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Atomic force microscopy investigation of the characteristic effects of silver ions on *Escherichia coli* and *Staphylococcus epidermidis*

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ABSTRACT

The influence of silver ions on *Escherichia coli* and *Staphylococcus epidermidis* has been investigated by atomic force microscopy (AFM). Both single cell and cell communities were visualized, and the nanoscale ultrastructure images of these two bacteria strains before and after stimulation of silver ions were obtained. The results showed that in the case of *E. coli* after treatment with silver ions, vesicles appeared on the cell walls, and the size of vesicles became larger with the increase of the incubation time. However, in the case of *S. epidermidis* after treatment with silver ions, irregular and deep grooves appeared on the cell walls, and the cytoplasm membrane shrank and became separated from the cell wall. The significant differences in cell wall changes between *E. coli* and *S. epidermidis* after treatment with silver ions were related to their structural characters. According to those results, it is shown that AFM can provide new insight into the antimicrobial mechanism of silver ions, and show a new methodological approach to understand the relationship between structure and function of microbial cell when it was exposed to external stimulation.

1. Introduction

It has been known that silver ions are effective antimicrobial agents. Silver ions have been reported to be lethal to a range of bacterial species, both Gram-positive bacteria and Gram-negative bacteria, so they have gained extensive applications in the fields of clinical medicine and daily life as antibacterial component [1,2]. In addition, silver ions have been re-evaluated as a new therapeutic strategy because of the increasing resistance of bacterial cells to most antibiotics [3]. However, the antimicrobial mechanism of silver ions is only partially understood. Therefore, it is important to study antimicrobial mechanism for further development and application of silver-based products.

In previous studies, silver ions have been reported to uncouple respiratory electron transport from oxidative phosphorylation [4], collapse the proton-motive force across the cytoplasm membrane [5], interfere with the outer membrane permeability [6], or interact with thiol groups of membrane-bound enzymes and protein [7,8]. Evidences for the above antimicrobial mechanisms were obtained by biochemistry techniques, which provided bulk measurements representing the average behavior of massive number of cells. The direct observation of morphological alterations has been completing the antimicrobial mechanism of silver ions by imaging techniques. The effects of silver ions on bacteria were studied by transmission electron microscopy (TEM) [8,9]. TEM observations were capable of visually revealing internal cellular changes and some apparent changes in cellular morphology, but no cellular surface change in great detail was examined after treatment with silver ions. Therefore, imaging techniques of surface analysis are appealed to investigate surface alterations of bacteria.

Atomic force microscopy (AFM) has opened up many new domains for surface analysis of biological specimens due to its advantages such as true three-dimensional data acquisition, measuring adhesive and mechanical properties at molecular level [10,11], and the possibility to study samples in different surroundings without pretreatments (chemical fixation, dehydration, dryness, and metal coating), which may lead to a change of surface properties. In recent years, AFM has been applied increasingly and successfully to investigate the microbial envelope [12–14]. Braga and Ricci studied the surface alterations of *Escherichia coli* induced by different concentrations of the beta-lactam antibiotic cefodizime [15]. Lu et al. observed the decomposition of cell walls and cell membranes of *E. coli* caused by an illuminated TiO_2 thin film [16]. Rautenbach and co-workers analyzed the membranolytic effects in the mechanisms of three antibacterial peptides [17]. In our previous work, the effects of natural and semisynthetic β-lactam antibiotics on *E. coli* were investigated, indicating that minor structural difference between them give rise to evidently different action effects [18]. As just mentioned, much work has

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been done to study the effect of antibacterial agents on microbial cell by AFM [19,20]. However, there is no attempt to investigate the antibacterial action of silver ions on bacteria by AFM, and the characteristic surface changes have never been visually revealed at high-resolution of Gram-negative bacteria and Gram-positive bacteria after treatment with silver ions.

Here, the effect of silver ions on bacteria was studied by AFM. *E. coli* and *Staphylococcus epidermidis* were chosen as model Gram-negative bacteria and Gram-positive bacteria, respectively. Both single cell and cell communities were visualized, giving some interesting results and a more complete understanding of related processes. The results showed that AFM imaging was able to discern the significant detailed differences in cell wall changes between *E. coli* and *S. epidermidis* after treatment with silver ions. This work demonstrated that AFM should be a useful tool in understanding the relationship between structural changes and physiological functions of cell, and provide visual evidences for clarifying antimicrobial mechanism of silver ions.

