Fructosyltransferases of Lactobacillus reuteri

van Hijum, Sacha Adrianus Fokke Taco

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Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides

S.A.F.T. van Hijum, G.H. van Geel-Schutten, H. Rahaoui, M.J.E.C. van der Maarel, and L. Dijkhuizen

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SUMMARY

Fructosyltransferase (FTF) enzymes produce fructose polymers (fructans) from sucrose. Here we report the isolation and characterization of an FTF encoding gene from *Lactobacillus reuteri* 121. A C-terminally truncated version of the *ftf* gene was successfully expressed in *Escherichia coli*. When incubated with sucrose, the purified recombinant FTF produced large amounts of fructo-oligosaccharides (FOS) with β(2→1) linked fructosyl units, plus a high molecular weight fructan polymer (>1×10^7) with β(2→1) linkages (an inulin). FOS, but not inulin, was found in supernatants of *L. reuteri* 121 cultures grown on media containing sucrose. Bacterial inulin production has been reported only for *Streptococcus mutans* strains. FOS production has been reported for a few bacterial strains. This paper reports the first-time isolation and molecular characterization of a (i) *Lactobacillus ftf* gene, (ii) an "inulosucrase" associated with a Generally Regarded As Safe (GRAS) bacterium, (iii) an FTF enzyme synthesizing both a high molecular weight inulin and FOS, and (iv) an FTF protein containing a cell-wall anchoring LPXTG motif. The biological relevance and potential health benefits of an "inulosucrase" associated with a *L. reuteri* strain remains to be established.

INTRODUCTION

Fructose polymers (fructans) are produced by a wide range of bacteria. Limited information is available about fructan synthesis by lactic acid bacteria. Most attention has been focussed on oral streptococci because of their role in dental caries formation [11]. Streptococci produce both fructans of the levan type [47, 66] with β(2→6) linked fructosyl units, and of the inulin type with ß(2→1) linked fructosyl units. *S. mutans* JC-2 for instance produces a fructan consisting mainly of ß(2→1) linked fructosyl units with 5% ß(2→6) branches [47, 151]. The fructan produced by *S. mutans* Ingbritt contains mainly ß(2→1) linked fructosyl units [3]. Previously, we have reported that *L. reuteri* 121 cultivated on media containing sucrose produces both a glucan and a fructan polymer [204]. The fructan polymer is a linear levan, consisting of only β(2→6) linked fructosyl residues, with an estimated size of 150,000 [204].

The enzymes responsible for the synthesis of fructans are generally referred to as fructosyltransferases (FTF), or more specifically, levansucrase (in case of levan synthesis) and inulosucrase (in case of inulin synthesis). Inulosucrase (sucrose: 2,1-β-D-fructan 1-β-D fructosyl-transferase, E.C. 2.4.1.9) catalyzes the following reaction:

$$\text{sucrose} + (\text{2,1-β-D-fructosyl})_n \rightarrow \text{D-glucose} + (\text{2,1-β-D-fructosyl})_{n+1}$$

Bacterial inulosucrases have only been described for *S. mutans* strains [36, 151, 162]. Levansucrase enzymes have been found in for instance *Zymomonas mobilis* [215], *Erwinia amylovora* [61], *Gluconacetobacter diazotrophicus* [71], *Bacillus amyloliquefaciens* [192], and *Bacillus subtilis* [183, 192]. Amongst lactic acid bacteria, levansucrase enzymes have been reported for *Leuconostoc mesenteroides* [150], *Streptococcus salivarius* [47, 66], and *L. reuteri* (**Chapter 3**). Recently, we have reported purification of the levansucrase enzyme responsible for levan formation in *L. reuteri* 121 (**Chapter 3**).

The *Lactobacillus* polysaccharides are of special interest because they may also contribute to human health as prebiotics [24], or because of their antitumoral [41], antiulcer [128], immunomodulating [164], or cholesterol lowering [146] activity. Moreover, some strains (e.g. *L. reuteri*) have been designated as probiotics [24, 58, 67].

Here we report the molecular characterization of a novel *ftf* gene from *L. reuteri* 121, including analysis of the products synthesized from sucrose by the enzyme expressed in *E. coli*. The enzyme is an inulosucrase, synthesizing both inulin fructo-oligosaccharides and a high molecular weight inulin.

MATERIALS AND METHODS

Strains, media, and growth conditions. *L. reuteri* 121 (culture collection TNO Nutrition and Food Research, Zeist, the Netherlands) was grown anaerobically at 37 ºC in MRS medium [40], or in modified MRS medium containing 50 $g.l^{-1}$ sucrose (MRS-S), instead of 20 $g.l^{-1}$ glucose. Because phosphate and citrate interfered with HPLC, fructo-oligosaccharide (FOS) production by *L. reuteri* was studied using cultures grown in modified MRS-S medium in which phosphate was omitted and ammonium citrate was replaced by ammonium nitrate.

E. coli strains JM109 (Phabagen, Utrecht, the Netherlands) and Top10 (Invitrogen, Carlsbad, Calif.) were used as hosts for cloning. *E. coli* strains were grown aerobically at 37ºC in Luria-Bertani (LB) medium [8], when appropriate supplemented with 50 $\mu g.ml^{-1}$ ampicillin for maintaining plasmids, or with 0.02% arabinose (w/v) for induction of *ftf* gene expression. Agar plates were made by adding 1.5% (w/v) agar to the media.

General molecular techniques. *L. reuteri* chromosomal DNA was isolated according to [208] as modified by [117]. *E. coli* plasmid DNA was isolated using a Wizard Plus SV plasmid extraction kit (Promega, Madison, Wisc.). General procedures for cloning, DNA manipulations, and agarose gel electrophoresis were as described [159]. Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers (GIBCO BRL Life Technologies, Breda, The Netherlands; New England Biolabs Inc., Beverly, Mass.; Roche Biochemicals, Basel, Switzerland). DNA was amplified with PCR techniques (DNA Thermal Cycler 480, Applied Biosystems, Foster City, Calif.), using *Pwo* DNA polymerase (Roche Biochemicals) for the standard PCR reactions and High-Fidelity DNA polymerase (Roche Biochemicals) for inverse PCR reactions. Oligonucleotides were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, N.Y.). DNA fragments were isolated from agarose gels using a Qiagen extraction kit, following the instructions of the supplier (Qiagen Inc., Chatsworth, Calif.). *E. coli* transformations were performed by electroporation in 0.2 mm cuvettes using the Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.) at 2.5 kV, 25 μF and 200 Ω, following the instructions of the manufacturer.

For Southern hybridization studies, DNA was restricted, separated by agarose gel electrophoresis, and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Probes were labelled using the DIG DNA random primed labelling and detection kit (Roche Biochemicals), following the manufacturer's instructions. Stringent hybridizations were done at 62 °C; non-stringent hybridizations were done at 45°C (followed by washing with 0.5 × SSC / 0.1 SDS).

