Charge Distribution on the S Layer of *Bacillus stearothermophilus* NRS 1536/3c and Importance of Charged Groups for Morphogenesis and Function

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The distribution and functional significance of charged groups on the outer and inner faces of the S layer from *Bacillus stearothermophilus* NRS 1536/3c was investigated. Chemical modification of the exposed amino or carboxyl groups was performed on whole cells, isolated S layers self-assembled in vitro, and cell wall fragments (S layer attached to the peptidoglycan-containing sacculus). Without chemical modification, S layer self-assembly products could be labeled with polycationic ferritin, while S layers on whole cells could not. Following treatment with glutaraldehyde, whole cells were uniformly labeled with polycationic ferritin. Whole cells treated with glutaraldehyde and glycine methyl ester in the presence of carbodimide did not bind polycationic ferritin significantly above background. Treatment of cell wall fragments with amino-specific, homobifunctional cross-linkers or with carbodimide alone rendered the S layer protein nonextractable with sodium dodecyl sulfate. After amidation of the accessible carboxyl groups, the modified, guanidine hydrochloride-extractable S layer protomers did not self-assemble into regularly structured lattices. N-Amidination with ethylacetimidate did not interfere with the self-assembly of the isolated protomers. N-Acetylation resulted in a considerable destabilization of the S layer lattice, as seen by the release of a large amount of modified protomers during the reaction. N-Succinylation led to a complete disintegration of the protein lattice. These results indicated that only the inner face of the S layer carried a net negative charge. On both faces, free amino and carboxyl groups of adjacent protomers were arranged in proximity so as to contribute by electrostatic interactions to the cohesion of the protomers in the two-dimensional array. The native charge of the protomers was required for both the in vitro self-assembly of the isolated subunits and the maintenance of the structural integrity of the S layer lattice. Among other functions, the biological significance of S layers may be in masking the electronegative charge of the cell wall proper.

Many eubacteria and archaeabacteria possess crystalline arrays of subunits (S layers) as the outermost component of their cell envelopes (3, 25, 29, 30). S layers completely cover the cell surface and exhibit hexagonal, square, or oblique lattices with a center-to-center spacing of the morphological units varying from 5 to 32 nm. Frequently, isolated S layer subunits show the ability to reassemble into lattices identical to those observed on intact cells. In vitro self-assembly leads to the formation of flat sheets, open-ended cylinders, or closed vesicles (1, 8, 16, 24, 32). Most of the S layers isolated from numerous organisms of different phylogenetic branches are composed of a single homogeneous polypeptide species of molecular weights ranging from 40,000 to 200,000. In contrast to this large variety of molecular weights, the amino acid compositions reported are rather similar: all S layer proteins show a large amount of acidic and hydrophobic amino acids, and among the basic amino acids lysine predominates (29). Conditions required for the disintegration of S layer lattices and for in vitro self-assembly indicated that electrostatic interactions, hydrogen bonds, and hydrophobic forces between adjacent protomers are responsible for the structural integrity of these protein arrays (29). So far, only a few studies on the role of free amino and carboxyl groups in cohesion of the S layer subunits and their attachment to the underlying cell envelope layer have been reported (17, 34).

In this report, chemical modification and labeling experiments were used to study the S layer from *Bacillus stearothermophilus* NRS 1536/3c for net charge at the inner and outer faces, arrangement of amino and carboxyl groups, and the importance of positive and negative charges for the stability of the S layer lattice and the in vitro self-assembly process.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** *B. stearothermophilus* NRS 1536/3c was cultivated as described previously (32). A detailed description on the S layer of this strain has been published (32).

**Preparation of cell wall fragments and S layer self-assembly products.** Cell wall preparation was produced as described (28). For the preparation of S layer self-assembly products, cell wall fragments (0.2 g; wet pellet as obtained by centrifugation at 20,000 × g) were extracted with guanidine hydrochloride (GHC) (20 ml; 5 M in 50 mM Tris hydrochloride buffer, pH 7.2) for 18 h and 57°C and centrifuged at 20,000 × g, and the clear supernatant was dialyzed against distilled water or a 10 mM CaCl₂ solution at 20 or 57°C for at least 24 h.
Electrophoresis of intact cells, cell wall fragments, and S layer self-assembly products. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in previous studies (19). For molecular weight determination, a set of high-molecular-weight standards (SDS-6H; Sigma Chemical Co.) was used.

