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Gene cloning, functional expression and secretion of the S-layer protein SgsE from *Geobacillus stearothermophilus* NRS 2004/3a in *Lactococcus lactis*

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Abstract

The ~93-kDa surface layer protein SgsE of *Geobacillus stearothermophilus* NRS 2004/3a forms a regular crystalline array providing a nanopatterned matrix for the future display of biologically relevant molecules. *Lactococcus lactis* NZ9000 was established as a safe expression host for the controlled targeted production of SgsE based on the broad host-range plasmid pNZ124*Sph*, into which the *nisA* promoter was introduced. *SgsE* devoid of its signal peptide-encoding sequence was cloned into the new vector and purified from the cytoplasm at a yield of 220 mg l^{-} of expression culture. Secretion constructs were based on the signal peptide of the *Lactobacillus brevis* SlpA protein or the *L. lactis* Usp45 protein, allowing isolation of 95 mg of secreted rSgsE l^{-1}. N-terminal sequencing confirmed correct processing of SgsE in *L. lactis* NZ9000. The ability of rSgsE to self-assemble in suspension and to recrystallize on solid supports was demonstrated by electron and atomic force microscopy.

Keywords: S-layer protein; *Geobacillus stearothermophilus*; Functional expression; Secretion; *Lactococcus lactis*

1. Introduction

The occurrence of two-dimensional crystalline surface layers (S-layer) as outmost bacterial cell envelope layer is well established [1]. The wealth of information accumulated on the general principles of S-layers has led to their exploitation for a wide range of applications in the fields of nanobiotechnology and biomedicine. Most envisaged applications are based on the unique feature of S-layer monomers to self-assemble in suspension and to recrystallize on solid supports (e.g., gold chips, silicon wafers, plastic materials), on liposomes, on Langmuir lipid films, and at the air–water interface, as well as on their periodic properties down to the subnanometer scale [2]. Whereas one direction of research utilizes the functional groups present on S-layer lattices at well-defined positions and orientations for the controlled chemical coupling of macromolecules (e.g., enzymes, ligands, antibodies) [3,4], a recent trend of S-layer research is directed towards the production of chimeric S-layer proteins. There, the native S-layer properties are tuned for specific applications by the incorporation of functional or structural domains by genetic engineering techniques. Such constructs may pro-

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vide self-assembling nanopatterned molecular matrices, and model vaccines, affinity microparticles or bioadsorbens may be developed on their basis.

In most cases, chimeric S-layer protein production is performed by using *Escherichia coli* [5–9] or *Caulobacter crescentus* [10], where the S-layer secretion system has been adapted for heterologous protein secretion, carrying an expression vector. However, Gram-negative bacteria have some drawbacks, including (i) the contamination of the produced recombinant proteins with endotoxin and, in the case of *E. coli*, (ii) the accumulation of most recombinant proteins in the cytosol and (iii) the possible deposition of recombinant proteins as insoluble inclusion bodies. Thus, the establishment of an endotoxin-free, high-yield expression system for nanobiotechnologically interesting self-assembling proteins, with the option for release into the growth medium as is preferred for some applications, is a highly emphasized aim in S-layer research. In the present study, *Lactococcus lactis* was exploited as a host for the intra- and extracellular production of the S-layer protein SgsE of *G. stearothermophilus* NRS 2004/3a for the following reasons [11]: (i) *L. lactis* is a Gram-positive, food-grade bacterium that is generally regarded as safe (GRAS); (ii) *L. lactis* has been extensively engineered for the production and export of heterologous proteins; (iii) *L. lactis* allows the stable production of heterologous proteins in the growth medium. SgsE is generated from a 903-aa preprotein by cleavage of a 30-aa signal peptide, and assembles into an oblique lattice [12]. Mature SgsE possesses two glycan chains attached at defined positions [13], opening up the possibility to construct next-generation (chimeric) S-layer proteins with surface-displayed glycosylation at the native, permissive sites with the glycans functioning, for instance, as recognition elements or targeting signals [14].

