Capsule of *Escherichia coli* K29: Ultrastructural Preservation and Immunoelectron Microscopy

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The polysaccharide capsule of *Escherichia coli* K29 fully surrounds the microorganism and thus occupies an extracellular space ca. 20 times larger in volume than that of the decapsulated cell. Since more than 95% of the capsule consists of water, dehydration for electron microscopy causes the material to collapse. We describe here a method for embedding the capsule in an uncollapsed form. Dehydration of gelatin-enrobed, glutaraldehyde-fixed cells was performed in dimethyl formamide. The cells were embedded in Lowicryl K4M with the "progressive lowering of temperature" method and UV polymerization. In ultrathin sections, the capsule can be identified by its low electron contrast. It occupies a layer 3/4 μm thick and shows fibrous strands embedded in a fine granular matrix. The thin strands extend radially from the cell wall and transverse the capsule. The entire capsule domain, as well as the outer membrane, binds specific anticapsular antibody, whereas the periplasmic space and most of the inner membrane lack capsule-specific immunostain.

Capsular material is found on most of the rod-shaped bacteria after they are freshly isolated from natural habitats and from pathogenic systems (5). *Escherichia coli* K29 originated from a clinical isolate (18) and was propagated for many years in our laboratory. The composition of polysaccharide capsules and the molecular conformation of a number of *E. coli* capsules have been determined, including that of strain K29 (6, 10). The polysaccharide capsule is composed of more than 95% water, and its presence adds considerably to the total dimension of the cell.

The capsule plays a major role in the survival of the bacterium, and poses a considerable barrier not only to the immunodefense of the animal host but also to the entry of macromolecules, bacteriophages, DNA, and drugs into the microorganism (5).

Dehydration for electron microscopy causes the capsular structure to collapse (2). Without protective measures, this collapse is unavoidable, since water is replaced by organic solvents such as alcohols or acetone. Freeze-etching revealed that the collapse of the capsular matrix has already occurred when 50% acetone concentration is achieved in the dehydration mixture (2). So far, the most successful stabilization of the capsule has been achieved by pretreatment of cells with anti-capsule antibody (2). After this treatment, dehydration does not cause structural collapse. However, the fine structure of such antibody-treated capsules is obscured, since most of the mass of the polysaccharide-antibody complex is composed of immunoglobulin G (IgG). Visualization of capsular polysaccharides (19) has been attempted with various degrees of success by the use of stains such as ruthenium red (2, 5, 9, 17) and a variety of postembedding stains, such as phosphotungstic acid (7, 13) and related compounds (14). These latter stains can be applied after embedding (8). However, the comparison of the size and shape of antibody-stained capsules does not seem to agree with the size of polysaccharide capsules observed with other methods. Antibody-treated capsules are larger in size and more homogeneous in their matrix and

show relatively smoother surface contours than capsules prepared, or stained, with ruthenium red (2, 5, 8, 17). *E. coli* K29 exhibits a relatively thick polysaccharide capsule as revealed in the light microscope after in vivo staining with India ink (2). We describe here a new method of the structural preservation of the capsule, a combination of dimethyl formamide (DMF) dehydration with progressive lowering of the temperature (14) and embedding in Lowicryl K4M (1). The cells show in ultrathin sections a well-delineated capsular domain, which is interspersed with fine, mostly radially oriented fibers. The capsule-specific antigenic activity is retained in the sections and can be demonstrated by immunolabeling methods.

