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Further Electron Microscopic Studies on the Expression of *Escherichia coli* Group II Capsules

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The de novo expression of *Escherichia coli* K1, K5, and K12 capsules was analyzed with immunoelectron microscopy in temperature upshift experiments, with upshift from 18°C (capsule restrictive) to 37°C (capsule permissive). Newly produced capsular polysaccharides appeared at the cell surface atop membrane adhesion sites (Bayer's junctions). After plasmolysis of the bacteria at an early expression stage, the capsular polysaccharides were labeled at discrete sites in the periplasm by the immunogold technique. After temperature upshift in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or chloramphenicol, the polysaccharides were labeled in the cytoplasm.

The capsular polysaccharides (K antigens) of *Escherichia coli* are divided into two groups on the basis of genetic, biochemical, and general microbiological characteristics (10, 11). Most *E. coli* strains causing extraintestinal infections have group II capsules. They are not expressed at a growth temperature of 18°C (16), and their formation is directed by the same or closely related expression systems (9, 18, 20, 21, 26).

The first electron microscopic studies on the expression of *E. coli* capsules were reported by Bayer and Thurow (5, 6). They studied the K29 antigen, a representative group I capsular polysaccharide. By using a temperature-sensitive mutant which expressed the K29 capsule at 25°C but not at 46°C, those authors showed that de novo expression of the capsule starts at discrete sites on the cell surface which coincide with membrane adhesion sites (Bayer's junctions) (2, 5, 7) and at which the cytoplasmic and outer membranes come into close apposition. Recently, we analyzed the expression of the K5 antigen, a medically important group II capsular polysaccharide, in expression mutants (12), and we provided evidence for discrete events during the export of capsular polysaccharide, viz., the translocation through the cytoplasmic membrane and transport across the periplasmic space.

The fact that *E. coli* does not express group II capsules at low temperatures provides a handle for the analysis of the process of capsule expression in wild-type *E. coli*. Such studies have been performed by Whitfield et al. (27) with the K1 (capsular group II) polysaccharide. In temperature upshift experiments, the expression of the K1 capsule over time could be observed, although the topography of surface expression (in patches, like that of the K29 capsule, or equally distributed over the surface) could not be demonstrated with certainty. However, Whitfield, et al. found that de novo expression of the K1 capsule depended on protein synthesis and transmembrane potential (27).

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In this study, we present further analyses of the surface expression of group II capsules. Immunoelectron microscopic studies using specific anticapsular gold-labeled antibodies in temperature upshift experiments are described for the following group II capsules: the K1 antigen [8)-αNeuNAc-(2] (13, 15), the K5 antigen [4)-βGlcA-(1,4)-αGlcNAc-(1,] (24), and the K12 antigen [3)-α-LRha-(1,2)-α-LRha-(1,5)-βKDO-(,2] (19), with KDO being 2-keto-3-deoxy-D-*manno*-octonic acid (23).

Chloramphenicol, CCCP, and agar VII for electron microscopy were purchased from Sigma, Deisenhofen, Federal Republic of Germany (FRG); Epon 812, dodecyl succinic anhydride, methylphthalic anhydride, 2,4,6-tri(dimethylaminomethyl)phenol, osmium tetroxide, uranyl acetate, and lysozyme were from Serva, Heidelberg, FRG; media for the cultivation of bacteria were from E. Merck AG, Darmstadt, FRG; the components for Lowicryl K4M resin were from Chemische Werke Lowi, Waldkrainburg, FRG; TSK-3000 was from LKB, Gräfelfing, FRG; and gold (15 nm)-conjugated anti-mouse antibody was from Janssen, Kaldenkirchen, FRG.

E. coli 2932 (O1:K1), 20026 (O10:K5), and 20025 (O4:K12) were grown in Merck standard I liquid medium at 18 and 37°C. For plasmolysis of the bacteria, the method of Smit and Nikaido (22) was used.

The K5- and K12-specific monoclonal immunoglobulin M antibodies have been described elsewhere (1, 8, 17). They were obtained from the respective hybridoma cell supernatants as reported previously (12) and purified on a TSK-3000 column. Horse serum H46 with anti-K1 specificity was a generous gift from J. B. Robbins (National Institutes of Health, Bethesda, Md.). To purify the immunoglobulin M anti-K1 antibody, this serum was dialyzed at 4°C against 10 mM Tris hydrochloride buffer (pH 8.0), and the precipitated material was dissolved in water and subjected to high-pressure liquid chromatography on MonoS (Pharmacia, Freiburg, FRG.). The temperature upshift experiments were performed as follows. The bacteria were adapted to growth at 18°C in two subsequent precultures in