Identification and nucleotide sequence analysis of the fructosyltransferase gene. Using the degenerated primers 5FTF and 6FTFi, based on conserved amino acid sequences of FTFs of Gram-positive origin (Fig. 1), chromosomal DNA of *L. reuteri* 121, and DNA polymerase, a PCR product of 234 bp was obtained (Fig. 2A). This fragment was cloned in *E. coli* JM109 using the pCR2.1 (Invitrogen) vector and sequenced. Primer 7FTF (Table 1) was designed based on this fragment, and primer 12FTFi was based on conserved amino acid sequences of FTFs of Gram-positive bacteria (Fig. 1). PCR with primers 7FTF and 12FTFi gave a product of 948 bp (Fig. 2B), which was cloned in pCR2.1 and sequenced. The 948 bp

product was used to design primers FTFC1i (Table 1) and FTFC2 (Table 1) for inverse PCR purposes. *L. reuteri* DNA was cut with *Nhe*I, ligated, and used in a PCR reaction with the primers FTFC1i and FTFC2 generating a 1438 bp fragment (Fig. 2C) which was cloned and sequenced.

The remaining 5' fragment of the putative *ftf* gene was isolated by standard and inverse PCR techniques. The 234 bp fragment (Fig. 2A) was used to design primer 8FTFi (Table 1) for inverse PCR purposes. *L. reuteri* DNA was cut with several restriction enzymes and ligated. PCR with primers 7FTF and 8FTFi, using the ligation product as template, yielded in all cases a PCR product of approximately 400 bp, which was cloned into pCR2.1 and sequenced (379 bp; Fig. 2D). This revealed that primer 8FTFi had annealed aspecifically (from position 1896 to 1917) as well as specifically (from position 2275 to 2254). This fragment was used to design primer 20FTFi (Table 1). Based on 7 amino acids (YNGVAEV) from an N-terminal amino acid sequence (QVESNNYNGVAEVNTERQANGQI) of a levansucrase enzyme purified from *L. reuteri* 121 (**Chapter 3**) a degenerated primer 19FTF (Table 1) was designed. PCR with primers 19FTF and 20FTFi gave a 754 bp PCR product (Fig. 2E), which was cloned into pCR2.1 and sequenced. Two inverse PCR primers FTFN1i (Table 1) and FTFN2 (Table 1) were designed based on this fragment. Strain 121 DNA was cut with *Bcl*I, ligated, and used in a PCR with primers FTFN1i and FTFN2 yielding a 1752 bp PCR product (Fig. 2F). Both DNA strands of the fragments comprising the entire contig were sequenced. In this way the sequence of a 3983 bp region of *L. reuteri* 121 genomic DNA, containing the *ftf* gene, was obtained.

5FTF

B. amy SacB	80	GL**DVWDSWP**LQNAD	93
B. sub SacB	82	GL**DVWDSWP**LQNAD	95
S. mut FTF	243	DL**DVWDSWP**VQDAK	256
S. sal FTF	282	EI**DVWDSWP**VQDAK	295
		:*******:*:*.	

6FTFi

B. amy SacB	56	QT**QEWSGSAT**FTSDGK	171
B. sub SacB	158	QT**QEWSGSAT**FTSDGK	173
S. mut FTF	312	LT**QEWSGSAT**VNEDGS	327
S. sal FTF	351	DD**QQWSGSAT**VNSDGS	366
		*:******...**.	

12FTFi

B. amy SacB	440	K**ATFGPSFL**MN	450
B. sub SacB	440	Q**STFAPSFL**LN	450
S. mut FTF	609	N**STWAPSFL**IQ	619
S. sal FTF	655	K**STWAPSFL**IK	665
		::*:.****::	

Figure 1. Parts of an alignment of amino acid sequences from four bacterial FTFs. The amino acid sequences are from *B. amyloliquefaciens* SacB (X52988), *B. subtilis* SacB (X02730), *S. mutans* FTF (M18954), and *S. salivarius* FTF (L08445). Sequences in bold indicate the consensus sequences used to construct the degenerated primers 5FTF (5'-GAYGTNTGGGAYWSNTGGGCC-3'), 6FTFi (5'-GTNGCNSWNCCNSWCCAYTSYTG-3') and 12FTFi (5'-ARRAANSWNGGNGCVMANGTNSW-3'). Sequences are according to International Union of Biochemistry (IUB) group codes (N=any base; M=A or C; R=A or G; W=A or T; S=C or G; Y=C or T; K=G or T; B= not A; D=not C; H=not G; V=not T).

Table 1. Primers used in this study. Degenerated bases are according to IUB codes shown in the legends to Fig. 1. In the primer sequences, *Nco*I and *Bgl*II restriction sites are underlined and stop codons introduced are in bold.

Primer[a]	Sequence (5' to 3')	Use
7FTF	GAATGTAGGTCCAATTTTTGGC	PCR
FTFC1i	GTGATACATTTCCATTATTATCAG	inverse PCR
FTFC2	CTATTACTACCAAACTTATGATCATG	inverse PCR
8FTFi	CCTGTCCGAACATCTTGAACTG	inverse PCR
20FTFi	CCGACCATCTTGTTTGATTAAC	PCR
19FTF	TAYAAYGGNGTNGCNGARGTNAA	PCR
FTFN1i	GAGAGTGTAACGACTGCCCAATTTTTACCGC	inverse PCR
FTFN2	CTGCTAATGCAAATAGTGCTTCTTCTGCCGC	inverse PCR
FTF1	CCATGGCCATGGTAGAACGCAAGGAACATAAAAAAATG	pBAD
FTF2i	AGATCTAGATCTGTTAAATCGACGTTTGTTAATTTCTG	pBAD
FTF2iB	AGATCTAGATCT**TTA**GTTAAATCGACGTTTGTTAATTTCTG	pBAD
FTFrp2	AGATCTAGATCTTTTTAATCCATAACCAATTAAG	pBAD
FTFrp2B	AGATCTAGATCT**TTA**TTTTAATCCATAACCAATTAAG	pBAD

[a] i, rp = reverse.