MATERIALS AND METHODS

E. coli Bi161/42 is serotype O9:K29(A):H⁻, called hereafter *E. coli* K29; the strain was given to us in 1974 by S. Stirm, Max-Planck-Institut für Immunobiologie, Freiburg, Federal Republic of Germany (18), and has since been maintained in our laboratory (2). The strain originated from a clinical case and was isolated by F. Ørskov and J. Ørskov, World Health Organization International *E. coli* Center, Copenhagen, Denmark. The growth medium was composed of 0.5% yeast extract, 0.5% tryptone (both Difco), 0.1% glucose, and 0.5% NaCl. Cells were harvested from aerated cultures growing at 37°C at cell densities (colony counts) of 2×10^8 to 4×10^8/ml. Antisera against K29 capsule antigen were produced in rabbits and were absorbed against *E. coli* O9:K9(L):H12; this strain contains the same (O9) lipopolysaccharide as the K29 strain but does not produce capsular antigen. The capsular antigen used for immunization was purified as described previously (2) by using phenol extraction and Catavlon (Ayerst) precipitation. After affinity purifications with an AH Sepharose 4B column and K29 antigen, the IgG showed in agglutination a dilution titer of >1:246 per mg of protein. For immunolabeling, the IgG was diluted between 10^2 and 10^3 times.

Immunolabel. Gold-protein A complexes were prepared as described (16). The colloidal gold particles averaged ~14 nm in diameter. Protein A was purchased from either Sigma

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Chemical Co. or Pharmacia Fine Chemicals and used without further purification.

Preparation of the cells for microscopy. Cultures (3 ml) in exponential growth phase at 37°C were fixed in 2% glutaraldehyde. After 60 min of fixation, the cells were allowed to cool to room temperature and were centrifuged at 1,500 × g. The pellet was suspended twice in 3 ml of 0.1 M phosphate buffer (pH 7; P-buffer). After the last centrifugation, the pellet was resuspended in 0.2 ml of P-buffer to which was added 10% melted (60°C) gelatin. After solidifying, the gel was cut into small cubes (side length, ~0.5 mm) and fixed a second time with 1% glutaraldehyde in P-buffer for 5 min. For dehydration, DMF was chosen, since we had observed that polysaccharides do not precipitate in this solvent. We have not detected a change in agglutination by capsule-antisera after brief dimethyl sulfoxide treatment and rehydration of the cells. Dehydration was performed by a procedure termed progressive lowering of temperature (4); its purpose is to dehydrate the specimen at the lowest possible temperature above the freezing point of the mixture. Dehydration steps consisted of treatment with 30% DMF for 30 min at 0°C, 50% DMF for 60 min at −20°C, 70% DMF for 60 min at −35°C, and two changes of 100% DMF for 1 h each at −35°C. Infiltration with Lowicryl K4M was executed at −35°C in steps of 33, 66, and 100% K4M for 1 h each and for 24 h in fresh 100% K4M at −35°C. Polymerization of the K4M was achieved by UV irradiation at −35°C for 24 h followed by 48 h of UV treatment at room temperature. Thin sections were cut with a diamond knife and placed on carbon-coated Formvar films on gold grids (Polaron). Immunostaining was performed as follows. Grids with the sections were placed on drops of ovalbumin in P-buffer (0.01 M; pH 7) followed by treatment for 20 min to a maximum of 2 h with the anti-capsule IgG or, for control, with noncross-reacting rabbit-IgG prepared from preimmune serum or from other nonimmunized control animals. After two washings of the grids in P-buffer, gold-protein A solution was applied for 30 to 60 min, the grids were washed three times in double-distilled water, and the sections were stained in half-saturated water-uranyl acetate at room temperature for various lengths of time (3 to 30 min). The gold-protein A solution was made as described previously (16) and was used with modifications in the time schedule. Electron microscopy was performed with a Philips 400 electron microscope and Kodak electron microscope film.

RESULTS

K29 capsular polysaccharide collapses during the early steps of conventional dehydration, long before being exposed to embedding media (Fig. 1a). We had observed earlier (2) that the collapse can be avoided by exposing the cells to anticapsular antibody (Fig. 1b). The progressive lowering of temperature method, in combination with gelatin enrobement, preserves the capsule without antibody treatment and reveals the capsule as a low-contrast domain (Fig. 2). The thickness of this domain varied between 0.5 to 0.8 μm. The outer contour of the capsule appeared to be smooth and well distinguishable from the more coarsely granulated gelatin (Fig. 2 and 3). Within the granular capsule matrix, thin fibers could be observed that extended radially through the entire capsule and reached its periphery (Fig. 3, arrows). The diameter of the fibers measured 2 to 4 nm; occasionally they were up to 15 nm thick; they appeared to associate with neighboring fibers and also to dissociate from a thicker fiber (Fig. 3). Frequently, fibers seemed to form small knots 8 to

10 nm in diameter. In contrast to the behavior of antibody-stabilized cells, which agglutinated, neighboring capsules usually retained a gap between each other (Fig. 4).

