Isolation and In Vitro Assembly of the Components of the Outer S Layer of Lampropedia hyalina

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The outermost component of the S layer of Lampropedia hyalina, the punctate layer, is assembled onto an inner perforate layer. The punctate layer is composed of long, tapered cylindrical units centered on p6 symmetry axes and connected by six fine linking arms, joining at the axis of threefold symmetry to create a hexagonal layer with a lattice constant of 25.6 ± 0.5 nm (J. A. Chapman, R. G. E. Murray, and M. R. J. Salton, Proc. R. Soc. London Ser. B 158:498-513, 1963; R. G. E. Murray, Can. J. Microbiol. 9:593-600, 1963). Extraction of cell envelopes with 100 mM Tris buffer (pH 8) containing 2% deoxycholate resulted in the release of several proteins, but left the S layers intact. The punctate layer was then extracted with 3 M guanidine hydrochloride or 6 M urea, leaving the perforate layer intact. This treatment led to the release of three polypeptides with molecular weights of 60,000, 66,000, and 240,000 (60K, 66K, and 240K polypeptides). These three polypeptides reassembled on the perforate layer as a template to form the S-layer complex or self-assembled to form the punctate layer alone after dialysis of the extract against 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.5) containing 10 mM CaCl₂. The self-assemblies were composed of a 240K polypeptide and a 60K polypeptide. The 240K and 60K polypeptides were separated by column chromatography and examined by electron microscopy. The 240K polypeptide appeared in negative stain as a long, flexible structure and assembled into loose arrays with sixfold symmetry with obvious Y-shaped linking elements, while fractions containing both the 60K and 240K polypeptides showed assemblies closely resembling the punctate layer. Immuno-electron microscopy was used to confirm the presence of both the 60K and 240K polypeptides as components of the punctate layer.

Materials and Methods

Organism and growth conditions. L. hyalina UWO 440 (originally obtained from R. E. Hungate, University of California, Davis, and of the same provenance as ATCC 43383) was used throughout and was chosen because it overproduced and shed its S layer into the growth medium. It was grown at 30°C on solid medium containing 0.3% each yeast extract and Bacto-peptone (Difco Laboratories, Detroit, Mich.), 0.05% sodium acetate (pH 7.3), and solidified with 1.5% Bacto-agar (Difco) (YPA agar). After 3 days the cells formed an uninterrupted lawn across the surface of the agar. Cells were also grown in YPA broth, with shaking at 150 rpm, at 30°C (Psychrotherm incubator-shaker; New Brunswick Scientific, New Brunswick, N.J.). Stock cultures were maintained on slants of YPA agar.

Preparation of cell envelopes. Isolation of the S-layer components was initiated from cell envelopes (i.e., a complex of plasma membrane, cell wall, and S layers). All steps were carried out at 4°C. Three-liter broth cultures in early stationary phase (24 h) were centrifuged (3,500 × g, 30 min). The pellet was washed once in 50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] buffer (Sigma Chemical Co., St. Louis, Mo.), pH 7.5, containing 10 mM CaCl₂ and suspended in 25 ml of the same buffer. The cells were disrupted by two passages through a French pressure cell (Aminco) at 16,000 lbs/in² (1 lb/in² = 6.895 kPa). Cell envelopes were sedimented by centrifugation (48,000 × g, 30 min) and washed in 100 mM Tris buffer (pH 8) containing 150 mM NaCl and 2% sodium deoxycholate to remove traces of cytoplasmic membrane and loosely associated proteins, leaving the outer membrane and the S layers.

Isolation of the components of the punctate layer. Cell
envelopes were suspended in 3 M guanidine hydrochloride for 15 min at room temperature to dissolve the punctate layer. The suspension was centrifuged (105,000 × g for 1 h), and the supernatant, which contained the soluble components of the punctate layer, was dialyzed against 10 mM sodium phosphate buffer (pH 7.5). The soluble components of the punctate layer were separated by column chromatography on hydroxyapatite (Bio-Rad Econocolumn, 5 ml bed volume) by elution with an increasing gradient (10 to 200 mM) of sodium phosphate buffer (pH 7.5), with a total volume of 60 ml. Column fractions (1 ml) were collected and assayed for protein concentration with the Coomassie blue reagent.

