Structures of Two Different Surface Layers Found in Six Bacteroides Strains

AGNETA SJÖGREN,1 SVEN HOVMÖLLER,1* GEORGE FARRANTS,1† HELENA RANTA,2,3 MARKUS HAAPASALO,2,4† KARI RANTA,2 and KARI LOUNATMAA5,6

Department of Structural Chemistry, University of Stockholm, S-106 91 Stockholm, Sweden,1 and Department of Cariology,7 Department of Bacteriology and Immunology,4 Department of General Microbiology,5 Department of Electron Microscopy, University of Helsinki,6 and National Public Health Institute,3 SF-00280 Helsinki, Finland

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The structures of crystalline layers from six Bacteroides strains were studied by electron microscopy. Two different hexagonal crystalline surface layers were found, one with a unit cell spacing of 21.5 nm and another with a spacing of 7.7 nm. A three-dimensional structure of the 21.5-nm layer and a two-dimensional projection of the 7.7-nm layer were determined to 3.0- and 3.8-nm resolution, respectively, by computerized image processing of electron micrographs. Both of these two crystalline layers were found in all of the six strains studied: B. pentosaceus NP333T and WPH61, B. capillus ATCC 33690T and ATCC 33691, and B. buccae ATCC 33574T and ES57. This further supports the identity of B. pentosaceus, B. capillus, and B. buccae as suggested by M. Haapasalo, K. Lounatmaa, H. Ranta, H. Shah, and K. Ranta (Int. J. Syst. Bacteriol. 35:65–72, 1985). The surface layer with 21.5-nm spacing is an intricate network with two classes of pores through the layer.

Many bacteria possess crystalline external surface layers (S-layers) outside their cell envelope (11). These S-layers completely cover the bacterial surface, even during cell duplication. The S-layers are composed of protein subunits forming an open network with pores running through the two-dimensional crystalline sheet. Several suggestions have been made for the function of such S-layers: a protective function acting as a barrier against external or internal factors, a supporting framework involved in maintaining cell shape, or a promotor for cell adhesion (surface recognition) (11). In the only case in which the function of an S-layer has been extensively studied, Aeromonas salmonicida, which causes the lethal disease furunculosis in fish, it was shown that only strains with S-layer are virulent; mutants devoid of S-layer were unable to multiply in the fish (7).

Structural knowledge about S-layers is in most cases limited to two-dimensional projections; three-dimensional structures of this type of crystalline protein layer have been published for only three species—S-layers in Sulfolobus acidocaldarius (3) and Synechocystis sp. CLII (8) and the outer envelope of Chlamydia trachomatis (2). To understand the principles of construction, function, and assembly of these layers, it is essential to have more information about the three-dimensional arrangement of the proteins.

Several Bacteroides species are frequently found in human periapical osteitis (infection of the jawbone). So far, three of these, B. capillus, B. buccae, and B. pentosaceus, have been shown to possess crystalline S-layers (Fig. 1). These bacteria are anaerobic, gram-negative rods with dimensions approximately 0.6 by 1.6 μm. We have recently suggested that these three species should be combined to a single species based on the homogeneity of various biochemical characteristics. The species are easily differentiated from other bile-sensitive Bacteroides spp. by their capability to ferment the pentose sugars arabinose and xylose and from B. oris by different DNA base composition and key enzyme electrophoretic mobilities (4). The structures of S-layers represent a further structural characteristic which can differentiate bacterial species. We have studied the structures of S-layers from five reference strains: B. pentosaceus NP333T and WPH61, B. capillus ATCC 33690T and ATCC 33691, B. buccae ATCC 33574T, and our own isolate, B. buccae ES57.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Our own isolate, B. buccae ES57, was isolated from a root canal infection as previously described (10). B. pentosaceus NP333T and WPH61 were received from Dr. H. Shah, London Hospital Medical College. The reference strains B. capillus ATCC 33690T and ATCC 33691 and B. buccae ATCC 33574T were obtained as freeze-dried cultures from the American Type Culture Collection, Rockville, Md. Pure cultures were preserved in glycerol-milk at −70°C for this study. For ultrastructural studies, all strains were grown on bacteriological agar no. 1 (Oxoid Ltd., London, England) supplemented with 5% horse blood, 5 g of yeast extract per liter, 500 mg of cysteine per liter, and 2 g of glucose per liter (MCG agar) for 3 days in an anaerobic chamber (Anaerobic system, model 1024; Forma Scientific). A gas mixture of 10% H2–5% CO2–85% N2 was used.

