

A recombinant bacterial cell surface (S-layer)-major birch pollen allergen-fusion protein (rSbsC/Bet v1) maintains the ability to self-assemble into regularly structured monomolecular lattices and the functionality of the allergen

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The mature crystalline bacterial cell surface (S-layer) protein SbsC of *Bacillus stearothermophilus* ATCC 12980 comprises amino acids 31–1099 and assembles into an oblique lattice type. As the deletion of up to 179 C-terminal amino acids did not interfere with the self-assembly properties of SbsC, the sequence encoding the major birch pollen allergen (Bet v1) was fused to the sequence encoding the truncated form rSbsC_{31–920}. The S-layer fusion protein, termed rSbsC/Bet v1, maintained the ability to self-assemble into flat sheets and open-ended cylinders. The presence and the functionality of the fused Bet v1 sequence was proved by blot experiments using BIP1, a monoclonal antibody against Bet v1 and Bet v1-specific IgE-containing serum samples from birch pollen allergic patients. The location and accessibility of the allergen moiety on the outer surface of the S-layer lattice were demonstrated by immunogold labeling of the rSbsC/Bet v1 monolayer, which was obtained by oriented recrystallization of the S-layer fusion protein on native cell wall sacculi. Thereby, the specific interactions between the N-terminal part of SbsC and a distinct type of secondary cell wall polymer were exploited. This is the first S-layer fusion protein described that had retained the specific properties of the S-layer protein moiety in addition to those of the fused functional peptide sequence.

Keywords: *Bacillus stearothermophilus*/major birch pollen allergen/recombinant fusion protein/S-layer protein

Introduction

Crystalline bacterial cell surface layers (S-layers) represent the outermost cell envelope component of many bacteria and archaea. S-layers completely cover the cell surface during all stages of bacterial growth and division and they are composed of a single protein or glycoprotein species (Sleytr *et al.*, 1999; Sára and Sleytr, 2000). In bacteria the S-layer subunits are linked to each other and to the underlying cell envelope layer by non-covalent interactions. Depending on the type of S-layer protein, the subunits show molecular masses of 40 000–200 000 Da. Isolated S-layer subunits frequently self-assemble in suspension (S-layer self-assembly products) or recrystallize into monolayers on various types of solid supports such as noble metals, plastics and silicon wafers or on interfaces including the air–water interface, Langmuir films and liposomes (Sleytr *et al.*, 1999).

The cell surface of *Bacillus stearothermophilus* ATCC 12980 is completely covered with an oblique lattice (Egelseer *et al.*, 1996) consisting of the S-layer protein SbsC. The *sbsC* gene encoding this S-layer protein has been cloned and sequenced (Jarosch *et al.*, 2000). The protein precursor includes a 30 amino acid long typical Gram-positive signal peptide and consists in total of 1099 amino acids. To elucidate the structure–function relationship of distinct segments of SbsC, various N- or C-terminal truncations were produced in *Escherichia coli* HMS174(DE3) (Jarosch *et al.*, 2001). After isolation from the host cells, the self-assembly and recrystallization properties of the truncated SbsC forms were investigated. It could be demonstrated that the N-terminal part (amino acids 31–257) recognizes a distinct type of secondary cell wall polymer as the proper anchoring structure in the rigid cell wall layer (Egelseer *et al.*, 1998; Jarosch *et al.*, 2000, 2001). In the C-terminal part, up to 179 amino acids could be deleted without interfering with the self-assembly properties of this S-layer protein. The deletion of 199 or even 219 C-terminal amino acids led to self-assembly products exhibiting either a faint or no regular lattice structure. Thus, rSbsC_{31–920} was the shortest C-terminal truncation still capable of forming self-assembly products with properties comparable to those obtained with the mature S-layer protein rSbsC_{31–1099} (Jarosch *et al.*, 2001).

As S-layer lattices consist of an identical protein or glycoprotein species, they exhibit repetitive surface properties down to the subnanometer scale (Sleytr *et al.*, 1999). In previous studies, the high density of functional groups on S-layer lattices was exploited for the immobilization of enzymes, ligands, immunoglobulins, antigens or haptens (Sára and Sleytr, 1996; Sleytr *et al.*, 1999). Most foreign molecules were linked to the carbodiimide-activated carboxylic groups of the acidic amino acids. However, in a few exceptions, coupling via sulfhydryl groups, which were introduced into the S-layer lattice by chemical modification or immobilization via the hydroxyl groups of the carbohydrate chains in S-layer glycoproteins, turned out to be advantageous (Sleytr *et al.*, 2001). Depending on the type of molecule immobilized, S-layers were either used as a matrix for diagnostic test systems (Breitwieser *et al.*, 1998), as escort particles in affinity cross-flow filtration (Weiner *et al.*, 1994) or as carrier/adjuvants for conjugated vaccines (Jahn-Schmid *et al.*, 1996, 1997). Both S-layer self-assembly products and S-layer-coated liposomes (Küpçü *et al.*, 1995; Mader *et al.*, 1999) can be considered as particulate adjuvants with dimensions comparable to those of bacteria or viruses that the immune system evolved to combat. Particulate adjuvants are naturally targeted for uptake by antigen-presenting cells to facilitate the induction of potent immune responses (Singh and O'Hagan, 1999).

