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AFM INVESTIGATION OF MORPHOLOGICAL CHANGES TO *STAPHYLOCOCCUS AUREUS* SURFACE INDUCED BY $AgNO_3$ AND OXACILLIN ADDITION

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ABSTRACT. *Staphylococcus aureus* is one of the most common pathogens in hospitals and can be responsible for infections ranging from minor to life-threatening ones, because its strains can acquire very fast resistance to clinically used antibiotics. A heterogeneous methicillin-resistant *S. aureus* (MRSA) strain, UCLA 8076 was selected as a model for this study. $AgNO_3$ or oxacillin were added to the culture medium and then incubated for 18 hours. Culture with no $AgNO_3$ and oxacillin addition served as control. For living cells, high-resolution imaging remains challenging, but a wealth of novel structural information can be obtained, such as visualization of surface structure in native conditions and monitoring of physiological changes in real time. The AFM technique was used in order to examine the structures and dynamics of bacteria because it is a powerful technique for imaging biological samples under nondestructive conditions and it was concluded that $AgNO_3$ inhibits the *S. aureus* development better than oxacillin.

Keywords: *AFM, S. aureus, silver ions, oxacillin*

INTRODUCTION

In recent years, in clinical settings, increases of skin and soft-tissue infections were encountered, particularly due to multidrug-resistant pathogens. The most serious and common infections produced by *S. aureus* are bacteremia, pneumonia, osteomyelitis, endocarditis [1-3], empyema, scalded skin syndrome, toxic shock syndrome [1] and abscesses of the muscle and various intra-abdominal organs [4,5]. It is known [6] that MRSA are inherently resistant to all β-lactam antibiotics, but some lineages (clones) have additionally

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evolved resistance to multiple antibiotic classes. The resistance to the most known antibiotic classes has occurred within the species due to mutation and horizontal gene transfer and this has led to anxiety regarding the future availability of effective chemotherapeutic options [6]. The characteristics of Oxacillin-Susceptible MRSA strains and the possible efficiency of oxacillin against them were strongly investigated [7-9] by in vitro and in vivo experiments, but the improved oxacillin treatment remains a topical issue among researchers [10].

It has been demonstrated [11-14] that silver ions have a strong inhibitory and bactericidal effect; the antimicrobial action of them is closely related to their interaction with thiol groups. Ag^+ has the ability to bind to functional groups of proteins, resulting in protein denaturation [16]. It is believed that cellular proteins become inactivated on Ag^+ treatment due to the loss of replication capacity of DNA [15]. Studies have reported [17-19] that the positive charge of the Ag ions is crucial for its antimicrobial activity through the electrostatic attraction since the microorganism cell membrane has a negative charge. The bacterial plasma membrane, associated with several housekeeping enzymes, is an important target site for silver ions.

The investigation of biological systems on the cellular level, rather than on the population level is permitted by atomic force microscopy (AFM). AFM is a powerful technique used in order to examine the morphology and dynamics of bacteria at the nanometer to micrometer scale under nondestructive conditions and has the resolution to observe small changes in cell morphology, even nanometer-scale features such as bacterial division septa [20-22]. This highly versatile microscopy technique is particularly well suited to the study of microorganisms, because it combines a greatly improved resolution compared to optical microscopy and has no requirement to scan in vacuum or need for a conductive coating compared to scanning electron microscopy. High-resolution images can be obtained with minimal sample fixation and staining. It has been shown that fixation can alter the mechanical properties of bacteria [23] and cells [24].

S. aureus cells treated with antibiotics and ovine antimicrobial peptides isolated from the blood neutrophils were reported in the literature [25, 26], but potential advantage of using Ag+ for the treatment of topical staphylococcal infections is strongly debated [27]. Beside the effect of silver ions on the viability and membrane integrity of S. aureus, the present study reports also an improvement in cell preparation for AFM investigation. This improvement is achieved by using a proper sized nylon filter instead of drying the culture on a glass slide which is reported to involve lysed cells and a covering with a thin layer of material as a result of the drying process and of the remains of the growth media, respectively [25].

