ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1978, p. 500-504
0066-4804/78/0014-0500$02.00/0

Vol. 14, No. 3
Printed in U.S.A.

Resistance of Spheroplasts and Whole Cells of *Pseudomonas cepacia* to Polymyxin B

JANE M. MANNIELLO,* HANS HEYMANN, AND FRANK W. ADAIR
Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Summit, New Jersey 07901

Received for publication 5 May 1978

Sucrose-lysozyme spheroplasts were prepared from two strains of *Pseudomonas cepacia* and tested for susceptibility to polymyxin B and benzalkonium chloride. Spheroplasts were more susceptible than whole cells to benzalkonium chloride but not to polymyxin B. Disruption of the outer membrane layer was not by itself sufficient to render *P. cepacia* susceptible to polymyxin B.

Pseudomonas cepacia, originally described as a plant pathogen (2), has been implicated in nosocomial infections with increasing frequency (3, 6). Although the organism is of low virulence, its omnivorous nutritional characteristic and natural resistance to many antimicrobial agents and antibiotics make it a potential clinical problem for severely compromised patients. *P. cepacia* is more resistant than most other gram-negative organisms to the commonly used antimicrobial agent benzalkonium chloride (BC). It is highly resistant to polymyxin B sulfate (PMB), another cationic agent that is effective against most gram-negative bacteria. BC and PMB are thought to act primarily against the cytoplasmic membrane. Resistance to them is postulated to be due to the impermeability of the outer trilaminar layer of the cell wall-membrane complex. Studies on the differential susceptibility of whole cells and wall-less forms to PMB tend to confirm this hypothesis (4, 8). We have examined the differential susceptibility of whole cells and spheroplasts of two strains of *P. cepacia* to PMB and BC. As a control, we also examined the differential susceptibility of *Escherichia coli* to PMB.

Sucrose-lysozyme spheroplasts were prepared from *P. cepacia* 153, a clinical isolate, and *P. cepacia* ATCC 25416, as described by Cheng et al. (5). Midlog-phase cells cultured in a semidefined medium were used (1). Spheroplasts of *E. coli* B ATCC 11303 were formed from midlog-phase cells, grown in nutrient broth according to the method of Cheng et al. (5) and by a modification of the method of Weiss and Frazer (9). Samples of cells were evenly divided; 90 ml was centrifuged (13,000 × *g*) and resuspended in 30 ml of 0.25 M sucrose containing 0.033 M tris-(hydroxymethyl)aminomethane buffer (Tris), pH 8.0; disodium ethylenediaminetetraacetate (EDTA) at a concentration of 15 mg, contained in 0.24 ml of a neutralized 6.25% (wt/vol) solution, and 300 μg of lysozyme (0.10 ml of a 0.3% solution) were added. Phase microscopy indicated the presence of 99.9 to 100% spheroplasts in all preparations. Control cells were washed with distilled water and resuspended in fresh medium prior to susceptibility testing. The susceptibility of cells and spheroplasts to PMB and BC was determined as follows. Samples (3 ml) of whole-cell or spheroplast suspensions were added to 7-ml portions of medium containing appropriate concentrations of the test compound. *P. cepacia* was tested in semidefined medium; *E. coli* was tested in nutrient broth. Assay tubes for Tris-EDTA-lysozyme spheroplasts were osmotically stabilized with 0.25 M sucrose. The number of viable whole cells and spheroplasts was determined by plate counts immediately after contact (0.5 min) with the antimicrobial agents and after 60 min of incubation at room temperature. Serial dilutions were made into tubes containing 0.01 M $MgCl_2$, 0.22% lecithin, 1.55% polysorbate 80, and 0.25 M sucrose. Samples were plated on Trypticase soy agar with lecithin and polysorbate 80 (BBL) and incubated at 30°C (*P. cepacia*) or 37°C (*E. coli*) for 48 h. Controls were plated in the same manner immediately prior to the addition of the antimicrobial agents.

