ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1976, p. 334-342

Vol. 9, No. 2
Printed in U.S.A.

Evaluation of Antibiotic Efficacy Using Electron Microscopy: Morphological Effects of Guanylureido Cephalosporin, Chlorobenzoylureido Cephalosporin, BL-P1654, and Carbenicillin on *Pseudomonas aeruginosa*

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Received for publication 22 July 1975

The response of *Pseudomonas aeruginosa* to carbenicillin, BL-P1654, and two cephalosporins (112384 and 112883) was evaluated by minimal inhibitory concentration determinations, [^{14}C]leucine uptake studies, morphological studies of colonial growth, and mouse intraperitoneal inoculations. Spheroplast formation and bacterial lysis were not the early response; instead, cell division was inhibited and long filaments were formed. Spheroplast formation and bacterial lysis were not observed in the first 7 h of incubation in minimal inhibitory concentrations of antibiotic.

Susceptibility of bacteria to antibiotics is commonly determined by broth or agar dilution and by disk-diffusion methods. End points in these routine susceptibility tests are made on the basis of presence or absence determinations of macroscopic bacterial growth. At the cellular level, a complex series of events may occur to give the final result. In studies with *Pseudomonas pseudomallei,* carbenicillin at 400 μg/ml inhibited septation and fission but caused lysis of only a small proportion of the filaments (2, 4). *Pseudomonas aeruginosa* treated with either carbenicillin or ticarcillin produce filaments when exposed to 0.1 to 10 times the minimal inhibitory concentration (MIC) and spheroplasts at higher antibiotic levels (10, 13). Sublethal concentrations of benzylpenicillin in *P. aeruginosa* induced filament formation, higher beta-lactamase production, slower growth rate, and sensitivity to cholate (7, 8).

Large discrepancies between the MIC and minimal bacterial concentration were reported for BL-P1654 (15, 16). It was found that the killing curves of *P. aeruginosa* by carbenicillin, ticarcillin, and BL-P1654 were similar and characteristic of a penicillin, but there was a small population more resistant to BL-P1654. These cells were resistant to BL-P1654 over a 16- to 64-fold concentration above the MIC, but this pattern of resistance was not observed with carbenicillin or ticarcillin.

In this study we compare the morphology and growth of *P. aeruginosa* treated with carbenicillin, BL-P1654, and cephalosporin compounds 112883 and 112384.

MATERIALS AND METHODS

Antibiotics. Carbenicillin (Geopen) was obtained commercially, BL-P1654 was provided by Bristol Laboratories, and 112883 and 112384 were prepared at the Lilly Research Laboratories. Chemical structures of these antibiotics are shown in Fig. 1.

Bacteria. All leucine uptake and cellular morphology studies were performed on *P. aeruginosa* X48. This strain was a clinical isolate with a typical antibiotic susceptibility profile and would cause infections in mice. Log-phase cultures were attained by daily passage in broth at high dilutions.

Susceptibility tests. Broth dilution susceptibility studies were performed in M9 medium (NH_4Cl, 1 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 5 g; $MgSO_4$, 0.1 g; glucose, 10 g; water, 1 liter) to determine appropriate antibiotic concentrations to be studied in the leucine uptake and cellular morphology experiments. Twofold serial concentrations of antibiotics were incorporated in 5-ml volumes of M9. All tubes were inoculated with 0.1 ml of a 10^{-3} dilution of an 18-h M9 broth culture of *P. aeruginosa* X48, resulting in a final inoculum density of 10^4 to 10^5 colony-forming units/ml of test broth. End points were determined visually after overnight incubation at 37 C in a water bath.

Leucine uptake. Inocula for the leucine uptake studies were prepared by diluting the log-phase cultures in prewarmed M9 media to attain either 10^5 or 10^7 colony-forming units/ml of test medium. Tests were performed in a 37 C water bath and all media were warmed prior to inoculation. A 1-μCi amount of L-[U-^{14}C]leucine (Amersham/Searle) was added to 6 ml of culture broth. Cold L-leucine was added at 1.8×10^{-4} mmol/6 ml for the 10^7 inoculum and 3.6×10^{-5} mmol/6 ml for the 10^5 inoculum. Antibiotic was dissolved in M9 medium and added to the culture broth just before inoculation.

