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Imaging Bacterial Shape, Surface, and Appendages Before and After Treatments With Antibiotics

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1. Introduction

Bacteria are typically smaller than eukaryotic cells. The average diameter of *Staphylococcus aureus* is 1 ± 0.5 μm, whereas *Escherichia coli* is on average 0.5×1.5 μm. The bacterial cell is also characterized by the presence of a complex external rigid structure called cell wall, which protects the internal protoplast and gives also the cellular shape, that generally falls into one of the, three basic morphologic categories, spherical (cocci), rod-shaped (bacilli), and spiral. Some bacteria show an atypical bacterial shape.

Bacteria are also able to extrude some material that collects outside the cell wall to form an additional, surface layer. Many genera of bacteria possess also filamentous structures projecting through the cell wall to form the so-called surface appendages. The most commonly observed bacterial appendages are flagella, fimbriae or pili, and filaments.

Antibiotics are particular type of drugs able to interfere in different ways to the metabolic pathways of bacteria. This causes also changes directly or indirectly in the structure of cell wall and consequent alterations in the shape of bacteria. The integrity of cell wall and bacterial shape are important to maintain the vitality and the virulence of bacteria. Morphostructural alterations not only cause bacteria to loose cytoplasm but also to be more easily phagocytized and killed by human phagocytic cells. A large amount of basic and clinical researches in microbiology, chemotherapy, and infectious diseases have been performed to investigate the morphology and structure of bacteria. These studies have been previously conducted by means of optical microscopy and scanning electron microscopy (SEM).

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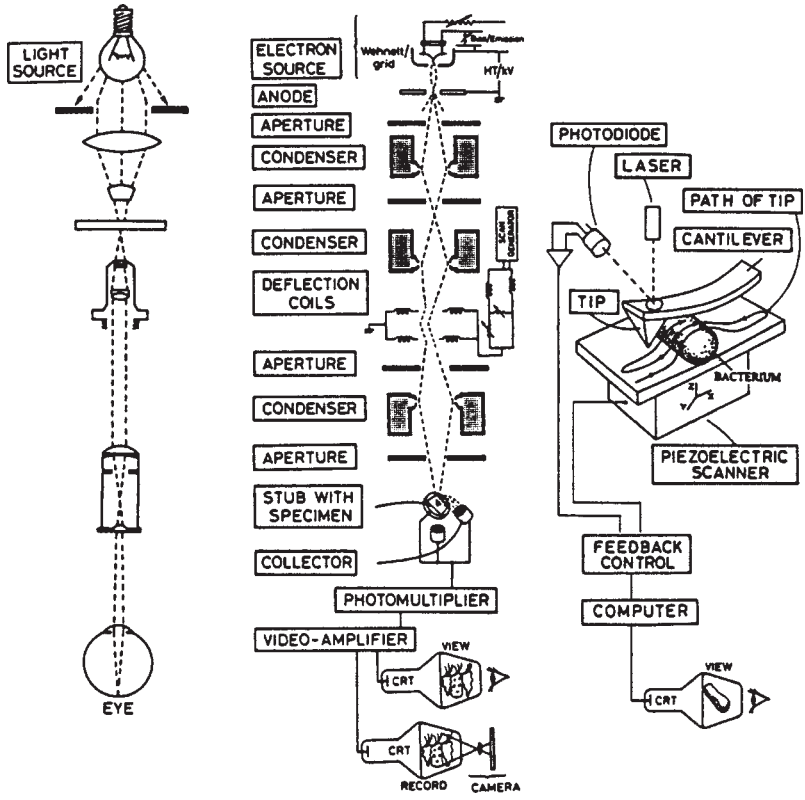
Optical and scanning (or transmission) electron microscopes are classified as far-field microscopes because the distance between the sample and the point at which the image is obtained is long in comparison with the wavelengths of the photons or electrons involved. In this case, the image is a diffraction pattern and its resolution is wavelength limited ***(1,2)***: in optical microscopy, resolution is determined by the Nyquist relation to the wavelength of the light used (typically about 1 μm); in a general purpose SEM, it is limited by the properties of the electromagnetic lenses (typically about 50 Å; **ref. *3***).

