Analysis of the structure–function relationship of the S-layer protein SbsC of *Bacillus stearothermophilus* ATCC 12980 by producing truncated forms

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The mature surface layer (S-layer) protein SbsC of *Bacillus stearothermophilus* ATCC 12980 comprises amino acids 31–1099 and self-assembles into an oblique lattice type which functions as an adhesion site for a cell-associated high-molecular-mass exoamylase. To elucidate the structure–function relationship of distinct segments of SbsC, three N- and seven C-terminal truncations were produced in a heterologous expression system, isolated, purified and their properties compared with those of the recombinant mature S-layer protein rSbsC_{31–1099}. With the various truncated forms it could be demonstrated that the N-terminal part (aa 31–257) is responsible for anchoring the S-layer subunits via a distinct type of secondary cell wall polymer to the rigid cell wall layer, but this positively charged segment is not required for the self-assembly of SbsC, nor for generating the oblique lattice structure. If present, the N-terminal part leads to the formation of *in vitro* double-layer self-assembly products. Affinity studies further showed that the N-terminal part includes an exoamylase-binding site. Interestingly, the N-terminal part carries two sequences of 6 and 7 aa (AKAALD and KAAYEAA) that were also identified on the amylase-binding protein AbpA of *Streptococcus gordonii*. In contrast to the self-assembling N-terminal truncation rSbsC_{258–1099}, two further N-terminal truncations (rSbsC_{343–1099}, rSbsC_{447–1099}) and three C-terminal truncations (rSbsC_{31–713}, rSbsC_{31–844}, rSbsC_{31–860}) had lost the ability to self-assemble and stayed in the water-soluble state. Studies with the self-assembling C-terminal truncations rSbsC_{31–880}, rSbsC_{31–900} and rSbsC_{31–920} revealed that the C-terminal 219 aa can be deleted without interfering with the self-assembly process, while the C-terminal 179 aa are not required for the formation of the oblique lattice structure.

Keywords: heterologous expression, self assembly, peptidoglycan, secondary cell wall polymer, exoamylase

INTRODUCTION

Crystalline bacterial cell surface layers (S-layers) are composed of identical protein or glycoprotein subunits with the ability to self-assemble into two-dimensional crystalline arrays, which completely cover the cell

surface of many bacteria and archaea during all stages of growth and division (Sára & Sleytr, 2000; Sleytr & Beveridge, 1999; Sleytr *et al.*, 1999). S-layer lattices exhibit oblique, square or hexagonal symmetry with a centre-to-centre spacing of the morphological units of 3.5–35 nm. In bacteria, the S-layer subunits are linked to each other and to the underlying cell envelope layer by non-covalent interactions and can therefore be isolated from whole cells or cell wall fragments with hydrogen-bond-breaking agents, by applying chelating agents or by changing the pH. During removal of the disrupting

Abbreviations: GHCl, guanidine hydrochloride; GPC, gel-permeation chromatography; HMMA, high-molecular-mass exoamylase; SLH, S-layer homologous.

agent, the S-layer subunits frequently reassemble in suspension, leading to self-assembly products which show the shape of flat sheets or open-ended cylinders and represent either monolayers or double layers (Sleytr *et al.*, 1999).

The S-layer protein from *Bacillus stearothermophilus* ATCC 12980 assembles into an oblique lattice type (Egelseer *et al.*, 1996). The gene encoding this S-layer protein (*sbsC*) was sequenced and cloned, showing that the full-length SbsC consists of 1099 aa, possesses a 30 aa signal peptide and has a calculated isoelectric point (pI) of 5.73 (Jarosch *et al.*, 2000). In the course of studies on the biological function, the oblique S-layer lattice was found to serve as an adhesion site for a cell-associated high-molecular-mass exoamylase (HMMA) which in the S-layer-deficient strain was bound to the peptidoglycan-containing rigid cell wall layer (Egelseer *et al.*, 1996). By using the whole S-layer protein, proteolytic cleavage fragments, native peptidoglycan-anionic polymer complex and peptidoglycan for affinity studies, it became apparent that the N-terminal part of SbsC (aa 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). This secondary cell wall polymer is composed of tetrasaccharide repeating units that contain glucose, N-acetylglucosamine and 2,3-dideoxy-2,3-diacetamido-D-mannuronic acid in the molar ratio of 1:1:2 and the polymer chains are covalently linked to the peptidoglycan backbone (Schäffer *et al.*, 1999). Due to the presence of uronic acids, this type of secondary cell wall polymer is strongly negatively charged and should be regarded as a member of the teichuronic acids (Archibald *et al.*, 1993). Typical of protein domains capable of interacting with carbohydrates (Transue *et al.*, 1997; Weis, 1997), the N-terminal part of the mature SbsC has a high content of arginine, lysine and tyrosine. In contrast to the mature SbsC, the N-terminal part is positively charged, with a calculated pI of 9.13 (Jarosch *et al.*, 2000). Moreover, the N-terminal part of the mature SbsC shows 85% identity to the N-terminal region of the mature SbsA, which assembles into a hexagonally ordered lattice and represents the S-layer protein of *B. stearothermophilus* PV72/p6 (Sára *et al.*, 1996). According to a common functional principle, the N-terminal part of the mature SbsA recognizes an identical type of secondary cell wall polymer as binding site in the rigid cell wall layer (Egelseer *et al.*, 1998; Sára & Sleytr, 2000).

