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Export, purification, and activities of affinity tagged *Lactobacillus reuteri* levansucrase produced by *Bacillus megaterium*

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Abstract Fructosyltransferases, like the *Lactobacillus reuteri* levansucrase, are important for the production of new fructosyloligosaccharides. Various His_6- and Strep-tagged variants of this enzyme were recombinantly produced and exported into the growth medium using the Gram-positive bacterium *Bacillus megaterium*. Nutrient-rich growth medium significantly enhanced levansucrase production and export. The *B. megaterium* signal peptide of the extracellular esterase LipA mediated better levansucrase export compared to the one of the penicillin amidase Pac. The combination of protein export via the LipA signal peptide with the coexpression of the signal peptidase gene *sipM* further increased the levansucrase secretion. Fused affinity tags allowed the efficient one-step purification of the recombinant proteins from the growth medium. However, fused peptide tags led to slightly decreased secretion of tested fusion proteins. After upscaling 2 to 3 mg affinity tagged levansucrase per liter culture medium was produced and exported. Up to 1 mg of His_6-tagged and 0.7 mg of Strep-tagged levansucrase per liter were recovered by affinity chromatography. Finally, the purified levansucrase was shown to synthesize new fructosyloligosaccharides from the novel donor substrates D-Gal-Fru, D-Xyl-Fru, D-Man-Fru, and D-Fuc-Fru.

R. Biedendieck and R. Beine contributed equally to this work.

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Introduction

Fructosyloligosaccharides are described as useful prebiotics due to the capacity of the β-linked fructose units to pass the gastrointestinal tract undigested. In the colon, they stimulate selectively the growth of beneficial gut bacteria, like *Bifidobacteria* or *Lactobacilli* (Salminen et al. 1996). In addition, fructans, like levan, are employed in the food and non-food industry as viscosifier, stabilizer, emulsifier, gelling, or water-binding agent. Fructooligosaccharides are produced by fructosyltransferases, like levansucrases. Levansucrases (EC 2.4.10) synthesize levan composed of β-2,6 linked fructose residues (van Hijum et al. 2001). Due to commercial interest, we investigated the Gram-positive bacterium *Bacillus megaterium* for the recombinant production and export of levansucrase into the growth medium. In contrast to *Bacillus subtilis*, *B. megaterium* does not possess alkaline proteases and is known for the stable replication and maintenance of plasmids (Vary 1992). Using the SEC pathway for protein export, the bacterium

readily secretes proteins. After the protein synthesis in the cytoplasm, the SEC system recognizes the N-terminal part of an exoprotein precursor, the so-called signal peptide, and transports the unfolded polypeptide chain through a channel in the cytoplasmic membrane. Outside the cytoplasm, a signal peptidase anchored to the cell membrane removes the signal peptide by cleavage at its recognition sequence, releasing the mature protein into the extracellular space. Recently, we investigated the production and secretion of a heterologous dextransucrase of high molecular mass (M_r=188,000) from *Leuconostoc mesenteroides*. For this purpose, a plasmid-based expression system with the xylose-inducible promoter of the *B. megaterium xylA* gene was employed (Malten et al. 2005a). Constructed shuttle vectors allow stable replication and antibiotic selection in *Escherichia coli* and in *B. megaterium*. The use of a *B. megaterium* strain deficient in the only detectable extracellular protease NprM significantly improved the production and extracellular stability of heterologous proteins (Wittchen and Meinhardt 1995). Moreover, the overproduction of the homologous signal peptidase SipM further increased protein export (Malten et al. 2005b).

Using *E. coli* as expression host, fast and simple purification of recombinant proteins is achieved using the

so-called affinity tags. These are chosen to have a minimal effect on the tertiary structure and the biological activity of the fusion protein. Therefore, often small peptide tags like polyhistidine (His-tag) or a streptavidin binding polypeptide (Strep-tag) are used. Recently, vectors for the production, export, and purification of recombinant proteins via affinity tags using *B. megaterium* were constructed (Malten et al. 2006). In this study, these vectors were systematically optimized for the recombinant production, export, and purification of *Lactobacillus reuteri* levansucrase.

Materials and methods

DNA manipulation for the construction of plasmids

Molecular biology methods were outlined before (Malten et al. 2006; Sambrook and Russell 2001). All constructed plasmids were amplified in *E. coli* DH10B and analyzed by a DNA sequence analysis of the relevant regions by MWG Biotech (Ebersberg, Germany). Plasmids are listed in Table 1.

Several vectors were constructed by encoding the signal peptide and parts of the *B. megaterium* penicillin amidase Pac. First, the coding sequence of the penicillin amidase was

Table 1 Plasmids used in this study

Name	Description	Reference or source
pMM1522	Shuttle vector for cloning in *E. coli* (Ap^r) and expression under xylose control in *B. megaterium* (Tc^r)-P_{xylA}-MCS	Malten et al. (2006)
pSTOP1522	pMM1522 derivative-P_{xylA}-MCS-Stop	Malten et al. (2006)
pRBBm23	pMM1522 derivative-P_{xylA}-SP_{pac}-*pac*	This study
pMM1525	pMM1522 derivative-P_{xylA}-SP_{lipA}-MCS	Malten et al. (2006)
pSTREP1522	pSTOP1522 derivative-P_{xylA}-StrepXa-MCS	Malten et al. (2006)
pHIS1525	pMM1525 derivative-P_{xylA}-SP_{lipA}-MCS-His	Malten et al. (2006)
pSTREP1525	pMM1525 derivative-P_{xylA}-SP_{lipA}-StrepXa-MCS	Malten et al. (2006)
pSTREPHIS1525	pMM1525 derivative-P_{xylA}-SP_{lipA}-StrepXa-MCS-His	Malten et al. (2006)
pRBBm26	pSTREP1522 derivative-P_{xylA}-SP_{pac}-StrepXa-MCS	This study
pRBBm27	pSTREP1522 derivative-P_{xylA}-SP_{pac}-α-*subunit″*-StrepXa-MCS	This study
pRBBm28	pSTREP1522 derivative-P_{xylA}-SP_{pac}-α-*subunit′*-StrepXa-MCS	This study
pRBBm29	pSTREP1522 derivative-P_{xylA}-SP_{pac}-α-*subunit*-StrepXa-MCS	This study
pRBBm13	P_{xylA}-SP_{lipA}-StrepXa-*levΔ773*	Malten et al. (2006)
pRBBm15	P_{xylA}-SP_{lipA}-*levΔ773*His	Malten et al. (2006)
pRBBm16	P_{xylA}-SP_{lipA}-StrepXa-*levΔ773*His	Malten et al. (2006)
pRBBm30	P_{xylA}-SP_{pac}-StrepXa-*levΔ773*	This study
pRBBm31	P_{xylA}-SP_{pac}-α-*subunit″*-StrepXa-*levΔ773*	This study
pRBBm32	P_{xylA}-SP_{pac}-α-*subunit′*-StrepXa-lev*Δ773*	This study
pRBBm33	P_{xylA}-SP_{pac}-α-*subunit*-StrepXa-*levΔ773*	This study
pMMBm7	P_{xylA}-SP_{lipA}-*levΔ773*MycHis	Malten et al. (2006)
pMGBm4	P_{xylA}-SP_{lipA}-*levΔ773*	Malten et al. (2006)
pEJBm7	P_{xylA}-SP_{lipA}-*levΔ773*His-terminator	This study
pEJBm2	P_{xylA}-SP_{lipA}-*levΔ773*Strep-terminator	This study
pMMBm3	P_{sipM}-*sipM*-xylR-P_{xylA}-*dsrS*	Malten et al. (2006)
pRBBm19	pMMBm7 with P_{sipM}-*sipM* inserted into *Afl*II	This study
pRBBm46	pEJBm2 with P_{sipM}-*sipM* inserted into *Afl*II	This study