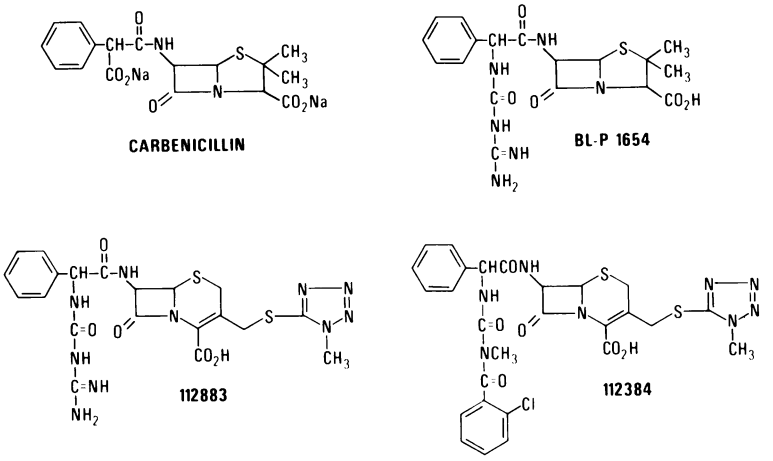

FIG. 1. *Chemical structures.*

Aliquots of 0.5 ml were removed at indicated times and filtered through 0.4-μm Nuclepore filters. Each filter was washed with 1 ml of M9 medium and shaken in a scintillation vial containing 10 ml of Aquafluor (New England Nuclear Corp.), with 0.5 ml of propylene glycol added to prevent freezing. The vials were counted in a Packard scintillation counter.

Morphology studies. Cells growing under the conditions described under leucine uptake were fixed with 2% glutaraldehyde overnight. Subsequently, the cells were filtered onto 0.4-μm Nuclepore filters, dehydrated in a graded alcohol series, and critical point dried with Freon 13 in a Bomar drier. The filter specimens were mounted on aluminum stubs, gold coated, and viewed with an ETEC Autoscan scanning electron microscope.

Mouse studies. A 15-h M9 broth culture of *P. aeruginosa* X48 was diluted 1:5 in M9 media containing 32 μg of 112384 per ml and then incubated at 37 C for 3.5 h. A control culture without antibiotic was also prepared. Four groups of 20 mice (15 g) were injected intraperitoneally with the following: group 1, 1 ml of treated culture and 1 ml of medium containing 250 μg of 112384; group 2, 1 ml of normal culture and 1 ml of medium containing 250 μg of 112384; group 3, 1 ml of treated culture and 1 ml of sterile medium; group 4, 1 ml of normal culture and 1 ml of sterile medium.

Mice were sacrificed at 10-min intervals starting 30 min postinfection, and approximately 0.2 ml of peritoneal fluid was removed. Each sample was diluted with 1 ml of 3% glutaraldehyde fixative, held at room temperature for 15 min, and filtered onto 0.4-μm Nuclepore filters after dehydration in a graded alcohol series. The samples were critical point dried and coated with gold for scanning electron microscopy.

Scanning electron microscopy of colonial growth. A 25-μl amount of a midlog culture, diluted 10^{-3} with warm M9 medium, was placed on 0.4-μm Nuclepore filters, previously laid on the surface of M9 agar (M9 broth solidified with 1% purified agar [Difco]). Antibiotic was incorporated into the agar medium at 4, 8, 16, 32, 64, 128, or 1,000 μg/ml, and control plates with no antibiotic were used. All plates were incubated at 37 C, and the lids were partially removed for the first hour to facilitate drying of the filter surface. At 5 to 7 h and 24 h postinoculation, filters were removed, placed in plastic filter holders, fixed with 3% glutaraldehyde, dehydrated in a graded alcohol series, critical point dried, and coated for scanning electron microscopy.

RESULTS

MIC determination. M9 broth dilution MICs for *P. aeruginosa* X48 were 32, 16, 16, and 16 μg/ml for carbenicillin, BL-P1654, 112384, and 112883, respectively. As the inoculum was increased, the MIC increased markedly, and above 10^6 cells/ml the culture was not inhibited by 1,000 μg of any of the compounds per ml. The level of 32 μg/ml was selected for further morphology and [^{14}C]leucine uptake experiments.

