Insights into polymer versus oligosaccharide synthesis: mutagenesis and mechanistic studies of a novel levansucrase from *Bacillus megaterium*

Arne HOMANN^{*1}, Rebekka BIEDENDIECK^{†1}, Sven GÖTZE^{*}, Dieter JAHN[†] and Jürgen SEIBEL^{*2}

^{*}Department of Technical Chemistry, Technical University Braunschweig, Section of Carbohydrate Technology, Hans-Sommer-Str. 10, D-38106 Braunschweig, Germany, and

[†]Department of Microbiology, Technical University Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, Germany

A novel levansucrase was identified in the supernatant of a cell culture of *Bacillus megaterium* DSM319. In order to test for the contribution of specific amino acid residues to levansucrase catalysis, the wild-type enzyme along with 16 variants based on sequence alignments and structural information were heterologously produced in *Escherichia coli*. The purified enzymes were characterized kinetically and the product spectrum of each variant was determined. Comparison of the X-ray structures of the levansucrases from Gram-positive *Bacillus subtilis* and Gram-negative *Gluconacetobacter diazotrophicus* in

conjunction with the corresponding product spectra identified crucial amino acid residues responsible for product specificity and catalysis. Highly conserved regions such as the previously described RDP and DXXER motifs were identified as being important. Two crucial structural differences localized at amino acid residues Arg³⁷⁰ and Asn²⁵² were of high relevance in polymer compared with oligosaccharide synthesis.

Key words: *Bacillus megaterium*, fructo-oligosaccharide, levansucrase, site-directed mutagenesis.

INTRODUCTION

Global regulatory responses allow bacilli to adapt to changes in environmental conditions [1]. These responses often include changes in the transcription rates of large sets of target genes and thus the control of a wide variety of cellular processes in these Gram-positive bacteria. One of these processes is the synthesis of degradative and polymer-building enzymes. The secretion of these enzymes enables the cell to build and utilize highly polymeric nutrients such as polysaccharides, nucleic acids, peptides and lipids. Polysaccharides, e.g. inulin, and non-digestible oligosaccharides are known to have human health benefits. They are used by the food industry as nutritional supplements [2,3] and their potential for immune stimulation is currently under investigation [4].

Levansucrases form polysaccharides by transfer of fructosyl units to oligosaccharides. This class of enzymes (E.C. 2.4.1.10) is common in eukaryotes and prokaryotes, e.g. plants, fungi (*Aspergillus niger* [5], *Saccharomyces cerevisiae* [6]) and bacteria (*Lactobacillus* spp. [7], *Bacillus subtilis* [8]). In these organisms they build up an extracellular energy reserve of fructans. These exopolysaccharides function as stabilizers of cell membranes protecting them against water stress caused by drought or temperature shift [9]. Furthermore, it is postulated that fructans may be important for survival of bacteria, for phytopathogenesis and symbiosis [10].

Fructosyl-transferring enzymes are divided into two classes based on the products formed. Inulosucrases (E.C. 2.4.1.9) mainly form $\beta(2\rightarrow1)$ bonds producing the polysaccharide inulin, whereas the second class, levansucrases (E.C. 2.4.1.10), form levan which is $\beta(2\rightarrow6)$ -linked. The oligosaccharides and polysaccharides are composed of a head group of α -D-glucopyranoside to which the fructosyl units are coupled.

One of the best characterized members of Gram-positive bacilli, *B. subtilis*, secretes the levansucrase, SacB, into its

environment if high amounts of sucrose are available [11]. Levansucrase catalyses the hydrolysis of sucrose as well as transglycosylation reactions with a growing oligosaccharide chain as acceptor. *B. subtilis* levansucrase dominantly synthesizes high-molecular-mass levan with a molecular mass of up to 3×10^6 Da. In contrast, the levansucrase from the Gram-negative *Gluconacetobacter diazotrophicus* mainly synthesizes short-chain oligosaccharides and low amounts of levan [12]. Until now, there has been no sufficient explanation for the formation of these different transfructosylation products.

In the present study, a novel levansucrase from *Bacillus megaterium*, which is within the same taxonomic group as *B. subtilis* [13,14], was isolated and its product spectra and reaction kinetics were characterized. Based on amino acid sequence alignments and structural comparisons with enzymes of the glycoside hydrolyse family 68 [15], 16 mutants have been designed by site-directed mutagenesis. A comparison with the X-ray structures of the levansucrases from *B. subtilis* (74% identity at the amino acid level) and *G. diazotrophicus* (26%) indicated an overall similar protein structure, carrying identical essential amino acid motifs (RDP and DXXER) but also important differences [16–19]. A detailed biochemical investigation of the 16 levansucrase variants identified common structure-based functional principles and catalytic specificity-modifying determinants.

MATERIALS AND METHODS

Identification of the levansucrase, SacB, from *B. megaterium*

B. megaterium was cultivated aerobically at 37°C in LB (Luria–Bertani) medium [20] supplemented with 0.5% (w/v) sucrose. Samples collected at indicated time points after the start of cultivation were separated by centrifugation (2600 g for 15 min at 4°C) into cells and cell-free growth medium. For SDS/PAGE

Abbreviations used: HPAEC, high-performance anion-exchange chromatography; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single-quantum coherence; LB, Luria–Bertani.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (email j.seibel@tu-bs.de).

[20] analysis of the secreted proteins, 2.5 ml of cell-free growth medium was desalted using PD-10 columns (GE Healthcare) prior to concentration by freeze-drying. Proteins were separated by SDS/PAGE [10% (w/v) gel] and stained with Coomassie Brilliant Blue.

Proteins were identified by peptide mass fingerprinting, as well as by using postsource decay fragmentation data recorded with a Bruker Ultraflex MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometer (Bruker Daltonics). The spectra were evaluated with the Biotoools software (Bruker Daltonics) along with the MASCOT search engine (version 1.9; Matrix Science). The criteria used to accept protein identifications included the extent of sequence coverage (minimum, 30%), the number of peptides matched (minimum number, 5) and the probability score (minimum Mowse score, 70).

Construction of the levansucrase variants by site-directed mutagenesis

Molecular biology methods have been outlined previously by Sambrook et al. [20]. The 1371 bp coding sequence of *B. megaterium* *sacB* lacking the signal peptide coding sequence at the 5' end of the gene was amplified by PCR using primers pRBec1_for (5'-TTATTCCATGGCTAAAGGAAACGACAGC-AAAG-3'; NcoI site italicized) and pRBec1_rev (5'-TATCAGGATCCGCTATTGCAAAGCGCTCAGTC-3'; BamHI site italicized). The PCR-product was digested with NcoI and BamHI and ligated into the previously NcoI/BamHI-cut pET11d (Novagen) yielding pRBec1.

