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Binding of Metals to Cell Envelopes of *Escherichia coli* K-12

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As representative of gram-negative bacteria, the isolated and purified envelopes of an *Escherichia coli* K-12 strain were used to determine metal-binding capacity. The envelopes were suspended in 5 mM metal solutions for 10 min at 23°C, separated and washed by centrifugation, and analyzed for metal by either atomic absorption or X-ray fluorescence spectroscopy. Of 32 metals tested, large amounts (>0.9 μmol/mg [dry weight]) of Hf and Os, intermediate amounts (0.1 to 0.4 μmol/mg [dry weight]) of Pb, Zn, Zr, Fe III, Mn, Mo, Mg, Co, and Ce IV, and small amounts (<0.1 μmol/mg [dry weight]) of Na, K, Rb, Ca, Sr, Cu, Sc, La, Pr, Sm, U, Fe II, Ru, Ni, Hg, Pt, Pd, Au, and In were detected. Li and V were not bound to the envelopes. Electron microscopy of unstained, thin-sectioned material provided an electron-scattering profile for localizing the bound metal within the envelope. Energy-dispersive X-ray analysis of thin sections detected all metals in single envelope vesicles. These data suggest that most metal deposition occurred at the polar head group regions of the constituent membranes or along the peptidoglycan layer. No leaching of envelope components was detected by monitoring radioactive probes within the lipopolysaccharide and peptidoglycan layers during metal uptake experiments, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from metal-loaded envelopes, or protein and carbohydrate determinations on the wash fluids. These results suggest that membrane integrity was not disturbed under these ionic conditions.

Surfaces of cells are usually anionic, and bacterial walls are no exception. Early work involving microelectrophoresis established that the isoelectric points of bacteria vary between pH 2 and 4 and that these can be altered by the addition of cationic surface-active agents (20, 28, 50). Accordingly, when soluble polyvalent metal (e.g., Fe^{3+}) is added to growing cells, their electronegative surface property can be converted to an electropositive surface property by bound metal (64). More recent electrokinetic studies have demonstrated that bacterial species, and often phenotypic strains, have a unique surface charge and conductivity (22) which can be attributed to the wall fabric (23). At low ionic levels, the conductivity of the bacterial cell is dominated by the fixed counterions in the wall, but at high ionic levels the wall becomes saturated with exogenous salt and has a conductivity which is roughly proportional to that of the environment (11). Gram-positive walls have a higher charge capacity than the gram-negative variety, but their isoelectric point is more variable (11, 28). The plasma membrane of the cell offers great resistance to electrical conductivity and effectively isolates the vital cellular contents from charge fluctuation (10).

All soluble and colloidal material, such as organic nutrients and essential metals, must contact and percolate through the wall substance before gaining access to the plasma membrane and, ultimately, the cytoplasm. Likewise, metabolic wastes must be transferred through the wall meshwork before extracellular liberation occurs. It is to be expected that the polyanions of the wall would interact with and bind the cations of the aqueous environment.

We now know that bacterial walls interact strongly with metal (5, 7, 18, 48) and that they must be important for the immobilization of dissolved metal in the environment. In fact, we could imagine a light but constant rain of bacteria and their products throughout the aqueous environment which would form a variable proportion of the sediment that accumulates at the bottoms of our lakes, rivers, and oceans.

Several studies have determined that gram-positive walls are potent metal chelators (5, 7, 8, 18, 26, 48), but gram-negative envelopes are structurally and chemically much different. They consist of two membrane bilayers (the outer and plasma membranes) which are chemically distinct from one another and which sandwich a thin peptidoglycan layer between them

in the periplasmic space. Because of these differences, we would expect a different metal-binding capability, in both quantitative and qualitative terms, from that of gram-positive walls. Most metal-binding studies on gram-negative bacteria have used whole cells for their determinations (37), but it is the cell envelope which is first in contact with metal in solution. For this reason, it is important to understand the reactivity between distinct metal aquo-ions and the fabric of the enveloping layers.

The chemistry and structure of the *Escherichia coli* K-12 envelope have been studied in detail and provide a good model system for metal-binding estimates. Since inappropriate metals can extract the organic components of the envelope, careful monitoring must be maintained during the binding process and subsequent washes. Radioisotopic labeling of both the outer membrane and the peptidoglycan layer of an auxotrophic mutant provided a rapid, sensitive assay for evaluating envelope degradation in our system. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the proteins in metal-loaded envelopes allowed the correlation of major proteins with those of the control preparations. For the first time, thin-section electron microscopy and energy-dispersive X-ray spectrophotometry allowed localization of the bound metal within the envelope fabric.

MATERIALS AND METHODS

Preparation of cell envelopes. *E. coli* AB264 (F^+ *ara-1* Mu^+), which was E. A. Adelberg's wild-type K-12 strain, was obtained from B. Bachmann of the *E. coli* Genetic Stock Center (Department of Human Genetics, Yale University School of Medicine, New Haven, Conn). These cells were grown in four 3-liter quantities of tryptone broth (Difco Laboratories) to a density of approximately 5×10^8 cells per ml and broken with a French press, and the envelopes were separated from the lysate by centrifugation at 48,000 $\times$ *g*. The envelopes were treated with 50 μg of deoxyribonuclease per ml (deoxyribonuclease type I; Sigma Chemical Co.) and 100 μg of ribonuclease per ml (ribonuclease type III-A; Sigma), washed five times in 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2) buffer (Calbiochem) and once in deionized distilled water, and then freeze-dried.

Radioactive labeling of the cell envelope. The lipopolysaccharide and peptidoglycan components of the envelopes were radioactively labeled by an auxotrophic mutant isolated in our laboratory (SFK11, *leu galE pps asd*). This mutant was selected by the conjugation of two parent strains kindly supplied by A. H. Goldie, Biochemistry Department, University of Western Ontario.

The mutant required diaminopimelic acid (DAP) for growth, owing to the *asd* mutation, and therefore incorporated exogenous DAP into peptidoglycan. However, it was a leaky mutant, since some DAP was decarboxylated to lysine. Lysine was added to the growth medium to minimize this reaction. SFK11 was grown in four 3-liter quantities of Difco nutrient broth containing 0.3% yeast extract, 50 μg of DAP per ml, 100 μg of L-lysine per ml, and 2 mg of D-galactose per ml. At the beginning of the exponential phase of growth, the radioactive amino acids [DL-(+)-*meso*]-2,6-diamino[*G*-^{3}H]pimelic acid dihydrochloride and D-[1-^{14}C]galactose (both from Amersham Nuclear Chemical Co.) were added to give a specific activity of 1 μCi/μmol for both amino acids. The cells were harvested 5 h later, and the envelopes were prepared as described above.