RESULTS AND DISCUSSION

1. *Staphylococcus aureus* (control)

The AFM imagines (topography and phase contrast) of the *S. aureus* culture without $AgNO_3$ or oxacillin addition used as control are presented in the Figure 1.

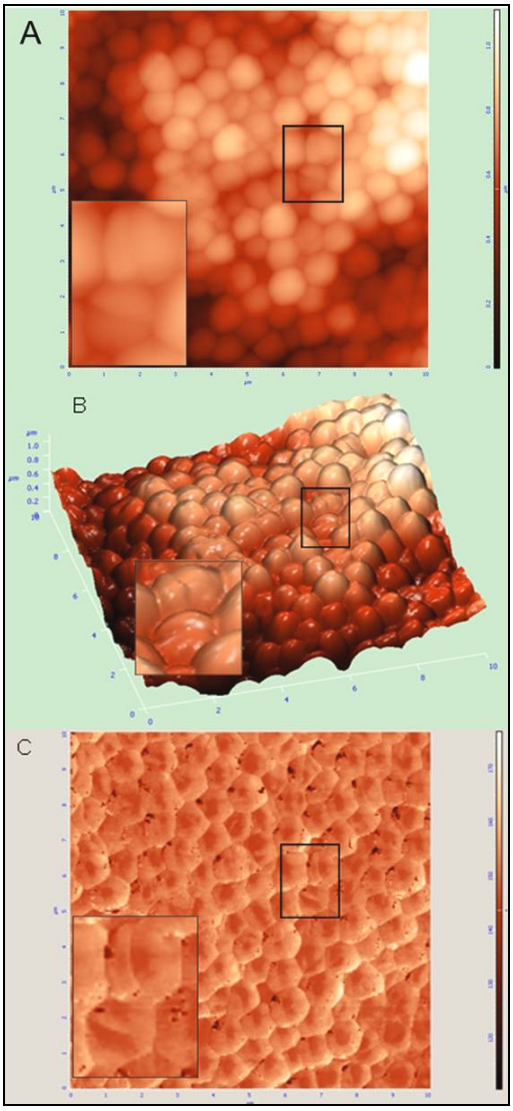

Figure 1. Tapping mode AFM images of *S. aureus* (control) in the 10μm range: A) two-dimensional topography, B) three-dimensional topography, C) phase contrast.

The phase contrast mode is used simultaneously with the tapping mode, so the topography is registered as well. For the phase contrast mode the phase lag of the cantilever oscillation sent to the piezo driver of the cantilever is monitored and recorded, being very sensitive to variations in material properties.

As it is well known, *S. aureus* is a spherical bacterium, about 1μm in size (Figure 1). The presence of a cross wall in some cells shown in the AFM imagines demonstrates that for these bacteria the early stage of cell division takes place. In the AFM imagines insets the cells division is more clearly expressed. Cells that are not in the process of dividing do not have a cross wall (Figure 1). This septum will continue its inward growth until a cross-wall is formed that completely separates the two daughter cells. At this point the cells remain joined together but eventually, through a process called splitting, will separate. During separation a small cleft is seen in the periphery of the wall (Figure 1). This stage is the first topological feature that is seen by AFM, where a shallow cleft is found in the relatively smooth homogeneous surface at the midpoint of a cell.

2. Morphological changes induced by oxacillin addition

The AFM imagines (topography and phase contrast) of the *S. aureus* with 8μg/ml oxacillin addition to the culture medium are presented in the Figure 2. The images obtained revealed not only the weak antibacterial effect of the oxacillin addition, but also the response strategies used by the bacteria. Cell wall collapse and morphological changes reflected some cell death.

3. Morphological changes induced by $AgNO_3$ addition

The AFM imagines (topography and phase contrast) of the *S. aureus* with 8μg/ml $AgNO_3$ addition to the culture medium are presented in the Figure 3 and Figure 4, for two different areas and scales, respectively. From these figures (Figure 3, 4) one can see that all the bacteria present big changes on their surfaces, unlike those subjected to oxacillin treatment (Figure 2).