The *sacB* gene was mutated using the QuikChange® site-directed mutagenesis kit (Stratagene). The forward primers used to generate the mutations were as follows (the exchanged amino acids are indicated as lower case): W94A, 5'-GACTTAGATGTAGctGACAGCTGGCC-3'; D95A, 5'-GACTTAGATGTATGGctAGCTGGCCATTAC-3'; V115A, 5'-CCTGCTAGGGCAA-AagcAATTTGATATCC-3'; L118A, 5'-CAAATTGTGTTTGCC-gctGCAGGTGATCCAAAG-3'; W172A, 5'-CAAACACAAG-AAgctTCTGGTTCTGGC-3'; S173A, 5'-CAAACACAAGAAT-GGgctTGTTCTGGCACG-3'; N252A, 5'-GACACAGGGGA-TgctCATACGCTAAGAG-3'; N252D, 5'-GACACAGGGGATg-ACCATACGCTAAGAG-3'; N252G, 5'-GACACAGGGGATggt-CATACGCTAAGAG-3'; N252H, 5'-GACACAGGGGATcACC-ATACGCTAAGAG-3'; R256A, 5'-GGATAACCATACgctAGCT-GATCCTCACTAC-3'; D257A, 5'-CCATACGCTAAGAGcTC-CTCACTACATAG-3'; E350A, 5'-CACAGTTACAGACgctATT-GAACGTGC-3'; E352A, 5'-GTTACAGACGAAATTGctCGTG-CAAACATTTTC-3'; R370A, 5'-GTTACCCGATTCTgctGGA-TCTAAAATGACG-3'; Y421A, 5'-GATAAAACATTTACGgc-TTCTCACTTTGCTG-3'.

All introduced mutations and the integrity of the residual *sacB* gene were confirmed by DNA sequencing.

Levansucrase production and purification

For cloning and cultivation, *Escherichia coli* cells carrying the corresponding plasmid were grown in LB medium [20] supplemented with 0.5 mM ampicillin at 37°C in shaking flasks. Recombinant gene expression was induced with 0.5 mM IPTG (isopropyl β -D-thiogalactoside) after 3 h and the temperature was lowered to 26°C for 20 h. For cell disruption, the cell culture was centrifuged at 4°C for 20 min at 2600 g and the supernatant discarded. The cells were washed and subsequently dissolved in 50 mM Sorensen's phosphate buffer (pH 6) and disrupted by sonication (Sonoplus, Bandelin electronics with sonotrode MS72)

Figure 1 Effect of sucrose on the secretome of *Bacillus megaterium* DSM319

B. megaterium DSM319 was cultivated aerobically in 100 ml of LB medium without (lane 1) or with (lane 2) the addition of 0.5% (w/v) sucrose at 37°C. At 6 h after the start of cultivation, 1.5 ml of cell-free growth medium was desalted using a PD-10 column and subsequently freeze-dried. The precipitated proteins were analysed via SDS/PAGE (10% gels) and stained with Coomassie Brilliant Blue G250. Lane M shows Precision Plus Protein Standard (Bio-Rad).

for 4 min at 70% power and 50% time. After centrifugation at 5200 g for 20 min at 4°C, the supernatant contained the recombinantly produced levansucrase. The enzyme was purified by FPLC using a 15 ml CM-Sepharose column with a controlled pump mix system (Liquid Chromatography Controller LCC-500 Plus, LKB Pump P-500, Pharmacia) and a UV-detector (Biotech UV-MII, Pharmacia). Loading was done with the crude cell extract in 50 mM Sorensen's phosphate buffer (pH 6.6) with a flow rate of 0.5 ml · min⁻¹. For elution, a linear gradient from 50 mM to 1 M of Sorensen's phosphate buffer (pH 6.6) was used. Fractions of 2 ml were collected (autosampler LKB Frac-100, Pharmacia) and analysed by SDS/PAGE (Figure 1). The protein concentrations of the fractions were measured photometrically at $\lambda = 280$ nm (NanoDrop Spectrophotometer ND-100,peqLab Biotechnology).

Characterization of the wild-type levansucrase

The optimal enzyme concentration was determined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37°C containing 500 mM sucrose. The tested enzyme concentrations were 73.60, 29.44, 14.72, 7.36 and 4.91 mg · l⁻¹ purified levansucrase respectively. Reactions were stopped after 1 h by heating (100°C for 10 min). The release of fructose and glucose from sucrose was analysed by TLC. The optimal substrate concentration for the transfructosylation was determined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37°C using 7.36 mg · l⁻¹ levansucrase. Sucrose concentrations tested were 1 M and 750, 500 and 250 mM respectively. Reactions were stopped after 1 h by heating (100°C for 10 min) and analysed as described above. The pH optimum was determined in 50 mM Sorensen's phosphate buffer at 37°C, containing 500 mM sucrose using an enzyme concentration of 7.36 mg · l⁻¹. The reactions at pH values of 5, 5.6, 6, 6.6, 7, 7.6 and 8 respectively, were stopped by heating (100°C for 10 min). Carbohydrate content was analysed by HPAEC (high-performance anion-exchange chromatography; pre column, CarboPac PA1, 4 mm × 50 mm; main column, CarboPac PA1, 4 mm × 250 mm Dionex; conductivity detector PAD-2, Dionex at 1 ml · min⁻¹). The optimal reaction temperature was determined in 50 mM Sorensen's buffer (pH 6.6) at various temperatures (25,

30, 37, 40 and 50 °C). The optimal reaction conditions for the wild-type and the variants were at an enzyme concentration of $7.36 \text{ mg} \cdot \text{l}^{-1}$, a substrate concentration of 500 mM sucrose and a reaction time of 60 min. The release of glucose was measured by HPAEC.

Determination of the kinetic parameters

The kinetic parameters of the wild-type and the variants were determined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C. The enzyme concentration was $7.36 \text{ mg} \cdot \text{l}^{-1}$ and the reaction time was 60 min. The substrate concentration varied from 500 mM to 1 M sucrose. The release of glucose was measured by HPAEC.

Determination of the product spectrum

The product spectrum was determined for wild-type levansucrase and each variant using 50 mM Sorensen's phosphate buffer (pH 6.6) containing 500 mM sucrose at 37 °C. The enzyme concentration was $100 \text{ mg} \cdot \text{l}^{-1}$. Samples were taken at the indicated times for a period of 9 days. The reaction products were identified using standards (1-kestose, 6-kestose and nystose; all $10 \text{ mg} \cdot \text{l}^{-1}$) by HPAEC. All unidentified oligosaccharides were separated by silica-gel-column chromatography ($3.5 \text{ mm} \times 500 \text{ mm}$, flow rate $500 \mu\text{l} \cdot \text{min}^{-1}$). The structures were analysed by NMR and MS.