In 1986, a completely new type of microscopy was proposed: without lenses, photons, or electrons, it involved the mechanical scanning of samples ***(4)*** and opened up unexpected possibilities for the surface analysis of biological specimens. Initially called the scanning force microscope (SFM), it was a development of the previous scanning tunneling microscope ***(5)***, which provided information at atomic resolution of specimens that are electrically conducing. Because SFMs involve interactions between atomic forces (about 10^{-9} Newton), they are also and more frequently called atomic force microscopes (AFMs; **ref. *3***).

These new types of 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. Located in the immediate vicinity of the sample itself (usually within a few nanometers), the probe records the intensity and not the interference signal, and this greatly improves resolution ***(1)***.

As shown in **Fig. 1**, AFM explores the surface of a sample not by means of a system of lenses that form an image using the diffraction patterns of rays of different wavelengths but by means of a very small sharp-tipped probe located at the free end of a cantilever driven by the interatomic repulsive or attractive forces (van der Waals forces) between the molecules at the probe tip and those on the surface of the specimen. This can be done by scanning the sample laterally (x, y) while a closed loop control system keeps the tip in proximity to the surface by adjusting the z position of the sample. In most AFMs, tip movements are monitored by reflecting a laser beam from the back of the cantilever on to a position-sensitive photodiode ***(6)***.

AFM is extremely useful for analyzing the three-dimensional structure of the surface of biological specimens, particularly bacteria. Although SEM is still frequently used, the introduction of the AFM technique offers substantial benefits in real quantitative data acquisition in three dimensions, minimal sample preparation times, flexibility in ambient operating conditions (i.e., no vacuum is necessary), and effective three-dimensional magnification at the submicron level ***(7,8)***.



	LIGHT MICROSCOPE	SCANNING ELECTRON MICROSCOPE	ATOMIC FORCE MICROSCOPE
•SAMPLE			
-preparation	none/ little	freeze drying gold sputtering vaccuum	none/ little
-characteristics:	thin/ trasparent	vacuum compatible	not excessive variation in height
-operating environment	dry/liquid/ vacuum	vacuum	dry/liquid/ vacuum
•BIOLOGICAL APPLICATION	many	surface analysis	surface analysis
•FOCUS (depth)	medium	small	small
•FIELD (depth)	small	large	medium
•RESOLUTION			
xy axis	1.0 μm	5 nm	0.1-1.0 nm
z axis	——	——	0.01 nm
•MAGNIFICATION	1-2x10³	10x10⁶	5x10²-10⁶

Fig. 1. Comparative schematic view of the elements characterizing light microscopy, scanning electron microscopy, and atomic force microscopy, together with their specific technical parameters.

To investigate the shape and the surface of bacteria offers the possibility of investigating the efficacy and the mechanism of action of antibiotics that disrupt this structure as an epiphenomenon of internal biochemical action ***(9–13)*** and at the same time the possibility of investigating their lack of activity, as in the case of resistance.

2. Materials

1. Test organisms. Both Gram-positive and Gram-negative bacteria are suitable for AFM.
2. Tryptic soy broth or other suitable medium.
3. Phosphate buffer saline (PBS): 0.02 *M* phosphate and 0.15 *M* NaCl, pH 7.3.
4. Glutaraldehyde: 2.5%.in 0.1 *M* cacodylate buffer, pH 7.0.
5. Graded alcohols (60, 70, 80, 90, and 100%).
6. Incubator.
7. Centrifuge.
8. Micropipette and sterilized disposables for culturing bacteria.
9. Round glass coverslides, diameter 6–7 mm (or mica).
10. AFM (including probe-tips, software for processing signals and three-dimensional rendering, and computer).