Isolation of cell wall components. Cells from the agar plates were suspended in 10 mM EDTA (pH 7.5) and mechanically broken with 0.2-mm glass beads for 5 to 15 min at room temperature. Cells were sedimented by centrifugation (1,000 x g for 10 min), and cell wall fragments were isolated by centrifugation at 10,000 x g for 10 min after fixation with 1% glutaraldehyde at 20°C for 1 h.

Electron microscopy. (i) Thin sections. For thin sections, cells were collected from the plates and fixed with 3% glutaraldehyde for 1 h at 20°C (Leiras, Turku, Finland) in 0.1 M sodium phosphate buffer (pH 7.2). The cells were postfixed for 2 h at 20°C with 1% osmium tetroxide in the same buffer. Sections were obtained from Ladd LX 112-embedded samples and stained with uranyl acetate and lead citrate in the 2168 Carsberg system (LKB Instruments, Inc.,

* Corresponding author.
† Present address: Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, England.
FIG. 1. Cell envelope of a thin-sectioned cell of B. buccae strain ES57. The S-layer (S), outer membrane (OM), peptidoglycan layer (PG), and cytoplasmic membrane (CM) are indicated. Bar, 200 nm.

Rockville, Md.). Micrographs were taken with a JEOL JEM-100 B operated at 80 kV.

(ii) Negative staining. Cell wall components were stained with 2% phosphotungstic acid (pH 6.5) on carbon-coated copper grids. Electron micrographs of the components were recorded at a magnification of ×38,000 in a Philips 301 electron microscope operating at 80 kV (calibrated against catalase crystals). Tilt series were taken with a eucentric tilt stage with a tilt range of +/−60°.

Computing. Crystalline areas in the images suitable for computer processing were selected by optical diffraction. The information in the micrographs, present as a variation in optical density, was digitized on a Joyce Loebl Microdensitometer 6. For each micrograph, an area of about 1 cm² was scanned on a 256 by 256 raster with a raster point size of 40 by 40 μm. This raster size corresponds to a sampling interval of 1.05 nm in the specimen and is sufficiently fine to preserve information to a resolution of 2.5 nm. The Fourier transforms of the digitized micrographs were calculated on a VAX 11/750 computer, and the amplitude parts of the complex Fourier transforms were displayed on a computer graphics screen. The diffraction patterns were indexed with the aid of a cursor and later refined by a least-squares procedure.

Three-dimensional reconstruction. A three-dimensional structure of the S-layer with 21.5-nm spacing was obtained by combining amplitudes and phases obtained from Fourier transforms of images of B. buccae ES57 taken at different tilt angles relative to the plane of the S-layer by the method of Henderson and Unwin (5). The three-dimensional reconstruction was based on 24 micrographs with tilt angles evenly spaced in the range from −40 to +56°. Each tilted view contained between 69 and 30 reflections (micrographs taken at higher tilt angles contained fewer reflections). The average phase error for the included reflections, compared with those of all other images, ranged from 7.0 to 19.2°. Symmetry relations for amplitudes and phases caused by the p6 symmetry were imposed. Data from the combined tilted images were used to plot variation of amplitude and phase in the Z* direction for each of the 31 unique (h, k, Z*) lattice lines. The resulting 1,188 amplitude and phase pairs from the combined data set were then interpolated by hand on a color graphics screen at intervals of 0.1 nm⁻¹ in Z*, fine enough to be used for membranes with a thickness of up to 10 nm. From this interpolation, 89 unique (h, k, l) reflections were obtained. These reflections were used in a three-dimensional Fourier synthesis on a 32 by 32 by 32 matrix to produce a three-dimensional map of the structure.