amplified using Oligo1 (5′-tacatatgtacaatgaagacgaagtggctaa tatca-3′) and Oligo2 (5′-tatcagagctcatcaatag tataggctctttatgc-3′) with the genomic DNA of *B. megaterium* ATCC14945 as the template. Oligo1 included a *Bsr*GI and Oligo2 a *Sac*I restriction site. The cloning of the *Bsr*GI/*Sac*I digested PCR fragment into the appropriately cut pMM1522 resulted in pRBBm23. Using pRBBm23 as template, the coding sequence of the signal peptide (sp_{pac}), and three parts of the α-subunit of the penicillin amidase Pac from *B. megaterium* ATCC14945 were amplified using Oligo3 (5′-ccgactagaccata aggg-3′) in combination with Oligo4 (5′-ccttgaccccttcactag tatcctccccagc-3′), Oligo5 (5′-ctttattactagttccatctcttctggattgc-3′), Oligo6 (5′-caatgcttgtaggagcactagtatcattttttccaca c-3′), and Oligo7 (5′-ccgactatggcggcactagtgcctatctttaacgg-3′), respectively. Oligo4, Oligo5, Oligo6, and Oligo7 included all *Spe*I restriction sites at their 5′-ends. The PCR products were digested using the restriction enzymes *Xho*I and *Spe*I, resulting in fragments of 633 bp (sp_{pac}), 840 bp (sp_{pac}-α-*subunit″*), 1,179 bp (sp_{pac}-α-*subunit′*), and 1,348 bp (sp_{pac}-α-*subunit*), respectively. They were cloned into the appropriately cut pSTREP1522 (Malten et al. 2006), resulting in pRBBm26, pRBBm27, pRBBm28, and pRBBm29, respectively. For fusing the coding sequences of the signal peptide derivatives to the levansucrase gene *levΔ773* from the *L. reuteri* strain 121 (culture collection TNO Nutrient and Food Research, Zeist, The Netherlands), the 2,222 bp *Bgl*II/*Sph*I-fragment from pRBBm13 (Malten et al. 2006) was subcloned into pRBBm26, pRBBm27, pRBBm28, and pRBBm29, resulting in pRBBm30, pRBBm31, pRBBm32, and pRBBm33, respectively. Oligo8 (5′-gcatgcggccgctcatcaccat caccatcactaaaaagccctcaatgaagagggctttttttaaccggt-3′) and Oligo9 (5′-accggttaaaaaaagccctcttcattgagggcttttttagtgatggt gatggtgatgagcggccgcatgc-3′) were hybridized to insert the DNA encoding a C-terminal His$_6$-tag and the terminator in *Sph*I and *Age*I restricted pRBBm15 (Malten et al. 2006) resulting in pEJBm7. Furthermore, Oligo10 (5′-gcatgcggccg cttggagccacccgcagttcgagaaataaaaagccctcaatgaagagggcttttttt aaccggt3′) and Oligo11 (5-accggttaaaaaaagccctcttcattgagggc tttttatttctcgaactg-cgggtggctccaagcggccgcatgc-3′) were hybridized to insert DNA encoding a C-terminal Strep-tag II and the terminator into *Sph*I and *Age*I restricted pRBBm15 resulting in pEJBm2. For the insertion of the *sipM* gene under the control of its own promoter, the *Afl*II fragment was subcloned from pMMBm3 (Malten et al. 2005b) into pMMBm7 (Malten et al. 2006) and pEJBm2, resulting to pRBBm19 and pRBBm46, respectively.

Analysis of levansucrase production and secretion in *B. megaterium*

Plasmids were transformed into *B. megaterium* MS941 cells as described before (Wittchen and Meinhardt 1995). Transformed *B. megaterium* was grown at 37°C in Luria-Bertani (LB) medium (Sambrook and Russell 2001) or in A5+4 medium (Malten et al. 2005a) with the addition of 10-μg tetracycline per ml. The induction of the recombinant gene expression, the preparation of the soluble, insoluble, and cell-free fractions by lyophilization, and their analysis were described in detail before (Malten et al. 2005a). Furthermore, proteins of 1.5 ml of cell-free growth medium were precipitated with four volumes of ice-cold acetone at −20°C overnight. Precipitated proteins were collected by centrifugation (10 min; 4,500 g; 4°C), washed, spun down again, and analyzed (Malten et al. 2005a). Proteins with levansucrase activity were detected by activity staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was washed three times in a washing buffer (20-mM sodium acetate, pH 5.4; 340-μM calcium chloride; 0.1% (v/v) Triton X-100) after electrophoresis. The incubation for 36 h at 50°C in the washing buffer substituted with 292-mM sucrose enabled the in situ formation of polymers by renatured active enzyme. The subsequent oxidation by periodic acid activated the polymer for a stain with Schiff´s reagent (Ferretti et al. 1987). For the immunochemical detection of affinity-tagged levansucrase, an anti-Strep antibody (IBA GmbH, Göttingen, Germany) or monoclonal mouse anti-His antibody (GE Healthcare, Freiburg, Germany) were used in Western blot experiments according to the instructions of the manufacturers.

Recombinant protein purification from the growth medium

Strep-tagged LevΔ773 was purified from the cell-free growth medium using a StrepTactin sepharose affinity column as described in the manufacturer's manual (IBA GmbH, Göttingen, Germany). After the ammonium sulfate precipitation (Englard and Seifter 1990), the precipitate was resolubilized in 100-mM TRIS-buffer (pH 8) with 1-mM EDTA and incubated with StrepTactin sepharose. Lev-Δ773His was purified directly from the cell-free growth medium via two alternative strategies: (1) 600 μl of MagneHis™ Ni-particles (Promega, Madison, USA) were incubated in a 50-ml cell-free growth medium for 1 h under soft shaking. The beads were separated from the solution and washed three times with a binding buffer (100-mM sodium phosphate buffer, pH 7). Proteins were eluted at different imidazole concentrations of 100 and 500 mM in 1-ml binding buffer. (2) 700 μl of Ni-loaded chelating sepharose FF (GE Healthcare, Uppsala, Sweden) were incubated with 50 ml of cell-free growth medium for 1 h. The sepharose was separated from the growth medium using a poly-prep chromatography column (BioRad, Hercules, USA). The column was washed with three column volumes of the binding buffer. Finally, proteins were eluted using 200-mM imidazole and 1-mM β-mercaptoethanol in the binding buffer. All purification steps were performed at

room temperature. All eluted fractions were stored at 4°C for further analysis via levansucrase activity test and SDS-PAGE. The protein concentration was determined using a Bradford assay kit (BioRad, Hercules, USA) with bovine serum albumin (Perbio, Rockford, USA) as standard.

Determination of levansucrase activity

The levansucrase activity of the various preparations described in this investigation was measured via the release of fructose and glucose from sucrose. The total amount of the reducing sugars, fructose and glucose, was determined using dinitrosalicylic acid as described in the dinitrosalicylic acid (DNS) method (Sumer and Howell 1935). Assays were performed at 37°C. Levansucrase-containing solutions were incubated in 25-mM sodium acetate buffer (pH 5.4) and 1-mM $CaCl_2$ with 100-mM sucrose, and kinetic parameters were determined as outlined before (van Hijum et al. 2004).

