JOURNAL OF BACTERIOLOGY, May 1993, p. 2762–2766
0021-9193/93/092762-05$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 175, No. 9

Large-Scale Recrystallization of the S-Layer of *Bacillus coagulans* E38-66 at the Air/Water Interface and on Lipid Films

D. PUM, M. WEINHANDL, C. HÖDL, AND U. B. SLEYTR*

Zentrum für Ultrastrukturforschung und Ludwig Boltzmann Institut für Molekulare Nanotechnologie, Universität für Bodenkultur, A-1180 Vienna, Austria

Received 11 November 1992/Accepted 24 February 1993

S-layer protein isolated from *Bacillus coagulans* E38-66 could be recrystallized into large-scale coherent monolayers at an air/water interface and on phospholipid films spread on a Langmuir-Blodgett trough. Because of the asymmetry in the physicochemical surface properties of the S-layer protein, the subunits were associated with their more hydrophobic outer face with the air/water interface and oriented with their negatively charged inner face to the zwitterionic head groups of the dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylethanolamine (DPPE) monolayer films. The dynamic crystal growth at both types of interfaces was first initiated at several distant nucleation points. The individual monocrystalline areas grew isotropically in all directions until the front edge of neighboring crystals was met. The recrystallized S-layer protein and the S-layer–DPPE layer could be chemically cross-linked from the subphase with glutaraldehyde.

Two-dimensional crystalline surface layers (S-layers) represent the outermost cell envelope component in many bacteria (for reviews, see references 2, 5, 9, 12, 23–25, 27, 28, and 32). S-layer lattices are formed of assemblies of a single protein or glycoprotein species (M_r, 40,000 to 200,000). Most S-layer lattices exhibit either oblique, square, or hexagonal symmetry with spacings between the morphological units of 3 to 30 nm. With respect to their inner and outer faces, S-layers are highly anisotropic structures with regard to their topography (1, 5) and physicochemical properties (14, 18, 19, 22). Permeability studies revealed that S-layers have the potential to function as selective isoporous molecular sieves in the ultrafiltration range (18, 20). Isolated S-layer subunits of many organisms have shown the inherent ability to reassemble into two-dimensional arrays either in suspension or on solid surfaces upon removal of the disrupting agents (for reviews, see references 26 and 30). Since S-layers are periodic structures, functional groups such as carboxyl, amino, or hydroxyl groups occur on each constituent protein or carbohydrate moiety in an identical position and orientation (for reviews, see references 15 and 17). Thus, the arrangement of macromolecules bound to an S-layer lattice frequently follows accurately the crystalline structure. Because of this characteristic feature, S-layers represent novel immobilization matrices and patterning structures for applications in molecular nanotechnology (15, 16, 21, 30).

The possibility for generating extended areas of coherent monolayers of the crystalline arrays appears of particular interest for studying the physicochemical surface characteristics and the ultrastructure of S-layers, their permeability properties, and their potentials for specific interactions with molecules, cells, and surfaces. As suggested previously, S-layers may also be used as supporting and stabilizing structures for Langmuir-Blodgett films and reconstituted biological membranes (13, 16, 20, 29). Such composite structures will not only mimic the molecular architecture of those archaebacterial cell envelopes which are exclusively composed of an S-layer and a plasma membrane but will also lead to new techniques for studying membrane functions (for a review, see references 12, 15, and 16). In this paper, we describe simple strategies for the reassembly of S-layer subunits isolated from *Bacillus coagulans* E38-66 at the air/water interface and on lipid films. Previous structural, labelling, adsorption, permeability, and chemical modification studies (14, 18, 22) have shown that the S-layer lattice from this organism is a highly specialized supramolecular structure. The characteristic features can be summarized as follows: (i) composition of identical nonglycosylated protein subunits (M_r, 100,000); (ii) oblique lattice symmetry with lattice constants of a = 9.4 nm, b = 7.4 nm, and base angle γ = 80°; (iii) anisotropic topographical surface properties; (iv) very precise molecular sieving properties with charge-neutral pores preventing unspecific molecular adsorption; (v) free negative charges within indentations on the outer surface allowing binding of small positively charged macromolecules or isolated polymer chains; (vi) a charge-neutral characteristic of the more elevated domains on the outer surface; (vii) a net negatively charged inner surface; and (viii) an outer surface which is more hydrophobic than the inner face.