In a previous study it was demonstrated that the S-layer protein isolated from *B. stearothermophilus* ATCC 12980 reassembles into small cylinders with a mean diameter of 100 nm, but independent of the applied experimental conditions, the self-assembly products did not exhibit a regular lattice structure in negatively stained preparations (Egelseer *et al.*, 1996, 1998). The absence of the oblique lattice structure was found to be due to the fact that *B. stearothermophilus* ATCC 12980 coexpresses a C-terminally truncated S-layer protein showing an apparent molecular mass of 60000 on SDS-

PAGE gels. This SbsC fragment, termed P₆₀, was incorporated into the S-layer lattice on whole cells and led to the loss of its regular structure. The amount of P₆₀ coexpressed with SbsC was strongly dependent on the growth conditions and on the growth stage of the cells. Moreover, the incorporation of P₆₀ into the S-layer lattice on whole cells was linked to the release of HMMA into the culture fluid (Egelseer *et al.*, 1996), suggesting that appropriate exoenzyme-binding sites in the S-layer lattice became blocked. Since HMMA recognized S-layer-carrying whole cells, S-layer-carrying cell wall fragments as well as self-assembly products as an adhesion site, it was concluded that the enzyme is not incorporated into the crystal lattice and does not participate in the self-assembly process, but binds to protein domains that are exposed on the outer S-layer surface. This was in clear contrast to P₆₀, which showed no affinity to the outer surface of the S-layer lattice (Egelseer *et al.*, 1996).

In order to investigate the self-assembly properties of SbsC in the absence of P₆₀ and to elucidate the structure-function relationship of distinct segments of this S-layer protein, the PCR products encoding the mature SbsC and three N- and seven C-terminal truncations were cloned and expressed in *Escherichia coli* HMS174(DE3). After isolation and purification of the various SbsC truncations, their ability to self-assemble, to bind to the native peptidoglycan-anionic polymer complex as well as to peptidoglycan and to function as adhesion site for HMMA was investigated.

METHODS

Growth of *B. stearothermophilus* ATCC 12980, preparation of cell wall fragments and isolation of the native peptidoglycan-anionic polymer complex. *B. stearothermophilus* ATCC 12980 was grown in batch culture on SVIII medium or on complex medium under conditions given in previous studies (Egelseer *et al.*, 1996, 1998). Preparation of cell wall fragments and extraction of the S-layer protein was performed as described by Egelseer *et al.* (1998). The native peptidoglycan-anionic polymer complex was purified according to the protocol established by Ries *et al.* (1997). For extraction of the secondary cell wall polymer, the native peptidoglycan-anionic polymer complex was treated with hydrofluoric acid (HF; 48%) for 96 h at 4 °C, which led to peptidoglycan of the A1 γ -chemotype (Egelseer *et al.*, 1998).

Other strains, plasmid, culture conditions and DNA manipulations. *E. coli* TG1 was used for transformations with the plasmid pET3a. For expression, *E. coli* HMS174(DE3) was chosen as a host strain for derivatives of pET3a as described by 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 harbouring pET3a, ampicillin was added to the medium to a final concentration of 50 μ g ml⁻¹. Chromosomal DNA of *B. stearothermophilus* ATCC 12980 was prepared by using Genomic Tips 100 (QIAGEN) according to the manufacturer's instructions. 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.* (1989).

PCR amplification, plasmid construction, cloning and expression. Derived from the repeated amino acid sequences within SbsC and from sequence comparison with other S-layer proteins (Jarosch *et al.*, 2000), various N- or C-terminal SbsC truncations (rSbsC₂₅₈₋₁₀₉₉, rSbsC₃₄₃₋₁₀₉₉, rSbsC₄₄₇₋₁₀₉₉, rSbsC₃₁₋₇₁₃, rSbsC₃₁₋₈₄₄, rSbsC₃₁₋₈₆₀, rSbsC₃₁₋₈₈₀, rSbsC₃₁₋₉₀₀, rSbsC₃₁₋₉₂₀ and rSbsC₃₁₋₉₃₀) were constructed by PCR amplification of the corresponding DNA fragments using the oligonucleotides listed in Table 1. For cloning, *Nde*I and *Bam*HI restriction sites were introduced during PCR at the 5' and 3' end, respectively. PCR amplification was performed under conditions given in a previous study (Jarosch *et al.*, 2000). The PCR products encoding the mature SbsC (rSbsC₃₁₋₁₀₉₉) or the various truncated SbsC forms were inserted into the corresponding restriction sites of pET3a and transformed into *E. coli* TG1 and *E. coli* HMS174(DE3). The plasmid stability test and expression were performed as described by Jarosch *et al.* (2000). Samples of cultures from *E. coli* HMS174(DE3) were taken 2–8 h after induction of expression. Preparation of biomass samples and SDS-PAGE were carried out as described by Laemmli (1970). Immunoblotting with polyclonal rabbit antiserum raised against the S-layer protein of *B. stearothermophilus* ATCC 12980 and N-terminal sequencing was performed as described by Egelseer *et al.* (1996). Whole cells of *E. coli* HMS174(DE3) induced to express the various pET3a constructs were fixed, embedded in Spurr resin and subjected to ultrathin sectioning according to procedures given by Messner *et al.* (1984). Preparations were examined with a Philips CM 100 transmission electron microscope, operated at 80 kV and equipped with a 30 µm objective aperture.

