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# Ultrastructural and Chemical Alteration of the Cell Envelope of *Pseudomonas aeruginosa*, Associated with Resistance to Ethylenediaminetetraacetate Resulting from Growth in a $Mg^{2+}$-Deficient Medium

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Cells of *Pseudomonas aeruginosa* became resistant to the lytic effect of ethylenediametetraacetate (EDTA) when grown in a $Mg^{2+}$-deficient medium. To correlate ultrastructural changes in the cell wall associated with the shift to EDTA-resistance, a freeze-etch study was performed. Upon fracturing, the outer cell wall membrane split down the hydrophobic center to reveal the outer (concave) and inner (convex) layers. The concave cell wall layer of EDTA-sensitive cells grown in $Mg^{2+}$-sufficient medium contained spherical units resting on an underlying smooth support layer. Upon EDTA treatment, approximately one-half of these spherical units were extracted. Cells grown in $Mg^{2+}$-deficient medium were resistant to EDTA. The concave cell wall layer of EDTA-resistant cells had increased numbers of highly compacted spherical units, giving this layer a disorganized appearance. The highly compacted appearance of this layer was unaltered by EDTA treatment. Thus, growth in $Mg^{2+}$-deficient medium resulted in cells which were resistant to EDTA and which possessed an ultrastructurally altered outer layer of the outer cell wall membrane. Cell envelopes from EDTA-resistant cells were found to possess 18% less phosphorus, 16.4% more total carbohydrate, and 13.3% more 2-keto-3-deoxyoctonate than cell envelopes from EDTA-sensitive cells. There were also qualitative, but not quantitative, differences in the protein content of cell envelopes from EDTA-resistant and EDTA-sensitive cells.

*Pseudomonas aeruginosa* is particularly sensitive to the lytic action of ethylenediaminetetraacetate (EDTA) (6, 10). The primary action of EDTA is believed to be the extraction of functional divalent cations from the cell envelope (1, 2, 13), which, in turn, causes the release of phospholipid and of a protein-lipopolysaccharide complex (13, 14). This complex has the ultrastructural appearance of rodlets 20 to 25 nm in length, which are made up of spherical units 7 ± 1 nm in diameter (14). These spherical units reside in the middle layer of the outer cell wall membrane (9). Thus, the removal of the spherical units from cells on exposure to EDTA resulted in cells that were osmotically fragile. These cells were termed osmoplasts (1, 2).

Brown and Melling (4) reported that growth of *P. aeruginosa* in a basal medium limited in $Mg^{2+}$ resulted in cells which were resistant to lysis by EDTA. The studies described herein were undertaken, therefore, to determine whether ultrastructural and chemical alterations of the cell envelope occurred in conjunction with the acquisition of EDTA-resistance. Since the spherical units in the middle layer of the outer cell wall membrane appear to represent an EDTA-sensitive site, then the acquisition of EDTA resistance might affect the ultrastructural appearance of this structure. Similarly, the chemical composition of the cell envelope of EDTA-sensitive and EDTA-resistant cells might also show differences. The data described herein, therefore, confirm that both ultrastructural and chemical alterations occurred.

## MATERIALS AND METHODS

**Preparation of chemically defined $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient media.** A basal medium (BM 1) was used as described previously by Brown and Melling (4), with the modification that the glucose concentration was raised to 0.05 M. Other

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substances in final concentration in the medium were: 0.01 M $(NH_4)_2HPO_4$, 0.01 M $(NH_4)_2SO_4$, 0.5 mM NaCl, and 0.5 mM KCl. To this basal medium, $MgSO_4$ was added to give a $Mg^{2+}$ concentration of 0.5 mM for the $Mg^{2+}$-sufficient medium and 0.005 mM for the $Mg^{2+}$-deficient medium. The pH of both media was 7.3.

A second basal medium (BM 2), previously described by Eagon and Phibbs (7), was also employed which contained: 0.03 M glucose, 0.04 M $K_2HPO_4$, 0.022 M $KH_2PO_4$, and 0.007 M $(NH_4)_2SO_4$. To this basal medium, $MgSO_4$ was added to give a $Mg^{2+}$ concentration of 0.5 mM for the $Mg^{2+}$-sufficient medium and 0.005 mM for the $Mg^{2+}$-deficient medium. The pH of both media was 7.0.

All glassware used for the preparation of the chemically defined media was cleaned in a dichromate-sulfuric acid solution, rinsed seven times in hot tap water, and then rinsed seven times in deionized water prior to use.

**Cultivation procedure.** *P. aeruginosa* strain OSU 64 was used throughout these studies. A starter culture was prepared by inoculating a 500-ml Erlenmeyer flask containing 100 ml of medium, either $Mg^{2+}$-sufficient or $Mg^{2+}$-deficient, with 0.1 ml of a 1-ml washing of an 18- to 24-h tryptic soy agar slant culture. This starter culture was incubated for 12 to 14 h at 30 C on a rotary shaker and then used to inoculate 500 ml- and 1-liter Erlenmeyer flasks containing one-fifth volume of the appropriate basal medium. A 0.1% inoculum size was used in all cases. These cultures were incubated at 30 C on a rotary shaker until the cells were in the late logarithmic phase of growth.