Isolation of S-layer subunits and assembly conditions. *B. coagulans* E38-66 was kindly provided by F. Hollaus (Österreichisches Zuckerforschungsinstitut, Fuchsenbigl, Austria). Growth of the bacteria in continuous culture is described in reference 31. Cell wall preparations and extraction of the S-layer protein with guanidine hydrochloride (5 M in 50 mM Tris hydrochloride buffer [pH 7.2]) were performed as described in reference 31. Guanidine hydrochloride extracts containing 5 mg of protein per ml of buffer were dialyzed against $CaCl_2$ solution (10 mM in distilled water, 2 h at 20°C) (14), and subsequently the self-assembly products were sedimented for 15 min at 40,000 × *g* at 4°C. The clear supernatant containing 2.5 mg of protein per ml with the unassembled S-layer subunits or oligomeric precursors (7) was used for all experiments. Recrystallization at air/water and lipid interfaces was carried out in a Fromherz-type

* Corresponding author.

Langmuir-Blodgett trough (made by Mayer Feintechnik, Göttingen, Germany) (4). The design of the trough shows eight circularly arranged compartments with a surface area of 45 cm^2 and a volume of 15 ml each. Thus, keeping the barriers at a fixed spacing allows transfer of a monolayer film between individual compartments and exposure of it to different subphases. The surface pressure was monitored with a Wilhelmi plate apparatus incorporated in the Fromherz trough (4). All experiments with the trough were carried out at room temperature (20°C). For the experiments, a volume of 2 ml of the clear supernatant of the S-layer solution (2.5 mg of S-layer protein per ml) was injected into the aqueous subphase in one compartment of the Fromherz trough. The advancing recrystallization at the interface could be monitored by measuring the increase in surface pressure with the Wilhelmi plate apparatus (increase of up to 15 to 20 mN/m). Recrystallization of S-layers on phospholipid films was carried out on monolayers of dipalmitoylphosphatidylcholine (DPPC; Sigma, no. P5911) and dipalmitoylphosphatidylethanolamine (DPPE; Avanti, no. 850705). A drop of solution of DPPC in hexane-ethanol solvent (1 mg of DPPC in 1 ml of hexane-ethanol [9:1, vol/vol]) or DPPE in chloroform-methanol solvent (1 mg of DPPE in 1 ml of chloroform-methanol [9:1, vol/vol]) was spread on the air/water interface and compressed to a surface pressure of 25 to 30 mN/m. Two milliliters of the clear supernatant of the S-layer solution as described above was injected into the water subphase of a neighboring compartment shortly before the compressed DPPC or DPPE film was carefully shifted over it. Cross-linking of the S-layer protein assembled at the interfaces and between the S-layer and the DPPE monolayer could be achieved by transferring the recrystallized S-layer or the S-layer–lipid film with the barriers of the Fromherz trough onto a further compartment containing 2.5% glutaraldehyde. This cross-linking step enhanced the stability of the films considerably for subsequent handling procedures.

S-layers at the air/water interface and on phospholipid films were transferred onto electron microscope grids coated with coherent or holey carbon films (33) which were carefully placed on the liquid surface and removed after 30 s by being lifted horizontally from the surface with a pair of tweezers (10).

Electron microscopical studies and digital image processing were done as described in reference 14. Image contrast was enhanced by negative staining of the protein and protein-lipid monolayer with uranyl acetate after fixation with glutaraldehyde as described in reference 11. To monitor the dynamic crystallization process at the interface, samples were taken after 20, 40, and 60 min.

