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Antibacterial Activity and Mechanism of Action of the Silver Ion in *Staphylococcus aureus* and *Escherichia coli*[▽]

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The antibacterial effect and mechanism of action of a silver ion solution that was electrically generated were investigated for *Staphylococcus aureus* and *Escherichia coli* by analyzing the growth, morphology, and ultrastructure of the bacterial cells following treatment with the silver ion solution. Bacteria were exposed to the silver ion solution for various lengths of time, and the antibacterial effect of the solution was tested using the conventional plate count method and flow cytometric (FC) analysis. Reductions of more than 5 $\log_{10}$ CFU/ml of both *S. aureus* and *E. coli* bacteria were confirmed after 90 min of treatment with the silver ion solution. Significant reduction of *S. aureus* and *E. coli* cells was also observed by FC analysis; however, the reduction rate determined by FC analysis was less than that determined by the conventional plate count method. These differences may be attributed to the presence of bacteria in an active but nonculturable (ABNC) state after treatment with the silver ion solution. Transmission electron microscopy showed considerable changes in the bacterial cell membranes upon silver ion treatment, which might be the cause or consequence of cell death. In conclusion, the results of the present study suggest that silver ions may cause *S. aureus* and *E. coli* bacteria to reach an ABNC state and eventually die.

Since ancient times, the silver ion has been known to be effective against a broad range of microorganisms. Today, silver ions are used to control bacterial growth in a variety of medical applications, including dental work, catheters, and the healing of burn wounds (17, 30, 31). Silver ions are also used for a number of nonmedical purposes, such as in electrical appliances (14, 36). The slow-release "nanosilver" linings of laundry machines, dishwashers, refrigerators, and toilet seats are also marketed and advertised. It is clear that we are exposed to a wide range of mostly unfamiliar uses of silver-containing products intended to function as antimicrobial biocides. Therefore, it is necessary to elucidate the antimicrobial activity of the silver ion, which is widely used in these products.

The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl) groups (1, 5, 9, 10), although other target sites remain a possibility (27, 34). Amino acids, such as cysteine, and other compounds containing thiol groups, such as sodium thioglycolate, neutralized the activity of silver against bacteria (18). By contrast, disulfide bond-containing amino acids, non-sulfur-containing amino acids, and sulfur-containing compounds, such as cystathione, cysteic acid, L-methionine, taurine, sodium bisulfate, and sodium thiosulfate, were all unable to neutralize the activity of silver ions. These and other findings imply that the interaction of silver ions with thiol groups in enzymes and proteins plays an essential role in its antimicrobial action, although other cellular components, like hydrogen bonding, may also be involved (10). Silver was also proposed to act by binding to key functional groups of enzymes. Silver ions cause the release of K^+ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes, is an important target site for silver ions (9, 22, 25, 29).

In addition to their effects on bacterial enzymes, silver ions caused marked inhibition of bacterial growth and were deposited in the vacuole and cell wall as granules (6). They inhibited cell division and damaged the cell envelope and contents of bacteria (27). Bacterial cells increased in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibited structural abnormalities. Finally, silver ions interact with nucleic acids (35); they interact preferentially with the bases in DNA rather than with the phosphate groups, although the significance of this in terms of their lethal action is unclear (12, 24, 34, 37).

The following silver compounds and silver are listed in *Martindale: the Extra Pharmacopoeia*: silver metal, silver acetate, silver nitrate, silver protein, and silver sulfadiazine (26a). The silver ion can be generated by electrolyzing the silver metal or dissolving the silver compounds. It is known that the electrically generated silver ion appeared to be superior to the silver compounds in antimicrobial activity (3, 4). However, most of the aforementioned studies which determined a mechanism of action of silver used silver ions produced from silver compounds like silver nitrate or silver sulfadiazine, and thus there has been limited research on the electrically generated silver ion. Recently, a laundry machine that emits electrically gener-

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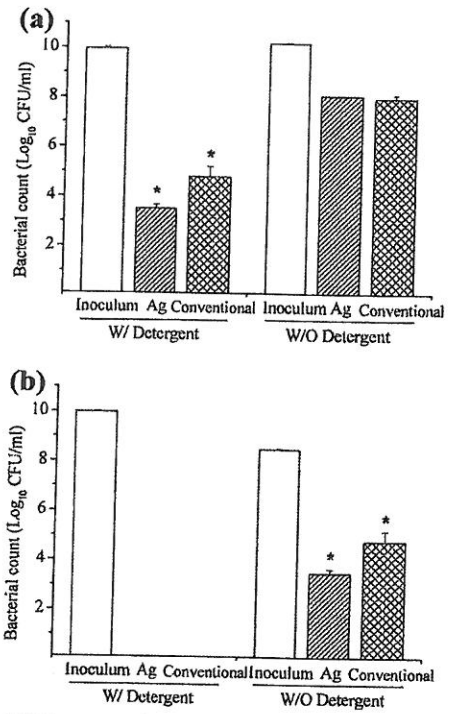


FIG. 1. Viable counts (mean ± standard error) of *Staphylococcus aureus* (a) and *Escherichia coli* (b) bacteria after washing bacteria-contaminated textile pieces using silver and conventional laundry machines with (W/) or without (W/O) detergent. Each group contained three pieces of test textiles. Inoculum, preinoculation bacterial count; Ag, result for silver laundry machine (Samsung); Conventional, result for conventional laundry machine (Samsung). Significant differences ($P < 0.05$) in viable counts of each bacteria between the silver and conventional laundry machines are denoted with asterisks.

