Biochimica et Biophysica Acta, 556 (1979) 233—243
© Elsevier/North-Holland Biomedical Press

BBA 78492

ARCHITECTURE OF THE OUTER MEMBRANE OF *ESCHERICHIA COLI* K12

IV. RELATIONSHIP BETWEEN OUTER MEMBRANE PARTICLES AND AQUEOUS PORES

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(Received December 13th, 1978)
(Revised manuscript received February 16th, 1979)

Key words: Outer membrane; Aqueous pore; Membrane protein; Freeze-fracture particle; (E. coli)

Summary

The hypothesis that intramembraneous particles, observed in the outer membrane of *Escherichia coli* by freeze-fracture electron microscopy, are the morphological representation of aqueous pores, was tested. A mutant which is deficient in five major outer membrane proteins, b, c, d, e and the phage λ receptor protein, contains a largely decreased number of intramembraneous particles and also shows a greatly decreased rate of uptake of several solutes. In derivatives of this strain which contain only one of these proteins in large amounts a strong decrease of the number of intramembraneous particles is observed, which is accompanied by a complete restoration of the rate of uptake of those solutes which use pores in which the protein in question is involved. The results provide strong evidence for the notion that an individual pore contains only one protein species, a property which has been found earlier for individual particles. The observed correlation between particles and aqueous pores strongly supports the hypothesis that the particles are the morphological representation of pores. Implications of this hypothesis for the structure of the particles are discussed.

Introduction

The outer membrane of Gram-negative bacteria contains proteins, phospholipids and lipopolysaccharide. From the various nomenclature systems, which

are in use for the major outer membrane proteins of *Escherichia coli* K12, we shall use the one of Lugtenberg et al. [1]. The major proteins synthesized by *E. coli* K12 during growth in various media include the proteins b, c, d and the lipoprotein, and, under special conditions, protein e and the receptor protein of bacteriophage λ [2—7].

Extensive electron microscopic investigations on the outer membrane of *E. coli* [7—12] have revealed that the concave or outer fracture face of the outer membrane (OM) is densely covered with particles with a diameter of 4—10 nm. On the corresponding convex or inner fracture face of the outer membrane (OM) many pits are visible which are complementary to the particles [9,13] and are thought to be due to the fact that lipopolysaccharide is a basic structural component of the particles [7,9—11,13]. Recently strong evidence was provided for the idea that the majority of the outer membrane particles of wild-type cells consists of subpopulations of b-lipopolysaccharide, c-lipopolysaccharide and d-lipopolysaccharide particles whereas e-lipopolysaccharide and phage λ receptor protein-lipopolysaccharide particles can be present in the membrane under special conditions [7]. These OM particles are possibly the morphological representations of hydrophilic pores [7].

Hydrophilic solutes with a molecular weight of up to about 700 pass the outer membrane through aqueous pores by a diffusion-like process in which proteins are involved [5,6,14—21]. The receptor protein of bacteriophage λ is involved in the permeation of maltose [22,23]. Proteins b, c and e are involved in the functioning of general aqueous pores, through which several unrelated solutes like ions, amino acids, sugars, nucleotides and antibiotics can permeate [5,6,18—21,24,25]. However, some 'specificity' exists among these general pores as 5'-adenosine monophosphate (5'-AMP) and related compounds can use the b- and e-pores but not, or much less efficient, the c-pore [5]. Similarly, β-lactam antibiotics can use the b- and c-pores but hardly, if at all, the e-pores [21]. Although results of one laboratory [26] suggest that protein d is involved in the uptake of glutamic acid and proline, later work in two other laboratories [6,25] contradict this suggestion. Protein d might be involved in the permeation of colicin L-JF246 or part of it [27]. So, pore functions have been established for at least four of the five proteins which are known to be involved in outer membrane particles [7].