Characterization of the polysaccharide

The formation kinetics of polysaccharides were examined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C containing 500 mM sucrose and at an enzyme concentration of $7.36 \text{ mg} \cdot \text{l}^{-1}$. Samples were taken after 30 s, 30 min, 60 min, 24 h and 72 h respectively, and analysed by TLC. The production of polysaccharides was performed in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C with $100 \text{ mg} \cdot \text{l}^{-1}$ levansucrase. After 10 min the polymer was precipitated with 1 vol. of ethanol [99 % (v/v)]. After centrifugation ($14\,000 g$ for 5 min at 4 °C) the supernatant was discarded and the precipitate dried by freeze-drying. Size distribution of formed polysaccharides was characterized by MALLD (multi-angle laser light diffraction; Dawn DSP, Wyatt Technologies) and RI (refractive index) analysis (RI-101, Shodex).

For the identification of the linkage type, the polysaccharides were produced as described above and dialysed against water at 4 °C overnight [Slide-A-Lyzer Dialysis cassettes, Pierce, MWCO (molecular mass cut-off) 30 kDa]. The sample was centrifuged for 5 min at $14\,000 g$ and the supernatant discarded. The precipitate was washed four times with 1 ml of ethanol in water [66 % (v/v)]. The polysaccharides were dried by freeze-drying and dissolved in 900 mM NaO^2H for NMR spectroscopy.

NMR spectroscopy

All samples for NMR analysis were freeze-dried from MilliQ water and dissolved in $^2\text{H}_2\text{O}$ (or 900 mM NaO^2H). Spectra were recorded on a Bruker DRX600 or Bruker DRX400 operating at 600 and 400 MHz respectively for ^1H . One-dimensional ^1H and ^{13}C spectra and phase-sensitive two-dimensional spectra [double-quantum-filtered COSY, NOESY, TOCSY, ^1H - ^{13}C HSQC (heteronuclear single-quantum coherence) and HMBC (heteronuclear multiple bond correlation)] were recorded using standard pulse programs at 38.8 °C, and data were analysed using the software package XWINNMR (Bruker). Chemical shifts are given in the δ -scale (p.p.m.) and coupling constants J in Hz.

RESULTS AND DISCUSSION

Characterization of the wild-type levansucrase SacB from *B. megaterium*

When *B. megaterium* was cultivated in LB medium with or without 0.5 % (w/v) sucrose, SDS/PAGE comparison of the secretome from both cultivations revealed a dominant protein solely produced in the presence of sucrose (Figure 1). This protein with a relative molecular mass of 52000 was identified as described in the Materials and methods section (Figure 2). The analysed peptide fragments were homologous with fragments predicted for the levansucrase SacB, a 473-amino-acid residue exoenzyme of *B. subtilis*. BLAST searches of the *B. subtilis* SacB against the translated genome data of *B. megaterium* led to the identification of the SacB protein sequence and the genomic position of the corresponding *sacB* gene [21,22]. *B. megaterium* SacB consists of 484 amino acids including the 29 amino acids of an N-terminal signal peptide. It shows 74 % identity at the amino acid sequence level with SacB from *B. subtilis*. Hence the *B. megaterium* protein was also named SacB. Nine of the 11 additional amino acids found in *B. megaterium* SacB compared with SacB of *B. subtilis* are adjacent to one another at location 82–90 (considering the numbering of *B. megaterium* SacB). In a similar manner to *sacB* of *B. subtilis*, *sacB* of *B. megaterium* is the first gene of an operon. It is followed by a gene consisting of 1563 bp. The deduced protein sequence shows 66 % identity with the levansucrase LevB from *B. subtilis*. *B. subtilis* *levB* is also the second gene in an operon [22]. The third and last gene in the operon in *B. subtilis* encodes a 520-amino-acid protein which might function as a permease [22]. Interestingly, the protein encoded by a gene sequence following the levansucrase gene *sacB* in *B. megaterium* was annotated as bacterioferritin (C. Hundertmark, K. Hiller, R. Münch, and D. Jahn, unpublished work). The *sacB* of *B. megaterium* was amplified lacking the coding sequence of the N-terminal signal peptide and cloned into an *E. coli* expression vector. After production and purification of the recombinant levansucrase (Figure 3) the optimal reaction conditions of *B. megaterium* SacB were examined as described in the Materials and methods section. A broad pH optimum between pH 6.0 and 7.0 for the levansucrase activity was observed. The enzyme activity significantly fell at pH values below 5.6 and above 7.6. The temperature optimum of the levansucrase was found to be 45 °C. In standard reactions, long-term temperature treatment revealed a high protein stability at 37 °C for at least 24 h. The optimal reaction conditions for fructosyl transfer were determined with the purified levansucrase by measuring the initial velocity of the reaction at 37 °C, at pH 6.6, with $7.36 \text{ mg} \cdot \text{l}^{-1}$ purified enzyme and with 500 mM sucrose.

Kinetic studies and characterization of the product spectrum of the wild-type levansucrase

The kinetic parameters were determined as described in the Materials and methods section (Table 1). The K_m value of 6.6 mM for hydrolysis measured for the wild-type levansucrase from *B. megaterium* is low compared with other levansucrases, e.g. SacB from *B. subtilis* with a K_m of 13.5–40 mM [23]. This indicates a high affinity for the substrate sucrose supported by a very high k_{cat} (2272 s^{-1}) and a high catalytic efficiency ($346 \text{ mM}^{-1} \cdot \text{s}^{-1}$, Table 1). The reaction conditions used for the determination of the product spectrum favour hydrolysis (58.4 %) followed by synthesis of polyfructan (22.1 %). Further analysis showed that polyfructan produced by the levansucrase from *B. megaterium* had a molecular mass of 2711 kDa and consisted mainly of $\beta(2 \rightarrow 6)$ linkages, whereas the polyfructan produced by the

Figure 2 Identification of the levansucrase by MALDI/MS
Mass spectrum of the peptide fragment VMKPLITSNTVTDEIER. The levansucrase was identified as described in the Materials and methods section.

Figure 3 SDS/PAGE analysis of the FPLC
Lane 1, crude cell extract; lane 2, flow-through of the FPLC (as described in the Materials and methods section); lane 3, first FPLC peak fraction; lane 4, protein marker (New England Biolabs, relative molecular masses are indicated in kDa); lanes 5–10, FPLC peak fractions. The apparently pure levansucrase containing fractions are boxed.

Table 1 Optimal reaction conditions

The optimal reaction conditions for the levansucrase from *B. megaterium* are shown in comparison with the levansucrase from *B. subtilis*.