2. Experimental

2.1. Reagents

Two experimental strains, Gram-negative *E. coli* and Gram-positive *S. epidermidis* were used to investigate the effect of silver ions in the form of $AgNO_3$ solution (Shanghai Chemical Reagents Co., China). The Luria–Bertani (LB) medium was purchased from Oxoid LID. Hampshtre, England. All solutions were prepared with ultrapure Millipore water (18.2 MΩ cm). The culture medium was sterilized in high-pressure steam at 120 °C for 30 min.

2.2. Preparation of bacterial samples for morphology studies

The culture of *E. coli* or *S. epidermis* was grown overnight to a log phase (~7.8×10^8 cell/ml) in LB medium at 37 °C on a shaker at 180 rpm. Silver ions at minimum inhibitory concentration (MIC) were added to the suspension, and the mixture was incubated at 37 °C for 1 h, 2 h, 3 h and 4 h with continuous shaking. Culture with silver-free served as control. The cells in 1.0 ml culture were isolated by centrifugation at 6000 rpm for 2 min in a centrifuge (Beckman CoulterMicrofuge 18) and washed twice with sterile water. Then, the cells were gently resuspended in sterile water for AFM analysis. The samples were applied on a freshly cleaved mica surface and allowed to dry for ~5 min before imaging.

2.3. Imaging cell

E. coli and *S. epidermis* cells were imaged in ambient air with a tapping mode AFM (SPI3800N-SPA400, Seiko Instrument) before and after being treated with silver ions. This microscope was equipped with a piezoscanner having a maximum scan range of 100 μm × 100 μm × 10 μm. A rectangular Si cantilever/tip (Seiko) with a spring constant of 12 N/m and a resonance frequency of ~130 kHz was used. A scan speed of 0.7–1.5 Hz was set and resulted in a final resolution of 256 by 256 pixels. A topography image and a phase image were obtained simultaneously in every single scan. Height and size information were acquired with the imaging software from Seiko.

3. Results and discussion

3.1. Morphological changes of E. coli after silver ions treatment

The effects of silver ions on *E. coli* cells for different incubation time were investigated, as shown in Fig. 1. Fig. 1A and B shows the topography image and phase image of typical untreated *E. coli* cells, respectively. They had rod-shaped morphologies and aligned side by side with adjacent cells in contact. A single *E. coli* cell had a relatively smooth surface with no ruptures or bulges. After incubation for 1 h with MIC of silver ions, *E. coli* cells maintained their rod-shaped morphologies, but some vesicles appeared on cellular surface, as shown in Fig. 1C and D. After incubation for 2 h, the number of the vesicles on cellular surface increased, although the *E. coli* cells remained relatively intact, as shown in Fig. 1E and F. After incubation for 3 h, not only the number of the vesicles increased, but also the vesicles became larger as the increase of the incubation time, as shown in Fig. 1G. In addition, the phase image (Fig. 1H) showed the cell surface became rough compared with that of the control cells. With the increase of the incubation time (4 h), the *E. coli* cells lost their clear-cut rod-shaped morphologies with appearance of numerous large patches, and the borders of the cells were diffuse, as shown in Fig. 1I and J. The height profile analysis provided quantitative measurement of the dimension of the vesicle on the cell surface, as shown in Fig. 2. After a short incubation time (2 h)

