Comparison of antimicrobial effect on biofilm of chlorine, silver ion and tobramycin

Authors: Jaeeun Kim, Betsey Pitts, Philip S. Stewart, Anne K. Camper, & Jeyong Yoon

This is a postprint of an article that originally appeared in Antimicrobial Agents and Chemotherapy on January 2008. DOI: 10.1128/aac.00054-07

Kim J, Pitts B, Stewart PS, Camper A, Yoon J, "Comparison of antimicrobial effect on biofilm of chlorine, silver ion and tobramycin," Antimicrob. Agents Chemother. 52(4):1446-1453

Made available through Montana State University's ScholarWorks
scholarworks.montana.edu

School of Chemical and Biological Engineering, Seoul National University, Sillim-dong, Gwanak-gu, Seoul, South Korea, and Center for Biofilm Engineering, Montana State University-Bozeman, Bozeman, Montana

The systematic understanding of how various antimicrobial agents are involved in controlling biofilms is essential in order to establish an effective strategy for biofilm control, since many antimicrobial agents are effective against planktonic cells but are ineffective when they are used against the same bacteria growing in a biofilm state. Three different antimicrobial agents (chlorine, silver, and tobramycin) and three different methods for the measurement of membrane integrity (plate counts, the measurement of respiratory activity with 5-cyano-2,3-ditolyl tetrazolium chloride [CTC] staining, and BacLight Live/Dead staining) were used along with confocal laser scanning microscopy (CLSM) and epifluorescence microscopy to examine the activities of the antimicrobials on biofilms in a comparative way. The three methods of determining the activities of the antimicrobials gave very different results for each antimicrobial agent. Among the three antimicrobials, tobramycin appeared to be the most effective in reducing the respiratory activity of biofilm cells, based upon CTC staining. In contrast, tobramycin-treated biofilm cells maintained their membrane integrity better than chlorine- or silver-treated ones, as evidenced by imaging by both CLSM and epifluorescence microscopy. Combined and sequential treatments with silver and tobramycin showed an enhanced antimicro-bial efficiency of more than 200%, while the antimicrobial activity of either chlorine or tobramycin was antagonized when the agents were used in combination. This observation makes sense when the different oxidative reactivities of chlorine, silver, and tobramycin are considered.

Bacterial biofilms are responsible for industrial biofouling, microbial regrowth in distribution systems, persistent infec-tions (6, 9, 11, 12), and many other expensive and life-threat-ening problems. Therefore, the control of biofilms is now un-derstood to be crucial. However, there are still few effective control strategies, and they are poorly understood in many contexts. Many antimicrobial agents that are effective against planktonic cells turn out to be ineffective against the same bacteria growing in a biofilm state (10, 12, 38). Whereas strong oxidizing biocides are usually reliably effective against plank-tonic cells, sometimes weak oxidants or nonoxidants are supe-rior for controlling biofilms (20, 41). Planktonic and biofilm cells also exhibit different susceptibilities to a certain antimi-crobial concentration. Bacterial adaptive responses play a role in the design of control strategies (31, 43). In microenviron-ments with the intensive and constant exposure of bacteria to antibiotics, "there is selective pressure for antibiotic-resistant bacteria to maintain those determinants, survive, and even dominate the bacterial populations" (43). The combined ap-plication of multiple antimicrobial agents may be a strategy to improve their performance and circumvent bacterial adapta-tion. This might involve the use of antimicrobials with different chemistries and modes of action together. Although only an-tibiotics or antiseptics are permitted for use in the human body, other biocides or combinations can be used to clean medical devices. Silver-coated medical devices are widely used (3, 19, 33), and the antiseptics chlorhexidine and silver sulfa-diazine and the antibiotics minocycline and rifampin have been used as catheter coatings. These were reported to reduce the risk of catheter-related bloodstream infections (26). Water treatment lines used for dialysis have been cleaned with hypo-chlorite solution (bleach) and with dilute acid solutions (nitric, citric, peracetic acid, etc.) (27, 29).

In order to choose appropriate antimicrobial agents and to optimize the dosing strategy on a case-by-case basis, it is nec-essary to improve our understanding of the interaction be-tween various antimicrobial agents and biofilm cells. Many studies have examined the efficacy of either antibiotics alone (5, 44, 48) or biocides alone (17, 40). However, very little work has been done on the efficacy of combinations of different categories of agents, for example, antibiotics and oxidative biocides. A quantitative literature survey of bacterial suscepti-bility to several antimicrobial agents, including oxidants, anti-biotics, and other biocides, was conducted by Stewart and Raquepas (39); and some biofilm resistance factors were sug-gested to be a measurement of biofilm susceptibility to anti-microbial agents. However, there was considerable variability in the resistance factor even for the same microbial species and the same antimicrobial agents, because they were collected from various studies conducted under different experimental conditions. Thus, a comparison of the susceptibilities of bio-films to various single agents of different categories has yet to be completed.

In this study, we selected three different antimicrobial agents (chlorine, silver, and tobramycin) to examine and compare their antimicrobial behaviors on biofilms. Chlorine, the most common disinfectant, is moderately oxidative and reacts with various components of bacterial cells (46). Silver has no oxi-

dizing capacity but is involved in rendering various enzymes inactive by binding to thiol (—SH) groups in a cell (24). Recently, interest in the antimicrobial efficiency of silver compounds has increased, and many studies on the use of silver and other inorganic compounds as biocides in industrial systems and medical devices have been conducted (19, 30). Tobramycin is frequently used in biofilm studies and reportedly inhibits protein synthesis and kills both growing and nongrowing cells (36). Three methods of assessment were used to compare the antimicrobial effects of chlorine, silver, and tobramycin on biofilm in this study: plate counts, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining, and BacLight Live/Dead staining. In addition, experiments with combined and sequential treatments with the three agents were conducted and the antimicrobial efficiencies were evaluated.

MATERIALS AND METHODS

Bacteria and media. *Pseudomonas aeruginosa* PAO1 was grown in tryptic soy broth (TSB) at 37°C. For the experiments with planktonic cells, the cells were harvested by centrifugation at 1,000 $\times$ *g* for 10 min and washed twice with phosphate-buffered saline (PBS; pH 7.0). A PAO1 suspension was prepared by resuspending the cell pellet in 50 ml of PBS.