In this paper we describe experiments designated to investigate the proposed correlation between aqueous pores and outer membrane particles. The freeze-fracture morphology and the permeability properties of mutants lacking all five mentioned proteins are compared with the properties of several mutant derivatives, each of which contains large amounts of only one of these proteins.

Materials and Methods

Strains and growth conditions. All strains used are derivatives of *E. coli* K12. Their origins and relevant characteristics are listed in Table I. Plasmid R1, coding for a very active periplasmic β-lactamase, was introduced into strain AB1133 and its derivatives by conjugation [30]. Cells were grown in yeast broth which had the composition described previously [2] except that it contained 135 mM NaCl instead of 85 mM. This higher NaCl concentration was

required to suppress the synthesis of substantial amounts of protein b [31] which are produced by strain P692*tut*2dI during growth in standard yeast broth medium. If the presence of the phage λ receptor protein was required, the medium was supplemented with 0.5% maltose. Cells were grown at 37°C under vigorous aeration and harvested at the end of the logarithmic growth phase.

Freeze-fracture electron microscopy. Cells were washed with 0.9% NaCl and resuspended in 0.9% NaCl to which 30% glycerol was added as a cryoprotectant. Samples were quenched from 0°C in a mixture of liquid and solid nitrogen and fractured in a Denton freeze-etch apparatus as described before [32]. Electron micrographs were made with a Philips 301 electron microscope. The number of particles/unit cell surface was estimated as described previously [9].

Uptake of 5'-AMP. The rate of uptake of ^{3}H-labeled 5'-AMP was measured according to the modified [5] procedure of Beacham et al. [19]. Permeation of the outer membrane is the rate-limiting step under these conditions [5].

Uptake of maltose. The procedure is based on the one described by Szmelcman and Hofnung [22]. Cells were washed at 4°C with minimal medium salt solution [2] supplemented with chloramphenicol (40 μg/ml final concentration) and resuspended in the same solution to an A_{660nm} value of 0.60. After preincubation of 2.0 ml of the suspension for 15 min at 37°C under gentle shaking, uptake was started by the addition of 100 μl 240 μM [U-^{14}C]maltose (8 Ci/mol) (The Radiochemical Centre, Amersham, U.K.). Samples (200 μl) were taken at various times during 3 min, filtered immediately (Millipore S.A., Molsheim, France, Type HA, diameter 0.45 μm) and washed twice with 5 ml of prewarmed minimal medium salt solution. After drying, the radioactivity on the filter was determined by liquid scintillation counting.

Assay of β-lactamase (EC 3.5.2.6). The rate of hydrolysis of cephaloridine by intact cells and the total β-lactamase activity, the latter measured in suspensions of spheroplasts, were determined as described previously [21]. Spheroplasts were prepared by the procedure of Osborn et al. [33], modified as described previously [34]. Under these conditions the rate of hydrolysis reflects the rate of permeation of cephaloridine through the outer membrane [34].

Assay of 5'-nucleotidase (uridine diphosphate-sugar hydrolase) (EC 3.1.3.5). The activity of 5'-nucleotidase was determined according to a modification [5] of the procedure of Nossal and Heppel [35].

Isolation and characterization of cell envelopes. After ultrasonic desintegration of cells, cell envelopes were isolated by differential centrifugation [1]. The protein patterns of cell envelopes were analyzed by sodium dodecyl sulphate gel electrophoresis [1]. Protein bands are indicated by their molecular weights multiplied by 10^{-3} and followed by the letter k.