Immunelectron microscopy. For immunolabeling, sections of L. R. White embeddings were mounted on grids and incubated on a drop of 100 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Tween 20 (Tris-NaCl-BSA-Tween-20) for 15 min to block nonspecific binding sites. The sections were then floated on a drop of a 1:10 dilution of stock IgG (OD280, 2.5) in Tris-NaCl-BSA-Tween-20 for 2 h and washed twice for 3 min each on drops of Tris-NaCl-BSA-Tween-20, followed by 2 h on a 1:20 dilution of protein A colloidal gold in Tris-NaCl-BSA-Tween-20. The nonspecifically adhering protein A-gold was then removed with repeated washes with Tris-NaCl-BSA-Tween-20 and finally with three washes in distilled water. The sections were then stained for 10 min with uranyl acetate and 3 min with lead citrate (31).

Electrophoresis. Discontinuous SDS-PAGE was done by the method of Laemmli (22). Samples were run in a minia-

ture electrophoresis chamber (Hoefer Scientific, San Francisco, Calif.) with a constant current of 12.5 mA for 0.75-

mm-thick slabs and 25 mA for 1.5-mm-thick slabs. For protein staining, gels were fixed and stained in a solution of 7% acetic acid–25% methanol–0.1% Coomassie blue R-250 at room temperature. Gels were destained in several changes of 7% acetic acid–25% methanol.

Electron microscopy. Micrographs were routinely taken with a Philips EM300 or Philips EM400T electron micro-
scope operated at an accelerating voltage of 60 kV.

For routine preparation for thin sectioning, cells were fixed by the method of Burdett and Murray (12). Fixed samples were washed with cacodylate buffer, enrobed in 2% Noble agar (Difco), and stained with 1.0% uranyl acetate (BDH Chemicals, Toronto, Ontario, Canada) in distilled water for 2 h. The samples were dehydrated through a 30 to 100% ethanol series and embedded in Spurr (Marivac Ltd., Halifax, Nova Scotia, Canada) or Vestopal W (Martin Jaeger Co., Geneva, Switzerland) embedding resin. Thin sections were cut on a Reichert OMU2 Ultra Microtome with glass knives and stained with lead citrate and uranyl acetate (31).

Samples for immunoelectron microscopy were fixed in 10 mM sodium phosphate buffer containing 2.5% glutaraldehyde (Polysciences, Warrington, Pa.) and 1% paraformaldehyde (BDH Chemicals) for 1.5 h. Cells to be used in immunolabeling experiments were usually not postfixed in OsO4. Fixed cells were enrobed in 2% Noble agar and washed several times with 10 mM sodium phosphate buffer (pH 7.5). The agar blocks were dehydrated through a graded series of ethanol to 95% ethanol and were infiltrated with L. R. White resin (Bio-Rad) overnight at room temperature. After several changes of the resin, blocks were polymerized at 60°C for 20 h.

Negative stains. To prepare the combined punctate and perforate layers for negative staining, a small amount of cells, grown on YPA agar, was vigorously suspended in a drop of water by aspirating the cells in a Pasteur pipette; this method appeared to release more of the combined layers. Carbon- and Formvar-coated grids were floated on the suspensions for 1 to 2 min and then floated on a drop of 1% ammonium molybdate–0.1% glycerol at pH 7.5 for ca. 2 min. Excess stain was removed by touching the edge of the grid to a torn edge of Whatman no. 1 filter paper. Uranyl acetate (pH 4.5) was not used because it disrupted the punctate layer.
FIG. 1. Electron micrographs showing the arrangement and structure of the S layers of *L. hyalina*. (A) Section showing the components of the envelope. pu, Punctate layer; pe, perforate layer; in, intercalated layer; om, outer membrane; pg, peptidoglycan; pm, plasma membrane. (B) The composite S layers (punctate plus perforate layers) in a negatively stained fragment shed from cells during growth. This shows the array of the spines of the punctate layer; the spaces between the punctate layer units are filled with stain, obliterating a view of linkers or the underlying perforate layer. (C) Isolated fragment of the perforate layer in negative stain, prepared as described in reference 1. Bars, 100 nm.

