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A freeze-fracture study of the membrane morphology of phosphatidylethanolamine-deficient *Escherichia coli* cells

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Abstract

Freeze-fracture electron microscopy was applied to study membrane morphology in a phosphatidylethanolamine-deficient *E. coli* strain. For growth, this strain requires millimolar concentrations of specific divalent cations like Mg^{2+} or Ca^{2+}. These cations bring the bilayer to nonbilayer phase transition temperature of the lipids back to wild type levels by shifting the phase preference of cardiolipin in the membrane towards the inverted hexagonal (H_{II}) phase. Under growth conditions, these cells show a bilayer based membrane with an intramembrane particle distribution as in wild type cells. Upon lowering the temperature, smooth areas are observed corresponding to gel state lipid bilayer domains. Ca^{2+} was used to manipulate the phase behavior of the membrane lipids in situ. Exposing the cells to Ca^{2+} up to 100 mM at 42°C did not result in the appearance of nonbilayer structures, despite the fact that in total lipid extracts under these conditions the hexagonal H_{II} phase was observed. However, the addition of a Ca^{2+} ionophore, which leads to exposure to Ca^{2+} of both faces of the plasma membrane, gives rise to formation of H_{II} phase, stacked bilayer domains and blebbing upon addition of 50 mM $CaCl_2$ at 42°C. We conclude that the asymmetrical localization of divalent cations in the periplasm of this strain allows them to be functionally effective while membrane stability is maintained.

Keywords: Lipid polymorphism; Freeze-fracture; (*E. coli*)

1. Introduction

In many biological membranes, lipid species are abundant which, upon isolation, are more favorably accommodated in a nonlamellar arrangement, such as the inverted hexagonal (H_{II}) phase. These lipids can be expected to greatly influence the structural and functional organization of the membrane. In wild type *Escherichia coli*, the zwitterionic nonbilayer lipid phosphatidylethanolamine (PE) forms approximately 70% of the phospholipids in the plasma membrane [1]. Whether the presence of such a nonbilayer lipid is important for membrane structure and function is studied with the mutant *E. coli* strain AD93, which lacks PE as a membrane constituent [2]. The two major lipid species remaining to constitute the bilayer backbone of the membrane are the negatively

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charged lipids phosphatidylglycerol (PG) and cardiolipin (CL). This strain requires millimolar concentrations of specific divalent cations for growth, such as Mg^{2+}, Ca^{2+} or Sr^{2+}. When isolated, the membrane lipids form a stable bilayer in the absence of divalent cations. However, in the presence of the growth promoting divalent cation, the lipids undergo a bilayer to nonbilayer phase transition slightly above the growth temperature, starting at approximately 40°C. Such a phase transition is observed in the same temperature range with lipids isolated from wild type (PE containing) cells in the absence of divalent cations [3]. This suggested that divalent cations are required to allow the strain to maintain a balance in bilayer and nonbilayer propensity of the membrane lipids, as has been proposed in the concept of polymorphic regulation of lipid composition. Several observations supported this hypothesis. It was found that in AD93 cells grown with Ca^{2+}, the level of CL was significantly lower (30 mol%) than in cells grown with Mg^{2+} or Sr^{2+} (50 mol%). This is consistent with the observation that Ca^{2+} has a stronger potency than Mg^{2+} to shift the phase preference of CL towards the H_{II} phase [4,5]. Indeed, when Ca^{2+} was added to a dispersion of AD93 lipids isolated from Mg^{2+} grown cells, a bilayer to H_{II} phase transition was observed approximately 10°C below the growth temperature. Further support was obtained by the observation that Ba^{2+}, which cannot induce formation of H_{II} phase in isolated CL or AD93 total lipid extracts in dispersion, cannot sustain growth of the mutant strain [3].

Changes in the Mg^{2+} concentration in the growth medium in the range of 1 μM to 0.1 M lead to only minor changes in the cellular concentration of Mg^{2+} in *E. coli* [6], indicating that the cytosolic Mg^{2+} level is actively regulated. This intracellular Mg^{2+} is dispersed throughout the cytoplasm and is not preferentially associated with the plasma membrane [7]. Ca^{2+} and Sr^{2+} are actively excluded from the cell by antiporter systems [8,9]. In AD93 cells, these growth promoting divalent cations are therefore most likely effective on the periplasmic side of the plasma membrane. This was supported by an in vitro assay with AD93 derived inverted membrane vesicles, in which the presence of growth promoting divalent cations at the periplasmic side of the membrane was shown to be required for efficient transport of a precursor protein [10].