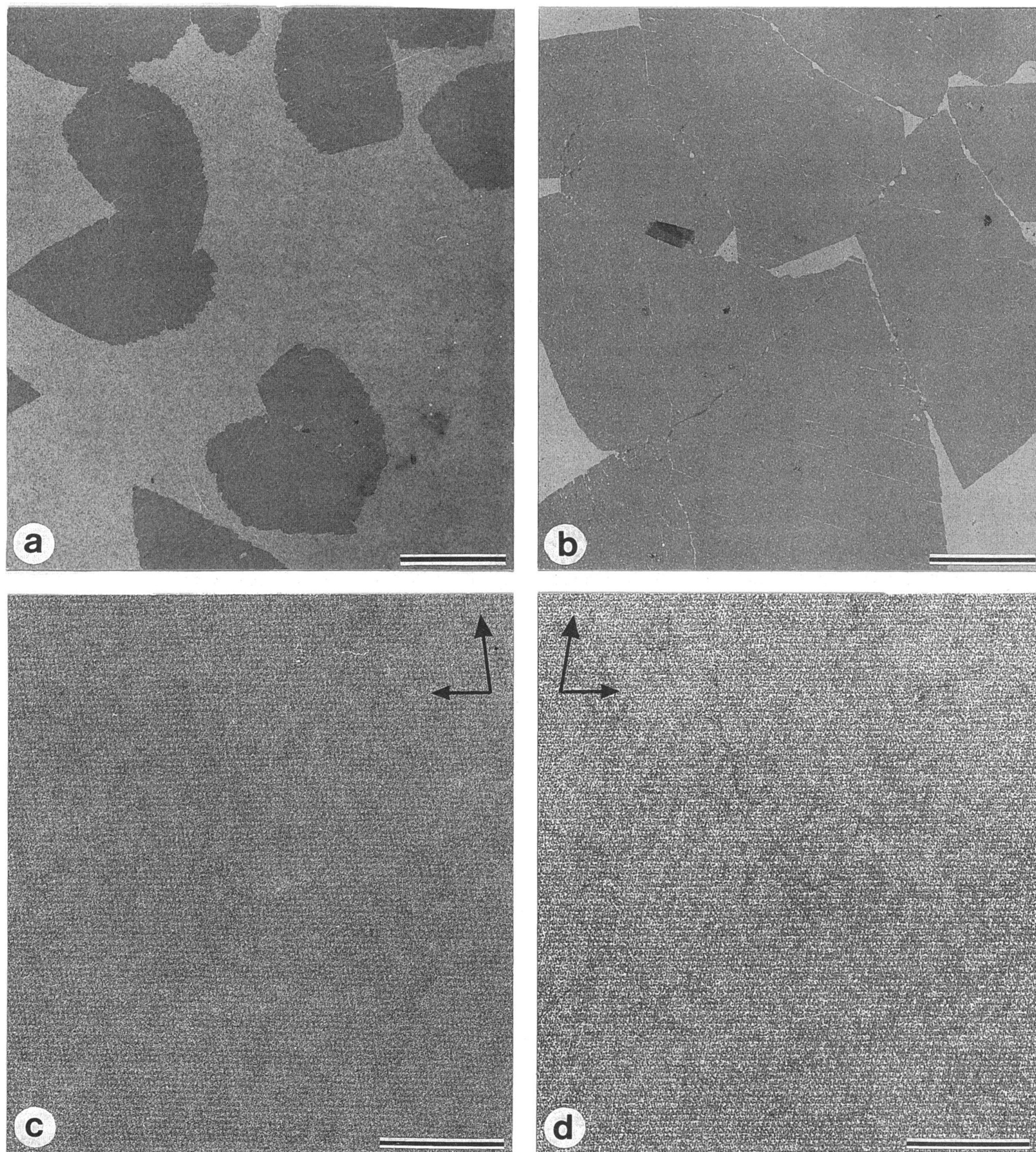
Recrystallization of S-layers at interfaces. Recrystallization experiments with the suspension of S-layer subunits isolated from *B. coagulans* E38-66 have shown that large-scale closed monolayers of crystalline arrays can be obtained after 1 h at the air/water interface. After transfer of the recrystallized S-layers onto carbon-supported films by horizontal lifting of the grids, the ordered protein domains appeared relatively undisturbed, completely covered holes up to a size of 1 to 5 μm, and were stable in the electron beam. Although the regularly structured layer had a crazy-paving appearance, with numerous crystal boundaries, all randomly oriented crystallites reaching an average size of several micrometers (5 to 10 μm) revealed the same handedness. Image processing and analysis of the optical diffraction pattern showed only a single primitive reciprocal lattice. Thus, we concluded that the oblique lattice represented a monolayer. The monolayer was also confirmed by digital image reconstructions (data not shown). Advanced stages of the dynamic process of formation of a closed monolayer at the air/water interface are shown in Fig. 1a to c. Negatively stained preparations of early stages of the assembly process (not shown) indicated that in the initial phase of lattice formation oligomers and/or small crystallites had randomly bound to the air/water interface. Concomitantly, crystal growth was initiated at several distant nucleation points and locally terminated when the advancing front regions of neighboring crystalline areas had met. No overlaps at the boundary lines were observed. A comparison of the handedness of the oblique lattice on intact cells (14, 22) and in the crystallites revealed that the monolayers were associated with the air/water interface with their outer face (Fig. 2a). The total size of the recrystallized protein layer was estimated by placing several grids at different locations onto the surface of the 45-cm^2 large compartment of the trough. Irrespective of where the grids had been placed on the interface, all were completely covered with an S-(mono)layer. Therefore, the size of the crystalline area must have been equivalent to the surface area of the compartment.

