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Morphological Expressions of Antibiotic Synergism Against *Pseudomonas aeruginosa* as Observed by Scanning Electron Microscopy

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Antibiotic-induced changes in *Pseudomonas aeruginosa* were observed by means of a scanning electron microscope. Seven frequent and five less frequent morphological changes were noted. The frequent changes were: (i) elongation; (ii) chain formation; (iii) nub formation; (iv) spheroplasts; (v) surface holes or pits; (vi) super-elongation; and (vii) increased filamentation. The less frequent changes were: (i) rounded ends; (ii) streptococcal forms; (iii) stalked nubs; (iv) surface bulges; and (v) convoluted surfaces. A morphological equivalent of antibiotic synergism was found in which changes were noted due to synergistic combinations of antibiotics that were not observed when the antibiotics were used alone or when a nonsynergistic combination of antibiotics was used.

The purpose of this investigation was to determine whether there are morphological equivalents visible by scanning electron microscope (SEM) of the phenomena of antibiotic synergism against gram-negative bacilli. We studied morphological changes in *Pseudomonas aeruginosa* that appeared when cells were exposed to antibiotics used alone and in synergistic combinations.

MATERIALS AND METHODS

The strain of *P. aeruginosa* used was a Fisher serotype 3,7, originally isolated from a patient with severe burns. The susceptibility of the strain to antibiotics used singly and in combination was determined by the tube dilution method (12). Minimal inhibitory concentrations (MICs) were determined by observing the lowest dilution of antibiotic that prevented visible growth of an inoculum of 10^5 organisms incubated for 18 h at 37°C. When pairs of antibiotics were tested in combination, 0.5 ml of each concentration of antibiotic in saline (total volume, 1 ml) was added to each tube, and 1 ml of Trypticase soy broth containing 10^5 organisms per ml was added to the antibiotic mixture. When three antibiotics were tested, 1.5 ml of the bacterial suspension in Trypticase soy broth was added to the 1.5-ml total volume of mixed antibiotic solution. The MICs were determined in the same manner as when single antibiotics were used. The Barenbaum criteria were used to determine synergism (2).

The antibiotic concentrations in the specimens observed in the SEM were one dilution lower than the MICs as determined by tube dilution susceptibility test, i.e., if the MIC was 12 μg/ml, the concentration of antibiotic in the specimen prepared for observation in the SEM was 6 μg/ml.

In control observations, chloramphenicol and tetracycline were used at 200 and 25 μg/ml, respectively, both singly and in combination.

Subcultures on thioglycolate medium were made from the tubes examined in the SEM to be sure no contaminating organisms were present that would give spurious morphological findings. For this purpose, 0.1 ml of the antibiotic-containing specimens was added to 10 ml of thioglycolate medium and observed for growth at 37°C after 48 h.

The SEM preparations were made as follows. At 18 h, the time at which the results of the tube dilution susceptibility tests were read, the bacteria in the tubes were fixed by the addition of 2 ml of 2.6% glutaraldehyde in phosphate buffer (pH 7.3) to each tube. The fixed bacteria were allowed to settle for 24 h at 4°C. Supernatants were decanted, and the undisturbed pellet was suspended in 2 to 4 ml of deionized water that had been filtered through a 0.20 μm membrane filter (Nalge Co., Rochester, N.Y.). A 0.4-ml sample of this suspension was transferred to a Gelman 0.45-μm TCM cellulose nitrate filter (Gelman Instrument Co., Ann Arbor, Mich.) in a funnel-type 25-m holder (Millipore Corp., Bedford, Mass.). The bacteria were washed with 125 ml of filtered deionized water, dehydrated in a graded ethanol series (50, 70, 85, 95, 100, 100, and 100%), and critical-point dried in a Samdri PVT-3 (Tousimis Research Corp., Rockville, Md.) (1). The filters were coated with gold-palladium in a high-vacuum evaporator while resting on an omnidirectional orbital rotator (Denton Vacuum, Cherry Hill, N.J.).

The specimens were examined in a JSM-35C SEM (JEOL USA, Medford, Mass.). The microscopic fields to be photographed were selected by random scanning, and at least 25 fields were photographed (Polaroid type 55 P/N film) for each concentration of antibiotic or antibiotics.