3. Methods

1. Prepare the cultures of chosen microorganism according to common standard procedures.
2. Wash the test microorganism from the suspension in broth (i.e., 10^6 cells/mL) three times with PBS.
3. Resuspend the final pellet in 1–2 mL of PBS.
4. Collect 0.1 mL (or less) of this suspended bacteria with a micropipet and place it on round glass coverslide (*see* **Note 1**).
5. Dry the coverslip in air.
6. Fix with 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.1).
7. Dehydrate in graded alcohols.
8. Dry the coverslip in air (*see* **Note 2**).
9. Repeat steps 1–8, incubating bacteria with various supra-minimum inhibitory concentrations (MICs) or sub-MICs of antibiotic.
10. AFM observation (*see* **Notes 3** and **4**). A typical AFM imaging session begins by firmly fixing the sample cover slide to the microscope holder to avoid even the slightest movement (*see* **Note 5)** and then positioning it under the probe tip and locating the area of interest by moving the *x*–*y* table.
11. A good-quality on-axis optical microscope is essential to be able to position the probe tip in the proximity of a bacterium to be imaged by AFM. Because bacteria are about 1 μm in size, it is necessary to have appropriate lighting conditions to distinguish them from any debris on the slide surface. In the experiments described here, a reflection optical microscope equipped with long-range objec-

tives was used. Although the cantilever bearing the probe partially obstructs the optical view of the underlying bacteria, it does allow the probe to be positioned sufficiently accurately in the area of interest (*see* **Notes 6** and **7**). Commercial AFM instrumentation coupled to a transmitted light optical microscope offers a higher degree of precision in the first approach of the probe to the sample (*see* **Note 8**).

12. Once an area has been located after the tip-to-sample approach, a first large scan (i.e., 30×30 µm) using a high scan speed and small number of pixels per line can be made in order to assess its exact position within the scanner coordinate system, identify the nature of the bacteria and select an interesting one. Further smaller scans may be necessary in order to position the bacterium exactly at the centre of the scanning area.
13. Record high-resolution images (*see* **Note 6**) by using appropriate instrument settings depending on the imaging mode selected (contact, intermittent contact, non-contact; *see* **Note 7**). In general, accurate feedback setting is necessary to obtain the maximum possible gain for the resolution of bacterial surface structures while avoiding oscillation when scanning along the cell sidewalls (*see* **Note 2**).
14. Acquire image (typically acquired at 512×512 pixels) and process by means of plane fitting, high-frequency filtering, and three-dimensional-shaded rendering (**Figs 2** and **3**).
15. Postprocessing analysis and the spatial representation of AFM-generated data are essential to extract all of the available information from the image dataset. Because the recorded data are an intrinsically three-dimensional digital matrix (the height of the sample recorded at each x, y coordinate), the software makes it easy to obtain numerical data of cross-sections of interesting features expressed with sub-nanometer accuracy (*see* **Note 8**). The same software allows three-dimensional rendering of the surface and rotation in space so that only one acquisition is needed to be able to observe the same object from many different points of view (*see* **Notes 9–11**).

4. Notes

1. It is better to use low concentrations of bacteria because they tend to concentrate in small areas during the air-drying phase, whereas a single bacterium provides a clearer image. Be sure to mark the location of your specimen on the upper surface of your round glass coverslide to avoid wasting time investigating the wrong side.
2. If the sample is kept dry, repeated sessions could generally be performed without any loss of resolution.
3. Bacterial sample preparation for AFM is very simple and rapid. There is no need for critical point drying, which also avoids shrinkage effects; there is no need for gold sputtering, a procedure that covers and smooths fine surface details. There is no need for vacuum conditions, as with SEM.
4. A recent technical evolution has also opened up the possibility of using AFM on wet samples, that is, living cells immersed in biological fluids in culture chambers ***(14,15)***.