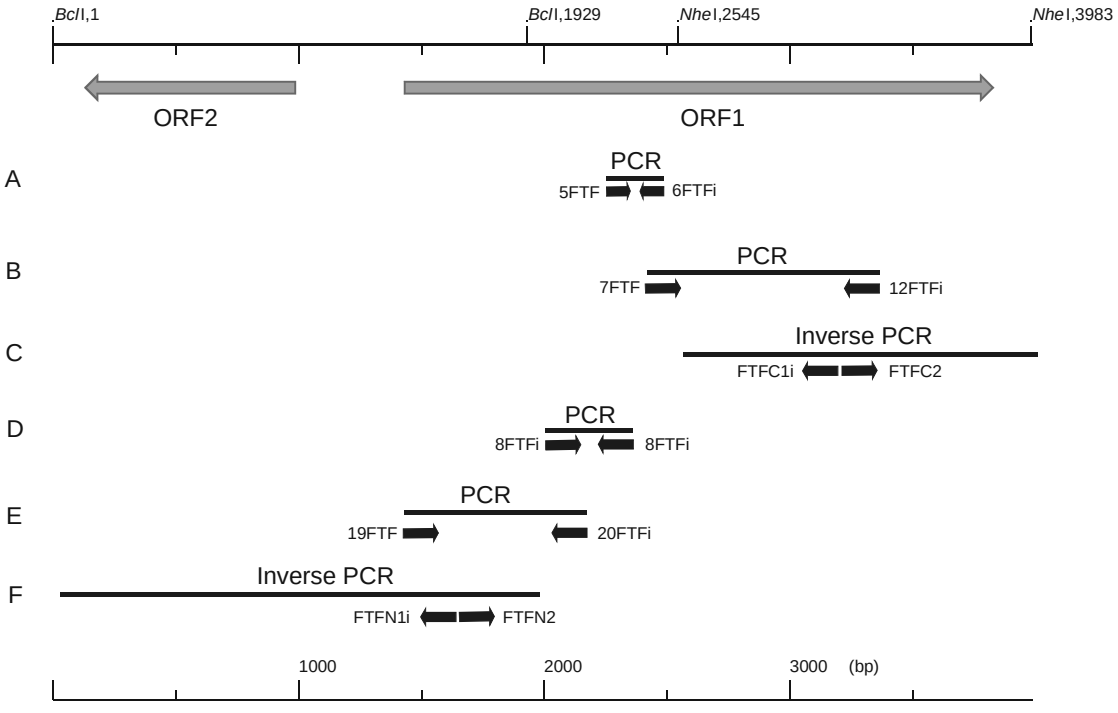


Figure 2. Strategy used for the isolation of the *ftf* gene from *L. reuteri* 121 chromosomal DNA.

Construction of plasmids for expression of the *ftf* gene in *E. coli*. Four primer sets were designed for expression of the *ftf* gene in *E. coli*, (i) a full-length *ftf* (nucleotides 1432 to 3825) with a C-terminal myc epitope and poly Histidine tag (InuHis) was generated with primers FTF1 (Table 1) and FTF2i (Table 1); (ii) a full-length *ftf* (Inu) was generated with primers FTF1 and FTF2iB (Table 1); (iii) a truncated *ftf* (nucleotides 1432 to 3525) encoding

an FTF C-terminally truncated from amino acid 699 onwards with a C-terminal myc epitope and poly Histidine tag (InuΔ699His) was generated with primers FTF1 and FTFrp2 (Table 1); (iv) a truncated *ftf* encoding an FTF C-terminally truncated from amino acid 699 onwards (InuΔ699) was generated with primers FTF1 and FTFrp2B (Table 1). Primer FTF1 introduces a mutation in the *ftf* sequence, changing the second (Leucine) codon into a Valine codon, necessary for introduction of the *Nco*I restriction site. PCR with *L. reuteri* genomic DNA (approximately 1 µg), *Pwo* DNA polymerase, and the primer sets, yielded the *ftf* gene derivatives flanked by *Nco*I and *Bgl*II restriction sites. The PCR products were cut with *Nco*I and *Bgl*II and ligated into the expression vector pBAD/myc-his C (Invitrogen), downstream of the inducible arabinose promoter and in frame upstream of a myc epitope and a poly Histidine tag. The resulting constructs were transformed to *E. coli* Top10 and used to study FTF expression. Correct construction of the plasmids containing the four *ftf* gene derivatives was confirmed by sequence analysis of both DNA strands of the inserts.

FTF purification. Cells of *E. coli* Top10 harbouring the *ftf* gene (InuHis and InuΔ699His constructs) were grown overnight in 400 ml LB medium with 0.02% arabinose to an OD_{600} of approximately 0.9. The pellets were washed with 50 ml binding buffer (50 mM Na_2HPO_4/NaH_2PO_4, pH 8.0) containing 5 mM β-mercaptoethanol, centrifuged, and resuspended in binding buffer containing 5 mM β-mercaptoethanol. Cells were broken by ultrasonication (7 × 10 sec at 6 microns with 20 sec intervals), followed by centrifugation for 30 min at 10,000 × *g*. The resulting cell free extracts (CE) were used in enzyme assays, or in the case of InuΔ699His, for Nickel-affinity purification. A bed volume of 600 µl Ni-NTA resin (Qiagen) was used to bind protein from 11.6 ml CE (3.0 mg protein ml^{-1}). The resin was washed with 6 ml of demineralized water and 3 ml binding buffer prior to applying CE. CE was added to the washed Ni-NTA agarose and gently shaken at 4 °C for 1 h. After washing with 3 ml binding buffer, the bound protein was eluted from the affinity resin with 3 ml of binding buffer containing 200 mM imidazole and 1 mM β-mercaptoethanol. The eluted fractions were dialyzed against a phosphate solution (5 mM, pH 8) and stored at 4 °C. The dialyzed Ni-NTA elution fractions (3 ml; 2.0 mg protein ml^{-1}) were adjusted to a volume of 5 ml in a Tris buffer (20 mM, pH 8.0). An anion exchange column (Amersham Pharmacia Biotech; Resource-Q; 1 ml column volume; flow rate 1 $ml.min^{-1}$) was equilibrated with a Tris buffer (20 mM, pH 8.0; A) and the sample (5 ml) was loaded on the column. After eluting the column with Tris buffer (20 mM, pH 8.0, 0.5 M NaCl; B), fractions were collected from 20% B to 80% B and screened for FTF activity (glucose release from sucrose; see below). Positive fractions were checked on SDS-PAGE [98], using a mini-Protean II slab gel system (Bio-Rad), and fractions showing only the FTF protein band were pooled (11.7 ml; 0.038 mg protein ml^{-1}), dialyzed overnight against a sodium acetate (NaAc) buffer (20 mM, pH 5.4), and stored at 4 °C for further analysis.

Biochemical characterization of the recombinant FTF enzyme. (i) N-terminal amino acid sequencing. Protein (approximately 10 µg) was run on SDS-PAGE and transferred to a PVDF membrane (Millipore Inc., Bedford, Mass.) by Western blotting [159]. After staining the PVDF membrane with Coomassie Brilliant Blue [159], the corresponding bands were cut from the PVDF membrane and subjected to amino acid sequence determination (Nucleic Acid Protein equence Unit, Biotechnology Laboratory, University of British Columbia, Vancouver, Canada) using standard Pulsed-Liquid Edman chemistry on a Procise protein sequencing system (model 494; Applied Biosystems).

(ii) Sucrase enzyme activity assays. Sucrose conversion by FTF yields glucose (and fructose, which is partly built into the fructan polymer) in a 1:1 molar ratio to the amount of sucrose used. The amount of glucose released allows determination of the overall enzyme activity (total amount of sucrose converted). Glucose was measured as described (**Chapter 3**). FTF enzyme activity was measured in a NaAc buffer (25 mM; pH 5.4) with 250 mM sucrose and 1 mM $CaCl_2$. Preliminary experiments showed that the recombinant FTF enzyme had highest glucose releasing activity from sucrose at 50 °C. Therefore, specific activity measurements were done at 50 °C. One unit of enzyme is defined as the release of 1 µmol glucose per min.

