Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*

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*Lactobacillus reuteri* strain 121 employs a fructosyltransferase (FTF) to synthesize a fructose polymer [a fructan of the levan type, with β(2→6) linkages] from sucrose or raffinose. Purification of this FTF (a levansucrase), and identification of peptide amino acid sequences, allowed isolation of the first *Lactobacillus* levansucrase gene (*lev*), encoding a protein (Lev) consisting of 804 amino acids. Lev showed highest similarity with an inulosucrase of *L. reuteri* 121 (Inu; producing an inulin polymer with β(2→1)-linked fructosyl units) and with FTFs from streptococci. Expression of *lev* in *Escherichia coli* resulted in an active FTF (LevΔ773His) that produced the same levan polymer [with only 2–3 % β(2→1→6) branching points] as *L. reuteri* 121 cells grown on raffinose. The low degree of branching of the *L. reuteri* levan is very different from bacterial levans known up to now, such as that of *Streptococcus salivarius*, having up to 30 % branches. Although Lev is unusual in showing a higher hydrolysis than transferase activity, significant amounts of levan polymer are produced both in vivo and in vitro. Lev is strongly dependent on Ca²⁺ ions for activity. Unique properties of *L. reuteri* Lev together with Inu are: (i) the presence of a C-terminal cell-wall-anchoring motif causing similar expression problems in *Escherichia coli*, (ii) a relatively high optimum temperature for activity for FTF enzymes, and (iii) at 50 °C, kinetics that are best described by the Hill equation.

INTRODUCTION

Lactic acid bacteria are Gram-positive, food-grade microorganisms consisting of many genera, e.g. *Lactococcus*, *Streptococcus* and *Lactobacillus*. Lactic acid bacteria possess the generally regarded as safe (GRAS) status find application in the production of food and feed (Lindgren & Dobrogosz, 1990). Lactic acid bacteria produce an abundant variety of polysaccharides (De Vuyst & Degeest, 1993). Moreover, some strains (e.g. *Lactobacillus reuteri*) have been designated as probiotics, i.e. they may have beneficial effects on the host by improving the properties of the indigenous population of gastrointestinal microorganisms (Havenaar & Huis in ’t Veld, 1992; Gibson et al., 1994).

Previously, it was reported that *L. reuteri* 121 cultivated on media containing sucrose produced large amounts of both a glucan and a fructan polymer (van Geel-Schutten et al., 1999). The fructan polymer was a levan containing β(2→6)-linked fructosyl residues, with two major fractions in the estimated size distribution of 150 000 Da and larger than 2 000 000 Da (van Hijum et al., 2001; van Geel-Schutten et al., 1999).

Enzymes responsible for the synthesis of fructan polymers of the levan type are generally referred to as fructosyltransferases (FTF) or levansucrases (sucrose:2,6-β-D-fructan 6-β-D-fructosyltransferase, EC 2.4.1.10). They catalyse the transfer of the fructosyl unit of sucrose to a number of acceptors including sucrose, water (resulting in hydrolysis) and fructan polymer. Levansucrases of *Zymomonas mobilis*...
and Bacillus species (Gunasekaran et al., 1995; Perez-Osengauer et al., 1996) have been studied in most detail. Levans are either linear or branched to various degrees at the C-1 position. The sizes of the bacterial levans vary from 20 kDa to several MDa. For lactic acid bacteria, fructan production by streptococci and several lactobacilli has been reported (Tieking et al., 2003). *Streptococcus salivarius* strains produce branched levans polymers [containing up to 30% β(2→1) branches] (Ebisu et al., 1975; Hancock et al., 1976; Simms et al., 1990) whereas *Streptococcus mutans* JC-2 produces a fructan of the inulin type consisting mainly of β(2→1)-linked fructosyl units with 5% β(2→6) branches (Rosell & Birkhed, 1974; Ebisu et al., 1975). Recently, inulosucrase genes from lactic acid bacteria were reported in *L. reuteri* 121 (van Hijum et al., 2002) and *Leuconostoc citreum* (Olivares-Illana et al., 2002, 2003).

Recently we described the purification of the levansucrase protein responsible for levan formation in *L. reuteri* 121, and determination of amino acid sequences of peptide fragments (van Hijum et al., 2001). Here we report the isolation and characterization of the levansucrase gene from the same strain. The gene was expressed in *E. coli* and its enzyme product was characterized. Structural characterization of the levan produced by the purified recombinant enzyme showed that this levansucrase is responsible for levan synthesis by *L. reuteri* 121 cells grown on raffinose.

