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Comparative studies of bacteria with an atomic force microscopy operating in different modes

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Abstract

Escherichia coli bacterial cells of two strains JM109 and K12 J62 were imaged with atomic force microscopy (AFM) in different environmental conditions. The AFM results show that the two strains have considerable difference in the surface morphology. At the same time after rehydration both strains show the loss of the topographic features and increase in lateral and vertical dimensions. Results obtained in different AFM modes (contact, tapping, MAC) were compared. Imaging in culture medium was applied for direct observation of the surface degradation effect of lysozyme. The treatment of the cells with the enzyme in the culture medium lead to the loss of surface rigidity and eventually to dramatic changes of the bacteria shape. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

A distinct feature of bacterial cells is a rigid cellular wall that is a critical factor in the survival of these organisms in a broad range of environmental conditions. The surface structure provides highly species-specific antigenic determinants of

the cells [1] and bacteria–host interaction. A unique property of bacterial cells is the formation of biofilms, very large aggregates during binding of bacteria to surfaces of different kind [2]. A great number of diseases are due to the biofilm formation. These surface features are not simple irregular cellular aggregates, but have unique biochemical characteristics different from isolated cells. Detailed structural studies of bacterial surface may help to understand molecular mechanisms of the biofilm formation and functioning.

Electron microscopy yields high resolution, but requires working in vacuum, staining and other

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special treatment. Novel techniques of scanning probe microscopy, particularly atomic force microscopy (AFM), have a great potential for structural studies in microbiology. Typical dimensions of bacterial cells (1 μm) are suitable for AFM, which yields resolution similar to electron microscopy. Being surrounded by cellular wall, bacteria have a surface much more rigid than that of animal cells, which simplifies AFM study. Moreover, AFM allows imaging in liquid without the sample drying and thus molecular and submolecular processes can be observed directly [3–5]. One of the main advantages of scanning probe microscopy over conventional structural research techniques is the possibility of imaging in liquid. In situ imaging procedure will allow the direct observation of biospecific interaction with biopolymers of bacterial cell, the destruction of bacteria by drugs, their growth and division in situ. There has been a number of works on AFM studies of bacterial cells [6–11] – including investigations of their morphological, adhesive and elastic properties [12].

In this paper, we present the results of AFM studies of *Escherichia coli* bacteria performed in various environmental conditions – air, water and bacterial culture medium. The term “in situ” is used here for imaging bacteria rehydrated after drying, as it has previously been done in Ref. [9]. It is believed [9], that such drying does not affect bacteria. Dried in ambient conditions, bacteria remain alive and being returned to a culture medium can continue their life cycle. In addition, AFM in situ was applied to monitor the degradation of a bacterial surface by lysozyme, an enzyme that is used for the removal of the walls of gram-negative bacteria.

2. Experimental section

2.1. Preparation of bacteria

The most famous and well-studied rod-shaped bacterium *Escherichia coli* (JM 109, K12 J62) was chosen for the experiments. Bacteria were cultured overnight on agarized 2 \times YT medium (pH 7.0 NaOH) containing (Bacto Triptone 16 g, Bacto

Yeast Extract 10 g and NaCl 5 g/l) on Petri dishes at 37°C. A layer of bacteria was clearly seen. Prior to imaging, bacteria were gently scratched off the agar surface with a metallic wire loop and dispersed in distilled water for no longer than 10 min. The concentration (10^9 – 10^{10} 1/ml) was used for AFM experiments.

2.2. Sample preparation

For imaging of dried samples a 5 μl droplet of bacteria suspension was applied to a freshly cleaved mica surface and left to dry.

For AFM in situ, mica was pre-treated with polylysine. A 10 μl drop of 10^{-2} M polylysine solution was applied to a freshly cleaved mica surface and left to dry. A 5–10 μl drop of bacteria suspension in distilled water was applied onto treated mica. After drying distilled water (pH 5.5) or bacteria culture medium 2 \times YT was injected into the fluid cell with a syringe. An O-ring was used only when imaging on pure mica.