When ultrathin sections were labeled with anti-capsule IgG and stained with gold-protein A, the capsular domains showed up very clearly (Fig. 5a and b). The nonspecific gold labeling of the background was relatively low. Where the stain aggregated, the distribution can be seen to follow linear tracks (Fig. 5b). These tracks coincide with the fibrillar structures previously mentioned (Fig. 3). Often, gold label seemed to extend a small distance into the gelatin beyond the contour of the capsular domain or into the cytoplasmic area (Fig. 5a). Studies of stereo views of tilted specimens suggest that some of these slightly extra-capsular gold accumulations may in fact be within the boundaries of the capsular domains. One of the reasons for this is that the immunostain interacted only with those areas of the section which were exposed at the surface of the section, whereas most of the contrast contribution of a section was derived from structures within the section. Thus, a mismatch may occur of structures on the section surface relative to the bulk of the structure within the section.

We tested the effect of another enrobement and dehydration system for the cells, namely agar in combination with ethanol dehydration. We found that (i) the capsule was not visibly preserved as a light halo and (ii) the arrangement of gold label was irregular and often in a few single tracks only. From these data, we conclude that agar embedding coupled
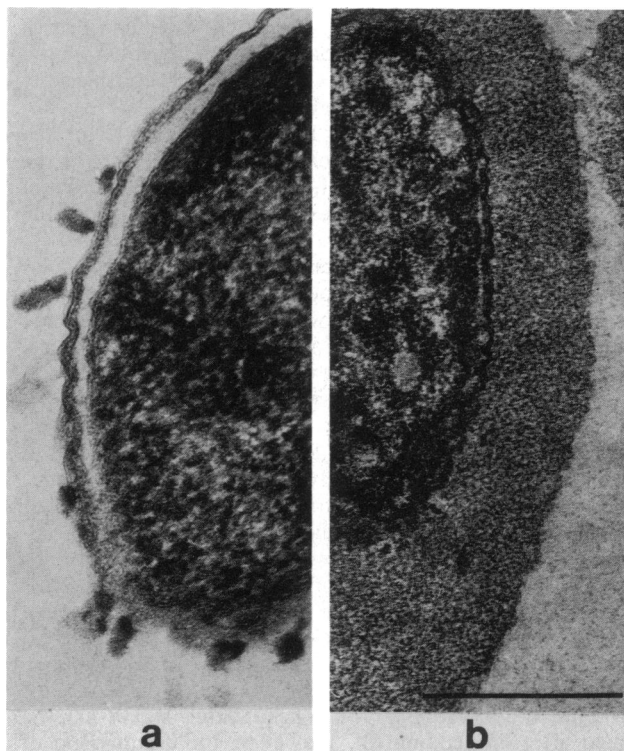