(iii) SDS-PAGE activity staining. In order to measure enzyme activity in SDS-PAGE gels, a periodic acid Schiffs reagent staining (PAS) [89] was done. Protein (approximately 1 µg) was run on SDS-PAGE, followed by washing in a preincubation buffer (25 mM NaAc pH 5.4; 1 mM $CaCl_2$; 0.5% Triton X-100), and overnight incubation in preincubation buffer with 50 mM sucrose or 50 mM raffinose. The gel was washed 30 min in a 12.5 % trifluoro acetic acid solution, in demineralized water, and incubated for 50 min in a 1 % periodic acid / 3 % HAc buffer. The periodic acid was washed away carefully with demineralized water, after which the gel was stained with Schiffs reagent (Sigma-Aldrich, St. Louis, MO) yielding purple spots where fructan polymer was produced.

Characterization of fructan and FOS. The FTF enzyme had an optimum temperature of 50 °C for the release of glucose from sucrose. *L. reuteri* 121, however, shows optimal growth at 37 °C. The reaction products of FTF were determined following incubations at the physiological temperature of strain 121 (37 °C). Fructan and FOS synthesis was studied in cell extracts of *E. coli* cells with InuHis (96 $U.l^{-1}$) and with the purified InuΔ699His (83 $U.l^{-1}$). FTF protein was incubated for 17 h at 37 °C in 250 ml NaAc buffer (25 mM, pH 5.4) containing 90 $g.l^{-1}$ sucrose and 1 mM $CaCl_2$. Polymeric fructan material was precipitated with 2 volumes of 96% ethanol followed by 10 min centrifugation at 10,000 × *g*. The pellet was resuspended in demineralized water during 16 h at 4 °C after which it was dialyzed against demineralized water. Subsequently, polymer was precipitated as described above and freeze dried for further analysis. Inulin and FOS synthesis was also studied in cultures of *L. reuteri* 121 grown overnight in modified MRS-S media. For **anion-exchange chromatography (Dionex)**, samples were centrifuged for 30 min at 10,000 × *g* and diluted 200 times in a 100% DMSO solution. An inulin digest (with a degree of polymerization of 1-20), 1-kestose (8 $mg.l^{-1}$), or nystose (6 $mg.l^{-1}$; Fluka Chemie, Milwaukee, Wisc.) were used as a standard. Separation was achieved with a CarboPac PA1 anion exchange column (250 × 4 mm; Dionex, Sunnyvale, Calif.) coupled to a CarboPac PA1 guard column (Dionex). The following gradient was used: 5 % eluent B (0 min); 35% (10 min); 45% (20 min); 65% (50 min); 100% (54-60 min); 5% (61-65 min). Eluent A was sodium hydroxide (0.1 M) and eluent B was NaAc (0.6 M) in sodium hydroxide (0.1 M). Detection was done with an ED40 Electrochemical Detector (Dionex) with an Au working electrode and a Ag/AgCl reference-electrode with a sensitivity of 300 nC. The pulse program used was: +0.1 Volt (0-0.4 s); +0.7 Volt (0.41-0.60 s); -0.1 Volt (0.61-1.00 s). Data were integrated using a Turbochrom (Applied Biosystems) data integration system. For **cation-exchange chromatography**, samples were centrifuged for 30 min at 10,000 × *g* and diluted 5 times in demineralized water. A cation exchange column in the calcium form (at 85°C; Benson BCX4, particle size 15-20 µm, column size 250 × 10 mm; 0.4 $ml.min^{-1}$; Benson polymeric / Sierra separations, Sparks, Nevada) with a mobile phase of calcium-EDTA in demineralized water (100 ppm) was used. Detection was done by using an 830-RI refractive index detector (Jasco International Co. Ltd., Tokyo, Japan; at 40 °C). The amounts of FOS, glucose, fructose, and sucrose were estimated by comparing the RI signals with those of glucose reference solutions. **Methylation analysis** of fructan was performed by permethylation using

methyl iodide and solid sodium hydroxide in methyl sulfoxide. After hydrolysis with 2 M TFA (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBD_4$. After neutralization, removal of boric acid by co-evaporation with methanol, and acetylation with acetic acid anhydride (3 h, 120 °C), the mixtures of partially methylated alditol acetates obtained were analyzed by gas-liquid chromatography on CP-Sil 43 CB (Merck, KGaA, Darmstadt, Germany) and by gas-liquid chromatography-mass spectrometry on DB-1 (Agilent Technologies Inc., Palo Alto, Calif.) [85, 87]. The molecular weight of the polymer was determined by **high performance size exclusion chromatography (HPSEC)** coupled on-line with a multi angle laser light scattering (MALLS) and differential refractive index detection (RI; Schambeck SDF, Bad Honnef, Germany). The HPSEC system consisted of an isocratic pump, an injection valve, a guard column, and a set of two size exclusion chromatography columns in a series (a Shodex SB806MHQ column [Showa Denko, K.K., Kawasaki, Japan] and a TSK gel 6000PW column [Thomson, Clearbrook, Vancouver, Canada]). A Dawn-DSP-F (Wyatt Technology, St. Barbara, Calif.) laser photometer HeNe (λ = 623.8 nm) equipped with a K5 flow cell, and thermostatted by a Peltier heating system, was used as MALLS detector. Samples were filtered through a 0.45 µm filter (Millipore Inc.) and the injection volume was 220 µl. Na_2SO_4 (0.1 M) was used as eluent at a flow rate of 0.8 $ml.min^{-1}$. Pullulan and dextran samples with sizes ranging from 40,000 to 2,000,000 were used as standards. Determinations were performed in duplicate.

Nucleotide sequence accession number. The Genbank nucleotide accession number for the *L. reuteri* 121 gene encoding the inulosucrase enzyme and its flanking regions is AF459437.

RESULTS

Isolation and nucleotide sequence analysis of the *L. reuteri* 121 *ftf* gene. PCR with degenerated primers, based on conserved amino acid sequences deduced from alignment of a number of FTFs of Gram-positive bacteria (Fig. 1), using chromosomal DNA of *L. reuteri* 121 as template, yielded a single amplicon of 234 bp (Fig. 2A). Sequence analysis confirmed its *ftf* identity. With (inverse) PCR techniques we obtained a large part of *ftf* ORF including its 3' end (Fig. 2B-C). The 5' of the *ftf* ORF was isolated in a PCR step with a degenerated primer based on the N-terminal amino acid sequence of the purified strain 121 levansucrase protein (**Chapter 3**; Fig. 2E). In total, a DNA fragment of 3983 bp was sequenced. This fragment contained an open reading frame (ORF1) of 2370 bp (starting at 1432 bp), encoding a putative FTF, and an ORF of 852 bp (ORF2), located upstream of, and divergently transcribed from, ORF1. To our surprise, we could not locate the N-terminal amino acid sequence of the strain 121 levansucrase protein in the ORF1 deduced amino acid sequence. The degenerated primer used had misannealed, nevertheless yielding an amplicon. ORF1 does not encode the strain 121 levansucrase protein. Southern hybridization under non-stringent conditions with the 234 bp (Fig. 2A) and the 1438 bp (Fig. 2C) PCR fragment and *L. reuteri* chromosomal DNA revealed only one hybridizing fragment.

