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Ultrastructural Localization of the Maltose-Binding Protein Within the Cell Envelope of *Escherichia coli*

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Abstract. Logarithmically growing cells of *Escherichia coli* were fixed with glutaraldehyde and incubated with anti-maltose-binding protein F_{ab} coupled to horseradish peroxidase (molecular weight of the complex 80,000). The position of this complex within the cell envelope was determined by reacting with diaminobenzidine-H_2O_2, staining with osmium tetroxide and processing for thin section electron microscopy. The following observations were made: (i) induction of the maltose-binding protein resulted in swelling and staining of the outer membrane; (ii) the swelling and staining was more prominent in short cells, less prominent or absent in long cells; (iii) rare examples exhibited granular staining in the space between the plasma membrane and the peptidoglycan layer. These stainings were observable mainly in pole caps; (iv) a mutant lacking the receptor for phage λ showed altered staining pattern. Treatment of glutaraldehyde-fixed cells with EDTA-lysozyme prevented the specific labelling of the maltose-binding protein.

Key words: Periplasmic binding proteins – Cell envelope – Outer membrane – Horseradish peroxidase staining

The maltose-binding protein (MBP) of *E. coli* (Kellermann and Szmelcman 1974) is an essential component of the maltose transport machinery (Wiesmeyer and Cohn 1960) that comprises at least five different proteins (Raibaud et al. 1979; Silhavy et al. 1979b). It establishes the recognition site of the transport system, it functions as chemoreceptor of maltose chemotaxis (Hazelbauer 1975) and it is located within the cell envelope. Evidence for its periplasmic location, outside the cytoplasmic membrane are its shock releasability (Kellermann and Szmelcman 1974) as well as the observation that its biosynthesis occurs through the plasma membrane (Silhavy et al. 1979a). A second well known component of the maltose transport system is the λ receptor located transmembranally in the outer membrane (Randall-Hazelbauer and Schwartz 1973). It is linked non covalently to the peptidoglycan layer (Endermann et al. 1978), has a subunit molecular weight of 47–50,000 and is composed in vivo of three identical subunits (Palva and Westermann 1979). Despite the observation that the λ receptor can function as a nonspecific diffusion pore for small hydrophilic molecules as demonstrated by in vitro systems using liposomes (Nakae 1979) or black lipid films (Boehler-Kohler et al. 1979) the λ receptor appears to show preferences for maltodextrins (Lukey and Nikaido 1980) and is infact a maltodextrin-binding protein (T. Ferenci et al. 1980). There are two lines of evidence indicating that this preference might be due to an interaction of the λ receptor with the maltose-binding protein. The first is the observation by Heuzenroder and Reeves (1980) that λ receptor can act in vivo as an unspecific pore for molecules other than dextrins preferentially in the absence of MBP. The second comes from the analysis of mutants defective in MBP that are phenotypically Mal^+ but $Maltodextrin^-$. Despite the inability to grow on dextrins the MBP isolated from these mutants still exhibits a rather high affinity for dextrins. In order to explain this paradoxical phenotype it has been concluded that the inability of these mutants to grow on maltodextrins was due to a disturbed interaction of MBP with λ receptor, caused by the structural alteration in MBP (Wandersman et al. 1979). These considerations prompted an attempt, described in this paper, to visualize the MBP within the cell envelope by electronmicroscopic techniques. In addition, MBP had recently been found to be synthesized only at certain times during the cell cycle, leading to the formation of pole caps (Dietzel et al. 1978). It was therefore of interest to search for specialized sites of MBP synthesis or secretion along the cell surface.

The method that we used to visualize the position of the MBP was to penetrate glutaraldehyde fixed bacteria with affinity purified F_{ab} against MBP that were coupled to horseradish peroxidase (αMBP-F_{ab})-HRPO, a molecule of about 80,000, and to stain for enzymatic activity.

