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Cell Envelope and Shape of *Escherichia coli*: Multiple Mutants Missing the Outer Membrane Lipoprotein and Other Major Outer Membrane Proteins

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Starting with an *Escherichia coli* strain missing the outer membrane lipoprotein, multiple mutants were constructed that in addition to this defect miss the outer membrane proteins II*, Ia and Ib, or Ia, Ib, and II*. In contrast to all single mutants or strains missing the lipoprotein and polypeptides Ia and Ib, drastic influences on the integrity of the outer membrane and cell morphology were observed in mutants without lipoprotein and protein II*. Such strains exhibited spherical morphology. They required increased concentrations of electrolytes for optimal growth, and Mg^{2+} or Ca^{2+} were the most efficient. These mutants were sensitive to hydrophobic antibiotics and detergents. Electron microscopy revealed abundant blebbing of the outer membrane, and it could clearly be seen that the murein layer was no longer associated with the outer membrane.

On the basis of several observations, it had been hypothesized that the assembly of the outer cell envelope membrane of *Escherichia coli*, specifically, of its major proteins, may participate in the expression of the genetic information determining cellular shape (10, 13). It later became possible to isolate mutants missing several of these major proteins (11), and these mutants did not exhibit a grossly altered morphology, although their outer membranes showed rather drastic changes in composition and ultrastructure (29). The original idea concerning shape determination was therefore dismissed. Recently, a mutant completely lacking the outer membrane lipoprotein (probably because of a deletion in the corresponding structural gene *lpp* [15]) has become available (14). Using this strain, we have constructed mutants that also lack other major outer membrane proteins. We report herein on some properties of such multiple mutants mainly because one class of them did exhibit spherical morphology.

MATERIALS AND METHODS

Culture media, bacterial strains, and selection for phage resistance. Cells were grown in nutrient broth (Difco; no glucose added) or antibiotic medium no. 3 (Difco). The Mg^{2+} concentration in these media was kindly determined by D. Geiseler (Medizinische Universitätsklinik, Tübingen) with a Perkin-Elmer atomic adsorption spectrometer, and it was found to be 0.13 mM in the former and 0.45 mM in the latter medium. Minimal medium (36) containing 0.05% glucose was used for selections in phage P1-mediated transductions (17). The lipoprotein-deficient mutant was that described by Hirota et al. (14), and the *aroD* strain (AB2848; *aroD352 tsx*) was a kind gift from H.-U. Schairer. (Genetic symbols are those of Bachmann et al. [1]). Selection for resistance to phages mentioned in the text was performed by plating 10^7 to 10^8 cells together with 10^9 to 10^{10} phages onto antibiotic medium no. 3, followed by incubation at 37°C.

Cell envelopes and electrophoresis. Cell envelopes were prepared by shaking cells with glass beads according to the method of Braun et al. (4). Sodium dodecyl sulfate-polyacrylamide gel electrophoreses (Laemmli-type slab gel electrophoresis) were performed as described previously (for conditions, see 26). Before electrophoresis, samples were kept for 5 min at 100°C in the presence of sodium dodecyl sulfate and mercaptoethanol.

Test for resistance to antibiotics and detergents. Resistance to antibiotics and detergents was determined by applying the substances in question onto filter paper disks. These were placed on plates (antibiotic medium no. 3) seeded with about 10^7 cells in an overlay of soft agar, and for each antibiotic (see below) 0.05 ml of a solution (1 mg/ml) in 0.85% NaCl was added to the filter paper. The same volume of aqueous solutions of sodium dodecyl sulfate (2%) or deoxycholate (2%) was used. Plates were incubated for 8 to 10 h at 37°C. It should be noted that the results greatly depended on the medium used. For example, *ompA* mutants lacking protein II* scored sensitive to ethylenediaminetetraacetic acid on nutrient broth but not on antibiotic medium no. 3. Similarly, such mutants scored sensitive to novobiocin on a proteose peptone-beef extract medium (5) but not on antibiotic medium no. 3. We have used the latter because it accentuated the difference between *lpp ompA* double mutants (see below) and the other strains investigated.

