JOURNAL OF BACTERIOLOGY, June 1978, p. 1089-1098
0021-9193/78/0134-1089$02.00/0

Vol. 134, No. 3
Printed in U.S.A.

Architecture of the Outer Membrane of *Escherichia coli*

III. Protein-Lipopolysaccharide Complexes in Intramembraneous Particles

LOEK VAN ALPHEN,[1]* ARIE VERKLEIJ,[2] JOSÉ LEUNISSEN-BIJVELT,[2] AND BEN LUGTENBERG[1]

Department of Molecular Cell Biology, Microbiology[1] and Electron Microscopy[2] Sections, and Institute for Molecular Biology, State University of Utrecht, Padualaan 8, Utrecht, The Netherlands

Received for publication 21 December 1977

In a previous paper (A. Verkleij, L. van Alphen, J. Bijvelt, and B. Lugtenberg, Biochim. Biophys. Acta **466**:269-282, 1977) we have hypothesized that particles on the outer fracture face of the outer membrane (OM), with corresponding pits on the inner fracture face of the outer membrane (OM), consist of lipopolysaccharide (LPS) aggregates stabilized by divalent cations and that they might contain protein and/or phospholipid. In the present paper the roles of LPS, cations, and proteins in these OM particles are described more extensively, using a strain that lacks the major outer membrane proteins, b, c, and d (b^- c^- d^-), and has a reduction in the number of OM particles of 75%. To study the role of divalent cations in the formation of OM particles, these b^- c^- d^- cells were grown or incubated with Ca^{2+}, Mg^{2+}, or putrescine. The presence of Ca^{2+} resulted in the appearance of many OM particles and OM pits. Mg^{2+} and putrescine were less effective than Ca^{2+}. Introduction of these particles was not accompanied by alterations in the relative amounts of LPS and cell envelope proteins. Ca^{2+} treatment of a heptoseless derivative of a b^- c^- d^- strain did not result in morphological changes. Incubation of Ca^{2+}-treated cells with ethylenediaminetetraacetate caused the disappearance of the introduced particles as well as the release of more than 60% of the cellular LPS. These results strongly support the hypothesis that LPS is involved in the formation of OM particles and OM pits. The roles of various outer membrane proteins in the formation of OM particles were studied by comparing the freeze-fracture morphology of b^- c^- d^- cells with that of cells which contain one of the outer membrane proteins b, c, d, and e or the receptor protein for bacteriophage lambda. The results showed that the presence of any of these five proteins in a b^- c^- d^- background resulted in a large increase in the number of OM particles and OM pits, indicating that these proteins are, independent of each other, involved in the formation of OM particles and OM pits. The simplest explanation for the results is that in wild-type cells each particle consists of LPS complexed with some molecules of a single protein species, stabilized by either divalent cations or polyamines. It is hypothesized that the outer membrane of the wild-type cell contains a heterogeneous population of particles, of which 75% consists of protein b-LPS, protein c-LPS, and protein d-LPS particles. A function of these particles as aqueous pores is proposed.

The outer membranes of a number of gram-negative bacteria have been investigated by freeze-fracture electron microscopy. In wild-type cells of *Escherichia coli* (1, 16, 21, 30, 31), *Salmonella typhimurium* (17, 24), *Pseudomonas aeruginosa* (6-8), and *Acinetobacter* (23), the concave or outer fracture face of the outer membrane (OM) is covered with particles 4 to 10 nm in diameter. In previous papers we have shown that on the corresponding convex or inner fraction face (OM) of *E. coli* K-12 many pits are visible, which are probably complementary to the particles (30, 31). Particles complementary to pits have been interpreted in freeze-fracture terms as micelle-like structures (32).

Studies with mutants of *E. coli* and *S. typhimurium* which are lacking one or more major outer membrane proteins showed that the number of OM particles is reduced (up to 75%) (17, 21, 30, 31). Moreover, some mutants with a shortened lipopolysaccharide (LPS) sugar chain showed the same phenomenon (24, 30, 31). In addition, we observed in *E. coli* K-12 that a reduction of the number of OM pits coincides with a reduction in OM particles (30).

Treatment of wild-type cells of *E. coli* K-12

with ethylenediaminetetraacetate (EDTA) caused (i) the release of half of the cellular LPS but no release of protein and (ii) a reduction of the number of $\breve{OM}$ particles and $\widehat{OM}$ pits of about 50% (30). A reduction of the number of $\breve{OM}$ particles coinciding with release of LPS was also observed in *P. aeruginosa* (6, 7). However, in the latter species part of the cell envelope protein is extracted together with LPS (6, 19, 24).

From the complementarity aspect of the $\breve{OM}$ particles and $\widehat{OM}$ pits and from the observed reduction in the number of particles in mutants and in wild-type cells treated with EDTA, it was hypothesized that in *E. coli* K-12 the $\breve{OM}$ particles contain aggregates of LPS stabilized by divalent cations and possibly containing protein and/or phospholipid (30).

In the present paper the roles of LPS, cations, and proteins in the $\breve{OM}$ particles are described more extensively. Strain P6922dI was chosen for these experiments, because it lacks major outer membrane proteins b, c, and d ($b^- c^- d^-$ mutant), and it has a reduction in particle density of 75% (9, 21, 30). Therefore, it allows the introduction of both particles and pits either by the presence of divalent cations or the polyamine putrescine or by the "insertion" of defined proteins in the outer membrane. The results strongly support the hypothesis mentioned previously and show that the hypothesis can be extended in that the majority of the particles contain protein-LPS aggregates.