AFM experiments in contact and tapping modes of operation were carried out using NanoscopeTM IIIa multimode scanning probe microscope equipped with a D-scanner and with a commercial tapping mode fluid cell (Digital Instruments, USA). Commercial silicon cantilevers NanoprobeTM with a spring constant 0.06 and 0.12 N/m were used for both contact and tapping modes in liquid. In contact mode the applied force was maintained at the level of 1 nN, gains of the feedback loop were 3.0 in air and 2.0 in liquid. Tapping mode images were collected in a broad range of frequencies, 10–170 kHz. The oscillation amplitude was 50–100 nm, setpoint ratio being 0.9. Experiments in acoustic and MAC modes were performed by means of a PicoSPM microscope (Molecular Imaging, USA) with Si₃N₄ cantilevers covered with a magnetic film, having a spring constant 0.5 N/m, resonant frequency 60–80 kHz in air and 20–30 kHz in liquid.

The data were analyzed with FemtoscanTM-001 [13] software (Advanced Technologies Center, Russia) and PicoScan image-processing software (Molecular Imaging, USA) for the data obtained on PicoSPM system (acoustic and MAC [14,15] modes).

3. Results and discussion

3.1. Imaging in air

The surface morphology of two strains *Escherichia coli* (JM 109 and K12 J62) in various environmental conditions (air and liquids) was compared. Freshly cleaved mica is an appropriate substrate for visualization of bacteria when the microscope is operating in air. Both strains form a flat patched layer on the surface and individual cells can be identified as well (Figs. 1 and 2). The preparation technique used gives a monolayer and each bacterium is distinguished in AFM images (Fig. 3). Most of bacteria are packed close to one another, forming compact coverings. Unlike the procedure described in Ref. [7], which employs culturing of bacteria in bulk and application of a suspension in culture medium our technique of preparation of bacteria provides clean AFM images without rinsing of samples, which is strongly necessary for the mentioned procedure to remove contamination.

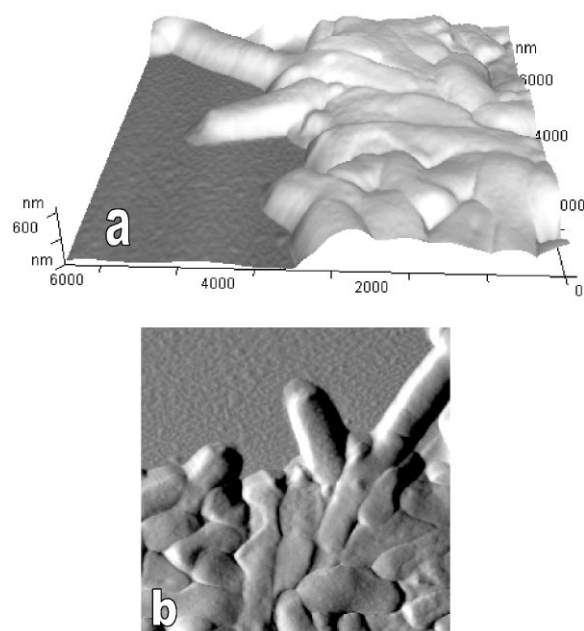


Fig. 1. AFM image of *E. coli* JM 109 bacteria. AFM data were obtained in air in contact mode. 3D height (a) and deflection mode (b) images. Image size $6.8 \times 6.8 \mu\text{m}^2$.

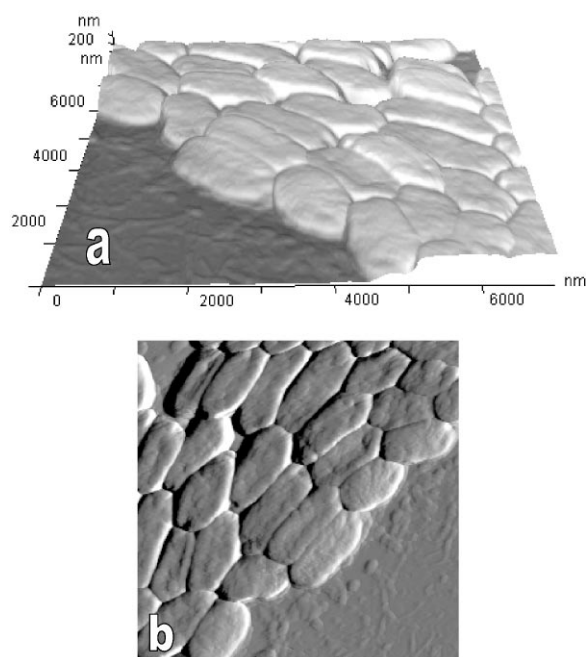


Fig. 2. AFM image of *E. coli* K12 J 62 bacteria. AFM data were obtained in air in contact mode. 3D height (a) and deflection mode (b) images. Image size $6.8 \times 6.8 \mu\text{m}^2$.

