

A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*

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Abstract: To investigate the mechanism of inhibition of silver ions on microorganisms, two strains of bacteria, namely Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Staphylococcus aureus* (*S. aureus*), were treated with AgNO₃ and studied using combined electron microscopy and X-ray microanalysis. Similar morphological changes occurred in both *E. coli* and *S. aureus* cells after Ag⁺ treatment. The cytoplasm membrane detached from the cell wall. A remarkable electron-light region appeared in the center of the cells, which contained condensed deoxyribonucleic acid (DNA) molecules. There are many small electron-dense granules either surrounding the cell wall or depositing inside the cells. The existence of elements of silver and sulfur in the

electron-dense granules and cytoplasm detected by X-ray microanalysis suggested the antibacterial mechanism of silver: DNA lost its replication ability and the protein became inactivated after Ag⁺ treatment. The slighter morphological changes of *S. aureus* compared with *E. coli* recommended a defense system of *S. aureus* against the inhibitory effects of Ag⁺ ions. © 2000 John Wiley & Sons, Inc. *J Biomed Mater Res*, 52, 662–668, 2000.

Key words: silver ions; antibacterial mechanism; DNA molecule; morphological changes; transmission electron microscopy

INTRODUCTION

Silver ions have long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities.¹ Some forms of silver have been demonstrated to be effective against burns, severe chronic osteomyelitis, urinary tract infections, and central venous catheter infections.^{2–5}

In one of our previous works, the antibacterial ceramics based on hydroxyapatite (HA) were successfully prepared in a wet chemical process with additions of AgNO₃.⁶ It has been proven that HA coatings on implant materials treated with silver exhibited excellent antibacterial effects.⁷ It is also reported that the incorporation of Ag⁺ ions into micro-porous HA coatings can be used as bioactive delivery systems for the slow release of antibiotics.⁸ Several proposals have been developed to explain the inhibitory effects of Ag⁺ ions on bacteria. It is generally believed that heavy

metals react with proteins by combining the SH groups, which leads to the inactivation of the proteins.⁹ Recent microbiological and chemical experiments implied that interaction of Ag⁺ with thiol groups played an essential role in bacterial inactivation.¹⁰ It is revealed that bulk silver in an oxygen-charged aqueous media catalyzes the complete destructive oxidation of microorganisms.¹¹ Silver and hydrogen peroxide acted synergistically on the viability of *E. coli* K-12. It appears that the combined toxic effect of silver and hydrogen peroxide may be related with damage to cellular proteins.¹² However, the mechanism of antimicrobial effects of silver is still not fully understood. The effects of silver ions on bacteria may be complicated; however, direct observation of the morphological and structural changes may provide useful information for understanding the comprehensive antibacterial effects and the process of inhibition of silver ions.

MATERIALS AND METHODS

Strains and silver ion treatment

Two experimental strains, Gram-negative *Escherichia coli* (*E. coli*, ATCC 23282) and Gram-positive *Staphylococcus au-*

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reus (*S. aureus*, ATCC 35696), were selected. *E. coli* is a widespread intestinal parasite of mammals,¹³ and *S. aureus* occurs on the body surface of mammals.¹⁴ They were cultivated at 37°C in a liquid LB medium at 200 rpm in a rotary shaker for 16 h. After 10 µg/mL AgNO₃ was added to the medium, cultivation was continued for 4–12 h. Culture with no AgNO₃ treatment served as control. 5 mL culture broth was centrifuged and washed with distilled water, and the biomass was subjected to Transmission Electron Microscopy (TEM).

TEM sample preparation

The collected control and Ag⁺ treated cells were fixed with 2% glutaraldehyde and 1% osmium tetroxide at room temperature. After eliminating the remaining glutaraldehyde and osmium tetroxide, the dehydration process was conducted with 30, 50, 70, 80, 95, and 100% of alcohol. The fixed cells were embedded with Epon, and small blocks of bacteria in the Epon were cut with an ultramicrotome (Leica ultracut). Ultrathin sections were then positively stained with uranylacetate and lead citrate for TEM observation.

