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Three-Dimensional Structure of the Surface Protein of *Desulfurococcus mobilis*

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The spherical cells of the thermophilic, sulfur-dependent archaebacterium *Desulfurococcus mobilis* are completely covered with a relatively poorly ordered, tetragonally arrayed surface protein. The structure of this surface protein was examined by using three-dimensional electron microscopy. The protein lattice forms an open meshwork composed of cross-shaped morphological units, which are released when glycerol is added. These subunits make contact at the distal ends of their four arms. The p4 symmetry requires that each of these morphological subunits represents a tetramer. The strong interaction of the monomers within the crosses and the relatively weak interaction of the intersecting arms of the crosses within the lattice structure suggest that the tetramers are assembled before their incorporation into the lattice.

Many species of archaebacteria live in extreme habitats reminiscent of the environmental conditions in archaean times. They may, therefore, be seen as living witnesses of early stages in the biotic evolution. At the root of the eubacterial and archaebacterial kingdoms, i.e., closest to the putative progenote, are the extreme thermophiles (1, 9, 17, 18). *Desulfurococcus mobilis* lives in solfataric hot springs of temperatures up to 97°C and at a pH between 2.2 and 6.5 (19). *D. mobilis* and its closest relative, *D. mucosus*, belong to the *Thermoproteales*, a group of thermophilic, anaerobic, and sulfur-dependent archaebacteria which also includes *Thermoproteus tenax* (20, 21). The *Thermoproteales* are, in turn, relatively close to the *Sulfolobales*, and both probably have the same origin.

Regularly arrayed surface (glyco)proteins, which are ubiquitous among procaryotes (15), appear to be a universal feature in sulfur-dependent archaebacteria. In this communication we describe the three-dimensional structure of the *D. mobilis* surface protein as obtained by electron microscopy in conjunction with digital image processing. The *D. mobilis* surface protein forms a tetragonal, almost filiform, network covering the entire cell surface; it looks distinctly different from any bacterial surface protein described so far.

MATERIALS AND METHODS

D. mobilis cells (type strain DSM 2161) were kindly provided by W. Zillig (Max-Planck-Institut für Biochemie). They were cultivated as described by Zillig et al. (19).

Cells were harvested after centrifugation at 5,000 × *g* for 10 min. For freeze-etching, the concentrated cell suspension was frozen between two copper platelets in a cryojet (Balzers Union, Liechtenstein). Freeze-etching was carried out in a modified freeze-etch unit (BAF 360; Balzers AG) at −100°C for 2 min. The fracture faces were shadowed with 1.5 nm of platinum-carbon at an elevation angle of 45°. The metal film was backed with a 20-nm-thick carbon layer. Replicas were cleaned with 70% sulfuric acid overnight.

The cells were lysed by freezing and thawing. The cell envelopes were pelleted by centrifugation at 39,000 × *g* for 15 min. The pellet was incubated at room temperature in 2% sodium dodecyl sulfate for 3 h. The isolated surface protein layers were adsorbed onto glow-discharged, carbon-coated grids and either negatively stained with 2% uranyl acetate or frozen in liquid nitrogen, freeze-dried in a BAF 360 apparatus (Balzers AG) between −80 and −40°C for 2 h, and unidirectionally shadowed with 1 nm of platinum-carbon at 45°. Some samples were mixed with thioglycerol (10%, vol/vol) and rotary shadowed at 30° with 0.8 nm of tantalum-tungsten at room temperature. A 5-nm-thick carbon layer was deposited at normal incidence onto the metal films.

All specimens were examined in a Philips EM 420 electron microscope at 100 kV and a magnification of ×35,000. Tilt series were recorded with tilt increments decreasing in proportion to cos Ψ and (nominal) tilt angles ranging from 0 to 80°. Optical diffractometry was used to select micrographs of well-preserved layers with correctly adjusted defocus and astigmatism.