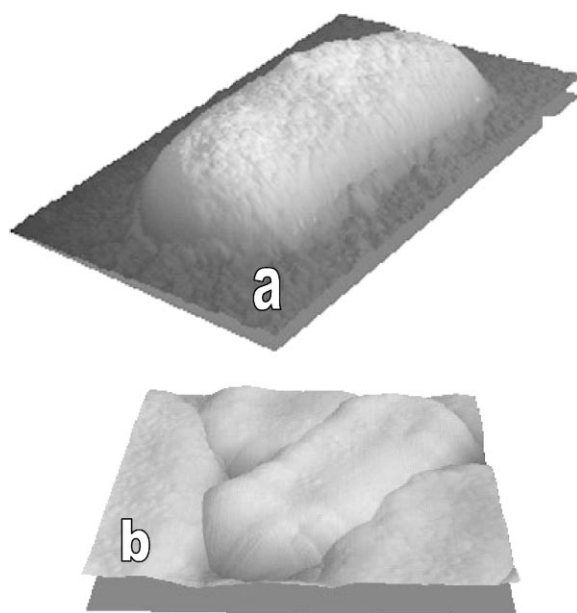


Fig. 3. Fragments of surface of bacteria *E. coli* strains JM 109 (a), K12 J62 (b). AFM data were obtained in air in contact mode. Image size (a) $3.7 \times 2.1 \mu\text{m}^2$, (b) $1.9 \times 1.9 \mu\text{m}^2$.

Both strains have different sizes. JM 109 cells are longer and thinner than those of K12 J62. The following obtained parameters characterizing the bacteria dimensions for the two *E. coli* strains are summarized in Table 1: length $L=1700\pm 1000$ nm, width $D=800\pm 100$ nm, height $H=280\pm 80$ nm. The length of the cells varies in a broad interval, which is in line with microbiological data

[16]. For K12 J62 these parameters were estimated to be $L=900\pm 100$ nm, $D=830\pm 60$ nm, $H=170\pm 40$ nm.

The data shown above were obtained with the Nanoscope IIIa instrument operating in contact mode. Similar data were obtained with PicoSPM microscope operating the microscope in acoustic (Fig. 4) or MAC (Table 2).

Table 1
Dimensions of *Escherichia coli* JM 109 and K12 J62 cells measured in air and liquid by AFM operating in contact mode

Environment	Strain	Cell length (nm)	Cell width (nm)	Cell height (nm)
Air	JM109	1700 ± 1000	800 ± 100	280 ± 80
	K12 J62	900 ± 100	830 ± 60	170 ± 40
Liquid	JM109	2000 ± 1000	1100 ± 500	500 ± 100
	K12 J62	1200 ± 400	1100 ± 200	260 ± 50