TEM and X-ray microanalysis

To directly observe the morphological changes of the internal structure of bacterial cells after silver ion treatment, TEM and X-ray microanalysis were employed. Pictures

ranging from 9,000–90,000× in magnification were taken with a CM120 TEM. X-ray microanalysis was performed using a Hitachi H800 TEM equipped with an energy-dispersive X-ray system.

RESULTS

Morphological changes of *E. coli* after Ag⁺ treatment

Figure 1 shows the internal structure of the untreated *E. coli* cells. It is clear that the cells show unanimous electron density, suggesting that the cells are in a normal condition without environment disturbance. DNA molecules, the electron-light material in the TEM picture [arrow in Fig. 1(b)], distributed randomly in almost all parts of the cells.

Significant morphological changes occurred in *E. coli* cells after the addition of Ag⁺ (Fig. 2). Figure 2(a) gives an overview of the silver treated cells, while Figs. 2(b)–(f) are enlarged magnifications of them. A remarkable electron-light region [arrow in Fig. 2(a)] often appears in the center of the *E. coli* cells treated with Ag⁺. Some tightly condensed substances were clearly visible in the center of the electron-light region. They were twisted together in the center of the electron-light region, like a twisted string [arrow in Fig. 2(b)].

A big gap exists between the cytoplasm membrane and the cell wall of the silver-treated *E. coli* cells [arrow in Fig. 2(c)]. Compared with the normal cytoplasm membrane shown in Fig. 1, detachment of the cytoplasm membrane

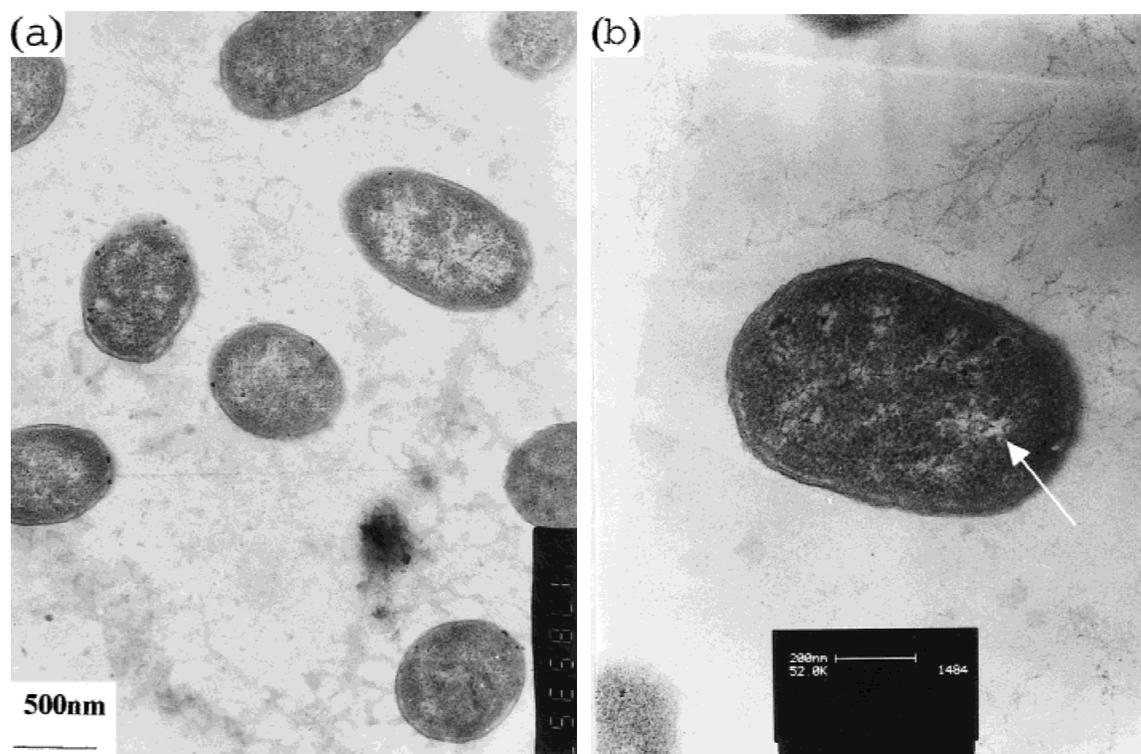


Figure 1. Internal structure of the untreated *E. coli* cells.