In this study, we analyze the membrane morphology of the mutant strain with freeze-fracture electron microscopy. So far all knowledge on polymorphic regulation of lipid composition in *E. coli* originates from in vitro approaches and observations. The unique possibility to manipulate the phase preference of the membrane lipids of the mutant strain with divalent cations was used to study the organization of the plasma membrane and test the implications of regulation of lipid phase behavior in vivo.

2. Materials and methods

2.1. Bacterial strains and growth

The mutant strain AD93 and its parental strain W3899 were used in this study. Strain AD93 (*pss93::kan recA srl*::Tn*10* $nadB^+$) is a P_1 derivative of W3899 (*pss*$^+$ *nadB7*) [2]. The strains were grown in LB (10 g tryptone (Sigma), 5 g yeast extract (Sigma) and 10 g of NaCl/l) at 37°C. AD93 cultures were supplemented with 50 mM $MgCl_2$. The cells were harvested in mid log phase of growth (A_{660} = 0.8) in a Sigma 302 K table top centrifuge at 700 × *g* for 3 min at 37°C. For light microscopy, cells were immobilized by suspending the wet pellet 1:1 in 25% (v/v) glycerol-0.1 M Tris (pH 8.5) containing 0.1 g/ml Mowiol and 1 mg/ml *para*-phenylenediamine (PPD), and studied with a Leitz Orthoplan phase contrast microscope. For electron microscopy, the cells were resuspended in a physiological salt solution (9 g NaCl/l) either without or with divalent cations at the indicated concentrations, incubated for 4 min at 37°C and quenched at 10, 37 or 42°C. Where mentioned, cells were pre-incubated on ice for 1 min and quenched at 37 or 42°C.

2.2. Extraction of lipids

AD93 membrane lipids were extracted according to Bligh and Dyer [11]. The lipids were converted to their sodium salts as described by Smaal et al. [12]. The lipids were dissolved in chloroform which was evaporated under a stream of nitrogen. Residual solvent was evaporated overnight under high vacuum. The lipid film was hydrated in a Pipes buffer (10 mM pH 7.4), where mentioned, supplemented with 50

mM $MgCl_2$ at room temperature and carefully vortexed. The dispersion was then centrifuged in an Eppendorf table-top centrifuge at maximum speed for 15 min and the supernatant was discarded. The lipid concentration was determined on the basis of P_i content according to Rouser et al. [13].

2.3. Incorporation of Ca^{2+} ionophore A23187

The total phospholipid content of cell suspensions was determined upon lipid extraction as described. A cell suspension equivalent to approximately 140 μM phospholipids was incubated with 5 mM EDTA for 4 min to destabilize the outer membrane, thereby facilitating the incorporation of the Ca^{2+} ionophore A23187 (Sigma). A23187 was added from an ethanol stock to a final ionophore concentration of 0.8 nM and a final ethanol concentration of 0.1% (v/v), and the suspensions were incubated for another 4 min. Cells were then washed to remove EDTA and pelleted in a table-top Eppendorf centrifuge for 30 sec at maximum speed, resuspended in a physiological salt solution containing 50 mM $CaCl_2$ and incubated at 37°C for 5 min. The cells were quenched at 37 or 42°C. The release of maltose binding protein (MBP) from the periplasmic space upon treatment with EDTA was quantified after incubation of the growth medium with a polyclonal antibody directed against MBP and subsequent incubation with protein A Sepharose. The precipitate was applied to SDS-PAGE and transferred to nitrocellulose and colored with standard Western blot techniques to increase the sensitivity of the detection. The MBP bands were quantified with densitometry. A 100% standard was obtained by treating cells with 0.5% Triton X-100, prior to immuno-detection.

2.4. Freeze-fracture electron microscopy

Cell samples or lipid dispersions, prepared as described, were applied to copper grids, placed between two copper holders (BAL-TEC) and quenched from different temperatures in a double sided propane jet freezer (BAL-TEC, JFD 030) at an average cooling rate of $3.9 \cdot 10^4$ Ks^{-1}. The samples were inserted in a hinged double replica table and fractured in a BAF 400 freeze-fracture unit at −108°C and 10^{-7} torr. Fractured samples were replicated with carbon-platinum from a 45° angle and with carbon from a 90° angle. The replicas were cleaned in Glorix®, washed in water and picked up on uncoated 400 mesh nickel grids. Electron micrographs were obtained with a Philips CM10 electron microscope.