Biofilm growth and biofilm reactors. Biofilms were grown in Centers for Disease Control and Prevention (CDC) reactors (Biosurface Technologies Inc., Bozeman, MT) (7). The CDC reactor contains eight rods which each hold three glass coupons. An overnight culture was prepared by incubating PAO1 in 1/10-strength TSB for 20 h at 37°C. The sterile reactor was inoculated with 3.5 ml of an overnight culture that had been added to 350 ml of 1/100-strength TSB. The initial PAO1 population in this batch medium was about 10^6 CFU per milliliter. The reactor was operated in batch mode for 24 h at 100 rpm and room temperature. After 24 h in batch mode, the reactor was connected via a nutrient feed line to a carboy containing 1/300-strength TSB and was operated in the continuous-flow mode at a flow rate of 11.67 ml/min for 24 h.

Antimicrobial experiments. For the antimicrobial experiments, a rod was removed from the reactor and rinsed with distilled water and was then soaked in 10 mg/liter of each antimicrobial agent solution (chlorine, silver, and tobramycin) in a glass bottle without stirring. This concentration was chosen because the maximum clinically achievable concentration of tobramycin is 10 mg/liter (34). The solutions were prepared by dilution of NaOCl solution, $AgNO_3$, and tobramycin sulfate salts (Aldrich Co.), respectively. The chlorine concentration was measured by the *N*,*N*-diethyl-*p*-phenylenediamine method with a DR/2010 portable datalogging spectrophotometer (Hach Co.). After disinfection, the rods were soaked in a neutralizing solution of 100 ml distilled water and 100 μl of neutralizing reagent (100 mM sodium thiosulfate for chlorine, 14.6% sodium thiosulfate and a 10% sodium thioglycolate solution for silver [18], distilled water for tobramycin) for 5 min and rinsed with distilled water. At each sampling time point, coupons for confocal laser scanning microscope (CLSM) were stained with BacLight Live/Dead stain or CTC–4′,6-diaminidino-2-phenylindole (DAPI). Those coupons to be used for plate counting and epifluorescence microscopy were collected in 10 ml of PBS solution in Falcon tubes. The tubes were sonicated for 1 min and vortexed for 2 min to remove the biofilm. One milliliter of each biofilm suspension was used for colony counting, and the rest was filtered onto black polycarbonate membranes (25 mm; pore size, 0.22 μm; Osmonics Inc.) and then stained for epifluorescence microscopy. The colonies on the plates were counted after 24 h of incubation at 37°C. The level of inactivation was expressed as the $\log_{10}$ reduction in the microbial survival ratio for the antimicrobial experiments. The antimicrobial efficacy in this study is given as the *CT* value (where *C* is the concentration of the antimicrobial agent [mg/liter] and *T* is the contact time [min]).

Experiments with the antimicrobials tested in combination and with the antimicrobials tested sequentially were conducted by following the same procedure used for the experiments with the single antimicrobials but with the following modifications: for the combined treatments, both agents (chlorine and silver, silver and tobramycin, chlorine and tobramycin) were put into the same glass bottle and mixed thoroughly before application; and for the sequential treatments, the biofilms were treated with the primary agent (chlorine, silver, or tobramycin), rinsed with distilled water, and then treated with the secondary agent.

Planktonic cell inactivation experiments were performed with overnight cultures of strain PAO1. Antimicrobial agent solutions were diluted to 1 mg/liter and 10 mg/liter for these experiments. The experiments were conducted with 50 ml of solution in flasks with an initial microbial population of 10^6 CFU/ml. In order to minimize the chlorine loss by volatilization, the contents were mixed slowly with a magnetic stirrer and the flasks were capped during stirring. The disinfection efficacy of each sample was evaluated only by plate counting.

Fluorescent stains. In order to evaluate cell membrane integrity, the BacLight Live/Dead bacterial viability kit (L-7012; Molecular Probes) was used. The kit contains Syto9 and propidium iodide to differentiate between cells with intact membranes (live) and membrane damaged cells (dead), respectively (15, 28). The stain was prepared by dilution of 3 μl of each component into 1 ml of distilled water. The respiratory activity of the cells was determined by staining with 1.6 μg/ml of CTC (Polysciences, Inc.) (37). Respiring cells were identified by the presence of intracellular, red CTC-formazan crystals. DAPI (10 μg/ml; Polysciences, Inc.) was used for the enumeration of the total cells (37).

For CLSM, the biofilm coupons were stained with 0.1 ml of each staining solution for 1 h in the dark. CTC-stained samples were incubated at 37°C. For epifluorescence microscopy, the cells were filtered onto black, polycarbonate membranes and then set on stain-soaked filter support pads for 20 min.

CLSM and epifluorescence microscopy. Biofilm samples were imaged with a Leica AOBS-SP2 confocal laser scanning microscope (Leica Microsystems Inc., Bannockburn, IL). A water immersion objective lens (63 by 0.9 numerical aperture) was used. The optimum photomultiplier setting was determined in a pre-experiment, and then the same photomultiplier setting was used for all untreated and treated samples. The suspended cells were enumerated, after filtration onto a membrane, by using an oil objective lens (100 by 1.4 numerical aperture) on a Nikon E800 microscope. When these preparations were analyzed, at least 2,000 cells were scored per sample. The image stacks collected by CSLM were analyzed with MetaMorph software (Molecular Devices Corporation, Downingtown, PA) and Imaris software (Bitplane, Zurich, Switzerland).

Normalization and statistics. All results were expressed as normalized cell ratios by setting the ratio for an untreated biofilm equal to 100%. The total cell density, plate count cell density, CTC-respiring cell density, and BacLight live cell density were defined as the number of DAPI-stained cells per unit area (number of cells/m^2), the number of CFU per unit area (number of CFU/m^2), the number of CTC-stained cells per unit area (number of cells/m^2), and the number of Syto9-stained cells per unit area (number of cells/m^2), respectively. In addition, we use the terms culturability ratio, respiratory ratio, and BacLight live cell ratio to refer to the plate count density/total cell density, the CTC-respiring cell density/total cell density, and the BacLight live cell density/total cell density, respectively. All ratios were averaged and expressed as the average $\pm$ standard error.

All antimicrobial experiments were repeated two or three times. Plate count analysis was conducted in triplicate, and more than five images per sample were taken for microscope analysis. The Student *t* test (two tailed) was performed for statistical analysis. A *P* value of less than 0.05 was used to indicate a significant difference, and a *P* value of more than 0.5 was used to indicate a similarity.