Large-scale recrystallization of S-layer subunits from *B. coagulans* E38-66 obtained on DPPC (Fig. 1d) and DPPE (not shown) monolayers was also successful. As on the air/water interface, the S-layer subunits assembled on the phospholipid films into closed, polycrystalline monolayers. As could be determined by the orientation of the base vectors of the oblique lattice (Fig. 1d), which was a mirror image of the crystalline arrays formed at the air/water interface, the subunits had bound with their net negatively charged inner face (14) to the hydrophilic head groups of DPPC and DPPE (Fig. 2b). The composite S-layer–lipid film structures could be transferred onto carbon-coated grids without destroying the crystalline structure. The random orientation of the individual crystallites and their boundary lines indicated that crystal growth was governed by mechanisms equivalent to those on the air/water interface.

The two dominant forces determining the orientation of the S-layer subunits with respect to the air/water and lipid interfaces must be hydrophobic and electrostatic interactions (6). Although proteins generally exhibit a strong tendency to unfold upon adhering to hydrophobic interfaces and to expose their hydrophobic parts, the S-layer proteins of *B. coagulans* E38-66 did not denature on the air/water interface and maintained their ability to form coherent monolayers. The observation that the recrystallized S-layer was associated with the air/water interface with its more hydrophobic outer face was in agreement with previous recrystallization experiments on solid surfaces (14).

The observation that the S-layer of *B. coagulans* E38-66 was oriented towards the phospholipid films with its inner face led to the conclusion that primarily electrostatic interactions between the exposed carboxyl groups on the inner face and the hydrophilic, zwitterionic head groups of DPPC and DPPE were responsible for the defined orientation of the subunits.

The possibility for generating large-scale coherent S-layers at an air/water interface or on lipid films and for handling such layers by standard Langmuir-Blodgett techniques (16, 34) opens a broad spectrum of applications. For studying the physicochemical surface properties of S-layers, the crystalline arrays may be attached to different solid supports. Depending on the surface properties (charge, hydrophobicity) of the support and because of the surface anisotropy of the S-layer or S-layer–lipid film, a vertical deposition will



•FIG. 1. Electron micrographs illustrating the dynamic crystallization process at the air/water interface of S-layer subunits isolated from *B. coagulans* E38-66 (a to c) and on lipid films (d). (a) Crystal growth is initiated at several distant nucleation points. (b) The individual monocrystalline areas grow isotropically in all directions until the front end of neighboring crystals is met. (c and d) Monocrystalline S-layer area at the air/water interface (c) and on a DPPC film (d). The vector pairs indicate the different orientations of the S-(mono)layers at the air/water interface and on lipid films. Bars, 2 μm in panels a and b and 200 nm in panels c and d.

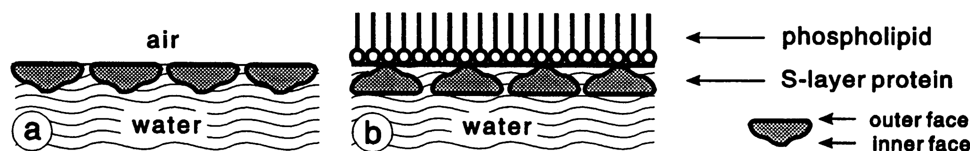


FIG. 2. Schematic representation of the orientation of the S-layer at the air/water interface (a) and on lipid films (b).

