Surface Layers of Bacteria
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INTRODUCTION

General Comments about Bacterial Surfaces

Procaryotes are extremely small unicellular life-forms; for example, Escherichia coli typically occupies a cell volume of only ca. 1.5 to 2.5 μm³ (18, 20). More than anything else, this minute size imposes on bacteria many of their so-called procaryotic features. Their small cytoplasmic space is not large enough to differentiate many cellular processes into compartmentalized organelles or to easily house a true nucleus. The small size also means that procaryotes cannot rely on the engulfment of particulate matter for nourishment but, rather, must depend on the diffusion of solutes across their boundary layers. It is very possible that the multitude of shapes and sizes we see in the procaryotic world are, at least in part, due to an absolute requirement for a high surface-area-to-volume ratio, which ensures efficient diffusion of nutrients and wastes (18). These broad surfaces increase the likelihood of solute impact with the cell periphery, and the interfacial forces concentrate diffusible substances from the local environment to this boundary layer.

With the possible exception of Mycoplasma and Spiroplasma spp. of the Tenericutes class (45, 129), and unlike eucaryotic cells, maintenance of bacterial shape is not determined by a cytoskeleton; instead, it is determined by a pressure-resisting cell wall (19, 107). Whether this wall is of the gram-positive, gram-negative, or gram-variable variety, it withstands the turgor pressure of the protoplast and contours the cell into a predetermined form (16, 18, 19). The estimated turgor pressures within bacteria are considerable (for gram-negative cells they can range from 2 to 5 atm [210 to 252 kPa] [18, 109], and for gram-positive cells they can be 5 to 10 times higher [18], since Bacillus subtilis walls, when hydrated, can withstand 2,431.2 kPa [128]) and attest to the tensile strength of the materials which make up various wall types. Indeed, as physical forces, these very pressures may be important for the growth and division of many bacteria (107, 108). Clearly, the growth of a bacterial cell requires surface expansion to accommodate increasing cellular mass, and, somehow, new wall polymers must be incorporated into the preexisting matrix to enable this expansion. Moreover, at a predetermined stage in the growth cycle, cell fission occurs and demands localized regions of tremendous wall growth. Whether division is through septation or constriction (20), large expanses of newly formed wall are exposed when daughter cells separate; for rods such as B. subtilis or E. coli, a new pole is manufactured by each daughter cell (135).

In addition to such features as diffusion efficiency, cell turgor resistance, maintenance of shape, and growth and division, bacterial surfaces are important since they make up the outermost boundary between the external milieu and the cell. It is these leading edges which must somehow withstand the brunt of environmental hostility, whether chemical (e.g., pH extremes, toxic substances, and hydrolytic enzymes) or physical (e.g., Bdellovibrio, phagocyte, bacteriophage, or bacteriocin attack). In an unalterable way, bacteria must somehow eke out their existence in natural environments where the competition is tough. Unless they have developed a way of resisting very harsh natural conditions in which monocultures are feasible (e.g., Halobacterium halobium in high salt concentrations), all bacteria in their natural settings must fight for survival; a good partition between the protoplast and the natural surroundings is one way to rebuff the "slings and arrows" from the external milieu. The boundary layers which compose these surfaces are major factors in determining bacterial survival and, consequently, can account for a substantial portion of each cell's mass (16).

The cell wall is considered to be the prime stress-bearing structure and resides above the plasma (or cytoplasmic) membrane (19, 20). For well-studied strains such as B. subtilis 168 and E. coli K-12, which are gram-positive and gram-negative bacteria, respectively, the walls are easily differentiated from one another in thin section. The B.
subtilis 168 wall is an amorphous matrix about 25 nm thick (Fig. 1), whereas that of E. coli is a more complex outer membrane-murein consortium (Fig. 2). However, for less extensively studied bacteria such as Clostridium thermosaccharolyticum (Fig. 3) or Methanococcus voltae (Fig. 4), the recognition is not so apparent. For C. thermosaccharolyticum, the wall consists of a peptidoglycan-bearing matrix which is only 3 to 6 nm thick and is overlaid by a proteinaceous surface array or S layer (24). M. voltae has only a single S layer as its wall structure (102). Our traditional idea that all walls are easily comparable to those of B. subtilis (gram positive) and E. coli (gram negative) is not justifiable any more, especially as our knowledge of gram-variable and archaeobacterial walls becomes more complete (24, 25, 35, 51, 111). Moreover, additional surface structures such as capsules, slimes, S layers, sheaths, or even pili (fimbriae), flagella, and spinae (16, 20) may complicate our view. Furthermore, new crypreparatory techniques for electron microscopy are changing our perception of the native polymeric organization of walls (55, 56, 79, 83, 88, 100, 120, 138, 168) and the advent of new nondenaturing plastics and immunogold labeling has provided a better impression of molecular placement of selected surface components (10, 40, 41, 106, 150, 155).

ELECTRON MICROSCOPY OF BACTERIAL SURFACES

Conventional Techniques and the Traditional View

Microbiology and electron microscopy have become strongly interrelated as the elucidation of high-resolution biological structure has progressed. Among the first cells viewed by the Toronto electron microscope in the late 1930s (the first electron microscope in North America was developed and built in 1938 by A. Prebus and J. Hillier in the Department of Physics, University of Toronto [73]) were the vole bacillus (Mycobacterium microti) and tubercle bacillus (Mycobacterium tuberculosi) in whole-mount preparations (110). These images and the breathtaking first thin-section images of a bacterium (B. cereus) by Chapman and Hillier in 1953 (43) ensured that the electron microscope was the tool of choice for the study of subcellular structure and, especially, of the macromolecular architecture of bacterial surfaces (16, 47, 92).

Certainly, there are problems associated with the use of conventional electron microscopy in elucidating bacterial structure since hydrated specimens cannot be observed in situ and only the “bare bones” of the cell are preserved (20). Yet, through the careful use of a variety of conventional methods including whole mounts, thin sections, freeze-etching, and negative stains in combination with one another, it is remarkable how accurately our microscopic impression of bacterial envelopes has correlated with the biochemical data. For example, the lipid bilayers of outer and plasma membranes are clearly seen in thin section (Fig. 2) and the weak bonding between opposing acyl lipid tails can be demonstrated by freeze-fracture (44), which also exposes many of the intrinsic protein complexes of the membrane (Fig. 5 and 6). Gram-positive bacteria, such as B. licheniformis, possess only one lipid bilayer (the plasma membrane) and show only the one membrane fracture (Fig. 5). Interestingly, plasma membranes possessing unusual tetraether phospholipids, such as in Methanospirillum hungatei, effectively cross-link the two membrane faces by the covalent bonds joining the acyl tails so that these bilayers cannot be cleaved in half during freeze-fracture (19, 29). The fracture of membranes in gram-negative bacterial surfaces is more difficult to interpret since both the outer membrane and plasma membrane reside in the cell envelope. The plasma membrane fractures with a higher incidence than the outer membrane, and these fractured surfaces resemble those seen in gram-positive bacteria (cf. Fig. 5 and 6). The outer membrane is not so easily fractured, which suggests that this membrane has stronger bonding forces which anneal the bilayer together. Concave fracture faces show a high particle density in the outer membrane (Fig. 6).

