*levG*), and the nucleotide sequence data reported for *inuGA* are available in the Third Party Annotation section of the GenBank/EMBL/DDBJ databases under the accession number TPA: BK006921.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

known lactic acid bacterial FTFs share a high amino acid sequence similarity (>60%), it is not known which structural features determine the linkage type in their fructan products. Various groups have attempted to elucidate structure–function relationships of FTF enzymes, following site-directed mutagenesis and FTF protein 3D structure analysis. At present, high-resolution 3D structures are only available for the levansucrase proteins of *Bacillus subtilis*, also with sucrose and raffinose bound in the active site (Meng & Futterer, 2003, 2008), and *Gluconacetobacter diazotrophicus* (Martinez-Fleites *et al.*, 2005). It has been proven by site-directed mutagenesis that the amino acid residues located at the –1 (donor) and +2 (acceptor) sugar-binding subsites are important in determining the size of the products synthesized and polymer versus oligosaccharide product ratio (Homann *et al.*, 2007; Ozimek *et al.*, 2006a). A recent study on the *B. subtilis* levansucrase mutant G342A complexed with raffinose (Frc-Glc-Gal) revealed that the galactosyl moiety of raffinose protruded out of the active site, while specificity-determining contacts were essentially restricted to the sucrosyl (Frc-Glc) moiety (Meng & Futterer, 2008).

Currently, only six inulosucrase genes/enzymes have been characterized from Gram-positive bacteria, namely in *Streptococcus mutans* (Rosell & Birkhed, 1974), *Leuconostoc citreum* CW28 (Olivares-Illana *et al.*, 2002), *Lactobacillus reuteri* 121 (van Hijum *et al.*, 2002), *Bacillus* sp. (Wada *et al.*, 2003), *L. reuteri* TMW 1.106 (Schwab *et al.*, 2007) and *Lactobacillus johnsonii* NCC 533 (Anwar *et al.*, 2008), while numerous levansucrases are known (<http://www.cazy.org/fam/GH68.html>) from both Gram-positive and Gram-negative bacteria. This limited knowledge of inulosucrase enzymes and lack of 3D structures hamper our efforts to understand the differences between inulo- and levansucrases.

Here, we describe the isolation and cloning of two *ftf* genes from two different *L. gasseri* strains, i.e. DSM 20604 and DSM 20077. These genes and the novel FTF enzymes that are encoded, an inulosucrase and levansucrase, are characterized in detail. We also report cloning (and restoration of enzyme activity by elimination of a preliminary stop codon) of an inulosucrase gene that was identified in the genome of the *L. gasseri* type strain DSM 20243.

METHODS

Amino acid sequence analysis. Multiple amino acid sequence alignments were made with CLUSTAL W 1.74 (Thompson *et al.*, 1994). Specific features and the catalytic core of *L. gasseri* FTFs were deduced from these alignments along with the Pfam database (<http://pfam.janelia.org>) and the signal peptide cleavage sites of the FTFs were predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>).

Bacterial strains, plasmids and culture conditions. The *L. gasseri* DSM 20243, 20604 and 20077 strains were obtained from the DSMZ culture collection. *L. reuteri* 121 (LMG 18388) was obtained from the culture collection of TNO Nutrition and Food Research, The

Netherlands. For genomic DNA isolation, the cells were cultivated anaerobically at 37 °C in MRS medium containing 20 g glucose l^{–1}. MRS with sucrose (200 g l^{–1}) instead of glucose, was used for polysaccharide production by these strains. *Escherichia coli* TOP10 (Invitrogen) and BL21 (DE3 star; Invitrogen) were used as hosts for cloning and expression purposes, respectively. Plasmid pET15b (Novagen) was used for expression of the *ftf* genes. *E. coli* transformations were performed by the heat shock method using chemically competent cells (Sambrook *et al.*, 1989). *E. coli* strains were grown at 37 °C and shaking at 210 r.p.m. in Luria–Bertani (LB) medium supplemented with the appropriate antibiotic in order to maintain plasmid integrity (100 µg ampicillin ml^{–1} or 50 µg kanamycin ml^{–1}). LB agar plates were made by adding 1.5% agar to the LB medium.

General molecular techniques. Genomic DNA of the three *L. gasseri* strains was extracted and purified using a genomic DNA purification kit (Sigma Aldrich). *E. coli* plasmid DNA was isolated using a plasmid miniprep kit (Sigma Aldrich). General procedures for cloning, DNA manipulations, transformations and agarose gel electrophoresis were performed as described by Sambrook *et al.* (1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the supplier (Fermentas). DNA was amplified by PCR (PTC-200 Thermal Cycler, MJ Research), using high-fidelity DNA polymerase (Fermentas) for the standard and inverse PCRs. The TOPO XL PCR cloning kit (Invitrogen) was used for cloning and sequencing of the *ftf* genes, following the supplier's instructions. Oligonucleotides (Supplementary Tables S1 and S2, available with the online version of this paper) were purchased from Eurogentec. DNA fragments were isolated from agarose gels using a Qiagen extraction kit, following the supplier's instructions (Qiagen).

Isolation, cloning and sequencing of *L. gasseri* *ftf* genes. The *ftf* gene fragment of *L. gasseri* DSM 20243 contained a naturally occurring early stop codon (TAA) at position 1954. Replacing the stop codon with a glutamine codon (CAA) resulted in a full-length *ftf* gene of 2367 bp (designated *inuGA*) as described below. Using the specifically designed PCR primer set Lgas-F/Lgas-R, a truncated version of *inuGA* encoding aa 137–702 of the FTF protein (including 50 aa downstream of the stop codon), with a 6 × C-terminal His tag, was amplified from genomic DNA. This amplicon was digested with *NcoI* and *BamHI* and cloned into the corresponding sites of the expression vector pET15b (Novagen). The resulting construct containing recombinant *inuGA* was designated pETInuGA-R. The stop codon in the recombinant gene was replaced with a codon for glutamate (CAA) by site-directed mutagenesis using primers LG-stop-f and LG-stop-r and the Quick Change protocol (Stratagene) with pETInuGA-R as template (Supplementary Table S2). The resulting construct (pETInuGA-RM) with the recombinant mutant gene (*inuGA*-RM) was transformed into *E. coli* BL21 (DE3 Star) for expression.