**METHODS**

**Strains, plasmids, media and growth conditions.** *L. reuteri* 121 (culture collection TNO Nutrition and Food Research, Zeist, The Netherlands) was grown anaerobically at 37 °C as described previously (van Hijum et al., 2002). *E. coli* Top10 (Invitrogen) was used as host for pCR-XL-TOPO (Invitrogen) and pBAD/myc-his C (Invitrogen) plasmids, which were used for cloning of inverse PCR fragments of the inulin type consisting mainly of β(2→1)-linked fructosyl units with 5% β(2→6) branches (Rosell & Birkhed, 1974; Ebisu et al., 1975). Recently, inulosucrase genes from lactic acid bacteria were reported in *L. reuteri* 121 (van Hijum et al., 2002) and *Leuconostoc citreum* (Olivares-Illana et al., 2002, 2003).

Degenerate bases are according to IUB codes (N, any base; W, A or T; S, C or G; Y, C or T).

**Degenerate primers used in this study**

**Isolation of the levansucrase gene.** Based on the amino acid sequences (QVESNNYNGVAEVNTERQANGQI and VYSPLVSTLM-ASDEVE) of two peptide fragments of the *L. reuteri* 121 levansucrase (van Hijum et al., 2001), degenerate primers Deg1 and Deg2i were designed (Table 1). PCR with *Pwo* DNA polymerase, these primers, and total DNA of *L. reuteri* 121 yielded an amplification product of 1385 bp (Fig. 1, A), which was used to design primers for two inverse PCR steps: (i) N1i and N2, and (ii) C1i and C2 (Table 1). *L. reuteri* 121 chromosomal DNA was digested with *Hin* cII and ligated, yielding circular DNA molecules. PCR with the ligation product as template and diverging primers (i) N1i and N2 yielded an amplicon of 1544 bp (Fig. 1, B) and (ii) C1i and C2 yielded an amplicon of 1542 bp (Fig. 1, C). The 1542 bp fragment was used to design inverse PCR primers IPBrevi and IPAfor (Table 1). *L. reuteri* 121 chromosomal DNA was digested with *Hind*III and ligated, yielding circular DNA molecules. PCR with primers IPBrevi and IPAfor with the circular ligation product as template yielded an amplicon of 1700 bp (Fig. 1, D). In total, a fragment of 4570 bp of *L. reuteri* 121 genomic DNA was cloned and sequenced (Fig. 1).

**Table 1. Primers used in this study**

Degenerate bases are according to IUB codes (N, any base; W, A or T; S, C or G; Y, C or T). *NcoI* and *BglII* restriction sites are underlined and stop codons are shown in bold.

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence (5’ to 3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
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<td>AAYTATAAYGGYGTGTCNGAAGT</td>
<td>PCR</td>
</tr>
<tr>
<td>Deg2i</td>
<td>TTCAACTCTCAGTNSNNGCAT</td>
<td>PCR</td>
</tr>
<tr>
<td>N1i</td>
<td>GTTGTATTACTGTAGCATAATATTTTTC</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>N2</td>
<td>GAAAGCAAAAAATCTACAATCTAGAATAATGC</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>C1i</td>
<td>CCATTAGCTTAACCCAGCAATTCAGCTGC</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>C2</td>
<td>CTCCACATTGCTATCTAATTTGTAGCGTTC</td>
<td>Inverse PCR</td>
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<tr>
<td>IPBrevi</td>
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<tr>
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<tr>
<td>BADFTFdC</td>
<td>AGATCTAGATCTTTTGTGATCTCGTATTTTTC</td>
<td>pBAD</td>
</tr>
</tbody>
</table>

*i = reverse. Primers BADFTFC and BADFTFdC are reverse.*
Expression of the \textit{L. reuteri} levansucrase gene in \textit{E. coli}.

