Atomic Force Microscopy: Application to Investigation of *Escherichia coli* Morphology before and after Exposure to Cefodizime

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Atomic force microscopy (AFM) is a recently developed technique that allows for the investigation of the surface morphology of a biological specimen at an unprecedented level of resolution. The aim of the present study was to explore some of the new opportunities offered by AFM by studying the morphological and surface alterations induced in *Escherichia coli* by supra-MICs and sub-MICs of a beta-lactam antibiotic (cefdizime). The underlying principle of AFM is the scanning and sensing of the topography of a sample by means of near-field microscopy that makes it possible to obtain simultaneous digital measurements of the x, y, and z coordinates of any point on the bacterial surface with great resolution (x and y, ~20 Å; z, ~1 Å). Unlike scanning electron microscopy, performance of AFM does not require a vacuum, drying to the critical point, or the coating of the bacterial surface with a metal layer. The digital storage of the information makes it easy to rotate the image, observe the bacterial surface and induced structural alterations from different points of view, and obtain a cross-section at any desired point with precise, automatic measurement of the heights and sizes of normal versus damaged bacteria. Use of the new and outstanding technique of AFM will make it possible for researchers to investigate biological samples immersed in biological fluids and will also make it possible for them to study the morphological alterations of living bacteria exposed to antibiotics as they are taking place.

β-Lactam antibiotics exert their antimicrobial activity by interfering with the enzymes specifically involved in peptidoglycan metabolism. These enzymes regulate cell walls, cell division, septation, elongation, and cell shape (14) and thus alter the surface morphology and structure of the bacteria. Each β-lactam antibiotic has its own binding affinity for these enzymes, called penicillin-binding proteins, and since penicillin-binding proteins vary among different bacterial species, a variety of morphological alterations and types of damage can occur (7–9). Evidence concerning the actions of β-lactam antibiotics has been obtained not only biochemically but also by means of the direct observation of morphological alterations by optical microscopy and scanning electron microscopy (SEM), and investigators have performed a series of studies on this subject (3, 5, 10, 11).

Optical and scanning (or transmission) electron microscopes are classified as “far-field microscopes” because the distance between the sample and the point at which the image is observed is long in comparison with the wavelengths of the photons or electrons involved. In this case, the image is a diffraction pattern, and its resolution is limited by the wavelength (4).

Scanning probe microscopes (SPMs) are based on the concept of “near-field microscopy,” which overcomes the problem of the limited diffraction-related resolution inherent in conventional microscopes because the probe, located in the immediate vicinity of the sample itself (usually within a few nanometers), records the intensity and not the interference signal, and this greatly improves the final resolution (4).

SPMs represent a new family of microscopes that are specifically dedicated to the investigation of surface morphology and provide details of unprecedented resolution without the need for complicated sample preparation.

Atomic force microscopy (AFM) is a recently developed technique that has extended the use of SPMs to the observation of nonconductive surfaces and that has thus opened up unexpected possibilities for the surface analysis of biological specimens (6) (Fig. 1).

The aim of this study was to investigate this new means of analyzing the surface and morphological alterations of bacteria by examining samples of *Escherichia coli* exposed to the β-lactam antibiotic cefodizime, one of the latest expanded-spectrum cephalosporins to enter clinical practice.

**MATERIALS AND METHODS**

Strains and culture conditions. *E. coli* ATCC 25922 and two strains of *E. coli* isolated from humans with urinary tract infections were used to investigate the effects of the antibiotic. Suspensions of each organism were prepared from overnight cultures in tryptic soy broth (Difco, Detroit, Mich.) at 37°C under static conditions. The organisms were harvested, washed in phosphate-buffered saline, and adjusted to 10⁷ organisms/ml as determined by direct microscopic counts in a Petroff-Hauser chamber.

Susceptibility testing. Cefodizime was the antimicrobial agent used in this study (Hoechst Marion Rousell). An inoculum of 10⁶ CFU of the organism was added to 4.5 ml of tryptic soy broth containing serial twofold dilutions of the antibiotic in order to determine the MIC. After incubation at 37°C for 18 h, the MIC was recorded as the lowest antibiotic concentration that completely inhibits visible growth of the organism. Because different antibiotic concentrations lead to different morphological changes in bacteria, the effects of cefodizime concentrations that were both higher and lower than the MIC were also examined.

Starting from the observed MICs, serial twofold dilutions of cefodizime (from 1/2 to 1/128 the MIC) were prepared in tryptic soy broth. All of the strains were grown in medium with and without antibiotic at different sub-MICs, without shaking, at 37°C for 18 h. The supra-MICs investigated were two, four, and eight times the MIC, with incubation times of 2, 4, and 6 h.