Isolation of *ftf* genes from *L. gasseri* DSM 20604 and 20077 was initiated by PCR using the degenerate primers 5FTF and 6FTFi, which are based on conserved amino acid sequences of FTFs of Gram-positive origin (van Hijum *et al.*, 2002). Amplicons of 228 and 235 bp were amplified this way from the genomic DNA of *L. gasseri* DSM 20604 and *L. 20077*, respectively. These fragments were cloned in *E. coli* Top10 cells using the pCR-XL-TOPO vector and five individual clones were sequenced from each reaction. Isolation of the complete *ftf* genes was accomplished by standard and inverse PCR techniques (Supplementary Fig. S1) using the primers listed in supplementary Table S1.

The *ftf* genes of *L. gasseri* DSM 20604 and 20077 were designated *inuGB* and *levG*, respectively. For FTF protein expression, 5' and 3' truncated *inuGB* and *levG* were amplified by PCR from genomic DNA

using primer sets Lg604-F3/Lg604-R2 and Lg077-F2/Lg077-R2, respectively (Supplementary Table S2). These truncated *fff* genes encode aa 137–703 of the InuGB protein (designated InuGB-R) and aa 128–689 of the LevG protein (LevG-R), both containing 6 × C-terminal His tags. These truncated genes were cloned into the expression vector pET15b using *Nco*I and *Bam*HI restriction sites. The resulting vectors pETInuGB-R and pETLevG-R were transformed to *E. coli* BL21 (DE3 Star) for protein expression. Correct construction of the plasmids was confirmed by nucleotide sequence analysis (GATC).

Expression and purification of FTF proteins. Protein expression in overnight cultures (600 ml each, grown at 37 °C in LB medium) of *E. coli* BL21 (DE3 star) harbouring pETInuGA-R, pETInuGA-RM, pETInuGB-R and pETLevG was induced with 0.1 mM IPTG for 1 h at 200 r.p.m. The cells were harvested by centrifugation at 3500 g for 15 min and the FTF proteins were purified to homogeneity by Ni-NTA and anion-exchange chromatography as described by van Hijum *et al.* (2002).

Biochemical characterization of the recombinant *L. gasseri* FTF enzymes.

All enzyme assays were performed at 50 °C in 25 mM sodium acetate buffer, pH 5.5, for InuGA-RM, pH 4.5, for InuGB-R and LevG-R, containing 1 mM CaCl₂ and using 0.45 µg protein ml⁻¹ unless specified otherwise. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad) with BSA as standard. One unit of enzyme activity is defined as the release of 1 µmol monosaccharide min⁻¹ from sucrose. The enzyme activity was measured as 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) (total activity). The amount of fructose (V_F) formed is a measure for the hydrolytic activity. The transglycosylation activity was calculated by subtracting the amount of free fructose from glucose ($V_G - V_F$).

(i) Effect of pH, temperature, CaCl₂ and EDTA. Michaelis' barbital sodium acetate buffer (Chipperfield & Taylor, 1970), pH 3.5–8.5 was used to study the effect of pH on the activity of recombinant FTF enzymes at 37 °C, as described previously (Anwar *et al.*, 2008). The effect of temperature on the enzyme activity was studied in 25 mM sodium acetate buffer supplemented with 500 mM sucrose and 1 mM CaCl₂. The effect on enzyme activity of scavenging calcium ions from the reaction buffer by EDTA (0–1000 µM) was studied as described previously (Anwar *et al.*, 2008).

(ii) Kinetic parameters. Kinetic parameters of the FTF reactions were determined by varying the sucrose concentration (5–1000 mM) at 37 and 50 °C. Data were fitted to the standard Michaelis–Menten formula: $V = (V_{max} [S] / (K_m + [S]))$ using Sigma Plot (version 10.0), where V is the specific activity (U mg⁻¹), S is the substrate concentration (mM sucrose), V_{max} is the maximum velocity (U mg⁻¹) and K_m is the Michaelis constant (mM sucrose).

Polysaccharide production and characterization. The polysaccharides synthesized by the three *L. gasseri* strains were produced by growing the strains anaerobically using a BBL gas pack in 100 ml MRS–sucrose medium at 37 °C. After 5 days of incubation, the cultures were centrifuged at 4000 g for 10 min and the supernatants were separated from the cells. The supernatants (3 µl each) were analysed by TLC (Silica gel 60 F₂₅₄; Merck). As mobile phase, 1-butanol:ethanol:water (5:5:3) was used and plates were run overnight. Plates were air-dried, sprayed with a urea developing solution specific for sugars containing fructose (Trujillo *et al.*, 2004) and developed at 80 °C. The polysaccharides were precipitated from the supernatant with 2 vols 96% cold ethanol followed by

centrifugation at 2500 g for 15 min. After dissolving in MilliQ water, the polymers were reprecipitated (Van Geel-Schutten *et al.*, 1999). After repeating this process twice, the polymers were freeze-dried and the weights of these samples were measured. The three *L. gasseri* strains and *L. reuteri* 121 were also inoculated in MRS medium containing 500 mM raffinose and incubated at 37 °C for 5 days; 3 µl culture broth was analysed by TLC as described above.