MATERIALS AND METHODS

Strains and growth conditions. All strains are derivatives of *E. coli* K-12. Origins and relevant characteristics are listed in Table 1. Strain P692*tut*2dI, subsequently abbreviated as strain P6922dI, is missing major outer membrane proteins b, c, and d, which are compensated for by increased amounts of (wild type) LPS and of phospholipids (28).

Unless otherwise indicated, bacteria were grown in standard tris(hydroxymethyl)aminomethane (Tris)-based medium (22, 30), supplemented with 0.5% glucose–0.2% Casamino Acids–20 μg of tryptophan, uracil, thymine, adenine, and guanine per ml–5 μg thiamine per ml. $CaCl_2$ or $MgCl_2$ was present in a final concentration of 0.2 mM. In some cases the concentration of $CaCl_2$ or $MgCl_2$ was increased to 2 mM, or 10 mM putrescine was added to the standard medium. Cells were grown to the late logarithmic phase at 37°C. The cells were harvested at 4°C, washed with 0.12 M Tris-hydrochloride (pH 8.0), and used for further incubation, chemical analyses, or freeze-fracturing.

Incubations and analytical procedures. To examine the influence of incubation with divalent cations or EDTA on the freeze-fracture morphology of the cells, the bacteria were washed with 0.12 M Tris-hydrochloride (pH 8.0), resuspended in 1/20 volume of the same buffer, and further treated either for 15 min at 37°C in the presence of 50 mM $CaCl_2$ or $MgCl_2$ or for 5 min at 37°C with 5 mM EDTA as described by Leive et al. (11). Cells were harvested by centrifugation for 15 min at 4°C at 3,000 × *g* and used for freeze-fracturing. Released LPS was quantitatively isolated from the supernatant solution by the addition of $CaCl_2$ and acetone (in final concentrations of 20 mM and 70%, respectively) at 4°C, followed by centrifugation. The pellet was washed once with 70% acetone.

Cell envelopes were isolated quantitatively as described before (13) except that EDTA was omitted. Protein was determined according to the method of Lowry et al. (12). Total cell protein was determined after ultrasonic treatment of the cells. The amount of cell envelope protein was expressed per milligram of total cell protein. 3-Deoxy-D-mannooctulosonic acid was determined by the thiobarbituric acid method of Weissbach and Hurwitz (34) as modified by Osborn (18) and corrected for the fact that only two of the three 3-deoxy-D-mannooctulosonic acid molecules are measured (5). The amount of LPS was calculated by using our previous data, which showed that LPS contains 11% 3-deoxy-D-mannooctulosonic acid (wt/wt). The protein patterns of the various fractions were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described before (13).

Freeze-fracture electron microscopy. To the cell suspension 25% glycerol in 0.9% NaCl was added as a cryoprotectant. The fracture faces were not influenced by the presence of glycerol. The samples were quenched from 4°C in a mixture of liquid and solid nitrogen and fractured in a Denton freeze-etch appa-

TABLE 1. *Strains and relevant characteristics*

Strain	Outer membrane protein abnormalities	Source, ref.
AB1133	Wild type	P. Reeves and ref. 28
P692*tut*2dI	Protein $b^- c^- d^-$ derivative of AB1133	U. Henning and ref. 9 and 28
CE1097	Heptoseless derivative of P6922dI	This paper
CE1096	Spontaneous protein b-proficient revertant of P6922dI	This paper
CE1150	Spontaneous protein c-proficient revertant of P6922dI	This paper
CE1071	d^+ exconjugant of P6922dI	Ref. 28
CE1145	$b^- c^- d^-$ derivative of PC0479	C. Verhoef and W. van Alphen
CE1146[a]	$b^- c^- d^- e^+$ derivative of PC0479	C. Verhoef and W. van Alphen

[a] Protein e is a major peptidoglycan-associated protein with an apparent molecular weight of 40,000. It is probably identical to protein Ic described by Henning et al. (10).

ratus as descibed before (33). Electron micrographs were made with a Siemens Elmiskop 1A. The particle density was estimated as described (30).

RESULTS

Influence of divalent cations and putrescine on the density of OM particles in a mutant lacking proteins b, c, and d. In our previous paper we provided strong evidence for the presence of LPS in OM particles (30). We reasoned that mutant strain P6922dI, which has a strong reduction in the number of OM particles (21, 30) despite its increased LPS content (28), could be used to see whether LPS, in the absence of three major proteins, could be forced to form OM particles by incubating the cells with divalent cations or putrescine. The complex growth media used in our previous studies (30, 31) did not allow us to study the influence of divalent cations, since a precipitate of Ca salts was formed. For the present study cells were grown in standard Tris-based medium. Growth in this medium resulted in essentially the same freeze-fracture morphology as described before in that the OM of the wild-type strain, AB1133, is almost completely covered with particles (Fig. 1) (30), whereas in the b^- c^- d^- mutant P6922dI a reduction of about 75% in the number of OM particles and OM pits (Fig. 2) (21, 30) was observed.