Production of oligofructoglycosides with alternative substrates

α-D-Galactopyranosyl-1,2-β-D-fructofuranoside (D-Gal-Fru), α-D-xylopyranosyl-1,2-β-D-fructofuranoside (D-Xyl-Fru), α-D-mannopyranosyl-1,2-β-D-fructofuranoside (D-Man-Fru), and α-D-fucopyranosyl-1,2-β-D-fructofuranoside (D-Fuc–Fru) were synthesized by enzymatic transfructosylation of D-galactose, D-xylose, D-mannose, and D-fucose as described elsewhere (Baciu et al. 2005; Seibel et al. 2006a). Reactions were performed at 37°C for 3 days with 3.3 U ml^{-1} of purified LevΔ773His in 25-mM sodium acetate buffer (pH 5.4), 1-mM $CaCl_2$, 250-mM donor substrate, and 500 mM of acceptor substrate D-galactose, D-xylose, D-mannose, or D-fucose. The high acceptor concentration avoided acceptor reactions with released fructose. Fructo-oligosaccharides were separated by thin layer chromatography (TLC). Chromatography was performed three times for 45 min on aluminium sheets covered with silica gel, using a solvent mixture of 80% acetonitrile and 20% water. Carbohydrate spots were stained with sulfuric acid and N-(1-naphtyl)ethylendiamine. Electrospray-ionization mass spectra were recorded with a Finnigan MAT 8340 (San Jose, CA, USA) on samples suspended in CH_3OH.

Results

Production and secretion of L. reuteri levansucrase by B. megaterium

The Gram-positive bacterium B. megaterium was employed for the recombinant production and secretion of levansucrase LevΔ773 from L. reuteri strain 121. First, B. megaterium

MS941 that was transformed with the levansucrase-encoding pMMBm7 (Malten et al. 2006) was grown in the A5+4 medium (Malten et al. 2005a), and the gene expression was induced with 0.5% (w/v) xylose at an OD$_{578nm}$ of 0.4. After the SDS-PAGE analysis of acetone-precipitated proteins from 1.5-ml growth medium, significant amounts of a protein with a relative molecular mass (M_r) of 110,000 were detected (Fig. 1a, lanes 4 to 6). As expected, this protein was not present in the growth medium of B. megaterium MS941 transformed with the target geneless the control plasmid pMM1522 (Fig. 1a, lanes 1 to 3). As reported previously, the
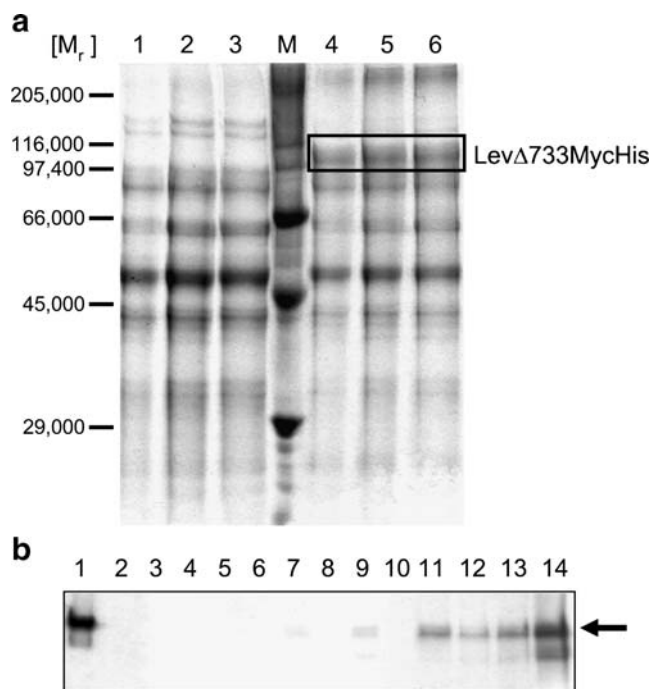

Fig. 1 Secretion of recombinant L. reuteri 121 levansucrase by B. megaterium MS941 carrying pMMBm7 (LevΔ773MycHis). a Acetone-precipitated proteins from 1.5-ml cell-free growth medium of MS941 carrying the target geneless pMM1522 (lanes 1 to 3) and MS941 carrying pMMBm7 (lanes 4 to 6) were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue G250. The SDS sample buffer contained 100 μM of β-mercaptoethanol. Lanes 1 and 4, 2 and 5, and 3 and 6 show proteins prepared 6, 9, and 12 h after induction of gene expression, respectively. Lane M shows the high molecular weight protein marker (Sigma-Aldrich, USA). b Activity stain for the intra- and extracellular localization of levansucrase LevΔ733MycHis. As control, lane 1 shows extracted proteins from E. coli DH10B transformed with pBAD-lev encoding LevΔ773MycHis. Lanes 2 to 4 depict the analysis of proteins from B. megaterium MS941 carrying target geneless pMM1522. The intracellular soluble (lane 2), insoluble (lane 3), and the secretome fractions (lane 4) are shown. Lanes 5 to 14 show levansucrase activity from B. megaterium carrying pMMBm7. Proteins from the intracellular soluble (lanes 5, 7, 9) and insoluble (lanes 6, 8, 10) fraction are shown. Lanes 5 and 6, 7 and 8, and 9 and 10 are from cells before and 1.5 and 3 h after induction of levansucrase gene expression, respectively. Secreted proteins were analyzed from the cell-free growth medium 3, 6, 9, and 12 h (lanes 11 to 14) after induction of levansucrase gene expression

recombinant levansucrase with a calculated molecular mass of 84,772 Da appears at a M_r of 110,000 in an SDS-PAGE analysis (van Hijum et al. 2004). Correct N-terminus formation was verified by the amino acid sequence determination (data not shown). The activity of the recombinant levansucrase was visualized in an enzyme activity stain after the SDS-PAGE analysis. The *L. reuteri* levansucrase produced by *B. megaterium* carrying pMMBm7 (Fig. 1b, lanes 11 to 14) revealed an identical relative molecular mass as the same enzyme recombinantly produced by *E. coli* DH10B (Fig. 1b, lane 1). No enzyme activity was visible in the culture medium of *B. megaterium* MS941 transformed with the target geneless pMM1522 (Fig. 1b, lanes 2 to 4). Recombinant levansucrase was continuously secreted by *B. megaterium* MS941 up to 12 h after the induction of the levansucrase gene expression with a maximum of 5.4 units per liter of growth medium (Fig. 1). The use of a protease deficient strain lacking the only detectable extracellular neutral protease NprM resulted in the absence of degradation until 9 h after induction. Twelve hours after induction, a degraded but active levansucrase form was detectable (Fig. 1b, lanes 11 to 14). Three hours after the induction of the gene expression, the enzyme was barely visible in the cell pellet (Fig. 1b, lane 9). All cell-associated recombinant levansucrase was found soluble, demonstrating the absence of severe protein-folding problems during protein production and secretion. The cell-associated recombinant levansucrase had the same relative molecular mass as the secreted one. Hence, all cell-associated recombinant levansucrase was found in its mature form. During SEC-dependent protein secretion, the mature form of a secreted protein is usually exclusively found outside the cytoplasm. Therefore, the residual amount of the detected mature cell-associated recombinant levansucrase protein was most likely localized between the cytoplasmic membrane and cell wall. No obvious cytoplasmic recombinant levansucrase accumulation was observed.