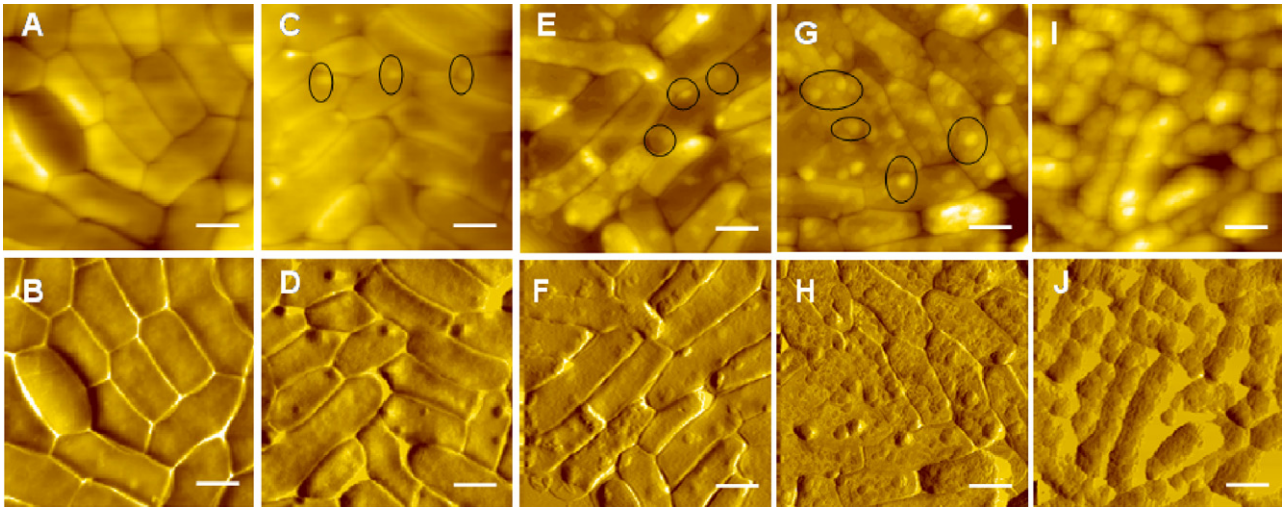

Fig. 1. Topography images *E. coli* cells before (A) and after treatment with silver ions for (C) 1 h, (E) 2 h, (G) 3 h and (I) 4 h, and their corresponding phase images (B), (D), (F), (H) and (J), Bar = 1 μm.

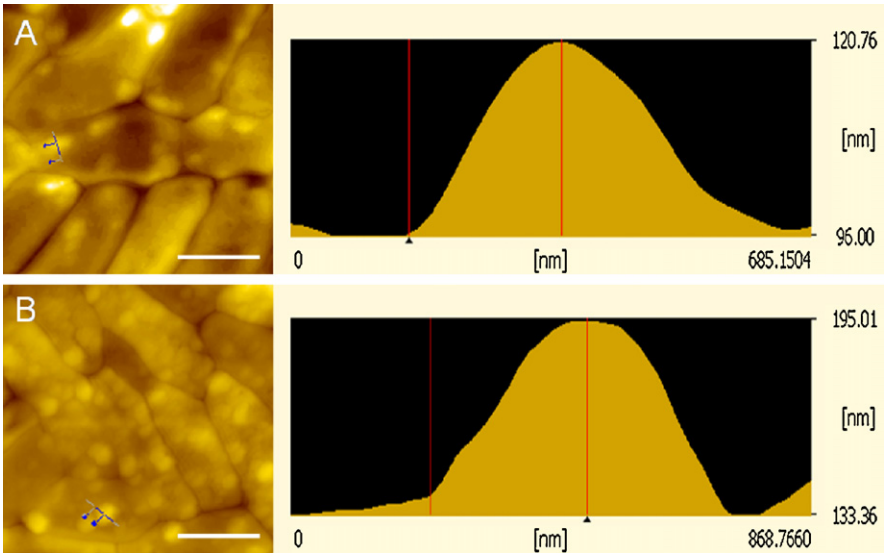

Fig. 2. Topography images and the corresponding height profiles of vesicles on cell surface of the *E. coli* after treatment with silver ions for (A) 2 h and (B) 3 h, Bar = 1 μm.

shown in Fig. 2A, the average height and the average radius of the vesicles were 25.05 ± 3.20 nm and 211.15 ± 13.67 nm, respectively. After a long time (3 h), the average height and the average radius of the vesicles increased to 55.25 ± 8.59 nm and 373.48 ± 61.98 nm (Fig. 2B), which is clearly larger than the average size of vesicle in Fig. 2A.