2.5. Differential scanning calorimetry

Approximately 20 μl of an AD93 derived lipid dispersion corresponding to 4–5 mg lipids was sealed in an aluminum sample pan. A Perkin Elmer DSC-4 differential scanning calorimeter was operated at a scan rate of 5°C/min. The dispersions were scanned in the range of −10 to 45°C for at least 5 times to demonstrate the reversibility of the transition. An empty sample pan was used as a reference.

3. Results

3.1. Membrane morphology and particle distribution

With light microscopy we have investigated whether the unusual membrane composition of the mutant strain AD93 leads to changes in overall cell shape. A distinguishable alteration was found, compared to the wild type parental strain W3899 (Fig. 1). The mutant cells form filaments and are estimated to exceed the wild type cell length up to a hundred times. To further study the cell morphology at the ultrastructural level, freeze-fracture electron microscopy was applied. Physical fixation of cells by rapid freezing can be regarded as a reliable method to preserve the overall cell shape and trap membrane molecules in their native state [14]. With freezing at rates of $4 \cdot 10^4$ Ks^{-1} or more, the formation of large ice crystals is suppressed and the use of chemical prefixation and cryoprotectants has become redun-

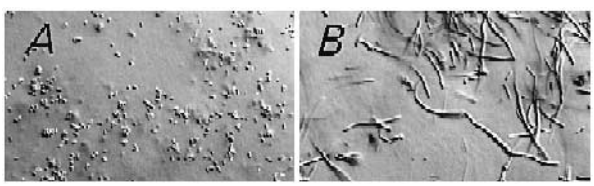

Fig. 1. Light micrographs of W3899 wild type (A) and AD93 *E. coli* cells (B). (Magnification 400×.)

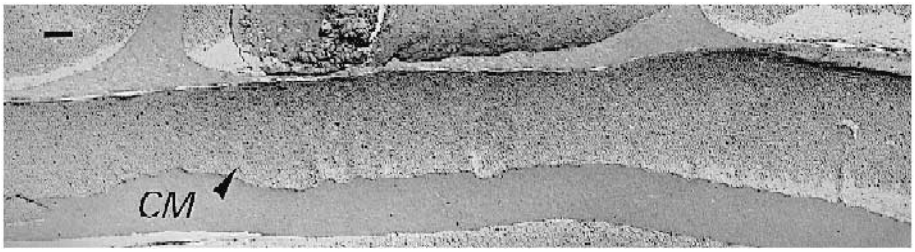

Fig. 2. Freeze-fracture electron micrographs of AD93 cells show that the filaments do not form septa but have a continuous plasma membrane. CM: cytoplasmic membrane. In all electron micrographs, the bar represents 100 nm.

dant [15]. Suspensions of the mutant strain were quenched under growth conditions at 37°C and freeze-fractured as described. From the electron micrographs, it can be concluded that the AD93 filaments do not have constrictions or septa, such as can be observed in normally dividing cells as well as in some filament forming mutants [16], but form a continuous cytoplasmic membrane (Fig. 2). The cytoplasmic as well as the complementary exoplasmic fracture faces of this membrane show a homogeneous distribution of membrane particles (Fig. 3A and B), similar to that observed in wild type cells [17,18]. The granular appearance of the outer membrane (Fig. 3B) reflects the high density of outer membrane porins [19].

3.2. Gel to liquid crystalline phase transition and lateral phase separation

The gel to liquid crystalline phase transition of AD93 lipids in dispersion was measured with differential scanning calorimetry. Scans of aqueous dispersions of AD93 lipids could not resolve the transition temperature in the absence of divalent cations (Fig. 4). This is probably due to the low enthalpy of the phase transition of these negatively charged lipids in combination with their heterogeneous fatty acid composition. However, in the presence of 50 mM Mg^{2+}, a broad transition peak was observed, starting at 12.0°C, with a maximum at 20.5°C and ending at 25.0°C. We therefore expect the AD93 membrane lipids in vivo to be in the gel state at temperatures below 12°C. Indeed, when quenched at 10°C in the presence of Mg^{2+}, many smooth particle denuded patches can be observed in the cytoplasmic membrane, reflecting the occurance of a lateral phase separation (Fig. 3C). Also when quenched at 10°C in the presence of Ca^{2+} or in the absence of divalent cations, this lateral phase separation can be observed (not shown).

