Biochimica et Biophysica Acta 466 (1977) 269—282

BBA 77679

ARCHITECTURE OF THE OUTER MEMBRANE OF *ESCHERICHIA COLI* K12

II. FREEZE FRACTURE MORPHOLOGY OF WILD TYPE AND MUTANT STRAINS

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(Received September 16th, 1976)

Summary

Freeze fracturing electron microscopy of *Escherichia coli* K12 cells showed that the outer fracture face of the outer membrane is densily occupied with particles. On the inner fracture face of the outer membrane, pits are visible, which are probably complementary to the particles at opposite fracture face. This observation suggests that the particles are micelle-like. In some mutants which lack one or more major outer membrane proteins the density of particles is reduced. The loss of protein d appeared to a prerequisite for this phenomenon. However, mutants which lack all glucose and heptose-bound phosphate in their lipopolysaccharide also have a reduction in particle density whereas, the amount of protein d is normal. Moreover, loss of lipopolysaccharide by EDTA treatment also caused a reduction in the density of particles. From these results it is hypothesized that the particles consist of lipopolysaccharide aggregates stabilized by divalent cations and probably complexed with protein and/or phospholipid.

Introduction

Research on the outer membrane of gramnegative bacteria has made much progress in the last years. This membrane is unusual in that it contains in addition to phospholipid and protein, lipopolysaccharide [1,2]. Now that the biochemistry of the individual constituents of the outer membrane has reached an advanced state, knowledge concerning the localization, organization and the mutual interaction of these components is the next step in the understanding of the physiological function of the outer membrane.

From X-ray and fluorescence studies [3] as well as from freeze fracture studies [4] it was concluded that the phospholipid of the outer membrane of *Escherichia coli* K12, consisting almost exclusively of phosphatidylethanolamine [5], partially exists as a mono- and/or bilayer. The outer membrane is asymmetric in that lipopolysaccharide is exclusively present at the outer surface [6]. Lipopolysaccharide is mobile along the cell surface [7,8]. The protein pattern of the outer membrane of *E. coli* is relatively simple. Many copies per cell are present from protein I, II*, III and Braun's lipoprotein [9]. The two former proteins belong to Schnaitman's major outer membrane protein [1] which can be resolved into four bands designated as a, b, c and d [10].

Some lipopolysaccharide mutants lack one or more of their major outer membrane proteins [4,11—15]. Also mutants with apparently normal lipopolysaccharide but lacking one or more major outer membrane proteins have been described [4,14—19]. These mutants could be very useful tools for the elucidation of the architecture of the outer membrane. Recently we reported the freeze fracture morphology of mutants of *E. coli* K12 with deficiencies in their lipopolysaccharide and/or in their individual major outer membrane proteins. Cells of both deep rough lipopolysaccharide mutants [4,20—22] as well as cells of mutants that lack protein d [4] are preferentially fractured through the outer membrane. The density of particles at the concave or outer fracture face of the outer membrane (OM) is reduced in heptoseless lipopolysaccharide mutants. A relation between the particles and lipopolysaccharide was suggested [4]. A similar reduction in particle density was reported for heptose-deficient mutants of *Salmonella typhimurium* [20]. In this case reduction in particle density is attributed to the decreased protein content of the outer membrane [20]. So far no clear answer has been obtained concerning the nature of the particles at the OM. Therefore, we have extended the freeze fracture experiments by studying other lipopolysaccharide mutants as well as mutants lacking combinations of proteins. In this paper the freeze fracture studies of these new mutants will be reported and the discussion will be focussed on the origin and nature of the particles on the outer fracture face of the outer membrane.

Materials and Methods

Only *E. coli* K12 strains were used. Their origins and sources are extensively described in the accompanying paper [23]. Table I summarizes their outer membrane defects. The tentative structure of *E. coli* K12 lipopolysaccharide is also shown in the accompanying paper [23]. The composition of yeast broth and brain heart has been described previously [14]. To suppress the biosynthesis of protein b, media were supplemented with 0.3 M NaCl (high salt). In order to check whether the mutants still had the described protein deficiencies, part of each batch of cells was used for the isolation of cell envelopes and the characterization of their envelope proteins according to the method described earlier [10]. The other part was used for electron microscopy. The cells were quenched from 0° C (unless otherwise stated) with a mixture of liquid and solid nitrogen and fractured in a Denton freeze-etch apparatus as described before [29]. Electron micrographs were made with a Siemens Elmiskop 1A.

EDTA treatment was carried out according to Leive et al. [26]. Cells were

grown in Tris-based medium [27] supplemented with 150 mg of each L-amino acid per l, washed with 0.9% NaCl and resuspended in 1/20 volume of 0.12 M Tris · HCl buffer, pH 8.0. After prewarming the cells at 37° C, EDTA was added (0.4 μmol/10^{10} cells). After incubation for 2 min 0.4 μmol $MgCl_2$/10^{10} cells was added, followed by centrifugation. Release of lipopolysaccharide was determined by measuring 3-deoxy-D-manno-octulosonic acid [28] in the supernatant as well as in cell envelopes of the cells obtained after centrifugation.