allow definition of the orientation of the layer (inner or outer face, lipid or protein side) on the support (16). In comparison to unsupported membranes, lipid films associated with S-layers are much more stable structures. Particularly after cross-linking with glutaraldehyde, S-layer-lipid composite structures can be spread over porous matrices and apertures. At present, we are studying S-layers which have recrystallized at the air/water interface or on lipid interfaces as supports for reconstituted biological and synthetic membranes. The latter are bimolecular phospholipid membranes and tetraether lipid membranes (3). Such composite structures generated by Langmuir-Blodgett techniques will closely resemble those archaebacterial envelope structures which are exclusively composed of an S-layer and a closely associated plasma membrane (for reviews, see references 2, 8, 12, 24, and 26). Spread over apertures, they allow combination of functional studies of membrane-associated or integrated molecules (16) with structural studies by transmission electron microscopy and scanning tunneling or atomic force microscopy.

This work was supported by grants from the "Österreichischer Fonds zur Förderung der wissenschaftlichen Forschung" Projekt S5705, the Österreichisches Bundesministerium für Wissenschaft und Forschung, and the Österreichische Bundeswirtschaftskammer.

We thank H. Strack, K. Heckmann, K. Lohner, F. Aussenegg, and H. G. Schindler for helpful discussions.

REFERENCES

1. **Baumeister, W., and H. Engelhardt.** 1987. Three-dimensional structure of bacterial surface layers, p. 109–154. *In* J. R. Harris and R. W. Horne (ed.), Electron microscopy of proteins, vol. 6. Academic Press Inc., London.
2. **Beveridge, T. J., and L. L. Graham.** 1991. Surface layers of bacteria. Microbiol. Rev. **55:**684–705.
3. **Choquet, C. G., G. B. Patel, T. J. Beveridge, and G. D. Sprott.** 1992. Formation of unilamellar liposomes from total polar lipid extracts of methanogens. Appl. Environ. Microbiol. **58:**2894–2900.
4. **Fromherz, P.** 1975. Instrumentation for handling monomolecular films at an air-water interface. Rev. Sci. Instrum. **46:**1380–1385.
5. **Hovmöller, S., A. Sjögren, and D. N. Wang.** 1988. The structure of crystalline bacterial surface layers. Prog. Biophys. Mol. Biol. **51:**131–161.
6. **Israelachvili, J.** 1985. Intermolecular and surface forces. Academic Press Inc., London.
7. **Jaenicke, R., R. Welsch, M. Sára, and U. B. Sleytr.** 1985. Stability and self-assembly of the S-layer protein of the cell wall of *Bacillus stearothermophilus*. Hoppe-Seyler's Z. Physiol. Chem. **366:**663–670.
8. **König, H.** 1988. Archaeobacterial cell envelopes. Can. J. Microbiol. **34:**395–406.
9. **Koval, S. F.** 1988. Paracrystalline protein surface arrays on bacteria. Can. J. Microbiol. **34:**407–414.
10. **Kubalek, E. W., R. D. Kornberg, and S. A. Darst.** 1991. Improved transfer of two-dimensional crystals from the air/water interface to specimen support grids for high-resolution analysis by electron microscopy. Ultramicroscopy **35:**295–304.
11. **Messner, P., F. Hollaus, and U. B. Sleytr.** 1984. Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains. Int. J. Syst. Bacteriol. **34:**202–210.
12. **Messner, P., and U. B. Sleytr.** 1992. Crystalline bacterial cell surface layers. Adv. Microb. Physiol. **33:**213–275.
13. **Pum, D., M. Sára, P. Messner, and U. B. Sleytr.** 1991. Two-dimensional (glyco)protein crystals as patterning elements for the controlled immobilization of functional molecules. Nanotechnology **2:**196–202.
14. **Pum, D., M. Sára, and U. B. Sleytr.** 1989. Structure, surface charge, and self-assembly of the S-layer lattice from *Bacillus coagulans* E38-66. J. Bacteriol. **171:**5296–5303.