Broth cultures with inocula of either 10^5 or 10^7 cells/ml were treated with each of the antibiotics, and aliquots from the 10^5 tubes were removed for scanning-electron microscopy. Untreated cells are shown in Fig. 2A. The cells vary in length from 1.5 to 4 μm and a large population is undergoing fission, as shown by the deep septal regions. In contrast, nearly all of the treated cells stop dividing but continue to grow as filaments. Carbenicillin-treated cells normally were converted to shorter filaments (Fig. 2B) than were those treated with either BL-P1654, 112384, or 112883 (Fig. 2C, D, and E). Also, there were definite septal regions with the carbenicillin-treated cells. BL-P1654, 112883, and 112384 filaments continue to grow through at least 4 h post-treat-

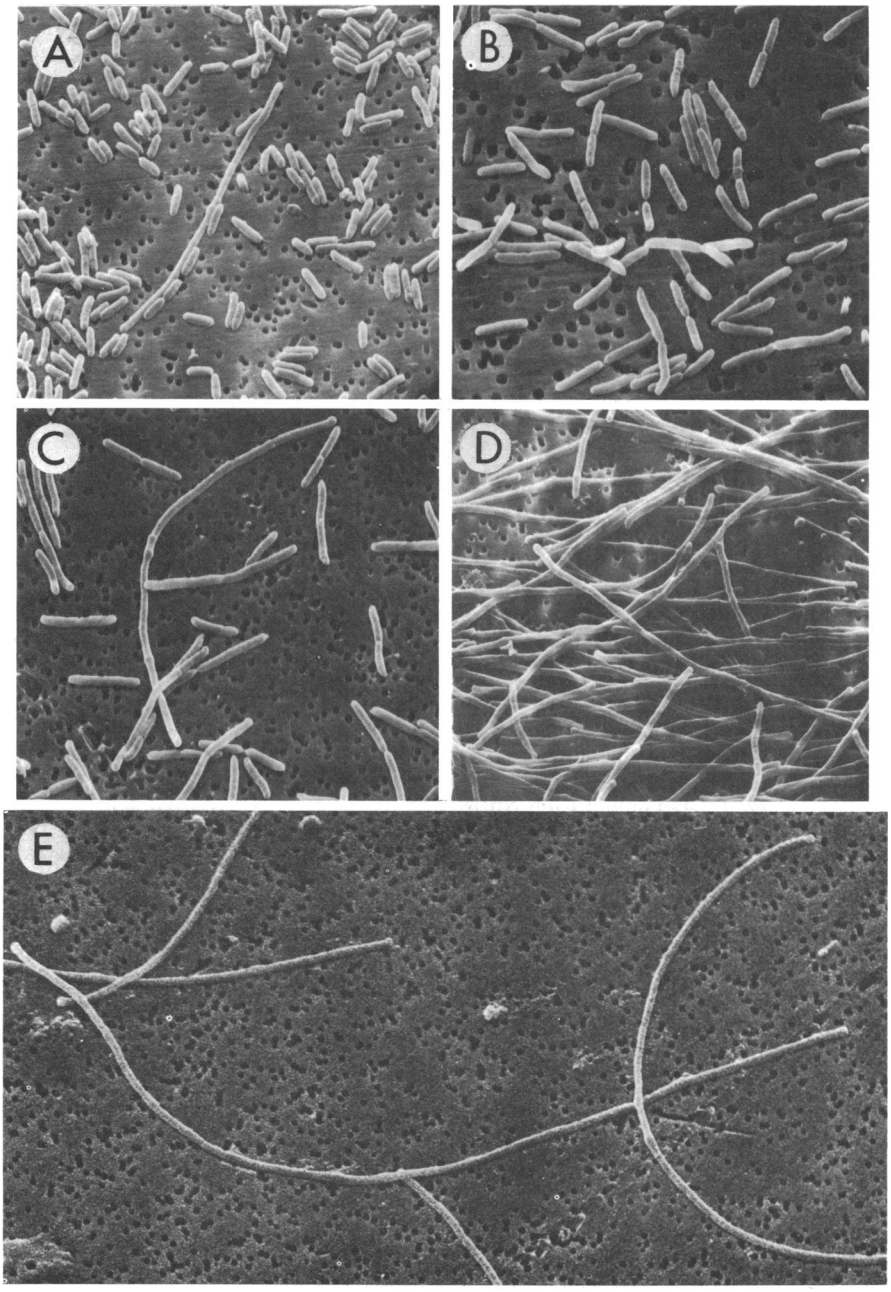

FIG. 2. *Scanning electron microscopy of P. aeruginosa at 10^5 cells/ml in broth culture on a 0.4-μm pore Nuclepore filter, except (B) is on a 0.8-μm pore filter. Antibiotic concentration was 32 μg/ml; incubation was for 240 min at 37 C. (A) Untreated control; (B) carbenicillin; (C) compound 112883; (D) compound 112384. (E) BL-P1654. ×2,500.*

ment, and the inoculum level did not affect the filamentous morphology. Measurements of filament lengths from 10^5 cells/ml cultures treated with 16 μg of 112384 per ml at various times indicate continued logarithmic growth through 6 h (Fig. 3). In contrast, stationary-phase cultures show a mixture of classic and filamentous cell forms after exposure to antibiotic. No bulbous regions were observed, although the cell wall was rough after 3 to 4 h of