No clear promoter and Shine-Dalgarno sequences that meet the consensus sequences could be identified in the DNA sequence upstream of ORF1. Two putative start codons were present in ORF1: (i) an ATG codon at position 1432, (ii) and an ATG codon located at position 1459. The ATG codon at position 1459 had an imperfect Shine-Dalgarno sequence (AAGGAA) 13 bp upstream. The consensus Shine-Dalgarno sequence is AGGAGG, which was found for instance for the Acyl CoA hydrolase from *L. reuteri* (AY082385). According to consensus promoter sequences described for lactobacilli [140] the consensus sequence for -35 is TTGCTG and the consensus sequence for -10 is AATAAT, although the -10 sequence

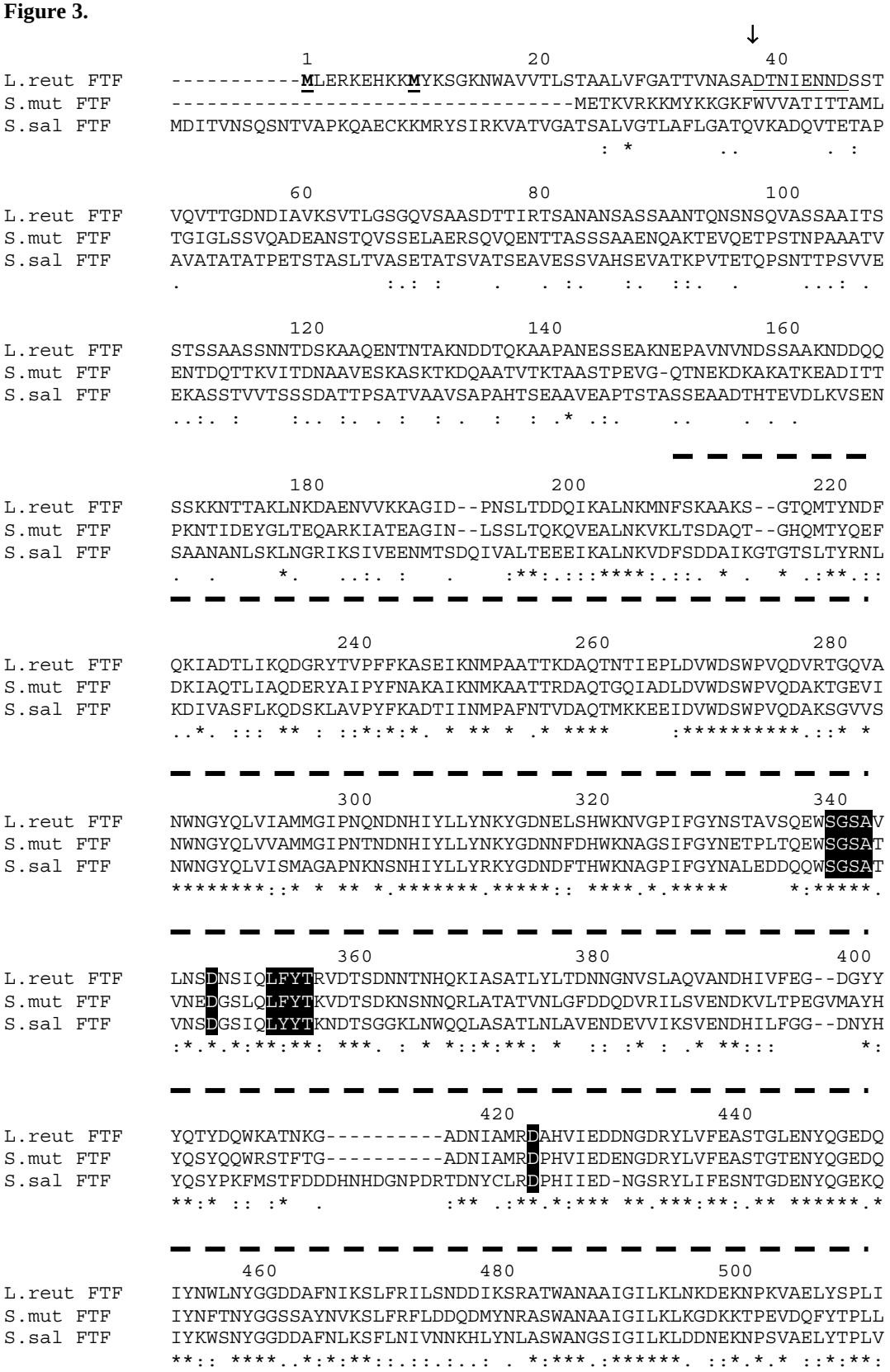
can vary among species. An imperfect promoter sequence could be identified (173 bp from ATG codon at position 1432) with putative –35 and –10 sequences (TTGATG and TTACAA, and a spacing of 19 nucleotides). Starting at position 1432, the putative protein (798 amino acids) had a deduced molecular weight of 86,778 Da and a pI of 4.62. Starting at position 1459, the putative protein (789 amino acids) had a deduced molecular weight of 85,598 Da and a pI of 4.51.

Amino acid sequence alignments and specific features of the strain 121 FTF. Blast searches with the deduced ORF1 amino acid sequence revealed highest similarities with *S. mutans* FTF (P11701; 58% identity and 73% similarity in 686 amino acids) and *S. salivarius* FTF (Q55242; 49% identity and 67% similarity in 756 amino acids). In the deduced ORF1 sequence, a core region of 436 amino acids could be identified belonging to the Glycoside Hydrolase family 68 of levansucrases and invertases (Fig. 3; 43% identity and 56% similarity in amino acid residues 210 to 661) (Pfam02435) (http://pfam.wustl.edu/). Family 68 was identified based on aligments between several FTFs and invertases. As of yet, no common structure could be assigned to family 68 enzymes. An alignment of the deduced ORF1 amino acid sequence with streptococcal FTFs is shown in Figure 3. This alignment revealed very limited similarity in the N-terminal amino acid sequences of the FTFs. Very limited information is available on the role of specific amino acids or regions in the catalytic mechanism of bacterial FTFs (Fig. 3). Based on literature, conserved amino acids found to be involved in catalysis were all present in the strain 121 FTF sequence (Fig. 3). A putative signal peptidase cleavage site [124] is present between amino acids ASA and DT (Fig. 3). The C-terminus of the deduced ORF1 amino acid sequence contains a 20-fold repeat of the motif PXX, an LPXTG motif, a hydrophobic stretch of amino acids, and the protein is terminated by 3 positively charged amino acids (Fig. 3). Also in the *S. salivarius* FTF a Proline-rich region is present. Alignment of the Proline-rich regions of both FTFs does not yield significant similarities (Fig. 3). A dendrogram (Fig. 4), constructed on the basis of alignments of the deduced ORF1 amino acid sequence with some bacterial FTFs, revealed that *L. reuteri* 121 FTF is most closely related with FTFs from Gram-positive bacteria, in particular with streptococcal FTFs. Lower similarities were observed with FTFs from Gram-negative bacteria. FTFs of Gram-positive bacteria form a separate group from FTFs of Gram-negative bacteria.

Blast searches with the deduced amino acid sequence of ORF2 revealed high similarity (41 – 57%) to several members of the uncharacterized protein family UPF0028 (Pfam entry: 01173). Similarities were also found with a protein with unknown function from *Pastereurella multocida* (AAK02722; 39% identity and 60% similarity in 273 amino acids) and with a predicted phosphoesterase from *Clostridium acetobutylicum* (AAK80379; 34% identity and 54% similarity in 279 amino acids).