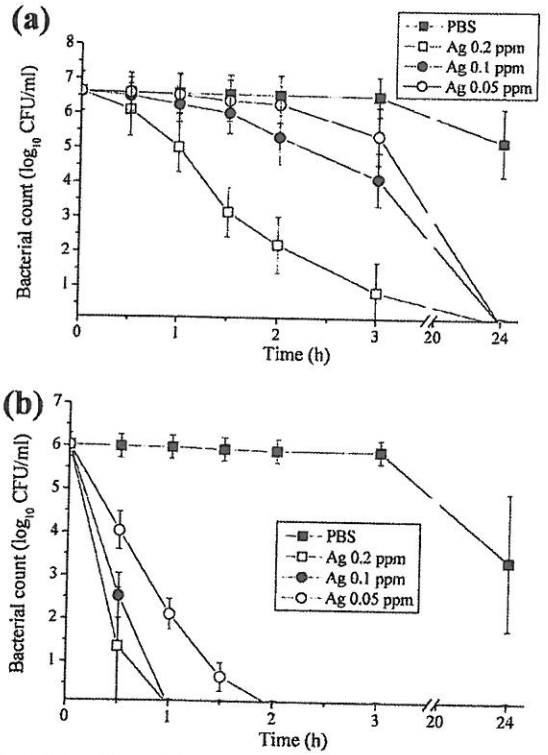


FIG. 2. The effect of the silver ion solution on *Staphylococcus aureus* (a) and *Escherichia coli* (b) was investigated by conventional plate counting. The tested silver ion concentrations were 0.2 ppm, 0.1 ppm, and 0.05 ppm, and PBS was used as a control.

ated silver ions was developed for hygiene, namely, in order to prevent easily transmissible bacterial and fungal skin infections from being transmitted by contaminated laundry. In particular, it can be beneficial to hospitals and homes in which immunocompromised people (the elderly, children, and medical patients) or pets may dwell. Our previous study demonstrated the antifungal activity of a laundry machine that electrically generates silver ions (14). In the present study, we used conventional plate counting, flow cytometry (FC), and transmission electron microscopy (TEM) to investigate the antibacterial activity and mechanism of action against *Staphylococcus aureus* and *Escherichia coli* bacteria of a silver ion solution generated from the laundry machine.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used in this study. The strains were grown in 5% sheep blood agar (Promed, Gyeonggi, Korea).

Antibacterial efficacy test of household laundry machines. A silver laundry machine (Samsung, Gyeonggi, Korea) and a conventional laundry machine (Samsung) which was the same as the silver laundry machine except for the fact that it did not emit silver ions were used as the experimental and control machines, respectively. The silver laundry machine is designed to release silver ions twice during the laundry process: once during the main washing step (for 30 min) and once during the final rinsing step (for 20 min). Powerclean Max (Oxy, Seoul, Korea) was used as the detergent.

The method for testing the antibacterial properties of household laundry machines was performed as previously described (17a) with minor revision. The bacteria were enumerated by the conventional plate count method. The test textile (100% cotton) was 5 cm × 5 cm. Three pieces of test textile were attached to the edge of a 1-m × 1-m laundry textile (100% cotton). Each test and laundry textile was autoclaved and dried, and then the test textiles were inoculated with *S. aureus* or *E. coli*. The bacteria were diluted to 10^9 to 10^{10} CFU/ml using 0.85% sterile saline. One milliliter of each adjusted bacterial culture was inoculated to the test textiles, and then textiles were washed in each laundry machine.

Two pieces of laundry textile with three pieces of test textile and 28 pieces of laundry textile without test textile, which were used to adjust the weight of the laundry to be 3 kg, were processed at the same time with or without detergent using the silver and the conventional laundry machine. The laundry textile with the test textile attached to it was taken out at the end of the laundry process. The test textile was then removed from the laundry textile and pummeled with 10 ml of sterile buffered peptone water (Becton Dickinson, Sparks, MD). The buffered peptone water rinse solution was then serially diluted with saline, and bacteria were counted using the conventional plate count method.

Silver ion preparation. A silver ion solution in phosphate-buffered saline (PBS; pH 7.4) was prepared from the silver laundry machine (Samsung), and this solution was used in all subsequent experiments (conventional plate counting, FC analysis, and TEM). The silver ions were produced from two silver plates while PBS was passed through the silver kit, which was made with polypropylene housing. The water from the tap passed through the silver kit housing and went down to the drum. Both the anode and cathode were 99.9% silver metal plates with surface areas of 12.5 cm^2, and two electrodes were installed parallel to each other with 5 mm of distance between them. The volume of the silver kit housing was 30 ml. The flow rate through the silver kit housing was regulated to be 10 liter/min, and the electric current was controlled at 80 mA by changing the input voltages from 2 to 24 V. The electric current was applied only during water supply. The concentration of the silver was determined by inductively coupled plasma mass spectrometry (ELAN 6100; Perkin-Elmer SCIEX, Norwalk, CT) at the National Center for Inter-University Research Facilities, Seoul National University, and it was approximately 0.2 ppm.