Lactococcus lactis strain NZ9000 was chosen for nisin-controlled [15] S-layer protein overexpression in combination with a new vector based on the broad host-range plasmid pNZ124. For secretion of SgsE, the efficiency of two signal peptides derived from the *L. brevis* S-layer protein SlpA and from the major secretory protein Usp45of *L. lactis* was compared. Using in vitro self-assembly and recrystallization studies of purified rSgsE in combination with Western-blot and N-terminal sequencing we demonstrate that intracellular and secreted SgsE is correctly processed and maintains its functionality in the heterologous host.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria–

Table 1
Bacterial strains and plasmids used in this study

Strains	Genotype	Reference or source
Escherichia coli DH5α	K-12 ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*) U169 F	Invitrogen
Escherichia coli BL21 (λDE3)	F^- *ompT* $HsdS_B$ ($r_B^- m_B^-$) gal dcm (DE3)	Novagen
Geobacillus stearothermophilus NRS 2004/3a	S-layer glycoprotein-covered neotype strain	[13]
Lactococcus lactis MG1363	Plasmid-free and prophage-cured derivative of NCDO712, Lac^- Prt^-	[39]
Lactococcus lactis NZ9000	*Lactococcus lactis* MG1363 pepN::nisRK	[40]
Lactococcus lactis DSM20729	Lac^+ (phospho-β-galactosidase$^+$, β-galactosidase$^-$) Suc^+ Nip^+ Nis^r Erm^s	DSMZ
Lactobacillus brevis DSM20556	Neotype strain	DSMZ
Plasmids	**Description**	**Reference or source**
pET-28a(+)	*E.coli* expression vector; Km^r	Novagen
pNGB100	pET-28a(+) : *sgsE*(-SP); pET-28a(+) containing *sgsE* from *G. stearothermophilus* NRS 2004/3a, devoid of its signal peptide; Km^r	This study
pNZ124*Sph*	Derivative of the broad host-range plasmid pNZ124, containing an additional *Sph*I restriction site; Cm^r	[26], Nestlé
pNGB101	Expression vector for *Lactococcus lactis*; pNZ124*Sph* containing the *nis*A promoter and the ATG start codon as part of the *Sph*I site; Cm^r	This study
pNGB102	pNGB101 containing *sgsE* from *G. stearothermophilus* NRS 2004/3a, devoid of the SP; Cm^r	This study
pNGB104	pNGB102 containing the SP sequence of the *Lactobacillus brevis* DSM20566 S-layer protein SlpA, translationally fused to SgsE; Cm^r	This study
pNGB106	pNGB102 containing the SP sequence of the *Lactococcus lactis* MG1363 Usp45 protein, translationally fused to SgsE; Cm^r	This study

Bertani broth at 37 °C and *G. stearothermophilus* NRS 2004/3a in modified SVIII-medium [13] at 57 °C, all with shaking. *Lactococcus lactis* strains MG1363, NZ9000 and DSM20729 were grown in M17 medium (Difco, Becton Dickinson, Vienna, Austria) supplemented with 0.5% glucose and 0.5% lactose at 30 °C. *Lactobacillus brevis* was grown in MRS medium (Oxoid, Basingstoke, Hampshire, UK) at 30 °C in a GasPak™ system (Oxoid). Media were supplemented with kanamycin (30 $\mu g\,ml^{-1}$) and chloramphenicol (5 $\mu g\,ml^{-1}$), when appropriate.

2.2. DNA manipulations and PCR experiments

Chromosomal DNA was isolated using Genomic Tips 100/G (Qiagen, Hilden, Germany), including pretreatment of *L. lactis* and *L. brevis* cells with lysozyme. Restriction enzymes were purchased from Invitrogen (Lofer, Austria) and digests were purified using the MinElute Reaction Cleanup Kit (Qiagen). Agarose gel electrophoresis was performed as described by Sambrook et al. [16]. DNA fragments were purified from agarose gels using the MinElute Gel Extraction Kit (Qiagen). Polymerase chain reaction (PCR) (PCR Sprint thermocycler, Hybaid, Ashford, UK) was performed using Pwo polymerase (Roche, Vienna, Austria), conditions were optimized for each primer pair and PCR products were purified using the MinElute PCR Purification Kit (Qiagen). *E. coli* and *L. lactis* strains were transformed by electroporation (Gene-PulserII apparatus, Bio-Rad, Vienna, Austria) [17,18]. Transformants were screened by in situ-PCR reactions; positive clones were analyzed by restriction mapping and confirmed by sequencing (Agowa, Berlin, Germany).