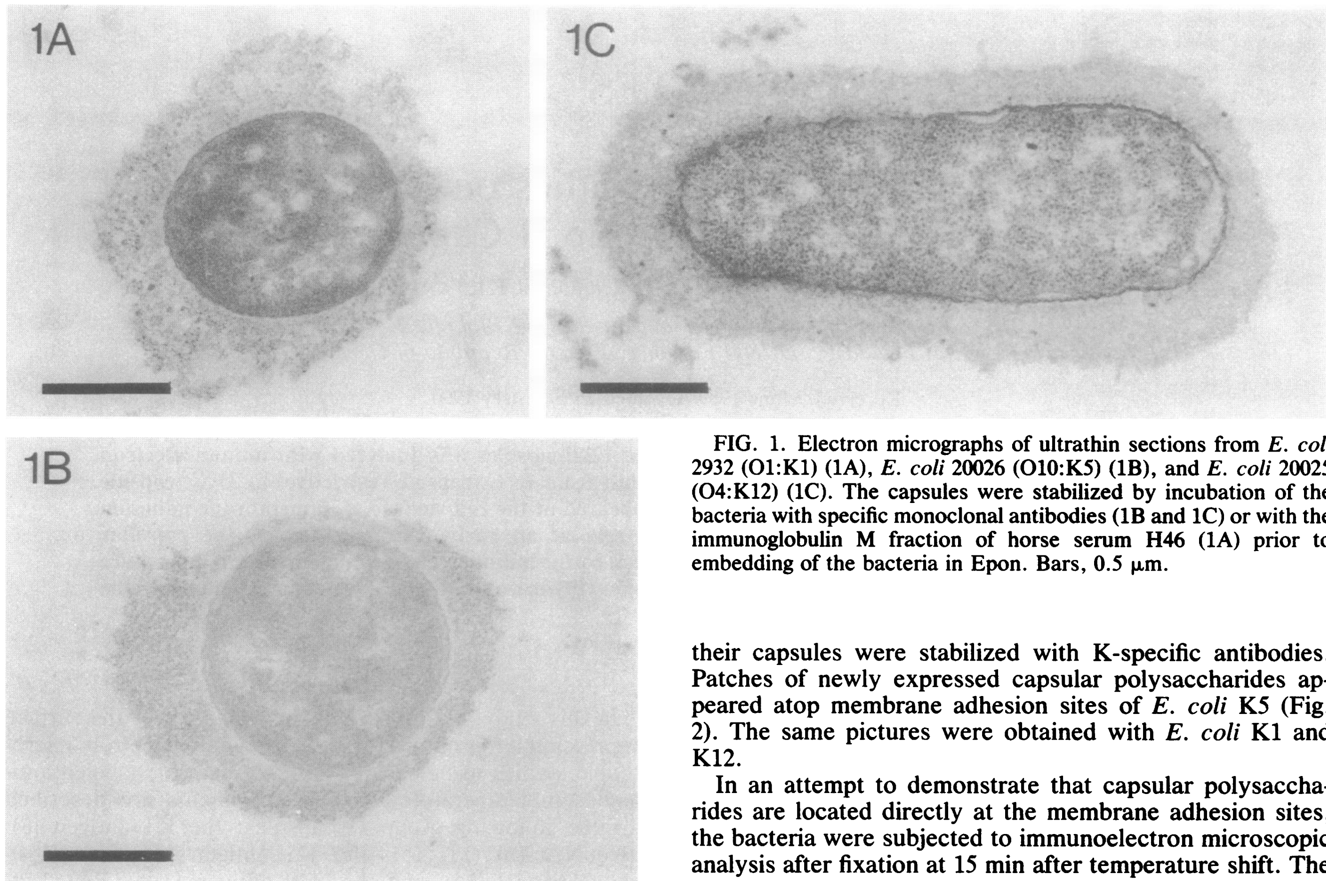


FIG. 1. Electron micrographs of ultrathin sections from *E. coli* 2932 (O1:K1) (1A), *E. coli* 20026 (O10:K5) (1B), and *E. coli* 20025 (O4:K12) (1C). The capsules were stabilized by incubation of the bacteria with specific monoclonal antibodies (1B and 1C) or with the immunoglobulin M fraction of horse serum H46 (1A) prior to embedding of the bacteria in Epon. Bars, 0.5 μm.

Merck standard I broth. A stationary preculture (incubated for about 16 h) was used for inoculation (1%) of 75 ml of the same medium and incubated at 18°C to a cell density of about 2×10^7 cells per ml (8 to 9 h). This suspension was centrifuged (16,000 × *g*, 4°C, 10 min), and the bacteria were suspended in 7 to 8 ml of the same medium, prewarmed to 37°C (cell density not more than 3×10^8 to 4×10^8 cells per ml). The suspensions were incubated at 37°C, and capsule expression was analyzed at various times after the temperature shift.

Sample preparation for electron microscopy, embedding in Epon or Lowicryl, specimen preparation, labeling with immunogold (postembedding procedure), and electron microscopy have been described in detail elsewhere (12).