Results

General characteristics of the outer membrane of E. coli K12

Before going into the freeze fracture morphology of the mutants we will describe and discuss the freeze fracture characteristics of the outer membrane of the wild type *E. coli* K12. The concave or outer fracture face of the outer membrane (OM) is densely occupied by particles with a diameter varying from 40 to 80 Å (Fig. 1A). The smaller ones can only be visualized with optimal replicas in which the resolution is close to the limit of Pt/C replication. They are also better resolved when the particle density is reduced by mutation. The convex or inner fracture face of the outer membrane (OM) shows a rough fracture face with large particles of about 100 Å in diameter and very interestingly, pits of a diameter between 60—80 Å (Figs. 1 and 2). We have suggested earlier [4] that the OM pits could be complementary to the particles on the complementary fracture face (OM) although less pits are visible on the OM (3000—5000/μm^2) than particles on the OM (6000—10 000/μm^2). This difference might be explained as follows: (i) visualization of the smallest pits is beyond the resolution of the Pt/C replication, (ii) snowing up of pits by carbon prevents a proper shadowing of these surface details, (iii) it is also possible that two or more populations of particles on the OM are present, of which only one population results in complementary impressions which are visible as pits.

The particle density on the OM of the 100 Å particles is about 500—700/μm^2. Pits complementary to these particles might be present as sometimes large pits on the OM can be found in mutants which have a reduction in particle density. The particles of 100 Å will not be taken further into consideration as none of the mutants showed a significant reduction in the density of these particles. The complementarity of the two membrane halves will be studied further on the hand of complementary replicas (Ververgaert, P.H.J.Th., in preparation).

We observed that the OM particles (and also the pits on the OM) aggregate when the cells are quenched from 22 and 0° C, but not when they are quenched from 37° C. This aggregation was explained as a reflection of a phase segregation induced by a lipid phase transition [4], as found for the cytoplasmic membrane of *E. coli* and other microorganisms [25]. This phenomenon of particle aggregation has been used for the estimation of the reduction of the particle density on the OM as after particle aggregation smooth areas became clearly visible. The ratio particulated area over smooth area at the OM at maximal aggregation (i.e. quenching from 0° C) has been used to determine the reduction in particle density. The seemingly better alternative, namely determination of the particle density by counting, may in our opinion cause erroneous values as

TABLE I

STRAINS AND RELEVANT PROPERTIES

Strain designation	Growth medium [a]	Defects in outer membrane		Freeze fracture characteristics	
		Lacking major outer membrane proteins, if not completely lacking, % decrease is indicated	Defects in lipolysaccharide	Reduction in particle density at $\widetilde{OM}$ [b]	Preference for fracture plane through [c]
CE 1052	YB	None	None	—	CM
CE 1052	YBHS	b	None	—	CM
CE 1056	YB	c	None	—	CM
CE 1054	YB	d	None	+	OM
CE 1054	BHHS	b,d	None	++	OM
CE 1058	YB	c,d	None	++	OM
CE 1058	BHHS	b,c,d	None	+++	OM exclusively
P 400	BH	None	None	—	CM
P 460	BH	d	None	+	OM
P 460 pr	BH	d (50%)	None	—	CM
CE 1071	BH	b,c	None	—	CM
P 692 2dI	BH	b,c,d	None	+++	OM exclusively
CE 1053	YB	b	Heptoseless	++	OM
CE 1057	YB	b,c	Heptoseless	++	OM
CE 1055	YB	b,d	Heptoseless	++	OM
CE 1059	YB	b,c,d	Heptoseless	+++	OM exclusively
CE 1023	YB	b	Heptoseless	++	OM
CE 1021	YB	b (50%)	Glucoseless	—	OM
D 21f1	YB	b (75%)	Glucoseless and lacking heptose-bound phosphate	+	OM
D 21e7	YB	b (75%)	Galactoseless and lacking heptose-bound phosphate	—	CM
CE 1002	BH	None	Galactoseless	—	CM
CE 1052	TBM	None	50% released	++	CM

[a] YB = yeast broth, BH = brain heart, YBHS = yeast broth high salt, BHHS = brain heart high salt, TBM = Tris based medium.

[b] —, no significant reduction; +, significant reduction (about 25%); ++, extensive reduction (about 50%); +++, extreme reduction (about 75%); $\widetilde{OM}$, outer fracture face of the outer membrane.

[c] CM, cytoplasmic membrane; OM, outer membrane.