Supplementation of the standard Tris-based medium with 2 mM $CaCl_2$ resulted in large changes in the morphology of the outer membrane of strain P6922dI. The OM of these Ca-grown cells was densely covered with particles, and numerous pits on the OM were seen (Fig. 3). Both the particles and the pits introduced by Ca^{2+} were stable in that they resisted washing of the cells with buffer without $CaCl_2$. Replacing Ca^{2+} in the growth medium with Mg^{2+} or putrescine resulted in a similar effect on the number of particles and pits, although the increase was lower (from 25 to 40%). Each of the three additions to the growth medium resulted in a particle density that differed from cell to cell. The mentioned results are averages of many individual cells. Such a heterogeneity in the cell population has been reported before for all mutants lacking protein d (30). Chemical analysis showed that the morphological differences caused by growth in standard Tris-based medium with various supplementations could be related neither to significant alterations in the amount of LPS or protein of the cell envelope per milligram of cell protein (Table 2) nor to the pattern of the cell envelope proteins on polyacrylamide gels (not shown). In the wild-type strain AB1133, no influence of the mentioned supplementations on the morphology and chemical composition of the outer membranes could be detected.

The morphological effects caused by growth in the presence of 2 mM $CaCl_2$ or $MgCl_2$ could also be observed after incubation of the mutant cells for 15 min in buffer supplemented with 50 mM $CaCl_2$ or $MgCl_2$. These effects were also observed in the presence of chloramphenicol (100 μg/ml), showing that de novo protein synthesis is not required for an increase of the number of particles. The particles and pits ob-

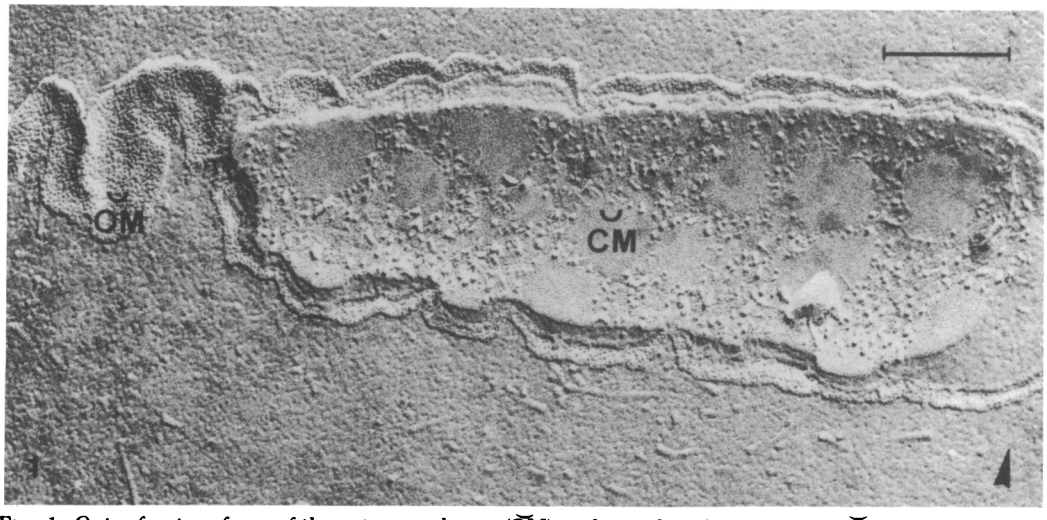

FIG. 1. *Outer fracture faces of the outer membrane (OM) and cytoplasmic membrane (OM) of E. coli K-12 strain AB1133 grown in standard Tris-based medium, quenched from 4°C. Bar = 200 nm. Arrow indicates direction of shadowing.*