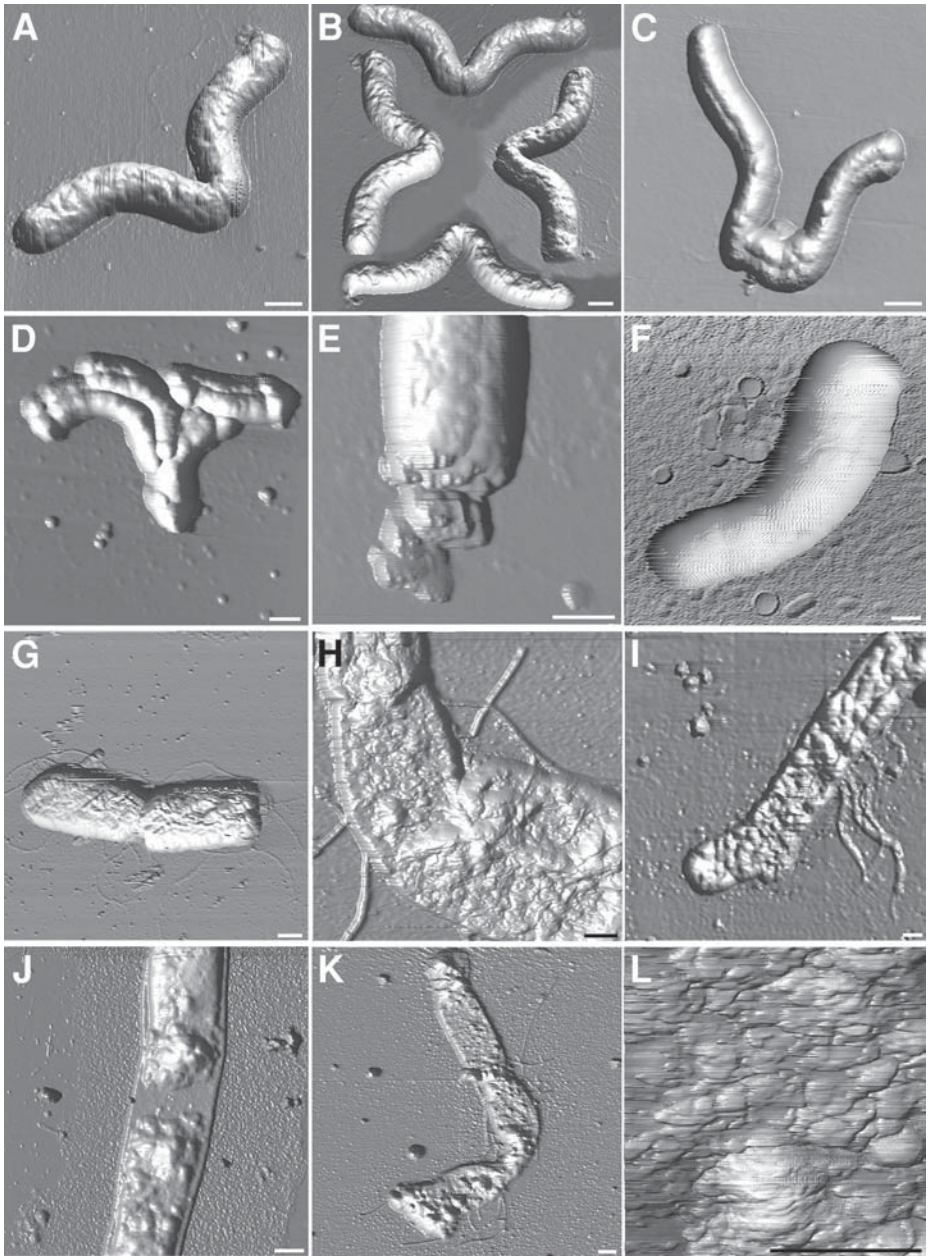


Fig. 2. Atomic force pictures of *Helicobacter pylori* and *E. coli*. The bar at the bottom right of each picture corresponds to 500 nanometers. **(A)** Common morphology of *H. pylori* without exposure to antibiotic. **(B)** Example of the different perspectives obtained by means of computer processing **(C–E)** Different alterations induced in *H. pylori* by