2.3. Analytical and general methods

Protein was determined by the method of Bradford [19]. Monosaccharide and amino acid analyses are described elsewhere [20]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [21] and staining of protein with Coomassie Blue R-250 was described previously [22]. The endotoxin content of S-layer protein was determined by the *Limulus* amoebocyte lysate-assay [23]. N-terminal sequencing of S-layer protein was done after semidry blotting onto a PVDF membrane (Immobilo-P™, Millipore, Badford, MA) [24]. Electron microscopic methods were outlined elsewhere [25].

2.4. Construction of an inducible expression vector *for* L. lactis

A 200-bp fragment containing the *nisA* promoter region [15] including the ribosome binding site (RBS) and the translational start codon at a *Sph*I restriction site was amplified by PCR from chromosomal DNA of *L. lactis* DSM20729 using the primer pair NisA_for (5′-AATCAAGATCTGTCTTATAACTATACTGACAATAGAAACATTAAC-3′; *Bgl*II)/NisA_rev (5′-AATCAGCATGCTGAGTGCCTCCTTATAA-3′; *Sph*I), introducing *Bgl*II and *Sph*I restriction sites (underlined). The purified amplification product was digested with the respective endonucleases and cloned into pNZ124 [26], yielding vector pNGB101.

2.5. Cloning and expression of *sgsE in* L. lactis

The *sgsE* gene devoid of its signal peptide (SP) encoding sequence (GenBank Accession No. AF328862) was amplified from chromosomal DNA of *G. stearothermophilus* NRS 2004/3a. The primers were sgsE_for (5′-AATCAgcATGcCGGACGTGGCGACGGT-3′; *Sph*I) and sgsE_rev (5′-AATCACTCGAGACAAGAAAGCATCTTGCTTGTG-3′; *Xho*I), introducing artificial restriction sites at the 5′- and 3′-end of the amplified DNA fragments (underlined; lowercase letters indicate nucleotides changed to construct appropriate restriction sites). The purified PCR product was digested with *Sph*I and *Xho*I and ligated into *Sph*I/*Xho*I-digested vector pNGB101, yielding pNGB102, which was propagated in *E. coli* DH5α and transformed into *L. lactis* NZ9000. For SgsE overexpression, an overnight culture of *L. lactis* NZ9000 (pNGB102) was diluted 1:20 into 400 ml of fresh growth medium and incubated at 30 °C to an OD_{600} of 0.6. Induction was carried out with 25 $ng\,ml^{-1}$ of nisin for 3 h. The intracellular organization of SgsE in *L. lactis* NZ9000 (pNGB102) cells, harvested in 1-h intervals after induction, was examined by electron microscopy.

2.6. Cloning and expression of *sgsE in* E. coli

For comparative reasons, *sgsE* was cloned into pET-28a(+) vector (Novagen, Vienna, Austria), yielding pNGB100, and expressed in *E. coli* BL21(λDE3) using standard conditions. A 2667-bp DNA fragment containing *sgsE*(-SP) was amplified from chromosomal DNA of *G. stearothermophilus* NRS 2004/3a with the oligo- nucleotides sgsE_for (5′-TTCCCccatgGCAACGGAC-GTGG-3′; *Nco*I) and sgsE_rev (5′-ATGGGAaGcTTGCTACATTGCTATAC-3′; *Hin*d III). Cells from a 3-h *E. coli* BL21(λDE3) (pNGB100) culture were harvested and samples were taken for ultrathin-sectioning.