Sucrose-lysozyme and Tris-EDTA-lysozyme spheroplasts of *E. coli* behaved in an identical manner when treated with PMB. Spheroplasts prepared by either method were slightly more susceptible to this agent than were whole cells (Fig. 1). This confirms the work of Koike et al. (7). However, with *P. cepacia,* there was no difference between whole cells and spheroplasts in their susceptibilities to PMB. In Fig. 2, it can be seen that the viability of spheroplasts of both

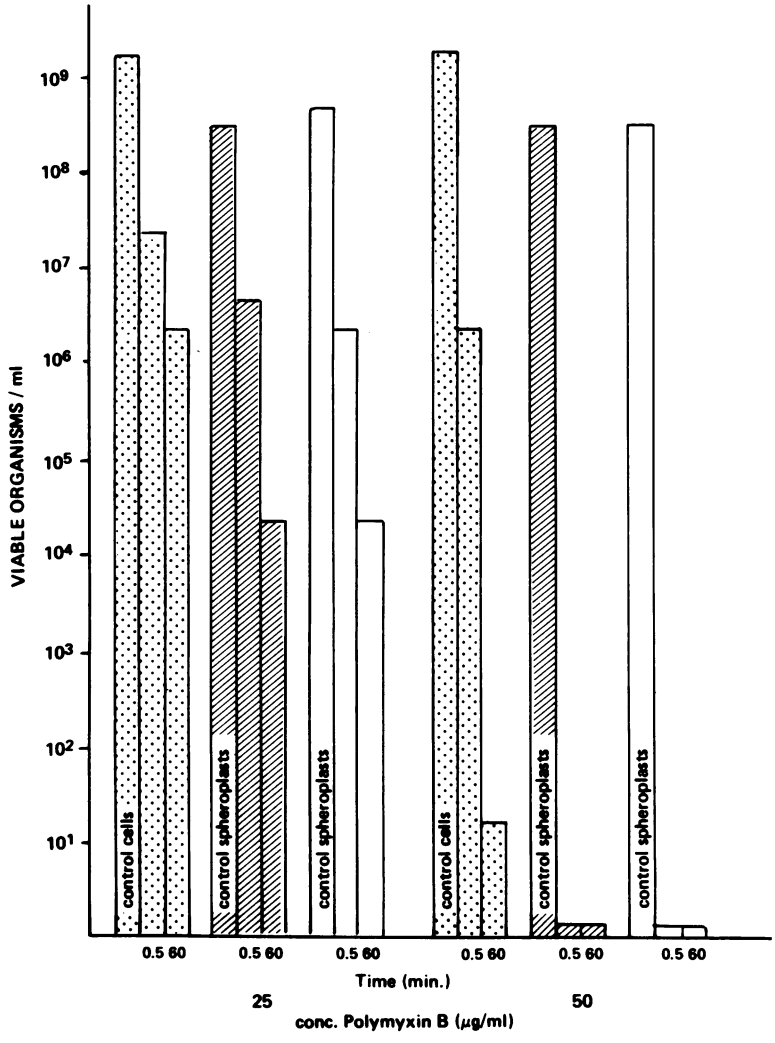

FIG. 1. *Effect of PMB on whole cells and spheroplasts of E. coli B. Organisms were treated with PMB as described in the text, and the number of viable organisms was determined at 30 s and 60 min also as described in the text. Control whole cells and spheroplasts were not exposed to PMB. Symbols:* (▩) *whole cells;* (▨) *sucrose-lysozyme spheroplasts;* (□) *Tris-EDTA-lysozyme spheroplasts.*

strains of *P. cepacia* was unchanged from that of whole cells even after treatment with 500 μg of PMB per ml for 60 min. Spheroplasts of these organisms were more susceptible to BC than were whole cells. Figure 3 shows that both spheroplasts and whole cells were killed on contact by a 0.5% solution of BC or at about the same rate on prolonged exposure to a 0.05% solution. At concentrations of 0.1 and 0.15%, there were 3- and 4-log differences in viability, between whole cells and spheroplasts to this agent.

