

## Lanthanide Accumulation in the Periplasmic Space of *Escherichia coli* B

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**Treatment of growing *Escherichia coli* B with lanthanide ions [lanthanum(III), terbium(III), and europium(III)] and subsequent aldehyde-OsO<sub>4</sub> fixation caused areas of high contrast to appear within the periplasm (the space between inner and outer membrane of the cell envelope). X-ray microanalysis of ultrathin sections of Epon-embedded or acrylic resin-embedded cells revealed the presence of the lanthanide and of phosphorus in the areas, whose contrast greatly exceeded that of other stained structures. Comparatively small amounts of the lanthanide were also present in the outer membrane and in the cytoplasm. The distribution of the periplasmic areas of high contrast was found to be random and not clustered at areas of current or future septum formation. Irregular cell shapes were observed after lanthanide treatment before onset of fixation. In contrast to glutaraldehyde-OsO<sub>4</sub> fixation, glutaraldehyde used as the sole fixer caused a scattered distribution of the lanthanide. Cryofixation (slam-freezing) and freeze substitution revealed a lanthanum stain at both the periplasm and the outer part of the outer membrane. Deenergization of the cell membrane by either phage T4 or carbonyl cyanide *m*-chlorophenylhydrazone abolished the metal accumulation. Furthermore, addition of excess calcium, administered together with the lanthanide solution, diminished the quantity and size of areas of high contrast. Cells grown in media of high NaCl concentration revealed strongly stained areas of periplasmic precipitates, whereas cells grown under low-salt conditions showed very few high-contrast patches in the periplasm. Terbium treatment (during fixation) enhanced the visibility of the sites of inner-outer membrane contact (the membrane adhesion sites) in plasmolized cells, possibly as the result of an accumulation of the metal at the adhesion domains. The data suggest a rapid interaction of the lanthanides with components of the cell envelope, the periplasm, and the energized inner membrane.**

Lanthanides (abbreviated Ln as a group) comprise the elements of the rare earth series. They occur only in trace amounts in organisms. A biological role for lanthanides is not known, and their interaction with bacterial cells has not yet been the subject of detailed studies. It is assumed that lanthanides can bind to the external surfaces of microorganisms and that they are not transported into the cytoplasm of bacteria, algae, or yeasts. In vitro, Ln ions are capable of replacing calcium in isolated eucaryotic organelles and in synthetic vesicles. This occurs without covalent binding (12). Lanthanides have been used extensively in nuclear magnetic resonance and fluorescence spectroscopy, including fluoroimmunoassays (25). The fluorescence properties of terbium, for example, were used to study binding sites of antibiotics and calcium displacement in neoplastic pituitary cells (9, 10). Cerium ions have been used in histochemical studies as capture agents for P<sub>i</sub> generated by phosphatase on aldehyde-fixed eucaryotic cells and tissues (23). From studies of calcium transport and transport inhibitors in eucaryotic cell systems, it has been suggested that lanthanides are unable to penetrate the membranes of living cells; however, lanthanides can be transported into pretreated eucaryotic cells and into isolated mitochondria, microsomes, and chloroplasts (12). Lanthanum has also been widely used in studies of receptor recognition and inhibition of cellular secretion processes (see reference 27 for references). Matsushita et al. (19) observed that lanthanum ions serve as blockers for the recently reported calcium channels in bacterial cells. Lanthanides have also been shown to bind to nucleic acids such as tRNA (26) and to membrane structures

such as tight junctions (22) and nuclear pores (24). Beveridge and Murray (8) described sites of metal deposition in the cell wall of *Bacillus subtilis*. Beveridge and Koval (7) showed that the capacity of isolated bacterial walls to bind metals differs widely between elements; binding appears to be stronger in gram-positive than in gram-negative cell walls. Isolated vesicles of the outer membrane of *Escherichia coli* were found to adsorb europium and also revealed in the electron microscope small randomly scattered deposits (13). In this report, the reaction of growing intact bacteria with a number of rare earth metal ions is described. Since the metals as well as phosphorus were observed to localize primarily to the clusters that formed within the periplasmic space and since the periplasm contains a variety of proteins, involved in substrate binding, in transmembrane transport, and in generating P<sub>i</sub> (22), the targets for lanthanides might be present in this cellular compartment. Another component of the periplasm is the peptidoglycan, whose molecular arrangement has been suggested to be an extended three-dimensional gel (15), whereas other models envisage the enterobacterial peptidoglycan as forming a narrower, more or less two-dimensional net that may be observed as being closely associated with the outer membrane or as a separate layer spanning the periplasm. While small molecules are assumed to be able to diffuse freely within a contiguous periplasm, the diffusion might be blocked by, for example, the periseptal annuli. These structures have been reported (20) to consist of inner and outer membrane contacts that seal off the present and future division areas. The presence of areas such as periseptal annuli would permit an uneven distribution of periplasmic contents. Furthermore, the Ln-containing precipitates described here could suggest the existence of still other microenvironmental domains in the

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periplasmic space. In this report, we describe the formation and distribution of Ln-containing precipitates and their responses to growth conditions, membrane polarization, and preparative procedures.

(Some of these data were reported previously [5].)

## MATERIALS AND METHODS

*E. coli* B and, in some cases, *E. coli* W3110 cells were grown to mid-logarithmic phase at 37°C in aerated nutrient medium containing 1% yeast extract, 1% tryptone (Difco), and 0.5% glucose. The low-osmolarity medium (55 mosM) consisted of 1% tryptone and 30 µg of uracil per ml (17). Medium of high osmolarity, containing 0.3 M NaCl, was established in the low-osmolarity medium by addition of NaCl, increasing the osmolarity to 620 mosM. Lanthanum(III) nitrate, terbium(III) nitrate, and europium(III) nitrate were purchased from Aldrich Chemical Co., Milwaukee, Wis. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma) was dissolved in ethanol at 100× concentration shortly before dilution into the cell culture to a final concentration of 20 µM.

Bacteriophage T4 ghosts (phages whose DNA had been released by osmotic shock) (1) were prepared in CsCl gradients, dialyzed against 0.1 M Tris buffer, and used as described earlier (3).