RESULTS

Antimicrobial efficacies of chlorine, silver, and tobramycin against planktonic and biofilm cells. In order to compare the treatment efficacies of the various antimicrobial agents against planktonic cells versus those against biofilm cells, inactivation curves were compared, as shown in Fig. 1. The data were derived from the plate count results. In Fig. 1a, for planktonic cells, the data for 1 and 10 mg/liter were averaged. For the biofilm experiment whose results are shown in Fig. 1b, only the data for 10 mg/liter were used since the 1-mg/liter treatment failed to cause any significant biofilm inactivation (less than 0.2 log unit of inactivation in 5 h). The inactivation efficiency of each antimicrobial agent was significantly different depending upon which state the cells were in: planktonic or biofilm ($P <$ 0.00001). Overall, the *CT* values for biofilm cells were a factor of 10 greater than those for planktonic cells. As expected, chlorine was the most effective among the three antimicrobial agents against planktonic cells, as shown in Fig. 1a. It required a *CT* value of 0.05 mg · min/liter to inactivate 1 log (90%;

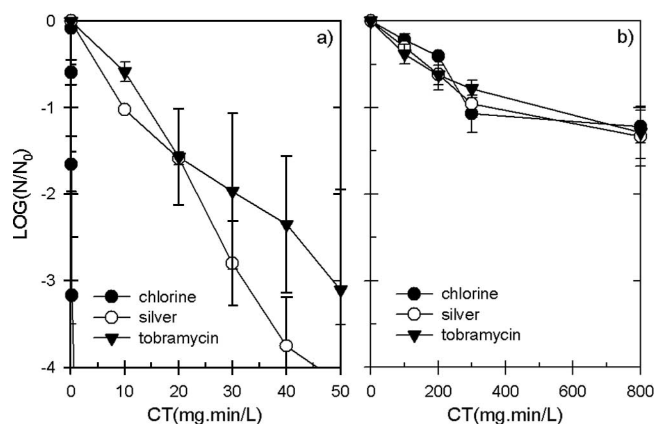

FIG. 1. Antimicrobial efficiencies of chlorine, silver ion, and tobramycin for inactivation of *P. aeruginosa* PAO1 planktonic cells (a) and biofilms (b).

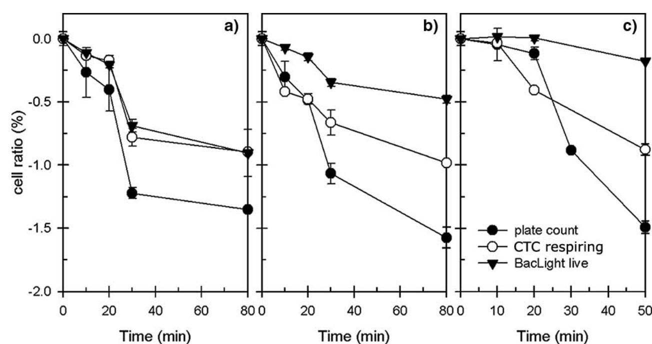

FIG. 3. Culturable, CTC-respiring, and BacLight live cell ratios of biofilms treated with 10 mg/liter of chlorine (a), silver (b), and tobramycin (c).

CT_{90}) of the planktonic cells with chlorine. On the other hand, the CT_{90} of the biofilm cells in chlorine was more than 300 mg · min/liter (Fig. 1b). A CT_{90} of 20 mg · min/liter was needed for planktonic cells with both silver and tobramycin. These *CT* values are much larger than the *CT* value of chlorine, indicating that silver and tobramycin are less effective antimicrobial agents than chlorine for planktonic cells. However, the CT_{90} values of silver and tobramycin for biofilm cells were similar to the *CT* value of chlorine ($P = 0.84$). As shown in Fig. 1b, the three different antimicrobial agents had roughly the same inactivation efficiencies against PAO1 biofilm cells ($P > 0.77$), even though they had significantly different antimicrobial efficiencies against planktonic cells.

Total, culturable, respiring, and BacLight Live/Dead live cell densities of untreated biofilm. An untreated biofilm was examined for total, culturable, respiring, and BacLight live cell densities (Fig. 2). The average total cell areal density was 2.5×10^{11} cells/m^2; and the average plate count cell density was roughly half of that, which means that half of the cells in the biofilm lost their culturability. For CTC-respiring cells, 2.3×10^{11} cells/m^2 was observed. The BacLight live cell density was about 1.8×10^{11} cells/m^2. Overall, for the untreated biofilm, 50% of the total cells maintained their culturability and 90% and 70% of the total cells were respiring and maintained intact cell membranes, respectively. It has been reported that biofilm cells can retain significant respiratory activity, even though they fail to form colonies (37).

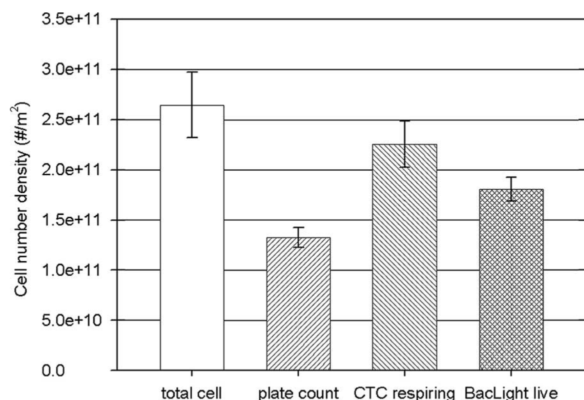

FIG. 2. Total, culturable, CTC-respiring, and BacLight live cell number densities of untreated biofilms.

The total cell density of the biofilm was $2.5 \times 10^{11}/m^2$, and the total growth surface area, which includes both sides of a coupon, was 1.9 cm^2 (coupon diameter, 1.1 cm). Thus, 4.8×10^7 cells were treated in total for each biofilm experiment. Planktonic cell experiments were done with 5×10^7 cells per test volume (10^6 CFU/ml $\times$ 50 ml). The populations of both biofilm and planktonic cells were so similar that comparison conditions were satisfied.

