JOURNAL OF BACTERIOLOGY, Aug. 1979, p. 664-667
0021-9193/79/08-0664/04$02.00/0

Vol. 139, No. 2

Decreased Binding of Polymyxin by Polymyxin-Resistant Mutants of *Salmonella typhimurium*

MARTTI VAARA,* TIMO VAARA, AND MATTI SARVAS

Central Public Health Laboratory, SF-00280 Helsinki 28, Finland

Received for publication 7 June 1979

Polymyxin-resistant *pmrA* strains were shown to absorb only about 25% of the amount of polymyxin absorbed by the corresponding polymyxin-sensitive parent strains. The lipopolysaccharide from the *pmrA* strains bound less polymyxin than the lipopolysaccharide from the parent strains.

A characteristic feature of the mode of action of polymyxins is the rapid binding of the drug to the target gram-negative bacteria (15, 18). High concentrations of polymyxin disrupt dramatically the ultrastructure of the outer membrane (9, 10, 13). The binding of polymyxin results in an increase of cell permeability and allows periplasmic contents and small cytoplasmic molecules to leak out (3, 15, 17), which is believed to be the primary lethal effect of polymyxins (15).

The isolated outer as well as inner membranes of *Escherichia coli* and *Salmonella typhimurium* bind large amounts of polymyxin, which is incorporated into their hydrophobic regions (19). Binding of polymyxins to the acidic lipid components of these membranes can likewise be demonstrated in vitro. Quantitatively, the most important acidic membrane lipid components are lipopolysaccharide (LPS), phosphatidylglycerol, and cardiolipin, which are presumed to be the main polymyxin receptors of the cell in vivo (19). The interaction of the anionic groups of these receptor lipids and the cationic amino groups of polymyxin is considered crucial for binding (6, 15, 19).

Polymyxin-resistant mutants can be expected to be useful tools in studying the structure of bacterial membranes. We have previously isolated polymyxin-resistant point mutants (*pmrA* mutants) of *S. typhimurium* (9, 11), and Dame and Shapiro have isolated polymyxin-resistant mutants of *E. coli* (4). Phenotypically polymyxin-resistant unstable variants of *Pseudomonas aeruginosa* have also been reported (2, 8). A prime question when dealing with mutants resistant to any agent is whether a receptor of that agent has been altered so that the resistance would be due to decreased binding. We demonstrate here that our polymyxin-resistant mutants indeed bind less polymyxin than do their polymyxin-sensitive parent strains. Furthermore, the decreased binding of polymyxin by the resistant bacteria corresponds to a decreased binding of polymyxin by their LPS.

Because the sensitivity of bacteria to polymyxin is dependent on growth conditions, medium, and cell density, we determined the polymyxin sensitivity of our strains in the exact conditions to be used in polymyxin-binding experiments. Bacteria at early logarithmic growth phase (40 Klett units, Klett-Summerson Colorimeter, red filter) in Luria broth (10 g of tryptone [Difco], 5 g of yeast extract [Difco], and 10 g of NaCl, supplemented with 0.25 g of $CaCl_2$ per liter) were washed in 0.07 M potassium phosphate buffer (pH 7.2) and resuspended to a final density of 10^9 cells per ml in the same buffer. One milliliter of this suspension was added to glass tubes containing 0 to 240 μg of polymyxin B sulfate in 50 μl of 0.9% NaCl. The suspensions were incubated for 10 min at 37°C and then rapidly diluted 1:100 into cold Luria broth. The number of viable bacteria was determined by plating appropriate dilutions of this suspension onto nutrient agar plates.

Figure 1 shows the effect of polymyxin on the polymyxin-sensitive parent strains SH5014 and SH6482 and on their polymyxin-resistant (*pmrA*) derivatives SH5357 and SH6497. About 2.5 μg of polymyxin per ml was required to kill 90% of the parent strain bacteria, whereas the mutants tolerated a 30 to 100-times-higher concentration of polymyxin. This difference agrees well with the corresponding sensitivity difference previously determined by plating the bacteria onto polymyxin-containing nutrient agar (11).