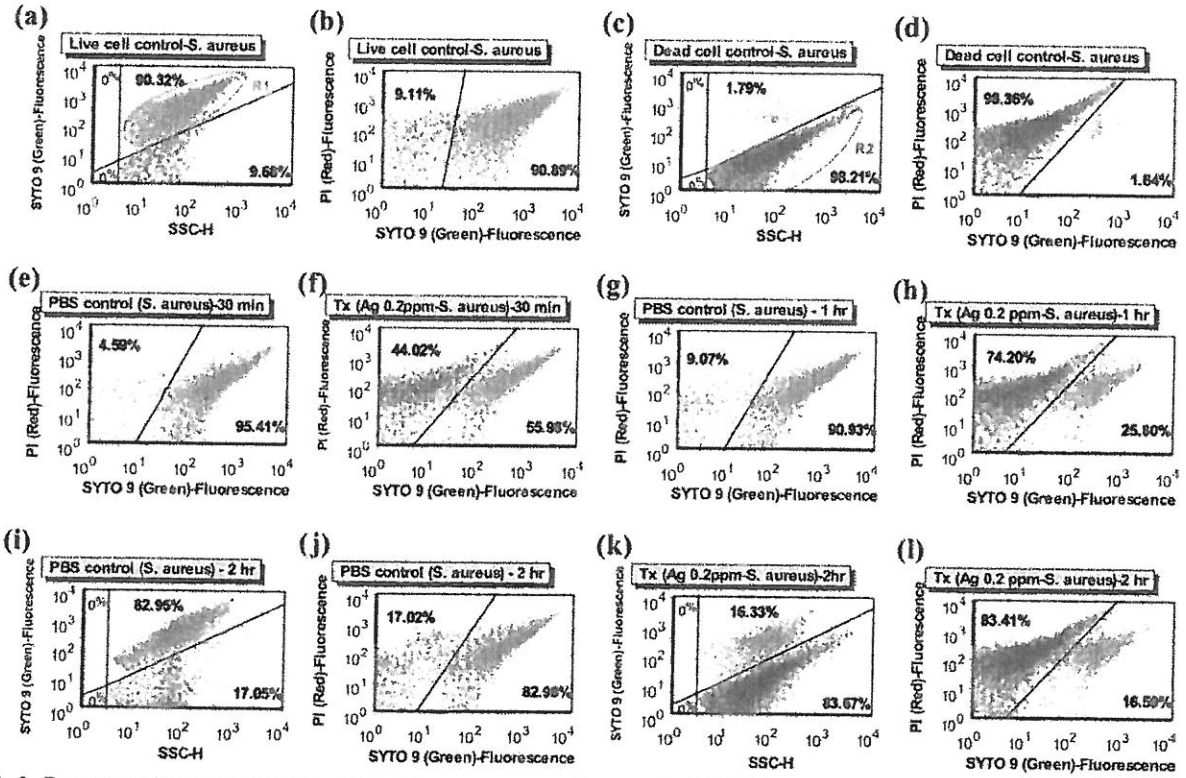


FIG. 3. Representative dot plot profiles of *Staphylococcus aureus* cells treated with PBS (e, g, i, and j) or silver ion solution (0.2 ppm) (Tx; f, h, k, and l) for 30 min, 1 h, and 2 h analyzed by FC after staining with SYTO 9 and PI. For controls, suspensions of fresh live (untreated) (a and b) and dead (70% isopropyl alcohol treated) (c and d) cells were also analyzed. The quadrants show the division between live cells in gate 1 (a; R1-green) and damaged or dead cells in gate 2 (c; R2-red) with the relative frequencies of cells in each gate before treatment with PBS or silver ion solution. All of the profiles were analyzed with gates placed on 1 and 2. SSC-H, side-scatter height.

Determination of antibacterial effect of silver ions by conventional plate counting. The silver ion solution made with PBS was autoclaved at 121°C for 15 min and tested for its antibacterial efficacy. The concentrations of silver ions tested were 0.2, 0.1, and 0.05 ppm. Ninety-nine milliliters of the test solution and 1 ml of the bacterial suspension in PBS were mixed to a final bacterial concentration of 10^5 to 10^6 CFU/ml. The mixture of solution and bacteria was incubated at 37°C with shaking and counted at 30-min intervals from 30 to 180 min and then again at 24 h using the conventional plate count method, with serial 10-fold dilutions with saline plated on plate count agar (Becton Dickinson).

FC analysis of antibacterial effect of silver ions. After the bacterial suspensions (10^5 to 10^6 CFU/ml) were treated with silver ion solution (0.2 ppm) or PBS for 30 min, 1 h, 1.5 h, 2 h, and 3 h, the bacterial cells (*S. aureus* or *E. coli*) were washed two times with PBS and resuspended in SYTO 9 and propidium iodide (PI) from a Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, Inc., Eugene, OR) (2, 28). The suspension was incubated for 15 min in the dark at room temperature. In the control group, suspensions of fresh live (untreated) and dead (70% isopropyl alcohol treated) cells were stained as described above, and the green and red fluorescence generated by SYTO 9 and PI staining, respectively, as well as the size (side scatter height) were also read by FC analysis. After reading the parameters of the live and dead cell controls, with the resulting live cells in gate 1 (R1-green) and damaged or dead cells in gate 2 (R2-red) as discriminated by FC analysis, the relative frequencies of cells in each gate before treatment with silver ion solution or PBS were determined, with all of the experimental profiles being analyzed with gates 1 and 2 by FC analysis. The green fluorescence of the SYTO 9 dyes (FL1) was collected using a 530-nm ± 30-nm band-pass filter. The red fluorescence emitted from PI (FL3) was collected using a 650-nm ± 13-nm band-pass filter. The proportions of live and dead cells were determined and analyzed by using a FACSCalibur with the CellQuest program (Becton Dickinson Immunocytometry Systems, San Jose, CA) and FCS Express software (De Novo Software, Ontario, CA), respectively.

For the enumeration of esterase-active bacteria, 900 μl of bacterial cells, which were treated with silver ion solution or PBS and washed as described above, were supplemented with 90 μl of sterile 1.0 M phosphate buffer (pH 8.0) and 10 μl of 50 mM EDTA. Then, carboxyfluorescein diacetate (CFDA; Molecular Probes, Inc.) stock solution in dimethyl sulfoxide was added to the sample at a final concentration of 10 μM, and the sample incubated at 35°C in the dark for 10 min (11). Following incubation, the cells were washed and resuspended in sterile 1.0 M phosphate buffer (pH 8.0), and esterase-active bacteria were enumerated by the enhanced-green-fluorescence intensity as determined by FC analysis. Positive-control live cells and negative-control dead cells were prepared and stained as described above.

TEM. Unstained cells of *S. aureus* and *E. coli* were observed for the presence of electron-dense precipitates by TEM. The two bacterial strains were diluted to a final concentration of 10^5 to 10^6 CFU/ml with silver ion solution (0.2 ppm) or PBS. The mixture of solution and bacteria was incubated at 37°C for 2 h with shaking, centrifuged at 1,320 × *g* for 30 min to obtain cell pellets, and then diluted with 1 ml of PBS. A drop of the mixture was placed on a glow-discharged Formvar-coated copper grid for 1 min. The excess liquid was drained off with a filter paper, and the preparation was air dried for 5 min. The specimens were examined with an energy-filtering TEM (LIBRA 120; Carl Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 120 kV. Zero-loss energy-filtered images were recorded with a 4 K slow-scan charge-coupled-device camera (4000 SP; Gatan, Pleasanton, CA).