Material and Methods

Bacterial Cells and Growth Conditions

Escherichia coli strain MC4100 (Silhavy et al. 1976) is wild-type in respect to the maltose transport system and contains the following genetic markers: F^- *araD rpsL lacU*169 *thi*. Strain HS2013 obtained from H. Shuman is a derivative of MC4100 and carries a deletion of the entire *malB* region. Strain TSL20 obtained from T.J. Silhavy also is a derivative of MC4100 and carries a Tn5 insertion in the *lamB* gene coding for the λ receptor. The strains were grown in minimal medium A (Miller 1972) with 0.2% maltose or glucose as carbon source supplemented with the appropriate requirements. Routinely, the cells were diluted 1:20 from an overnight culture and grown for 3–4 h prior to harvesting and treatment with glutaraldehyde.

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Lists of Non Common Abbreviations: MBP, maltose-binding protein; (αMBP-F_{ab})-HRPO, F_{ab} fragments against maltose-binding; protein coupled to horseradish peroxidase; IgG, immunoglobulin; PBS, phosphate buffered saline

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Maltose-binding protein Sepharose column was prepared according to Cuatracasas (1970). Five milliliter preswollen Sepharose 2B300 was washed twice with 1 l destilled water and suspended in 12 ml water. 0.5 g cyanogen bromide was added and pH 11 was maintained by addition of 2 M NaOH. The suspension was washed extensively with 0.1 M bicarbonate. To 3 ml of slurry 2 ml of purified MBP (1 mg/ml, dialyzed extensively against water) was added and gently agitated at 4° C overnight. Five milliliter of 0.5 M lysine in 0.1 M bicarbonate, pH 9.0 was added for 8 h. Subsequently, the material was transferred onto a small column and washed with phosphate (0.01 M) buffered saline, pH 7.2 (PBS). When not in use the column was stored at 4° C in PBS containing 1 mM sodium azide. The column contained about 3 ml Sepharose and 2 mg MBP.

Isolation of F_{ab} Fragments Specific for Maltose-Binding Protein and Coupled to Horseradish Peroxidase

A rabbit was immunized with 0.2 mg maltose-binding protein (Ferenci and Klotz 1978) in 0.1 M Tris-HCl, pH 7.0 in a 1:1 mixture with complete Freund adjuvant. The immunization was repeated after 5 weeks and bleeding was begun after the 8th week. After that time the serum contained precipitating antibodies against MBP as judged by Ouchterlouny immunodiffusion.

The immunoglobulin (IgG) fraction of 20 ml serum was twice precipitated with 1.6 g/ml Na_2SO_4 while the precipitate was dissolved in 10 ml PBS and dialyzed against the same buffer. To free this preparation from IgG's that carry specificity towards any bacterial surface antigens other than MBP the IgG fraction was incubated for 3 h with glutaraldehyde-treated and washed bacteria of strain HS2013 that lack the maltose transport machinery. The final density of bacteria was about 10^{10}/ml; they were removed by centrifugation. The solution was passed through the MBP-Sepharose column. The breakthrough fraction was saved as control serum not containing anti-MBP antibodies. The column was washed with 50 ml PBS and eluted with 4 ml 6 M guanidinium hydrochloride. After washing with PBS the column was readily reactivated. The breakthrough fraction was two more times passed through the column in order to ensure complete absorption of anti MBP-antibodies. The guanidinium-HCl containing eluates were dialyzed against PBS. From a total of 80 mg IgG, 8.9 mg were obtained that carry specificity against MBP. As judged by Ouchterlouny immunodiffusion these IgG retained their precipitating activity.

Five milligram activated, insoluble papain was washed 3 times with 20 ml PBS. It was resuspended in 12 ml PBS containing 8.9 mg (αMBP)-IgG and agitated for 8 h at 37° C and subsequently for 12 h at room temperature. To remove the cleaved off Fc portion of the IgG the supernatant was passed twice through the MBP-Sepharose column. αMBP-F_{ab} and uncleaved αMBP-IgG were eluted from the column by a total of 8 ml 6 M guanidinium-HCl and dialyzed against PBS. After concentration to 2 ml the solution was passed through a Biogel-P-200 column (1.5 × 30 cm) where separation of F_{ab} fragments was achieved. The coupling of the F_{ab} fragments to activated horseradish peroxidase was done according to the standard procedure of Nakane and Kawaoi (1974). The concentrated final solution was passed through a Biogel-P-200 column. Fractions corresponding to a molecular weight of 80,000 were collected. They contained peroxidase activity (diaminobenzidine-H_2O_2) as well as anti MBP specificity (retention by MBP Sepharose). A total of 36 ml containing 40 µg/ml (αMBP-F_{ab})-HRPO were obtained.

