Chapter 2

The probiotic *Lactobacillus johnsonii* NCC 533 produces high molecular mass inulin from sucrose using an inulosucrase enzyme

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Abstract

Fructansucrase enzymes polymerize the fructose moiety of sucrose into levan or inulin fructans, with β(2-6) and β(2-1) linkages, respectively. The probiotic bacterium *Lactobacillus johnsonii* strain NCC 533 possesses a single fructansucrase gene (orf AAS08734) annotated as a putative levansucrase precursor. However, $^{13}$C NMR analysis of the fructan product synthesized *in situ* revealed that this is of the inulin type. The *ftf* gene of *L. johnsonii* was cloned and expressed to elucidate its exact identity. The purified *L. johnsonii* protein was characterized as an inulosucrase enzyme, producing inulin from sucrose, as identified by $^{13}$C NMR analysis. TLC analysis of the reaction products showed that InuJ synthesized besides inulin polymer a broad range of fructose oligosaccharides (FOS). Maximum InuJ enzyme activity was observed in the pH range of 4.5 to 7.0, decreasing sharply at pH 7.5. InuJ exhibited highest enzyme activity at 55 ºC, with a drastic decrease at 60 ºC. Calcium ions were found to have an important effect on enzyme activity and stability. Kinetic analysis showed that the transfructosylation reaction of the InuJ enzyme does not obey Michaelis-Menten kinetics. The non-Michaelian behavior of InuJ may be attributed to the oligosaccharides that were initially formed in the reaction, and which may act as better acceptors than the growing polymer chain. This is only the second example of the isolation and characterization of an inulosucrase enzyme and its inulin (oligosaccharide) product from a *Lactobacillus* strain. Furthermore, this is the first *Lactobacillus* strain shown to produce inulin polymer *in situ*.

1. Introduction

Levansucrase and inulosucrase enzymes, collectively called fructansucrases (FSs) or fructosyltransferases (FTFs), polymerize the fructose moiety of their substrate sucrose into fructans which possess either levan or inulin structures with β(2-6) and β(2-1) linkages, respectively. Possible applications of inulin and its oligosaccharides have been reviewed earlier (18). Inulin-type fructans of higher degree of polymerization are of particular interest due to their demonstrated pronounced *in vitro* prebiotic effects (44). In the food industry, inulin is used as fat replacer, and for providing texture and stability in several products, such as desserts, bakery and fermented dairy products, as well as infant formula (35). Inulin polymers also have potential application as surfactants. Carbamoylated inulin has the
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property to reduce the interfacial tension, thus providing a biodegradable surface-active agent (39).

Inulosucrase enzymes (EC 2.4.1.9) are classified in glycoside hydrolase family GH68 along with other bacterial FTF enzymes (http://www.cazy.org). Recently, a few mutagenesis studies focused on determining the structure-function relationship among FTF enzymes have been published. For instance, modification of residues located at the -1 sugar binding subsite of inulosucrase from *Lactobacillus reuteri* 121 strongly affected the size of products synthesized (31). Mutagenesis of the *Bacillus megaterium* levansucrase Arg370 and Asn252 amino acids revealed that these residues are crucial for the polymer versus oligosaccharide product ratio of the enzyme (15). However, in spite of the availability of two high resolution 3-D structures for the levansucrase proteins of *Bacillus subtilis* (23) and *Gluconoacetobacter diazotrophicus* (22), also with sucrose bound in the active site, still little is known concerning the structure-function relationships in these enzymes responsible for the specificity of the glycosidic linkage in the fructan products.

The type of linkages formed in fructans is most certainly based on differences in identity and position of specific amino acid residues present in the active sites of FTF enzymes. All lactic acid bacteria FSs share a high amino acid sequence similarity (>60%) which does not allow straightforward discrimination between inulosucrase and levansucrase proteins (20,46) by amino acid sequence alignments as it has been done for the identification of residues determining linkage specificity in glucansucrase enzymes (21). Furthermore, no structural information for inulosucrase enzymes is available yet, and only a limited number of inulosucrase enzymes have been characterized. As reviewed by (46), levansucrase genes/enzymes have been characterized from more than 17 species of Gram-positive and Gram-negative bacteria, whereas inulosucrase genes/enzymes have only been found in a few species of lactic acid bacteria, namely in *Streptococcus mutans* (36), *Leuconostoc citreum* CW28 (28) and *Lb. reuteri* 121 (50). An inulin producing enzyme also has been characterized from *Bacillus sp.*, but the gene involved has not been identified yet (52).