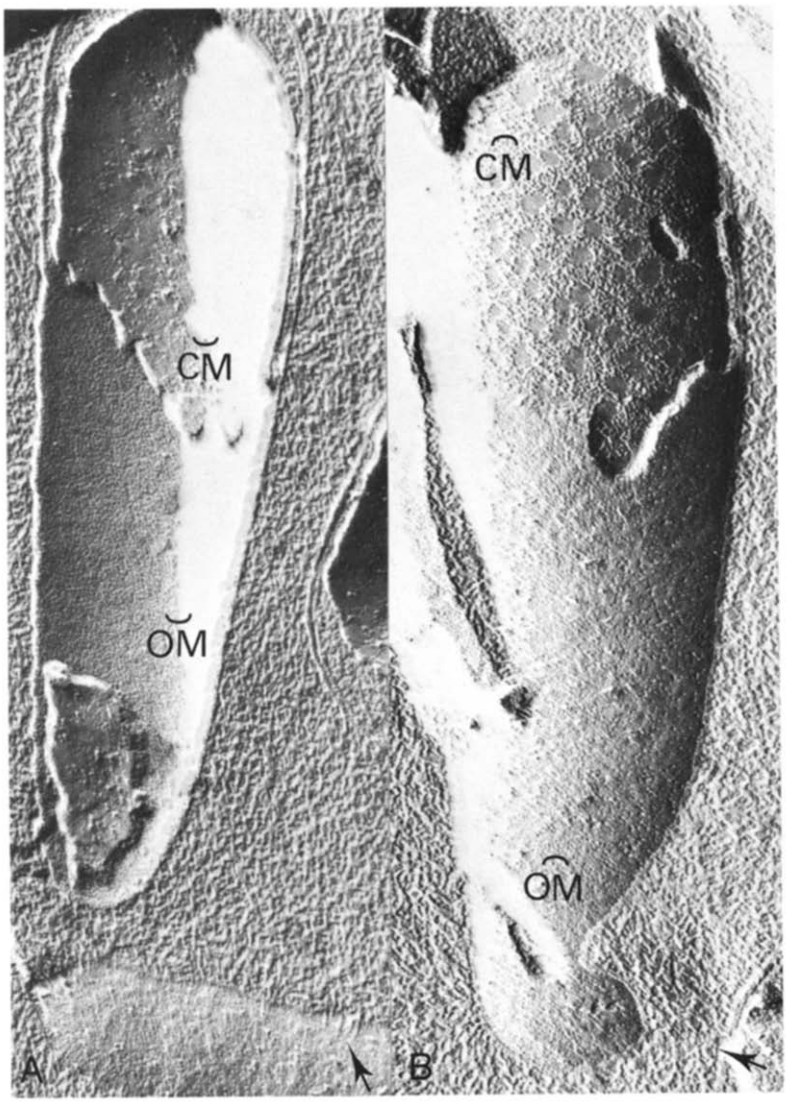

Fig. 1. Fracture faces of *E. coli* K12 strain CE 1052 quenched from 20° C. $\overset{\frown}{OM}$ and $\overset{\smile}{OM}$ are convex (inner) fracture faces and concave (outer) fracture faces of the outer membrane respectively. $\overset{\frown}{CM}$ and $\overset{\smile}{CM}$ are the convex (inner) fracture faces and concave (outer) fracture faces of the cytoplasmic membrane, respectively (X64 000).

the visible number of particles and pits is dependent on the quality of the Pt/C replica. Moreover it is found that the reduction in particle density is not homogenous in one population of cells. Therefore, we have distinguished the degree in particle reduction into significant, extensive and extreme reduction, corresponding with about 25, 50 and 75% smooth outer fracture face of the outer membrane, respectively.

As it appeared that the preference for fracturing through the cytoplasmic membrane observed in wild type cells can change to a preference to fracturing