Thin sectioning and freeze-etching are usually not considered to be high-resolution techniques since the grain size of the embedding plastic (Fig. 1 to 4) or shadowing substance (Fig. 5 and 6) obscures fine detail (92). Narrow structures such as flagella and pili (Fig. 7) and thin boundary layers such as S layers (Fig. 8) are difficult to image in thin section and are better defined by negative staining, from which high-resolution structural information can sometimes be extracted by Fourier computer filtration and image reconstruction (159, 160, 162). For this type of analysis, the structural object must possess some degree of periodicity and optical diffraction, or, preferably, electron diffraction can be used as an index of the retention of high-order structure prior to computer processing. It is by this technique that porin complexes (see, e.g., reference 101), purple membrane (see, e.g., reference 100), and S layers (Fig. 9) have been so accurately defined. Although platinum shadowing and freeze-etching are not usually considered high-resolution techniques because they coat surfaces and their appendages with a layer of metal, the visualization of these structures is amplified. S layers are particularly well suited for this technique since their topography and in situ lattice defects can be ascertained (151–154). For nonperiodic objects such as ribosomes or highly distorted lattices, computer correlation averaging can be used (13, 49, 159, 160, 162).

It is to these techniques of negative staining, thin sectioning, shadow-casting, freeze-etching, and computer enhancement of image that we owe much of our present-day idea of the macromolecular architecture of bacterial surfaces (160, 162). These have shown accurately the juxtaposition of the boundary layers and have given, at least, a glimpse of their predicted macromolecular architecture. Cytocchemical approaches using thin sections in which the molecular substance is identified have also allowed us to recognize that there is a chemical distribution within bacterial envelopes (118, 119).

Surface Layer Organization and the Gram Stain

The Gram stain remains the major cytological technique in use today for readily deciphering the shape, form, and intactness of bacterial cells by light microscopy; conveniently, it also distinguishes among gram-positive, gram-negative, and gram-variable eubacteria. However, it cannot be used so readily to distinguish among different types of archaeobacteria (19, 35).

Although a variety of distinguishing features or chemical differences in eubacteria have been suggested as being responsible for differentiating between gram-positive and gram-negative varieties (see reference 51 for a list), it is apparent that the murein content of the cell wall is of primary importance (24, 25, 51, 143). Except for regional wall weaknesses related to septation in some genera (24), gram-positive walls have enough cross-linked peptidoglycan in their matrices to withstand the trauma of Gram staining and retain the initial purple staining deposit (25, 51). Even though
it is becoming apparent that more than one peptidoglycan layer makes up gram-negative murein (88, 115, 119), these envelopes are more harshly disturbed by the Gram reagents; the outer membrane dissolves, the murein layer breaks apart, and the cells are decolorized and stained red with either safranin or carbol fuchsin (25). Bacteria which are notorious for their Gram-staining variability (e.g., *B. brevis*, *C. thermosaccharolyticum*, or *C. tetani*) frequently have walls that are intermediate between those of their more unequivocally staining counterparts (cf. Fig. 3 with Fig. 1 and 2). In such cells, the murein layer is of intermediate thickness and its absolute thickness can be growth phase dependent; as cells enter exponential growth the layer becomes thinner and the frequency of gram-positive cells decreases (24). So far, regardless of the Gram response in eubacteria, the reaction can be interpreted in terms of cell wall chemistry and organization (24, 25, 51, 143).

The Gram reaction is not as straightforward for archaeobacteria. These cells are encompassed by a wide range of simple to complex boundary layers, and their envelopes can contain unusual macromolecules (111). For example, *M. voltae* (Fig. 4), *Sulfolobus acidocaldarius*, and *H. halobium* have single proteinaceous or glycoproteinaceous S layers as their sole cell walls, whereas the thick, amorphous walls of *Methanobacterium* and *Methanosarcina* spp. are made of polyurein [alternating N-acetyl-D-glucosamine β(1,3)-linked N-acetyl-L-talosaminuronic residues with tripeptide stems of t-Glu, t-Ala, and t-Lys] and of methanochondroitin (a loose, fibrillar matrix of uronic acid β(1,3)-linked to an N-acetylgalactosamine dimer), respectively (111). The most complex envelopes found in archaeobacteria belong to *Methanospirillum* and *Methanoseta* (Methanotherixi) spp.; these bacteria are bound by single-layered walls (presumably S layers [28, 29, 33]) and enrobed in a proteinaceous sheath in which individual cells are separated by spacer plugs (Fig. 10). The sheaths possess a periodic surface array and are also thought to be S layers (28, 29, 33, 161).

An encompassing statement about the relationship of the Gram reaction to archaeobacterial enveloping structures cannot yet be made; precise experiments monitoring the staining response for a wide variety of archaeobacteria possessing representative envelope profiles have not yet been performed. So far, *Methanospirillum hungatei* is the best studied of this group, and a mechanistic view of its Gram stain has recently been presented (35). This bacterium grows as chains of cells within a sheath (Fig. 10), and only the terminal cells of each filament stain gram positive; the remainder of the filament stains gram negative. The sheath is so impervious that the Gram reagents cannot penetrate it; they can penetrate only the more porous terminal plugs at the filament ends and, consequently, can form large staining deposits which cannot be removed by decolorization only within the terminal cells (35). The entire filament of cells would be gram positive if only the staining reagents could get in! The counterstain, carbol fuchsin, also cannot enter the filament but sticks to the outside of the sheath by charge interaction and colors the rest of the filament red. Clearly, the Gram stain reveals a striking difference between *Methanospirillum* and other bacteria. In a general sense, the impermeability of this bacterium implies that only very small molecules (e.g., those with \( M_c \) less than 50; e.g., \( \text{H}_2, \text{CO}_2, \) and \( \text{CH}_4 \)) can penetrate the sheath and that larger molecules must diffuse inward from the ends of the filament (35). This seems to be a unique design strategy for the bacterium (18, 20) and emphasizes how relatively simple light-microscopic stains, such as the Gram stain, can outline fundamental characteristics of the kingdom *Prokaryotae*.

**Surface Layers External to Cell Wall**

Frequently, there are external surface layers above the cell wall; this is especially evident with bacteria growing naturally in the environment or those recently isolated and grown in the laboratory. For the latter group, after serial passage on laboratory media, selection pressure generally results in unadorned cell walls which are, presumably, an economical adaptation made possible by the undemanding confines of an artificial environment.

