

ELECTRON MICROSCOPIC OBSERVATIONS OF POLYSACCHARIDE
COMPONENTS IN POLYMYXIN B TREATED OUTER
MEMBRANES FROM *SERRATIA MARCESCENS**

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Treatment of outer membranes of *Serratia marcescens* with polymyxin B results in the formation of blebs. This effect is thought to be due to the action of the antibiotic on the lipopolysaccharides, proteins, phospholipids or a combination thereof. It is unclear whether this effect is dissociative, degradative or due to an inhibition of the assembly of outer membrane components. Prior studies showed that lipopolysaccharides and polymyxin B form complexes, but direct visualization of the *in situ* action of polymyxin B had not been accomplished. Isolated outer membranes normally exhibit a periodicity of the polysaccharide molecules when stained by the thiosemicarbazide-silver technique. Polymyxin B treated outer membranes display a change in their basic morphology. This effect is very drastic in the sensitive strain as demonstrated by the large gaps in the deposition of the granules in the modified outer membrane structure. Thus it appears that the polysaccharide molecules (probably the lipopolysaccharide) either alone or in association with protein or phospholipids are the primary targets of the antibiotic.

Polymyxin B, a cationic cyclicpeptide antibiotic, interacts with various anionic cellular components such as lipopolysaccharides (LPS),^{1,2)} phospholipids (PL),³⁻⁶⁾ deoxyribonucleic acid (DNA),⁷⁻⁹⁾ and ribosomes⁸⁻¹⁰⁾ of Gram-negative bacteria. When whole cells are treated with the antibiotic, blebs are formed on the cell surface,^{7,11-13)} and subsequently, disorganization of the cell envelope occurs if susceptible cells are exposed to a lethal dose of the antibiotic. Although the formation of the blebs has been suggested to be caused by an aggregation and/or complex formation of the antibiotic with outer membrane components such as PL⁹⁾ and LPS,^{9,14)} direct *in vivo* evidence in polymyxin B treated outer membranes has not been available. Recently, the polysaccharide components in the blebs of polymyxin B treated cells of *Serratia marcescens* have been localized by the silver proteinate method of THIERY.¹⁵⁾ This method involves oxidation by periodic acid (PA) of 1:2 glycol linkages of the polysaccharide to yield aldehydes, which on reaction with thiosemicarbazide (TSC) form hydrazones. Reduction of the silver proteinate reagent by the thiol groups of the hydrazones results in the deposition of silver at the site of original 1:2 glycol linkages. Electron microscopy has been used to observe the deposition of silver as dark granules. In this study we attempted to use this method to observe the effect of polymyxin B on the polysaccharide components in outer membranes isolated from 2 strains of *Serratia marcescens*.

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Materials and Methods

Serratia marcescens strain 08 (polymyxin B resistant) and strain Bizio (polymyxin B sensitive) were used in this study. Cells were grown in an enriched medium with aeration at ambient temperature and harvested at an optical density between 0.50 and 0.55 units (after approximately 18 hours of growth).¹⁶⁾ Cells from 1 liter of culture medium were treated with 20 mg of polymyxin B sulfate (Burroughs Wellcome) in 75 ml of 0.9% NaCl, pH 7.8 for 1 minute at 37°C. Outer membranes were isolated from treated cells according to the method of OSBORN *et al.*,¹⁷⁾ as described by TSANG *et al.* for cells of *S. marcescens*.¹⁸⁾

Ultrathin sections of isolated outer membranes were prepared by using a modified method of FORSBERG *et al.*¹⁹⁾ Samples were embedded in 1% agar (Difco Noble Special Agar), fixed with 5% glutaraldehyde and 2% osmium tetroxide, dehydrated, and embedded in Epon 812. Post section staining was accomplished with uranyl acetate-lead citrate. Staining for polysaccharide components was performed according to the method of THIERY²⁰⁾ as described previously.¹⁵⁾ Thin sections of all samples on gold grids were floated on 1% PA for 1 hour and washed with distilled water. They were subsequently floated on a 1% thiosemicarbazide solution in phosphate buffer (0.05 M, pH 7.4) and washed with distilled water. Finally the grids were placed on a 1% aqueous solution of silver proteinate reagent (Electron Microscopy Sciences) for 30 minutes in the dark. All sections were examined in either a Hitachi HU-11A or an RCA EMU-3G electron microscope operating at an accelerating voltage of 50 kV.