Effect of detergents on the cell envelope. Envelopes incubated at room temperature for up to 16 h with various nonionic detergents or bile salts still possessed a well-ordered punctate and perforate layer (Table 1), although the membrane components disappeared after detergent extraction of the envelopes. Detergent treatment proved useful as a pretreatment before extraction of the S-layer proteins.

While nonionic detergents and bile salts had no effect on the S layer, most anionic and cationic detergents disrupted the punctate layer, leaving the perforate layer intact. The dipolar-ionic derivative of cholic acid, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate), did not affect the structure of the punctate layer. The punctate layer was disrupted by lithium 3,5-diiodosalicylate (LIS). Several cationic detergents, including cetyltrimethylammonium bromide (CTAB), dodecytrimethylammonium bromide (DTAB), and tetradeutrimethylammonium bromide (TDTAB), selectively disrupted the punctate layer, causing it to form long fibers about 3 nm in thickness (Fig. 2A). These fibers were seen only in extracts with cationic detergents which removed the 60K polypeptide from envelopes (Fig. 3, lane 7). Incubation of the S layer in 100 mM Tris buffer (pH 8.0) containing 10 mM EDTA or 10 mM EGTA disorganized but did not appreciably dissolve the punctate layer (Fig. 2B). Detergent extraction of cell envelopes was assayed by SDS-PAGE of extracted cell envelopes (Fig. 3). Very few of the envelope proteins were dissolved by nonionic detergents. The bile salts (sodium deoxycholate and cholic acid) and the cationic detergents removed more proteins than did the nonionic detergents, but bile salts did not release the 60K polypeptide. Extraction of cell envelopes with 2% deoxycholate offered the best method to remove contaminating proteins while keeping the S layer intact.

Effect of urea and guanidine hydrochloride on cell envelopes. Treatment with urea or guanidine hydrochloride released the punctate layer from cell envelopes. The predominant polypeptides dissolved by urea and guanidine hydrochloride were 32K, 60K, 66K, and 240K polypeptides (as shown in a preliminary study [2]). Electron microscopy

TABLE 1. Effect of various treatments on the integrity of the punctate layer

| Treatment (conc)          | Punctate layer     |
|---------------------------|--------------------|
| Distilled water           | Intact             |
| HEPES buffer (pH 7.5) (50 mM) | Intact             |
| HEPES buffer (pH 7.5) (50 mM) + CaCl₂ (10 mM) | Intact             |
| Tween 20 (2%)             | Intact             |
| Tween 80 (2%)             | Intact             |
| Brij 58 (2%)              | Intact             |
| CHAPS (2%)                | Intact             |
| Sodium deoxycholate (2%)  | Intact             |
| Cholic acid (2%)          | Intact             |
| SDS (2%)                  | Soluble            |
| DTAB (2%)                 | Formation of strands |
| CTAB (2%)                 | Formation of strands |
| TDTAB (2%)                | Formation of strands |
| LIS (5%)                  | Dissolved          |
| Guanidine hydrochloride (6 M) | Dissolved          |
| Urea (6 M)                | Dissolved          |
| EDTA (20 mM)              | Disorganized       |
| EGTA (20 mM)              | Disorganized       |

* Envelopes were incubated for 1 h at room temperature and examined by electron microscopy after negative staining. Overnight incubation showed no change.
indicated that extraction of cell envelopes with 3 M guanidine hydrochloride or 6 M urea caused disappearance of the punctate layer and partial solubilization of the perforate layer, the latter effect leading to the appearance of the 32K polypeptide (1). The insoluble residue of the envelope remaining after extraction with 3 M guanidine hydrochloride consisted of perforate layer and outer membrane pieces with strands, perhaps from the intercalated layer, radiating from them (Fig. 4A). Extraction with 3 M urea led to only partial dissolution of the punctate layer (Fig. 4B), which was easily recognized because of its distinctive structure (1, 13, 24).