CONCLUSIONS

By exploiting the natural tendency of many bacteria to thrive on solid surfaces, the air drying of a suspension of *S. aureus* on a filter is a suitable method for imaging bacteria at high resolution by atomic force microscopy. This technique allows simultaneous mapping of the topography and phase contrast. The fact that the cells appear healthy and vigorous when imaging bacteria in air convinced that the cells imaged on filters are quite native. In fact, in this hydrated air–solid interface, the cells grow, divide, multiply, move, and spread outward across the filter.

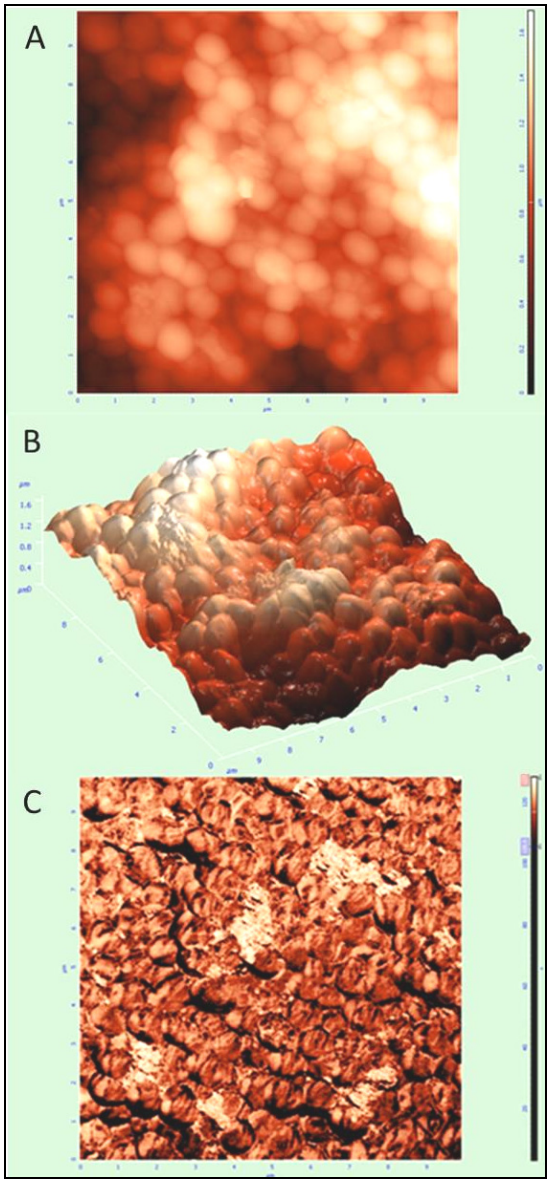

Figure 2. Tapping mode AFM images of *S. aureus* with 8µg/ml oxacillin addition in the 10µm range: A) two-dimensional topography, B) three-dimensional topography, C) phase contrast.