	Levansucrase, SacB, from <i>B. subtilis</i> [23]	Levansucrase, SacB, from <i>B. megaterium</i>
K_m (mM)	13.5–40	6.6
TO number (k_{cat} ; s ⁻¹)	0.6–60	2272
pH optimum for hydrolysis	6	6.6
Temperature optimum (°C)	37–50	45
Stability	pH 3.5–7.5 at 4°C, no time indicated	>6 months at pH 6.6 and –20°C

levansucrase from *B. subtilis* had a molecular mass of 6295 kDa and was described as $\beta(2\rightarrow6)$ -linked levan with some $\beta(2\rightarrow1)$ branches [8]. Besides the polyfructan formation, the wild-type

Figure 4 Identification of the transfructosylation products
The carbohydrates up to tetrasaccharides were identified by HPAEC. The dotted line is the oligosaccharide standard (1-kestose, 6-kestose and nystose; all 0.01 mg · l⁻¹), the solid line is the wild-type levansucrase reaction (stopped after 19 h at 37°C and 0.5 M sucrose in 50 mM Sorensen's phosphate buffer, pH 6.6; enzyme concentration was 0.1 mg · l⁻¹), and the dashed line is the oligosaccharide standard (concentration as above) mixed with the same wild-type levansucrase sample.

levansucrase of *B. megaterium* also synthesized five different detectable oligosaccharides. Three products were identified by HPAEC as 1-kestose (isokestose), 6-kestose and nystose which are known acceptors for the transfer of fructosyl units [7] (Figure 4). The two unknown by-products were isolated and analysed by one and two-dimensional NMR and MS (Figure 5, Tables 2 and 3). The one-dimensional proton spectrum of the first compound showed two anomeric protons (4.6–5.2 p.p.m.), which corresponded to an anomeric mixture of two non-reducing sugar residues. The H-1 of the β -configured glucoside residue resonated at higher field (4.59 p.p.m.) with a large coupling constant (d, 7.96Hz), whereas the H-1 (5.16 p.p.m.) of the α -configured glucose appeared as minor signal of a reducing sugar in a ratio of α/β 1:2. Based on the results of the two-dimensional COSY

Figure 5 Correlation spectrum of α,β blastose (HMBC)

Table 2 NMR data for the identification of blastose

C-atom	α -Blastose		β -Blastose	
	δ_C	δ_H	δ_C	δ_H
C-1	94.94	5.16 (d, 3.70)	98.79	4.59–4.57 (d, 7.96)
C-2	74.27	3.49–3.46 (m)	76.89	3.20–3.16 (t, 8.70)
C-3	75.48	3.63–3.68 (m)	78.45	4.42–3.40 (m)
C-4	72.55	3.34–3.40 (m)	72.49	3.88–3.85 (m)
C-5	73.49	3.85–3.88 (m)	77.79	3.84–3.80 (m)
C-6	63.61	3.96–3.89, 3.67–3.62	63.61	3.96–3.89, 3.67–3.62
C-1'	62.99	3.70–3.62 (m)	62.99	3.70–3.62 (m)
C-2'	106.52	—	106.55	—
C-3'	79.68	4.58–4.56 (d, 8.26)	79.78	4.58–4.56 (d, 8.26)
C-4'	77.31	4.07–4.03 (t, 7.60)	77.45	4.07–4.03 (t, 7.60)
C-5'	83.97	3.82–3.76 (m)	84.02	3.82–3.76 (m)
C-6'	65.21	3.77–3.72 (m)	65.32	3.77–3.72 (m)

spectrum, correlations were observed between the H-1s of the glucose residues and the corresponding H-2s (α 3.8–4.1 p.p.m.; β 3.20–3.16 p.p.m.), which was coupled to H-3s (α 3.63–3.68 p.p.m.; β 3.42–3.40 p.p.m.). The assignments for the anomeric carbon signals (α 94.94 p.p.m.; β 98.79 p.p.m.) and C-2s (α 74.27 p.p.m.; β 76.89 p.p.m.) were achieved by two-dimensional HSQC spectrum analysis. In addition, $^1\text{H}/^{13}\text{C}$ -HMBC long-range 2J and 3J correlations were observed for the H-6 signal of glucose with the ^{13}C signals at δ_C 106.52 p.p.m. (α , C-2') and 106.55 p.p.m. (β , C-2') of the fructofuranoside respectively, which showed cor-

Table 3 NMR data for the identification of neokestose

C-atom	Neokestose	
	δ_C	δ_H
C-1	62.89	3.91–3.87, 3.76–3.73
C-2	106.45	—
C-3	79.47	4.16–4.13 (d, 8.5)
C-4	77.03	4.11–4.07 (t, 7.4)
C-5	83.88	3.84–3.80 (m)
C-6	65.17	3.83–3.73
C-1'	94.74	5.35 (d, 3.88)
C-2'	73.74	3.53–3.49 (dd, 3.90, 10.00)
C-3'	75.15	3.69–3.66 (m)
C-4'	74.76	3.92–3.88 (m)
C-5'	71.89	3.48–3.45 (m)
C-6'	62.03	3.58 (s)
C-1''	64.14	3.73–3.69, 3.64–3.61
C-2''	106.47	—
C-3''	78.90	4.18–4.16 (d, 8.49)
C-4''	76.64	4.04–4.00 (t, 8.5)
C-5''	84.08	3.87–3.83 (m)
C-6''	65.08	3.80–3.76, 3.68–3.64

relations for the fructofuranoside H-3' positions (β 79.78 p.p.m.; α 79.68 p.p.m./4.58 p.p.m.) and H-4' positions (β 77.45 p.p.m.; α 77.31 p.p.m./4.07 p.p.m.) (Figure 4). All other carbon and proton signals were assigned from a combination of two-dimensional COSY, TOCSY, HMBC and HSQC experiments. All experiments

