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Bacterial surface proteins

Some structural, functional and evolutionary aspects

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The structure of several eubacterial and archaebacterial surface (glyco)proteins as determined by three-dimensional electron microscopy is described. Particular emphasis is placed on surface proteins which interact with membranes. Some structure-function relationships deduced from the structural information, such as shape maintenance and molecular recognition phenomena, are discussed.

1. Introduction

Although the discovery of regularly arrayed surface proteins ('S-layers', 'RS layers') dates back to 1953 when Houwink [1] first described a macromolecular monolayer on the cell wall of an unidentified *Spirillum* species, and in spite of the great number of reports published since then describing similar structures for other species, they have for long been viewed as mere 'oddities' not deserving any great attention. This belief, still held in some quarters, is mistaken, as we hope to demonstrate in this short review.

2. Regular surface proteins: distribution and definitions

Regularly arrayed surface proteins are a rather heterogeneous class of proteins defined merely by their location at the surface of prokaryotes. They appear to be an almost universal feature of archaebacterial cell envelopes and are common in eubacteria where they are found in most branches of the evolutionary tree (fig. 1). The presence of regular arrays of surface proteins has been attested to for almost 200 bacterial species (for recent compilations see refs. 2 and 3), mostly by (low-resolution) electron microscopy. It should be emphasized here that for various reasons regular arrays of surface proteins quite easily escape attention. Freeze-etching, the most successful tool for detecting S-layers, often fails in the presence of surface carbohydrates which tend to mask the underlying lattice. Other techniques, such as negative staining, require the isolation of the cell envelope but surface arrays frequently dissociate upon removal from their normal environment. Moreover, it is not uncommon in laboratory cultures for the synthesis of surface proteins to be discontinued or for the bacteria to become unable to assemble the layer properly.

Often enough it is not a trivial matter to decide whether a regular surface array as visualized by electron microscopy represents an S-layer or a regularly arrayed (outer) membrane protein (rOmp). S-layers in the strict sense have protein-

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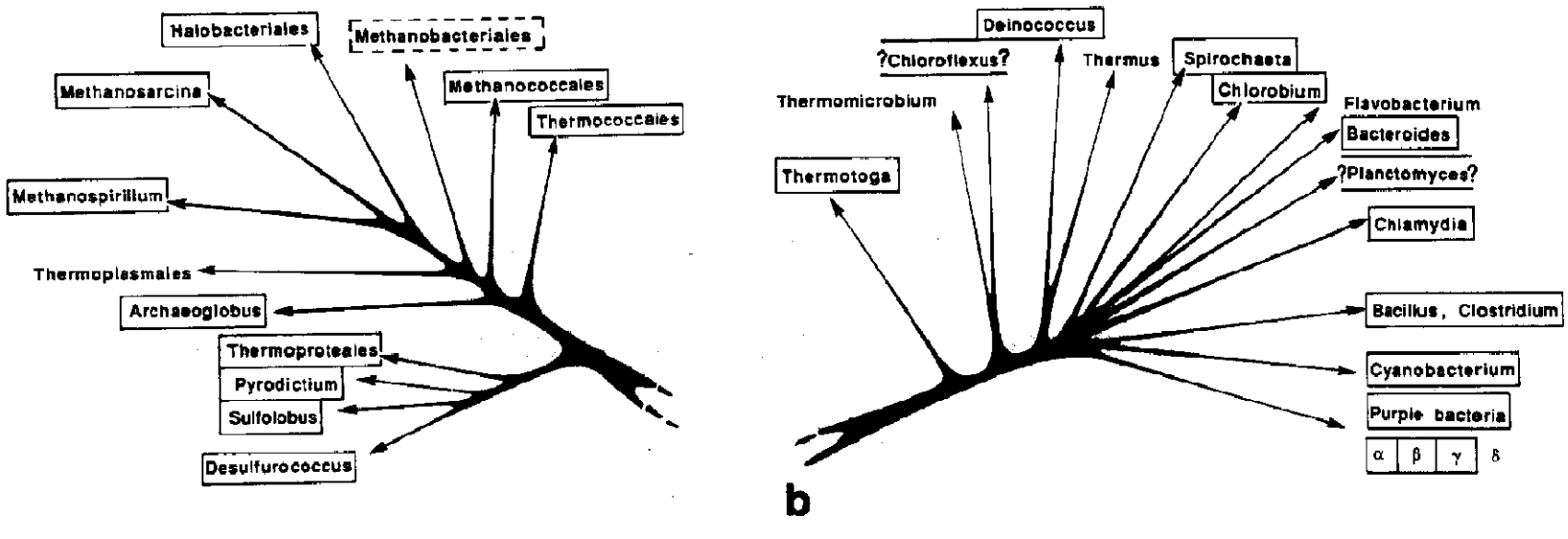

Fig. 1. Distribution of archaebacteria (a) and eubacteria (b) possessing regular surface proteins over the ramifications of the phylogenetic tree. Boxes indicate that S-layers or rOmps are common in the corresponding phylogenetic lines.

protein interactions which are sufficiently strong to maintain the integrity of the lattice without relying on a membranous support. Nevertheless, S-layers may be intimately associated with membranes either by specifically interacting with integral membrane components or via a membrane anchor and the association with the membrane might add to the stability of the protein surface network. In contrast, true rOmps have a significant portion of their volume incorporated into the membrane and the lipid matrix is an essential structural component of the supramolecular assembly; any removal of the lipid 'glue' will lead to the dissociation of the lattice [4].

S-layers and rOmps may share some functions and we would not be surprised to see phylogenetic relationships emerging from future sequence data. Most investigations of channel-forming outer membrane proteins ('porins') have been confined to S-layer-deficient Enterobacteriaceae (γ-subdivision of purple bacteria). If the occurrence of porins were indeed restricted to this group, this would lend some support to the hypothesis that porins are descendants of the more widespread S-layers, which in a distinct evolutionary step have been integrated into the outer membrane. However, more recently, rOmps with the structural and chemical characteristics of porins were also found in several other branches of the phylogenetic tree (e.g., the β-subdivision of purple bacteria, Chlamydiae [5]). Most unexpected was the discovery of a regular surface protein strikingly reminiscent of the 'classical' porins OmpC or OmpF of *Escherichia coli* in *Thermotoga maritima* (manuscript in preparation), an organism representing the earliest branching-off point in the evolution of eubacteria with a particularly slow evolutionary clock [6]. This only serves to demonstrate that our present knowledge of the structure of bacterial surface proteins in the various phylogenetic lines is too fragmentory to propose any pathways for the evolution of bacterial cell envelopes.