A corresponding control preparation of F_{ab}-HRPO conjugate was obtained with that portion of the IgG's that has passed through the MBP affinity column. However, for this preparation the Fc portion was not separated and the final preparation contained besides F_{ab}-HRPO also Fc-HRPO conjugate

Specifically Staining for the Maltose-Binding Protein

25 ml of logarithmically growing cells were harvested by centrifugation at room temperature and resuspended in 25 ml PBS. One milliliter of 50% glutaraldehyde was added and slowly stirred at room temperature for 2 h. The suspension was washed five times with PBS and resuspended to a cell density of about 10^{10}/ml. To 300 µl of such a suspension 1 ml (αMBP-F_{ab})-HRPO conjugate (~ 40 µg protein) was added and the suspension gently agitated at 4° C overnight. The cells were then washed three times in PBS, resuspended in 30 ml PBS and gently agitated overnight. After centrifugation they were resuspended in 5 ml 0.05 M Tris-HCl, pH 7.6 containing 0.075% diaminobenzidine (Sigma) and 0.001% H_2O_2. This solution was freshly prepared before use and was filtered through Millipore, 0.45 µ pore size. After washing in PBS the pellet was resuspended in 300 µl PBS and 100 µl 4% osmiumtetroxide (in water) was added. After 90 min the pellet was washed in PBS and resuspended for 1 h in 2% uranylacetate in water. The samples were then dehydrated with ethanol and embedded according to Spurr (1969). Thin sections were prepared with a Reichert ultratome. Without further staining the sections were examined in a Philips 300 electronmicroscope at 60 KV. Control experiments were done by using either F_{ab}-HRPO conjugates lacking anti MBP specificity or by HRPO with or without washing in PBS before reacting in diaminobenzidine-H_2O_2. In addition, controls for background staining were obtained by omitting incubation with F_{ab}-HRPO and diaminobenzidine-H_2O_2.

Results

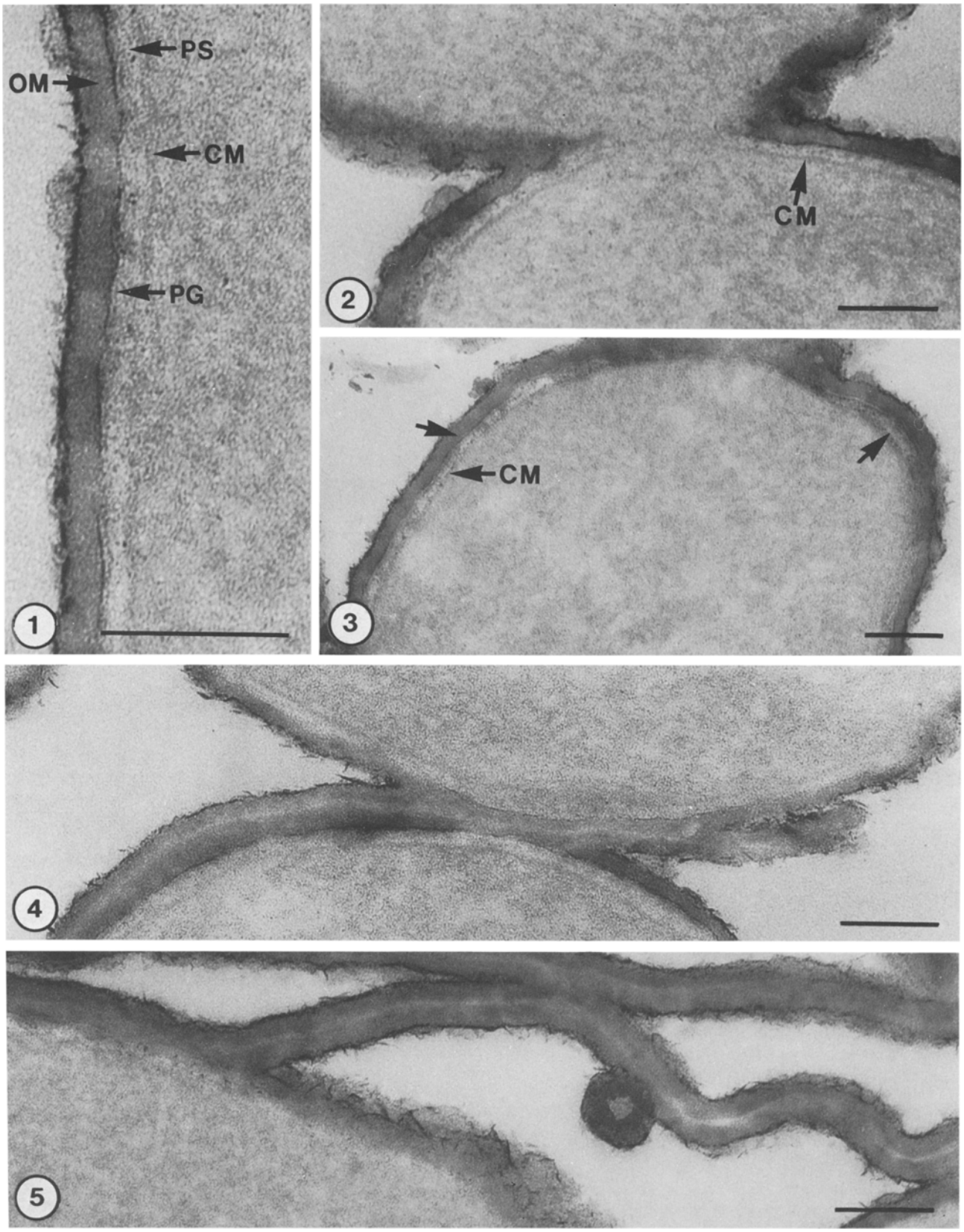
Appearance of Escherichia coli Wild-Type Bacteria Induced with Maltose