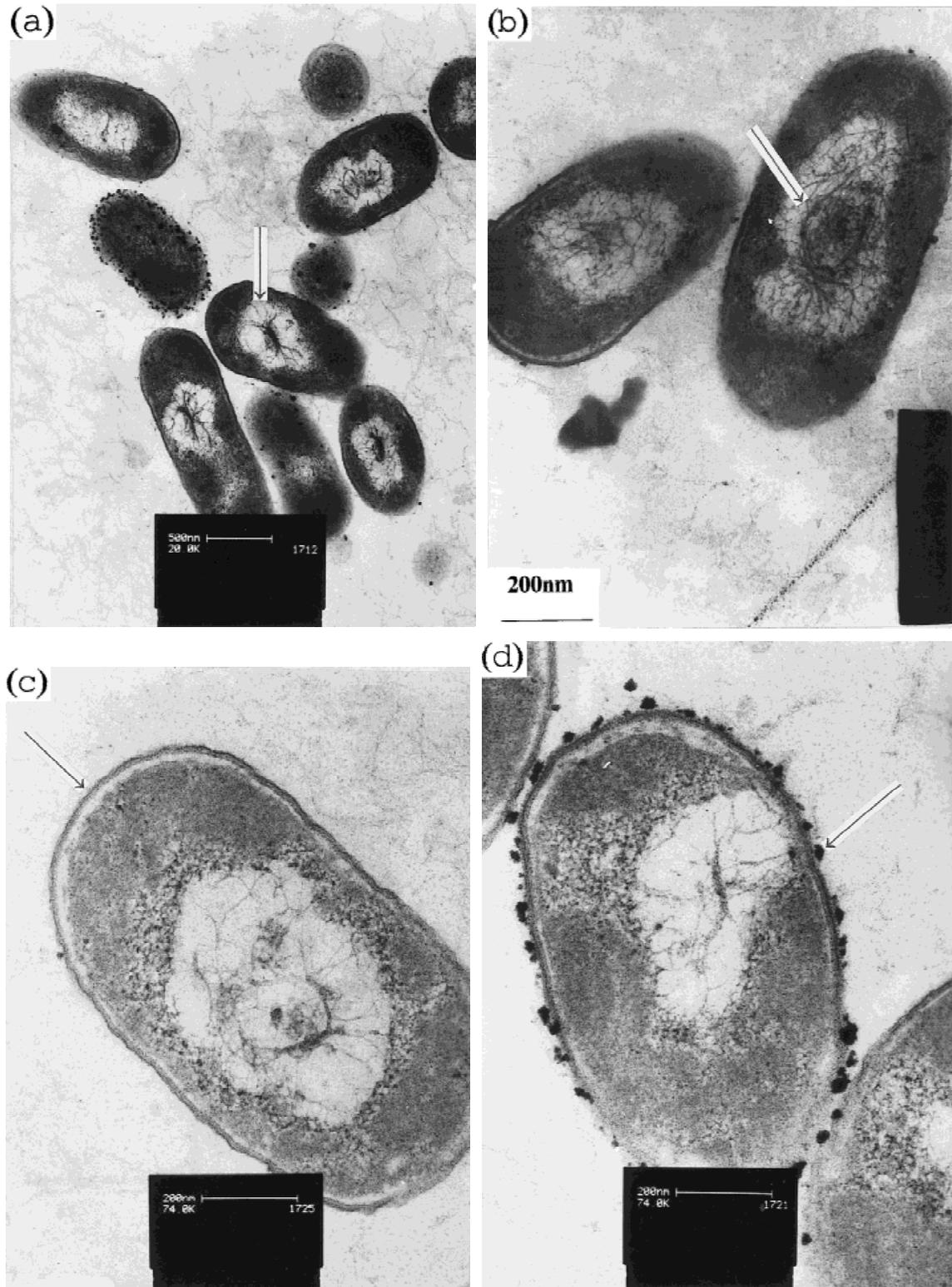


Figure 2. Internal structure of the silver ion treated *E. coli* cells. (a) A remarkable electron-light region (arrow) in the center of the cell. (b) Condensed form of DNA (arrow) in the center of the electron-light region. (c) A big gap between the cytoplasm membrane and the cell wall (arrow). (d) Electron-dense granules around the cell wall (arrow). (e) A cell composed of a great amount of large electron-dense granules. (f) The cell wall was seriously damaged (arrow).