Four primer sets were designed for expression of the levansucrase gene in \textit{E. coli}: (i) BADFTFN and BADFTFC1 (Table 1) giving the full-length mature levansucrase (Lev; residues 37 to 804; see Fig. 2), (ii) BADFTFN and BADFTFC (Table 1) giving a full-length mature levansucrase with a C-terminal His tag (LevHis; residues 37 to 804; Fig. 2), (iii) BADFTFN and BADFTFdC1 (Table 1) giving a levansucrase truncated from the LPXTG motif at position 773 onwards (LevΔ773; residues 37 to 773), and (iv) BADFTFN and BADFTFdC (Table 1) yielding a truncated levansucrase from the LPXTG motif at position 773 onwards with a C-terminal His tag (LevΔ773His; residues 37 to 773). The four levansucrase gene derivatives started with an ATG codon (vector sequence) followed by the \textit{ftf} gene sequence encoding the amino acids found in the N-terminus of the strain 121 purified mature levansucrase protein (van Hijum \textit{et al.}, 2001), starting at amino acid residue 37 (Fig. 2). PCR with \textit{L. reuteri} genomic DNA (approx. 1 \textmu g), Pwo DNA polymerase, and the primer sets, yielded the \textit{ftf} gene derivatives flanked by Nco and BglII restriction sites. Using the Nco and BglII restriction sites, the amplicons were cloned into the expression vector pBAD/myc-his C. The resulting pBAD vectors were transformed to \textit{E. coli} Top10 for expression studies. Correct construction of the plasmids was confirmed by nucleotide sequence analysis of both DNA strands.

Protein purification

(i) Preparation of cell-free extracts. Cells of \textit{E. coli} Top10 harbouring the \textit{ftf} gene were grown overnight at 37 °C in 500 ml LB with 0.02% (w/v) arabinose to an OD$_{600}$ of approximately 1-5. Cell extracts were obtained by ultrasonication as described previously (van Hijum \textit{et al.}, 2002).

(ii) Nickel affinity purification. Ni-NTA resin (500 \textmu l; Qiagen) was used to bind protein from 26 ml cell extract (3.6 mg protein ml$^{-1}$). The resin was washed with 5 ml demineralized water and 2.5 ml binding buffer (50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 8.0) prior to applying the cell extract. The suspension was gently shaken at 4 °C for 1 h. Unbound material was washed away with 2-5 ml binding buffer, and bound protein was eluted from the affinity resin with 2 ml binding buffer containing 200 mM imidazole and 1 mM \textit{β}-mercaptoethanol. The eluate was dialysed against phosphate buffer (5 mM, pH 8.0) and adjusted to a volume of 5 ml in Tris buffer (20 mM, pH 8.0).

(iii) Resource-Q column chromatography. An anion-exchange column (Resource-Q; Amersham Pharmacia Biotech; 1 ml column volume; flow rate 1 ml min$^{-1}$) was equilibrated with Tris buffer (20 mM, pH 8.0; A) and the sample (5 ml) was loaded on the column. The column was eluted with Tris buffer (20 mM, pH 8.0-0.5 M NaCl; B) and eluted fractions, collected from 20% B to 80% B, were screened for levansucrase activity (glucose release from sucrose; see below). Positive fractions were run on SDS-PAGE and peak fractions containing one protein band were pooled (4 ml) and stored at 4 °C for further analysis.

Biochemical characterization of the recombinant levansucrase

(i) N-terminal amino acid sequencing. This was performed as described previously (van Hijum \textit{et al.}, 2002).

(ii) Mass analysis. Matrix-assisted laser desorption-ionization mass spectrometry was used to determine the protein molecular masses. The adjusted Ni-NTA eluate (5 \textmu l; \approx 100 \textmu g ml$^{-1}$) was mixed with matrix (5 \textmu l; 20 mg sinapinic acid ml$^{-1}$ in acetonitrile/0-1% trifluoroacetic acid; 40/60, v/v), and 2 \textmu l of the mixture was dried on a target. Spectra were recorded on a ToFSpec MALDI E and SE spectrometer (Micromass).