3.3. Lipid polymorphic phase behavior

Since there are no phase separations observed in the electron micrographs at 37°C (Fig. 3A and B) it is concluded that the membrane lipids form a liquid crystalline bilayer. This is consistent with the DSC data described in the previous paragraph. To learn more about the polymorphic behavior of the lipids in situ, conditions were applied which were expected to shift the phase equilibrium of the lipids towards nonbilayer (type II) structures. First, this was carried out by increasing the temperature. Up to 42°C, which is the highest temperature at which samples were quenched, no nonbilayer structures could be detected in Mg^{2+} grown cells in Mg^{2+} containing medium (not shown), despite the fact that the initiation of a bilayer to non-bilayer phase transition was observed in the isolated lipids at 40°C [3]. This indicated that in intact cells the membrane lipids are more strongly constrained to a bilayer configuration, compared to the isolated lipids. In order to probe the extent of this constraint, conditions were required which further shift the phase preference of the membrane lipids towards the H_{II} phase. Since it is technically difficult to quench cells from higher temperatures, we chose to use Ca^{2+} as a tool to influence the the phase preference of the lipids. The propensity of the lipids to adopt a nonbilayer conformation is more enhanced by Ca^{2+} than by Mg^{2+}, which was shown with ^{31}P-NMR measurements with AD93 derived lipids [3]. First the isolated lipids from Mg^{2+} grown cells

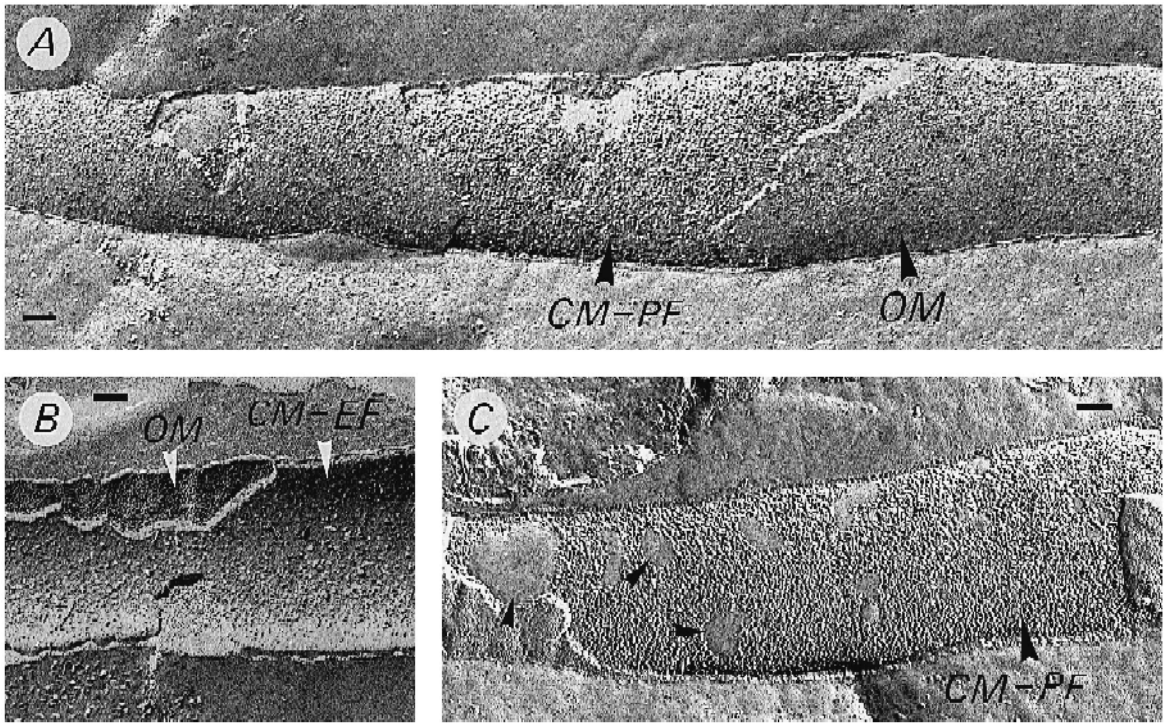

Fig. 3. Freeze-fracture electron micrographs of *E. coli* AD93 grown with 50 mM Mg^{2+}, quenched at 37°C (A and B). When quenched at 10°C, the cytoplasmic membrane displays extended areas which are devoid of intramembrane particles (C; small arrows). CM: cytoplasmic membrane; OM: outer membrane; PF and EF: protoplasmic and exoplasmic hydrophobic face of the cytoplasmic membrane.

were quenched in the presence of 50 mM Ca^{2+} at 42°C. Indeed, the formation of nonbilayer structures was observed, bordered by stacked bilayers (Fig. 5A). A striated pattern can be recognized which is characteristic for the inverted hexagonal phase in which the lipids form cylinders with aqueous cores, arranged in a hexagonal lattice [20]. However, when Mg^{2+} grown intact cells were treated with 50 mM Ca^{2+} and quenched at 42°C, the cells were able to accommodate this increased pressure towards formation of nonlamellar structures without an actual phase transition taking place. No structural reorganizations were observed. Identical results were obtained with 100 mM Ca^{2+} (Fig. 5B).