Previous work has demonstrated an increase in susceptibility of *Proteus mirabilis* to PMB upon removal of the outer trilaminar membrane (8). Whole cells of this organism were intrinsically resistant to high concentrations of PMB. In these organisms, the outer membrane serves as a barrier to penetration of the compound. In *P. cepacia,* however, removal of most of the outer membrane layer was not sufficient to render the cells susceptible to PMB. The methods we used to prepare spheroplasts do not completely remove all of the outer cell wall. This was true for *E. coli* preparations as well as for those from *P. cepacia.* In the former, disruption of the outer membrane layer resulted in an

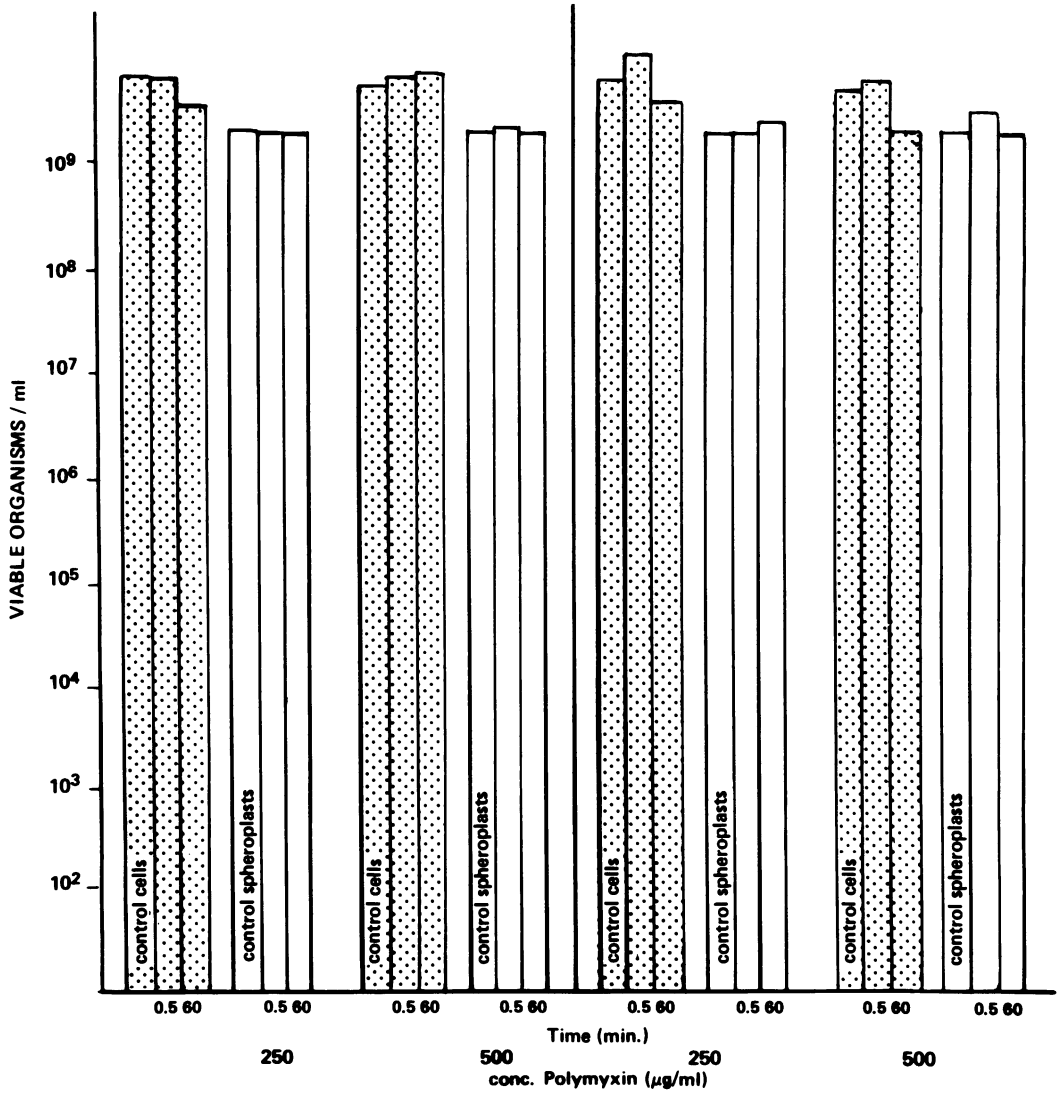

FIG. 2. *Effect of PMB on whole cells (▨) and sucrose-lysozyme spheroplasts (□) of P. cepacia ATCC 25416 (left) and P. cepacia 153 (right). Whole cells and spheroplasts were treated with 250 and 500 μg of PMB per ml as described in the text. After 30 s and 60 min of contact, suitable dilutions were plated and colonies were counted, also as described in the text. Control whole cells and spheroplasts were not exposed to PMB.*