5. Care must be taken when choosing the adhesive used for fixing the glass slide to the sample holder. Avoid using thick double-sided adhesive tape, as this can expand for a long time after pressure and thus cause instability in the vertical position of the tip. The specially produced sticky tabs made by different manufacturers are fine.
6. To obtain the best results, it is necessary to be thoroughly familiar with the characteristics of different cantilevers and tips, how these can be used and how they suit to the different kinds of samples investigated. For high-resolution work, tip sharpness is essential: tip properties can vary significantly within the same batch of cantilevers. Fine tuning of the feedback loop and set point, together with the chosen scan speed, is critical for good surface tracking.
7. As mentioned above, AFM offers different imaging modes for investigating the sample. There is the contact mode in which the tip of the probe makes soft physical contact with the sample, which should be used with harder and stiffer materials than biological samples as it can easily give rise to undesirable effects as the result of tip-to-sample interactions (**Fig. 2L**). Tip pressure can indent and deform the sample surface, and lateral forces can stretch the sample, drag away loosely bound fragments, or even detach the whole bacterium from the substrate ***(3)***. These drawbacks of the contact AFM mode are overcome by using the intermittent-contact mode, also called tapping mode. In this case, the AFM feedback loop constantly dampens the high-frequency oscillations of the vibrating cantilever due to the tip coming into contact with the surface for a very short time ***(16)***. For this reason, indentation effects are less invasive, lateral forces are greatly reduced, and a high lateral resolution can be maintained. In the third noncontact mode, small amplitude and high-frequency oscillations induced on the cantilever allow the feedback control loop to maintain the tip-to-sample distance within the range of attractive Van der Waals forces. Tip-to-sample interactions are greatly reduced at the expense of lateral resolution and the scanning speed ***(2)***. For biological specimens the noncontact and intermittent contact are the most suitable, although the contact mode may be used for high-resolution work on very small areas.
8. To make accurate dimensional measurements, the calibration of the AFMs piezoelectric scanner has to be periodically checked. The procedures are usually described in the instrument manual. Lateral dimension calibration is relatively straightforward, but special care must be taken when calibrating height. We used a VLSI standard calibration grid (NIST traceable) with a 100-nanometer nominal step height and an in-house developed statistical analysis procedure for calibration.

Fig. 2. *(continued)* exposure to sub-MICs and supra-MICs of rokitamycin. **(F)** Example of artifacts. High-frequency oscillations and lack of bacterium surface detail can be caused by feedback instabilities induced by an electrostatically charged sample. **(G)** Common morphology of *E. coli* without exposure to antibiotic. **(H–K)** Different alterations induced in *E. coli* by exposure to sub-MICs and supra-MICs of cefodizime. **(L)** Example of artefact produced by excessive tip-to-sample interactions in contact mode.