In intact wild type *E. coli* cells, the formation of multilayer and hexagonal arrangements in the membrane can be induced by preincubating cells at 0°C, followed by a heatshock at 42°C in the presence of Ca^{2+} [18]. These conditions are usually applied to achieve the uptake of plasmid DNA. The same prototcol was carried out with AD93. Mg^{2+} grown cells were cold-treated on ice for 1 min and subsequently subjected to a heat shock at 42°C for 1 to 5 min in the presence of 50 or 100 mM Ca^{2+} and immediately quenched at 42°C. Under none of these conditions, membrane morphology of the mutant strain was affected (not shown).

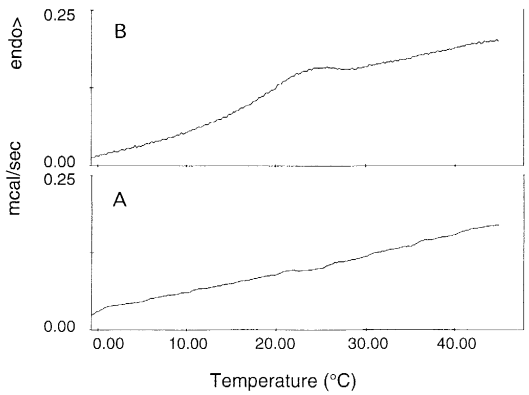

Fig. 4. Calorimetric scans of aqueous dispersions of AD93 derived total lipid extracts in 10 mM Pipes (pH 7.4) without divalent cations (A) or with 50 mM $MgCl_2$ (B). A heating rate of 5°C/min was employed.

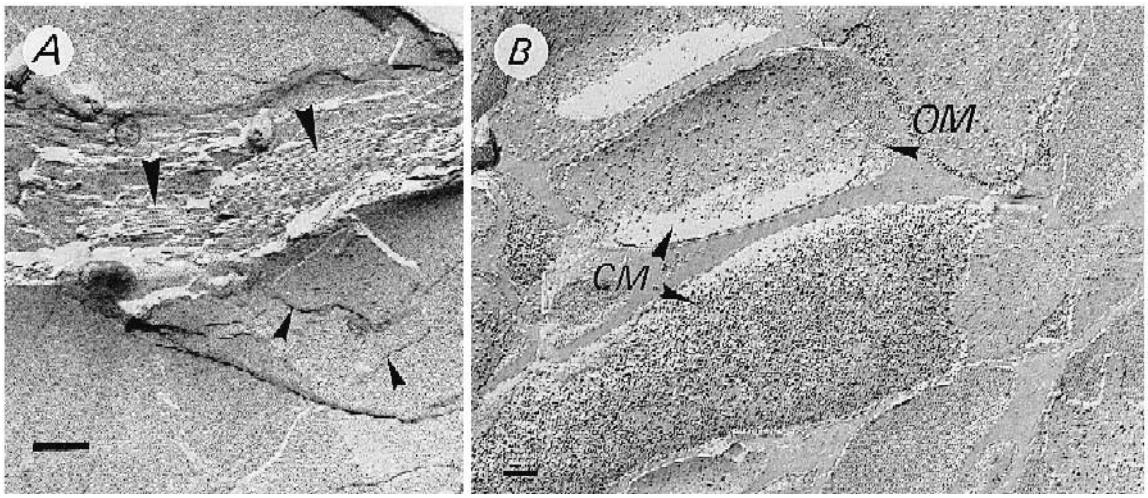

Fig. 5. A dispersion of AD93 derived lipids in 10 mM Pipes (pH 7.4) was incubated with 50 mM Ca^{2+} and quenched at 42°C. Extended H_{II} domains (large arrows) border the stacked bilayers (small arrows) (A). Mg^{2+} grown AD93 cells were incubated with 100 mM Ca^{2+} at 42°C. A transition to the H_{II} phase was not observed under these conditions (B). CM: cytoplasmic membrane; OM: outer membrane.