For nuclear magnetic resonance (NMR) spectroscopy, the purified polymer products were dissolved in 99.9 atom % D₂O (Sigma-Aldrich). One-dimensional ¹³C-NMR spectra were recorded at 125 MHz on a 500 MHz Varian Inova NMR spectrometer at a probe temperature of 80 °C. Chemical shifts are expressed in p.p.m. relative to the methyl group of internal acetone ($\delta = 31.07$). Carbon spectra were recorded in 38K datasets, with a spectral width of 30 166 kHz. Prior to Fourier transformation, the time-domain data were apodized with an exponential function, corresponding to a 0.5 Hz line broadening.

To study the product profile of the purified recombinant levan- and inulosucrases of the *L. gasseri* strains, the enzymes (100 U l⁻¹) were incubated at 37 °C with sucrose (600 mM) for 72 h in 25 mM sodium acetate buffer pH 4.5 (for InuGB-R and LevG-R) and pH 5.5 (for InuGA-RM) containing 1 mM CaCl₂. To analyse product formation, 1 µl aliquots from these reaction mixtures (4 × diluted) were run on TLC plates overnight. Activities of these enzymes were also tested on raffinose (500 mM) using the same pH and temperature conditions, but with 5000 U l⁻¹ enzyme concentration and 18 h incubation time. Recombinant *L. reuteri* 121 inulosucrase (InuΔ699His) (van Hijum *et al.*, 2002) was used as a positive control.

High-pressure anion-exchange (HPAE) chromatography (Dionex) was used to separate oligosaccharides produced by growing (for 5 days) cultures of *L. gasseri* strains DSM 20604 and 20077, and incubating InuGA-RM with sucrose for 72 h at 37 °C, as described above. Separation of these fructo-oligosaccharides (FOS) was achieved as described previously (Ozimek *et al.*, 2006a) using the following gradient (with eluent B): eluent A (0 min, 100%); (10 min, 78%); (25 min, 60%); (80 min, 10%); (83 min, 0%); (91 min, 100%). Eluent A was 0.1 M sodium hydroxide and eluent B was 0.1 M sodium hydroxide in 0.6 M sodium acetate. As standards, a 1:1 mixture of Raffiline ST-Gel and Raffiline HP (Orafti) representing chicory inulin were used.

Quantitative analysis of the products synthesized by the FTF enzymes. For quantitative analysis of the FOS and polymers synthesized, the enzymes were incubated under similar conditions to those described above and the enzyme activity was monitored by determining the glucose released from sucrose after every 24 h. After 7 days of incubation, when glucose concentration did not further increase, 0.1 ml sample aliquots were taken and subjected to analysis by HPAE (see below). The remaining part of the reaction mixture was treated with 2 vols cold ethanol for polymer isolation and purification, as described above. Fructan polymer solutions (1% w/v) were prepared in demineralized water by heating at 50 °C for 10 min. For complete degradation, the inulin solutions (100 µl) were incubated for 4 h in 100 mM acetate buffer at pH 4.5 (100 µl) with 20 units exo-inulinase (Megazyme) and 1 unit endo-inulinase (Megazyme) to a total volume of 300 µl with MilliQ water. Similarly, the levan was treated with 5 units endo-inulinase and 20 units inulinase of *Aspergillus niger* (Fluka) for 48 h under sterile conditions. After complete degradation, the fructose concentration of the samples was determined.

HPAE chromatography (see above) was used to quantitatively determine the remaining sucrose, and the glucose and fructose produced by the purified recombinant enzymes. The amount of free glucose (G_f) produced is a measure of total FTF activity, which

includes hydrolysis and transglycosylation reactions. Subtracting the amount of free fructose (F_f), produced as a result of hydrolytic activity, from that of free glucose (G_f) gave the amount of fructose (F_t) involved in transglycosylation reactions ($F_t = G_f - F_f$). The amount of fructan polymer was determined by weight (see above) and its fructose content (F_p) was determined as described above. The fructose incorporated in FOS (F_{fos}) is defined as the difference between the amount of fructose (F_t) involved in transglycosylation reactions and fructose (F_p) incorporated into the polymer product ($F_{fos} = F_t - F_p$). It is worth mentioning that, in our studies, the values for the amount of fructose incorporated in the polymer product are likely to be slightly underestimated due to small losses of the product during purification steps, with a concomitant overestimation of the fructose in FOS that was calculated indirectly (see above). However, the results still allow a comparative analysis.

Enzyme localization studies. *L. gasseri* strains were grown in MRS medium with 30 g sucrose l^{-1} at 37 °C and the cultures were diluted with MRS medium to $OD_{600}=1.0$ before being harvested by centrifugation (10 000 g for 15 min) in the late exponential growth phase. Cells were washed twice with saline solution (8.5 g l^{-1}) and resuspended to the original volume in 25 mM sodium acetate buffer (pH 5.5) containing 1 mM $CaCl_2$. Culture supernatants were dialysed for 24 h against this sodium acetate buffer, which was replaced four times during dialysis. Total, hydrolysis and transglycosylation activities in the washed cells and supernatants were measured as described previously (Anwar *et al.*, 2008) at 37 °C in 25 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$, using 62 μ l cell suspension or supernatant in the reaction mixture as the source of enzyme. Sucrose was used as substrate at 500 mM final concentration.

RESULTS AND DISCUSSION

Isolation and nucleotide sequence analysis of three putative *L. gasseri* *ftf* genes

Previous work (Pridmore *et al.*, 2004; van Hijum *et al.*, 2006) suggested that the genome of *L. gasseri* DSM 20243 contains a putative *ftf* gene. Supplementary material related to the published genome sequence of DSM 20243 (Azcarate-Peril *et al.*, 2008) indicated the presence of two gene loci, LGAS_1281 and LGAS_1282, with assigned functions in fructan synthesis. However, analysis of the genome sequence (GenBank accession no. CP000413)

revealed that the genes with these identities have no similarity with *ftf* genes. Instead, a locus tag, LGAS_1266, annotated as a pseudogene, shows high similarity to the inulosucrase (*inuJ*, orf LJ0913; GenBank accession no. AE017198) of *L. johnsonii* NCC 533 (Anwar *et al.*, 2008; Pridmore *et al.*, 2004). The complement of this gene spans the region 1262127–1264493 bp of the *L. gasseri* DSM 20243 genome.