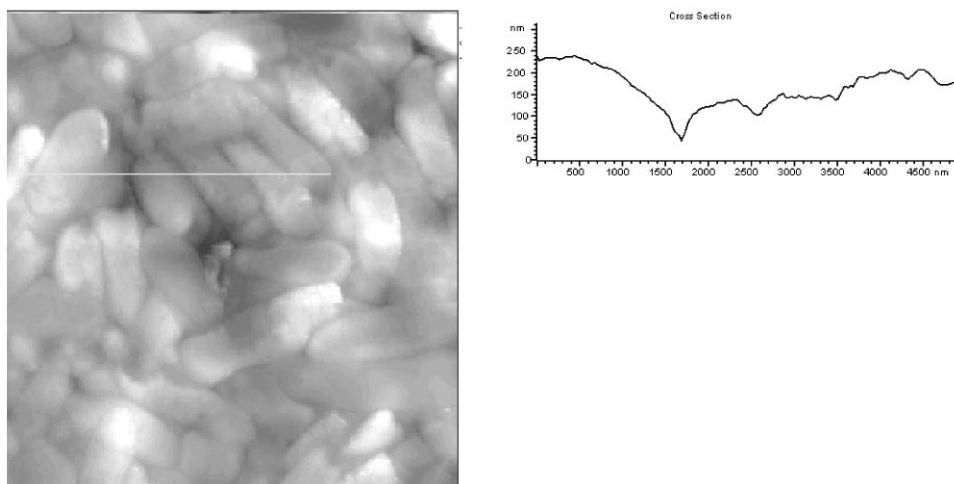


Fig. 4. AFM image of *E. coli* AFM data were obtained in air in acoustic mode (height image). Image size – $7.5\times 7.5\mu\text{m}^2$.

Table 2
Dimensions of *Escherichia coli* JM 109 cells measured in air and liquid by AFM operating in contact, tapping and MAC modes

Mode of operation		Scan rate, Hz	Cell width, nm	Cell height, nm
Contact	Air	up to 30	800 ± 100	280 ± 80
	Liquid	1–2	1100 ± 500	500 ± 100
Tapping	Air	1–2	800 ± 200	300 ± 70
	Liquid	1–2	1000 ± 400	80 ± 40
MAC	Air	2–4	800 ± 100	250 ± 90
	Liquid	0.5–1.0	1000 ± 500	500 ± 100

3.2. Imaging in liquid

The samples prepared by drying on bare mica were not stable and some cells were swept away during scanning, after injection of distilled water or physiological solution into the AFM fluid cell. Since the mica surface is negatively charged in liquid, poor adhesion could be explained by the electrostatic repulsion. To overcome this difficulty we used treatment of mica with aminopropyl-3-ethoxy silane (AP) [17], which has previously been successfully used for the immobilization of negatively charged nucleic acids. Nevertheless, in the case of bacteria that did not give positive results and it looks like, that for holding bacteria one needs longer hooks, than AP-tail.

Successful results were obtained on mica subjected to polycationic treatment. *E. coli* is a gram-negative bacterium. Its surface is formed by lipopolysaccharides, negatively charged at physiological pH. For immobilization we chose polylysine, a synthetic polypeptide, homopolymer of α -amino acid lysine. Each residue of lysine contains an amino-group (pK 10.63). Polymer chain of polylysine is positively charged and remains in stochastic globule conformation at pH under 12.0 [18]. Polylysine readily adsorbs to mica and gives a smooth coverage in a form of monolayer, composed of tightly packed globules with average lateral dimension of 20 nm (data not shown). Such coverage is inappropriate for imaging individual macromolecules, but is smooth enough (RMS roughness 1.0–1.5 nm) for imaging “huge” objects, like bacteria. Polylysine is biocompatible and should not affect bacteria. Further experiments showed that bacteria have much better adhesion to mica surface covered with polylysine than on freshly cleaved mica.

The experiments were performed under conditions favorable for bacteria: the samples were imaged in $2 \times$ YT cultivating medium (pH 7.0). The data for contact mode imaging are shown in Fig. 5. The images remain stable during repetitive scanning over the area.

The average ($N = 80$) dimensions were obtained for bacteria imaged in liquid. The dimensions of *E. coli* JM 109 (height $H = 540 \pm 180$ nm and width $D = 1100 \pm 500$ nm) exceed the same parameters