Isolation and purification of rSbsC₃₁₋₁₀₉₉ and of the various N- or C-terminal SbsC truncations. Cultures of *E. coli* HMS174(DE3) cells induced to express the *sbsC* gene or the various *sbsC* derivatives were centrifuged (6000 g, 10 min, 4 °C) and 1 g wet pellet of biomass was resuspended in 100 ml 0.1 M CaCl₂ solution. After incubation on ice for 30 min, the suspensions were centrifuged (6000 g, 5 min, 4 °C) and the pellets were resuspended in 100 ml 0.1 M Tris/HCl (pH 8.0) containing 1% (v/v) glycerol, 10 mM EDTA and 10 mg lysozyme. The mixtures were heated in a water bath until 42 °C was reached and then incubated at 30 °C for 15 min. This was followed by the addition of 10 ml 1% Triton X-100 in 50 mM Tris/HCl (pH 7.8) and by a further incubation step for 10 min at 20 °C. After sonication of the suspensions (Branson Sonifier, 10 × 10 s), the soluble and insoluble fractions were separated by centrifugation (20000 g, 30 min, 4 °C) and investigated by SDS-PAGE. The insoluble pellets were washed once with 1% Triton X-100 in 50 mM Tris/HCl (pH 7.8) and twice with 50 mM Tris/HCl (pH 7.8). The pellets were then resuspended in a fivefold volume of 5 M guanidine hydrochloride (GHCl) in 50 mM Tris/HCl buffer (pH 7.2) and the suspensions were stirred for 20 min on ice. For removal of peptidoglycan fragments, the suspensions were centrifuged at 36000 g for 30 min at 4 °C. The supernatant was diluted to a final GHCl concentration of 2 M and 2 ml of this solution was subjected to gel-permeation chromatography (GPC) using a Superdex 200 column (Pharmacia) for separation and 2 M GHCl in 50 mM Tris/HCl buffer (pH 7.2) for elution. Fractions containing S-layer protein were pooled, dialysed against distilled water for at least 12 h at 4 °C, frozen at –20 °C and lyophilized. The procedure for the isolation of the truncated SbsC forms from the soluble fraction (cytoplasm fraction of the *E. coli* cells) was as follows. After separation of the soluble and the insoluble fraction, the soluble S-layer proteins were precipitated with ammonium sulphate (35–45 %

or 45–65 % saturation). The resulting pellets were dissolved in 2 ml distilled water and the solutions were dialysed against distilled water for at least 24 h at 4 °C. Material which was still insoluble was removed by centrifugation (36000 g, 30 min, 4 °C) and the clear supernatants were applied onto a Superdex 200 column using 150 mM NaCl in 50 mM Tris/HCl buffer (pH 7.2) for elution. Fractions containing S-layer protein were pooled, dialysed against distilled water for at least 24 h at 4 °C and lyophilized.

Investigation of the self-assembly properties of rSbsC₃₁₋₁₀₉₉ and of the various N- or C-terminal SbsC truncations. To evaluate the self-assembly properties, 1 mg of the purified and lyophilized S-layer proteins was dissolved per ml 2 M GHCl in 50 mM Tris/HCl buffer (pH 7.2). The solutions were dialysed against distilled water, 10 mM CaCl₂ or 10 mM KCl for at least 24 h at 20 °C. The suspensions were negatively stained and prepared for ultrathin sectioning according to procedures described by Messner *et al.* (1984). Labelling of self-assembly products formed by rSbsC₃₁₋₁₀₉₉ with polycationized ferritin as a positively charged topographical marker was carried out as described in a previous study (Messner *et al.*, 1986).

Isolation of HMMA from the S-layer deficient strain, and affinity studies. Extraction of HMMA from cell wall fragments of the S-layer-deficient strain of *B. stearothermophilus* ATCC 12980 with 4 M GHCl in 50 mM Tris/HCl buffer (pH 7.2) was performed as described by Egelseer *et al.* (1996). After centrifugation of the suspension at 36000 g for 10 min at 4 °C, the clear supernatant was diluted with 50 mM Tris/HCl buffer to a final GHCl concentration of 2 M and the solution was subjected to GPC using a Sephacryl S-200 column for separation. Fractions containing HMMA were pooled, dialysed against 50 mM Tris/HCl buffer (pH 7.2) for 24 h at 4 °C and the protein content of the dialysed solution was adjusted to 100 µg per ml buffer. The purity of HMMA was checked by SDS-PAGE and the amylolytic activity was visualized *in situ* on SDS-gels by the method of Lacks & Springhorn (1980). To perform the binding assay, 1 mg lyophilized self-assembly products consisting of rSbsC₃₁₋₁₀₉₉, rSbsC₃₁₋₈₈₀ or rSbsC₂₅₈₋₁₀₉₉ were dissolved in 1 ml 2 M GHCl in 50 mM Tris/HCl buffer, and the solutions were dialysed for 18 h at 20 °C against 50 mM Tris/HCl buffer. After the addition of 100 µl HMMA solution, the suspensions were incubated for 20 min at 20 °C, centrifuged at 36000 g for 10 min at 4 °C and the pellets were washed twice with buffer. The pellets and the supernatants were collected and analysed by SDS-PAGE. For comparison, self-assembly products formed by the S-layer protein from *Bacillus sphaericus* CCM 2177 were used (Ilk *et al.*, 1999). The affinity of the HMMA to the native peptidoglycan–anionic polymer complex and to peptidoglycan devoid of the secondary cell wall polymer from *B. stearothermophilus* ATCC 12980, *B. stearothermophilus* PV72/p2 (Sára *et al.*, 1996) and *B. sphaericus* CCM 2177 (Ilk *et al.*, 1999) was also investigated.