3. Surface protein functions: Some general considerations

Typically, surface proteins account for 7–12% of the total cell protein of prokaryotes, i.e., where present, surface proteins represent the predominant protein species. The abundance of these proteins implies that each cell must devote a quite considerable proportion of its energy budget to their production; this simple fact and, no less important, the presence of surface proteins in almost all ramifications of the evolutionary tree are strong indications of their vital importance. Nevertheless, their precise function is still rather enigmatic. It would clearly be a misconception to infer from our present ignorance that regularly arrayed surface proteins are an aesthetically pleasing, but dismissible adornment! Since a priori also the relatedness of the various types of eubacterial and archaebacterial surface proteins must be ques-

tioned, any search for common functions might also be misleading.

Intuitively, one may suppose that, by virtue of their exposed location, the function of surface proteins must in one way or another be related to the interplay between the cell and its environment. In this context it is a notable observation that S-layers are a common feature of cells from fresh isolates but tend to become lost upon prolonged cultivation in the laboratory. Even though this observation cannot be generalized, it suggests that S-layers serve an essential function in natural habitats which becomes dispensible in less competitive environments.

Fig. 2 is a synopsis of the principal functions of surface proteins currently under scrutiny. Shape maintenance and shape determination – which are not identical phenomena [7] – are thought of as primordial functions of perpetual importance for some archaebacteria with rather simple cell envelopes devoid of a rigid murein or pseudomurein layer. The most conspicuous feature in electron micrographs of isolated S-layers, however, is their porous structure which prompted the view that their primary function is that of a molecular sieve. S-layers are, indeed, effective molecular sieves which led Sleytr and Sara [8] to explore the possibility of using them commercially for ultrafiltration; the cut-off of these filters is apparently in the range 20–40 kDa. While the ability of isolated S-layers to perform molecular sieving is undisputed, the biological implications are much less clear.

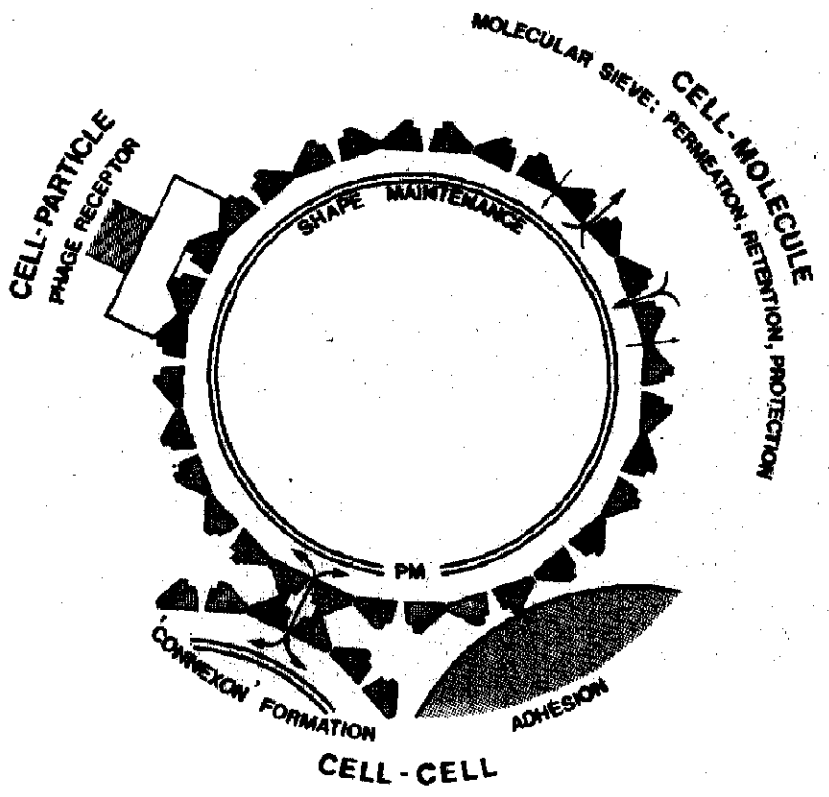

Fig. 2. Synopsis of (putative) functions of regular bacterial surface proteins.

Molecular sieving has various aspects: controlling the permeation of low- and medium-molecular-mass solutes, retention of endogenous enzymes and protection of critical cellular targets by excluding exogenous, lytic enzymes. The quality of a molecular sieve, of course, depends critically on its selectivity. The molecular mass cut-off of 20–40 kDa measured with isolated S-layers means that only macromolecules (proteins, DNA) are excluded from free diffusion across it. This might be sufficient for conferring a protective effect or for retaining endogenous proteins. The latter possibility has the interesting implication that in the case of Gram-positive eubacteria and also in many archaebacteria the S-layer and the plasma membrane delineate a 'compartment' which may be considered as being analogous to the periplasmic space of the Gram-negative eubacteria. For critically controlling permeation, however, S-layers do not seem to be endowed with the necessary molecular selectivity which is characteristic of outer membranes, for example. It is important, however, to bear in mind that in many species S-layers are intimately associated with a membrane and possibly exert a strong influence on the molecular organization of this membrane; thus, they form a functional entity, which, rather than one of the components alone, will determine the discriminatory properties.

A property which S-layers have in common with some outer membrane proteins such as OmpA of *E. coli* is that they can act as phage receptors. Phage-S-layer interactions have been studied in some detail with *Bacillus sphaericus* using mutants with partial deletions [9], as well as with *Aeromonas salmonicida* [10].

Owing to their location at the cell surface, S-layers are predisposed to mediating adhesion, again comprising a whole spectrum of phenomena, ranging from unspecific adherence to inanimate surfaces to more specific targeting and, ultimately, to highly specific cell-cell interactions.