RESULTS

Two different types of hexagonal S-layers were found in all six studied strains. Both types of S-layer were found associated with a supporting membrane, presumably the outer membrane. One type of S-layer was frequently found to extend outside the borders of the supporting membrane (Fig. 2a). Very often the supporting membrane was folded over, giving a straight edge along the fold. Sometimes this layer grew into cylinders with a diameter of about 100 nm. This layer was found on the outside of intact bacteria, in both negatively stained and freeze-etched bacteria. The other type of S-layer was also associated with a membrane, often with large protrusions, but this layer never extended outside the edges of the membrane. In rare cases, the two types of crystalline layer were found together on the supporting membrane, indicating that they were both associated with the same membrane (Fig. 2b). The two crystalline forms of S-layer did not exist on top of each other. When they were both found on the same supporting membrane, they were in contact with each other. This indicates that they grow on the same side of the supporting membrane.

The two-sided plane group (6) and cell dimensions were determined from projections of the two types of crystalline layers for the six strains. Computer-calculated Fourier transforms of digitized areas of the two different crystalline layers are shown in Fig. 3. The two crystalline layers proved to have the two-sided plane group p6. The S-layers had unit cell spacings of 21.5 +/− 0.5 nm and 7.7 +/− 0.3 nm, respectively. The projected structures (Fig. 4) obtained by inverse Fourier transformation were identical for the six strains, which supports the suggestion put forward by Haapasalo et al. (4) that these strains are identical. The packing of protein subunits in the S-layer with 21.5-nm spacing can be described as a chicken wire arrangement, with an extra ring of protein inside each hexagon. In the other S-layer, with 7.7-nm spacing, the protein was present hexameric over the sixfold axes, with pores at the threefold axes. These pores had a diameter of approximately 3 nm. It is not yet clear whether the two layers represent two different proteins or one protein which can rearrange from one lattice to the other. Notably, the length of the unit cell of the larger S-layer lattice was very close to three times that of the other layer. Although the two forms of S-layer can coexist on the same supporting membrane (Fig. 2b), analysis by optical diffraction showed that they never occurred on top of each other.

A balsa wood model built from the three-dimensional map of the S-layer with 21.5-nm spacing in B. buccae is shown in Fig. 5. This structure also represents the S-layers of B. pentosaceus and B. cavallis, since they are identical to this resolution. In the map, the protein density extended over 16 sections in the c direction, from sections −7 to 9, giving a total thickness of the S-layer protein of 5 nm. The value for the thickness of the S-layer obtained by observation of thin-sectioned, stained material was 8 nm. This difference could be due to either overestimation in the thin-sectioned material caused by positive staining or by shrinkage in the z direction of the negatively stained S-layer during electron
irradiation. Such shrinkage has been found for stained crystalline membrane proteins, and values of up to 50% for the degree of shrinkage have been reported (1). The structure of the S-layer was an open network with pores occupying more than one-third of the area. There were two distinct classes of pore through the S-layer. In every unit cell, there was one round pore of approximately 5 nm diameter situated around the sixfold axis, and six elongated pores (5 by 2.5 nm) surrounding the larger round pore. The pores were big enough to allow molecules up to the size of small proteins to pass through the S-layer. The elongated pores were skewed, such that one cluster of seven pores (six elongated pores surrounding the central round pore) looked like a cogwheel. The hand of the S-layer is easily visualized as the hand of a cogwheel. The main protein mass was at the border between the cogwheels, with a fine protein structure separating the elongated pores from one another and from the central pore. Protein was situated around the threefold and the twofold axes, with the protein protruding to different sides of the layer at these two positions. The simplest possible unit that can generate the whole S-layer is indicated in Fig. 4a.