The complete genome sequence (2.0 Mb in size) of the probiotic bacterium *Lactobacillus johnsonii* strain NCC 533 (formerly *Lactobacillus acidophilus* La1) has been published (34), which encodes a total of 1821 proteins (5). *Lb. johnsonii* strain NCC 533 is a member of the acidophilus group of intestinal lactobacilli that have been extensively
studied for their probiotic activities, pathogen inhibition, epithelial cell attachment, and immunomodulation (7,11-13,16,19,26). The food company Nestlé, which elucidated the genome sequence of the organism, uses it in a yogurt-like dairy product called LC1. According to the company, LC1 strengthens the body's natural defenses and keeps the bowel healthy (10). The published genome sequence of *Lb. johnsonii* NCC 533 contains an orf, AAS08734 (GenBank accession no. AE017198), predicted to encode a levansucrase. Here we report a detailed molecular and biochemical characterization of this novel fructansucrase from the probiotic strain *Lb. johnsonii*. We clearly show that *Lb. johnsonii* is capable of synthesizing an inulin polymer *in situ*. Furthermore, it uses orf AAS08734 (GenBank accession no. AE017198), in fact encoding an inulosucrase, to synthesize this inulin.

2. Materials and methods

2.1. Amino acid sequence alignment of Inulosucrase (*InuJ*) from *Lb. johnsonii* NCC 533 and phylogenetic tree construction

Multiple amino acid sequence alignments of *InuJ* and known inulosucrases and levansucrases from other lactic acid bacteria were made with ClustalW 1.74 (41). Characteristic features and the catalytic core of *InuJ* were deduced from these alignments together with the aid of http://pfam.janelia.org (1). A phylogenetic tree of all known fructansucrases of lactic acid bacteria was constructed with MEGA version 4 using the neighbor joining method (40).

2.2. Bacterial strains and culturing conditions

The *Lb. johnsonii* NCC 533 strain was obtained from the Nestlé Research Center, Lausanne, Switzerland. For genomic DNA isolation, the cells were cultivated anaerobically at 37 °C in MRS medium containing 200 g l\(^{-1}\) glucose. MRS with sucrose (200 g l\(^{-1}\)) was used for polysaccharide production by *Lb. johnsonii*. *Escherichia coli* TOP10 (Invitrogen) and BL21 (DE3 star) (Invitrogen) were used as host for cloning and expression purposes, respectively. *E. coli* strains were grown at 37 °C at 210 rpm in Luria–Bertani (LB) medium supplemented with 50 µg ml\(^{-1}\) of ampicillin in order to maintain plasmid integrity. LB-agar plates were made by adding 1.5% agar to the LB medium.
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2.3. Cloning of the *inuJ* gene

Total genomic DNA from *Lb. johnsonii* NCC 533 was extracted and purified by the method described by (51) and modified by (25). DNA was amplified on a DNA thermal cycler PTC-200 (MJ Research) using high fidelity DNA polymerase (Fermentas, Germany). The *Lb. johnsonii* genomic DNA and the primers, FTF-Lj-F

5'-TATGTCAACCATGGATGAAAAACAAGTTGAAAAGAAAGAC-3’, containing an *Nco*I site (underlined) and FTF-Lj-R

5'-TATGTCAAGGATCCTTAATGGTGATGGTGATGGTGTTGTTGGCTTCAA-3’, containing a *Bam*HI site (underlined) and a C-terminal His tag (italics), were used in a PCR reaction to amplify the 5’ and 3’ truncated *Lb. johnsonii* fructansucrase (*ftf*) gene. This truncated *ftf* gene encodes amino acids 144 to 709 of the InuJ protein, with a C-terminal His tag (designated InuJΔ144-709His). Using the *Nco*I and *Bam*HI restriction sites, the *inuJ* amplicon was cloned into the expression vector pET15b (Novagen). The resulting vector (pETInuJ) was transformed to *E. coli* BL21 (DE3 Star) for expression studies. Correct construction of the plasmid was confirmed by nucleotide sequence analysis (GATC, Germany).