Growing cells were harvested at densities of  $2 \times 10^8$  to  $3 \times 10^8$ /ml, fixed for 30 min in 2.5% glutaraldehyde (GA) made from 25% GA (Electron Microscopy Sciences, Fort Washington, Pa.), and diluted in 0.1 M cacodylate buffer (Sigma Corp.) (pH 7.2) that also contained 1% lanthanide. The mixture measured 440 mosM by freezing-point depression. For plasmolysis, the buffer was made to contain 12% sucrose (935 mosM). In most cases, the freshly prepared Ln was either added 30 s to 1 min before addition of fixer or administered simultaneously with the fixation liquid. In a number of experiments, Ln concentrations of 0.1 and 0.5% were also used and caused no changes in the distribution of high-contrast patches, although the extent of the contrast areas was diminished relative to that of cells treated with 1% Ln. Instead of cacodylate buffer, 0.1M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.2) was occasionally used. There was no difference in action observed between the buffers. After 30 min of fixation at room temperature, the cells were sedimented at  $1,500 \times g$  in a Sorvall tabletop centrifuge, resuspended in the buffer plus Ln, and centrifuged again; the pellet was postfixed for 30 min in freshly prepared mixtures of 1% osmium tetroxide in cacodylate plus Ln. After centrifugation, the pellet was washed twice in cacodylate buffer, slowly dehydrated in acetone, and embedded in either Spurr medium, Epon 812 resin, or LR white (Polysciences, Warrington, Pa.). Polymerization at 50°C was carried out for 2 days. Ultrathin sections were cut on a Sorvall MT2 microtome and were stained with uranyl acetate (saturated) and lead citrate (10 min each). In a number of experiments, the embedding in Lowicryl K4M (Polysciences) was performed after GA fixation at 4°C with 1% lanthanum nitrate present and acetone dehydration, with progressive lowering of the temperature (PLT procedure [15]).

For cryofixation and cryosubstitution, the  $\text{La}^{3+}$ -treated cells were rapidly frozen either in liquid propane (kept at ~120 K) or by slam-freezing procedures (using a Life-Cell apparatus [Life-Cell Corp., The Woodlands, Tex.]), with liquid nitrogen as the coolant. After freeze substitution in acetone at -92°C, the cells were embedded in either Epon, LR white, or Lowicryl HM20 (Polysciences). Ultrathin

sections were stained as described above. Micrographs were taken with Philips 400 and 420 electron microscopes. The X-ray microanalysis was performed in the Philips 420, using the EDAX system in the transmission electron microscopy mode with a specimen tilt of 25°, 60- to 80-kV acceleration, 20- to 60-µA current, and 10- to 100-nm-diameter probe, 0- to 20-keV range, 10 eV per channel, usually between 100 and 400 lifetime s. The detector was Si with Be window, with 0.13-sr solid angle of the detector, and the EDAX 9800 Plus analyzer.

In a statistical analysis, we addressed the question of whether the centers of the Ln patches were clustered at sites of previous, current, or future (prospective) divisions. The analysis was executed in the statistics laboratory of S. Litwin, Fox Chase Cancer Center, and was designed to test whether patches were preferentially localized to areas within 1/16 of the cell length at either the half or quarter length of a nondividing cell or in dividing cells near the division sites or near half and quarter lengths of the daughter cells.

## RESULTS

**Areas of high contrast.** When growing cells were treated with Ln ions either shortly before addition of fixer or simultaneously with the aldehyde fixation mixture, a patchy deposition of highly electron scattering material in the periplasm of the bacterium was observed. The extent and appearance of the Ln deposition as well as the shape of the cells were found to be affected by the timing of Ln exposure and by the fixation procedures and growth condition of the cells. Figure 1 shows cells that had been fixed in the presence of La with a combination of aldehyde and  $\text{OsO}_4$  treatment. Serial sections revealed that in such preparations almost all cells produced high-contrast patches, each cell showing a few (two to five) larger patches. The sizes of the patches varied from cell to cell. Some cells exhibited larger numbers of relatively small patches (Fig. 1b). Many cells were slightly plasmolized as a result of the increased osmotic pressure of the fixation liquid. The ultrastructure of briefly treated cells was well preserved when double-fixation procedures were used together with the Ln. The three Ln tested [La(III), Tb(III), and Eu(III)] generally revealed similar patterns of deposition. However, treatment with the terbium ion caused frequently a deposit of small high-contrast patches at membrane contact sites (see below). Shown here are mostly the data for lanthanum. The extent of patch formation was highest in lanthanum-treated cells and lowest in terbium-treated cells. A decrease in patch density (but not distribution) was observed when the Ln concentration was reduced to 0.5 and 0.1%. Figure 1c shows cells treated with 1% Eu(III); these preparations occasionally showed small vesicular structures protruding from the cell surface. La- or Eu-treated cells revealed no differences in the membrane features and in the distribution of high-contrast domains between unplasmolized and slightly plasmolized cells. The highest electron density in the deposition area was achieved when the Ln-treated cells were postfixed with  $\text{OsO}_4$  and the sections were stained with uranyl acetate and lead citrate. Cells that were not postfixed with osmium revealed the structural aspect of a slight plasmolysis (Fig. 2a). The presence of lanthanum during GA fixation caused a fine precipitate to occur in the envelope (Fig. 2b). Although there is a lack of membrane contrast in these preparations, it appears that the precipitate occurred in the area of periplasm and outer membrane. In addition to this effect, the combination of lanthanide and aldehyde fixation caused a distortion of the cell shape (Fig. 2b). As demonstrated above (Fig.