Electron microscopy. Cells were prefixed with 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, (both at 4°C for 1 h), dehydrated with ethanol, stained with 2% uranyl acetate, and finally embedded in Epon. Thin sections were stained with lead citrate and observed at 60 kV with a Philips 201 electron microscope.

RESULTS

Construction of transductants. During initial attempts to obtain a mutant without lipoprotein and protein II* (the latter is identical with proteins 3a [27], B [24], d [18], 0-10 [22] and 7 [30]) selection for resistance to phage TuII* (11) was performed in the *lpp* strain. Such selection normally results in mutants, most of which lack or possess greatly reduced amounts of protein II* (structural gene: *ompA*; 7, 12, 20). Phage TuII*-resistant mutants of this type were not recovered in the *lpp* background. *lpp* is cotransducible with *aroD* (14). Therefore, an *aroD* strain was made resistant to phage TuII*, and several such *ompA* mutants analyzed lacked protein II*. Phage P1 grown on the *lpp* (*aroD*$^+$) mutant was used to infect the *aroD* and *aroD ompA* strains. Selection for *aroD*$^+$ in the former case yielded *lpp* transductants with the reported frequency (14). However, *lpp ompA* recombinants were not recovered from the latter transduction, although 55 transductants were examined. The *aroD-lpp* cotransduction frequency is about 50% (14), and thus it should have been possible to find the desired double mutants among these transductants. Before concluding that this type of double mutant is not viable, several protective media were tested (osmotic protection by 0.3 or 0.4 M sucrose, addition of horse serum, increasing the concentration of divalent cations, and combinations of these additives). The desired *lpp-ompA* recombinant was, via the transduction mentioned, obtained by increasing the Mg^{2+} concentration of the minimal medium from 1 to 10 mM. Colonies of wild-type, *lpp*, and *lpp-ompA* mutants were discernible on such minimal glucose plates. *lpp* colonies were more pale and translucent than wild type, and *lpp ompA* colonies were smaller and had a glistening, somewhat mucoid appearance.

lpp mutants missing the closely related polypeptides Ia and Ib (26; these porins [21] are identical with proteins 1a/1b [2], b/c [18], 0-9/0-8 [22], A_1/A_2 [24], 4 [30], and Rosenbusch's matrix protein [25]) were obtained without difficulty. The original *lpp* mutant (then called *lpo* [14]) was made resistant to phage TuIb and, subsequently, to phage TuIa. As expected, most of these TuIa^r TuIb^r strains, in addition to their lacking the lipoprotein, lacked the two proteins in question (6). To obtain all mutants in a nearly isogenic state, *lpp* TuIa^r TuIb^r (Ia$^-$ Ib$^-$) strains were also constructed in the *aroD* background, following the same procedure outlined for *lpp ompA* transductants. Finally, by selection for resistance to phage TuII* in an *aroD* TuIa^r TuIb^r (Ia$^-$ Ib$^-$) mutant, derivatives also missing protein II* were obtained, and transduction with the *aroD*$^+$ *lpp* strain as donor yielded recombinants without proteins Ia, Ib, and II* and the lipoprotein. The same increased requirement for Mg^{2+} was found as mentioned above for *lpp ompA* transductants. Cell envelope protein patterns of the three types of mutants are shown in Fig. 1.

Properties of multiple mutants. In nutrient broth or antibiotic medium no. 3 at 30 or 37°C, *lpp ompA* transductants (in contrast to all parental strains or *lpp* Ia$^-$ Ib$^-$ mutants) grew with an almost uniform spherical morphology. In nutrient broth such strains required about 30