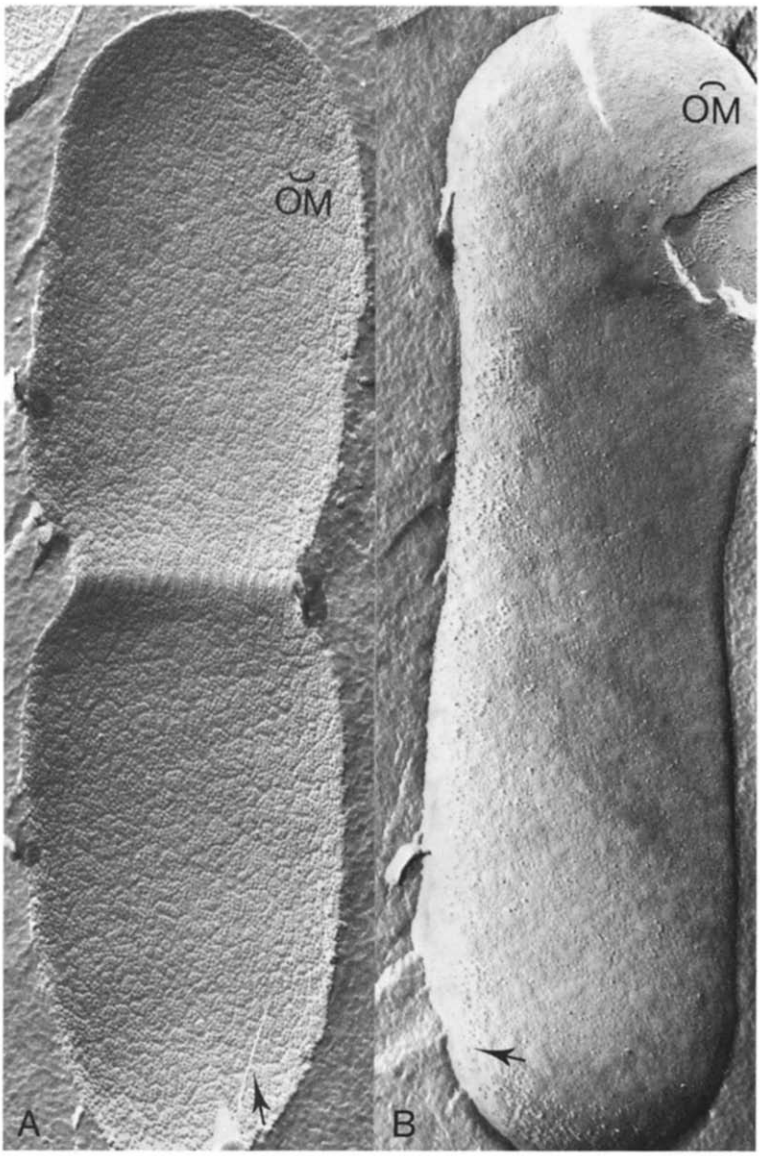

Fig. 2. Fracture faces of *E. coli* K12 strain CE 1054 grown in yeast broth; preferential cleavage of the outer membrane (×64 000). Note the linear arrangement of the $\overset{\frown}{OM}$ particles in the septum.

through the outer membrane in mutants, this behaviour is mentioned in Table I.

Mutants with deficiencies in outer membrane proteins

The defects of the outer membrane as well as the freeze fracture analysis of all strains which are relevant to this study are summarized in Table I. All differences have been related to the corresponding wild type strain. As we have reported earlier [4], the lack of one of the individual proteins b or c (e.g. CE

1052 grown in yeast broth high salt and CE 1056, respectively) does not change the freeze fracture characteristics. Mutants that lack only d show a preferential fracturing through the outer membrane (Figs. 2A and B). Moreover in strains CE 1054 and P 460 (both grown in yeast broth) one can find cells (about 70%) which hardly show a reduction in particle density on the OM (Fig. 3A) next to cells of which the reduction is significant to extensive (Figs. 3B and C, respectively). The same heterogeneity is found when these mutants are grown in brain heart except that after growth in the latter medium the particle density is somewhat more reduced. The heterogeneity is not understood. That protein d is really of importance is confirmed by the fact that mutant P 460 pr, which is a partial d^+ revertant, shows the wild type freeze fracture characteristics.

The influence of the lack of two major outer membrane proteins was studied in double mutants. The lack of both b and c (e.g. CE 1071) still results in a