Figures 1–7 and 9 represent thin sections of induced wild-type bacteria that have been specifically labelled with F_{ab} fragments against maltose-binding protein coupled to horseradish peroxidase. The following features are apparent:

1. The different components of the envelope: outer membrane (OM), peptidoglycan (PG), periplasmic space (PS), and cytoplasmic membrane (CM) can be recognized (Figs. 1–3). The outer membrane has become greatly enlarged due to the hydrophobic precipitate originating from oxidized diaminobenzidine. The thickening of the OM probably occurs within the lipid bilayer. Occasionally the outer membrane becomes so large that it splits through its plane as seen in Fig. 4. Occasionally large sheets of these thick layers peal off and form their own membrane aggregates as seen in Fig. 5. In these aggregates the hydrophobic but not the hydrophilic sides form the aggregates. This dramatic thickening of the outer membranes is clearly an artifact of the labelling procedure caused by the formation of oxidized diaminobenzidine by specifically bound horseradish peroxidase. The staining within the outer membrane appears smooth (Figs. 1–3). In contrast, granular staining can be seen at locations where MBP is in a predominantly polar environment, i.e. between the outer membrane and the cytoplasmic membrane (Fig. 3). Thus, it appears that MBP is located in two compartments: the periplasm, but predominantly in the outer membrane.