Bet v1 is the major birch pollen allergen and it shares IgE epitopes with all major tree pollen allergens from closely related species such as alder, hazel, hornbeam, beech and European chestnut (Breiteneder *et al.*, 1989). Due to the high

sequence identities among these allergens and the well studied cross-reactions with B-cell epitopes, Bet v1 represents a model allergen. The construction of conjugate vaccines for the treatment of IgE-mediated allergies has been suggested as a possibility to modulate or redirect the T_H2 -like immune responses of atopic patients towards the T_H0/T_H1 -like cellular pattern typical of non-allergic individuals (Wiedermann *et al.*, 1998). To produce a particulate conjugated vaccine, rBet v1 was chemically coupled to S-layer self-assembly products in previous studies (Jahn-Schmid *et al.*, 1996, 1997). Although the use of such conjugates was very promising regarding the immune response, major drawbacks were seen in the application of potentially toxic chemicals during the coupling procedure and in the low resuspendibility of the rBet v1–S-layer conjugates.

In the present study, an S-layer fusion protein comprising the sequence of Bet v1 was produced. For this purpose, the S-layer protein was reduced to its minimum length as required for the formation of the oblique lattice structure (amino acids 31–920) and the deleted part was replaced by a functional protein sequence.

Materials and methods

Growth of B.stearothermophilus ATCC 12980, preparation of cell wall fragments and isolation of peptidoglycan-containing sacculi

B.stearothermophilus ATCC 12980 was grown in batch culture on SVIII medium under conditions described in previous studies (Egelseer *et al.*, 1996, 1998). Preparation of cell wall fragments and purification of native cell wall (peptidoglycan-containing sacculi) were performed as described by Egelseer *et al.* (Egelseer *et al.*, 1998). Peptidoglycan-containing sacculi of *B.stearothermophilus* ATCC 12980 consist of peptidoglycan of the A1 γ -chemotype and a secondary cell wall polymer. The latter is composed of tetrasaccharide repeating units that contain glucose, *N*-acetylglucosamine and 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid in a molar ratio of 1:1:2 (Egelseer *et al.*, 1998; Schäffer *et al.*, 1999).

Other strains, plasmid, culture conditions and DNA manipulations

E.coli TG1 (Stratagene) was used for transformations with the plasmid pET28a. For expression, *E.coli* BL21star(DE3) (Invitrogen) was chosen as a host strain for derivatives of pET28a as described by Studier *et al.* (Studier *et al.*, 1990). *E.coli* was grown on Luria–Bertani medium (Gibco BRL Life Technologies) or on modified M9ZB medium (Studier *et al.*, 1990) at 37°C. For selection of transformants harboring pET28a, kanamycin was added to the medium to a final concentration of 30 μ g/ml. Chromosomal DNA of *B.stearothermophilus* ATCC 12980 was prepared by using Genomic Tips 100 (Qiagen) according to the manufacturer's procedures. Digestion of PCR fragments with restriction endonucleases, separation of DNA fragments by agarose gel electrophoresis, ligation of DNA fragments and transformation procedures were performed as described by Sambrook *et al.* (Sambrook *et al.*, 1989).

Cloning of sbsC, bet v1 and the chimeric sbsC/bet v1 gene

To obtain the desired *sbsC/bet v1* PCR product, the 'overlap extension PCR' (Ho *et al.*, 1989) was performed. This method requires the use of three separate PCR steps and four primers. For PCR amplification of the *sbsC* derivative encoding

rSbsC_{31–920}, total *B.stearothermophilus* ATCC 12980 DNA was used as a template and the oligonucleotide primers, primer 1 (CGGAATTCATGGCAACGGACGTGGCGAC) and primer 2 (GTAATTGAAAACACCCATACCACCAGCA-TAATATAATTTGTGACCAGATTG) were chosen. The forward primer (primer 1) introduced the restriction site *Nco*I at the 5' end of the coding sequence, while the reverse primer (primer 2) carried an overlapping complementary *bet v1* sequence. For PCR amplification of the *bet v1* gene from plasmid pMW175 (Hoffmann-Sommergruber *et al.*, 1997), the oligonucleotide primers primer 3 (ACAAATTATATTATGCT-GGTGGTATGGGTGTTTTCAATTACGAAACTGAG) and primer 4 (GACCGCTCGAGTTAGTTGTAGGCATCGGAG-TG) which introduced the restriction site *Xho*I at the 3' end of the coding sequence were used. Primer 3 carried a complementary *sbsC* overlap at the 5' end. In primers containing overlapping sequences, the sequence encoding two glycine residues (GGTGGT) was inserted as a spacer between SbsC_{31–920} and Bet v1. PCRs were performed as described by Jarosch *et al.* (Jarosch *et al.*, 2000). For amplification of the chimeric *sbsC/bet v1* gene encoding the S-layer fusion protein, the gel-purified PCR products were used as templates together with primer 1 and primer 4. The chimeric gene encoding the S-layer fusion protein was amplified using a PCR touchdown protocol (Don *et al.*, 1991). For cloning, the gel-purified PCR fragments were inserted into the corresponding restriction sites of the plasmid pET28a and transformed into *E.coli* TG1.

