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Outer Membrane Permeability Barrier Disruption by Polymyxin in Polymyxin-Susceptible and -Resistant *Salmonella typhimurium*

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In contrast to their polymyxin-susceptible parent strains, polymyxin-resistant *Salmonella typhimurium* mutants (*pmrA* strains) did not lose their outer membrane permeability barrier to macromolecules such as lysozyme and periplasmic proteins upon polymyxin treatment. The sensitization of *pmrA* strains to deoxycholate-induced lysis required 10-times-higher polymyxin concentrations than did the sensitization of the parent strains. These findings indicate that the *pmrA* mutation affects the outer membrane and decreases its susceptibility to polymyxin. By contrast, the *pmrA* mutants did not differ from their parents in the uptake of gentian violet after treatment with polymyxin, suggesting a degree of specificity in the *pmrA* effect in the outer membrane.

Polymyxin, a polycationic amphipathic antibiotic, binds to the outer membrane (OM) of gram-negative bacteria (22). Electron microscopic evidence suggests an intercalation of polymyxin in the outer leaflet of the OM (8). We have isolated polymyxin-resistant mutants of *Salmonella typhimurium*, all mutated at the same locus, called *pmrA* (10), and shown that polymyxin-induced electron microscopic alterations in the OM of these strains are less extensive and also qualitatively different from those in the parent strains (8, 9). The *pmrA* strains and also the lipopolysaccharides (LPS) isolated from them bind less polymyxin than do the corresponding parent strains and their LPS (23). These findings suggest that the reduced susceptibility of the *pmrA* strains to polymyxin may be due to the reduced susceptibility of their OM to polymyxin.

The OM functions as a permeability barrier to macromolecules, hydrophobic antibiotics and dyes, and hydrophilic substances above approximately 600 daltons in molecular size (see reference 13 for review). It also prevents the leakage of periplasmic proteins. Several findings suggest that the intercalation of polymyxin into the OM disrupts the permeability barrier function of the OM. Thus polymyxin treatment makes the OM permeable to lysozyme (16, 21, 24) and periplasmic proteins (1, 2). Studies in which polymyxin was immobilized on agarose beads showed a synergism of polymyxin with bacitracin and rifampin, probably indicating that polymyxin facilitates their entry through the OM (16).

If the OM of our polymyxin-resistant *pmrA* mutants are less susceptible to polymyxin, as we have suggested, they may retain their permeability barrier function after polymyxin treatment. We therefore compared the extent of polymyxin-induced permeability barrier disruption of the OM in polymyxin-susceptible and -resistant strains. We used four different probes to which all of the strains were equally impermeable or resistant in the absence of polymyxin. These were lysozyme, periplasmic proteins, an anionic detergent (sodium deoxycholate [DOC]), and a basic hydrophobic dye (gentian violet).

MATERIALS AND METHODS

Bacterial strains. The strains used were derivatives of *S. typhimurium* LT2. SH 5014 and SH 6482 and their corresponding *pmrA* mutants SH 5357 and SH 6497 have been described earlier (8, 10). SH 9178 is SH 5014 carrying the β-lactamase-coding R plasmid R471a (4, 14) and was a kind gift from H. Nikaido (University of California, Berkeley, Calif.). SH 7426 is a *pmrA* mutant of SH 9178 isolated after diethyl sulfate mutagenesis and mapped as described by Mäkelä et al. (10).

Bacterial suspensions. The bacteria were grown in L broth (10 g of tryptone [Difco Laboratories. Detroit, Mich.], 5 g of yeast extract [Difco], and 5 g of NaCl per liter, pH 7.0) in a rotary shaker (220 rpm) at 37°C into the early logarithmic growth phase (40 Klett units; Klett-Summerson colorimeter, red filter). They were washed at room temperature in 0.07 M potassium phosphate buffer, pH 7.2, and resuspended to a final absorbance of 120 Klett units in the same buffer.

Polymyxin treatment. If not otherwise stated, the treatment was done in glass tubes containing the 4.5-

ml bacterial suspensions and increasing amounts of polymyxin (Sigma Chemical Co, St. Louis, Mo.) The tubes were incubated for 10 min at 37°C, and viable counts were made as described by Vaara et al. (23).