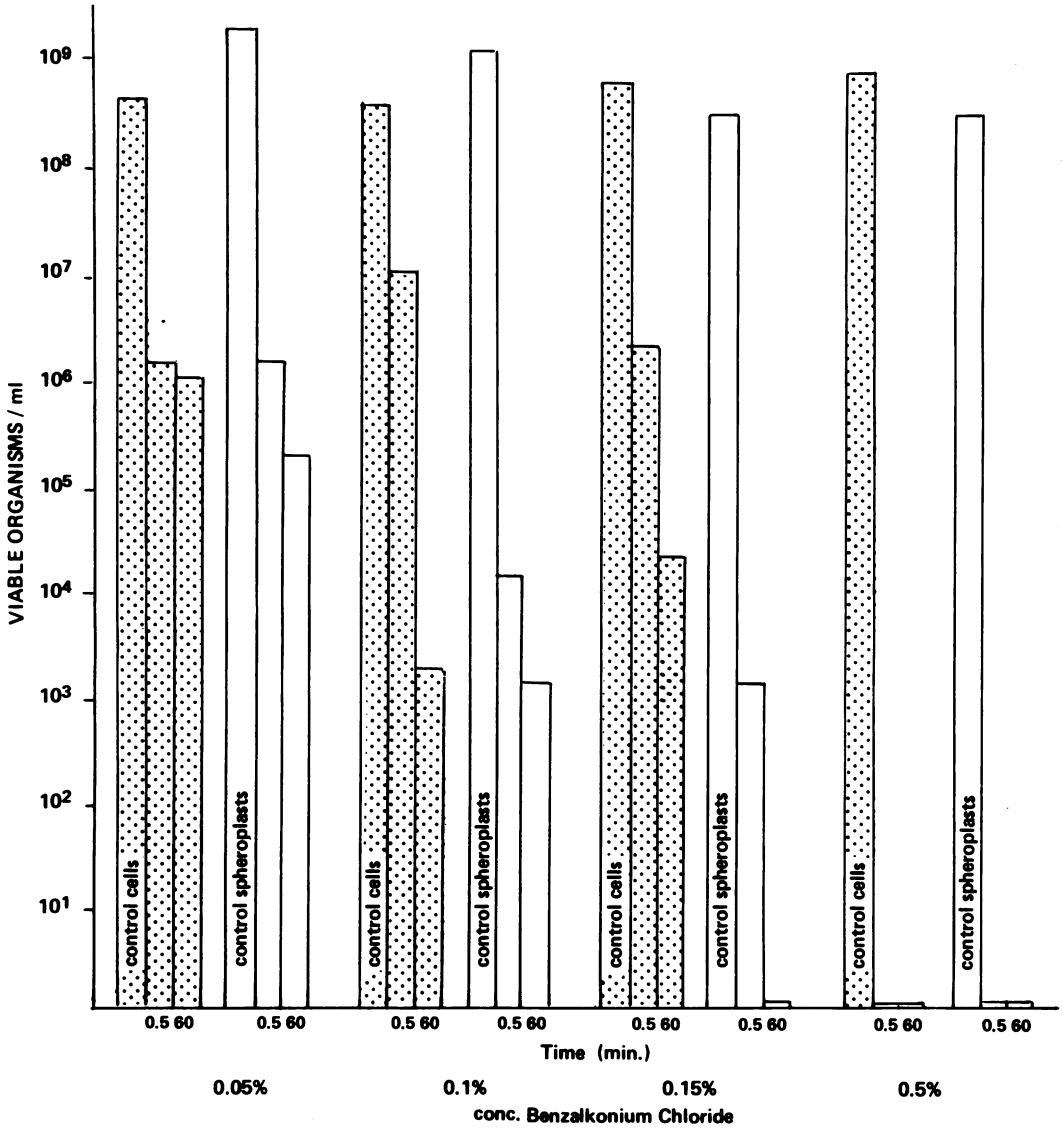

FIG. 3. *Effect of BC on whole cells (▨) and spheroplasts (□) of P. cepacia 153. Whole cells and spheroplasts of P. cepacia 153 were exposed to the indicated concentrations of BC as described in the text. After 30 s and 60 min of contact, suitable dilutions were plated and colonies were counted, also as described in the text. Control whole cells and spheroplasts were not exposed to BC.*

increased susceptibility to PMB. In the latter, it did not. We believe that resistance to PMB by *P. cepacia* involves more than just a barrier effect.

LITERATURE CITED

1. **Adair, F. W., S. G. Geftic, and J. Gelzer.** 1971. Resistance of *Pseudomonas* to quaternary ammonium compounds. II. Cross resistance characteristics of a mutant of *Pseudomonas aeruginosa*. Appl. Microbiol. **21**:1058–1063.
2. **Ballard, R. W., N. J. Palleroni, M. Doudoroff, R. Y. Stanier, and M. Mandel.** 1970. Taxonomy of the aerobic Pseudomonads: *Pseudomonas cepacia, P. marginata, P. alliicola* and *P. caryophylii*. J. Gen. Microbiol. **60**:199–214.
