SELF-ASSEMBLY, ADHESION, AND CHEMICAL PROPERTIES OF TETRAGONARLY ARRAYED S-LAYER PROTEINS OF CLOSTRIDIUM

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The S layer of Clostridium difficile GAI 0714, which was composed of squarely arrayed, two-subunit proteins with respective molecular weights of 32 kDa and 45 kDa, was examined for its morphological, physico-chemical, and biological properties. Optical diffraction analysis of the S layer showed that the surface-arrayed rhombus had four sides of 8.1 nm with interior angles of 88°. The two proteins were heterogeneous to each other in respect to the first ten N-terminal amino acid sequences except for residues No’s. 4 and 9. Self-assembly of the subunit proteins into a regular array was dependent on such divalent cations as Ca²⁺ or Zn²⁺, but not Ba²⁺ or Mg²⁺. When the mixture, made of purified 32 and 45 kDa subunits in an equal concentration ratio on a protein basis was submitted to self-assembly, flattened paracrystalline sheetlike fragments were generated. However, neither sheetlike fragments nor regular arrays was observed for any self-assembled products derived from each subunit alone or their quantitatively heterologous mixtures. Intact cells of the organism adhered significantly to HeLa cells or mouse fibroblast 929 cells, whereas 8 M urea- or 4 M guanidinehydrochloride-extracted bacteria, from which both S layer protein subunits were removed, scarcely adhered to both cells. Fab fragments of anti-32 kDa or anti-45 kDa subunits antibodies effectively inhibited adhesion of the organism to HeLa cells, suggesting that the S layer had a direct role in adhesion.

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Many gram-positive and gram-negative bacteria, including archaeabacteria, possess a superficial regularly arrayed structure, designated as the S layer (12, 13). The S layer is composed of a single protein or glycoprotein species of molecular weights of 40 to 200 kDa (10) and the subunits are generally noncovalently attached either to each other or to underlying cell wall components. Therefore, such chaotropic agents as urea or guanidine hydrochloride (GHCl) were preferably used for the solubilization or extraction of S layer or S-layer proteins (12). Due to external surface localization, the S layer is directly involved in the interactions between the cell and its environment and consequently play an important role in host-parasite interaction, ranging from adherence to immune reactions (8, 17).

Clostridium difficile GAI 0714, an important pathogen causing human pseudomembranous colitis, has a squarely arrayed S layer, which is composed of two protein subunits with respective molecular weights of 32 kDa and 45 kDa (15). Recently, we reported concerning to the purification and characterization of S-layer proteins of C. difficile GAI 0714 and demonstrated that the two subunits were dissimilar in their chemical and immunological properties (16).

The present paper describes a structural unit and the self-assembly of the S-layer proteins, together with their physicochemical properties. Furthermore, the relationship between S-layer proteins and bacterial adherence to HeLa cells is discussed.

MATERIALS AND METHODS

**Bacterial strains and growth conditions.** C. difficile GAI 0714 was grown in GYPT medium (14) at 37°C overnight. The cells were harvested by centrifugation at 10,000 × g for 15 min and washed with cold water.

**Preparation of cell walls.** The cell walls of the organism were prepared by the same method as described previously (15). The cell wall preparation, which was obtained by differential centrifugation, was rinsed up by sequential treatments with 1 M NaCl, 2% Triton X-100, and cold water. The cell wall preparation was stored at −20°C until use.

**Purification of S-layer proteins.** All procedures used were described in a previous paper (16). Briefly, the cell wall preparations from 10 l culture of C. difficile GAI 0714 were suspended in 50 mM Tris-HCl buffer (pH 8.3) containing 8 M urea (urea buffer) and extracted by stirring for 1 h at room temperature before ultracentrifugation at 100,000 × g for 30 min. The supernatant (8U-sup) was applied on a column (2.5 × 30 cm) of DEAE-Sepharose CL-6B (Pharmacia LKB biotechnology, Uppsala, Sweden) equilibrated with the urea buffer, and the S layer proteins, 32 kDa, and 45 kDa proteins, were separately eluted with a linear gradient of 0 to 0.3 M NaCl in the urea buffer. After rechromatography on the same column, both proteins were finally purified by gel filtration using TSY G3000 SW column (7.5 mm × 60 cm; TOSOH Co., Tokyo, Japan) coupled to high performance liquid chromatography (HPLC; Hitachi 6200 system, Hitachi Co., Tokyo, Japan).
Preparation of the native S-layer fragments. The cell wall pellets were suspended in 50 mM Tris-HCl buffer (pH 7.2) containing 10 mM CaCl₂ and 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and allowed to autolize at 37°C for 30 min. After the autolysis, the cell wall residues were removed by centrifugation at 3,000 × g for 15 min. The S-layer fragments thus obtained in the pellet were rinsed up with cold water containing 10 mM CaCl₂ and negatively stained for electron microscopical observation.

