The Role of Multivalent Cations in the Organization and Structure of Bacterial Cell Walls[1]

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Recently, Eagon and Carson (1965) and Eagon, Simmons and Carson (1965) reported that Ca^{++}, Mg^{++} and Zn^{++} were components of the cell wall of Pseudomonas aeruginosa strain OSU 64. They concluded that the binding of divalent cations was essential for the integrity of the cell wall of this microorganism. Michaels and Eagon (unpublished observations) obtained evidence inferring that these cations were present in the lipopolysaccharide fraction of the cell wall. Thus, the investigations described in this paper were undertaken to provide further information on the role of multivalent cations in the organization and structure of the cell wall of P. aeruginosa strain OSU 64.

EXPERIMENTAL METHODS

P. aeruginosa was cultivated for 14-16 hr at 37 C on a rotary shaker in a basal salts-glucose-yeast extract medium as used for previous experiments (Eagon and Carson, 1965). After harvesting and washing, the cells

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were incubated with ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (tris) buffer, lysozyme and cations as described in the protocols for the tables. Protocols for other experimental procedures discussed herein are also given with the tables.

RESULTS

Microscopic examination of cells of P. aeruginosa incubated with EDTA or with EDTA plus lysozyme in a hypertonic sucrose solution revealed rod-shaped bacilli indistinguishable from normal cells; these were, however, osmotically fragile. Voss (1964) has previously observed this phenomenon. Thus, we propose the term, osmoplast, to describe these osmotically fragile rods. Restored cells are defined as the osmotically stable cells resulting from the incubation of osmoplasts with multivalent cations.

The experimental results depicted in Table I clearly indicate that EDTA-induced osmoplasts were restored to an osmotically stable state by the addition of a variety of multivalent cations as evidenced by the absence of lysis when the restored cells were removed from hypertonic sucrose and resuspended in water. Whereas 10 μmoles of cations were used per ml in several experiments in order to ensure an excess of cations, it was evident that 1 μmole of cations per ml was equally effective. Lower quantities than this were not used.

The data indicate that all multivalent cations employed in these experiments restored osmoplasts to an osmotically stable state irrespective of whether they were di- or trivalent cations. Similarly, the variations in the atom diameters of the multivalent cations appeared not to be important in the restoration process. Finally, cations that are not normally found in the cell wall of P. aeruginosa were also able to restore osmoplasts to the osmoti-

TABLE I

Restoration of EDTA-Induced Osmoplasts of Pseudomonas aeruginosa by Multivalent Cations

System	O.D. (660 mμ)
Complete	0.33
minus EDTA	0.61
minus EDTA + 30 μg Lysozyme	0.60
+ 30 μg Lysozyme	0.05
+ 150 μmoles Na^{+}	0.25
+ 150 μmoles Li^{+}	0.27
+ 150 μmoles K^{+}	0.26
+ 10 μmoles Ba^{++}	0.58
+ 1 μmole Ca^{++}	0.59
+ 10 μmoles Ca^{++}	0.61
+ 10 μmoles Fe^{++}	*0.71
+ 10 μmoles Mg^{++}	0.58
+ 1 μmole Mn^{++}	0.60
+ 10 μmoles Mn^{++}	0.59
+ 10 μmoles Sr^{++}	0.58
+ 10 μmoles UO^{++}	0.64
+ 10 μmoles VO^{++}	0.52
+ 10 μmoles Zn^{++}	0.57
+ 10 μmoles Bi^{+++}	*0.90
+ 10 μmoles Fe^{+++}	0.67
+ 1 μmole La^{+++}	0.60
+ 10 μmoles La^{+++}	0.64
+ 1 μmole Ca^{++}; 1.3 μmoles Mg^{++}; 0.6 μmole Zn^{++}	0.58
+ Basal Salts	0.60
+ 10 μmoles Ca^{++}; 30 μg Lysozyme	0.22