**Capsules.** Capsules are one of the most frequently encountered superficial layers. Extending great distances from the cell surface (often up to several micrometers [14]), capsules are used as cementing substances to bind bacteria together into microcolony formats, to bind the cells to substrata, or to form flocs, eventually developing large biofilms which shielded visible surfaces (48, 121). The organic constituents of capsules are branched or unbranched homo- or heteropolymers which are typically polysaccharidic but can be proteinaceous (16, 127, 173). Surprisingly, their most abundant constituent is water, and this characteristic makes them very difficult to preserve intact for ultrastructural study; consequently, they readily collapse during dehydration for electron microscopy. Capsule polymers are highly flexible, extend radially from the cell wall, are entirely bathed in water, and are rarely cross-linked to one another by strong bonding forces. The most effective linkages between capsule polymers seem to be electrostatic; highly acidic types (e.g., those rich in carboxyl groups) can be naturally cross-linked by divalent metal ions such as \( \text{Mg}^{2+} \) or \( \text{Ca}^{2+} \). However, these metals are highly exchangeable and can be displaced by other multivalent species (127). This trait has been used for many years by electron microscopists since highly electro-positive electron-dense stains, such as ruthenium red or alcin blue, add chemical stability through salt linkage between the polymers, thereby reducing the degree of collapse during processing for thin sectioning (20, 48, 92). Diamines can also be used for this purpose (99). A more selective method of stabilization is the use of specific antisera or lectins which cross-link and maintain the extended state of the capsular polymers. Care must be taken in

*FIG. 1.* Note the amorphous wall fabric of *B. subtilis* 168 (arrow), which lies directly above the plasma membrane.

*FIG. 2.* *E. coli* possesses a thin peptidoglycan or murein layer above the plasma membrane (arrow), over which lies a wavy outer membrane. The region between the outer and plasma membranes is called the periplasmic space; this is where the periplasm resides. The waviness of the outer membrane is believed to be an artifact of the conventional fixation-embedding technique, and much of the periplasm has been leached out.

*FIG. 3.* *C. thermosaccharolyticum* is an example of a gram-variable bacterium which has a wall profile intermediate between the gram-positive and gram-negative formats in Fig. 1 and 2. The peptidoglycan layer (arrow) is thinner than that of *B. subtilis* but thicker than that of *E. coli*. Above this layer is a proteinaceous S layer of periodically arrayed subunits.

*FIG. 4.* *Methanococcus voltae* is an archaeobacterium and possesses only a thin S layer (arrow) above the plasma membrane as its sole wall layer.
FIG. 5 and 6. Freeze-fractured and -etched cells of B. licheniformis and E. coli, illustrating the envelope fracture planes. The large arrowhead in each figure points out the shadow direction. Bars, 100 nm.

FIG. 5. B. licheniformis fractures through the plasma membrane (PM), exposing proteinaceous particles in this convex cleavage plane. The outline of the cell wall can be seen at the periphery of the cell.

FIG. 6. Fractures through two E. coli cells are shown to demonstrate concave ( ~/ upper cell) and convex ( ~/ lower cell) cleavage planes through the envelope layers. Fracture through the outer membrane (OM) is relatively rare in E. coli and indicates that the bonding forces are stronger here than those which anneal the two plasma membrane (PM) faces together. Of all the fracture faces, the concave hydrophobic outer membrane face is the most particulate and emphasizes the incidence and arrangement of outer membrane proteins within this membrane. The plasma membrane of E. coli resembles that of B. licheniformis in particle distribution.
FIG. 7. Piliated *E. coli* strain negatively stained with 2% (wt/vol) uranyl acetate. This strain was isolated from an infected animal at the Ontario Veterinary Clinic, University of Guelph. These pili (arrows) are among the thinnest structures emanating from the surfaces of bacteria, and although this particular *E. coli* strain has not been serotyped, they presumably act as adhesins. Bar, 25 nm.

FIG. 8. Negative stain (2% uranyl acetate) of the S layer of *Aquaspirillum putridiconchylum* showing the linear array of proteinaceous subunits which are arranged with p2 symmetry. Bar, 25 nm.

FIG. 9. Computer-enhanced image of the S layer of *A. putridiconchylum* shown as a contour map of the proteinaceous subunits. The unit cell is drawn with $a = 11.8$ nm, $b = 7.8$ nm, and $\gamma = 73^\circ$. Fourier analysis was used to produce the image. Reproduced from M. Stewart, T. J. Beveridge, and R. G. E. Murray, Structure of the regular surface layer of *Spirillum putridiconchylum*, J. Mol. Biol. 137:1–8, 1980, with permission of the Journal of Molecular Biology.
interpreting stabilized capsules since all stabilizing agents add extra mass to these lean polymers and can contribute substantial "substance" to their electron-microscopic profiles. A recent study of the capsules of *Streptococcus suis* in which several different stabilizing techniques were used revealed that the capsules could vary from 20 nm to 375 nm depending on the preparatory technique (98).

**External slimes.** Like capsules, bacterial slimes include a wide range of externalized homo- and heteropolymers (16, 20). They are abundant in nature, often floating freely in the aqueous milieu, and are a frequent constituent of biofilms. Sometimes bacteria overproduce their capsular materials or, for some reason, fail to anchor them securely to their surfaces. Such polymers are sloughed off into the surroundings and float freely until they become associated with other solid surfaces. Electron microscopy of natural biofilms or suspended flocs frequently reveals envelope and other cellular debris from lysed bacteria intermixed with intact cells throughout a polymeric matrix (Fig. 11); this matrix was initially part of a capsule but is now more appropriately classified as extracellular slime since it is no longer entirely associated with the dynamic turnover processes of viable cells (173).

**Sheaths.** Sheaths are less frequently encountered than capsules in the bacterial world. They are tubes enclosing linear chains of cells and may be composed of an amorphous polymeric network, such as those of *Thiothrix* or *Thioploca* spp. or some cyanobacteria (117, 158), or a highly ordered surface array, such as those of *Methanosaeta* or *Methanospirillum* spp. (29, 33, 34, 161). In this context, the latter group have sheaths consisting of an S layer. Those which exhibit gliding motility (e.g., *Thioploca* spp.) can glide within the sheath (117). Other genera such as *Leptothrix* and *Sphaerotilus* can develop flagella in the cells at the filament termini. These cells, called swarmers, swim away to form new filaments (76), frequently leaving partially empty sheaths. The sheaths of both genera are noted for their tremendous precipitation of iron (*Sphaerotilus*) and manganese (*Leptothrix*) oxides (76), especially in the older, empty regions of the sheath (21, 22, 76). At least for *Leptothrix discophora*, the manganese-oxidizing capability is determined by a protein within the sheath (3, 39). Recently, cryofixation and freeze-substitution have shown that this species possesses a capsule which is structurally differentiated from the sheath and outer membrane (21, 83) (Fig. 12) (also see the section on freeze-substituted surface layers).

The sheaths of *Methanospirillum hungatei* and *Methanosaeta conciliae* are very different from those previously discussed. They are proteinaceous, regularly arrayed (and therefore similar to S layers), and resistant to degradation (33, 36, 136). Small (2.8-nm) particles are arranged with p2 symmetry on the outer face of both *Methanospirillum* and *Methanosaeta* sheaths. Specialized partitions (cell spacers) close off the sheath along its length, keeping the cells separated, and are associated with cell division (28, 29). In addition, these archaeobacterial sheaths are instrumental in resisting cell turgor pressure and are at least partially responsible for contouring individual cells into a rod shape (29, 33). Clearly, these sheaths are an integral peripheral structure of these genera and have far-reaching design purposes over and above those of more usual eubacterial varieties (18).