Comparison of biofilm inactivation efficiencies by three methods: determination of plate counts, respiration activity, and membrane integrity. The inactivation curves for the biofilms treated with chlorine, silver, and tobramycin were evaluated by three methods of measurement, shown in Fig. 3. For chlorine, the plate count curve declined sharply over the treatment time. After 80 min of treatment, a reduction of 1.4 log units was observed in the plate count curve. The CTC-respiring and BacLight live cell curves almost overlapped, as cell reductions of 0.9 log unit were observed for both curves after 80 min. The inactivation curve of the plate counts decreased most sharply with silver treatment, while the CTC-respiring and BacLight live cell curves decreased more smoothly. A total of 1.6 log units of cells in the plate count experiments were inactivated during 80 min of silver treatment. For both CTC-respiring and BacLight live cells, 1.0-log-unit and 0.5-log-unit cell reductions were shown, respectively. In the experiments with tobramycin, the qualitative trends were similar to those of silver inactivation, although the magnitudes were different. After 50 min of tobramycin treatment, the cells in the plate count experiments were reduced 1.5 log units, and 0.9-log-unit and 0.2-log-unit cell reductions were observed for the CTC-respiring and BacLight live cell ratios, respectively.

Comparison of respiration activity and membrane integrity of biofilms treated with three antimicrobial agents. In order to compare the different results obtained with each antimicrobial agent more easily, the data in Fig. 3 were reorganized according to the CTC-respiring cell ratios and the BacLight live cell ratios. The plate count cell ratio is shown on the *x* axis, and the CTC-respiring cell ratio and the BacLight live cell ratio are shown on the *y* axis in Fig. 4a and b, respectively. For example,

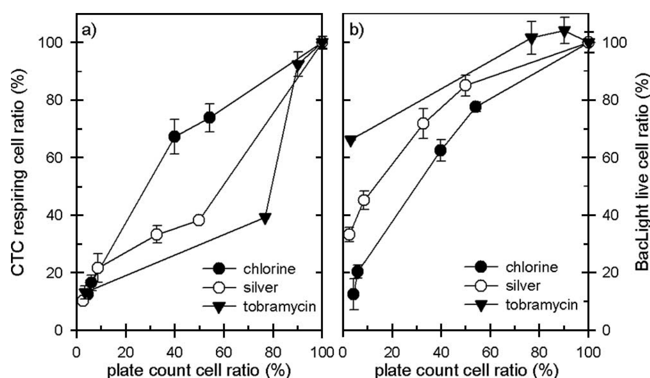

FIG. 4. CTC-respiring and BacLight live cell ratios of biofilms treated with chlorine, silver, and tobramycin on the basis of equal culturability. (a) CTC-respiring cell ratio versus plate count cell ratio; (b) BacLight live cell ratio versus plate count cell ratio. The data pairs in Fig. 4 were collected from Fig. 3 and reorganized.

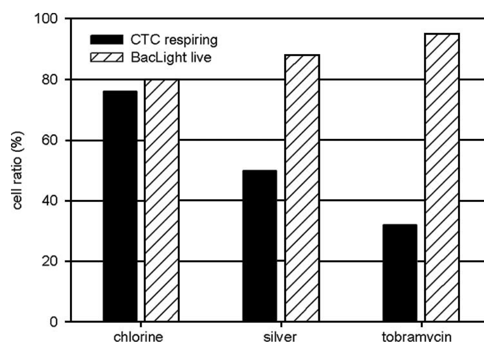

FIG. 5. CTC-respiring and BacLight live cell ratios of chlorine-, silver-, and tobramycin-treated biofilms with 60% culturability from Fig. 4.

each pair of CTC-respiring cell and plate count cell data at the same time point from Fig. 3a were collected and plotted for chlorine in Fig. 4a. Similarly, the BacLight live cell and plate count cell pairs from Fig. 3a are shown for chlorine in Fig. 4b. In the same manner, the pairs from Fig. 3b and c are shown for silver and tobramycin in Fig. 4.

All results were normalized by setting the ratio for the untreated biofilm to 100%; therefore, cell ratios close to 100% mean fewer damaged cells. Overall, there was a contrary tendency between the CTC-respiring cell ratio and the BacLight live cell ratio. For the CTC-respiring cell ratio, the activity of the tobramycin-treated biofilm decreased sharply and that of the chlorine-treated biofilm decreased gently as the plate count cell ratio decreased. The opposite trend was observed for the BacLight live cell ratio. Membrane integrity was retained up to 70% in the tobramycin-treated biofilm, while the membrane integrity was not retained in chlorine-treated cells when the plate count cell ratio decreased toward 0%. We found another phenomenon that showed their different reactivities (Fig. 4). The CTC-respiring activities of the three plots in Fig. 4a decreased as the plate counts decreased, and then the three curves met at the point of the 20% viable cell ratio. In contrast, in Fig. 4b, the membrane integrity determined from the three plots decreased in a different way, and the plots did not meet at any point. This is plausible, in that chlorine can react with cell inner components as well as with the cell membrane (16). On the contrary, silver and tobramycin are likely to be more reactive with inner components than with the cell membrane after intracellular uptake.

A more quantitative comparison was carried out (Fig. 5). At the point of 60% culturability, a set of CTC-respiring cell ratios was collected for chlorine, silver, and tobramycin, as shown in Fig. 4a. A set of BacLight live cell ratios (Fig. 4b) was collected, and these sets of ratios were compared in Fig. 5. As shown in Fig. 5, 75% of the cells in the chlorine-treated biofilm were respiring and 80% of the cells in the chlorine-treated biofilm had healthy cell membranes, while only 30% of the cells in the tobramycin-treated biofilm, in which 60% of the cells were culturable, were respiring and 90% of the cells in the tobramycin-treated biofilm maintained their cell membrane integrity. In the case of the silver-treated biofilm, 50% of the cells retained their respiring activity, while 10% of the cells had damaged cell membranes. These results were visualized by CLSM imaging, detailed in the following section.

CLSM visualization of untreated and treated biofilms with 60% culturability inactivated by chlorine, silver, and tobramycin. Figures 6 and 7 show the CLSM images, analyzed by using Imaris software, of untreated and treated biofilms with 60% culturability. BacLight Live/Dead-stained biofilms were imaged and are shown in Fig. 6. Live cells, which have intact cell membranes, are stained with Syto9 and emit a green fluorescence when they are stained with the BacLight Live/Dead stain. Otherwise, cells with damaged membranes stained with propidium iodide and showed a red fluorescence. As shown in Fig. 6, the untreated biofilm was stained mostly green, with a few red cells being present. In the chlorine-treated biofilm, most cells were red, which means that chlorine intensively damaged the cell membrane integrity. On the other hand, there were half green cells and half red cells in the silver-treated biofilm and mostly green cells with a few red cells in the tobramycin-treated biofilm. The interpretation is that the cell membrane integrity was less damaged by silver and tobramycin than by chlorine.