2.7. Investigation of the self-assembly properties of rSgsE

The isolation of rSgsE from *L. lactis* NZ9000 (pNGB102) and *E. coli* BL21(λDE3) (pNGB100), using ~5.5 g of cells (wet weight) essentially followed a published protocol [27], except that EDTA and glycerol

were omitted during treatment with lysozyme. To allow formation of recombinant SgsE (rSgsE) self-assembly products, the final supernatant was dialyzed (molecular mass cut-off of tubing, 20 kDa) at 4 °C, twice against 10 mM $CaCl_2$ and four times against distilled water, 1 l each. rSgsE was lyophilized and verified by N-terminal sequencing.

2.8. Recrystallization of rSgsE

2.8.1. Reattachment of rSgsE on peptidoglycan of G.stearothermophilus NRS 2004/3a

Peptidoglycan preparation of *G. stearothermophilus* NRS 2004/3a was carried out as published previously [28]. For reattachment experiments, 1 mg of lyophilized rSgsE isolated from *L. lactis* NZ9000 (pNBG102) and *E. coli* BL21 (λDE3) (pNBG100), respectively, was resuspended in 4 ml of 5 M GHCl, 50 mM Tris/HCl, pH 7.2, with 1, 2, and 5 mg of peptidoglycan, stirred for 30 min (25 °C), and dialyzed for self-assembly formation as described above. Peptidoglycan of *L. lactis* NZ9000 served as a negative control. Recrystallization of rSgsE was investigated by electron microscopy.

2.8.2. Recrystallization of rSgsE on silicon surfaces

rSgsE monomers were obtained after treatment of lyophilized rSgsE (2 mg ml^{-1}) with 3.2 M GHCl, 50 mM Tris/HCl, pH 7.4 (30 min, 25 °C), centrifugation, and dialysis of the supernatant against 2 mM EDTA. SgsE was recrystallized at a concentration of ~0.6 mg ml^{-1} from 5 mM Tris/HCl, pH 10.5, 10 mM $CaCl_2$, and incubated with a silicon wafer (MEMC, Italy) at 25 °C (2.5 h for hydrophobic silicon, 6 h for oxygen plasma-treated silicon). Pre-treatment of silicon wafers and atomic force microscopy (AFM) were performed according to reference [29].

2.9. Construction of sgsE secretion vectors for L. lactis

The 30-aa SP encoding sequence of the *L. brevis* surface layer protein SlpA (SP_{SlpA}) (GenBank Accession No. Z14250) and the 27-aa SP encoding sequence of the Usp45 protein of *L. lactis* MG1363 (SP_{Usp45}) (GenBank Accession No. M60178) were used as secretion signals for *sgsE*(-SP). SP_{SlpA} was amplified from chromosomal DNA of *L. brevis* DSM20556 with *Sph*I sites added at the 5′- and 3′ ends, using the primers SP_{slpA}_for (5′-AATCAGCATGCAATCAAGTTTAAAGAAATCTC-3′) and SP_{slpA}_rev (5′-AATCAGCATGCACTTAGCTGAAGCAGTCGTTGAAACGGCAGC-3′); SP_{usp45} was amplified from *L. lactis* MG1363 chromosomal DNA with the primer combination SP_{usp45}_for (5′-AATCAGCATGCAAAAAAAGATTATCTCAGCTAT-3′) SP_{usp45}_rev (5′-AATCAGCATGCTGTCAGCGTAAACACCTGAC-3′), introducing *Sph*I restriction sites. The *Sph*I-cut PCR fragments were alternatively ligated into pNGB102, yielding pNGB104 (SP_{SlpA}) and pNGB106 (SP_{Usp45}). The correct orientation as an in-frame fusion to SgsE was verified by PCR with primers pNZ124_forward (5′-GTTTCCCAAAACACCTATACC-3′) and SP_{slpA}_rev or SP_{usp45}_rev.

2.10. Preparation of cellular and supernatant fractions of L. lactis

Cells from 8 ml of *L. lactis* overexpression cultures, harboring pNGB104 or pNGB106, were harvested, and pellet and supernatant were processed separately following the procedure by Le Loir et al. [30], except that cells were broken by sonication instead of treatment with lysozyme. Either fraction was analyzed for its protein content, resuspended in Laemmli sample buffer [21], and equal volumes were loaded on SDS-PA gels for Western-blotting.