Expression of the *ftf* gene in *E. coli* and recombinant FTF purification. Analysis of ORF1 showed that it contained two putative translation start codons. No data is available on the N-terminal amino acid sequence of the mature FTF protein. We decided to express ORF1 in *E. coli* starting from the start codon at position 1432. In total, four *ftf* derivatives were constructed for expression studies in *E. coli*. A full-length Inu construct did not yield any transformants. Transformants were obtained with the InuHis construct. Extracts of *E. coli* Top10 cells containing the InuHis construct clearly possessed sucrose hydrolyzing activity (1050 $U.l^{-1}$). SDS-PAGE of cell extracts showed the InuHis protein to be present as a smear (results not shown). Furthermore, the protein could not be purified with nickel column purification and antibodies against the poly Histidine tag could not detect the His-tag.

Figure 3.



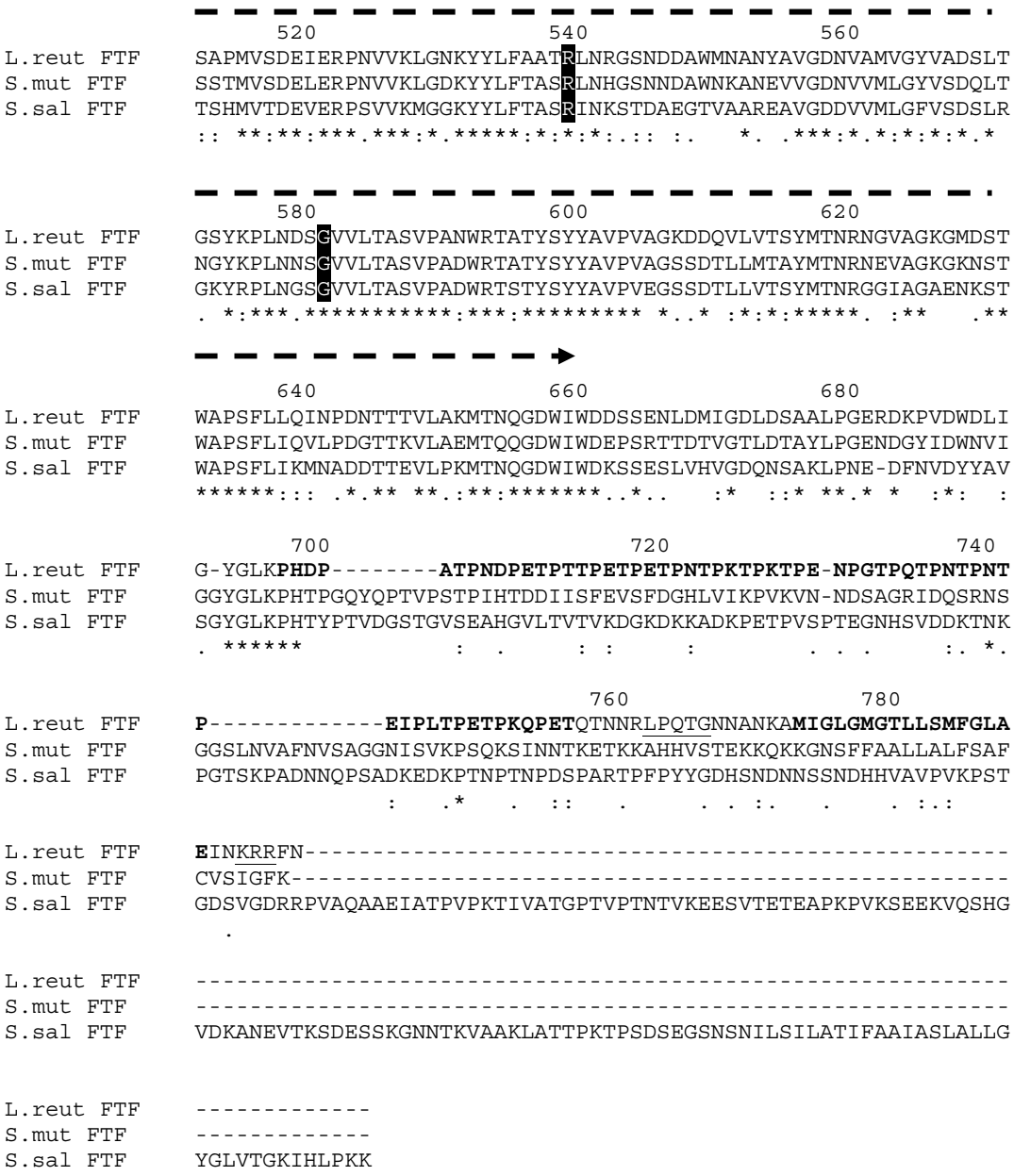


Figure 3. Multiple sequence alignments of bacterial FTFs. Alignments were made with ClustalW 1.74 [194] using a gap opening penalty of 30 and a gap extension penalty of 0.5. FTF amino acid sequences are from *L. reuteri* 121 (L. reut FTF; the ORF1 deduced amino acid sequence from 1432 to 3825 bp), *S. mutans* (S. mut. FTF; M18954), and *S. salivarius* (S. sal. FTF; L08445). (*) indicates a position with a fully conserved amino acid residue, (:) indicates a position with a fully conserved 'strong' group: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW, and (.) indicates a position with a fully conserved 'weaker' group: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. Amino acid groups are according to the Pam250 residue weight matrix [4]. Key amino acids D349 [176], D424 [15], R541 [33], G583 [136], and regions with strong homologies among FTFs and invertases (at positions 341 and 354) [163] are shown by letters in boxes. Two N-terminal amino acids (at positions 1 and 10) are underlined and in boldface. The N-terminal amino acid sequence determined from the recombinant FTF is underlined starting at position 39. A signal peptidase cleavage site (between position 38 and 39) is indicated with an arrow above the sequence. The core region belonging to Glycoside Hydrolase family 68 (residues 210 to 661) is indicated with a dashed arrow above the sequence. Other features for the strain 121 FTF are: a putative spacer region with twenty times the amino acid motif PXX (in bold at position 699-758), a cell-wall anchoring LPXTG motif (underlined at position 764), a hydrophobic stretch of amino acids (in bold at position 776-791), and three positively charged amino acids KRR (underlined at position 794).

