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Role of a Major Outer Membrane Protein in *Escherichia coli*

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Mutants of *Escherichia coli* B/r lacking a major outer membrane protein, protein b, were obtained by selecting for resistance to copper. These mutants showed a decreased ability to utilize a variety of metabolites when the metabolites were present at low concentrations. Also, mutants of *E. coli* K-12 lacking proteins b and c from the outer membrane were shown to have an identical defect in the uptake of various metabolites. These results are discussed with regard to their implications as to the role of these proteins in permeability of the outer membrane.

The cell envelope of gram-negative bacteria consists of an inner cytoplasmic membrane and an outer membrane separated by a thin layer of peptidoglycan. The cytoplasmic membrane contains all of the active transport systems investigated thus far, the components of the respiratory chain, and enzymes of the tricarboxylic acid cycle (12, 16). The function of the outer membrane, however, remains unclear, although it does have a molecular sieving property (11). The outer membrane must also play a more specific role in permeability, since it contains several proteins involved in the transport of vitamin B_{12} (2), siderochrome iron (3), and maltose (17).

A distinctive feature of the outer membrane is the presence of a few proteins in very large numbers. Recently, the proteins found in *Escherichia coli* K-12 with molecular weights of 35,000 to 40,000 have been resolved into four bands, designated a, b, c, and d (8). In contrast, *E. coli* B contains only b, d, and small amounts of a (8). The b and c proteins in the K-12 strains have been shown to be very similar; the only difference has been traced to a peptide fragment that does not originate from either of the ends (14).

The functions of these proteins are now being elucidated. It was first proposed that they play a role in shape determination (5), but the isolation of mutants lacking one or all of these proteins and showing no shape defects has made this unlikely (4). Protein d plays a role in conjugation, since its removal by mutation decreases the receptor ability of recipient strains (15). Nakae and Nikaido (11) concluded that the permeability of the outer membrane to low-molecular-weight hydrophilic molecules is due to the presence of protein molecules in the outer membrane. It was shown from reconstitution experiments (10) that the presence of a complex of three major outer membrane proteins with molecular weights between 35,000 and 40,000 was sufficient to make vesicles, composed of phospholipid and lipopolysaccharide, permeable to low-molecular-weight saccharides. More recently (9) it was determined that, with *E. coli* B, this permeability of reconstituted vesicles could be restored by adding just one protein with a molecular weight of 36,500, presumably protein b. This is the matrix protein described by Rosenbusch (13).

In this paper I demonstrate the role of proteins b and c in the uptake of various metabolites. Since there is little specificity and protein b shows no affinity for these metabolites in binding experiments in vitro, I conclude that proteins b and c independently form pores in the outer membrane that allow small molecules to diffuse rapidly into the periplasmic space.

MATERIALS AND METHODS

Bacterial strains. The strains used are listed in Table 1. The column marked "relevant phenotype" refers to proteins b and c of the outer membrane, according to the nomenclature of Lugtenberg et al. (7). Strains CuR_7, CE1036R, and CE1061R were selected on minimal medium plates containing 20 μM $CuSO_4$.

Radiochemicals. L-[^{35}S]methionine (270 Ci/mmol), L-[^{3}H]leucine (57 Ci/mmol), and [^{3}H]thymidine were obtained from the Radiochemical Centre, Amersham, England.

Growth of bacteria. Bacteria were grown in a rotary shaking bath at 37°C. In all experiments, a minimal medium (M9; 1) supplemented with 0.4% glucose was employed. Growth was measured by following the optical density at 540 nm (OD_{540}) in a Hilger-Gilford spectrophotometer.

Uptake measurement. To measure the uptake of various labeled compounds, cells were grown to an OD_{540} of 0.2. Then, the labeled compound was added, at the appropriate specific activity and concentra-

TABLE 1. *E. coli strains used*

Strain	Relevant phenotype	Source
B/r	b^+ (no c)	C. Helmstetter
CuR_7	b^- (no c)	This laboratory
CE1061	b^+ c^+	B. Lugtenberg
CE1036	b^+ c^-	B. Lugtenberg
CE1061R	b^- c^+	This laboratory
CE1036R	b^- c^-	This laboratory

tion, to 1 ml of culture. At various times, 100-μl volumes were removed, pipetted onto 3 MM paper disks, and placed in 5% trichloroacetic acid. After a minimum of 30 min, the disks were washed twice with 5% trichloroacetic acid and once with 80% ethanol, dried, and counted in a liquid scintillation counter.