Electron microscopy. Freeze-etching, negative staining, thin-sectioning, and electron microscopy were performed as described (19).

Investigation of net charge of inner and outer face of the S layer. Native cells, S layer self-assembly products, and GHCl-treated cell wall fragments (peptidoglycan-containing sacculi) were labeled with polycaiton ferritin (PCF) at pH 5.7 as described in previous studies (18).

Investigation of arrangement of charged groups on the outer S layer face. Accessible primary amino groups were modified through reaction with glutaraldehyde. For this purpose native cells (0.1 g, wet pellet) were suspended in a solution of glutaraldehyde (10 ml; 0.5% in 0.1 M sodium cacodylate hydrochloride buffer, pH 7.2), and the mixture was stirred at 500 rpm for 20 min at 20°C. After centrifugation at 5,400 x g, the pellet was thoroughly washed with distilled water. Carboxyl groups exposed on the outer S layer face of glutaraldehyde-modified whole cells were subsequently converted into neutral groups by amidation (5). The chemically modified S layer material was used for PCF labeling.

To determine the distance between primary amino groups on the outer S layer face, amino-specific, homobifunctional cross-linkers (23, 33) such as dimethyladipimidate (cross-linking span, 0.9 nm), dimethylpeeliminimide (cross-linking span, 1.0 nm), dimethylsuberimidate (cross-linking span, 1.1 nm), and the N-hydroxysuccinimide ester of suberic acid (cross-linking span, 1.1 nm) were applied to clean cell wall fragments (0.1 g, wet pellet). The cell wall material was mixed with the imideol ester solutions (2 ml; 2% in 0.1 M triethanolamine hydrochloride buffer, pH 8.5) or with an N-hydroxysuccinimide ester solution (2 ml; 1 mM in 0.1 M sodium phosphate buffer, pH 7.2) and incubated for 1 h at 20 or 37°C. The zero-length cross-linker 1-ethyl-3,3′-(dimethyl-aminopropyl) carbodiimide (EDC) can activate free carboxyl groups for reaction with adjacent amino groups, which leads to the formation of amide bridges. For this purpose cell wall fragments (0.1 g, wet pellet) were suspended in distilled water, EDC (60 mg) was added, and the reaction was allowed to proceed at pH 4.75 (21). After application of the different cross-linkers, the SDS-extractable S layer material was analyzed by SDS-PAGE.

Chemical modification of the S layer protein. Cell wall fragments (0.3 g, wet pellet) with the outer face of the S layer lattice accessible and S layer self-assembly products (0.1 g, wet pellet) with the outer and the inner faces of the S layer lattice accessible were used for modification studies.

Amidation of the carboxyl groups. Amidation of the carboxyl groups was performed at pH 4.75 with EDC (60 mg) and glycine methyl ester hydrochloride (1.32 g) (5). After 1 h of reaction time, the suspension was centrifuged at 20,000 x g, and the clear supernatant was discarded. The pellet was washed three times with distilled water. In general, changes in the molecular weight of the S layer protein induced by chemical modification were determined by SDS-PAGE. To extract the modified S layer subunits, cell wall fragments and S layer self-assembly products were incubated with 5 M GHCl, and the extracted subunits were reconstituted as described for native S layer protein. After dialysis, the protein content of the suspension was assayed by the method of Lowry et al. (15). The structure of the S layer material was investigated by negative staining. Both the molecular weight and the amount of GHCl-extractable, modified S layer protein were compared with the results obtained for native S layer material. The amidated S layer material was also used for PCF labeling.

Amidination, acetylation, and succinylation of the primary amino groups. Amidination of the primary amino groups was performed as described (10). The S layer material was treated with ethylacetimidate (10 ml; 1.6% in 0.5 M triethanolamine hydrochloride buffer, pH 8.5) for 2 h at 37°C. For complete amidination, more ethylacetimidate (160 mg) was added, and the reaction was allowed to proceed for 2 h at 37°C. The suspension was treated as described for the amidated S layer material. In addition, the free amino groups of native and amidinated, GHCl-extractable S layer protein were determined by the trinitrobenzenesulfonic acid (TNBS) test (9).

Acetylation of the primary amino groups was performed as described (20). The S layer material was suspended in 0.1 M sodium phosphate buffer (pH 7.4), and acetic anhydride (30 µl) was added six times at 10-min intervals. During the reaction the pH was kept at 7.4 and the mixture was cooled in an ice bath. Subsequently, the suspension was centrifuged at 20,000 x g, and the pellet was treated as described above. The clear supernatant containing the S layer protein liberated during the acetylation procedure was dialyzed for 72 h at 4°C and concentrated by ultrafiltration. The retentate was analyzed by the methods cited above.