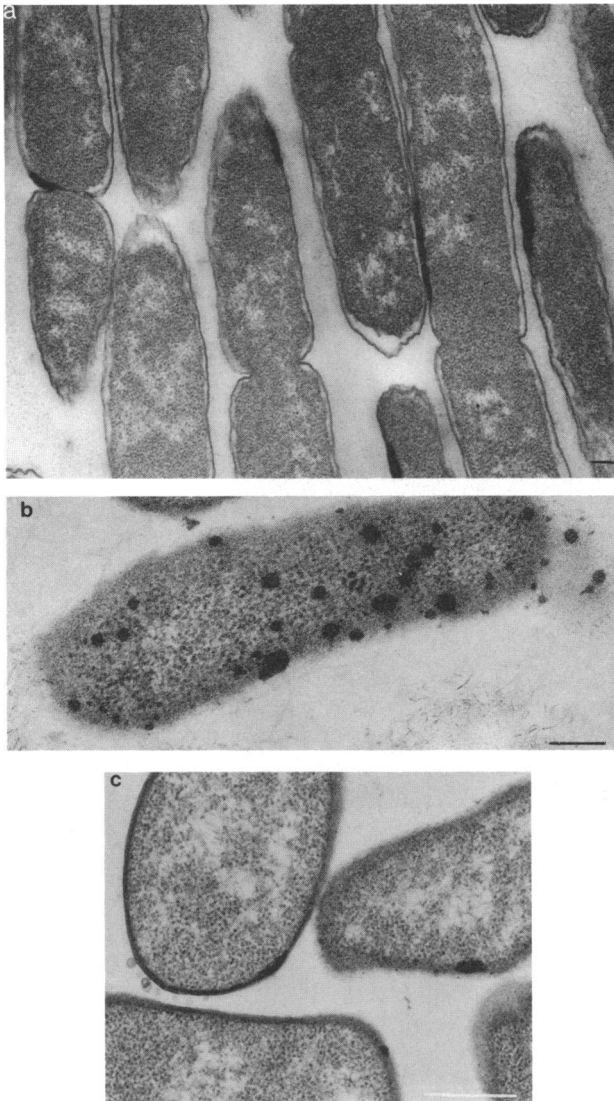


FIG. 1. (a) Ultrathin section of *E. coli* after treatment with lanthanum nitrate during GA-OsO<sub>4</sub> fixation. High-contrast deposits are localized to the periplasmic domain. Spur embedding; uranyl acetate-lead citrate staining. The bar in this and subsequent micrographs represents 0.25  $\mu$ m. (b) Grazing section of envelope of lanthanum-treated *E. coli* B showing high-contrast deposits of different sizes scattered over the entire area of the cell surface. (c) Eu(III)-treated *E. coli* B showing deposit areas similar to those seen in panels a and b. Small vesicular surface structures are visible on the cell at the left.

1), most aldehyde-OsO<sub>4</sub>-fixed cells exhibited patches that often extended over 1  $\mu$ m in length but generally measured several 100 nm in length and were usually of round or oval shape, with serrated edges visible in grazing sections of the periplasm (Fig. 3a). Cross sections through the cell envelope revealed the borders of high-contrast patches to be usually well defined. Often bridgelike elements were seen spanning the space between outer and inner membrane (Fig. 3b and c). Occasionally the edges of Ln patches did not terminate abruptly but thinned out. At these areas, a dark (high-contrast) linear structure with a beaded deposition (Fig. 3d) was often visible. It showed a bead repeat of 2 to 3 nm and was positioned adjacent to the outer membrane. Terbium

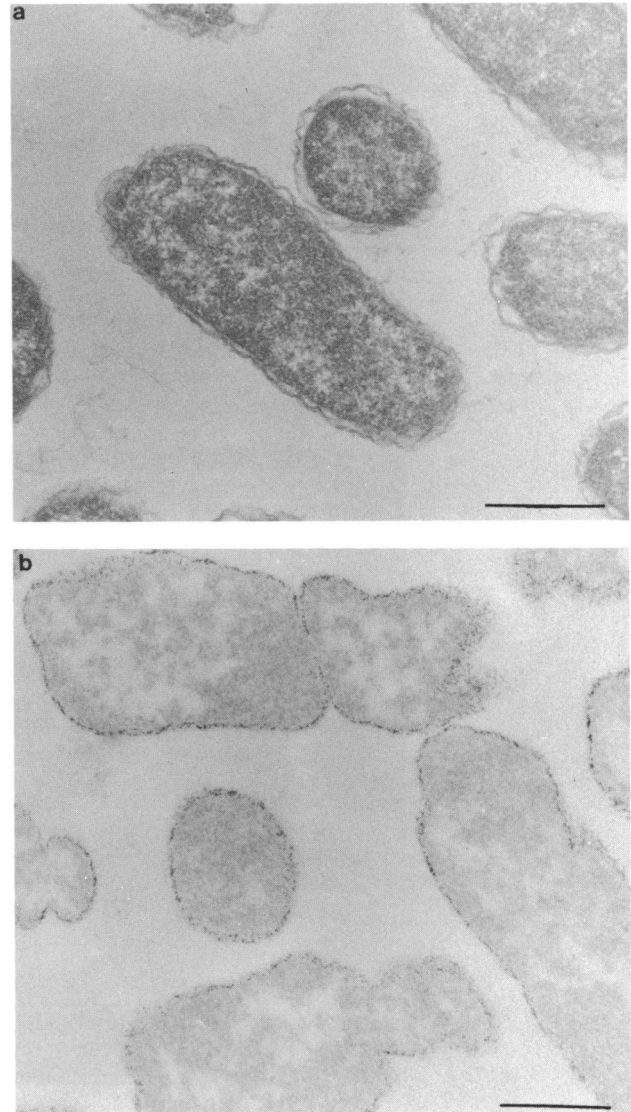


FIG. 2. Effects of lanthanum treatment and GA fixation. (a) *E. coli* B after GA fixation and brief staining of the section. (b) Cells after lanthanum treatment plus GA fixation; section unstained. The cell envelope shows larger deposits and numerous smaller deposits of high-contrast material. The periplasmic space is absent, and the cell shape is uneven.

treatment showed in general much smaller patches of high contrast in the periplasm of unplasmolized cells. In plasmolized cells, the smaller patches were localized to contact areas between inner and outer membrane (see Fig. 7a and b), whereas Tb deposits were absent from the (osmotically widened) periplasmic space. This result contrasts with the generally periplasmic localization of lanthanum and europium.