Teuber and Bader (18, 19) have shown that mono-*N*-acetyl-[^{14}C]polymyxin B can be used to measure the binding of polymyxin to bacteria. This labeled derivative was prepared by acetylation of polymyxin B (Dumex, Copenhagen) with [1-^{14}C]acetic anhydride (The Radiochemical Centre, Amersham, England) as described by

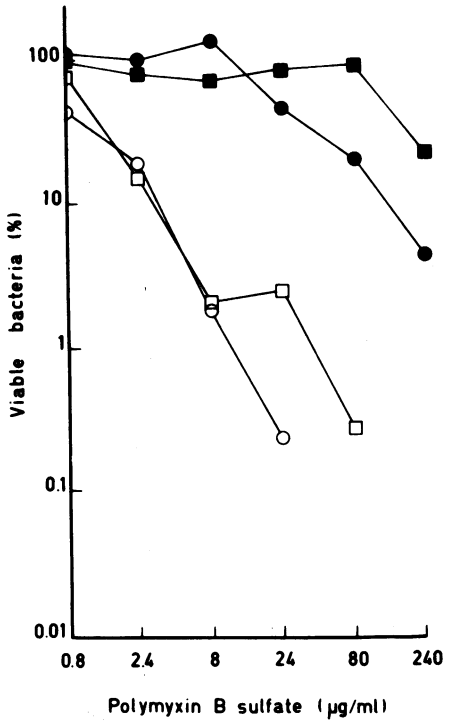

FIG. 1. *Effect of polymyxin on the polymyxin-sensitive rfaJ strains (SH5014, ○, and SH6482, □) and their polymyxin-resistant rfaJ pmrA derivatives (SH5357, ●, and SH6497, ■). Viability after 10 min of incubation in 0.07 M potassium phosphate buffer (pH 7.2).*

Teuber (16). The reaction products were separated by thin-layer chromatography on cellulose-coated aluminum foil (E. Merck, Darmstadt, West Germany) using the solvent system *n*-butanol–pyridine–acetic acid–water (30:20:6:24, vol/vol/vol/vol). The slowest moving of the five radioactive spots ($R_f = 0.70$) was eluted from the cellulose with pyridine–acetic acid–water (10:4:86, vol/vol/vol). The eluant was evaporated, and the residue was dissolved in 0.9% NaCl. Only one radioactive spot ($R_f = 0.70$) was found when samples of the product (up to 30,000 cpm as detected with a Tracerlab strip scanner [division of LFE Electronics, Richmond, Calif.]) were rechromatographed as described above.

In the polymyxin-binding experiments, 1 ml of the bacterial suspension (10^9 bacteria per ml, made as described above) was mixed in glass tubes with 50 μl of 0.9% NaCl solution containing increasing amounts of mono-*N*-acetyl-[^{14}C]polymyxin B (specific activity, 7.4 mCi/mmol). After 10 min at 37°C the bacteria were pelleted (5,000 rpm for 15 min at 0°C) and the supernatant was removed. The pellet was washed in the buffer and resuspended in 1.0 ml of distilled water, and radioactivity was measured with a Beckman LS100 liquid scintillation spectrometer. Some of the labeled polymyxin was found to bind to the glassware. This amount did not exceed 15% of the total radioactivity in the system, and, in contrast to Teuber and Bader (19), we could not reduce it by substituting plastic materials, including polypropylene, for glass.

Figure 2 shows the absorption by polymyxin-sensitive and -resistant strains. A clear correlation between the resistance to polymyxin and the amount of polymyxin bound is evident. The polymyxin-resistant *pmrA* strains bound only 25% of the amount of labeled polymyxin absorbed by the polymyxin-sensitive parent strains, suggesting that some of the major polymyxin receptors were missing in *pmrA* mutants or were changed in a way decreasing their affinity to polymyxin.