3.2. Morphological changes of *S. epidermidis* after silver ions treatment

The effects of silver ions on *S. epidermidis* cells for different incubation time were also investigated, as shown in Fig. 3. Fig. 3A showed the topography image of typical untreated *S. epidermidis* cells. They had spherical shapes and aligned side by side with adjacent cells in contact. The corresponding phase image (Fig. 3B) with a smaller scan area showed a single *S. epidermidis* cell with smooth and regular surface, and the periphery between adjacent cells was sharp. After incubation for 1 h with MIC of silver ions, *S. epidermidis* cells maintained their spherical morphologies, and some dark regions presented on the periphery of cell, as shown within circles of Fig. 3C. The corresponding phase image (Fig. 3D) with a smaller scan area showed that dense protrusions were clearly visible on the periphery of *S. epidermidis* cells (dark circle), indicating a mild cell surface perturbation. After incubation for 2 h, the dark regions (dark arrows in Fig. 3E) located at the periphery between neighboring cells were pronounced, and the number of the protrusions (dark circle in Fig. 3F) increased as shown in their corresponding phase image with a smaller scan area. After incubation for 3 h, the *S. epidermidis* cells had a distinct feature (Fig. 3G): appearance of some grooves on cell wall, and the shape of *S. epidermidis* cell became irregular sphericity. The corresponding phase image of a single *S. epidermidis* cell showed that deep and irregular groove on the cell surface (dark arrows in Fig. 3H), and the cell walls had separated from the cytoplasm membrane (white arrows in Fig. 3H). With the increase of the incubation time (4 h), besides grooves distributed on almost every cell, some ruptures occurred on the cell walls, as shown in Fig. 3I. The corresponding phase image of a single *S. epidermidis* cell (Fig. 3J) gave a good illustration of above changes. The white arrows showed the region between the cell wall and cytoplasm membrane, and the grooves and rupture were marked with dark arrows and a white circle, respectively. The height profile analysis provided quantitative measurement of the distance between

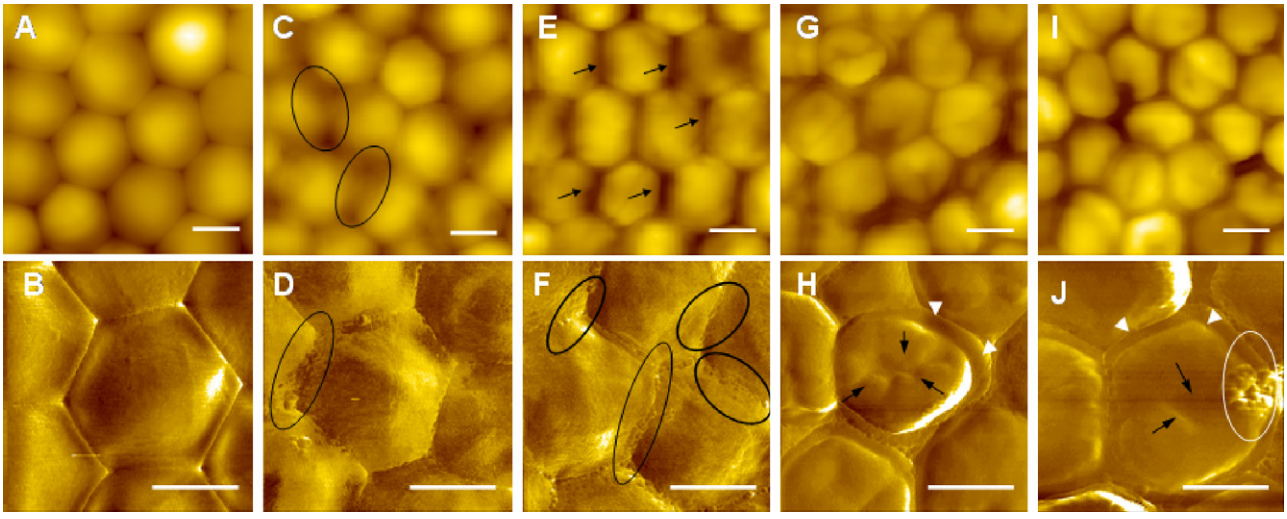

Fig. 3. Topography images *S. epidermidis* cells before (A) and after treatment with silver ions for (C) 1 h, (E) 2 h, (G) 3 h and (I) 4 h, and their corresponding phase images with a smaller scan area (B), (D), (F), (H) and (J), Bar = 500 nm.