FIG. 1. *E. coli* K29. (a) Conventional aldehyde-osmium tetroxide fixation, alcohol dehydration, and Epon embedding (uranyl acetate stain of section). The capsule polysaccharide has collapsed to small clumps of high density. (b) In this preparation, the capsule was stabilized by exposure to anti-capsule IgG before aldehyde-osmium tetroxide fixation, dehydration, Epon embedding, and uranyl acetate staining. Bar represents 0.5 μm.
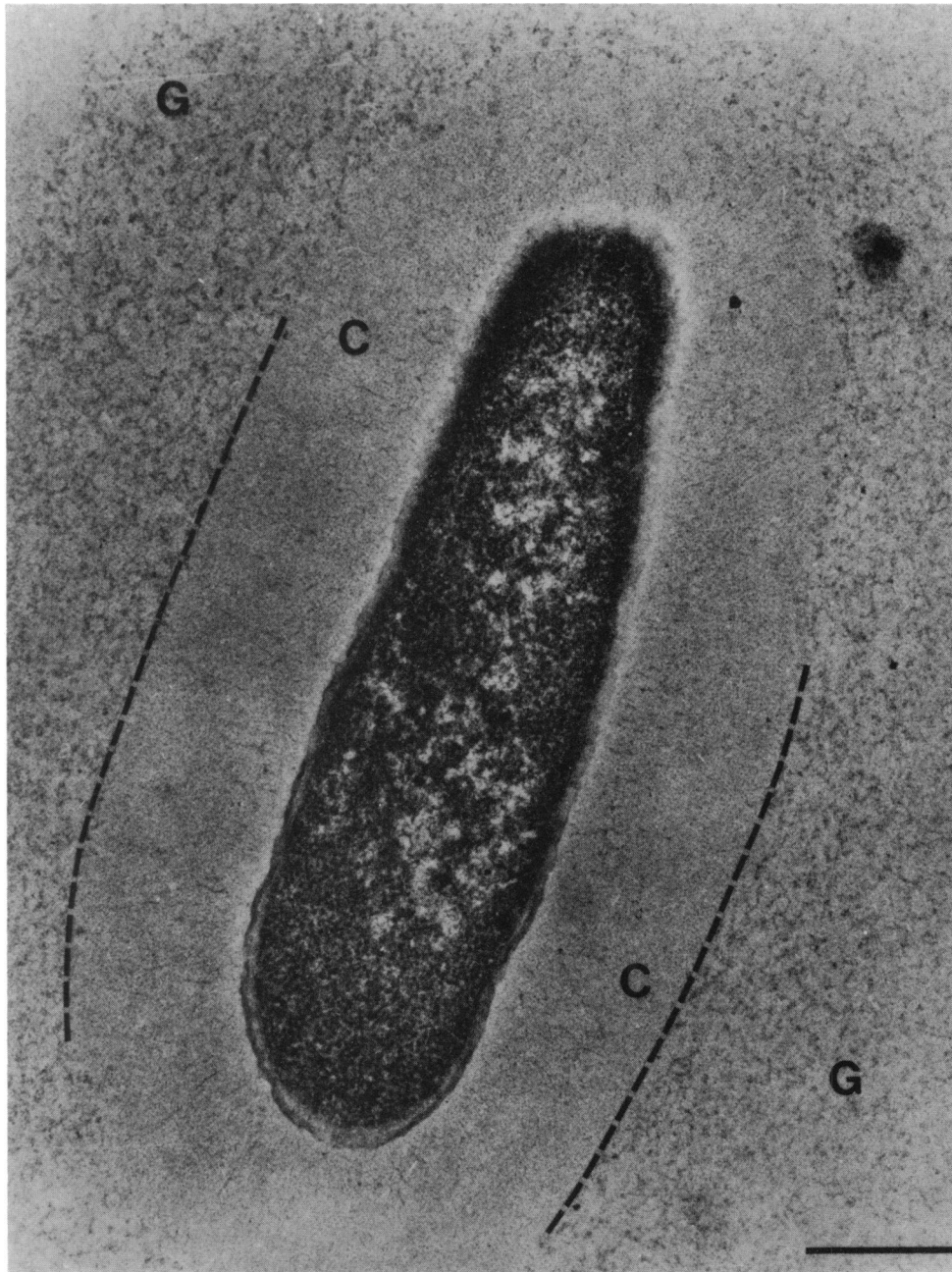
FIG. 2. *E. coli* K29 after glutaraldehyde fixation, gelatin enrobement, and K4M embedding at low temperature (uranyl acetate stain of section). Note the fine-granular capsule domain (C) surrounding the cell, forming a layer of 0.6 μm; the gelatine (G) is recognizable by its coarser grain. A portion of the C-G boarder is indicated by the dotted line. Bar represents 0.5 μm.

with ethanol dehydration is not feasible for preservation of the capsular domain.