A search for an altered membrane component in *pmrA* mutants has previously given only negative results. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis did not reveal changes in the polypeptide composition of the isolated cell envelopes (M. Nurminen and M. Vaara, unpublished data). No qualitative or quantita-

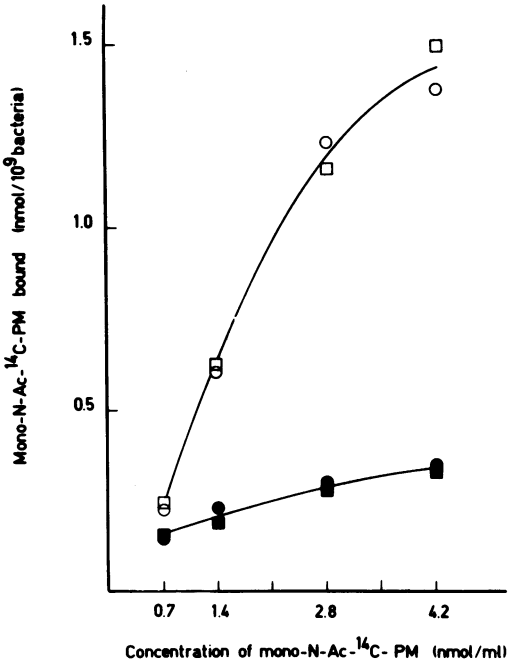

FIG. 2. *Absorption of mono-N-acetyl-[^{14}C]polymyxin by polymyxin-sensitive strains (SH5014, ○, and SH6482, □) and their polymyxin-resistant pmrA derivatives (SH5357, ●, and SH6497, ■). Incubation for 10 min in 0.07 M potassium phosphate buffer (pH 7.2).*

tive differences could be detected in the phospholipid composition or in the fatty acids between the envelopes of our *pmrA* mutants and their parent strains (T. Korhonen, M. Sci. thesis, University of Helsinki, Helsinki, Finland, 1975). Similarly, Dame and Shapiro did not find any gross phospholipid or fatty acid alterations in their polymyxin-resistant *E. coli* strains (5).

Because the LPS is the only one of the suggested polymyxin receptors (19) that is exposed on the cell surface (14), special attention was paid to it. LPS was extracted from the mutant and parent strains (all of them rough, *rfaJ*) by the method of Galanos et al. (7). Quantitative analysis of the monosaccharide components (glucose, galactose, L-glycero-D-manno-heptose, and keto-deoxy-mannooctonate) of the isolated LPS revealed no differences between the *pmrA* mutants and their parents (unpublished data). Since also the sensitivity of the *pmrA* mutants to a set of LPS-specific phages was unaltered (9), it is unlikely that the oligosaccharide core of the LPS is affected by the *pmrA* mutation. These methods would not, however, detect an alteration in the lipid A or deep-core domains of the LPS, and in fact these are exactly the parts of the molecule likely to interact with the basic, amphiphilic polymyxin molecule. Therefore we compared the polymyxin-binding capacities of the mutant and parent strain LPS. This was performed by bioassay (1) as follows: 15 nmol of polymyxin B sulfate and increasing amounts of LPS (quantitated by its heptose content as determined with H_2SO_4–cysteine; 12) were incubated together in 0.5 ml of 0.9% NaCl at 37°C for 1 h. A 4.5-ml sample of a log-phase culture of polymyxin-sensitive bacteria (SH5014, Luria broth) was then added, and the incubation was continued for 2 h before the optical density was measured (Table 1). Without any added LPS the polymyxin concentration used completely inhibited the growth of the indicator bacteria. This growth inhibition was gradually prevented by increasing amounts of LPS, presumably because of complex formation between LPS and polymyxin. More LPS from the polymyxin-resistant strains was needed for this prevention as compared to LPS derived from the polymyxin-sensitive parent strains, indicating a lower polymyxin-binding capacity of the mutant-strain LPS. We suggest that the *pmrA* mutation has caused an alteration of the LPS, probably in the lipid A or deep core. Such an LPS alteration has not been previously described, and we are now attempting to define it chemically.

TABLE 1. *Growth inhibition of indicator bacteria by polymyxin-LPS mixtures*[a]

LPS (heptose, nmol)	Growth inhibition (%) by LPS from:			
	Polymyxin-sensitive strains		Polymyxin-resistant strains	
	SH5014	SH6482	SH5357	SH6497
0	100	100	100	100
15	100	100	100	100
30	32	7	100	93
60	9	0	63	23
120	0	0	0	0

[a] A 15-nmol sample of polymyxin B was preincubated for 1 h with increasing amounts of LPS and then diluted 1:10 with log-phase culture of polymyxin-sensitive *Salmonella* strain. After 2 h, growth inhibition was determined as a percentage of maximal growth without added polymyxin.