Polymyxin-lysozyme treatment. To the bacterial suspension, lysozyme (5 μg/ml; Boehringer, Mannheim, West Germany) and polymyxin (9 μg/ml) were added, and the decrease in absorbance was continuously followed.

Release of periplasmic proteins by polymyxin. The bacterial suspensions in 0.07 M sodium phosphate buffer, pH 7.2, were treated with polymyxin (9 μg/ml) for 10 min at 37°C and centrifuged at room temperature. The supernatant was filtered through a cellulose acetate membrane filter (Millipore Corp., Bedford, Mass.; pore size, 0.45 μm) and properly concentrated by ultrafiltration (Millipore immersible CX ultrafiltration unit; molecular size cutoff of 10,000 daltons).

Release of periplasmic proteins by osmotic shock. The osmotic shock method of Neu and Chou (11) was used as slightly modified. The bacteria (Luria broth-grown SH 9178, Klett 120, 50 ml) were washed twice in cold 10 mM tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (pH 7.4)-30 mM NaCl, incubated at room temperature for 10 min in 4 ml of 30 mM Tris-hydrochloride (pH 7.4)-5 mM ethylenediaminetetraacetate (EDTA)-20% sucrose, and suspended rapidly after centrifugation in 6 ml of ice-cold 0.5 mM $MgCl_2$. The cells were removed by centrifugation, the supernatant was filtered through a cellulose acetate membrane filter (Millipore; 0.45 μm), concentrated by ultrafiltration (Millipore immersible CX; cutoff, 10,000 daltons), evaporated into dryness under vacuum, and dissolved in electrophoresis sample buffer.

Preparation of cell envelope and cytoplasmic material. Cell envelope and cytoplasmic material were obtained from the pellet and the supernatant, respectively, after brief sonication of exponentially grown SH 9178 in 0.07 M potassium phosphate buffer, followed by centrifugation at 8,000 × *g* for 10 min (15).

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out in 10% acrylamide (thickness, 0.8 mm) by the method of Laemmli (5).

Release of β-lactamase. Polymyxin-treated suspensions of SH 9178 and SH 7426 were centrifuged at room temperature, and samples from the supernatants were assayed for β-lactamase (17) and glucose 6-phosphate dehydrogenase (6). Polymyxin did not interfere with these determinations. The total amount of these enzymes in the bacterial suspensions used was estimated as described by Nikaido et al. (14) after brief sonication in an ice-water bath, using the microtip of an MSE sonic oscillator.

Polymyxin-DOC treatment. Polymyxin-treated bacteria were centrifuged at room temperature and resuspended in the same buffer into the same volume. After the optical density of the suspension was measured, DOC (Schwarz/Mann, Orangeburg, N.Y.) was added to a final concentration of 0.25%. The suspension was incubated for 10 min at 37°C before the absorbance was measured.

Polymyxin-gentian violet treatment. The gentian violet treatment was basically similar to that of Gustafsson et al. (3). Polymyxin-treated bacteria (1 ml) were centrifuged at room temperature as for polymyxin-DOC treatment, resuspended in 2 ml of the same buffer containing gentian violet (10 μg/ml; gentian violet B, E. Merck AG, Darmstadt, West Germany), and incubated for 10 min at 37°C under vigorous shaking. The cells were removed by centrifugation, and the amount of gentian violet remaining in the supernatant was measured at 590 nm in a Hitachi 100-60 spectrophotometer.

RESULTS

Polymyxin-induced lysozyme susceptibility. Neither lysozyme (5 μg/ml) nor polymyxin (9 μg/ml) alone caused any decrease in absorbance of the polymyxin-susceptible or -resistant bacterial suspensions (Fig. 1 and data not shown). When, however, polymyxin (9 μg/ml) was added to the bacterial suspensions incubated in the presence of lysozyme (Fig. 1), the polymyxin-susceptible strain lysed with an 80% decrease in optical density in 10 min. The time course of the lysis indicated that polymyxin had caused an immediate disruption of the OM permeability barrier to lysozyme.

The corresponding polymyxin-resistant mutant strain, on the other hand, was still fully resistant to lysis by lysozyme under the same conditions (Fig. 1). Increasing amounts of polymyxin (up to 240 μg/ml) did not sensitize any of the *pmrA* strains to lysozyme-induced lysis (data not shown). Lysis was not found to occur even when the lysozyme concentration was increased up to 100 μg/ml (data not shown).