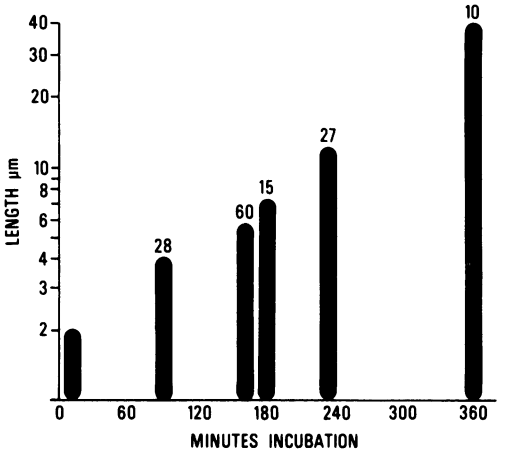

FIG. 3. *Filament length of P. aeruginosa at 10^5 cells/ml treated with compound 112384 at 16 µg/ml. Number of cells measured on scanning micrographs are above the bars. Normal cell length is indicated by cell at zero time.*

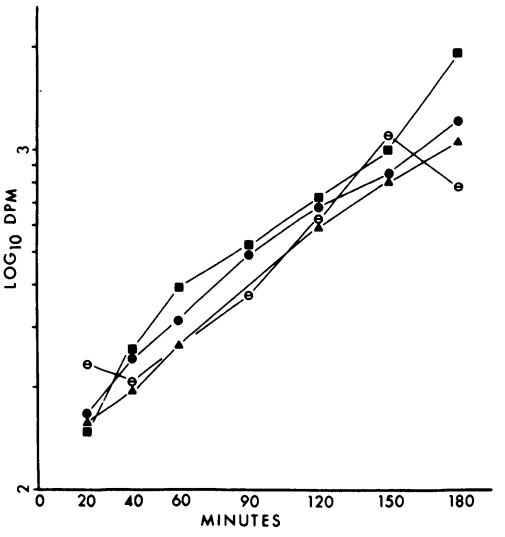

FIG. 4. *[^{14}C]leucine uptake by P. aeruginosa broth culture; 1 µCi of [^{14}C]leucine and 3.8 × 10^{-5} mmol of cold leucine per 6 ml, 10^5 colony-forming units/ml inoculum. Symbols: ■, 112883; ▲, BL-P1654; ●, carbenicillin; ⊖, control.*

exposure to these antibiotics at 32 µg/ml. Cellular debris or cell wall ghosts were generally not observed in any of the treated broth cultures.

Growth as measured by [^{14}C]leucine uptake shows an exponential increase over the 3-h period with either a 10^5 or 10^7 inoculum in the untreated control tubes. The slope of the line was dependent on the amount of cold leucine added to the broth. Whereas MIC determinations show that exposure of the 10^5 inoculum to 32 µg/ml of any of the four antibiotics does inhibit growth for 18 h, none of the antibiotics at this level inhibited leucine uptake during the first 3 h of treatment (Fig. 4). At the 10^7 inoculum level, conditions under which MIC determinations show no growth inhibition by 32 µg/ml, leucine uptake was also not inhibited. Other experiments show that none of these antibiotics at 32µg/ml inhibit leucine uptake at either inoculum level for up to 7 h.

Filamentous cells reverted to the classic form when the antibiotic was removed. A culture was incubated for 2 h in 16 µg of 112883/ml of broth, washed free of antibiotic-containing medium, and reincubated in fresh broth medium. Aliquots removed after 60 min of incubation contained a significant number of classic cells and shorter filaments than thsose observed in the presence of antibiotic. Only a few filaments remained after 120 min of incubation.

Filamentous bacterial cells were injected intraperitoneally into mice to determine if reversion occurs in an animal system. Reversion of the filaments to the classic form was observed after 60 to 90 min. When antibiotic 112883 or carbenicillin was also injected intraperitoneally at the time of infection with filamentous cells, the filaments did not revert by 120 min. Also, when mice were pretreated with either antibiotic, and then infected with untreated bacteria, the classic cells did not divide and were observed as two to four cell filaments by 120 min.