Protocol: Complete system contained 1 μmole EDTA, pH 8, 33 μmoles tris buffer, pH 8 and 2.9×10^{9} cells per ml of 0.55 M sucrose. After incubation of complete system for 10 min at room temperature, cations were added to give a final concentration per ml as indicated above. The resulting reaction mixtures were then incubated for an additional 10 min. Restored cells or osmoplasts were collected by centrifugation at 3,500 x g for 15 min and resuspended in water. Lysozyme was added to complete system where indicated at time "0" to give a final concentration of 30 μg per ml. Basal salts were the same multivalent cations in the same final concentration as used in media for propagation of P. aeruginosa (i.e., Ca^{++}, 1 μmole; Fe^{++}, 0.07 μmole; Mg^{++}, 0.5 μmole; Mn^{++}, 0.01 μmole; and, Zn^{++}, 0.05 μmole).

*High readings were due to colored solutions or to insoluble materials.

cally stable state. For example, Mn^{++} is known not to be a component of the cell wall of P. aeruginosa (Eagon, Simmons and Carson, 1965). It is unlikely,

TABLE II

Effect of Cation Restoration of Viability of Pseudomonas aeruginosa

System	No. Bacteria/ml	% Survivors
Complete	8×10^4	< 0.01
No EDTA	2.9×10^9 *4.1×10^9	100 *100
+ 1 μmole Ca^{++}	*8.7×10^7	*2.1
+ 10 μmoles Ca^{++}	4.4×10^6	0.2
+ 1 μmole Mn^{++}	*6.6×10^7	*1.4
+ 10 μmoles Mn^{++}	2.3×10^7	0.8
+ 1 μmole La^{+++}	*1.5×10^7	*0.4
+ 10 μmoles La^{+++}	2.2×10^5	< 0.01
+ Basal Salts	*1.5×10^8	*3.7
+ 1 μmole Ca^{++}; 1.3 μmoles Mg^{++}; 0.6 μmoles Zn^{++}	*2.4×10^8	*5.9

Protocol: Experimental conditions were the same as described for Table I. The reaction mixtures were diluted into sterile water blanks and numbers of bacteria were estimated by the plate count method with nutrient agar as the culture medium. The cultures were incubated at 35 C for 48 hr.

*Results from two separate experiments are recorded. Results from one experiment are indicated by asterisks while those from the other are uncharacterized.

furthermore, that cations such as Sr^{++}, UO^{++}, VO^{++}, Bi^{+++} or La^{+++} occur naturally in the cell wall.

The data in Table I also indicate that monovalent cations were not able to restore osmoplasts even when used in high concentrations. Finally, lysozyme alone was not effective in inducing osmoplast formation, confirming

previous reports (Eagon and Carson, 1965; Carson and Eagon, 1966). When this reagent was used in combination with EDTA, however, irreversible osmoplasts were formed which could not be restored with Ca^{++} nor, presumably, with other multivalent cations as well. This indicates that the damage done to the cell wall by the combination of EDTA and lysozyme was too extensive for repair by multivalent cations. It is considered unlikely, moreover,

TABLE III

Comparison of Rate and Extent of Glucose Dissimilation by Resting Normal Cells and by Restored Cells of Pseudomonas aeruginosa

	µl O_2 uptake/hr	Total O_2 uptake	% Theoretical
Normal Cells	230	455	85
Cells restored with:			
1 µmole Ca^{++}	136	355	66
10 µmoles Ca^{++}	75	387	72
Basal Salts	140	398	74

Protocol: Experimental procedures for the preparation of restored cells were the same as described for Table I. Normal cells are defined as those cells that were handled in the same manner as restored cells but were not exposed to EDTA or to cations. Oxygen uptake was measured by the Warburg respirometer at 30 C. Each Warburg vessel contained 4 µmoles glucose, 800 µmoles phosphate buffer, pH 7, 2 ml cell suspension (4.1×10^9 cells/ml) and 0.2 ml of 40% KOH in the center well to give a total volume of 3.2 ml.

that the sensitive linkages cleaved by lysozyme could be repaired by multivalent cations.