The combination of our fixation and embedding procedure with gold-protein A labeling produced a resolution high-enough to study the distribution of the capsular antigen in cross-sectioned views of the cell (Fig. 5b): label was almost absent in the cytoplasm. The inner membrane was labeled only to a small extent, in small areas, whereas the outer membrane accumulated a relatively high concentration of label. The periplasmic space, visible in slightly plasmolized cells, did not contain label, with the exception of a few sites at which the inner membrane also showed localized accumulation of label.

Labeled extracellular domains could often be observed which apparently lacked a cell. In these cases, the section missed the cell body entirely (Fig. 5a, lower left). Occasionally, cell surface and embedding medium separated. This represents an artifact and can be reduced by placing the sections on a support film, and gradually and slowly exposing them to the electron beam. Whenever the separation of the embedding matrix involved larger portions of the cell surface, we had reasons to blame an incomplete penetration of the K4M for the artifact.

In conclusion, dehydration of gelatin-enrobed *E. coli* K29 in dimethyl formamide at low temperature plus embedding at minus 35°C in Lowicryl K4M permits (i) the observation of
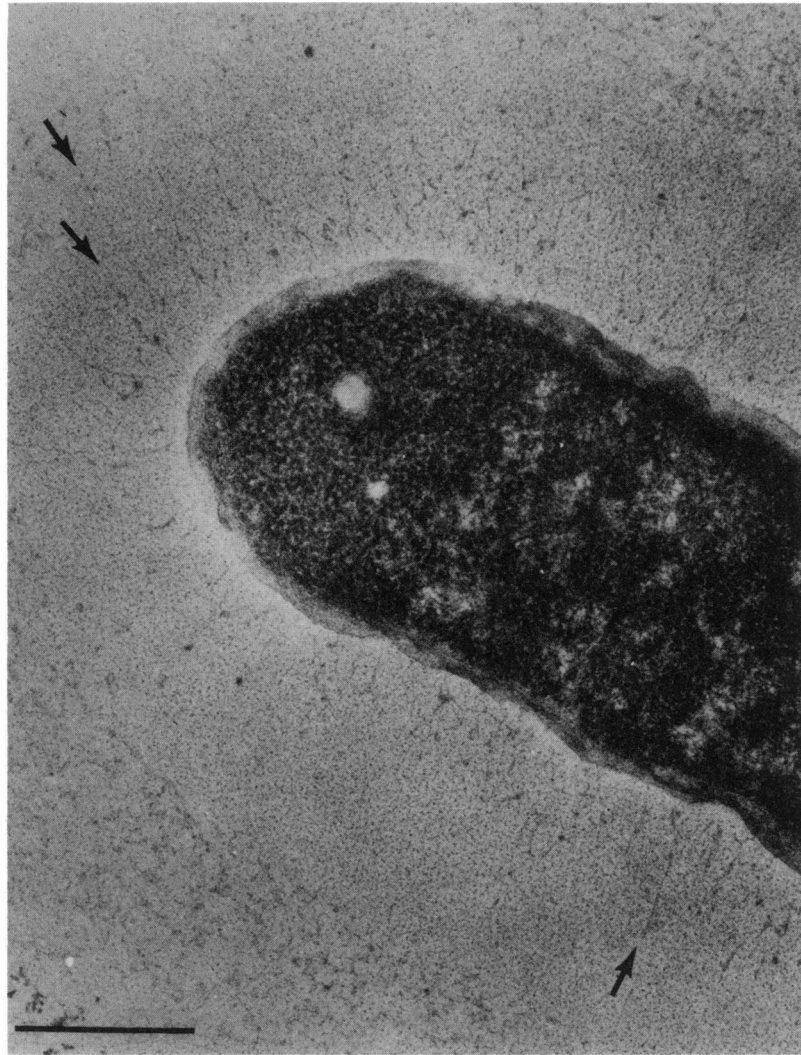
FIG. 3. *E. coli* K29, treated as described in the legend to Fig. 2. Note filaments (arrows) crossing the entire capsule. Bar represents 0.5 μm.

an uncollapsed polysaccharide capsule containing a filamentous substructure, (ii) the localization of the capsular domain with immunostaining, and (iii) the study of the distribution of the capsular antigen in the layers of the cell envelope.