Improved levansucrase secretion in nutrient-rich medium

The secretion of the recombinant levansucrase into the growth medium was enhanced by growing *B. megaterium* in a nutrient-rich LB broth supplemented with 2.5-mM CaCl$_2$. Compared to the previous cultivation in A5+4 medium, a 4.2-fold higher recombinant levansucrase secretion with a maximum of 22.6 units per liter of growth medium was observed 6 h after the induction of the levansucrase gene expression. The nutrient-rich medium suppressed the export of host exoproteins. As a consequence, the levansucrase was the dominant protein of the secretome. CaCl$_2$ was added to the growth medium due to a reported levansucrase requirement for calcium cations (van Hijum et al. 2004).

Comparison of different signal peptides for the secretion of *L. reuteri* levansucrase LevΔ773 by *B. megaterium* MS941

Two different *B. megaterium* signal peptides were tested for their ability to direct the recombinant target protein to the growth medium. The first signal peptide was from the extracellular esterase LipA (Ruiz et al. 2002) and the second from the penicillin amidase Pac (Panbangred et al. 2000). The penicillin amidase is produced as a pre-protein, secreted, and autocatalytically cleaved into two active subunits. We used the α-subunit of the penicillin amidase to systematically investigate the contribution of the exported protein itself on the leader peptide-mediated export. For this purpose, N-terminal fusions of the levansucrase to the partial and complete α-subunit, including the corresponding leader peptide, were investigated. In all cases, the maximal secreted amount of levansucrase was observed 3 h after induction. Both a densitometric analysis of the SDS-PAGE and enzyme activity tests revealed that the levansucrase fused to the 28 amino acid residues of SP$_{lipA}$ (pRBBm13) was secreted best (25.3 U l^{-1}). The fusion of levansucrase to the 24 amino acid residues of SP$_{pac}$ (pRBBm30) yielded 65% (8.9 U l^{-1}) less export. The fusion to 74 (pRBBm31), 184 (pRBBm32), or 241 (pRBBm33) amino acid residues of the α-subunit of penicillin amidase further decreased the amount of secreted levansucrase by 72% (7.1 U l^{-1}), 87% (3.3 U l^{-1}), and 89% (2.8 U l^{-1}), respectively, compared to the SP$_{lipA}$-mediated export. The longer the α-subunit part, the less levansucrase was secreted.

Export of affinity tagged forms of levansucrase

The recently constructed *B. megaterium* vectors (Malten et al. 2006) pMMBm7 (LevΔ773MycHis), pMGBm4 (LevΔ773), pRBBm13 (StrepLevΔ773), pRBBm15 (LevΔ773His), and pRBBm16 (StrepLevΔ773His) were used to study the effect of a fused His$_6$-tag, Strep-tag II, and Myc-epitope on the secretion of levansucrase. To investigate the integrity of the exported fusion proteins in the growth medium, levansucrase fused to affinity tags was analyzed by Western blot experiments using antibodies against the Strep-tag II (IBA GmbH, Göttingen, Germany) and the His$_6$-tag (GE Healthcare, Freiburg, Germany), respectively (Fig. 2a). For StrepLevΔ773, the immunological detection of the Strep-tag II revealed a strong defined protein with an approximate M_r of 108,000 and three minor proteins with an apparent M_r of 97,000, 75,000, and 68,000 (Fig. 2a, lane 5). The levansucrase variant carrying two tags resulted in a single protein with an approximate M_r=110,000 when analyzed with a Strep-tag II antibody (Fig. 2a, lane 4). The anti His-tag antibody solely detected levansucrase molecules with
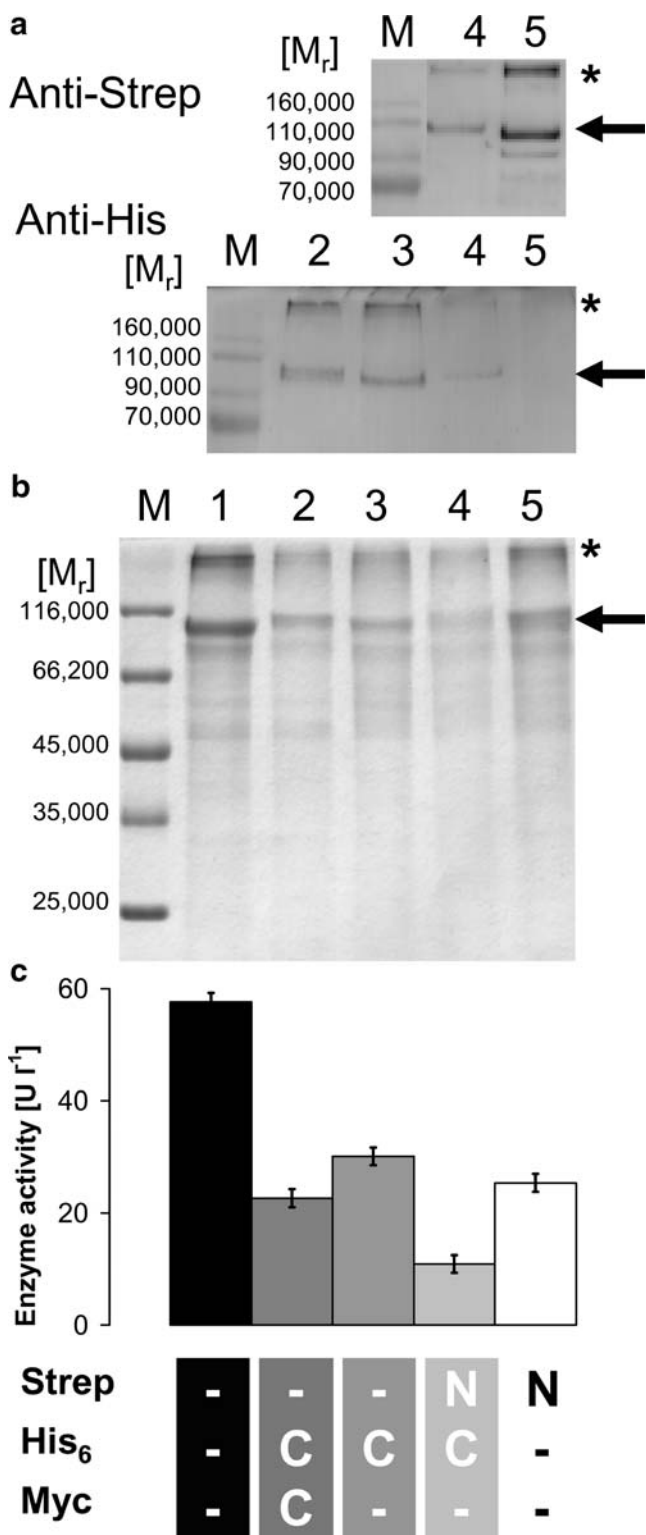
Fig. 2 Influence of affinity tags fused to LevΔ773 on secretion and enzyme activity. *B. megaterium* MS941 carrying plasmids coding for LevΔ773 (lane 1), LevΔ773MycHis (lane 2), LevΔ773His (lane 3), StrepLevΔ773His (lane 4), or StrepLevΔ773 (lane 5) were cultivated in LB medium. **a,b** Six hours after induction of levansucrase gene expression proteins of 1.5-ml cell-free growth medium were separated by SDS-PAGE. SDS sample buffer contained 100 μM of β-mercaptoethanol. An *arrow* and an *asterisk* show the position of LevΔ733His monomer and dimer, respectively. **a** The tags fused to the LevΔ773 were detected using anti-Strep or anti-His antibodies in Western blot experiments. Lane M shows prestained protein ladder (MBI Fermentas, St. Leon-Rot, Germany). **b** The SDS-PAGE gel was stained with Coomassie Brilliant Blue G250. Lane M shows the protein molecular weight marker (MBI Fermentas). **c** The levansucrase activity in the cell-free growth medium was determined using the DNS method. One unit of enzyme was defined as the release of 1-μmol glucose per minute, describing the transferase and hydrolase activity. N- and C-terminal positions of fused affinity tags are shown