2.4. InuJ expression and purification

*E. coli* BL21 harboring pETInuJ was grown overnight in 600 mL LB medium equally divided in three 1 L Erlenmeyer flasks. The medium was supplemented with 0.1 mM IPTG to induce protein expression. The cells were harvested by centrifugation at 3500 × g for 15 min and the pellet was resuspended in 20 ml binding buffer (20 mM Na₂HPO₄/NaH₂PO₄, pH 8.0) containing 5 mM β-mercaptoethanol and 4 mM imidazol. After sonication the extract was centrifuged (20,000 × g for 20 min) and protein present in the supernatant was purified to homogeneity by Ni-affinity (Sigma, USA) and anion exchange chromatography (6 ml ResourceQ column Amersham Pharmacia, Sweden). The purest enzyme fractions (as judged by SDS-PAGE) after anion exchange chromatography possessing fructansucrase activity were pooled, dialyzed overnight against sodium acetate buffer (25 mM, pH 5.4), and stored at 4 °C for further studies.
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2.5. Biochemical characterization of the recombinant inulosucrase

All assays were performed at 55 °C and pH 7.0 in Michaelis’ barbital sodium acetate buffer (6), unless described otherwise. Purified enzyme (0.45 µg ml⁻¹) was used for biochemical characterization and kinetic studies. One unit of InuJ enzyme activity is defined as the release of 1 µmol of monosaccharide per min from sucrose. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad, Germany) with bovine serum albumin as standard.

2.5.1. Effect of pH, temperature, CaCl₂ and EDTA

Michaelis’ barbital sodium acetate buffer in the range of pH 4 to 8 was used to study the effect of pH on the activity of recombinant InuJΔ144-709His. Enzymatic incubations were performed in reaction mixtures containing 200 mM sodium acetate-sodium barbital buffer supplemented with 500 mM sucrose and 1 mM CaCl₂. Activity of the enzyme (0.45 µg ml⁻¹) was measured at 50 °C. After pre-incubation of the assay mixture at the assay temperatures for 10 min, reactions were started by enzyme addition. Samples were taken every 3 min and used to determine the amount of glucose and fructose released from sucrose (47). 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 (\( V_G - V_F \)). Effect of temperature on the enzyme activity was studied in 200 mM sodium acetate-sodium barbital buffer pH 7.0, supplemented with 500 mM sucrose and 1 mM CaCl₂.

To study the effects of calcium ions on enzyme activity, the experiments initially were conducted in buffer without CaCl₂, and all solutions used were prepared in MilliQ water. However, no differences in InuJ activities were observed in the presence or absence of Ca²⁺ ions. Therefore, further studies were conducted in the presence of gradually increasing concentrations of EDTA (from 0 to 800 µM), but without addition of CaCl₂. Subsequently, the effect of Ca²⁺ ions (at concentrations of 0 to 800 µM) was studied in the presence of 600 µM of EDTA in the reaction medium.
2.5.2. Kinetic parameters

For the determination of kinetic parameters, activity assays were performed with 0.45 µg ml\(^{-1}\) of protein and sucrose concentrations ranging from 5 mM to 1000 mM. The ‘Sigma Plot’ program (version 10.0) was used for curve fitting of the data, either with the standard Michaelis-Menten formula: 
\[
y = \frac{a \cdot x}{c + x}
\]
or the 3 parameter Hill formula: 
\[
y = \frac{a \cdot x^b}{c + x^b}
\]
In these formulas, \(y\) is the specific activity (U mg\(^{-1}\)), \(x\) is the substrate concentration (mM sucrose), \(a\) is the \(V_{max}\) (U mg\(^{-1}\)), \(b\) is the Hill factor, \(c\) is the \(K_{m50}\) (mM sucrose; \(K_m\) in case of Michaelis-Menten type of kinetics; \(K_{50}\) in case of Hill type of kinetics).

2.5.3. Polysaccharide production and characterization

The polysaccharide synthesized by \(Lb.\ johnsonii\) NCC 533 was produced by growing the strain anaerobically in 20 ml of MRS-sucrose medium for 7 days at 37 °C. The culture was centrifuged at 4000 \(\times\) g for 10 min and the supernatant was separated from the cells. The supernatant was run on a TLC plate (Silica gel 60 F\(_{254}\); Merck, Germany) overnight using 1-butanol: ethanol: water (5:5:3) as mobile phase. The plates were air-dried, sprayed with a urea developing solution specific for sugars containing fructose (43) and developed at 80 °C. \(In situ\) \(Lb.\ johnsonii\) products were degraded by exo-inulinase of \(Aspergillus\ niger\) (Megazyme, Ireland). For this purpose, the exo-inulinase enzyme (final concentration: 10 U ml\(^{-1}\)) was added to a mixture of the sample (the supernatant), 0.3 M phosphate buffer of pH 4.5 and MilliQ water (100 µl each). The reaction mixture was incubated at 40 °C for 2 h and 3 µl of this solution was analyzed by a TLC plate as described above.