Results

Characteristics of the bacterial strains

The correlation between individual pore functions and intramembraneous particles was investigated for cells of the wild-type strain AB1133 ($b^+c^+d^+$), cells of various mutant strains containing large amounts of only one of the proteins

b, c, d, e and the λ receptor proteins and cells of the $b^-c^-d^-$ strain P692*tut*2dI which is deficient in all five mentioned proteins since protein e is not produced by this strain and the receptor protein of phage λ is only induced by the presence of maltose in the growth medium (Table I). Derivatives of this strain which possess only one of the protein b, c and d in large amounts have been described [7]. P692*tut*2dI cells containing large amounts of the λ receptor protein were obtained by supplementation of the growth medium with maltose. As a protein e^+ strain was not present in an AB1133 background, the effect of the presence of protein e was studied with strain PC0479 and its derivatives (Table I). In order to be able to measure the rate of permeation of β-lactam antibiotics through the outer membrane, plasmid R1 was introduced in some strains (Table I).

Cell envelopes were prepared of cells from the same suspensions which were used for freeze-fracture studies and uptake experiments. The polyacrylamide gel electrophoresis patterns of cell envelope proteins of the strains is shown in Fig. 1. Compared with the parental strains AB1133(R1) (Fig. 1A) and PC0479 (Fig. 1G) the mutants P692*tut*2dI(R1) (Fig. 1C) and CE1145 (Fig. 1I) are deficient in protein b, c and d. The presence of maltose in the growth medium results in the appearance of a 50k protein, the λ receptor protein (Figs. 1B and 1F). The b^+ revertant (Fig. 1D), the c^+ revertant (Fig. 1E) and the $e^+b^-c^-d^-$ derivative of strain PC0479 contain large amounts of proteins b, c and e, respectively. In addition to the differences in major outer membrane proteins, small differences in the amounts of some unidentified minor proteins can be observed (Fig. 1).

TABLE I

STRAINS AND RELEVANT CHARACTERISTICS

For the strains plasmid R1 was introduced by conjugation.

Strain	Origin	Major outer membrane protein abnormalities	Source, reference
AB1133(R1)		None (wild type)	P. Reeves, and Ref. 28
P692*tut*2dI(R1)	d^- derivative of ab^-c^- mutant of AB1133	$b^-c^-d^-$	U. Henning, and Refs. 9, 12 and 29
CE1096(R1)	Spontaneous b^{++} revertant of P692*tut*2dI	$b^{++}c^-d^-$	[7]
CE1150(R1)	Spontaneous c^{++} revertant of P692*tut*2dI	$b^-c^{++}d^-$	[7]
PC0479		None	PC *
CE1145	d^- derivative of ab^-c^- mutant of PC0479	$b^-c^-d^-$	[5, 7]
CE1146	d^- derivative of CE1108, an e^+ derivative of CE1107	$e^+b^-c^-d^-$	[6,7]

* PC, Phabagen Collection, c/o Miss H.S. Felix, Department of Molecular Cell Biology, State University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

