Ultrastructural Changes in *Escherichia coli* Grown in Divalent Cation-deficient Medium

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*Escherichia coli* strains B and K12 could grow in very limiting conditions of divalent cation deficiency. Growth curves showed a long lag period of about 30 h, followed by an exponential phase bringing the bacterial concentration to about $10^7$ ml$^{-1}$, with a 24 min doubling time, while the growth curves of control cultures were characterized by short lag periods, maximum populations of about $10^9$ ml$^{-1}$ and an 18 min doubling time. The DNA/protein ratio in bacteria grown in deficient medium was 0.48 compared with 0.21 for control bacteria. Significant differences were found in the ultrastructure of the two types of bacteria. Freeze-etched control cells showed the typical appearance with the protoplasmic fracture face of the cytoplasmic membrane (PFC) having a random distribution of intramembranous particles. Bacteria growing in deficient medium in exponential phase presented several particle-free areas on the PFC. At the beginning of the stationary phase, the particle-free zones became larger and crystalline structures were formed. These structural modifications, which increased with culture age, were never observed in bacteria grown in control medium. Optical diffraction analysis of the crystalline structures in freeze-etched cells revealed regular periodic arrays with a rhomboid repeating unit approximately $7.6 \times 5.4$ nm in dimension and an angle between the axes of about 73°. Negative staining of isolated membranes of bacteria grown in deficient medium showed a more complex organization of the crystalline arrays, each unit being clearly composed of subunits.

INTRODUCTION

A number of studies have been made on the specific role of Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$ in cell structure and function, apart from their role in maintaining ionic strength. Results *in vitro* for bacterial systems indicate specific requirements for divalent cations for some biosynthetic processes (Gesteland, 1966; Schlessinger *et al.*, 1967) as well as the capacity of Ca$^{2+}$ and Mn$^{2+}$ to substitute for Mg$^{2+}$ (Donelli *et al.*, 1973; Weiss *et al.*, 1973). Divalent cations are also responsible for the structural stability of nucleic acids (Ott *et al.*, 1975). The essential role of divalent cations in bacteria has also been demonstrated *in vivo* (Webb, 1949). Mg$^{2+}$ requirements of Gram-negative species are greater than those of Gram-positive species (Webb, 1966).

The only available information on the correlation between divalent cations and the most important structural and biochemical features of bacterial cells is from experiments on bacteria starved of divalent cations (Fil & Branton, 1969; Weiss, 1977).

Visicato (1968) showed that a streptomycin-resistant strain of *Escherichia coli* could grow in a culture medium to which no Mg$^{2+}$ had been added. In the present paper, the general validity of such an unexpected result has been verified both by extending the experiments to *E. coli* wild-type strains B and K12 and by enhancing the conditions of cation deficiency. An ultrastructural study was made of both intact bacterial cells and isolated cell envelopes.
METHODS

Organisms and growth conditions. Escherichia coli strains B and K12 (ATCC 11303 and ATCC 14948) were used. Bacteria were grown in a chemically defined divalent cation-deficient medium (DM) containing 5·0 g Na₂HPO₄·12H₂O, 2·5 g KH₂PO₄, 3·0 g NaCl, 2·0 g NH₄Cl, 0·1 g Na₂SO₄ and 1·0 g glucose in 1 l double-distilled water. The composition of the control medium (CM) was the same as DM but supplemented with MgSO₄·7H₂O (1 mm). To obtain the least concentrations of divalent cation impurities, Merck chemicals of ‘Suprapur’ and ‘Pro analysi’ grade were used.

Cultures were grown in a fermentation apparatus designed for the automatic sampling of microbial cultures (Donelli, 1974). The culture vessel (3 l) and all the glassware used was made of French Pyrex. The growth of each culture was monitored by viable counts using the double layer technique.

Analytical methods. DNA and proteins were extracted following the methods described by Marmur (1961) and Lowry et al. (1951), respectively. Purified ribosomes were obtained according to Gesteland (1966). Absorbance measurements were made with a Cary 15 spectrophotometer. Contamination levels of divalent cations were determined by atomic absorption with a Perkin-Elmer 603 spectrophotometer.

Isolation of cell envelopes. Samples of bacterial cultures (200 ml) were centrifuged at 2000 g for 15 min. The resulting pellet was suspended in a small volume of 0·2 M-ammonium acetate and then sonicated for 30 s with a Branson sonifier. The cell envelopes were collected by centrifugation at 25000 g for 30 min and resuspended in 0·2 M-ammonium acetate.