**Determination of growth characteristics in the basal media.** A 1-liter Erlenmeyer flask containing 200 ml of medium was inoculated with 0.2 ml of the starter culture and incubated at 30 C on a rotary shaker. Samples of 3 ml of culture fluid were removed aseptically at the time of inoculation, at 2-h intervals between 10 and 24 h of incubation, and at hourly intervals between 25 and 33 h of incubation. Absorbancy readings of the 3-ml samples were taken at 470 nm. Growth of the organism in both $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 1 and BM 2 was monitored in this manner.

**Determination of EDTA sensitivity.** Cells grown in both $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 1 and BM 2 were harvested by centrifuging while in the late logarithmic phase of growth. The cells were tested for sensitivity to EDTA by incubation in a system of 3 ml total volume which contained a final concentration of 1 mM EDTA and 33 mM tris(hydroxymethyl)aminomethane (Tris) buffer. Cells suspended in deionized water were added to give the complete system an initial absorbancy at 660 nm ($A_{660}$) of approximately 0.60. The pH was 8.6.

**Freeze-etching procedures.** Freeze-etching of cells grown in both $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 1 and BM 2 was performed by suspending the cells after centrifuging in a BM 1- or BM 2-glycerol (7:3, vol/vol) mixture. The cells were held in this mixture for 2 to 3 h at 4 C and then freeze-etched according to the standard procedure described previously (9). Cells grown in both $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient media were also subjected to the procedure for the formation of osmoplasts as described previously (9), and then freeze-etched. Cells grown in the $Mg^{2+}$-sufficient media were converted to osmoplasts and required freeze-etching in 0.55 M sucrose containing BM 1- or BM 2-glycerol. Cells grown in the $Mg^{2+}$-deficient basal media remained osmotically stable and could be freeze-etched in the basal medium-glycerol mixture alone.

**Preparation of cell envelopes.** Cell envelopes were prepared as described previously from cells which were harvested in the late logarithmic phase (15).

**Quantitative analyses.** Total protein was estimated by the biuret method (11) with bovine serum albumin as the standard. Carbohydrate was determined by the anthrone method (3) with glucose as the standard. Total phosphorus of acid-hydrolyzed samples was determined by the method of Chen et al. (5). 2-Keto-3-deoxyoctonate (KDO) was estimated in samples after hydrolysis at 100 C for 30 min in 0.25 N $H_2SO_4$ by the method of Weissbach and Hurwitz (16), as modified by Osborn (12). Supernatant fractions from suspensions of osmoplasts were analyzed as described previously (9).

**Polyacrylamide gel electrophoresis.** Cell envelopes were extracted with acidic *N,N'*-dimethylformamide (DMF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed with the extract as described previously (15). Molecular weight determinations of proteins extracted from cell envelopes by DMF were carried out as described previously (15).

## RESULTS

**Growth characteristics of P. aeruginosa grown in the various basal media.** The organism grew slowly and poorly in both $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 1 (Fig. 1A). When grown in $Mg^{2+}$-sufficient BM 1, the cells exhibited a lag phase lasting 10 h, after which the cells grew logarithmically until the stationary phase was reached at 27 h. At this point, the culture had an $A_{470}$ of 0.25. Cells grown in the $Mg^{2+}$-deficient BM 1 had a lag phase of 12 h, then grew logarithmically until the stationary phase was reached at 30 h. The $A_{470}$ in this case was 0.18.

The organism grew well in $Mg^{2+}$-sufficient BM 2 but poorly in $Mg^{2+}$-deficient BM 2 (Fig. 1B). In $Mg^{2+}$-sufficient BM 2, the stationary phase was reached by 20 h with an $A_{470}$ of 0.90. In $Mg^{2+}$-deficient BM 2, the organisms remained in the lag phase for 10 h after which they grew logarithmically until the stationary phase was reached at 30 h of incubation. The $A_{470}$ was 0.30.

**EDTA sensitivity of P. aeruginosa grown in the basal media.** Cells grown in both $Mg^{2+}$-sufficient media retained their sensitivity to the lytic action of EDTA-Tris, as evidenced by a rapid decrease in absorbance when incubated with EDTA-Tris (Fig. 2). Cells grown in

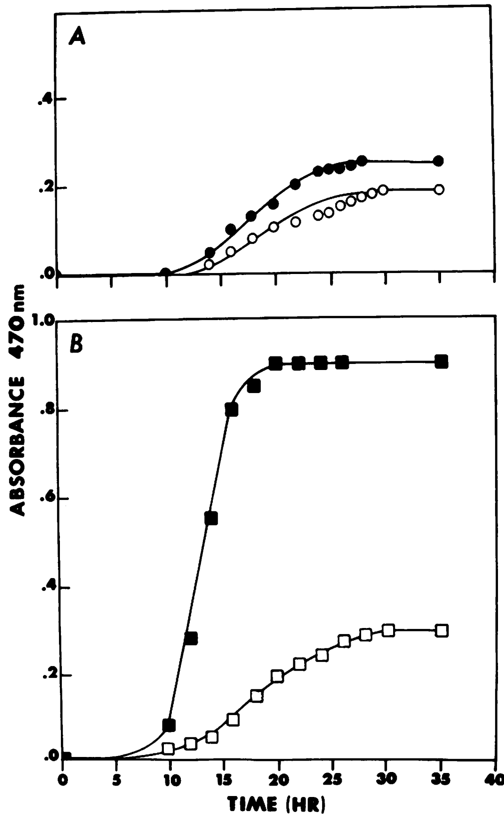


FIG. 1. *Growth of P. aeruginosa in the various media. (A) Growth in BM 1: ●, growth in $Mg^{2+}$-sufficient BM 1; ○, represent growth in $Mg^{2+}$-deficient BM 1. (B) BM 2: ■, growth in $Mg^{2+}$-sufficient BM 2; □, growth in $Mg^{2+}$-deficient BM 2.*

$Mg^{2+}$-deficient media, on the other hand, were resistant to the lytic action of EDTA-Tris, as evidenced by no decrease in absorbance when incubated with EDTA-Tris.

