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Specific Localization of the Lysis Protein of Bacteriophage MS2 in Membrane Adhesion Sites of *Escherichia coli*

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Specific localization of the lysis (L) protein of bacteriophage MS2 in the cell wall of *Escherichia coli* was determined by immunoelectron microscopy. After induction of the cloned lysis gene, the cells were plasmolyzed, fixed, and embedded in either Epon or Lowicryl K4M. Polyclonal L-protein-specific antiserum was purified by preabsorption to membranes from cells harboring a control plasmid. Protein A-gold was used to label the protein-antibody complexes. Between 42.8% (Lowicryl) and 33.8% (Epon) of the label was found in inner and outer membranes, but 30.3% (Lowicryl) and 32.8% (Epon) was present mostly in clusters in the adhesion sites visible after plasmolysis. The remaining label (26.9 and 33.4%, respectively) appeared to be present in the periplasmic space but may also have been part of membrane junctions not visible because of poor contrast of the specimen. In contrast, a quite different distribution of the L protein was found in cells grown under conditions of penicillin tolerance, i.e., at pH 5, a condition that had previously been shown to protect cells from L-protein-induced lysis. At tolerant conditions, only 21.0% of the L protein was in the adhesion sites; most of the protein (68.2%) was found in inner and outer membranes. It is concluded that lysis of the host, *E. coli*, was a result of the formation of specific L-protein-mediated membrane adhesion sites.

The RNA bacteriophage MS2 codes for a low-molecular-weight hydrophobic protein that is sufficient to trigger lysis of the host, *Escherichia coli* (6, 15). This lysis (L) protein has no enzymatic activity by itself (13). Similar proteins have been described for other small phages, including ΦX174 and G4 (7, 10, 21), but their modes of action remain unclear. It has been proposed that the L protein of phage MS2, which was shown to dissipate the proton motive force in *E. coli* membrane vesicles, may trigger murein hydrolases by reducing the electrochemical gradient across the membrane (8). However, in vivo evidence is still lacking.

Recent results on the mode of action of the MS2 L protein suggested that the phage protein unleashes endogenous murein hydrolases of the host by a specific interaction with the inner and outer membrane system (12, 13, 20). Mutants impaired in the synthesis of the major osmoregulatory compound of the periplasmic space, the membrane-derived oligosaccharides, have been found to be less sensitive to L-protein-induced lysis by preventing a stable insertion of the hydrophobic phage protein into the membranes (12). In particular, it has been shown that the number of sites of membrane adhesion between inner and outer membranes increases by approximately 50 per cell when the cloned lysis gene (15) is induced (20).

Sites at which cytoplasmic and outer membranes adhere to each other are numerous in *E. coli* (about 400 per cell) and can be demonstrated clearly after gentle plasmolysis of the cells (1). These membrane adhesion sites are considered to be involved in a number of uptake and export processes of quite different compounds (2). Many phages have been shown to bind to receptors in the outer membrane specifically arranged at membrane junctions (2). Adhesion sites are thought to be involved in the uptake of the nucleic acid into the cytoplasm of the bacterium (2). On the other hand, adhesion sites have also been demonstrated to take part in the assembly and release of phages (3, 16).

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By using the immuno-gold labeling technique, we provide evidence that the MS2 L protein itself is a structural part of the unique adhesion sites that are formed by specific insertion of the phage protein into the bacterial cell wall.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* M5219 carrying a defective lambda prophage coding for the temperature-sensitive *c*I repressor (18) was used as a host for plasmids. The plasmids used were all pPLa831 derived and kindly given to us by Jan van Duin (University of Leiden, Leiden, The Netherlands). Plasmid pMS16 carries the *Eco*RI-*Bam*HI segment 869 to 2057 of MS2, coding for the coat and lysis genes downstream of the lambda p_L promoter (15). Plasmid pMS20 is identical to pMS16 except for a small deletion of 58 base pairs (1764 to 1822) in the lysis gene which results in premature out-of-phase termination. Bacteria were grown in Luria broth in the presence of 50 μg of kanamycin per ml at 30°C in a water bath shaker. For induction of the L protein, cultures in the logarithmic growth phase (10^8 cells per ml) were quickly transferred to a 42°C water bath shaker. Under these conditions, lysis commenced at about 35 min after the temperature shift.