Preparation of bacterial samples for morphology studies. For each antibiotic concentration and each incubation time samples (2 ml) of each *E. coli* strain were collected, washed three times with phosphate-buffered saline, and centrifuged. The final pellet was divided, and each part was placed on a round microscope cover slide. The first cover slide was simply dried in air, whereas the second one

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FIG. 1. Schematic diagram of an atomic force microscope.

was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) and dehydrated in graded alcohol. None of the samples underwent drying to the critical point or gold coating.

AFM. An Autoprobe CP atomic force microscope (Park Scientific Instruments, Sunnyvale, Calif.) was used for all imaging by AFM. The microscope was equipped with a scanner that had a maximum $\times y$ scan range of 100 by 100 $\mu$m and a $z$ range of 7 $\mu$m and was operated by means of a ScanMaster (Park Scientific Instruments), a real-time closed-loop scanning control system that allows for the accurate measurement, repositioning, and zooming in on selected features. The scanner was calibrated in the three directions by using a VLSI reference standard (VLSI Standard, San José, Calif.). The images were acquired by using silicon cantilevers with high-aspect-ratio conical silicon tips (Ultralevers; Park Scientific Instruments); the force constants were 0.03 N/m for contact-mode imaging and 7.4 N/m for intermittent-contact-mode imaging. In order to be able to locate the area of interest on the samples and identify any bacteria showing damage, we used the built-in long-range on-axis microscope, capable of a 5:1 zoom and $\times 3,500$ magnification. Intermittent-contact-mode imaging, which makes it possible to use higher scan rates and which can cope more easily with steep features, was used to acquire larger scans of whole bacteria at scan speeds of between 5 and 50 mm/s. Contact-mode imaging was used at scanning speeds of between 1 and 10 mm/s for higher-resolution images of less steep features. In both imaging modes, accurate feedback tuning was necessary in order to obtain the maximum possible gain allowing for the resolution of bacterial surface structures while avoiding oscillations when scanning along the side walls of the cell. All of the images were acquired at 512 by 512 pixels and were processed by means of plane fitting, high-frequency filtering, and three-dimensional shaded rendering. Cross-sections of interesting features were obtained by using the image analysis software of the microscope to acquire numerical topographical information. A typical imaging session began by using the built-in optical microscope and moving the $x$-$y$ table in the search for bacteria showing signs of damage; the AFM cantilever was then moved toward the surface in the proximity of the chosen bacterium. A large scan (50 by 50 $\mu$m) was made in order to assess the exact position and nature of the bacterium, with further smaller scans being used to zoom in on any interesting features.

RESULTS

Because the aim of the study was to highlight the possibilities offered by AFM in investigating the damage to bacterial morphology induced by an antimicrobial agent (i.e., cefodizime), the results will be confined to the images obtained, and no numerical data will be given. An example of the normal morphology of $E. coli$ not exposed to cefodizime is shown in Fig. 2.

Supra-MICs of cefodizime induce the death of $E. coli$, and Fig. 3 shows an example of the type of damage observed to lead to lysis of the bacterium; a hole can be seen on the surface, and the cell wall has disappeared to reveal the fine structure of the underlying cytoplasm, whereas the remaining part of the bacterium seems to be intact. The image can be analyzed from different points of view (Fig. 3a to d). The image appears clear and sharp and includes details with submicron-sized dimensions (Fig. 3e and f).

We found that the quality of the probe tip is essential in order to obtain this level of detail: only very sharp high-aspect-ratio tips make it possible to follow the lateral walls of the cell and obtain fine surface details at this level.

A second type of damage observed in the experiments with cefodizime at supra-MICs is shown in Fig. 4 and 5: a flattened empty cell with numerous rough surface patches that are signs of the small amount of cytoplasm remaining after lysis and the discharge of most of the intracellular content. This can be considered a further step in the damage process following the damage shown in Fig. 3.

It is worth underlining the fact that AFM reveals the real roughness of the surface of the bacterial envelope, which other types of microscopy frequently shown as being relatively smooth.

This type of damage could be seen in all of the experiments with supra-MICs, but its frequency varied according to the antibiotic concentrations and the incubation times. Compared with the types of damage induced by supra-MICs of cefodizime, the types of damage induced by sub-MICs of cefodizime had a different appearance. One of the most common shape alterations induced by $\beta$-lactams at sub-MICs is the filamentation, and an example is shown in Fig. 6. Part of the bacterial filament is still partially filled, although damage to the cell wall has caused a hole in the surface; the loss of cytoplasm through the hole leads to a flattened filament, such as that shown in Fig. 7. Figure 7 also shows the fine morphological and surface alterations that take place after exposure to sub-MICs of cefodizime.