Financial support from the Sigrid Jusélius Foundation (to M.S.) is gratefully acknowledged.

LITERATURE CITED

1. **Bader, J., and M. Teuber.** 1973. Action of polymyxin B on bacterial membranes. I. Binding to the O-antigenic lipopolysaccharide of *Salmonella typhimurium*. Z. Naturforsch. **28c**:422–430.
2. **Brown, M. R. W., and W. M. Watkins.** 1970. Low magnesium and phospholipid content of cell walls of *Pseudomonas aeruginosa* resistant to polymyxin. Nature (London) **227**:1360–1361.
3. **Cerny, G., and M. Teuber.** 1971. Differential release of periplasmic *versus* cytoplasmic enzymes from *Escherichia coli* B by polymyxin B. Arch. Microbiol. **78**:166–179.
4. **Dame, J. B., and B. M. Shapiro.** 1976. Use of polymyxin B, levallorphan, and tetracaine to isolate novel envelope mutants of *Escherichia coli*. J. Bacteriol. **127**:961–972.
5. **Dame, J. B., and B. M. Shapiro.** 1979. Lipid and lipopolysaccharide composition of *Escherichia coli* surface-altered mutants selected for resistance to levallorphan, tetracaine, and polymyxin. J. Bacteriol. **137**:1043–1047.
6. **Feingold, D. S., C. C. Hsu-Chen, and I. J. Sud.** 1974. Basis for the selectivity of action of the polymyxin antibiotics on cell membranes. Ann. N. Y. Acad. Sci. **237**:480–492.
7. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for the extraction of R-lipopolysaccharide. Eur. J. Biochem. **9**:245–249.
8. **Gilleland, H. E., Jr., and R. G. E. Murray.** 1976. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **125**:267–281.
9. **Lounatmaa, K., P. H. Mäkelä, and M. Sarvas.** 1976. The effect of polymyxin on the outer membrane of *Salmonella*: ultrastructure of wild-type and polymyxin-resistant strains. J. Bacteriol. **127**:1400–1407.
10. **Lounatmaa, K., and N. Nanninga.** 1976. Effect of polymyxin on the outer membrane of *Salmonella typhimurium* studied in freeze-fractured cells. J. Bacteriol. **128**:665–667.
11. **Mäkelä, P. H., M. Sarvas, S. Calcagno, and K. Lounatmaa.** 1978. Isolation and characterization of polymyxin resistant mutants of *Salmonella*. FEMS Microbiol. Lett. **3**:323–326.
12. **Osborn, M. J.** 1963. Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxy-octonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. **50**:499–506.

13. **Schindler, P. R. G., and M. Teuber.** 1975. Action of polymyxin B on bacterial membranes: morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. Antimicrob. Agents Chemother. **8**:95–104.
14. **Smit, J., Y. Kamio, and H. Nikaido.** 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. **124**:942–958.
15. **Storm, D. R., K. S. Rosenthal, and P. E. Swanson.** 1977. Polymyxin and related peptide antibiotics. Annu. Rev. Biochem. **46**:723–763.
16. **Teuber, M.** 1970. Preparation of biologically active mono-N-acetyl(^{14}C)-derivatives of the membrane specific polypeptide antibiotic polymyxin B. Z. Naturforsch. **25b**:117.
17. **Teuber, M.** 1974. Action of polymyxin B on bacterial membranes. III. Differential inhibition of cellular function in *Salmonella typhimurium*. Arch. Microbiol. **100**: 131–144.
18. **Teuber, M., and J. Bader.** 1971. Quantitative correlation of uptake with antibiotic activity of polymyxin B in *Salmonella typhimurium*. FEBS Lett. **16**:195–197.
19. **Teuber, M., and J. Bader.** 1976. Action of polymyxin on bacterial membranes. Binding capacities for polymyxin B of inner and outer membranes isolated from *Salmonella typhimurium G30*. Arch. Microbiol. **109**:51–58.