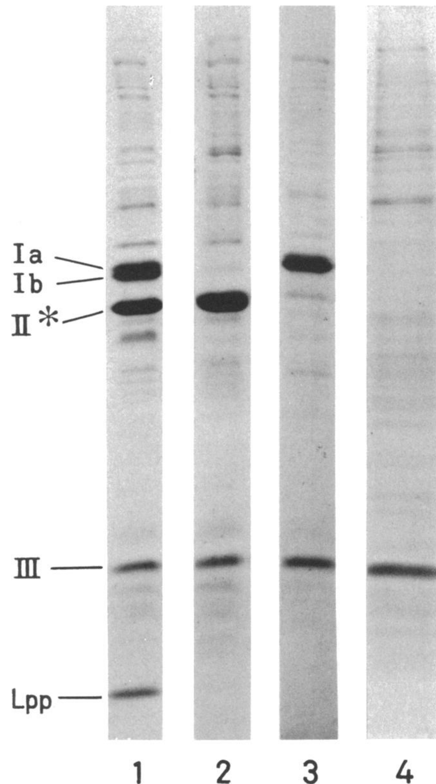

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoreses. All strains were grown at 30°C in a complete medium (antibiotic no. 3, Difco) containing 20 mM $MgSO_4$. Envelopes were prepared and subjected to electrophoresis as described in the text. 1, Wild type (aroD); 2, aroD$^+$ lpp TuIa^r TuIb^r; 3, aroD$^+$ lpp ompA; 4, aroD$^+$ lpp ompA TuIa^r TuIb^r. Lpp, Lipoprotein. For the outer membrane protein III, see reference 9.*

mM $MgCl_2$ or $MgSO_4$ for optimal growth, and 30 mM $CaCl_2$ supported growth equally well. NaCl or KCl at the same ionic strength also supported growth, although the divalent cations allowed three to four times faster growth rates. Without added electrolyte, cells lysed. In antibiotic medium no. 3 the double mutant grew, albeit very slowly, and increasing the concentration of Mg^{2+} ions to 20 to 30 mM stimulated growth as in nutrient broth; the main difference between the two media concerning growth of the mutant probably was in their Mg^{2+} content (see Materials and Methods). Osmotic protection was not required on any medium; on the contrary, addition of 0.4 M sucrose strongly inhibited growth of the double mutant.

Upon repeated subculturing, spherical morphology was lost in several cases, and such strains grew as a mixture of swollen rods and eggs. The mechanism of this suppression has remained unknown because changes were not detected in the electrophoretic protein profiles of envelopes from such derivatives.

Electron microscopy of the *lpp ompA* transductant revealed abundant formation of outer membrane blebs (Fig. 2), a defect known to occur in the *lpp* strain or in another mutant containing greatly reduced amounts of the murein-bound form of the lipoprotein (37) only upon Mg^{2+} starvation. From Figs. 2 and 3 it also appears quite clear that the murein layer is no longer associated with the outer membrane. This