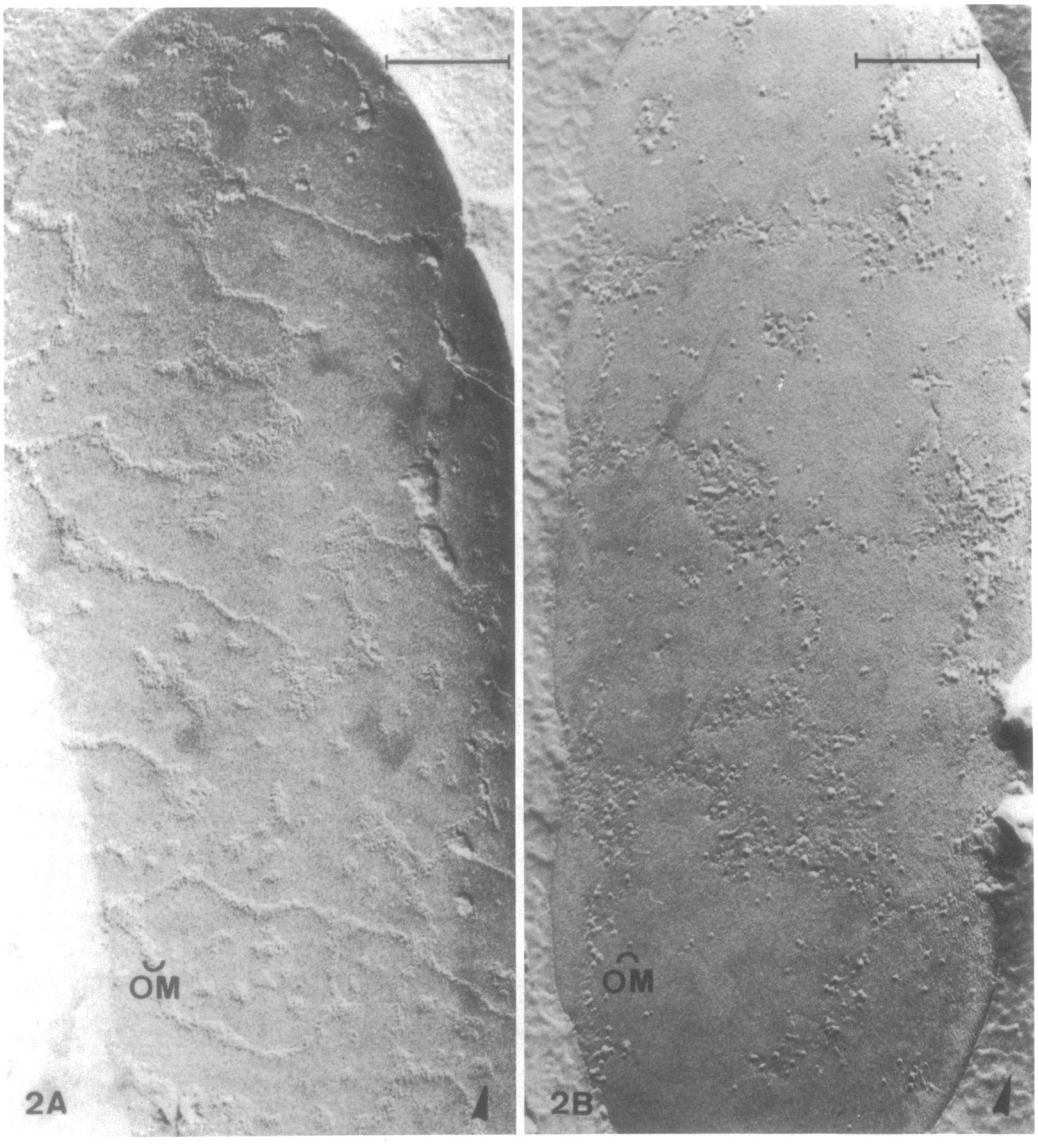

FIG. 2. *Fracture faces of the outer membrane of strain P6922dI, grown in standard Tris-based medium, quenched from 4°C. (A) $\breve{OM}$; (B) $\hat{OM}$. Bar = 200 nm. Arrow indicates direction of shadowing.*

served after incubation with Ca^{2+} or Mg^{2+} were less stable, since they disappeared after washing of the cells with buffer without divalent cations.

In summary, in the outer membrane of strain P6922dI particles and corresponding pits can be introduced either by growth in the presence of or by incubation with divalent cations without detectable alteration in the biochemical composition of the cell envelope. It is likely that in the absence of the major outer membrane proteins b, c, and d, these particles consist of aggregates of LPS stabilized by divalent cations. It is likely that the core region of LPS (possibly the negatively charged phosphate groups) is involved in the aggregation process, since an increase in the number of the $\breve{OM}$ particles and $\hat{OM}$ pits, either by growth or by incubation with Ca^{2+}, could not be observed in strain CE1097, a heptoseless mutant of strain P6922dI.

The roles of LPS and divalent cations in the $\breve{OM}$ particles introduced by divalent cations were further studied by treating the cells with EDTA, which is known to result in the release of about half of the cellular LPS (11, 30). After

