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Tobramycin Resistance of *Pseudomonas aeruginosa* Cells Growing as a Biofilm on Urinary Catheter Material

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When disks of urinary catheter material were exposed to the flow of artificial urine containing cells of *Pseudomonas aeruginosa*, a thick adherent biofilm, composed of these bacteria and of their exopolysaccharide products, developed on the latex surface within 8 h. After this colonization, sterile artificial urine containing 1,000 μg of tobramycin per ml was flowed past this established biofilm, and a significant proportion of the bacterial cells within the biofilm were found to be still viable after 12 h of exposure to this very high concentration of aminoglycoside antibiotic. Planktonic (floating) cells taken from the test system just before the exposure of the biofilm to the antibiotic were completely killed by 50 μg of tobramycin per ml. The MIC of tobramycin for cells taken from the seeding cultures before colonization of the catheter material, and for surviving cells recovered directly from the tobramycin-treated biofilm, was found to be 0.4 μg/ml when dispersed cells were assayed by standard methods. These data indicate that growth within thick adherent biofilms confers a measure of tobramycin resistance on cells of *P. aeruginosa*.

Pseudomonas aeruginosa is the predominant aerobic inhabitant of natural aquatic systems, including the oceans, rivers, and streams. In these natural systems, this organisms, with other aerobic bacterial species, colonizes available surfaces and proliferates to form adherent biofilms (8, 12), within which the sessile (attached) cells grow in a hydrated matrix partially composed of their own uronic acid containing alginate exopolysaccharides (29). We devised direct recovery and microscopy methods to establish that sessile cells of these aquatic organisms constitute the numerically predominant bacterial population (10) in all natural aquatic systems examined to date (4). Direct examination of established biofilms by autoradiography (9) and by the heterotrophic potential technique (14) have indicated that >70% of the cells within these biofilms are metabolically active and that very active cells are found deep within the biofilm, adjacent to the originally colonized surface.

Because growth within these adherent biofilms appears to confer upon *P. aeruginosa* a measure of protection from environmental antibacterial factors (6) and because biofilm bacteria in industrial systems have been shown to resist 20- to 50-fold higher concentrations of biocides than their planktonic (floating) counterparts (26), we resolved to assess the degree of resistance of biofilm bacteria to antibiotics in a medically important system.

MATERIALS AND METHODS

Modified Robbins device. The observation that traditional sampling methods measure free-floating planktonic bacteria rather than the more important adherent sessile bacteria in industrial and environmental situations led to the development of the Robbins Biofilm Sampler (21). We modified the original Robbins device to develop an artificial multiport sampling catheter (Fig. 1). The modified Robbins device was constructed of an acrylic block, 41.5 cm long, with a lumen of 2 by 10 mm. Twenty-five evenly spaced sampling ports were devised so that catheter material (0.5 cm^2) attached to sampling plugs would lie flush with the inner surface, without disturbing flow characteristics. The sampling plugs could be removed and replaced aseptically.

This modified Robbins device was connected to a 2-liter reservoir held in a 37°C water bath. Medium containing bacteria or antibiotic was pumped from the reservoir (in vitro bladder) through the modified Robbins device (artificial catheter) by a peristaltic pump set to deliver 60 ml/h. The modified Robbins device exposed disks cut from a urinary catheter (Silko-latex Rusch gold balloon catheter/Canada Ltd.) to the bacteria- or antibiotic-containing urine medium, and these disks could be aseptically removed from the system for analysis. Before each experiment, the entire experimental apparatus (shown in Fig. 1) was sterilized with ethylene oxide.

Microbiology. The strain of *P. aeruginosa* used in these experiments was isolated from a patient with a catheter-associated urinary tract infection and identified by routine microbiological methods. The medium used was artificial urine adapted from that of Minuth et al. (22) supplemented with 0.4% nutrient broth (Difco Laboratories). The bacteria were stored on slants at −70°C and serially cultured at 8-h intervals to provide an inoculum for the reservoir. Based on standard growth curve data developed in batch cultures of this organism in this medium, the reservoir was inoculated with a 2% inoculum so that the reservoir delivered logarithmic-phase cells to the modified Robbins device throughout the 8-h colonization period. Bacterial growth within the reservoir was monitored by using standardized turbidity as a growth parameter with a Unicam SP1800 spectrophotometer at 600 nm. The MIC of tobramycin for the logarithmic-phase cells of this *P. aeruginosa* strain was determined by using a 2% inoculum in Mueller-Hinton broth (31).