**Distribution of lanthanum deposits.** We asked whether the high-contrast patches would coincide with the localization of the sites of septation, including septum domains of past, current, or future cell division. The statistical analysis of the distribution of the centers of patches of medium and large size tested the null hypothesis that there is no clustering of the centers of lanthanum patches at sites of either recent, current, or prospective divisions. Under this hypothesis,

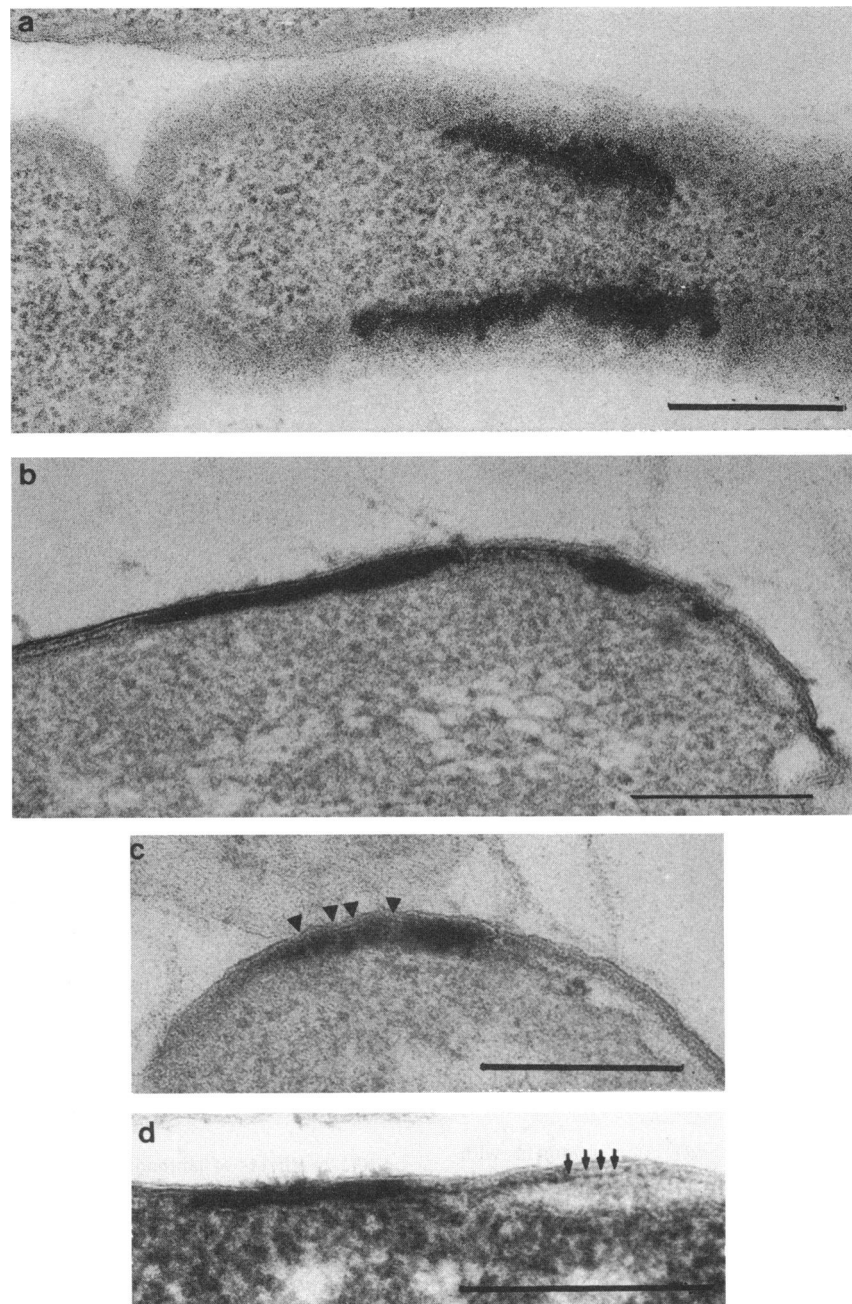


FIG. 3. *E. coli* after lanthanum treatment and fixation in GA and  $\text{OsO}_4$  (see legend to Fig. 1). (a) Grazing section showing serrated edges of deposit. (b) Cross-sectional view. Note the boundaries of the areas of high contrast. (c) High-contrast area with membranous material bridging the periplasmic space (arrowheads). (d) Stained layer with beaded appearance (arrows) bordering the inner face of the outer membrane.

patch centers are assumed to be uniformly and independently distributed over the length of the dividing or nondividing cell. The chance that a randomly chosen center of a patch is located within  $1/16$  (of the total length of the cell) to one of the division sites (at 0,  $1/4$ ,  $1/2$ ,  $3/4$ , and 1) is 50%. Under these conditions, the number of centers within this set is binomially distributed [ $b(k, n, p)$ , where  $k$  is the number of centers inside the set,  $n$  is the total number of patches, and  $p$  is 0.5]. Our analysis included 75 sectioned cells. Of these, 46 were nondividing cells revealing 25 centers close (in the

above-stated sense) to the quarter points out of 46 centers. The chance of observing at least this number is 0.329 and is thus not statistically significant. In the 29 dividing cells counted, we found 19 centers close to the quarter points out of 40 observations. Eleven cells had two deposits, one on either side of the septum. The chance of at least 19 close centers is 0.682 and is likewise not statistically significant. We conclude that our observations support the null hypothesis; we found no evidence to reject it. Therefore, the centers of the patches appear to be randomly distributed.