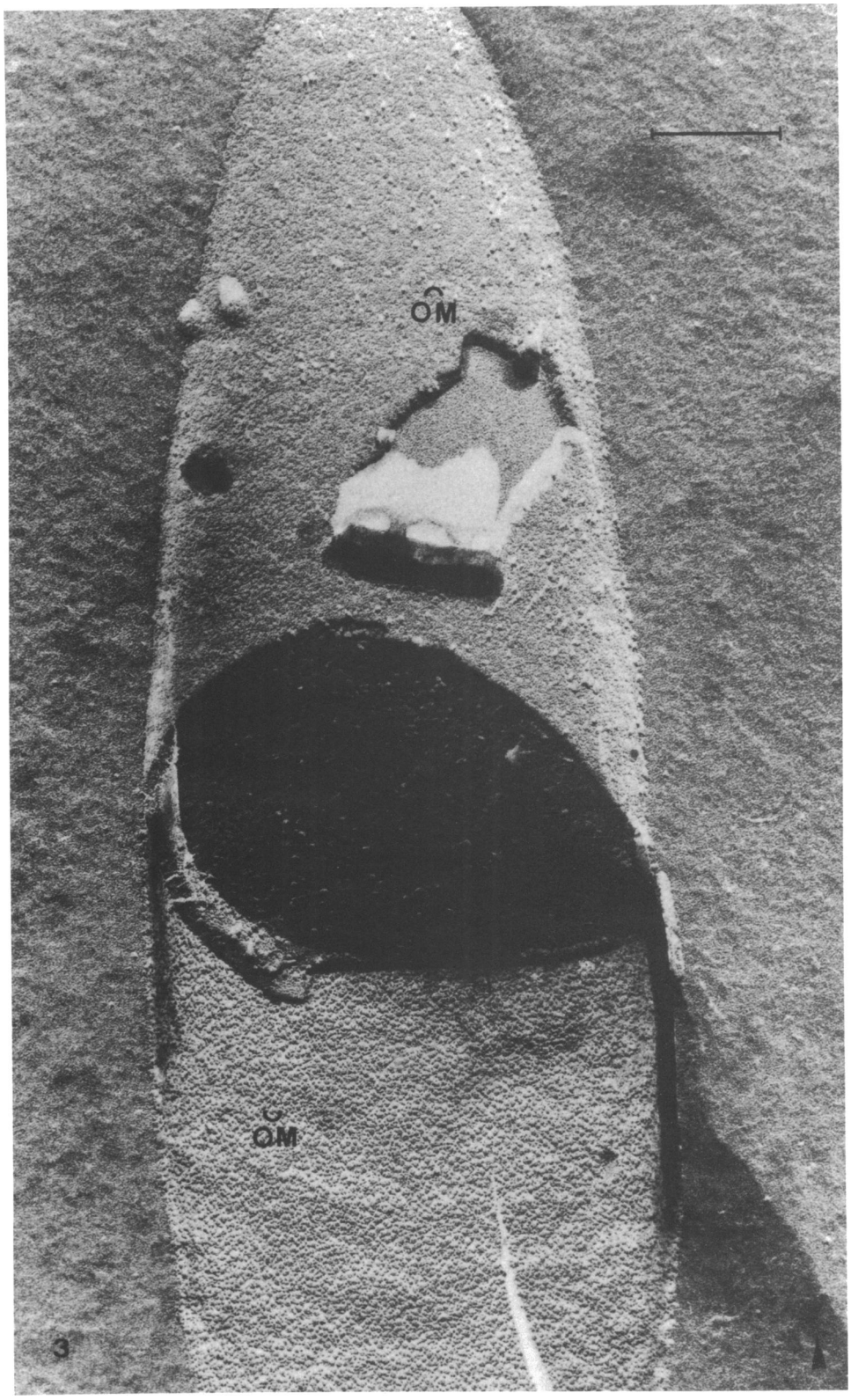

FIG. 3. *Fracture faces of strain P6922dI grown in Ca-supplemented medium, quenched from 4°C. Bar = 200 nm. Arrow indicates direction of shadowing.*

TABLE 2. *Influence of growth medium and EDTA treatment on the relative amounts of LPS and cell envelope protein*[a]

Strain	Addition to growth medium	Outer membrane protein defects	Cell envelope component per mg of cell protein		LPS released after EDTA treatment[b] (% of total LPS)
			LPS (nmol)	Protein[c] (mg)	
AB1133	None	None	6.4	0.25	35
AB1133	$CaCl_2$	None	6.8	0.27 (0.26)[d]	43
P6922dI	None	$b^- c^- d^-$	10.8–12.0	0.19	63
P6922dI	$CaCl_2$	$b^- c^- d^-$	10.6–12.8	0.19 (0.20)[d]	63
P6922dI	$MgCl_2$	$b^- c^- d^-$	13.3	0.20	67
P6922dI	Putrescine	$b^- c^- d^-$	10.7	0.24	ND[e]
P6922dI	Maltose	$b^- c^- d^-$	7.8	0.22	ND
CE1096	None	$b^+ c^- d^-$	8.1	0.20–0.25	ND
CE1150	None	$b^- c^+ d^-$	8.1	0.19	ND

[a] Cells were grown in Tris-based medium supplemented, if indicated, with 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM putrescine, or 0.5% maltose and washed with 0.12 M Tris-hydrochloride, pH 8.0. In some cases the cells were treated with 5 mM EDTA for 5 min at 37°C. Cell envelopes were isolated, and the amounts of LPS and cell envelope protein were determined.

[b] The amounts of LPS were determined in the untreated cell envelopes, in the released material, and in the cells after EDTA treatment. The sum of the latter two values was always equal to the first.

[c] The amount of cell envelope protein per milligram of total cell protein is quite high, compared with the values obtained before (29), since EDTA was omitted during the isolation of the cell envelopes. In this case also some cytoplasmic and periplasmic protein is obtained in the membrane fraction.

[d] The numbers in parentheses represent the values after EDTA treatment of the cells.

[e] ND, Not determined.

growth or incubation with divalent cations, more than 60% of the cellular LPS of strain P6922dI was released by the EDTA treatment (Table 2). The amount of cell envelope protein did not change (Table 2), and the protein patterns of cell envelopes isolated before and after EDTA treatment were identical. EDTA treatment caused the disappearance of the OM particles and the OM pits which were introduced by divalent cations (Fig. 4). The same freeze-fracture morphology was observed after EDTA treatment of cells grown in standard Tris-based medium (not shown). This treatment did not result in a significant reduction in the number of particles. In the latter case EDTA treatment resulted in the release of the same fraction of the cellular LPS as was released from cells grown in the presence of divalent cations (Table 2) and also in the release of 3% of the cellular protein. The gel patterns of the protein released did not resemble those of cell envelopes. Since most bands on the gel also occur in the cytoplasmic fraction, the released protein was due to lysis of a small fraction of cells, most likely. The results described so far with strain P6922dI are schematically summarized in Fig. 5.

Role of proteins in the formation of OM particles. Reports from several laboratories (21, 24, 30) suggest that the outer membrane proteins of *Enterobacteriaceae* play a role in the formation of the OM particles, although the role of the proteins was not understood in detail. The role of different outer membrane proteins in particle formation can be illustrated by studying the effect of inserting a single protein species into the outer membrane of the $b^- c^- d^-$ strain P6922dI, since this strain has a reduction of 75% in particle density. Compared with strain P6922dI, each of the following five strains contained one extra major outer membrane protein: (i) the spontaneous protein b-proficient partial revertant of strain P6922dI (strain CE1096); (ii) the spontaneous protein c-proficient partial revertant of strain P6922dI (strain CE1150); (iii) the protein d-proficient exconjugant CE1071; (iv) strain P6922dI, in which the receptor protein of phage lambda was induced by replacing the glucose of the growth medium with maltose; (v) the $b^- c^- d^- e^+$ strain CE1146 (as protein e is not present in the AB1133 background, the results of strain CE1146 were compared with the $b^- c^- d^-$ parent strain CE1145).