Investigation of the affinity of rSbsC₃₁₋₁₀₉₉ and of the various N- or C-terminal SbsC truncations to the native peptidoglycan–anionic polymer complex as well as to peptidoglycan. For affinity studies, 1 mg lyophilized rSbsC₃₁₋₁₀₉₉ and of the self-assembling SbsC truncations was dissolved in 1 ml 2 M GHCl in 50 mM Tris/HCl buffer (pH 7.2) and either 1 mg lyophilized native peptidoglycan–anionic polymer complex or 1 mg peptidoglycan (HF-extracted peptidoglycan–anionic polymer complex) was added. The suspensions were then stirred for 15 min at 20 °C. After dialysis against distilled water for at least 24 h at 4 °C, the suspensions were negatively stained and prepared for ultrathin sectioning. To investigate the binding properties of

Fig. 1. SDS-PAGE patterns of whole-cell extracts of *E. coli* HMS174(DE3) harbouring either the pET3a plasmid (lane 1) or the various pET3a gene constructs encoding the mature S-layer protein (rSbsC_{31–1099}, lane 2) or the truncated SbsC forms (rSbsC_{258–1099}, lane 3; rSbsC_{343–1099}, lane 4; rSbsC_{447–1099}, lane 5; rSbsC_{31–713}, lane 6; rSbsC_{31–844}, lane 7; rSbsC_{31–860}, lane 8; rSbsC_{31–880}, lane 9). The molecular masses are indicated on the left.

the soluble truncated S-layer proteins, 1 mg of the lyophilized material was dissolved in 1 ml 50 mM Tris/HCl buffer (pH 7.2) and 1 mg native peptidoglycan–anionic polymer complex or peptidoglycan was added. The suspensions were incubated for 30 min at 20 °C and centrifuged at 36000 *g* for 20 min at 4 °C. Subsequently, the pellets and the supernatants were analysed by SDS-PAGE.

DNA sequencing. PCR products encoding the mature SbsC or the various truncated SbsC forms were sequenced as described elsewhere (Jarosch *et al.*, 2000).

RESULTS

Expression of the *sbsC* gene and of the various *sbsC* derivatives in *E. coli* HMS174(DE3), and isolation and purification of rSbsC_{31–1099} and the various SbsC forms

The PCR products encoding the mature SbsC and the various N- or C-terminal SbsC truncations (Table 1) were cloned and expressed in *E. coli* HMS174(DE3).

Table 1. Survey of the properties of the recombinant mature SbsC and the various truncated SbsC forms

rSbsC form	Primer* sequence (5' to 3')	Theoretical mol. mass (Da)	Apparent mol. mass on SDS-PAGE (kDa)	pI†	N-terminal sequence‡
rSbsC _{31–1099}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTACGATGCTGATTTTGTACCAATTTG reverse	112 471	122	5.40	ATDVA
rSbsC _{258–1099}	CGCGGATCCCATATG/GCAGCATTGACGCCGAAGG CGCGGATCC/TTACGATGCTGATTTTGTACCAATTTG reverse	87 259	91	4.89	AALTP
rSbsC _{343–1099}	CGCGGATCCCATATG/GCTGGTAAAGAATTTGAACTAGC CGCGGATCC/TTACGATGCTGATTTTGTACCAATTTG reverse	78 271	82	4.80	AGKEF
rSbsC _{447–1099}	CGCGGATCCCATATG/GCAGAAGTTAGTGAATTAATAAAC CGCGGATCC/TTACGATGCTGATTTTGTACCAATTTG reverse	67 091	66	4.71	AEVSE
rSbsC _{31–713}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTATGCATTAACGATTACACCATTTGTATC reverse	73 137	73	6.55	ATDVA
rSbsC _{31–844}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTAAGCATCATCTTTTAAGTTATCAAC reverse	86 624	90	5.41	ATDVA
rSbsC _{31–860}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTATGTTAATGTTACTGTACCTGCTTTTC reverse	88 152	91	5.62	ATDVA
rSbsC _{31–880}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTAAGCAGGAGCTTTAACTGTGATTG reverse	90 231	93	5.62	ATDVA
rSbsC _{31–900}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTATACACCAGTTACACCTGGCAG reverse	92 113	96	5.42	ATDVA
rSbsC _{31–920}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTAAGCATAATATAATTTGTGACCAGATTTG reverse	94 353	98	6.09	ATDVA
rSbsC _{31–930}	CGCGGATCCCATATG/GCAACGGACGTGGCGAC CGCGGATCC/TTAAGCTGGCGCAGGTACTG reverse	95 276	100	5.74	ATDVA

* Oligonucleotide primers used for amplification in PCR.

† Calculated isoelectric point.

‡ Determined experimentally.

Fig. 2. Electron micrographs of ultrathin sections of *E. coli* HMS174(DE3) demonstrating the presence of self-assembly products consisting of (a) rSbsC₃₁₋₁₀₉₉ or (b) rSbsC₂₅₈₋₁₀₉₉ in the cytoplasm of the host cells. Bars, 200 nm.

After induction of expression by the addition of IPTG, biomass samples were harvested at various times and analysed by SDS-PAGE and ultrathin sectioning. In comparison to *E. coli* HMS174(DE3) carrying pET3a (Fig. 1, lane 1), an additional high-molecular-mass protein band was observed on SDS-PAGE gels in samples from *E. coli* HMS174(DE3) cells induced to express the *sbsC* gene or the various *sbsC* derivatives (Fig. 1, lanes 2–9). In such samples, the apparent molecular mass of the additional protein band corresponded approximately to the calculated molecular mass of the mature SbsC or of the respective N- or C-terminal truncation (Table 1). On immunoblots, a strong cross-reaction was observed between these protein bands and the polyclonal rabbit antiserum raised against the S-layer protein of *B. stearothermophilus* ATCC 12980 (data not shown). The identification of the additional protein bands as the mature SbsC or as the N- or C-terminal SbsC truncations was accomplished by N-terminal sequencing (Table 1).