products detectable only for the N-terminal Strep-tagged levansucrase indicated that the protein is partly susceptible to degradation from its C-terminus. Therefore, using affinity purification via the C-terminal, His$_6$-tag should lead to the purification of intact levansucrase. The Western blot experiments (Fig. 2a) also visualized tagged levansucrase with approximately twice the calculated molecular mass (M_r=170,000) of the monomer. Increasing the β-mercaptoethanol concentration in the loading buffer from 0.1 to 1 mM abolished this protein dimerization. Next, the amount of exported levansucrase by the various tested strains was visualized via SDS-PAGE (Fig. 2b). The highest amount of secreted levansucrase was found for the strain producing untagged levansucrase. About half the amount was observed for strains producing LevΔ773MycHis, LevΔ773His, and StrepLevΔ773. Even lower amounts were detected for the strain producing StrepLevΔ773His. All produced enzyme variants were found active. The comparison of the protein amounts from the SDS-PAGE analysis with the obtained enzyme activities clearly demonstrated that enzyme activities increased in the same magnitude as the detected protein amounts (Fig. 2b,c). In accordance to the specific activity of the affinity chromatographically purified levansucrase, variants revealed almost identical values (data not shown). Most protein and enzyme activity in the growth medium was detected for the untagged form of levansucrase (57.6 U l^{-1}). LevΔ773His, StrepLevΔ773, and also LevΔ773MycHis revealed about half of the activity of the untagged levansucrase (30.1 U l^{-1}, 25.3 U l^{-1}, and 22.6 U l^{-1}, respectively). In this group of fusion proteins, small but interesting differences were seen in dependence of the size of the fused affinity tag. The His$_6$-tag consists of 6, the Strep-tag II of 8, and the Myc-epitope plus His$_6$-tag of 21 amino acid residues. We observed that the longer the amino acid sequence of a fusion tag was, the lower was the measured activity and the detected protein amount. The lowest amount

an approximate M_r of 110,000, 106,000, and 110,000 in the growth medium of *B. megaterium* strains carrying pMMBm7 (LevΔ773MycHis; Fig. 2a, lane 2), pRBBm15 (LevΔ773His; Fig. 2a, lane 3), and pRBBm16 (StrepLevΔ773His; Fig. 2a, lane 4), respectively. The degradation
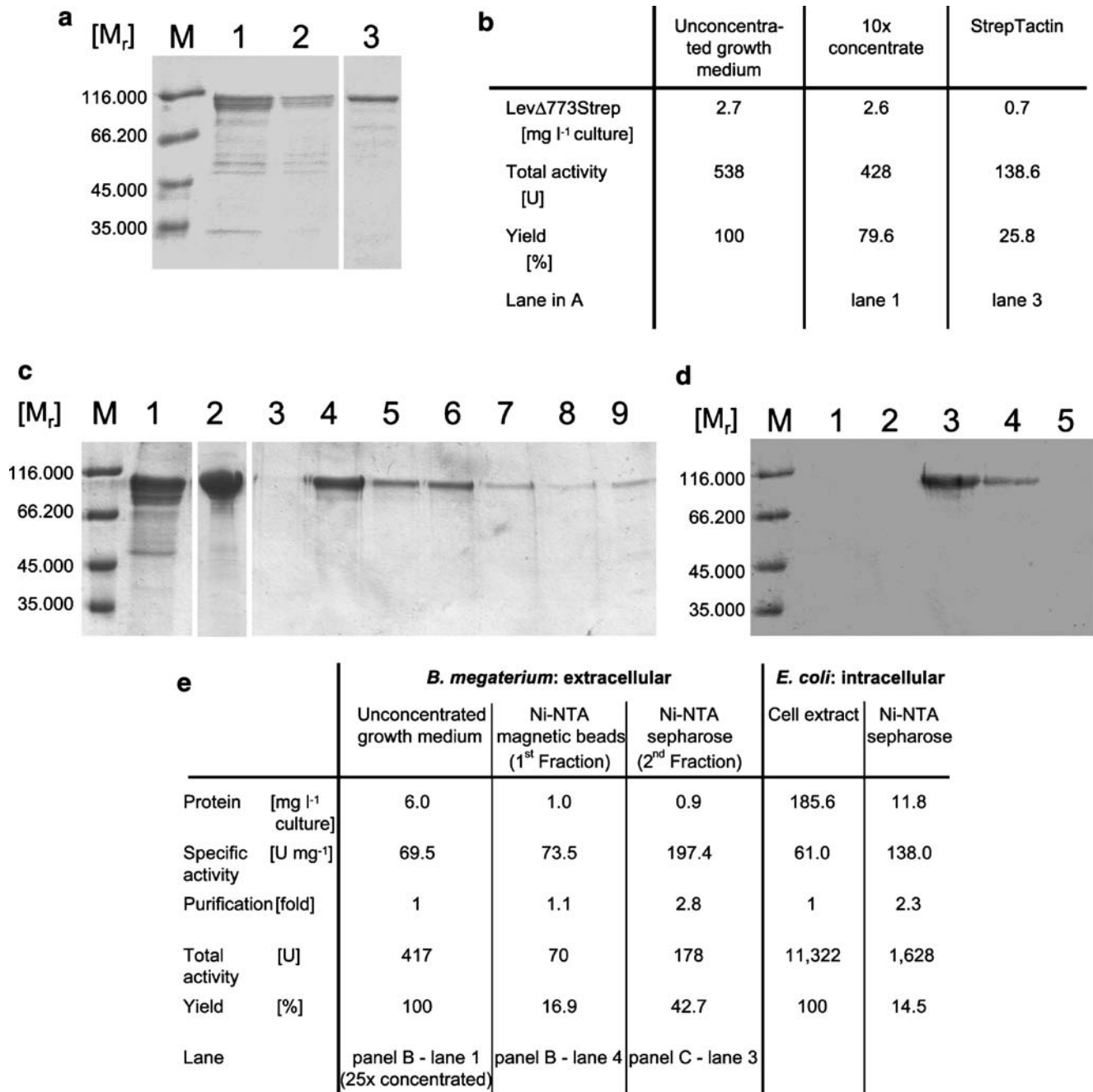
b	Unconcentrated growth medium	10x concentrate	StrepTactin
LevΔ773Strep [mg l⁻¹ culture]	2.7	2.6	0.7
Total activity [U]	538	428	138.6
Yield [%]	100	79.6	25.8
Lane in A		lane 1	lane 3

e	B. megaterium: extracellular			E. coli: intracellular	
	Unconcentrated growth medium	Ni-NTA magnetic beads (1ˢᵗ Fraction)	Ni-NTA sepharose (2ⁿᵈ Fraction)	Cell extract	Ni-NTA sepharose
Protein [mg l⁻¹ culture]	6.0	1.0	0.9	185.6	11.8
Specific activity [U mg⁻¹]	69.5	73.5	197.4	61.0	138.0
Purification [fold]	1	1.1	2.8	1	2.3
Total activity [U]	417	70	178	11,322	1,628
Yield [%]	100	16.9	42.7	100	14.5
Lane	panel B - lane 1 (25x concentrated)	panel B - lane 4	panel C - lane 3		