DISCUSSION

Despite the great biological significance of bacterial capsules (5, 18), information on their ultrastructure remains relatively scarce. The reason for this is the fact that the visualization of capsular material has been hampered by a lack of adequate preparatory techniques. The stabilization of highly hydrated biological substances such as eucaryotic glycocalyces and procaryotic capsules poses a major problem to the electron microscopist. Stabilization of capsular material has been achieved by exposing the capsulated organism to specific antiserum which causes the capsule to form a gel-like product with the antibody. Eighty-three years ago (12), the increase in mass density of antibody-treated capsules was observed in the light microscope. Antibody treatment for purposes of contrast enhancement in capsules of other microorganisms has also been used early on as a tool for diagnostic purposes (15). Employment of this approach made it possible to visualize pneumococcal capsule

materials to some degree in the early years of electron microscopy (11). More recently, antibody treatment was employed to examine with high-resolution microscopy the size of *E. coli* capsules, their polysaccharide strands, and their interaction with capsule-specific phages (3). These studies were performed on ultrathin sections of plastic-embedded cells as well as on freeze-etched replicas of unfixed cells. The question as to the extent of volume changes of capsules during the reaction between capsular polysaccharide and IgG remained unanswered, and the notion of a capsular response in terms of "Quellungsreaktion" (12) or swelling reactions (11) would suggest that the size of an antibody-treated capsule has increased. Other methods to stabilize the capsule had also been used: capsular structures seem to be stabilized to some extent in the presence of ruthenium red (2, 5, 9). After such treatment, however, the thickness of the capsules seems to be considerably reduced relative to the thickness of (i) antibody-treated cells and (ii) cells treated by the technique described in our present paper.

Postembedding staining by the phosphotungstic acid method (13) and variants thereof (14) was reported to reveal capsular domains of up to 260 nm thickness in K4M-
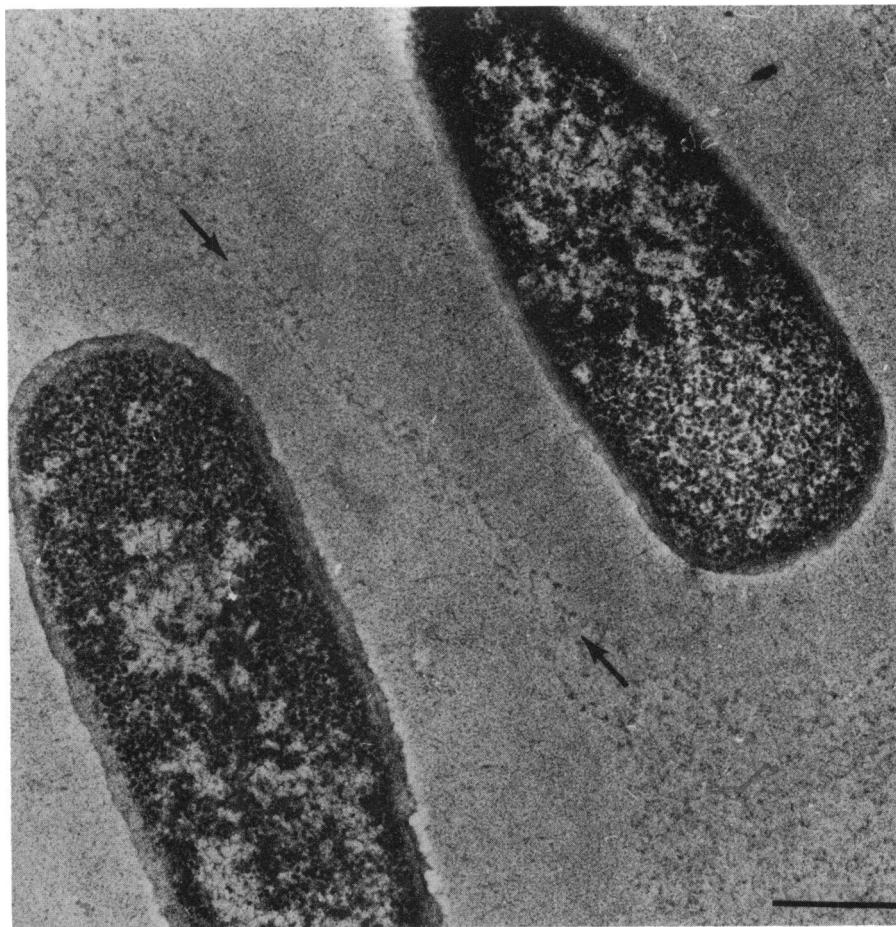
FIG. 4. Another view of *E. coli* K29, treated as described in the legend to Fig. 2. Neighboring capsules are kept apart by a gap (arrows) which is occupied by gelatin. Bar represents 0.5 μm.