Preabsorption of anti-L-protein serum. Antiserum raised against a synthetic L-protein fragment consisting of the 25 amino acids at the C terminus of the L protein (75 amino acids) was kindly provided by Jan van Duin. For purification, the serum was preabsorbed to envelopes of *E. coli* M5219 induced for the control plasmid pMS20. Cells suspended in 20 mM sodium phosphate buffer (pH 7.2) containing 0.13 M sodium chloride (phosphate-buffered saline [PBS]) were broken mechanically by sonication (three bursts of 15 s each at 200 W). The broken cells were sedimented at 100,000 × *g* for 2 h at 4°C and resuspended in PBS, and the same volume of fixative (4% paraformaldehyde, 0.2% glutardialdehyde in PBS) was added. The envelopes were centrifuged as described above and suspended in PBS containing 1% bovine serum albumin. For preabsorption of the serum,

the membranes were divided into four equal portions (10 mg of protein per ml) and incubated sequentially with 1 ml of serum for 1 h at room temperature. The envelopes were removed by centrifugation (see above) between each step. The purified serum was kept frozen at −20°C in appropriate portions; it was tested on a Western blot (immunoblot) of membrane proteins of induced cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein transfer on nitrocellulose were done as described previously (12).

Sample preparation for immunolabeling. Cells induced for 30 min at 42°C were plasmolyzed with an equal volume of 60% sucrose in Luria broth for 10 min at room temperature, fixed for 1 h at room temperature with 2% paraformaldehyde–0.1% glutardialdehyde (final concentration), and washed three times with PBS. Two different methods were used to prepare ultrathin sections for immunolabeling.

(i) Cryosubstitution and embedding in Lowicryl K4M. Rapid freezing of the samples was done by using a homemade plunging device (generously supplied by Heinz Schwarz). The samples on grids were shot (with compressed air) at a speed of 4 m/s into fluid propane. The grids were transferred into fluid nitrogen, dehydrated at −90°C with methanol in three steps of 8 h each, and embedded at −35°C in Lowicryl K4M, which was polymerized under UV light for 72 h.

(ii) Epon embedding. After dehydration of the samples with ethanol, the method described by Luft (17) was followed.

Specific immunolabeling of ultrathin sections. Immunolabeling of ultrathin sections prepared with an LKB ultramicrotome was done as described by Tommassen et al. (19). Nonspecific binding sites were blocked by incubating the ultrathin sections with 50 mM glycine in PBS for 15 min and by two 10-min incubations in 0.2% gelatin–0.5% BSA in PBS (PBG). The thin sections were incubated for 2 h with preabsorbed serum diluted 1:10 or 1:2 in PBS. After six washing steps with PBG, the sections were incubated with protein A-gold (8 nm in diameter) for 60 min. The samples were washed six times with PBG, two times with PBS, and four times with distilled water before being stained for 2 min with 1% aqueous uranyl acetate solution to enhance contrast. Finally, the samples were rinsed with distilled water and examined with a Siemens 1A or Siemens 102 electron microscope at 80 kV.

RESULTS

Specificity of preabsorbed anti-L-protein serum. Serum raised in rabbits against a synthetic lysis peptide fragment (8) could be increased in specificity by preabsorption to membranes of *E. coli* M5219 harboring plasmid pMS20, which carries a deletion in the L protein that inactivates the protein (15). All bands that were visible in addition to the L protein in immunoblots made with crude antiserum were successfully removed by the preabsorption step (Fig. 1). With use of this purified antiserum, very little background label (about one grain per 20 thin sections) was observed with cells not induced for the L protein. Although the yield of labeling (Fig. 2 and 3) was relatively low, it was highly specific.