In addition, the detailed ultrastructural changes induced by the silver ion treatment in embedded bacterial cells were examined. The cell pellets of the two bacterial strains were fixed with modified Karnovsky's fixative consisting of 2% (vol/vol) glutaraldehyde and 2% (vol/vol) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4°C for 2 h (15). They were then washed three times with the same buffer for a period of 10 min. The specimens were postfixed with 1% (wt/vol) osmium tetroxide in the same buffer at 4°C for 2 h and washed briefly with distilled water twice. The postfixed specimens were dehydrated in a graded ethanol series (once in 30, 50, 70, 80, and 95% and three times in 100% for 10 min each). The specimens were further treated with propylene oxide twice each for 10 min as a transitional fluid and then embedded in Spurr's resin (33).

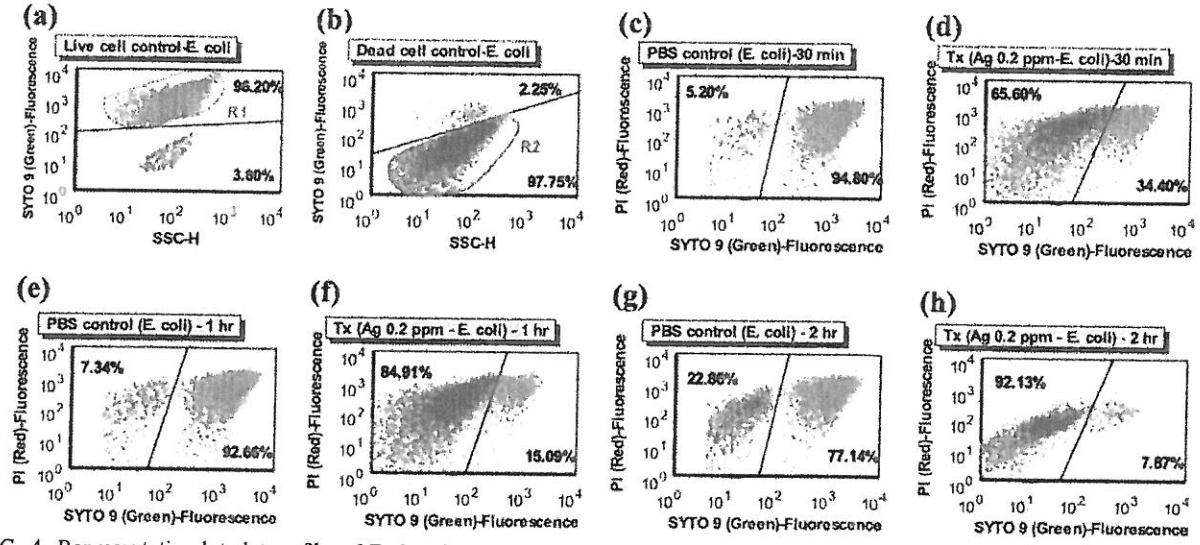


FIG. 4. Representative dot plot profiles of *Escherichia coli* cells treated with PBS (c, e, and g) or silver ion solution (0.2 ppm) (Tx; d, f, and h) for 30 min, 1 h, and 2 h analyzed by FC after staining with SYTO 9 and PI. For controls, suspensions of fresh live (untreated) (a) and dead (70% isopropyl alcohol-treated) (b) cells were analyzed. The quadrants show the division between live cells in gate 1 (a; R1-green) and damaged or dead cells in gate 2 (b; R2-red) with the relative frequencies of cells in each gate before treatment with PBS or silver ion solution. All the profiles were analyzed with gates placed on 1 and 2. SSC-H, side-scatter height.

Ultrathin sections (approximately 60-nm thickness) were cut with a diamond knife using an ultramicrotome (MT-X; RMC Inc., Tucson, AZ) and then mounted on bare copper grids. They were stained with 2% uranyl acetate and Reynolds' lead citrate (26) for 7 min each, followed by examination with the electron microscope.

Statistical analysis. The data from triplicate experiments are presented as the mean ± standard error of the mean. An unpaired *t* test analysis was performed using Origin 6.1 (OriginLab, Northampton, MA) to compare the viable bacterial counts within different samples that underwent different washing treatments (detergent and laundry machines) and to compare the viable bacterial counts between the silver ion treatment and nontreatment groups. The proportions of live or dead *E. coli* or *S. aureus* determined by FC analysis in the silver ion treatment groups treated for different periods of time (30 min, 1 h, 1.5 h, 2 h, and 3 h) were compared with those in the control (PBS) group using the Kruskal-Wallis one-way analysis of variance by rank. Significant differences in the data that originated from the same group but were determined at different times were analyzed by the Wilcoxon signed-rank test using Analyze-it software (Analyze-it Software Ltd., Leeds, United Kingdom). The level of significance was set at a P value of <0.05.

RESULTS

Antibacterial efficacy of household laundry machines. The efficacy test results of the two laundry machines against *S. aureus* and *E. coli* conducted with or without detergent are shown in Fig. 1. The *S. aureus* bacterial count was significantly reduced by the silver laundry machines with detergent in comparison to the results with the conventional laundry machine ($P < 0.05$). All of the inoculated *E. coli* bacteria were eliminated when detergent was used in both the silver and conventional laundry machines. In the absence of detergent, *E. coli* was significantly reduced by the silver laundry machine in comparison to the results with the conventional laundry machine ($P < 0.05$).

Effect of the silver ions on the bacterial reduction rate. The antibacterial effects of the silver ion solution at different concentrations of silver ions against *S. aureus* and *E. coli* bacteria as determined by the conventional plate count technique are shown in Fig. 2. The total number of *S. aureus* bacteria was reduced by over 5 $\log_{10}$ CFU/ml after treatment with the original silver ion solution (0.2 ppm) for 90 min, demonstrating that the antibacterial activity of the silver ion solution was significantly greater than that of PBS treatment ($P < 0.05$). The *E. coli* bacterial count was reduced from the inoculum size (10^5 CFU/ml) to the limit of detection (<20 CFU/ml) within 30 min at a silver ion concentration of 0.2 ppm. All of the tested silver ion solutions (0.2, 0.1, and 0.05 ppm) significantly eliminated *E. coli* cells in comparison to PBS treatment ($P < 0.05$).