Fig. 3 Affinity chromatography purification of recombinant LevΔ773 from the cell-free growth medium of *B. megaterium*. **a** Purification of StrepLevΔ773. The proteins from 30-ml cell-free growth medium were precipitated by ammonium sulfate (lane 1). As a 10-fold concentrate, redissolved proteins were applied onto a StrepTactin sepharose column. Washing fractions (lane 2) and elution fractions (lane 3) are shown. **b** Purification table of StrepLevΔ773. **c** Purification of LevΔ773His. Lane 1 shows 20 μl of 25-times concentrated cell-free growth medium. Lane 2 represents proteins from 10 ml of cell-free growth medium bound to 15-μl Ni–NTA magnetic beads. Lanes 3 to 8 show the elution of LevΔ773His from 50 ml of cell-free growth medium bound to 1 ml of Ni–NTA magnetic particles. Proteins attached to 15-μl Ni-charged magnetic particles after the elution of the proteins are shown in lane 9. **d** Purification of LevΔ773His. 700 μl of Ni-NTA sepharose were added to 50 ml of cell-free growth medium in a batch process. Washing fractions (lane 1) and elution fractions (lanes 2–5) are shown. Each fraction at 20 μl was analyzed by SDS-PAGE. Lane M shows the protein molecular weight marker (MBI Fermentas). The SDS-PAGE gels were stained with Coomassie Brilliant Blue G250. SDS sample buffer contained 1 mM of β-mercaptoethanol. **e** The comparison of the purification of extracellular produced LevΔ773His by *B. megaterium* and intracellular produced LevΔ773MycHis by *E. coli* (van Hijum et al. 2004)

of secreted levansucrase (10.9 U l^{-1}), however, were observed when both Strep-tag II and His$_6$-tag, with a total of 14 amino acid residues, were added C- and N-terminally.

Influence of a transcriptional terminator and coexpression of signal peptidase gene *sipM*

To investigate the influence of transcriptional termination on protein production in *B. megaterium*, a rho-independent terminator sequence previously identified at the end of the *B. megaterium cobI* operon (Raux et al. 1998) was introduced into the expression of plasmid-encoding LevΔ773His, resulting in pEJBm7. However, no positive effect on the production and secretion of levansucrase was observed.

The coexpression of *sipM* was previously reported to enhance the secretion of a heterologous dextransucrase via a more effective cleavage of the signal peptide in *B. megaterium* (Malten et al. 2005b). The coexpression of *sipM* under control of its own promoter increased the amount of LevΔ773MycHis (plasmid pRBBm19) and LevΔ773Strep (plasmid pRBBm46) to 1.9- and 1.3-fold, respectively (data not shown).

Upscaling of levansucrase production and export

To upscale the established recombinant levansucrase production and export process, *B. megaterium* harbouring pRBBm13 (StrepLevΔ773), pRBBm15 (LevΔ773His), and pEJBm2 (LevΔ773Strep) was cultivated in a five-time higher volume of LB medium (500 ml) with an extension of the culture time to 12 h after the induction of the gene expression. Tagged levansucrase variants were chosen to allow for subsequent affinity chromatography purification. Twenty-one, 14, and 13 times more secreted and tagged levansucrase (538 U l^{-1}, 416 U l^{-1}, and 523 U l^{-1}) per liter of growth medium, respectively, were observed. Calculated from the determined specific activity, 2.7-mg StrepLevΔ773, 2.1-mg LevΔ773His, and 2.7-mg LevΔ773Strep were secreted per liter of growth medium. The total protein concentration of the growth medium was measured as 6 mg l^{-1} after the elimination of the small peptides from the complex medium by the dialysis of the growth medium against the phosphate buffer. Hence, the heterologous tagged levansucrase accounts for 35 to 45% of the total amount of secreted proteins and peptides.

Affinity chromatographic purification of Strep-tagged levansucrase from the cell-free growth medium

Next, the affinity chromatographic purification of StrepLevΔ773 and LevΔ773Strep found in milligram amounts in the growth medium was tested. Running the growth medium directly over the affinity chromatography column leads to the rapid coloring and blocking of the material. The batch incubation of the chromatographic StrepTactin material with the enzyme containing growth medium yielded the purification of only 3% of StrepLevΔ773 and 0.1% of LevΔ773Strep from the growth medium. Only microgram amounts of StrepLevΔ773 and LevΔ773Strep per liter of culture broth were purified. To enhance affinity material binding, StrepLevΔ773 was first concentrated by ammonium sulfate precipitation (Englard and Seifter 1990) before purification. A tenfold concentration and recovery of 98% (Fig. 3b) of native StrepLevΔ773 was achieved. The solubilized protein precipitate was incubated in a batch with the StrepTactin sepharose, and 34% of the Strep-tagged levansucrase found in the growth medium was bound to the StrepTactin material. After elution, 0.7-mg StrepLevΔ773 per liter of growth medium were obtained in the main elution step (Fig. 3a,b).

Affinity chromatographic purification of His$_6$-tagged levansucrase from the cell-free growth medium

First, a purification of LevΔ773His directly from the cell-free growth medium was persued. From initial binding studies using commercially available paramagnetic pre-charged nickel particles (MagneHis™, Promega), a demand of 600 μl of magnetic Ni particles was calculated for 50 ml of cell-free growth medium. After incubation for 1 h under soft shaking, the beads were separated from the solution and washed with binding buffer. About 1-mg LevΔ773His per liter of culture was obtained in the elution steps (Fig. 3c,e). Most protein was detected after the first elution step using 100 mM of imidazole (Fig. 3c, lane 4). Still, considerable amounts of LevΔ773His eluted at higher imidazole concentration (Fig. 3c, lanes 7 and 8) or remained bound to the magnetic Ni particles (Fig. 3c, lane 9). Hence, a complete dissociation of LevΔ773His from the magnetic Ni-particles was not achieved. The purified protein LevΔ773His recovered in the first elution step showed a low specific activity of 73.5 U mg^{-1}. Only a 1.1-fold purification with a yield of 16.9% was calculated from the obtained data (Fig. 3e).