Molecular analysis showed that this putative *ftf* gene was terminated by a stop codon (TAA), 4 aa before the end of the catalytic domain, whereas all other lactobacilli *ftf* genes possess a glutamine codon (CAA) at this position. Cloning and subsequent exchange of the stop codon with the glutamine codon resulted in an *ftf* gene (*inuGA*) encoding an inulosucrase enzyme with similar domain organization and size as the previously described fructansucrases of lactobacilli (Fig. 1). It is interesting to note that strain *L. gasseri* DSM 20243 contains a heteropolysaccharide biosynthesis gene cluster (LGAS_1156 to LGAS_1172) composed of 16 genes (Azcarate-Peril *et al.*, 2008), in addition to the *ftf* gene. To our knowledge, this is the first example of both an *ftf* gene (homopolysaccharide) and a heteropolysaccharide gene cluster in a single bacterium.

The *ftf* genes from *L. gasseri* strains DSM 20604 and DSM 20077, of which the genome sequences are unknown, were obtained as described previously (van Hijum *et al.*, 2002, 2004; Waldherr *et al.*, 2008), using degenerate and specific primers. Consequently, a 2707 bp DNA fragment containing an FTF open reading frame of 2283 bp (*inuGB*) was sequenced from the genomic DNA of *L. gasseri* DSM 20604 (Supplementary Fig. S1a). Similarly, a 2960 bp DNA fragment containing a single FTF open reading frame of 2304 bp (*levG*) was obtained from the genomic DNA of *L. gasseri* DSM 20077 (Supplementary Fig. S1b). Sequence analysis of the genes confirmed their *ftf* identities. Sequencing of five separate clones from each strain, obtained with the degenerate primers 5FTF and 6FTFi, yielded identical sequences, indicating that each strain contains a unique single *ftf* gene. The putative inulosucrase protein InuGB has a deduced molecular mass of 83 kDa

Fig. 1. Schematic representation of the domain organization of FTF enzymes of *L. gasseri* strains showing four different protein domains: (i) signal sequence, (ii) N-terminal variable region, (iii) catalytic domain, (iv) C-terminal domain with PXX repeats (black hatched boxes) and cell-wall-anchoring LPKAG motifs (grey boxes surrounded by a thick black line).

and pI of 5.44, while the putative levansucrase protein LevG has a deduced molecular mass of 84 kDa and pI of 5.22.

Amino acid sequence analysis of the three isolated FTFs

Alignments of the full-length amino acid sequence of InuGA with other fructosyltransferases revealed that it had the highest similarity with the inulosucrase (InuJ) of *L. johnsonii* NCC 533 (81 % identity) (Anwar *et al.*, 2008). The amino acid sequence of LevG exhibited the highest similarity with *L. johnsonii* NCC 533 InuJ inulosucrase (59 % identity) and InuGA (58 % identity). Among other levansucrases, LevG showed the highest similarity to *L. reuteri* 121 (50 % identity) (van Hijum *et al.*, 2004) and *L. sanfranciscensis* (49 % identity) (Tieking *et al.*, 2005) levansucrases. Similarly, InuGB had the highest similarity (86 % identity) with InuGA. However, a major difference between these two proteins is the preliminary stop codon in the latter, as already mentioned above.

Analysis of the deduced FTF amino acid sequences encoded by the three FTFs revealed the presence of: (i) a signal peptide, (ii) an N-terminal variable region, (iii) a catalytic domain and (iv) a C-terminal domain (Fig. 1).

(i) Signal peptides and N-terminal variable regions.

As predicted by SignalP, all three FTFs possess a typical Gram-positive signal peptide of 36 aa for secretion (Fig. 1), in agreement with the extracellular location of FTF enzymes. The N-terminal variable regions of these *L. gasseri* FTFs were similar in size to FTFs from other lactobacilli, ranging from 152 to 167 aa (Fig. 1). Including the InuGA, InuGB and LevG sequences, the amino acid sequence of the FTF N-terminal region is clearly variable among the FTFs of lactobacilli (not shown).

(ii) Catalytic domains.

The catalytic domains of the three *L. gasseri* FTF enzymes are very similar in size, approximately 450 aa (Fig. 1), corresponding very well to the sizes observed in other lactobacilli FTF enzymes. The amino acids reported to be involved in catalysis (Homann *et al.*, 2007; Meng & Futterer, 2003; Ozimek *et al.*, 2004) are conserved in the catalytic cores of the *L. gasseri* FTFs, and correspond to D266, D419 and E518 in InuGA; D257, D410 and E509 in InuGB; and D251, D404 and E505 in LevG.

(iii) C-terminal domains.

The C terminus of InuGA contains an amino acid sequence with a 13-fold repeat of the motif PXX, a Gram-positive cell wall anchoring domain of 41 aa (from aa 744 to 784) containing an LPKAG motif and a hydrophobic stretch of 23 aa (from aa 760 to 782). A C-terminal variable region of 43 aa is present between the catalytic core and the cell wall anchoring domain and the protein is terminated by six positively charged amino acids (Fig. 1). The LPKAG motif

was also reported to occur in *L. johnsonii* NCC 533 inulosucrase InuJ (Anwar *et al.*, 2008) and is similar to the consensus sequence LPXTG, which is well-conserved in Gram-positive cell-wall-associated proteins (Fischetti *et al.*, 1990; van Hijum *et al.*, 2002, 2004; Waldherr *et al.*, 2008). The C terminus of InuGB contains a 17-fold repeat of the motif PXX preceded by a C-terminal variable region of 43 aa. The protein terminated abruptly at aa 761 and it lacked the cell-wall-anchoring LPXTG/LPKAG motif. In LevG, a proline-rich spacer region of 82 aa with a 13-fold repeat of the motif PXX was present following the catalytic core. A Gram-positive anchor spanned from aa 724 to 764 on the C terminus of LevG and contained the cell-wall-anchoring motif LPKAG.