Electron microscope. Negative staining was carried out with 4% uranyl acetate (pH 4.1) as described previously (16). Specimens were examined with a Hitachi HU-11E electron microscope operating at 75 kV.

Optical diffraction. The optical diffraction was measured with an Eiko optical diffractometer (Eiko Engineering Co., Tokyo, Japan) according to the method of Klug and Rosier (5). The negatively stained image of the S-layer fragments was reversed onto photographic film by contact printing. Optical diffraction was carried out upon areas of the reversed images.

In vitro self-assembly of S-layer proteins. The 8U-sup at a concentration of 500 µg protein/ml was dialyzed at 4°C overnight against the dialysis buffer (50 mM Tris-HCl buffer, pH 7.4) in the presence or absence of CaCl₂, ZnCl₂, BaCl₂, MgCl₂ or KCl and LiCl. In the case of the purified 32 and 45 kDa subunits were mixed with each other in several combination of concentration ratios of 0.5 to 2-fold or each subunit alone on a protein basis (Table 1) and dialyzed as described above. The dialyzates were centrifuged at 8,000 × g for 10 min and the pelleted materials were rinsed up with cold water before negative staining for electron microscope observation.

In vitro adhesion assay. The adhesion of C. difficile GAI 0714 to HeLa cells was carried out according to the method of Wood-Helie et al. (16). Both types of

<table>
<thead>
<tr>
<th>Ratio of subunits</th>
<th>Sheet</th>
<th>Self-assembly</th>
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<tbody>
<tr>
<td>32 kDa : 45 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 : 0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>0 : 1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1 : 1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2 : 1</td>
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<td>No</td>
</tr>
<tr>
<td>1 : 2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Control (8U-sup)</td>
<td></td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Protein concentration of each subunits was adjusted to 2 mg/ml. Total volumes of the reaction mixtures changed from 0.1 ml to 0.3 ml depending on the combination ratios.

* The reaction mixtures were dialyzed against 50 mM Tris/HCl buffer (pH 7.4), and the products were negatively stained before an electron microscopic examination.

* The 8U-sup was adjusted to 0.5 mg protein/ml and allowed to self-assembly as described in the text.

"Yes" and "No" mean a positive and negative results, respectively.
cells were maintained in Han’s F12 media with 10% fetal calf serum (FCS) (Gibco, USA) and penicillin/streptomycin (Difco, USA), 50 units and 50 μg/ml, respectively. Approximately 5 × 10^2 cells were added to each well of an eight-chambered tissue culture slide (Lab. Tek. Products, USA). Before the assay, the media were changed to Han’s F12 with 1% FCS without antibiotics. *C. difficile* GAI 0714 cells grown overnight in GYPT medium were harvested by centrifugation and suspended in Eagle’s minimal essential media (MEM) (Flow Labs, USA) with 1% FCS at approximately 1 × 10^9 organisms/ml. Each well of the chambered slide was mixed with 0.1 ml of the bacterial suspension and incubated at 37°C for 15 min. Slides were then freed and immersed in MEM once and flushed with a pasteur pipette once. The cells on the slides were fixed with ethanol and stained with Gimsa stain. For the assay of adhesion, 50 HeLa cells were examined for a count of the average number of adhered bacterial cells per HeLa cell. Similar procedures were carried out glutaraldehyde-fixed (at 4°C overnight) bacterial cells.

Inhibition of bacterial adhesion to HeLa cells with Fab fragments was performed as follows: 0.1 ml of bacterial suspension was mixed with 0.1 ml of Fab fragment, and the mixture was incubated at 37°C for 15 min before adding to HeLa cells and followed by an additional 15 min incubation.

**Preparation of Fab fragments.** Fab fragments were prepared from rabbits antisera against, respectively, 32 kDa and 45 kDa proteins by the method of Poter (11), using immunoglobulin G, which was purified according to the method of Murphy and Bartos (9).

**SDS-PAGE.** Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a separation gel of 12% acrylamide (16). Samples were routinely heated in the sample buffer at 100°C for 5 min according to the method of Laemmli (6). The gels were stained and fixed in 0.1% Coomassie brilliant blue R 250–25% methanol–10% acetate.

**Chemicals.** N-terminal amino acid sequence was determined using a model 470 gas phase sequenater (Applied Biosystem, Foster city, CA, USA) with an in-line Applied Biosystem model 120 PTH amino acid analyzer. Protein was determined by the method of Lowry et al. (7) using bovine serum albumin (Sigma) as a standard.