**S layers.** S layers were relatively unknown three decades ago and were, at that time, restricted to a few *Acinetobacter*, *Aqua spirillum* (*Spirillum*), *Bacillus*, and *Clostridium* spe-
cies; by 1983 S layers had been identified on almost 150 bacteria, and today the number approaches 250 (151–154). Unlike most other surface structures, these arrays can be unequivocally identified only by electron microscopy, and this has inhibited their routine study. They seem to be a common feature of archaeobacterial surfaces (111, 154) and are being identified with increasing frequency on a range of eubacteria (153).

S layers are planar arrays of proteinaceous or glycoproteinaceous subunits which can be aligned in unit cells of p1, p2, p4 or p6 symmetries (Fig. 8 and 9) (13, 16, 93, 112, 114, 151–154). The regularity of their arrangement makes them convenient structures for crystallographic analysis and computer image enhancement (13, 159, 160); three-dimensional analyses have been produced for several S layers (13). Our overall impression is that, once the S layers are self-assembled on the cell, between their regularly arranged subunits there exists a network of identically sized pores able to selectively filter macromolecules according to size and charge. In support of this are experiments which show some S layers to have sharp exclusion cutoffs for molecules with $M_w$’s between $5 \times 10^4$ and $8 \times 10^4$ (145, 146); indeed, they seem suitable candidates for a new generation of industrial ultrafiltration membranes or immobilization matrices (146, 148).

Possibly even more important is the discovery that S layers can contribute to virulence when present as a structural component of pathogens. The S layer of Aeromonas salmonicida A450 has two slightly different p4 lattice formats which affect the size of their pores and, presumably, their filtration characteristics (162). Since this S layer is required for infection (97), it is possible that the structural transformation is influenced by host factors as the bacterium attempts to cope with antagonistic serum products. Campylobacter fetus also possesses a linear lattice (58), and recent information suggests that the bacterium produces a family of S-layer proteins of different $M_w$’s but with common chemical, structural, and antigenic traits (57). S layers have also been identified on Treponema spp., Helicobacter (Campylobacter) pylori, Aeromonas hydrophila, Cardiobacterium hominis, Bacteroides spp., Wolinella spp., Rickettsia spp., Chlamydia spp., and pathogenic Clostridium and Bacillus spp. (153). S layers may prove to be much more important in pathogenesis than was originally thought.

Archaeobacterial and eubacterial S layers share a unique characteristic: they represent the only incidence of surface glycoproteins in the procaryotic world. Certainly, most S layers do not have carbohydrate moieties, but the chemical structures of small glycosubstituents in Halobacterium halobium, Bacillus stearothermophilus, Clostridium thermohydrodsulfuricum, and Clostridium symbiosum have been determined (131, 133, 174). Since the glycosubstituents are frequently only a small percentage of the total glycoprotein mass, they can be missed by periodic acid-Schiff staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and more rigorous chemical analyses may be required (e.g., hydrolytic separation from the protein and nuclear magnetic resonance analysis [131]). It is very possible that reanalysis of existing proteinaceous S layers by these new techniques will show that several are glycoproteins.

For electron microscopy, freeze-etching and negative staining are the best methods of determining S-layer struc-
FIG. 12. Thin section of a cryofixed, freeze-substituted cell of *Leptothrix discophora*. This is a swarmer cell caught in the process of leaving its sheath (S). A capsule (C) surrounds the bacterium, and no periplasmic gel is seen between the outer membrane and the plasma membrane. Manganese oxide precipitates are associated with the sheath in the background (M). Bar, 500 nm. The culture was kindly supplied by W. Ghiorse, Cornell University. Reproduced from reference 18 with permission of the *Canadian Journal of Microbiology*. 
ture. Freeze-etching relies on the fact that frequently a fracture plane will occur along the ice-cell interface and etching will expose the subunit profiles. Because the grain of the shadowing metal (e.g., Pt) is relatively large (ca. 2 to 5 nm), freeze-etching reveals only the general form and alignment of the S-layer subunits around the cell. Crystal defects are often seen by this technique (151, 152). Negative staining is a better resolving technique and relies on the fact that heavy metal salts such as sodium phosphotungstate or uranyl acetate can ensnarl the subunits in an electron-dense "glass" revealing contours and linkages of the proteinaceous components. Unfortunately, the best imaging occurs only with extremely thin specimens, and this requires that intact S layers be, somehow, sloughed off of the cells as single layers before a negative stain is applied. On negatively stained whole cells, an S layer can usually be seen only as a thin, periodic fringe at the cell periphery. Accordingly, it is very difficult to directly determine cell wall–S-layer interactions on cells by electron microscopy without fixation, embedding, and thin sectioning. Because this technique requires dehydraion with organic solvents, correct protein conformation can be altered and organic constituents can be extracted; this latter point is especially critical for gram-negative bacteria (79).

It is also becoming clear that some S layers are neutrally charged on their external face at physiological pH (144, 147). Others, such as Thermoproteus spp., are anionic (132). S layers are deficient in highly electronegative thiol groups, and many of their electronegative carboxylates are buried within the subunit core after molecular folding and self-assembly has taken place (16, 112, 114, 152). Since the visibility of S layers in thin-section profiles relies on the availability of electronegative chemical groups and their binding of heavy-metal cations, the most intensely stained regions would be expected to be the subunit cores.

Since S layers are self-assembly systems, their subunits are usually held to each other and to the underlying wall by weak bonding forces; electrostatic and hydrophobic interactions prevail. In some cases, salt bridging by divalent metalloids (e.g., Ca$^{2+}$ and Mg$^{2+}$) is important (16, 93, 112, 114, 151, 152). Consequently, relatively mild extraction procedures (such as pH extremes, cation displacement, nonionic detergents, or hydrogen bond-breaking agents) can be used for their removal (113). However, the very fact that some archaeobacteria, such as Thermoproteus, Sulfolobus, and Methanococcus spp., rely on a single S layer as their sole cell wall suggests that these bonding forces are strong enough to form a protective barrier for these cells. Indeed, for Thermoproteus spp. the S layer maintains the rod shape of the bacterium (132).

New Cryotechniques for Electron Microscopy

One of the most exciting advances in electron microscopy of biological structure has been the recent development and use of cryotechniques which supersede the techniques of freeze-etching. Unlike traditional chemical fixation methods, these new techniques rely on ultrarapid freezing to physically fix cellular substance so quickly that all vital features are retained. This technique, combined with low-temperature scanning electron microscopy, has been used to provide excellent topographical views of yeasts and molds (15). For more extreme resolution, transmission electron microscopy is used, and it is critical that the freezing is so rapid that crystalline ice is not formed (140, 165). Instead, an amorphous or vitreous glass is necessary so that small, growing ice crystals do not puncture cells and deform their native structure. Theoretically, cooling rates much greater than thousands of degrees per second are required to form vitreous ice, but these are extremely difficult to measure accurately and obtain since the rate will be affected by the cell type (140). Typically, for microbiological samples, thin films of cells are either plunged into an ultracold cryogen (e.g., propane or ethane) or slammed onto a smooth metal surface (e.g., gold or copper). The cryogen (or the surface) is held at either liquid nitrogen or liquid helium temperature and, being highly conductive, acts as a "heat sink" to extract thermal energy from the specimen. This extraction must be so efficient that all of the latent heat of crystallization is removed from the cell, otherwise there may be localized melting, recrystallization of the amorphous ice, and structural damage (140, 165). Since microorganisms are so small, usually an ultrarapid freezing depth of 5 to 15 μm is suitable.