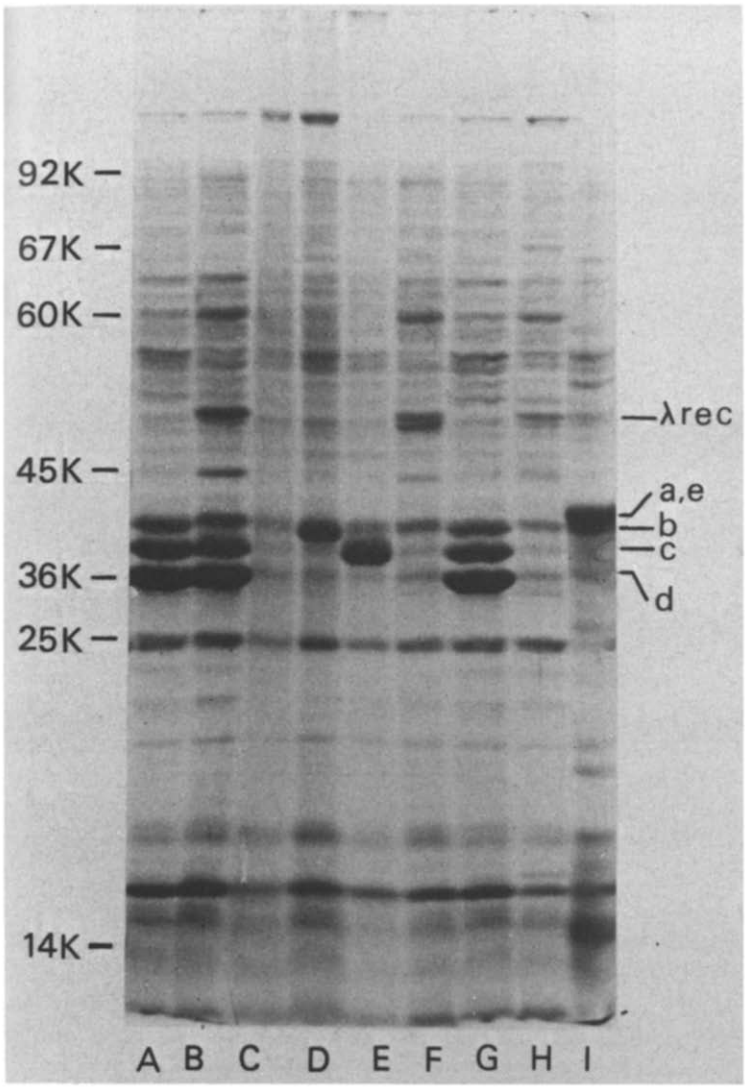

Fig. 1. SDS-polyacrylamide gel electrophoresis of proteins of cell envelopes of the parental strain AB1133(R1), grown in the absence (A) and in the presence (B) of maltose, the $b^-c^-d^-$ strain P692*tut*2dI-(R1) grown in the absence (C) and presence (F) of maltose, the b^+ revertant strain CE1096(R1) (D), the c^+ revertant strain CE 1150(R1) (E), the parental strain PC0479 (G), its $b^-c^-d^-$ mutant CE 1145 (H) and $e^+b^-c^-d^-$ derivative CE1146 (I). The positions of the relevant proteins are indicated at the right, those of molecular weight standard proteins at the left. A protein band just below the λ receptor protein appears as a result of growth in the presence of maltose (see for instance lane F). This protein is unrelated to the λ receptor protein as the former protein in contrast to the latter one is not peptidoglycan-associated and is present in *lamB* derivatives.

Freeze-fracture morphology of the outer membrane

Although relatively few cleavage planes are observed through the outer membrane of wild-type cells in comparison to the inner membrane (see also Refs. 8, 10 and 12), it is clear that the OM of these cells is covered with particles (Fig. 2A). The outer membranes of strain AB1133(R1), grown with or without maltose, and of strain PC0479 had the same morphological appearance. A