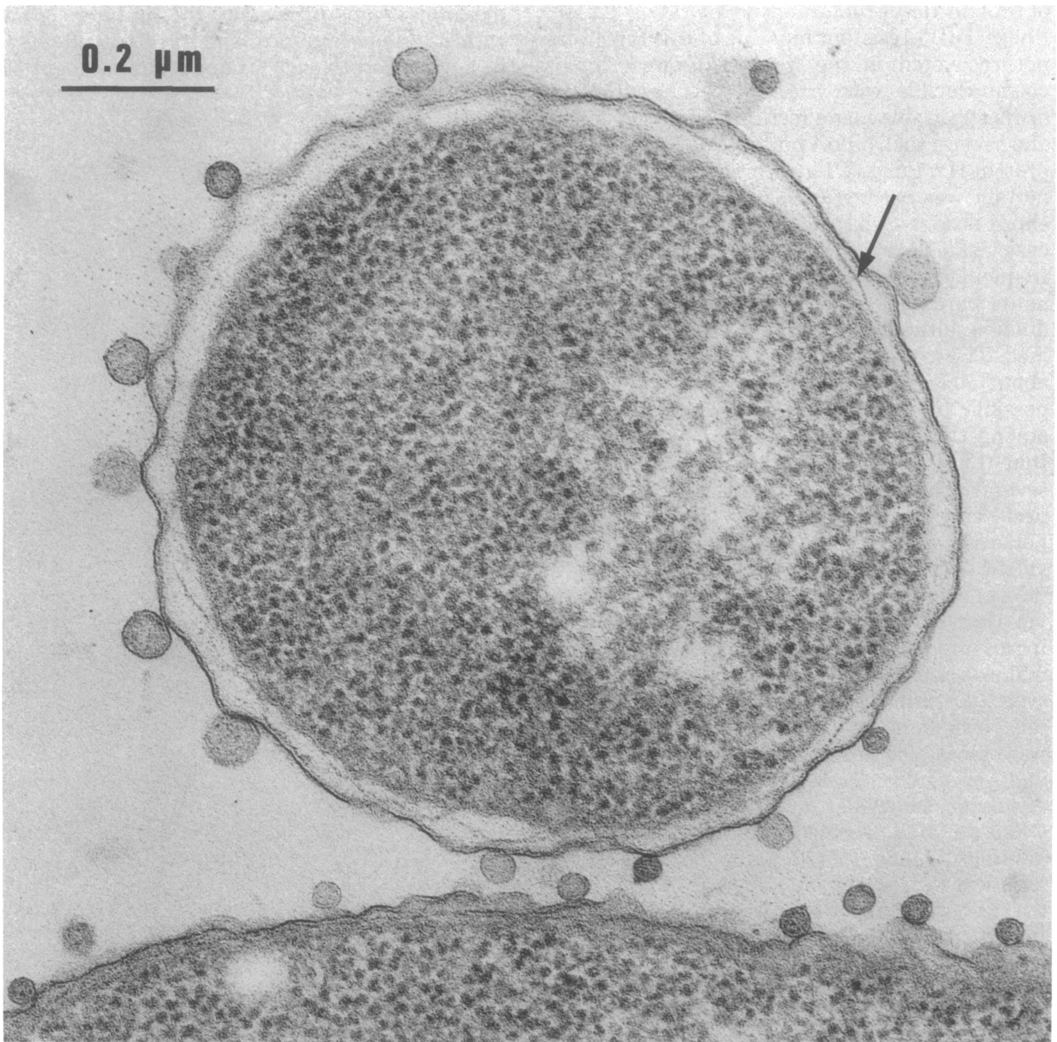

FIG. 2. *Electron micrograph of a thin section of the lpp ompA double mutant showing extensive outer membrane blebbing and a "free-floating" murein layer (arrow).*