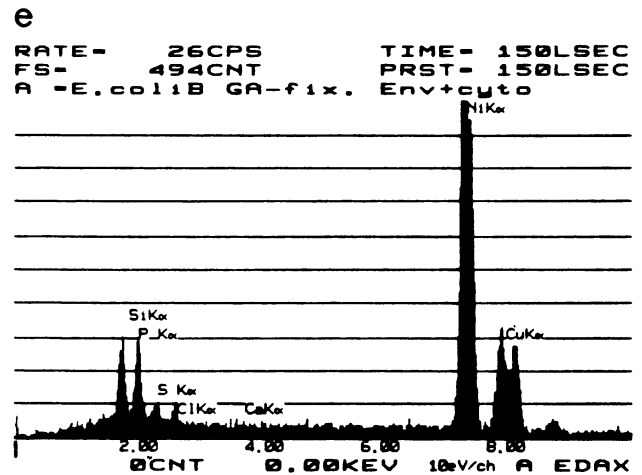
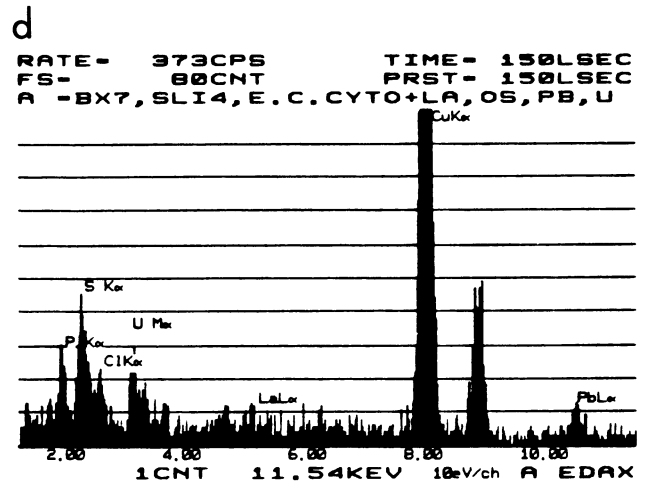
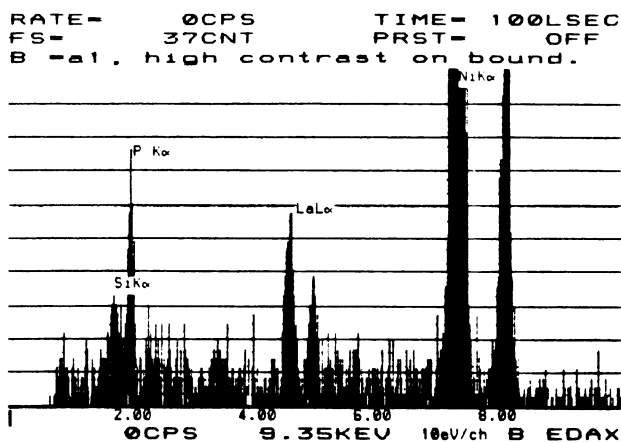
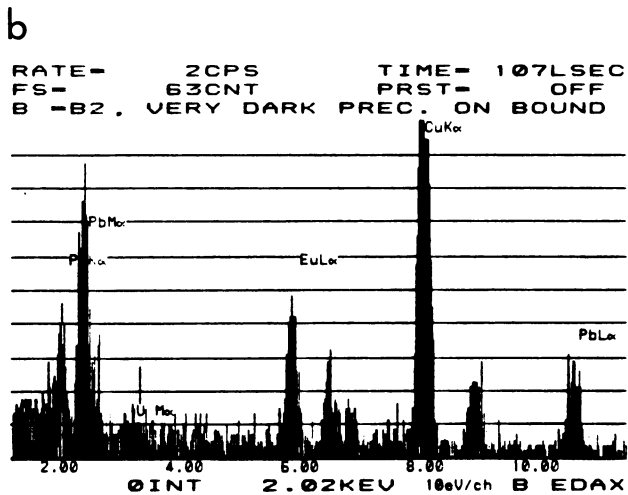
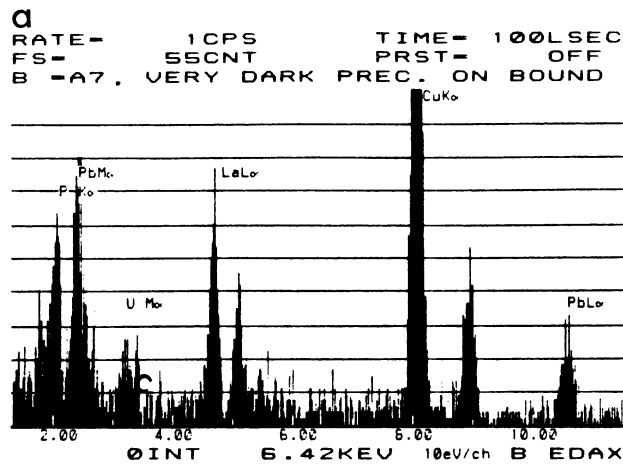


FIG. 4. Elemental analysis of periplasmic deposits and other areas measured in ultrathin sections of *E. coli* B. (The full scale of the ordinate counts is given for each panel as FSC.) Spot size was 10 to 20 nm. (a) Lanthanum treatment during aldehyde and  $\text{OsO}_4$  fixation plus subsequent staining (see legend to Fig. 1). The analysis was from a high-contrast area of the periplasm. The elements lanthanum (La), phosphorous (P), uranium (U), and lead (Pb) are present. The copper (Cu) is from the support grids; silicium is (Si) from the embedding medium. An osmium peak is not visible because of an overlap with the predominant copper peak. (b) Europium treatment (same procedure as for panel a). Note the presence of the elements Eu, P, Pb, U, Cu, and Si. (c) High-contrast patch of a La-treated cell that was fixed in GA only; no further staining procedures were used. La, P, Ni (from support grid), and Si are shown. (The large peak to the right of Ni is Cu from the specimen carrier.) (d) Cytoplasmic area of La-treated cell, fixed as for panel a). Compared with P and Pb, La is present in small amounts. (e) Spectrum of envelope and adjacent cytoplasmic area of *E. coli* after GA fixation only, without Ln treatment or additional staining (spot size, 100 nm). Ni is from the support grid; Cu is from the specimen holder.



**Elemental analysis.** Elemental analysis of ultrathin sections of cells treated with the lanthanide nitrate of either La(III) or Eu(III) showed the element to be present mainly in the high-contrast area of the periplasm (Fig. 4a to c). Very little lanthanum signal was obtained over the outer membrane regions outside a high-contrast patch. Data from terbium(III)-treated cells showed identical results when the analysis included a membrane-associated deposit. High-contrast patches contained in addition significant amounts of phosphorus and of the elements that were used in the postfixation (Os) and staining (U and Pb) processes. Cells that were fixed with glutaraldehyde only and not stained subsequently showed clearly the Ln residing in the larger high-contrast patches (Fig. 4c) and in much smaller amounts localized to the outer membranes (data not shown) and the cytoplasmic area (Fig. 4d). In Ln-treated cells, fixed with either GA alone or postfixed in  $\text{OsO}_4$ , the uncontrasted compartments of the periplasm revealed no measurable amount of Ln. Often, a silicon peak was observed; it was present also in the cell-free sectioned embedding medium but was not found in the Formvar support film. We assume that the material originated from the resin preparation. The metal of the support grid (copper or nickel) was strongly visible in all of the spectra. Copper lines often hide the less intense osmium lines. The envelope and cytoplasm of cells that were fixed in GA only and neither treated with Ln nor stained with uranyl acetate and lead citrate showed a phosphorus signal as well as Si and Ni (from the support grid) and Cu (from the specimen holder) (Fig. 4e).