Reaction of envelopes with metal solutions. A total of 5 mg (dry weight) of envelopes was incubated in 2 ml of a 5 mM metal solution under our standard conditions (5, 7). A total of 32 different metal salts were used and all were of ultrapure quality (see reference 5). After reaction with the metals, the envelopes were immediately cooled to 4°C, pelleted at 40,000 $\times$ *g* for 20 min at 4°C, and washed with six 10-ml quantities of deionized, distilled water by similar centrifugation.

Most metals were detected by atomic absorption analysis, using a Perkin-Elmer model 403 spectrophotometer in either the flame or graphite furnace modes as previously described (7). The heavy-metal analyses were done by the X-ray fluorescence technique with a Philips PW 1450 atomic sequential X-ray spectrophotometer (5).

Electron microscopy and energy-dispersive X-ray analyses. Envelopes were processed as for quantitative metal analyses, except that after the washing procedure they were either used as unstained whole mounts for electron microscopy or dehydrated into Epon 812 and sectioned. For unstained whole mounts, a grid was floated on a drop of wall suspension for 15 s and blotted dry before insertion into the electron microscope. For plastic embeddings, both unfixed and 4% glutaraldehyde-fixed samples were used. For the former, walls were immediately dehydrated through an ethanol-propylene oxide series into Epon 812. For the latter, walls were fixed in 4% aqueous glutaraldehyde for 60 min at 22°C, washed with four 3-ml volumes of water, and processed into Epon 812 in the same manner. Thin sections (ca. 60 nm thick) were obtained and mounted on carbon-Formvar-coated, 200-mesh copper grids. For electron-scattering profiles of the metal bound to the envelopes, no staining reagents other than the original metals were used. Figure 2 was obtained by conventional 4% glutaraldehyde to 1% OsO_4 fixation, Epon 812 embedding, and uranyl acetate-lead citrate staining of the thin section. Electron microscopy was done with a Philips EM 300 electron microscope (equipped with a gonimeter stage) operating at 60 kV under standard conditions with a liquid nitrogen cold trap to help preserve the specimens. Energy-dispersive X-ray analyses were performed with a Zeiss EM10C electron microscope equipped with STEM and KEVEX μX700 accessories. The analyses were performed at 20 kV with an electron beam diameter of 10.0 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein content of metal-loaded envelopes was analyzed by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis, using the discontinuous buffer system described by Laemmli (39). Electrophoresis was performed in slabs 0.75 mm thick and 10 cm long, using the Bio-Rad model 220 vertical slab gel electrophoresis cell. Samples were electrophoresed at room temperature at a constant current of 10 mA per slab until they had entered the stacking gel and then at 25 mA per slab. Gels were fixed and stained simultaneously in 0.1% Coomassie brilliant blue R250–25% isopropanol–7% acetic acid and destained by diffusion in several change of 10% isopropanol–7% acetic acid.

Analysis of wash fluids from metal-binding reactions. For each metal-binding experiment, the metal supernatant and the washing fluids were pooled and freeze-dried. The resulting residue from *E. coli* SFK11 envelopes was digested in 1 ml of Protosol (New England Nuclear Corp.) for 1 h at room temperature. This fluid was dissolved in 15 ml of Aquasol (New England Nuclear), and liquid scintillation for ^{3}H and ^{14}C was performed in a Beckman model LS 250 counter.

RESULTS

The envelopes used in this study were free from contaminating cytoplasmic debris, as judged by electron microscopy. Chemical analyses of the envelope fraction revealed ketodeoxyoctonate (a marker for the lipopolysaccharide of the outer membrane), DAP (a marker of the peptidoglycan layer), and succinic dehydrogenase (a marker of the plasma membrane) (B. Hoyle, University of Guelph, personal communication); thus the envelope fraction was a three-component system. Negatively stained samples of preparations before freeze-drying revealed collapsed membrane vesicles of between 25 and 250 nm in diameter as a consequence of the mechanical shearing process. Thin sections of this material confirmed these observations and demonstrated their membranous nature (Fig. 1 and 2). Most vesicles appeared to be constructed of one membrane, but occasional vesicles contained internal membrane fragments (Fig. 2, arrows). Presumably, the single format represents the annealed product of either the plasma membrane or the outer membrane, whereas double vesicles could represent both membrane products.

Mucopeptide could not be demonstrated to be a distinct layer in either electron microscopic preparation (although hot 2% sodium dodecyl sulfate treatment of the envelopes left this layer as an insoluble residue). We believe the peptidoglycan to be collapsed against the inner face of the outer membrane in our preparation.

Metal-binding experiments. Thirty-two metals were used for metal-binding reactions. The results are given in Table 1, which shows the metals that were bound so tightly to the envelopes that they could not be displaced by water during the washing procedure. Of all of the metals used, only Li and V were not bound to the envelopes. Os and Hf were bound in the largest amounts (1.040 and 0.940 μmol/mg, but, in general, smaller amounts of metal were bound.

Electron microscopy. Electron microscopy was used to monitor the electron-scattering power of the envelopes after metal binding. Whole amounts and thin sections of control envelopes (no metal) were not distinguishable owing to their low contrast. The electron-scattering capacity of the envelopes coincided with the atomic number (Z) and the quantity of the bound metal. For example, both Cu and Mg gave detectable electron-scattering profiles (Fig. 3 and 4). Mg has a low atomic number ($Z = 12$) but was bound in large amounts (0.256 μmol/mg), whereas Cu has a greater atomic number ($Z = 29$) but had minimal binding (0.090 μmol/mg). These two metals as did most metals, produced the bilayer profile which is typical for membranes stained by more conventional means. In all cases, the membrane thickness was 7.5 ± 1.0 nm, which was also comparable to results produced by conventional methods (cf. Fig. 2 to 4). Current thought suggests that the heavy metal used as a stain for electron microscopy binds along hydrophilic domains of membranes (29, 67). Clearly, our results are analogous, and we believe that our metals are complexed with the polar head groups in these regions.

A few of the transition elements I produced more asymmetric profiles (i.e., Hf, Zr, Pr, and Sm). Of these, the asymmetry of Hf staining was most pronounced, and in several instances the bilayer track was indistinct (Fig. 5, arrows). In this case, the outer face of the membrane was more densely stained than that of the innerface. Since these metals are unstable aquo-ions in solution (13), it is possible that metal accretion proceeds, and is complete, at the external surface before the soluble ions can traverse the membrane fabric. At least, with respect to the outer membrane, the external (lipopolysaccharide) face is highly anionic and should interact strongly with and immobilize these metals.