	W94A				W172A				N252A, N252D R256A			
	D95A		L118A		S173A		N252G, N252H		D257A			
Gd	70	VWVWDTWTLLDKH	82	89	NGWEVIFCLTADP	101	160	QAEWSGSSRLMQ	171	240	QNEF-----FNFRDPPTTFED	254
Ps	42	VFIWDTMPLRELD	54	61	NGWSVIVTLTADR	73	128	TREWAGTPVLLN	139	194	QNST-----WNFRDPSPFFID	210
Ra	42	VFIWDTMPLRLSLD	54	61	DGWSVIFTLTAQR	73	128	SREWAGTPILLN	139	194	QNPY-----WNFRDPSPFFID	210
Ea	42	VFIWDTMPLRDFD	54	61	NGWCIIFTLTADR	73	128	TREWAGTPILLN	139	194	QNAF-----WNFRDPSPFFID	210
Zm	40	LWLWDTWPLRDIN	52	59	KGNWVIFSLVADR	71	110	TAEWSGGTIMAP	121	183	ENNL-----WNFRDPHVFIN	199
Ss	284	IDVWDSWFPVQDAK	295	303	NGYQLVISMAGAP	315	352	DQWWSGSATVNS	363	424	KFMStf [11] rtdnYCLRDPHIIED	456
Pp	91	FDVWDSWPLQNAD	103	110	KGYNIVFGLAGDP	122	169	SEEWSSGSATLTS	180	245	QSVGvdm----dnHTFRDPHYVED	265
Ba	82	LDVWDSWPLQNAD	94	101	NGYHVVFALAGSP	113	160	TQEWSSGSATFTS	171	229	QFIDegnyt--sgdnHTLRDPHYVED	253
Sm	243	LDVWDSWFPVQDAK	254	262	NGYQLVVAMMGIP	274	311	TQEWSSGSATVNE	322	384	QWRStftg--adnIAMRDPHYVED	406
Bs	56	LDVWDSWPLQNAD	68	75	HGYHIVFALAGDP	87	134	TQEWSSGSATFTS	145	203	QFIDegnys--sgdnHTLRDPHYVED	227
Bm	91	LDVWDSWPLQNAD	103	110	HGYQIVFALAGDP	122	169	TQEWSSGSGTLTK	180	239	QFIDeggyd--tgdnHTLRDPHYIED	263
	E350A				R370A				Y421A			
	E352A				R370A				Y421A			
Gd	333	ANCVNDQTERPQVYLHNGKYYIFTISHRTTFA	363	420	FQSYSHYVMPG	430						
Ps	279	AVGVNDQTERPHYVFQDGKYYLFTISHKFTYA	309	349	FQTYSHCVMPN	359						
Ra	279	AVGVNDQTERPHFVFQDGKYYLFTISHKFTYA	309	349	FQTYSHCVMPN	359						
Ea	279	AVGVNDQTERPHFVFQDGKYYLFTISHKYTYA	309	349	FQTYSHYVMPN	359						
Zm	268	AFGVNDQMERPHVIFQNGLTLYLFTISHDSTYA	298	339	TEAYAHYIMNN	349						
Ss	539	SHMVTDEVERPSVVKMGKKYYLFTASRINKST	569	620	TSTYSYAYVPV	630						
Pp	346	SNLVTDEIERANVFKMNGWLWYFTSTRGSKVT	376	420	TWTYAHFAIPQ	430						
Ba	334	SNTVTDEIERANVFKMNGKWYLFDSRSGSKMT	364	408	TFTYSHFAVPQ	418						
Sm	491	STMVSDLEIRPNVVKLGDKYYLFTASRLNHGS	521	572	TATYSYAYVPV	582						
Bs	304	SNTVTDEIERANVFKMNGKWYLFDSRSGSKMT	334	382	TFTYSHFAVPQ	392						
Bm	344	SNTVTDEIERANIFKKDKWYLFDSRSGSKMT	374	418	TFTYSHFAVPQ	428						

Figure 6 Sequence alignment of related levansucrases

Gd is *Gluconacetobacter diazotrophicus* [19], Ps is *Pseudomonas syringae* [28], Ra is *Rahnella aquatilis* [29], Ea is *Erwinia amylovora* [30], Zm is *Zymomonas mobilis* [31], Ss is *Streptococcus salivarius* [32], Pp is *Paenibacillus polymyxa* [33], Ba is *Bacillus amyloliquefaciens* [34], Sm is *Streptococcus mutans* [35], Bs is *Bacillus subtilis* [18], and Bm is *Bacillus megaterium*. The amino acids used for the mutagenesis studies are indicated and the resulting variant is labelled above the sequence.

provide strong evidence of an 1/2 α : β mixture of β 6Fru- α , β -Glc (6-*O*- β -D-fructofuranosyl- α , β -D-glucopyranoside, blastose).

The MS of the second compound showed the molecule peak at $m/z = 527$ [M^+Na^+]. This trisaccharide is a non-reducing sugar. In the one-dimensional proton spectrum the anomeric proton resonated at δ_H 5.35 p.p.m. with a small J_{12} value of 3.90 Hz. This proton showed correlation in the two-dimensional HMBC spectrum with quaternary carbon C-2' (106.45 p.p.m.) of a fructofuranoside residue. In the two-dimensional HMBC spectrum protons corresponding to the H-6 glucose residue showed correlations to the quaternary carbons C-2'' of a second fructofuranoside residue. Taken together, all signals assigned from the one-dimensional and correlation spectroscopy (i.e. COSY, TOCSY, HMBC, DEPT and HSQC) identified the trisaccharide as β 2,6Fru- α Glc- β 1,2Fru (2,6-*O*- β -D-fructofuranosyl- α -D-glucopyranosyl-1,2-*O*- β -D-fructofuranoside or neokestose).

Functional role of the exchanged amino acids and insights into the reaction mechanism of the levansucrase from *B. megaterium*

Levansucrases belong to glycoside hydrolase family 68 (GH 68) according to CAZy [15], which bind the substrate sucrose in the -1 (fructosyl residue) and +1 site (glucosyl residue). In this family, two X-ray structures of the *B. subtilis* and *G. diazotrophicus* enzymes have been elucidated and both show a 5-fold β -propeller topology [18,19].

Amino acid sequence alignments of various levansucrase enzymes (Figure 6) in combination with structural data of family members pointed towards functionally relevant residues. Subsequent site-directed mutagenesis of Trp⁹⁴, Asp⁹⁵, Val¹¹⁵, Leu¹¹⁸, Trp¹⁷², Ser¹⁷³, Asn²⁵², Arg²⁵⁶, Asp²⁵⁷, Glu³⁵⁰, Glu³⁵², Arg³⁷⁰ and Tyr⁴²¹ was performed in order to explore the structural determinants which are responsible for the transfructosylation activity in *B. megaterium* SacB.

The residues Asp⁹⁵, Glu³⁵² and Asp²⁵⁷ are proposed to form the catalytic triad of the levansucrase from *B. megaterium*

(Figure 7). The substitution of an alanine residue for Asp⁹⁵ inhibited the enzyme activity (Table 4). Sequence alignments with other levansucrases from various bacteria showed that this amino acid residue acted as a nucleophile. It usually attacks the glucopyranosyl residue of the sucrose and forms an enzyme-fructosyl intermediate with inversion of the glycosidic bond in the intermediate state. The corresponding acid/base catalyst was Glu³⁵². Consequently, an exchange into an alanine residue led to a high decrease in enzyme activity. The variant D257A showed almost no activity. The residue Asp²⁵⁷ might have been co-ordinated by hydrogen bonds to the fructofuranoside in positions 3-OH and 4-OH.