(iii) Levansucrase activity assays. Sucrose conversion by levansucrase yields (a) fructose, which is (partly) built into the growing polymer, and (b) glucose, in a 1:1 ratio to the amount of sucrose converted. In control experiments the glucose formed reflected the total amount of sucrose utilized, since the residual sucrose (measured by hydrolysing sucrose with invertase and enzymically measuring the free glucose and fructose), fructan (measured by a mild 0-5 M trifluoroacetic acid hydrolysis followed by the enzymic detection of fructose), free glucose and free fructose formed added up to the amount of sucrose added to the reaction mixture (results not shown). Based on the above-mentioned experiments, the amount of glucose formed reflects the total amount of sucrose utilized by the enzyme (total activity). The amount of fructose formed is a measure of the hydrolytic activity of the enzyme (transfer of fructosyl units to water). The amount of glucose minus the amount of free fructose reflects the transerase activity (the transfer of fructosyl units to an acceptor other than water). Glucose and fructose were measured enzymically as described by van Hijum \textit{et al.} (2001). Levansucrase activity was measured in a sodium acetate buffer (25 mM; pH 5.4) with 100 mM sucrose and 1 mM calcium
chloride at 50 °C, unless stated otherwise. The optimal temperature and pH for \(L. reuteri\) 121 Lev (at 3 \(\mu\)g ml\(^{-1}\)) total enzyme activity (glucose release from sucrose) were determined from 20 to 55 °C and pH 3.0 to 6.5 (from pH 5.5 to 6.5 a 25 mM MES buffer was used), respectively. One unit of enzyme activity is defined as the release of 1 \(\mu\)mol glucose or fructose min\(^{-1}\). All experiments were performed in triplicate and, where appropriate, the results are presented as the means±SEM. The ‘Sigma Plot’ program (version 4.0) was used for curve fitting of the data, either with the standard Michaelis–Menten formula: \(y=(a\times x)/(c+x)\), or a Michaelis–Menten formula with a substrate inhibition constant: \(y=(a\times x)/(c+x+(c^2/d))\). In these formulae, \(y\) is the specific activity (U mg\(^{-1}\)), \(x\) is the substrate concentration (mM sucrose), \(a\) is the \(V_{\text{max}}\) (U mg\(^{-1}\)), \(b\) is the Hill factor, \(c\) is the \(K_m\) (mM sucrose; \(K_m\) in the case of Hill-type kinetics), and \(d\) is the substrate inhibition constant (mM sucrose).

(iv) Levanusacrase activity assays in SDS-PAGE gels. Protein (approx. 5 \(\mu\)g) was run in duplicate on SDS-PAGE. A duplicate part of the gel was stained with Coomassie brilliant blue to identify the position of the proteins in the gel. Protein was cut from the corresponding unstained duplicate part of the gel. To determine enzyme activity in gel slices, protein was renatured by adding a sodium acetate buffer containing sucrose and 0.5-5 % (v/v) Triton X-100, and incubated at 50 °C. Glucose and fructose formation in the samples were determined as described by van Hijum et al. (2001).

Fructan analysis

(i) Fructan production and purification. Reaction products of FTF were produced at the optimal growth temperature of strain 121 (37 °C), by incubating the purified levansucrase in a sodium acetate buffer (25 mM, pH 5.4; 1 mM CaCl\(_2\)) with 100 g sucrose 1\(^{-1}\), at 37 °C for 16 h. For comparison, fructan produced by \(L. reuteri\) cells grown overnight on MRSr was used. Polymer was precipitated and cleaned as described by van Hijum et al. (2001).

(ii) Molecular mass and methylation analysis. Polymer characteristics (i) molecular mass by HPSEC/MALLS and (ii) the fructose linkage type by methylation were determined as described by van Hijum et al. (2001).

RESULTS

Nucleotide sequence analysis of an \(L. reuteri\) genomic fragment encoding a levansucrase

Using PCR and inverse PCR a fragment of 4570 bp was cloned from the genomic DNA of \(L. reuteri\) 121. On this fragment three open reading frames (ORFs; Fig. 1) were present. A putative protein encoded by ORF1 (804 amino acids) had a deduced molecular mass of 87 602 Da and a pI of 4.81. The deduced N-terminal amino acid sequence of ORF1 carried a putative signal peptide sequence of 36 amino acids, followed by a putative signal peptidase cleavage site, most likely located between amino acid 36 (alanine) and amino acid 37 (aspartic acid) (as determined by SignalP; http://genome.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997). The amino acid sequence following amino acid 37 matched the complete N-terminal peptide sequence determined from the purified \(L. reuteri\) levansucrase, with the exception of the first amino acid (Fig. 2). Furthermore, the three amino acid sequences determined for the internal peptide fragments of the purified \(L. reuteri\) levansucrase enzyme (van Hijum et al., 2001) were present in the ORF1 deduced amino acid sequence. We therefore concluded that ORF1 (hereafter referred to as the lev gene) encodes the \(L. reuteri\) 121 levansucrase (Lev). The GenBank accession number for the \(L. reuteri\) 121 lev gene and its flanking regions is AF465251.