**Pre-treatments affecting Ln deposition.** The Ln deposition could be changed by means that affect periplasm and inner membrane: addition of calcium in twice the amount of simultaneously added lanthanum diminished the size and number of the high-contrast areas. Cells grown in high-salt conditions and treated with lanthanum during fixation showed very pronounced periplasmic (high-contrast) depositions of La (Fig. 5a), whereas cells grown at a low salt concentration did not contain the deposits (Fig. 5b). The organisms growing in high-salt medium also revealed round spots of high contrast in the cytoplasm (Fig. 5a). Elemental analysis showed Pb and Os peaks over the spots, whereas La was not represented.

We examined the effect of membrane deenergization on the formation of Ln patches and used bacteriophage infection to reduce the proton gradient across the inner membrane (3, 18). When phage T4 ghosts or complete T4 phages were used to infect *E. coli* B at a multiplicity of 4 to 10 and the cells were exposed to 1% lanthanum 4 min later, high-contrast deposits were not formed. Furthermore, a different method for dispersal of the proton gradient, namely, treatment with CCCP (20  $\mu\text{M}$ , added 2 min before addition of the Ln) had an identical effect: high-contrast deposits were absent.

**Timing of the Ln treatment.** Ln treatment must begin either at the start of fixation or within 30 s before fixation. A greater delay in the onset of fixation had a significant effect on the shape of the bacterium (Fig. 6b). *E. coli* washed in 0.9% NaCl or 0.1M PIPES buffer and exposed to lanthanum nitrate 1 to 20 min before addition of aldehyde fixer and subsequent  $\text{OsO}_4$  fixation showed highly deformed shapes, often revealing bulging surface areas. These preparations had a striking similarity to cells that were lanthanum treated and immediately fixed in GA as the sole fixer. Signs of cell lysis, such as empty cells or extruding DNA, were observed in the preparations after 5 to 10 min of Ln treatment.

**Effect of terbium.** An unexpected effect was encountered when *E. coli* B was exposed to terbium nitrate during aldehyde fixation in the presence of 12 to 15% sucrose: the procedure

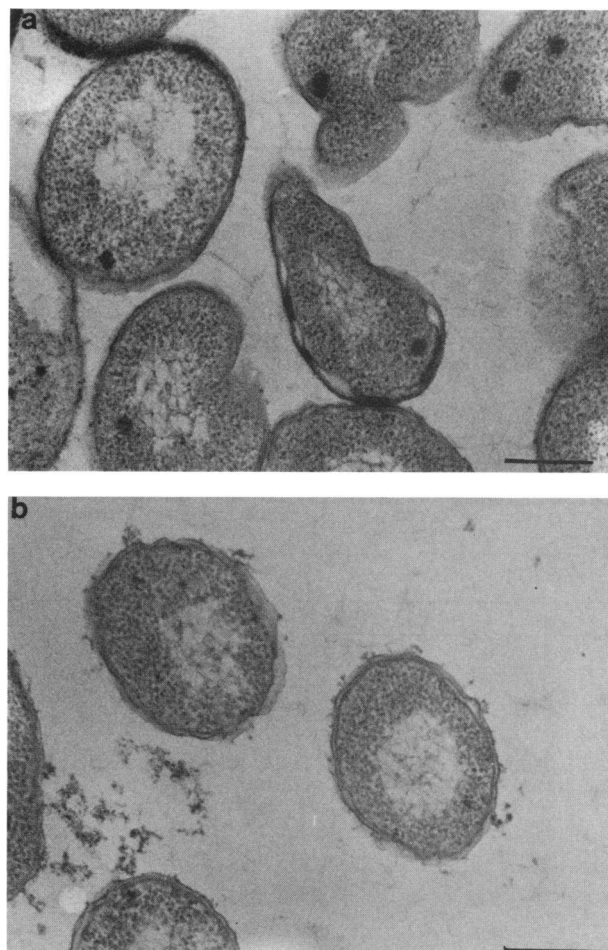


FIG. 5. (a) Cells grown at a high salt concentration, showing periplasmic deposits that contain La, Pb, and Os, as measured by EDAX. (The dark cytoplasmic areas do not contain La but contain Pb and Os.) (b) Cells grown at a low salt concentration, which show no periplasmic deposit.

enhanced visibility and structural definition of the typical membrane adhesion sites (2) (Fig. 7). Frequently, the portion of the extended membrane pointing toward the outer membrane was seen to be thinned out, most likely because of an overstretching of the outer portions of the adhesion site material. Preliminary counts showed that in Tb-treated cells the number of adhesion sites was not drastically altered in comparison with the number in untreated plasmolized *E. coli* B.

**Other embeddings.** In addition to Epon embedding, Lowicryl K4M and HM20 resins were used for conventional embedding as well as for cryosubstitution experiments. In both settings, the performance of the latter resins was often unsatisfactory and unreliable. Ln-treated cells that were treated with the PLT method (15) and embedded in Lowicryl K4M showed at many of the areas of metal deposition a total separation of cell envelope and embedding medium. The areas of contrast often revealed a crystalline, apparently brittle composition which seemed to also involve the adjacent membranes of the envelope. Nevertheless, areas of high contrast were observed to occur in patches localized to the periplasm (data not shown). The distribution and X-ray data of the metal patches were similar to those of Epon-embedded material.