Experimental. Artificial urine containing logarithmic-phase cells of *P. aeruginosa* was passed through the modified Robbins device for 8 h, and the development of bacterial biofilm was monitored by regular sampling of catheter material surfaces by scanning electron microscopy (SEM). At time 0, individual sample disks bearing sessile (adherent) bacteria were aseptically removed for SEM, epifluorescence microscopy, and viable counts. At the same time, a speci-

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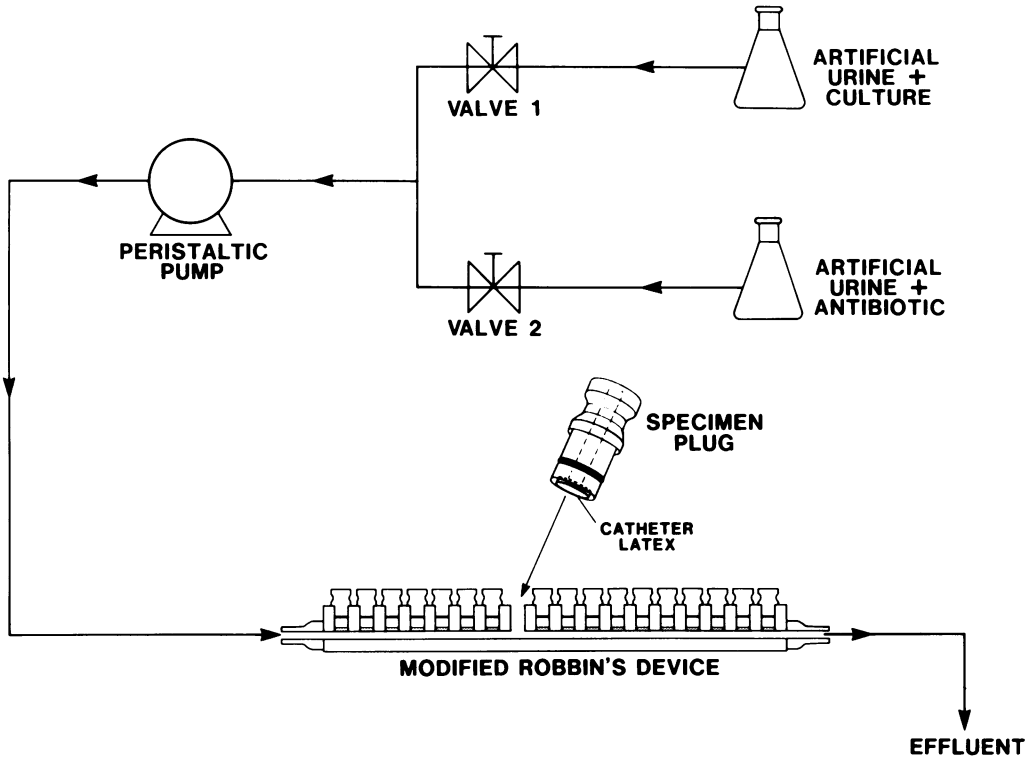
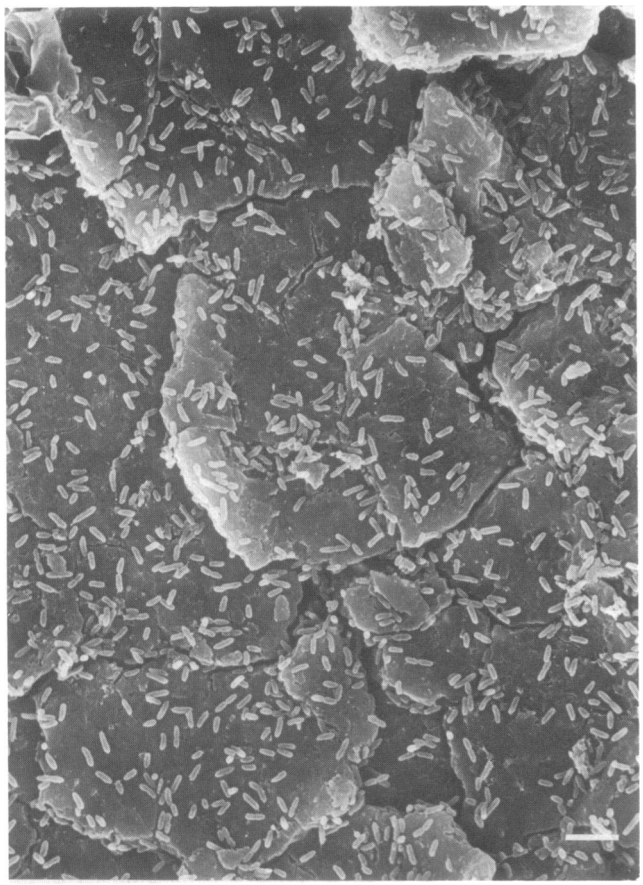