Biofilms stained with CTC-DAPI were visualized through CLSM (Fig. 7). Respiring cells can reduce the redox stain CTC and fluoresce red, while nonrespiring cells are stained only by DAPI and fluoresce blue. The untreated biofilm showed mostly red-stained cells and a few blue cells (Fig. 7). Similarly, most cells in the chlorine-treated biofilm were red, which means that cell respiring activity was not as affected by chlorine. In contrast, the silver-treated biofilm showed some blue fluorescent cell clumps surrounded by red cells. Very few red cells were visible in the tobramycin-treated biofilm, which indicates that tobramycin reduced the cell respiring activity more than silver and chlorine did.

Enhanced efficiencies in combined or sequential antimicrobial treatment of biofilm. In order to compare the antimicrobial efficiencies of single treatments, combined treatments, and sequential treatments, the log inactivation results based on the plate cell count after 30 min of each treatment are summarized in Table 1. In the combined and sequential treatments, most treatments showed enhanced activity compared to the activity of the single treatments; the exception was the combination of

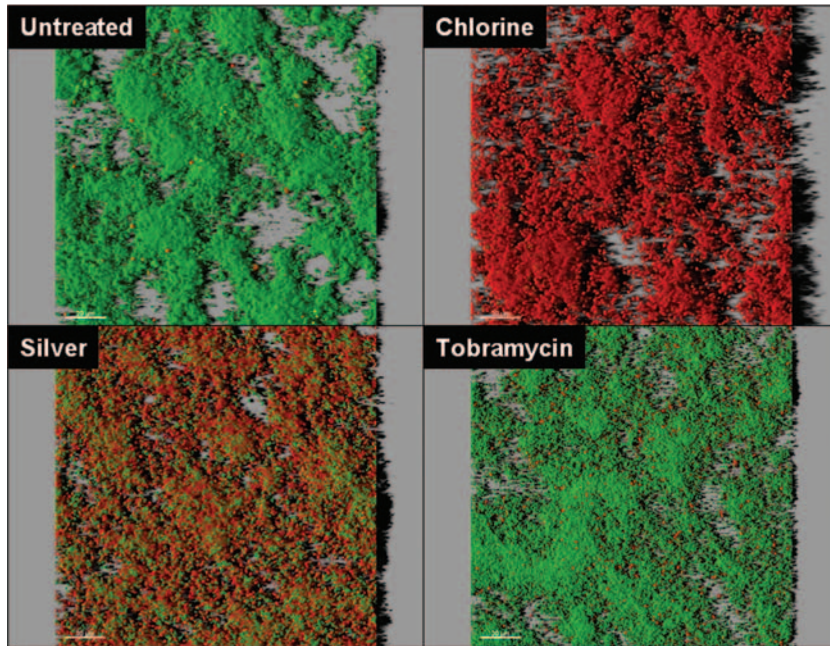

FIG. 6. CLSM images of untreated and chlorine-, silver-, and tobramycin-treated PAO1 biofilms stained with BacLight Live/Dead stain. The culturability of each treated biofilm was 60%.

chlorine and tobramycin. Overall, silver and tobramycin, applied either in combination or sequentially, was the most effective at inactivating biofilm cells. This combination treatment showed almost 300% enhanced efficiency. Enhancements of 290% and 222% were observed when silver was used as the primary and as the secondary agent, respectively ($P = 0.00006$). Chlorine and silver combined showed a mild enhancement of 118%. The sequential application of chlorine and silver was more effective than the use of chlorine and silver combined, and the enhancements were 257% (when chlorine was used as the primary agent) and 197% (when chlorine was used as the secondary agent) ($P = 0.00008$). On the other hand, the antimicrobial activities of chlorine and tobramycin were decreased when they were used in combination. Their

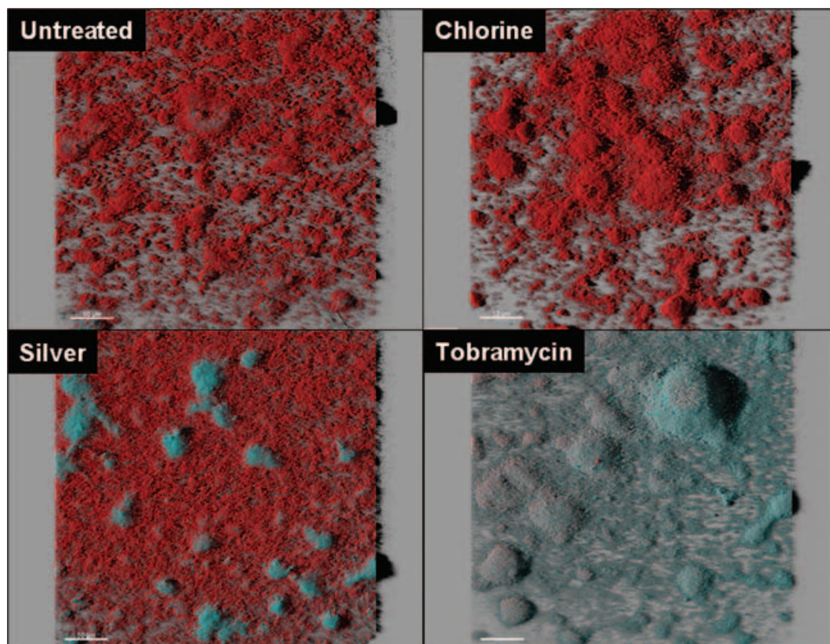

FIG. 7. CLSM images of untreated and chlorine-, silver-, and tobramycin-treated PAO1 biofilms stained with CTC-DAPI. The culturability of each treated biofilm was 60%.