Results

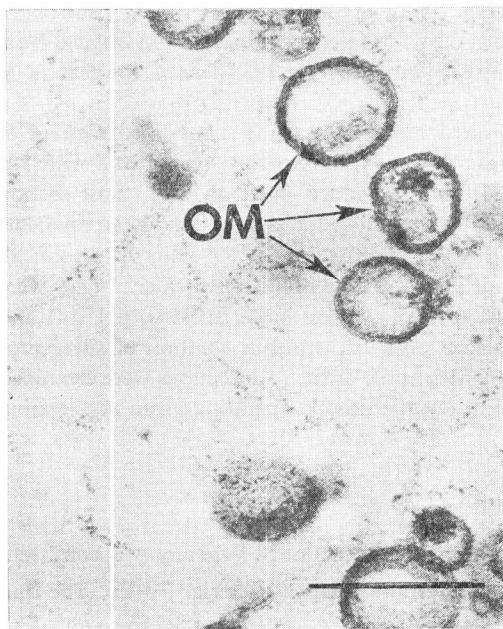
The basic morphology of the isolated outer membranes of 2 strains of *S. marcescens* is consistent with the spheroidal shape of the outer membranes of other Gram-negative bacteria.^{21,22,23)} The thin sections of outer membranes stained with uranyl acetate-lead citrate of strain 08 (Fig. 1A) and strain Bizio (Fig. 1B) showed the spheroidal trilaminar structures with smooth periphery. However, the configurations of the morphology of outer membranes isolated from polymyxin B treated cells were modified to some extent. In preparations of strain 08 (Fig. 1C), elongated as well as ribbon-like structures were readily seen. One of the blebs formed on the outer membrane was seen attached to the ribbon-like structure after the outer membrane was disrupted. Fig. 1D shows the high degree of pleiomorphic diversity with numerous shapes in the treated outer membranes of strain Bizio. Although the configurations of most of the structures deviated from the normal spheroidal shape of the untreated outer membranes, the site(s) of the dissociative/degradative action of polymyxin B on the treated outer membrane is not discernible.

When the morphology of untreated outer membranes was observed by the silver proteinate technique of THIERY,²⁰⁾ the normal spheroidal structures were again observed (Figs. 2A and 2B). The silver granules were deposited on the periphery of the double-track structures. The location of the granules, most likely represents the presence of the lipopolysaccharides in the outer membranes. In the treated outer membranes of strain 08 many granules are seen distributed along the periphery of the blebs (Fig. 2C). However, in the strain Bizio the general integrity of the treated outer membranes was lost in addition to a decrease in the number of silver granules deposited on these disrupted membranes. Those granules that were still present were widely dispersed at points along the membrane periphery or on the blebs that were dissociated from the outer membrane proper. Furthermore, the presence of the dark granules in the ribbon-like structures clearly demonstrates the lipopolysaccharide nature of the material released by polymyxin B treatment.

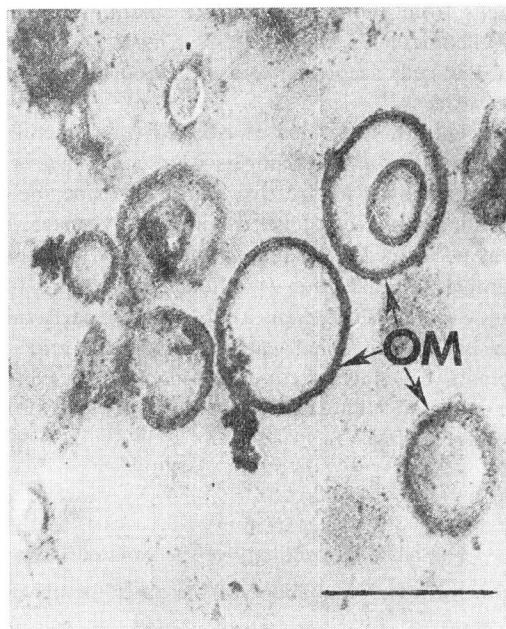
Fig. 1. Morphological appearance of outer membranes from *Serratia marcescens* before and after polymyxin B treatment (stained with uranyl acetate-lead citrate).

