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Mode of Action of Polymyxin B: Physiological Studies with a *Bacillus subtilis*-Resistant Mutant

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Polymyxin B resistance in *Bacillus subtilis* can be suppressed by the synergistic action of lysozyme or of an analogous cell wall lytic activity released by *B. subtilis* spores during germination.

Such a synergistic effect is probably due to partial cell-wall digestion by lysozyme that allows polymyxin to reach its site of action and is therefore distinct from the analogous synergistic effect described by other authors in *Escherichia coli*. In the latter case polymyxin B probably damaged the outer membrane, allowing lysozyme to reach and digest the cell wall.

Polymyxin B is a peptide antibiotic containing an hydrophobic side chain, particularly active against gram-negative bacteria (1, 2). In *Pseudomonas aeruginosa*, Newton (4) demonstrated that polymyxin B is adsorbed on sensitive cells and that the inhibiting dose corresponds to 2×10^5 molecules per cell, whereas adsorption saturation is reached only at 10^6 molecules per cell. The interaction is probably at the level of the lipoproteic bilayer of the cytoplasmic (or outer) membrane leading to structural aberrations and impairment of the osmotic barrier. Pache et al. (5) have confirmed that the side chain (isopelargonic acid) interacts with phospholipids forming a hydrophobic bond in artificial membranes.

The natural resistance of many bacterial strains (mainly gram positive) seems to be due to characteristics of their cell wall that does not allow contact of the antibiotic with the cytoplasmic membrane. Teuber (7) has shown that spheroplasts and L forms of the naturally resistant species *Proteus mirabilis* are susceptible to the antibiotic. The interaction between polymyxin and membrane structures can explain the reported synergism between polymyxin B and lysozyme on bacterial species (like *Escherichia coli* and *Neisseria catarrhalis*) naturally resistant to the lytic action of the enzyme because of an outer membrane impermeable to lysozyme (8).

B. subtilis is a gram-positive bacterium very sensitive to polymyxin B. Resistant mutants have been selected and analyzed to gain further insights into the mechanism of action of the antibiotic.

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MATERIALS AND METHODS

Bacterial strains and culture media. Strain PB 920 (*pur*A16 *leu*8 *met*B5; Mu16u8u5 of N. Sueoka) was used to obtain resistant mutants and as the "susceptible" control.

PB 3412 (*pur*A16 *leu*8 *met*B5 *plx*) and several other analogous spontaneous resistant mutants were isolated by plating approximately 10^9 cells from exponential cultures of PB 920 on nutrient agar containing 50 μg of polymyxin B per ml.

All bacterial growths were carried out in NB medium (nutrient broth; Difco) at 37 C with agitation, or on the same medium solidified with 2% agar (Difco). Sporulation was carried out on solid NB medium containing $MnCl_2$ (10^{-5} M) at 37 C for 3 days.

Abbreviations and symbols. *pur*, *leu*, and *met* indicate auxotrophy for adenine, leucine, and methionine, respectively; *plx* indicates resistance to polymyxin B. Absorbance at 540 nm was measured with a Zeiss PMQ II spectophotometer. FG indicates filtered NB (through membrane filters [millipore], 0.45 μm in diameter) in which spores (inoculated at an absorbance of 540 nm of 0.600) have germinated and outgrown for 60 min. FG 920 and FG 3412 are the filtrates of the NB media in which spores of PB 920 or PB 3412, respectively, have germinated.

Chemicals. Polymyxin B sulfate (7675 U/mg; Schwarz/Mann Co., Orangeburg, N.Y.) and lysozyme (egg white, grade I; Sigma Chemical Co., Saint Louis, Mo.) were dissolved in NB at the concentration of 1 mg/ml before use.

RESULTS

Effect of polymyxin B on growth of parental and resistant strains. Vegetative cells of both parental and resistant strains were exposed to increasing doses of polymyxin B in NB medium. As is shown in Fig. 1, 10 μg of polymyxin B per ml completely inhibits growth of the parental strain PB 920, causing lysis and

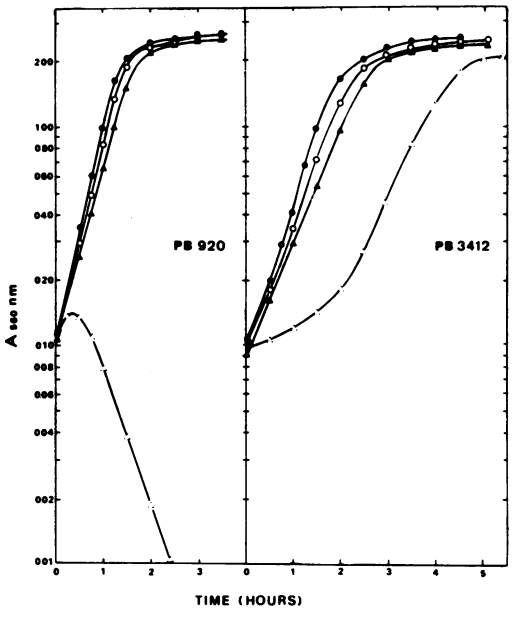

FIG. 1. *Growth of vegetative cells of PB 920 and PB 3412 (plx mutant) in the presence of different doses of polymyxin B. Cells in logarithmic phase of growth were diluted to an absorbance at 560 nm of 0.100 in NB medium containing in the case of strain PB 920: 0 (●), 2 (○), 5 (▲), and 10 (△) μg of polymyxin B per ml, respectively, and in the case of PB 3412 strain: 0 (●), 5 (○), 10 (▲), and 20 (△) μg of polymyxin B per ml.*