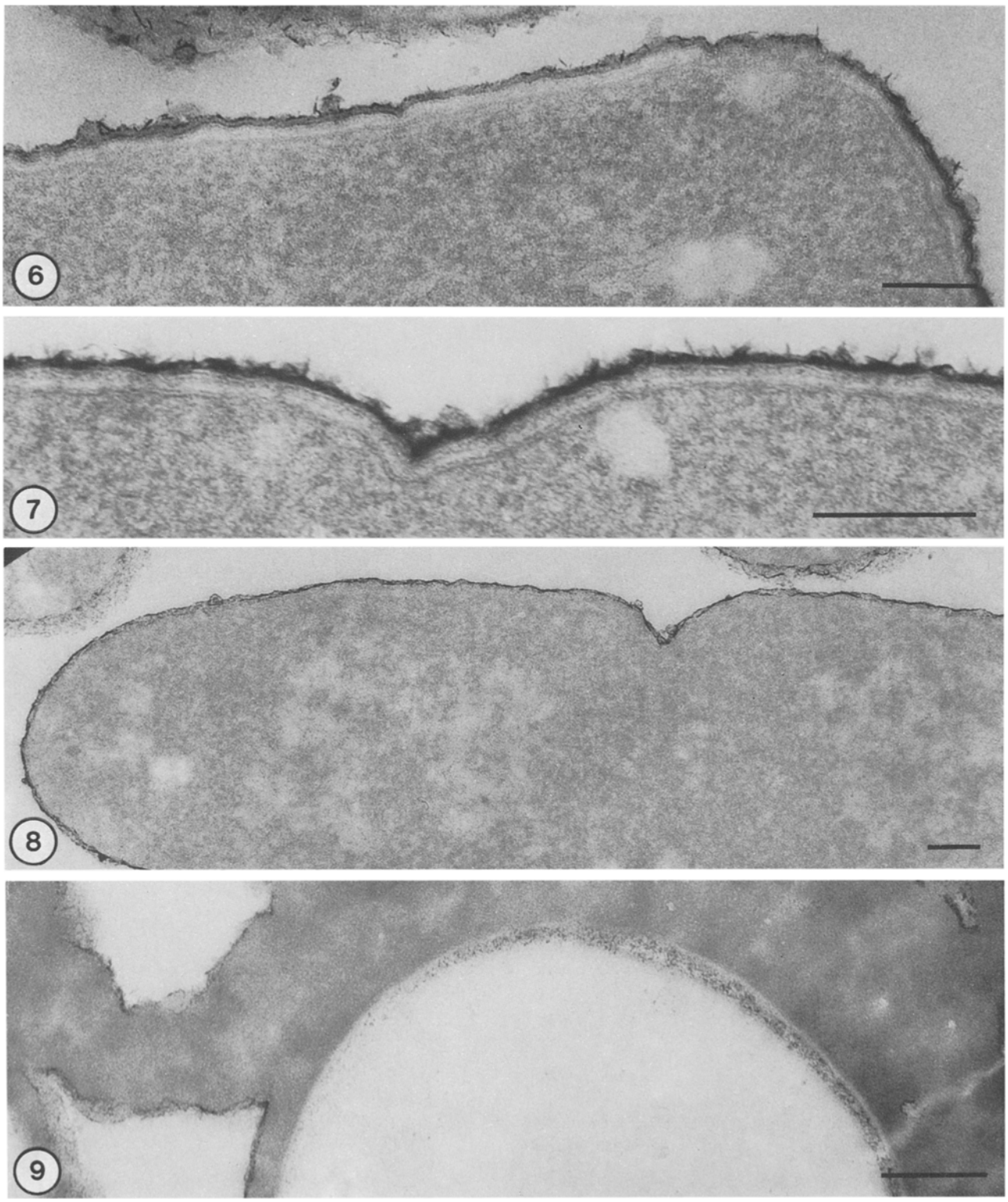
2. Looking at a large number of individual bacteria it appears that the specific staining of the outer membrane occurs preferentially in small cells (Figs. 3, 4) but is much less dramatic in longer cells (Fig. 6) or cells that start to divide (Fig. 7). However, as seen in Fig. 2, pairs of cells that have nearly finished their cell division exhibit strong staining even around the septum area. As discussed later, this is a consequence of the cell cycle dependent synthesis of the maltose transport machinery.

3. The outside surface of the bacteria appears to be stained to varying degrees. This staining consists sometimes of a sharp line but has mostly a fuzzy appearance (Fig. 7). This surface stain is derived partly from staining by OsO_4 that occurs even in the absence of the diaminobenzidine precipitate (Fig. 8) and partly from an unspecific interaction of



Figs. 1–3. Thin sections of *Escherichia coli* wild-type bacteria grown on maltose. The cells were fixed with glutaraldehyde and treated with F_{ab} fragments against maltose-binding protein coupled to horseradish peroxidase. The presence of maltose-binding protein is indicated by the dark precipitate resulting from the reaction with diaminobenzidine-H_2O_2 and staining with OsO_4-uranylacetate. The following structures can be recognized: *CM*, cytoplasmic membrane; *PG*, peptidoglycan; *OM*, outer membrane; *PS*, periplasmic space. In Fig. 3 smooth staining can be seen within the outer membrane and much rarer granular staining within the periplasmic space. Magnifications: Fig. 1, 336,000 × ; Fig. 2, 174,000 × ; Fig. 3, 135,000 × . The bars indicate 0.1 μm