Preparation of cell envelope and total cell extracts. For preparation of membranes, 50 ml of culture at an OD_{540} of 0.2 was centrifuged at 4°C. The cell pellet was suspended in a 5-ml solution containing 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), 5 mM ethylenediaminetetraacetate (pH 7.8), and 1 mM β-mercaptoethanol. The cells were then disrupted by three 20-s bursts at the full power of an MSE 100-W ultrasonic disintegrator. Cell debris was removed by low-speed centrifugation, and membranes were pelleted from the supernatant by centrifugation at 100,000 $\times$ *g* for 45 min. The membrane pellet was resuspended in the same buffer with sonic oscillation and again pelleted. The final pellet was suspended in 50 μl of sodium dodecyl sulfate (SDS) sample buffer, which contained 62.5 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8), 1% SDS, 5% β-mercaptoethanol, and 10% glycerol. To prepare total cell protein, 5 ml of culture at an OD_{540} of 0.2 was pelleted. The pellet was suspended in 50 μl of SDS sample buffer and stored in the cold.

SDS-polyacrylamide gel electrophoresis. Proteins were analyzed on SDS-polyacrylamide gels essentially by the procedure of Laemmli (6).

Separation of both total cell protein and membrane proteins employed gels containing 16% acrylamide and 0.094% bisacrylamide. Gels of this composition are used routinely in this laboratory because of their extreme stability during drying. All slab gels were run for 15 h at a constant current of 8 mA. Samples in SDS sample buffer were heated in a boiling-water bath for 4 min before being applied to the gel.

Proteins were fixed by immersing the gel for 10 min in a mixture of 45% ethanol and 9% acetic acid, stained for 10 min in 0.25% Coomassie brilliant blue in 7% ethanol and 5% acetic acid, and destained in 7% ethanol and 5% acetic acid with several changes. All steps were carried out at 37°C.

RESULTS

Isolation of mutants lacking protein b. Mutants resistant to Cu^{2+} were selected on minimal medium plates containing 20 μM $CuSO_4$ and appeared with a frequency of 10^{-5}. Membrane preparations from Cu^{2+}-resistant mutants of strain B/r contained little if any protein b (Fig. 1). To determine if this mutation to Cu^{2+} resistance resulted in a decrease in the synthesis of protein b or prevented incorporation of protein b into the membrane, gels of total cell

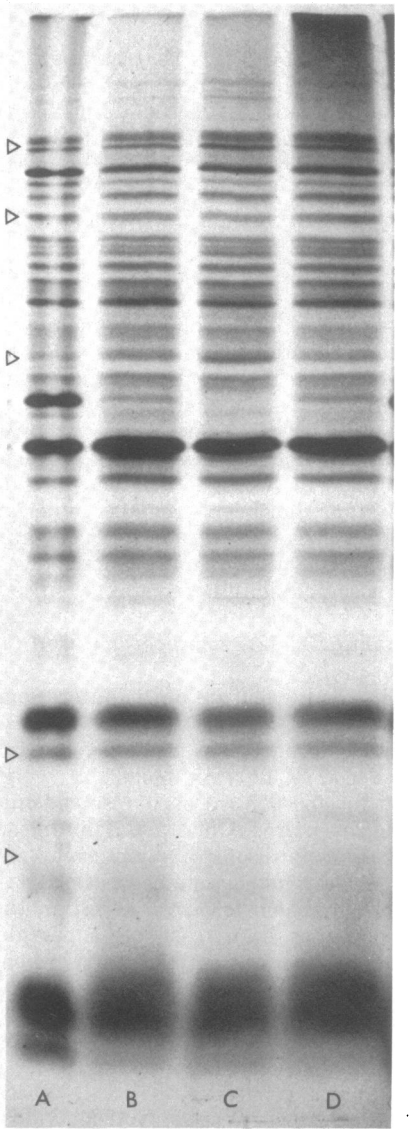

FIG. 1. *SDS-polyacrylamide gel electrophoresis of the cell envelopes of strain B/r and three of its copper-resistant mutants. Cell envelope preparation and gel electrophoresis were as described in the text. (A) B/r (wild type); (B) CuR_3; (C) CuR_7; and (D) CuR_{10}. The molecular weight standards are as follows: phosphorylase a (92,000), bovine serum albumin (68,000), oval albumin (43,000), β-lactoglobulin (18,000), and lysozyme (14,000).*

protein were run. Fortunately, protein b was in a relatively clear region of the gel, and from Fig. 2 it can be seen that it was not synthesized in the mutants examined, or, if it was synthesized, it was very unstable.