Morphological studies of colonial growth on agar plates containing antibiotic were conducted to observe single colony-forming units out of a large population. One can observe classic cells, filaments, lysed cells, and colonies of variable sizes on the Nuclepore filter. There was good correlation between morphological responses of the cells in broth culture and agar plates at comparable antibiotic levels. On antibiotic-free agar, the diluted cell cultures rapidly multiplied to form small colonies by 4 h (Fig. 5) and later became macroscopically visible. Cultures grown on agar with 16 µg of 112384 per ml became filamentous and tended to coil (Fig. 6). Colonial growth on plates with 8 µg/ml was macroscopically visible by 24 h of incubation, but rough surface filament comprised the colony (Fig. 7). At concentrations of 32, 64, or 128 µg/ml, the 5-h colony appears similar to those at the lower concentrations, but by 24 h the cells had lysed and only the debris was observed (Fig. 8). Carbenicillin treatment caused shorter filaments, generally of two to four cell lengths, after 5 h of incubation (Fig. 9). Cellular growth had continued,

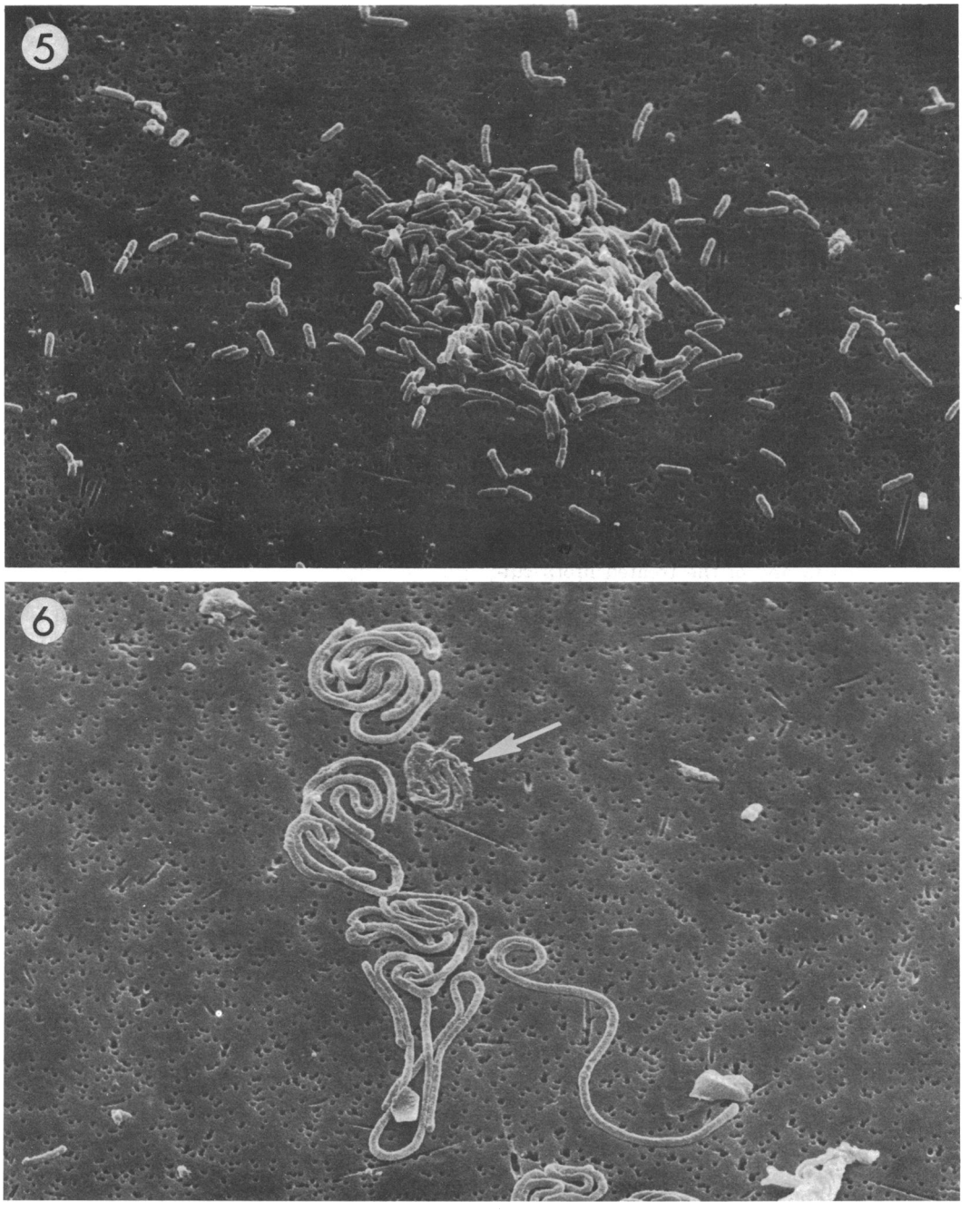

FIG. 5. *Five-hour colonial growth on Nuclepore filter placed on agar medium; diluted inoculum was used to give separated colonies. Note the scattering of the cells from the colony.*

FIG. 6. *Five-hour colonial growth with filter on ;ar medium with 32 μg of 112384 per ml. Lysed filament (arrow). ×2,500.*

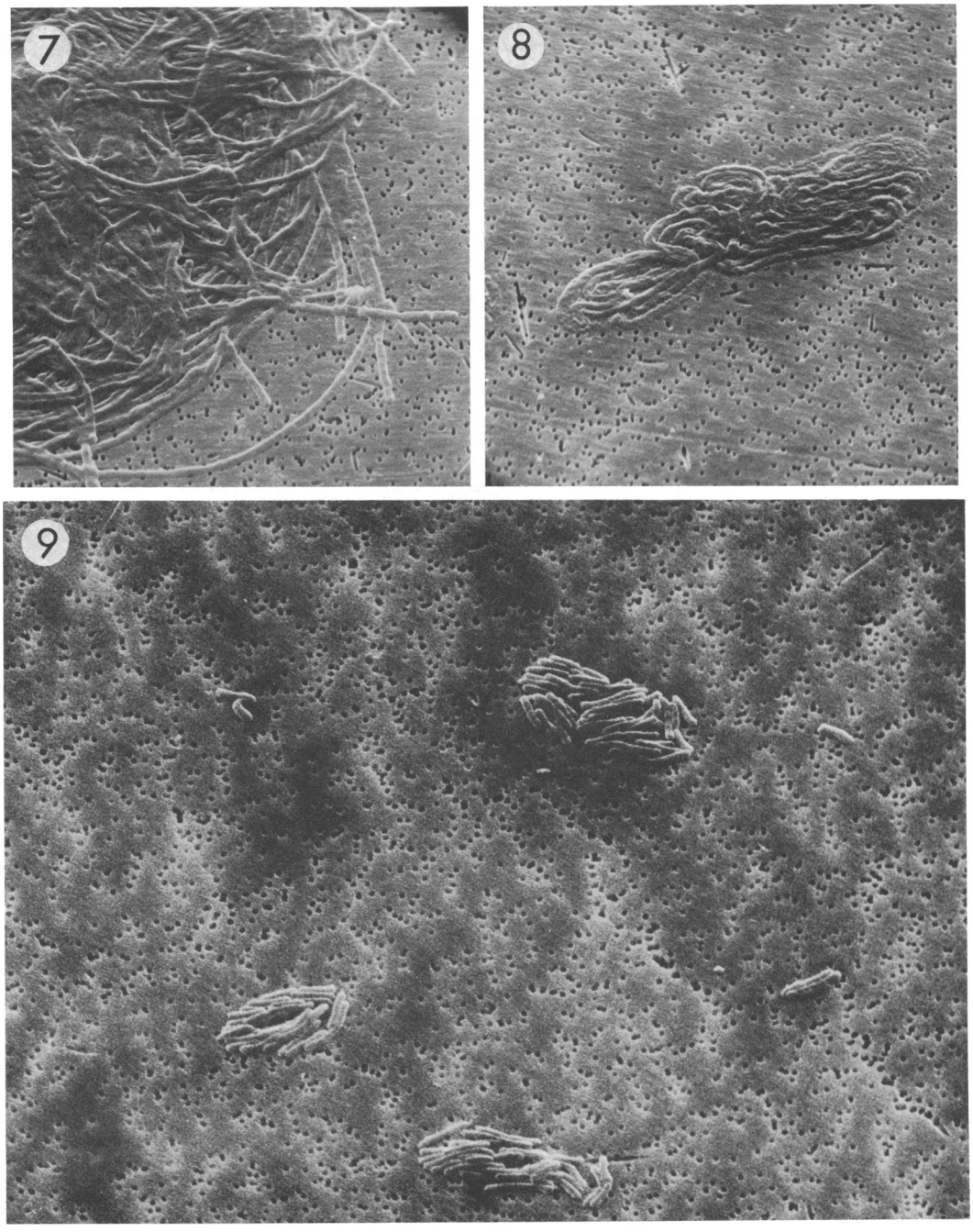

FIG. 7. *Colony edge on filter from plate with 8 μg of 112384 per ml; 24-h incubation.*
FIG. 8. *Colony on filter from plate with 32 μg of 112384 per ml; 24-h incubation.*
FIG. 9. *Colonies on filter from plate with 32 μg of carbenicillin per ml; 5-h incubation.* ×2,500.