Adhesion prevents bacteria from being swept away from locations for which they are adapted, and assists them in reaching and accumulating at favourable locations. Homotypic cell-cell interactions as proposed in the bacterial connexon hypothesis [11] require a mechanism of molecular recognition. Three-dimensional electron microscopy of several eubacterial and archaebacterial S-layers has revealed a variability and species or even strain specificity which are adequate for such a purpose. Of course, adsorption is not exclusively effected by surface proteins; there are other candidates to be considered such as carbohydrate capsules and appendages (fimbriae, pili).

In the following sections we shall describe the three-dimensional structure of a number of surface proteins from eubacteria and archaebacteria, as

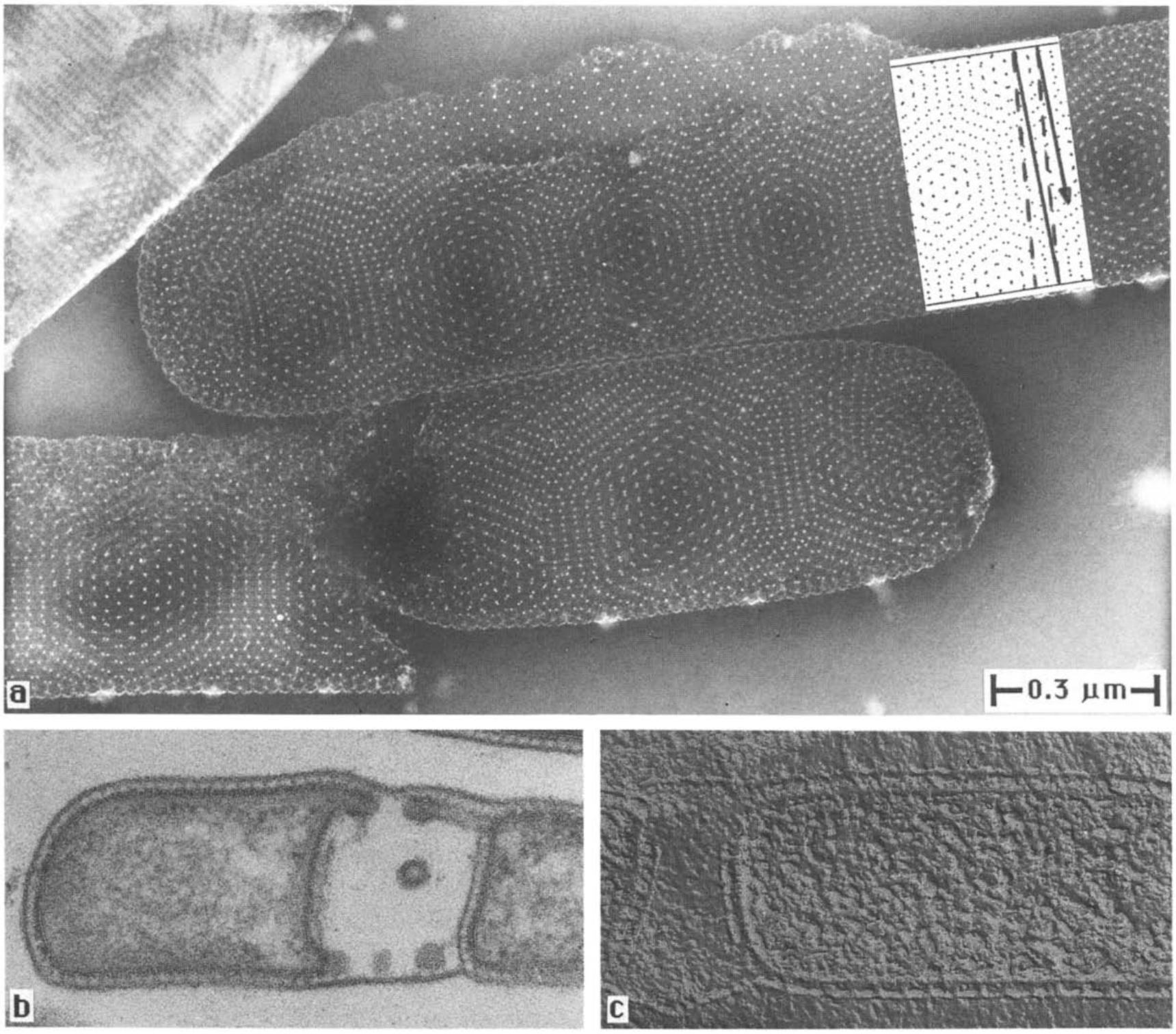

Fig. 3. (a) Negatively stained isolated S-layer from *T. tenax*. The prominent white dots represent needle-like spikes protruding from the inner surface of the layer and anchoring it via their distal ends to the cell membrane. Analysis of the Moiré pattern reveals the helical nature of the layer which is useful for explaining the mechanism of shape maintenance [18]. This sections (b) and freeze-fractures (c) show the layer at a distance of approx. 25 nm above the membrane level thus creating a distinct interspace.

determined by three-dimensional electron microscopy, and discuss some structure-function relationships deduced from these investigations. The examples have been chosen to emphasize one frequently ignored aspect of surface proteins, i.e., their association with the plasma or outer membrane. Although many S-layers exist free of a membrane, such as the peptidoglycan-associated S-layers of the Gram-positive eubacteria, many others interact, at various levels of intimacy, with membranes, ranging from a relatively loose peripheral association to an intrinsic location.

4. Surface proteins of *Thermoproteus tenax* and *Desulfurococcus mobilis*

The Thermoproteales [12–14] constitute a group of archaebacteria adapted to extreme habitats reminiscent of the environmental conditions in Archean times; they may, therefore, be viewed as living witnesses of early stages in biotic evolution. Both of these species, *T. tenax* and *D. mobilis*, possess S-layers, which despite their close phylogenetic relationship, appear quite different in structure.