Trp⁹⁴, Arg²⁵⁶, Glu³⁵⁰ and Tyr⁴²¹ were selected as their corresponding amino acid residues of the levansucrase from *B. subtilis* [18,24] form hydrogen bonds to sucrose and direct it in a productive orientation. In the present study, the substitution of an alanine residue for each of these amino acid residues in the levansucrase from *B. megaterium* showed different effects. The variants R256A and E350A were nearly inactive. This agreed with the structural information obtained for the levansucrase from *B. subtilis* where the corresponding Arg²⁴⁶ formed hydrogen bonds with the 3-OH of the fructofuranoside and also with 4-OH of the glucopyranoside while Glu³⁴⁰ co-ordinated the 3-OH and 4-OH of the glucopyranoside. The levansucrase variant W94A still carried 9% of the catalytic efficiency k_{cat}/K_m , whereas variant Y421A showed 3% of the wild-type activity. Considering the X-ray structure of the levansucrase from *B. subtilis*, this tryptophan residue formed a hydrogen bond with 6-OH of the fructofuranoside and the tyrosine residue formed a hydrogen bond with the 2-OH of the glucopyranoside [18]. Owing to the low reaction rate, hydrolysis was predominantly observed (Tables 5 and 6). The substituted amino acid residues Trp¹⁷² and Ser¹⁷³ were proposed to be part of interactions with amino acids of the active site. In the levansucrase of *B. subtilis*, this tryptophan residue is situated in the +1 region and functioned to form a pocket for the glucopyranoside of the C-6-OH. Our experiments with the

Figure 7 The proposed transfructosylation reaction mechanism

The mechanism is based on SacB from *B. megaterium* with sucrose as substrate.

Table 4 Kinetic parameters of the wild-type levansucrase and its variants

The reaction was performed with $7.36 \text{ mg} \cdot \text{l}^{-1}$ enzyme in 50 mM Sorensen's phosphate buffer (pH 6.6) with 500 mM sucrose. For the variants D95A, R256A, D257A, E350A and E352A the determination of kinetic parameters was not possible due to missing or too slow release of glucose under these reaction conditions.

Variant	K_m (mM)	V_{max} (mM $\cdot$ min $^{-1}$)	k_{cat} (s $^{-1}$)	k_{cat}/K_m (mM $^{-1} \cdot$ s $^{-1}$)
Wild-type	6.6 ± 1.1	19.6 ± 0.6	2272	346
W94A	31.9 ± 11.0	8.6 ± 0.9	1000	31
D95A	—	—	—	—
L118A	66.6 ± 22.1	10.6 ± 1.1	1231	19
W172A	480.4 ± 124.8	20.7 ± 3.2	2396	5
S173A	2.3 ± 1.2	3.1 ± 0.3	363	159
N252A	4.1 ± 1.7	12.8 ± 1.1	1480	361
R256A	—	—	—	—
D257A	—	—	—	—
E350A	—	—	—	—
E352A	—	—	—	—
Y421A	51.9 ± 16.3	2.9 ± 0.3	335	6.5

levansucrase variant W172A showed a 72-fold increase of the K_m and thus confirmed this function. It was very interesting to note that substitution of an alanine residue for Ser¹⁷³ affected the catalytic efficiency dramatically, whereas the K_m was comparable with the wild-type levansucrase. Thus it is proposed that Ser¹⁷³ does not influence the binding mode of sucrose, but it must somehow participate in subsequent catalysis.

Polysaccharide compared with oligosaccharide synthesis

The substitution of an alanine residue for Arg³⁷⁰ of *B. megaterium* SacB resulted in fundamental changes in product formation. The corresponding residue in the levansucrase from *B. subtilis* in subsite -1 (Arg³⁶⁰), was previously described as a key residue for the formation of 1-kestose [25]. We observed the time-dependent accumulation of different oligosaccharides during levansucrase variant R370A catalysis. After a reaction time of 60 min an accumulation of neokestose (2,6- β -Fru- α -Glc-1,2- β -Fru, 32.7 mM) was determined, whereas after 19 h, blastose (2,6- β -Fru- α , β -Glc) was the main reaction product (69.7 mM). A 4-fold K_m value decrease compared with the wild-type enzyme supported the interactions of Arg³⁷⁰ with the 2-OH and 3-OH of the glucosyl residue in the active site. It is possible that the substitution of an alanine residue favoured an unspecific substrate binding and enabled a fructosylation at C-6 of the glucosyl residue of sucrose-yielding neokestose. When the trisaccharide neokestose was accumulating and entering the active site of the *B. megaterium* levansucrase variant R370A, the $\beta(2 \rightarrow 1)$ linkage is cleaved leading to the hydrolysis product blastose. Blastose was not supposed to be a donor substrate of the enzyme indicated by its strong accumulation due to a unfavourable $\beta(2 \rightarrow 6)$ linkage between glucose and fructose. Residue Asn²⁵² obviously plays an important role for transfructosylation. A substitution of an alanine or glycine residue completely stopped the polysaccharide production, although the K_m and k_{cat} values were not affected (Table 7). Catalysis of this variant was switched from mainly polysaccharide synthesis to hydrolysis (Tables 5 and 6). In contrast, mutation of Asn²⁵²

Table 5 Product spectrum after 60 min reaction time

All values are given in mM · l⁻¹. The sample used for the identification of neokestose is indicated.

	Wild-type	L118A	W172A	N252A	N252D	N252G	N252H	R370A	Y421A
Glucose	428.0	85.1	13.0	447.9	463.4	334.9	118.6	138.7	12.2
Fructose	292.2	71.6	11.3	391.4	362.0	279.9	102.3	103.0	9.6
Sucrose	41.9	407.1	487.0	17.1	0.0	141.8	381.4	321.3	487.5
1-Kestose	15.9	3.4	0.0	5.2	3.5	6.9	0.0	3.4	0.0
α,β-Blastose	6.4	0.0	0.0	7.3	17.3	9.7	0.0	3.9	0.0
6-Kestose	3.5	0.0	0.0	15.0	4.2	6.8	0.0	0.0	0.0
Neokestose	2.8	3.5	0.0	5.8	7.4	6.6	0.0	32.7	0.0
Nystose	1.5	0.5	0.0	1.7	4.1	0.0	0.0	0.0	0.0

Table 6 Product spectrum after 19 h reaction time

All values are mM · l⁻¹. The sample used for the identification of blastose is indicated.