Energy-dispersive X-ray analyses. Thin sections of select preparations (i.e., control, U, Zr, Hf, Fe, and Os envelopes) were used for qualitative energy-dispersive X-ray analysis. Since an electron beam diameter of 10.0 nm was used as the probe, individual membrane vesicles could be analyzed for bound metal. Analysis of control envelopes revealed Mg, K, and Ca as the major constituent metals (Fig. 6A). The metal-loaded envelopes (Fig. 6B is representative) contained predominantly the bound metal with

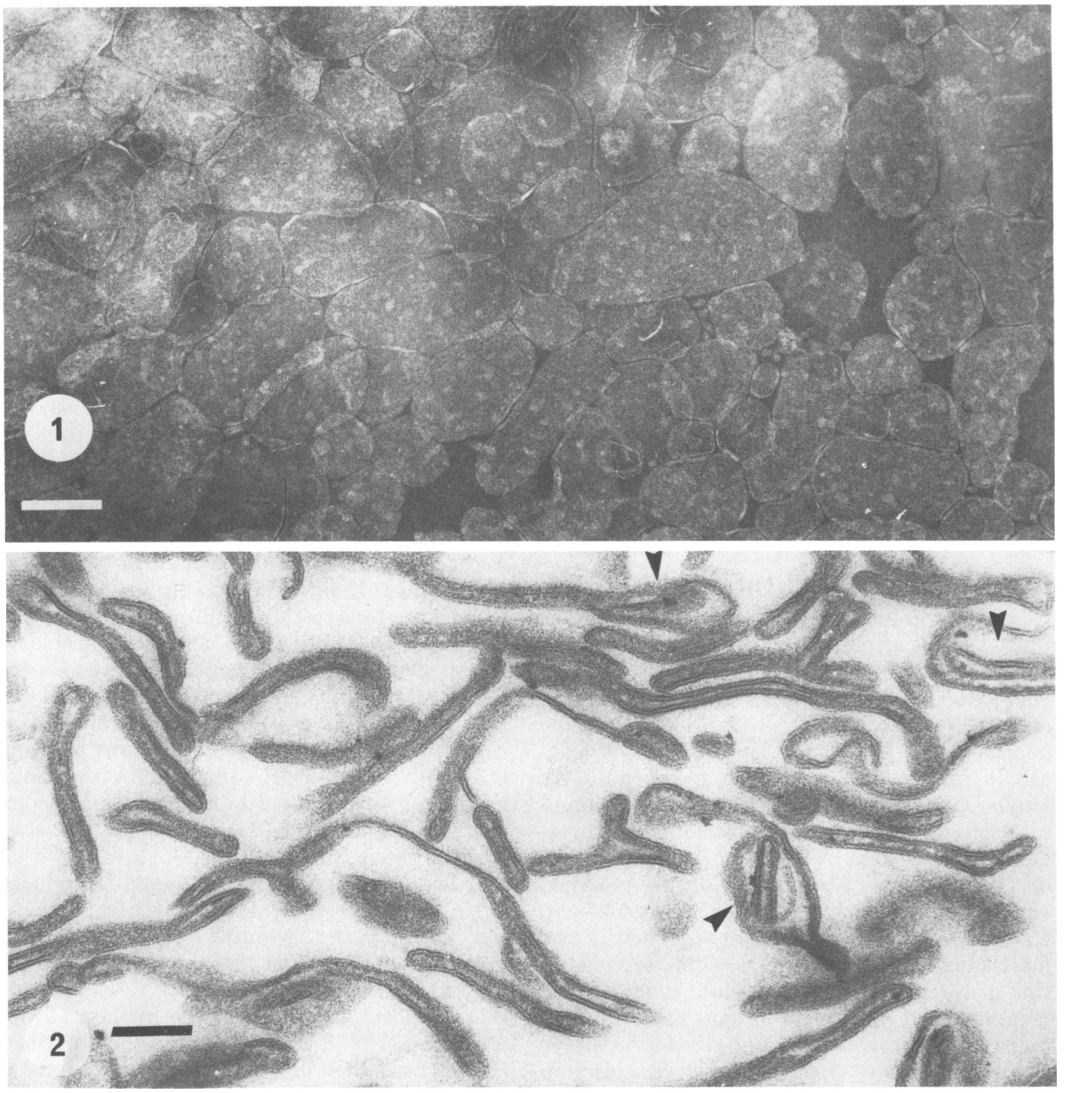

FIG. 1. *Envelope preparation negatively stained with 2% ammonium molybdate. Bars in Fig. 1 through 5 = 100 nm.*

FIG. 2. *Thin section of the envelope preparation prepared by conventional glutaraldehyde-osmium tetroxide fixation and uranyl acetate-lead citrate staining methods.*

small amounts of Mg, K, and Ca. Although these results were not quantitative, it appeared that approximately one-half (or more) of the Mg had been removed during metal treatment (cf. Fig. 6A and B).

All analyses revealed small Si peaks in the preparations. Since these peaks were also detected on grids without sections, we suspect that the Si readings were due to background emission from the carbon-Formvar support film. The high peaks for Cu (Cu[1], Cu[Kα], Cu[kβ]) seen in all preparations were due to the copper grid.

Release of envelope material during metal binding. The outer membrane of the gram-negative wall contains magnesium, and sometimes calcium, as integral components (6). These metals contribute to the outer membrane, and if they are extracted or replaced by exogenous counterions, the loss of protein or lipopolysaccharide may result. Therefore, it was important to monitor the metal-binding experiments for loss of these components, which could have produced artificially low metal uptake values.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of metal-loaded envelopes suggested that there was no extraction of

TABLE 1. *Metal binding by E. coli AB264 envelopes*[a]

Element	μmol/mg (dry wt) of envelopes bound to metal[b]
Alkali metal	
Li	0.000
Na	0.042
K	0.082
Rb	0.001
Alkaline-earth metal	
Mg	0.256
Ca	0.035
Sr	0.001
Transition element I	
Sc	0.096
La	0.078
Ce IV	0.100
Pr	0.058
Sm	0.011
U (as UO_2^{2+})	0.066
Zr (as ZrO_2^{2+})	0.212
Hf (as HfO_2^{2+})	0.940
V (as VO_2^{2+})	0.000
Mo (as MoO_2^{2+})	0.225
Mn	0.140
Transition element II	
Fe III	0.200
Fe II	0.057
Ru	0.090
Os {as OsO_4^{2-} or $[OsO_4(OH_2)]^{2-}$}	1.040
Co	0.178
Ni	0.002
Pd	0.010
Pt	0.002
Transition element III	
Cu	0.090
Au III	0.056
Zn	0.390
Hg	0.064
Group III	
In	0.001
Group IV	
Pb	0.152

[a] See reference 5 for a complete description of the metal ions and their polymerization products which would be available for interaction with the envelopes.