It is possible, by using specially adapted electron microscopes with cryospecimen holders, to maintain thin films of small particles, such as viruses, in a vitrified state during examination and to rely on the mass difference between the particle and ice to provide contrast for viewing (4, 55, 165, 166). Since flagella, S layers, and purple membranes approach the thinness of virus particles, they, too, can be imaged (77, 100, 120, 167). In fact, it is possible to produce vitreous thin sections of frozen hydrated bacteria. These sections reveal the outer and plasma membranes of E. coli to be of uniform density and the periplasm to be ca. 33 nm thick (56). Staphylococcus aureus walls, representing those of gram-positive cells, appear as relatively amorphous material ca. 40 nm thick. Since cryospecimens are embedded in frozen water (ice), they remain hydrated, and it is likely that retention of the hydration shells encompassing macromolecules enhances preservation of native structure (105) and is responsible for the better images obtained.

Cryofixation also seems to better retain highly mobile ions and their distribution in cells and, when used with energy-dispersive X-ray spectroscopy (EDS), provides accurate compositional mapping (163). The instrumental analysis (163) can be used with elemental mapping in the scanning transmission electron microscopy (STEM) mode. In this way the distribution of Na, K, Ca, Mg, etc., in exponential-phase E. coli B cells and Bacillus spores has been determined (42, 103, 163, 164). The spore studies have proven especially interesting. Ca, Mn, Mg, and P were concentrated in the cores of all species studied, but the cores can possess a range of other metalloids. Besides the usual mono- and divalent ions in the coat, Si was detected in B. cereus (163), Si and Zn in B. megaterium (103), and Fe and S (but no Si) in B. coagulans (164).

In addition to elemental mapping, cryosections can be used for immunolabeling to locate and determine the distribution of specific macromolecules throughout cellular space. This technique has been used to identify lipopolysaccharide (LPS) on Neisseria gonorrhoeae (172) and common antigen on E. coli and Yersinia enterocolitica (1). Some microscopists question the accuracy of this method since, unlike cryoelemental analysis, immunolabeling requires thawing or freeze-drying of the specimen and exposure to an antibody fluid phase, which could affect antigen distribution. Prefixation with low aldehyde concentrations prior to vitrification can retard this macromolecular dispersion, but can also denature the antigenic structure (150).

For electron microscopists without devoted cryomicroscopes, a tremendous advance, combining rapid freezing with conventional plastic-embedding techniques, has be-
come available; it is called freeze-substitution. Although the technique was first used almost 30 years ago (61, 62, 169), it has been slow to gain popularity in microbiology. Much of the initial work was with fungi and produced starting detail (90, 94, 175); cellular ground substance was well preserved and distributed through cellular space; internal membrane systems, organelles, and the nucleus were well preserved; and apical vesicles were intact and spherical (see, e.g., reference 91). The first reports of freeze-substituted bacteria came much later (7-9, 27, 88, 89).

For freeze-substitution of bacteria, thin layers of cells (5 to 15 μm deep) are ultrarapidly frozen by either the plunging or slaming technique. Then, at low temperature (−80°C), the vitreous ice is slowly cryosubstituted by an organic solvent (e.g., acetone) containing chemical fixatives (e.g., glutaraldehyde or osmium tetroxide). At the low substitution temperature, the cells remain frozen but the ice is gradually replaced by the solvent fluid (dehydration), which carries the chemical fixatives with it (fixation). Although most fixatives are poorly reactive at −80°C, the specimen in its frozen state is immobile and diffusion of organic solvent into the specimen is slow. In all possible fixatives, it is suggested that there can be no restricted penetration of fixative by previously cross-linked material, when temperatures are raised all molecules will be fixed simultaneously (96). Since the entire process occurs at −80°C, macromolecular hydration shells are maintained (105) until fixation preserves the native molecular conformations. There is very little information on the size of hydration shells surrounding macromolecules, but it must be affected by electrolyte concentration (e.g., each DNA nucleotide is surrounded by 20 to 50 water molecules in pure water but by fewer than 10 in physiological buffers [86]). It is possible that substitution does not entirely remove all hydration water at these low temperatures and that this is also a reason for the remarkable preservation produced by the freeze-substitution technique. Indeed, the low-temperature Lowicryl methacrylates (10, 40) and the low-temperature embedding techniques (40, 41) of the researchers in Basel produce not only superior structural preservation but also good retention of cellular antigenicity (106), so they can be used for immunogold labeling (155). Recently, Graham and Beveridge have compared freeze-substitution with conventional embedding by using E. coli and B. subtilis, have established the superiority of freeze-substitution for both preservation of structure and cell composition, and have developed a simple methodology involving plunging cryofixation for the freeze-substitution of bacteria (79, 80).

Freeze-Substituted Surface Layers

Freeze-substitution has irreversibly altered our perception of bacterial surfaces. The first images made by using this method revealed the periplasmic space of E. coli (7, 88) to be completely filled with matter in which a thin peptidoglycan layer could not be seen; the concept of a periplasmic gel was thus established (88). The periplasm was so well preserved that its enzyme machinery, the molecules in transit (presumably including de novo cell wall macromolecules), and the peptidoglycan were left intact. It is still unclear whether peptidoglycan fills the entire space, but small-angle neutron diffraction measurements in a separate study (115) suggest that the layer may be more than one molecule thick in some regions of the cell.

Close scrutiny of the freeze-substituted E. coli K-12 outer membrane, which possesses only core carbohydrate on its LPS, suggested that it was asymmetrically stained, with the bulk of the heavy-metal deposition occurring on the outer face of the bilayer (Fig. 13) (18, 19, 21). This confirms an asymmetrical lipid distribution across the outer membrane, in which most of the LPS resides on the outer face whereas phospholipid is mostly on the inner face (74, 134). The higher phosphate-to-lipid ratio of LPS compared with phospholipid ensures that the outer face is more electronegative and binds more stain (64-66). Bacteria, such as P. aeruginosa PA01, which possess long O side chains on their LPS show an entirely different outer membrane profile. A fringe of fine fibrils extends ca. 40 nm above the outer membrane face (Fig. 14) (116). The PA01 strain, which does not express alginate as a capsule, possesses two distinct kinds of LPS in its outer membrane, a low-M, A-band LPS and a high-M, B-band LPS, but only B-band LPS binds uranyl ions (139) for visualization by electron microscopy. An isogenic rough mutant (strain AK 1414) lacking both A and B bands and a mutant (strain AK 1401) containing only A-band LPS do not show the fringe (116). It is very possible that the fringe on strain PA01 seen by freeze-substitution is the long-chain B-band LPS. This visual discrimination of LPS types shows the remarkable preservative power of the technique. Recently, Meno and Amako, using immunogold on freeze-substituted Klebsiella pneumoniae, detected two distinct fibrous domains on the outer membrane; the immunoprobe was able to penetrate the expoly saccharide and label the underlying O antigen (130).