The marker bars in all micrographs represent 0.2 μm .

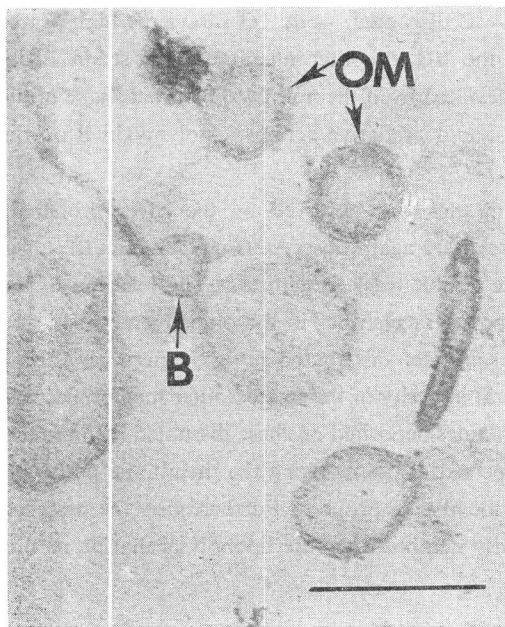
(A) Untreated outer membrane of the resistant strain 08



(B) Untreated outer membrane of the sensitive strain Bizio



(C) Polymyxin B treated resistant strain 08: note the bleb (B) protruding from the elongated outer membrane.



(D) Polymyxin B treated sensitive strain Bizio: note the bleb (B), the modified outer membrane (M-OM) and the degraded/dissociated (D/D-OM).

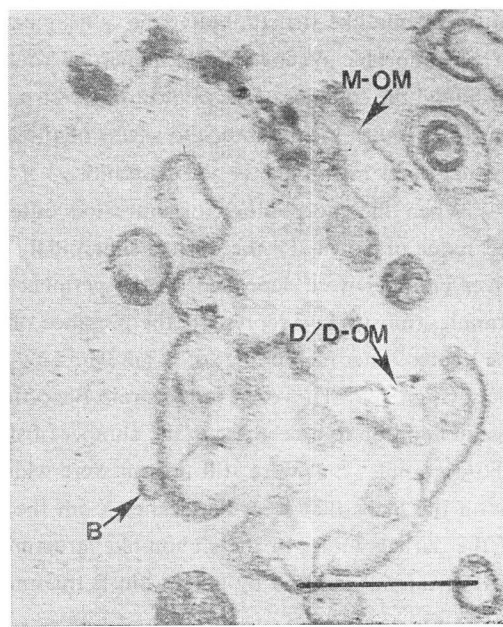
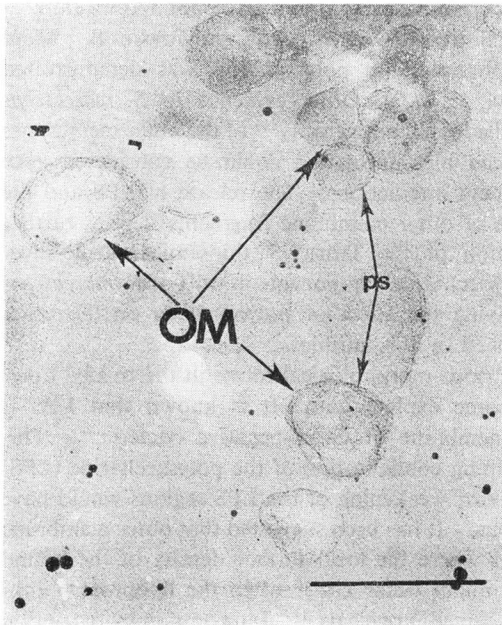
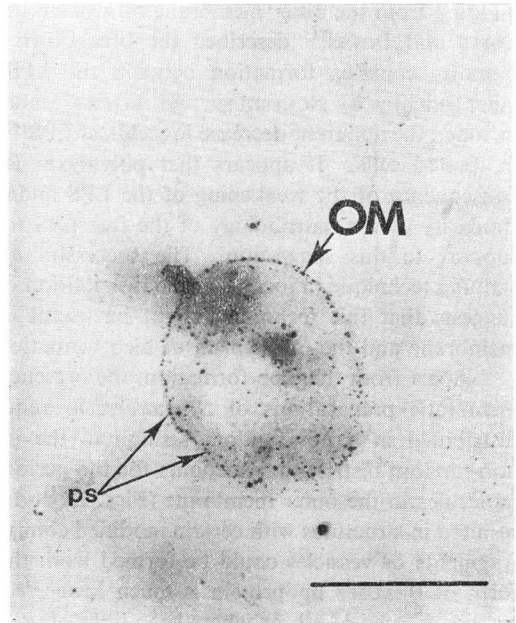