Figs. 4 and 5. Thin sections of *Escherichia coli* wild-type bacteria grown on maltose. The cells were treated in the same way as described in the legend to Figs. 1–3. Figure 4 demonstrates splitting of the diaminobenzidine (*DAB*) precipitate within the outer membrane. Figure 5 shows an example of an outer membrane filled with DAB precipitate and in the process of pealing off from the cell surface. Magnifications: Fig. 4, 174,000 × ; Fig. 5, 174,000 × . The bars indicate 0.1 μm



Figs. 6–9. Thin sections of *Escherichia coli* wild-type bacteria grown on maltose. In long cells (Fig. 6) the staining within the outer membrane is much less dramatic. The cell shown in Fig. 7 was treated as described in the legend to Figs. 1–3. At the beginning of septum formation, the typical swelling of the outer membrane is not observed. Figure 8, cell fixed with glutaraldehyde and stained with OsO_4 and uranylacetate, but not treated with the F_{ab}-horseradish peroxidase conjugate and allowed to react with diaminobenzidine-H_2O_2. The cell shown in Fig. 9 was treated as described in the legend of Figs. 1–3. This picture illustrates a rare example of heavy granular staining within the periplasmic space. The outer membrane is completely distorted in the pole region due to the large amounts of reaction product formed. Magnifications: Fig. 6, 160,000×. Fig. 7, 273,000×; Fig. 8, 84,000×; Fig. 9, 174,000×. The bars indicate 0.1 μm