ORF1 contained a putative start codon (TTG, encoding a formylmethionine at position 1193), with a perfect Shine-Dalgarno ribosome-binding site (AGGAGG) 8 bp upstream. Furthermore, two putative promoter sequences could be identified, according to the consensus promoter sequences described for \textit{Lactobacillus} genes (Pouwels & Leer, 1993): (i) 238 bp upstream of the formylmethionine the sequences TTGAAA (−35) and TATAAA (−10) with a spacer region of 11 nucleotides, (ii) 199 bp upstream of the formylmethionine the sequences TTGATA (−35) and TAAATAA (−10) with a spacer region of 12 nucleotides. A strong terminator hairpin structure (AG −22.6 kcal mol\(^{-1}\)) was found between ORF1 (68 nucleotides downstream) and ORF3 (172 bp downstream). The hairpin comprised a stem of 18 bp and a loop of 11 unpaired bases.

BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) with the deduced Lev amino acid sequence showed highest similarity with: \(L. reuteri\) 121 inulosucrase (Inu; AF459437; 56 % identity and 86 % similarity in 768 amino acids), \textit{S. mutans} FTF (P11701; 48 % identity and 65 % similarity in 773 amino acids), and \textit{Salivarius} FTF (Q55242; 48 % identity and 66 % similarity in 735 amino acids). Lev contained the core regions of Glycoside Hydrolase family 68 of levansucrase and invertases (Fig. 2; 41 % identity and 55 % similarity in amino acid residues 187 to 640; Pfam entry at 02435; http://pfam.wustl.edu/) and family 32 of invertases, levanases and inulinases (Fig. 2; 24 % identity and 36 % similarity in amino acid residues 274 to 437; Smart entry at 00640; http://smart.embl-heidelberg.de/).

A striking feature of the Lev protein is the presence of direct repeats in the N- and C-terminal regions (Fig. 2). BLAST searches with the amino acid sequences of these repeats yielded no significant similarity with any known protein sequence. These repeats were not observed in the amino acid sequences of Inu and other FTFs from Gram-positive bacteria (Fig. 2) or FTFs from Gram-negative bacteria. The C-terminal amino acid sequence of the Lev protein contained a proline-rich putative spacer region (Fig. 2; 72 amino acids with 13 proline residues). Furthermore, a Gram-positive LPXTG cell-wall anchor was identified (Fig. 2; Pfam entry PF00746 at http://pfam.wustl.edu/).

The isolated DNA fragment also contained ORF2, encoding a putative protein of 272 amino acids (from ATG start codon at position 133; Fig. 1), and ORF3, encoding a putative protein of 134 amino acids (from ATG start codon at position 4299; Fig. 1). BLAST searches with the translated amino acid sequence of ORF2 showed highest similarity to hypothetical protein NMA1791 from \textit{Neisseria meningitidis}
Characterization of an L. reuteri levansucrase

**Fig. 2.** Alignments of amino acid sequences from *L. reuteri* 121 Lev, *L. reuteri* 121 Inu (AF459437), *S. mutans* FTF (M18954), *S. salivarius* FTF (L08445) and *B. subtilis* SacB (X02730). Alignments of amino acid sequences were made with CLUSTALW 1.74 (Thompson *et al.*, 1994) using a gap opening penalty of 30 and a gap extension penalty of 0.5. Amino acid groups are according to the Pam250 residue weight matrix (Altschul *et al.*, 1990). Homologous regions of Lev with enzymes from the Glycoside Hydrolase families 68 (dotted) and 32 (dashed) are indicated with thick arrows. The N-terminal amino acid sequence of levansucrase purified from *L. reuteri* culture supernatants as reported previously (van Hijum *et al.*, 2001) is shown above the sequence at position 37. Indicated with arrows are direct repeats of 14 (at position 86–127) and of 13 amino acids (at position 727–751). The C-terminal cell-wall-anchoring motifs of Lev and Inu are shown as follows: (i) a putative spacer region (underlined, position 666–759); (ii) an LPXTG motif (bold, position 773); (iii) a stretch of hydrophobic amino acids (underlined, position 781–800); and (iv) three positively charged amino acids KRH (bold, position 801).

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**Recombinant enzyme expression and purification**

Cell extracts of *E. coli* Top10 harbouring the four Lev derivatives (Lev, LevHis, LevD773 and LevD773His) clearly possessed sucrase activity (glucose release from sucrose)
when incubated in a buffer with sucrose as substrate. The highest sucrase activity with all four constructs was observed when *E. coli* cells were incubated overnight with 0.02% arabinose (approx. 11 000 U l⁻¹ in the cell extracts). No activity was detected without arabinose induction. The Lev and LevHis proteins showed smearing on SDS-PAGE gels (results not shown), whereas distinct bands were observed with the LevΔ773 and LevΔ773His proteins on SDS-PAGE gels. LevΔ773His was selected for further purification using the polyhistidine tag.