Expression of recombinant *ftf* genes in *E. coli*

Based on the obtained sequences, the three *ftf* genes were cloned and successfully expressed in *E. coli*. To enable high level protein expression in *E. coli*, truncated versions of the *L. gasseri* *ftf* genes were made in accordance with the strategies used in previous studies (Anwar *et al.*, 2008; Tieking *et al.*, 2005; van Hijum *et al.*, 2002), where the truncations did not affect enzyme activity and the composition of the polysaccharide products. The recombinant InuGA-RM contained a glutamine residue instead of the stop codon. Attempts to express the native sequence with the stop codon yielded only inclusion bodies that could not be solubilized. Purified proteins of ~63 kDa each were obtained from all the constructs and subjected to further characterization.

Biochemical characterization of the recombinant FTF enzymes of *L. gasseri*

(i) Effect of pH, temperature and CaCl₂ on enzyme activity. The effect of pH on the FTF enzyme activities was studied at 37 °C. The highest total inulosucrase activity of InuGA-RM was observed in the pH range from 4.5 to 6.0, with maximum activity between pH 4.5 and 5.5. This enzyme exhibited highest activity at 50 °C. Both the LevG-R and InuGB-R exhibited the highest activities in a relatively lower pH range, i.e. at pH 3.5–4.5, and at a temperature of 55 °C, with a drastic activity decrease at 60 °C.

The pH optima of LevG-R and InuGB-R are relatively low compared with those of several other FTF enzymes. For instance, the levansucrase of *L. sanfranciscensis* has an optimum pH of 5.4 (Tieking *et al.*, 2005) and the FTF enzymes of *L. reuteri* display an optimum range of pH 4.5–5.5 (van Hijum *et al.*, 2003, 2004). In contrast, levansucrase from *L. mesenteroides* exhibited highest activity at pH 6.5–7 (Olvera *et al.*, 2007), while the inulosucrase of *L. johnsonii* NCC 533 exhibited maximum transglycosylation activity in two pH regions, pH 4.5–6.0 and at pH 7.0, and maximum hydrolytic activity at pH 7.0 (Anwar *et al.*, 2008). Optimal enzymic activity for levansucrase of *L. panis* (Waldherr *et al.*, 2008) was observed in the pH range 4–4.6, which is

almost the same as those of InuGB-R and LevG-R (this study). Higher optimum temperatures for activity have also been reported for the FTF enzymes from *L. reuteri* (50 °C) (van Hijum *et al.*, 2003, 2004), *L. sanfranciscensis* (35–45 °C) (Tieking *et al.*, 2005), *Bacillus* sp. (60 °C) (Ben Ammar *et al.*, 2002) and *L. panis* (45–50 °C) (Waldherr *et al.*, 2008).

Enzyme activity of InuGA-RM was most adversely affected by addition of EDTA in the reaction mixture, reaching a 92 % reduction at 800 µM EDTA. By comparison, EDTA less strongly affected the InuGB-R and LevG-R activity, causing a 30 and 48 % drop in activity, respectively, at 800 µM EDTA. Enzyme activities remained unaffected when 400 µM Ca²⁺ ions was added in the presence of 800 µM EDTA. These results indicated that calcium ions are required for optimal activity of the *L. gasseri* FTF enzymes, in agreement with the proven role of calcium ions for stability in other FTF enzymes. Using site-directed mutagenesis, Ozimek *et al.* (2005) demonstrated that Asp520 in inulosucrase of *L. reuteri* 121 plays an important role in Ca²⁺ binding. The equivalent amino acid residues in *L. gasseri* InuGA, InuGB and LevG are Asp515, 506 and 505, respectively. This Asp residue is highly conserved in family GH68 proteins of Gram-positive origin.

(ii) Kinetic parameters.

Kinetic parameters for the FTF enzyme activities were determined at 37 and 50 °C (Table 1). However, at both temperatures, the kinetic constants could not be determined for the total (V_G) and transglycosylation (V_{G-F}) activities of InuGA-RM and InuG-R, because these enzymes did not become saturated at the higher sucrose

concentrations. Consequently, high standard errors were obtained with curve fits.

The non-Michaelis–Menten kinetic behaviour exhibited by InuGA-RM and InuGB-R was similar to that reported for inulosucrases of *L. reuteri* 121 (van Hijum *et al.*, 2002) and *L. johnsonii* NCC 533 (Anwar *et al.*, 2008). This typical behaviour has been attributed to the oligosaccharides that were formed at an early stage of the reaction that might act as better acceptor substrates than the growing polymer chain (Anwar *et al.*, 2008). In spite of its high similarity (86 % identity) with InuGA-RM, InuGB-R exhibited significantly higher total and transglycosylation enzyme activities (Table 1).

All three enzymes exhibited higher total FTF activity values at 50 °C compared with 37 °C. The K_m and k_{cat} values were also higher at 50 °C. For InuGA-RM and LevG-R, the transglycosylation/hydrolysis ratios remained almost constant when the temperature was increased to 50 °C. However, LevG-R was highly hydrolytic at both temperatures with relatively low k_{cat} values for transglycosylation activity. Its K_m value for hydrolysis at 37 °C is comparable to that of the levansucrase of *B. megaterium* (Homann *et al.*, 2007). At 37 °C, InuGB-R exhibited about three times higher transglycosylation activity than hydrolysis, with a 1.3-fold increase at 50 °C.