Suitable areas of the micrographs were digitized (512 by 512 pixel arrays with a pixel size of 0.57 nm at the specimen level) and processed by using the SEMPER (14) or the EM (8) image processing system. Correlation averaging was performed by standard procedures (11). For three-dimensional reconstructions, the hybrid real space-Fourier space approach (13) was applied, starting with unit cells extracted from correlation averages of the tilt series. Surface relief reconstructions from shadowing experiments were carried out using the procedure described in reference 7.

RESULTS

Figure 1 shows cell surfaces of *D. mobilis* as revealed by freeze-etching. The bacteria appear spherical in shape, with a diameter of approximately 1 μm. The entire cell surface is covered with a poorly ordered tetragonal molecular array. On some of these cells the tetragonal surface layer is, in addition, obscured by superimposed irregular slime threads. In Fig. 1a the tetragonal surface lattice appears more prominent than in Fig. 1b, which is due to prolonged etching.

Surface layer fragments isolated by detergent extraction exhibit the same basic features as the surface arrays on freeze-etched cells: the lattice has clear p4 symmetry and a lattice constant of 18 nm. Negatively stained patches showed a varying mass distribution (especially at the point of intersection), depending on the orientation with respect to the

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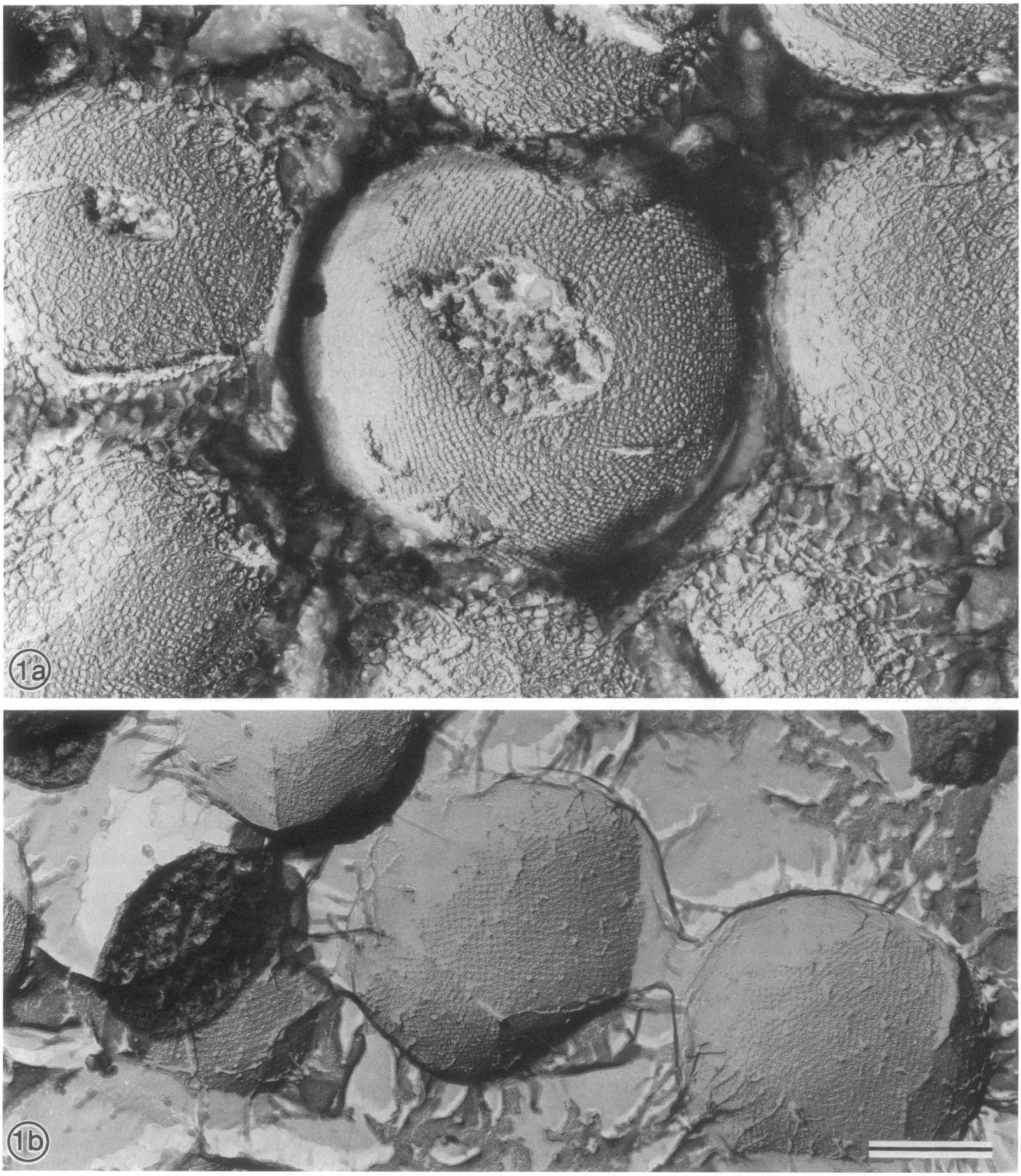