Fig. 2. Morphological appearance of outer membranes from *Serratia marcescens* before and after polymyxin B treatment (stained by the thiosemicarbazide silver proteinate method).

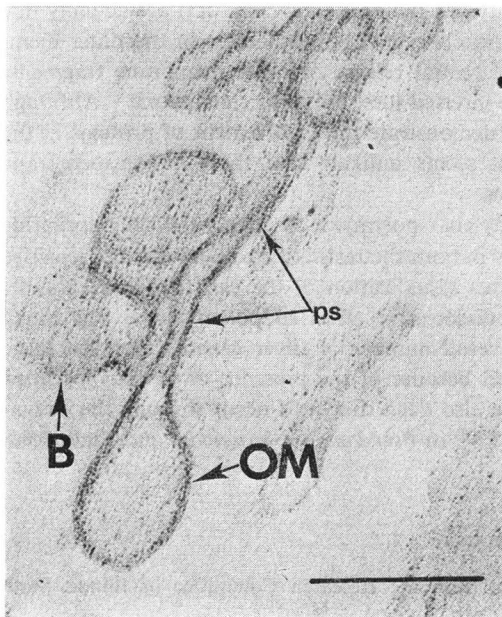
(A) Untreated outer membranes of the resistant strain 08: note the intense staining along the periphery of the outer membrane (ps).



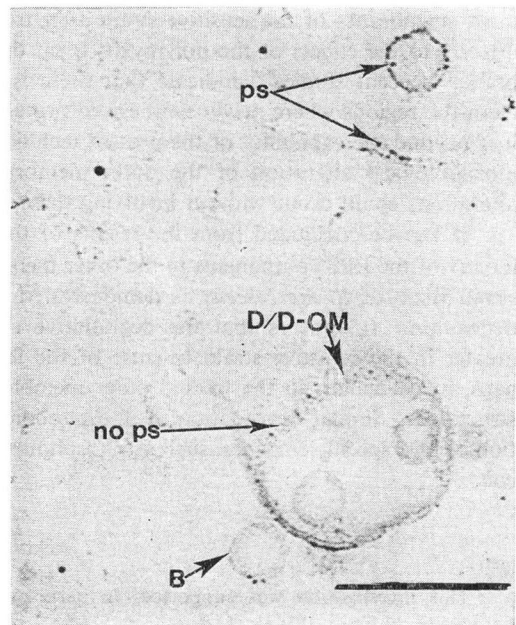
(B) Untreated outer membranes of the sensitive strain Bizio: note the intense staining along the periphery of the outer membrane (ps).