**Freeze-etching of cells grown in the $Mg^{2+}$-sufficient media.** The micrographs shown in Fig. 3 to 5 are of cells grown in $Mg^{2+}$-sufficient BM 1. The fracture surface of particular interest in these cells is the concave cell wall layer. This layer had the same appearance as previously shown by the tryptic soy broth (TSB)-grown cells (9), i.e., the presence of spherical units resting upon, or embedded in, an underlying smooth layer (Fig. 3). The concave cell wall was organized in such a manner that the underlying smooth layer was clearly seen; the spherical units appeared to be resting upon, or embedded in this layer; and, in some cases, the spherical units comprised small rodlets. All of the other fracture surfaces were also identical to those exhibited by TSB-grown cells (9).

Osmoplasts formed from cells grown in $Mg^{2+}$-sufficient BM 1 showed extraction of spherical units from the concave cell wall layer similarly to osmoplasts formed from TSB-grown cells (9). Approximately one-half of the spherical units appeared to have been extracted (Fig. 4). This was ascertained by determining in highly enlarged micrographs the average number of spherical particles per unit area in the concave cell wall layer of osmoplasts versus untreated cells. Thus, the unaltered appearance of the untreated concave cell wall layer and the extraction of spherical units from this layer in the formation of osmoplasts showed that growth in $Mg^{2+}$-sufficient BM 1 did not cause ultrastructural variations in the concave cell wall layer. This was as expected.

The appearance of the convex cytoplasmic membrane of cells grown in $Mg^{2+}$-sufficient BM 1 is shown in Fig. 5; all cells which exhibited the convex cytoplasmic membrane fracture showed the netlike arrangement of particles previously described for TSB-grown cells (9).

Cells grown in $Mg^{2+}$-sufficient BM 2 medium exhibited the same ultrastructure as cells grown in $Mg^{2+}$-sufficient BM 1.

**Freeze-etching of cells grown in $Mg^{2+}$-deficient media.** Cells shown in Fig. 6 to 8 were grown in $Mg^{2+}$-deficient BM 1. Such cells

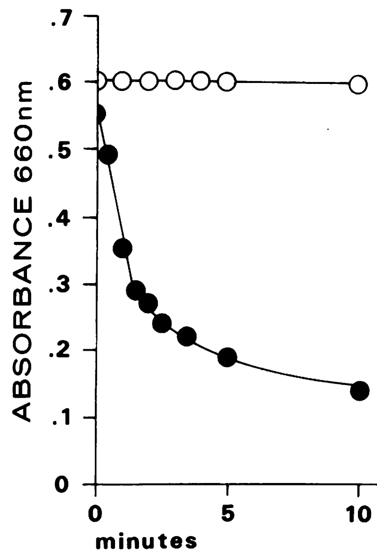


FIG. 2. *EDTA sensitivity of cells grown in $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient media. Symbols: ●, cells grown in $Mg^{2+}$-sufficient BM 1; ○, cells grown in $Mg^{2+}$-deficient BM 1. The cells were incubated in a system of 3 ml total volume which contained 1 mM EDTA and 33 mM Tris. Sensitivity to lysis by EDTA-Tris is indicated by a drop in $A_{660}$ readings. Similar results were obtained for cells grown in $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 2.*