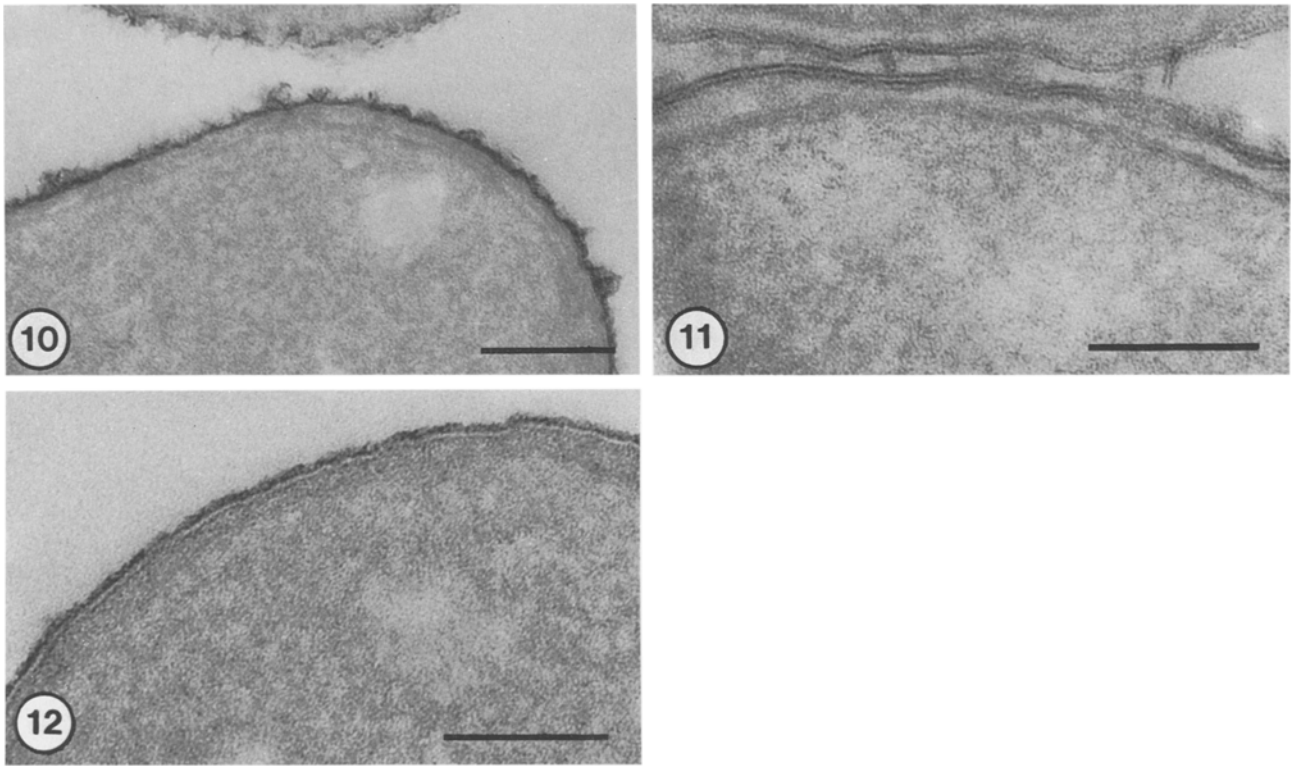


Fig. 10. Control experiment. Wild-type cells of *Escherichia coli* were treated as described in the legend to Figs. 1–3. The cells were grown on glucose. Under these conditions the maltose transport machinery including MBP is not induced. Magnification: 174,000 ×. The bar indicates 0.1 μm

Figs. 11 and 12. Staining patterns in an *Escherichia coli* mutant lacking the λ receptor and in cells with damaged outer membrane. Figure 12, mutant bacterium lacking the λ receptor. It does contain maltose-binding protein. Treatment as described in the legend to Figs. 1–3. The typical swelling of the outer membrane does not occur, but granular staining, particularly in the region of the pole caps, is regularly observed. Figure 11 shows a wild-type bacterium treated with lysozyme-EDTA after glutaraldehyde fixation. Incubation with F_{ab} fragments against MBP, coupled to horseradish peroxidase and reaction with diaminobenzidine-H_2O_2 as well as staining with OsO_4-uranylacetate were done as usual. No swelling of the outer membrane is observed and the periplasm appears empty. Magnifications: Fig. 11, 219,000 ×; Fig. 12, 219,000 ×. The bars indicate 0.1 μm

the (αMBP-F_{ab})-HRPO with the surface layer of the bacteria. Most likely the HRPO-part of the conjugate is responsible for the reaction since horseradish peroxidase alone elicits the same staining pattern (not shown).

4. The periplasmic space, here defined as the space between cytoplasmic membrane and peptidoglycan layer (Figs. 1–3, 7) usually does not stain to any great extent. Occasionally, single grains can be detected, so in Fig. 3. A rare picture is depicted in Fig. 9. Here, the periplasmic space of pole caps is heavily stained with granular material. In addition, the outer membrane is artificially blown up by an accumulation of oxidized diaminobenzidine. No particular staining has been detected in any preparation at or within the cytoplasmic membrane.

Control Experiments

Several control experiments were performed in order to establish the specific nature of the staining process. Figure 10 shows the thin section of a wild-type strain that has been grown on glucose to repress the synthesis of the maltose transport machinery. As can be seen, neither the thickening of the outer membrane, nor granular staining in the periplasmic space can be observed. However, as in the induced strain, the surface staining is still visible. The same picture was obtained when a mutant lacking the entire *malB* region was used in the standard procedure (not shown).

The same type of staining patterns was obtained with any strain, induced or uninduced for the maltose transport, when a control conjugate of F_{ab}-HRPO was used that did not contain αMBP specificity (not shown). Figure 8 shows a section of a bacterium that had been fixed with glutaraldehyde, but had not been treated with any F_{ab}-conjugate nor with diaminobenzidine-H_2O_2. As in the standard procedure it was stained with OsO_4 and uranylacetate before embedding and thin sectioning. Only the surface layer of the cell is stained and no resolution of the envelope layers can be seen.

Staining for the Maltose-Binding Protein in a Strain that Lacks the λ Receptor

Figure 12 shows a thin section of a strain that lacks λ receptor but contains normal amounts of maltose-binding protein. As can be seen, the usual thickening of the outer membrane routinely observed in the wild-type does not occur or only to a minor extent. However, the periplasmic space, particularly in the pole region, reveals the presence of MBP. It thus appears, as if the presence of λ receptor is necessary for the proper positioning of MBP at the outer membrane. In addition, no staining of either the outer membrane, nor the periplasm

could be observed when the wild-type cells, after glutaraldehyde fixation, were treated with lysozyme-EDTA (Fig. 11).