3. **Bassett, D. C. J., J. A. S. Dickson, and G. H. Hunt.** 1973. Infection of Holter valve by *Pseudomonas*-contaminated chlorhexidine. Lancet **i**:1263–1264.
4. **Brown, M. R. W.** 1975. The role of the cell envelope in resistance, p. 71–107. *In* M. R. W. Brown (ed.), Resistance of *Pseudomonas aeruginosa*. John Wiley & Sons, London.
5. **Cheng, K.-J., J. W. Costerton, A. P. Singh, and J. M.**

Ingram. 1973. Susceptibility of whole cells and spheroplasts of *Pseudomonas aeruginosa* to actinomycin D. Antimicrob. Agents Chemother. **3**:399–406.

6. **Gelbart, S. M., G. R. Reinhardt, and H. B. Greenlee.** 1976. *Pseudomonas cepacia* strains isolated from water reservoirs of unheated nebulizers. J. Clin. Microbiol. **3**:62–66.
7. **Koike, M., K. Iida, and T. Matsuo.** 1969. Electron microscopic studies on mode of action of polymyxin. J. Bacteriol. **97**:448–452.
8. **Teuber, M.** 1969. Susceptibility to polymyxin B of penicillin G-induced *Proteus mirabilis* L forms and spheroplasts. J. Bacteriol. **98**:347–350.
9. **Weiss, R. L., and D. Fraser.** 1973. Surface structure of intact cells and spheroplasts of *Pseudomonas aeruginosa*. J. Bacteriol. **113**:963–968.