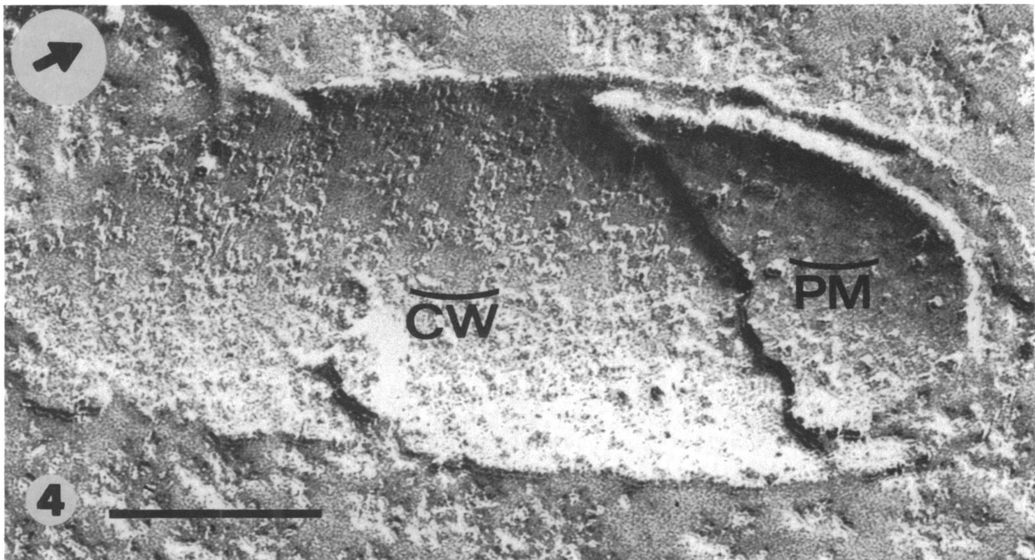


FIG. 3. *Freeze-etched cells grown in $Mg^{2+}$-sufficient medium. The small arrow in the upper left corner of this and of all the following electron micrographs indicates the direction from which the metal was evaporated in the production of the replica. The horizontal bar in all electron micrographs represents 200 nm. Abbreviations used in this and the following electron micrographs are as follows:* $\overset{\smile}{CW}$, *concave cell wall;* $\overset{\smile}{PM}$, *concave cytoplasmic membrane;* $\overset{\frown}{PM}$, *convex cytoplasmic membrane;* $\overset{\frown}{CW}$, *convex cell wall. This cell shows the concave cell wall layer, which is comprised of spherical units resting upon, or embedded in, an underlying smooth layer. Note that the underlying layer can be clearly seen in many areas.*

FIG. 4. *Freeze-etched osmoplast formed from cell grown in $Mg^{2+}$-sufficient medium. Concave cell wall and concave cytoplasmic membrane are shown. The loss of spherical units from the concave cell wall is evident.*

revealed an ultrastructurally altered concave cell wall layer upon freeze-etching (Fig. 6). The spherical units in this layer appeared more crowded and disorganized than the concave cell wall layer of cells grown in TSB (9) or grown in $Mg^{2+}$-sufficient medium. There appeared to be greater numbers of the spherical units present. The spherical units were so closely compacted

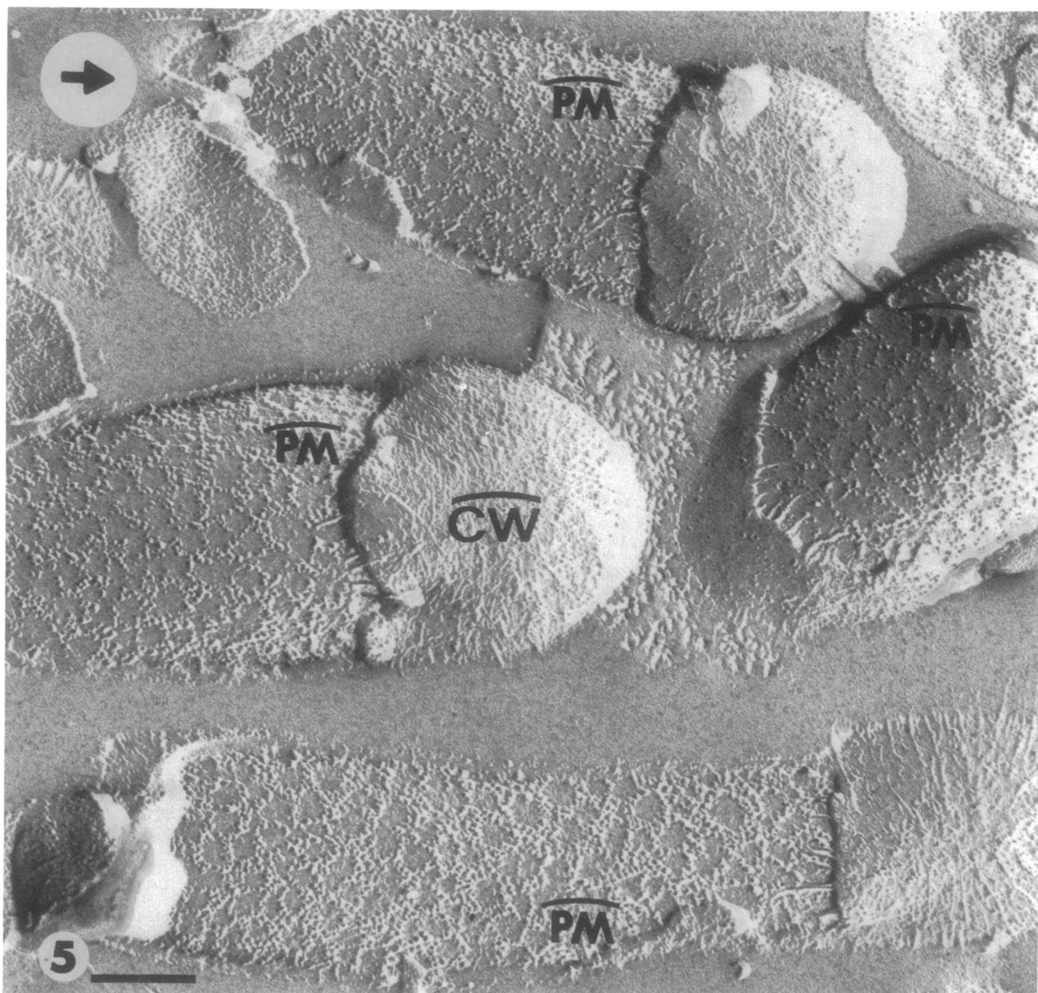


FIG. 5. *Freeze-etched cells grown in $Mg^{2+}$-sufficient medium. Convex cell wall and convex cytoplasmic membrane are shown. All the cells which reveal the convex cytoplasmic membrane show a netlike array of particles resting upon, or embedded in, an underlying smooth layer.*

that individual spherical units or rodlets could not be clearly discerned and the underlying smooth layer could not be seen clearly.