[b] Each value is the mean of at least three separate binding reactions. In no instance was the standard deviation greater than 5%.

the major proteins of the outer membrane by most metals (Fig. 7). Only two heavy metals, uranium (uranyl acetate) and osmium (tetroxide), showed unique electrophoretic patterns. With the former there was a decrease in the TolG protein, whereas in the latter several minor, low-molecular-weight proteins were absent. There was no detectable protein in the wash fluids from the metal-binding experiments. Less than 5% of the total carbohydrates were released into the wash fluids, as detected by chemical analysis (19) or radioactive labeling techniques. Degradation of the peptidoglycan was less than 1% as determined by release of [^{3}H]DAP.

DISCUSSION

The transport mechanisms of potentially toxic metals and their various organic derivatives throughout nature are at present poorly understood. Recently, there has been an increasing awareness that much of the soluble metal in our environment has been chelated by organic members to form naturally sedimentable, metal-rich particulates (12, 34). As a consequence of industrial loading, synthetic fertilizers, and the acid leaching of natural sources, the types and concentrations of toxic metals have increased in our environment. In fact, Mangini et al. have estimated the average soluble uranium content of our rivers to now be approximately 0.3 μm/liter (46).

Bacteria are ubiquitous throughout nature and consist of a variety of highly charged homo- and heteropolymers, some of which (e.g., those making up the walls) are remarkably resilient to degradation. Cell walls have a high anionic charge density and consequently interact strongly with the electropositive metal ions dissolved in the environment to accumulate large quantities of bound metal (5, 7, 15, 18, 25, 26, 48). In fact, traces of microbes and high concentrations of associated metal have been found in sediments from ancient geological horizons (4, 21, 38, 57, 58). More recently, microorganisms have been implicated in the concentration of radioelements, such as uranium, plutonium, and polonium, from marine waters (31). The microbial associations with manganese concretions in both marine (27) and freshwater systems (9, 14) are well documented.

Bacteria require distinct metals in solution for their growth and for their ability to withstand environmental stress (2, 43, 44, 53, 65). It is well known that Mg is required for ribosomal structure and function (71), that intracellular K is essential for the Na-K gradient which exists over the plasma membrane (several transport systems are driven as a result of the gradient, e.g., see reference 69), and that metals, particularly Mg and Ca, are essential for the maintenance of membrane structure (6, 68). As a consequence, bacteria act as "biological exchange resins" (16) and are surprisingly rich sources of particulate metal, especially in older cultures (37). Some bacteria even synthesize and secrete molecules

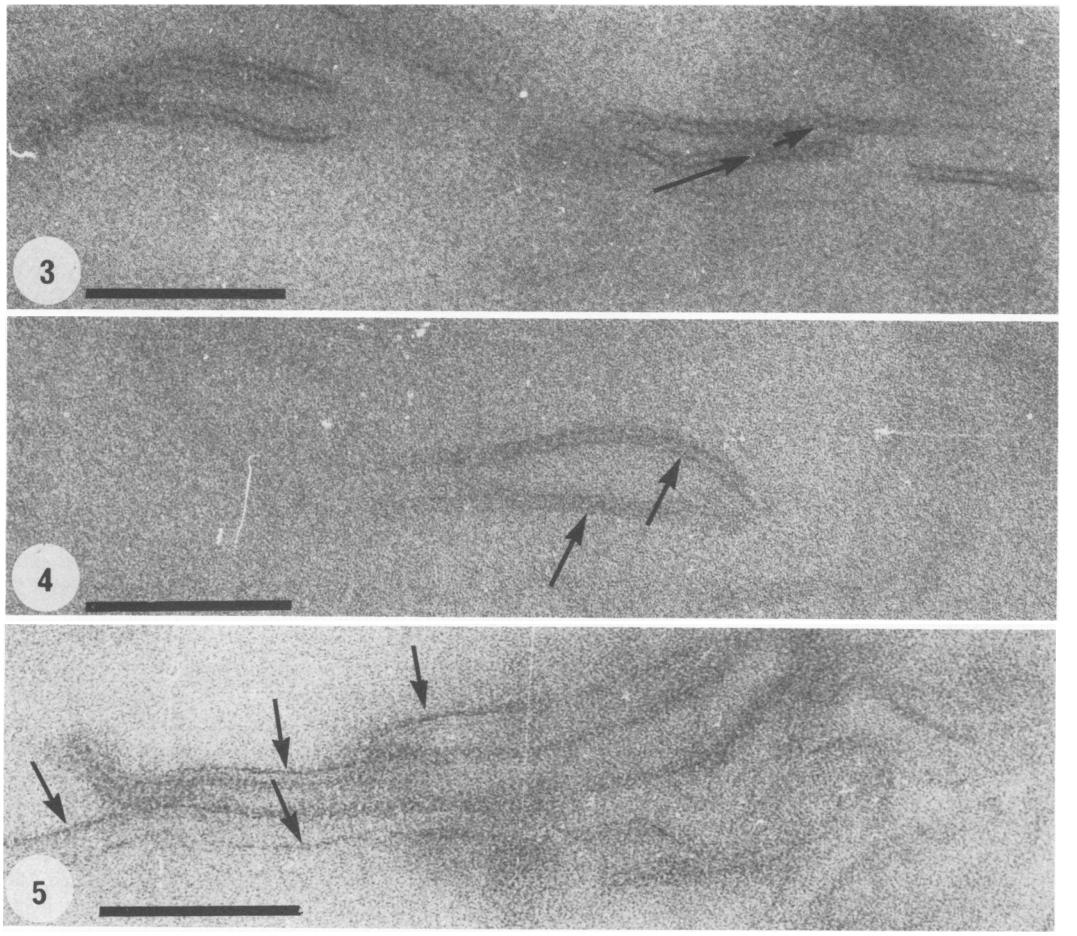

FIG. 3. *Thin section of Cu envelopes. The arrows point to the membrane of a single vesicle. In this micrograph and those in Fig. 4 and 5, no stain other than the initial binding metal was used for contrast.*

FIG. 4. *Mg envelopes. The arrows point to the membrane of a single vesicle.*

FIG. 5. *Hf envelopes. The arrows point out the asymmetry of the deposition product since only the external membrane face is visible in these regions.*

which efficiently scavenge and chelate essential metal from the environment (e.g., the enterochelin and ferrochrome systems of *E. coli* [42, 59], the pyoveridines or pyocyanines of *Pseudomonas* spp. [51, 52], deferrischizokinen, deferriaerobactin, and deferriferrioxamine B of *Bacillus megaterium* [3], the nocobactins of *Nocardia* spp. [60], the exochelins of *Mycobacterium*, *Staphylococcus*, and *Streptococcus* spp. [45, 47, 49], and the MoO_4^{-2} chelator of *Bacillus thuringiensis* [35]). Even the virulence of pathogenic bacteria can be stimulated by the addition of exogenous metal (2, 36, 55).