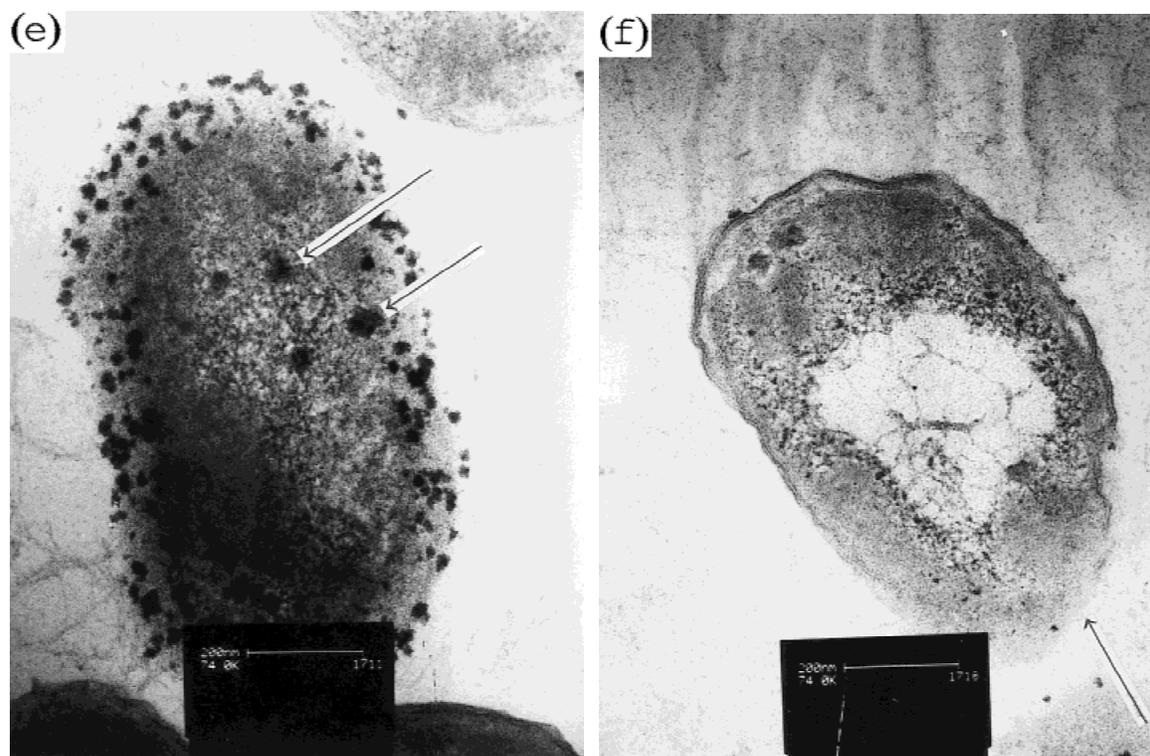


Figure 2. (Continued).

from the cell wall may be attributed to the effect of silver ions.

There are some electron-dense granules around the cell wall, seen clearly in Fig. 2(d) (arrow). These granules also existed in the cytoplasm [Fig. 2(a)–(f)], but there was no electron-dense granule in the electron-light region. It is very interesting to observe that a large amount of electron-dense granules appears to surround the electron-light region, but not within the region. This may indicate that the electron-light region has a function of preventing the electron-dense granules from entering into it. In contrast to this, no electron-dense granule was found in the control samples.

However, a contradictory phenomenon is shown in Fig. 2(e). A cell composed of a great amount of pervasively large electron-dense granules was observed to have no electron-light region and condensed DNA molecules. There is no cell wall at all in this cell. It is a cell just like a region containing a large amount of electron-dense granules and cytoplasm, which may be a characteristic form of the last life stage of the silver treated cells: homogeneous again with only the silver combined material.