When these cells were subjected to the osmoplast procedure, osmoplasts were not formed. When the treated cells were freeze-etched, no spherical units appeared to have been extracted, and the appearance of the concave cell wall layer remained unchanged from that of untreated cells (Fig. 7).

The convex cytoplasmic membrane layer also appeared altered in cells grown in $Mg^{2+}$-deficient BM 1 (Fig. 8). The netlike array of particles appeared disorganized. There was an apparent clumping of the spherical particles and larger areas of the underlying smooth layer were exposed. $Mg^{2+}$-starvation of *Escherichia coli* has also been reported to cause similar alterations in the convex cytoplasmic membrane layer (8).

Freeze-etching of cells grown in $Mg^{2+}$-deficient BM 2 medium revealed ultrastructural alterations identical to cells grown in $Mg^{2+}$-deficient BM 1.

**Analysis of supernatant fluid from osmoplast preparations.** Osmoplasts were prepared from cells grown in $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 1. The supernatant fluid from osmoplasts prepared from cells grown in $Mg^{2+}$-sufficient medium contained 81.0 $\mu$g of protein per ml and 66.3 $\mu$g of carbohydrate per ml (Table 1). On the other hand, the supernatant fluid from cells grown in $Mg^{2+}$-deficient medium and subjected to the osmoplast-prepara-

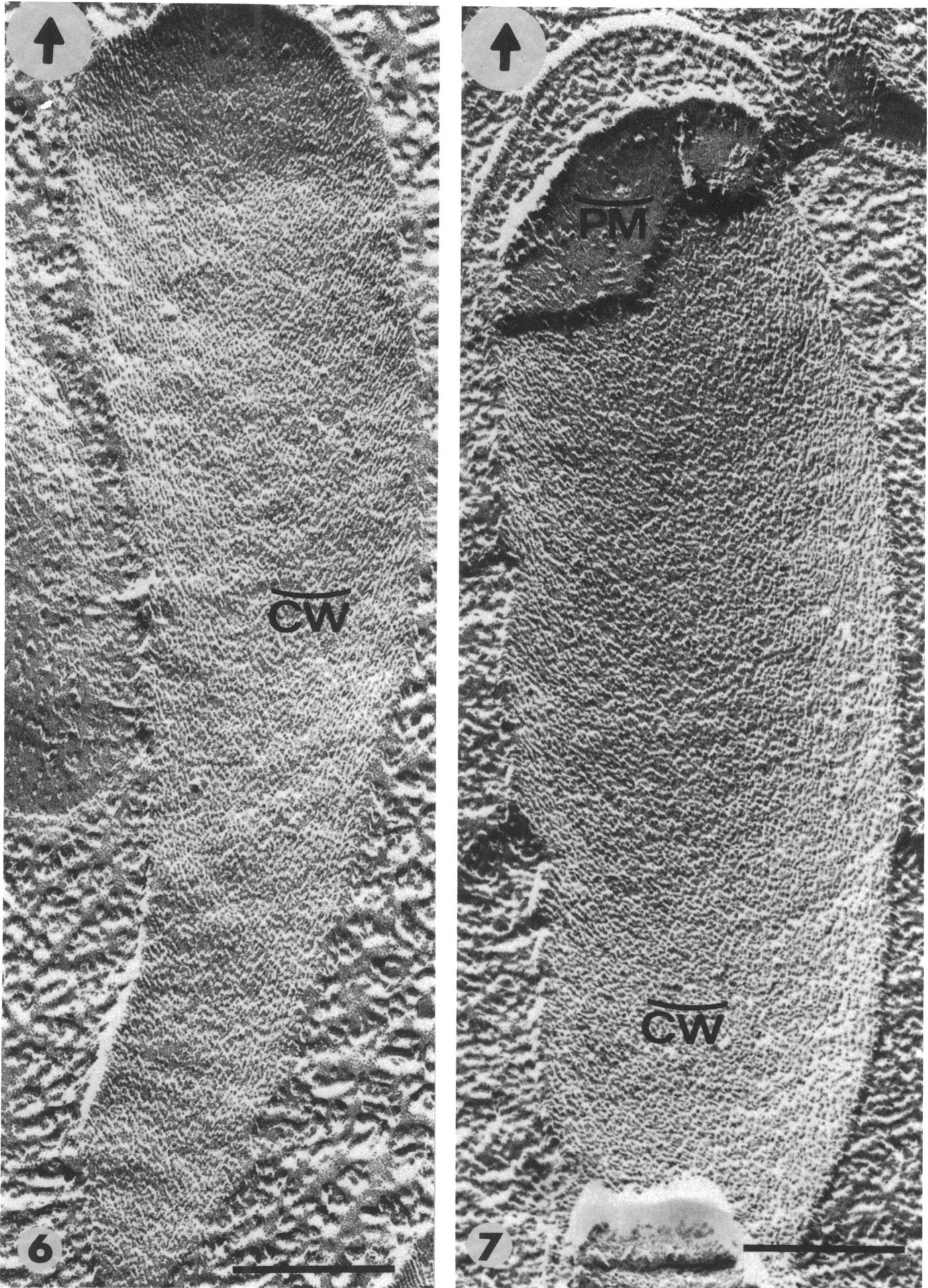


FIG. 6. *Freeze-etched cell grown in $Mg^{2+}$-deficient medium. The concave cell wall fracture appears disorganized and more crowded with spherical units. The underlying smooth layer cannot be seen clearly.*