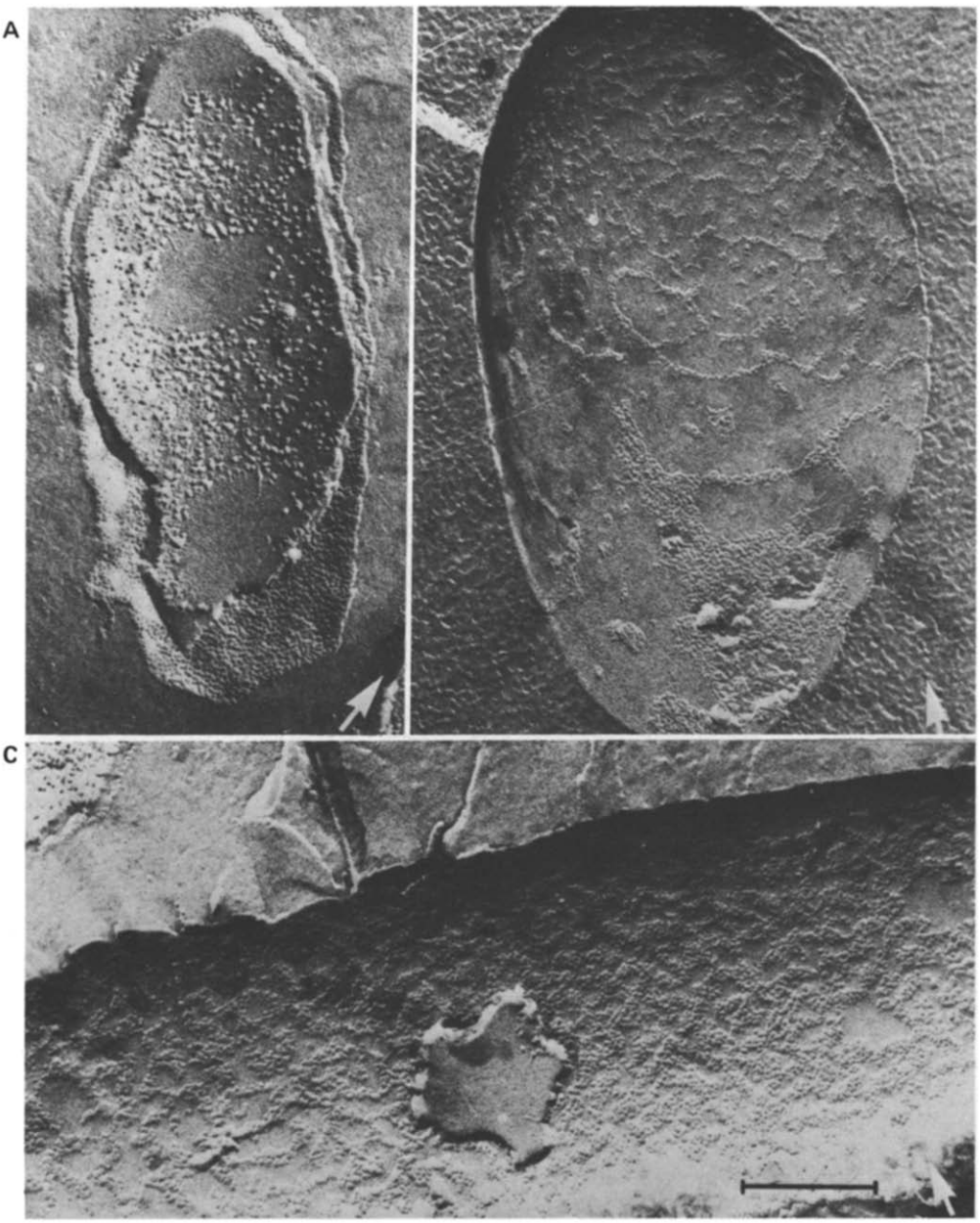

Fig. 2. Outer fracture faces of the outer membrane (OM) of cells of parental strain AB1133(R1) ($b^+c^+d^+e^-\lambda rec^-$) (A), its $b^-c^-d^-e^-\lambda rec^-$ derivative P692*tut*2dI(R1) (B) and the protein b^+ derivative of the latter mutant, strain CE1096(R1) (C). The bar represents 200 nm. The arrows indicate the direction of shadowing.

reduction in the number of OM particles of approximately 60% was observed in strains P693*tut*2dI(R1) (Fig. 2B) and CE1145, both of which lack all five studied proteins. When either protein b (strain CE1096(R1)), protein c (strain CE1150(R1)), protein d (not shown), protein e (strain CE1146) or the λ receptor protein were introduced in such a particle-poor background, the

number of OM particles increased from 40% to 70–100% of the value observed for the wild-type strains, somewhat depending on the protein present (Table II). Some variation in the particle density was observed, as was reported earlier [7,9]. Fig. 2C shows a representative example of strain CE1096(R1). Compared with a previous publication, in which the plasmid-less cells were grown in another medium [7], we now observed a slightly less extreme reduction in the number of OM particles in strain P692*tut*2dI(R1) and a higher number of particles in the R1 strains which contain one of the major proteins. These differences were found to be due to the growth medium and not to the presence of the plasmid (not shown). As observed earlier [7,9,13], also in the present experiments a decrease or increase in the number of OM particles was accompanied by a corresponding change in the number of OM pits [7,9,13].