To investigate whether rSbsC₃₁₋₁₀₉₉ and the various truncated SbsC forms assembled in the cytoplasm of the host cells, whole cells of *E. coli* HMS174(DE3) carrying the constructs were fixed and embedded and the ultrathin sections were examined by electron microscopy. In longitudinal ultrathin sections of cells producing rSbsC₃₁₋₁₀₉₉, sheet-like structures were visible which were arranged in a parallel fashion at high density, but cross-sections showed that these sheet-like structures were organized as closely packed spirally wound sheets (Fig. 2a). Similar structures were observed in cells synthesizing the C-terminal truncations rSbsC₃₁₋₈₈₀, rSbsC₃₁₋₉₀₀, rSbsC₃₁₋₉₂₀ and rSbsC₃₁₋₉₃₀. In longitudinal sections of whole cells producing the N-terminal truncation rSbsC₂₅₈₋₁₀₉₉, monolayer cylinders with a diameter of about 50 nm were visible (Fig. 2b). A

similar observation was made for the N-terminal truncation rSbsC₃₄₃₋₁₀₉₉. No structures indicating the formation of self-assembly products were observed in ultrathin sections of cells producing the truncated forms rSbsC₄₄₇₋₁₀₉₉, rSbsC₃₁₋₇₁₃, rSbsC₃₁₋₈₄₄ and rSbsC₃₁₋₈₆₀ (not shown).

As revealed by electron microscopy, self-assembly in the cytoplasm of the host cells was a characteristic of rSbsC₃₁₋₁₀₉₉, of the N-terminal truncations rSbsC₂₅₈₋₁₀₉₉ and rSbsC₃₄₃₋₁₀₉₉, and of the C-terminal truncations rSbsC₃₁₋₈₈₀, rSbsC₃₁₋₉₀₀, rSbsC₃₁₋₉₂₀ and rSbsC₃₁₋₉₃₀. These findings were basically confirmed by data from SDS-PAGE, since with the exception of rSbsC₃₄₃₋₁₀₉₉, the mature recombinant S-layer protein and all other truncated SbsC forms that assembled in the cytoplasm of the host cells had accumulated in the insoluble fraction of the lysed *E. coli* cells. On the other hand, the truncated forms rSbsC₃₄₃₋₁₀₉₉, rSbsC₄₄₇₋₁₀₉₉, rSbsC₃₁₋₇₁₃, rSbsC₃₁₋₈₄₄ and rSbsC₃₁₋₈₆₀ were exclusively detected in the soluble fraction of the host cells, and they could be separated from other soluble proteins by ammonium sulphate precipitation and purification by GPC. All truncated SbsC forms that were enriched in the insoluble fraction were extracted with 5 M GHCl and subjected to GPC. Each purified SbsC form migrated as a single protein band with the expected molecular mass on SDS-gels (e.g. Fig. 3d, inset; Table 1).

Self-assembly products formed by rSbsC₃₁₋₁₀₉₉ and by the various N- or C-terminal SbsC truncations

To investigate the self-assembly properties, the purified S-layer proteins were disintegrated in GHCl, which was subsequently removed by dialysis against distilled water or solutions of monovalent or bivalent cations. As shown by negative staining, rSbsC₃₁₋₁₀₉₉ reassembled

Fig. 3. Electron micrographs of negatively stained preparations of self-assembly products formed by rSbsC₃₁₋₁₀₉₉ and by the various truncated SbsC forms. In the case of self-assembly products formed by rSbsC₃₁₋₁₀₉₉, double-layer sheets (a) and double-layer cylinders (b) are shown. The N-terminal truncation rSbsC₂₅₈₋₁₀₉₉ reassembled exclusively into monolayer cylinders (c) exhibiting an oblique lattice structure. Self-assembly products formed by the C-terminal truncation rSbsC₃₁₋₈₈₀ did not show a regular lattice structure (d). A faint lattice structure was observed on self-assembly products consisting of rSbsC₃₁₋₉₀₀ (e), while self-assembly products formed by rSbsC₃₁₋₉₂₀ clearly exhibited the oblique lattice structure (f). Bars, 100 nm. Inset in (d): SDS-PAGE pattern of purified rSbsC₃₁₋₈₈₀.

Fig. 4. SDS-PAGE patterns demonstrating the purity (lane 1) and activity (lane 2) of HMMA and its affinity to self-assembly products consisting of rSbsC₃₁₋₁₀₉₉ (lanes 3 and 6), rSbsC₃₁₋₈₈₀ (lanes 4 and 7) and rSbsC₂₅₈₋₁₀₉₉ (lanes 5 and 8). Lanes 1 and 3–5, silver staining; lanes 2, 6–8, *in situ* detection of amylolytic activity. The position of the HMMA is indicated.

into double-layer sheets (Fig. 3a) and into open-ended monolayer and double-layer cylinders. The diameter of the monolayer cylinders was in the range of 70–100 nm, while the double-layer cylinders (Fig. 3b) were generally larger, with a diameter of 220–280 nm. As determined by computer image reconstruction of negatively stained preparations, the self-assembly products clearly exhibited the oblique (p2) lattice structure with the lattice constants of $a = 11.6$ nm, $b = 7.2$ nm and $\gamma = 81^\circ$ (D. Pum, personal communication). Labelling with polycationized ferritin revealed that the self-assembly products did not bind this positively charged topographical marker (not shown). Since some of the self-assembly products were monolayers, it became apparent that neither the outer nor the inner S-layer surface has a net negative charge. In contrast to rSbsC₃₁₋₁₀₉₉, the N-terminal truncation rSbsC₂₅₈₋₁₀₉₉ reassembled exclusively into monolayer cylinders with a maximum diameter of 130 nm, which in negatively stained preparations exhibited an oblique lattice structure very weak in contrast (Fig. 3c).