A three-dimensional structure determination of the S-layer with 7.7-nm spacing gave no information apart from what
species are highly conserved (9). It is not yet known whether the three-dimensional structure is conserved to the same degree. The symmetry of an S-layer can be described in the same way as for a three-dimensional crystal one unit cell thick, or (which is equivalent) a two-sided plane group. There are 80 two-sided plane groups, but only 17 of them are allowed for chiral molecules such as proteins. Of these 17, only 5 have all molecules facing the same way, namely p1, p2, p3, p4, and p6. The other 12 two-sided plane groups have twofold axes or 2 screw axes in the plane, leading to the inside and outside being identical. Although this is not impossible, it seems rather unlikely, considering the different environments the two sides of an S-layer have, one facing the bacterium and the other the surrounding medium. Of the five most likely symmetries, most of the bacterial S-layers studied show hexagonal p6 symmetry, with a smaller number showing tetragonal p4 symmetry (11). For the comparison of structures of S-layers it can be useful to classify them according to plane group, unit cell size, and the positions of protein and pores relative to the symmetry elements. The structures of the S-layers of the Bacteroides strains were compared with those of the other S-layers and the outer cell envelope for which three-dimensional data are available (Table 1). All these S-layers have the same twosided plane group, p6, but the packing of the protein subunits

was already seen in the projection (Fig. 4b). The main reason for this is that there were only three unique reflections 1, 0, 1, 1, and 2, 0 to this resolution (Fig. 3b).

**DISCUSSION**

Structural studies of S-layers may in the future become a valuable tool in the classification of bacterial species. Relationships between species may be found in the three-dimensional structures or amino acid sequences of the proteins or both. It has been found that the amino acid sequences of S-layers of *A. salmonicidia* from a variety of host fish

![FIG. 4. Two-dimensional projection maps of the two different crystalline layers. (a) S-layer with 21.5-nm unit cell spacing. (b) S-layer with 7.7-nm unit cell spacing. Contours represent protein. One asymmetric unit is indicated for each layer along with the following symmetry elements: , sixfold; , threefold; and , twofold. Bars, 10 nm.](image)

![FIG. 5. Balsa wood model of the three-dimensional structure of the S-layer with 21.5-nm unit cell spacing, showing the two sides of the S-layer.](image)
is remarkably different in all cases. In the two-sided plane group p6, the following symmetry elements are present in every unit cell: one sixfold, two threefold, and three twofold axes. The chemical surroundings of the three twofold axes are identical, as are the surroundings of the two threefold axes. As a consequence, the structures can be described to a great extent by the surroundings of only the three symmetry axes in the unit cell. In Table 1, the distributions of protein and pores around these three symmetry axes are given for the four structures investigated so far. These four S-layers are so different in structure that no common scheme can be found, except that they are all very open and have large pores running through them. The S-layers of the Bacteroides strains with 21.5-nm spacing are the thinnest and most open of these layers.

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**TABLE 1. Structural characteristics of crystalline protein layers of bacteria**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Two-sided plane group</th>
<th>Unit cell spacing (nm)</th>
<th>Protein (+) or pore (−) at symmetry axis:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis sp. CL II</td>
<td>p6</td>
<td>15.2</td>
<td>+ − +</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>p6</td>
<td>17.5</td>
<td>− − +</td>
</tr>
<tr>
<td>S. acidocaldarius</td>
<td>p6</td>
<td>22.0</td>
<td>− − +</td>
</tr>
<tr>
<td>B. buccae ES57</td>
<td>p6</td>
<td>21.5</td>
<td>− + +</td>
</tr>
<tr>
<td>B. buccae ES57</td>
<td>p6</td>
<td>7.7</td>
<td>+ − +</td>
</tr>
</tbody>
</table>

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