Pore properties of the strains

Comparison of the rates of 5′-AMP uptake of strain AB1133(R1) and P692*tut*2dI(R1) showed that the rate of uptake is considerably lower for the mutant strain (Table II). The rate of uptake of 5′-AMP by cells of strain CE1096(R1), which possesses protein b as the only major outer membrane protein, is even higher than that of AB1133(R1) cells. Comparison of the rate of 5′-AMP uptake by strains PC0479, CE1145 and CE1146 shows that when protein e is present as the only major outer membrane protein, the rate of uptake is almost restored to the wild-type level, which is even better than the stimulation by protein e in a $b^-c^-d^+$ background measured earlier [5]. This

TABLE II

PORE PROPERTIES AND NUMBER OF OUTER MEMBRANE PARTICLES OF VARIOUS OUTER MEMBRANE PROTEIN MUTANTS

The values are expressed as percentages relative to values found for the corresponding parental strains (data in italics). The absolute values measured for uptake of 5′-AMP, maltose and cephaloridine by cells of the parental strains were approximately 0.04, 1.7 and 360 nmol/min per mg dry weight cells. All values are the average of at least three determinations. The largest deviation from the given values for the uptake for 5′-AMP and maltose was 13% of the indicated value. Higher values were much more accurate. The accuracy of the rate of hydrolysis of cephaloridine was within 5%. In freeze-fracture experiments the number of particles/unit cell surface was estimated from at least 20 different cells of each strain. In some mutants the measured values vary considerably from cell to cell, even in one batch of cells. Therefore a range is given for the number of OM particles. n.d., not determined.

Strain	Major outer membrane protein(s) present	Relative rate of uptake of		Relative rate of hydrolysis of cephaloridine	Particle density at OM
		5′-AMP	Maltose		
AB1133(R1)	b, c, d	*100*	8	*100*	*100*
AB1133(R1) *	b, c, d, λ rec	n.d.	*100*	68	100
P692*tut*2dI(R1)	none	18	8	27	40
CE1096(R1)	b	135	8	132	70–90
CE1150(R1)	c	39	n.d.	92	80–100
P692*tut*2dI(R1) *	λ rec	n.d.	102	18	70–90
PC0479	b, c, d	*100*	n.d.	n.d.	*100*
CE1145	none	37	n.d.	n.d.	40–50
CE1146	e	86	n.d.	n.d.	70–90

* The λ receptor protein (λ rec) was induced by growing the cells in yeast broth supplemented with 0.5% maltose.

result as well as the high rate of 5′-AMP uptake in strain CE1096(R1) is probably caused by the very high levels of proteins e and b in strains CE1146 and CE1096(R1), respectively. Strain CE1150(R1), which produces protein c, a protein which seemed not to be involved in the uptake of 5′-AMP [5], was tested as an aspecific control in order to check whether the presence of high amounts of an arbitrary 5′-AMP nonspecific pore protein in the outer membrane would not result in an increased rate of uptake. The result (Table II) shows that the rate of uptake of 5′-AMP by cells of strain CE1150(R1) is significantly higher than that of cells of strain P692*tut*2dI(R1). This increase can be explained by the substantial amounts of protein b present in strain CE1150(R1) (see Fig. 1E). Therefore the contribution of protein c in the uptake of 5′-AMP is probably negligible.

The rate of maltose uptake is strongly stimulated in cells grown in the presence of maltose (Table II), which results in derepression of the maltose transport system, including a 50k outer membrane protein which is the receptor for phage λ (Fig. 1B and Refs. 5, 21 and 36). As mutants deleted in the *lamB* gene incorporate maltose under the used conditions with a very low rate (Ref. 22 and unpublished observations), the results of Table II show that the λ receptor protein in the absence of proteins b, d and e functions as a perfect pore for maltose. The presence of protein b, tested as an arbitrary pore protein, does not stimulate the rate of maltose incorporation (strain CE1096(R1)).