FIG. 1. Diagrammatic representation of the modified Robbins device and the apparatus used to provide a flow of artificial urine, with and without bacteria or tobramycin, or both.



men of the artificial urine medium containing the logarithmic-phase bacterial cells was transferred to flasks in which these planktonic (floating) bacteria were exposed to various concentrations of tobramycin for either 8 or 12 h.

At time 0, flow was started from the antibiotic medium reservoir (Fig. 1) so that artificial urine containing tobramycin flowed through the modified Robbins device during the treatment period. Treatment of the sessile bacterial population colonizing the catheter material in the modified Robbins device was carried out with tobramycin concentrations of 100 and 1,000 μg/ml. Disks of catheter material were removed from the treated device at 8 and 12 h, and the sessile bacteria in the biofilm were examined by SEM, epifluorescence, and viable counting. At 12 h, a disk bearing sessile bacteria was removed from the system and rinsed in the artificial urine medium, and the adherent cells were aseptically scraped into fresh artificial urine medium in which the MIC of tobramycin was determined.

Catheter disk surface examination. Quantitative sessile bacterial counts were obtained by using a modified quantitative epifluorescence technique (10). An epifluorescence count shows all cells and is usually 1 log higher than viable counts due to the clumping artifacts in most culture systems. The glutaraldehyde-fixed catheter specimens were stained with 0.01% acridine orange for 2 min and subsequently destained with isopropanol. Because of problems due to fluorescing catheter material, we modified the technique by

FIG. 2. SEM of the surface of a disk of latex catheter showing the plate-like topography of this material and the large number of adherent bacteria 5 min after contact with artificial urine containing 3.0×10^6 cells per ml of a uropathogenic strain of *P. aeruginosa*. Bar, 5 μm.

counterstaining the specimens with a 1% aqueous solution of malachite green for 10 min. The specimens were air dried, mounted on slides, and examined with a Zeiss standard 16 microscope fitted for epifluorescence microscopy. Cells were counted and numbers of sessile cells per cm^2 were calculated. However, the technique does not distinguish between live or dead bacteria. Quantitative counts of viable adherent bacteria were obtained by low-output ultrasonication of surface scraping and the catheter disk itself in a sterile phosphate-buffered saline solution. Dilution series were made up to 10^{-4} and spread on nutrient agar from which quantitative plate counts were obtained. The catheter specimens designated for SEM were gently removed from the Robbins stud and placed in a fixative solution consisting of 5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 1 h at 22°C, followed by dehydration in a series of aqueous ethanol solutions (20 to 100%) and Freon 113-ethanol solutions (30 to 100%) and then air dried. Samples were coated with gold in a sputter coater and examined by using a Hitachi S450 scanning electron microscope.

Before being processed for transmission electron microscopy, morphological stabilization of the biofilm glycocalyx with specific antibody was performed on the biofilm specimens. We have previously described this technique (3, 15). Briefly, this involves exposing the washed catheter surface to undiluted antisera for 1 h. The antisera was developed by serial injection of washed Formalin-treated cultures of *P. aeruginosa* into New England white rabbits. The immune response of the rabbits was monitored with a modification of the precipitin test (25), and serum specificity for expolysaccharide was monitored with counter immunoelectrophoresis (3). The phosphate-buffered saline-washed surfaces were fixed in 5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) with 0.15% ruthenium red, enrobed in 4% agar (Difco), postfixed in 2% OsO_4, and dehydrated through a series of acetone washes. All the solutions used in processing the specimen after glutaraldehyde fixation contained 0.05% ruthenium red. After further dehydration in propylene oxide, the specimens were embedded in Spurr (27) low-viscosity embedding resin, sectioned, stained with uranyl acetate and lead citrate, reinforced with evaporated carbon, and examined with a Hitachi 600 transmission electron microscope at an acceleration voltage of 60 kV.