As observed with the C-terminal truncations rSbsC₃₁₋₈₈₀, rSbsC₃₁₋₉₀₀ and rSbsC₃₁₋₉₂₀, the length in this part of the sequence had a significant influence on the formation of the oblique lattice structure. The C-terminal truncation rSbsC₃₁₋₈₈₀ reassembled into flat sheets or open-ended cylinders and the self-assembly process was as quantitative as that of rSbsC₃₁₋₁₀₉₉, since no S-layer protein could be detected in the supernatant after the removal of self-assembled material by centrifugation. In contrast to rSbsC₃₁₋₁₀₉₉, self-assembly products formed by rSbsC₃₁₋₈₈₀ did not exhibit a regular lattice structure (Fig. 3d). Self-assembly products consisting of rSbsC₃₁₋₉₀₀ (Fig. 3e), rSbsC₃₁₋₉₂₀ (Fig. 3f) or rSbsC₃₁₋₉₃₀ (not shown) had a comparable shape, but differences were observed in the quality of the oblique lattice structure. For example, self-assembly products formed by rSbsC₃₁₋₉₀₀ exhibited only a diffuse lattice

structure in negatively stained preparations (Fig. 3e), while self-assembly products consisting of rSbsC₃₁₋₉₂₀ (Fig. 3f) or rSbsC₃₁₋₉₃₀ clearly showed the oblique lattice structure. Considering the water solubility of rSbsC₃₁₋₈₈₀ and the formation of first self-assembly products by rSbsC₃₁₋₈₈₀, an elongation by 20 aa was sufficient to establish the contact sites necessary for subunit-to-subunit interactions. Based on the results obtained with rSbsC₃₁₋₈₈₀ and rSbsC₃₁₋₉₂₀, only 40 additional aa in the C-terminal part were required to completely restore the oblique lattice structure (Fig. 3e, f).

Affinity studies with HMMA

In addition to self-assembly products formed by rSbsC₃₁₋₁₀₉₉, those consisting of rSbsC₂₅₈₋₁₀₉₉ or rSbsC₃₁₋₈₈₀, which represented either the shortest N- or the shortest C-terminal truncation capable of self-assembly, were used for affinity studies with HMMA. As shown by SDS-PAGE, the exoenzyme (Fig. 4, lanes 1 and 2) could bind to self-assembly products formed by rSbsC₃₁₋₁₀₉₉ (Fig. 4, lanes 3 and 6) or rSbsC₃₁₋₈₈₀ (Fig. 4, lanes 4 and 7), but not to those consisting of rSbsC₂₅₈₋₁₀₉₉ (Fig. 4, lanes 5 and 8), which indicated that the N-terminal part of SbsC must comprise the binding region for HMMA. Actually, two sequences of 6 and 7 aa (AKAALD and KAAYEAA), which are located between aa 223–228 and 254–260 in the N-terminal part of SbsC, were previously identified in the amylase-binding protein AbpA of *Streptococcus gordonii* (Rogers *et al.*, 1998). Further affinity studies revealed that HMMA recognized the native peptidoglycan–anionic polymer complex from *B. stearotheophilus* ATCC 12980, as well as pure peptidoglycan of the A1 γ -chemotype from which the secondary cell wall polymer had been extracted, as binding site. Moreover, HMMA could bind to the native peptidoglycan–anionic polymer complex of *B. stearotheophilus* PV72/p2, which possesses the same peptidoglycan chemotype (A1 γ), but reveals a different type of secondary cell wall polymer (Sára & Sleytr, 2000). In contrast, the exoenzyme did not bind to native peptidoglycan–anionic polymer complex or to peptidoglycan from *B. sphaericus* CCM 2177, which has a different peptidoglycan chemotype (A4 α) (Ilk *et al.*, 1999) as well as a different type of secondary cell wall polymer than *B. stearotheophilus* ATCC 12980 (Egelseer *et al.*, 1998). Furthermore, the exoenzyme did not bind to self-assembly products formed by the S-layer protein from *B. sphaericus* CCM 2177 (not shown).

Affinity of rSbsC₃₁₋₁₀₉₉ and of the various N- or C-terminal SbsC truncations to the native peptidoglycan–anionic polymer complex and to peptidoglycan

For affinity studies, rSbsC₃₁₋₁₀₉₉ and all self-assembling SbsC truncations were dissolved in 2 M GHCl and incubated with the native peptidoglycan–anionic poly-

Fig. 5. Electron micrographs of ultrathin sections demonstrating the ability of the C-terminal truncation rSbsC₃₁₋₉₀₀ to bind to (a) native peptidoglycan-anionic polymer complex but not (b) peptidoglycan. os, outer S-layer; pg, peptidoglycan-containing layer; is, inner S-layer. Bars, 200 nm.