Effect of protein b loss. In initial experiments with various Cu^{2+}-resistant B/r mutants, in which attempts were made to label proteins with radioactive amino acids, it was noticed that the mutants incorporated very little label when compared with the parent strain. However, in two separate experiments the incorporation into the mutant varied 10-fold. A possible explanation for this was that the concentration of methionine was different in the two experiments. To examine this further, the experiment presented in Fig. 3 was carried out with strains B/r and CuR_7, one of the Cu^{2+}-resistant mutants. The concentration of methionine was varied 100-fold. As the external concentration decreased, the difference in the rate of incorporation between the mutant and the parent increased (Fig. 3). This indicates that CuR_7 is unable to utilize methionine when it is present at a low concentration. Other compounds were also tested to examine further the effect of protein b loss on incorporation. Similar differences in incorporation between the mu-

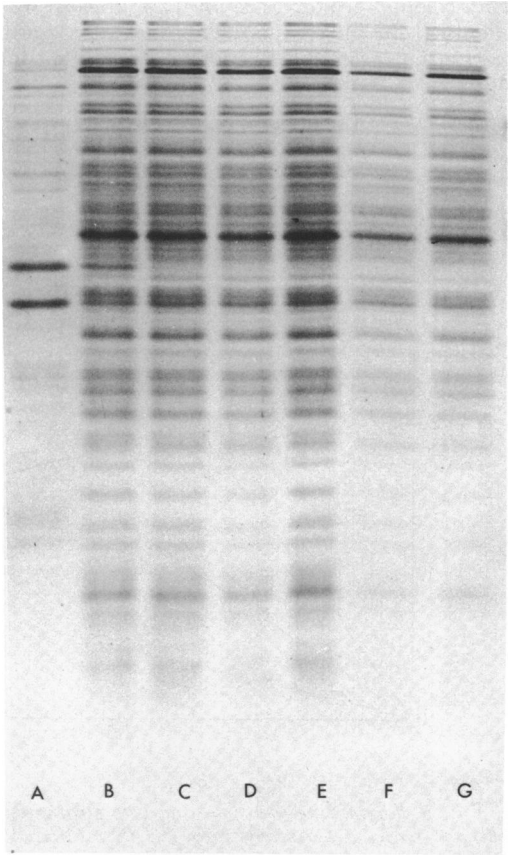

FIG. 2. *Whole-cell extracts from strain B/r and six of its copper-resistant mutants. (A) Cell envelope preparation from strain B/r; (B) strain B/r; (C) CuR_3; (D) CuR_7; (E) CuR_8; (F) CuR_9; and (G) CuR_{10}.*