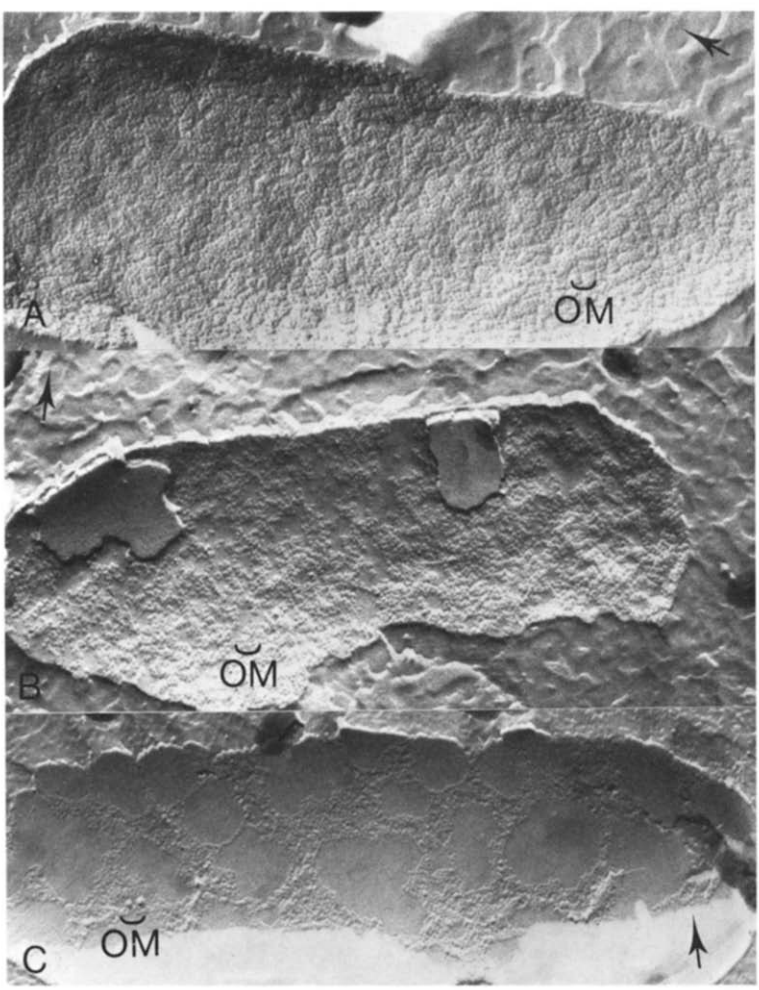

Fig. 3. Fracture faces of *E. coli* K12 strain CE 1054 grown in yeast broth (×64 000). Similar results were obtained with strain P 460.

wild type outer membrane morphology. The additional lack of b or c in mutants that lack d (e.g. strains CE 1054 grown in brain heart high salt and CE 1058 grown in yeast broth), leads to a further decrease in particle density (Table I, Fig. 4A). In both strains this additional reduction in particle density is accompanied by a reduction in the number of pits on the $\widetilde{OM}$ (Fig. 4B). It can be concluded that, although no effect of the lack of b and/or c on the outer membrane morphology was observed, the lack of one of these proteins in a mutant lacking d results in a further reduction of the particle density. In mutants that lack all three proteins b, c and d (e.g. CE 1058 grown in brain heart high salt and P 692 2dI) an extreme reduction of the number of $\breve{OM}$ particles (Fig. 5A) and corresponding pits on the $\widetilde{OM}$ (Fig. 5B) is observed. Moreover the fracture plane is exclusively through the outer membrane.

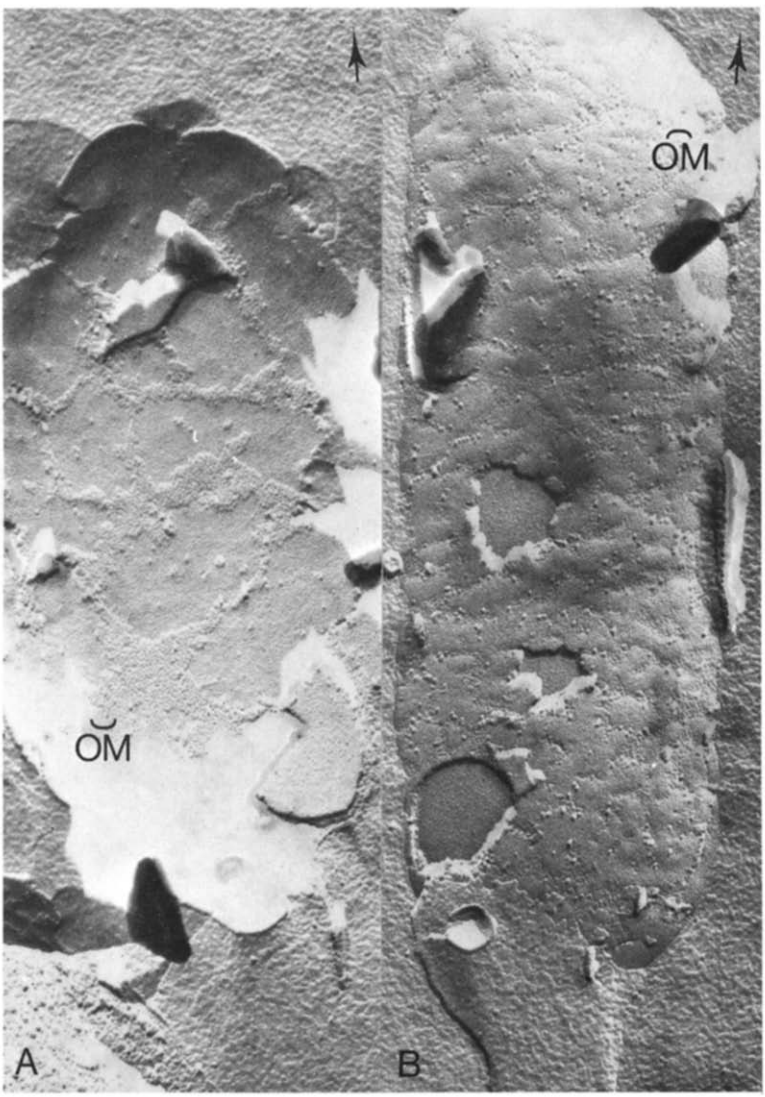

Fig. 4. Fracture faces of *E. coli* K12 strain CE 1054 grown in brain heart high salt (×64 000).