Morphological characteristics seen in the photographs were classified and quantitated without the observer's knowing the antibiotics to which the bacteria had been exposed. Morphological changes in the bacteria were designated as "frequent" if they were seen in more than 10% of the bacteria and as "less frequent" if they were seen in 2 to 10% of the bacteria. The entire experiment was performed using prepara-

tions fixed and prepared for the electron microscope on two or more separate occasions, and the findings were consistent.

RESULTS

The MICs of the various antibiotics were: amikacin, 6.25 μg/ml; carbenicillin, 200 μg/ml, polymyxin B, 0.78 μg/ml; tetracycline, 50 μg/ml; and chloramphenicol, 1,000 μg/ml. The MICs of the synergistic antibiotics were: amikacin-carbenicillin, 0.78–25 μg/ml; amikacin-polymyxin B, 1.56–0.19 μg/ml; carbenicillin-polymyxin B, 50–0.19 μg/ml; amikacin-carbenicillin-polymyxin B, 1.56–50–0.19 μg/ml. The MICs of the nonsynergistic combination of chloramphenicol and tetracycline were 1,000 and 50 μg/ml, respectively.

The concentrations of antibiotics in the specimens selected for observation were: amikacin, 3.12 μg/ml; carbenicillin, 50 μg/ml; polymyxin B, 0.39 μg/ml; tetracycline, 25 μg/ml; chloramphenicol, 200 μg/ml; amikacin-carbenicillin, 0.39–12.5 μg/ml; amikacin-polymyxin B, 0.78–0.09 μg/ml; carbenicillin-polymyxin B, 25–0.09 μg/ml; amikacin-carbenicillin-polymyxin B, 0.78–25–0.9 μg/ml; tetracycline-chloramphenicol, 25–200 μg/ml.

A control culture typical of untreated *P. aeruginosa* is shown in Fig. 1. Seven frequent morphological changes were seen in cells exposed to the concentrations of antibiotics indicated above: (i) increased filamentation, in which the number of filaments between bacterial cells increased to three or more times normal (Fig. 2); (ii) small, roughly circular holes or pits in cell surfaces (Fig. 3): (iii) nub formation, consisting of small, roughly spherical surface outpouchings (Fig. 4); (iv) super-elongation, in which the bacteria were from 60 to 100 times normal length (Fig. 5); (v) elongation, in which the bacteria grew to from 3 to 10 times normal length (Fig. 6 and Table 1); (vi) spheroplasts, or polar or central spherical surface structures of between 0.1 and 0.5 μm (Fig. 7); and chain formation, in which there were three or more incompletely separated bacteria (Fig. 8).

Five less frequent morphological changes were: (i) rounded ends of the cells (Fig. 9); (ii) stalked nubs of 0.1 to 0.3 μm (Fig. 10); (iii) surface bulges, which consisted of semi-spherical projections distributed over the length of the cell (Fig. 11); (iv) streptococcal chains of rounded bacteria (Fig. 12); (v) convoluted surfaces, consisting of depressions and troughs running along the length of the cell (Fig. 13).

No abnormal forms that resembled any of the frequent or less frequent changes were observed in the thousands of bacteria inspected that were grown in media with no antibiotics added.

The morphological changes seen in the nonsynergistic antibiotics and combinations consisted of the appearance of occasional coccal forms, some chain formation, some increased filamentation, and some elongation in the preparations containing tetracycline. This is not surprising since some tetracyclines have been shown to have moderate activity against *P. aeruginosa* (4). No unique morphological changes were seen in the nonsynergistic combination of chloramphenicol and tetracycline.

There were four changes in the morphology of organisms subjected to the synergistic combinations of antibiotics. Surface holes and pits were noted only when amikacin was combined with carbenicillin or polymyxin B, and super-elongation was only noted in the combination of carbenicillin and polymyxin B. Among the less frequent changes occurring only in synergistic combinations of antibiotics were rounded ends, which appeared only in the polymyxin B-amikacin-carbenicillin combination, and surface bulges, which were seen only in the amikacin-carbenicillin combinations. Of interest was the fact that the synergistic combinations also seemed to inhibit certain morphological changes normally seen with the individual antibiotics. These changes included chain formation due to polymyxin B or carbenicillin, nub formation due to amikacin, streptococcal configurations seen with polymyxin B or amikacin, and the stalked nubs seen with amikacin.