A comparison of the freeze-fracture morphology of strain P6922dI with those of the five other strains is schematically shown in Fig. 6. Starting from a $b^- c^- d^-$ mutant, we find that in all five cases the insertion of each single protein species is accompanied by a strong increase of the number of particles on the OM (from 25% to 50 to 80%) and of the number of pits on the OM. For instance, the effect of the insertion of the phage lambda receptor protein in strain P6922dI can be observed by comparison of Fig. 2 and 7. The insertion of single proteins, all of which were present in high amounts (Fig. 8), resulted in a reduction of about 20% in the amount of LPS

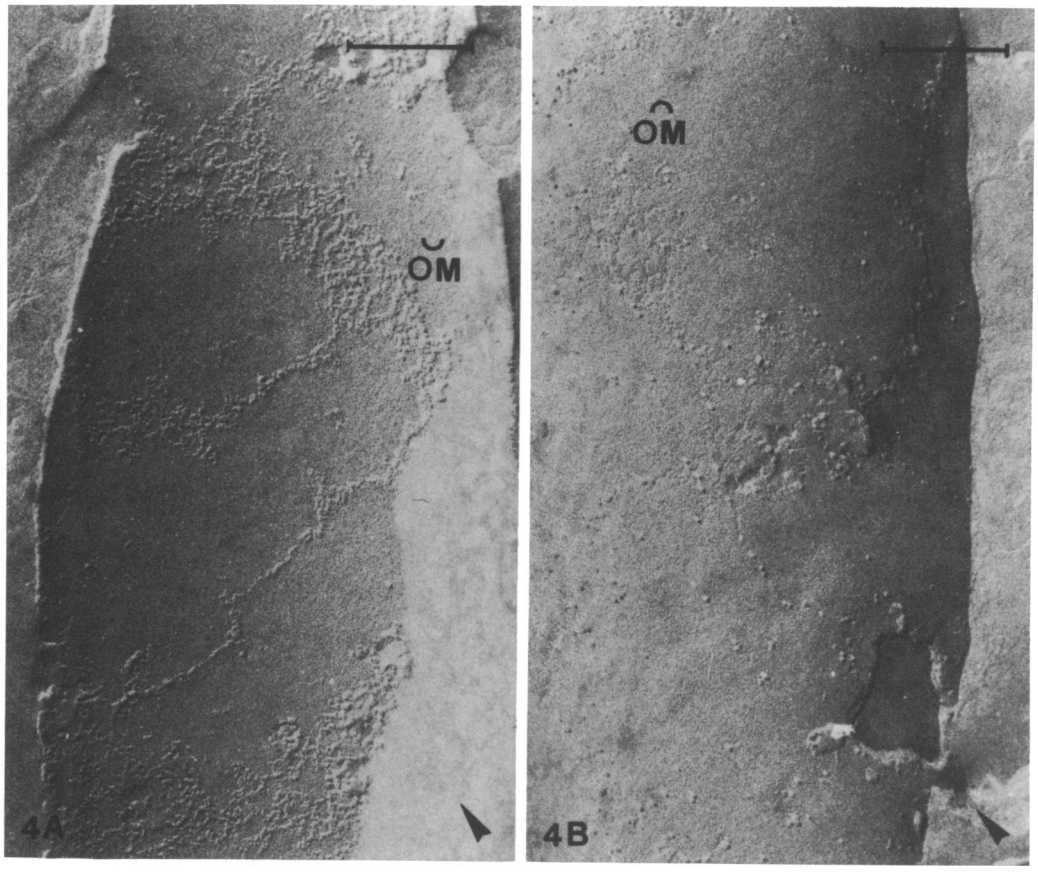

FIG. 4. *Fracture faces of strain P6922dI grown in Ca-supplemented medium, after treatment of the cells with EDTA. (A) OM; (B) OM. Bar = 200 nm. Arrow indicates direction of shadowing.*

per milligram of cell protein (Table 2) (28). The results clearly show that each of the tested outer membrane protein species is involved in the formation of OM particles and OM pits.

DISCUSSION

Involvement of LPS and proteins in OM particles. The results obtained after growing or incubating cells of the b^- c^- d^- mutant strain P6922dI in the presence of divalent cations and after treating these cells subsequently with EDTA, summarized in Fig. 5, strongly suggest that the introduced OM particles consist of LPS aggregates stabilized by Ca^{2+}. The observation that Ca^{2+} introduces both particles and pits in b^- c^- d^- cells strongly suggests that LPS is responsible for the pits in the OM, although it cannot be excluded that other proteins may be marshalled into particles. This observation supports the hypothesis (30) that in non-Ca^{2+}-treated cells pits complementary to particles are caused by micelle-like structures of LPS. The OM particles introduced by Ca^{2+} probably do not occur in substantial amounts in wild-type cells, since in these cells most of the LPS is expected to be complexed with protein.