Clearly, the bacterial enveloping layers are first in contact with exogenous metal in solution. These layers must somehow maintain and control the type and quantity of metal which reaches the vital constituents in the cell. For this reason, it is important to define the mechanisms involved with metal-envelope interaction. This phenomenon has been most clearly studied in the case of gram-positive walls (8, 18, 30, 40); it has not been studied in the case of gram-negative envelopes. This system is more complex in structural and chemical terms in that it consists of at least three chemically distinct layers.

The chemistry of the bacterial plasma membrane is a complex issue. As with most membranes, phospholipids (especially phosphatidylethanolamine) and proteins (both structural and enzymatic) are important. Bacterial plasma membranes do not contain cholesterol (6). We do know that many enzymes are closely associated with it and that nucleic acids (both deoxyribonucleic acid and ribonucleic acid) can be attached (62). All material predestined for the

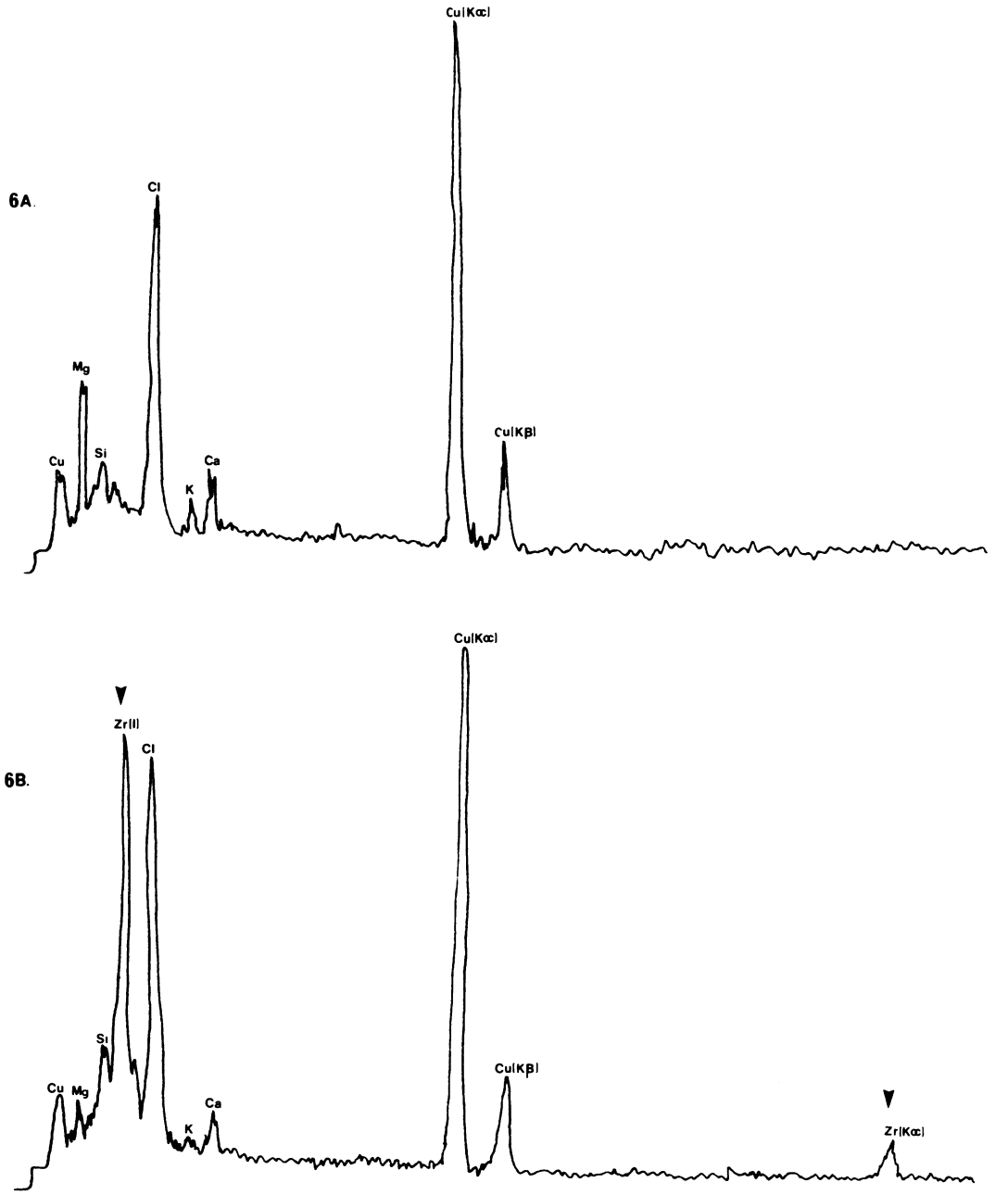

FIG. 6. *Energy-dispersive X-ray analysis of Zr envelopes at 20 kV with a 30-mm² detector window. Measuring time, 50 s. (A) Data from a control envelope vesicle without bound metal. (B) Data from a Zr envelope. The arrows point to the Zr peaks. Notice that the Mg peak has been reduced from that in (A). The strong Cu peaks in both (A) and (B) are due to the copper grid.*

cell wall must either pass through or be processed by this membrane.