The S-layer of *T. tenax* (fig. 3) forms a rigid, remarkably perfect p6 lattice. The interaction of the protomers in the layer is so strong that all attempts at dissociation by chemical means have been met with frustration [12,15]. Mass measurements with a scanning transmission electron microscope have yielded a molecular mass of 3240 kDa per unit cell [15]; supposing that six monomers are contained in the unit cell, we arrive at a molecular mass of 540 kDa per monomer (the carbohydrate content is unknown). The monomers must have an extremely elongated shape, spanning a distance of at least 50 nm. They form a relatively thin (3–4 nm) filamentous network (fig. 4a) with long protrusions emanating around the 6-fold symmetry axis and extending towards the cell membrane [16]; the distal ends of these protrusions appear to penetrate the membrane, thus serving as a membrane anchor. These protrusions, which might be hollow tubes rather than solid pillars, maintain a 25 nm wide interspace between the cell membrane and the S-layer proper. The existence of such a distinct interspace between the cell membrane and surface layer appears to be rather common among archaebacteria [17] and one could imagine this interspace as harbouring a

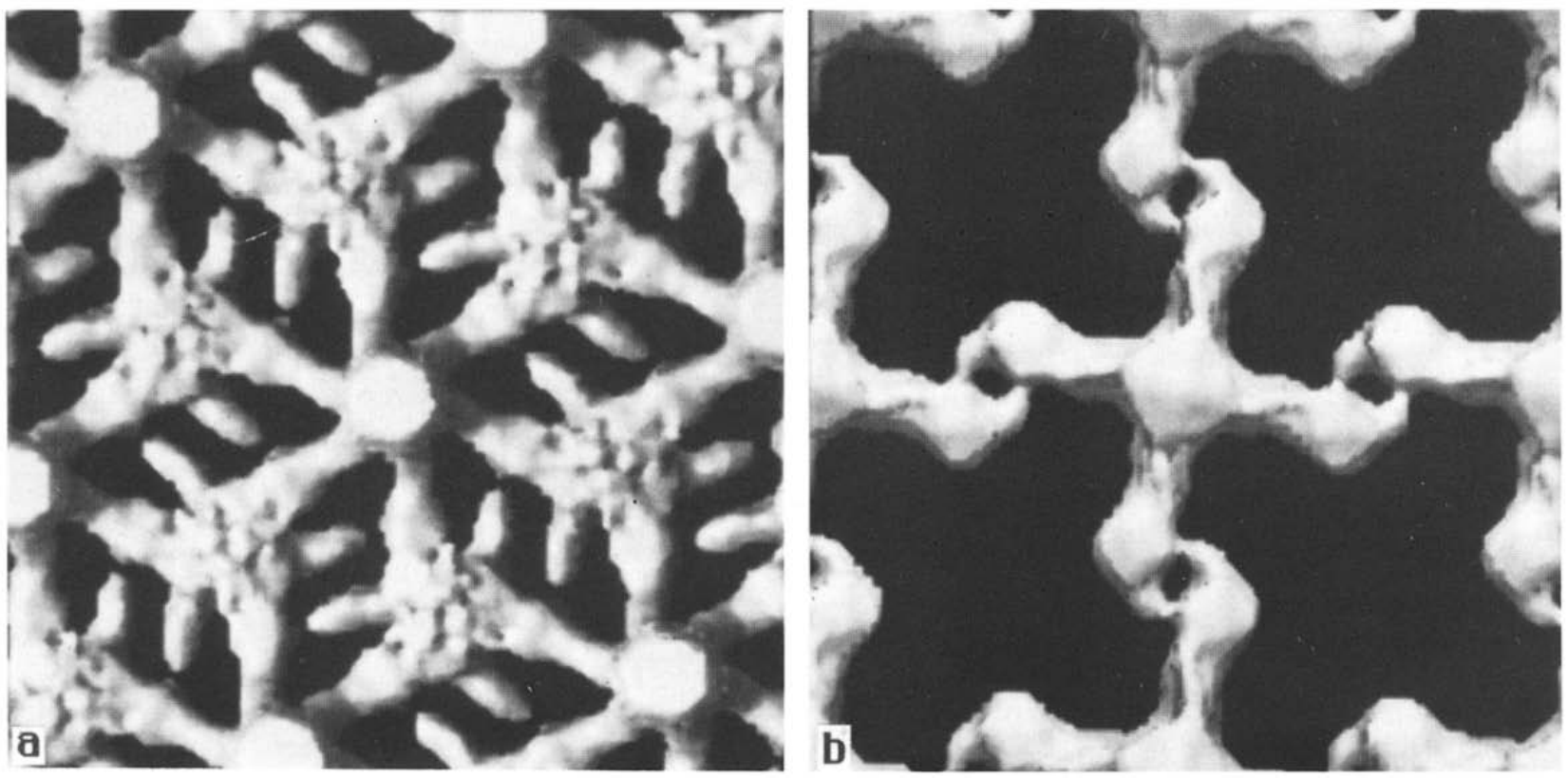

Fig. 4. Views showing the three-dimensional organization of the surface layers of (a) *T. tenax* and (b) *D. mobilis*. With *T. tenax* the protrusion on the 6-fold axis which is directed towards the cell membrane has been 'removed' for the sake of clarity.

distinct consortium of enzymes, analogous to the periplasmic space in Gram-negative eubacteria.

A characteristic feature of *T. tenax* cells is the rather constant cell diameter (0.41 ± 0.03 μm); the length of the cells may vary between 1 and 80 μm [12]. Moreover, the lattice base vectors show a preferred orientation with respect to the longitudinal axis of the cell. Hence, the surface lattice can be described in terms of a helix, more specifically as a two-stranded parallel helix with a pitch of approx. 58 nm (fig. 3). The notion of the helical nature of the S-layer, though somewhat trivial, is useful for rationalizing the mechanism of shape maintenance in a fairly simple manner. Closed surface crystals can only grow via 'intussusception' of new units which requires a departure from perfect crystallinity. With *T. tenax* no lattice defects are observed in the cylindrical part, but a distinct number have been shown to exist near the cell poles [18]. The helical template model [16] suggests that these disclinations [44] act as points of growth or the start of the helix, thus propagating the surface cylinders unidirectionally. Synchronisation of growth of the two strands of the helix poses no problem, provided they have a common start, which could be accomplished through the insertion of pre-assembled trimers.