In some cells, the cell walls were seriously damaged [Fig. 2(f)]. Inside the cell there are many granules around the electron-light region.

X-ray microanalysis of the small electron-dense granules and the cytoplasm outside the electron-light region [Figs. 3(a), 3(b)] showed a significant amount of silver and sulfur. It can be assumed that silver ions entered the cells and combined some cell components containing sulfur.

Morphological changes of *S. aureus* after Ag^+ treatment

S. aureus is a typical Gram-positive bacterium, which has a thicker cell wall compared to *E. coli*, a typical gram-

negative bacteria. Figure 4 shows normal *S. aureus* cells. There is a comparatively apparent nuclear region in the center of the cells in contrast to *E. coli* cells, in which DNA molecules distribute randomly.

After Ag^+ treatment, similar morphological changes occur in *S. aureus* (Fig. 5). An electron-light region appeared after Ag^+ treatment [long arrow in Fig. 5(a)], the condensed substances positioned in the center of the electron-light region [short arrow in Fig. 5(a)] exactly the same as the *E. coli* cells. The cytoplasm membrane shrank and detached from the cell wall slightly [arrow in Fig. 5(b)] compared with *E. coli*. X-ray microanalysis confirmed the existence of silver and sulfur in the cytoplasm [Fig. 6(a)]. A large amount of phosphorus was detected in the condensed region in the middle of the cells [Fig. 6(b)]. From that, we propose it to be the condensed form of DNA molecules, while phosphorus is a primary component of DNA molecules.

Besides the similar morphological changes between these two typical types of bacteria, minor differences were also observed between *S. aureus* and *E. coli* after identical treatment. *S. aureus* remained integral, the amounts of the electron-dense granules inside the cells were smaller, and the electron-light region was comparatively darker than that of *E. coli*. All these phenomena suggested that *S. aureus* may have a stronger defense system against silver ions.

DISCUSSION

The same phenomenon occurred in the Ag^+ treated cells of typical Gram-negative *E. coli* cells and typical Gram-positive *S. aureus* cells, That is: the cytoplasm membrane shrank or detached from the cell wall; an

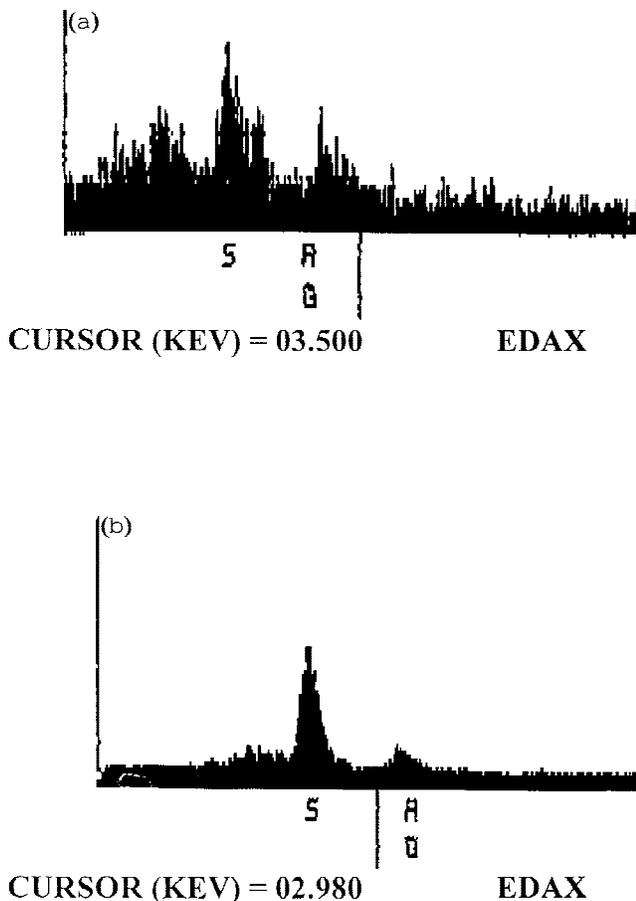


Figure 3. X-ray microanalysis on the electron-dense granules (a) and the cytoplasm outside the electron-light region (b) of the silver ion treated *E. coli* cells.

electron-light region appeared in the center of the cells, with condensed DNA molecules positioned in the center of it; and silver ions were detected inside the cells. All these phenomena suggested possible mechanisms of silver ions to attack bacteria, as well as a general defense system of both the Gram-negative and Gram-positive bacteria against silver ions.