To verify secreted rSgsE by N-terminal sequencing, it was purified from the supernatant of a 100-ml expression culture of *L. lactis* NZ9000 (pNGB104). The supernatant was passed through a 0.22-μm filter (Millipore), dialyzed against distilled water (4 °C), concentrated to dryness, and resuspended in 15 ml of 5 M GHCl, 50 mM Tris/HCl, pH 7.2, for S-layer protein extraction and self-assembly formation as described above. Assembled rSgsE was resuspended in Tris–HCl buffer containing 2 M GHCl and purified on a 50-ml FPLC-column (Amersham Biosciences, Vienna, Austria) of Sephacryl S-400 (Amersham Biosciences) run in the same buffer (flow rate 0.25 ml min^{-1}). Fractions (2 ml) were pooled according to their rSgsE content as determined by SDS-PAGE.

2.11. Preparation of polyclonal antibodies and immunoblotting

For raising polyclonal anti-SgsE antibodies, rSgsE was purified by preparative electrophoresis (PrepCell model 491, Bio-Rad) on a 6% PA gel (10 h, 12 W) using running buffer according to Laemmli [21] and 60 mM of Tris/HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, as elution buffer. Rabbit immunization and Western blot analysis was done as previously described [31].

3. Results and discussion

3.1. Vector and cloning strategy considerations for L. lactis

To establish a safe, high-yield protein production system for applied S-layer research, the non-pathogenic and non-invasive, Gram-positive bacterium *L. lactis*

NZ9000 was used in combination with the nisin-controlled expression system [32]. The constructed vector pNGB101 allowed regulated overexpression of genes amplified with an *Sph*I site on the 5′-end from their own ATG translational start, located at an optimal distance from the *nisA* RBS. The S-layer protein SgsE from *G. stearothermophilus* NRS 2004/3a was chosen for expression, because it is regarded prime matrix to construct a next-generation, chimeric, functionalized S-layer proteins [33].

The expression constructs were addressed for export by comparing the signal peptides of the *L. brevis* S-layer protein SlpA [34] and of the *L. lactis* major secreted protein Usp45 [35]. Either SP has been reported to be efficiently recognized by the lactococcal Sec machinery. However, secretion efficiency is known to be dependent on the nature of the expressed protein and, thus, is non-predictable. In this study, the SP-encoding sequences were translationally fused with the expression constructs via the unique *Sph*I site. Primers were designed in such a way that the native protease cleavage site required for signal peptide processing was maintained.

3.2. Production of SgsE *in* L. lactis *and* E. coli

Electron microscopy of cross-sections of *L. lactis* NZ9000 (pNGB102) cells revealed rSgsE assembling in concentric arrangement in the cytoplasm, with an outer diameter of about one tenth of a typical *L. lactis* cell (Fig. 1(a)). Samples taken from the time-course indicated that S-layer protein assembly was initiated already 1 h after addition of nisin to the culture medium, and a maximum package of rSgsE inside the cells was reached after 5 h. This was supported by the SDS-PAGE evidence. As Western blot analysis using polyclonal anti-SgsE antibodies revealed minor amounts of rSgsE degradation products after 5 h, 3-h expression cultures were used for rSgsE production in a preparative scale. The time-course of rSgsE expression in *L. lactis* was compared with that of pET-based expression in *E. coli* BL21 (λDE3), which has been so far the most frequently used system for expressing (chimeric) S-layer proteins from *Bacillaceae*. As evident form SDS-PAGE analysis (not shown), a significant increase of rSgsE production occurred 2 h after induction, remaining constant for the rest of the expression. As demonstrated for rSgsE expression in *L. lactis* NZ9000, densely packed S-layer protein assemblies of about four times the size as compared to *L. lactis* were formed in *E. coli* BL21 (λDE3) (pNGB100) cells (not shown).

