JOURNAL OF BACTERIOLOGY, Sept. 1976, p. 1400-1407
Vol. 127, No. 3

Printed in U.S.A.

Effect of Polymyxin on the Ultrastructure of the Outer Membrane of Wild-Type and Polymyxin-Resistant Strains of *Salmonella*

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Received for publication 14 June 1976

The effect of polymyxin on two sets of *Salmonella* mutants was studied by thin-section and scanning electron microscopy. Polymyxin (in increasing concentrations, starting just below bactericidal effect) caused the appearance of the previously described rodlike projections on the cell surface of wild-type (smooth, polymyxin-sensitive) bacteria. These projections seemed to involve the outer membrane of the cell wall. In rough mutants, which are deficient in lipopolysaccharide, the projections were much smaller and flat. Higher concentrations of polymyxin were required to produce morphological effects in polymyxin-resistant mutants of both smooth and rough forms. Furthermore, in these mutants polymyxin caused vesicle-like bulging of the total outer membrane quite different in appearance from the rodlike projections of the wild type.

Antibiotics that affect membranes may be used as probes to study the structure and properties of microbial membranes. Polymyxin acts mainly on gram-negative bacteria (15), and their outer membrane (OM), which is absent in gram-positive bacteria, seems to be one of its main targets. Polymyxin seems to increase the permeability of the OM (5). Several electron microscope studies (6, 9, 10, 19, 21) have demonstrated that polymyxin alters the ultrastructure of the OM, whereas changes are not seen in the cytoplasmic membrane.

In the present report we describe the morphological effects of polymyxin on mutants with increased resistance to polymyxin (*pmr* mutation) compared with their polymyxin-sensitive parents. Both smooth and rough (*rfa* mutation) derivatives of *Salmonella typhimurium* were used. Lipopolysaccharide (LPS) is a major component of the OM. In the complete, smooth form it provides the cell with a hydrophilic surface polysaccharide layer, which is largely missing in rough mutants with defective LPS (13). We show here that both the *pmr* and the *rfa* mutations influence in characteristic ways the ultrastructural changes caused by polymyxin. Based on these findings we suggest a possible mechanism for this polymyxin effect.

MATERIALS AND METHODS

Bacterial strains. Strains used were derivatives of *S. typhimurium* LT2, strain SH4247. Table 1 shows their relevant properties and their sensitivity to polymyxin under the conditions used for electron microscopy and in ordinary culture media.

Polymyxin treatment. Bacteria grown in L-broth (1% tryptone [Difco]-0.5% yeast extract [Difco]-1% NaCl – 0.025% $CaCl_2$, pH 7.0) were harvested in the logarithmic phase of growth (Klett 40) by centrifugation and suspended in 5 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, containing polymyxin B sulfate (Pfizer or Dumex) in the amounts indicated in the figure legends. The suspensions were incubated at 37°C on a rotatory shaker.

Preparation for TEM and SEM. After 30 min of polymyxin treatment, the samples were prefixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Samples for transmission electron microscopy (TEM) were postfixed for 2 h with 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2). The sections were obtained from Epon 812-embedded samples and stained with uranyl acetate and lead citrate. For scanning electron microscopy (SEM) the samples were prefixed as above and then dehydrated by the critical-point method (2) in an Aminco apparatus before evaporation with gold in a Balzers (Liechtenstein) apparatus (Micro-BA3).

LPS was extracted by the hot phenol-water method (23). It was dissolved in Tris buffer (pH 7.2) containing 15 or 150 μg of polymyxin B sulfate per ml, prefixed as above, and ultracentrifuged at 39,000 rpm for 1 h. Postfixation and staining were then carried out as above.

The TEMs were taken with a JEOL-100B electron microscope operating at 80 kV, and the SEMs were taken with a JSM-U3 electron microscope operating at 15 or 20 kV.