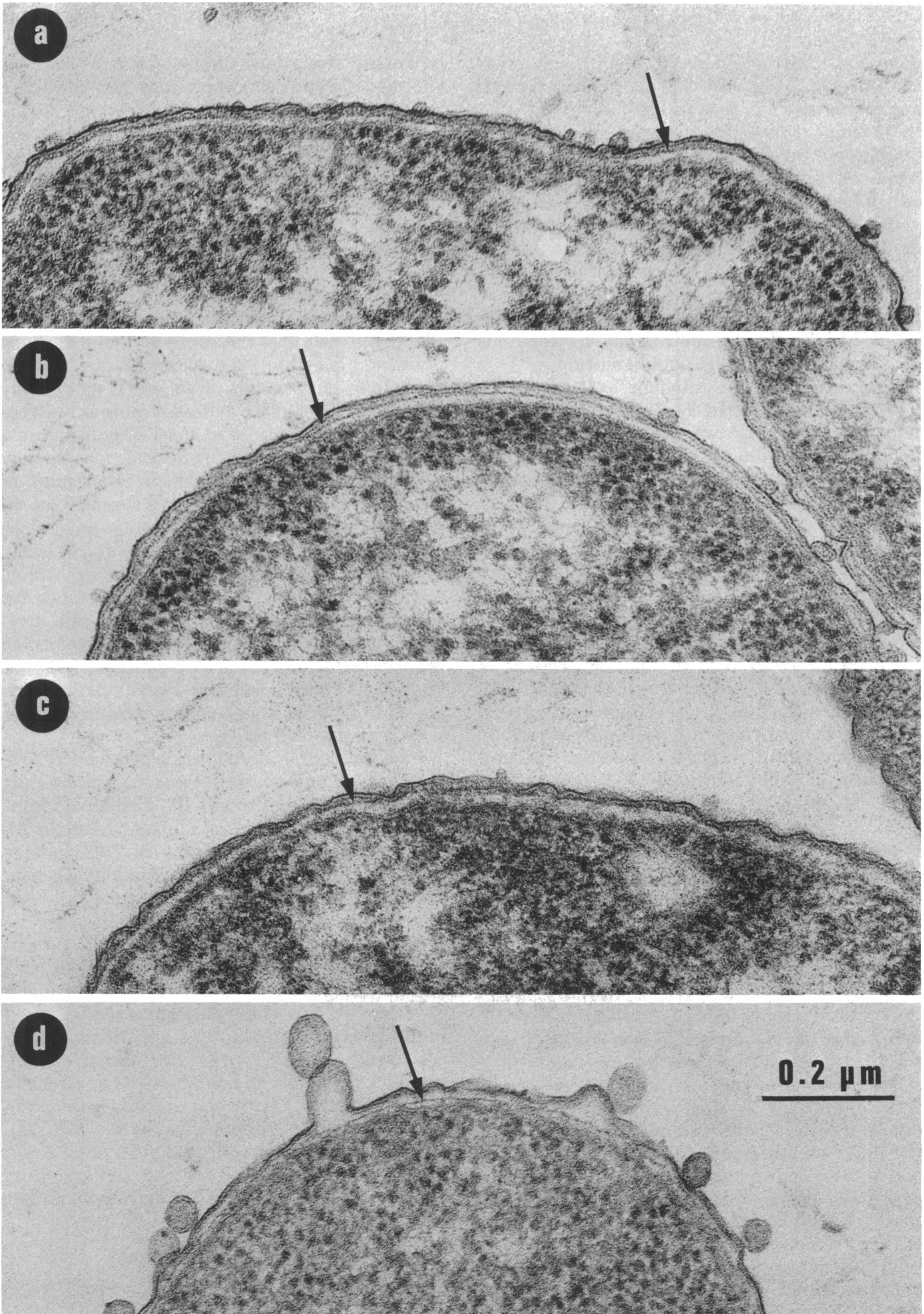

FIG. 3. *Comparison of thin sections of (a) wild type (the aroD strain used for the construction of the following mutants) with the: (b) lpp mutant; (c) lpp mutant also missing polypeptides Ia and Ib; and (d) lpp ompA mutant. Arrows show the murein layer.*

defect has only occasionally (Fig. 3), and never that clearly, been observed in the *lpp* parent, and it was also not seen in *lpp* TuIa^r TuIb^r (Ia$^-$ Ib$^-$) strains. Freeze-fracturing of the *lpp ompA* double mutant did not show differences in the behavior of the parents or other *E. coli* strains (e.g., see 3, 29, 35).

lpp TuIa^r TuIb^r (Ia$^-$ Ib$^-$) mutants showed no defects other than those described for the *lpp* parent (14) concerning physiological or structural parameters mentioned so far. Likewise, the mutant missing the lipoprotein and polypeptides Ia, Ib, and II* did not differ in any very obvious way from the *lpp ompA* double mutant.

The rather drastic influence exerted on the outer membrane by the loss of both the lipoprotein and protein II* was also apparent in the behavior of this double mutant towards several hydrophobic antibiotics (cf. 23) and detergents. According to the simple spot test used (see Materials and Methods), all parental mutants as well as *lpp* TuIa^r TuIb^r (Ia$^-$ Ib$^-$) mutants scored resistant to actinomycin D, bacitracin, novobiocin, rifampin, deoxycholate, and dodecyl sulfate. The *lpp ompA* double mutant was clearly sensitive to all agents.

Finally, and because of the involvement of penicillin-binding protein 2 in the expression of cell shape (32, 33), the penicillin-binding proteins were analyzed (33) in the *lpp ompA* strain, and differences from the two parents were not detected (data not shown).

DISCUSSION

It has not been formally excluded that the *lpp* mutant is in fact a double mutant, that a second mutant site is cotransduced with *lpp*, and that the combination of *ompA* with this hypothetical second site is responsible for, e.g., the altered morphology. However, spherical morphology and outer membrane blebbing have also been observed in double mutants missing protein II* and harboring greatly reduced amounts of only the murein-bound form of the lipoprotein (37).