**Fractionation of the components.** The components of the punctate layer were obtained in solution by treating sodium deoxycholate-extracted cell envelopes in 3 M guanidine hydrochloride for 15 min at room temperature. The suspension was centrifuged, and the supernatant containing the soluble punctate layer was dialyzed overnight versus 10 mM sodium phosphate buffer (pH 7.5) and fractionated on a hydroxyapatite column. The bound proteins were eluted with a linear gradient of sodium phosphate. Four peaks were obtained (Fig. 5A). Peak 1 contained small amounts of protein which did not adsorb to the hydroxyapatite. The remaining peaks contained the components of the punctate layer. SDS-PAGE (Fig. 5B) indicated that peak 2 (fraction 25) contained the isolated 240K polypeptide, peak 3 contained predominantly the 66K polypeptide, and peak 4 contained a mixture of the 240K and 60K polypeptides.

Negative stains of samples from these fractions showed distinctive components of the punctate layer; the fractions containing the isolated 240K polypeptide contained long, slender, and slightly curved structures (Fig. 6A). Fractions containing both the 60K and the 240K proteins contained assemblies which closely resembled the native punctate layer. The center of each unit cell had a well-defined stain-excluding region in the shape of a ring, and these rings were connected by Y-shaped linking elements. In contrast, the assemblies formed by the isolated 240K protein had centers which appeared to contain the junction of six linking elements without the central ring.

Antibody made to the 240K polypeptide reacted only with that protein and showed no reaction, in Western blots, with either the 66K or 60K polypeptide (data not shown; J. W. Austin, Ph.D. thesis, University of Western Ontario, London, Ontario, Canada, 1989). There was, therefore, no indication that the larger protein was a multimeric form of
either or both of the smaller polypeptides. Furthermore, the antibody to the 60K polypeptide did not react with the 240K polypeptide in Western blots.

Immunoelectron microscopy. When sections of *L. hyalina* were labeled with anti-240K or anti-60K IgG, the antibody bound specifically to the punctate layer (Fig. 7A and B). All dilutions of IgG could be used without nonspecific binding to the resin or other cell constituents. Some label was found in the cytoplasm, and clusters of gold particles were often observed in the area of the cytoplasmic membrane or periplasm.

Since fixation in aldehydes without postfixation in OsO₄ gave poor contrast for the cell envelope layers, cells were processed for section immunolabeling with postfixation in 1% OsO₄ in 10 mM sodium phosphate buffer (pH 7.5). The cell envelope layers were then stainable with lead citrate and uranyl acetate. Cells fixed in 1% OsO₄ labeled intensely with anti-240K IgG but did not label with anti-60K IgG, suggesting that the epitopes on the 60K polypeptide were destroyed by osmium tetroxide.

**Reassembly of the punctate layer.** When the soluble punctate layer proteins were mixed with intact perforate layer

![Image](http://jb.asm.org/)

**FIG. 4.** Effect of urea and guanidine hydrochloride on the structure of cell envelopes. Extraction of the punctate layer was more effective with guanidine hydrochloride than with urea. (A) Electron micrograph of a negatively stained preparation of cell envelopes after extraction with 3 M guanidine hydrochloride. The punctate layer is in large part destroyed, yet the perforate layer remains intact. (B) Electron micrograph of a negatively stained preparation of cell envelopes after extraction with 3 M urea. The punctate layer has been damaged but is still recognizable. Bars, 100 nm.