TABLE 1. *Bacterial strains used and their polymyxin sensitivity*[a]

Strain no. (relevant genotype)[b]	Time of incubation at 37°C (min)	No. of colonies on plates after treatment[c]					MIC[d] (IU/ml) on nutrient agar
		1[e]	3	10	30	100	
SH4247	1	+	+	150	–	–	10
(smooth)	10	+	1,000	118	–	–	
	30	+	1,000[f]	87	–	–	
SH5014	1	+	+	8	–	–	10
(*rfa*)	10	+	+	16	–	–	
	30	+	+[f]	127	–	–	
SH4420	1	+	+	+	1,000	200	300
(smooth *pmrA5*)	10	+	+	300	300	35	
	30	+	+	81[f]	700	–	
SH5357	1	+	+	150	67	1	100–300
(*rfa pmrA19*)	10	+	+	100	87	–	
	30	+	+[f]	250	150	–	

[a] All strains are derivatives of *S. typhimurium* LT2.
[b] Smooth, rough (*rfa*) LPS character; *pmrA*, locus for polymyxin resistance (to be published).
[c] Bacterial suspensions were treated as for electron microscopy; 0.1-ml samples were spread at times indicated on nutrient agar plates, and colonies were counted (+, confluent growth; –, no growth).
[d] MIC, Minimal inhibitory concentration.
[e] Concentration (micrograms/milliliter) of polymyxin B sulfate in Tris buffer.
[f] Lowest polymyxin concentration at which ultrastructural changes were detectable in the OM.

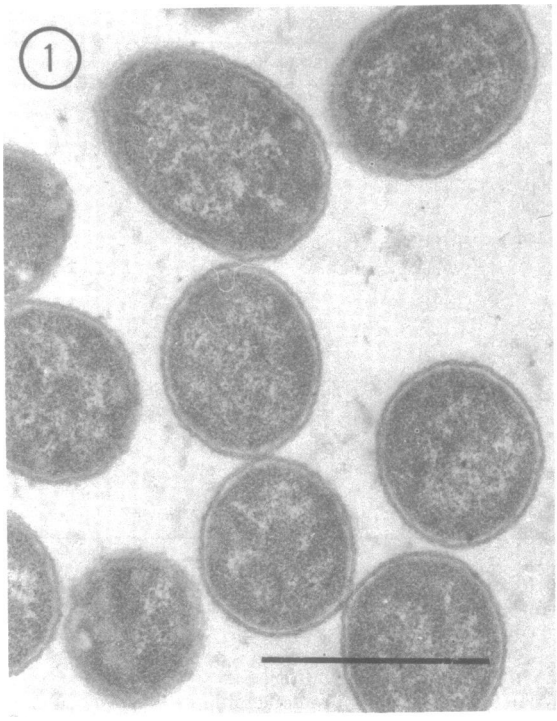
RESULTS

Effects of polymyxin on sensitive strains. (i) Smooth strain SH4247. The general morphology and the ultrastructure of the cell envelope of untreated SH4247 (Fig. 1 and 2) are quite similar to those of gram-negative rods in general (16). Incubation for 30 min in Tris buffer in the presence of less than 1 μg of polymyxin per ml did not cause visible changes. The concentration of 1 μg/ml is slightly below the concentration that causes a decrease in the number of viable bacteria (Table 1). At 1 μg of polymyxin per ml, some rodlike projections appeared on the surface of the OM (not shown). These projections became more numerous and longer with increasing concentrations of polymyxin (Fig. 3 and 4). No other changes of cellular ultrastructure were found below 30 μg/ml; at polymyxin concentrations of 30 μg/ml or higher extensive contraction of cytoplasmic material was visible (Fig. 3). At these concentrations all bacteria were rapidly killed (Table 1). Similar deformation of cytoplasmic material can also be seen after treatment with detergents like sodium dodecyl sulfate (22), and they probably reflect the breakdown of the integrity of the cell envelope.

The overall structure of the projections (Fig. 3 and 4) was that of a thin rod. Their diameter was about 8 nm at all concentrations of polymyxin studied, whereas their length increased with increasing concentration up to about 100 nm at 100 μg/ml. Cross sections (Fig. 3) show that the projections were cylindrical rather than cuts from longitudinal folds. There was no consistent internal structure visible in the projections, and in this respect they resembled simple lipid micelles (7).