The rate of hydrolysis of cephaloridine by cells of strain P692*tut*2dI(R1) is low compared with that by cells of strain AB1133(R1) (Table II). The presence of either protein b or protein c as the only major protein in the outer membrane strongly stimulates the rate of cephaloridine hydrolysis. Consistent with earlier observations [5] the presence of protein b is more effective than that of protein c. The presence of the λ receptor protein as an arbitrary pore protein (strain P692*tut*2dI(R1) grown in yeast broth supplemented with maltose) is accompanied by a rate of hydrolysis of cephaloridine which is even lower than in cells of strain P692*tut*2dI in which the λ receptor was not induced. This low rate of hydrolysis is probably due to catabolite repression caused by the presence of maltose as the specific activity of β-lactamase (and also of 5′-nucleotidase) decreased by 25—45% when this sugar was present in the growth medium (Table II).

All strains grew with the same rate before and after the uptake experiments, showing that cell death cannot be responsible for low rates of uptake. Leakage of 5′-nucleotidase or β-lactamase was not significant, indicating that extracellular conversion of 5′-AMP and cephaloridine, respectively, could not influence the uptake. The activities of these enzymes, expressed per mg dry weight cells, were the same for all strains (except when grown in medium supplemented with maltose), indicating that differences in rates of hydrolysis of 5′-AMP or cephaloridine are due to differences in the permeability of the outer membrane.

Discussion

The results of our freeze-fracture experiments show that the 'introduction' of a major protein in a particle-poor background ($b^-c^-d^-e^-\lambda rec^-$ cells) results in

a considerable increase in the number of $\widetilde{OM}$ particles/unit cell surface (Table II and Fig. 2). This correlation was observed earlier under slightly different conditions [7]. Moreover, a greatly enhanced pore activity was observed after 'introduction' of a pore protein in the $b^-c^-d^-e^-\lambda rec^-$ background (Table II). Various mutants which contain only one protein species of the five proteins under study overproduce this protein in the absence of the other major proteins (Fig. 1 and text), a phenomenon observed previously in outer membrane protein mutants [2,29,31,37,38]. This phenomenon probably explains why in some mutants the particle density approaches the density of wild-type cells.

The overproduction probably is the reason why the pore activity of such mutants for certain solutes is higher than the corresponding activity of wild-type cells (compare for instance the rates of uptake of strains AB1133(R1) and CE1096(R1) for 5′-AMP and cephaloridine in Table II. The results presented in Table II indicate that the individual pore proteins in the absence of four other major proteins function normally. If individual pores contained two or more of these protein species one would expect that the pore activity in a mutant which contains only one of these proteins would not exceed that of cells lacking all proteins under study. The results therefore most likely exclude the possibility of a substantial contribution of hypothetical mixed pores to the total pore activity for the used solutes. Our results are consistent with the notion that a single pore contains only one major protein species. The results of uptake experiments with single mutants are also best explained by this hypothesis [5,21]. Also recent results of experiments which demonstrate the function of purified single pore protein species in vitro suggest that it is more likely that a single pore contains only one protein species instead of being more complex since under certain conditions the rate of permeation of glucose through the b-pore in vitro is in the same order of magnitude as the rate measured in whole cells [39]. Moreover Palva and Randall showed that proteins b, c and d can be cross-linked to themselves, but not to each other, which makes the occurrence of mixed pores very unlikely [40]. The presence of minor proteins in these major outer membrane protein pores is unlikely for reasons of stoichiometry but our results do not exclude the possibility that the only major protein which is present in amounts comparable with the proteins studied, the lipoprotein [3], is involved in the functioning of at least one of these types of pores. Also this possibility seems not very likely as lipoprotein-deficient mutants function well in the uptake of 6-aminopenicillanic acid [41] and of 5′-AMP (unpublished results). Moreover, $\widetilde{OM}$ particles can also be introduced by Ca^{2+} [7]. It is of interest to note that these particles (which probably do not contain protein) are inactive as pores for 5′-AMP (unpublished results) supporting the notion that the presence of a protein is a prerequisite for pore activity.