The surface layer of *D. mobilis* is a rather filiform network composed of tetrameric cross-shaped units [19] which leave exceptionally large holes (12×12 nm) in between (fig. 4b). If these holes are not at least partially occluded by flexible protein masses or carbohydrate, remaining invisible in the averages, the S-layer as such can hardly be regarded as an efficient molecular sieve; one might infer from the structure that it allows the passage of molecular species up to approx. 500 kDa in molecular mass. One must bear in mind, however, that the surface protein is, as in other archaebacteria, closely associated with the cell membrane and elucidating this interaction may provide the key to an understanding of its functional significance.

One may consider it as a prokaryotic type of exoskeleton, structurally reminiscent of the spectrin network in erythrocytes or the nuclear lamina in eukaryotes [21,22] but also of the proteoglycan-collagen-fibronectin complexes of the extracellular matrix [23]. In fact, it was speculated several years ago [24,25] that archaebacterial cell wall proteins represent the original 'pro-eukaryotic' glycoprotein from which the glycoproteins of plant cell walls [26] and the collagens have evolved. It is noteworthy in this context that some similarities in the glycosylation patterns of the surface glycoprotein of *Halobacterium halobium* and collagen have recently been found [27].

In contrast to the situation in *T. tenax*, the layer appears very flexible, shows lattice defects in abundance and the interaction between the tetramers is so weak that even exposure to glycerol dissociates the lattice [19]. In fact, the cell shape and dimensions appear not to be stringently controlled in *D. mobilis*. Nevertheless, such an exoskeleton could determine the topography of intrinsic membrane proteins and if it possesses a membrane-traversing domain it could interact, directly or indirectly, with intracellular components.

5. Surface proteins of some *Sulfolobus* species

During the course of investigating the structure of various eubacterial S-layers, it already began to transpire that their outer surfaces are quite variable while the inner surfaces including the pore-forming portions are rather more conservative. *Sporosarcina ureae* [28] and *B. sphaericus* [29] are particularly good examples to demonstrate this point (fig. 5). These two species, which are comparatively closely related, exhibit an inner surface structure which appears to be identical within the limits of experimental error. The outer surfaces, however, show distinct differences in the spatial disposition of mass.

We have scrutinized this phenomenon more closely, selecting several species and strains of the genus *Sulfolobus* since their mutual phylogenetic relationship is quite well-established. We have, in the first instance, used freeze-etching, or more specifically deep-etching, in conjunction with surface relief reconstruction [30] to screen the various *Sulfolobus* isolates. This technique has the merit of being able to depict S-layers in situ, i.e., in their correct orientation on the cell surface undisturbed by any isolation procedure. In spite