It is expected that the high concentration of divalent cations required for growth of the mutant strain function in the periplasm and thus should exert pressure towards type II structures only in the outer leaflet of the inner membrane. The preference of the lipids in the inner leaflet to adopt a bilayer configuration could counteract this pressure towards formation of nonbilayer structures. If it is assumed that CL is present in both leaflets, then allowing entry of divalent cations into the cytosol would exert a stronger destabilization of the bilayer structure. This was tested by incorporation of the calcium specific ionophore A23187. The cells were treated with EDTA to render the plasma membrane accessible for the ionophore. To test this accessibility, the release of the periplasmic maltose binding protein (MBP) was quantified under the applied conditions. Upon treatment with EDTA, with and without the ionophore and Ca^{2+}, 70 to 80% of the MBP was released from the periplasmic space, compared to 7% release from control cells which were not treated with EDTA (Fig. 6). This was taken as an indication that the ionophore will have access to the plasma membrane. After incubation with A23187, the cells were incubated at 37°C in the presence of 50 mM Ca^{2+} and quenched at 42°C. Under these conditions, a variety of changes occur in the cytoplasmic membrane (Fig. 7A and B). Large domains can be observed in which the lipids are in H_{II} phase or in stacked bilayers. In the H_{II} domains, the hexagonal packing of the cylinders gives rise to very symmetrically striated appearance, whereas in a cross-section of stacked bilayers, the linepattern is more irregular. The release of blebs and small vesicles (Fig. 7B) indicates that also the structure of the outer membrane is disturbed. This is consistent with

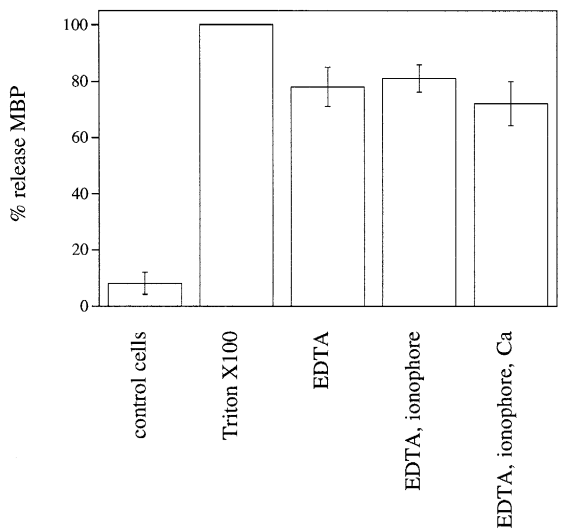

Fig. 6. The release of MBP was quantified as described. Control: cells which were not treated with EDTA; Triton X-100: 100% release standard of Triton X-100-treated cells; Bars 3 to 5: cells treated with EDTA, with and without ionophore and Ca^{2+} as indicated.