To produce fructan oligosaccharides and polymer, the purified recombinant inulosucrase (4.5 µg ml\(^{-1}\)) was incubated with sucrose (600 mM) at 55 °C in Michaelis’ barbital sodium acetate buffer (pH 7.0) containing 1 mM CaCl\(_2\) and samples were taken at different time intervals. To characterize the oligosaccharide and polymer products formed, 1 µl aliquots from this reaction mixture (4 times diluted) were run on TLC plates overnight, as described above. The polymer was precipitated from the rest of the reaction mixture with two volumes of 96% cold ethanol and separated by centrifugation at 2500 \(\times\) g for 15 min. After dissolving in MilliQ water, the polymer was precipitated (45). This process was repeated two more times and the polymer was freeze dried finally. For NMR spectroscopy,
samples were dissolved in 99.9 atom % D$_2$O (Aldrich). One-dimensional $^{13}$C-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 ppm relative to the methyl group of internal acetone ($\delta$=31.07). Carbon spectra were recorded in 38K data sets, 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.

HP anion-exchange (HPAE) chromatography (Dionex, Sunnyvale, USA) was used to separate oligosaccharides produced by incubation of InuJ with sucrose for 48 h at 37 ºC as described above. Separation of oligosaccharides was achieved as described previously (31) using the following gradient: 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 Raftiline ST-Gel and Raftiline HP (Orafti, Tienen, Belgium) representing chicory inulin were used.

Molecular weight of the inulin 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). A Dawn-F-DSP (Wyatt Technology, Santa Barbara, Calif.) laser photometer He-Ne ($\lambda$ = 690 nm) equipped with a K5 flow cell and 18 detectors at angles ranging from 12.8 to 164.7° was used as MALLS detector. Samples were filtered through a 0.45 µm filter (MILLEX) and the injection volume was 240 µl. NaNO$_3$ (0.1 M) was used as eluent at a flow rate of 1.0 ml min$^{-1}$. Pullulan (psspulkitL, Polymer Standards Service, USA) and dextran (T2000, Amersham Pharmacia Biotech., Sweden) samples with molecular mass ranging from $4 \times 10^4$ to $2 \times 10^6$ Da were used as standards.

3. Results and discussion

3.1. Amino acid sequence analysis of Inulosucrase (InuJ) from *Lb. johnsonii* NCC 533

The putative *ftf* gene of *Lb. johnsonii* consists of 2394 bp and coded for a 797 amino acid protein with a deduced molecular weight of 87.2 kDa. A putative signal peptidase cleavage site is present between amino acids 36 and 37.
Inulosucrase from Lactobacillus johnsonii

(\url{http://www.cbs.dtu.dk/services/SignalP/}). Following the N-terminal variable domain of 172 amino acids, a core region of 453 amino acids (residues 210 to 662) was identified (\url{http://pfam.janelia.org}) (9) belonging to the Glycoside Hydrolase family 68 (\url{http://www.cazy.org}). A Gram-positive cell wall anchoring domain of 41 amino acids (from amino acid 753 to 794) was detected at the C-terminal end of InuJ, following a C-terminal variable region of 89 amino acids. The C-terminus of InuJ also contains a 17-fold repeat of the PXX motif and a hydrophobic stretch of 23 amino acids (residues 769 to 791), and the protein is terminated by 6 positively charged amino acids. This organization is similar to the protein anchoring system reported for \textit{Lb. reuteri} 121 inulosucrase (50) except that its LPQTG motif is replaced by an LPKAG motif in InuJ. The LPKAG motif also has been reported in an immunoglobulin light chain-binding protein of a few strains of \textit{Peptostreptococcus magnus} (17) where it is considered to be similar to the consensus sequence LPXTG, which is well conserved in Gram-positive bacterial cell wall associated proteins \{Fischetti, 1990 576 \textit{id}; Van Hijum, 2002 477 \textit{id}; Van Hijum, 2004 560 \textit{id}\}.