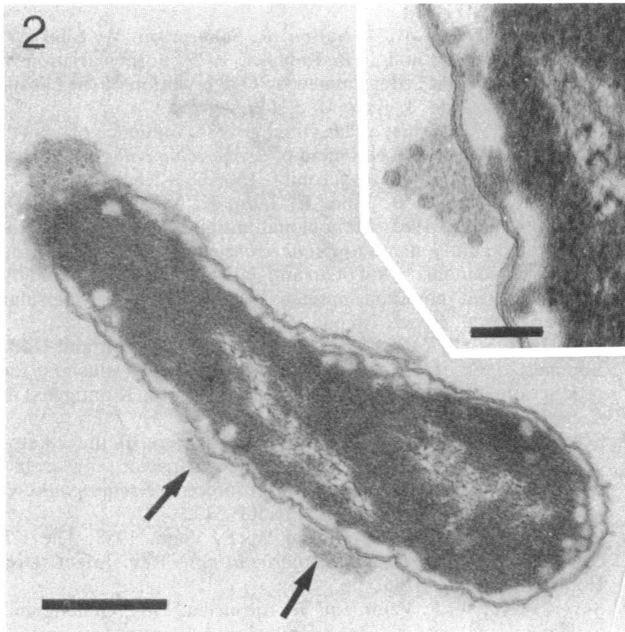
The appearance of the K1, K5, and K12 capsules in the early logarithmic phase at 37°C (1×10^6 to 2×10^6 cells per ml) is shown in Fig. 1. The capsules developed under these conditions have a diameter of about 300 to 400 nm. Samples taken at the mid-logarithmic phase (1×10^8 to 2×10^8 cells per ml) expressed much thinner capsules with diameters of about 50 to 100 nm. This observation, which is in agreement with a previous report (25), may be important for in vitro studies of defense mechanisms.

After temperature shift from 18°C (capsule restrictive) to 37°C (capsule permissive), K1-, K5-, and K12-specific capsules appeared on the bacterial surfaces in patches and were not equally distributed over the entire surface. To see whether the export sites corresponded to membrane adhesion sites, the bacteria were plasmolyzed at an early stage of expression (15 min after temperature shift), before their capsules were stabilized with K-specific antibodies. Patches of newly expressed capsular polysaccharides appeared atop membrane adhesion sites of *E. coli* K5 (Fig. 2). The same pictures were obtained with *E. coli* K1 and K12.

In an attempt to demonstrate that capsular polysaccharides are located directly at the membrane adhesion sites, the bacteria were subjected to immunoelectron microscopic analysis after fixation at 15 min after temperature shift. The K5-specific capsular polysaccharides were labeled with gold particles at sites which span the periplasmic space and which may be adhesion sites (Fig. 3). The weak contrast of membranes in Lowicryl-embedded samples does not allow a more definite statement about membrane adhesion sites. The same observations were made with the capsular K12 polysaccharides (not shown). The capsular K1 polysaccharides could not be labeled sufficiently with the polyclonal antibodies.

These results are in accord with previous reports on the role of membrane adhesion sites in the export of macromolecules (3–5, 14), and they underscore the implication of these specialized surface areas in capsule expression (5, 6). Whitfield et al. have reported that inhibition of protein synthesis with chloramphenicol and reduction of membrane energy with CCCP inhibit the de novo expression of the K1 capsule (27). For an electron microscopic analysis of these inhibitory actions, we performed the temperature upshift experiments in the presence of chloramphenicol and in the presence of CCCP. After temperature shift, the bacteria were kept at 37°C for 1 h, which is sufficient for complete capsule expression in the absence of inhibitors. The bacteria were then fixed, embedded in Lowicryl, and subjected to electron microscopy by the immunogold technique (12).

When CCCP (50 μM) was added prior to the temperature shift, practically no gold label could be seen in the bacterial preparation during subsequent immunoelectron microscopy. Addition of CCCP (50 μM) 15 min after the temperature shift resulted in distinct cytoplasmic labeling of the capsular polysaccharides (Fig. 4). These results indicate that for de novo capsule expression, membrane energy may be required at two stages: (i) in an early reaction of the polysaccharide



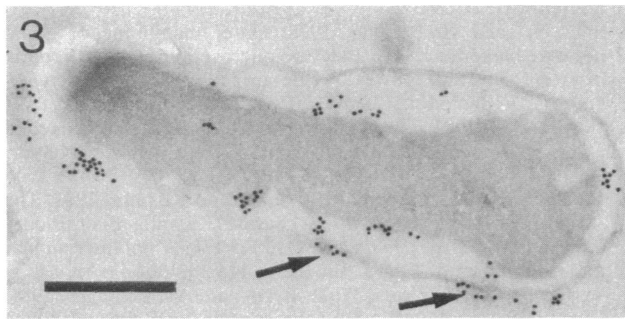


FIG. 2. Electron micrograph of *E. coli* 20026 (O10:K5) that was plasmolyzed in 20% sucrose 15 min after temperature upshift from 18 to 37°C. Extracellular capsular polysaccharides located over membrane adhesion sites are indicated (→). Bar, 0.5 μm. Insert: Magnification of one of the export sites. Bar, 100 nm.