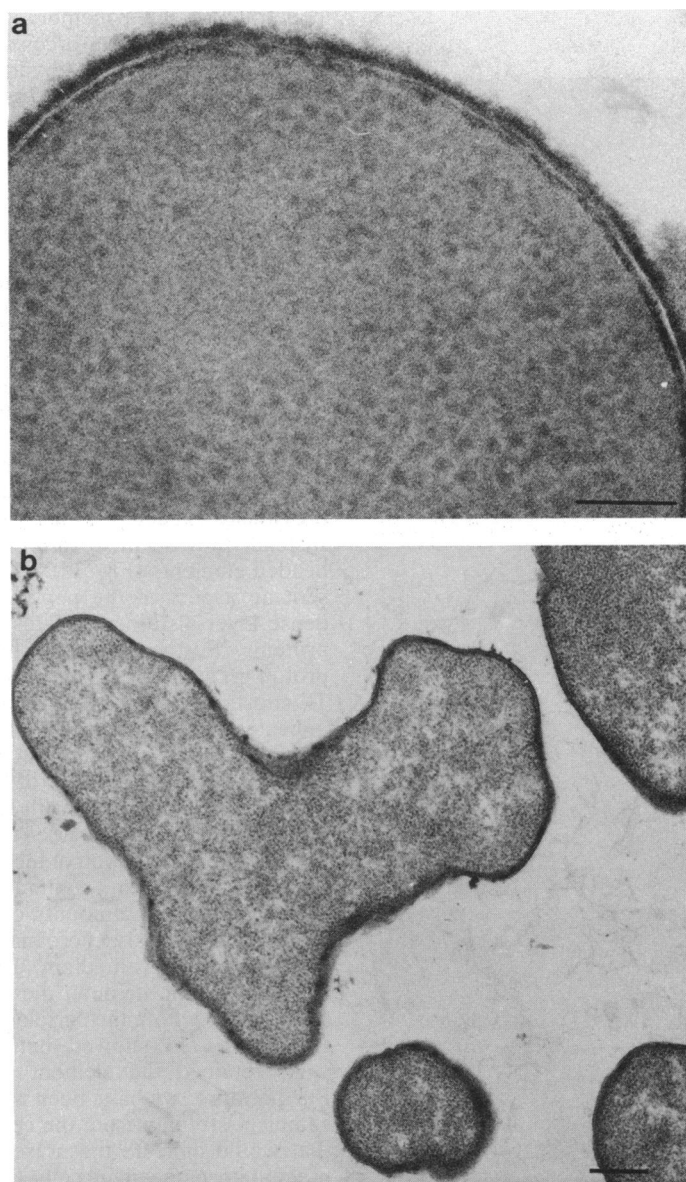


FIG. 6. (a) Unfixed lanthanum-treated, cryofixed, and cryosubstituted cell. In addition to a periplasmic stain, high-contrast material is visible at the outside of the envelope. Note the many structures crossing the periplasmic space. (b) Section showing that lanthanum treatment 30 s before onset of fixation (GA-OsO<sub>4</sub>) causes cells to assume bizarre shapes.

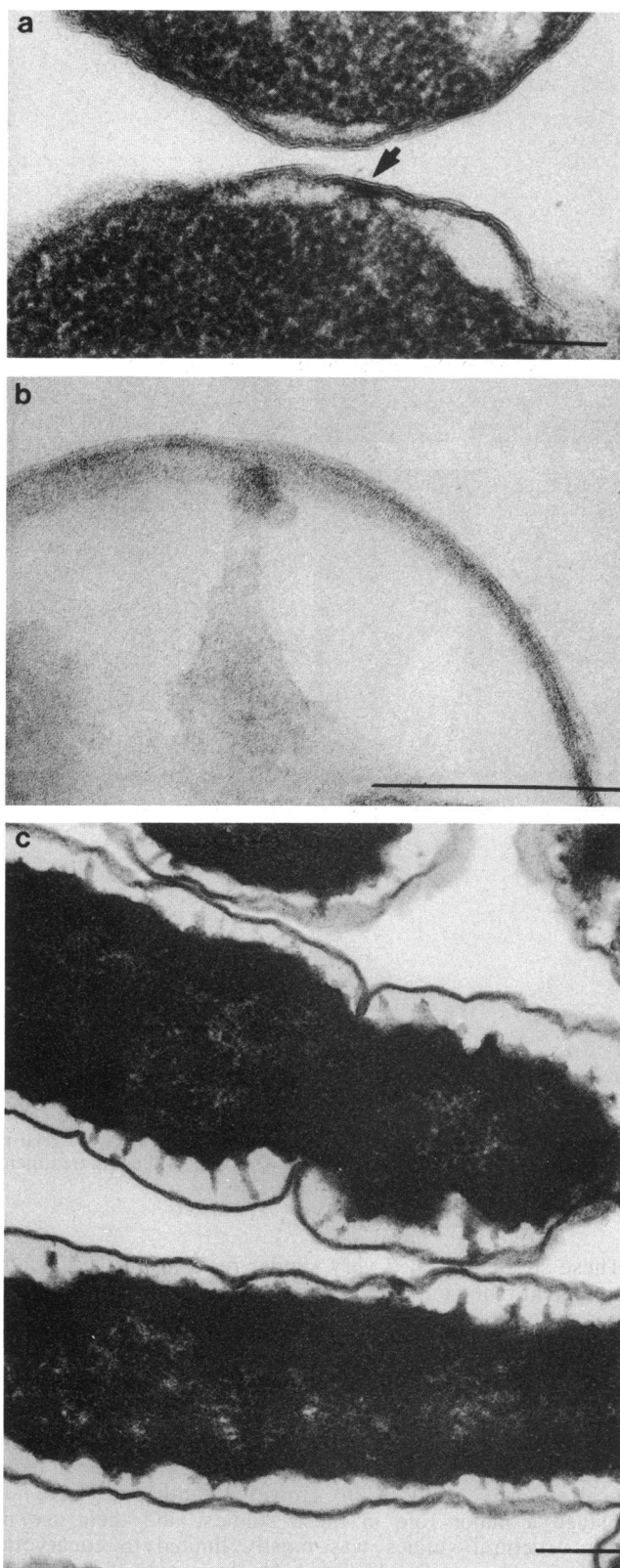
The use of cryofixation and cryosubstitution of lanthanum-treated *E. coli* revealed material of high contrast that resided not only in larger areas of the periplasm (Fig. 6a) but also at the outside of the outer membrane. The result was the same when the cryofixed cells were substituted into Lowicryl HM20 or Epon. X-ray analysis demonstrated the presence of lanthanum in the extracellular deposits as well as in the high-contrast areas of the periplasm. The contrast domains in the periplasm appeared to be more extended than those observed in conventional fixation regimens. Close inspection of these high-contrast domains showed that they were frequently interrupted by clusters of material of lesser contrast (Fig. 6a) that may very well represent periplasmic protein or ill-preserved inner-outer membrane bridges.

**Other gram-negative cells.** In a number of experiments, *Salmonella anatum* and *Cytophaga* U67 (14) were studied.

These strains revealed very similar reactions to lanthanum treatment, showing intensely contrasted patches within the periplasmic domain of aldehyde-fixed and OsO<sub>4</sub>-postfixed cells (micrographs not shown).