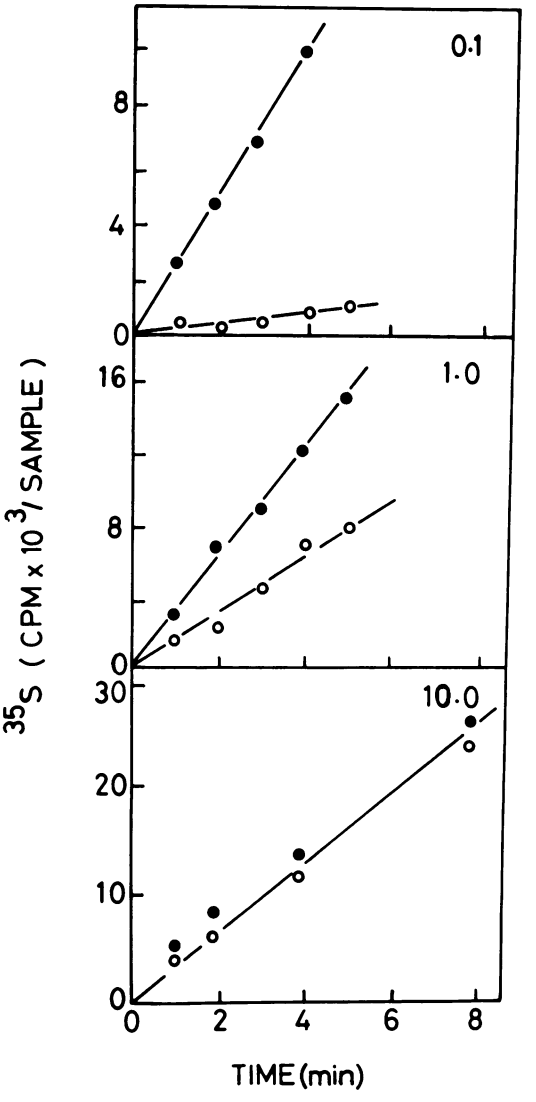

FIG. 3. *Methionine incorporation into strains B/r (●) and CuR_7 (○). At time zero, [^{35}S]methionine (2.0 μCi/μg) was added to exponentially growing cells to give the concentrations (micrograms per milliliter) indicated. At the times indicated, samples were removed and precipitated with trichloroacetic acid, and the amount of radioactivity was determined in a liquid scintillation counter.*

tant and the wild type were obtained with leucine and histidine (data not shown). Utilization of glucose was also examined by growing cells on various concentrations of glucose. The growth rate of CuR_7 decreased gradually as the concentration of glucose fell from its initial limiting value of 0.05% (Fig. 4). Furthermore, when the cultures growing on 0.05% glucose were diluted into glucose-free medium to lower the glucose concentration, CuR_7 was unable to utilize the glucose present, whereas the parent was able to grow and deplete the glucose from the medium.

However, different results were obtained when the uptake of thymidine was investigated. Strain CuR_7 was able to incorporate thymidine at the same rate as the parent, even at low concentrations of thymidine (Fig. 5).

Isolation of *E. coli* K-12 mutants lacking protein b. K-12 strain CE1061, which was chosen as the parent, contains the two nearly identical proteins, b and c, which I have been unable to resolve in a one-dimensional gel system. Analysis of gels of total cell protein from Cu^{2+}-resistant CE1061 mutants did not show any changes from that of the parent strain. These mutants, however, were still sensitive to phage Mel, indicating that they still contain protein c (17).

CE1036, which was isolated as resistant to phage Mel and lacks protein c (17), showed the same sensitivity on plates to Cu^{2+} as the parent. Mutants of CE1036 that were selected as resistant to Cu^{2+} and analyzed on gels lacked a band at a molecular weight of 36,000 presumably because they lacked proteins b and c (Fig. 6).

Effect of the loss of proteins b and c. Since the loss of protein b in strain B/r affected the uptake of several metabolites, we investigated the ability of the Cu^{2+}-resistant CE1061 mutant to incorporate various metabolites. The loss of either protein b or c did not affect the ability of the cells to take up methionine at low concentrations (Fig. 7). However, in the double mutant, lacking both proteins, the incorporation of methionine was severely curtailed at the lower concentrations.

The effect of these mutations on glucose transport was also investigated by examining the size of colonies formed on minimal medium plates supplemented with only 0.01% glucose. Both the protein b and the protein c mutants formed colonies the same size as those of the parent. However, the double mutant formed tiny colonies (colony size on 0.2% glucose was identical for all strains). This indicates again that either protein b or c alone is sufficient for efficient transport.

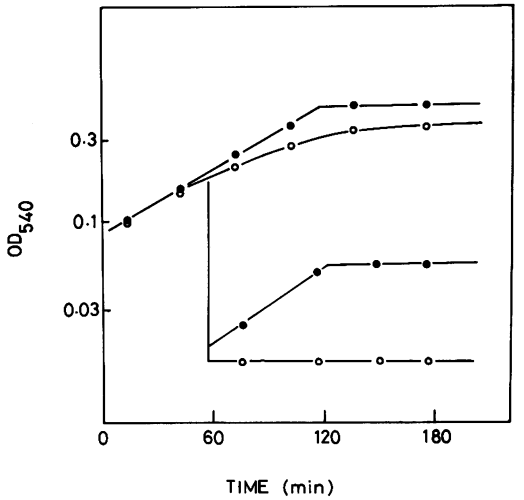

FIG. 4. *Growth of strains B/r (●) and CuR_7 (○) on limiting glucose. Cells growing in minimal medium supplemented with 0.05% glucose were diluted 10-fold at 60 min into glucose-free medium.*