(ii) Rough strain SH5014. The rough mutant of SH4247 is defective in the synthesis of the core part of LPS (*rfa* mutation) and thus lacks most of the polysaccharide of LPS (13). This degree of LPS alteration does not increase the permeability of the cell to various antibiotics or its sensitivity to detergents, nor does it cause a loss of OM proteins as do some other *rfa* ("heptoseless") mutations (1). The sensitivity of this strain to polymyxin was very similar to that of the smooth parent SH4247 (Table 1).

Polymyxin again caused the formation of projections on the OM (Fig. 5 and 6); the threshold concentration was 1 μg/ml, the same as with the smooth strain SH4247 and somewhat below the concentration detectably affecting viability (Table 1). However, the morphology of the projections was very different from those on the smooth strain (Fig. 3 and 4). The projections were flat and wide, and their shape was ill defined (Fig. 5 and 6). Both their number and



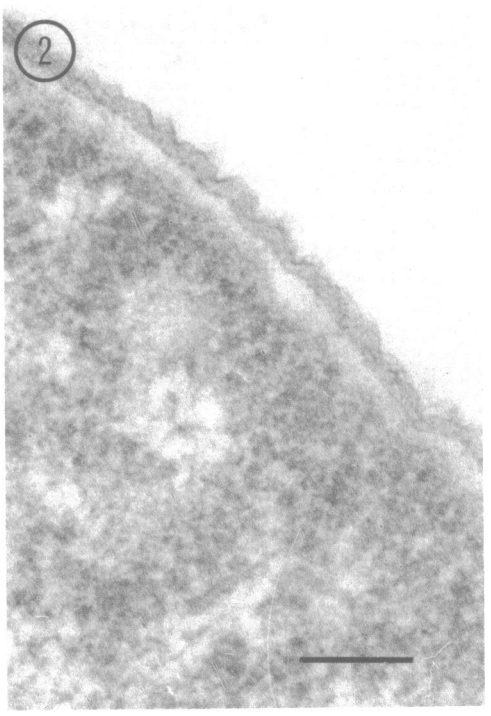


FIG. 1. *Untreated cells of S. typhimurium smooth strain SH4247. Bar, 1 μm.*
FIG. 2. *Cell envelope of S. typhimurium SH4247. Bar, 0.1 μm.*

size increased with increasing concentrations of polymyxin.

Effects of polymyxin on resistant strains. The polymyxin-resistant mutants isolated from either SH4247 or SH5014 all had point mutations at one locus, *pmrA*. Their increased sensitivity to deoxycholate suggested that the mutation had affected the structure of the cell envelope.

Table 1 shows that on nutrient agar plates the polymyxin-resistant strains SH4420 and SH5357 tolerated concentrations of polymyxin about 30 times higher than their parents. In Tris buffer the difference was smaller. No difference was found between the smooth strain SH4420 and the rough strain SH5357.

Morphological changes of the OM started appearing in both these strains only at higher polymyxin concentrations than in the sensitive strains. The first detectable changes appeared at a polymyxin concentration of 10 μg/ml, which is again somewhat lower than the lowest concentration impairing viability (Table 1).

Figures 7 and 8 show the effect of 30 μg of polymyxin per ml on strain SH4420. The alterations appear to be small vesicles, attached to the OM with a narrow shaft. A double track, typical of a unit membrane, is visible in their walls. With increasing concentrations of polymyxin, both the number and the size of the vesicles increased; in each case they were irregular in distribution (Fig. 7), in contrast to the projections seen on the polymyxin-sensitive parent (Fig. 3).

The effect of 30 μg of polymyxin per ml on the rough, polymyxin-resistant strain SH5357 is also seen as vesicles on the OM (Fig. 9 and 10). The unit membrane structure of their wall is very prominent, and some of them seem to be peeling off from the membrane. Thus, in both the polymyxin-resistant mutants SH4420 and SH5357 the effect of polymyxin is strikingly different from that in the polymyxin-sensitive parents.