#### DISCUSSION

The toxicological, biochemical, industrial, and environmental aspects of interactions of bacteria with metals have been extensively studied (6). However, lanthanides have not played a major role in these studies, and their use in ultrastructural studies was mostly limited to eucaryotic systems. The reaction leading to the formation of patches of high electron-scattering capability in the periplasm of gram-negative bacteria has, to our knowledge, not been reported before. We conclude from our data that rare earth metals



penetrate the outer membrane and react with substances of the periplasm to form precipitates that are insoluble in the subsequent buffer and dehydration environments. Robinson and Karnovsky (23) have used the lanthanide cerium as capture agent for the  $P_i$  released during the action of phosphatase on phosphate-containing substrates. Since the periplasm contains these enzymes (see reference 21), we propose that an analogous reaction takes place between the Ln ions and components of the bacterial cell envelope. The distribution of the areas of high contrast is apparently random, as indicated by our statistical evaluation. The existence of a sharp border that surrounds a patch could indicate the presence of a larger, more or less circular area of junction of outer and inner membrane. The random distribution of the large patches seems to exempt the periplasmic bays (20) from being the only sites of Ln deposition, although in some cases a patch was observed at areas of cell division (Fig. 1a). Thus, the areas of metal deposition might suggest a periplasmic organization of higher complexity than previously expected. Not all patches have a sharp border. Instead, they seem to thin out along a layer composed of beaded elements (Fig. 3d). The layer plus beads may represent an aspect of the peptidoglycan in which a relatively dense layer also incorporates a portion of outer membrane proteins. Furthermore, the finding that dissipation of the proton gradient of the inner membrane (by CCCP or phage T4 ghosts) prevents the formation of high-contrast patches indicates the participation of the inner membrane in the localized enhancement of phosphate-rich substrates.

With regard to the effect of the NaCl concentration on the formation of precipitates with La, we speculate that the membrane-derived oligosaccharide that is produced in high amounts at low salt concentration of the growth medium (16) may serve as protection against La interaction and precipitation.

Relatively small amounts of La(III), Eu(III), and Tb(III) were found by X-ray microanalysis in the outer membrane of *E. coli* grown in L medium. The presence of small amounts of lanthanide in the outer membrane may be independent of the reaction within the periplasm and at the inner membrane, since Ferris (13) showed that europium-treated isolated cell walls retained the element in small crystalline deposits. Furthermore, we have been unable to detect an La signal of sufficient strength from the cell wall of GA-OsO<sub>4</sub>-fixed cells that would indicate the presence of a finely distributed La precipitate as seen in cells fixed with GA only (Fig. 2b). Unless the OsO<sub>4</sub> fixation and staining processes selectively extract the precipitates from the cell surface, the data can be interpreted as suggesting that the distribution of Ln complexes after GA fixation is the product of a reorganization of the metal deposit during subsequent dehydration and embedding procedures. The cytoplasmic content of Ln (although small) indicates a diffusion of the metal into the cell. However, the penetration of the inner membrane barrier might be a postmortem event as a consequence of leakiness after fixation of the membrane.

The negatively stained bridges observed in cross sections of a high-contrast patch (Fig. 3c) can be interpreted as

FIG. 7. Terbium-treated cells, plasmolized in 10% sucrose. (a) Deposition of high-contrast material at membrane adhesion (arrow). (Energy-dispersive X-ray analysis revealed the presence of Tb at this area.) (b) Membrane adhesion site showing deposit in section plane slightly tilted relative to axis of adhesion. (c) Group of plasmolized cells, all of which show a multitude of well-preserved adhesion sites.



representing inner-outer membrane connections spanning the periplasmic space. This suggestion would need additional exploration, especially in view of the proposed existence of a rather extended homogeneous periplasmic gel that is seen to leave no space for adhesion sites between the envelope membranes (15). One has to remember in this context that the data pertaining to an uninterrupted periplasmic gel have been recently challenged (11), and new methods of rapid freezing with and without cross-linking have established the existence of well-defined sites of adhesion connecting inner and outer membrane (4). The current uncertainty of effects of some of the freezing methods also requires a cautious interpretation of the *in vivo* structure of the peptidoglycan within the periplasm. Thus, a possible correlation between the high contrast of an Ln patch and peptidoglycan will not be addressed here. The beaded structure seen in some of the micrographs such as Fig. 3d might represent a structural element of the peptidoglycan and its associated outer membrane constituents.

The precipitation of the Ln within the periplasm is most dramatic when the standard fixation procedures are followed. Lanthanides enhance the contrast of membrane structures and appear to stabilize membrane adhesion. However, Ln treatment of cells before fixation causes the cells to assume bizarre shapes (Fig. 6b). We interpret this effect tentatively as caused by an increased activity of lytic enzymes of the cell envelope. Cells fixed in GA alone showed a fine precipitate in the cell envelope. This finding raises the question of whether subsequent fixation in OsO<sub>4</sub> causes a contraction of the originally diffuse precipitate or whether the fine precipitate is an artifact due to the failure of the GA fixation to stabilize envelope components. We do not have a conclusive answer to this question. We hypothesize that the aldehyde fixation fails to stabilize the material that composes a high-density patch during washing, dehydration, and embedding process.

Our expectation that cryofixation by slam-freezing and cryosubstitution would allow a localization of the Ln complex within the cell envelope was not fulfilled, since the Ln appeared also at the outside of the outer membrane (Fig. 6a). At present we have no explanation except that during freezing the Ln is driven outward from the periplasm to the surface of the outer membrane, where the metal forms new aggregates.

In conclusion, the structural effect of Ln ions on the bacterial cell is rapid and consists of the formation of periplasmic precipitates containing lanthanum and phosphorus. Terbium appears to form small deposits at contact sites of outer and inner membrane. The reaction is dependent on membrane energization and can be blocked by an excess of calcium. Growth at low ionic concentration of the medium also prevents formation of the periplasmic precipitate. We have also observed that cells were killed by Ln and that the killing followed a response pattern analogous to that of the generation of precipitate (5). The results described in this report suggest that the periplasm and the inner membrane comprise early interactive sites in reactions between cell envelope and Ln.

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