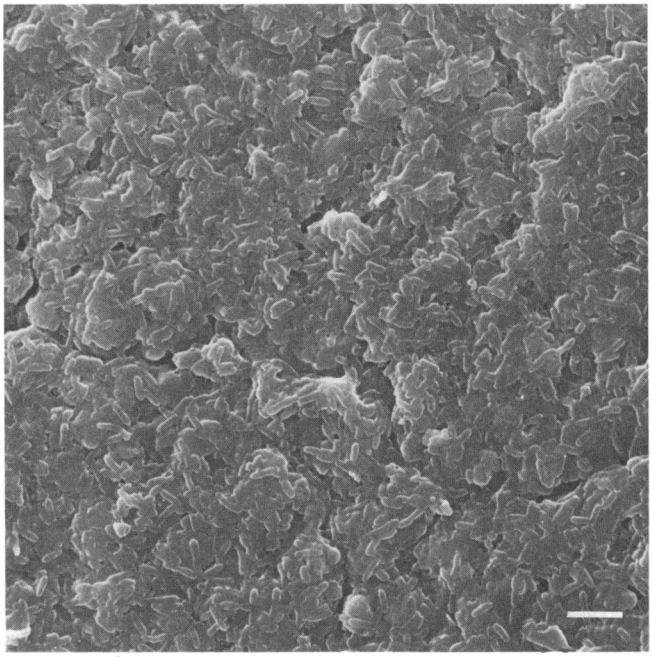


FIG. 4. SEM of a catheter latex disk 8 h after contact with bacteria-containing artificial urine showing the development of a thick bacterial biofilm that has completely occluded the latex surface. Bar, 5 μm.

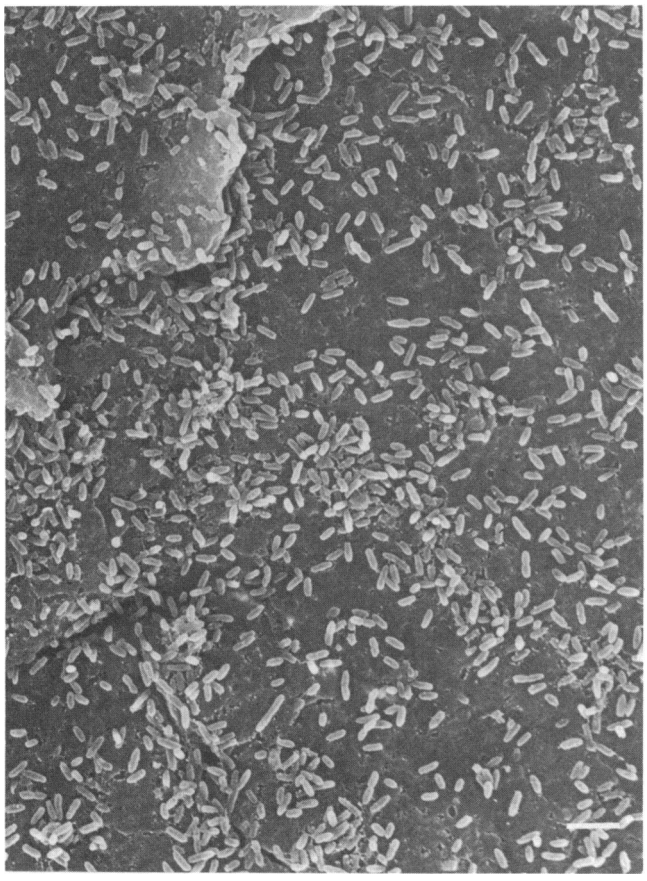


FIG. 3. SEM of a catheter latex disk 2 h after contact with bacterial cells in artificial urine showing heavy colonization and partial occlusion of the latex surface. Bar, 5 μm.