mer complex (and in some selected examples with peptidoglycan). After removal of GHCl by dialysis, the pellets obtained by centrifugation were prepared for ultrathin sectioning. It could be demonstrated that rSbsC₃₁₋₁₀₉₉ and all self-assembling C-terminal truncations (rSbsC₃₁₋₈₈₀, rSbsC₃₁₋₉₀₀, rSbsC₃₁₋₉₂₀, rSbsC₃₁₋₉₃₀) recognized the native peptidoglycan-anionic polymer complex, but not peptidoglycan as binding site (Fig. 5a, b; shown only for SbsC₃₁₋₉₀₀). If the native peptidoglycan-anionic polymer complex was used as matrix for recrystallization, a complete outer and inner S-layer was formed (Fig. 5a). In contrast to the C-terminal self-assembling truncations, the N-terminal truncation rSbsC₂₅₈₋₁₀₉₉ did not bind to the native peptidoglycan-anionic polymer complex and re-assembled into the characteristic monolayer cylinders (not shown). The affinities of the water-soluble N- or C-terminal truncations to the native peptidoglycan-

Fig. 6. SDS-PAGE patterns demonstrating the results of the affinity studies of selected water soluble N- or C-terminal truncations (rSbsC₄₄₇₋₁₀₉₉ and rSbsC₃₁₋₈₄₄) to the native peptidoglycan-anionic polymer complex. After centrifugation of the suspensions, the whole amount of rSbsC₄₄₇₋₁₀₉₉ remained in the clear supernatant (lane 1) and no protein could be detected in the pellet (lane 2). In contrast, no rSbsC₃₁₋₈₄₄ was detected in the supernatant (lane 3), and the whole amount was concentrated in the pellet consisting of the native peptidoglycan-anionic polymer complex (lane 4). The molecular masses are indicated.

anionic polymer complex and to peptidoglycan were the same as those of the self-assembling N- or C-terminal SbsC truncations. As demonstrated by SDS-PAGE, neither rSbsC₃₄₃₋₁₀₉₉ (not shown) nor rSbsC₄₄₇₋₁₀₉₉ (Fig. 6, lanes 1 and 2) showed affinity to the native peptidoglycan-anionic polymer complex, whereas rSbsC₃₁₋₇₁₃, (not shown), rSbsC₃₁₋₈₄₄ (Fig. 6, lanes 3 and 4) and rSbsC₃₁₋₈₆₀ (not shown) recognized them as binding site. As expected, the water soluble C-terminal truncations did not bind to peptidoglycan (not shown).

DISCUSSION

Up to now, only a little information has been available on the self-assembly and cell wall-binding properties of truncated S-layer proteins and there exist no data as to which parts of the S-layer proteins could be responsible for exoenzyme binding. In the case of *Aeromonas hydrophila* (Thomas *et al.*, 1992), a Gram-negative organism exhibiting a square S-layer lattice, a mutant producing a C-terminally truncated S-layer protein was isolated. The molecular mass of the truncated form comprising the protease-resistant N-terminal domain was 39 kDa instead of 52 kDa for the mature S-layer protein. Although the truncated form was exported via the periplasm, it could not be anchored to the outer membrane nor could it self-assemble into the square lattice structure (Trust, 1993). A truncated form of the S-layer protein SlpA from *Thermus thermophilus* which was devoid of the S-layer-homologous (SLH) motifs located in the N-terminal part resulted in a protein unable to attach to the cell surface and to assemble into the hexagonally ordered lattice type (Olabarriá *et al.*,

1996). In contrast, the N-terminal parts of the S-layer proteins Sap and EA1 of *Bacillus anthracis* (Mesnage *et al.*, 1999) and SlpA of *Clostridium thermocellum* (Lemaire *et al.*, 1998; Chauvaux *et al.*, 1999) comprising the three SLH motifs were able to attach to the rigid cell wall layer via a peptidoglycan-associated cell wall polymer.

As demonstrated in previous studies, a proteolytic cleavage fragment of the S-layer protein SbsC from *B. stearo-thermophilus* ATCC 12980 lacking the N-terminal 227 aa had lost the ability to bind to the native peptidoglycan–anionic polymer complex, but was still able to self-assemble (Egelseer *et al.*, 1998; Jarosch *et al.*, 2000). From affinity studies it became apparent that the N-terminal part of SbsC recognizes the secondary cell wall polymer as the proper anchoring structure in the rigid cell wall layer. Studies on the biological function revealed that the oblique S-layer lattice from *B. stearo-thermophilus* ATCC 12980 serves as an adhesion site for HMMA, an exoamylase that was produced by the S-layer-carrying as well as by the S-layer-deficient strain (Egelseer *et al.*, 1996). Several studies indicated that HMMA does not participate in the self-assembly process but recognizes protein domains that are exposed on the outer S-layer surface (Egelseer *et al.*, 1996, 1997).

To obtain more insight into the structure–function relationship of distinct segments of the S-layer protein SbsC, the PCR products encoding three N- or seven C-terminal truncations were cloned and expressed in *E. coli* HMS174(DE3). In agreement with the results obtained from previous studies (Egelseer *et al.*, 1998; Jarosch *et al.*, 2000) with the proteolytic cleavage fragment of the S-layer protein SbsC lacking the N-terminal 227 aa, all N-terminal truncations had lost the ability to bind to the native peptidoglycan–anionic polymer complex, while all C-terminal truncations recognized them as a binding site. Thus, it is obvious that the N-terminal region comprising aa 31–257 is responsible for cell wall binding.