Polymyxin-induced release of periplasmic proteins. The supernatants of polymyxin-treated bacterial suspensions were subjected to electrophoresis in SDS-slab gels (Fig. 2). A set of proteins different from both that of

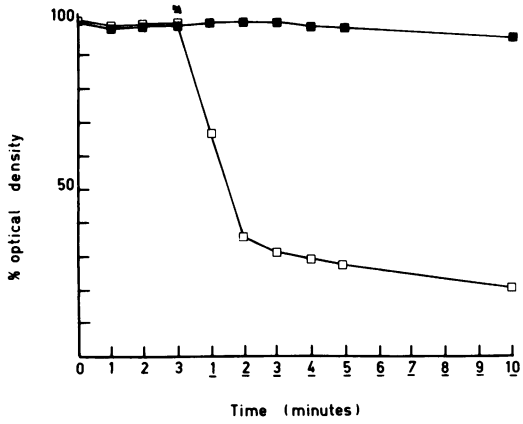

FIG. 1. *Polymyxin-induced lysozyme sensitivity in the polymyxin-sensitive strain SH 6482* (□) *and its pmrA derivative SH 6497* (■). *Both suspensions contained 5 μg of lysozyme per ml; at 3 min, 9 μg of polymyxin per ml was added.*

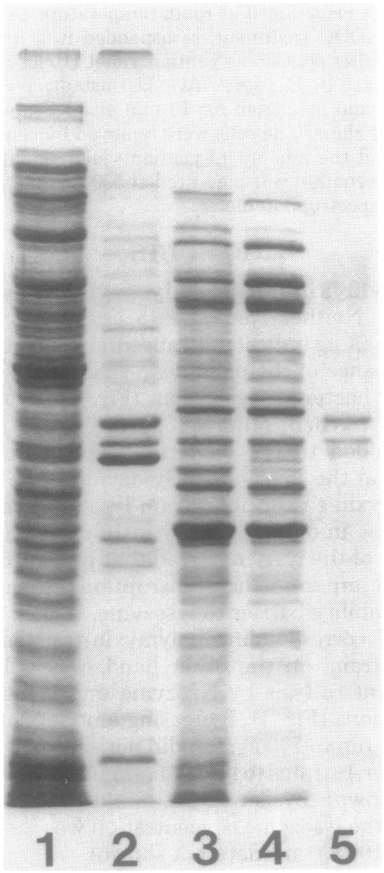

FIG. 2. *SDS-polyacrylamide gel electrophoresis of proteins released into the supernatant during polymyxin treatment (9 μg/ml) of polymyxin-susceptible strain SH 9178 (slot 4) and its polymyxin-resistant derivative SH 7426 (slot 5). These slots contain the protein released from approximately 1.5 × 10^{10} cells. The proteins from the cytoplasm (slot 1, approximately 10^8 cells), cell envelope (slot 2, approximately 0.5 × 10^9 cells), and osmotic shock fluid (slot 3, approximately 7.5 × 10^9 cells) of SH 9178 are presented for comparison.*

the cell envelope and that of the cytoplasm was released during polymyxin treatment from the polymyxin-susceptible bacteria. A very similar pattern of released proteins was obtained by using the osmotic shock procedure of Neu and Chou (11). Therefore, most of the polymyxin-released proteins probably originated from the periplasm. Virtually no proteins were released from the polymyxin-resistant bacteria.

For a quantitative assay of periplasmic protein release, we measured the β-lactamase enzyme release by using the polymyxin-susceptible strain SH 9178, which harbors the plasmid R471a coding for the periplasmic β-lactamase of the TEM type (14), and its polymyxin-resistant *pmrA* mutant SH 7246. About 50% of the β-lactamase was released into the medium from the polymyxin-susceptible strain after addition of polymyxin at concentrations of ≥3 μg/ml (Table 1). Polymyxin did not, in any concentration, cause leakage of the glucose 6-phosphate dehydrogenase, indicating that the cytoplasmic membrane remained impermeable to proteins. By contrast, the polymyxin-resistant SH 7426 did not release periplasmic β-lactamase upon polymyxin treatment even at the lethal concentration of 240 μg/ml (Table 1).