FC analysis in conjunction with a *Bac*Light kit was also performed to examine the antibacterial effect of the original silver ion solution (0.2 ppm) against *S. aureus* and *E. coli* bacteria in terms of damage to the cell membrane, shown in different colors (green in live cells and red in damaged or dead cells). In addition, CFDA staining was used for the enumeration of esterase-active bacteria because CFDA is cell permeant and undergoes hydrolysis of the diacetate groups into fluorescent carboxyfluorescein by intracellular nonspecific esterases. Based on the side light scatter and green (FL1) fluorescence, the R1 and R2 gates were used to identify live and damaged or dead cells, respectively. The proportions of damaged or dead cells (both *S. aureus* and *E. coli*) in the silver ion solution-treated groups were significantly greater ($P < 0.05$) at 30 min, 1 h, 1.5 h, and 2 h of treatment than with the control (PBS) groups (Fig. 3 and 4). Longer treatment times (from 30 min to 2 h) had a positive effect on the antibacterial effect of the silver ion solution ($P < 0.05$); however, there were no significant differences in the proportions of live or dead cells when both *S. aureus* and *E. coli* cells were treated with the silver ion solution for 2 or 3 h ($P > 0.05$).

The antibacterial-efficacy results determined by conventional plate count and FC analyses are compared in Fig. 5. For

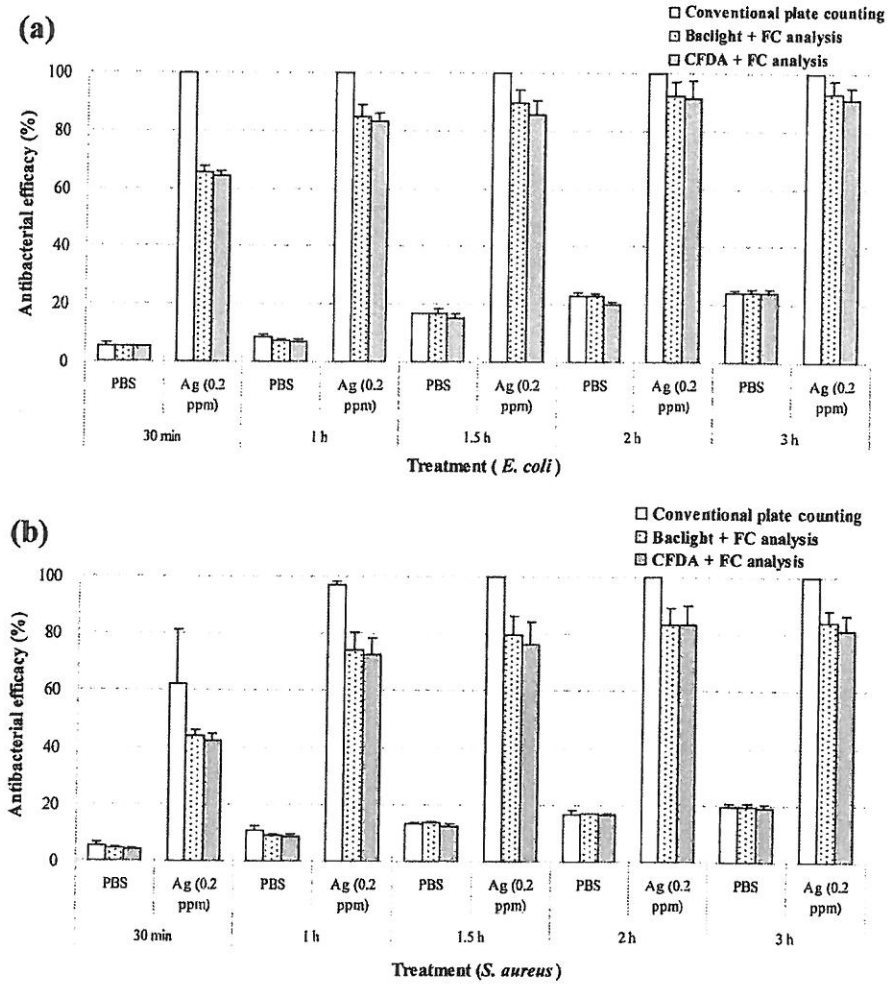


FIG. 5. Comparative analysis of the antibacterial efficacy of the silver ion solution (0.2 ppm) against *Staphylococcus aureus* (a) and *Escherichia coli* (b) bacteria as determined using the conventional plate count method and FC analysis. PBS was used as a control. Antibacterial efficacy was calculated using the following formula: antibacterial efficacy = $[(A - B)/A] \times 100$, where A is the preinoculation bacterial count (CFU/ml) and B is the bacterial count after treatment with silver ion solution or PBS (CFU/ml).

the PBS-treated control group and silver ion-treated experimental groups tested, both the *Bac*Light kit and the CFDA assay gave similar antibacterial efficacies ($P > 0.05$). The number of physiologically active bacteria enumerated by FC analysis in conjunction with the *Bac*Light kit or the CFDA assay was relatively higher than the bacterial count determined by conventional plate counting ($P < 0.05$), except for *E. coli* bacteria after 2 and 3 h of treatment. This difference appeared to be nonlinear across different treatment times, suggesting that the difference in antibacterial efficacy determined by the two analyses decreased as the silver ion treatment times approached 2 and 3 h.