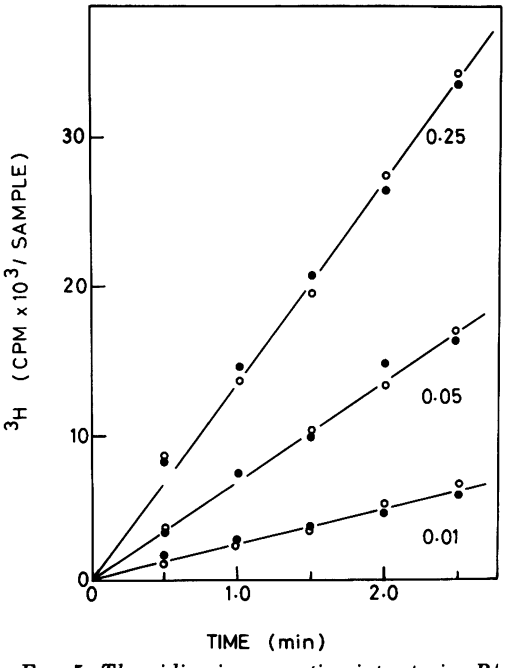

FIG. 5. *Thymidine incorporation into strains B/r (●) and CuR_7 (○). At time zero, [^{3}H]thymidine (52 mCi/mmol) was added to exponentially growing cells to give the concentrations (micrograms per milliliter) indicated. At the times indicated, samples were precipitated with trichloroacetic acid, and the amount of radioactivity was determined in a liquid scintillation counter.*

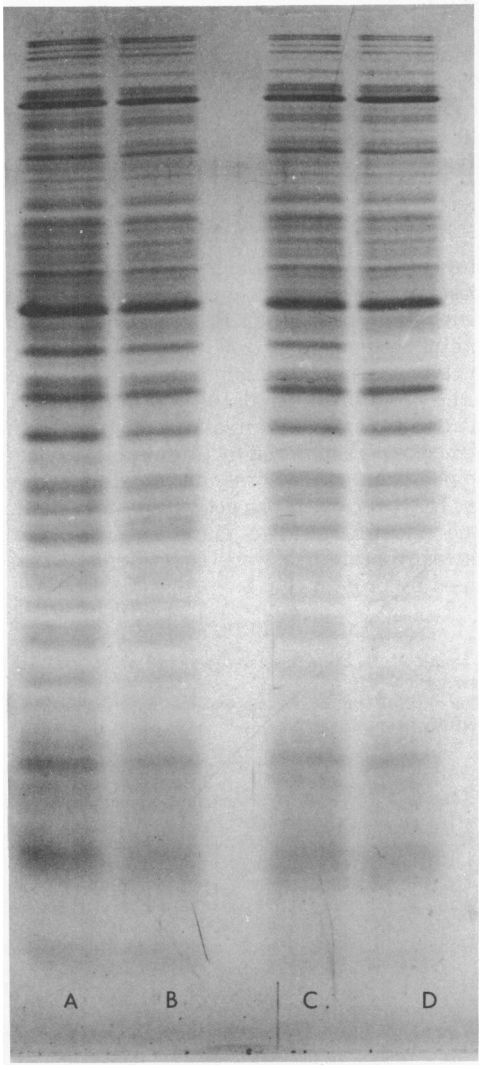

FIG. 6. *Whole-cell extracts from a K-12 strain and several of its mutants. (A) CE1061 (b^+ c^+); (B) CE1061R (b^- c^+); (C) CE1036 (b^+ c^-); and (D) CE1036R (b^- c^-).*

DISCUSSION

The involvement of protein b in strain B/r and proteins b and c in strain K-12 in the incorporation of several metabolites has been demonstrated by the inability of mutants to utilize low concentrations of these compounds. In the wild type, the presence of these proteins gives a selective advantage over the mutant under poor nutritional conditions. However, with the usual laboratory concentrations of 0.4% glucose and 20 to 40 μg of amino acids per ml, these mutants are phenotypically identical to the parent and cannot be detected.