Electron microscopy. For negative staining of whole bacteria, a small drop of the cells (suspended in 0·2 M-ammonium acetate) was placed on a 400-mesh grid coated with a thin carbon film. After a few seconds of air drying the specimen was stained with 1·% (w/v) sodium phosphotungstate, pH 7·0. Isolated cell envelopes were observed after negative staining with 2·% (w/v) sodium phosphotungstate, pH 7·0.

For the experiments with OsO₄, the bacteria were suspended after centrifugation in 1·% (w/v) OsO₄ and then directly observed after periods varying from 10 min to 3 h.

Bacteria were fixed and embedded according to the technique of Kellenberger et al. (1958). To ensure a good penetration of the resin in the case of cells grown in DM, the infiltration times of Araldite were considerably extended as follows: Araldite/acetone (1:3), 8 h; Araldite/acetone (1:1), 15 h; Araldite/acetone (3:1), 8 h; Araldite, 80 h. Ultrathin sections were mounted on 300-mesh uncoated copper grids and observed either directly or after 20 min staining with lead citrate.

For freeze-etching, the pelleted samples of intact bacteria or isolated membranes were directly transferred, without pretreatment, to gold–nickel specimen holders and then quickly frozen from the growth temperature (37 °C) in Freon 22 pre-cooled by liquid nitrogen and finally stored under liquid nitrogen. The specimens were transferred rapidly to the pre-cooled table of a Balzers 360M apparatus (equipped with electron gun). The frozen specimens were fractured at −100 °C, then etched for 2 min and shadowed with platinum/carbon at an angle of 45°. Replicas were cleaned by overnight sodium hypochlorite treatment followed by double washing with distilled water. The replicas were mounted on 300-mesh uncoated grids.

Micrographs were taken on Kodak Electron Image plates with a Siemens Elmiskop 102 electron microscope operating at 60 or 80 kV.

The diffraction patterns were obtained with a Polaron optical diffractometer equipped with a 2·0 mW helium/neon laser and recorded on Polaroid film, type 55 P/N. Measurements were taken with a comparing microscope, with an accuracy of 0·01 mm.

RESULTS

 Cultures of E. coli strains B and K12 were grown either in divalent cation-deficient medium (DM) or in the control medium (CM) containing about 1 mmol Mg²⁺ l⁻¹. To determine the actual content of divalent cations in DM, several stocks were tested by atomic absorption spectrophotometry, which indicated an average value of 2 µmol l⁻¹ for Mg²⁺ and even lower values for Ca²⁺ and Mn²⁺.

Figure 1 shows typical growth curves obtained in CM and DM. The curves for CM cultures showed a short lag period and a maximum bacterial concentration of about 10⁹ ml⁻¹. The doubling times were 24 min for bacteria grown in DM and 18 min for those grown in CM. For DM cultures the growth curves always showed a long lag period (about 30 h) followed by an exponential phase reaching a maximum bacterial concentration of 5·0 × 10⁷ ml⁻¹ or a little less. When the exponential phase was complete, a sharp fall in viable count was observed, the rate of cell death being higher in these bacterial populations.

DNA, proteins and ribosomes were extracted from exponentially growing cultures and
E. coli in divalent cation deficiency

Fig. 1. Growth curves for *Escherichia coli* B in control medium (CM, ●) and in divalent cation-deficient medium (DM, ○). The doubling time in DM is 6 min longer than in CM.

quantitatively determined: values were related to $10^{10}$ bacteria. Similar results were found for DNA and ribosomes in bacteria from the two different cultural conditions. However, bacteria grown in DM had a lower protein content; the DNA/protein ratio was 0.48 in DM compared with 0.21 in CM.

Bacteria grown in DM showed poor resin infiltration and good preparations for electron microscopy were obtained only when infiltration times of about 100 h were used. Figure 2 shows a particularly significant comparison between ultrathin sections of bacteria grown in CM and DM. In the sections of bacteria grown in DM the nucleoid appeared as easily distinguishable DNA filaments in a wider and almost fully electron-transparent area, while the protoplasmic matrix was significantly denser. The cell envelopes showed a little increase in the periplasmic space and some signs of internal membranes (Fig. 2b). The envelopes differed in their reactivities to uranyl acetate used before embedding. Without lead citrate staining, the sections of bacteria grown in CM showed (Fig. 3b) a lower electron density of the outer membrane than bacteria grown in DM (Fig. 3a). No difference was revealed between the two cell types when intact bacteria were negatively stained with sodium phosphotungstate. However, differential behaviour was observed using OsO₄: bacteria grown in CM appeared much more electron-dense (Fig. 4b) than those grown in DM (Fig. 4a).