![Phylogenetic tree of FTF proteins from lactic acid bacteria](image)

**Fig. 1.** Unrooted phylogenetic tree of FTF proteins from lactic acid bacteria. Proteins are labeled LEV: levansucrase (β 2-6), INU: inulosucrase (β 2-1) and FTF: unknown linkage specificity. Alignments and dendrogram construction were done (using the complete amino acid sequences) with MEGA4 using the neighbor joining method. Bootstrap values (in percentage) are indicated at the branching points. The scale bar corresponds to a genetic distance of 0.1 substitution per position.
Alignments of the amino acid sequence of InuJ with FTF proteins of other lactic acid bacteria revealed highest similarities with \textit{Lb. reuteri} 121 inulosucrase (60\% similarity) and \textit{Lactobacillus gasseri} FTF (82\% similarity); InuJ clustered most closely with the latter in the phylogenetic tree (Fig. 1). The conserved amino acids reported to be involved in catalysis in FTF enzymes were all present in the InuJ sequence. From the 3D structure of the \textit{B. subtilis} levansucrase, the residues D86, D247 and E342 have been identified as the catalytic nucleophile, the transition state stabilizer and the general acid/base catalyst, respectively (23). In \textit{Lb. reuteri} 121 inulosucrase, the importance of the equivalent amino acids D272, D424 and E523, which correspond to D272, D425 and E524 in InuJ of \textit{Lb. johnsonii}, have been proven by site-directed mutagenesis (33).

3.2. Cloning, Expression and purification of InuJ

An N- and C-terminally truncated version of InuJ was constructed, aiming for high protein expression in \textit{E. coli}. Previously, it has been reported that cloning and expression of the full length \textit{ftf} genes from \textit{Lb. reuteri} 121 in \textit{E. coli} led to expression problems. Only an \textit{ftf} construct with a C-terminal truncation from the PXX amino acid residues onwards (deleting the cell wall anchor) yielded good levels of protein expression (50). In addition, we made an N-terminal truncation in InuJ at position previously shown not to affect the \textit{Lactobacillus sanfranciscensis} levansucrase enzyme activity, nor the composition of its polysaccharide products (42). The position of the truncation (at both the N- and C-terminal ends) was also based on the amino acid sequence of the \textit{B. subtilis} SacB protein that has been crystallized (23), taking care not to delete conserved sequences.

The truncated \textit{inuJ} gene of 1724 bp with a C-terminal His-tag was successfully cloned in pET15b after amplification with primers FTF-Lj-F and FTF-Lj-R. This truncated gene encodes a recombinant FTF protein (InuJA144-709His) of 63 kDa exhibiting very high expression levels in \textit{E. coli}, yielding about 25 mg of His-tag purified protein from 1 l culture.

3.3. Biochemical characterization of the InuJ enzyme

3.3.1. Effect of pH, temperature and CaCl\textsubscript{2} on enzyme activity

In order to define the best conditions for subsequent kinetic studies, the pH and temperature optima of InuJ, and the influence of Ca\textsuperscript{2+} ions on InuJ activity were determined.
The effect of pH on enzyme activity was studied at 50 ºC (Fig. 2a). The highest total inulosucrase activity was observed at pH 7.0, with a rapid decrease in activity at higher pH values. More than 85% of activity was retained in the pH range 4.5 to 6.0. The pH optima of several related enzymes have been reported previously, i.e. levansucrase of *Lb. sanfranciscensis* (optimum pH of 5.4) (42) and the FTF enzymes of *Lb. reuteri* 121 (pH optima 4.5-5.5) (48). Levansucrase (LevC) of *Leuconostoc mesenteroides* exhibited highest activity in the pH range 6.5-7 (29). The InuJ exhibited maximum transglycosylation activity in a broad range of pH 4.5 to 7.0 (65-71 %). Maximum hydrolytic activity was also observed in the same pH range with a peak (29%) at pH 7.0.

![Fig. 2. Effect of pH and temperature on InuJ enzyme activity.](image)

The highest total InuJ enzyme activity was found at 55 ºC, with a drastic decrease at higher temperatures (Fig. 2b). A large part (84%) of this total activity was coming from the transglycosylation activity. Maximum hydrolytic activity (28% of total activity) was observed at 40 ºC. Relatively high optimum temperatures have also been reported for the FTF enzymes from *Lb. reuteri* 121 (50 ºC) (49), *Lb. sanfranciscensis* (35-45 ºC) (42) and
Bacillus sp. (60 ºC) (3). The increased transglycosylation activity observed at higher temperatures may be due to oligosaccharide formation (see below).