RESULTS

Examination by SEM showed that the disks of catheter latex used in this study did not bear significant numbers of ethanol-killed bacterial cells. Five minutes after exposure to cells of *P. aeruginosa* in artificial urine, significant numbers of adherent bacterial cells were seen (Fig. 2), and these plate-like latex surfaces were almost confluently colonized by adherent bacteria after 2 h of colonization (Fig. 3). After 8 h of colonization, the characteristic plate-like surface of the latex disks was completely occluded by large numbers of adherent bacteria (Fig. 4), which were embedded in large amounts of their own amorphous exopolysaccharides to form a thick adherent biofilm (Fig. 5). When biofilm material was scraped from the latex surface after 8 h of colonization and stained with ruthenium red after stabilization with specific antibody, it was evident that the cells of *P. aeruginosa* within the biofilm were embedded in an extensive fibrillar matrix (Fig. 6). Because the biofilm was disturbed during its removal from the latex disk, we cannot ascertain the original position or orientation of this adherent biofilm,

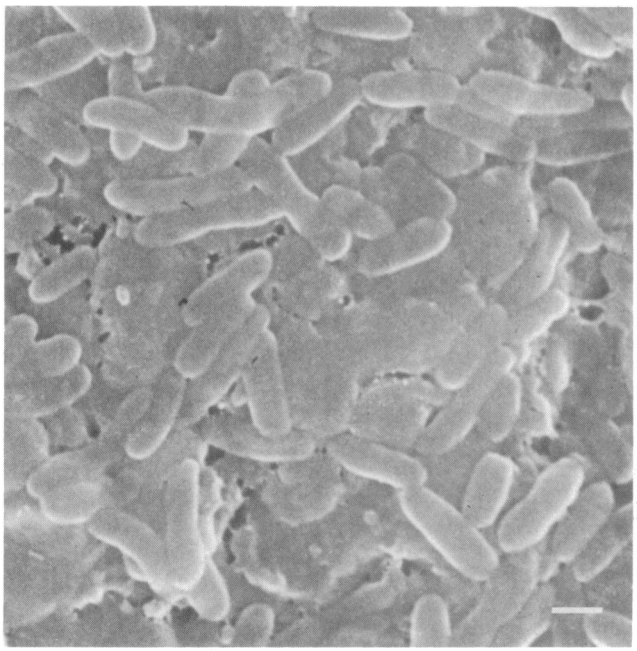


FIG. 5. SEM, at higher magnification, of the latex disk seen in Fig. 4 showing the extent to which the biofilm bacteria are "buried" in the amorphous residue of their dehydration-collapsed glycocalyces. Bar, 1.0 μm.

but it is clear that the sessile bacteria lie enmeshed in a very extensive fibrous glycocalyx. The optical density at 600 nm of the reservoir culture monitored throughout the 8-h colonization phase rose from 0.065 (3.0×10^6 cells per ml) to 1.400 (2.1×10^9 cells per ml), indicating that the *P. aeruginosa* cells entering the modified Robbin device were in the logarithmic phase of growth throughout the colonization period.

After colonization was complete, at 8 h, the mature biofilms on the catheter material were exposed to flowing solutions of two concentrations of tobramycin. Exposure to 1,000 μg/ml of this antibiotic for 12 h did not kill all of the bacteria within the thick adherent biofilm (Table 1). Whereas the viable count of sessile biofilm bacteria on the surface of the latex disks showed some decrease after exposure to 1,000 μg of tobramycin per ml for 12 h, the number of adherent cells (alive and dead) seen by epifluorescence microscopy remained constant. When planktonic cells of *P. aeruginosa* were removed from the modified Robbins device and incubated in flasks with various concentrations of tobramycin, the MBC of this antibiotic for these free-floating cells was found to be 50 μg/ml (Table 2). Examination of these planktonic cells by epifluorescence microscopy, after 8 or 12 h of exposure to tobramycin, showed no decrease in total cell numbers (alive and dead) due to antibiotic treatment. The MIC of tobramycin for the planktonic cells in the colonization system was 0.4 μg/ml, and this same MIC was obtained when surviving cells from the biofilms treated with 1,000 μg of tobramycin per ml were rinsed in medium, dispersed, and tested by the same method.

DISCUSSION

The original Robbins device (Biofilm Sampler) was made of admiralty brass, with steel specimen plugs, and it is now very widely used to monitor the development of biofilms harboring corrosion-causing bacteria in pipelines and oil production facilities (7). In industry, an illusion of control of bacterial growth had been produced by data showing total killing of planktonic bacteria in systems treated with low levels of commercial biocides, but bacterial corrosion and souring continued unabated in these treated systems (5). Thick bacterial biofilms could be developed on the surfaces of industrial materials in the Robbins Device (21), and studies using this apparatus have shown that biofilm bacteria are resistant to biocide concentrations 20- to 50-fold higher than those that kill planktonic organisms in the same systems (26). The use of biocide concentrations and dosing strategies based on Robbins device data has enabled industrial microbiologists to control corrosion and souring problems caused by biofilm bacteria adherent to metal surfaces (7).