The results obtained after the insertion of only one of the protein species b, c, d, and e or of the receptor protein of bacteriophage lambda in a b^- c^- d^- background show that this insertion results in an outer membrane which contains many more OM particles with corresponding OM pits (Fig. 6 and 7). The simplest interpretation of these results is that these additional particles represent protein-LPS aggregates and that the protein component of the complex is only one protein species. With respect to the roles of the individual proteins, this interpretation extends our previous hypothesis (30).

Nature of OM particles in wild-type cells. According to our hypothesis, the outer membrane of wild-type cells contains a heterogeneous population of particles, consisting of about 75% of a mixture of b-LPS, c-LPS, and d-LPS particles. Previous freeze-fracture studies with mutants deficient in one, two, or all three of the

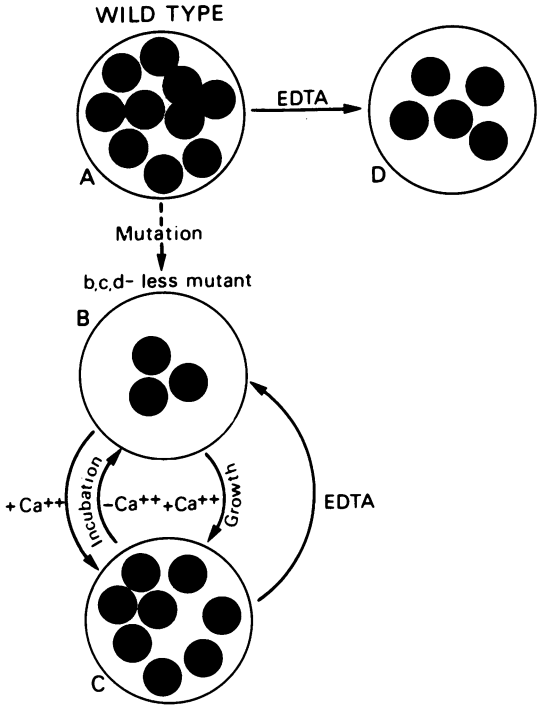

FIG. 5. *Schematic illustration of the effects of divalent cations and EDTA on the freeze-fracture morphology of the outer membrane. OM particles with corresponding OM pits are indicated by black spheres. The OM of parent strain AB1133 is covered with particles (A). The number of OM particles in the b⁻ c⁻ d⁻ strain P6922dI is reduced to 25% (B). Supplementation of the growth medium of strain P6922dI with Ca^{2+} results in an increase in the particle density to 75 to 80% (C). Incubation with Ca^{2+} has the same effect. The particles introduced by growth in the presence of Ca^{2+} or by incubation with Ca^{2+} are removed by EDTA treatment, resulting in the morphology observed for strain P6922dI grown in standard Tris-based medium (B). EDTA treatment of the latter cells hardly influences the morphology (not shown). In contrast, EDTA treatment of wild-type cells results in the disappearance of about half of the OM particles and OM pits (D). EDTA treatment releases more than half of the cellular LPS (see Table 2).*

proteins b, c, and d showed that the number of particles in strains missing a single protein or in b⁻ c⁻ mutants was almost normal, whereas the number of particles in a b⁻ c⁻ d⁻ strain was strongly decreased (21, 30, 31). Although the latter result suggested a role of these outer membrane proteins, the results obtained with the single-protein mutants and with b⁻ c⁻ strains did not allow solid conclusions with respect to the roles of the individual proteins (21, 30, 31). However, it has been reported that the lack of one or two of the proteins b, c, and d is often compensated for by increased amounts of one or two of the remaining proteins, but such a compensation was not observed when all three, proteins b, c, and d, are lacking (3, 9, 20, 21, 29). With our present hypothesis, the mentioned compensation effects by proteins are interpreted in terms of compensation by OM particles. Taking a b⁻ c⁻ strain as an example, it is known that such a strain contains increased amounts of protein d (9, 20, 21, 29), whereas its outer membrane contains a normal number of OM particles (21, 30). According to our hypothesis, the latter result was obtained because the missing b-LPS and c-LPS particles are compensated for by increased amounts of (morphologically similar) d-LPS particles.

The involvement both of LPS and of the proteins b, c, d, and e and the lambda receptor protein in particle formation predicts that these proteins interact with LPS. Interactions of the proteins b, c (4; unpublished results cited in

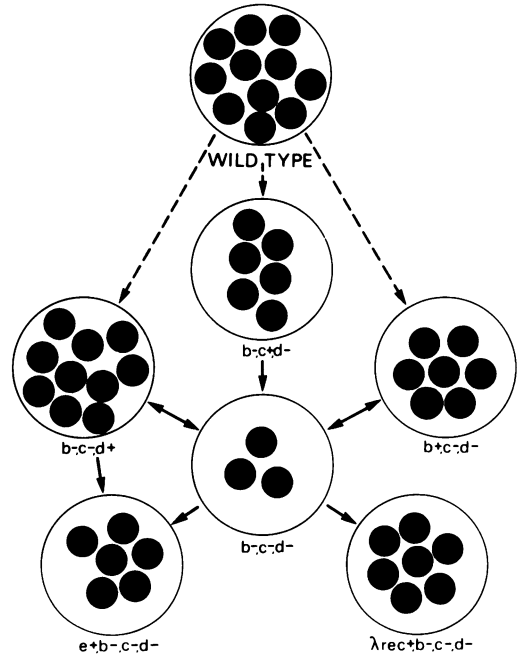

FIG. 6. *Schematic representation of the effect of the presence of proteins b, c, d, and e and the phage lambda receptor protein on the freeze-fracture morphology of the outer membrane. Symbols are as in Fig. 5. The b⁻ c⁻ d⁻ strain P6922dI, a derivative of wild-type strain AB1133, has an extreme reduction in the density of OM particles and corresponding OM pits. This figure illustrates that, starting from the b⁻ c⁻ d⁻ strain, the acquisition of protein b, c, d, or e or the lambda receptor protein results in a strong to extensive increase in the numbers of OM particles and OM pits.*