FIG. 1. Freeze-etched cells of *D. mobilis* showing the tetragonal surface protein meshwork. The more pronounced relief in panel a is due to prolonged (i.e., deeper) etching of the specimen. Bar, 300 nm.

supporting carbon film. The opposite handedness of the two surface layer fragments shown in Fig. 2 is clearly displayed by the inserted diffractograms. By correlation with surface relief reconstructions from freeze-etched samples (see Fig. 4c), the sidedness of the two projections can be determined. The layer in Fig. 2a is adsorbed via its inner surface (faced towards the cell membrane), and the layer shown in Fig. 2b is adsorbed with its outer (faced towards the environment)

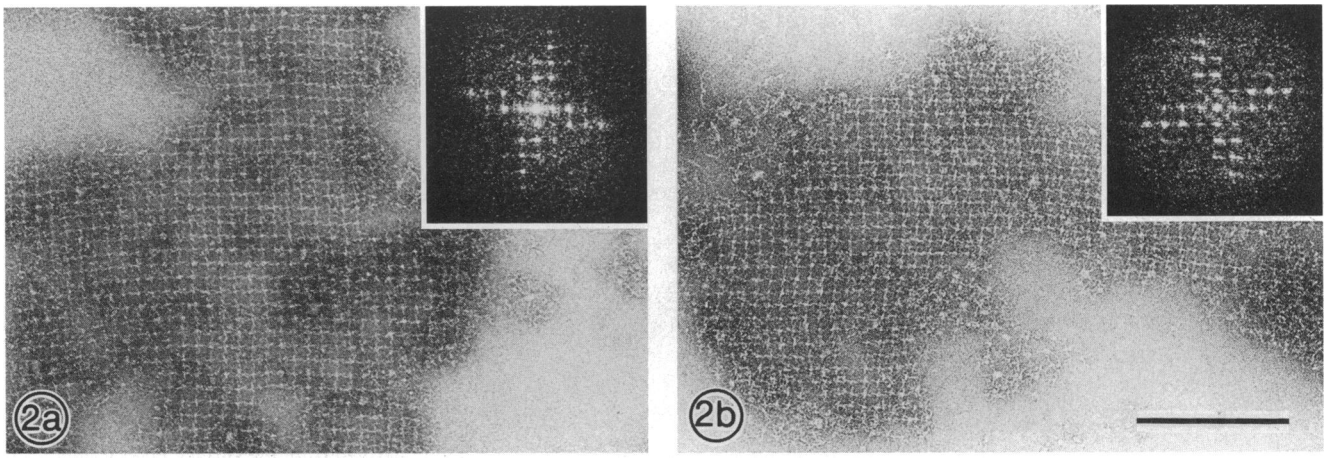

FIG. 2. Negatively stained, isolated surface protein layers in two different orientations with respect to the carbon supporting film. The layer in panel a is adsorbed via the inner surface, and that in panel b is adsorbed with its outer surface onto the supporting film. The opposite handedness is clearly shown by the inserted diffractograms. Bar, 200 nm.