The direct examination of the surfaces of plastic and metal prostheses removed from patients in whom they had become foci of infection has shown extensive bacterial biofilm development on intravenous catheters (18, 23), intraperitoneal catheters (20), intrauterine contraceptive devices [illegible], cardiac pacemakers (19), and urinary catheters (22a). In vitro studies have described the formation of extensive bacterial biofilms (1, 24) on the surfaces of biomaterials used in the manufacture of these catheters and other prostheses. Catheter-acquired urinary tract infections account for as much as 35% of all nosocomial infections (28) and are notoriously refractory with respect to antibiotic therapy (13). The biofilms developed in situ on the surfaces of catheters (Nickel et al., in press) and of urine collection systems (17) are very similar to those developed on latex catheter surfaces in this present study (Fig. 4 and 5), in that numerous biofilm bacteria are embedded in a very extensive and amorphous matrix that occludes the colonized surface even after extensive condensation during dehydration in preparation for electron microscopy. The anionic alginate exopolysaccharide produced by cells of *P. aeruginosa* is composed of uronic acid molecules and is highly hydrated (99% water) (29).

In this study, we have shown that cells of a strain of *P. aeruginosa* that have a tobramycin MIC of <1.0 μg/ml and

TABLE 1. Effects of high concentrations of tobramycin on *P. aeruginosa* cells growing within biofilms on latex catheter material

Tobramycin concn (μg/ml)	Viable no. of cells/cm²[b] at time:			Total no. of cells/cm²[d] at time:		
	0[c]	8 h	12 h	0	8 h	12 h
0[a]	4.7×10^8	2.3×10^9	2.7×10^9	2.9×10^9	8.7×10^9	9.1×10^9
100	5.8×10^8	5.3×10^7	4.2×10^7	2.9×10^9	2.4×10^9	2.9×10^9
1,000	2.0×10^8	1.1×10^7	6.1×10^6	2.9×10^9	2.9×10^9	2.9×10^9

[a] Sterile artificial urine, without antibiotic, was passed through the colonized device.
[b] Viable sessile cell numbers were determined by serial dilution and plating after the removal of the biofilm from the latex surface.
[c] Time 0 samples were taken immediately before the exposure of the biofilms to the antibiotic.
[d] Total cell numbers (live and dead) were determined by epifluorescence microscopy after removal of the biofilm from the latex surface and dispersal by ultrasonication.