![Image](http://jb.asm.org/)

**FIG. 5.** Separation of the soluble components of the punctate layer (3 M guanidine hydrochloride extract of envelopes dialyzed against 10 mM sodium phosphate) by column chromatography on hydroxyapatite. (A) Column elution profile obtained (OD₅₉₅) with 1-ml fractions; the absorption peaks are numbered from 1 to 4. (B) SDS-PAGE of the fractions containing protein. Lane 1 is the sample applied to the column; lanes 4 to 20 are fractions 25 to 41 from panel A, respectively, showing a degree of separation of the components of the punctate layer (see text). Positions of the 240K, 66K, and 60K proteins are indicated.
and dialyzed versus 50 mM HEPES buffer (pH 7.5) containing either 10 mM CaCl₂ or 10 mM SrCl₂, the punctate layer reassembled onto the perforate layer (Fig. 8A). Dialysis against 50 mM HEPES buffer (pH 7.5) without divalent cations resulted in very limited reassembly, in the form of small patches (Fig. 8B). Dialysis against 50 mM tetrasodium EDTA in 50 mM HEPES buffer (pH 7.5) did not allow any assembly of the punctate layer.

The punctate layer self-assembled without the perforate layer present as a template when the soluble proteins were dialyzed against 50 mM HEPES buffer (pH 7.5) containing 10 mM CaCl₂ (Fig. 8C). Addition of 150 mM NaCl to the HEPES buffer-Ca²⁺ did not affect the reassembly. The assemblies were collected in a centrifuged pellet and proved to consist almost entirely of the 240K and 60K polypeptides. Reassembled punctate layer could be negatively stained with 1% ammonium molybdate or 1% phosphotungstate; uranyl acetate could not be used because the low pH disrupted the reassemblies.

Very limited reassembly could be obtained by dialysis of the guanidine hydrochloride-soluble extract against 50 mM HEPES buffer (pH 7.5) without added CaCl₂, probably the result of small amounts of calcium remaining in the extract. This calcium may have remained bound to the protein or dissolved at trace levels. Addition of EDTA or EGTA to 50 mM HEPES buffer prevented all reassembly.

**DISCUSSION**

The identification of the components of a visibly complex S layer was done by differential extraction and monitored by electron microscopy, associated with SDS-PAGE analysis of the polypeptides in each fraction. The insolubility of the S layer in several detergents, including sodium deoxycholate, allowed extraction of membrane proteins and loosely associated proteins but left the S layer intact. Further extraction with guanidine hydrochloride or urea dissolved the structural proteins of the S layer with little contamination from other envelope proteins. It is not unusual for nonionic detergents and bile salts to remove specifically some envelope proteins without affecting the S layer, because these detergents do not usually affect protein-protein interactions and do not usually denature proteins (23). The S layers of *Aeromonas salmonicida* A400 and A461 (18) and *Aqua spirillum serpens* VHA (20) also are not soluble in sodium deoxycholate.

The punctate layer was selectively extracted from cell envelopes by urea or guanidine hydrochloride; the latter was preferable because it extracted predominantly the punctate layer, leaving the perforate layer intact. The guanidine hydrochloride extract contained three polypeptides (60K, 66K, and 240K polypeptides). Reassemblies of the punctate layer showed that two of these three polypeptides, the 60K and 240K polypeptides, were essential to that structure. The precise role of the 66K polypeptide in the punctate layer has not been determined, but preliminary evidence indicates that the reassemblies of the punctate layer, formed by using the perforate layer as a template, contained the 66K polypeptide in addition to the 60K and 240K polypeptides. Sequential assembly-dissolution-assembly experiments were persuasive that these were the necessary components of the S-layer complex. Immunocytochemical studies with antibodies to both the 60K and 240K polypeptides showed that the punctate layer contains both of these polypeptides.

Chromatography on hydroxyapatite allowed separation of
the 240K, 66K, and 60K polypeptides in dilute mixtures of unassembled punctate layer components. The eluted protein was concentrated, and this probably facilitated the observed reassembly of the isolated components. The fractions containing the isolated 240K polypeptide showed long, slightly curved molecules in negative stain. The limited and loose assemblies of these were convincing evidence that this molecule comprises both the delicate Y-shaped linking ele-

FIG. 7. Immunolabel electron microscopy of the punctate layer. Thin sections of *L. hyalina* were labeled with antibodies (IgG) specific to the 60K and the 240K polypeptides; binding was detected with protein A complexed to 5-nm colloidal gold particles. The results show that these polypeptides are components of the S layer. (A) Section labeled with anti-60K IgG. (B) Section labeled with anti-240K IgG. Bars, 100 nm.