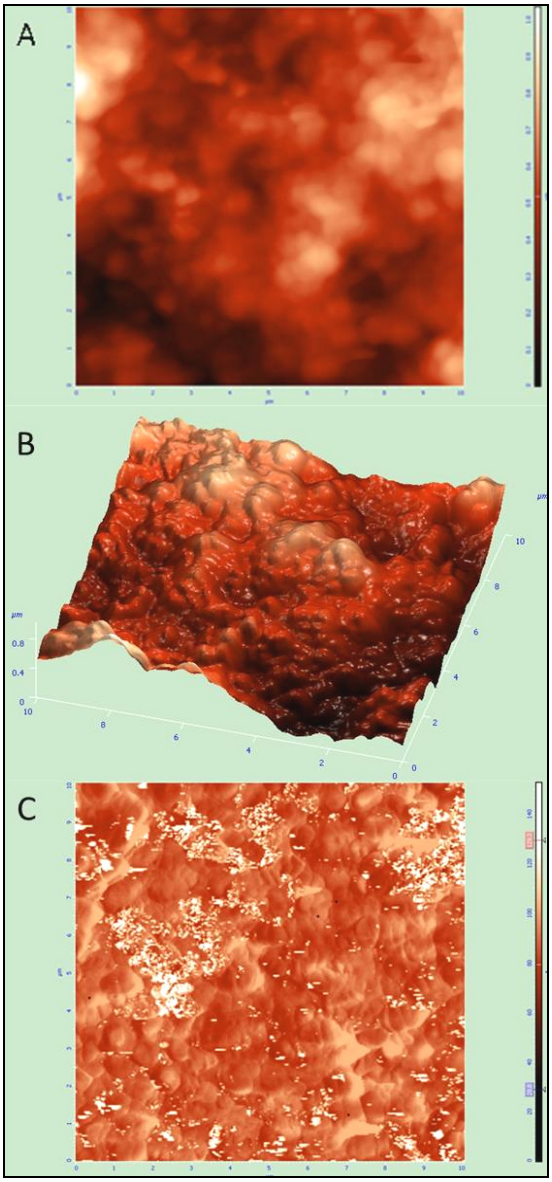

Figure 3. Tapping mode AFM images of *S. aureus* with 8µg/ml $AgNO_3$ addition in the 10µm range: A) two-dimensional topography, B) three-dimensional topography, C) phase contrast.

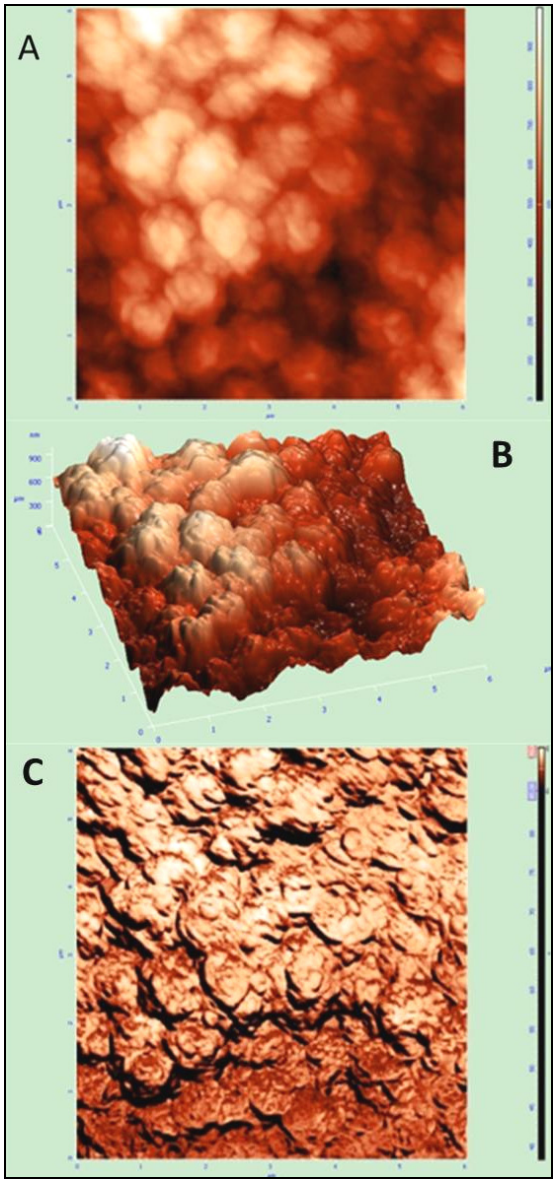

Figure 4. Tapping mode AFM images of *S. aureus* with 8μg/ml $AgNO_3$ addition in the 5μm range: A) two-dimensional topography, B) three-dimensional topography, C) phase contrast.

For the *S. aureus* (control), the AFM technique was able to follow the division process with precision and confirm the initial splitting of the septum.