clumping of the bacteria. Viable counts at 60 and 120 min show a 6 and 2% survival, respectively. The same dose of 10 μg/ml is barely effective on the resistant derivative PB 3412. Spore preparations from both strains were germinated in the presence of an analogous dose range of the antibiotic giving, in this instance, an indistinguishable result; both show a sensitivity similar to that of vegetative cultures of the parent PB 920 (Fig. 2).

Thus the germinating spores of the "resistant" strain are just as susceptible as the ones of the "susceptible" strain, although they show again their characteristic resistance once they are outgrown, washed, and reinoculated in fresh medium. Such a lack of resistance during germination suggests two possibilities: the mutant structure that confers resistance to the antibiotic appears only at a later stage of the differentiative process of spore outgrowth, or germinating spores secrete into the medium "factors" able to act synergistically with polymyxin B.

To distinguish between the two mentioned hypotheses we tested the suceptibility of an exponential culture of the resistant mutant PB 3412 after inoculum into nutrient broth where spores had previously been germinated (FG medium; see above). In FG media, irrespective of which spores germinated in it, PB 3412 becomes fully susceptible to 10 μg of polymyxin B per ml (Fig. 3A). The growth of PB 3412 in FG media challenged with a dose range of antibiotic (see Fig. 3B) shows that the resistant cells are susceptible to doses as low as 1 μg of polymyxin B per ml, i.e., much more susceptible than the "susceptible" parental strain PB 920 in NB media (see Fig. 1).

Thus, *B. subtilis* germinating spores release in the medium factors that render both spores and vegetative cells much more susceptible to polymyxin B, and such factors are able to overcome the resistance acquired by mutation in strain PB 3412.

The synergistic factor is probably a cell-wall lytic enzyme. Strange and Dark (6) described a lytic activity (very similar to lysozyme) released by germinating spores, suggesting for it a physiological function in the dissolution of spore envelopes before spore outgrowth.

The synergistic factor we have indicated above might correspond to the "Strange and Dark enzyme;" in fact it has some properties of a high-molecular-weight protein, since it can not be dialyzed, and it is heat labile.

Assuming that the synergistic factor could be a lysozyme-like activity we have performed a "reconstruction experiment" with purified lysozyme. Vegetative cells of PB 3412 were grown in medium containing 0.1 μg of lysozyme per ml, a dose that does not affect growth in the presence of several doses of polymyxin B; 2 μg of polymyxin B per ml (see Fig. 4) in this medium is sufficient to cause immediate lysis of the bacterial culture.

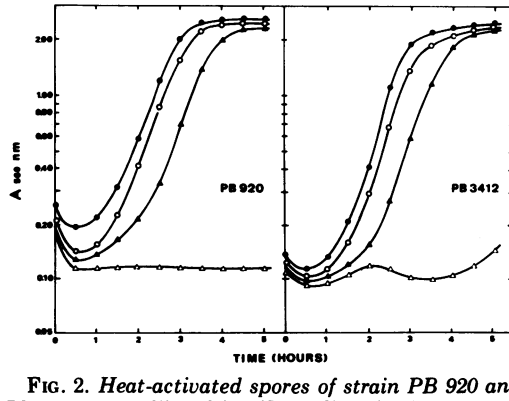

FIG. 2. *Heat-activated spores of strain PB 920 and PB 3412 were diluted in NB medium in the presence of 0 (●), 2 (○), 5 (▲), and 10 (△) μg of polymyxin B per ml, respectively.*