surface oriented towards the supporting film. Two independent three-dimensional reconstructions were carried out with crystal fragments exhibiting opposite orientation (with respect to the supporting film) to get an estimate of the extent of adsorption-induced local flattening. The resolution was almost isotropic within the range of 2 to 2.5 nm. The highest actual tilt angles obtained were 79° (after correction of the goniometer offset). The lattice line data in Fourier space from the two reconstructions are presented in Fig. 3. The modulus of the Fourier coefficient is proportional to the area enclosed by the corresponding circle. The projections, in the z direction, through the complete three-dimensional reconstructions are presented in Fig. 4a and b. The protein lattice appears to be composed of cross-shaped morphological units which are interconnected, via the distal ends of their arms, near the twofold crystallographic axis. Thus the structure can be regarded as prototypical for the M_2C_4 S-layer type according to the notation of Saxton and Baumeister (12). The crosses, which are rotated between 12 and 15° with respect to the axes of the array, have a diameter of 20 nm and a thickness of 6 nm at the center. The thickness of the four arms of the crosses is 2.5 nm, and since they are somewhat bent near their ends, they each have a total length of 11 nm. On the central fourfold axis of each cross, a knob-shaped protrusion with a diameter of 3.5 nm is located which faces the plasma membrane in intact cells. The dimensions of this protrusion may be incorrect; this could occur with a rather delicate structure which could collapse during preparation. In one of the two reconstructions (Fig. 4a), the protrusion appears flattened and its mass is, to some extent, spread into the arms.

The covering of a sphere with a tetragonal lattice will, inevitably, result in a departure from perfect crystallinity. In fact, freeze-etching of *D. mobilis* cells already reveals a degree of lattice disorder that is unusual for bacterial surface layers. Lattice defects, dislocations in particular, are abun-

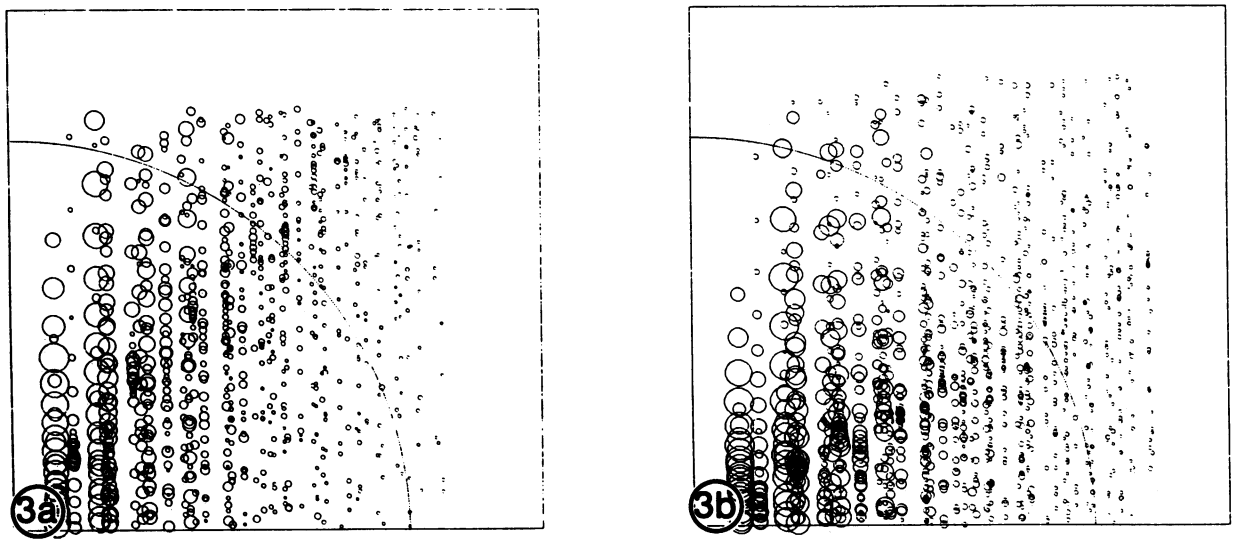

FIG. 3. Spatial distribution of data in Fourier space. Panels a and b correspond to the reconstructions presented in Fig. 4a and b, respectively. The area enclosed by each circle is proportional to the modulus of the corresponding Fourier coefficient. The "missing cone," where no data are measured owing to the restricted tilt range, is very small, and hence resolution is nearly isotropic. The curves represent a 2-nm resolution level.