Clearly, the appearance and extent of the periplasm and the retention of outer membrane asymmetry depend on the cryofixatives used during substitution (80); we recommend the use of osmium tetroxide and uranyl acetate unless Lowicryl resins are to be used (40, 41, 80). All gram-negative eu bacteria that we have studied possess asymmetric outer membranes, but not all exhibit an E. coli-type envelope profile after freeze-substitution (Fig. 13). Proteus, Pasteurella, Campylobacter, Aeromonas, Klebsiella, and Hae morhopsis spp. all possess periplasmic gels, but Leptothrix and Caulobacter spp. do not; these two bacteria have only thin peptidoglycan layers (see, e.g., Fig. 12) (83). P. aeruginosa (Fig. 14) and Vibrio cholerae (Fig. 15) have intermediate periplasmic densities (83). No doubt these details reiterate, in surface-structural terms, that not all gram-negative bacteria are entirely equal to E. coli. A more detailed discussion on periplasms appears in reference 82.

Freeze-substituted gram-positive walls are also very different from their conventionally fixed counterparts. Instead of an amorphous matrix, thin-sectioned freeze-substituted walls of B. subtilis (Fig. 16), Lactobacillus casei, Lactobacillus acidophilus, and S. aureus reveal a fibrous matrix radiating outward and extending 10 to 30 nm beyond the plasma membrane (9, 46, 80, 138, 168). In B. subtilis, the wall density was graduated, appearing darkest at its inner face and decreasing in electron density as the distance from the cell increased (80). Since each fiber was well stained, the decreasing electron density was presumed to indicate a decrease in mass at the most peripheral wall regions. This fits the concept of wall growth and turnover for this bacterium (108); the innermost wall polymers are tightly packed since they are under low lateral stress, whereas those at the periphery are more loosely bonded and are being shed into the external milieu as soluble products. The long polymers in the middle region are strongly bonded and are under high stress and therefore are presumably more spread out than the innermost polymers. Recent analyses of exponential-phase B. subtilis cells revealed that the wall thickness...
FIG. 13 through 16. Thin sections of cryofixed, freeze-substituted bacteria which are representative of gram-negative and gram-positive envelope types. Not all gram-negative bacteria show periplasmic gels, but their asymmetric LPS distribution within the outer membrane is well preserved. Bars, 50 nm.

FIG. 13. *E. coli* K-12 has a well-defined periplasmic gel, and the outer face location of the LPS is intensely stained.

FIG. 14. *P. aeruginosa* PAO1 possesses an intermediate-sized periplasmic gel and long-chain LPS, which is seen as a fringe above the outer membrane profile.

FIG. 15. *Vibrio cholerae* shows a more intermediate type of periplasm in which less substance is seen than in *E. coli* (i.e., Fig. 13), but more than in *Leptothrix discophora* (i.e., Fig. 12). Reproduced from reference 83 with permission of the American Society for Microbiology.

FIG. 16. *B. subtilis* 168 is representative of all of the gram-positive bacteria that we have freeze-substituted and shows the fibrous nature of the wall.
was greatest at cylinder-pole junctions (38.2 nm) and over developing septa (40.5 nm); the cylindrical wall (a region of high turnover [108]) and cell poles (regions of low turnover [108]) were consistently thinner at 33.8 nm (81). Clearly, there are distinct disparities in regional thickness within the *B. subtilis* wall which can be seen by freeze-substitution and which indicate regions of active growth and turnover of the constituent polymers.

Capsules are among the most difficult surface structures to stabilize for electron microscopy and, as stated above, usually require stabilizing agents for preservation. Because capsules are so dependent on water for their extended state, freeze-substitution (or other methods which use ultrarapid freezing) is the technique of choice for their accurate visualization. Capsules have been visualized by freeze-substitution of *E. coli* (K1, K30 [Fig. 17], and His 1 strains), *K. pneumoniae*, *V. vulnificus*, *Proteus mirabilis*, *Haemophilus pleuronopneumoniae*, and *Leptothrix discophora* without the aid of stabilizing agents (6, 8, 83). Those capsules of *E. coli* and *K. pneumoniae* extend 100 to 200 nm from the cell as thin fibrils (83, 130). Interestingly, for *Leptothrix discophora*, freeze-substitution revealed a previously unseen fibrous capsule which extended from the outer membrane to the sheath, the outermost structure for these cells (Fig. 12) (83).

*S* layers have not been the subject of much study using freeze-substitution. However, the few studies that have been performed are quite informative. For example, the *S* layer of *Aeromonas salmonicida* A450 was shown to be continuously attached to and not separated from the underlying outer membrane (83) as conventional embedding techniques had implied. The *S*-layer subunits of *Aeromonas salmonicida* are thought to be attached to the lipid A core of the LPS (104). *S* layers that are very difficult to preserve, such as that of *Caulobacter crescentus*, also show attachment to the outer membrane, and their periodicity is exquisitely preserved (Fig. 18). Intuitively, for layers which are supplied by protomers from below and which, at least with gram-negative bacteria, require a wall surface on which to self-assemble (151, 152), the attachment seems reasonable. It is possible that freeze-substitution will allow wall-*S*-layer interactions to be better understood. Very dynamic and loosely bonded *S* layers, such as that of *B. thuringiensis*, which are not retained by conventional embedding techniques, are also preserved by freeze-substitution (122).

In the freeze-substitution study of Graham et al. (83), subunit periodicity in *Aeromonas salmonicida* was partially obscured by a narrow band of "fuzz" which emanated from the outer membrane to a level slightly above the outer *S*-layer face (Fig. 19). Since freeze-substitution has managed to preserve the extended LPS O side chains of *P. aeruginosa* (116), it is likely that the *Aeromonas salmonicida* fuzz is due to its LPS side chains. Implicit in this interpretation is the recognition that the termini of the LPS O side chains are surface available on this microorganism; this has been confirmed by enzyme-linked immunosorbent assay (137).

Freeze-substitution and other cryotechniques are proving to be powerful tools in redefining our traditional perception of bacterial surface organization. It is not so much that our original ideas were wrong, but more that conventional techniques could take us only so far. Now we have managed to take a few steps farther, and some of the organizational gaps have been filled. As these techniques become more and more routine, we are confident that even more gaps will be filled. Indeed, the recent application of immunocytochemical labeling to freeze-substituted specimens has enabled the localization of specific cell surface antigens (1, 2, 130, 149) and is a method which avoids the difficulties of cryoultramicrotomy. Clearly, rapid freezing preserves epitopes accurately and the substitution process renders samples into more easy-to-handle plastic blocks.