	Wild-type	L118A	W172A	N252A	N252D	N252G	N252H	R370A	Y421A
Glucose	449.8	304.1	101.1	459.9	443.8	448.7	431.1	414.4	97.8
Fructose	359.8	226.7	89.5	399.4	375.5	360.3	360.3	357.2	83.8
Sucrose	0.0	172.2	389.7	0.0	0.0	0.0	27.4	0.0	389.5
1-Kestose	7.6	4.5	2.1	4.0	7.7	7.0	6.0	6.0	4.7
α,β-Blastose	24.5	7.4	1.1	14.1	21.5	26.3	16.7	69.7	1.1
6-Kestose	4.9	4.1	0.6	11.9	14.8	6.7	4.5	1.2	0.0
Neokestose	9.5	5.3	5.4	7.0	9.3	15.0	12.3	13.6	6.9
Nystose	3.7	2.5	0.0	3.2	3.5	3.4	2.1	2.1	0.0

Table 7 Kinetic parameters of the wild-type levansucrase and its variants

The reaction was performed with 7.36 mg · l⁻¹ enzyme in 50 mM Sorensen's phosphate buffer (pH 6.6) with 500 mM sucrose.

Variant	K _m (mM)	V _{max} (mM · min ⁻¹)	k _{cat} (s ⁻¹)	k _{cat} /K _m (mM ⁻¹ · s ⁻¹)
Wild-type	6.6 ± 1.1	19.6 ± 0.6	2272	346
N252A	4.1 ± 1.7	12.8 ± 1.1	1480	361
N252D	8.4 ± 2.3	32.3 ± 1.6	3743	445
N252G	7.5 ± 0.8	19.5 ± 0.4	2256	301
N252H	35.1 ± 30.7	13.2 ± 3.4	1529	43.5
R370A	29.2 ± 11.6	1.5 ± 0.2	179	6.1

to Asp²⁵² affected just down-regulation of polysaccharide synthesis (Figure 8), also without significantly changing K_m and k_{cat} values. The X-ray structure of the levansucrase from *B. subtilis* (74 % identity on amino acid level) enabled insights into the function of this residue. Owing to its position in the + 2 site, it might stabilize the third fructosyl unit of the growing oligosaccharide chain and direct it as an acceptor substrate in the optimal position for further transfructosylation. It is located in a channel where the oligosaccharide chain is able to enter the active site for the transfructosylation reaction. Indeed, based on the levansucrase structure of *B. subtilis*, it seems probable that no other possibilities exist for the oligosaccharide chain to access the catalytic site (Figure 9).

The mutation of Asn²⁵² to Asp²⁵² obviously reduced the co-ordination of a fructosyl unit in the + 2 site. However, it was still possible as indicated by the formation of polysaccharides (Figure 8). The mutations to alanine, glycine and histidine residues led to a complete knock-out of polysaccharide formation. Very recently, Ozimek et al. [26] proposed a model of the sugar-binding subsites in two *Lactobacillus reuteri* 121 fructosyltransferases. For the fructan polymer synthesis the subsites + 2, and + 3 need

Figure 8 TLC analysis of polymer compared with oligosaccharide formation activity

The reaction was performed using wild-type levansucrase SacB from *B. megaterium* and indicated variants carrying amino acid exchanges at position 252. The reaction conditions were 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C. The enzyme concentration was 7.36 mg · l⁻¹ each. Std1 and Std2 are carbohydrate standards containing glucose (Glc), fructose (Fru), sucrose (suc) and maltose (not indicated) top down. The sample times are indicated above in h. The detectable polymer is encircled. Os, oligosaccharide; Ps, polysaccharide.

a high affinity for binding the growing fructan polymer chain. No structural determinants have so far been reported.

Residues Arg³⁷⁰ and Asn²⁵², which seem to be crucial for the polyfructan synthesis, are found to be conserved in levansucrase from Gram-positive bacteria. In contrast, the endophytic Gram-negative bacterium *G. diazotrophicus* SRT4 secretes a constitutively expressed levansucrase (LsdA, EC 2.4.1.10), which mainly converts sucrose into fructo-oligosaccharides. It contains histidine instead of Arg³⁷⁰ at the equivalent position (419). This histidine residue is found strictly conserved in Gram-negative levansucrases. Furthermore Asn²⁵² is strictly conserved in Gram-positive bacteria, whereas in Gram-negative bacteria this region shows variability. The corresponding part of the three-dimensional structure of LsdA was almost perfectly superimposable with the equivalent residues of the *B. subtilis* SacB in the active site. In contrast the region + 2 was not perfectly superimposable (Figure 9).

Figure 9 Local identification of the crucial amino acid residues

This superimposed Figure shows the levansucrases from *B. subtilis* (Bs, purple) and *G. diazotrophicus* (Gd, cyan) with sucrose in the active site [18,19,36,37]. The analogue amino acid residues of the *B. megaterium* (Bm) levansucrase are given in parentheses.

In conclusion the present study reports the full characterization of a novel levansucrase, SacB, from *B. megaterium* and its product spectra. Site-directed mutagenesis clarified the functional roles of different amino acids which are involved in the formation of different reaction products. There are only few studies existing which concern the oligosaccharide compared with polysaccharide formation of levansucrases describing a processive or non-processive mechanism [24–26]. So far, no structural insights or amino acids have been identified to give conclusions or clarify differences regarding polysaccharide and oligosaccharide synthesis among the levansucrases in detail. In the present study it was shown for the first time that a single amino acid substitution at the +2 subsite switches the enzymatic reaction from polysaccharide to oligosaccharide formation conserving a high enzyme activity by changing from a processive to a non-processive mechanism. Based on these studies in future more structural details of the +2 and +3 subsites may enable the redesign of this enzyme for the synthesis of tailor-made short-chain fructo-oligosaccharides.

This project is supported by DFG (Deutsche Forschungsgemeinschaft; SFB 578) and AIF (Arbeitsgemeinschaft industrieller Forschungsvereinigungen; Project 14777).