Immunoelectron microscopy of *E. coli* cells induced for the L protein and grown under lysis-permissive conditions. The best way of embedding and dehydrating biological material is still a matter of controversy. In particular, the demonstration of membrane adhesion sites has been criticized as representing preparative artifacts not seen when appropriate techniques are used (11). However, we could clearly demonstrate such sites of adherence of inner and outer membranes with ultrathin sections prepared by two different techniques, low-temperature Lowicryl embedding and cryosubstitution (Fig. 2) and Epon embedding (Fig. 3). Trials to increase contrast by treating the samples with lead citrate unfortunately resulted in obscuring of the visibility of gold grains and was therefore considered inappropriate. The total number of adhesion sites after plasmolysis of the cells was found to be comparable in the two preparation methods, i.e., between 5 and 10 per thin section (Fig. 2 and 3). The distribution of gold label among the substructures of the cell wall was basically the same for the two procedures (Table 1). The Lowicryl technique showed 42.8% of the specific L-protein label to be present in inner and outer membranes, as compared with 33.8% in preparations embedded in Epon. The ratio of label present in inner and outer membranes differed in the two preparations, with a predominance of label in the outer membrane in the Epon-treated samples. The adhesion sites in the two preparations were labeled to similar degrees, 30.3% in the Lowicryl samples and 32.8% in the Epon samples. This high density of label in the junctions (label per volume) led to a clustering of the gold particles in these structures (Fig. 2 and 3).

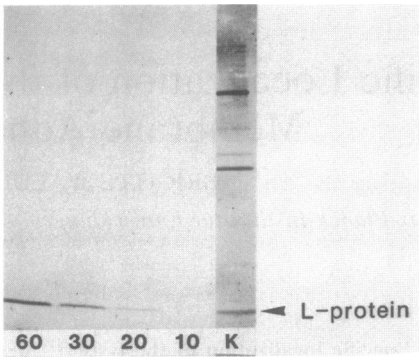

FIG. 1. Specificity of preabsorbed anti-L-protein serum. Membrane proteins of *E. coli* M5219 harboring the L-protein-coding plasmid pMS16 were separated by sodium dodecyl sulfate-gradient polyacrylamide (10 to 18%) gel electrophoresis, and the L protein was visualized by the Western blot technique, using either crude (lane K) or preabsorbed (lanes 10 to 60) antiserum. Cells were induced for the indicated times (10, 20, 30, or 60 min); cells in lane K were induced for 30 min.

Immunoelectron microscopy of *E. coli* cells induced for the L protein and grown under conditions of penicillin tolerance. The distribution of the L protein in the various membrane structures was also determined for cells grown under lysis-restrictive conditions. The immunolocalization of the L protein in *E. coli* M5219(pMS16) grown at pH 5, which prevents lysis (13), revealed a much lower percentage of label in the membrane adhesion sites (36% less than at pH 7). The amount apparently localized in the periplasm was reduced by 68% (Table 1).

DISCUSSION

Previous results have demonstrated an increase by 17% in the overall number of membrane adhesions after expression of the cloned L protein (20). The results presented here show the L protein to be part of the adhesion sites. Either the hydrophobic phage protein stabilizes preexisting adhesion sites by its insertion into the structure, resulting in an overall increase in the number of detectable adhesion sites after