Expression of the chimeric sbsC/bet v1 gene and immunoblot analyses of rSbsC/Bet v1

Plasmid stability test and heterologous expression in *E.coli* were performed as described by Jarosch *et al.* (2000). Samples (1 ml) of the *E.coli* BL21star(DE3) cultures were taken 2 h (OD at 600 nm = 3.0) and 5 h (OD at 600 nm = 4.5) after induction of *sbsC/bet v1* gene expression with 1 mM IPTG (GERBU). Preparation of samples and SDS–PAGE were carried out as described by Laemmli (Laemmli, 1970).

Immunoblotting with polyclonal rabbit antiserum raised against the S-layer protein SbsC of *B.stearothermophilus* ATCC 12980 was performed as described by Egelseer *et al.* (Egelseer *et al.*, 1996). The presence of Bet v1 epitopes in the S-layer fusion protein was checked by immunoreactivity with BIP1, a monoclonal mouse anti-Bet v1 antibody (Jarolim *et al.*, 1989). After separation on SDS gels and blotting to a nitrocellulose membrane (Protran; Schleicher and Schüll), blocking with 3% BSA in 0.15 M TBS (Tris-buffered saline) was applied for 18 h at 4°C. The membrane was then incubated with BIP1 (diluted 1:500 in blocking solution) for 2 h and washed four times with wash solution (0.5% Tween 20 in 0.15 M TBS) and once with 0.15 M TBS. Subsequently, the membrane was incubated with an anti-mouse IgG alkaline phosphatase conjugate (Sigma; diluted 1:5000 in blocking solution) for 45 min. After five washing steps, bound antibody was detected by using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (= BCIP/NBT; Roche) as precipitating chromogenic substrate.

Isolation of rSbsC/Bet v1 from the host cells and purification

Cultures of *E.coli* BL21star(DE3) cells (250 ml; OD at 600 nm = 4.5) induced to express the *sbsC/bet v1* gene were centrifuged at 6000 g for 15 min at 4°C. Subsequently, 2.5 g biomass pellets were resuspended in 100 ml of 0.1 M CaCl₂ in distilled water. After incubation on ice for 30 min, the suspension was centrifuged at 6000 g for 5 min at 4°C. The

pellet was then resuspended in 100 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glycerol, 50 mM EDTA, 75 mM NaCl and 20 mg of lysozyme. The mixture was heated in a water-bath kept at 72°C until the suspension had reached 42°C. This was followed by an incubation step at 30°C for 20 min. Subsequently, 10 ml of 1% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.2) were added and the suspension was incubated for 10 min at 20°C. After sonification (Branson Sonifier, 5×15 pulses) in an ice-bath, the soluble and insoluble fractions of the cell pellet were separated by centrifugation at 30 000 g for 20 min at 4°C. The insoluble pellet was washed once with a solution of 1% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.2) and twice with 50 mM Tris-HCl buffer (pH 7.2). The pellet then was resuspended in 4 ml of 5 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) and the suspension was stirred for 20 min at 20°C. For removal of peptidoglycan and membrane fragments, the suspension was centrifuged at 36 000 g for 30 min at 4°C. The supernatant containing rSbsC/Bet v1 was diluted 1:2.5 with 50 mM Tris-HCl buffer (pH 7.2) and filtered through a 0.45 µm RC-membrane (Minisart RC 25). The clear filtrate was finally subjected to gel permeation chromatography (GPC) using a Superdex 200 column (Pharmacia, Uppsala, Sweden). For elution, 2 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) was used. Fractions containing rSbsC/Bet v1 were pooled, dialyzed against distilled water for 18 h at 4°C, lyophilized and stored at -20°C. The purity of rSbsC/Bet v1 was examined by SDS-PAGE and immunoblotting under the conditions described above. For obtaining self-assembly products, 2 mg of purified lyophilized rSbsC/Bet v1 were solubilized in 1 ml of 5 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) and dialyzed against distilled water for 18 h at 4°C. The formation of self-assembly products was checked by negative staining (Messner *et al.*, 1984). Self-assembly products formed by rSbsC₃₁₋₉₂₀ (Jarosch *et al.*, 2001) were used for comparison.

Transmission electron microscopy

Ultrathin sectioning of whole cells and negative staining were performed as described by Messner *et al.* (Messner *et al.*, 1984). Preparations were examined with a Philips CM100 transmission electron microscope, operated at 80 kV and equipped with a 30 µm objective aperture.