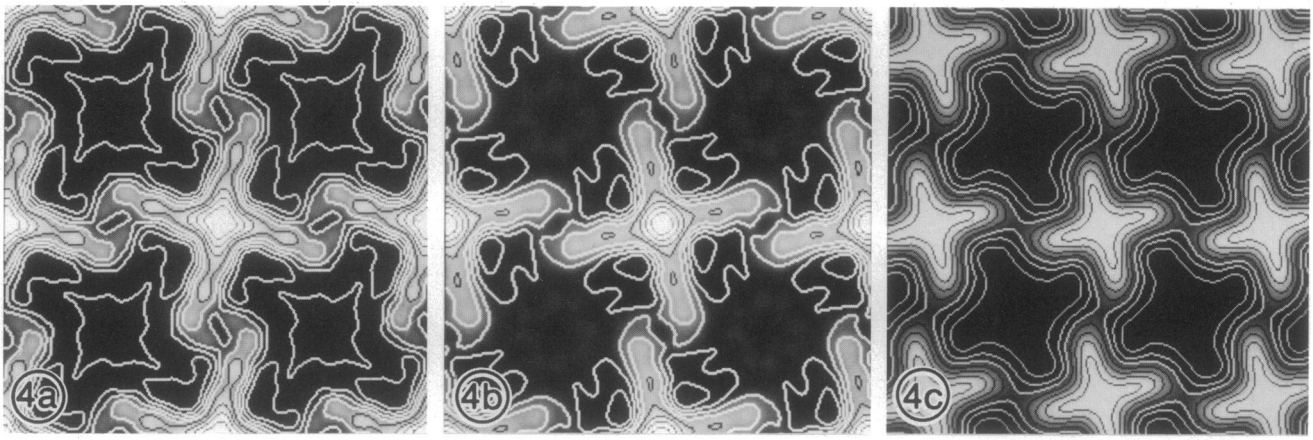

FIG. 4. (a and b) Projections, in the z direction, through the three-dimensional reconstruction derived from the surface layer fragments in Fig. 2. (c) Surface relief reconstruction (4) of an outer surface revealed by freeze-etching, used to determine the sidedness of the layer. Note that in panel c, bright areas represent elevations.

dant in isolated layers (see Fig. 6a, e.g.). These distortions leave relatively large gaps in the protein network.

Figure 6b shows a surface layer fragment freeze dried and unidirectionally shadowed. There is no evidence from these preparations that the meshes of the network are filled by proteinaceous or nonproteinaceous matter. Adding glycerol to isolated layers resulted in a partial disintegration of the lattice. The released fragments are uniformly shaped crosses with equal-length arms, confirming that they represent the morphological units observed in the lattice. The small increase in dimensions of the individual crosses (as compared to those incorporated into the lattice) is due to the applied preparation technique; glycerol treatment combined with rotary shadowing tends to increase the apparent dimensions.

Three-dimensional reconstructions allow one to obtain a rough estimate of molecular volumes and hence of molecular masses. Our reconstructions yield a unit cell volume (which is equivalent to the volume of one cross-shaped unit) of 180 nm^3. Assuming a protein density of 1.33 g/cm^3, the molecular mass of this morphological unit is 140 kilodaltons (kDa). When taking into account that, for the sake of clarity in displaying structural models, the threshold is usually chosen at a fairly restrictive level (3) (such that the models include only 50 to 80% of the true volume), the true molecular mass is probably closer to 200 kDa. The p4 symmetry requires that the unit cell is composed of four subunits, and therefore the protein monomer might be about 50 kDa. Preliminary biochemical investigations have shown one band on sodium dodecyl sulfate gels with an apparent molecular mass of over 200 kDa. We suggest that this band corresponds to the cross-shaped subunits discussed above.