FIG. 7. *Freeze-etched cell grown in $Mg^{2+}$-deficient medium and submitted to the technique for the preparation of osmoplasts. The concave cell wall fracture appears to be unaltered by this treatment, with no spherical units being extracted.*

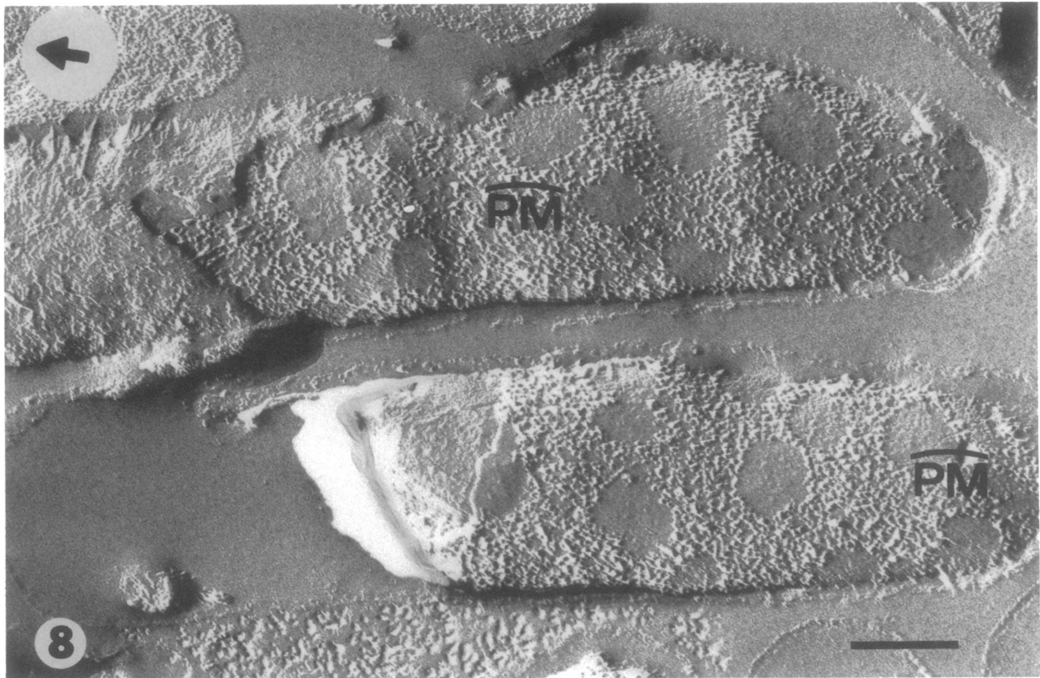


FIG. 8. *Freeze-etched cells grown in $Mg^{2+}$-deficient medium. The convex cytoplasmic membrane of these cells appears disorganized with the disruption of the netlike array of particles and the appearance of large smooth areas of exposed underlying layer.*

TABLE 1. *Analysis of the supernatant fluid from cells of P. aeruginosa grown in $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient medium after exposure to the osmoplast-preparation technique*[a]

| Growth medium | Protein | Carbohydrate |
|---|---|---|
| $Mg^{2+}$-sufficient | 81.0 ± 1 | 66.3 ± 5 |
| $Mg^{2+}$-deficient | 11.0 ± 3 | 12.0 ± 7 |

[a] Results expressed as micrograms of material per milliliter.

tion technique contained 11.4 μg of protein per ml and 12.0 μg of carbohydrate per ml. It is interesting that the quantity of material solubilized from cells grown in $Mg^{2+}$-deficient medium is almost identical with that remaining in the supernatant after restoration of osmoplasts formed from TSB-grown cells to osmotic stability by the addition of divalent cations (9).

**Chemical analysis of cell envelopes.** Cell envelopes were prepared from cells grown in $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient BM 2, then analyzed for total protein, total phosphorus, total carbohydrate, and KDO. No difference in the total protein content of the two envelopes was observed. However, the cell envelopes of cells grown in $Mg^{2+}$-deficient medium contained only 1.50% phosphorus compared with 1.83% in cell envelopes of cells grown in $Mg^{2+}$-sufficient medium (Table 2). Conversely, envelopes prepared from cells grown in $Mg^{2+}$-deficient medium had higher carbohydrate (15.2%) and KDO (1.5%) concentrations than envelopes prepared from cells grown in $Mg^{2+}$-sufficient medium (12.7 and 1.3%, respectively).

**Polyacrylamide gel electrophoresis of cell envelope proteins.** Considerable qualitative differences were found in the protein content of the cell envelopes (Fig. 9). Cell envelopes prepared from cells grown in $Mg^{2+}$-sufficient medium contained four distinct bands (Fig. 9, panel 1), including proteins A, B, and C which we have previously described (15). In addition, a new major band (protein E) having a molecular weight of approximately 100,000 was found. Cell envelopes prepared from cells grown in $Mg^{2+}$-deficient medium (Fig. 9, panel 2), however, had almost no protein E, and a new band (protein D) having an approximate molecular weight of 50,000 appeared in greater proportion.