Effect of polymyxin on isolated LPS. LPS is one of the major components of OM. Further, isolated LPS forms lamellar or vesicular structures, which seem to be composed of bilayers and, in TEM, look like typical unit membranes (17). Since LPS also binds large amounts of polymyxin (3), it was of interest to study the effect of polymyxin on the morphology of such LPS preparations. Figure 11 shows the appearance of extracted smooth LPS, and Fig. 12 shows the same preparation after 30 min in the presence of 150 μg of polymyxin per ml. In

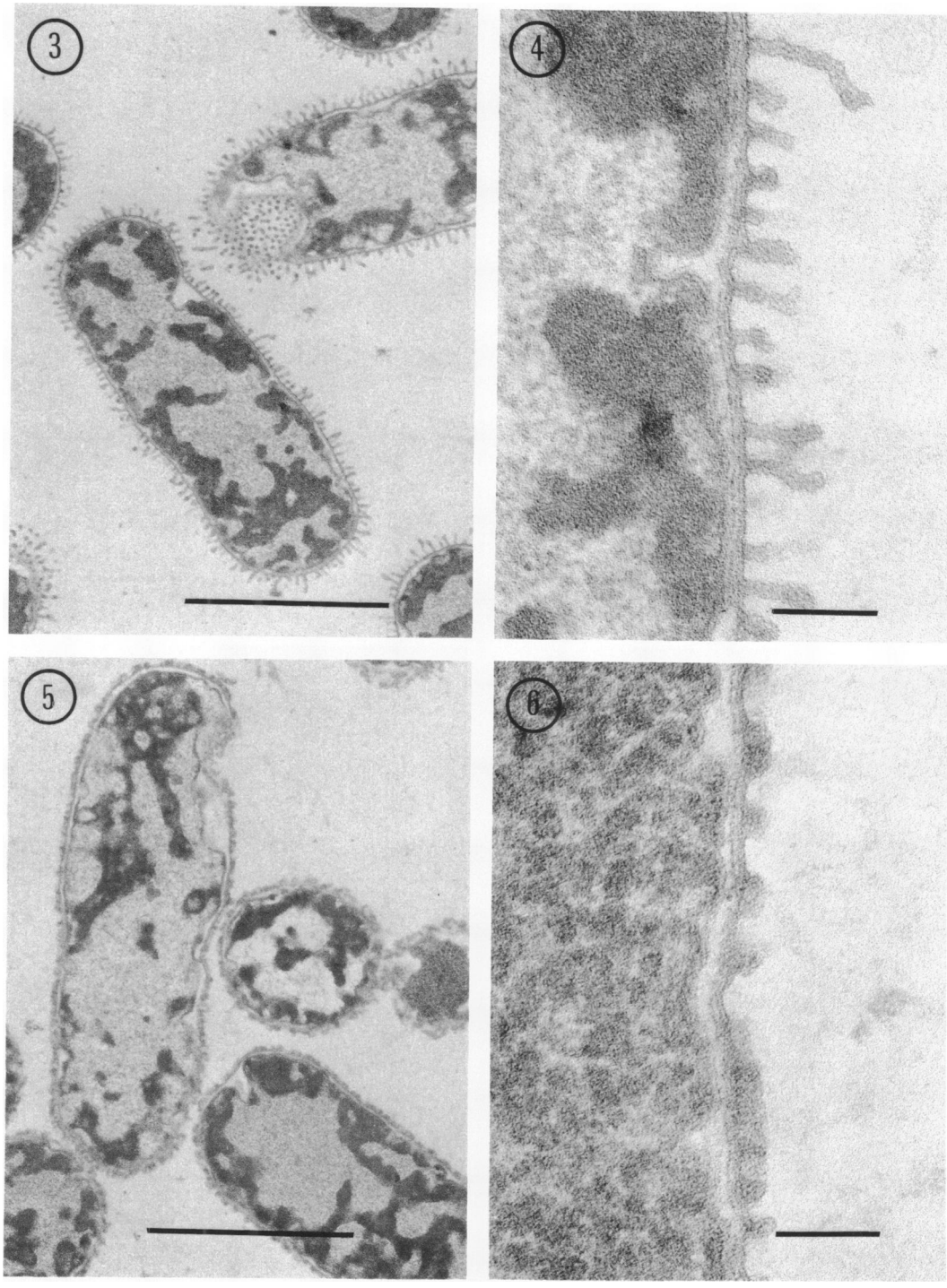


FIG. 3. *Polymyxin-sensitive smooth (S) strain SH4247 treated with 30 μg of polymyxin per ml. Bar, 1 μm.*
FIG. 4. *Higher magnification of strain shown in Fig. 3. Bar, 0.1 μm.*
FIG. 5. *Polymyxin-sensitive rough (R) strain SH5014 treated with 30 μg of polymyxin per ml. Bar, 1 μm.*
FIG. 6. *Higher magnification of strain shown in Fig. 5. Bar, 0.1 μm.*