3.3. Investigation of the self-assembly capability of rSgsE

To investigate the self-assembly capability of rSgsE produced by *L. lactis* NZ9000 and *E. coli* BL21 (DE3), it was extracted from biomass of expression cultures harboring pNGB102 and pNGB100, respectively. Yields were ~220 mg l^{-1} of rSgsE in the case of *L. lactis* and 240 mg l^{-1} in the case of *E. coli*. N-terminal sequencing revealed the sequence mpDVATVV (the native SgsE sequence is written in upper case) for rSgsE isolated from *L. lactis* NZ9000 (pNGB102), with the first two amino acids changed due to the artificial *Sph*I site introduced by the chosen primer, and mATDVATV for the material produced in *E. coli* BL21 (λDE3) (pNGB100), with the methionine residue originating from the *Nco*I cloning site. This confirmed that cytoplasmic production of

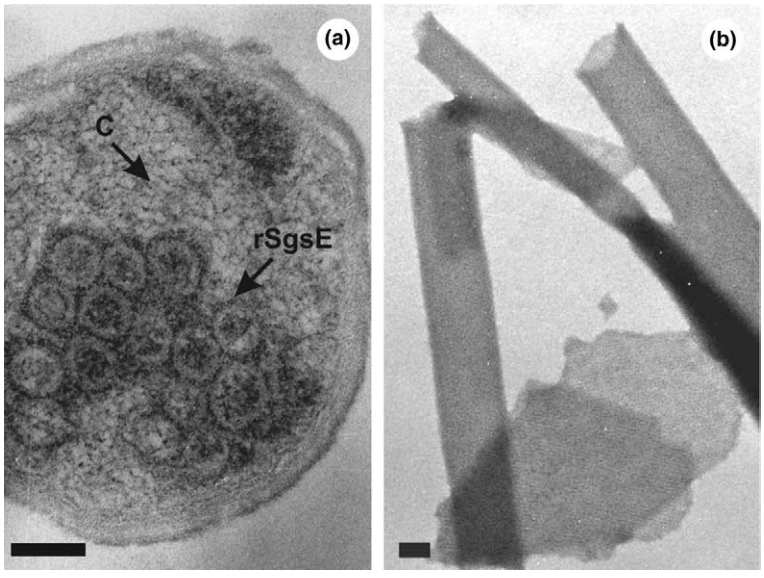

Fig. 1. Electron microscopic characterization of the S-layer protein SgsE from *G. stearothermophilus* NRS 2004/3a overexpressed in *L. lactis* NZ9000. (a) Cross-section through a *L. lactis* cell showing rSgsE assembled in the cytoplasm; (b) Negatively stained rSgsE self-assembly product in the form of cylindrical and sheet-like structures exhibiting oblique lattice symmetry. C, cytoplasm. Bars, 100 nm.

rSgsE is fully compatible with either host. Upon dialysis during the last step of the S-layer protein isolation procedure, rSgsE assembled preferably into open ended mono- or double-layered cylinders and, to a lower extent, into flat sheets (Fig. 1(b)). All self-assembly products exhibited oblique lattice symmetry with lattice parameters identical to those present in native SgsE ($a = 11.6$ nm, $b = 9.4$ nm, $\gamma \sim 78°$) [12]. The forms and sizes of the assemblies were independent of the source of rSgsE, with the cylindrical structures varying between 700 nm–3 μm in length and 90–300 nm in diameter, and the average size of the sheets being 4 μm^2.

3.4. Investigation of the recrystallization properties of rSgsE

3.4.1. Reattachment on peptidoglycan sacculi

rSgsE was capable of recrystallizing into a monolayer on A1γ-type peptidoglycan sacculi isolated from *G. stearothermophilus* NSR2004/3a, when a 1:2 (w/w) mixture of rSgsE and peptidoglycan was used for reattachment studies (Fig. 2(a)). No reattachment was observed using a peptidoglycan preparation of *L. lactis* NZ9000 (A4α-type) as recrystallization support (not shown).

3.4.2. Recrystallization on silicon wafers

As demonstrated by AFM analysis, rSgsE recrystallized into a monolayer on both hydrophobic (Fig. 2(b)) and hydrophilic silicon (not shown). The monolayer consisted of randomly orientated patches, all of which exhibited oblique lattice symmetry. This opens up the opportunity of recrystallizing future rSgsE-based proteins on supports with various surface properties.