Mutants with deficient lipopolysaccharide

The freeze fracture characteristics of various lipopolysaccharide mutants are summarized in Table I. In our previous study [4] we have shown that heptoseless mutants showed an altered freeze fracture morphology i.e. a preference of the fracture plane for the outer membrane as well as a reduction in the number of $\breve{O}\breve{M}$ particles. Experiments with other heptoseless strains confirm that heptose deficiency leads to these phenomena (Table I). Remarkable is the fact that the lack of protein d, which is a prerequisite for the reduction of the particle density in the outer membrane protein mutants described above, is not required for the reduction of $\breve{O}\breve{M}$ particles in heptoseless strains (e.g. CE 1053 and CE 1057).

It was already concluded [4] that lipopolysaccharide is involved in the

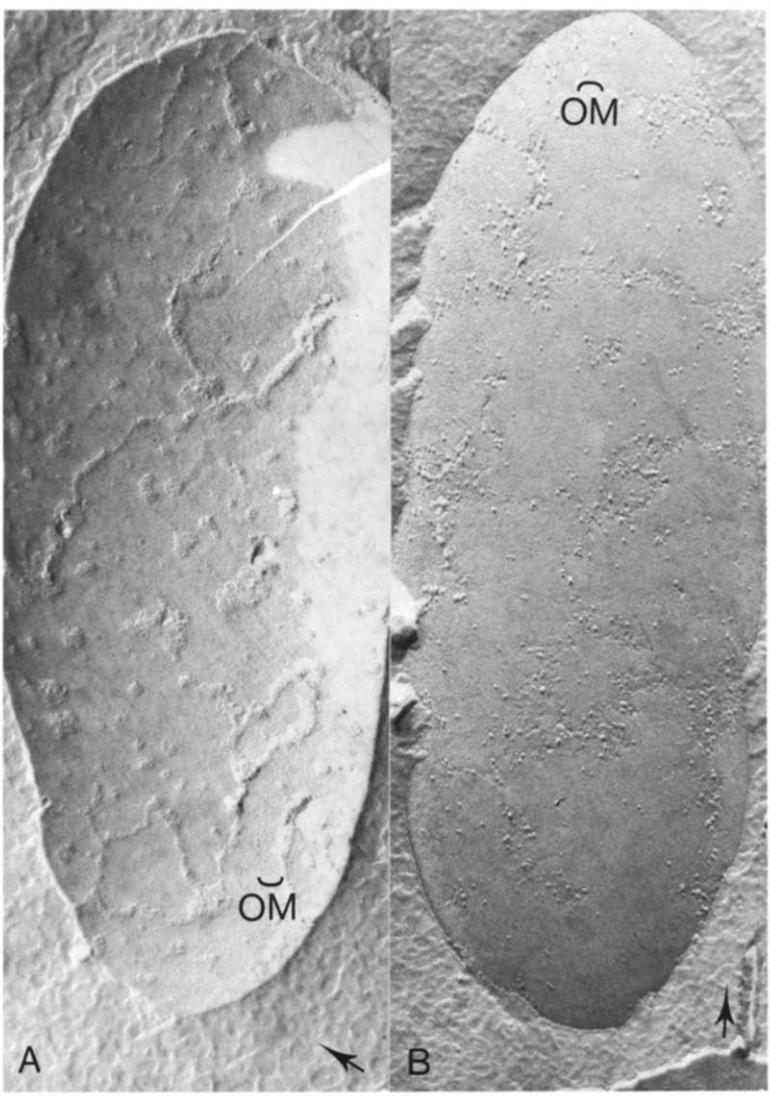

Fig. 5. Fracture faces of *E. coli* K12 strain CE 1058 grown in brain heart high salt (×64 000).

reduction in particle density. In determining which part of the lipopolysaccharide molecule is involved, we have investigated lipopolysaccharide mutants with other deficiencies. The lack of galactose alone (strain CE 1002) or the additional lack of two of the three glucoses and heptose-bound phosphate (strain D21e7) does not change the freeze fracture morphology. The lack of all glucose (strain CE 1021) results in a preferential cleavage through the outer membrane but not in a reduction of the $\breve{O}\breve{M}$ particle density. When in addition to glucose, heptose-bound phosphate is also lacking (strain D21f1), a significant reduction in $\breve{O}\breve{M}$ particle density is observed (Fig. 6).