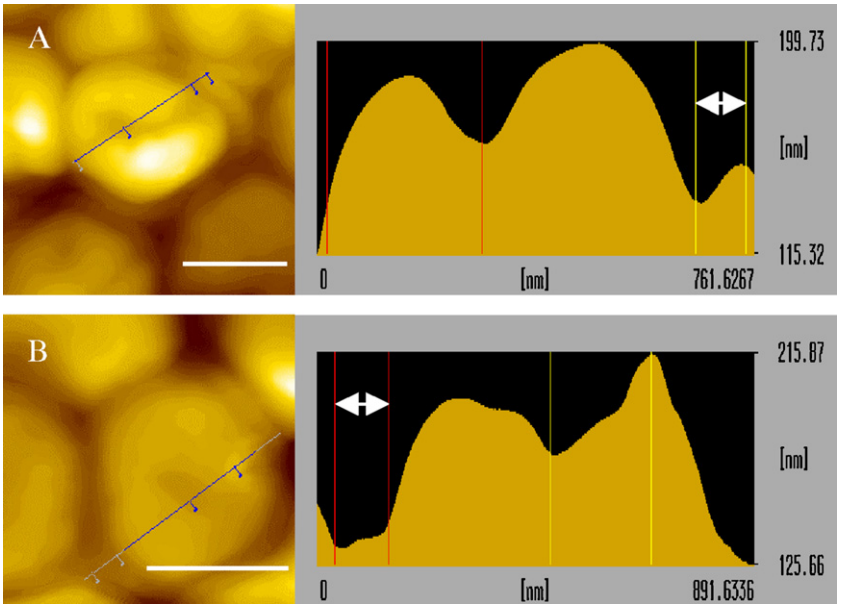

Fig. 4. Typical single cell surface height profiles of the long axis of the cell body with corresponding topography images exhibited by *S. epidermidis* cells after treatment with silver ions for (A) 3 h and (B) 4 h, Bar = 500 nm.

the cell wall and cytoplasm membrane. As shown in Fig. 4A, after incubation 3 h, the distance was ca. 87.42 nm. After a long time incubation (4 h), the distance increased to ca. 108.55 nm (Fig. 4B), which is larger than the distance in Fig. 4A.

3.3. Analysis of characteristic damages on different types of bacteria

The effects of silver ions were investigated on *E. coli* and *S. epidermidis* using AFM. High-resolution images of AFM illustrated different features on two types of bacteria after exposure to silver ions (Fig. 5). As shown in Fig. 5A, vesicles appeared on the cell walls of *E. coli*, and the corresponding surface height profile of a single vesicle on cell wall provided quantitative measurements of the dimensions. However, deep grooves appeared on cell walls of *S. epidermidis*, and the cytoplasm membrane shrank and became separated from the cell wall, as shown in Fig. 5B. The corresponding "peaks-valleys" liked surface height profile indicated appearance of grooves on cell walls, and the detached region (green lines) between the cell wall and cytoplasm membrane. Here, the distinct morphological changes of two typical bacteria probably were a consequence of their different structure of cell wall.

The *E. coli* belongs to the Gram-negative bacteria, which has a relatively complex cell wall: an outer membrane and a thin peptidoglycan layer (~2 nm) [21]. The outer membrane consists of lipopolysaccharide (LPS) which acts as a selective permeability barrier. Wall shape and strength is primarily due to peptidoglycan. In contrast, the *S. epidermidis* belongs to the Gram-positive bacteria, which has a simple cell wall: a thick peptidoglycan layer (20–80 nm), lacking the outer membrane [21]. Because of the thicker peptidoglycan layer, the walls of Gram-positive cells are stronger than those of Gram-negative bacteria. Moreover, the thicker peptidoglycan contains a peptide interbridge and teichoec acids which are not present in Gram-negative bacteria. These