DISCUSSION

The structure of quite a number of bacterial surface proteins has been investigated by electron microscopy over the past few years, some of them at the level of a three-dimensional analysis (3). Some principles of organization have

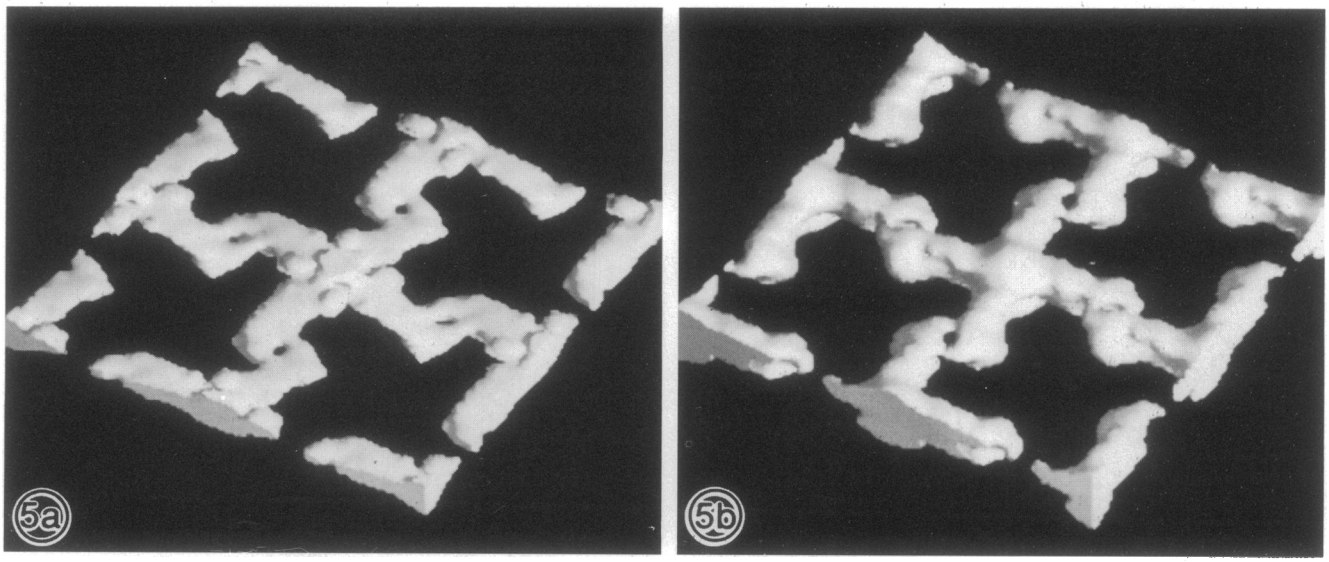

FIG. 5. Computer-generated view representation of three-dimensional reconstructions of the two-layer fragments shown in Fig. 2. Owing to the opposite orientation of the two samples in the original preparation, the inner (a) and the outer (b) surface displayed in the views correspond to surfaces not in contact with the supporting film. This rules out the possibility of distortions due to interface denaturation.