Morphological changes in *S. aureus* and *E. coli* cells after silver ion treatment. TEM analysis of unstained bacteria showed the external morphological features of the two bacterial strains. The untreated *S. aureus* cells retained their coccal morphology (ca. 600 nm in diameter) and seemed to be normal (Fig. 6a). In contrast, *S. aureus* cells treated with the silver ion solution for 2 h appeared to undergo lysis, resulting in the release of their cellular contents into the surrounding environment, and finally became disrupted (Fig. 6b to d). It was common to find electron-dense particles or precipitates around damaged bacterial cells that were electron translucent in comparison to undamaged cells. In cross section, the untreated cells of *S. aureus* showed normal cell characteristics and homogeneous electron density in the cytoplasm. Their cell walls and membranes were intact, showing a well-preserved peptidoglycan layer and cytoplasmic membrane (Fig. 7a and b). However, significant morphological changes were observed in *S. aureus* cells treated with the silver ion solution. They showed lysed cells with broken walls and membranes and decreases and heterogeneity in electron density in the cytoplasm (Fig. 7c and d). The localized separation of the cell membrane from the cell wall could be discerned.

E. coli cells diluted in PBS showed normal morphology having many filaments, such as flagella and fimbriae (Fig. 8a). The fimbriae were peritrichous, approximately 7 nm wide, and up to 900 nm long. Meanwhile, the bacterial cells after silver ion

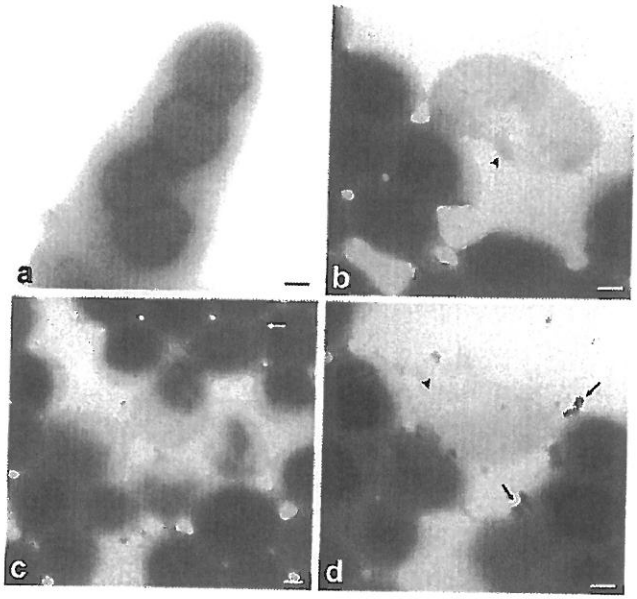


FIG. 6. External morphology of unstained *Staphylococcus aureus* cells observed by TEM. (a) Untreated bacteria. (b, c, and d) Bacteria treated with silver ion solution (0.2 ppm). Electron-dense particles were found around damaged cells (arrows). Note the release of cellular contents (arrowheads).

treatment for 2 h appeared to be seriously damaged (Fig. 8b to d). The cells showed aberrant morphology; they were cracked and ruptured. Electron-dense particles or precipitates were also observed around damaged bacterial cells. The internal

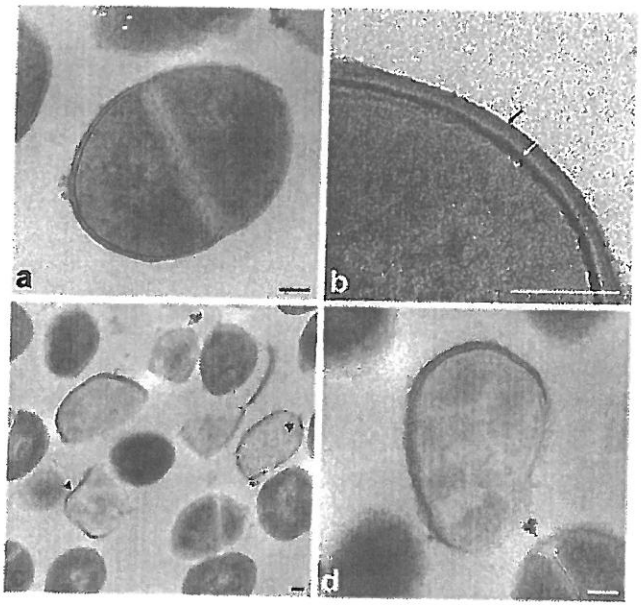


FIG. 7. Internal structure of *Staphylococcus aureus* observed by TEM. (a and b) Untreated bacteria. (c and d) Bacteria treated with silver ion solution (0.2 ppm). Black and white arrows indicate peptidoglycan layer and cytoplasmic membrane, respectively. Note the separation of cell membrane from the cell wall (arrowheads).

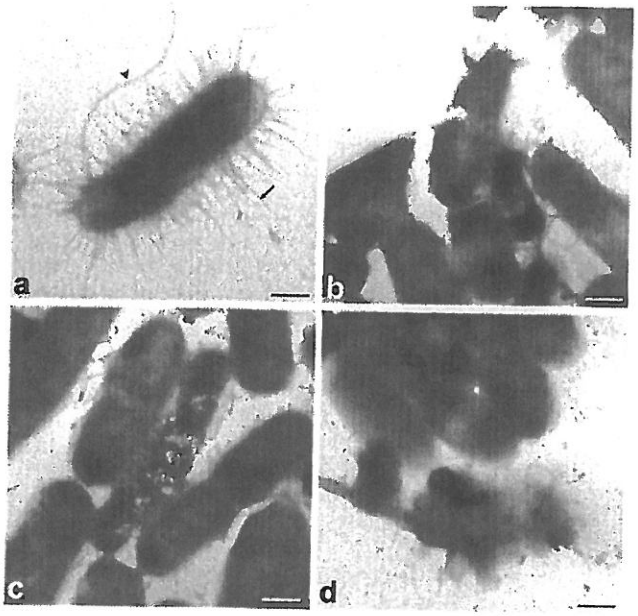


FIG. 8. External morphology of unstained *Escherichia coli* observed by TEM. (a) Untreated bacteria. An arrow and an arrowhead indicate fimbriae and a flagellum, respectively. (b, c, and d) Bacteria treated with silver ion solution (0.2 ppm).

structure of the untreated *E. coli* cells appeared to be normal, showing a multilayered cell surface consisting of an outer membrane, a peptidoglycan layer in the periplasmic space, and a cytoplasmic membrane (Fig. 9a and b). Damaged cells showed either localized or complete separation of the cell membrane from the cell wall (Fig. 9c). The cellular degradation was also accompanied by electron-translucent cytoplasm and cellular disruption in the damaged cells (Fig. 9d).