The LevΔ773His protein was purified to homogeneity from *E. coli* cell extracts by two column chromatography steps (Table 2). The yield of protein after purification was relatively low due to loss of protein in the washing steps of the Ni-NTA column. In *E. coli* cell-free extracts and Ni-NTA fractions, a second, smaller and less abundant protein band was found next to the dominant protein band. The smaller band had an apparent size of 75 000 Da, smaller than the calculated molecular mass (84 676 Da) of LevΔ773His. SDS-PAGE of cell extract, Ni-NTA and resource-Q fractions showed that the dominant protein band had an apparent size of 110 000 Da (results not shown), larger than the calculated molecular mass (84 676 Da) of LevΔ773His. Similar sizes and ratios as the LevΔ773His protein were observed for LevΔ773. Mass spectrometry analysis of the adjusted Ni-NTA eluate showed that the protein running at 110 000 Da had a mass of 84 772 Da and that the protein running at 75 000 Da had a mass of 63 841 Da. The N-terminal amino acid sequence of the protein running at 110 000 (MDQVES) corresponded to the *lev* translated amino acid sequence starting at position 37 (Fig. 2). The N-terminal amino acid sequence of the protein running at 75 000 Da (MPATYTVDA) corresponded to the translated amino acid sequence of *lev* starting from an alternative start codon (ATG) at position 1877 (amino acid residue 229 in Fig. 2). The deduced molecular mass of the Lev protein variant translated from the alternative start codon was 63 891 Da, corresponding to the size of the smaller protein determined by mass spectrometry. An imperfect ribosome-binding site (AAG-GAA; at position 1863) and no consensus promoter sequence could be identified. We conclude that the *L. reuteri* 121 *lev* gene contains a second start codon that corresponded to the translated amino acid sequence starting from an alternative start codon (ATG) at position 1877 (amino acid residue 229 in Fig. 2). The deduced molecular mass of the Lev protein variant translated from the alternative start codon was 63 891 Da, corresponding to the size of the smaller protein determined by mass spectrometry. An imperfect ribosome-binding site (AAG-GAA; at position 1863) and no consensus promoter sequence could be identified. We conclude that the *L. reuteri* 121 *lev* gene contains a second start codon that is recognized by *E. coli*. Staining for sucrase activity in SDS-PAGE gels showed a clearly positive activity band for the LevΔ773His protein, whereas the N-terminally truncated Lev protein showed no detectable activity.

**In vitro fructan production by LevΔ773His and fructan analysis**

Incubation of nickel-column-purified LevΔ773His protein (53 U l⁻¹) for 16 h at 37 °C with 100 g sucrose l⁻¹ yielded a total amount of 1.4 g fructan l⁻¹ with 18 g sucrose l⁻¹ consumed. Methylation analyses of fructan produced by raffinose-grown cells of *L. reuteri* 121 revealed the presence of 98 % 1,3,4-tri-O-methylfructose units [β(2→6) linkages] and 2 % 3,4-di-O-methylfructose units [β(1→2→6) linked branchpoints]. Methylation analyses of fructan produced by recombinant LevΔ773His protein revealed the presence of 97 % 1,3,4-tri-O-methylfructose units [β(2→6) linkages] and 3 % 3,4-di-O-methylfructose units [β(1→2→6) linked branchpoints]. Thus, both fructans were linear levens with only low amounts of β(2→1→6) branching points (2 % and 3 %, respectively). HPSEC/MALLS elution profiles of both fructans (those of LevΔ773His and raffinose-grown cells of *L. reuteri* 121) were also comparable, showing in both cases two major fractions with molecular masses of 20 000 Da (97 % w/w) and (3→4) × 10⁶ Da (3 % w/w).

**Recombinant enzyme characterization**

The purified LevΔ773His enzyme showed highest activity (glucose release from sucrose; total activity) at 50 °C and around pH 4–5–5.5. The enzyme was almost inactive without addition of 1 mM Ca²⁺ (2.28 ± 0.19 % residual activity); no cations other than calcium could restore enzyme activity. Lev enzyme activity was (almost) completely inhibited by Hg²⁺, Fe³⁺ and Cu²⁺. Partial inhibition was observed with Fe²⁺ (Table 3).