15. **Pum, D., M. Sára, and U. B. Sleytr.** 1992. Two-dimensional (glyco)protein crystals as patterning elements and immobilisation matrices for the development of biosensors, p. 141–160. *In* U. B. Sleytr, P. Messner, D. Pum, and M. Sára (ed.), Immobilised macromolecules: application potential. Springer-Verlag, London.
16. **Pum, D., and U. B. Sleytr.** Molecular nanotechnology with S-layers. *In* T. J. Beveridge and S. F. Koval (ed.), NATO-ARW: advances in bacterial paracrystalline surface layers, in press. Plenum Publishing Corp., New York.
17. **Sára, M., S. Küpcü, C. Weiner, S. Weigert, and U. B. Sleytr.** 1992. Crystalline protein layers as isoporous molecular sieves and immobilization and affinity matrices, p. 71–86. *In* U. B. Sleytr, P. Messner, D. Pum, and M. Sára (ed.), Immobilised macromolecules: application potential. Springer-Verlag, London.
18. **Sára, M., D. Pum, and U. B. Sleytr.** 1992. Permeability and charge-dependent adsorption properties of the S-layer lattice from *Bacillus coagulans* E38-66. J. Bacteriol. **174:**3487–3493.
19. **Sára, M., and U. B. Sleytr.** 1987. Charge distribution on the S-layer of *Bacillus stearothermophilus* NRS 1536/3c and the importance of charged groups for morphogenesis and function. J. Bacteriol. **169:**2804–2809.
20. **Sára, M., and U. B. Sleytr.** 1988. Membrane biotechnology: two-dimensional protein crystals for ultrafiltration purposes, p. 615–636. *In* H. J. Rehm (ed.), Biotechnology, vol. 6b. VCH Verlagsgesellschaft, Weinheim, Germany.
21. **Sára, M., and U. B. Sleytr.** 1989. Use of regularly structured bacterial cell envelope layers as matrix for the immobilization of macromolecules. Appl. Microbiol. Biotechnol. **30:**184–189.
22. **Sára, M., and U. B. Sleytr.** 1993. Relevance of charged groups for the integrity of the S-layer from *Bacillus coagulans* E38-66 and for molecular interactions. J. Bacteriol. **175:**2248–2254.
23. **Sleytr, U. B.** 1978. Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly, and function. Int. Rev. Cytol. **53:**1–64.
24. **Sleytr, U. B., and P. Messner.** 1983. Crystalline surface layers on bacteria. Annu. Rev. Microbiol. **37:**311–339.
25. **Sleytr, U. B., and P. Messner.** 1988. Crystalline surface layers in procaryotes. J. Bacteriol. **170:**2891–2897.
26. **Sleytr, U. B., and P. Messner.** 1989. Self assembly of crystalline bacterial cell surface layers (S-layers), p. 13–31. *In* H. Plattner (ed.), Electron microscopy of subcellular dynamics. CRC Press, Inc., Boca Raton, Fla.
27. **Sleytr, U. B., P. Messner, and D. Pum.** 1987. Analysis of crystalline bacterial surface layers by freeze-etching, metal shadowing, negative staining, and ultra-thin sectioning, p. 29–60. *In* F. Mayer (ed.), Methods in microbiology, vol. 20. Academic Press Inc., London.
28. **Sleytr, U. B., P. Messner, D. Pum, and M. Sára (ed.).** 1988. Crystalline bacterial cell surface layers. Springer-Verlag, Berlin.
29. **Sleytr, U. B., P. Messner, D. Pum, and M. Sára.** Crystalline

bacterial cell surface layers: general principles and application potential. J. Appl. Bacteriol., in press.
30. **Sleytr, U. B., D. Pum, M. Sára, and P. Messner.** 1992. Two-dimensional protein crystals as patterning elements in molecular nanotechnology, p. 167–177. *In* A. Aviram (ed.), AIP Conference Proceedings 262, Molecular Electronics—Science and Technology. American Institute of Physics, New York.
31. **Sleytr, U. B., M. Sára, Z. Küpcü, and P. Messner.** 1986. Structural and chemical characterization of S-layers of selected strains of *Bacillus stearothermophilus* and *Desulfotomaculum nigrificans*. Arch. Microbiol. **146:**19–24.
32. **Smit, J.** 1987. Protein surface layers of bacteria, p. 343–376. *In* M. Inouye (ed.), Bacterial outer membranes as model systems. John Wiley and Sons, New York.
33. **Toyoshima, C.** 1989. On the use of holey grids in electron crystallography. Ultramicroscopy **30:**439–444.
34. **Ulman, A.** 1991. An introduction to ultrathin organic films. Academic Press Inc., Boston.