DNA is the most important genetic information stored in the cells. Any harm to DNA may lead to either mutation or death of an organism. Naturally, some cells act to protect DNA molecules from harm. The electron-light region [Fig. 2(a)] may provide such a function to protect DNA molecules. The evidence of silver ions in the electron-dense granules [Fig. 3(a)] and almost no electron-dense granules in the electron-light region may explain the protective function of the electron-light region on DNA. However, until now, we did not know how this electron-light region formed, and what the major components, except for DNA molecules, were in the electron-light region.

Nover et al.¹⁵ reported that, against a harmful heat stimulus to the living cells, some small molecular weight proteins were produced and conglomerated surrounding the nuclear region. Although heat shock may be different than silver ions in interfering with

the cells, they are both attacks from the outer environment for microorganisms. So it is possible that some of the stimulated proteins produced by the cells after the attack of silver ions conglomerate, surrounding the nuclear region to protect DNA molecules, as shown in Fig. 2(b). However, if the attack is so harmful that the exigent response cannot operate effectively, the electron-light region, or even the cell wall, may collapse and the electron-dense granules pervade the cell, as the cell shown in Fig. 2(e).

It is known that the replication of DNA molecules is effectively conducted only when DNA molecules are in a relaxed state. In a condensed form, DNA molecules lose their replicating abilities, as was confirmed by the present experiment. During continuous cultivation by inoculating the silver-treated cells into a fresh liquid LB medium, no cell growth or multiplication was observed (results not shown). This experiment demonstrated one possible antibacterial mechanism of silver: the condensed form of DNA loses its replicating ability.

The existence of sulfur in the EDAX spectrum [Figs. 3(a), 3(b), and 6(a)] supported another possible antibacterial mechanism of silver ions by interacting with thiol groups in proteins and inactivating the enzyme activity. Thiol group is an important group of proteins responsible for enzymatic activity. It was reported that heavy metals reacted with proteins by combining the thiol groups, which leads to the inactivation of the proteins.¹⁰ On the other hand, silver is a kind of heavy metal that can cause the deposition of proteins *in vitro*. Therefore, the entrance of silver into bacterial cells may lead to the deposition of proteins in cells. Considering this, the small electron-dense granules outside the electron-light region should be a combination of silver and the deposition of proteins.

The slight morphological changes of *S. aureus* may be due to its structural character. Gram-positive and Gram-negative cells differ markedly in their cell walls. Obviously, the peptidoglycan in the cell walls of Gram-positive cells is much thicker than that in the Gram-negative ones. The thicker cell wall of *S. aureus* is of immense practical importance in protecting the cell from penetration of silver ions into the cytoplasm.

The present study gives a suggestion for the practical use of antimicrobial implants. There is an antibacterial effect only when silver ions can release from the implants. The released silver ions penetrate the cell wall and enter into the cells, subsequently turning DNA into a condensed form, which at the same time reacts with proteins. All these phenomena lead to the damage or even the death of the microorganisms (Fig. 2). However, the broad use of silver as a powerful clinical tool against infections is still in the future, because its full range of activity remains to be elucidated.