**Table 3.** Effects of various compounds on LevΔ773His enzyme activity in the presence of 1 mM CaCl₂

<table>
<thead>
<tr>
<th>Compound (1 mM)</th>
<th>Enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>MgCl₂</td>
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<tr>
<td>HgCl₂</td>
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<tr>
<td>FeCl₃</td>
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<tr>
<td>FeCl₂</td>
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</tr>
<tr>
<td>CuCl₂</td>
<td>ND*</td>
</tr>
<tr>
<td>NaCl</td>
<td>105.8 ± 5.1</td>
</tr>
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</table>

*Enzyme activity could not be detected.*
Kinetic properties determined for LevA773His were compared to those reported for FTFs from *S. salivarius*, *Bacillus subtilis* and *Gluconacetobacter diazotrophicus* (Table 4). Because for Lev the optimal temperature for the release of glucose from sucrose was 37 °C and the optimal temperature for growth of *L. reuteri* was 37 °C, kinetic constants were determined at both temperatures. At both temperatures, enzyme activity versus substrate concentration curves (Fig. 3) were determined, measuring the release of glucose ($V_G$) and fructose ($V_F$) from sucrose. Curve fitting was done for each of the three curves ($V_G$, $V_F$ and $V_G - V_F$) presented in Fig. 3. Michaelis–Menten-type kinetics was observed for the hydrolysis reaction, with minor sucrose substrate inhibition. Both transferase and overall enzyme activity followed normal Michaelis–Menten kinetics at 37 °C but displayed kinetics best described by the Hill equation at 50 °C (with Hill factors of 0.51 ± 0.07 and 0.84 ± 0.1, respectively). The $K_{cat}/K_m$ quotients (hydrolysis $F^T = 10^{-4} \text{M s}^{-1}$, and transferase $G-F = 2.43 \text{M s}^{-1}$) showed that at 37 °C, Lev favours the hydrolysis reaction over the transferase reaction (Table 4). Total and hydrolytic activity of the Lev enzyme increased with incubation temperature (reflected in the higher $K_{cat}^{G}$ and $K_{cat}^{F}$ values; Table 4).

### DISCUSSION

Previous attempts to clone the *L. reuteri* 121 levansucrase gene failed when using degenerate primers based on conserved regions in FTFs of Gram-positive bacteria (van Hijum *et al.*, 2002). The *ftf* gene isolated in this way

![Figure 3](http://mic.sgmjournals.org)

**Fig. 3.** $V$ versus $[S]$ relationship for Lev enzyme at 37 °C (a; 8.0 µg ml⁻¹) and 50 °C (b; 2.0 µg ml⁻¹). ●, $V_G$ (total activity); Δ, $V_F$ (hydrolytic activity); ⬇, $V_G-V_F$ (transferase activity).
turned out to encode an inulosucrase (Inu). In earlier work we purified the levansucrase responsible for levan synthesis in *L. reuteri* 121 (van Hijum et al., 2001). Here, we report the isolation of the corresponding gene (*lev*) and its expression in *E. coli*. The recombinant LevΔ773His enzyme produced levan polymer that was identical to that of *L. reuteri* 121. Furthermore, we have shown that the levansucrase activity occurs cell-associated as well as a free supernatant protein (van Geel-Schutten et al., 2001). The biochemical properties and the products formed by the levansucrase purified from *L. reuteri* (van Hijum et al., 2001) are comparable. Obviously, the C-terminal truncation of Lev from amino acid 773 onwards (Fig. 2) and the addition of a C-terminal Myc epitope and polyhistidine tag did not have significant effects on the products formed. The full-length recombinant Lev protein (LevHis), containing the membrane-spanning region, showed smeared on SDS-PAGE gels, suggesting that the C-terminal membrane-spanning region in *E. coli* interfered with protein expression or protein stability. Similar effects of the C-terminal domain were observed for the *Inu* enzyme (van Hijum et al., 2002). With the HPSEC/MALLS method two major fractions with molecular masses of 200 000 Da (97 %, w/w) and (3–4) × 10^5 Da (3 %, w/w) were found for fructan polymers produced by both *L. reuteri* and LevΔ773His. Earlier we reported molecular masses of 150 000 and more than 2 × 10^6 Da when using gel-filtration chromatography (van Hijum et al., 2001). The discrepancy between these results can be explained by an increased accuracy with the HPSEC/MALLS method (Blennow et al., 2001; Turquois & Gloria, 2000).