TABLE 1. Enhanced efficiencies in combined and sequential treatments of biofilms

Treatment	Log inactivation after 30 min of treatment: Observed	Log inactivation after 30 min of treatment: Theoretical sum[a]	% Enhancement
Single treatment			
Chlorine	0.92 ± 0.06		
Silver	0.84 ± 0.11		
Tb[b]	0.71 ± 0.03		
Combined treatment			
Chlorine + silver	2.08 ± 0.29	1.76	118
Silver + Tb	4.51 ± 0.23	1.55	291
Tb + chlorine	0.87 ± 0.23	1.63	53
Sequential treatment[c]			
Chlorine→silver	4.53 ± 0.11	1.76	257
Silver→chlorine	3.47 ± 0.02	1.76	197
Silver→Tb	4.50 ± 0.11	1.55	290
Tb→silver	3.44 ± 0.30	1.55	222
Chlorine→Tb	1.03 ± 0.32	1.63	63
Tb→chlorine	1.78 ± 0.25	1.63	109

[a] Predicted if enhancement is absent.
[b] Tb, tobramycin.
[c] Thirty minutes of primary treatment and 30 min of secondary treatment were conducted for the sequential treatments.

efficiency when they were used in combination (53%) was much less than the sum of the individual efficiencies (100%). Similar antienhancing effects were observed when chlorine and ciprofloxacin or chlorine and carbenicillin were combined (data not shown). A slightly enhanced efficiency of 109% was observed only when tobramycin was applied before chlorine was applied.

DISCUSSION

Biofilm resistance to various antimicrobial agents. We observed significantly increased levels of resistance of biofilm cells to chlorine (an oxidant), silver ion (a biocide), and tobramycin (an antibiotic) compared with those of planktonic cells. We also observed different mechanisms of action based upon the antimicrobial agent used. It is well known that the susceptibility of cells to antimicrobial agents is diminished in biofilms (1, 12, 13, 38, 40); and a literature survey of the susceptibilities of biofilms to several antimicrobial agents, including oxidants, antibiotics, and other biocides, was conducted (39), even though a direct comparison of biofilm susceptibilities to various antimicrobial agents which belong to different groups was not carried out. In this study we tried to quantitatively compare biofilm resistance to various antimicrobial agents in parallel. We used the resistance factor and the *CT* ratio to compare their resistance. The *CT* ratio was defined as the *CT* value of biofilm cells over that of planktonic cells required to achieve a 2-log-unit inactivation with an antimicrobial agent. Several *CT* values and *CT* ratios collected from previous studies (16) and from Fig. 1 of this study are summarized in Table 2. *CT* values required for a 2-log-unit inactivation of bacteria in a planktonic state were 0.1, 25, and 30 mg · min/liter for chlorine, silver ion, and tobramycin, respectively (Fig. 1a). If we extrapolate these curves to 2-log-unit reductions, we get the *CT* value for the

TABLE 2. Antimicrobial efficiencies of chlorine, silver, and tobramycin for 2-log-unit inactivation of planktonic and biofilm cells

Agent	*E. coli* ATCC 8739: *CT* value for 2-log inactivation (mg · min/liter), Planktonic cells	*E. coli* ATCC 8739: *CT* value, Biofilm cells	*E. coli* ATCC 8739: *CT* ratio[a]	*P. aeruginosa* PAO1: *CT* value for 2-log inactivation (mg · min/liter), Planktonic cells	*P. aeruginosa* PAO1: *CT* value, Biofilm cells	*P. aeruginosa* PAO1: *CT* ratio
Chlorine	0.1[b]	830[b]	8,300[b]	0.1	1,000	10,000
Silver	28[b]	780[b]	28[b]	25	1,000	40
Tobramycin				30	1,000	33

[a] The CT ratio is the *CT* for the biofilm cells/CT for planktonic cells.
[b] Data collected from Kim et al. (16).

2-log inactivation of the biofilm, which is about 1,000 mg · min/liter for all three antimicrobial agents (Fig. 1b). Thus, the *CT* ratio is calculated to be 10,000 for chlorine, 40 for silver ion, and 33 and tobramycin. As shown in Table 2, similar results were reported for *Escherichia coli*. The susceptibility of *E. coli* biofilm cells to chlorine decreased by 8,300 times compared with that for planktonic cells, while for silver the decrease was only 28 times. For another *P. aeruginosa* strain (ATCC 700829), the *CT* ratios were reported to be 10,750 for chlorine and 29 for silver (16).

Different reactivities of antimicrobial agents cause different kinds of damage in biofilm cells. After antimicrobial treatment, many cells retained significant respiratory activity or membrane integrity, even though they lost the ability to form colonies on agar medium, as shown in Fig. 2 to 4. A previous study reported that bacterial numbers based on plate counts were less than the numbers based on direct viable counts, bacterial luminescence, and CTC or Syto9 staining (8). Among the three antimicrobial agents used in this study, chlorine appeared to damage the cell membrane most effectively, while tobramycin affected cell respiratory activity. Silver had characteristics between those of chlorine and tobramycin. This might be because of their different reactivities with the cells. Chlorine destroys microorganisms by chlorinating the lipid protein substance in the bacterial cell wall to form toxic chloro compounds (14, 42) and induces the leakage of macromolecules from the cells. One of the major bactericidal functions of silver is its interaction with the ribosome and the ensuing inhibition in expression of the enzymes and proteins essential to ATP production (47). Silver is also reported to react with the respiratory chain and inhibit the oxidation of glucose, glycerol, fumarate, etc. (4) and to make an Ag-DNA complex, which was reported to exhibit antibacterial activity (2). Tobramycin is one of the aminoglycosides, which are hydrophilic sugars with multiple amino groups that are protonated at physical pH to function as polycations and target accessible regions of polyanionic 16S rRNA on the 30S ribosome (43).

According to previous studies, the effects of the limited penetration of antimicrobial agents (38), changes in the bacterial phenotype of biofilm cells (25, 32, 40), and biofilm cells in persister states (21, 22, 36, 45), as well as the different reactivities of antimicrobial agents, were widely considered to be factors which affect biofilm resistance to antimicrobial agents. It was shown that chlorine reacted with the cellular

biomass fast enough that the diffusion of the disinfectant into the biofilm was limited and that tobramycin could penetrate the *P. aeruginosa* biofilm but failed to inactivate the bacteria (44). A silver penetration study has not yet been reported; and only the adsorption of heavy metal ions, such as Cu^{2+} and Zn^{2+}, by biofilms has been reported (5). Therefore, limited penetration cannot explain the different antimicrobial actions of the three agents at present. In addition, the differences in the reactivities of different phenotypes, i.e., dormant or persister cells, with oxidative antimicrobial agents have not yet been reported, so our results cannot be explained by these factors until further studies are conducted.