Succinylation of the free amino groups was performed in 0.1 M sodium phosphate buffer (pH 8.2) by addition of succinic anhydride (140 mg) (7). After 2 h of reaction time at pH 8.2, the suspension was treated as described for the acetylation procedure.

RESULTS

Charged functional groups on the outer and inner S layer faces. In freeze-etched preparations, intact cells of B. stearothermophilus NRS 1536/3c appeared completely covered with a square lattice. The morphological units showed a center-to-center spacing of 13.8 nm (Fig. 1a). As investigated by negative staining, the isolated subunits were able to reassemble into regularly structured sheets in both the presence and absence of divalent cations (Fig. 2a). The size of the sheet like self-assembly products formed during dialysis strongly depended on the temperature maintained during the in vitro self-assembly process. Sheets of up to 15 µm were obtained at 57°C, whereas considerably smaller sheets (maximum size, 1.5 µm) were formed at 20°C.

In freeze-etched preparations of native, PCF-treated cells, the square lattice was clearly visible (Fig. 1a), indicating that the outer face of the S layer was unable to bind the positively charged marker molecules. Contrary to the results for whole cells, the sheet like self-assembly products were completely labeled with PCF (Fig. 2b). Thin-section preparations demonstrated that they were monolayers (Fig. 3), with both the outer and the inner faces directly contacting the ambient environment. As shown in freeze-etched preparations of GHCl-extracted cell wall fragments, the outer face of the peptidoglycan-containing sacculus was also capable of PCF binding (Fig. 4).

In freeze-etched preparations, the outer face of the S layer was uniformly labeled with PCF after treatment of the whole cells with glutaraldehyde (Fig. 1b). On the average, 1 PCF molecule (12 nm diameter) was bound per morphological unit of the S layer lattice. Obviously, the net negative charge
resulted from the conversion of the exposed amino groups into neutral groups by their reaction with glutaraldehyde. Moreover, the altered binding properties strongly suggested the presence of carboxyl groups. Accordingly, in freeze-etched preparations, the outer face of the S layer of the glutaraldehyde-treated and subsequently carboxyl-amidated whole cells was only sparsely labeled with PCF (Fig. 1c), corresponding to the unspecific background labeling.

After treatment of cell wall fragments with the homobifunctional, amino-specific cross-linkers dimethyladipimidate, dimethylpimelimidate, dimethylsuberimidate, and the N-hydroxysuccinimide ester of suberic acid, no S layer protein could be extracted with SDS. This indicates the formation of covalent linkages between adjacent protomers. According to the cross-linking span, the distance between primary amino groups was in the range of 1 nm. Treatment of cell wall fragments with the zero-length cross-linker EDC also resulted in the introduction of covalent linkages between the constituent subunits of the S layer lattice. The ability of EDC to form amide bridges between adjacent protomers indicates the presence of electrostatically interacting amino and carboxyl groups in the native array.

**Amidation of the carboxyl groups.** All modification procedures were performed on cell wall fragments (S layer bound to the peptidoglycan-containing sacculus) and on S layer self-assembly products. After performing the amidation reaction on cell wall fragments, 60% of the S layer protein was extractable from the peptidoglycan layer with 5 M GHCl. The fact that 40% of the S layer subunits had been cross-linked was attributed to the reaction of EDC-activated carboxyl groups with closely opposed amino groups on adjacent protomers. The EDC-activated carboxyl groups could obviously react with either the amino group of the added glycine methylester or the protein-bound amino groups.

On SDS gels, the native S layer protein migrated as a single band with an apparent molecular weight of 117,000 (Fig. 5, lanes a and b). The apparently decreased molecular weight of the amidated S layer protein (110,000, lane c) was thought to reflect the difference in migration properties due to a more compact protein structure resulting from the introduction of intramolecular cross-links. The presence of an additional weak band at the boundary between the stacking and separation gels (not shown; molecular weight >
PCF binding, showing that the amidation reaction involved only the carboxyl groups originally exposed on the outer face of the S layer lattice. Contrary to this, the carboxyl-amidated S layer self-assembly products were unable to bind the positively charged PCF molecules. Disintegration of the amidated S layer self-assembly products was not achieved with SDS or GHC. Apparently, the EDC-activated carboxyl groups had reacted with protein-bound amino groups on both S layer faces.