Production and identification of the polymer products

The oligo- and polysaccharide products synthesized during growth of *L. gasseri* DSM 20604 and DSM 20077 cultures,

Table 1. FTF activities [U (mg protein)⁻¹] and apparent kinetic constants for the *L. gasseri* FTF enzymes

The kinetic constants are K_m (mM) and k_{cat} (s⁻¹) for formation of glucose (^G; total enzyme activity), formation of fructose (^F; hydrolytic enzyme activity) and glucose minus fructose (^{G-F}; transglycosylation enzyme activity). ND, Kinetic parameters could not be determined due to the fact that these enzymes did not become saturated with sucrose, resulting in high standard errors with curve fits. Values indicated are means ± SD of the results from two independent experiments.

Kinetic parameters	InuGA-RM		InuGB-R		LevG-R	
	37 °C*	50 °C†	37 °C‡	50 °C‡	37 °C‡	50 °C‡
Total activity§	316 ± 9.9	411 ± 17	378 ± 9.9	538 ± 6.4	238 ± 19.8	280 ± 2.1
Transglycosylation§	189 ± 11.3	243 ± 19.1	291 ± 3.5	430 ± 8.5	43 ± 0.7	50 ± 0.7
Hydrolysis§	127 ± 21.2	168 ± 2.1	88 ± 4.9	108 ± 13.4	195 ± 19.8	230 ± 1.4
Transglycosylation/hydrolysis§	1.5	1.5	3.0	4.0	0.2	0.2
K_m^G	ND	ND	ND	ND	6.7 ± 0.3	8.3 ± 0.9
k_{cat}^G	ND	ND	ND	ND	250 ± 20.8	294 ± 2.2
K_m^F	4.8 ± 1.0	5.8 ± 0.9	14.7 ± 4.3	9.4 ± 1.5	7.3 ± 1.5	8.3 ± 1.0
k_{cat}^F	133 ± 22.3	177 ± 2.2	92 ± 5.2	113 ± 14.1	204 ± 20.8	242 ± 0.7
K_m^{G-F}	ND	ND	ND	ND	3.2 ± 1.2	6.9 ± 1.6
k_{cat}^{G-F}	ND	ND	ND	ND	45 ± 0.7	53 ± 1.5

*Enzyme assays were carried out with 0.55 µg ml⁻¹ enzyme (final concentration).

†Enzyme assays were carried out with 0.25 µg ml⁻¹ enzyme (final concentration).

‡Enzyme assays were carried out with 0.45 µg ml⁻¹ enzyme (final concentration).

§Activity values measured at 500 mM sucrose.

and the purified recombinant InuGA-RM enzyme, with sucrose or raffinose at 37 °C for 5 days, were analysed by TLC. The TLC pattern showed that InuGA-RM and *L. gasseri* DSM 20604 convert sucrose into a range of FOS in addition to polymeric material (Fig. 2). ¹³C NMR spectra of the polysaccharides produced by InuGA-RM, the growing culture of *L. gasseri* DSM 20604 and its recombinant enzyme InuGB-R, revealed the presence of β2-1 linkages only, thus identifying the fructan formed as an inulin (Supplementary Fig. S2a; Table 2) (Anwar *et al.*, 2008; Morales-Arrieta *et al.*, 2006; Olivares-Illana *et al.*, 2002; Shimamura *et al.*, 1987; van Hijum *et al.*, 2001). *L. gasseri* 20077, in contrast, produced a fructan of the levan type, as revealed by ¹³C NMR spectroscopy of the purified polysaccharide product (Supplementary Fig S2b; Table 2). *L. gasseri* 20243 did not produce any FOS or polymer product in spite of its growth on MRS medium containing sucrose.

HPAEC analysis showed that the FOS produced by *L. gasseri* 20604 and the InuGA-RM enzyme was of the inulin-type, and oligosaccharides ranging from GF2 to GF13 were clearly detected on the chromatogram (Fig. 3a and c). In contrast, *L. gasseri* DSM 20077 synthesized a polymer product and kestose only (Fig. 2 and Fig. 3b). In a recent report (Azcarate-Peril *et al.*, 2008), none of the nine *L. gasseri* strains tested, including the type strain (DSM 20243), was found to ferment raffinose. We also could not detect any growth or product formation by the three *L. gasseri* strains used in our studies, not even after 5 days of incubation with 500 mM raffinose (Fig. 4a). However, all three strains showed copious growth on MRS–raffinose medium supplemented with 50 mM sucrose, indicating that the growth was not inhibited by raffinose.

Also, the recombinant purified *L. gasseri* inulosucrases InuGA-RM and InuGB-R produced inulin polymer and FOS (data not shown), with similar TLC patterns to those observed for the products of whole cells of the respective strains. These TLC patterns were similar to those observed

Fig. 2. TLC analysis of oligo/polysaccharides produced by *L. gasseri* strains DSM 20604 (1) and DSM 20077 (2), and by the recombinant purified InuGA-RM enzyme from *L. gasseri* DSM 20243 (3). ST, Sucrose, kestose and nystose standards.

for the *L. reuteri* 121 Inu products (Ozimek *et al.*, 2006b), but were clearly different from those of the *L. johnsonii* NC533 InuJ products (Anwar *et al.*, 2008), due to the absence of the unidentified product spot observed for the latter between the sucrose and kestose spots. Moreover, HPAEC chromatograms of InuGA-RM and InuGB-R FOS products exhibited single peaks, whereas double peaks were observed for InuJ products (Anwar *et al.*, 2008). The data

Table 2. ¹³C NMR chemical shift values of fructans produced by *Lactobacillus* strains (S) and recombinant FTF enzymes (R)