Our envelopes have been treated with nucleases and have been thoroughly washed of contaminating cytoplasmic debris (Fig. 2). We therefore would expect that metal from solution should interact with distinct polar head groups of phospholipids and with acidic groups on exposed (hydrophilic) polypeptides of plasma membrane vesicles. The bilayer electron-scatter-

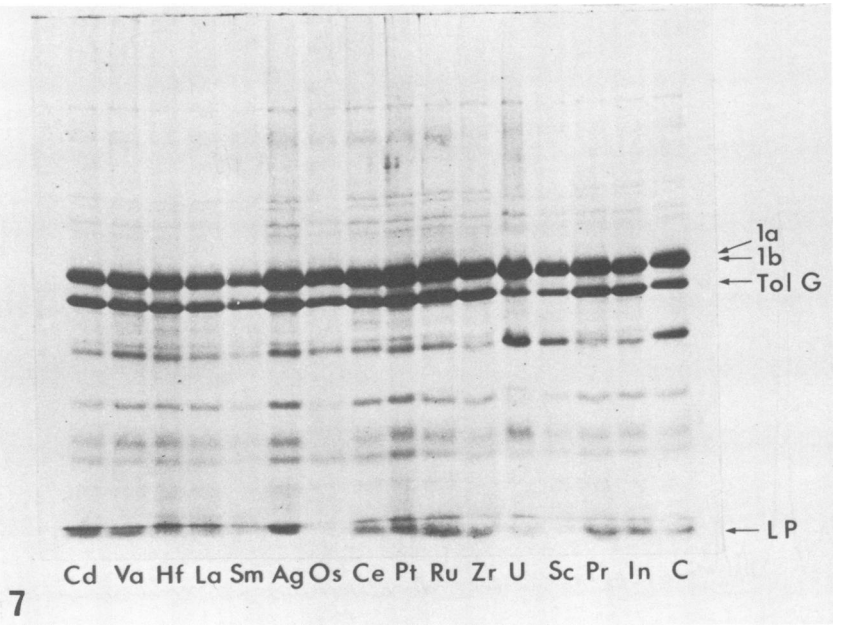

FIG. 7. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins of metal-loaded E. coli AB264 envelopes. Proteins were separated on a 10% acrylamide gel. The specific metal is indicated at the bottom of each lane. The lane at the extreme right (C) is the control preparation of envelopes without metal addition. The major outer membrane proteins of E. coli are indicated at the right by arrows.*

ing profiles of metal-loaded envelopes is consistent with this idea. Metal is important to this membrane because metal depletion produces distinct ultrastructural changes within the membrane fabric: "bracket junctions" between plasma membrane and internal membranes can occur (72), particle-free patches are seen on freeze-cleaved surfaces (1), and planar crystalline arrays can be produced (1).

The peptidoglycan layer of *E. coli* consists of a meshwork of linear glycan strands which contain repeating units of β-(1,4)-linked *N*-acetylglucosamine-*N*-acetylmuramic acid dimer and which are intermittently cross-linked by direct linkage between (L)-*meso*-DAP and D-alanine residues of adjacent peptide stems [L-Ala-D-Glu-(L)-*meso*-DAP-D-Ala]. Approximately 30% of these strands are cross-linked in *E. coli* (66).

The peptidoglycan of all gram-negative bacteria is of chemotype I, as is the peptidoglycan of *Bacillus subtilis* (63). It should be a potent site for metal deposition, owing to the anionic carboxyl groups of the D-glutamic acid residues (7). A distinct layer of peptidoglycan could not be distinguished in the envelope vesicles (see Fig. 2), but sodium dodecyl sulfate digestion of the envelopes at 100°C revealed that the layer was an insoluble end product. Therefore, the layer was present and was probably collapsed against the inner face of the outer membrane in our preparation. Consequently, we were not able to distinguish this layer by a distinct electron-scattering profile (Fig. 3 to 5), and we cannot give presently a definitive value to its metal binding capacity.

The outer membrane of *E. coli* AB264 contains three major components: phospholipids, lipopolysaccharide, and proteins. The lipopolysaccharide of *E. coli* K-12 strains is located asymmetrically in the outer half of the membrane (24) and contains a complete core but lacks O-antigenic side chains (54). Most phospholipid is restricted to the inner half of the membrane.

This membrane contains major proteins (Ia, Ib, TolG, and lipoprotein) plus a number of minor proteins (Fig. 7; references 6, 17, 32, and 56). Some of these proteins are transmembranous, whereas others are found only on the membrane surfaces. It is the lipoprotein (covalent linkage) and the Ia or Ib protein (salt linkage) which chemically binds the outer membrane to the peptidoglycan layer.

All outer membrane components are fitted together to form a bilayer structure, and, like the plasma membrane, the principal sites for metal interaction must exist in the hydrophilic regions. This restricts us to the probability that the metal interacts with the polar head groups of the phospholipids (mainly on the inner face),

available anionic sites of the lipopolysaccharide (mainly on the outer face), and the acidic groups of exposed polypeptides. The bilayer electron-scattering profiles or metal-loaded envelopes are, once again, consistent with this view.

Galdiero et al. have previously reported some preliminary results for the binding of Na, K, Ca, and Mg to cell walls of *E. coli* (25). Since the preparation used by those authors must have included plasma membrane, their wall material would have been analogous to our envelope preparation. We have expanded their results to a total of 32 metals and have ensured the accuracy of the binding results by methodically monitoring all steps of the procedure for released envelope constituents. The results of Galdiero et al. and our results confirm that gram-negative envelopes do not bind as much metal on a dry-weight basis as do gram-positive walls. Often the binding capacity of gram-negative envelopes is approximately one-tenth that of gram-positive walls (T. Beveridge, Proc. 2nd Annu. Chem. Congr. North Am. Cont. Div. Microbiol. Biochem. technical paper no. 33).

Metal-chelating agents such as ethylenediaminetetraacetic acid are able to free Mg from the outer membrane of *E. coli* with concomitant extraction of a lipopolysaccharide-protein complex (41, 61, 70). This implies that some membrane-bound Mg is instrumental in maintaining structural integrity and that this Mg must be closely apposed to the outer surface. This Mg, then, may be available to other (exogenous) metal species for displacement. On the other hand, the addition of Mg to a rough (heptose-deficient) strain of *Salmonella typhimurium* cemented the outer membrane fabric tightly together and reduced its ability to cleave during the freeze-etching process (33). Therefore, some Mg must be able to enter the lipophilic domain and tighten intermolecular associations by salt linkage. This Mg would not be easily displaced or replaced under the conditions used in this study. Our energy-dispersive X-ray analysis data suggest that although large quantities of exogenous metal are bound to the envelopes some residual Mg remains in the system (Fig. 6A and B). Possibly this is a more deeply bound Mg.

This study has demonstrated that the gram-negative envelope of *E. coli* is able to interact with and immobilize many metal ion species. It has provided a starting point for elucidating the principle sites of metal deposition. Work is already in progress to partition these envelopes into their constituent layers and to then study the contribution of each to the metal-binding process. Our knowledge of the chemistry of at least two of these layers (the murein and the outer membranes) may permit distinct chemical localization of the reactive groups.