and, by 24 h, macroscopically invisible clumps were formed on plates with 16, 32, or 128 μg of carbenicillin per ml (Fig. 10A, B, and C).

DISCUSSION

The effects of these four antibiotics on *P. aeruginosa* differ markedly from their effects on *Escherichia coli*, which we have taken to indicate effective activity against *E. coli* (3). Against *Pseudomonas*, growth is not inhibited up to 7 h, cell division is inhibited, filamentous forms are produced, and lysis occurs only with 112384. But agar dilution MIC values indicate susceptibility to all four antibiotics, and efficacy in therapy of experimental *P. aeruginosa* infections in mice generally parallel the in vitro

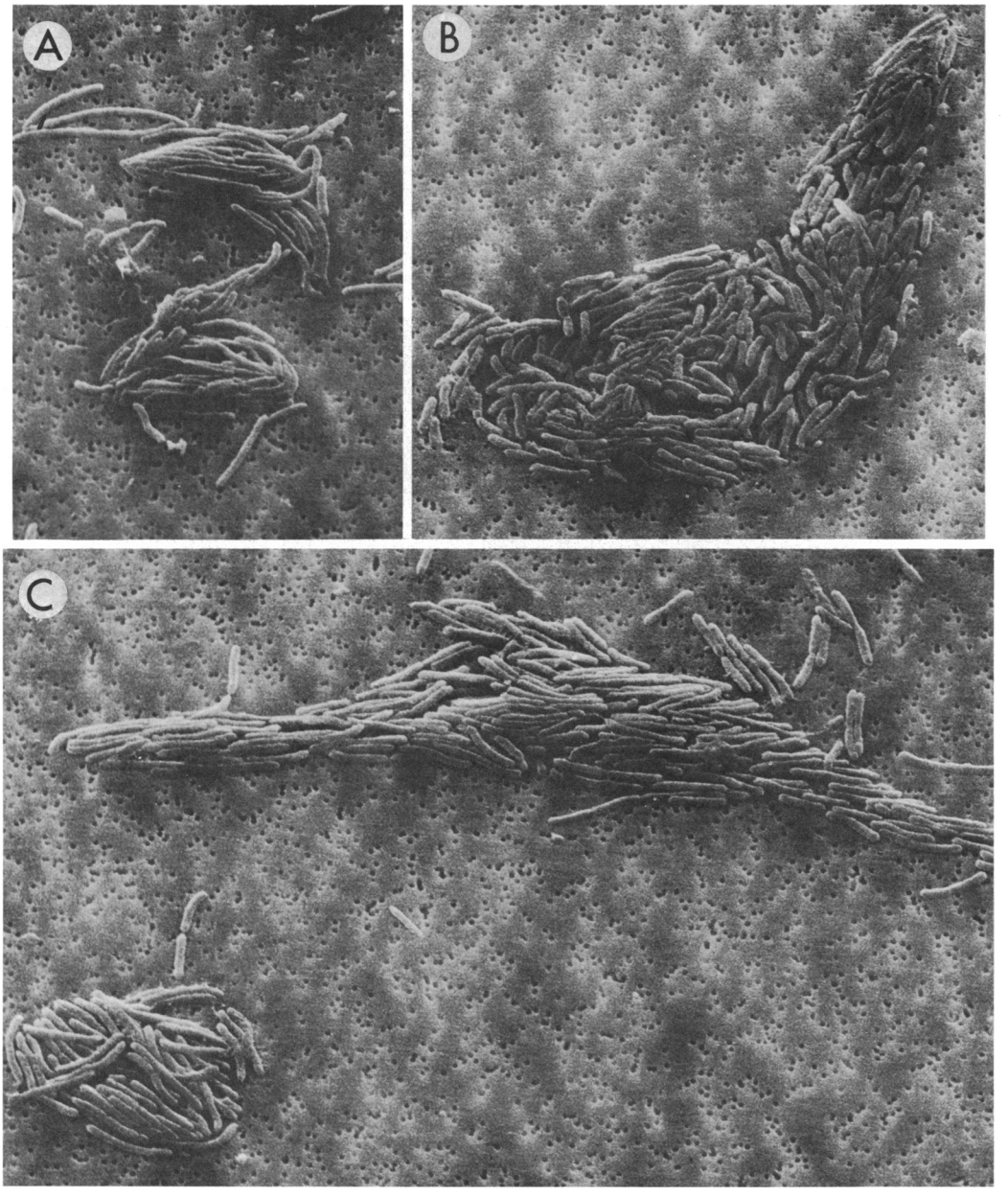

FIG. 10. *Colonies on filter from carbenicillin plate; 24-h incubation. (A) 16 μg/ml; (B) 32 μg/ml; (C) 128 μg/ml.*

susceptibilities to the four antibiotics (unpublished data). The subtle differences in the morphological effects produced by the four antibiotics may manifest themselves in subtly different degrees of efficacy, but more animal testing will be required to show these differences.

Discrepancies between MIC and minimal bactericidal concentration of beta-lactam antibiotics have been reported (15, 16). These studies of cell morphology, leucine uptake, and in vivo experiments add further data to support proof of this phenomenon. Comparison of the killing curves of carbenicillin and BL-P1654 did not show a major difference, except for the small resistant population that grows out. Serial passage in the presence of BL-P1654 attained a bacterial population less antibiotic susceptible than the parent strain (15). Price et al. (12) have hydrolyzed BL-P1654 with crude beta-lactamase preparations from *Pseudomonas*. Their studies lend indirect evidence that beta-lactamase induction occurs with *Pseudomonas*, which may be a possible mechanism for reduced antibiotic susceptibility.

Cell morphology of the treated broth cultures shows that carbenicillin forms shorter filaments than BL-P1654, 112883, or 112384. The enzymes involved in septation-fission can be inhibited without cessation of growth (1). Greenwood and O'Grady (5) have shown that cephalexin causes *E. coli* to form filaments over a wide range of antibiotic concentration. We demonstrate that these three compounds cause filaments as the first response to the antibiotics.