Detection of IgE reactivity of rSbsC/Bet v1 by immunoblotting

The IgE reactivity of 11 patients suffering from birch pollen allergy (radioallergosorbent test >3) was tested. Recombinant Bet v1 (1 µg/lane) was separated on 12% SDS gels. Purified rSbsC/Bet v1 fusion protein representing the equivalent amount of Bet v1 per lane and rSbsC₃₁₋₉₂₀ were separated on 7.5% SDS gels. Proteins were blotted on to nitrocellulose membranes which were subsequently incubated with patients' sera or plasma at 4°C for 16 h. After incubation with a ¹²⁵I-labeled anti-human IgE antibody (IBL, Hamburg, Germany), bound IgE was visualized by autoradiography (Jarolim *et al.*, 1989).

Immunogold labeling of self-assembly products and of the monolayer generated on peptidoglycan-containing sacculi by oriented recrystallization of rSbsC/Bet v1

For investigation of the accessibility of the Bet v1 portion at the C-terminus of the S-layer fusion protein, self-assembly products formed by rSbsC/Bet v1 and the monolayer generated by oriented recrystallization of rSbsC/Bet v1 on peptidoglycan-containing sacculi were incubated with the monoclonal

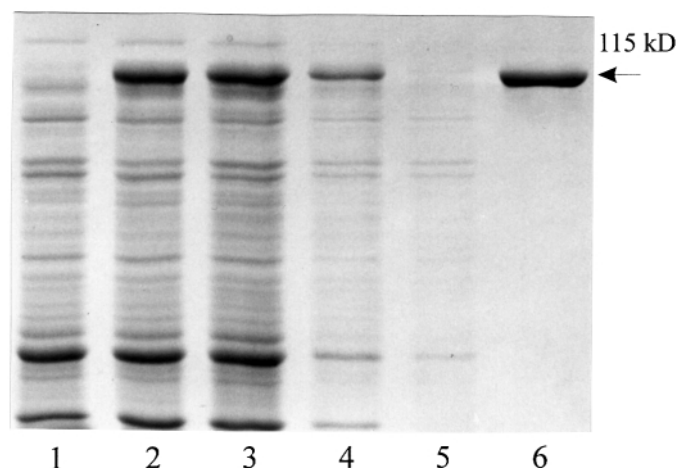


Fig. 1. SDS-PAGE patterns of whole-cell extracts of *E.coli* BL21star(DE3) harboring the pET28a-*sbsC/bet v1* gene construct before (lane 1) and after 2 and 5 h induction of *sbsC/bet v1* expression (lanes 2 and 3). The rSbsC/Bet v1 fusion protein had assembled in the cytoplasm of the host cells and therefore accumulated in the insoluble fraction of the cell lysate (lane 4). No rSbsC/Bet v1 protein could be seen in the soluble fraction (lane 5). After extraction with 5 M GHCl, rSbsC/Bet v1 was purified by GPC and dialyzed against distilled water. On SDS gels only a single protein band could be observed (lane 6). The molecular mass is indicated on the right.

mouse antibody BIP1 (Jarolim *et al.*, 1989). Recrystallization of rSbsC/Bet v1 on peptidoglycan-containing sacculi of *B.stearothermophilus* ATCC 12980 was performed as described previously (Jarosch *et al.*, 2000, 2001). After centrifugation of the suspensions containing either self-assembly products or peptidoglycan-containing sacculi carrying the rSbsC/Bet v1 monolayer at 16 000 g for 20 min at 4°C and five washing steps with PBS (phosphate-buffered saline), the pellets were suspended in 30 µl of anti-mouse IgG-colloidal gold conjugate (Amersham) and incubated for 60 min at 20°C. Subsequently, unbound gold-labeled antibody was removed by centrifugation under the conditions described above and three washing steps with PBS. The pellets were resuspended in 40 µl of distilled water and subjected to negative staining. As a control, the same procedure was carried out with rSbsC₃₁₋₉₂₀.

Results

Cloning and expression of the *sbsC/bet v1* gene

A PCR product, obtained by PCR amplification using primer 1 and primer 4, comprising the chimeric gene *sbsC/bet v1* was ligated into the pET28a vector. The plasmid was first cloned in *E.coli* TG1 and then established in *E.coli* BL21star(DE3) for expression. After inducing expression by the addition of IPTG, biomass samples of *E.coli* BL21star(DE3) were harvested at distinct points in time and subjected to SDS-PAGE and ultrathin sectioning. In comparison with *E.coli* BL21star(DE3) cells harvested before the addition of IPTG (Figure 1, lane 1; final concentration of IPTG 1 mM), an additional high molecular mass protein band was observed on SDS gels in samples from *E.coli* BL21star(DE3) cultures induced to express the *sbsC/bet v1* gene (Figure 1, lanes 2 and 3). This additional protein band had an apparent molecular mass of 115 000 Da. On immunoblots, a strong cross-reaction was observed between the high molecular mass protein band and the polyclonal rabbit antiserum raised against the S-layer protein SbsC of *B.stearothermophilus* ATCC 12980, and also with the monoclonal mouse antibody BIP1 recognizing epitopes