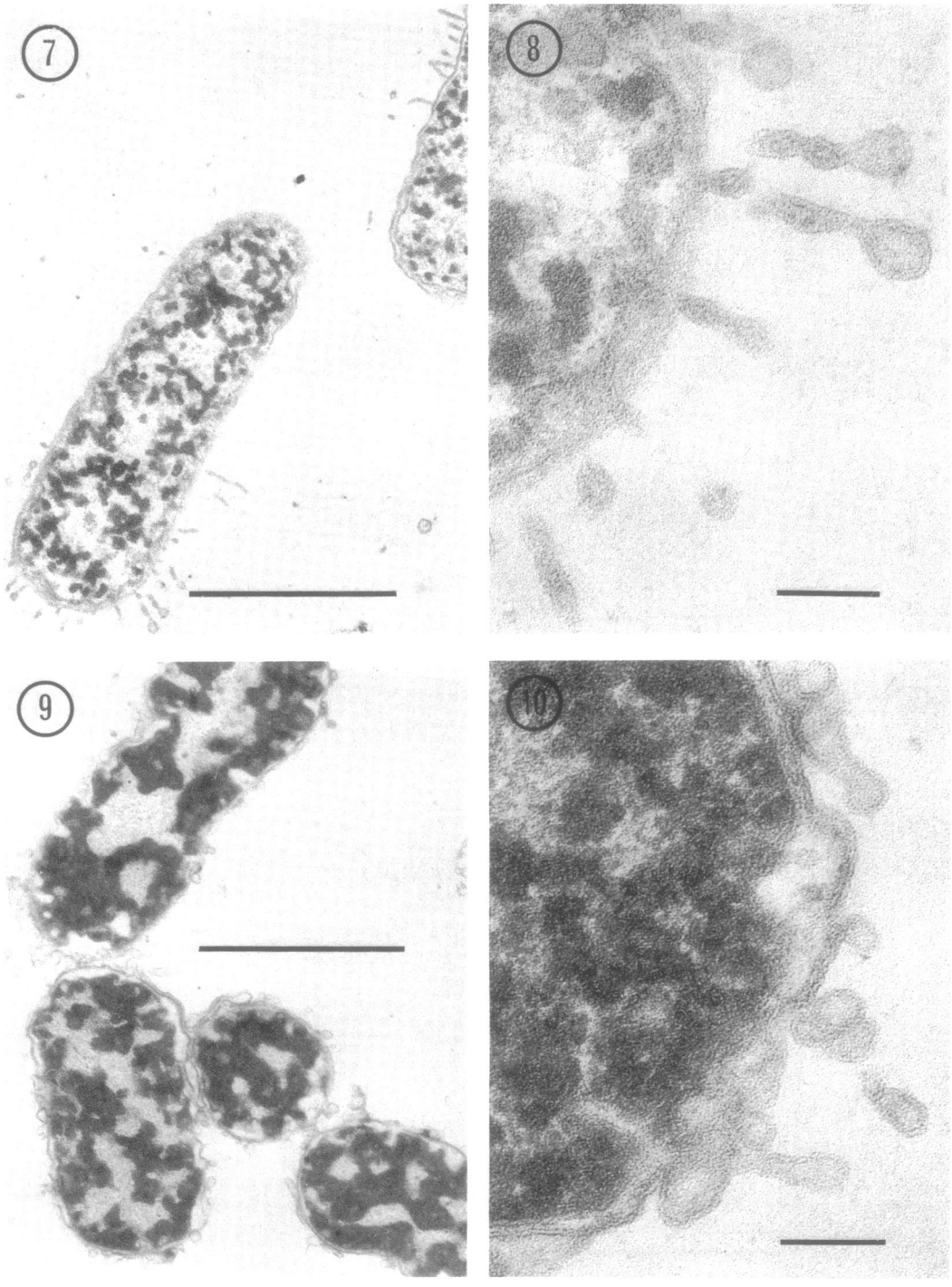


FIG. 7. *The polymyxin-resistant smooth (S) strain SH4420 treated with 30 μg of polymyxin per ml. Bar, 1 μm.*

FIG. 8. *Higher magnification of strain shown in Fig. 7. Bar, 0.1 μm.*

FIG. 9. *Polymyxin-resistant rough (R) strain SH5357 treated with 30 μg of polymyxin per ml. Bar, 1 μm.*

FIG. 10. *Higher magnification of strain shown in Fig. 9. Bar, 0.1 μm.*

agreement with Lopes and Inniss (12), LPS sheets and vesicles were broken to smaller pieces by polymyxin, and the trilaminar membrane structure became fuzzy. No projections were seen on the surface of the LPS sheets.

SEM of polymyxin-treated *Salmonella*. In SEM the surface of all untreated bacteria looked smooth without any visible structure (data not shown, but similar to Fig. 14). Figure 13 shows the effect of 30 μg of polymyxin per ml (same concentration as in TEM [Fig. 3]) on the polymyxin-sensitive smooth strain SH4247. The surface of the bacteria seems to be densely covered by granules or projections, in agreement with the thin projections seen in TEM (Fig. 3), although the width of single projections is below the resolution of SEM (20 nm).

No changes are seen in SEM preparations of the rough polymyxin-sensitive strain SH5014 at the same concentration of polymyxin (30 μg/ml) (Fig. 14); apparently, the flat projections seen in TEM (height of about 10 to 15 nm [Fig. 6]) are below the resolution of SEM. The polymyxin-resistant strain SH4420, treated with 100 μg of polymyxin per ml, is shown in Fig. 15. The surface looks very irregular, with projections of different sizes corresponding roughly to the appearance in TEM (large vesicles, Fig. 8).

DISCUSSION