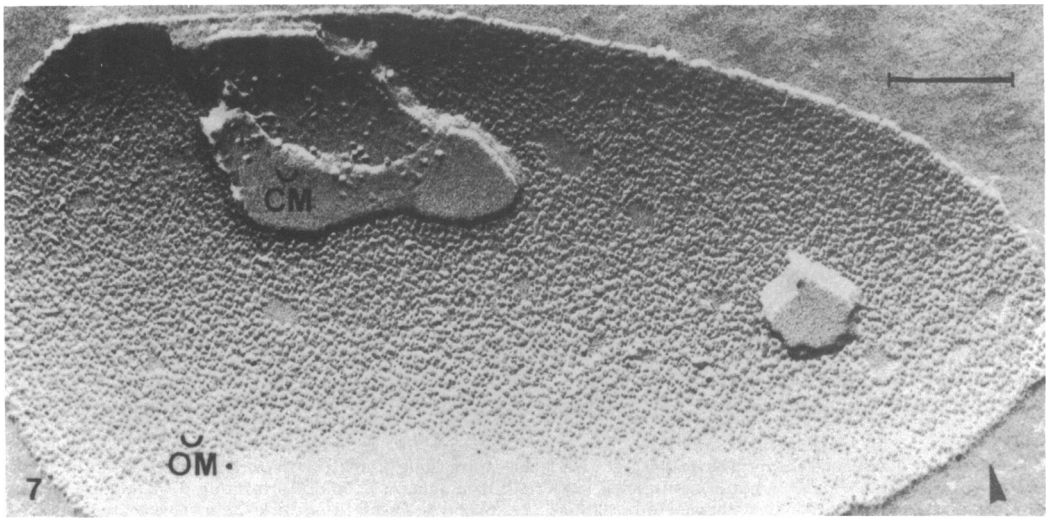

FIG. 7. *OM of strain P6922dI after growth in Tris-based medium, supplemented with maltose to induce the phage λ receptor. Bar = 200 nm. Arrow indicates direction of shadowing.*

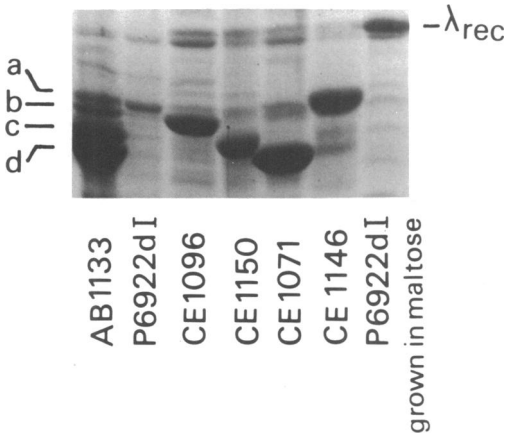

FIG. 8. *Polyacrylamide gel pattern of the cell envelope proteins of the mutants in Fig. 6. Only the relevant part of the gel is shown. Strain AB1133 is wild type with respect to outer membrane composition; strain P6922dI lacks proteins b, c, and d. Strain CE1096 has the phenotype $b^+ c^- d^-$, strain CE1150 is $b^- c^+ d^-$, strain CE1071 is $b^- c^- d^+$, strain CE1146 is $b^- c^- d^- e^+$, and in strain P6922dI grown on maltose the receptor protein for phage lambda has been induced.*

reference 35), and d (4, 27) with LPS in vitro have indeed been reported.

In wild-type cells about half of the OM particles are stabilized by divalent cations (30). The ability of putrescine to introduce OM particles in strain P6922dI suggests that putrescine and possibly other polyamines could be involved in the stabilization of the other half of the OM particles.

From studies involving the extraction of *P. aeruginosa* with EDTA it was concluded that the OM particles in this species are also protein-LPS complexes (6). This conclusion was based on the observations (i) that EDTA treatment causes a reduction in the number of OM particles and (ii) that the released material consisted of a complex containing 60% protein (in contrast to *E. coli* K-12), 30% LPS, and 10% loosely bound lipid. The protein fraction consisted mainly of two major outer membrane proteins (6, 19, 25).

Proteins b, c, d, and e and the phage lambda receptor protein, which are involved in particle formation (Fig. 6), are reported to be involved in the functioning of hydrophilic pores with different specificities (2, 14, 15, 26, 29a, 29b). Therefore, it is tempting to speculate that the OM particles are morphological reflections of hydrophilic pores through the outer membrane of *E. coli* in which the specificity of the pore function is determined by the protein. The pits observed on the surface of *E. coli* cells (1) could represent the entrances of these pores.

ACKNOWLEDGMENTS

We thank C. Verhoef and W. van Alphen for their gift of strains and P. G. de Haan and P. H. J. T. Ververgaert for critical reading of the manuscript.

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