(C) Polymyxin B treated resistant strain 08: note the intense staining of the polysaccharides (ps) that remain after treatment.



(D) Polymyxin B treated sensitive strain Bizio: note the lack of stained polysaccharides (no-ps) along the portion of the degraded/dissociated outer membrane (D/D-OM). Also note a few polysaccharide staining sites in a residual membrane fragment (ps).



Discussion

Induction of bleb formation by polymyxin B has been observed in various Gram-negative bacteria.^{7,11-13}) It has been suggested that bleb formation may be related to the interactions of the LPS of the outer membranes with polymyxin B. An LPS-like material was first observed to be budding from the outer membrane of a strain of *Escherichia coli* grown in a lysine-limited medium.²⁴) LOPES and INNIS²⁵) described the breakdown of LPS treated *in vitro* with polymyxin B. More recently, complex formation between the LPS-phospholipid and polymyxin B was demonstrated independently by SCHINDLER and TEUBER⁹) and TSANG *et al.*¹⁴) Other evidence in *S. marcescens* included the apparent decrease in yields of LPS¹⁶) and the loss of antigenicity²⁶) of the outer membranes in treated cells. It appears that polymyxin B induced bleb formation could be considered as a consequence of the weakening of the LPS and/or protein interactions. The release of LPS and the similarity of the morphology of the free LPS to those of outer membrane fragments²⁷) gives further support to this suggestion. The successful application of the THIERY²⁰) thiosemicarbazide-silver staining technique in localizing the distribution of polysaccharide components in cells of *S. marcescens* suggests that this technique might be useful in studying the effect of polymyxin B on the outer membrane and the mechanism of bleb formation induced by this antibiotic.

Apart from the bleb formation, the presence of various morphological forms in the treated outer membrane preparations of *S. marcescens* requires some explanations. It is known that LPS is distributed in a non-random fashion in the outer membrane of Gram-negative bacteria.²⁹) This non-random distribution accounts for the periodic staining configuration of the polysaccharide (LPS) molecules in the outer membrane (Figs. 2A and 2B). Any weakening of the LPS regions would have resulted in structures with certain modified configurations. It has been suggested that outer membrane fragments or vesicles could be formed from the areas where the local surface density of the bound form of BRAUN's lipoprotein is much lower.²⁹) This might occur either when the lipoprotein links are not formed at all, or where such links after being formed, are cleaved. Interruption by the action of polymyxin B of the insertions of LPS, phospholipids and/or proteins into specific outer membrane zones would have resulted in various sizes and forms of the outer membrane fragments. The presence and intensity of the silver deposition at these affected areas provides information not only on the localization of the site of action of polymyxin B with the polysaccharides, but also the extent of the damage of these affected areas. Consistent with this premise is the idea that the large gaps seen in the outer membranes of the sensitive strain after treatment with the antibiotic (Fig. 2D) are actually due directly to the effects of the polymyxin B on the polysaccharide (LPS) molecules in the outer membrane. Recent studies³⁰) indicate that there is a preferential release of outer membrane fragments from the regions where newly synthesized proteins are inserted into the outer membrane. Although it is beyond the capability of the present technique to demonstrate the involvement of proteins in the morphological alteration of the outer membranes, it seems unlikely that these major membrane alterations could occur without involving these proteins.

It can be concluded from the results of this study that polymyxin B affects the polysaccharide moiety of the LPS components in the outer membranes in both the resistant strain 08 and the sensitive strain Bizio of *S. marcescens* as demonstrated by direct visualization of the electron dense granule deposition. It appears that the degradative and/or dissociative effect of polymyxin B was much greater in the sensitive strain because of the fewer overall number of silver granules and the large gaps, in particular, in the treated outer membrane and because of the presence of various modified structures. Similar degradation of phospholipids have also been shown to occur through the activation of OM specific enzymes, such as phospholipase A₁^{31,32}) in *Pseudomonas aeruginosa* and *Escherichia coli*.

Acknowledgements

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