**RESULTS AND DISCUSSION**

**Morphological properties of the S layer**

Negatively stained cell walls of *C. difficile* GAI 0714 exhibited the characteristic fold edge seen as light margins composed of the subunits arranged like the teeth of a saw (Fig. 1A). When the cell walls were extracted with 8 M urea the teethlike structure completely disappeared, leaving underlying peptidoglycan components with a smooth surface (Fig. 1B). The tetragonally arrayed structure of the S layer was clearly shown by electron microscopic observation on partially autolyzed cell wall fragments (Fig. 2A). Optical diffraction analysis demonstrated
that a morphological unit of the S layer was composed of a rhombus with side measuring 8.1 nm and interior angles of 88° (Fig. 2A, Inset). By SDS-PAGE analysis, the protein components 32 kDa and 45 kDa were found as the wall fragments (Fig. 2B).

**In vitro self-assembly of the S layer**

One of the characteristic features of the bacterial S layer is a self-assembling ability in vitro (9). When the 8U-sup was dialyzed against 50 mM Tris-HCl buffer, pH 7.4, supplemented with 10 mM CaCl₂, sheetlike fragments possessing a tetragonally arrayed pattern (Fig. 3A), which was characteristic for the native S-layer fragment (Fig. 2A), were recovered from the dialysate. In contrast, amorphous debris was separated from the dialysis when the Tris dialysis buffer was used (data

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**Fig. 1.** Negatively stained native cell wall fragments (A) and 8 M urea-extracted cell wall fragments (B) of *C. difficile* GAI 0714. Bar, 100 nm.

**Fig. 2.** A, negatively stained partially autolyzed cell wall fragments of *C. difficile* GAI 0714. Inset: typical optical diffraction pattern from the field circled in A. Bar, 100 nm. B. SDS-PAGE of the wall preparation.
not shown). Dialysis against the dialysis buffer supplemented with 10 mM ZnCl₂ generated some assembled fragments having regular lattice patterns (Fig. 3B). In the case of the dialysis against 10 mM BaCl₂ (Fig. 3C) or 10 mM MgCl₂, no fragments having a regular lattice structure were observed. No self-assembled layer or fragment was separated from the dialysate against monovalent cations, including KCl and NaCl (data not shown). These results demonstrated that Ca²⁺ or Zn²⁺ is an essential factor in self-assembly of the S layer in vitro. Azotobacter vinelandii (2) grown in a defined medium lacking calcium did not exhibit the tetragonally arranged surface layer that was present on calcium-sufficient cells. Aquaspirillum serpens MW5 specifically requires Ca²⁺ for S layer assembly (4), whereas Aq. serpens VHA was able to self-assemble in the presence of both Mg²⁺ and Ca²⁺ (1). The requirement of divalent cations for S-layer assembly may be related to the generally acidic nature of proteins constituting the arrays.

The two purified subunits, 32 kDa and 45 kDa, were also tested for their ability to reform the regular array under the condition of supplementation with 10 mM CaCl₂. These results are listed in Table 1. When the two subunits were mixed in an equal concentration ratio and subjected to self-assembly, large or small flattened sheetlike fragments were observed (Fig. 4A), although no regular arrayed structures could be seen on the sheets. Neither regular arrays nor sheetlike fragments were observed in the dialyzed products derived from either dialyzed mixtures or the single subunit alone (data not shown). It seems likely that in vitro self-assembly of
the two subunits into the S layer requires one or more unknown essential factors contained in 8U-sup. Alternatively, the subunits lost their self-assembling ability during their isolation procedures. For a quantitative understanding of the relation between the two protein subunits in self-assembly of the S layer, three self-assembled products prepared from the partially autolyzed cell walls, the 8U-sup, the mixture (1:1) of the purified 32 kDa, and 45 kDa, were compared by SDS-PAGE (Fig. 4B). All three products were composed of two protein subunits corresponding to the 32 kDa and 45 kDa at a rough rate of 1:1. These results strongly suggest that a quantitative ratio between the two subunits plays an important role for the reconstitution.

**Chemical properties of S-layer proteins**

The S layer of *C. difficile* GAI 0714 is composed of 32 kDa and 45 kDa proteins (14, 15). On the other hand, when the molecular weights of the two proteins were estimated in comparison with marker proteins on HPLC gel filtration, their molecular weights were calculated to be 61 kDa for the 32 kDa and 99 kDa for the 45 kDa (Fig. 5), suggesting that the two proteins exhibit dimeric forms in the absence of SDS. The molecular weight values estimated in the presence of 1 M urea seem to be incorrect, but the molecular weights of the marker proteins used fell into a linear log plot, and the resulting determinations agreed with separations in the absence of 1 M urea on the same column.