The observed problem of low recovery was solved using Ni-NTA sepharose (NTA, nitrilotriacetic acid) instead of magnetic particles in the batch process. Slurry of 700 μl of Ni-charged chelating sepharose FF (GE Healthcare, Uppsala, Sweden) was incubated with 50 ml of cell-free growth medium for 1 h before the sepharose was separated from the growth medium. After washing the column, proteins were eluted using 200-mM imidazole and 1-mM β-mercaptoethanol, resulting in 0.9 mg of pure LevΔ773His per liter of culture (Fig. 3d,e). The purified protein LevΔ773His of the second elution step showed a specific acti-
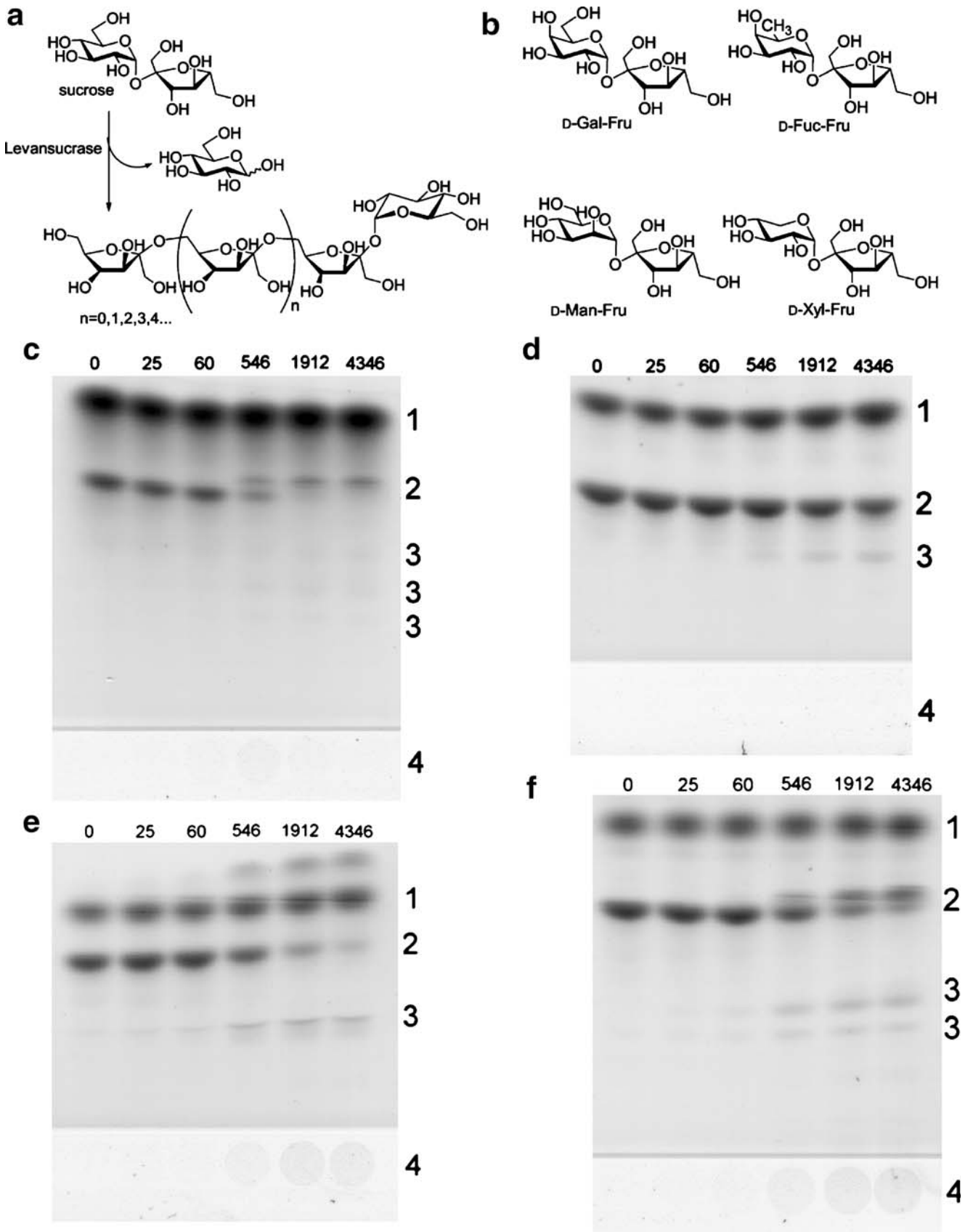
◀ **Fig. 4 a** Enzymatic synthesis of oligofructosides and levan from sucrose. **b** Sucrose analogues as alternative substrates for recombinant purified levansucrase. **c** to **f** Sucrose analogues were incubated with recombinant purified levansucrase. Samples were taken after 0, 25, 60, 546, 1,912, and 4,346 min and separated by TLC. **c** Samples from D-Xyl-Fru incubated with levansucrase and D-xylose showed on the TLC spots of xylose (*1*), D-Xyl-Fru (*2*), different xylosyloligofructosides (*3*), and levan (*4*). After 546 min, D-fructose appeared above spot 2. The donor substrate D-Xyl-Fru diminished and was completely consumed until 1,912 min. **d** Samples from D-Man-Fru incubated with levansucrase and D-mannose showed D-mannose (*1*), D-Man-Fru (*2*), and mannosyloligofructoside (*3*), the major product of the reaction. Levan was not detectable (*4*). The D-fructose release was not visible because it was located at the same place as the D-mannose. The reaction is slow because the major amount of the D-Man-Fru substrate is still not consumed after 3 days. **e** Incubation of D-Gal-Fru with levansucrase and D-galactose: D-galactose (*1*) and D-Gal-Fru (*2*). After 25 min, D-fructose appeared above D-galactose, and the analogue was consumed. One major galactooligofructoside (*3*) was formed. Later, a second product (*3*) appeared close below the main product spot. Also, levan was formed (*4*). **f** Incubation of D-Fuc-Fru with levansucrase and D-fucose. D-Fucose (*1*), D-Fuc-Fru (*2*). After 546 min, D-fructose appeared above this spot. The analogue was consumed and different fucosyloligofructosides (*3*) formed. The yield of fucosyloligofructosides was comparable to that of levan (*4*)

vity of 197.4 U mg^{-1} that is comparable to the 138.0 U mg^{-1} obtained for the *E. coli* produced from LevΔ773MycHis (van Hijum et al. 2004). In this study, we found a 2.8-fold purification with a yield of 42.7% (Fig. 3e). Hence, for the His$_6$-tagged levansucrase, the batch application of Ni-NTA sepharose is the preferred method of purification.

Production of oligosaccharides using purified levansucrase

The purified *L. reuteri* levansucrase enabled the study of the production of new oligosaccharides from sucrose analogues. Recently, we developed a route to sucrose analogues, such as α-D-galactopyranosyl-1,2-β-D-fructofuranoside (D-Gal-Fru), α-D-xylopyranosyl-1,2-β-D-fructofuranoside (D-Xyl-Fru), α-D-mannopyranosyl-1,2-β-D-fructofuranoside (D-Man-Fru), and α-D-fucopyranosyl-1,2-β-D-fructofuranoside (D-Fuc-Fru) (Baciu et al. 2005; Seibel et al. 2006a; Fig. 4b). The sucrose analogues carry a high-binding energy in the glycosidic bond similar to that of sucrose that is utilized to drive the synthesis of novel oligo- and polysaccharides via fructofuranosyltransfer by fructosyltransferases (Fig. 4a). In a proof of concept, sucrose analogues D-Gal-Fru, D-Xyl-Fru, D-Man-Fru, and D-Fuc-Fru (Fig. 4b) have been successfully tested as alternative substrates for levansucrase from *B. subtilis* and *B. megaterium* (Seibel et al. 2006b). The glycoside residues of these sucrose analogues were varied systematically with respect to the positions (2, 4, and 6) of the hydroxyl group. A change from equatorial to axial allowed a detailed structural study of donor substrate specificity of *L. reuteri* levansucrase. Interestingly, the recombinant- and affinity-purified levansucrase accepted all of the tested sucrose analogues as substrates. Transfructosy-

lation of these analogues resulted in the formation of new fructooligosaccharides (Fig. 4c to f). Different migration patterns indicate the formation of specific oligofructoglycosides for each sucrose analogue. Although with D-Man-Fru (Fig. 4d) as substrate, only one mannosyloligofructoside was formed; fructooligosaccharides and levan were formed using each of the other sucrose analogues (D-Gal-Fru, D-Xyl-Fru, and D-Fuc-Fru; Fig. 4c,e,f). Besides the major product, levan, the enzyme produced two galactosyloligofructosides with D-Gal-Fru (Fig. 4e), two fucosyloligofructosides with D-Fuc-Fru (Fig. 4f), and at least three different xylosyloligofructosides with D-Xyl-Fru (Fig. 4c). The ESI-MS analysis result ([D-Xyl-Fru-Fru+Na]$^+$ at m/z 497) obtained from one isolated xylosyloligofructoside is in agreement with the structural assignments proposed from high performance anion-exchange chromatography and TLC analysis. Thus, it has been demonstrated that the levansucrase accepts a wide variety of novel alternative substrates, yielding new oligosaccharides and polysaccharides. Further work on the isolation and characterization of these new oligosaccharides is going on.