Initially, no differences in InuJ activities were observed in the presence or absence of Ca²⁺ ions. However, the InuJ enzyme activity decreased with increasing EDTA concentrations, with only 5% activity remaining at 700 µM EDTA. The enzyme activity was completely recovered upon addition of 300 µM of Ca²⁺ ions (Fig. 3). This result is in agreement with the proven essential role of calcium ions for FTF activity (30). The later study used site directed mutagenesis to demonstrate that Asp520 in inulosucrase of Lb. reuteri 121 plays an important role in Ca²⁺ binding. This residue is highly conserved in family GH68 proteins of Gram-positive origin. In the 3D structure of B. subtilis levansucrase, the corresponding Asp339 is involved in calcium binding (23). In case of InuJ, the equivalent amino acid residue is Asp521. The data show that the purified InuJ protein contained bound Ca²⁺ ions, which were scavenged when EDTA was added, thus decreasing enzyme activity. Calcium ions were also found to have a significant effect on the enzyme stability. In the absence of EDTA, the enzyme retained 80% of its total activity after incubation at 55 ºC for 8 h, whereas it completely lost its activity within 3 h of incubation in the presence of 600 µM of EDTA.

Fig. 3. Effects of EDTA (▲) and Ca²⁺ (at 600 µM EDTA) (●) on InuJ enzyme activity. The reaction was carried out in Michaelis’s barbital sodium-acetate buffer pH 7.0 containing 500 mM sucrose. The reaction was started by the addition of 0.45 µg ml⁻¹ enzyme in 250 µl reaction mixture. The results are presented with standard error of the mean (SEM; N = 3).
3.3.2. **Kinetic parameters.** Like many other lactobacilli, *Lb. johnsonii* NCC 533 grows optimally at 37 °C, while its FTF enzyme exhibited maximum activity at 55 °C. Therefore, kinetic studies with InuJ were carried out at both temperatures (Table 1). The total, transglycosylation and hydrolytic activity values of InuJ were determined in a reaction buffer with 500 mM sucrose. The ratio between transglycosylation and hydrolysis activities was almost 1:1 at 37 °C, but increased about 26 fold at 55 °C. Total activity of InuJ at 37 °C was about 2.5 and 23,000 fold higher than the activities of the C-terminally truncated inulosucrases from *Lb. reuteri* 121 (31) and *Leuc. citreum* (27), respectively. The C-terminally truncated inulosucrase of *Leuc. citreum* also displayed higher activity than the wild type, full-length, enzyme (27). The significantly higher activity of InuJ, as compared to other inulosucrases, may be the result of its N- and C-terminal truncation.

The kinetic constants for the InuJ activities were determined with substrate concentrations ranging from 5 mM to 1000 mM sucrose (Table 1). Even at higher substrate concentrations, the total (V<sub>G</sub>) and transfructosylation (V<sub>G-F</sub>) activities of the enzyme were not saturated by sucrose. Consequently, high standard errors were obtained with curve fits. Only the hydrolytic activity (V<sub>F</sub>) followed standard Michaelis-Menten kinetics at 37 °C. The K<sub>m</sub> value of InuJ for hydrolysis was similar to that of inulosucrase from *Leuc. citreum* CW28 (8) and *Lb. reuteri* 121 (49) while its k<sub>cat</sub> value was significantly higher (176 ± 3.4 s<sup>-1</sup>), indicating that this enzyme has a relatively higher efficiency. The InuJ hydrolysis reaction suffered from a strong sucrose substrate inhibition at 55 °C. However, the inhibition constant could not be determined due to high standard errors with the curve fit. A similar but lower substrate inhibition effect has been observed for the *Lb. reuteri* 121 Inu enzyme (49).

The InuJ V<sub>G</sub> and V<sub>G-F</sub> activities did not obey Michaelis-Menten kinetics and the curve fit data with the 3 parameter Hill equation could not be used to calculate the catalytic turnover rate (k<sub>cat</sub>). However, its Hill factor values were similar to those obtained for the *Lb. reuteri* 121 inulosucrase (49), except the value (0.76 ± 0.06) for total activity at 55 °C. Hill type kinetics is based on the assumption that there is more than one binding site present in the enzyme and/or that the enzyme exists in the multimeric form. A high Hill factor value reflects a positive cooperativity, which indicates a positive interaction of the enzyme binding sites, and/or multimers, and vice versa. The Hill factor value for transfructosylation activity at 55 °C was closer to 1, indicating that there were neither negative nor positive cooperativity.
effects. The non-Michaelian behaviour of InuJ may be attributed to the oligosaccharides that were formed at an early stage of the reaction (Fig. 4), which may act as better acceptors than the growing polymer chain. Similar observations were made for the *Lb. reuteri* 121 inulosucrase (32).