3.5. Secretion of SgsE from L. lactis

Based on the SgsE-expression plasmid pNGB102, the S-layer protein was targeted for secretion by in-frame fusion of the SP_{SlpA} or SP_{Usp45} encoding sequence to *sgsE*. Probing the sterile-filtered cell-free supernatant fraction of a *L. lactis* NZ9000 (pNGB104) culture with anti-SgsE antibodies demonstrated secretion of rSgsE. The supernatant revealed one distinct protein band of expected size (molecular mass calculated from the aa-sequence, 93.96 kDa) (Fig. 3, lane 2). A comparable amount of recombinant protein was retained in the cytoplasm migrating slightly slower than the supernatant fraction on the SDS-PA gel (calculated molecular mass, 96.87 kDa) (Fig. 3, lane 1). The supernatant fraction was indistinguishable from the cytoplasmic fraction of the intracellular expression culture *L. lactis* NZ9000 (pNGB102) (calculated molecular mass 93.73 kDa) (Fig. 3, lane 3). The supernatant of that culture revealed no signal with anti-SgsE antibodies (Fig. 3, lane 4), demonstrating that no unspecific release of cytoplasmic rSgsE had occurred. This strongly suggested that the SP_{SlpA} was proteolytically processed, allowing membrane translocation of mature rSgsE. Analysis of the *L. lactis* NZ9000 (pNGB106) expression culture, in

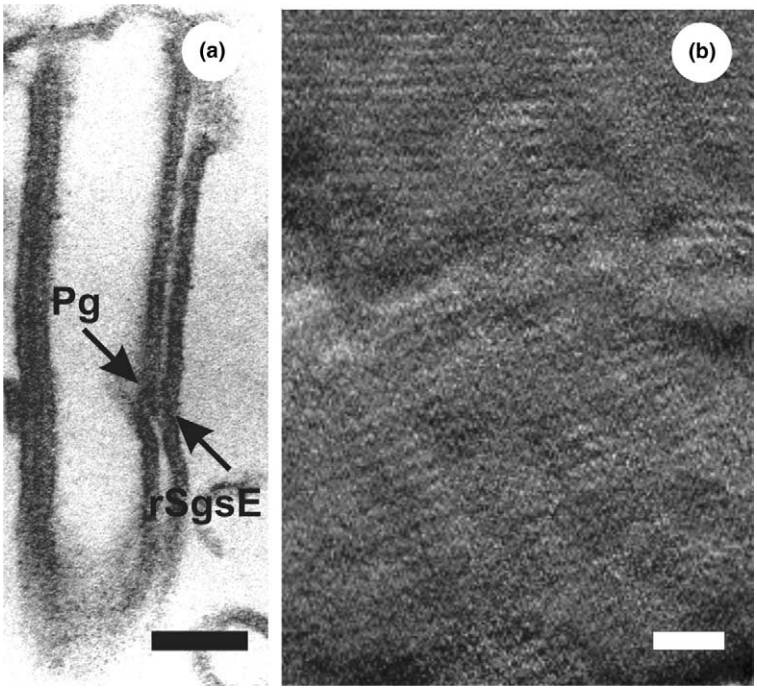

Fig. 2. Analysis of the recrsytallization properties of rSgsE from *L. lactis* NZ9000. (a) Electron micrograph of ultrathin-sectioned peptidoglycan sacculi of *G. stearothermophilus* NRS 2004/3a covered with recrystallized rSgsE. (b) AFM image of rSgsE recrystallized on a hydrophobic silicon surface. Pg, peptidoglycan. Bars, 100 nm.