DISCUSSION

Although it is beyond the scope of this presentation to review the extensive literature on the morphological changes caused by antibiotics in bacteria as seen by SEM, a search of this literature revealed that elongation, spheroplast formations, and nub formations have been observed by others, whereas surface holes, super-elongation, increased filamentations, and less frequent changes, as reported here, have not been previously reported. The reason for this is probably that the vast majority of previous observations by SEM were made after only 2 to 4 h of exposure at inhibitory concentrations of antibiotics, whereas ours were made after 18 h of exposure to subinhibitory concentrations (5, 6).

It would not be sound to try to relate our findings in the SEM to transmission electron microscopy changes reported in the literature, since these comparisons will better be made on the specimens studied in parallel. However, transmission electron microscopy findings should be mentioned in the frame of reference that many of these may turn out to be equivalents of our SEM observations when parallel

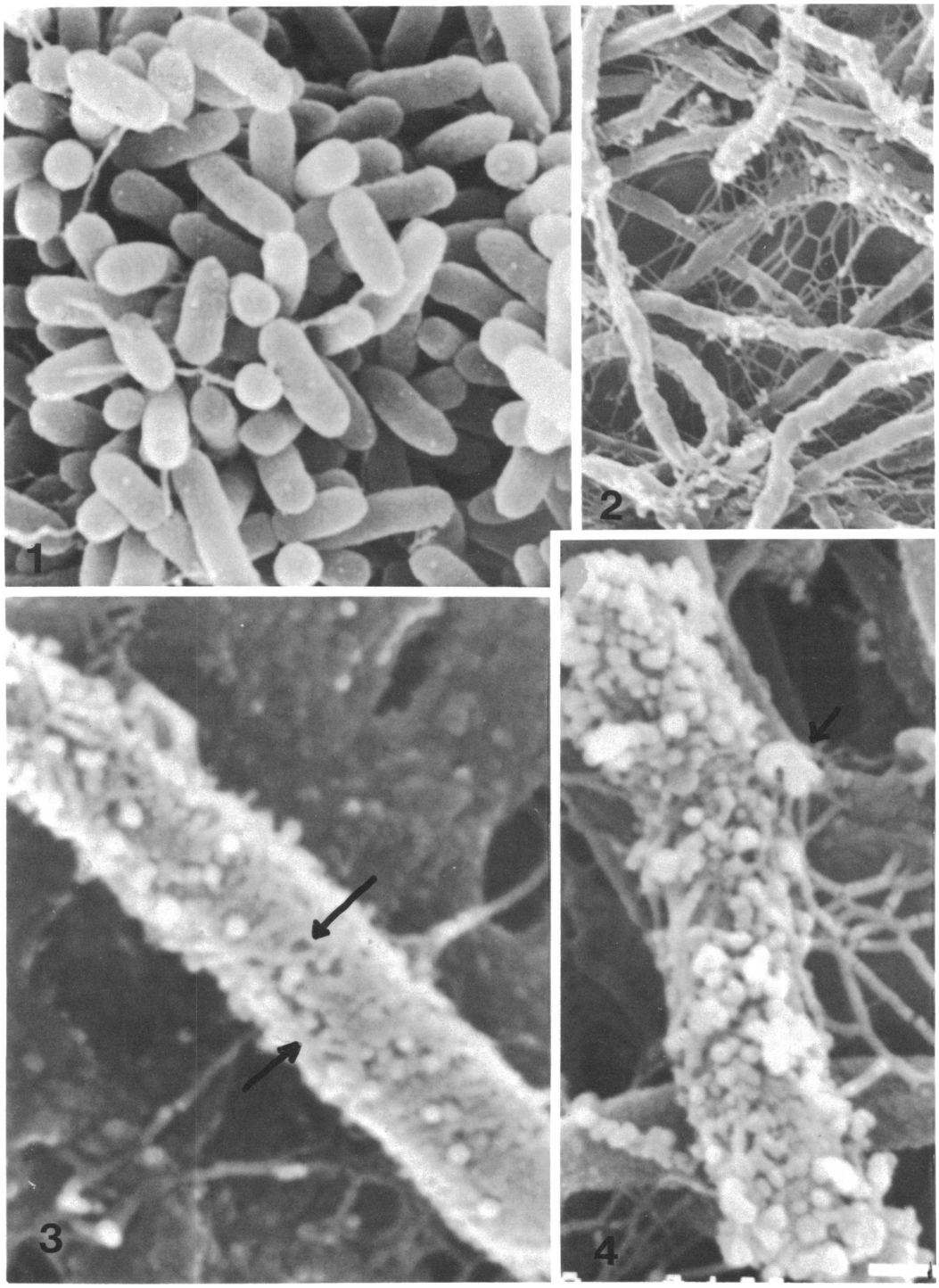
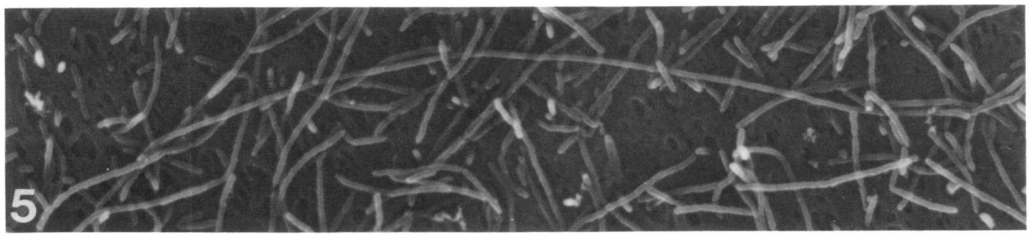