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LITERATURE CITED

1. **Arancia, G., S. Belli, G. Donelli, and P. Trovalusci.** 1980. Ultrastructural changes in *Escherichia coli* grown in divalent cation-deficient medium. J. Gen. Microbiol. **119**:155–164.
2. **Archibald, F. S., and I. W. DeVoe.** 1978. Iron in *Neisseria meningitidis*: minimum requirements, effects of limitation, and characteristics of uptake. J. Bacteriol. **136**:35–48.
3. **Aswell, J. E., A. H. Haydon, H. R. Turner, C. A. Dawkins, J. E. L. Arceneaux, and B. R. Byers.** 1977. Specificity of siderophore receptors in membrane vesicles of *Bacillus megaterium*. J. Bacteriol. **130**:173–180.
4. **Banghoorn, E. S., and S. A. Tyler.** 1965. Microorganisms from the gunflint chert. Science **147**:563–577.
5. **Beveridge, T. J.** 1978. The response of cell walls of *Bacillus subtilis* to metals and to electron microscopic stains. Can. J. Microbiol. **24**:89–104.
6. **Beveridge, T. J.** 1981. Ultrastructure, chemistry and function of the bacterial wall. Int. Rev. Cytol. **72**:229–317.
7. **Beveridge, T. J., and R. G. E. Murray.** 1976. Uptake and retention of metals by cell walls of *Bacillus subtilis*. J. Bacteriol. **127**:1502–1518.
8. **Beveridge, T. J., and R. G. E. Murray.** 1980. Sites of metal deposition in the cell wall of *Bacillus subtilis*. J. Bacteriol. **141**:876–887.
9. **Burns, N. M., and J. O. Nriagu.** 1976. Forms of iron and manganese in Lake Erie waters. J. Fish. Res. Board Can. **33**:463–470.
10. **Carstensen, E. L.** 1967. Passive electrical properties of microorganisms. II. Resistance of the bacterial membrane. Biophys. J. **1**:493–503.
11. **Carstensen, E. L., H. A. Cox, Jr., W. B. Mercer, and L. A. Natale.** 1965. Passive electrical properties of microorganisms. I. Conductivity of *Escherichia coli* and *Micrococcus lysodeikticus*. Biophys. J. **5**:289–300.
12. **Chester, R., A. Griffiths, and J. H. Stoner.** 1978. Minor metal content of surface sea water particulates and organic-rich shelf sediments. Nature (London) **275**:308–309.
13. **Cotton, F. A., and G. Wilkinson.** 1962. Advanced inorganic chemistry: a comprehensive text. John Wiley & Sons, Inc., New York.
14. **Cronan, D. S., and R. L. Thomas.** 1972. Geochemistry of ferromanganese oxide concretions and associated deposits in Lake Ontario. Geol. Soc. Am. Bull. **83**:1493–1502.
15. **Cutinelli, C., and F. Galdiero.** 1967. Ion-binding properties of cell wall of *Staphylococcus aureus*. Riv. Biol. (Perugia) **60**:297–305.
16. **Damadian, R.** 1973. Cation transport in bacteria. Crit. Rev. Microbiol. **2**:377–422.
17. **DiRienzo, J. M., K. Nakamura, and M. Inouye.** 1978. The outer membrane proteins of Gram-negative bac-

teria: biosynthesis, assembly, and functions. Annu. Rev. Biochem. **47**:481–532.