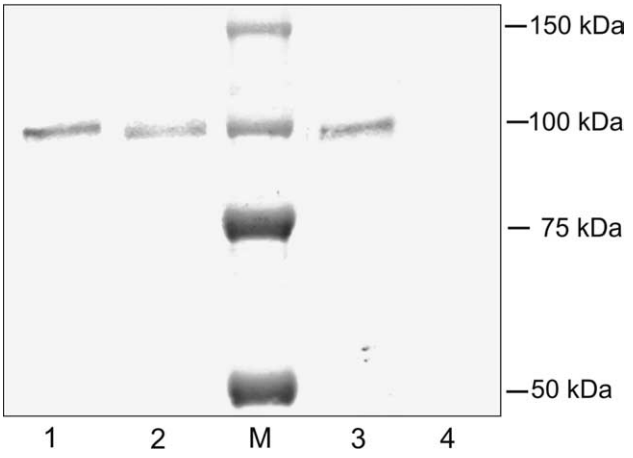

Fig. 3. Detection of recombinant SgsE in cell extracts and the supernatant of induced *L. lactis* NZ9000 cultures by Western-blot using anti-SgsE antibodies. Lanes 1 and 2 correspond to the intracellular and the supernatant fraction of *L. lactis* NZ9000 (pNGB104). Lanes 3 and 4 correspond to the intracellular and the supernatant fraction of *L. lactis* NZ9000 (pNGB102). M, Precision Plus Protein All Blue Standard (Bio-Rad). Positions of molecular masses are indicated on the right.

which SgsE was exported via SP_{Usp45} corroborated the above result (not shown). To confirm the Western blot result, rSgsE was purified from the supernatant of a *L. lactis* NZ9000 (pNGB104) culture in a quantity of ~95 mg l^{-1}, and N-terminally sequenced. The amino acid sequence kcmpDVAT, where K and C correspond to additional amino acids introduced during the cloning procedure, and M and P replace the first two amino acids (AT) of the native SgsE protein, indicated that the A–K cleavage site was correctly recognized by the *L. lactis* processing enzyme.

The established system possesses secretion efficiency for the tested S-layer protein of ~50%. rSgsE could be easily purified from the culture supernatant, avoiding time-consuming cellular lysis and purification steps for protein delivery. No pH-controlled cultivation was necessary to prevent degradation of recombinant protein; the buffer capacity of the culture medium was sufficient to maintain the pH at a constant value of ~7.0. As inferred from the immunoblot, there was no detectable activity of the unique extracellular housekeeping protease HtrA of *L. lactis*. However, for secretion of other proteins, the use of the protease-deficient mutant *L. lactis* NZ9000Δ*htrA* may be advantageous [36,37].

This data makes *L. lactis* a promising host for the secretion of heterologous, high-molecular mass S-layer proteins. Callegari and coworkers [38] have reported the switch from the *E. coli* expression host to *L. lactis* MG1363, because the *Lactobacillus helveticus* S-layer gene *slpH* was unstable in *E. coli*. To our knowledge, this is so far the only report of the expression of an S-layer protein in *L. lactis*. There, constitutive protein expression was accomplished under the strong lactococcal P32 promoter. Due to cloning the entire *slpH* gene, including its RBS and SP sequence, accumulation of rSlpH in the growth medium was accomplished. The observation that small clumps of rSlpH were attached to some sites on the lactococcal cell wall, could not be confirmed with rSgsE by electron microscopy using ultrathin-sectioned *L. lactis* NZ9000 (pNGB104) cells (not shown). This phenomenon, however, may be valid only within the *Lactobacillaceae* family.

3.6. Determination of endotoxicity

rSgsE isolated from *L. lactis* NZ9000 exhibited an endotoxin contamination of 3.2 EU mg^{-1} (dry weight), whereas the value for rSgsE from *E. coli* BL21 (λ DE3) was about two orders of magnitude higher (481.0 EU mg^{-1}). Thus, *L. lactis* is suitable for the production of biocompatible S-layer protein, without additional treatment for endotoxin removal.

In conclusion, this work demonstrates that *L. lactis* is a very potent host for the safe, controllable, high-yield production of a functional S-layer protein, capable of replacing the *E. coli* expression host, especially if GRAS-status and endotoxin-free material is required as is in the field of biomedicine. In addition, *L. lactis* offers the possibility of exporting the heterologous S-layer protein into the medium, implicating a purification step already during protein production.

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