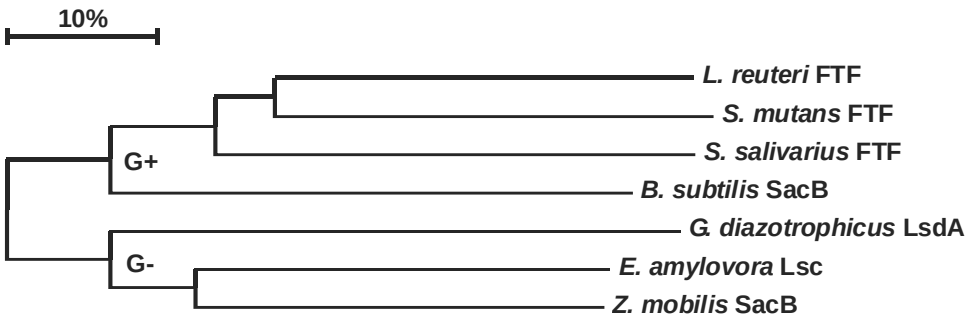


Figure 4. A dendrogram of bacterial FTFs. Alignments were made with ClustalW 1.74 [194] using a gap opening penalty of 30 and a gap extension penalty of 0.5. Dendrogram construction was done with TreeCon 1.3b [199] using the neighbour joining method with no correction for distance estimation. The length of the bar indicates 10% difference at the amino acid level. Bootstrap values were all 100% (100 samples). FTFs from Gram-positive (G+) bacteria are *L. reuteri* 121 FTF (this work), *S. mutans* FTF (M18954), and *S. salivarius* FTF (L08445), *B. subtilis* SacB (X02730). FTFs from Gram-negative (G-) bacteria are *G. diazotrophicus* LsdA (L41732), *Z. mobilis* SacB (L33402), and *E. amylovora* Lsc (X75079).

Because expression and cloning problems were encountered with the full-length FTF, an FTF variant was constructed with C-terminal truncation from the PXX amino acid residues onwards. Expression of the two truncated *ftf* derivatives (InuΔ699 and InuΔ699His) in *E. coli* yielded protein present on SDS-PAGE gels as intense bands, which did not appear to be smeared, at about 85,000 and clear FTF activities in *E. coli* extracts (both at about 1600 U.l^{-1}). The InuΔ699His protein was purified to homogeneity in two chromatography steps (Table 2). From SDS-PAGE the M_r of InuΔ699His was estimated to be 84,000 (results not shown). The N-terminal sequence of InuΔ699His was determined as DTNIEN(N)(D) (with ambiguous residues shown between parentheses). These amino acids corresponded to the deduced FTF amino acid sequence following the predicted signal peptidase cleavage site (Fig. 3).

Table 2. Purification of InuΔ699His enzyme from *E. coli* cells.

Purification step	Total activity (U)	Specific activity (U.mg^{-1} protein)	Purification (-fold)	Yield (%)
Cell lysate	631	18.0	1	100
Ni-NTA[1]	538	88.3	4.9	85
Resource-Q	46.0	103.7	5.8	7

[1] Nickel resin chromatography purification step

Basic recombinant FTF enzymatic properties. The InuΔ699His enzyme showed highest activity at 50 °C and pH 5 to 5.5 (measured as glucose release from sucrose in the presence of 1 mM calcium chloride). At 37 °C, 50 ± 0.4 % enzyme activity was observed (in the presence of 1 mM calcium chloride). In the absence of calcium chloride the enzyme activity decreased to 79 ± 0.6 %.

Analysis of products synthesized from sucrose by *L. reuteri* wild type and the recombinant FTF. Upon incubation with sucrose the InuΔ699His protein produced both fructo-oligosaccharides (FOS) and fructan. After 17 h of incubation with 90 g.l^{-1} sucrose, 16.5 g.l^{-1} of the sucrose was consumed, and 5.1 g.l^{-1} FOS and 0.8 g.l^{-1} fructan were synthesized. Furthermore, 2.6 g.l^{-1} fructose and 6.0 g.l^{-1} glucose were produced from sucrose. Similar amounts of polymer and FOS were produced by cell extracts of arabinose induced *E. coli* with the full-length FTF (InuHis), which might indicate that the C-terminal truncation of

FTF does not have a significant effect on product formation. Anion-exchange chromatography (Dionex; Fig. 5) revealed that the FOS produced were 1-kestose (a β(2→1) linked fructosyl unit to the fructosyl of sucrose; at 6.5 min) and nystose (a β(2→1) linked fructosyl unit to the terminal fructosyl of 1-kestose; at 8.0 min). In Fig. 5, the large peaks in panels A and B at 5.2 to 6.0 min represent sucrose. Cation exchange chromatography showed that the majority of FOS was 1-kestose (95% w/v) and a minor amount of nystose (5% w/v). Methylation analysis of the polymer revealed the presence of 93% 3,4,6-tri-O-methylfructose units (β(2→1) linkages) and 7% 3,4-di-O-methylfructose units (β(1→2→6) linked branchpoints). HPSEC/MALLS analysis of the *in vitro* produced fructan polymer indicated that it was of high molecular weight (> 10^7 Da). In view of the production of inulin polymer by the recombinant FTF, we designated the *L. reuteri* 121 FTF as Inu and the corresponding gene as *inu*.

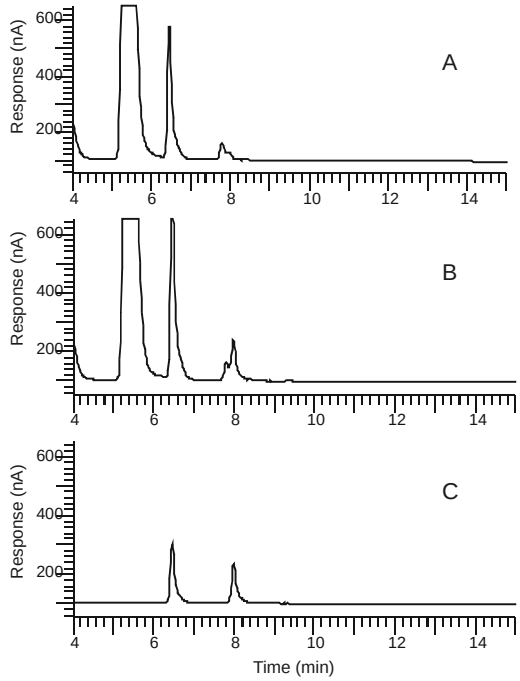


Figure 5. Dionex analysis of the fructo-oligosaccharides synthesized by recombinant InuΔ699His. Panel A: InuΔ699His products; panel B: InuΔ699His products spiked with 1-kestose (6.5 min) and nystose (8.0 min); Panel C: 1-kestose (6.5 min) and nystose (8.0 min).

As shown previously, *L. reuteri* culture supernatants incubated with sucrose produced a single fructan (7 $g.l^{-1}$) with β(2→6) linked fructosyl units only (a levan) with an average molecular weight of 150,000 [204]. Searching for Inu activity in *L. reuteri* strain 121, we analyzed the fructan products of strain 121 grown on media containing sucrose. In the present study we observed that *L. reuteri* incubated with sucrose also produced approximately 10 $g.l^{-1}$ fructo-oligosaccharides (FOS). These FOS consisted of 1-kestose (95%) and of nystose (5%). Previous experiments have shown that the strain 121 levansucrase protein produces a levan polymer that is not PAS-stainable [204]. The recombinant InuΔ699His protein produced an inulin polymer that was PAS stainable (results not shown) on SDS-PAGE gels. However, no PAS stainable polymeric bands were observed with *L. reuteri* 121 cells, cell extracts, and fractionated cell-wall material. Also incubations of the same fractions in a buffer containing sucrose did not yield inulin polymer.