## DISCUSSION

Brown and Melling (4) reported that cells of *P. aeruginosa* became resistant to the lytic effect of EDTA when grown in a $Mg^{2+}$-deficient

TABLE 2. *Analysis of cell envelopes from P. aeruginosa grown in $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient media*[a]

| Growth medium | Phos-phorus | Carbo-hydrate | KDO | Protein |
|---|---|---|---|---|
| $Mg^{2+}$-sufficient | 1.83 ± 0.2 | 12.7 ± 0.5 | 1.3 ± 0.1 | 50.2 ± 9 |
| $Mg^{2+}$-deficient | 1.50 ± 0.2 | 15.2 ± 0.3 | 1.5 ± 0.1 | 51.0 ± 5 |

[a] Results expressed as dry weight percentage.

medium. It seemed probable that, in conjunction with the acquisition of EDTA resistance, there might occur an ultrastructural alteration of the EDTA-Tris-sensitive cell wall component which we recently described in *P. aeruginosa* (9). To investigate this possibility, cells grown in two $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient media were freeze-etched.

Cells grown in the two $Mg^{2+}$-sufficient media were found to respond to EDTA-Tris treatment similarly to TSB-grown cells and to have a similar ultrastructural appearance. The cells were fully susceptible to the lytic action of EDTA-Tris (Fig. 2); the concave cell wall layer was unaltered in appearance (Fig. 3); osmoplasts possessing a concave cell wall layer with extracted spherical units could be formed (Fig. 4); and, the convex cytoplasmic membrane exhibited a typical netlike array of particles (Fig. 5). These results indicated that cells grown in $Mg^{2+}$-sufficient media were ultrastructurally comparable to cells grown in TSB.

It is of interest that electron microscope evidence indicated that approximately one-half of the spherical units were extracted by EDTA-Tris from the concave cell wall layer of cells grown in $Mg^{2+}$-sufficient media. This correlates well with chemical evidence that EDTA-Tris treatment extracted approximately 50% of the lipopolysaccharide from the cell envelope of *P. aeruginosa* (H. Thota, Ph.D. dissertation, University of Georgia, Athens, 1972).

Cells grown in the two $Mg^{2+}$-deficient media, on the other hand, were resistant to lysis by EDTA-Tris (Fig. 2). When such cells were exposed to the osmoplast-preparation technique, no spherical units appeared to be extracted from the concave cell wall fracture (Fig. 7), the cells could still be freeze-etched without the presence of sucrose in the medium-glycerol mixture, and the amount of protein released into the supernatant fluid by the cells appeared to be comparable to that released from TSB-grown cells in the presence of sucrose plus Tris but in the absence of EDTA (9).

Untreated cells grown in $Mg^{2+}$-deficient medium also revealed an ultrastructurally altered concave cell wall layer (Fig. 6) and convex cytoplasmic membrane layer (Fig. 8). The fact that the concave cell wall layer in these EDTA-resistant cells had an altered ultrastructural appearance of the spherical units in this layer further supports the identification of this layer as an EDTA-sensitive site in the cell wall. The convex cytoplasmic membrane appeared to undergo a disorganization of the netlike array of particles on its surface upon growth in a $Mg^{2+}$-deficient medium. The manner in which this is accomplished is uncertain. Fiil and Branton (8)

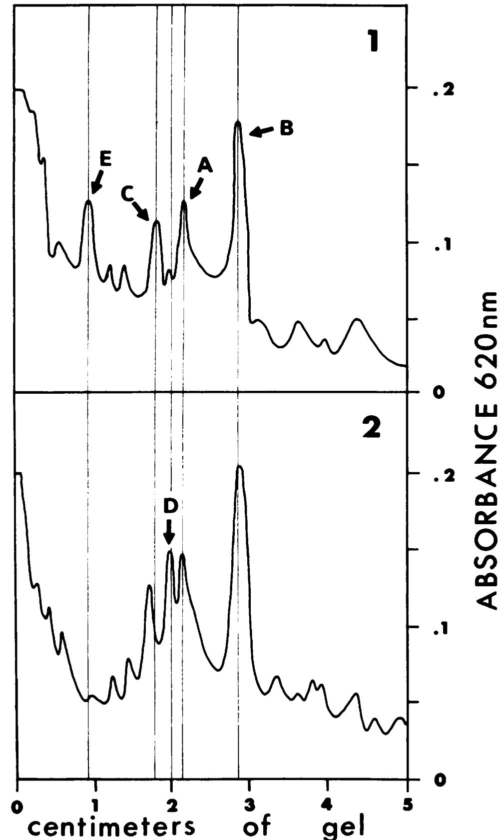


FIG. 9. *Densitometric tracing of electrophoresis gels to which 50 μg of protein was applied. All gels were stained with Coomassie blue. Panel 1: proteins extracted from envelopes of cells grown in $Mg^{2+}$-sufficient medium. Panel 2: proteins from envelopes of cells grown in $Mg^{2+}$-deficient medium. The tops of the gels are to the left. The tracing was done with a Gilford scanner at 620 nm. Protein A (43,000 daltons), protein B (16,500 daltons), protein C (72,000 daltons), and protein E (100,000 daltons) are identified in panel 1. This figure shows that envelopes from cells grown in $Mg^{2+}$-deficient medium (panel 2) differ from those of cells grown in $Mg^{2+}$-sufficient medium (panel 1) by having almost no protein E and having a greater proportion of protein D (50,000 daltons).*

suggested that, since the $Mg^{2+}$ concentration within the envelope itself did not decrease, the mechanism in *E. coli* may be indirect due to disturbances in cellular metabolism such as ribosome breakdown.