Effective combination of antimicrobial agents for biofilm control. Combined or sequential treatment with silver and tobramycin, which have relatively similar antimicrobial behaviors among three agents, was the most effective for biofilm control (Table 1). A significant enhancement of activity might be achieved by targeting of the antimicrobial actions of agents in combination, although in this case, the actions of both agents involve respiratory activity. On the other hand, chlorine was found to be less effective in combination with antibiotics. It is possible that chlorine reacted with the antibiotics (tobramycin, carbenicillin, ciprofloxacin), decreasing the available concentrations of both chlorine and the antibiotics. When 10 mg/liter of chlorine and 10 mg/liter of tobramycin were mixed, the chlorine concentration decreased to 5 mg/liter after 5 min and remained at 5 mg/liter for 30 min. Chlorine at 10 mg/liter is 191 μM and tobramycin at 10 mg/liter is 21 μM, so it is possible that 4.5 molecules of chlorine reacted with a molecule of tobramycin. This is plausible because tobramycin has five secondary amine groups per molecule which can react with chlorine. Chlorine demand was observed with carbenicillin and ciprofloxacin. In sequential treatment, the total antimicrobial efficiency was found to be enhanced when the biofilm was treated with a more reactive and less selective agent (i.e., chlorine) prior to treatment with a less reactive and more selective agent (silver ion). This was also applicable to silver and tobramycin but not to chlorine and tobramycin because they react with each other. It is supposed that a more reactive and less selective agent could alter the cell structure physically or chemically in such a way that the antimicrobial action of a secondary agent might be facilitated.

In conclusion, the antimicrobial activities of an oxidant, a metal ion, and an antibiotic (chlorine, silver ion, and tobramycin, respectively) on biofilm cells were investigated by three methods, each of which used a different analytical principle for the determination of antimicrobial activity. The resistance of the biofilm cells to an oxidant was increased almost 250 and 300 times compared with the resistance to the metal ion and the antibiotic, as assessed by viable plate counts. The components of biofilm cells principally affected were related to cell membrane integrity in the case of oxidant treatment and respiratory activity in the case of antibiotic treatment. Metal ion treatment affected both components similarly. The use of combinations of agents which have similar antimicrobial behaviors but which are not too oxidative, i.e., silver and tobramycin, might be an effective strategy for preventing microbial adaptation and facilitating the antimicrobial actions of the agents. This study might contribute to a better understanding of the antimicrobial interactions between biofilm cells and antimicrobial agents and help to establish better strategies for the use of antimicrobials against biofilms through the appropriate choice and the use of the appropriate combinations of agents.

ACKNOWLEDGMENTS

This study was supported by the Brain Korea 21 program (of the Ministry of Education) and the Center for Biofilm Engineering, MSU.

We are grateful to Kelli Buckingham-Meyer and Katherine Sossa for their technical assistance.

REFERENCES

1. **Allison, D. G., T. Maira-Litran, and P. Gilbert.** 2000. Antimicrobial resistance of biofilms. p. 149–166. *In* L. V. Evans (ed.), Biofilms: recent advances in their study and control. Harwood Academic Publishers, Amsterdam, The Netherlands.
2. **Arakawa, H., J. F. Neault, and H. A. Tajmir-Riahi.** 2001. Silver complexes with DNA and RNA studied by Fourier transform infrared spectroscopy and capillary electophoresis. Biophys. J. **81:**1580–1587.
3. **Borschel, D. M., C. E. Chenoweth, S. R. Kaufman, K. V. Hyde, K. A. VanDerElzen, T. E. Raghunathan, C. D. Collins, and S. Saint.** 2006. Are antiseptic-coated central venous catheters effective in a real-world setting? Am. J. Infect. Control **34:**388–393.
4. **Bragg, P. D., and D. J. Rainnie.** 1973. The effect of silver ions on the respiratory chain of *Escherichia coli*. Can. J. Microbiol. **20:**883–889.
5. **Brooun, A., S. Liu, and K. Lewis.** 2000. A dose-response study of antibiotics resistance in *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. **44:**640–646.
6. **Camper, A. K., K. Brastrup, A. Sandvig, J. Clement, C. Spencer, and A. J. Capuzzi.** 2003. Effect of distribution systems materials on bacterial regrowth. J. Am. Water Works Assoc. **95:**107–121.
7. **Center for Biofilm Engineering, Standardized Biofilm Methods Team.** 2003. CDC biofilm reactor operator's manual. Center for Biofilm Engineering, Montana State University, Bozeman, MT.
8. **deBeer, D., R. Srinivasan, and P. S. Stewart.** 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. **60:**4339–4344.
9. **Donlan, R. M.** 2000. Biofilm control in industrial water systems: approaching an old problem in new ways, p. 333–360. *In* L. V. Evans (ed.), Biofilms: recent advances in their study and control. Harwood Academic Publishers, Amsterdam, The Netherlands.
10. **Drenkard, E.** 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microbes Infect. **5:**1213–1219.
11. **Ellis, B. D., P. Butterfield, W. L. Jones, G. A. McFesters, and A. K. Camper.** 2000. Effects of carbon source, carbon concentration, and chlorination on growth related parameters of heterotrophic biofilm bacteria. Microb. Ecol. **38:**330–347.
12. **Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley.** 2005. Survival strategies of infectious biofilms. Trends Microbiol. **13:**34–40.
13. **Griebe, T., C.-I. Chen, and R. Stinivasan.** 1994. Analysis of biofilm disinfection by monochloramine and free chlorine, p. 151–162. *In* G. G. Geesey, L. Zbigniew, and H.-C. Flemming (ed.), Biofouling and biocorrosion in industrial water systems. Lewis Publishers, Boca Raton, FL.
14. **Haas, C. N., and R. S. Engelbrecht.** 1980. Physiological alterations of vegetative microorganisms resulting from chlorination. J. Water Pollut. Control Fed. **52:**1976–1989.
15. **Jefferson, K. K., D. A. Goldmann, and G. B. Pier.** 2005. Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. Antimicrob. Agents Chemother. **49:**2467–2473.
16. **Kim, J., J. Y. Kim, and J. Yoon.** 2006. Disinfection efficiency of silver disinfectants for biofilm. J. Korean Soc. Environ. Eng. **28:**81–87.
17. **Lambert, R. J. W., M. D. Johnston, and E. A. Simons.** 1999. A kinetic study of the effect of hydrogen peroxide and peractic against *Staphylococcus aureus* and *Pseudomonas aeruginosa* using the bioscreen disinfection method. J. Appl. Microbiol. **87:**782–786.
18. **Landeen, L. K., M. T. Yahya, and C. P. Gerba.** 1989. Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila*. Appl. Environ. Microbiol. **55:**3045–3050.
19. **Lawrence, E. L., and I. G. Turner.** 2005. Materials for urinary catheters: a review of their history and development in the UK. Med. Eng. Phys. **27:**443–453.
20. **LeChevallier, M. W., C. D. Lowry, and R. G. Lee.** 1990. Disinfecting biofilms in a model distribution system. J. Am. Water Works Assoc. **82:**87–95.
21. **Lewis, K.** 2000. Programmed death in bacteria. Microbiol. Mol. Biol. Rev. **64:**503–514.
22. **Lewis, K.** 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45:**999–1007.
23. Reference deleted.
24. **Lin, Y. E., R. D. Vidic, J. E. Stout, and V. L. Yu.** 1996. Individual and combined effects of copper and silver ions on inactivation of *Legionella pneumophila*. Water Res. **30:**1905–1913.