Because AFM images are acquired in digital form, they can easily be rotated in space and observed from different points of view (left to right laterally combined with up and down views), as can be seen from Fig. 2 to 7. This is a useful feature that makes it possible to obtain an overall picture and a better comprehension of alterations which other techniques generally show only from one side.

A further outstanding aspect of AFM is the fact that it is possible to obtain a cross-section of the image and measure the height and size of the observed features precisely. Figure 8 shows that the collapsed cell along the cross-section is 160 nm thick and that the thickness of the fimbriae is 9 and 14 nm. It is also easy to make comparative measurements of modified and unmodified bacterial regions.

DISCUSSION

The basic principle of AFM is the scanning and sensing of the topography of a sample by means of a very sharp tip (cross-section, $< 10$ nm) placed at the end of a flexible cantilever (6, 12, 13).

Depending on the surface of the sample, the interactions between the atoms or molecules of both the tip and the sample can be explained in terms of forces of attraction or repulsion: when the tip is rastered across the sample by means of piezoceramic devices, these forces cause the cantilever to deflect up or down according to the fine tridimensional topography of the surface of the sample (6).

A laser beam focused on the reflective back of the cantilever makes it possible to measure any deflection with high degrees of precision and sensitivity. During scanning, a feedback control loop maintains the selected level (or force) of cantilever deflection by applying a driving voltage to the vertical piezoceramic actuator that ensures that it will follow the topography of the explored surface, and it is this signal which is used to generate the image by AFM.

Two distinctly different modes of operation of AFM have been proposed: the contact (or repulsion) mode described above and the noncontact (or attraction) mode, during which...
FIG. 2–4. AFM images of *E. coli* under different conditions.

FIG. 2. Common morphology of *E. coli* without exposure to cefodizime. Bars, 500 nm.

FIG. 3 and 4. Example of bacterial damage after exposure to supra-MICs of cefodizime. Bars, 500 nm.
FIG. 5-7. AFM images of E. coli under different conditions.

FIG. 5. Example of bacterial flattening and emptying after exposure to supra-MICs of cefodizime. Bars, 500 nm.

FIG. 6. E. coli filamentation following exposure to sub-MICs of cefodizime. Bars, 500 nm.

FIG. 7. Filamentation and bulge formation in E. coli after exposure to sub-MICs of cefodizime. Bars, 500 nm.
small-amplitude, high-frequency oscillations that are applied to the cantilever and that are subsequently damped according to surface proximity are used to drive the feedback control loop in order to ensure very low tip-sample interaction at the expense of a lower lateral resolution.

A third operational mode, called the “tapping mode,” has recently been introduced. This involves the application of larger-amplitude cantilever oscillations that induce the tip to touch the surface intermittently. “Clipping” (or the reduction of the amplitude of the oscillations on the basis of the topography of the surface) is then used to drive the feedback loop and build up a high-resolution image while reduced lateral, shear, or frictional forces are exerted across the surface.

Like optical microscopes, SEMs measure only the x and y (not the z) dimensions of samples. The resolution limit of a general-purpose SEM may reach, under optimal conditions, 50 to 70 Å (depending on the specific properties of the electromagnetic lenses).

Atomic force microscopes simultaneously measure surfaces in the x, y, and z dimensions. The x and y resolutions are typically about 20 Å or less, and the z resolution can reach about 1 Å. Furthermore, the fact that the data are collected in digital rather than analog form greatly helps with the measurement of dimensions and the treatment of the images without any loss of resolution.

AFM not only provides a true three-dimensional map of the surface of bacterial samples on a submicron scale but also avoids the need for vacuum conditions or the coating of surfaces with layers of metal that may interact with samples and cover their fine natural structure. The bacteria can be prepared simply and quickly.

To our knowledge, this is the first study that has used AFM to investigate the bacterial surface damage induced by exposure to an antibiotic, and the images presented here clearly support the remarkable possibilities that the technique offers.

Two new and very interesting improvements in AFM have recently been introduced. The first is the miniaturization of the basic mechanical structure to the dimensions of an optical microscope objective that can be directly screwed into the revolver of an optical microscope, thus transforming it into an atomic force microscope that allows for both optical observation and AFM scanning of the sample. The second improvement is the possible use of AFM to analyze samples immersed in biological fluids, which means opening up the possibility of working with living samples at the submicron level (1, 2), an opportunity that has never existed before.

The application of AFM in biology, microbiology, and chemotherapy is only just beginning, and we think that it will not be long before we will be able to investigate morphological alterations in living bacteria exposed to antibiotics while they are taking place.

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REFERENCES