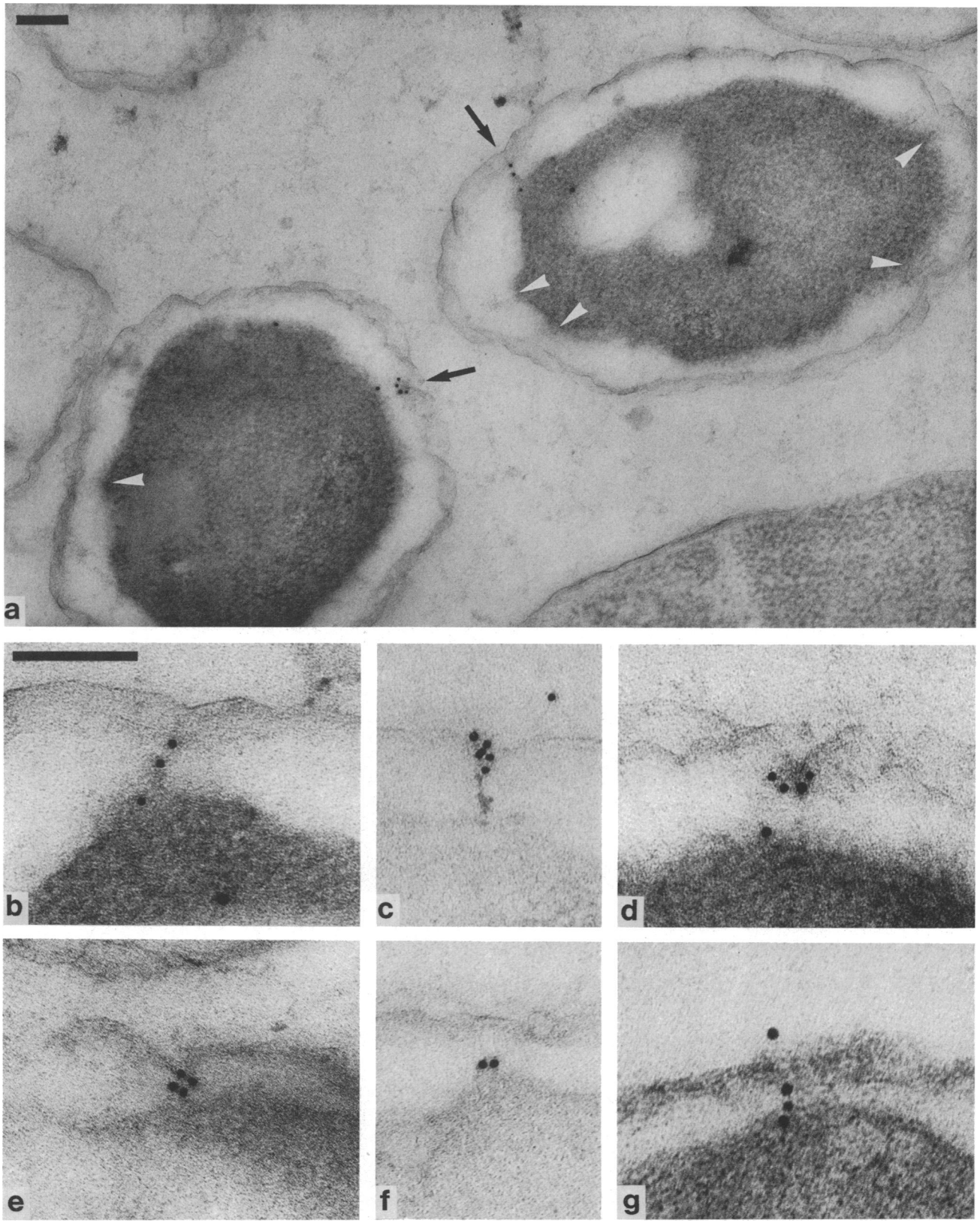

FIG. 2. Localization of the MS2 L protein in ultrathin sections of *E. coli* embedded in Lowicryl. *E. coli* M5219(pMS16) induced for the L protein was embedded in Lowicryl K4M as described in Materials and Methods. Sections were incubated with preabsorbed anti-L-protein serum and protein A-gold (8 nm) to label the L protein. Labeled (arrows) and unlabeled (arrowheads) adhesion sites are indicated. Bars, 0.1 μm.