The results shown in Table II indicate that restored cells were not able to multiply extensively as evidenced by their failure to form colonies. The highest percentage of survivors was noted when a mixture of Ca^{++}, Mg^{++} and Zn^{++} in the same ratio as found in the cell wall was used to restore osmo-

plasts. Microscopic examinations of suspensions of restored cells revealed, however, that they were normal in appearance and that they were motile as well.

That restored cells are capable of respiration is shown by data in Table III. Osmoplasts restored either by 1 μmole Ca^{++}/ml or by the mixture of divalent cations used in the basal salts solution took up oxygen at a lower rate and to a lesser extent than did normal cells. Osmoplasts restored with 10 μmoles Ca^{++}/ml aggregated into large spherules (approximately 1-2 mm in diameter) when added to phosphate buffer in the Warburg vessel, possibly due to the formation of a calcium-phosphate complex at the cellular surface. Thus, the slow rate of oxygen uptake may have been due to slow diffusion of glucose throughout these spherules. Nevertheless, these data show that the respiration of restored cells has not been extensively impaired.

DISCUSSION

Sensitivity to EDTA is not restricted to P. aeruginosa. Gray and Wilkinson (1965a; 1965b) reported that, in addition to P. aeruginosa, Alcaligenes faecalis was highly sensitive to EDTA while Escherichia coli was moderately sensitive. Lipopolysaccharide was solubilized by this reagent. These authors also indicated that EDTA sensitized a wide variety of bacterial species to chloroxylenol preparations or potentiated its effects. Leive (1965) reported that EDTA liberated lipopolysaccharide from E. coli. Michaels and Eagon (unpublished observations) obtained evidence that lipopolysaccharide per se from P. aeruginosa was altered by EDTA. Burton and Carter (1964) detected Ca^{++} and Mg^{++} in the lipid A component of lipopolysaccharide of E. coli.

Divalent cations have also been implicated in the structural organization of lipoprotein-type of membranes. Abram and Gibbons (1961) and Brown

(1963) concluded that the lipoprotein cell walls of the halophilic Halobacterium may be held together in part by divalent cations. Similarly, Razin, Morowitz and Terry (1965) reported that the lipoprotein cell membranes of the pleuropneumonia-like organism Mycoplasma could be dissolved into subunits with sodium lauryl sulfate. These subunits could be reaggregated to form membrane-like structures in the presence of multivalent cations. Thus, there is sufficient evidence to postulate a unitary role for the association of divalent cations with the lipopolysaccharide and, perhaps, lipoprotein components of cell walls of gram negative bacteria.

We have presented evidence here and elsewhere that a component of the cell wall of P. aeruginosa can be liberated as subunits by EDTA. There is strong inference from our studies that this is the lipopolysaccharide component. Moreover, reaggregation was shown to occur in the presence of multivalent cations. Thus, lipopolysaccharide may be composed of subunits cross-linked via divalent cations. Similarly, the lipopolysaccharide layer per se may be cross-linked to other components of the cell wall via multivalent cations.

It may be speculated, furthermore, that the formation of a complete bacterial cell wall sacculus containing lipopolysaccharide and lipoprotein may be formed in vivo via physico-chemical properties by which negatively charged subunits are "trapped" by forming ionic and coordinate bonds intermediated by multivalent cations.

In our experiments, cells with reaggregated walls (i.e., restored cells) were no longer capable of multiplication. This indicates that either (1) a high degree of selectivity for specific multivalent cations is exhibited by the growing cell or (2) that a delicate balance between cell wall formation and biosynthesis of subunits was destroyed.

SUMMARY

Incubation of P. aeruginosa with EDTA induced the formation of osmotically fragile rods. These could be restored to osmotically stable forms by multivalent cations. The lipopolysaccharide component of the cell wall was inferred to be the site of attack by EDTA. A mechanism for the synthesis of the lipopolysaccharide sacculus was proposed.

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