In contrast to rSbsC_{31–1099} and to the self-assembling C-terminal truncations which formed *in vitro* monolayers as well as double-layer self-assembly products, the N-terminal truncation rSbsC_{258–1099} reassembled exclusively into monolayer cylinders exhibiting an oblique lattice structure (Fig. 3c). This observation indicated that the N-terminal region of SbsC is responsible for double-layer formation, which was independent of the presence of bivalent cations. In a previous study, conditions leading to the formation of monolayer or double-layer self-assembly products, or both, were investigated in detail for the wild-type S-layer glycoprotein from *B. stearo-thermophilus* NRS 2004/3a (Messner *et al.*, 1986). Interestingly, in double-layer self-assembly products the individual monolayers had bound to each other with the inner S-layer surface. Double-layer formation occurred only in the presence of bivalent cations. Because polycationized ferritin was densely bound by the monolayer self-assembly products, but not at all by the double layers, it was concluded that the inner S-layer surface has a net negative charge. These

findings are in clear contrast to the results obtained in the present study for the self-assembly products consisting of rSbsC_{31–1099}, since on the one hand, the monolayers did not bind polycationized ferritin, while on the other hand, the formation of double-layer self-assembly products was independent of the presence of bivalent cations. Moreover, sequence analysis revealed that the N-terminal part of SbsC which must be located on the inner S-layer surface and is conserved among S-layer proteins from *B. stearo-thermophilus* wild-type strains (Sára & Sleytr, 2000) reveals a positive net charge with a pI > 9. These data strongly indicate that the net negative charge determined on the inner S-layer surface of self-assembly products formed by the wild-type S-layer proteins might result from adherent secondary cell wall polymer (Egelseer *et al.*, 1998; Sára *et al.*, 1998; Sára & Sleytr, 2000).

Affinity studies with the HMMA and self-assembly products formed by rSbsC_{31–1099}, rSbsC_{258–1099} or rSbsC_{31–880} demonstrated that the exoenzyme requires the N-terminal part for binding to this S-layer protein. Comparison of the N-terminal part of SbsC with the amylase-binding protein AbpA from *Streptococcus gordonii* (Rogers *et al.*, 1998) revealed the existence of common sequences comprising 6 aa (AKAALD) and 7 aa (KAAYEAA). In SbsC, the 7 aa sequence is located in the N-terminal part between aa 254 and 260, and carries a glutamic acid residue which is attacked by endo-proteinase Glu-C and can therefore be expected to be exposed on the surface of the S-layer subunits (Egelseer *et al.*, 1998). Derived from secondary-structure prediction, the N-terminal part is mainly organized as α -helices connected by loops and seems to fold independently of the remainder of the protein sequence. Although the N-terminal part is involved in cell wall binding and a major portion must therefore be located on the inner S-layer surface, it cannot be excluded that certain sequences are exposed on the outer S-layer surface, thereby functioning as adhesion site for HMMA. It was interesting to observe that the 6 and 7 aa sequences which were identical to those on AbpA of *S. gordonii* (Rogers *et al.*, 1998) were located at the end of the N-terminal part, which connects this structurally defined domain to that part of the sequence responsible for the formation of the oblique lattice structure. In contrast to the S-layer protein, HMMA could bind to peptidoglycan of the A1 γ -chemotype, but it is not currently known whether the exoenzyme also shows affinity to the secondary cell wall polymer. The pullulanase of *Thermoanaerobacterium thermo-sulfurigenes* EM1 was demonstrated to bind via its SLH motifs to the native peptidoglycan–anionic polymer complex. However, a reduced amount of this exoenzyme was able to attach to the HF-extracted peptidoglycan–anionic polymer complex, which most probably represented pure peptidoglycan (Brechtel & Bahl, 1999). Surface plasmon resonance, a method to determine molecular interactions, further revealed that HMMA immobilized on the sensor chips could bind the native peptidoglycan–anionic polymer complex as well as the

Fig. 7. Schematic drawing of the S-layer protein SbsC according to Jarosch *et al.* (2000). Arrows indicate the beginning of N-terminal truncations (N) and the end of C-terminal truncations (C). All N-terminal truncations stopped with aa 1099; all C-terminal truncations started with aa 31. The S-layer protein SbsC consists of several repeats: R_M (aa 447–700), R_C (844–1089), 23 % identity; R_{M1} (aa 271–600), R_{M2} (aa 348–706), 23 % identity; R_{C1} (aa 831–978), R_{C2} (aa 931–1089), 28 % identity.

peptidoglycan, but the exoenzyme was not able to interact with non-assembled monomeric rSbsC_{31–1099} (unpublished results). These findings strongly indicated that in addition to appropriate HMMA-binding sites on the S-layer subunits, the oblique lattice structure or at least the dimers are required for exoenzyme binding. This is in agreement with observations on whole cells of *B. stearothermophilus* ATCC 12980 (Egelseer *et al.*, 1996).

As described for the S-layer proteins of *A. hydrophila* and *C. glutamicum* (Thomas *et al.*, 1992; Chami *et al.*, 1997), the C-terminal part of SbsC is of major importance for the self-assembly process and for the formation of the oblique lattice structure. With the various C-terminal SbsC truncations it could be demonstrated that the C-terminal 219 aa do not determine the shape of the self-assembly products and can be deleted without interfering with the self-assembly process, while the C-terminal 179 aa are not required for the formation of the oblique lattice structure (Fig. 7).

In contrast to Sap of *B. anthracis* (Etienne-Toumelin *et al.*, 1995) and CTC of *Bacillus thuringiensis* (M. Sun & Z. Yu, GenBank accession no. AJ012290), SbsC possesses a longer N-terminal part and no SLH motifs, as well as a longer C-terminal part. From sequence comparison it was suggested that the additional C-terminal amino acids on the SbsC sequence (beyond aa 880) are not required for the self-assembly of this S-layer protein (Jarosch *et al.*, 2000), which could actually be confirmed by experimental data in the present study with the various C-terminal truncations.

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