EDTA treatment of E. coli

In order to investigate the effect of the removal of part of the lipopolysac-

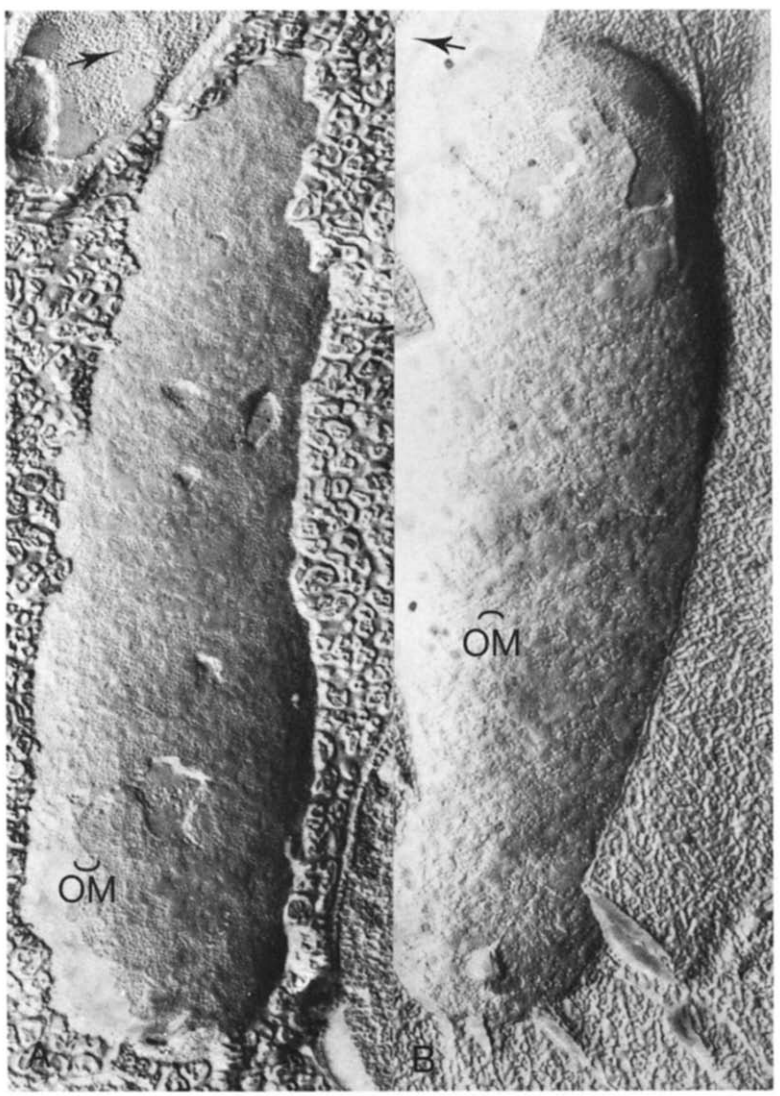

Fig. 6. Fracture faces of *E. coli* K12 strain D21f1 grown in yeast broth (×64 000).

Fig. 7. Fracture faces of *E. coli* K12 strain CE 1052 treated with EDTA (×64 000).

charide on the density of OM particles, we treated *E. coli* K12 strain CE 1052 with EDTA as it is known that this treatment reduces the particle density in the OM of *Pseudomonas aeruginosa* [30] and also extracts lipopolysaccharide from the cell [26]. 50% of the lipopolysaccharide, but no protein, was extracted by this procedure. Freeze fracturing showed that the particle density of the OM is clearly reduced (Fig. 7).

Discussion

Preferential cleavage through the outer membrane

Preferential cleavage through the outer membrane is observed in two types

of apparently unrelated mutants, namely in mutants lacking protein d and in a number of lipopolysaccharide mutants (Table I). In the latter case the change to a preferential cleavage through the outer membrane coincides with the lack of heptose-bound glucose. The reduced amount of protein b in heptoseless and glucoseless lipopolysaccharide mutants is not responsible for the preferential cleavage through the outer membrane as this effect is not observed in some other strains that lack this protein (CE 1052 in yeast broth high salt and CE 1071). Moreover, mutants lacking d contain slightly increased amounts of protein b [14].

Preferential cleavage through the outer membrane in the mutants mentioned above can be explained in two ways. (i) Increased amounts of phospholipid per unit surface area have been detected in heptoseless and glucoseless lipopolysaccharide mutants [20] as well as in mutant P 460 [23] which lacks protein d. It is likely that an increase in phospholipid content results in more phospholipid bilayer and therefore in an outer membrane that is easier to cleave. (ii) If one assumes that in wild type cells protein d interacts with the heptose-bound glucose of lipopolysaccharide, the loss of this interaction can explain the preferential cleavage through the outer membrane in the mutants.