18. **Boyle, R. J., T. A. Matthews, and U. N. Streips.** 1980. Chemical basis for selectivity of metal ions by the *Bacillus subtilis* cell wall. J. Bacteriol. **143**:471–480.
19. **Dubois, M., R. A. Gillies, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
20. **Dyar, M. T., and E. J. Ordal.** 1946. Electrokinetic studies on bacterial surfaces. I. The effects of surface-active agents on the electrophoretic mobilities of bacteria. J. Bacteriol. **51**:149–167.
21. **Eglinton, G., P. M. Scott, T. Belsky, A. L. Burlingame, and M. Calvin.** 1964. Hydrocarbons of biological origin from a one-billion-year-old sediment. Science **145**:263–264.
22. **Einolf, C. W., Jr., and E. L. Carstensen.** 1967. Bacterial conductivity in the determination of surface charge by microelectrophoresis. Biochim. Biophys. Acta **148**:506–516.
23. **Einolf, C. W., Jr., and E. L. Carstensen.** 1969. Passive electrical properties of microorganisms. IV. Studies of the protoplasts of *Micrococcus lysodeikticus*. Biophys. J. **9**:634–643.
24. **Funahara, Y., and H. Nikaido.** 1980. Asymmetric localization of lipopolysaccharides on the outer membrane of *Salmonella typhimurium*. J. Bacteriol. **141**:1463–1465.
25. **Galdiero, F., M. Lembo, and M. A. Tufano.** 1967. Sulla capacità legante ioni del cell-wall di bacteri Gram negativi. In corso di stampa su Boll. 1st sieroter. Milan **46**:41–45.
26. **Galdiero, F., M. Lembo, and M. A. Tufano.** 1968. Affinity of various cations for *Staphylococcus aureus* cell wall. Experimentia (Basel) **24**:34–36.
27. **Greenslate, J.** 1974. Manganese and biotic debris associations in some deep-sea sediments. Science **186**:529–531.
28. **Harden, V. P., and J. O. Harris.** 1953. Isoelectric point of bacterial cells. J. Bacteriol. **65**:198–202.
29. **Hayat, M. A.** 1975. Positive staining for electron microscopy. Van Nostrand Reinhold Co., New York.
30. **Heptinstall, S., A. R. Archibald, and J. Baddiley.** 1970. Teichoic acids and membrane function in bacteria. Selective destruction of teichoic acid reduces the ability of bacterial cell walls to bind Mg^{2+} ions. Nature (London) **225**:519–521.
31. **Hodge, V. F., M. Koide, and E. D. Goldberg.** 1979. Particulate uranium, plutonium and polonium in the biogeochemistries of the coastal zone. Nature (London) **277**:206–209.
32. **Inouye, M.** 1979. Bacterial outer membranes. John Wiley & Sons, Inc., New York.
33. **Irvin, R. T., A. K. Chatterjee, K. E. Sanderson, and J. W. Costerton.** 1975. Comparison of the cell envelope structure of a lipopolysaccharide-defective (heptose-deficient) strain and a smooth strain of *Salmonella typhimurium*. J. Bacteriol. **124**:930–941.
34. **James, A. M.** 1979. Molecular aspects of biological surfaces. Chem. Soc. Rev. **8**:389–418.
35. **Ketchum, P. A., and M. S. Owens.** 1975. Production of a molybdenum-coordinating compound by *Bacillus thuringiensis*. J. Bacteriol. **122**:412–417.
36. **Kochan, I.** 1977. Role of siderophores in nutritional immunity and bacterial parasitism, p. 251–288. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcel Dekker, Inc., New York.
37. **Kung, F.-C., J. Raymond, and D. A. Glaser.** 1976. Metal ion content of *Escherichia coli* versus cell age. J. Bacteriol. **126**:1089–1095.
38. **LaBerge, G. L.** 1967. Microfossils and precambrian iron-formations. Geol. Soc. Am. Bull. **78**:331–342.
39. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**:680–685.
40. **Lambert, P. A., I. C. Hancock, and J. Baddiley.** 1975. The interaction of magnesium ions with teichoic acid. Biochem. J. **149**:519–524.
41. **Leive, L., V. K. Sholvin, and S. E. Mergenhagen.** 1968. Physical, chemical and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediamine tetraacetate. J. Biol. Chem. **243**:6384–6391.
42. **Leong, J., and J. B. Neilands.** 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. **126**:823–830.
43. **Ljunger, C.** 1973. Further investigations on the nature of the heat resistance of thermophilic bacteria. Physiol. Plant. **28**:415–418.
44. **Ljunger, C., and K. Larsson.** 1973. On the nature of the heat stabilizing effect of inorganic ions on *Escherichia coli*. Physiol. Plant. **28**:299–303.
45. **Macham, L. P., and C. Ratledge.** 1975. A new group of water-soluble, iron-binding compounds from Mycobacteria: the exochelins. J. Gen. Microbiol. **89**:379–382.
46. **Mangini, A., C. Sonntag, G. Bertsch, and E. Müller.** 1979. Evidence for a higher natural uranium content in the world rivers. Nature (London) **278**:337–339.
47. **Marcelis, J. H., H. J. denDaas-Slagt, and J. A. A. Hoogkamp-Korstanje.** 1978. Iron requirement and chelator production of staphylococci, *Streptococcus faecalis* and *Enterobacteriaceae*. Antonie Leeuwenhoek J. Microbiol. **44**:257–267.
48. **Marquis, R. E., K. Mayzel, and E. L. Carstensen.** 1976. Cation exchange in cell walls of Gram-positive bacteria. Can. J. Microbiol. **22**:975–982.
49. **McCullough, W. G., and R. S. Merkal.** 1976. Iron-chelating compound from *Mycobacterium avium*. J. Bacteriol. **128**:15–20.
50. **McQuillen, J.** 1950. The bacterial surface. I. Effect of cetyl-trimethylammonium bromide on the electrophoretic mobility of certain Gram positive bacteria. Biochim. Biophys. Acta **5**:463–471.
51. **Meyer, J. M., and M. A. Abdallah.** 1978a. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. **107**:319–328.
52. **Meyer, J. M., and J. M. Hornsperger.** 1978b. Role of pyoverdine$_{pf}$, the iron-binding fluorescent pigment of *Pseudomonas fluorescens*, in iron transport. J. Gen. Microbiol. **107**:329–331.
53. **Mosley, G. A., G. L. Card, and W. L. Koostra.** 1976. Effect of calcium and anaerobiosis on the thermostability of *Bacillus stearothermophilis*. Can. J. Microbiol. **22**:468–474.
54. **Orskov, I., F. Orskov, B. Jann, and K. Jann.** 1977. Serology, chemistry and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. **41**:667–710.
55. **Osada, Y., T. Une, T. Ikeuchi, and H. Ogawa.** 1975. Divalent cation stimulation of cell infectivity of *Shigella flexneri* 2a. Jpn. J. Microbiol. **19**:163–166.
56. **Osborn, M. J., and H. C. P. Wu.** 1980. Proteins of the outer membrane of Gram-negative bacteria. Annu. Rev. Microbiol. **34**:369–422.
57. **Pflug, H. D., and H. Jaeschke-Boyer.** 1979. Combined structural and chemical analysis of 3,800-million-year-old microfossils. Nature (London) **280**:483–486.
58. **Pierce, D., and P. Cloud.** 1979. New microbial fossils from ~1.3 billion-year-old rocks of Eastern California. Geomicrobiol. J. **1**:295–309.
59. **Pugsley, A. P., and P. Reeves.** 1976. Iron uptake in colicin B-resistant mutants of *Escherichia coli* K-12. J. Bacteriol. **126**:1052–1062.
60. **Ratledge, C., and P. V. Patel.** 1976. The isolation, properties and taxonomic relevance of lipid-soluble,

iron-binding compounds (the Nocobactins) from *Nocardia*. J. Gen. Microbiol. **93**:141–152.

61. **Rogers, S. W., H. E. Gilleland, Jr., and R. G. Eagon.** 1973. Characterization of a protein-lipopolysaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. Can. J. Microbiol. **15**:743–748.
62. **Salton, M. R. J., and P. Owen.** 1976. Bacterial membrane structure. Annu. Rev. Microbiol. **30**:451–482.
63. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–471.
64. **Schott, H., and C. Y. Young.** 1953. Electrokinetic studies of bacteria. III. Effect of polyvalent metal ions on electrophoretic mobility and growth of *Streptococcus faecalis*. J. Pharm. Sci. **62**:1797–1801.
65. **St. John, A. C., and A. L. Goldberg.** 1980. Effects of starvation for potassium and other inorganic ions on protein degradation and ribonucleic acid synthesis in *Escherichia coli*. J. Bacteriol. **143**:1223–1233.
66. **Takebe, I.** 1965. Extent of cross linkage in the murein sacculus of *Escherichia coli* B cell wall. Biochim. Biophys. Acta **101**:124–126.
67. **Ting-Beall, H. P.** 1980. Interactions of uranyl ions with lipid bilayer membranes. J. Microsc. (Oxford) **118**:221–227.
68. **Traüble, H., and H. Eibl.** 1974. Electrostatic effects on lipid phase transitions: membrane structure and ionic environment. Proc. Natl. Acad. Sci. **71**:214–219.
69. **Tsuchiya, T., S. M. Hasan, and J. Raven.** 1977. Glutamate transport driven by an electrochemical gradient of sodium ions in *Escherichia coli*. J. Bacteriol. **131**: 848–853.
70. **Voll, M. J., and L. Leive.** 1970. Release of lipopolysaccharide in *Escherichia coli* resistant to the permeability increase induced by ethylene diaminetetraacetate. J. Biol. Chem. **245**:1108–1114.
71. **Webb, M.** 1970. Effects of magnesium deficiency on ribosomal structure and function in certain Gram-positive and Gram-negative bacteria. Biochim. Biophys. Acta **222**:416–427.
72. **Weiss, R. L.** 1977. Site-specific membrane particle arrays in magnesium-depleted *Escherichia coli*. J. Cell Biol. **73**:505–519.