The N-terminal amino acid sequence of the first 10 amino acid residues of the
two S-layer proteins were listed in Table 2, along with corresponding sequences for several other bacterial S layer proteins. A marked dissimilarity in the amino acid sequence was found between the 32 and 45 kDa proteins, in which identical residues were only two: the residue numbers of 4 (glutamic acid) and 9 (alanine). The first ten amino acids of the 45 kDa proteins consisted of six hydrophobic amino acids (M, 2V, I, 2A) and four acidic ones (2E, 2D), whereas those of the 32 kDa consisted rather of miscellaneous amino acids, including four polar but uncharged (N, S, T, G), three acidic (D, 2E), two basic (H, R), and one hydrophobic (A).

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**Table 2. N-terminal amino acid sequences of S-layer proteins from *C. difficile* GAI 0714 and other bacteria.**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Residue</th>
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<tbody>
<tr>
<td><em>C. difficile</em> GAI 714</td>
<td>D N S E T G E H A R</td>
</tr>
<tr>
<td>32 kDa</td>
<td>M V I E V D A E A D</td>
</tr>
<tr>
<td>45 kDa</td>
<td>A P K D G I Y I G G</td>
</tr>
<tr>
<td><em>B. brevis</em> 47 (18)</td>
<td>D V V I G P N D N T</td>
</tr>
<tr>
<td><em>A. salmonicida</em> A450 (10)</td>
<td>M I S K S E V S E L</td>
</tr>
<tr>
<td><em>C. fetus</em> VC119 (3)</td>
<td>M I S K S E V S E L</td>
</tr>
</tbody>
</table>

*Amino acid residues are designated by the single-letter nomenclature. Common amino acids in the sequences are blocked. The number in parentheses indicates the reference in the text.*
amino acids. It can be said in general that the 45 kDa protein should be more hydrophobic than the 32 kDa protein. This is well agreed with the fact that the 32 kDa was water-soluble, whereas the 45 kDa was water-insoluble (15).

Remarkable differences in N-terminal amino acid sequences were also observed among several bacterial S layers, listed in Table 2. These results probably reflect strain-specific taxonomical features of eubacterial S layer (13). If the diversity among S layers suggests a nonconservative character of bacterial cell surface components, then sequence homology studies on S-layer proteins appear essential to elucidation of the taxonomical significance of the S layer as well as the structural, functional, and evolutionary relationships among S layers.

**Participation of the S layer in bacterial adhesion to HeLa cells**

According to the report of Wood-Helie et al. (19), *C. difficile* strains adhered significantly to human embryonic intestinal cells and adult colon cells. Here, whether or not the S layer or its subunit proteins participates in bacterial adherence to HeLa cells was examined. Overnight cultures of *C. difficile* GAI 0714 in GYPT medium were harvested and suspended in MEM containing 1% FCS, and adherence tests were carried out according to the procedure described in Materials and Methods. The assay was run in triplicate. The results showed that 34.4 bacterial cells adhered per HeLa cell when intact bacteria were used (Fig. 6A), whereas 2.2 bacterial cells adhered per HeLa cells when glutaraldehyde-fixed cells were tested.

![Fig. 6. Adherence of *C. difficile* GAI0714 viable cells (A) and GA-fixed cells (B) to HeLa cells.](image)

<table>
<thead>
<tr>
<th>Fab fragment</th>
<th>No. of bacterial cells attached/HeLa cell*</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>32.5</td>
</tr>
<tr>
<td>Fab-32</td>
<td>12.2</td>
</tr>
<tr>
<td>Fab-45</td>
<td>14.7</td>
</tr>
<tr>
<td>Fab-32 + Fab-45</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* Each number is the arithmetic mean of three determinations.
Fab fragments prepared from antisera against the 32 kDa or 45 kDa protein were used to test for their ability to inhibit bacterial attachment to HeLa cells. Fab fragments were selected for this study to avoid bacterial agglutination. The results are summarized in Table 3. Fab fragments from the 32 kDa antiserum (Fab-32) or the 45 kDa antiserum (Fab-45) significantly inhibited bacterial attachments to levels of 38% or 45%, respectively. Fab-32 showed a tendency to be more effective than Fab-45 in the inhibitory reaction. When the mixture of the two Fab fragments were used for the inhibition test, a considerable inhibitory effect was seen. Thus, coating of the S layer with Fab fragments resulted in neutralization of adhesiveness of the bacteria. These data demonstrate an intrinsic role for the S layer or its constituent proteins in in vitro bacterial adhesion.

REFERENCES
15) Takumi, K., Takeoka, A., and Kawata, T., Purification and immunochemical properties of a wall