DISCUSSION

The electrically generated silver ion solution exhibited good bactericidal efficacy against *S. aureus* and *E. coli* both in experiments using the silver laundry machine with contaminated fabric and in those using the silver ion suspension generated from the silver laundry machine. The efficacy of the silver ion solution showed better activity against the gram-negative *E. coli* than against the gram-positive *S. aureus*. This was possibly due to the thickness of the peptidoglycan layer, which may prevent the action of the silver ions through the bacterial cell wall, and this result was consonant with the results of other studies (8, 23). Although the *S. aureus* and *E. coli* bacteria were effectively eliminated from the contaminated fabric by the silver washing course, it was not confirmed that the silver ions killed the bacteria. It is possible that the bacteria were removed from the fabric by the washing course. Therefore, the antibacterial effect of the silver ions was confirmed by the conventional plate count, FC, and TEM analyses in this study.

The number of bacteria determined by conventional plate counting, which counts only culturable colonies in media, was significantly lower than the number determined by FC analysis,

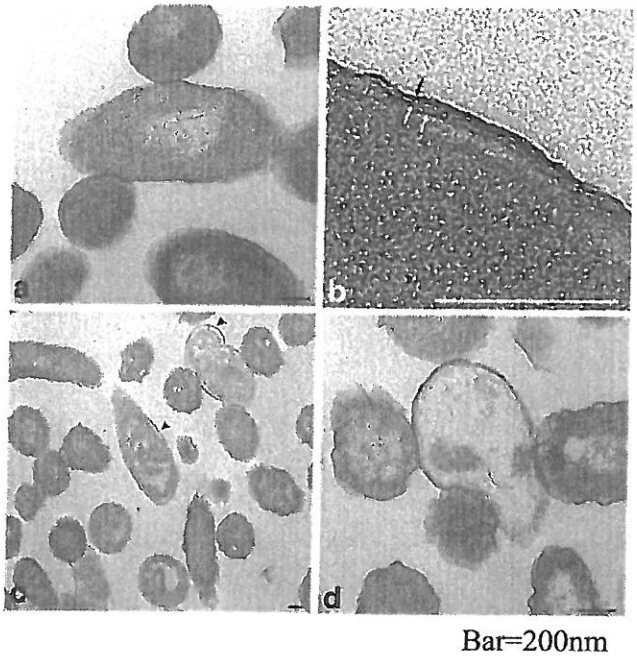


FIG. 9. Internal structure of *Escherichia coli* observed by TEM. (a and b) Untreated bacteria. (c and d) Bacteria treated with silver ion solution (0.2 ppm). Arrows indicate outer membrane, peptidoglycan layer, and cytoplasmic membrane from the outside of the cell. Arrowheads indicate separation of the cell membrane from the cell wall.

suggesting that the cell membrane and intracellular esterase activity of the bacteria treated with the silver ion solution might be damaged. Bacteria in the environment are exposed to various conditions that lead to survival stress. To counter this condition, some bacteria are capable of maintaining metabolic activity while developing recalcitrance to culture. Such a state in bacteria is often defined as an "active but nonculturable (ABNC)" state, a state in which the bacteria exhibit measurable traits of physiological activity but fail to grow to a detectable level (16). A state of ABNC or sublethal injury of bacteria seems to be induced by exposure to silver ions, thus rendering bacteria nonculturable in media (7, 21). This may serve as a possible explanation for the discrepancy in the results determined by the two methods used in this study, and this observation is consistent with the findings of other studies (11, 13). This finding may be expected because bacteria previously exposed to environmental stresses may only be able to divide a limited number of times, which would give a positive result in the FC analysis, but they would be unable to produce visible colonies on solid media.

The differences between the results of the conventional plate count and FC analyses were nonlinear, and the difference rate between the results of the two methods was reduced as time progressed. The reason for this aspect might be that the bacteria in the ABNC state started to die after 2 h of treatment with the silver ions.

Similar phenomena were also observed in the silver ion-treated cells of *S. aureus* and *E. coli* by the TEM studies. Following the silver ion treatment, the cytoplasm membrane shrank and became separated from the cell wall. Cellular contents were then released from the cell wall, and the cell wall was degraded. These phenomena suggest possible antibacterial mechanisms by which silver ions inhibit bacterial growth, as well as cellular responses of both the gram-positive and gram-negative bacteria to the silver ion treatment. Although the mechanisms underlying the antibacterial actions of silver are still not fully understood, several previous reports (20, 23, 32) showed that the interaction between silver and the constituents of the bacterial membrane caused structural changes and damage to the membranes and intracellular metabolic activity which might be the cause or consequence of cell death, as demonstrated in this study. Analytical electron microscopy remains to be done to identify the elemental composition of the electron-dense particles or precipitates around damaged bacterial cells. In conclusion, the results of the present study clearly show that the electrically generated silver ion solution exerts its antibacterial effect by inducing bacteria into a state of ABNC, in which the mechanisms required for the uptake and utilization of substrates leading to cell division were disrupted at the initial stage and caused the cells to undergo morphological changes and die at the later stage. These findings suggest that the use of the silver ion solution may have valuable applications in various fields, such as the manufacture of household appliances and medical devices.