A possible explanation as to how proteins b and c function in transport is that they form an aqueous pore in the outer membrane, which allows rapid diffusion of low-molecular-weight hydrophilic molecules into the periplasmic space. Such a model was proposed for protein b by Nakae (9) on the basis of observations with reconstituted membrane vesicles. The model is consistent with our observations. First, proteins b and c affect the uptake of a variety of

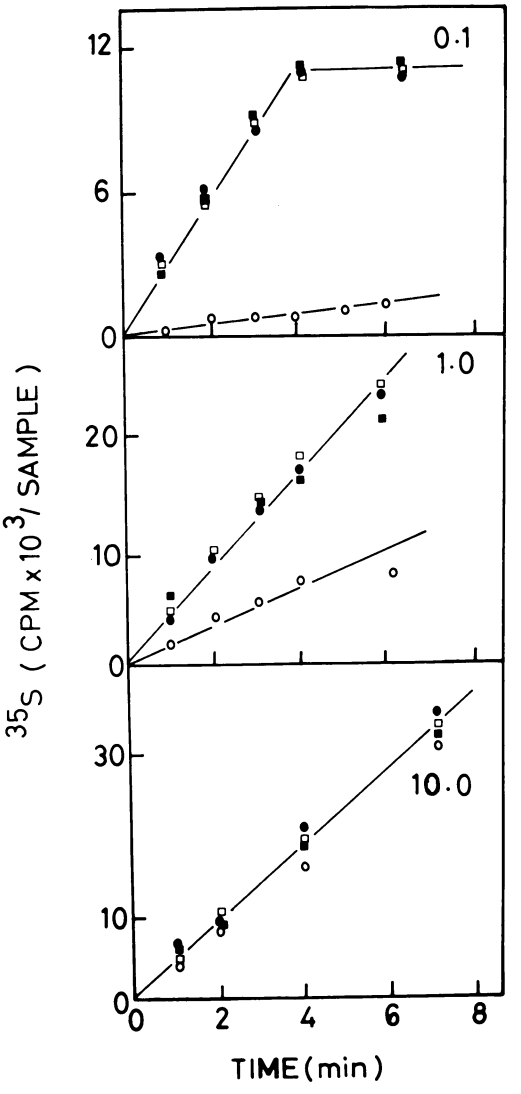

FIG. 7. *Methionine incorporation into strain CE1061 and its mutants. The experimental details are the same as those described in the legend of Fig. 3, except that the specific activity of [^{35}S]methionine was 10 μCi/μg. Symbols: ●, CE1061 (b^+ c^+); ■, CE1036 (b^+ c^-); □, CE1061R (b^- c^+); and ○, CE1036R (b^- c^-).*

compounds and, therefore, appear to show little specificity. Second, protein b prepared by the Rosenbusch procedure (13) did not bind methionine in equilibrium dialysis experiments (data not shown).

A pore formed by proteins b and c, however, cannot be the only mechanism by which these small hydrophilic molecules permeate the outer membrane. This can be clearly seen at high concentrations of metabolites when the lack of proteins b and c does not affect the rate of incorporation (Fig. 3 and 7). In addition, the rate of incorporation of thymidine was not affected by the lack of proteins b and c at any of the concentrations examined. Another possibility is that small amounts of proteins b and c are produced which are sufficient. This seems extremely unlikely, however, since with one of the mutants, CuR_7, no trace of protein b could be observed in the membrane preparations.

A more likely explanation is that other pores might also exist in the outer membrane. Based upon the observed effect of concentration on uptake, hydrophilic molecules would be expected to have a lower rate of diffusion in these other pores. In other words, at high concentrations of metabolites, diffusion through these secondary pores would be sufficient to saturate the cytoplasmic transport systems, whereas at low concentrations it would not be sufficient. In the wild type, diffusion through the protein b-c pore would be rapid enough to saturate the cytoplasmic transport systems even at low concentrations of metabolites.

It is likely that the Cu^{2+}-resistant mutants isolated from the B/r strain are the same as that selected by von Meyenberg (20) for slow growth on low concentrations of glucose. This is because that mutant is also defective in the uptake of the compounds tested here, as well as of other sugars, phosphate, and sulfate. Assuming that the mutants are the same, the pores formed by proteins b and c would also be involved in the uptake of these compounds. It is interesting that no defect was observed in the uptake of uracil or uridine while we observed no defect in thymidine uptake, as was shown by Hantke (3a).

In view of the apparent lack of specificity of the protein b-c pores, it is difficult to understand why maltose at low concentrations requires a separate outer membrane protein, the *lamB* gene product (16). In addition, our results suggest that thymidine uses a separate site for penetration of the outer membrane.