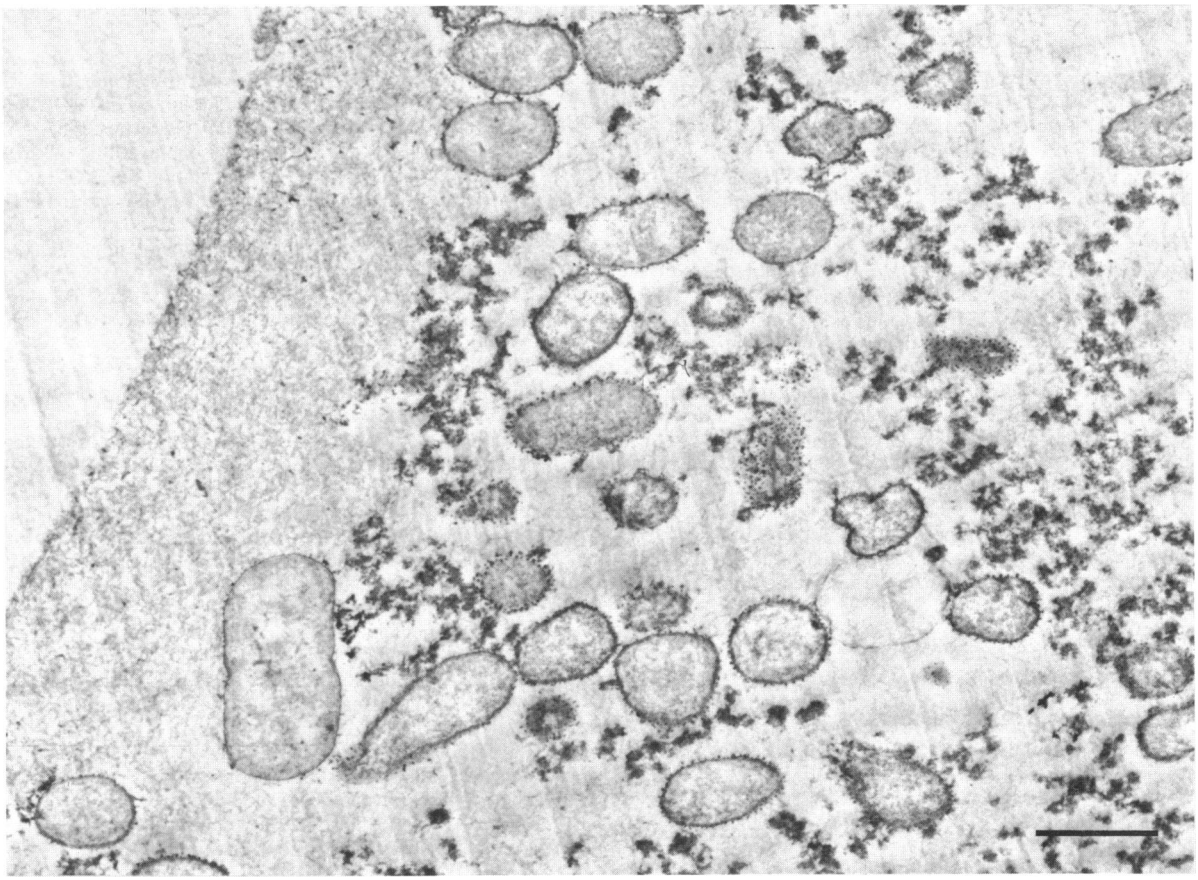


FIG. 6. TEM of a ruthenium red-stained antibody-stabilized preparation of biofilm material scraped from the surface of a latex disk after an 8-h exposure to bacteria-containing artificial urine. Note the partial stabilization of the fibrous anionic matrix between rod-shaped cells and its partial collapse with dehydration to form coarse electron-dense aggregates in some areas. Bar, 1.0 μm.

an MBC of 50 μg/ml survive exposure to 1,000 μg/ml when they are living within thick biofilms on the surface of catheter latex. Under usual clinical conditions, levels of plasma tobramycin rarely exceed 8 μg/ml, which usually results in urine tobramycin concentrations of 50 to 200 μg/ml (11). Thus, this present data may contribute to the explanation of the paradoxical resistance of catheter-associated urinary tract infections to doses of antibiotics that are effective in standard in vitro tests (30) and may help to stimulate the development of colonization-resistant biomaterials.

TABLE 2. Effects of various concentrations of tobramycin on planktonic *P. aeruginosa* cells[a]

Tobramycin concn (μg/ml)	No. of viable cells/ml[b] at time:		
	0[c]	8 h	12 h
0	1.1×10^9	9.8×10^8	1.4×10^9
5	7.8×10^8	9.1×10^8	2.8×10^8
10	1.0×10^9	1.2×10^9	3.0×10^6
20	1.2×10^9	9.5×10^8	3.6×10^6
40	8.2×10^8	1.1×10^9	3.0×10^6
50	1.2×10^9	0	0
100	9.0×10^8	0	0
500	1.2×10^9	0	0

[a] Cells were incubated in artificial urine without addition of the antibiotic.
[b] Viable cell numbers were determined by serial dilution and plating.
[c] Time 0 samples were taken immediately before the addition of the antibiotic.

The mechanism of the effective protection of biofilm bacteria from the bactericidal effects of tobramycin is unknown, but it is unlikely to result from metabolic inactivity of biofilm bacteria, because in other systems biofilm bacteria have been shown to be more metabolically active than their planktonic counterparts (14). We are presently using newly developed physiological techniques to study the metabolic activity (heterotrophic potential) of uropathogenic bacteria, growing in biofilms in urine-containing systems, in response to exposure to various antibiotics. We are also using direct chemical techniques to study the binding of antibiotics to the anionic exopolysaccharide materials that constitute the glycocalyx of cells of *P. aeruginosa* to determine whether binding to matrix components retards the penetration of antibiotics through these bacterial biofilms (2).

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