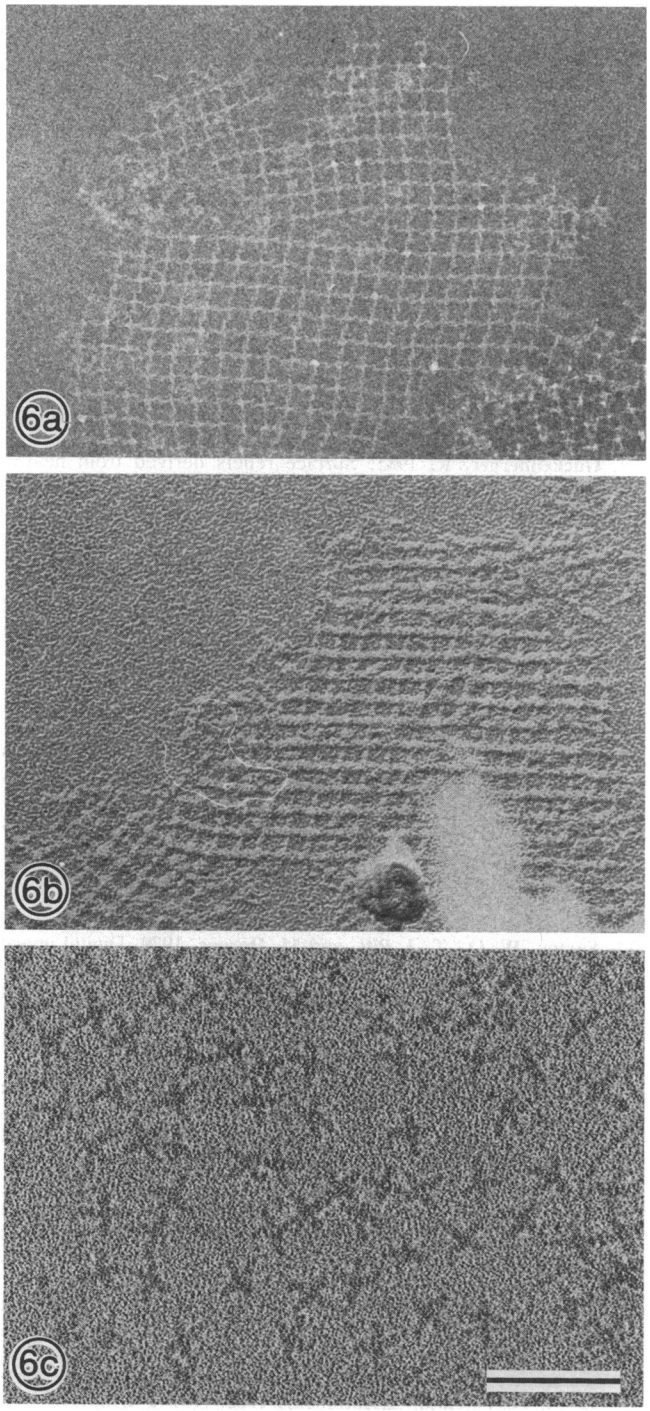

FIG. 6. (a) Negatively stained layer fragment showing a dislocation, the predominant type of lattice defect in *D. mobilis*. (b) Layer fragment, freeze-dried and undirectionally shadowed. (c) Addition of 10% glycerol to the layer preparation before rotary shadowing induces structural perturbations in the contact regions, which disintegrates the lattice and releases tetrameric complexes. Bar, 100 nm.

emerged, restricting the number of basic motifs (12). Although the *D. mobilis* surface protein is accommodated by the classification scheme and can be regarded as prototypical for the M_4C_2 type, it is so far a unique type of surface array; the most conspicuous feature is its unusual porosity. A bacterial surface protein remotely reminiscent of the *D. mobilis* structure is the tetragonal surface layer of *Azotobacter vinelandii* (5), which is, however, of the M_4C_4 type; this means that the connectivity between the cross-shaped subunits is established via the fourfold axis instead of the twofold axis. As a consequence, the gaps between the subunits are smaller.

When extending the comparison beyond procaryotic surface proteins, we find a protein meshwork on the inner nuclear membrane of eucaryotes that looks strikingly similar, at first glance, to the *D. mobilis* surface protein. Triton X-100-extracted, freeze-dried, and metal-shadowed nuclear envelopes show a quasiregular meshwork, the nuclear lamina (2), closely resembling the surface lattice seen on deep-etched *D. mobilis* cells (Fig. 1a). Closer examination reveals, however, that the dimensions as well as the basic design are different. The nuclear lamina is composed of intermediate-type filaments which appear to form a meshwork with a "woven" topology and cross-overs similar to those seen in tropomyosin crystals (10). The *D. mobilis* surface layer is made up of distinct cross-shaped subunits, as demonstrated by the glycerol experiments (Fig. 6c), with the distal ends of the four arms in contact. The structure of the dislocations (Fig. 6a) also indicates that the crosses are the building blocks of the lattice. The p4 symmetry requires that each morphological subunit represents a tetramer and that the regions of contact are equivalent. (Since proteins are chiral molecules, a single monomer per morphological unit establishing quasiequivalent contacts is an extremely unlikely possibility.) The monomers (M_r ~50,000) can be arranged in the cross-shaped tetramers in various ways (Fig. 7). The simplest arrangement is one in which each monomer makes up one arm of the cross and contributes to the globular central domain, but, in principle, other topologies with more extended overlaps of the individual polypeptide chains are also possible. Contacts at the twofold axes appear to be relatively weak, and it is perhaps surprising that glycerol, which is frequently used as an (indispensable) additive in electron microscopy of filamentous proteins, is sufficient to disintegrate the lattice, probably by inducing local structural perturbations. The relatively weak interactions of the crosses in the lattice, as opposed to the much stronger interaction of the monomers within the crosses, suggest that tetramers are assembled before their incorporation into the