As others have noted, it is interesting that strain K-12 contains two nearly identical proteins and yet strain B/r contains only one. One possibility is that one of the proteins is post-translationally modified to form the other. This, however, seems to be ruled out, since initially the loss of either protein can be elicited without loss of the other (although not always [19]). The other possibility, which seems more likely, is that in the K-12 strain the gene for protein b has undergone a gene duplication with very little subsequent mutation.

So far we do not know why selection for resistance to Cu^{2+} specifically leads to a loss of protein b. It is possible that Cu^{2+} penetrates the outer membrane through the pores formed by protein b to reach a sensitive site. That the sensitive site is not protein b itself is suggested by the observation that the mutants are still sensitive to high concentrations of Cu^{2+}. In the absence of pores formed by protein b, Cu^{2+} may also penetrate other pores, although at a lower rate. However, with respect to uptake of methionine and glucose, we found that the pores formed by protein b were equivalent to those formed by protein c.

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LITERATURE CITED

1. **Adams, M. H.** 1959. Bacteriophages, 1st ed., p. 466. Interscience, New York.
2. **DiMasi, D. R., J. C. White, C. A. Schnaitman, and C. Bradbeer.** 1973. Transport of vitamin B_{12} in *Escherichia coli*: common receptor sites for vitamin B_{12} and the E colicins on the outer membrane of the cell envelope. J. Bacteriol. 115:506-513.
3. **Hancock, R. E., K. Hantke, and V. Braun.** 1976. Iron transport in *Escherichia coli* K-12: involvement of the colicin B receptor and a citrate-inducible protein. J. Bacteriol. 127:1370-1375.

3a. **Hantke, K.** 1976. Phage T6-colicin K receptor and nucleoside transport in *Escherichia coli*. FEBS Lett. 70:109-112.

4. **Henning, U., and I. Haller.** 1975. Mutants of *Escherichia coli* K-12 lacking all "major" proteins of the outer cell envelope membrane. FEBS Lett. 55:161-164.
5. **Henning, U., K. Rehn, and B. Hohn.** 1973. Cell envelope and shape of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. 70:2033-2036.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
7. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K-12 into four bands. FEBS Lett. 58:254-258.
8. **Lugtenberg, B., R. Peters, H. Bernheimer, and W. Berendsen.** 1976. Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Mol. Gen. Genet. 147:251-262.
9. **Nakae, T.** 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem. Biophys. Res. Commun. 71:877-884.

10. **Nakae, T.** 1976. Outer membrane of *Salmonella*. Isolation of a protein complex that produces transmembrane channels. J. Biol. Chem. **251**:2176–2178.
11. **Nakae, T., and H. Nikaido.** 1975. Outer membrane as a diffusion barrier in *Salmonella typhimurium*. Penetration of oligo- and polysaccharides into isolated outer membrane vesicles and cells with degraded peptidoglycan layer. J. Biol. Chem. **250**:7359–7365.
12. **Osborn, M. J., J. F. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly on the outer membrane of *Salmonella typhimurium*: isolation and characterisation of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
13. **Rosenbusch, J.** 1974. Characterisation of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulphate binding. J. Biol. Chem. **249**:8019–8029.
14. **Schmitges, C. J., and U. Henning.** 1976. The major proteins of the *E. coli* outer cell-envelope membrane: heterogeneity of protein I. Eur. J. Biochem. **63**:47–52.
15. **Skurray, R. A., R. E. W. Hancock, and P. Reeves.** 1974. Con$^-$ mutants: class of mutants in *Escherichia coli* K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J. Bacteriol. **119**:726–733.
16. **Schnaitman, C. A.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104**:890–901.
17. **Szmelcman, S., and M. Hofnung.** 1975. Maltose transport in *Escherichia coli* K-12: involvement of the bacteriophage lambda receptor. J. Bacteriol. **12**:112–118.
18. **van Alphen, W., B. Lugtenberg, and W. Berendsen.** 1976. Heptose-deficient mutants of *Escherichia coli* K-12 deficient in up to three major outer membrane proteins. Mol. Gen. Genet. **147**:263–269.
19. **Verhoef, V., P. J. de Graaf, and E. J. J. Lugtenberg.** 1977. Mapping of a gene for a major outer membrane protein of *Escherichia coli* K-12 with the aid of a newly isolated bacteriophage. Mol. Gen. Genet. **150**:103–105.
20. **von Meyenburg, K.** 1971. Transport-limited growth rates in a mutant of *Escherichia coli*. J. Bacteriol. **107**:878–888.