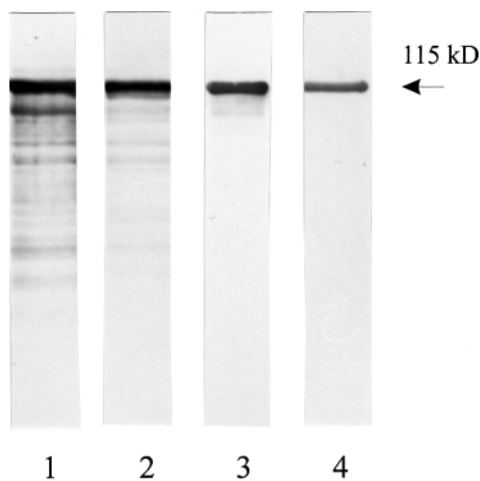


Fig. 2. Immunoblot analysis of SDS extracts of whole *E. coli* BL21star(DE3) cells (lanes 1 and 2), induced to express the *sbsC/bet v1* gene and of purified rSbsC/Bet v1 after GPC and dialysis (lanes 3 and 4). The presence of the SbsC portion was examined with anti-SbsC antiserum and anti-rabbit IgG-alkaline phosphatase conjugate (lanes 1 and 3); the presence of Bet v1 epitopes was examined with BIP1 and anti-mouse IgG-alkaline phosphatase conjugate (lanes 2 and 4).

of Bet v1 (Figure 2, lane 1 and 2). Ultrathin sections of whole cells from *E. coli* BL21star(DE3) induced to express the *sbsC/bet v1* gene revealed the presence of intracellular sheet-like or cylindrical structures (not shown). As derived from previous studies (Jarosch *et al.*, 2001), these sheet-like or cylindrical structures indicated that the recombinant S-layer fusion protein had assembled in the cytoplasm of the host cells.

Isolation and purification of rSbsC/Bet v1 and formation of self-assembly products by purified rSbsC/Bet v1

As derived from SDS-PAGE, the recombinant S-layer fusion protein had accumulated in the insoluble fraction of the lysed *E. coli* BL21star(DE3) cells (Figure 1, lane 4). This was in agreement with data from ultrathin sectioned whole cells, indicating the formation of self-assembly products by rSbsC/Bet v1 in the cytoplasm of the host cells. The insoluble fraction of the lysed *E. coli* BL21star(DE3) cells was extracted with 5 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) and the GHCl extract was subjected to GPC. After unifying the eluted fractions containing rSbsC/Bet v1 and removing GHCl by dialyses against distilled water, only a single major protein band was observed on SDS gels (Figure 1, lane 6). Immunoblotting confirmed the ability of the monomeric rSbsC/Bet v1 fusion protein to bind antibodies against both SbsC and Bet v1 (Figure 2, lanes 3 and 4). The identification of the high molecular mass protein band as the rSbsC/Bet v1 fusion protein was finally accomplished by N-terminal sequencing.

To investigate the self-assembly properties of isolated rSbsC/Bet v1, purified and lyophilized S-layer fusion protein was denatured at a concentration of 2 mg/ml with 2 M GHCl in 50 mM Tris-HCl buffer (pH 7.2), which was subsequently removed by dialysis against distilled water at 4°C for 18 h. As shown by negative staining, rSbsC/Bet v1 reassembled into flat sheets and open-ended cylinders with a dimension of up to 100 nm. On all self-assembly products, the oblique lattice structure was clearly visible (Figure 3B).

Reaction of the Bet v1 moiety in rSbsC/Bet v1 with Bet v1 specific IgE on immunoblots

The functionality of the fused Bet v1 sequence was demonstrated by IgE binding to rSbsC/Bet v1. IgE from all 11 serum

or plasma samples from patients suffering atopic allergy caused by birch pollen recognized rSbsC/Bet v1 and showed a comparable reaction to the positive control, for which rBet v1 was taken. As a negative control, rSbsC₃₁₋₉₂₀ was used which did not exhibit IgE binding (Figure 4).

Immunogold labeling of self-assembly products formed by rSbsC/Bet v1 and of the monolayer generated by oriented recrystallization of rSbsC/Bet v1 on peptidoglycan-containing sacculi

From a previous study (Jarosch *et al.*, 2001), it was known that rSbsC₃₁₋₁₀₉₉ and rSbsC₃₁₋₉₂₀ form monolayer and double-layer self-assembly products. In double-layer self-assembly products, the individual monolayers faced each other with the inner S-layer surface comprising the N-terminal region. To investigate the accessibility of the fused Bet v1 sequence, self-assembly products formed by rSbsC/Bet v1 were labeled with BIP1 and the bound antibody was visualized by an anti-mouse colloidal gold conjugate. As shown in Figure 5A, self-assembly products consisting of rSbsC/Bet v1 were densely labeled with the colloidal gold conjugate, whereas self-assembly products formed by rSbsC₃₁₋₉₂₀ remained completely unlabeled (not shown). To guarantee binding of rSbsC/Bet v1 via the N-terminal region of SbsC and to ensure blocking of the inner S-layer surface, rSbsC/Bet v1 was recrystallized on peptidoglycan-containing sacculi of *B. stearotheophilus* ATCC 12980 and subsequently labeled with BIP1. Because BIP1 could densely bind to the monolayer formed by rSbsC/Bet v1, evidence was provided that the Bet v1 portion was located on the outer surface of the S-layer lattice (Figures 5B and 6). When samples of rSbsC₃₁₋₉₂₀ were used for recrystallization on peptidoglycan-containing sacculi, no gold labeling could be observed in negatively stained preparations (not shown).