Carbon atom	Levan <i>L. reuteri</i> 121*		Inulin <i>L. johnsonii</i> NCC 533†		Levan <i>L. gasseri</i> DSM 20077		Inulin <i>L. gasseri</i> DSM 20243	Inulin <i>L. gasseri</i> DSM 20604	
	S	R	S	R	S	R	R	S	R
C-1	61.7	59.6	62.1	62.2	61.7	61.6	62.0	62.1	62.1
C-2	105.0	104.0	104.1	104.2	105.1	105.1	104.1	104.1	104.1
C-3	78.1	76.0	78.3	78.4	78.0	77.9	78.2	78.2	78.3
C-4	76.6	74.9	75.6	75.6	76.6	76.5	75.6	75.6	75.6
C-5	81.2	80.0	82.2	82.2	81.3	81.2	82.1	82.1	82.1
C-6	64.3	63.2	63.2	63.1	64.3	64.3	63.1	63.1	63.1

*van Hijum *et al.* (2001).

†Anwar *et al.* (2008).

Fig. 3. HPAE chromatography analysis of the FOS products synthesized by *L. gasseri* strains DSM 20604 (a) and DSM 20077 (b) *in situ*, and by the purified recombinant InuGA-RM (c) enzyme. Dotted lines, inulin standards, a 1 : 1 mixture of Raftiline ST-Gel and Raftiline HP representing chicory inulin.

reported here for the *L. gasseri* strains DSM20604 and DSM20077, and the purified recombinant InuGA-RM, InuGB-R and LevG-R enzymes, also provide further evidence that inulosucrase, but not levansucrase, enzymes of lactobacilli synthesize a range of FOS molecules, in addition to the inulin polymer (Fig. 2). The broad range of inulin-type FOS synthesis by the growing culture of *L. gasseri* DSM 20604 (and by the InuGB-R enzyme) has never been reported for any other bacteria (and inulosucrase enzymes). These results thus reveal interesting variations in the composition of FOS synthesized by the recombinant inulosucrases of various lactobacilli. Although none of the three *L. gasseri* strains was able to ferment raffinose, their respective recombinant enzymes were clearly active with raffinose as a substrate. The enzymes InuGA-RM and InuGB-R converted raffinose into a polymer and into FOS with a TLC pattern comparable to that of *L. reuteri* InuΔ699His, whereas LevG-R produced a polymer and a single oligosaccharide (most likely Gal-Glc-Frc-Frc) (Fig. 4b). It remains unclear why the *L. gasseri* and *L. reuteri* 121 strains differ in their ability to grow on raffinose.

Quantitative analysis of the products synthesized by the FTF enzymes

After 7 days of incubation with 600 mM sucrose at 37 °C, equal units (100 U l⁻¹) of the InuGA-RM, InuGB-R and LevG-R produced 11.0 ± 1.6 g l⁻¹, 13.0 ± 0.5 g l⁻¹ and 15.0 ± 0.4 g l⁻¹ of the fructan polysaccharide product, respectively. These values were significantly higher than those reported for *L. reuteri* 121 Inu and Lev, which produced 0.8 g l⁻¹ and 1.4 g l⁻¹ polymers, respectively (van Hijum *et al.*, 2001, 2002). However, it is noteworthy that lower sucrose and enzyme concentrations were used and less incubation time was given in the *L. reuteri* 121 studies. Our HPAEC data (Table 3) revealed that the maximum amount of added sucrose was consumed by InuGB-R, of which the major portion was integrated into FOS, while about 20 ± 1.8 g l⁻¹ of fructose was incorporated into the inulin polymer product. A similar trend was observed for InuGA-RM, where 41 ± 2.9 g l⁻¹ of fructose was incorporated into FOS. FOS production was also the dominant reaction of *L. reuteri* 121 Inu (van Hijum *et al.*, 2002), constituting about 86 % of the transglycosylation reaction. The levansucrase, LevG-R, consumed about 66 % of the total sucrose, with the major part being hydrolysed and about 22 ± 0.9 g l⁻¹ of fructose incorporated into polysaccharide product. The extent of sucrose hydrolysis (determined as free fructose) for LevG-R is about 2.0–2.5 times higher than that for InuGA-RM and InuGB-R (Table 3). Higher hydrolytic activity (51.6 % of sucrose consumed) has also been reported for *L. reuteri* 121 Lev, which is about three times higher than the hydrolytic activity exhibited by inulosucrase of the same strain (at a sucrose concentration of 840 mM) (Ozimek *et al.*, 2006b). On the contrary, levansucrase of *L. panis* yielded higher amounts of fructan and kestose, indicating predominant transgly-

Fig. 4. TLC analysis of the products synthesized (a) after 5 days of incubation in MRS-raffinose medium by cells of *L. reuteri* 121 (control) (1) and cells of *L. gasseri* strains DSM 20243 (2), DSM 20604 (3), and DSM 20077 (4), and (b) after 18 h incubation of recombinant FTF enzymes with 500 mM raffinose by *L. reuteri* 121 Inu (control) (1), InuGA-RM (2), InuGB-R (3) and LevG-R (4).

cosylation reaction at 500 mM sucrose concentration (Waldherr *et al.*, 2008). Studies with truncated versions of *L. sanfranciscensis* levansucrase, Lev Δ His and LevCD Δ His, have shown that environmental conditions affect product ratios, with low temperature and high sucrose concentration favouring production of kestose (Tiekink *et al.*, 2005). Nonetheless, significantly high amounts of free fructose were produced in all incubations with these Lev enzymes, indicating high hydrolytic activity. The differences in transglycosylation/hydrolysis activities of levan- and inulosucrase enzymes also indicate that these enzymes diverge at the structural level.