FIG. 1. *Control culture typical of the untreated Pseudomonas (×14,000).*
FIG. 2. *Increased filamentation in the presence of amikacin (×8,000).*
FIG. 3. *Hole formation (arrows) in the presence of amikacin and carbenicillin (×42,000).*
FIG. 4. *Nub formation (arrow) in the presence of amikacin (×27,000).*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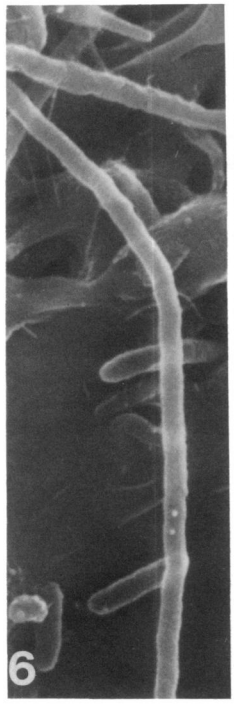
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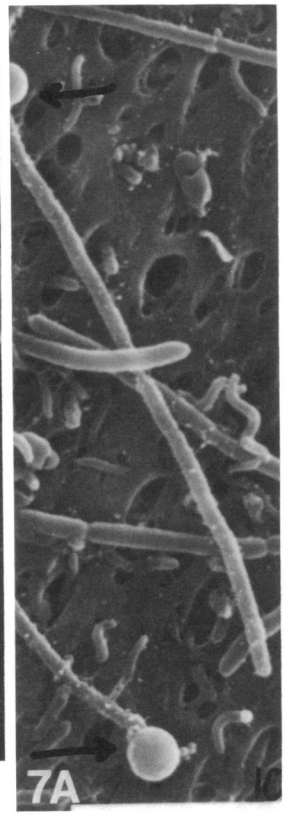
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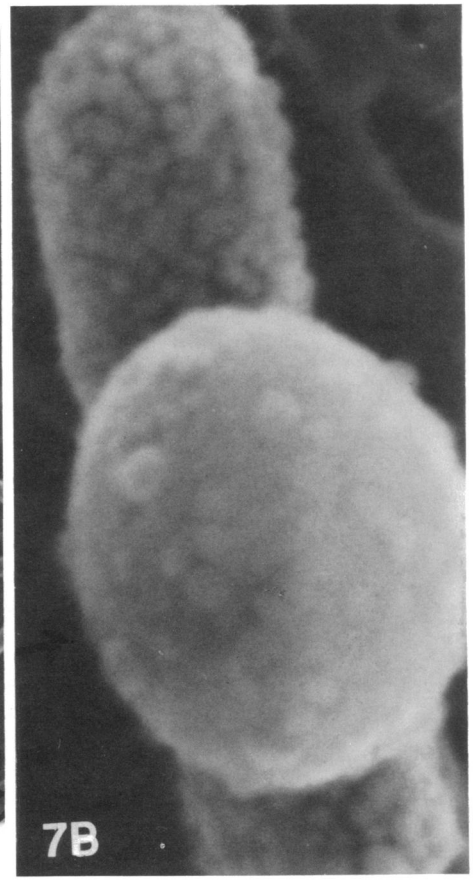
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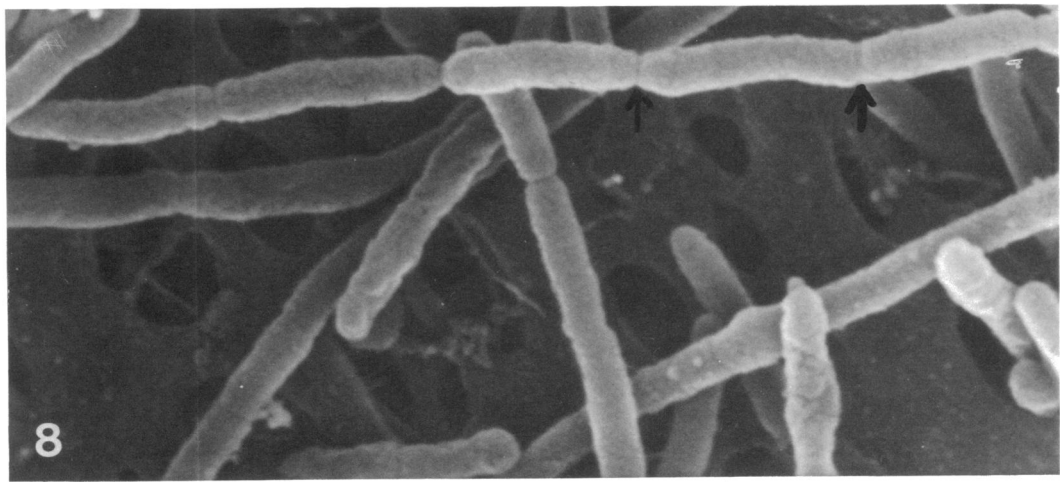
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TABLE 1. *Patterns of morphological changes visible by SEM P. aeruginosa induced by single and combined antibiotics*