Discussion

The elucidation of the structure-function relationship of the S-layer protein SbsC (Jarosch *et al.*, 2000, 2001) allowed the construction of the first S-layer fusion protein, which had retained the specific properties of the S-layer protein moiety, as well as those of the fused functional peptide sequence. In the rSbsC/Bet v1 fusion protein, 179 C-terminal amino acids of SbsC were deleted and replaced by the 161 amino acid long Bet v1 sequence.

As described for rSbsC₃₁₋₉₂₀ (Jarosch *et al.*, 2001), rSbsC/Bet v1 formed sheet-like and cylindrical structures in the cytoplasm of the host cells and therefore accumulated in the insoluble fraction of the cell lysate. The isolated and purified S-layer fusion protein was capable of self-assembling *in vitro* into open-ended cylinders and flat sheets, which clearly exhibited the oblique lattice structure in negatively stained preparations. These findings were in agreement with data from a previous study with rSbsC₃₁₋₉₂₀ showing that the C-terminal deleted 179 amino acids are not required for the formation of the oblique lattice structure (Jarosch *et al.*, 2001). The ability of self-assembly products formed by rSbsC/Bet v1 to bind BIP1 further demonstrated the accessibility of the fused Bet v1 sequence. Since at least in the case of rSbsC₃₁₋₉₂₀ the self-assembly products consisted of monolayers or double layers (Jarosch *et al.*, 2001), binding of the monoclonal antibody did not allow us to draw conclusions whether the fused Bet v1 portion was located on the outer or on the inner S-layer surface. To answer this question, the specific interactions

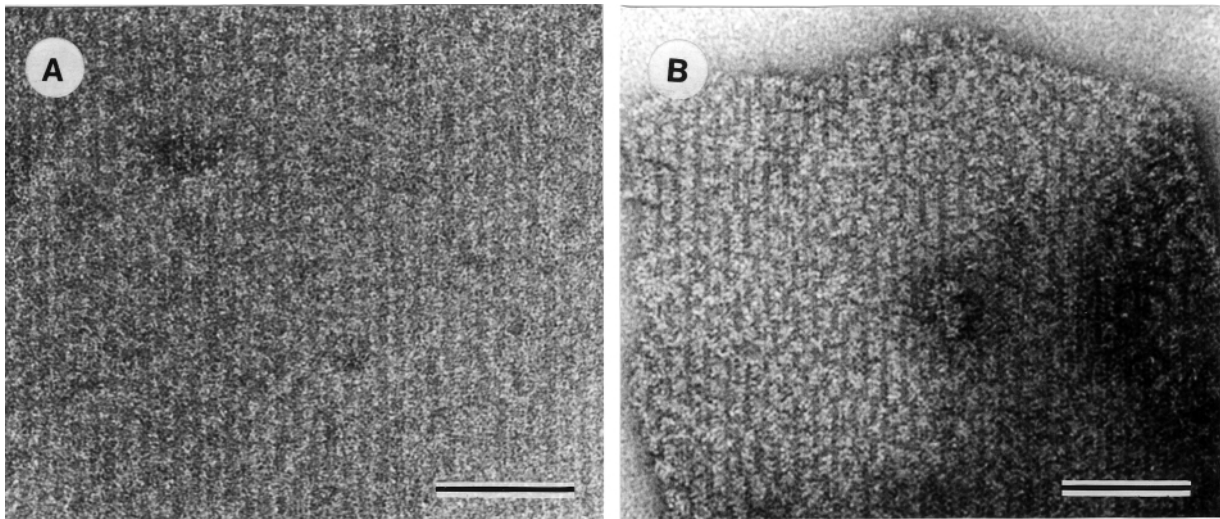


Fig. 3. Electron micrographs of negatively stained preparations of self-assembly products formed by (A) rSbsC₃₁₋₉₂₀ and (B) GPC-purified rSbsC/Bet v1. Bars, 100 nm.