**Surface Physicochemistry**

Because bacteria are so small, the physicochemical attributes of their surfaces have been exceedingly difficult to
bacteria, for groups are present to external substances such as metal
capsule, sheath, or a combination, is in immediate contact
with the external milieu, and its physicochemical relationship
with the local environment is of the utmost importance
(18, 19). In the preceding sections of this review we have
concentrated on the structural organization of cell surfaces.
For this section we must recognize that this organization
provides a framework by which surface-exposed chemical
groups are presented to external substances such as metal-
oions and other solutes. Clearly, external pH is important
for the ionization of surface groups and can even affect the
conformation of constituent polymers. We envision the cell
surface to be undergoing a constant interplay with its envi-
ronmental constituents, in which the cell surface macromol-
ecules flex in response to pH and electrolyte concentration
as weak bonding forces are affected and ions are inter-
changed. Clearly, loosely knit polymers such as those in
capsules would be profoundly sensitive (75, 126), but even
more robust networks such as peptidoglycan layers can be
altered (22, 31, 32, 52, 54, 95). In fact, it is becoming
apparent that we have been shortsighted when we think of
bacterial surfaces only in terms of their organoconstituents;
their very design ensures an active association with environ-
mental agents so that the agents become an integral part of
the surface structure (17, 21–23). This is especially true of
inorganic agents such as select divalent alkaline earth metal
ions (Mg$^{2+}$ and Ca$^{2+}$) which are essential for the correct
packing of LPS in outer membranes (65); if they are dis-
placed, the outer membrane is forced to adopt tighter curvatures at discrete localities (i.e., it blebs and is sloughed
off). A prime example of this phenomenon is the effect of
aminoglycoside antibiotics on the P. aeruginosa outer mem-
brane; gentamicin (123) and amikacin (170) displace essential
Mg$^{2+}$ and Ca$^{2+}$, bleb the outer membrane (Fig. 20) and
create minute transient holes in the wall, allowing additional
antibiotic to reach the plasma membrane. This correlates
well with the self-promoted uptake concept suggested by
Hancock (85). Although the major killing action of these
antibiotics is at the ribosomal level, it is possible that
localized antibiotic effects on the cell envelope also contrib-
ute to cell lysis and death.

Because bacteria depend on diffusion of nutrients and
wastes for their livelihood (18), their surfaces must not only
be permeable to these substances, but must also be wettable.
Most bacterial surfaces studied so far have possessed an
overall electronegative charge (18, 23). Certainly some sur-
faces have a higher charge than others (i.e., the γ-glutamyl
capsule of B. licheniformis ATCC 9945 has a higher charge
density [126] than the outer S-layer face of B. stearothermo-
philus [147]), but the undeniable fact remains that some
hydrophilicity must exist in all bacterial surface structures.
In fact, it is very possible that distinct regional charge
disparities exist both above and throughout the surface
matrix. Certainly, if a bacterium possesses a gram-negative
envelope with a capsule on top, there is no reason why the
charge of the capsule must equal that of the outer membrane
or the peptidoglycan. Indeed, because they are composed of
different chemical constituents, equal charges would be most
unlikely. Furthermore, since turnover rates for these struc-
tures differ, it is possible that the progressive development of
new charge sites on each structure alters through time.

A simple example of charge differential is the polycationic
ferritin (PCF) labeling of B. subtilis walls under limiting PCF
concentrations at neutral pH, which has revealed that cer-
tain regions of the wall surface, especially those close to
polar caps are more electronegative than others (81, 156).
Under higher saturating conditions, PCF labels the entire
outside of intact cells (81); the outer face of isolated walls is
also labeled, but not the inner face (Fig. 21) (157). This
asymmetric charge distribution across the wall is controlled
by the placement and integrity of both peptidoglycan and
tetrahydroxy polymers (157). Other bacilli, such as B.
licheniformis, B. megaterium, B. cereus, and B. thuringien-
sis (Fig. 22), do not share the wall charge asymmetry of B.
subtilis. For this reason, there must be a fundamental
difference in the organization of the wall polymers of these
other bacilli compared with B. subtilis, and this is reflected in
the availability of exposed electronegative sites. Interest-
ingly, when isolated B. subtilis walls are freeze-substituted,
both the inner and outer faces of the walls exhibit a fibrous
topology similar to that seen only on the outer face of intact
cells (e.g., Fig. 16). However, the asymmetrical charge
character seen in conventional embeddings is conserved (81).

PCF is a useful probe for electronegative surface sites since it has a thick electron-dense iron core that is easily identified by electron microscopy. However, as a probe for labeling distinct molecularly based electronegative sites, it is an extremely large macromolecule. Together with M. Sára, D. Pum, and U. B. Sleytr (Zentrum für Ultrastrukturenforschung, Vienna, Austria), we have used much smaller molecular probes, such as cytochrome c, for this purpose. The high isoelectric point of this molecule (pl = 10.8) at pH 7.0 and its small molecular dimensions (2.5 by 2.5 by 3.7 nm) allow it to fit into very small spaces on bacterial surfaces (22). Cytochrome c was used to map the periodic exposed COO− sites on the sheath of Methanospirillum hungatei to a resolution of 0.9 nm; this showed them to lie within the holes between the protein subunits of the S-layered sheath (Fig. 23) (22).

Although these electron-microscopic techniques enable the examination of individual cell characteristics, other methods which rely on large numbers of cells (i.e., bulk hydrophobicity-hydrophilicity tests) are also useful in determining bacterial surface physicochemistry. Contact angle measurements and the partitioning of cells into the solvents of biphasic systems (e.g., dextran and polyethylene glycol or water and hexadecane) have all been used (65, 142). In this way, capsular polysaccharides have been shown to make a bacterial (Acinetobacter) surface more hydrophilic than the outer membrane alone (141). The wettability of the relatively hydrophobic bacterium Serratia marcescens is dependent on the relative abundance of three surface constituents, a 70-kDa protein (serrapathin), a pigment (prodigiosin) (both of which are hydrophobic), and an amphiphatic aminolipid (serratamolide) (11, 12, 87). Recently, two new surface-active lipids have been identified in Serratia rubidaea: rubiwettin RG1, which is a glycolipid, and rubiwettin R1, which is a mixture of linked 3-hydroxy fatty acids (124).

Indeed, even the simple sorption of metallic ions to gram-negative surfaces can profoundly alter their physicochemistry; the addition of Mg2+ or Mn2+ to E. coli K-12 makes the surfaces much more hydrophobic than if they were in the Ca2+ or Na+ salt form (65). LPS is the prime surface candidate for metal sorption to this bacterium because of the availability of its phosphoryl groups (64) and, possibly, one of three existing 2-keto-3-deoxyoctulosonic acid carboxylate sites (66). Metallic ion-induced hydrophobicity occurs in an order related to (i) the quantity of metal bound to the outer membrane and (ii) the valence and hydration energy of the cation (63). If LPS is removed from the surface, there is an overall increase in hydrophobicity since this molecule is crucial for hydrogen bonding to the liquid-water lattice which surrounds the cell. Consequently, interfacial free energy increases, with a resultant loss in wettability (65). The entire metalloion effect is particularly important since surface hydrophobicity must influence outer membrane permeability and cell-cell, cell-substrate, or cell-substratum adhesion; the properties of the entire water-solid interface are affected.