FIG. 8. Reassembly of the punctate layer. (A) Reassembly onto the isolated perforate layer in 50 mM HEPES buffer with addition of 10 mM CaCl₂. (B) Reassembly onto the isolated perforate layer in 50 mM HEPES buffer without divalent cations. Note the assembly of small patches of the punctate layer on the perforate layer. (C) Reassembly of the punctate layer without a template. The soluble components of the punctate layer were dialyzed against 50 mM HEPES buffer with addition of 10 mM CaCl₂. Bars, 100 nm.
ments and the spines of the punctate layer but not the central structure of the natural unit. When the 60K protein was present, the reassembly was enhanced and closely resembled the native punctate layer. The 66K polypeptide was only incorporated in self-assemblies of the punctate layer when the perforate layer was present. This suggests that the 66K polypeptide attaches and forms a bridge between the punctate and perforate layers. While addition of the perforate layer as a template improved the reassembly of the punctate layer, the two layers exhibited a degree of independence. This is in contrast to the behavior of the outer layers of both Aquaspirillum serpens MW5 (19) and Bacillus brevis (36, 39), which fail to reassemble into even small patches if the inner layer is not present as a template.

Addition of exogenous CaCl2 assisted but was not necessary for assembly of the punctate layer; however, chelation of Ca2+ with EDTA or EGTA inhibited reassembly and denatured the punctate layer. It is likely that enough Ca2+ remained bound to the soluble proteins to allow proper reassembly, and when this Ca2+ was removed by chelation, the proteins were unable to assemble into the punctate layer. It seems probable that Ca2+ contributes to the conformation of the components and thus indirectly contributes to assembly. The presence of Ca2+ has been found to be essential for the assembly of the S layers of A. serpens (9–11, 19, 21), A. putridiconchylium (4, 5), A. metamorphum (3), Aquaspirillum “Ordal” (6), and Azotobacter vinelandii (7, 15).

Several bacteria possess double S layers, including A. metamorphum (3), Aquaspirillum “Ordal” (6), A. serpens MW5 (19, 34), A. sinusum (S. Smith, master’s thesis, University of Western Ontario, London, Ontario, Canada, 1989), Nitrocystis oceanus (30, 38), and B. brevis (36, 39). Even more complex S layers exist; Walsby’s “square bacterium” is covered by three S layers (17, 27, 35, 37). The superficial wall structure of Aquaspirillum “Ordal” consists of five layers, in which there are two S layers, including an inner S layer arranged in tetragonal symmetry and an outer hexagonal S layer (6). When multiple S layers have been characterized biochemically, they have been found to have different polypeptide subunits in each layer, e.g., A. serpens MW5 (19) and B. brevis (36, 39).

Complex morphological units in S layers have been described, notably those of the S layer of Flexibacter polymorphus (32), which was shown to consist of four major polypeptides, 80K, 74K, 29K, and 13K. Others have been observed, but not characterized biochemically, on the surfaces of Amoebobacter bacillus (14), Methylo monas albus (30), and Chromatium inderi (14, 30). The freshwater photosynthetic bacteria Chromatium weissii and C. okennii possess hexagonal S layers made up of hollow cone-shaped units 25 nm long and 13 nm in diameter, with a center-to-center spacing of 19 nm (16).

L. hyalina has an unusual and complex S layer that is amenable to isolation and analysis. The function of this complex structure remains unknown. Variants of L. hyalina which lack the S layer grow as individual cells rather than in tablets (24), suggesting that the S layer somehow holds the cells together within a tablet. These naked variants are susceptible to predation by Bdellovibrio bacteriovorus, while the covered strains are resistant to B. bacteriovorus (S. J. Lynch, master’s thesis, University of Western Ontario, London, Ontario, Canada, 1972). While the S layer is not required for growth in culture, the layer must have an important function in nature, especially when one considers the large investment in energy required to synthesize such a complex S layer.

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