Polymyxin-induced DOC sensitivity. DOC (0.25%) alone had no effect on the viability or optical density of either the polymyxin-susceptible or -resistant bacteria under the test conditions. In contrast, if the bacteria were preincubated with polymyxin, the addition of DOC led to lysis (Fig. 3). Both polymyxin-susceptible and -resistant strains were lysed, but at different polymyxin concentrations (about 10-fold higher for the polymyxin-resistant strains).

Polymyxin-induced uptake of gentian violet. The gentian violet uptake of all strains was 18 to 28% in the absence of polymyxin, but increased to 49 to 55% after polymyxin treatment, irrespective of the polymyxin resistance of the strains (Fig. 4). Gentian violet uptake values were not corrected for adsorption of gentian violet to the glass tubes used in the uptake

TABLE 1. *Leakage of a periplasmic enzyme (β-lactamase) and an intracytoplasmic enzyme (glucose 6-phosphate dehydrogenase) from polymyxin-susceptible SH 9178 and its polymyxin-resistant mutant SH 7426 after polymyxin treatment*

Polymyxin treatment[a] (μg/ml)	Leakage			
	Penicillinase[b]		Glucose 6-phosphate dehydrogenase[c]	
	SH 9178	SH 7426	SH 9178	SH 7426
0	<7	<7	0.08	0.04
1	10	<7	0.16	ND[d]
3	290	<7	0.20	0.04
9	430	<7	0.08	0.08
27	440	20	0.16	<0.04
81	410	<7	0.12	0.04
240	500	10	0.12	0.12
Total[e]	820	880	19	16

[a] One milliliter of bacterial suspension was treated with polymyxin for 10 min at 37°C; the amount of enzyme released is given for 1 ml of the supernatant.

[b] Expressed as nanomoles of penicillin hydrolyzed per minute.

[c] Expressed as nanomoles of reduced nicotinamide adenine dinucleotide phosphate formed per minute.

[d] Not done.

[e] From 1 ml of sonicated bacterial suspension.

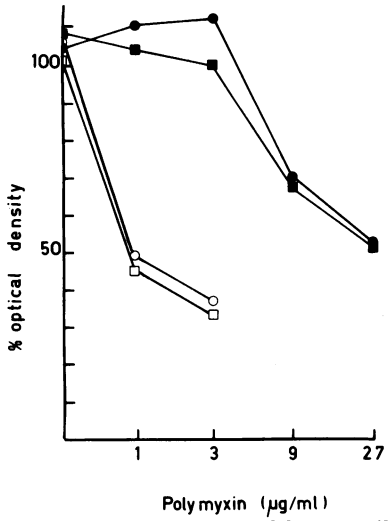

FIG. 3. *Polymyxin-induced DOC susceptibility in the polymyxin-susceptible strains (SH 5014* [○] *and SH 6482* [□]*) and in their polymyxin-resistant derivatives (SH 5357* [●] *and SH 6497* [■]*). Polymyxin-treated bacteria were centrifuged, suspended in 0.07 potassium phosphate buffer, and treated with 0.25% DOC for 10 min at 37°C.*

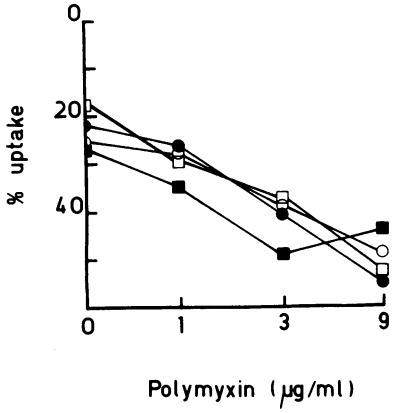

FIG. 4. *Gentian violet uptake of polymyxin-treated polymyxin-susceptible strains (SH 5014* [○] *and SH 6482* [□]*) and their polymyxin-resistant derivatives (SH 5357* [●] *and SH 6497* [■]*). The measurement was done as specified in the text.*

experiments. This adsorption varied but did not exceed 10% of the total amount of gentian violet in the system.