Changes	Change induced by drug[a]:									
	PB	AK	CB	TC	CL	CB-PB	AK-CB	PB-AK	PB-AK-CB	TC-CL
Frequent										
Elongation	+		+	+		+	+	+	+	+
Chain formation	+[b]		+	+		+				
Nub formation		+[b]								
Spheroplasts			+[b]	+			+		+[c]	
Surface holes and pits							+[d]	+[d]		
Super-elongation						+[d]				
Increased filamentation	+	+	+				+	+	+	
Less frequent										
Round ends									+[d]	
Streptococcal configurations	+[b]	+[b]		+						+
Stalked nubs		+[b]		+						
Surface bulges							+[d]		+[d]	
Convoluted surfaces		+				+			+	

[a] Abbreviations: PB, polymyxin B; AK, amikacin; CB, carbenicillin; TC, oxytetracycline; CL, chloramphenicol.
[b] Morphological changes that disappeared in synergistic combinations.
[c] Spheroplasts appeared at cell poles in this combination only.
[d] Morphological changes that appeared in synergistic combinations.

studies are done. Koike et al. studied the mode of action of polymyxin B with *Pseudomonas* and *Escherichia coli* and found that projections erupted from the cell wall and membrane after 30 min of exposure to polymyxin B and that this was associated with inhibition of normal septum (8). Lopes and Inniss demonstrated, using transmission electron microscopy, the disruption of lipopolysaccharide structure in the presence of polymyxin B (9). Koike and Iida showed the effect of lipopolysaccharide on polymyxin B by demonstrating that it caused disruption of lipopolysaccharide receptors for bacterophage (7). Traub et al. showed membrane projections and blebs in *Serratia* exposed to polymyxin B (11). Lorian and Atkinson found many elongations, surface distortions, and coccus-to-rod and rod-to-coccus transformations to be caused by penicillins and aminoglycosides (10). Many of these shape distortions were found to be directly related to a substantial decrease in the number of ribosomes in the affected cells (10). Burdett and Murray described the formation of surface bulges and holes as a result of antibiotic exposure of staphylococci and *E. coli*, citing these changes as evidence of incomplete septum formation (3).

That some of these transmission electron microscopy findings will be altered in synergistic combinations is a hypothesis that will be relatively easy to test; experiments in this mode may help explain our findings further.

Of particular interest is that overall there were fewer SEM changes in the morphology of bacteria exposed to combinations of antibiotics than in those exposed to antibiotics alone. This supports a concept that some of the changes observed were in the nature of protective mechanisms of which the organisms were deprived when exposed to synergistic combinations of antibiotics.

These initial morphological demonstrations should stimulate others besides ourselves to attempt to add further morphological equivalents to the actions of antibiotics used alone and in combination.

FIG. 5. *Super-elongation in the presence of carbenicillin and polymyxin B (×1,800).*
FIG. 6. *Elongation in the presence of carbenicillin (×6,500).*
FIG. 7. *(A) Polar spheroplasts (arrows) in the combination of polymyxin B and amikacin (×5,000). (B) Central spheroplast (arrow) in the presence of carbenicillin (×63,000).*
FIG. 8. *Chain formation with incomplete separation (arrows) in the presence of polymyxin B (×14,000).*