Freeze-etched *E. coli* cells from an exponentially growing culture in CM showed (Fig. 5) the well-known structural features previously observed (Bayer & Remsen, 1970; Nanninga, 1970) in Gram-negative bacteria. The convex protoplasmic fracture face of the cytoplasmic membrane (PFC) was characterized by a dense covering of randomly distributed intramembranous particles (IMP), about 8.0 nm in diameter. The cell envelope structure did not change in bacteria observed in the stationary phase, even after several days of culture. In contrast, the cytoplasmic membrane of bacteria grown in DM appeared clearly modified during the exponential growth phase (44 h): several roundish particle-free areas were present on the PFC (Fig. 6). During the stationary phase (68 h), particle-free areas became larger and it was possible to observe some clustering of the IMPs which tended to form crystalline arrays (Fig. 7). In bacteria cultured for 120 h or more (Fig. 8) the extension of these arrays increased to occupy almost the whole surface of the PFC.

No structural differences in the outer membrane were noted between the two cell types in the different phases of growth.

Optical diffraction analysis of the crystalline arrays demonstrated the presence of a periodic structure consisting of a rhomboid repeating unit (Fig. 9). The average values of
Fig. 2. Ultrathin sections of *E. coli* B grown in CM (a) and DM (b). The nucleoid of bacteria grown in DM appears as well-defined DNA filaments in a wider and almost fully electron-transparent area, while the protoplasmic matrix is significantly denser. Bar markers represent 0.5 μm.

Fig. 3. Unstained ultrathin sections of bacteria treated with uranyl acetate during dehydration. The outer membrane of bacteria grown in DM (a) is more electron-dense than that of bacteria grown in CM (b). Bar markers represent 0.1 μm.

the lattice parameters, with standard deviations, were \( a = 7.6 \pm 0.5 \text{ nm} \), \( b = 5.4 \pm 0.5 \text{ nm} \) and \( \theta = 73 \pm 9^\circ \).

Crystalline arrays were also observed in the negatively stained envelopes of bacteria grown in DM (Fig. 10). The better resolution obtained with negative staining compared with the freeze-etching technique revealed a more complex organization of the crystalline structures (Fig. 11). These were composed of subunits, each about 3 nm in diameter, arranged to form the lattice schematically shown in Fig. 12, the unit cell containing a pair of such subunits. These data were confirmed by optical diffraction (Fig. 13a) and by analysis of the optically reconstructed images (Fig. 13b). The average values of \( a \), \( b \) and \( \theta \), with standard deviations, were \( 8.0 \pm 0.5 \text{ nm} \), \( 5.9 \pm 0.4 \text{ nm} \) and \( 70 \pm 4^\circ \), respectively.
**Fig. 4.** Bacterial cells stained with 1% OsO₄ for 3 h. Bacteria grown in DM (a) are much less electron-dense than those grown in CM (b). Bar markers represent 1 μm.

**DISCUSSION**

Our results indicate the need for a long adaptation period for bacteria growing in a medium containing a sub-physiological concentration of divalent cations, and a slower metabolism revealed by an increase in doubling time as well as by reduced protein synthesis.

The ultrastructural results suggest a role for divalent cations at different levels. The ‘relaxation’ of the nuclear material could confirm the involvement of Mg²⁺ in the intramolecular bonding of DNA (Sander & Ts'O, 1971).

The high electron-density of the outer membrane observed in sections of bacteria grown...
Fig. 6. Freeze-etched cell from a culture growing exponentially (44 h) in DM. Several roundish particle-free areas are present on the PFC. Bar marker represents 0.1 \( \mu m \).

Fig. 7. Freeze-etched cell from a culture grown to the stationary phase (68 h) in DM. Arrows indicate the start of the crystallization process. Bar marker represents 0.1 \( \mu m \).

Fig. 8. Freeze-etched cell from a culture grown to stationary phase (120 h) in DM. The crystalline structures and large particle-free areas occupy almost the entire fracture face of the cytoplasmic membrane. Bar marker represents 0.1 \( \mu m \).
in DM may be interpreted (Costerton et al., 1974) by considering that the negative charges of lipopolysaccharides are not neutralized, due to the lack of positive ions in the medium, so that uranyl ions are bound instead. In bacteria grown in CM the negative charges are almost all neutralized by cations in the medium and consequently not available to bind uranyl ions.

The lower osmiophilia of bacteria grown in DM could be due to a reduced penetration of OsO₄ or, assuming the same degree of penetration, to a lesser reactivity of OsO₄ with the cytoplasm. Since OsO₄ reacts with the double bonds of unsaturated lipids and various amino acids and peptides, the lower electron-density could be related to the lower protein content of these cells.