DISCUSSION

This paper reports the isolation and characterization of the first *Lactobacillus* (*L. reuteri*) gene (*inu*) encoding a FTF (Inu), producing in *E. coli* a high molecular weight inulin with β(2→1) glycosidic bonds only and inulin fructo-oligosaccharides (FOS; mainly consisting of 1-kestose). FOS production was also observed with *L. reuteri* 121 cells but no inulin formation was detected. In further work, we have raised antibodies in rabbits against the purified recombinant Inu protein (unpublished results). Unfortunately, no specific immunostaining was observed with *L. reuteri* cells, culture supernatants, or cell-wall

material. In these studies we observed a high background response. Very likely, the rabbits used to raise antibodies contained endogenous lactobacilli. Northern blot hybridization experiments, with a probe comprising the region in *inu* corresponding to the family 68 core region, did not reveal presence of *inu* mRNA. In summary, FOS synthesis was observed in *L. reuteri* culture supernatants, but the inulin type of fructan produced by the recombinant Inu has not been observed in *L. reuteri* 121. Possible explanations for this clear discrepancy are: (i) the *inu* gene is silent in *L. reuteri* or not expressed under the growth conditions tested, (ii) the Inu enzyme only synthesizes FOS under the conditions tested in its natural host, (iii) the inulin polymer already has been degraded at the time of harvesting of the cultures (no evidence, however, has been found for the presence of inulin-degrading activities in *L. reuteri* 121 supernatants), or (iv) Inu has other activities in *E. coli* extracts than in *L. reuteri* 121. InuHis protein produced in *E. coli* showed smearing on SDS-PAGE gels, and the His-tag could not be detected. These observations may suggest that in *E. coli* the InuHis protein is in fact truncated at its C-terminus. At present we cannot exclude the possibility that the products synthesized by the *L. reuteri* Inu protein are different from the products synthesized by the recombinant Inu(Δ699)His protein.

In the process of cloning the *inu* gene from *L. reuteri* 121, a PCR step was performed with a specific primer based on the (incomplete) *inu* sequence (20FTFi) and a degenerate primer based on an N-terminal amino acid sequence of a previously purified levansucrase protein from *L. reuteri* 121. Misannealing of the degenerate primer (19FTF) yielded an amplicon overlapping with the *inu* DNA sequence. This PCR was done at 50 °C, with a proof-reading DNA polymerase. The calculated melting temperature of the DNA sequence to which the primer 19FTF annealed (5'-TAAACGTTTAGCAAAAAGGTAAA-3') was 36 °C (based on the formula melting temperature = 2×AT + 4×CG + 4). This result might be explained by misannealing of the primer at the start of the PCR reaction. A "hot-start" involving separation of the DNA polymerase from the PCR mixture at temperatures lower than 90 °C assures that no misannealing takes place [14]. Conceivably, no PCR product had been obtained when such a "hot-start" had been used in the PCR reaction involving primers 19FTF and 20FTFi.

A typical secretion signal peptide [124] is present in the N-terminus of the Inu protein, with a possible initiation from either translation start codons at positions 1432 and 1459. The N-terminal amino acid sequence of the purified recombinant InuΔ699His protein corresponded to the amino acid sequence following the predicted signal peptidase cleavage site in the deduced Inu sequence. The lack of FTF activity (glucose release from sucrose) in arabinose induced *E. coli* (harbouring the *inu* gene) culture supernatants (results not shown) indicates that the recombinant InuΔ699His protein is not secreted by *E. coli*. N-terminal amino acid sequence analysis of purified InuΔ699His protein from *E. coli* cells shows that *E. coli* does cleave the signal sequence from the Inu protein, and thus that the *E. coli* protein export machinery recognizes the signal sequence. A similar observation was done for the *S. salivarius* FTF [176]. Most likely the Inu protein is present either in the cell membrane, or in the periplasmic space of the *E. coli* cells.

A cell-wall anchoring motif reported for various Gram positive cell-wall associated proteins (**Chapter 1**), was present at the C-terminus of the deduced Inu amino acid sequence. It consisted of a 20 times repeat of the amino acids PXX, an LPXTG motif, a hydrophobic domain, and was ended by three positively charged amino acids (Fig. 3). This is the first report of this motif for a FTF enzyme. Major problems arose, when attempting to introduce and express the full-length Inu (no transformants) and InuHis (protein smears on gel) constructs in *E. coli*. Introduction and expression in *E. coli* of the InuΔ699 and InuΔ699His contructs was straightforward. Apparently the C-terminal region of Inu is problematic for the *E. coli* protein expression machinery.

The products of *L. reuteri* 121 Inu incubated with its substrate sucrose were mainly 1-kestose and an inulin of high molecular weight. High molecular weight inulin production was reported before only in *S. mutans* sp. [36, 47, 151]. The inulin polymers produced by *L. reuteri* and streptococci, contain β(2→6) branches (5-7%). Exclusive production of 1-kestose has been reported for *Aspergillus niger* [76]. Plant FTFs are known to synthesize 1-kestose as primer for the production of inulin polymers [210]. The combined production of 1-kestose and a levan polymer has been reported for the *G. diazotrophicus* levansucrase enzyme [71]. The combination of the production of 1-kestose and levan is remarkable, because levan polymers consist of β(2→6) linked fructosyl units, while 1-kestose consists of a β(2→1) coupled fructosyl unit to sucrose. The production of 1-kestose by Inu is the first elongation step in the polymerization reaction. The large amounts of FOS formed under the incubation conditions used thus may represent aborted polymerization attempts of the Inu enzyme.

The deduced amino acid sequence of the Inu protein of the Generally Regarded As Safe (GRAS) bacterium *L. reuteri* shows highest homology to FTFs from streptococcal origin. Streptococci are well-studied inhabitants of the oral cavity, with fructan synthesized from sucrose most likely contributing to the cariogenicity of dental plaque formation [152]. *L. reuteri* strains, in contrast, are residents of the mammalian gut system. It will be interesting to study the *in situ* functional properties of *L. reuteri* 121 and the fructans produced and their possible roles in the probiotic properties attributed to *L. reuteri* strains [41, 146].

Previously, we reported the presence of a levansucrase in *L. reuteri* 121 [204] (**Chapter 3**). Here we report the isolation and characterization of a novel inulosucrase encoding gene from *L. reuteri* 121. Southern hybridization studies under non-stringent conditions with two probes against the inulosucrase gene and strain 121 chromosomal DNA revealed one hybridizing band. The N-terminal sequence as well as the internal amino acid sequences determined for the purified levansucrase from *L. reuteri* 121 (**Chapter 3**) could not be identified in the deduced strain 121 inulosucrase sequence. *L. reuteri* 121 thus contains both a levansucrase gene and an inulosucrase gene. Apparently, these two *ftf* genes are significantly different not only in products formed, but also in amino acid sequence.

Future work will involve (i) a detailed biochemical characterization of the recombinant Inu enzyme, and (ii) *inu* gene expression studies in *L. reuteri* 121. This will enable a more detailed investigation of the catalytic mechanism of FTF enzymes producing inulin polymers, and analysis of the Inu activities and products in *L. reuteri* itself. The biological relevance and potential health benefits of an “inulosucrase” associated with a *Lactobacillus reuteri* strain remains to be established.

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