The characteristic effect of polymyxin on the morphology of *Salmonella* seems to be the formation of various kinds of projections and blebs on the outer membrane of the cell envelope. Alterations of other components of the cell are seen only at considerably higher concentrations of polymyxin, which probably causes extensive damage. These findings are in good general agreement with earlier reports on the effect of polymyxin on the ultrastructure of gram-negative bacteria (6, 9, 10, 19, 21). In these reports a variety of different kinds of projections on the bacterial surface have been described. Our finding that the structure of the cell envelope influences the alterations caused by polymyxin may explain some of the differences between these reports: in most cases the bacterial strains studied were not thoroughly characterized.

The general appearance of the solid-looking projections on polymyxin-sensitive bacteria is that of an exogenous structure attached to the cell surface. In thin sections the projections appeared continuous with the outer leaflet of the OM. A comparison of polymyxin-treated smooth and rough strains showed, however, that the structure of the LPS had a strong influence on the morphology of these projections. This suggests that LPS is a component of these projections, perhaps together with other components of the OM.

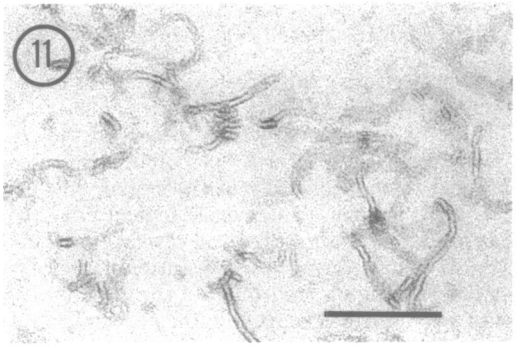
The *pmr* mutations, which increased the resistance of the bacteria to polymyxin, also changed the morphology of the polymyxin effect, although the first morphological changes appeared in both sensitive and resistant strains at just below lethal concentrations of polymyxin. Polymyxin treatment of the polymyxin-resistant mutants resulted in the appearance of clearcut vesicles on the OM. The walls of the vesicles consisted of a unit membrane clearly continuous with the whole OM, without participation of the entire cell envelope.