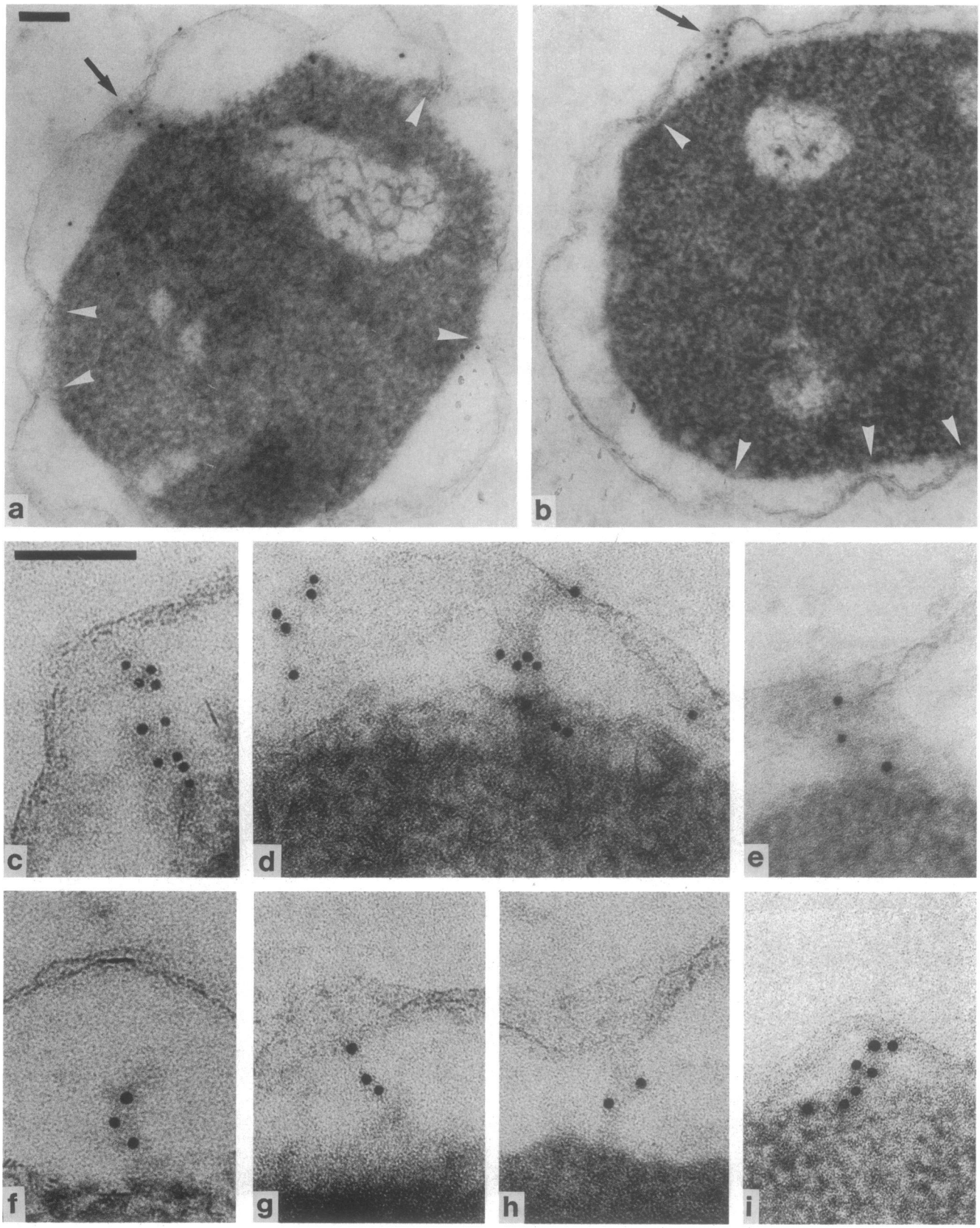

FIG. 3. Localization of the MS2 L protein in ultrathin sections of *E. coli* embedded in Epon. Specimens were prepared as for Fig. 2 except that embedding was in Epon as described in Materials and Methods. Bars, 0.1 μm.