The smooth areas seen on the PFC of freeze-etched divalent cation-deficient bacteria have been observed in several biological membranes and it is now accepted that they are lipid domains produced by a phase transition of lipids from the liquid phase to the gel phase. The lipid phase transition can be triggered by change of chemico-physical parameters such as temperature, pH, tonicity and divalent cation concentration (Verkleij & Ververgaert,
Fig. 11. Negatively stained crystalline array. The subunits are clearly visible. Bar marker represents 0.05 μm.

Fig. 12. Schematic model of subunit arrangement in the crystalline areas.

Fig. 13. Optical diffraction pattern of a negatively stained crystalline array (a) and the related optically reconstructed image (b).

1975; Bayer et al., 1977). Since we never observed these smooth areas in bacteria grown in CM, we believe these zones to be due to a lipid phase transition induced by the divalent cation deficiency. In cells grown in DM, prefixed with glutaraldehyde before freezing, we noted the disappearance of the smooth areas. This result suggests a redistribution of the IMPs as a result of prefixation. Such an effect of glutaraldehyde on freeze-etched E. coli has been described by Arancia et al. (1980).

The structural modification observed in the cytoplasmic membrane can explain the altered permeability of bacteria grown in DM in accordance with the hypothesis proposed by other authors concerning the relationship between some membrane functions, such as permeability, and the lipid phase transition (Haest et al., 1972; Papahadjopoulos et al., 1973).

On the PFC of bacteria growing exponentially in DM we never noted the paracrystalline patterns described by Fiil & Branton (1969) in E. coli cells grown in complete medium and then transferred to Mg²⁺-free medium. The start of the IMP aggregation process was not observed in our experiments before the end of the exponential growth phase, i.e. cells cultured for 60 to 70 h. The extent of the aggregation zones increased with culture age up to the formation of large crystalline arrays and, consequently, of neighbouring particle-free
areas. On the PFC of cells grown for 120 h or more, ‘lipid domains’ alternating with ‘protein domains’ were observed while the free IMPs had almost completely disappeared.

It is likely that these crystalline structures are composed of components of the protoplasmic half-membrane: we never found patterns of pits corresponding to the crystalline arrays observed on the PFC on the concave fracture face of the cytoplasmic membrane.

With regard to the lattice parameters, the differences between negatively stained and freeze-fractured patterns are not statistically significant though a tendency of vectors $a$ and $b$ towards higher values was noticeable after negative staining.

Regarding the ultrastructure of the crystalline arrays, neither the data from the direct observation of negatively stained preparations nor those obtained from the micrographs through optical diffraction and filtering (Donelli & Paoletti, 1977) seem to allow an interpretation such as that proposed recently by Weiss (1977). He observed similar crystalline arrays in Mg$^{2+}$-starved *E. coli* and suggested that Mg$^{2+}$ starvation leads to the development of a membrane junction (bracket junction) which, like the vertebrate gap junction, contains a stable array of IMPs. The junction particles are visualized as elongated units separated by a central stain-filled line whereas in our analysis the repeating units are composed of a pair of rounded subunits forming the lattice shown in Fig. 12. It is possible that the smaller particles forming the lattice are derived from the larger pre-existing IMPs by dissociation. This would mean that the IMPs observed under normal conditions are complexes of two to four subunits held together with divalent cations.

The interpretation of the crystalline arrays in terms of a membrane junction is doubtful for the following reasons. (1) In sectioned control bacteria it is quite frequently possible to observe folded cytoplasmic membranes, while crystalline arrays were never found after freeze-etching. (2) We have not verified in quantitative terms a satisfactory relationship between the folded membranes in sectioned bacteria grown in deficient medium and the crystalline arrays in the same freeze-etched bacteria. In the latter, most of the PFC appears occupied by the crystals, while in the corresponding sections the membrane folding necessary to justify the proposed junction is quite modest (Fig. 2b). (3) Freeze-etched bacteria should always exhibit at least the thickness of the two coupled membranes where the fracture plane passes through the cytoplasmic membrane in correspondence with the crystalline arrays. It is unlikely that the extremely low frequency with which this is observed can support the above-mentioned hypothesis.

On the basis of our results we suggest that the cytoplasmic membrane folding is simply the consequence of a rate difference between the two processes of cell division and membrane synthesis. In this respect, our experimental conditions, clearly indicating a decreased rate of cell division, are quite compatible with those (Mg$^{2+}$-starved cells at 37 °C for several hours) adopted by Weiss. Moreover, Weiss’ hypothesis seems to suggest, in keeping with the analogy of the vertebrate gap junction, a role for ‘bracket junctions’ in facilitating the permeation of the cell. However, our experimental data suggest a reduced permeability of *E. coli* grown in the virtual absence of divalent cations.

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