DISCUSSION

In enteric bacteria, polymyxin disrupts the OM permeability barrier to macromolecules such as extracellular lysozyme (21) and periplasmic proteins (1, 2). We studied this disruption in *S. typhimurium* with lysozyme (Fig. 1), periplasmic proteins visualized in SDS-gel electrophoresis (Fig. 2), and the periplasmic enzyme β-lactamase (Table 1). In the polymyxin-susceptible wild-type strains, disruption of the OM permeability barrier to both lysozyme and β-lactamase took place at nearly the same polymyxin concentration. At this same concentration, the majority (90%) of the bacteria were killed (data not shown), but there was no leakage of a cytoplasmic enzyme (glucose 6-phosphate dehydrogenase was tested) in 10 min. In contrast, polymyxin-resistant mutants of the same strains did not lose this barrier after polymyxin treatment. They did not become susceptible to lysozyme (Fig. 1), nor did they release periplasmic β-lactamase (Table 1) or other periplasmic proteins (Fig. 2). Even at the lethal concentration (240 μg/ml) of polymyxin, this barrier remained intact.

We have previously presented the following data suggesting that the *pmrA* mutation affects the OM of the bacteria in a way that makes it more resistant to polymyxin action. (i) *pmrA* strains absorb much less polymyxin than do their parents (23). (ii) Electron microscope studies show characteristic alterations of the OM upon treatment with polymyxin, and these changes are much less extensive in the *pmrA* mutants (8, 9). The morphological changes suggest preferential intercalation of polymyxin in the outer leaflet of the OM; this outer leaflet consists mainly of protein and LPS (13). (iii) LPS extracted from *pmrA* strains bind less polymyxin than do the LPS from parent strains (23). The results presented in this paper provide proof of this suggestion, showing that *pmrA* mutants are resistant to polymyxin-induced disruption of the OM permeability barrier to macromolecules like proteins.

Additionally, we studied the effect of polymyxin on the OM permeability barrier to gentian violet and DOC. These agents penetrate the intact enterobacterial OM only poorly, but do so easily when the OM is defective, as is the case in "heptoseless" LPS mutants or EDTA-treated bacteria (12, 13). The *pmrA* strains were sensitized to DOC-induced lysis by sublethal concentrations of polymyxin, although these concentrations were still 10 times higher than those required for sensitization of the parent strains. Polymyxin was also able to facilitate bacterial uptake of gentian violet by both *pmrA* strains and their parents. The uptake was measured as removal of gentian violet from the medium; thus, no distinction was made between possible adsorption to the surface and penetration into or through the OM. The uptake increased after polymyxin treatment to values (approximately

50%) comparable to those for heptoseless *S. typhimurium* mutants (18). In contrast to the polymyxin-induced sensitization to DOC, the amounts of polymyxin required to increase gentian violet uptake were the same for both *pmrA* strains and their parents. The sensitization of *pmrA* strains to DOC and gentian violet by polymyxin thus indicates that their OM structure is not totally unaffected by polymyxin. Consistent with this finding is that *pmrA* strains still absorb polymyxin (23), even in amounts sufficient to produce electron microscopic alterations in their OM structure (8, 9).

It has been postulated that interactions between LPS and the major OM proteins might play a crucial role in the structural integrity of the OM (13). Such a highly organized interaction has recently been demonstrated by using *Escherichia coli* K-12 LPS and the major protein O-8 in the presence of Mg^{2+} (25). Although the mechanism of OM permeability barrier disruption by polymyxin remains undetermined, the complex formation of polymyxin with LPS (22, 23) can be expected to alter this interaction. This, in turn, could conceivably account for the permeability changes observed. Although macromolecules of the size of large proteins can pass through the polymyxin-treated OM, no visible holes could be observed by electron microscopy (8). Also, Tris-EDTA is believed to destabilize the complexes in the outer membrane by removing divalent cations (7). The effects of polymyxin and Tris-EDTA on OM seem to be similar in many respects. Both increase the gentian violet uptake of bacteria and sensitize them to DOC and lysozyme. EDTA does not, however, cause leakage of periplasmic proteins (2), suggesting that the effects of polymyxin on OM are more profound.

Interestingly, the mechanism of the naturally occurring polymyxin resistance of *Proteus* species has also been attributed to the OM, although the OM component or components causing the polymyxin resistance have not yet been identified. Polymyxin treatment of *Proteus* does not sensitize it to lysozyme (19) nor cause leakage of periplasmic proteins (2), but does sensitize it to DOC (19). Spheroplasts of *Proteus* are polymyxin susceptible (20). Data in this paper show that the *pmrA* mutation in *S. typhimurium* leads to a polymyxin resistance which is in many respects similar to that of *Proteus*.

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