Table 1. FTF activities (U mg\(^{-1}\) of protein) and comparison of the apparent kinetic constants for FTF activity of InuJΔ144-709His inulosucrase protein of *Lb. johnsonii* at different temperatures using sucrose concentrations varying from 5-500 mM. The kinetic constants are \(K_{50}\) and \(k_{cat}\) for (a) formation of glucose (\(G\); total enzyme activity), (b) formation of fructose (\(F\); hydrolytic enzyme activity), and (c) glucose minus fructose (\(G-F\); transglycosylation enzyme activity).

<table>
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<th>Kinetic parameters</th>
<th>Temperature</th>
<th>37 °C(^b)</th>
<th>55 °C(^b)</th>
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<tr>
<td>Total Activity(^a)</td>
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<td>(k_{cat}) (^G) (s(^{-1}))</td>
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\(^a\) Activity values measured at 500 mM sucrose concentration.

\(^b\) Enzyme assays were done with 0.45 µg ml\(^{-1}\) enzyme; final concentration.

* Kinetic parameters could not be determined due to the fact that the enzyme was not saturated with sucrose, resulting in high standard errors with curve fits.

Φ Kinetic parameters could not be determined due to high substrate inhibition.
3.4. Production and Identification of the polymer by InuJ

Clear evidence was obtained for in situ production of poly- and oligosaccharides by *Lb. johnsonii* cells in MRS-sucrose medium (Fig. 4), although the levels remained relatively low under the experimental conditions used in the present studies. About 150 mg of polysaccharide product was obtained from 100 ml of *Lb. johnsonii* culture after 7 days of incubation with 20% sucrose in MRS-sucrose medium. The poly- and oligosaccharides
material produced was degraded when inulinase was added (Fig. 4). Furthermore, $^{13}$C NMR analysis confirmed the polysaccharide to be an inulin (Table 2). The data thus indicate that *Lb. johnsonii NCC 533 in situ* produces inulin polymer and inulin oligosaccharides (Fig. 4), the first example of a probiotic strain that synthesizes prebiotic FOS and inulin *in situ*. It has already been reported that *Lb. johnsonii* possesses a cell-surface associated protein (GroEL) having activities for attachment to mucus and epithelial cells, in addition to other cellular functions (4), and this strain also shares carbohydrate-binding properties with several entero pathogenic bacteria (26). *In situ* inulin and FOS production by *Lb. johnsonii* may further contribute to its probiotic properties. Sucrose is abundant in many fruits and is the most abundant carbon source in ungerminated cereal grains (37). The dietary sucrose that has escaped digestion in the upper gastrointestinal tract may serve as a substrate for inulin and FOS production by *Lb. johnsonii* cells in the colon.

Table 2. Comparison of $^{13}$C NMR chemical shift values of fructans produced by lactic acid bacteria.

<table>
<thead>
<tr>
<th>Carbon Atom</th>
<th>Levan*</th>
<th>Levan*</th>
<th>Inulin</th>
<th>Inulin</th>
<th>Inulin*</th>
<th>Inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Lb. reuteri 121</em></td>
<td><em>Leuc. mesenteroides</em></td>
<td><em>S. mutans</em></td>
<td><em>Leuc. citreum</em></td>
<td><em>Lb. reuteri 121</em></td>
<td><em>Lb. johnsonii NCC 533</em></td>
</tr>
<tr>
<td>C-1</td>
<td>61.7 (59.6)*</td>
<td>60.1</td>
<td>60.9</td>
<td>61.4</td>
<td>62.2</td>
<td>62.1 (62.2)*</td>
</tr>
<tr>
<td>C-2</td>
<td>105.0 (104.0)*</td>
<td>104.3</td>
<td>103.2</td>
<td>103.60</td>
<td>104.2</td>
<td>104.1 (104.2)*</td>
</tr>
<tr>
<td>C-3</td>
<td>78.1 (76.0)*</td>
<td>76.5</td>
<td>77.0</td>
<td>77.5</td>
<td>78.9</td>
<td>78.3 (78.4)*</td>
</tr>
<tr>
<td>C-4</td>
<td>76.6 (74.9)*</td>
<td>75.4</td>
<td>74.3</td>
<td>74.9</td>
<td>75.6</td>
<td>75.6 (75.6)*</td>
</tr>
<tr>
<td>C-5</td>
<td>81.2 (80.0)*</td>
<td>80.5</td>
<td>81.1</td>
<td>81.8</td>
<td>82.1</td>
<td>82.2 (82.2)*</td>
</tr>
<tr>
<td>C-6</td>
<td>64.3 (63.2)*</td>
<td>63.6</td>
<td>62.2</td>
<td>62.7</td>
<td>63.1</td>
<td>63.2 (63.1)*</td>
</tr>
</tbody>
</table>