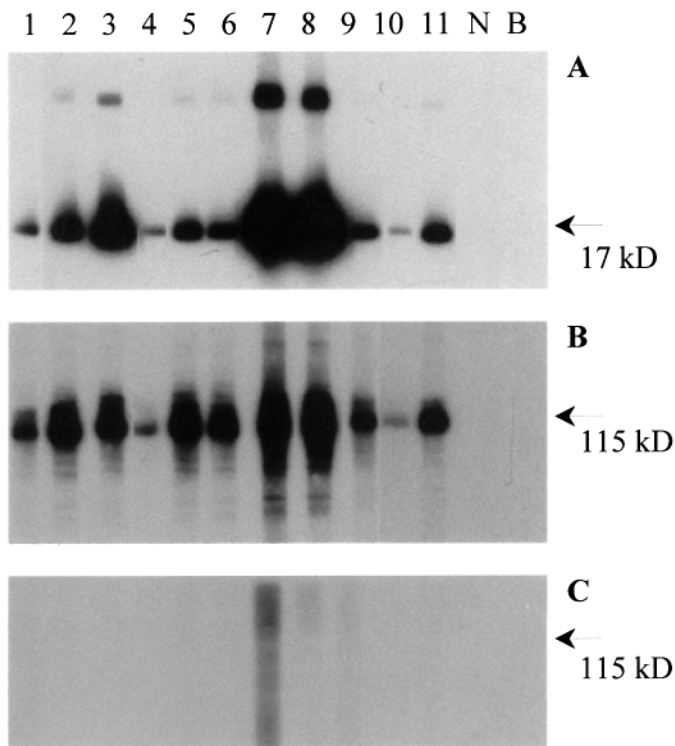


Fig. 4. Results from immunoblots using recombinant Bet v 1 (A), rSbsC/Bet v1 (B), rSbsC₃₁₋₉₂₀ (C) and Bet v1-specific IgE-containing serum and plasma samples from 11 patients. N = negative control serum pool; B = buffer control.

between the N-terminal part of SbsC and the secondary cell wall polymer were exploited to generate an rSbsC/Bet v1 monolayer in defined orientation on peptidoglycan-containing sacculi of *B.stearotherophilus* ATCC 12980 (Figure 6). The ability of BIP1 to bind to the rSbsC/Bet v1 monolayer finally revealed that the Bet v1 portion is located on the outer surface of the crystal lattice.

To demonstrate the IgE reactivity of the rSbsC/Bet v1 fusion protein, serum and plasma samples from birch pollen allergic patients were subjected to immunoblotting experi-

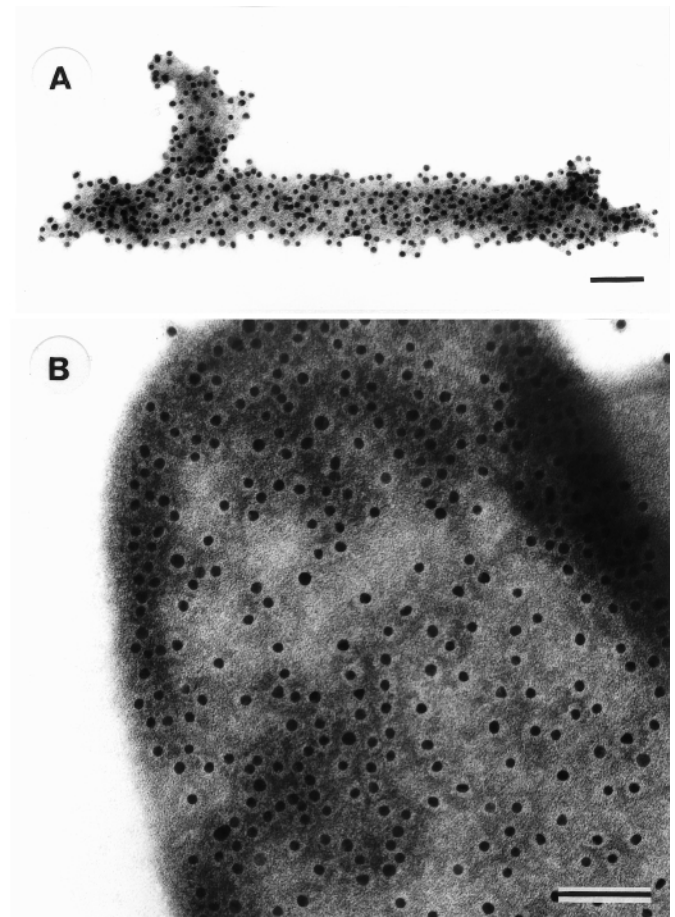


Fig. 5. Immunogold-labeling of (A) self-assembly products formed by rSbsC/Bet v1 and (B) the rSbsC/Bet v1 monolayer generated by oriented binding and recrystallization of the S-layer fusion protein on peptidoglycan-containing sacculi of *B.stearotherophilus* ATCC 12980. Bound BIP1 was detected by using anti-mouse IgG colloidal gold-labeled antibodies. Bars, 100 nm.

ments. Binding of IgE to the S-layer fusion protein confirmed the functionality of the fused Bet v1 moiety.