Nature of the $\breve{O}\breve{M}$ particles

Smit et al. [20], studying lipopolysaccharide mutants of *S. typhimurium*, observed a close correlation between the extent of protein reduction in the outer membrane and that of reduction of the number of $\breve{O}\breve{M}$ particles. They suggest that at least a large portion of these particles is composed of protein and conclude that the particles are at least protein-containing entities [20]. Some of our results do not agree with their suggestion [20] that the particles are largely proteinaceous in nature. This can be illustrated by comparing the following two strains. The heptoseless strain CE 1057, lacking b and c, shows an extensive reduction in particle density, whereas strain CE 1071, lacking the same proteins, has wild type freeze fracture characteristics (Table I). Moreover, in some strains no quantitative correlation exists between the extent of the deficiency (at least 95%) and the extent of the reduction in particle density. A similar lack of quantitative correlation has been observed independently by Schweizer et al. [31]. These authors conclude that in a mutant that lacks b, c and d, the remaining $\breve{O}\breve{M}$ particles are either not proteinaceous in nature or contain other proteins. They suggest the possibility that in the wild type the composition of the particles is not homogenous. They also suggest that protein d (II*) is part of the particles whereas protein b and c (I) are not. However, this suggestion seems to be contradicted by their observation that no reduction in particle density was observed in a mutant that lacks protein d [31].

Also our data (Table I) show that the nature of the $\breve{O}\breve{M}$ particles is complex. However, in trying to understand the freeze fracture morphology in itself we found an overal interpretation for the nature of the $\breve{O}\breve{M}$ particles. The freeze fracture morphology of the outer membrane is characteristic in that, complementary to the particles on the $\breve{O}\breve{M}$, pits on the $\breve{O}\breve{M}$ are visible. The observation that less pits are present on the $\breve{O}\breve{M}$ (3000—5000/μm^2) might be explained either by the assumption that visualization of the smallest pits is beyond the resolution of the Pt/c replication (about 30 Å) or by the snowing effect of

carbon. Another possible explanation cannot be excluded namely that more than one population of $\breve{OM}$ particles exists of which only one causes complementary impressions in the $\widehat{OM}$ which are visible as pits.

Pits complementary to particles are very unusual in freeze fracturing [25]. For instance, no clear pits or depressions, complementary to the particles on the inner fracture face, can be found on the outer fracture face of the erythrocyte membrane. However, globules of the same size as particles present on the fracture faces of sphingomyelinase-treated erythrocytes have clearly complementary depressions [25]. It was suggested that these globules represent micelles of ceramides formed by sphingomyelinase action. With respect to micellar structures with a hydrophilic core it was suggested that the cleavage plane will not deform the structure, as is proposed for penetrating proteins [25], but will give a fracture which results in a fracturing characteristic, complementary with respect to particles. Therefore we assume that the $\breve{OM}$ particles complementary to pits on the $\widehat{OM}$ of *E. coli* reflect micelle-like structures that are deeply embedded in the inner monolayer of the outer membrane.

Only two outer membrane constituents can be responsible for micelle-like structures, namely phospholipid and lipopolysaccharide. It is conceivable that phospholipids alone cannot be responsible for the $\breve{OM}$ particles and the corresponding OM pits. It is also unlikely that a complex of phospholipid, with one or more major outer membrane proteins b, c and d causes particles and pits, as a quantitative correlation is lacking between the extent of the deficiency and the extent of the reduction in particle density. To us it seems that lipopolysaccharide, which is exclusively located in the outer leaflet [6], is most likely responsible for the micelle-like nature of the $\breve{OM}$ particles and for the corresponding $\widehat{OM}$ pits.

Nikaido [32] proposed three possible arrangements for lipopolysaccharide in the outer membrane: (i) free molecules with the fatty acyl chains inserted in a monolayer of phospholipid molecules, (ii) aggegates, most likely stabilized by divalent cations in order to neutralize the negatively charged carboxyl groups of the 3-deoxy-D-manno-octulosonic acid and phosphate residues, (iii) lipopolysaccharide-protein aggregates, most likely stabilized by divalent cations. We assume that lipopolysaccharide molecules can be present in all three arrangements and that these are in equilibrium with each other.

We hypothesize that the $\breve{OM}$ particles (corresponding with pits) represent lipopolysaccharide aggregates, possibly complexed with protein and/or phospholipid. In mutants with a reduction in particle density a number of particles is dissociated. In such a dynamic situation the reduction in particle density is not necessarily quantitatively correlated with the reduction in protein content. The following considerations support this hypothesis. (i) Our hypothesis explains that the observed reduction in particle density in mutants is caused by dissociation of particles. (ii) Newly synthesized lipopolysaccharide has a lateral mobility constant which is much lower than expected from single lipopolysaccharide monomers [7,8]. From this result Mühlradt et al. [7] suggested the possibility that lipopolysaccharide in the outer membrane is either cross-linked or linked to other components in this membrane. This observation fits well in our model and also suggests that a large part of the lipopolysaccharide of a wild type is present in aggregates. (iii) EDTA treatment releases 50% of the

cellular lipopolysaccharide [26]. The released material consists of 85% lipopolysaccharide [26]. The observation that EDTA treatment [30] causes a reduction in particle density (Fig. 7) agrees with the hypothesis if one assumes that the particles are stabilized by divalent cations.

Acknowledgements

We thank Prof. P.G. de Haan and Dr. P.H.J.Th. Ververgaert for critical reading of the manuscript.

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