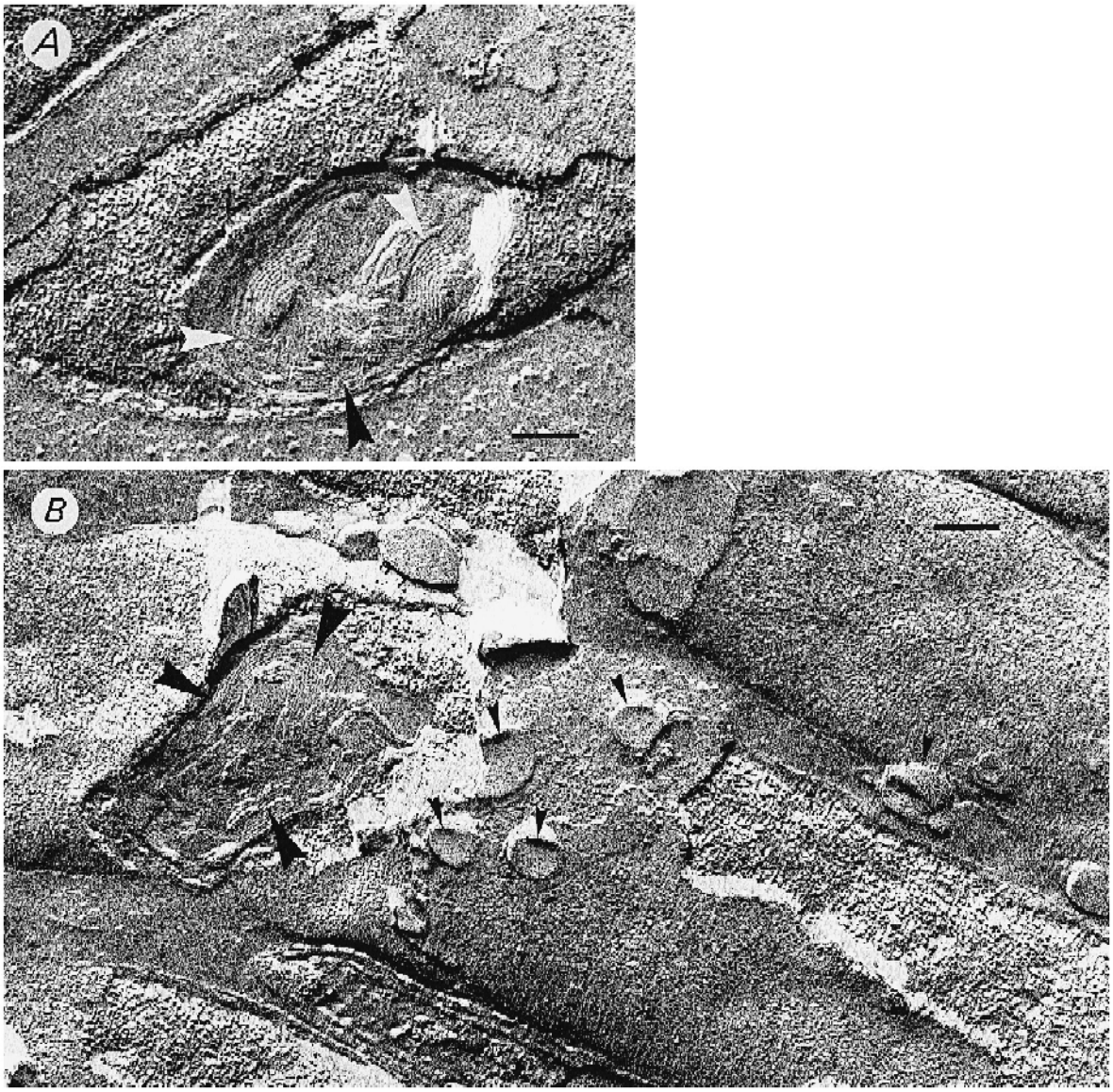

Fig. 7. Mg^{2+} grown AD93 cells were treated with 50 mM Ca^{2+} after incorporation of the Ca^{2+} ionophore A23187, and quenched at 42°C (A and B). Large disrupted domains are present in the cytoplasmic membrane where H_{II} structure can be observed (large black arrows) as well as the stacking of bilayers (large white arrows). The cells also show frequent blebbing (small arrows) (B).

the observed release of MBP under these conditions. The same treatment either without the ionophore or without Ca^{2+} does not lead to any such changes in membrane morphology (not shown). This excludes the possibility that EDTA or the ionophore itself induce formation of nonbilayer structures. Thus, the disruption of the cytoplasmic membrane is observed only in the presence of both ionophore and Ca^{2+}, from which we conclude that incorporation of the ionophore does occur and an influx of Ca^{2+} is achieved. This leads to a double sided exposure to Ca^{2+} and the subsequent formation of extended non-bilayer and multilayer domains in the membrane.

4. Discussion

The cell and membrane morphology of PE-deficient *E. coli* cells were studied with light microscopy

and freeze-fracture electron microscopy. The filamentation of these cells is a feature which is observed in the light micrographs. In *E. coli*, mutations of variable nature have been reported to cause filamentation, which may be considered as a more general stress response [21]. It is also conceivable that in this strain, filamentation is caused specifically by the absence of PE. For instance in the initiation of septum formation, PE may be necessary for the function of FtsZ, a cytosolic GTPase which supposedly initiates the membrane invagination by forming a membrane associated cytokinetic ring [22]. A correct interaction of FtsZ with the membrane may now be hindered by the overall negative charge of the interface or by conformational changes in FtsZ or a putative receptor protein, either of which may be sensitive to the lipid environment. It is also possible that divalent cations, present in the periplasm at high concentration, cause the cross-linking of components of the two opposing leaflets of the cytoplasmic and outer membrane, bridging the periplasm. Such an interaction can be imagined to obstruct the invagination of the membrane. Although wild type *E. coli* cells divide normally in the presence of 50 mM Ca^{2+}, the negative charge of the opposing membrane leaflets in the mutant strain will obviously cause a stronger interaction with divalent cations, possibly together with other components of the periplasmic space [23].