TABLE 1. Distribution of L-protein-specific immunolabel

pH for growth	Embedding material	No. of cross sections counted	Total no. of gold particles counted	Proportional distribution of gold particles (%)[a]			
				IM	OM	P	AS
7	Lowicryl	440	1,027	29.8	13.0	26.9	30.3
	Epon	282	889	12.3	21.5	33.4	32.8
5	Epon	373	787	24.1	44.1	10.8	21.0

[a] IM, Inner membrane; OM, outer membrane; P, periplasmic space; AS, adhesion site.

plasmolysis of the cells, or the L protein itself promotes the formation of L-protein-specific adhesion sites that differ from other membrane adhesions. The latter assumption would explain our finding that not all adhesions could be immunolabeled.

Thioredoxin, another low-molecular-weight protein involved in the release of phages (the filamentous phages fd and f1 [3, 16]), was found likewise to reside in membrane adhesion sites (4). However, a chemical cross-linking step was essential to ensure adequate recovery of the protein in thin sections, since thioredoxin, in contrast to the MS2 L protein, shows only weak membrane association. In addition to thioredoxin, the gene *I* protein of phage f1 may also be involved in the formation of adhesion sites for phage assembly (16). Thioredoxin and the gene *I* protein seem to interact directly with each other (4). Recent results by Horabin and Webster (14) indicated that, similar to the MS2 L protein, the f1 protein also may be inserted in the membrane in adhesion sites. The actual presence of the f1 phage protein in adhesion sites has not yet been demonstrated by immunoelectron microscopy.

For evaluation of the relative distribution of the L protein among the various membrane structures (cytoplasmic membrane, outer membrane, and sites of membrane adhesion), the volume of these different compartments available for insertion of the phage protein must be taken into consideration. The proportion of membrane adhesion sites as compared with the inner and outer membrane systems can be estimated from the thin sections presented. On the basis of direct measurements of the width and length (circumference) of the different membrane structures, the total area of adhesion sites (about five per thin section) can be calculated to be roughly 5% of the area of the two membrane layers together. Thus, a value of 33% gold label as found for the adhesion sites indicates a high specificity for insertion of the L protein into membrane junctions. If indeed, as discussed above, insertion of the L protein into the envelope induces the formation of new L-protein-specific adhesion sites, these sites would represent a subpopulation among the adhesion sites. The calculated value for the label density, that is, the number of gold particles per unit area available, would then increase considerably.

In addition, a major portion of the label apparently located in the periplasm might well be part of adhesion sites not visible because of the poor contrast of the specimens. The L protein has never been found in soluble cell fractions (5). As a highly hydrophobic protein, it is expected to be present exclusively in the membranes of the cell. Therefore, we assume that the label found in the periplasm originates from membrane structures that either have been damaged during preparation or are not visible, rather than indicating an actual localization of the L protein in the periplasmic space. Consistent with this interpretation, we found a rather low label density in adhesion sites as well as in the periplasmic space after L-protein induction in cells grown under lysis-restrictive conditions. It was previously shown that growth of *E. coli* at pH 5, which is known to result in penicillin tolerance (9), also protected the cells from MS2 L-protein-induced lysis (13). Whereas the protein was inserted into the envelope in normal amounts, the total number of adhesion sites was found not to increase after induction of the L protein in cells grown at pH 5 (20).

Although it has been shown that murein degradation is the earliest defect that can be observed (20), it is not clear by what kind of mechanism murein hydrolases become unleashed from the cellular control circuits. Since we have shown that failure of the L protein to induce lysis of *E. coli* always correlates with the absence of the formation of L-protein-specific membrane adhesions (20), it is tempting to speculate that these structures trigger lysis.

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