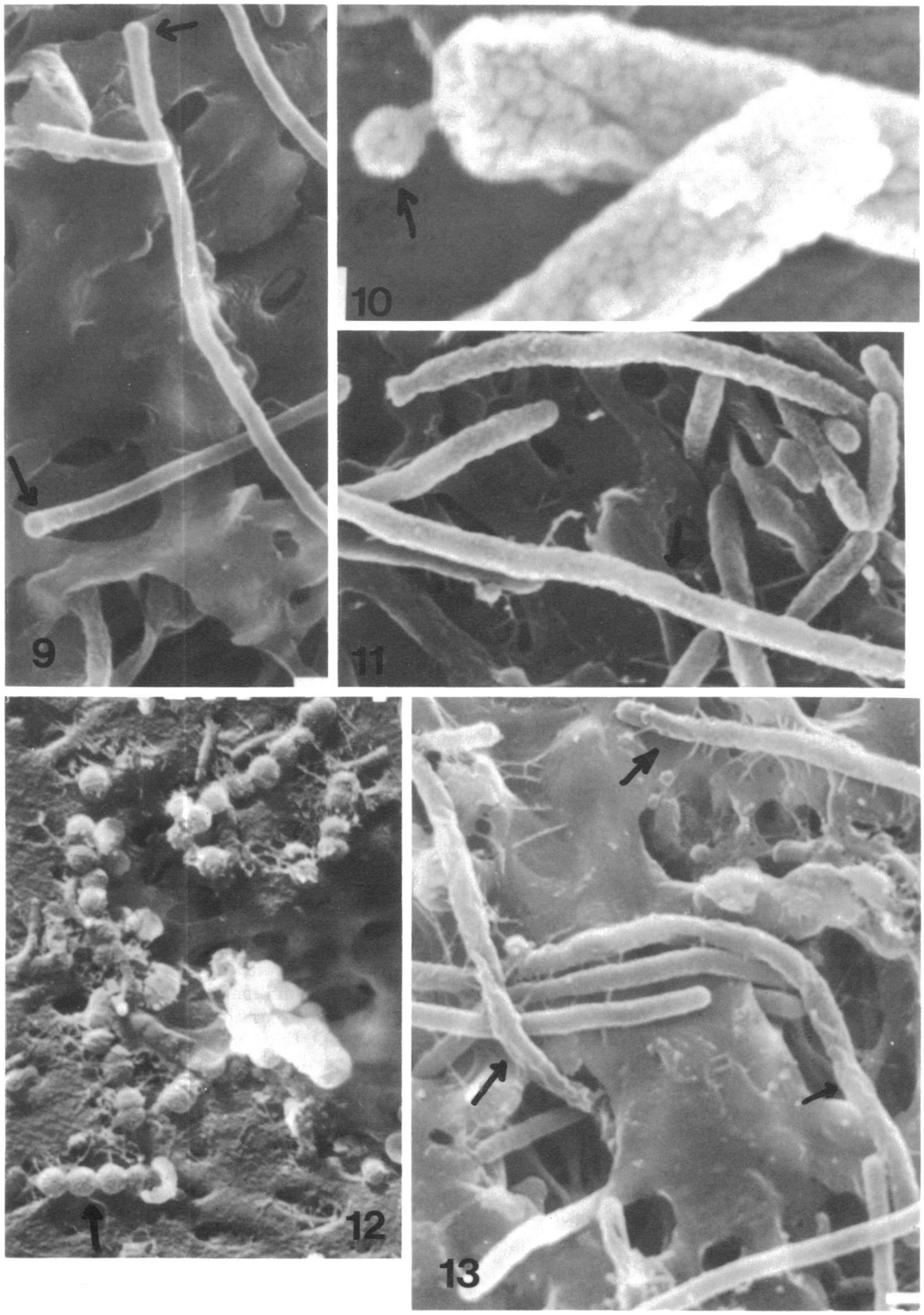

FIG. 9. *Rounded ends (arrows) in the triple combination (×7,000).*
FIG. 10. *Stalked nubs (arrow) in the presence of amikacin (×44,000).*
FIG. 11. *Surface bulges (arrow) in the triple combination (×10,000).*
FIG. 12. *Streptococcal form (arrow) in the presence of polymyxin B (×8,000).*
FIG. 13. *Convoluted surface (arrows) in the presence of carbenicillin (×7,000).*

LITERATURE CITED

1. **Anderson, T.** 1950. Techniques for the preservation of the three dimensional structure in preparing specimens for the electron microscope. Trans. N.Y. Acad. Sci. **13:** 130–145.
2. **Berenbaum, M. C.** 1978. A method for testing synergy with any number of agents. J. Infect. Dis. **137:**122–130.
3. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Septum formation in *Escherichia coli*: characterization of septal structure and effects of antibiotics on cell division. J. Bacteriol. **119:**303–324.
4. **Erwin, C., B. Waisbren, and R. Kruse.** 1953. Clinical and laboratory studies of infections due to *Pseudomonas aeruginosa* and *Pseudomonas* species. Am. J. Med. Sci. **225:**525–532.