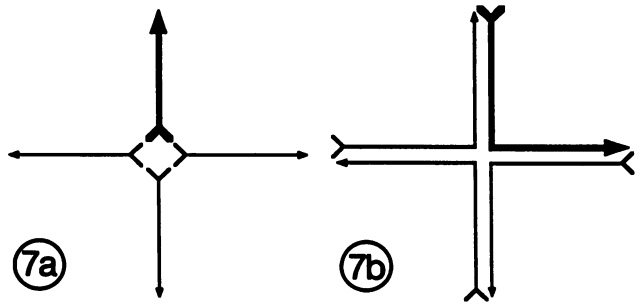

FIG. 7. Schematic representation of two possible arrangements of monomers in the tetrameric complexes, supposing equivalence of the distal ends of the arms which establish the connectivity in the p4 lattice. Panel a represents the simplest case, i.e., each arm represents a single monomer; in panel b each monomer extends over two of the arms. Although more complex configurations are allowed (such as each monomer contributing to each of the four arms), these are, in principle, less likely.

lattice. Incorporation of preassembled oligomers was also postulated for the S layers of *T. tenax* (16) and *A. vinelandii* (6).

An obvious question to ask is, of course, what function such a protein surface array might serve. The most frequently voiced supposition, clearly inspired by the porous structure of S layers, is that they act as a molecular sieve at the cell surface, controlling the passage of small and medium-sized molecules into and out of the cell. In the case of *D. mobilis* the meshes of the network are so large (12 by 12 nm) that the S layer can hardly be regarded as an efficient barrier. Even relatively large molecular species (up to 700 kDa) would be able to pass the network, although one cannot totally disregard the possibility that very small features, remaining invisible because of the 2-nm resolution limit, partially occlude the holes. One must keep in mind, however, that the surface layer is, as in other archaebacteria, intimately associated with the plasma membrane, which of course represents a selective barrier; hence the conception of an open network, dissecting the S layer intellectually from the membrane, is misleading.

In the case of *T. tenax* it has been shown that a regular surface lattice can provide shape maintenance; possibly it can also determine the shape (16). In *D. mobilis* the cell shape and dimensions do not appear stringently controlled, nor is it likely that a surface array with such an abundance of lattice faults is capable of determining a unique shape. The design of the lattice, as revealed by our three-dimensional reconstruction and the behavior of the isolated fragments, indicates a high degree of flexibility rather than rigidity. Nevertheless, the layer could be thought of as a procaryotic type of exoskeleton possibly providing some protection against osmotic stress.

There are other factors which should be considered in contemplating surface protein functions. Adsorption, specific or nonspecific, is important for ensuring that bacteria reach and remain in environments they are adapted for. Due to their exposed location, surface proteins are natural candidates for mediating adsorption.

Although the nature of the interaction between the *D. mobilis* surface protein and the underlying membrane is as yet unclear, it is nevertheless likely that, analogous to other archaebacteria, the protruding domain on the central, fourfold axis serves as a membrane anchor and perhaps penetrates the membrane. Thus the S layer could exert an organizing effect on other membrane proteins, or it may, analogous to molecules of the extracellular matrix of eucaryotes or the nuclear lamina, interact directly or indirectly with intracellular molecules. Also it cannot be ruled out that surface proteins have an as yet unidentified enzymatic (proteases, protease-inhibitor, glycosidases, nucleosidases) or receptor function.

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