Atomic force microscopy (AFM) imaging was used to obtain high-resolution images of the effect of silver ions and oxacillin on the bacterial morphology. The mechanism of antibacterial activity of Ag ions and oxacillin were studied by AFM, analyzing the growth and morphology of the bacterium wall.

The topographical imaging completed in tapping mode produced high resolution images demonstrating dramatic differences in surface morphology of the staphylococcal strains, strongly dependent on the $AgNO_3$ and oxacillin addition. Conformational changes induced by the additions of $AgNO_3$ and oxacillin were remarked.

The AFM technique, used in order to examine the structures and dynamics of bacteria may prove to be a valuable tool for probing microbial surface at high resolution under nondestructive conditions and it was concluded that $AgNO_3$ inhibit the *S.aureus* development much better than the oxacillin, confirmed by the fact that the cells walls damage had indeed occurred more in the presence of silver ions than under the oxacillin treatment.

However, researchers should keep in mind that the use of AFM in microbiology remains delicate and that accurate data collection and interpretation require specific expertise. In particular, great care should be taken to optimize sample preparation procedures and imaging conditions when exploring new specimens. Also, the best results will be obtained when AFM is combined with complementary biochemical and structural techniques.

EXPERIMENTAL SECTION

S. aureus growth: The first step was to grow fresh, dense cultures of bacteria (greater than 107 CFU/ml). A heterogeneous methicillin-resistant *S. aureus* strain, UCLA 8076 was selected for this study. The culture was maintained on Mueller-Hinton agar (Fluka, Buchs, Switzerland) plates. Incubation at room temperature is generally sufficient to initiate growth and complex behaviours but the agar plate can also be placed in an incubator, refrigerator, or other controlled environment at this step to encourage the cells to grow. Therefore the strain was cultured at 37°C in Mueller-Hinton broth (Fluka, Buchs, Switzerland) at 150 rpm in a rotary shaker (CERTOMAT BS-T, Sartorius Stedim, Aubagne, France). 8 μg/ml $AgNO_3$ and oxacillin respectively were added to the culture medium and then were incubated for 18 hours in dark room condition. Culture without $AgNO_3$ or oxacillin addition served as control.

Preparation for AFM investigation: For AFM investigation bacteria must be immobilized on a surface. Without this strong attachment, cells can move in response to the lateral movement of the tip and can be pushed across the surface during imaging. Air drying a suspension of *S. aureus* on a filter provides sufficient immobilization because this bacterium does not have any appendages

that permit motility. Therefore, the possibility to move out of the area of investigation is not a problem. 5 ml culture broth was centrifuged and washed with sterile, deionized water, the pellet resuspended in 1000 µl ultrapure water and filtered on 0.45 µm pore-sized nylon filter (ADVANTEC MFS, Pleasanton, USA). This kind of pore-size filter is recommend since the *S. aureus* diameter is known to be around 0.7 – 1 µm. The solution containing bacteria, suspended in culture medium is deposited central onto the filters. The solution makes a rounded bubble on top of the filter that soaks through into the plates in about 30 minutes. As long as the pore size is smaller than the bacteria, the bacteria remain on top while the cell debris goes through the filter. If necessary, the excess filter can be cut off with a razor or scissors. The microorganisms can be imaged immediately without any further treatment. This method for imaging bacteria on a nylon filter is a suitable one because even after removing the filter and imaging the bacteria for half an hour, a large fraction of the cells can be scraped off and grown in solution.

Experimental device: The *Staphylococcus aureus*' shape and morphology were accessed using an Atomic Force Microscope: NTEGRA Vita from NT-MDT, combining the strengths of SPM with an inverted optical microscope for biological and medical applications. The topography and phase imagines were recorded in tapping mode at room temperature in air, using a chemically stable Au reflective coating silicon probe cantilever a of 100 µm length, 35 µm width, 1.2 µm thickness and 90 kHz resonance frequency.

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