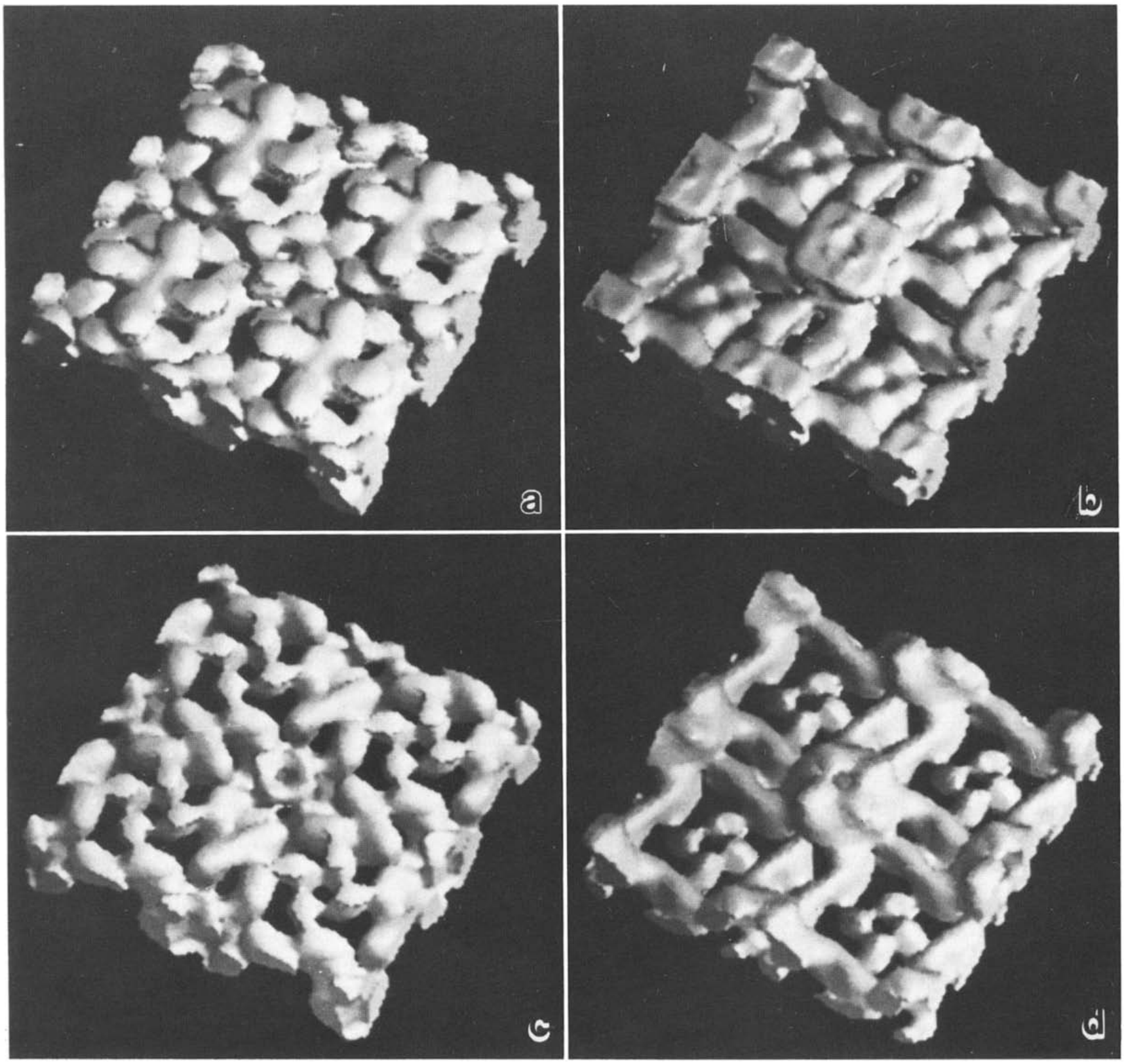

Fig. 5. Views of three-dimensional reconstructions of the surface layers of *Sp. ureae* (a,b) and *B. sphaericus* (c,d). The outer surfaces (a,c) are distinctly different whereas the inner surfaces (b,d) are remarkably similar. (The three-dimensional data for *B. sphaericus* were kindly provided by J. Lepault and K. Leonard.)

of the relatively low resolution attainable (2.5–3 nm) these investigations clearly revealed variations in the structure of the cell surface reflecting quite accurately the phylogenetic status and indicating species specificity [31].

Subsequently, complete three-dimensional reconstructions from tilt series have been performed with three selected species: *Sulfolobus solfataricus* [32], *Desulfurolobus ambivalens* (fig. 6) and *S. brierleyi* (manuscript in preparation). A three-dimensional reconstruction of the *S. acidocaldarius* S-layer was already available [33]. In comparing these four structures it was striking to observe how similar they are from the centre towards the inside, even to the extent of local congruence.

This is particularly remarkable in the case of *S.*

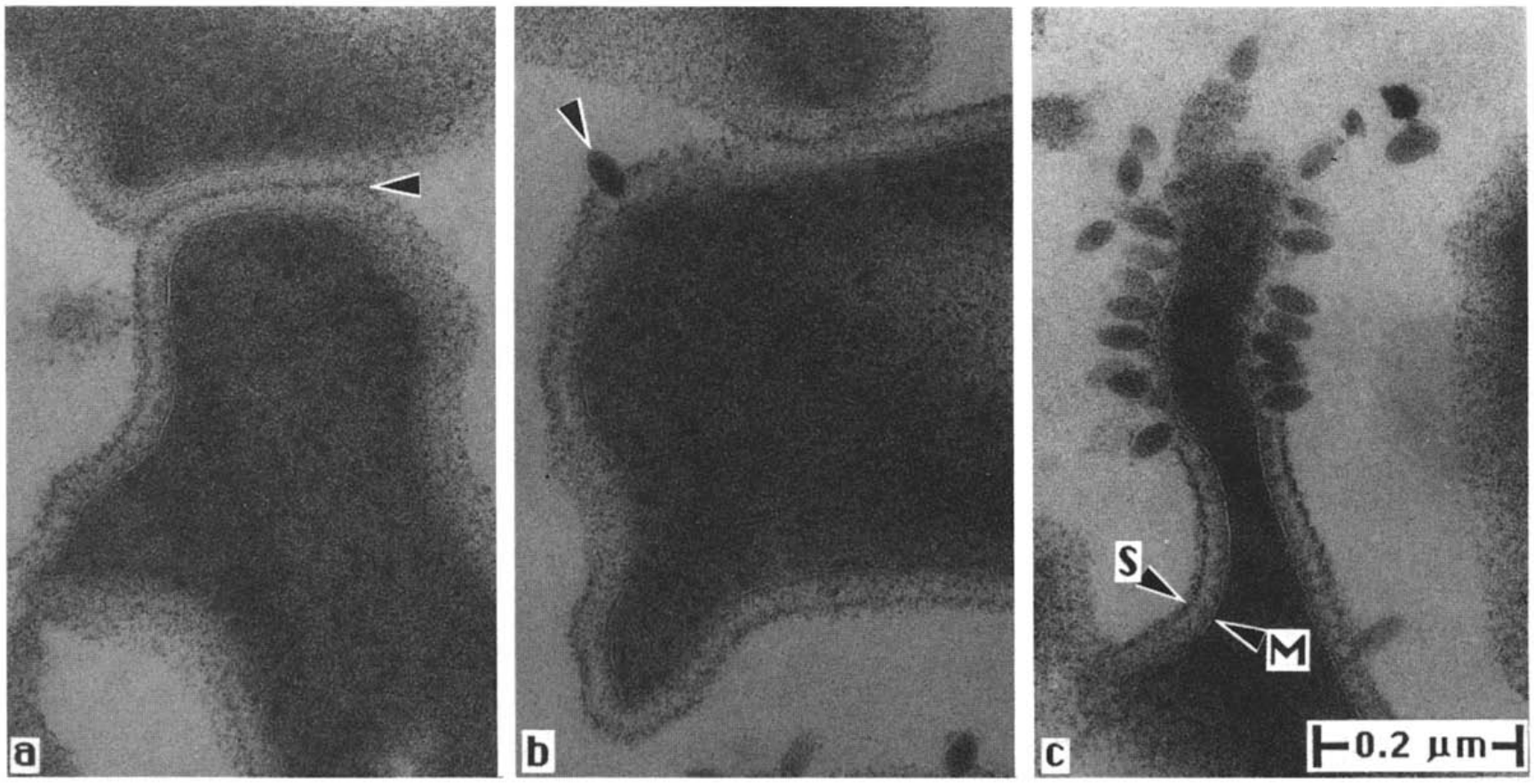

Fig. 6. Micrographs of thin sections of *Sulfolobus* sp. (isolate B 12) showing (a) cell-cell contacts, (b) SSV 1 virus penetrating the surface layer and being attached to the cell membrane and (c) another cell covered with virus particles. In panel c the distinct interspace between the S-layer proper and the membrane (20 nm) typical of many archaebacteria is clearly visible.

brierleyi, which deviates from the other species with regard to the symmetry of the S-layer; it is p3 instead of p6 with the implication of three monomers (or heterodimers) in the unit cell instead of six. Nevertheless, the mass disposition is very similar, pointing to very strong pressure in favour of this particular configuration. Towards the outer surface, however, the structures begin to diverge.