**Amidination, acetylation, and succinylation of amino groups.** Amidination of the free amino groups did not alter their positive charge. Ideally, for maximum amidination of amino groups with monofunctional imidoesters, a pH ≥10 is required. Under weakly alkaline conditions (pH 8), cross-linking can occur through activation by the imidoester (11, 23). Since the S layer protein does not tolerate pH 10, the amidination had to be performed at pH 8.5, as suggested previously (11).

Following amidination of cell wall fragments or S layer self-assembly products, only 60% of the S layer protein was extractable with 5 M GHCl. This showed that under the experimental conditions, a remarkable degree of cross-linking had occurred. Considering the cross-linking span of ethylacetimidate, it was deduced that the distance between primary amino groups of adjacent protomers is ca. 0.3 nm. On SDS gels, the amidated SDS-extractable S layer protein showed an apparent molecular weight of 120,000 (Fig. 5, lane d). The presence of a weak protein band at the boundary between the stacking and the separation gel (not shown; molecular weight ≥ 200,000) was attributed to the formation of dimers or higher oligomers. As determined by the TNBS test, the amidinated, GHCl-extractable S-layer protein contained 5 or 8% unmodified, primary amino groups when the amidination was performed on cell wall fragments and S layer self-assembly products, respectively. As observed for native S layer protein, the amidinated S layer protomers reassembled into regularly structured sheets, displaying a square lattice, similar to that shown in Fig. 2a.

Unlike amidination, acetylation converts the positively charged amino groups into neutral acetamido groups. During acetylation, 90% of the S layer protein bound to the peptidoglycan-containing sacculi, and 50% of the S layer protein

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**FIG. 3.** Thin section of sheet like self-assembly products of *B. stearothermophilus* NRS 1536/3c. Bar, 100 nm.

**FIG. 4.** Freeze-etching preparation of GHCl-extracted cell wall fragments (peptidoglycan-containing sacculi) of *B. stearothermophilus* NRS 1536/3c (a) before and (b) after labeling with PCF. Bar, 150 nm.

**FIG. 5.** SDS-PAGE electropherograms of native and chemically modified S layer protein of *B. stearothermophilus* NRS 1536/3c. Lanes: a, whole-cell extract; b, native S layer protein; c, amidated S layer protein; d, amidinated S layer protein; e, acetylated S layer protein remaining in the protein lattice; f, acetylated, liberated S layer protein; g, succinylated S layer protein. Molecular weights are shown to the left.
arranged in S layer self-assembly products was released. In the liberated S layer protein, 7% of the originally free amino groups had escaped acetylation. On SDS gels, a diffuse band (apparent molecular weight, 123,000 to 137,000) was observed (Fig. 5, lane f). The liberated S layer subunits showed no tendency to form aggregates. Evidently, in the absence of primary amino groups, considerable electrostatic repulsion between the isolated protomers occurred, preventing the formation of protein associates. The S layer protomers which were not liberated during the acetylation procedure could be extracted with 5 M HCl. During removal of the disrupting agent, the subunits aggregated into irregularly structured material. In the modified S layer protein, 17% of the originally free primary amino groups had escaped acetylation. These protomers had an apparent molecular weight of 120,000 (Fig. 5, lane e).

Succinylation converted the primary amino groups into negatively charged succinylmonoamido groups. Application of this procedure led to complete disintegration of the S layer lattice. In water-soluble succinylated S layer protein, 7% of the original free amino groups had remained. On SDS gels, it could be separated into three bands with apparent molecular weights of 138,000, 145,000, and 148,000 (Fig. 5, lane g).

DISCUSSION

In vitro self-assembly experiments with S layers of a variety of gram-positive and gram-negative organisms have clearly shown that the information for the formation of the regular arrays and the morphology of the self-assembly products is exclusively determined by the morphology and the binding properties of the lattice protomers (26). From disintegration and reassembly conditions it was concluded that hydrogen bonds, hydrophobic forces, and ionic interactions between the amino and carboxyl groups are responsible for the cohesion of the protomers and their adhesion to the rigid cell wall layer (29).

Recent studies on Lactobacillus buchneri involving chemical modification procedures confirmed that the native charge of the S layer protomers plays a crucial role in the morphogenesis of the crystalline array (17).