Discussion

The localization of transport related binding proteins in the periplasm, outside the cytoplasmic membrane of Gram-negative bacteria has been deduced from their releasability by osmotic shock (Heppel 1971), by their inactivation via reagents that cannot penetrate the cytoplasmic membrane (Pardee and Watanabe 1968), and by their exclusive way of biosynthesis through the cytoplasmic membrane (Smith et al. 1977) via membrane bound ribosomes (Randall and Hardy 1977). However, the final localization of these proteins within the architecture of the cell envelope is less well defined. Reaction product staining as successfully employed in case of periplasmic enzymes such as alkaline phosphatase (MacAllister et al. 1972) and 5′-nucleotidase (Nisonson et al. 1969) cannot be done with binding proteins due to the lack of enzymatic activity.

Based on their essential role in transport it is now generally assumed that binding proteins interact with specific components in the cytoplasmic membrane that are part of the transport system. Indeed, genetic evidence suggests that the histidine-binding protein from *Salmonella* has to interact directly with a component of the histidine transport system bound to the cytoplasmic membrane (Ferro-Luzzi Ames and Spudich 1976). In the maltose transport system there is at least one protein located in the cytoplasmic membrane, the gene product of the *malF* gene (Shuman et al. 1980) that could specifically interact with the maltose-binding protein. Moreover, in vitro immobilized MBP does specifically bind to a methylated chemotaxis protein solubilized from the cytoplasmic membrane (Koiwai and Hayashi 1979). Thus, the maltose-binding protein has to be at least temporarily in close contact with the cytoplasmic membrane. However, it has also become apparent that MBP can interact with the λ receptor of the outer membrane (Heuzenroder and Reeves 1980; Wandersman et al. 1979).

The staining procedure used here to visualize MBP within the cell envelope has several shortcomings. The first is the problem of limitations in the permeability of the F_{ab} horseradish peroxidase conjugate. It is not clear whether this molecule can freely penetrate to all compartments of the cell envelope, particularly the space below the peptidoglycan layer. The second is a possible modification of the maltose-binding protein by glutaraldehyde fixation which may affect its ability to specifically bind the F_{ab} portion of the conjugate depending on its local environment. The third is the distortion of the true location of the protein due to a "moving away" of the reaction product formed by the attached enzyme. From the pictures it is clear that particularly the latter point is important. Two types of staining can be seen: One, granular, that is found exclusively in the periplasm, presumably where the binding protein is situated in a hydrophilic environment, and the other, smooth, most likely due to the production and deposition of the hydrophobic diaminobenzidine precipitate within or close to the lipid phase of the outer membrane. Such an accumulation of hydrophobic molecules in the non-polar interior of biological membranes and resulting in split membrane bilayers has recently been demonstrated in *Bacillus cereus* cells exposed to lipophilic substances such as phenethyl alcohol and tetracain-HCl (Silva et al. 1979). Whatever the cause, it is clear that the deposition of the reaction product and the resulting dramatic swelling of the outer membrane is closely related to the presence of the binding protein since it is absent in cells lacking the maltose-binding protein. Since mutants lacking the λ receptor do not exhibit this type of staining the interaction of λ receptor with the maltose-binding protein seems to be required for its positioning close to or within the outer membrane.

The maltose-binding protein (Dietzel et al. 1978) as well as the λ receptor (Ryter et al. 1975) are only synthesized at certain times in the cell cycle of the bacterium, during or shortly after the cell has divided. This agrees well with the observation that small cells that presumably just have divided exhibit in the staining procedure a larger swelling in their outer membrane than long cells. Furthermore, cells in the initial stages of cell division cannot be distinguished from cells lacking MBP. Thus, MBP after being synthesized in a short pulse appears to be diluted out in the outer membrane during cell elongation. This seems not to be the case for MBP in the periplasm. When seen at all, it is mostly found at the pole end of the bacteria (Fig. 9).

The technique described here to localize the transport related periplasmic protein is not without ambiguity. But it will allow to analyze the various mutants that are defective in the maltose transport machinery, in particular those that are defective in the secretion process (Bassford et al. 1979) and those that might carry defects in the interaction of MBP with the λ receptor (Wandersman et al. 1979).

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