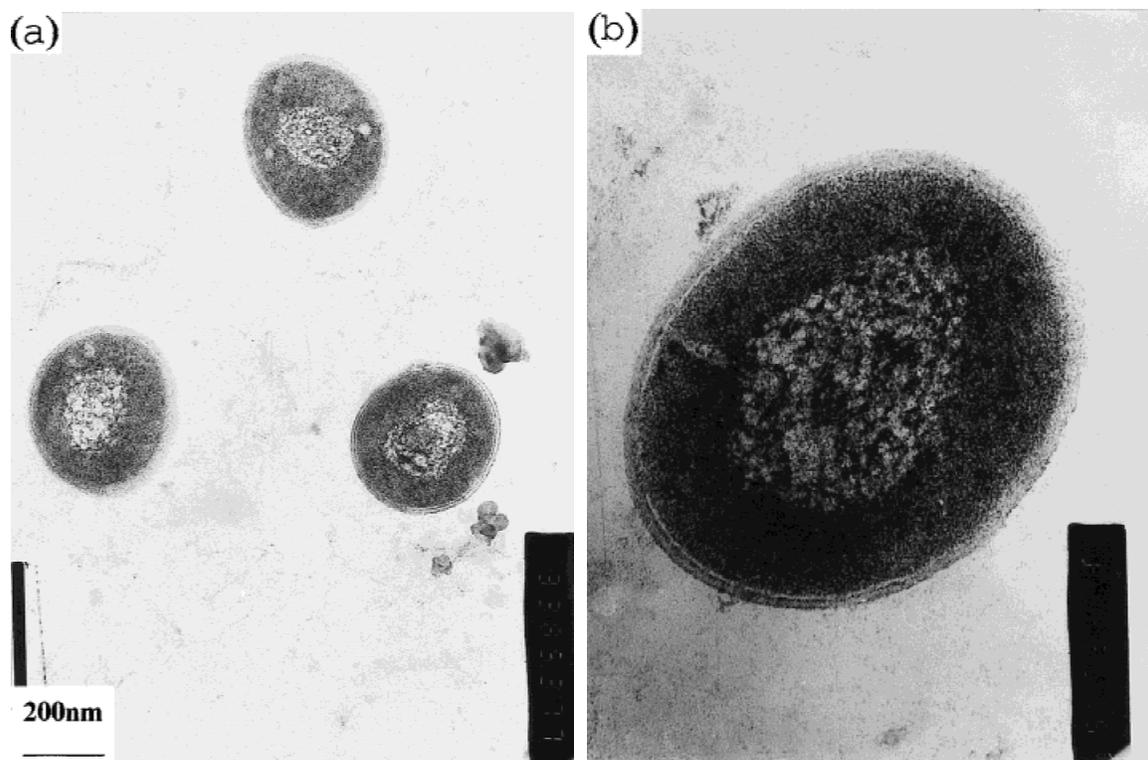


Figure 4. Internal structure of the untreated *S. aureus* cells.