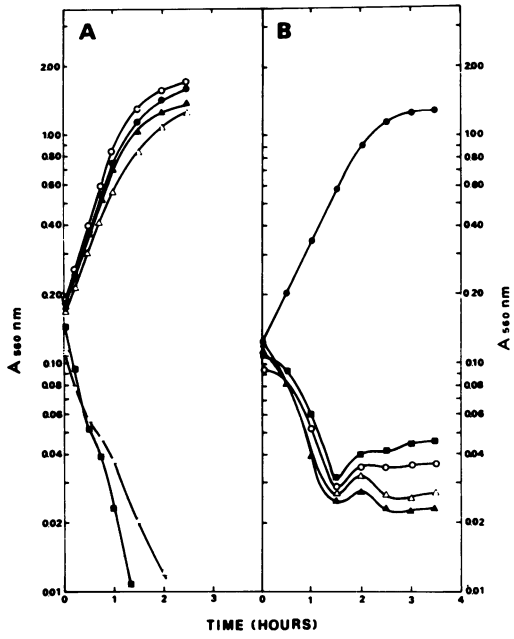

FIG. 3. *(A) Cells of strain PB 3412 in the logarithmic phase of growth were washed by centrifugation and resuspended at an absorbance at 560 nm of about 0.150 in different media: NB (●), FG 3412 (○), FG 920 (▲), NB containing 10 μg of polymyxin per ml (△), FG 3412 containing 10 μg of polymyxin per ml (■), and FG 920 containing 10 μg of polymyxin per ml (□). (B) Growth of vegetative cells of strain PB 3412 in FG 3412 medium in the presence of increasing doses of polymyxin B. The doses tested were: 0 (●), 1 (■), 2 (○), 5 (△), and 10 (▲) μg/ml.*

The same synergistic effect is demonstrable on polymyxin-susceptible wild-type strains of *B. subtilis*, and it is clearly visible also on a plate assay where sublethal doses of polymyxin B are included in the agar, and lysozyme on disks is put on the surface of a bacterial lawn.

DISCUSSION

Doses of 10 μg of polymyxin B per ml completely inhibit growth of *B. subtilis* strains both during the vegetative phase and spore germination.

A mutant strain, whose vegetative cells are resistant to 20-μg/ml doses of polymyxin B, produces spores that, during germination, are just as susceptible to polymyxin B as wild-type spores. The lack of resistance displayed by the resistant mutant during germination is not due to peculiar properties of its spores, but rather it is an effect of the germination process itself during which a compound that acts synergistically with polymyxin B is released into the medium.

The existence and activity of such a compound is demonstrated by the fact that even vegetative cells of the resistant strain become very susceptible to polymyxin if grown, instead of in fresh medium, in media where *B. subtilis* spores have germinated. The synergistic compound is not dialyzable and is heat labile, which would indicate its macromolecular nature.

Strange and Dark (6) demonstrated a cell wall lytic enzyme associated with spores of *Bacillus* species that is released into the medium during spore germination. We suggest that an analogous cell wall lytic activity might be the synergistic compound we have described. To support this hypothesis we have performed reconstruction experiments evaluating the poly-

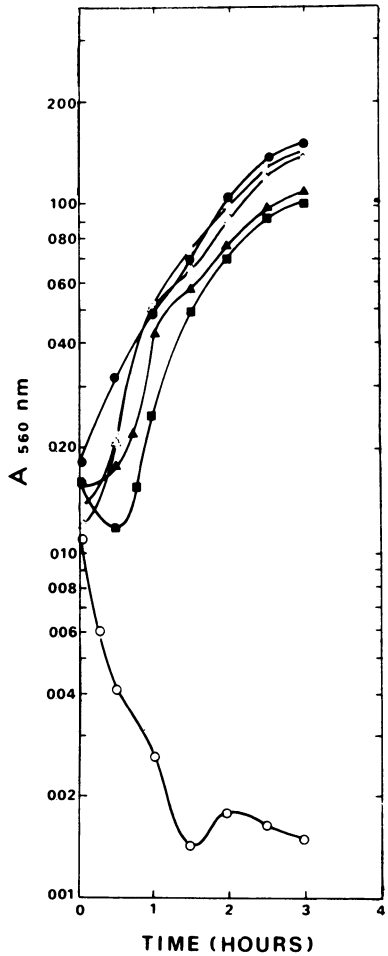

FIG. 4. *Synergic effect between lysozyme and polymyxin B. Cells of strain PB 3412 were resuspended in NB medium containing 0.1 μg of lysozyme per ml and increasing concentrations of polymyxin: 0 (●), 0.05 (□), 0.1 (△), 0.5 (▲), 1 (■), and 2 (○) μg/ml.*

myxin B susceptibility of resistant vegetative cells grown in the presence of lysozyme concentrations that do not, by themselves, affect bacterial growth, and have found that lysozyme sensitizes vegetative cells to the action of very minute doses of polymixin B.

We conclude that the *plx* phenotype of the resistant mutant might be due to a cell wall alteration that prevents polymyxin B from reaching its site of action, the cytoplasmic membrane. A normal (or exaggerated) cell wall permeability can be restored by the action of cell wall lytic enzymes.

The synergy between lysozyme and polymyxin B observed in *B. subtilis* must recognize a mechanism opposite to the one described in *E. coli* and *N. catarrhalis* (8). In the latter cases, polymyxin B permitted lysozyme to gain access to its substrate through the damaged outer membrane; in our case lysozyme damages the peptidoglycan and allows polymyxin to reach the cytoplasmic membrane.

A possible applicative use of our studies is a biological test to assay the activity of cell wall lytic enzymes in minute amounts on vegetative cells growing in presence of noninhibitory concentrations of polymyxin B.

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