In the present study, labeling experiments with PCF indicated that only the inner face of the S layer lattice of B. stearothermophilus NRS 1536/3c, which adheres to the net negatively charged peptidoglycan-containing sacculus, exhibits a net negative charge and that therefore the S layer is a highly polar, anisotropic membrane. These results are in accordance with previous studies on S layers of different members of the family Bacillaceae (27). The negatively charged carboxyl groups exposed on the inner face are obviously involved in the formation of salt bridges between the S layer and the adjacent peptidoglycan sacculus. In fact S layers were found to be capable of binding cations and may function as a natural buffering barrier between the cell and its environment (2, 3). Moreover, S layers were also reported to substitute for the cation binding teichoic or teichuronic acid present in the cell wall of gram-positive bacteria (3, 14). In addition to the carboxyl groups required for adhesion of the protomers to the underlying cell envelope layer, carboxyl groups directly interacting with primary amino groups were found exposed on the outer and inner S layer faces. Since these amino and carboxyl groups are located on adjacent protomers, it can be concluded that their electrostatic interaction is required for the cohesion of the subunits in the two-dimensional protein array. As reported earlier (28), acid treatment (pH 2.2) of cell wall fragments of two closely related Clostridium species converted their regularly structured S layers into layers of random granular substructure. This was presumably induced by protonation of the dissociated carboxyl groups, which led to a breakdown of the electrostatic interactions occurring in the protein lattice.

Experiments with chemically modified S layer protein of B. stearothermophilus NRS 1536/3c confirmed that the native charge of the protomers plays a crucial role in cohesion of subunits as well as in the in vitro self-assembly process of isolated subunits. Amidination of the free amino groups did not interfere with the refolding of the denatured S layer protein and reassembly of the isolated subunits, since the positive charge was preserved. Similar observations were reported for the in vitro self-assembly of the amidinated S layer protein of L. buchneri (17), for tubulin (6), and for the capsid protein of the tobacco mosaic virus (22). Unlike amidination, acetylation of the amino groups and amidation of the carboxyl groups converted the charged groups into neutral groups. In both cases, the native charge pattern of the S layer protomers was significantly altered. As a consequence, the modified subunits lost the ability to reassemble into regularly structured lattices. Alterations of the net charge obviously prevented the refolding of the denatured polypeptide chain or correct alignment of the correctly refolded protomers. Furthermore, conversion of the free amino groups into neutral or negatively charged groups by either acetylation or succinylation led to a remarkable destabilization of the S layer lattice, as seen by the release of modified protomers. The loss of the positive charges after chemical modification is normally linked to an increase in repulsive forces within the polypeptide chain and dissociation of supramolecular structures into their constituent subunits (13). This was also observed for the S layer of B. stearothermophilus NRS 1536/3c.

As reported for the S layers of gram-negative bacteria, electrostatic interactions involving divalent cations are also required for the maintenance of the integrity of these protein arrays. S layer subunits are liberated when the divalent cations are replaced by monovalent cations (4, 12).

Labeling with PCF clearly showed that the outer face of the S layer lattice of B. stearothermophilus NRS 1536/3c did not display a net negative charge. Thus, the S layer surface exposed to the ambient environment has a net charge different from that of the peptidoglycan-containing sacculus, which has been observed for other Bacillaceae members (27). Consequently, the presence of an S layer distal to the peptidoglycan alters the cell surface properties and determines the nature of the interactions between the cell and its environment. Cell adhesion and surface recognition processes in particular would be mediated to a high degree by the exposed functional groups. On the other hand, S layers frequently tend to be lost after prolonged continuous cultivation under optimal laboratory conditions (29). This observation indicates that masking the electronegative peptidoglycan-containing sacculus appears to provide no selective advantage in noncompetitive environments.

Permeability studies showed that S layers of bacteria from different phylogenetic branches of both eubacteria and archaeabacteria function as accurate molecular sieves (31). The molecular weight cutoff level (90% retention) of a particular molecule of known molecular weight of the S layer of B. stearothermophilus NRS 1536/3c was in the range of 45,000, indicating a pore size of 4 to 5 nm. Similar values have been reported for S layers of other Bacillaceae (31; M.
Sára and U. B. Sleytr, submitted for publication). Since the sieving properties of S layers will be determined exclusively by the mass and charge distribution of the protein meshwork, a more detailed study on the importance of charged amino and carboxyl groups for the rejection characteristics and the molecular sieve function of S layers is in progress.

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