An interaction between this murein-bound form and protein II* thus appears to be of great importance for the maintenance of integrity of the outer membrane and generation of normal cell shape. Such an interaction for the expression of cellular shape cannot, however, be represented by any simple linear sequence in membrane assembly, because the aberrations mentioned were not observed in the parental single mutants. In line with this notion is the fact that partial suppression of spherical morphology has been found without the lipoprotein or protein II* being present.

Loss of the two proteins described clearly led to drastic alterations of the outer membrane, and the effect on cell morphology may be rather indirect. For example, the morphological evidence shown in Fig. 2 and 3 would be consistent with an unbalanced synthesis of outer membrane constituents in relation to murein synthesis. That is, loss of the two proteins may affect murein synthesis, and such an alteration in turn may have still other effects ultimately leading to spherical morphology. There are, however, topological relationships which it may be worthwhile to consider. The involvement of penicillin-binding protein 2 in the expression of cell shape is beyond question (32). Although the function of this protein remains unknown, it most likely represents an enzyme acting in murein metabolism, and it is likely that most *E. coli* mutants described that exhibit spherical morphology (*rod* mutants; see 16) in one way or another affect this metabolism. We have recently shown that protein II* in the cell envelope is in close contact with the murein layer (8), and it is the murein-bound form of the lipoprotein which, together with protein II*, is of importance for normal morphology. Thus, the three cell envelope components now known to be involved in shape expression are close neighbors. It is not known, of course, whether these relationships are of functional importance regarding generation of normal cell shape.

Finally, a fairly interesting aspect concerning functions of major outer membrane proteins has become apparent. Protein II*, besides its nonphysiological function as a phage receptor (6, 34), can serve as a mediator in F-dependent conjugation (28, 31, 34), appears to play a role in the uptake of amino acids (19), and, at least together with the lipoprotein, is required for the structural integrity of the outer membrane and the generation of normal cell shape. Thus, this protein is multifunctional at very different levels of organization. The receptor activities need nothing but the protein together with lipopolysaccharide (6, 34). Likewise, its (mechanistically unknown) function in conjugation can partially be demonstrated with the isolated protein in combination with lipopolysaccharide (28, 34). The structural role described here, however, represents an integral cellular function requiring interaction (not necessarily direct) with another polypeptide.

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LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.