In the isolated membrane lipids, a gel to liquid crystalline phase transition was observed in the temperature range of 20 to 25°C in the presence of Mg^{2+} at the same concentration as present during growth, similar to the phase transition in wild type *E. coli* derived lipid dispersions [24]. This can be explained by the observation that the fatty acid composition of the mutant strain is unchanged compared to wild type cells, grown at 37°C [25]. It is the fatty acid length and degree of saturation which mainly determine the gel to liquid crystalline phase transition temperature [26]. The electron micrographs of cells quenched at 37°C show that the bilayer structure and the distribution of membrane proteins was not distinguishably altered relative to wild type PE containing cells. Also, when quenched at 10°C, the cells exhibit a lateral phase separation in the plasma membrane, similar to that observed in wild type cells at this temperature. Thus, under normal growth conditions, the mutant strain possesses a bilayer based membrane which is not discernibly altered in overall organization by the absence of PE.

With increasing the temperature, exposing the cells to a high Ca^{2+} concentration or applying conditions used to induce the formation of nonbilayer structures in wild type *E. coli*, the phase equilibrium of the membrane lipids was shifted towards the nonlamellar phase. Contrary to expectations, no polymorphic changes could be detected in the mutant under these conditions. A specific feature of the mutant strain which likely influences the lipid phase behavior is the asymmetric presence of divalent cations. When Ca^{2+} is allowed into the cell with the use of a Ca^{2+}-ionophore, large nonbilayer domains are formed. As a control, cells were treated with EDTA to disturb the outer membrane and subsequently exposed to excess Ca^{2+}. This treatment did not lead to structural changes in the cytoplasmic membrane, which demonstrated that it is the additional exposure of the cytoplasmic leaflet of the membrane to Ca^{2+} which triggers the formation of H_{II} phase. These experiments show that maintenance of an asymmetric distribution of divalent cations is a critical determinant in lipid polymorphic behavior in this strain. With an asymmetric transmembrane distribution, divalent cations can shift the preference of the membrane lipids towards a nonbilayer phase only in the outer leaflet of the inner membrane. In vitro it was shown that a stable bilayer can be maintained despite a high content of nonbilayer preferring lipids in one leaflet. Stollery and Vail [27] report that unilamellar vesicles were formed with pure egg PE. Already at 25°C this lipid undergoes a transition to an H_{II} phase under physiological conditions [28]. However, vesicles can be formed at low ionic strength and high pH (9.2). After formation of the vesicles, the external pH could be lowered to 7.2 by means of dialysis without causing the vesicle morphology to change. Analogous to this observation, in the mutant strain nonbilayer preferring lipids can be present in the outer leaflet of the plasma membrane while a stable bilayer is maintained. A situation in which the two membrane leaflets have distinct properties also exists in the outer membrane. Lipopolysaccharide (LPS) is a major component of the outer leaflet, while PE is the main constituent of the inner leaflet in wild type cells [29]. In the mutant strain, the phospholipid composition of the inner leaflet of the outer membrane is equal to the compo-

sition of the total inner membrane (Rietveld, unpublished results), containing CL and PG. This leaflet will also be exposed to divalent cations which results in a propensity to form nonbilayer structures. This pressure will be counteracted by the LPS in the outer leaflet, providing the outer membrane with a stable bilayer structure.

The presence of nonbilayer preferring lipids in the outer leaflet of the cytoplasmic membrane can have several functions. We have demonstrated previously that in the process of protein translocation across the cytoplasmic membrane in AD93 membrane vesicles, divalent cations markedly increase the translocation efficiency when they are present at the periplasmic side of the membrane. This increased efficiency of transport was also achieved by the incorporation of the nonbilayer preferring lipid DOPE [10]. Another possible function of nonbilayer lipids in the outer leaflet of the plasma membrane is to facilitate contact formation between the inner and outer membrane in *E. coli*. It has been shown in vitro that the fusogenic tendency of lipid bilayer vesicles is largely dependent on the properties of the external monolayers at the fusion interface [30]. Nonbilayer preferring (type II) lipids, and also lipid mixtures containing type II lipids, can form complex bicontinuous liquid crystalline cubic phases which possess a net concave water–lipid interface and allow for a lateral diffusion of lipids in the same order of magnitude as a liquid crystalline bilayer [31]. It is conceivable that such structures occur as contact sites between the two proximate membranes in *E. coli*, thus creating a possibility for lipid flow from inner to outer membrane without the loss of the plasma membrane barrier function. Such a semifusion event has been proposed earlier to occur in the formation of membrane contact sites [20]. In the mutant strain, this semifusion could be facilitated by the presence of divalent cations. This idea is consistent with the functional location of divalent cations in the periplasmic space.

Acknowledgements

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