Enzyme localization studies

The LPXTG/LPKAG motifs are known as cell-wall-anchoring motifs for proteins in many bacteria. Presence of an incomplete cell-wall-anchoring domain and lack of the LPXTG/LPKAG motif raised questions about the cellular location of the *L. gasseri* 20604 inulosucrase protein. The impact was clearly indicated by the tendency of the enzyme to be secreted into the growth medium in contrast with the *L. gasseri* 20077 levansucrase, which was exclusively detected associated with the cell surface (Table 4).

Washed cell suspensions and dialysed culture supernatants of *L. gasseri* DSM 20604 exhibited nearly equal total FTF activities (Table 4). However, the transglycosylation:hy-

drolisis ratio was about four times higher for the cell-associated enzyme than that of the free enzyme present in the supernatant. By contrast, virtually all the FTF activity of *L. gasseri* DSM 20077 was cell-wall-associated, displaying equal transglycosylation and hydrolytic activity. Although relatively low, FTF activity (measured as glucose and fructose release) was also detected for *L. gasseri* 20243 cells. Neither fructan nor FOS production was observed for this strain, not even after long incubation times with sucrose, reflecting the inactive status of the InuGA enzyme. The measured activity might have resulted from the action of two putative glycoside hydrolase family GH32 proteins (<http://www.cazy.org/fam/GH32.html>) in *L. gasseri* DSM 20243, LGAS_0399 (GenBank accession no. ABJ59804.1) and LGAS_1779 (GenBank accession no. ABJ61066.1), both annotated as sucrose-6-phosphate hydrolases, causing sucrose hydrolysis. In our FTF enzyme activity assays, we used 0.1 vols 1 M NaOH to stop the reactions. Although the absence of signal sequences suggests that these enzymes are intracellular, it is most likely that their products become released due to cell lysis by NaOH treatment and that they contributed to the measured sucrose hydrolytic activity.

Conclusions

Synthesis of fructose polysaccharides (fructans) and/or oligosaccharides (FOS) from sucrose, involving fructansucrase enzymes, is one of the potential factors that confer a

Table 3. Quantitative analysis of the various products synthesized by the purified *L. gasseri* FTF enzymes (each supplied at 100 U l⁻¹) from sucrose (600 mM) during incubation for 7 days at 37 °C

Values shown are means $\pm$ SD of the results obtained from three independent experiments.

FTF enzyme	Sucrose consumed (%) [*]	Free glucose (g l ⁻¹)	Free fructose (g l ⁻¹)	Fructose incorporated in fructan polymer (g l ⁻¹)	Fructose incorporated in FOS (g l ⁻¹)
InuGA-RM	78 $\pm$ 0.6	73 $\pm$ 1.7	14 $\pm$ 0.8	18 $\pm$ 2.1	41 $\pm$ 2.9
InuGB-R	91 $\pm$ 1.2	85 $\pm$ 1.3	16 $\pm$ 0.7	20 $\pm$ 1.8	49 $\pm$ 3.3
LevG-R	66 $\pm$ 1.7	63 $\pm$ 1.0	30 $\pm$ 0.8	22 $\pm$ 0.9	11 $\pm$ 1.6

^{*}Percentage of the total (600 mM) sucrose used.

Table 4. FTF enzyme activities (U ml⁻¹) of *L. gasseri* strains (with sucrose as substrate) on the surfaces of washed *L. gasseri* cells (the DSM 20604 FTF enzyme has no cell anchoring LPXTG motif), as well as in their dialysed culture supernatants

Values are the means $\pm$ SD of the results from three independent experiments.

Activity	<i>L. gasseri</i> DSM 20243		<i>L. gasseri</i> DSM 20604		<i>L. gasseri</i> DSM 20077	
	Cells	Supernatant	Cells	Supernatant	Cells	Supernatant
Total	6.7 $\pm$ 0.6	0	27 $\pm$ 0.8	24 $\pm$ 1.3	23 $\pm$ 0.3	0
Hydrolysis	4.7 $\pm$ 0.1	0	6.0 $\pm$ 2.2	13 $\pm$ 1.7	11 $\pm$ 1.7	0
Transglycosylation	2.0 $\pm$ 0.7	0	21 $\pm$ 2.9	11 $\pm$ 0.4	12 $\pm$ 2.0	0
Transglycosylation/ hydrolysis	0.42	–	3.5	0.85	1.1	–

probiotic role(s) to several lactobacilli. In this work, we have studied three different *L. gasseri* strains from the DSMZ culture collection, including the type strain, and found considerable heterogeneity among their *ftf*FTF genes/enzymes and the fructan products synthesized. These data provide important novel insights into the variation in properties occurring between closely related strains. The *L. gasseri* type strain (DSM 20243) did not synthesize any fructan product, apparently because it harbours an *ftf* gene with a preliminary stop codon rendering it inactive. Replacement of the stop codon with a glutamine codon resulted in an active inulosucrase gene of a normal length; the recombinant InuGA-RM enzyme converted sucrose into inulin poly- and oligosaccharides. The *L. gasseri* strain DSM 20604 clearly synthesized both inulin poly- and oligosaccharides, products of its inulosucrase enzyme InuGB. Strain DSM 20604 produced a broad range of FOS of the inulin type ranging from DP2–DP13; the larger FOS particularly are not produced in similar quantities by other bacteria, nor by their purified FTF enzymes. The InuGB protein lacks the cell-anchoring-motif resulting in a partly free and partly cell-associated status. Strain DSM 20077 synthesized a levan polysaccharide, the product of its levansucrase enzyme LevG. Sequence analysis showed that the overall domain organization of the InuGA, InuGB and LevG proteins was similar to those for FTFs of other lactobacilli. An interesting feature of the *L. gasseri* strains is that they were unable to ferment raffinose, whereas their respective recombinant enzymes used raffinose as a substrate.

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