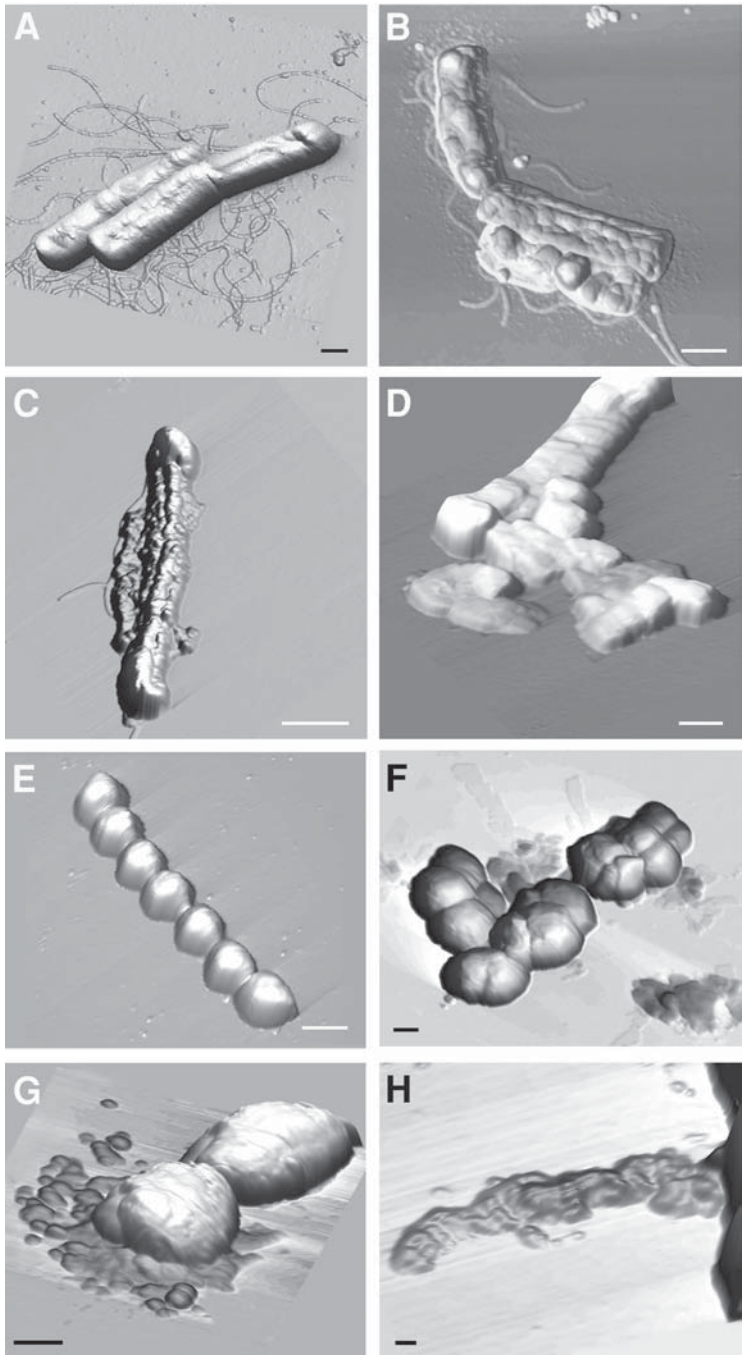


Fig. 3. Atomic force pictures of *Bacillus cereus* and *Streptococcus pyogenes*. The bar at the bottom right of each picture corresponds to 1 μm. **(A)** Example of untreated common rod-shaped morphology with flagella of *B. cereus*. **(B)** Morphostructural

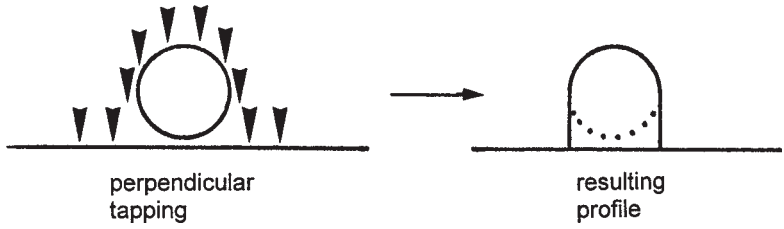


Fig. 4. Example of the AFM rendering of a spherical bacterium.

9. The images may sometimes be blurred as a result of poor washing procedures, an electrostatic charge on the specimen, improper feedback parameter settings, debris on the tip, or an eroded tip (**Fig. 2F**).
10. The images of spherical bacteria, such as *S. aureus*, will suffer from little lateral resolution along the perimeter because of the perpendicular direction of analysis. In general, the shape of the tip and its lateral walls will limit the detection of steep elevated features (**Fig. 4**).
11. After image acquisition, the built-in software allows the rendering of the picture to be greatly improved by means of shadowing, rotation, different illumination, and different points of view (**Fig. 2B**).

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Fig. 3. *(continued)* alterations of *B. cereus* after 2-h incubation with daptomycin at 8× MIC. **(C)** Example of alterations induced in *B. cereus* after 4-h incubation with daptomycin 8 × MIC. **(D)** Lateral view of altered structure of *B. cereus* after 8-h incubation with daptomycin 4× MIC. **(E)** Example of common morphology of *S. pyogenes* phenotype M without exposure to antibiotic. **(F–H)** After 6-h of incubation with rokitamycin (2 µg/mL, abnormally enlarged cells, loss of typical chain structure, formation of clusters, flattening of the cells, and ghost formation were present.

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