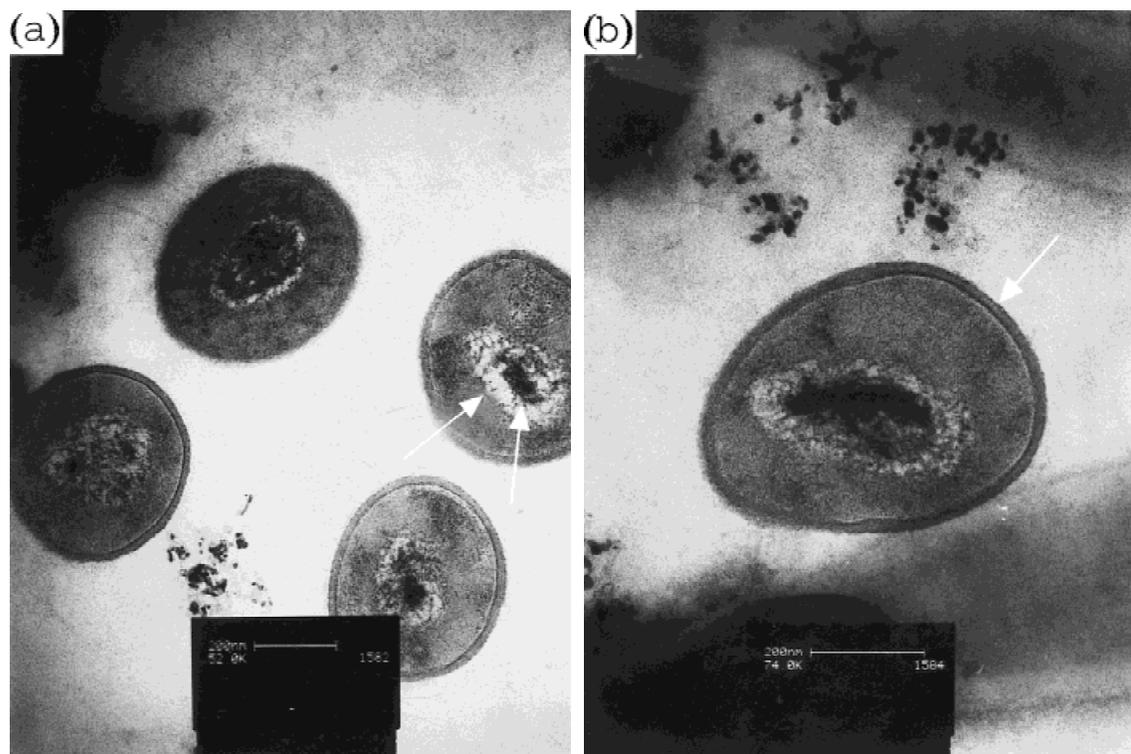


Figure 5. Internal structure of the silver ion treated *S. aureus* cells. (a) An electron-light region (long arrow) in the center of the cell with condensed and concentrated DNA molecules in the center of it (short arrow). (b) Cytoplasm membrane detached from the cell wall (arrow).

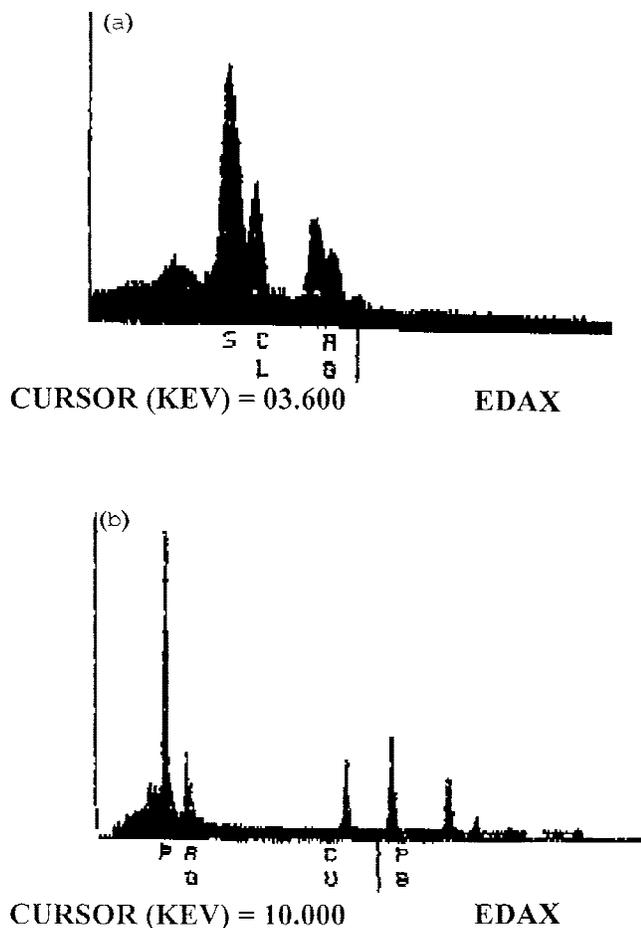


Figure 6. X-ray microanalysis on (a) the cytoplasm and (b) the conglomerated DNA molecules of the silver ion-treated *S. aureus* cells.

CONCLUSION

Comparing the AgNO_3 treated *E. coli* and *S. aureus* with the control setting, it was found that:

1. The free state of DNA changed to a condensed form in the center of the electron-light region in the cells;
2. Many electron-dense granules appeared surrounding the cell wall or electron-light region;
3. X-ray microanalysis demonstrated the existence of silver in electron-dense granules, cytoplasm, and DNA molecules.

The above results lead to the following suggestions about the bactericidal mechanism of silver ions against *E. coli* and *S. aureus*:

1. As a reaction against the denaturation effects of

silver ions, DNA molecules become condensed and lose their replication abilities;

2. Silver ions interact with thiol groups in protein, which induce the inactivation of the bacterial proteins.

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