embedded *E. coli* E68/K88 (8). However, the coat of these cells is composed not only of a capsular antigen but also of proteinaceous pili, and it is unfortunate that the contribution of the proteins to staining and to capsular stability had not been established. When we tested the phosphotungstic acid method (13) with *E. coli* K29 sections, we did not observe an increase in contrast of the capsular domain. The complexities in the staining of complex carbohydrates and proteins have been discussed in detail (7, 13). Cell surfaces composed of polysaccharide only, such as the capsule of *E. coli* K29, are probably much more sensitive to the manipulations during embedding than are the protein-polysaccharide-containing capsules of other strains.

In the ultrathin section of K4M-embedded *E. coli* K29, the thickness of the capsule (0.5 to 0.8 μm) appears to be increased over that of the antibody-stabilized capsules (0.3 to 0.4 μm [2]). One might suggest several reasons for the size differences. (i) Antibody might cross-link neighboring strands of the capsular polysaccharide in such a way that the strands would be tied to each other in a nonextended form, due to the reduced electrical charge of the polysaccharide chains after attachment of antibody. Thus, the capsule might somewhat shrink during the cross-linking by antibody. (ii) DMF might do the opposite, namely extend a normally relaxed capsule polysaccharide strand. This appears to be less likely, though, since the capsule has been enrobed in gelatin. (ii) Lowicryl K4M might expand after sectioning. While cell

organelles such as ribosomes seem to be well preserved after embedding in K4M, the embedding medium in the capsular domain might indeed be less stabilized due to the low concentration of reinforcing biological structures in that area.

The micrographs clearly demonstrate that the antigenicity is preserved over the entire area of the capsule. However, the staining is not totally random, since clusters of gold particles or linear arrangements are visible in the capsular domains. Closer inspection reveals that underlying fibrillar elements seem to be responsible for this localized distribution of label (Fig. 5b). Obviously, not all of the fibrillar structures visible in the capsular domain will be capable of binding the label. As pointed out before, the immune reaction can only take place at the surface of a section, and large portions of visible cell structures are unaccessible within the volume of the section.