Filaments can revert to the classic form rapidly when the antibiotics are removed from the growth medium. Cultures exposed to 1 or 2× MIC levels for up to 4 h revert to the classic form in 60 to 90 min. We have not observed inhibition of cell wall synthesis, as demonstrated by spheroplast formation, nor have bulbous formations indicative of *E. coli*-like transpeptidase inhibition by penicillins been observed (11). The leucine uptake studies show no inhibition by any of these antibiotics in the first few hours. The filament-to-classic form reversion was demonstrated in either broth or agar plate cultures or in mouse infections.

Inhibition of macroscopic cellular growth was attained with low inocula in broth or on agar plates with each of these antibiotics. If high levels of inocula were used, then the MICs were very high. The long filaments become a tangled clump after 3 h, which makes the colony-forming unit a difficult unit to define. The filament continues to grow at a log rate for at least 7 h, but the plate assays show the colony size was very restricted by the antibiotics. Microcolonies that were not visible to the unaided eye were formed on the agar surface. Cellular growth continued for the 7 h, but then the inhibition and/or lysis occurred by 24 h. Inhibition seems to occur without spheroplast formation. Previous reports of bulbous formations and cell wall weakening were not observed (6, 9). After 24 h of incubation, cells grown on agar containing 112384 flattened, indicating empty cells.

Carbenicillin-treated cells generally did not lyse completely. Twenty-four-hour broth cultures contained both classic and filamentous forms, which supports Sanders' idea of a less susceptible population (15). In the rapidly growing culture nearly all the cells were converted to filaments in broth or plate observations. A low intracellular concentration of the antibiotic due to either the permeability barrier or beta-lactamase activity would explain the filamentous growth, if filamentous growth simply results from low antibiotic concentrations in the periplasmic region; however, it is also possible that the novel structures of these antibiotics simply produce a greater affinity for one target (involved in septation) than for another target (involved in elongation), and that antibiotic concentrations within the outer membrane are not particularly low.

ACKNOWLEDGMENTS

The technical assistance of the following persons is gratefully acknowledged: P. Craig, P. Ensminger, D. Holmes, Barbara Simmons, and June Wood. We thank Robin Cooper and Felicitas José of the Lilly Research Laboratories for samples of their compound, 112883.

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