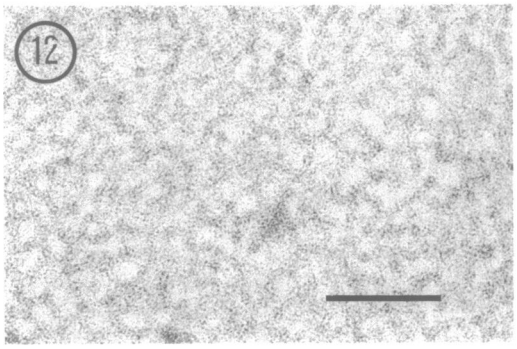
This difference in the morphology of the changes caused by polymyxin between sensitive and resistant strains suggests that the *pmrA* mutation affects the structure of the OM. This is also suggested by the increased sensitivity of these mutants to deoxycholate, a property shared by many types of mutants with defective OM (11).

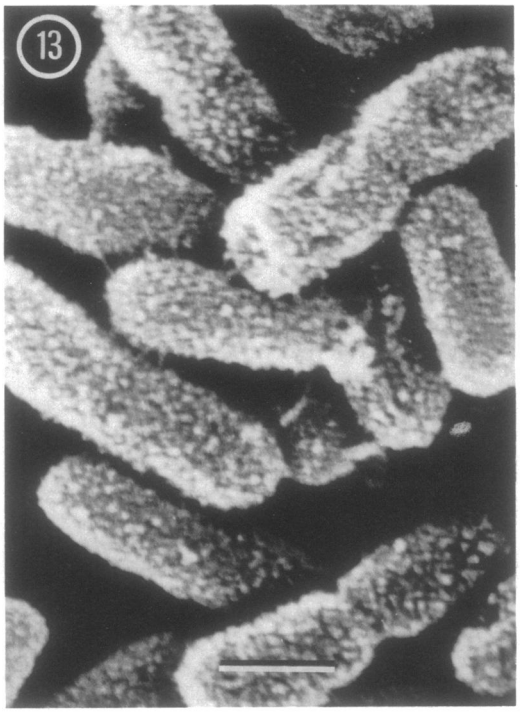
Studies on the binding of polymyxin by synthetic lipid bilayers indicate that, like other amphipathic molecules, the polymyxin is intercalated in the membrane, perhaps in close association with negatively charged phospholipids and LPS (8). This readily suggests a possible mechanism for the morphological changes caused by polymyxin. The intercalation of large amounts of polymyxin in the OM (20) must increase its surface area. Due to the tight binding of OM to the rigid peptidoglycan layer of the cell envelope (4), the OM most probably is not free to expand. The increase of the surface area can, under such conditions, be expected to force the membrane into folds and outbuddings.

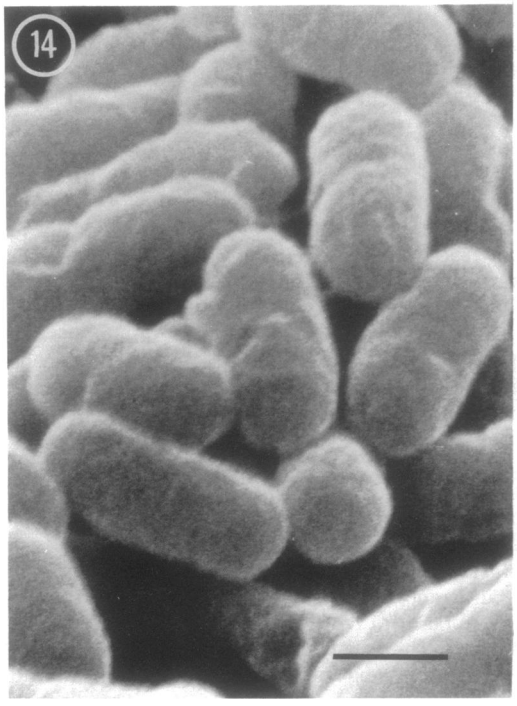
Recently Sheetz and Singer (18) have suggested a somewhat different, alternative mechanism. These authors found that certain amphipathic molecules, while bound by erythrocyte ghosts, caused either outbuddings or pits on the surface of the membrane. They suggested that this was due to asymmetric binding of the particular amphipathic substance to only one of the monolayers of the membrane, thus increasing the surface area of one layer of the membrane more than the other. The consequence would be local bending or budding of the membrane, either outwards or inwards.