The intensity of the label varies widely between the components of the cell envelope (Fig. 5a and b): the outer membrane exhibits the highest label density. In contrast, the periplasmic space does not show any significant label, except at those areas at which the inner membrane also shows clusters of gold particles. Otherwise, the inner membrane and the cytoplasm do not bind capsular label. These data suggest that the polysaccharide production sites of the inner membrane are clustered and export occurs from these clusters to the outer membrane.
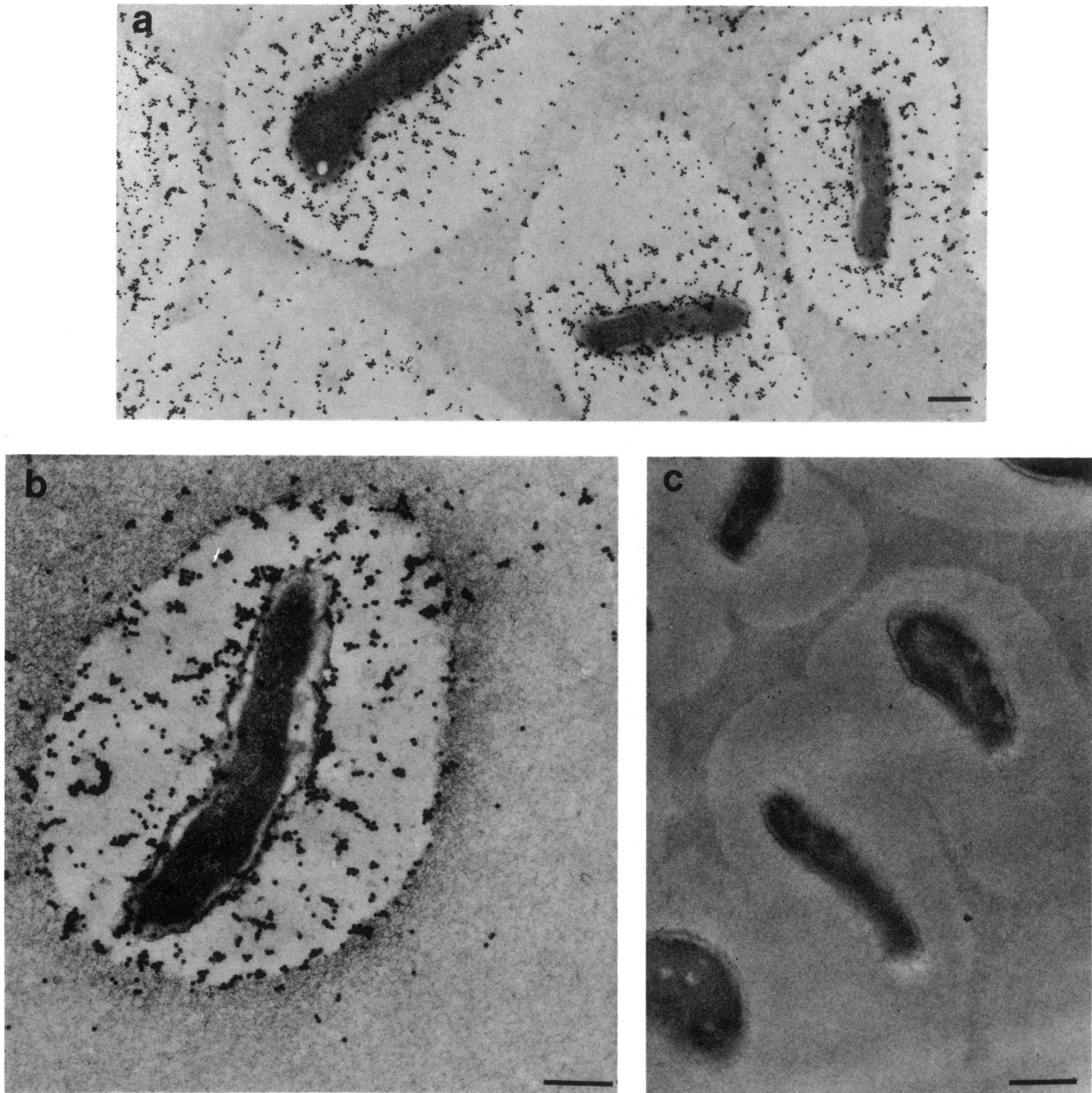
FIG. 5. (a) Section of K29 cells after exposure to anti-capsule IgG and treatment with gold-protein A. Note the large capsular domains. (b) Label distribution in capsular domain and at outer membrane of slightly plasmolized cell. Periplasmic space is sometimes bridged by label. (c) Control. Section was exposed to staining procedure described in the legend to Fig. 5a, with IgG treatment omitted. Bar represents 0.5 μm in all three micrographs.

In conclusion, the method described in this paper reveals a large polysaccharide capsule containing filamentous elements which retain antigenic sites. Immunoelectron microscopy allows one to study with sufficiently high resolution the distribution of the antigen within the elements of the cell envelope.

ACKNOWLEDGMENTS

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