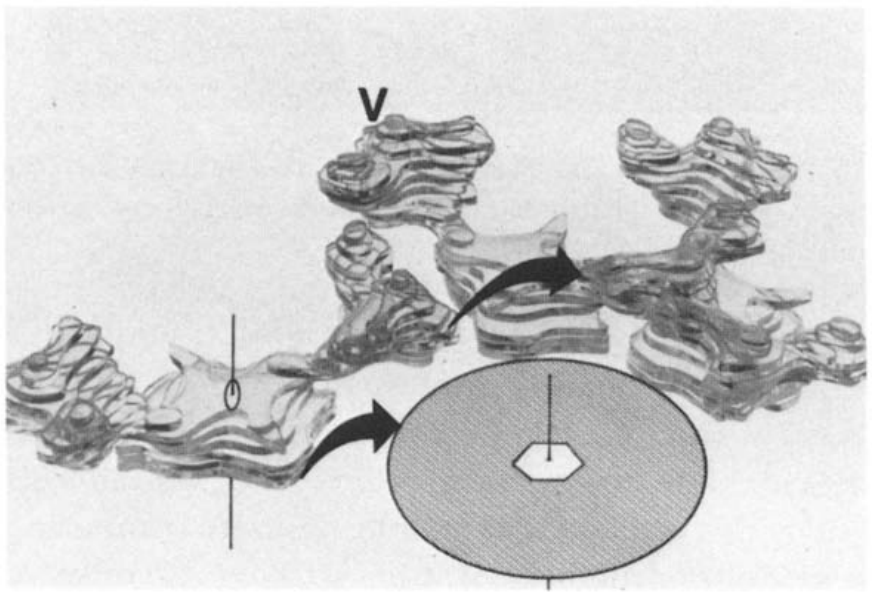

Fig. 7. 'Exploded' model of the *D. ambivalens* surface layer showing its dimeric organization. The domain marked 'V' varies in shape and position in layers from different species.

The differences can actually be ascribed to one distinct domain varying in shape and relative position. Thus, the 'species specificity' appears to be expressed at the level of the protein domain structure.

A species specificity imprinted on the outer surface, as is apparently common in eubacteria and archaebacteria, is an indication of a molecular recognition function. It could endow cells with the necessary specificity for establishing homotypic cell-cell contacts as observed in *Sulfolobus* (fig. 7), without ruling out the possibility of other types of specific interactions which are important, for example, in host-pathogen interactions.

6. Surface proteins of *Deinococcus radiodurans* and *Azotobacter vinelandii*

The surface layers of these two species have, at first glance, very little in common, the first one forming a hexagonal array on the bacterium with an unusually complex cell envelope and the second giving rise to a tetragonal array on a 'typical' Gram-negative bacterium. However, both serve as

useful examples, emphasizing in different ways the importance of the interaction with the underlying membrane.

The structure determination of the surface protein from *D. radiodurans* (HPI-layer) is particularly advanced; the three-dimensional structure has been determined via several independent routes [34,35] and in two dimensions a resolution of 0.8 nm has recently been attained [36]. The gene coding for the 105 kDa HPI polypeptide has been cloned and expressed in *E. coli* [37] and the primary structure has been derived from the nucleotide sequence. One of the interesting features of the HPI polypeptide is the occurrence of a tightly bound fatty acid located in the N-terminal region which is believed to act as a membrane anchor. The outer membrane of *Dc. radiodurans*, which has evolved independently of that of the Enterobacteriaceae, is very unusual. It contains several high-molecular-mass components to some of which enzymatic activities have been assigned [39]. The most abundant outer membrane protein is a 120 kDa 5′-3′ exonuclease [40] which is in direct contact with the HPI-layer forming 1 : 1 stoichiometric complexes in an epitaxial fashion [41]. This is the first demonstration of a supramolecular complex between an integral membrane protein and a surface protein and the challenge for the future is to unravel the functional implications of their association.

Although the nature of the interaction is less clear with *Az. vinelandii*, there is little doubt about its importance. When S-layer sheets are detached from the membrane they undergo a conformational change which entails a change in the apparent porosity (fig. 8). Even though the release of the layer from a support is a highly artificial situation, two conclusions can obviously be drawn: The association with the membrane is one determinant of the structure of the layer (and vice versa) and, from a mechanistic point of view, the layer is capable of carrying out distinct conformational changes [42].

7. The surface protein of *Comamonas acidovorans*

C. acidovorans (formerly *Pseudomonas acidovorans*) possesses an outer membrane displaying over its entire surface a rather simple tetragonal 'cobblestone' pattern, a structural motif which is not uncommon in the β-subdivision of purple bacteria