We have also observed an alteration in the netlike arrangement of granules in the convex cytoplasmic membrane of 24-h stationary phase, TSB-grown cells of *P. aeruginosa* similar to that of cells grown in $Mg^{2+}$-deficient media (unpublished observations). Thus, the appearance of granules in the convex cytoplasmic membrane appears to reflect the physiological state or metabolic unbalance of the bacterial cells.

It is of interest to note that $Mg^{2+}$ deficiency appeared to affect the organization of both membranes in the cell envelope, and the layer involved in each case was the layer tentatively identified as a protein middle layer (9).

Growth in either BM 1 or BM 2 was found to have the same effect on the cells. Freeze-etching of cells grown in the two $Mg^{2+}$-sufficient and $Mg^{2+}$-deficient media gave identical results. Similarly, the response to EDTA-Tris treatment was the same for cells grown in the two $Mg^{2+}$-sufficient media and for cells grown in the two $Mg^{2+}$-deficient media. The growth of the organisms in $Mg^{2+}$-sufficient BM 1, however, gave lower cell yields than growth in the $Mg^{2+}$-deficient BM 2 (Fig. 1). Thus, due to the poor growth of *P. aeruginosa* in BM 1, it would have been difficult to obtain sufficient quantities of cells to prepare cell envelopes for chemical analyses. For this reason, chemical analyses were carried out on envelopes prepared from cells grown on BM 2.

Chemical analysis of the cell envelopes also reflected changes as a result of this switch to EDTA resistance (Table 2). Our analyses compare favorably with the reports of Wilkinson (17, 18), who found lower phosphorus and higher carbohydrate content in cell envelopes of EDTA-resistant species of *Pseudomonas*. Wilkinson (18) also reported that cell walls prepared from *P. aeruginosa* grown on nutrient agar had less sensitivity to EDTA and contained less phosphorus than walls prepared from cells grown on tryptone-glucose extract agar. Thus, as Wilkinson (18) pointed out, a correlation between the phosphorous content of the envelope and sensitivity to EDTA is not surprising when the probable role of the cation-binding properties of phosphate groups in lipopolysaccharide is considered.

The absence of a quantitative change in protein content of the cell envelope appeared to be inconsistent with our ultrastructural findings. The appearance of the concave cell wall of cells grown in $Mg^{2+}$-deficient medium was more crowded with spherical units. This might have been due to a change in the conformation of the protein of the spherical units (e.g., the protein was not so tightly coiled). Thus, this would explain the disorganized, crowded appearance of the spherical units without a significant quantitative increase in the protein content.

The absence of a quantitative change in protein content, on the other hand, might indicate instead that the total protein of the cell envelope was the same, but there was more protein in the cell wall and less protein in the cytoplasmic membrane of cell envelopes from cells grown in $Mg^{2+}$-deficient medium. As noted herein, large plaque areas appeared on the ctyoplasmic membrane of these cells. The large plaque areas were apparently the result of a loss of cytoplasmic membrane protein. Thus, the increase in outer cell wall membrane protein under conditions of $Mg^{2+}$-deficiency was accompanied by a decrease in cytoplasmic membrane protein. The chemical analyses used did not discriminate between cytoplasmic and outer membrane protein. Thus, the net affect would appear to be no change in the overall quantity of cell envelope protein.

The ultrastructural changes in the cell wall layer which we consider to be the protein middle layer of the outer membrane would infer qualitative changes in protein composition. By using gel electrophoresis we have shown a qualitative change in the protein content of the cell envelope as a result of growth in $Mg^{2+}$-deficient medium. Protein E, a 100,000-molecular weight protein, was almost completely absent from cell envelopes of cells grown in $Mg^{2+}$-deficient medium, and another protein (protein D, 50,000 molecular weight) was present in increased amounts in cell envelopes of cells grown in $Mg^{2+}$-deficient media. Since protein D is one-half the molecular weight of protein E, perhaps protein D, when formed by cells grown in $Mg^{2+}$-deficient medium, cannot be dimerized to protein E. Such a dimerization, however, would involve a covalent linkage, since the samples were reduced with mercaptoethanol prior to electrophoresis and electrophoresis was carried out in the presence of sodium dodecyl sulfate and urea.

We have previously identified proteins A, B, and C as major proteins in cell envelopes from TSB-grown cells (15). We have also shown that proteins A and B are associated with the protein-lipopolysaccharide complex (15). Proteins D and E, however, have not been observed previously as major proteins in cell envelopes of

TSB-grown cells. Thus, the proteins of cell envelopes from cells grown in the $Mg^{2+}$-deficient and $Mg^{2+}$-sufficient media described herein and grown in TSB as described previously (15) differ qualitatively. Changes in cell envelope proteins of other bacteria as a result of growth in different media have also been noted by other workers (R. L. Bennett et al. Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 155, 1973).

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