FIG. 3. Electron micrograph of an immunogold-labeled ultrathin section from *E. coli* 20026 (O10:K5) plasmolyzed in 20% sucrose 15 min after temperature shift from 18 to 37°C. Immunogold-labeled extracellular capsular polysaccharides and capsular polysaccharides in membrane adhesion sites are marked (→). Bar, 0.5 μm.

biosynthesis and (ii) in subsequent translocation of the finished polysaccharide across the cytoplasmic membrane. This interpretation implies that polymerization occurs at the inner face of the cytoplasmic membrane.

When present directly after temperature shift, chloramphenicol inhibits the de novo expression of group II capsules only (27; K.-D. Kröncke and K. Jann, unpublished data). We therefore added chloramphenicol (150 μM) prior to the temperature upshift. Immunogold electron microscopy of the bacteria after 1 h at 37°C showed that the capsular polysaccharides were labeled in the cytoplasm (Fig. 4). Thus, under these conditions chloramphenicol seemed to affect the translocation of the polysaccharides across the cytoplasmic membrane. Our interpretation of this finding is that translocation requires the action of a protein that is not synthesized at 18°C, and whose synthesis at 37°C is an early reaction, and that is necessary for the de novo expression of group II capsules.

Our morphological observations of the expression of *E. coli* group II capsules provide a basis for further study of these important *E. coli* virulence determinants.

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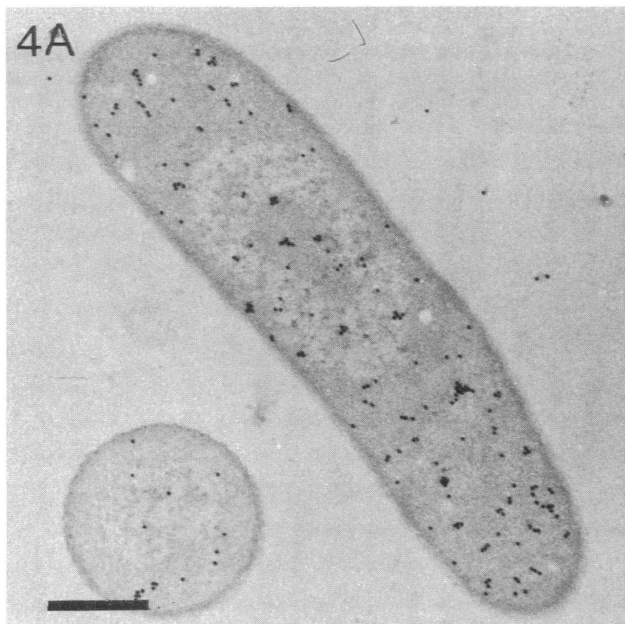

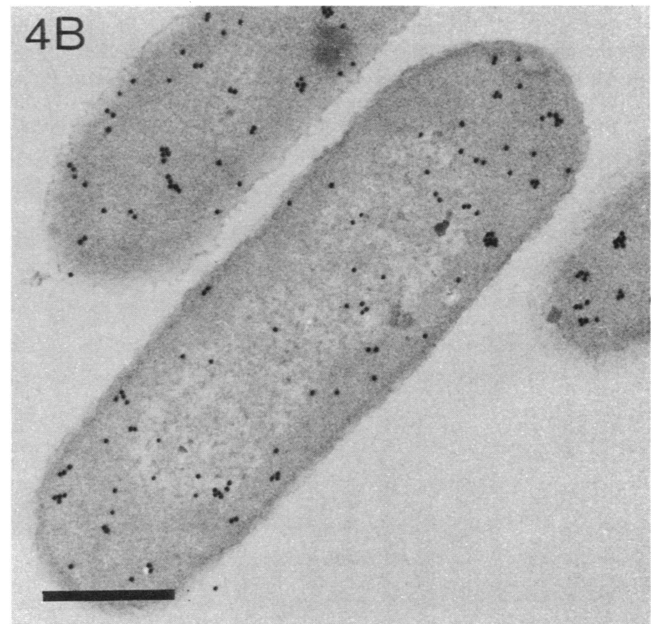

FIG. 4. Electron micrograph of an immunogold-labeled ultrathin section from *E. coli* 20025 15 min after temperature shift from 18 to 37°C, in the presence of 150 μM chloramphenicol (4A) or 50 μM CCCP (4B). Bars, 0.5 μm.

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