25. **Mah, T.-F., B. Pitts, B. Pellock, G. C. Walker, P. S. Stewart, and G. A. O'Toole.** 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature **426:**306–310.
26. **Marciante, K. D., D. L. Veenstra, B. A. Lipsky, and S. Saint.** 2003. Which antimicrobial impregnated central venous catheter should we use? Modeling the costs and outcomes of antimicrobial catheter use. Am. J. Infect. Control **3:**1–8.
27. **Marion-Ferey, K., M. Pasmore, P. Stoodley, S. Wilson, G. P. Husson, and J. W. Costerton.** 2003. Biofilm removal from silicone tubing: an assessment of the efficacy of dialysis machine decontamination procedures using an in vitro model. J. Hosp. Infect. **53:**64–71.
28. **Maukonen, J., H.-L. Alakomi, L. Nohynek, K. Hallamaa, S. Leppämäki, J. Mättö, and M. Saarela.** 2006. Suitability of the fluorescent techniques for the enumeration of probiotic bacteria in commercial non-diary drinks and pharmaceutical products. Food Res. Int. **39:**22–32.
29. **Morin, P.** 2000. Identification of the bacteriological contamination of a water treatment line used for hemodialysis and its disinfection. J. Hosp. Infect. **45:**218–224.
30. **Rupp, M. E., T. Fitzgerald, N. Marion, V. Helget, S. Puumala, J. R. Anderson, and P. D. Fey.** 2004. Effect of silver-coated urinary catheters: efficacy, cost-effectiveness, and antimicrobial resistance. Am. J. Infect. Control **32:**445–450.
31. **Sanderson, S. S., and P. S. Stewart.** 1997. Evidence of bacterial adaptation to monochloramine in *Pseudomonas aeruginosa* biofilms and evaluation of biocide action model. Biotechnol. Bioeng. **56:**201–209.
32. **Saucer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. **184:**1140–1154.
33. **Schierholz, J. M., L. J. Lucas, A. Rump, and G. Pulverer.** 1998. Efficacy of silver-coated medical devices. J. Hosp. Infect. **40:**257–262.
34. **Schulz, M., and A. Schmoldt.** 1997. Therapeutic and toxic blood concentrations of more than 500 drugs. Pharmazie **52:**895–911.
35. Reference deleted.
36. **Spoering, A. L., and K. Lewis.** 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J. Bacteriol. **183:**6746–6751.
37. **Stewart, P. S., T. Griebe, R. Srinivasan, C. I. Chen, F. P. Yu, D. DeBeer, and G. A. McFesters.** 1994. Comparison of respiratory activity and culturability during monochloramine disinfection of binary population biofilms. Appl. Environ. Microbiol. **60:**1690–1692.
38. **Stewart, P. S., G. A. McFeters, and C.-T. Huang.** 2000. Biofilm control by antimicrobial agents, p. 373–405. *In* J. D. Bryers (ed.), Biofilms II: process analysis and applications. Wiley-Liss, Inc., New York, NY.
39. **Stewart, P. S., and J. B. Raquepas.** 1995. Implication of reaction-diffusion theory for the disinfection of microbial biofilms by reactive antimicrobial agents. Chem. Eng. Sci. **50:**3099–3104.
40. **Stewart, P. S., J. Rayner, F. Roe, and W. M. Rees.** 2001. Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorsulfamates. J. Appl. Microbiol. **91:**525–532.
41. **Van der Wende, E.** 1991. Biocide action of chlorine on *Pseudomonas aeruginosa* biofilm. Ph.D. thesis. Montana State University, Bozeman.
42. **Venkobachar, C., L. Itengar, and A. V. S. P. Rao.** 1976. Mechanism of disinfection: effect of chlorine on cell membrane functions. Water Res. **11:**727–729.
43. **Walsh, C.** 2003. Antibiotics: actions, origins, resistance, vol. 1. ASM Press, Washington, DC.
44. **Walters, M. C., III, F. Roe, A. Bugnicourt, M. J. Franklin, and P. S. Stewart.** 2003. Contribution of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob. Agents Chemother. **47:**317–323.
45. **Webb, J. S., L. S. Thompson, S. James, T. Charlton, T. Tolker-Nielsen, B. Koch, M. Givskov, and S. Kjelleberg.** 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. J. Bacteriol. **185:**4585–4592.
46. **White, G. C.** 1992. The handbook of chlorination and alternative disinfections, 3rd ed. Van Nostrand Reinhold, New York, NY.
47. **Yamanaka, M., K. Hara, and J. Kudo.** 2005. Bactericidal actions of a silver ion solution on *Escherichia coli*, studied by energy-filtering transmission electron microscopy and proteomic analysis. Appl. Environ. Microbiol. **71:** 7589–7593.
48. **Zheng, Z., and P. S. Stewart.** 2004. Growth limitation of *Staphylococcus epidermidis* in biofilms contributes to rifampin tolerance. Biofilms **1:**31–35.