2. **Bassford, P. J., D. L. Diedrich, C. A. Schnaitman, and P. Reeves.** 1977. Outer membrane proteins of *Escherichia coli*. VI. Protein alteration in bacteriophage-resistant mutants. J. Bacteriol. **131**:608-622.
3. **Bayer, M. E., and C. C. Remsen.** 1970. Structure of *Escherichia coli* after freeze-etching. J. Bacteriol. **101**:304-313.
4. **Braun, V., H. Gnirke, U. Henning, and K. Rehn.** 1973. Model for the structure of the shape-maintaining layer of the *Escherichia coli* cell envelope. J. Bacteriol. **114**:1264-1270.
5. **Chai, T. and J. Foulds.** 1974. Demonstration of a missing outer membrane protein in *tolG* mutants of *Escherichia coli*. J. Mol. Biol. **85**:465-474.
6. **Datta, D. B., B. Arden, and U. Henning.** 1977. Major proteins of the *Escherichia coli* outer cell envelope membrane as bacteriophage receptors. J. Bacteriol. **131**:821-829.
7. **Datta, D. B., C. Krämer, and U. Henning.** 1976. Diploidy for a structural gene specifying a major protein of the outer cell envelope membrane from *Escherichia coli* K-12. J. Bacteriol. **128**:834-841.
8. **Endermann, R., C. Krämer, and U. Henning.** 1978. Major outer membrane proteins of *Escherichia coli* K-12: evidence for protein II* being a transmembrane protein. FEBS Lett. **86**:21-24.
9. **Garten, W., I. Hindennach, and U. Henning.** 1975. Major proteins of the *Escherichia coli* outer cell envelope membrane. Characterization of proteins II* and III, comparison of all proteins. Eur. J. Biochem. **59**:215-221.
10. **Haller, I., and U. Henning.** 1974. Cell envelope and shape of *Escherichia coli* K12. Crosslinking with dimethyl imidoesters of the whole cell wall. Proc. Natl. Acad. Sci. U.S.A. **71**:2018-2021.
11. **Henning, U., and I. Haller.** 1975. Mutants of *Escherichia coli* K12 lacking all "major" proteins of the outer cell envelope membrane. FEBS Lett. **55**:161-164.
12. **Henning, U., I. Hindennach, and I. Haller.** 1976. The major proteins of the *Escherichia coli* outer cell envelope membrane: evidence for the structural gene of protein II*. FEBS Lett. **61**:46-48.
13. **Henning, U., K. Rehn, and B. Hoehn.** 1973. Cell envelope and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. **70**:2033-2036.
14. **Hirota, Y., H. Suzuki, Y. Nishimura, and S. Yasuda.** 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. U.S.A. **74**:1417-1420.
15. **Inouye, S., N. Lee, M. Inouye, H. C. Wu, H. Suzuki, Y. Nishimura, H. Iketani, and Y. Hirota.** 1977. Amino acid replacement in a mutant lipoprotein of the *Escherichia coli* outer membrane. J. Bacteriol. **132**:308-313.
16. **Iwaya, M., W. Jones, J. Khorana, and J. L. Strominger.** 1978. Mapping of the mecillinam-resistant, round morphological mutants of *Escherichia coli*. J. Bacteriol. **133**:196-202.
17. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virol. **1**:190-206.
18. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoeck, 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.
19. **Manning, P. A., A. P. Pugsley, and P. Reeves.** 1977. Defective growth functions in mutants of *Escherichia coli* K12 lacking a major outer membrane protein. J. Mol. Biol. **116**:285-300.
20. **Manning, P. A., A. Puspurs, and P. Reeves.** 1976. Outer membrane of *Escherichia coli* K-12: isolation of mutants with altered protein 3A using host range mutants of bacteriophage K3. J. Bacteriol. **127**:1080-1084.
21. **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.
22. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. **80**:1411-1422.
23. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta **433**:118-132.
24. **Reithmeier, R. A. F., and P. D. Bragg.** 1977. Crosslinking of the proteins in the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta **466**:245-256.
25. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. J. Biol. Chem. **249**:8019-8029.
26. **Schmitges, C. J., and U. Henning.** 1976. The major proteins of the *Escherichia coli* outer cell envelope membrane. Heterogeneity of protein I. Eur. J. Biochem. **63**:47-52.
27. **Schnaitman, C. A.** 1974. Outer membrane proteins of *Escherichia coli*. III. Evidence that the major protein of *Escherichia coli* O111 outer membrane consists of four distinct polypeptide species. J. Bacteriol. **118**:442-453.
28. **Schweizer, M., and U. Henning.** 1977. Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. J. Bacteriol. **129**:1651-1652.
29. **Schweizer, M., H. Schwarz, I. Sonntag, and U. Henning.** 1976. Mutational change of membrane architecture. Biochim. Biophys. Acta **448**:474-491.
30. **Sekizawa, J., S. Inouye, S. Halegoua, and M. Inouye.** 1977. Precursors of major outer membrane proteins of *Escherichia coli*. Biochem. Biophys. Res. Commun. **77**:1126-1133.
31. **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**:726-735.
32. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. **72**:2999-3003.
33. **Spratt, B. G., and A. B. Pardee.** 1975. Penicillin-binding proteins and cell shape in *E. coli*. Nature (London) **254**:516-517.
34. **van Alphen, L., L. Havekes, and B. Lugtenberg.** 1977. Major outer membrane protein d of *E. coli* K12. Purification and *in vitro* activity on bacteriophage K3 and F-pilus mediated conjugation. FEBS Lett. **75**:285-290.
35. **van Gool, A. P., and N. Nanninga.** 1971. Fracture faces in the cell envelope of *Escherichia coli*. J. Bacteriol. **108**:474-481.
36. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97-106.
37. **Yem, D. W., and H. C. Wu.** 1978. Physiological characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein. J. Bacteriol. **133**:1419-1426.