The possibility of producing S-layer fusion proteins has

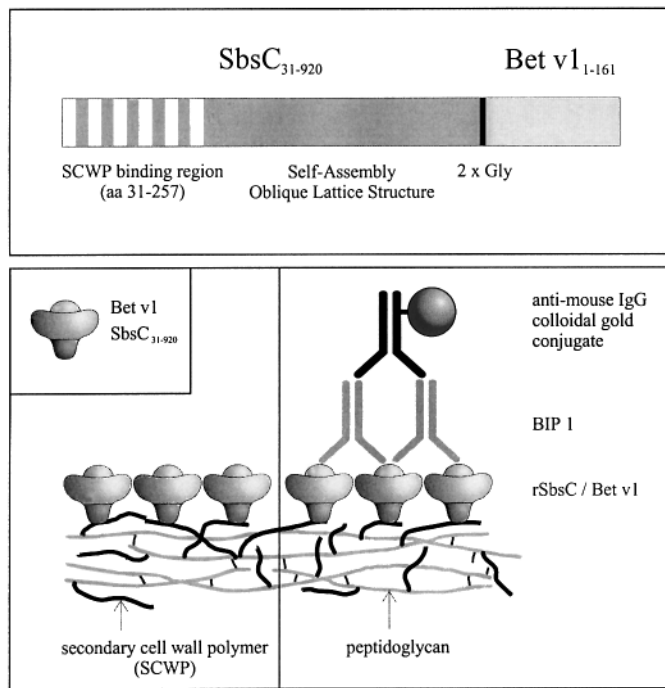


Fig. 6. Schematic drawing demonstrating the oriented binding of rSbsC/Bet v1 on peptidoglycan-containing sacculi of *B. stearotheophilus* ATCC 12980. The N-terminal part of SbsC recognizes the secondary cell wall polymer (SCWP) as the proper anchoring structure in the rigid cell wall layer.

already been described by Truppe *et al.* (Truppe *et al.* 1997). According to this study, sequences of up to 600 amino acids were inserted at different positions of the S-layer proteins SbsA and SbsB (Kuen *et al.*, 1994, 1996, 1997), but the formation of sheet-like and cylindrical structures in the cytoplasm of the host cells was taken as the only indication whether the S-layer fusion proteins had retained the ability to self-assemble or not. Data based on ultrathin sections of *E. coli* cells showed that insertions into the N-terminal part did not inhibit the formation of intracellular self-assembly products, while insertions into the middle part had a negative effect. More detailed analysis of the S-layer proteins SbsA, SbsB and SbsC (Egelseer *et al.*, 1998; Howorka *et al.*, 2000; Jarosch *et al.*, 2000, 2001; Sára, 2001) revealed that the N-terminal part of these S-layer proteins recognizes a distinct type of secondary cell wall polymers as the proper anchoring structures in the rigid cell wall layer, but the N-terminal part is not involved in the self-assembly process. Considering the concept of building up 3-D self-assembling supramolecular structures (Sleytr *et al.*, 2001), the N-terminal part shall be exploited for an oriented binding of S-layer fusion proteins on various types of solid supports. This implies that the secondary cell wall polymer must be covalently attached to solid supports or that secondary cell wall polymer-containing glycolipids are incorporated into Langmuir films or liposomes. To ensure that interactions with the secondary cell wall polymer are not reduced, modifications in the N-terminal part must be avoided. In the case of rSbsC/Bet v1, an oriented binding via the N-terminal region is of particular relevance for building up arrays for diagnostic test systems to determine the concentration of Bet v1 specific IgE in patients' whole blood, plasma or serum samples (Breitwieser *et al.*, 1998).

In various other S-layer fusion proteins, only the cell wall

targeting domains such as the three S-layer homologous (SLH) motifs (Lupas *et al.*, 1994) were exploited. For example, the chimeric genes encoding the sequences of the SLH domains of the S-layer proteins Sap or EA1 of *Bacillus anthracis* and levansucrase of *Bacillus subtilis* were cloned in *B. anthracis* (Mesnage *et al.*, 1999a). The fusion proteins were secreted and could attach to the bacterial cell surface and levansucrase retained its enzymatic activity, but such S-layer fusion proteins did not self-assemble into a regular lattice structure. The SLH motifs were also incorporated into the fusion protein SLH-Tox, carrying fragment C of the tetanus toxoid of *Clostridium tetani* (Mesnage *et al.*, 1999b). Another example is the green fluorescence-SAC fusion protein, comprising the cell wall targeting domain of *Lactobacillus acidophilus* ATCC 4536 (Smit *et al.*, 2001).

To conclude, the elucidation of the structure-function relationship of the S-layer protein SbsC (Jarosch *et al.*, 2000, 2001) allowed the construction of an S-layer fusion protein with the desired properties: the ability to recrystallize into a regularly structured lattice, an oriented binding via the N-terminal part of the S-layer protein moiety due to specific interactions with the secondary cell wall polymer, the functionality of the fused protein sequence and its location on the outer surface of the protein lattice. Owing to the crystalline structure of S-layer lattices, introduced functional sequences have a pre-defined distance in the nanometer range, which can be considered of great relevance for applications in nano(bio)technology.

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