The estimated affinities of the Lev enzyme for sucrose are clearly higher than those of the *S. salivarius* FTF (*K_{cat}^G = 63.5 ± 3.6 s^{-1} and K_{cat}^{F0} = 28.9 ± 1.2 s^{-1}, respectively*). This corresponded with the high hydrolytic activity that we also observed for the purified *L. reuteri* levansucrase (van Hijum et al., 2001). The Lev enzyme apparently transfers the fructosyl unit of sucrose relatively efficiently to water. Nevertheless, the enzyme produced significant amounts of levan both *in vivo* (van Hijum et al., 2001) and *in vitro* (this study).

A striking feature of the LevΔ773His protein is its high optimal temperature of 50 °C and that at 50 °C a shift occurs from Michaelis–Menten to kinetics best described by the Hill equation. Speculatively, with increasing temperatures, the enzyme can use sucrose more efficiently as acceptor than (oligo) fructan molecules. Only for the *L. reuteri* Inu has a temperature optimum of 50 °C been reported (van Hijum et al., 2002, 2003); other FTFs show lower optimal temperatures. Regardless of the optimal temperature, no Hill-type kinetics has to our knowledge been observed for FTFs previously, except for Inu (van Hijum et al., 2003). The Hill factors calculated from activities at 50 °C for the LevΔ773His (total activity and transferase reactions) were lower than 1. This was also observed for the *Inu* enzyme (van Hijum et al., 2003) and indicates a negative cooperativity for these reactions. With Hill-type kinetics it is assumed that there is more than one binding site present in the enzyme and/or multimeric forms of the enzyme. For Hill-type kinetics a positive cooperativity indicates a positive interaction of binding sites present in the enzyme and/or multimers. Alternatively a negative cooperativity indicates a negative interaction of enzyme binding sites and/or multimers. In FTFs, it is not known how many binding sites are present for substrate and product binding due to the lack of detailed structural protein information. Multimeric forms of FTFs were reported only for the levansucrase from *Actinomyces viscosus* T14 (Pabst et al., 1979). Thus, we cannot draw conclusions on the nature of the negative cooperativity suggested by the best-fit for the total and transferase activities at 50 °C observed in this *L. reuteri* Lev enzyme.

Two non-levan-producing mutants of *L. reuteri* 121 have been described (strains 35-5 and K24), isolated during continuous culture experiments (van Geel-Schutten et al., 1999). Under the growth conditions applied, levansucrase activity became lost in a few generations, suggesting that the *lev* gene in *L. reuteri* 121 is located on a transposable element, or on a plasmid. Interestingly, ORF3 (Fig. 1) shows strong homology to a transposase from a *Lactobacillus casei* strain (CAA05973). Transposable elements have been described for a number of lactic acid bacteria (Davidson et al., 1996). When comparing genomic maps of *Lactococcus lactis* MG1363 and *Streptococcus thermophilus*, a large number of inversions and translocations are present. These genomic rearrangements are partly attributed to the presence of mobile elements in the genomes of lactic acid bacteria such as transposons (Davidson et al., 1996). In view
of the presence of ORF3 downstream of ORF1, the possible location of lev on a transposable element, flanked by recognition sequences for a transposase encoded by ORF3, warrants further investigation. No transposon insertion sequences could be identified in the DNA sequence flanking ORF1. Therefore, the mechanism of inactivation of the levansucrase activity in L. reuteri 121 mutants remains unclear.

This is believed to be the first report of the identification of a Lactobacillus levansucrase gene (lev) and the characterization of the recombinant protein. The L. reuteri 121 levansucrase is most closely related to L. reuteri Inu and to levansucrases of streptococci, based on biochemical characteristics and sequence homologies. L. reuteri 121 levan contains significantly lower amounts of β(2→1) branches thanlevans produced by Streptococcus spp. The L. reuteri 121 levansucrase is unusual in displaying a relatively high rate of sucrose hydrolysis. The lev gene was successfully expressed in E. coli, enabling production of relatively larger amounts of levansucrase and its levan polymer. Our current studies focus on a detailed biochemical and structural characterization of the L. reuteri Lev and Inu enzymes, to identify features that determine (i) the percentage of β(2→1→6) branches, (ii) product size, (iii) the β(2→1) versus β(2→6) product specificity, and (iv) hydrolysis versus transglycosylation specificity.

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