In conclusion, the combined results of freeze-fracturing experiments and uptake studies show that the 'introduction' of one of the proteins b, c, e or the phage λ receptor protein into a $b^-c^-d^-e^-\lambda rec^-$ background is accompanied by a strong increase in the amount of the individual protein, in the number of $\widetilde{OM}$ particles and in the permeability of the outer membrane for a specific solute. These results provide strong support (but no proof) for our hypothesis that particles containing protein are the morphological representations of aqueous pores. It is very likely that the outer membrane of wild-type cells contains sub-

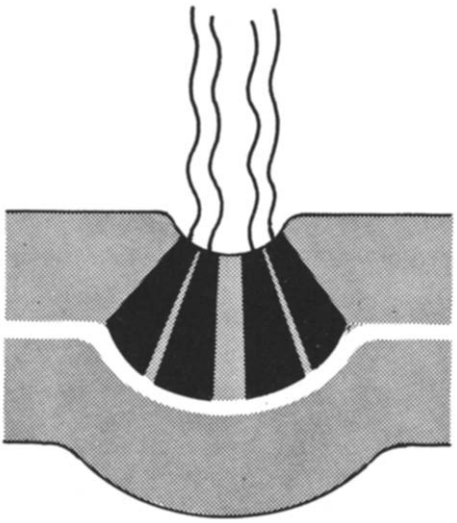

Fig. 3. Schematic representation of a transversal section of an $\breve{OM}$ particle with corresponding $\hat{OM}$ pit. This figure shows only the wedge-shaped organization of lipopolysaccharide molecules in $\breve{OM}$ particles and their embedding in the lipid matrix due to a proposed interaction with divalent cations, protein and/or polyamines. Protein has not been included in this figure. For explanation see text.

populations of b-, c- and e-pores and, when maltose is present in the growth medium, also of λ receptor protein pores, all being represented by particles.

It was argued that aggregates of lipopolysaccharide molecules are the structural basis of the morphological appearance of freeze-fracture particles [7,9]. A schematic drawing of this idea is presented in Fig. 3. We propose that in a particle neighbouring lipopolysaccharide molecules are organized in a wedge-shaped structure with a relatively more bulky apolar than polar part as a result of charge neutralization of the negatively charged phosphate and/or 3-deoxy-D-manno-octulosonic acid residues [42,43], thereby creating hemi-micelles ($\breve{OM}$ particles) in the outer monolayer. In a $b^-c^-d^-$ mutant such lipopolysaccharide particles can be induced by Ca^{2+}, except in heptoseless strains which lack some phosphate residues [7]. In wild-type cells proteins, divalent cations and/or polyamines can be responsible for the charge neutralization thereby creating hemi-micelles in which the pore protein is located, although it is not necessary that the protein is visible in the particles [44]. The hemi-micelles make impressions in the inner monolayer ($\hat{OM}$ pits), complementary to the $\breve{OM}$ particles. Cleavage would take place between the two monolayers. There is some evidence that lipopolysaccharide plays not only a role in the morphological appearance of $\breve{OM}$ particles but also in the functioning of pores as protein preparations of Enterobacteriaceae which have been used for the in vitro demonstration of pore activity all contain lipopolysaccharide [15,16,40].

Acknowledgements

The technical assistance of Ria van Boxtel, Irma van Die and José Leunissen-Bijvelt is greatly acknowledged. This work was supported in part by the Nether-

lands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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