Discussion

In this study, the *B. megaterium* vector-based protein production, secretion, and purification system was systematically optimized for the synthesis of recombinant levansucrase LevΔ773 from *L. reuteri*. One crucial point of protein export is the nature of the employed signal peptide. In *B. subtilis*, many different signal peptides were tested for the secretion of heterologous proteins (Tjalsma et al. 2000). Significant differences in the secretion efficiency were observed for the various, investigated signal peptides, but were not clearly related to their structures. Besides their length, the here used signal peptides of the *B. megaterium* esterase LipA and penicillin amidase Pac differ in their signal peptidase recognition sites. The SP$_{lipA}$ carries an AGA site whereas the corresponding amino acid sequence of SP$_{pac}$ consists of the amino acid residues of VFA (Panbangred et al. 2000; Ruiz et al. 2002). The presence of the small amino acid residues, alanine and glycine, in SP$_{lipA}$ represents a better adaptation to the published consensus recognition sequence AXA (Tjalsma et al. 2000). This might be responsible for an enhanced cleavage by the signal peptidase I and contributed to the improved secretion of the levansucrase. The importance of the leader peptide cleavage process for secretion was further demonstrated with the signal peptidase *sipM* coexpression experiments where, again, an increased export of levansucrase was observed. The further optimization of the recombinant secretion signal seems to provide a suitable target for further optimization strategies (Ravn et al. 2000).

A second systematically investigated crucial point of protein production using *B. megaterium* was the affinity chromatographic purification of the tagged levansucrase from the growth medium. Besides the commonly used column chromatography on Ni-NTA agarose for the purification of recombinant His-tagged proteins, Ji et al. (1996) investigated the use of Ni-NTA super paramagnetic beads. Both methods led to comparable amounts of purified His_6-tagged levansucrase. For future application, magnetic beads provide an easy method to optimize purification schemes using microtiter plates during high through-put screening. For common large-scale protein purifications, the column chromatography seems more economically due to the recycling of the used agarose. When comparing the purification of His_6- and Strep-tagged levansucrase from the cell-free growth medium, it became obvious that His_6-tagged levansucrase did not require concentration prior chromatographic purification. One possible explanation for this observation is the significant differences in the affinity parameters of the tested tags towards their immobilized binding partners. The dissociation constants (K_D) of the His_6-tag to nickel and of the nickel to nitrilotriacetic acid are in the range of 10^{-11} and 10^{-9} M, respectively (Dawson et al. 1989). For the Strep-tag II ligand and its binding partner StrepTactin K_D values close to 10^{-6} M (Voss and Skerra 1997) were measured, allowing efficient and quantitative binding only in a more concentrated protein solution.

In general, the gentle purification method for recombinant proteins directly from the cell-free growth medium provides an alternative to *E. coli* production and purification systems, where cell disruption, several centrifugation steps, and often more than one purification step are necessary. The outlined differences between *B. megaterium* and *E. coli* were also obvious for the intracellular production and subsequent purification of *L. reuteri* levansucrase. Solubility and protein stability problems were observed for *E. coli* produced levansucrase. In contrast to that, the one-step purification of the levansucrase from the growth medium of *B. megaterium* yielded comparable amounts of pure and active enzyme.

References

Baciu IE, Joerdening HJ, Seibel J, Buchholz K (2005) Investigations of the transfructosylation reaction by fructosyltrans-ferase from *Bacillus subtilis* NCIMB 11871 for the synthesis of the sucrose analogue galactosyl-fructoside. J Biotechnol 116:347–357

Dawson RMC, Elliott DC, Elliott WH, Jones KM (1989) Data for biochemical research, 3rd edn. Clarendon, Oxford

England S, Seifter S (1990) Precipitation techniques. In: Deutscher MP (ed) Methods in enzymology 182. Academic, San Diego, pp 285–301

Ferretti JJ, Gilpin ML, Russell RR (1987) Nucleotide sequence of a glucosyltransferase gene from *Streptococcus sobrinus* MFe28. J Bacteriol 169:4271–4278

Malten M, Hollmann R, Deckwer WD, Jahn D (2005a) Production and secretion of recombinant *Leuconostoc mesenteroides* dextransucrase DsrS in *Bacillus megaterium*. Biotechnol Bioeng 89:206–218

Malten M, Nahrstedt H, Meinhardt F, Jahn D (2005b) Coexpression of the type I signal peptidase gene *sipM* increases recombinant protein production and export in *Bacillus megaterium* MS941. Biotechnol Bioeng 91:616–621

Malten M, Biedendieck R, Gamer M, Drews A-C, Stammen S, Buchholz K, Dijkhuizen L, Jahn D (2006) A *Bacillus megaterium* plasmid system for the production, export, and one-step purification of affinity-tagged heterologous levansucrase from growth medium. Appl Environ Microbiol 72: 1677–1679

Panbangred W, Weeradechapon K, Udomvaraphant S, Fujiyama K, Meevootisom V (2000) High expression of the penicillin G acylase gene (*pac*) from *Bacillus megaterium* UN1 in its own *pac* minus mutant. J Appl Microbiol 89:152–157

Raux E, Lanois A, Warren MJ, Rambach A, Thermes C (1998) Cobalamin (vitamin B12) biosynthesis: identification and characterization of a *Bacillus megaterium cobI* operon. Biochem J 335:159–166

Ravn P, Arnau J, Madsen SM, Vrang A, Israelsen H (2000) The development of TnNuc and its use for the isolation of novel secretion signals in *Lactococcus lactis*. Gene 242: 347–356

Ruiz C, Blanco A, Pastor FI, Diaz P (2002) Analysis of *Bacillus megaterium* lipolytic system and cloning of LipA, a novel subfamily I.4 bacterial lipase. FEMS Microbiol Lett 217: 263–267

Salminen S, Isolauri E, Salminen E (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie van Leeuwenhoek 70:347–358

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor

Seibel J, Beine R, Moraru R, Behringer C, Buchholz K (2006a) A new pathway for synthesis of oligosaccharides by the use of non-Leloir glycosyltransferases. Biocatal Biotransform 24: 157–165

Seibel J, Moraru R, Gotze S, Buchholz K, Na'amnieh S, Pawlowski A, Hecht HJ (2006b) Synthesis of sucrose analogues and the mechanism of action of *Bacillus subtilis* fructosyltransferase (levansucrase). Carbohydr Res 341:2335–2349

Sumer JB, Howell SF (1935) A method of determination of invertase activity. J Biol Chem 108:51–54

Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM (2000) Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol Mol Biol Rev 64:515–547

van Hijum SA, Bonting K, van der Maarel MJ, Dijkhuizen L (2001) Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiol Lett 205:323–328

van Hijum SA, Szalowska E, van der Maarel MJ, Dijkhuizen L (2004) Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. Microbiology 150:621–630

Vary PS (1992) Development of genetic engineering in *Bacillus megaterium*. In: Doi R, McGloughlin M (eds) Biology of bacilli: application to industry Butterworths–Heinemann, Boston, pp 251–310

Voss S, Skerra A (1997) Mutagenesis of a flexible loop in streptavidin leads to higher affinity for the Strep-tag II peptide and improved performance in recombinant protein purification. Protein Eng 10:975–982

Wittchen KD, Meinhardt F (1995) Inactivation of the major extracellular protease from *Bacillus megaterium* DSM319 by gene replacement. Appl Microbiol Biotechnol 42:871–877