REFERENCES

- Priest, F. G. (1977) Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.* **41**, 711–753
- Yun, J. W. (1996) Fructooligosaccharides: occurrence, preparation and application. *Enz. Microb. Technol.* **19**, 107–117
- Kaur, N. and Gupka, A. K. (2002) Applications of oligofructose in health and nutrition. *J. Biosci.* **27**, 703–714
- Bornet, F., Brouns, F., Tashiro, Y. and Duvillier, V. (2002) Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications. *Dig. Liver Dis.* **34**, 111–120
- Hidaka, H., Hirayama, M. and Sumi, N. (1988) A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.* **52**, 1181–1187
- Straathoff, A. J. J., Kieboom, A. P. G. and van Bekkum, H. (1986) Invertase-catalysed fructosyl transfer in water-organic solvent systems. *Carbohydr. Res.* **146**, 154–159
- van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L. and van Geel-Schutten, I. G. (2006) Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. Rev.* **70**, 157–176
- Chambert, R., Treboul, G. and Dedonder, R. (1974) Kinetic studies of levansucrase of *Bacillus subtilis*. *Eur. J. Biochem.* **41**, 285–300
- Vijn, I. and Smeeke, S. (1999) Fructan: more than a reserve carbohydrate? *Plant Physiol.* **120**, 351–360
- Cote, G. A. and Ahlgren, J. A. (1993) Metabolism in microorganisms, part I: levan and levansucrase. In *Science and Technology of Fructans* (Suzuki, M. and Chatterton, N. J., eds), pp. 141–168. CRC Press, Boca Raton
- Steinmetz, M. (1993) Carbohydrate Catabolism: Pathways, Enzymes, Genetic Regulation, and Evolution *Bacillus subtilis*, and Other Gram-Positive Bacteria (Sonenshein, A. L., Hoch, J. A. and Losick, R., eds), pp. 157–170. American society for Microbiology, Washington DC
- Hernandez, L., Arrieta, J., Menendez, C., Vazquez, R., Coego, A., Suarez, V., Selman, G., Petit-Glatron, M. F. and Chambert, R. (1995) Isolation and enzymic properties of levansucrase secreted by *Acetobacter diazotrophicus* SRT4, a bacterium associated with sugar cane. *Biochem. J.* **309**, 113–118
- Priest, F. G., Goodfellow, M. and Todd, C. (1988) A numerical classification of the genus *Bacillus*. *J. Gen. Microbiol.* **134**, 1847–1882
- Vary, P. S. (1994) Prime time for *Bacillus megaterium*. *Microbiology* **140**, 1001–1013
- Coutinho, P. M. and Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach. In *Recent Advances in Carbohydrate Bioengineering* (Gilbert, H. J., Davies, G., Henrissat, B. and Svensson, B., eds), pp. 3–12. The Royal Society of Chemistry, Cambridge
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
- Schäffer, A. A., Aravind, L., Madden, T. L., Shavirin, S., Spouge, J. L., Wolf, Y. I., Koonin, E. V. and Altschul, S. F. (2001) Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res.* **29**, 2994–3005
- Meng, G. and Futterer, K. (2003) Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. *Nat. Struct. Biol.* **10**, 935–941
- Martinez-Fleites, C., Ortiz-Lombardia, M., Pons, T., Tarbouriech, N., Taylor, E. J., Arrieta, J. G., Hernandez, L. and Davies, G. (2005) Crystal structure of levansucrase from the Gram-negative bacterium *Gluconacetobacter diazotrophicus*. *Biochem. J.* **390**, 19–27
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1999) *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchet, S. et al. (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256
- Sun, J., Wang, W., Hundertmark, C., Zeng, A. P., Jahn, D. and Deckwer, W. D. (2006) A protein database constructed from low-coverage genomic sequence of *Bacillus megaterium* and its use for accelerated proteomic analysis. *J. Biotechnol.* **124**, 486–495
- Schomburg, I., Hofmann, O., Bansch, C., Chang, A. and Schomburg, D. (2000) Enzyme data and metabolic information: BRENDA, a resource for research in biology, biochemistry, and medicine. *Gene Funct. Dis.* **3–4**, 109–118
- Seibel, J., Moraru, R., Götz, S., Buchholz, K., Na'ammeh, S., Pawlowski, A. and Hecht, H. J. (2006) Synthesis of sucrose analogues and the mechanism of action of *Bacillus subtilis* fructosyltransferase (levansucrase). *Carbohydr. Res.* **341**, 2335–2349
- Chambert, R. and Petit-Glatron, M. F. (1991) Polymerase and hydrolase activities of *Bacillus subtilis* levansucrase can be separately modulated by site-directed mutagenesis. *Biochem. J.* **279**, 35–41
- Ozimek, L. K., Kralj, S., van der Maarel, M. J. and Dijkhuizen, L. (2006) The levansucrase and inulosucrase enzymes of *Lactobacillus reuteri* 121 catalyse processive and non-processive transglycosylation reactions. *Microbiology* **152**, 1187–1196
- Marchler-Bauer, A., Anderson, J. B., Derbyshire, M. K., DeWeese-Scott, C., Gonzales, N. R., Gwadz, M., Hao, L., He, S., Hurwitz, D. I., Jackson, J. D. et al. (2007) CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res.* **35**, 237–240
- Hettwer, U., Jaekel, F. R., Boch, J., Meyer, M., Rudolph, K. and Ullrich, M. S. (1998) Cloning, nucleotide sequence, and expression in *Escherichia coli* levansucrase genes from the plant pathogens *Pseudomonas syringae* pv. *glycinea* and *P. syringae* pv. *phaseolicola*. *Appl. Environ. Microbiol.* **64**, 3180–3187
- Song, K. B., Seo, J. W., Kim, M. G. and Rhee, S. K. (1998) Levansucrase of *Rahnella aquatilis* ATCC33071. Gene cloning, expression, and levan formation. *Ann. N.Y. Acad. Sci.* **864**, 506–511
- Geier, G. and Geider, K. K. (1993) Characterization and influence on virulence of the levansucrase gene from the fireblight pathogen *Erwinia amylovora*. *Physiol. Mol. Plant Pathol.* **42**, 387–404

- 31 Song, K. B., Lee, S. K., Joo, H. K. and Rhee, S. K. (1994) Nucleotide and derived amino acid sequences of an extracellular sucrose gene (*invB*) of *Zymomonas mobilis* ZM1 (ATCC10988). *Biochim. Biophys. Acta* **1219**, 163–166
- 32 Rathsam, C., Giffard, P. M. and Jacques, N. A. (1993) The cell-bound fructosyltransferase of *Streptococcus salivarius*: the carboxyl terminus specifies attachment in a *Streptococcus gordonii* model system. *J. Bacteriol.* **175**, 4520–4527
- 33 Bezzate, S., Aymerich, S., Chambert, R., Czarnes, S., Berge, O. and Heulin, T. (2000) Disruption of the *Paenibacillus polymyxa* levansucrase gene impairs its ability to aggregate soil in the wheat rhizosphere. *Environ. Microbiol.* **2**, 333–342
- 34 Tang, L. B., Lenstra, R., Borchert, T. V. and Nagarajan, V. (1990) Isolation and characterization of levansucrase-encoding gene from *Bacillus amyloliquefaciens*. *Gene* **96**, 89–93
- 35 Shiroza, T. and Kuramitsu, H. K. (1988) Sequence analysis of the *Streptococcus mutans* fructosyltransferase gene and flanking regions. *J. Bacteriol.* **170**, 810–816
- 36 Kanz, C., Aldebert, P., Althorpe, N., Baker, W., Baldwin, A., Bates, K., Browne, P., van den Broek, A., Castro, M. and Cochrane, G. (2005) The EMBL Nucleotide Sequence Database. *Nucleic Acids Res.* **33**, D29–D33
- 37 DeLano, W. L. (2002) The PyMOL Molecular Graphics System. DeLano Scientific LLC, San Carlos, CA

Received 4 May 2007/14 June 2007; accepted 4 July 2007

Published as BJ Immediate Publication 4 July 2007, doi:10.1042/BJ20070600