The high surface area-to-volume ratio of bacteria, their electronegative surface character, their metal-binding ability, and their ubiquitousness throughout water, soil, sediment, and the deep subsurface give procaryotic cells a special relationship with the initial phases of the global cycling of hard minerals. Microbial acids can leach metals from minerals and speed up weathering processes, but the ability of bacteria to sorb and immobilize metals in different waters, sediments, and soils is, perhaps, not as well known. It is probable that bacterial sorption has a profound effect on
The initial immobilization and concentration of soluble environmental metals and, through lithification, is an important first step in the diagenesis of metals. Several reviews and chapters about metal-biosurface interactions with microorganisms and their eventual mineralization have been written (17, 21–23, 26, 52, 63, 84, 127), and they provide a more in-depth treatment than can be provided here. Sufficient to say that bacterial surfaces, more than any other biological surface we are aware of, are tailor-made to enshroud themselves with metallic coats (23). Samples from a number of natural sites, whether they be from freshwater streams, hydrothermal vents, mine tailings, or industrially contaminated soils (67–71, 125), reveal that the metal deposit around each cell can approach and surpass cellular dimensions (Fig. 24). In fact, laboratory simulations involving clay-bacterial wall composites in metal sorption experiments, revealed that organoconstituents dominate the immobilization process (171) and that the mineralized product, especially that associated with the bacterial fractions, was difficult to remobilize under a variety of leaching conditions (72).

The overall effect of this phenomenon on a global scale is not trivial (26) and could approach Gaian proportion, especially when annual microbial biomass production rates are considered along with the fact that these microbiogeochemical reactions have, presumably, been under way since the dawn of life (21, 23, 26). It is very possible that the ancient remnants of prokaryotic life embedded in certain cherts and shales, such as the Gunflint Chert of the northern Superior region of Canada, owe their very origin to this innate ability
of the cells to sorb and mineralize the electrolytes which surrounded them (30, 70).

GENERAL FUNCTIONAL ATTRIBUTES OF BACTERIAL SURFACES

Over the last decade there have been tremendous advances in the understanding of the molecular disposition of bacterial surfaces. Certainly, the ability of molecular genetics to decode and control the expression of various surface components has been a most powerful tool in this understanding, but recent developments in microscopy have also played an important role; these, unlike other approaches, have been able to discern minute surface disparities on individual cells. In this review we have concentrated on the microscopic approach.

The broad surfaces of procaryotes have allowed bacteria a most effective livelihood and have made them some of the most successful lifeforms. They are definitely the most ancient lifeform, and they will be around for a long, long time to come; they will most probably outlive mankind.

The procaryote approach is simple: remain small and adopt a high surface area-to-volume ratio so that simple diffusion can be relied on to stoke the metabolic fires. A single chromosome, high mutation rates, and extrachromosomal elements provide a malleable genetic code and have ensured tremendous diversity and ubiquitousness. However, one common theme has been preserved through time: almost all bacteria rely on external surface layers which lie above a limiting membrane. They may be single S layers, gram-positive or gram-negative walls, capsules, sheaths, or composites thereof. Two overriding attributes of these layers are that they help provide cellular form by contouring the protoplast into a wide variety of possible bacterial shapes (18) and that they provide a barrier against the external milieu. More than you and I, whose individual cells are arranged into multicellular consortia and are protected in tissues and serum, bacteria must have external devices to contend with fluctuations in pH, electrolytes, nutrients, and toxic or hydrolytic substances. This is one reason why microbial biofilms (48) or microbial flocs (121) are preferred in nature. However, we cannot underestimate the importance of individual bacterial surfaces to procaryotic life. Cell walls represent a major proportion of the mass of each cell (16), and their upkeep and expansion must be a major energy drain. Additional layers must be even more expensive. Each surface layer is exquisitely engineered to ensure an economy of design and function. When the competition is tough for growth, a singular wall may not be enough. A better strategy may be to have a series of layers composed of different macromolecular matrices of different porosities and possessing different charge groups, all of which are piled one on top of another. In this way the cell would have a number of intermediate layers between itself, its neighbours, and the external milieu. For example, Leptothrix discophora surrounds itself by a gram-negative wall, a capsule, and a sheath (Fig. 12). On the other hand, for bacteria such as Thermoanaerobacterium spp. that thrive in a high-temperature, high-H₂S environment (132), where few other bacteria can survive and compete, perhaps a single S layer will do. Our awareness of surface structure and physicochemistry is just now approaching the level at which some association can be made between the kinds and numbers of surface layers observed on cells isolated from different natural environments.
In this review, we have not had time to point out all the new and exciting approaches to the study of bacterial surfaces. Compositional analyses of fine-grain mineral particulates associated with bacteria by energy-dispersive X-ray spectroscopy (5, 24, 25, 51, 67-69, 171) and selected-area electron diffraction (67-69, 161, 171) are physical techniques which are becoming powerful tools. Immunogold labeling continues to be important for the localization of specific epitopes of various peripheral molecules (155), and crystallographic approaches (159-162) have steadily increased our resolution of paracrystalline surfaces such as S layers and regularly arranged porins (see, e.g., reference 101). The enormous discriminative power of monoclonal antibodies adsorbed to colloidal gold makes cryoimmunolabeling one of the most promising techniques for correctly identifying surface components and attributing function to them. Another exciting approach to viewing bacterial surfaces at submolecular resolution has been the advent of scanning tunneling microscopy and atomic force microscopy, neither of which has yet been used extensively in microbiology. Scanning tunneling microscopy has had tremendous success in discerning the atomic structure of inorganic surfaces (see, e.g., references 60 and 78), but the natural electrical resistance and molecular pliability of biological surfaces has made high resolution of them difficult. So far, there have been few scanning tunneling microscopy studies of bacterial surfaces (37, 38, 50), but a new design and new scanning mode hold promise; this approach has already produced a 0.4-nm topographical map of Methanospirillum hungatei (cf. Fig. 25 and 26) (34). Atomic force microscopy does not require tunneling currents and can more readily be performed on hydrated objects such as lipid-protein films (59); it may prove to be the better tool for bacterial surfaces. Real-time, submolecular, dynamic images of these surfaces may be feasible.

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FIG. 25 and 26. Surfaces of small fragments of the Methanospirillum hungatei sheath.

FIG. 25. Platinum-shadowed fragment showing the topography of the outer and inner surfaces and presents their electron-microscopic views to orient the reader. Bar, 100 nm.

FIG. 26. High-resolution scanning tunneling microscopic view of the topography of the inner sheath surface. The picture is not computer enhanced, so that every scan of the microscope tip can be seen. The X, Y, and Z dimensions are indicated, and the height resolution is at least 0.4 nm. To obtain this image of a non-metal-coated biological specimen, a new scanning tunneling technique was required; this technique is described in reference 34. Reproduced from reference 34 with permission of the American Society for Microbiology.
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