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REFERENCES

1. **Belly, R. T., and G. C. Kydd.** 1982. Silver resistance in microorganisms. Dev. Ind. Microbiol. **23**:567–577.
2. **Ben-Amor, K., H. Heilig, H. Smidt, E. E. Vaughan, T. Abee, and W. M. de Vos.** 2005. Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. Appl. Environ. Microbiol. **71**:4679–4689.
3. **Berger, T. J., J. A. Spadaro, R. Bierman, S. E. Chapin, and R. O. Becker.** 1976. Antifungal properties of electrically generated metallic ions. Antimicrob. Agents Chemother. **10**:856–860.
4. **Berger, T. J., J. A. Spadaro, S. E. Chapin, and R. O. Becker.** 1976. Electrically generated silver ions: quantitative effects on bacterial and mammalian cells. Antimicrob. Agents Chemother. **9**:357–358.
5. **Bragg, P. D., and D. J. Rainnie.** 1974. The effect of silver ions on the respiratory chain of *Escherichia coli*. Can. J. Microbiol. **20**:883–889.
6. **Brown, T., and D. Smith.** 1976. The effects of silver nitrate on the growth and ultrastructure of the yeast *Cryptococcus albidus*. Microbios Lett. **3**:155–162.
7. **du Preez, M., R. Kfir, and P. Coubrough.** 1995. Investigation of injury of coliforms after chlorination. Water Sci. Technol. **31**:115–118.
8. **Feng, Q. L., J. Wu, G. Q. Chen, F. Z. Cui, T. N. Kim, and J. O. Kim.** 2000. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J. Biomed. Mater. Res. **52**:662–668.
9. **Fuhrmann, G. F., and A. Rothstein.** 1968. The mechanism of the partial inhibition of fermentation in yeast by nickel ions. Biochim. Biophys. Acta **163**:331–338.
10. **Furr, J. R., A. D. Russell, T. D. Turner, and A. Andrews.** 1994. Antibacterial activity of Actisorb Plus, Actisorb and silver nitrate. J. Hosp. Infect. **27**:201–208.
11. **Hoefel, D., W. L. Grooby, P. T. Monis, S. Andrews, and C. P. Saint.** 2003. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. J. Microbiol. Methods **55**: 585–597.
12. **Izatt, R. M., J. J. Christensen, and J. H. Rytting.** 1971. Sites and thermo-

dynamic quantities associated with proton and metal ion interaction with ribonucleic acid, deoxyribonucleic acid, and their constituent bases, nucleosides, and nucleotides. Chem. Rev. **71**:439–481.
13. **Joux, F., and P. Lebaron.** 1997. Ecological implications of an improved direct viable count method for aquatic bacteria. Appl. Environ. Microbiol. **63**: 3643–3647.
14. **Jung, W. K., S. H. Kim, H. C. Koo, S. Shin, J. M. Kim, Y. K. Park, S. Y. Hwang, H. Yang, and Y. H. Park.** 2007. Antifungal activity of the silver ion against contaminated fabric. Mycoses **50**:265–269.
15. **Karnovsky, M. J.** 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. **27**:137–138.
16. **Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer.** 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek **73**:169–187.
17. **Klasen, H. J.** 2000. Historical review of the use of silver in the treatment of burns. I. Early uses. Burns **26**:117–130.
17a. **Korea Testing and Research Institute.** 2001. SC9608-01. The method for testing the antibacterial properties of a household laundry machine. Korea. Testing and Research Institute, Seoul.
18. **Liau, S. Y., D. C. Read, W. J. Pugh, J. R. Furr, and A. D. Russell.** 1997. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett. Appl. Microbiol. **25**:279–283.
19. Reference deleted.
20. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. **12**:147–179.
21. **McFeters, G. A., M. W. LeChevallier, A. Singh, and J. S. Kippin.** 1986. Health significance and occurrence of injured bacteria in drinking water. Water Sci. Technol. **18**:227–231.
22. **Miller, L. P., and S. E. A. McCallan.** 1957. Toxic action of metal ions to fungus spores. Agric. Food Chem. **5**:116–122.
23. **Pal, S., Y. K. Tak, and J. M. Song.** 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*. Appl. Environ. Microbiol. **73**: 1712–1720.
24. **Rahn, R. O., and L. C. Landry.** 1973. Ultraviolet irradiation of nucleic acids complexed with heavy atoms. II. Phosphorescence and photodimerization of DNA complexed with Ag. Photochem. Photobiol. **18**:29–38.
25. **Rayman, M. K., T. C. Lo, and B. D. Sanwal.** 1972. Transport of succinate in Escherichia coli. II. Characteristics of uptake and energy coupling with transport in membrane preparations. J. Biol. Chem. **247**:6332–6339.
26. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
26a. **Reynolds, J. E. F.** 1993. Martindale extra pharmacopoeia, p. 201, 1412. Pharmaceutical Press, London, England.
27. **Richards, R. M. E., H. A. Odelola, and B. Anderson.** 1984. Effect of silver on whole cells and spheroplasts of a silver resistant *Pseudomonas aeruginosa*. Microbios **39**:151–158.
28. **Sachidanandham, R., K. Y. Gin, and C. L. Poh.** 2005. Monitoring of active but non-culturable bacterial cells by flow cytometry. Biotechnol. Bioeng. **89**:24–31.
29. **Schreurs, W. J., and H. Rosenberg.** 1982. Effect of silver ions on transport and retention of phosphate by *Escherichia coli*. J. Bacteriol. **152**:7–13.
30. **Silver, S., and L. T. Phung.** 1996. Bacterial heavy metal resistance: new surprises. Annu. Rev. Microbiol. **50**:753–789.
31. **Slawson, R. M., M. I. Van Dyke, H. Lee, and J. T. Trevors.** 1992. Germanium and silver resistance, accumulation, and toxicity in microorganisms. Plasmid **27**:72–79.
32. **Sondi, I., and B. Salopek-Sondi.** 2004. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J. Colloid Interface Sci. **275**:177–182.
33. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
34. **Thurman, R. B., and C. P. Gerba.** 1989. The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. CRC Crit. Rev. Environ. Control **18**:295–315.
35. **Yakabe, Y., T. Sano, H. Ushio, and T. Yasunaga.** 1980. Kinetic studies of the interaction between silver ion and deoxyribonucleic acid. Chem. Lett. **4**:373–376.
36. **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.
37. **Zavriev, S. K., L. E. Minchenkova, M. Vorlickova, A. M. Kolchinsky, M. V. Volkenstein, and V. I. Ivanov.** 1979. Circular dichroism anisotrophy of DNA with different modifications at N7 of guanine. Biochim. Biophys. Acta **564**: 212–224.