* Chemical shift values for the fructans produced by recombinant enzymes

TLC analysis showed that pure recombinant InuJ synthesized a range of fructo-oligosaccharides (FOS) in addition to inulin polymer (Fig. 4). Synthesis of FOS started within 5 min of incubation. The synthesis of a range of FOS has also been observed for the
Inulosucrase from Lactobacillus johnsonii

Inu of *Lb. reuteri* 121 (32) and thus appears to be a typical property for inulosucrase enzymes. Oligosaccharides ranging from GF2 to GF15 were clearly detected by HPAEC, although synthesis of higher DP FOS (DP > 15) cannot be excluded as evident from the small peaks after 35 min retention (Fig. 5). In addition, several unknown products, eluting next to the GFn oligosaccharides, were also present which were virtually not detectable in the FOS synthesized by *Lb. reuteri* 121 Inu (31).

The main signals in the $^{13}$C NMR spectrum of the fructan synthesized by InuJ corresponds to a fructose polymer with $\beta$-(2-1) linked fructose units, typical for the structure found in inulin (Fig. 6). The NMR data also indicate that either the inulin product is linear, only one signal is present at C2 (2), or the degree of branching is too low to be detected.

Therefore, the *Lb. johnsonii* *ftf* gene encodes an inulosucrase enzyme (Table 2). The *ftf* gene present on the genome of *Lb. johnsonii* NCC 533 has been annotated (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=AAS08734.1) to encode a levansucrase precursor. Our data clearly show that this *ftf* actually encodes an inulosucrase enzyme.

![Fig. 5. HP anion-exchange chromatography analysis of the FOS products synthesized by purified recombinant InuJΔ144-709His. Solid line: FOS produced by InuJ; Dotted line: inulin standards.](image)

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Fig. 6. $^{13}$C-NMR spectrum of inulin produced by purified recombinant InuJΔ144-709His recorded in D$_2$O at 80º C. Chemical shifts are given in parts per million (ppm) relative to the signal ($\delta=31.07$) of internal acetone reference.

Using HPSEC-MALLS, the average molecular mass of the inulin produced by recombinant InuJ of \textit{Lb. johnsonii} was determined as $4 \times 10^7$ Da, which is comparable to the values, $6-9 \times 10^7$ Da and $1 \times 10^7$ Da, reported for the molecular mass of the inulin produced by \textit{S. mutans} GS-5 (14) and \textit{Lb. reuteri} 121 (50), respectively. The polymer also showed a remarkable small polydispersity index ($M_w/M_n$; where $M_w$ and $M_n$ denote, in gmol$^{-1}$, the average molar mass by weight and the average molar mass by number respectively) value of 1.05-1.17, indicating that there is not much variation in the $M_w$ of polymer chains and they all contain approximately the same number of monosaccharide units.

4. CONCLUSIONS

Our results unequivocally show that the putative levansucrase gene present in the genome of \textit{Lb. johnsonii} NCC 533 actually encodes an inulosucrase enzyme that is responsible for the synthesis of an inulin type fructan. Using our truncation strategy for improved \textit{E. coli} protein expression, we were able to obtain high yields of pure inulosucrase protein. The InuJ catalytic properties, $Ca^{2+}$ dependence and FOS synthesis largely resemble that of the Inu enzyme of \textit{Lb. reuteri} 121.

This is only the second \textit{Lactobacillus} inulosucrase which has been purified and characterized in detail, the first being reported in \textit{Lb. reuteri} 121 (50). The available FTF
amino acid sequence information does not allow yet straightforward identification of
inulosucrase or levansucrase enzymes. Characterization of more inulosucrase sequences,
and/or crystallographic information for an inulosucrase protein, may provide this
information in future. The isolation and characterization of the inulosucrase gene/enzyme
that we have reported here may be an important step in this direction.

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Chapter 2

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