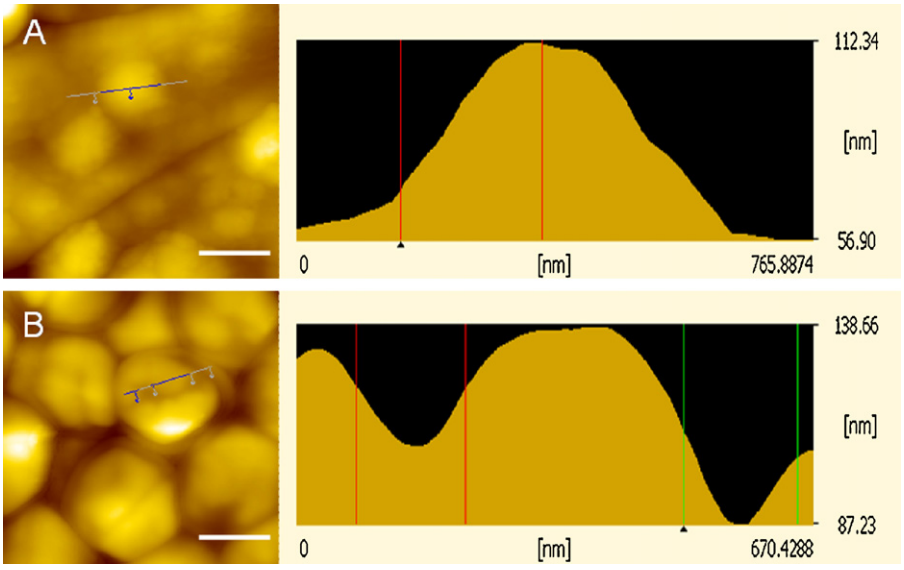

Fig. 5. Characteristic changes of (A) *E. coli* cells and (B) *S. epidermidis* cells after treatment with silver ions, Bar = 500 nm.

molecules may be important in maintaining the structure of the wall. Both Gram-negative bacteria and Gram-positive bacteria have the same cytoplasmic membrane inside the cell wall.

In the case of *E. coli*, the characteristic changes were relative to the structure of *E. coli*. The outer membrane lay outside the *E. coli*, which prevented or slowed the entry of toxic substances that might kill or injure the bacterium. As reported, release of outer membrane vesicles by Gram-negative bacteria was a novel envelope stress response [22]. When the *E. coli* cells were attacked, increased vesiculation can get rid of undesirable and toxic components by the outer membrane. In this study, some vesicles appeared on the cell surface of *E. coli* after treatment with silver ions. The results indicated that vesicle production might be a mechanism for the *E. coli* cell to protect itself from the attack of silver ions. Because the outer membrane was to serve as a protective barrier, silver ions cannot reach the peptidoglycan layer at the beginning. Therefore, the *E. coli* cells remained their rod-shapes with appearance of some vesicles. With the increase of the incubation time, not only the number of the vesicles increased, but also the vesicles became larger. Moreover, it was worth noting that cell surface of *E. coli* became rough compared with that of native cells. The results indicated that the physiological function of the outer membrane was weakened severely. This was to facilitate the further damage of the thin peptidoglycan layer, leading to disappearance of rod-shape morphology of *E. coli* and subsequent damage of inside cytoplasmic membrane or intracellular metabolic activity.

In the case of *S. epidermidis*, the characteristic changes were also relative to the structure of *S. epidermidis*. The *S. epidermidis* lacked the outer membrane, but its thick peptidoglycan layer had a specific peptide interbridge to result in an enormous, dense and interconnected network, differing from that of Gram-negative bacteria. These networks were strong enough to remain their shape and integrity. When the *S. epidermidis* cells were attacked by toxic substances, the firm peptidoglycan layer was a stronger defense system [8,21]. In this study, some protrusions were clearly visible on the periphery of *S. epidermidis* cells, but the cells maintained their spherical morphologies after treatment with silver ions for a short incubation time. The results indicated a mild perturbation of the peptidoglycan layer. With the increase of the incubation time, many irregularly shaped grooves appeared on the surface of *S. epidermidis*, which was the consequence of progressive damage of the peptidoglycan layer. These grooves became active sites to facilitate the attack of silver ions. Silver ions can reach the inside cytoplasmic membrane through the grooves. The inside cytoplasmic membrane shrank and became separated from the cell wall in order to protect the cells as the second barrier. After a longer incubation time, besides grooves, some ruptures occurred on the cell walls of *S. epidermidis*, indicating the severer damage of the peptidoglycan layer. A large of silver ions attacked the cytoplasmic membrane of *S. epidermidis* through the ruptures, and cellular contents were then released from these ruptures.

4. Conclusion

This is the first the visual demonstration of different damages to the cell wall between *E. coli* and *S. epidermidis* caused by silver ions. AFM imaging was able to discern the significant detailed differences in cell wall changes of two typical bacteria due to their structural characters. It is clear that AFM can provide useful information for understanding the antimicrobial mechanism of silver ions, and show great potential for understanding the relationship between structure and function of microbial cell.

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