Three-Dimensional Structure of the T-Layer of *Bacillus sphaericus* P-1

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The three-dimensional structure of the regular surface layer of *Bacillus sphaericus* P-1 (T-layer) was determined to a resolution of ca. 2.5 nm by electron microscopy and image analysis. The T-layer has P4 symmetry, a lattice constant of 13 ± 0.2 nm, and a thickness of ca. 8 nm. The reconstruction revealed three distinct domains: a major, a minor, and an arm domain. In the z-direction, the domains are arranged in two planes creating two different surface reliefs.

Many bacteria are surrounded by a regular protein layer generally referred to as the S-layer (14). Although extensively studied, the specific function of S-layers is not fully understood. They may act as a selective barrier, as has been proposed (16, 17). They are excellent specimens for study by electron crystallography (5). They exhibit different crystal systems (14). The three-dimensional reconstruction of S-layers having an hexagonal lattice has been determined for four bacteria: *Sulfolobus acidocaldarius* (18), *Deinococcus radiodurans* (2), *Chlamydia trachomatis* (3), and the cyanobacterium *Synechocystis* sp. strain CL11 (8). In this paper we report on the three-dimensional structure of an S-layer having a square lattice: the S-layer of *Bacillus sphaericus* P-1. This layer is generally referred to as the T-layer (1, 9, 15). *B. sphaericus* P-1, originally identified as a *Bacillus brevis* species, was later identified as a *B. sphaericus* species (7). The molecular weight of T-layer subunits is 140,000 (1). We studied the T-layer to compare its structure with that of other S-layers having a square lattice, such as those of *B. sphaericus* wild type or *Sporosarcina ureae* (4). Such comparisons may reveal structural features which lead to a better understanding of S-layer function.

**MATERIALS AND METHODS**

T-layer from *B. sphaericus* P-1 was kindly donated by J. Dubochet and was prepared as described by Aebi et al. (1).

Electron microscopy. Negatively stained specimens were prepared as follows. The T-layer suspension was deposited onto an air-glow-discharged carbon-coated grid. After 30 s, a drop of 2% phosphotungstic acid (pH 7.0) solution was added. Excess liquid was blotted with a filter paper, and the grid was dried in air. Micrographs were taken in a Philips EM400T electron microscope equipped with a eucentric stage, using the Philips low-dose unit for off-specimen focusing and astigmatism correction. The micrographs in the tilt set were recorded either on the same specimen or on different specimens.

Freeze-dried specimens were prepared as described earlier (13). A drop of T-layer suspension was applied to an air-glow-discharged carbon-coated grid. After 30 s, the excess was partially removed with a filter paper, and the grid was plunged into liquid nitrogen. Eight grids were then mounted into a Reichert holder and transferred into a Reichert cryo-Former. In the cryo-former, the grids were main-

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Freeze-dried and shadowed T-layers show either a rough or a smooth structure (Fig. 2). As described previously (9), these two structures correspond to opposite sides of the T-layer. The optical diffraction of the rough surface extends as in the case of negatively stained T-layer to the seventh order (Fig. 2, insert). From the shadow length of a single T-layer, the layer thickness can be estimated to be $7 \pm 2$ nm.

Three three-dimensional reconstructions were calculated. The first was carried out with a tilt data set recorded on the same specimen. The second was done with a data set recorded on different crystals to minimize beam damage. The results of these reconstructions being similar, the two data sets were combined. Typical lattice lines are shown in Fig. 3. Figure 4 shows nine central sections of the Fourier synthesis of the interpolated lattice lines. Density maps were contoured at a level where a clear edge to the structure was apparent in the direction perpendicular to the plane of the cell wall. Assuming values of 810 daltons/nm$^3$ for hydrated protein density (10), the lower contour level we used defines a unit cell volume containing 60% of a T-layer tetramer weight. Figure 5 shows the projection of the three-dimensional reconstruction as well as different sections perpendicular to the plane of the layer.

A hidden surface representation of the three-dimensional reconstruction is shown in Fig. 6. The thickness of the reconstructed T-layer is 8 to 9 nm. As expected from the
FIG. 2. Freeze-dried T-layer shadowed with a tantalum-tungsten layer. The T-layer displays a rough surface (R) and a smooth surface (S). The thickness of the T-layer is better estimated when the rough surface lies toward the support (double arrow). The circle arrow indicates the shadowing direction. Inserted is the optical diffraction pattern corresponding to the rough and smooth surfaces. The arrow indicates the seventh order diffraction spot. Bar, 100 nm.

FIG. 3. Amplitude (+) and phase (○) variations for representative lattice lines. The ordinate unit is in degrees for phases and arbitrary for amplitudes. The abscissa units are for z*.
shadowing data (Fig. 2), three-dimensional reconstruction of negatively stained T-layer displays a smooth and a rough surface. According to Aebi et al. (1), the T-layer structure can be divided into three domains: a major domain (M), a minor domain (m), and an arm (A). These domains are shown with arrows in Fig. 6. In the direction perpendicular to the plane of the layer the structure can be divided into two halves. The first half contains only the major domain; the second half contains both minor and arm domains.

At the resolution of our reconstruction it is not possible to determine whether the central channel in the major domain is fully open or closed in its apical area. If this channel is open, its opening diameter is less than 2.0 nm.

**DISCUSSION**

The projected structure of the *B. sphaericus* P-1 T-layer has been extensively studied (1, 9, 12, 15). So far, the only available three-dimensional information has been deduced from freeze-dried and shadowed T-layer images (9, 15). In this work, we present a three-dimensional reconstruction calculated from tilted negatively stained T-layer images. Since unstained and negatively stained T-layer projections are similar (12), it is reasonable to assume that negative staining does not introduce major artifacts into the T-layer native structure. This reconstruction shows that the major, minor, and arm domains (1) are located in different planes. The minor domain is made of conelike structures having a height of ca. 3 nm. The arm domain is slightly thicker than the major domain (ca. 4 nm). Both arm and minor domains form a densely packed domain. The major domain protrudes about 4 or 5 nm from the arm domain. The three-dimensional reconstruction of negatively stained T-layer thus displays a rough surface on one side and a smoother surface on the other. This is in agreement with the results of shadowing experiments (9, 15).

By following the connectivity of highest density through the three-dimensional reconstruction it should be possible to outline the shape of individual subunits. Although this is possible in the arm and minor domains, the resolution of our reconstruction is not sufficient to outline clearly the individual subunits in the major domain. The propositions made by Aebi et al. (1) about negatively stained T-layer projections are compatible with our three-dimensional reconstruction.

The T-layer of *B. sphaericus* P-1 has a structure similar to that of the closely related species *Sporosarcina ureae*, and freeze-etching shows that the layer rough surface faces the bacteria cell wall (4). It is therefore reasonable to extrapolate the freeze-etching results obtained with *Sporosarcina ureae* to *B. sphaericus* P-1. The dome-shaped major domain is then in interaction with the cell wall constituent.
Minor and arm domains form a basketlike structure raised away from the cytoplasm. Such a feature seems to be characteristic for most S-layers (2, 3, 18). In the case of the T-layer the basketlike structure is highly perforated. The diameter of the channels is less than 3 nm. These relatively small openings are in agreement with the hypothesis stating that S-layer function is a selective molecular barrier (16, 17). However, if we are to understand fully the function of the
T-layer, knowledge of a higher-resolution structure is necessary. We hope, nevertheless, that comparison of the T-layer structure with that of other S-layers will provide some information on the cell wall function.

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LITERATURE CITED