An analogous mechanism could explain the projections caused by polymyxin, if the drug is mainly bound by the outer leaflet of the OM. There is no direct proof of such asymmetric binding, but it seems plausible. It is probable that the composition of both leaflets of OM is not similar (14). When first trapped by the outer leaflet, a large amphipathic molecule like polymyxin may not be expected to readily flip









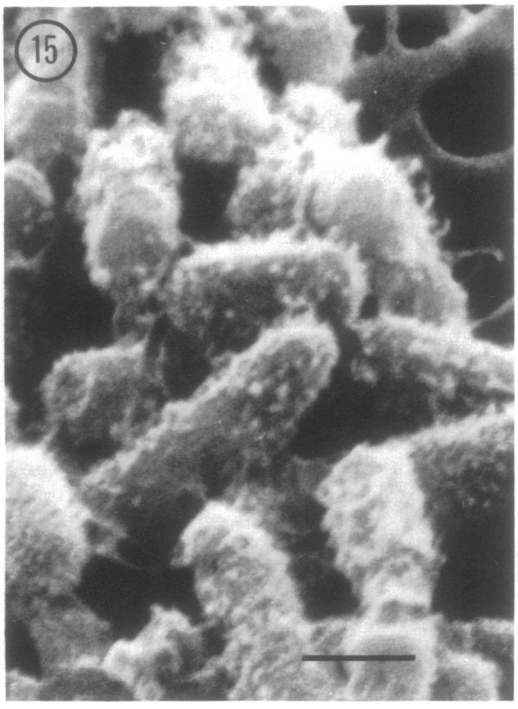


FIG. 11. *Isolated LPS of smooth S. typhimurium. Bar, 0.1 μm.*

FIG. 12. *Same LPS treated with 150 μg of polymyxin per ml. Bar, 0.1 μm.*

FIG. 13. *SEM of the same strain (SH4247) and treatment (30 μg of polymyxin per ml) as shown in Fig. 3. Bar, 1 μm.*

FIG. 14. *SEM of the same strain (SH5014) and treatment (30 μg of polymyxin per ml) as shown in Fig. 5. Bar, 1 μm.*

FIG. 15. *SEM of the same strain (SH4420) as shown in Fig. 7 treated with 100 μg of polymyxin per ml. Bar, 1 μm.*

over to the inner leaflet. The appearance of the projections in thin sections of polymyxin-sensitive bacteria is consistent with a major role of the outer leaflet of the OM in the structure of the projections. However, the resolution of the micrographs is not good enough in this respect. In the polymyxin-resistant strains, such asymmetric contribution of different leaflets of the OM was not apparent – the whole OM appeared to participate in the vesicles seen in them.

The importance of an asymmetry of the OM for the formation of the polymyxin-caused projections is also suggested by the effect of polymyxin on isolated LPS. The LPS forms membranous bilayers, both surfaces of which are similar and similarly exposed to polymyxin. No projections or blebs were seen in polymyxin-treated LPS, although the binding of polymyxin to LPS was indicated by extensive fragmentation of the LPS sheets (Fig. 12).

ACKNOWLEDGMENTS

We would like to thank S. Vene, E. Kujamäki, and T. Koro for excellent technical assistance.

Financial support from the Emil Aaltonen Foundation (to K. L.) and from the Sigrid Juselius Foundation (to M. S.) is gratefully acknowledged.

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