5. **Greenwood, D., and F. O'Grady.** 1969. Antibiotic-induced surface changes in microorganisms demonstrated by scanning electron microscopy. Science **163:**1076–1078.
6. **Klainer, A., and I. Geis.** 1973. Agents of bacterial disease. Harper & Row, Hagerstown, Md.
7. **Koike, M., and K. Iida.** 1971. Effect of polymyxin B on bacteriophage receptors of the cell walls of gram-negative bacteria. J. Bacteriol. **108:**1402–1411.
8. **Koike, M., K. Iida, and T. Matsuo.** 1969. Electron microscopic studies on mode of action of polymyxin B. J. Bacteriol. **97:**448–452.
9. **Lopes, J., and W. E. Inniss.** 1969. Electron microscopy of polymyxin on *Escherichia coli* lipopolysaccharide. J. Bacteriol. **100:**1128–1130.
10. **Lorian, V., and B. Atkinson.** 1975. Abnormal forms of bacteria produced by antibiotics. Am. J. Clin. Pathol. **64:**678–688.
11. **Traub, W., G. Acker, and I. Klebben.** 1976. Ultrastructural surface alterations of *Serratia marcescens* after exposure to polymyxin B and/or fresh human serum. Chemotherapy **33:**104–113.
12. **Waisbren, B., C. Carr, and J. Dannette.** 1951. The tube dilution method of determining bacterial sensitivity to antibiotics. Am. J. Clin. Pathol. **21:**884–891.