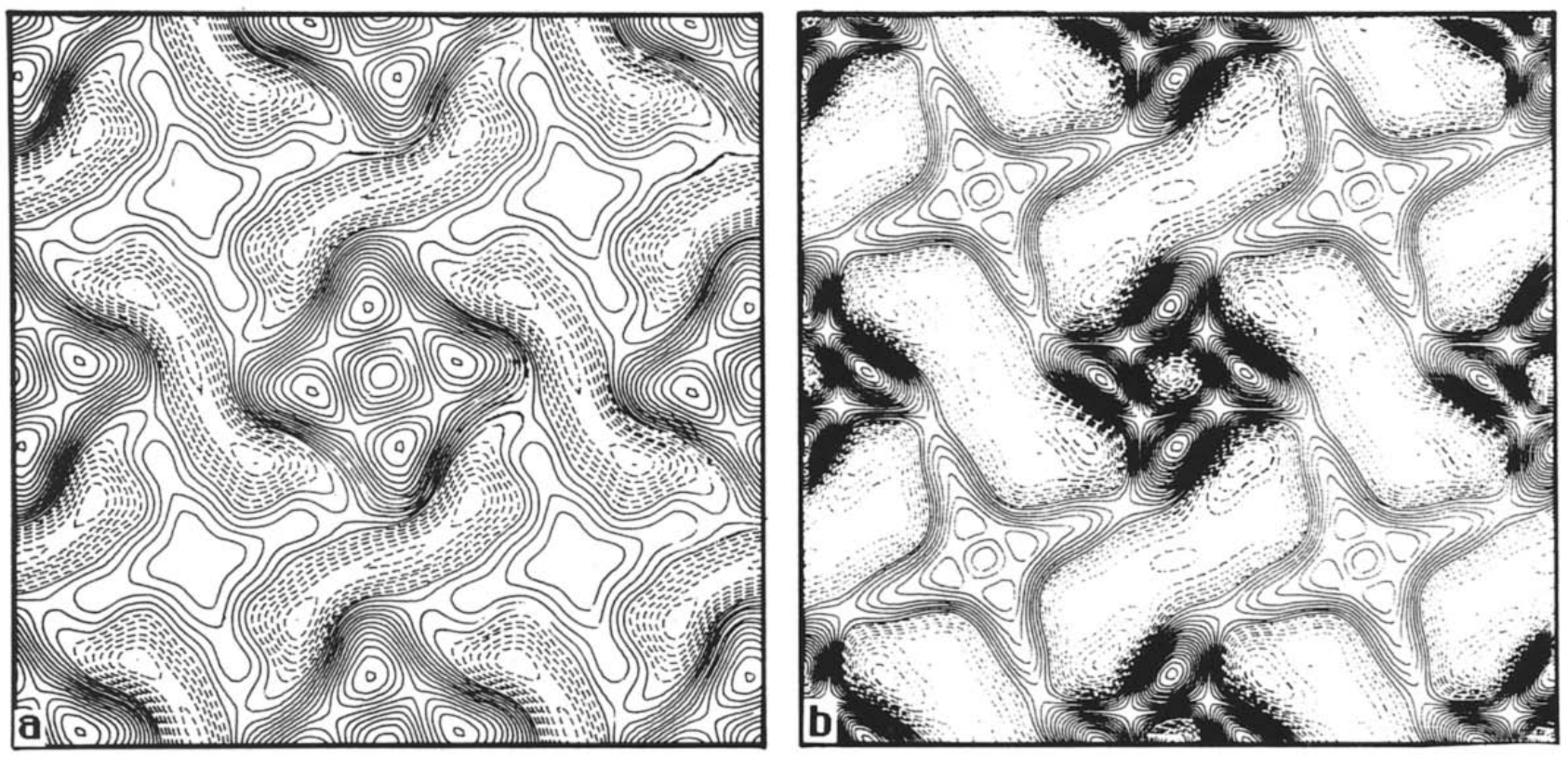

Fig. 8. Projection structures of the *Az. vinelandii* S-layer showing two conformational states. (a) From the membrane-associated layer; (b) from a layer dislodged from the membrane. From ref. 42.

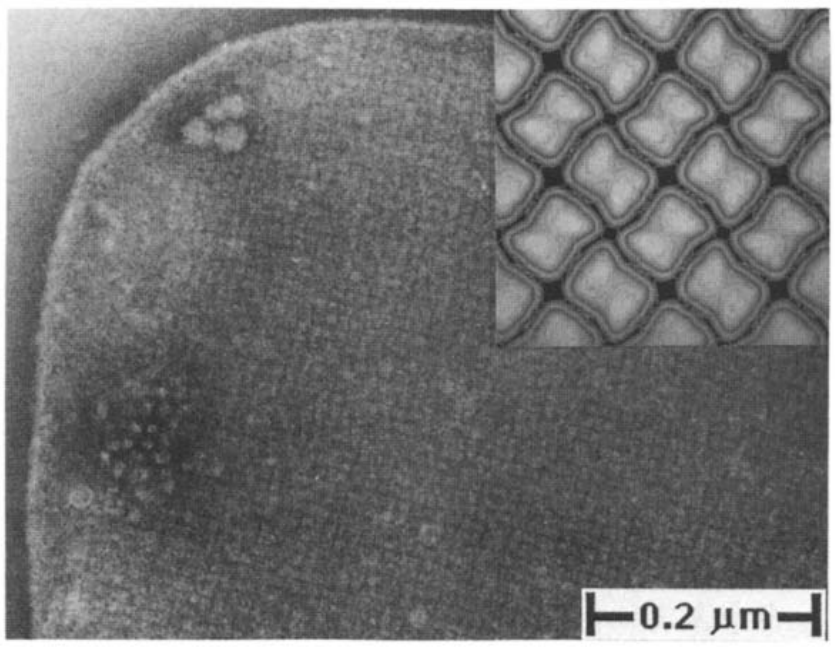

Fig. 9. Micrograph of a negatively stained outer membrane fragment of *C. acidovorans* with the corresponding correlation average showing the 'cobblestone' pattern. This surface protein might be classified as an rOmp and shares several features with the classical porins.

(fig. 9). While all attempts to dissect the protein lattice from the outer membrane have failed, treatment with SDS disintegrates the lattice. This immediately suggests that the protein is an integral component of the outer membrane (an 'rOmp'). Probably, the lattice is composed of a single polypeptide species with an apparent molecular mass of 32 kDa; since each of the 2-fold symmetric morphological units has a molecular mass of approx. 270 kDa according to STEM mass measurements, it could accommodate two tetramers of the 32 kDa protein [43]. It is reasonable to assume that the 32 kDa protein endows the outer membrane in one form or another with the necessary permeability to small solute molecules. Hence, it may be referred to as a porin even though the oligomeric complexes appear monolithic on electron micrographs without any evidence of a pore at a resolution of 2 nm. Without disregarding the possibility of a pore emerging at higher resolutions, one could also imagine pores as being formed in a fashion different from the classical porins. One could envisage, for example, pores to be formed in between the cobblestones, assuming that the protein is capable of organizing the lipid phase such that it departs from a simple bilayer organization. It is noteworthy that the 32 kDa protein shares several characteristic structural features with bona fide porins such as OmpF or OmpC from *E. coli*, including a β-structure content of 65% (manuscript in preparation). However, final proof of the relatedness must be obtained from sequence data. One of the attractions of this membrane is its rather simple composition and architecture, offering a good chance of obtaining a highly detailed and yet 'holistic' model of a bacterial outer membrane.

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