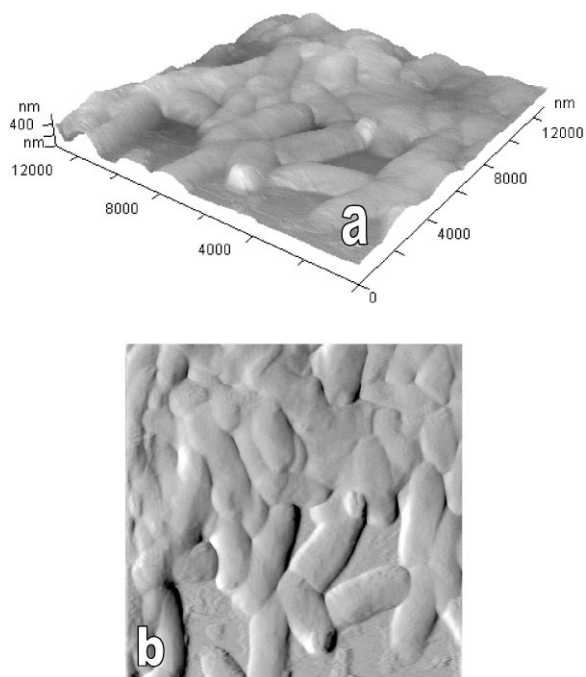


Fig. 5. 3D (a) and deflection (b) AFM images of *E. coli* bacteria. AFM data were obtained in liquid (bacteria cultivation medium) in contact mode. Image size $13.4 \times 13.4 \mu\text{m}^2$.

obtained for dried samples in air. For *E. coli* K12 J62 height and width measurements give $H = 260 \pm 50$ nm, $D = 1100 \pm 200$ nm (see Table 1). Since the length of these bacterial cells (L) varies in the interval of 2000 nm, the expected 100–200 nm difference of the value of this parameter for dried and hydrated samples cannot be correctly detected due to lack of statistics.

These data indicate that the height of bacterial cells in liquid exceeds considerably their height in air. Since in liquid tip–sample forces can be maintained at lower rates, than in air, it is difficult to explain the decrease of height in air by compression due to tip–surface interaction. If bacteria were compressed when imaged in air, that would lead to width augmentation, whereas it decreases. We suppose that 200 nm height and width reduction in air is due to drying of the polysaccharide layer on the surface of bacteria.

Another important point of comparative air–liquid studies is the topography of the cells.

Imaging in air reveals many topographic features that are missing for the cells imaged in liquid (compare Figs. 1 and 5). Typically imaging in liquid increases the resolution [4,5], so the loss of topographic features on bacteria surfaces is very likely due to the dynamics of cellular filaments – carbohydrate chains of lipopolysaccharides, that form the outer surface of gram-negative bacteria [6]. These inherent features of bacterial cells are collapsed onto the wall surface creating a strain-specific topography of the surface.

It is noteworthy, that the imaging was very sensitive to the applied force value, which had to be maintained in a very narrow interval. The changes in applied force caused by thermal drift have to be adjusted every other frame in our experimental conditions. If the applied force was higher than 10^{-9} N, some bacteria were swept away by the tip.

In tapping mode, minimization of lateral forces diminishes sweeping and distortion of objects. Despite the fact that no essential difference was noticed between dimensions of bacteria imaged in tapping and contact mode in air (Table 2), tapping mode images of bacteria in liquid demonstrated abnormally flat picture (data not shown). The height of bacteria was $80 \text{ nm} \pm 40 \text{ nm}$, 6 times less than in contact mode. In both modes, images were acquired at the same scan rate, so this difference

cannot be related to the feedback errors. The origin of this phenomenon is yet unclear. Figures obtained in contact mode seem much more realistic, taking into consideration the structure of bacteria.

Imaging in MAC mode in liquid provided proper images (Fig. 6), bacteria having the same dimensions as in contact mode images. Nevertheless, the imaging force also had to be minimized. Proper MAC mode images could be acquired at a scan rate of 0.4 Hz, which is 4–5 times more slowly than in contact mode (2.0–2.5 Hz). Image quality in MAC mode was the same as in contact mode.

3.3. The effect of lysozyme on the shape of bacteria

Lysozyme is an enzyme that causes the lysis of bacterial cellular walls, so after the partial degradation of murein framework only fragments of the gram-negative bacteria cellular walls remain attached to the cellular surface. A few microliters of saturated lysozyme solution was injected into the fluid cell. After 1 h of incubation dramatic changes of the bacteria shape were observed (Fig. 7), suggesting bacteria become spheroplasts [18]. Spheroplasts of gram-negative bacterium *E. coli* remained stable for a considerable period of time in the hypertonic medium $2 \times \text{YT}$, which

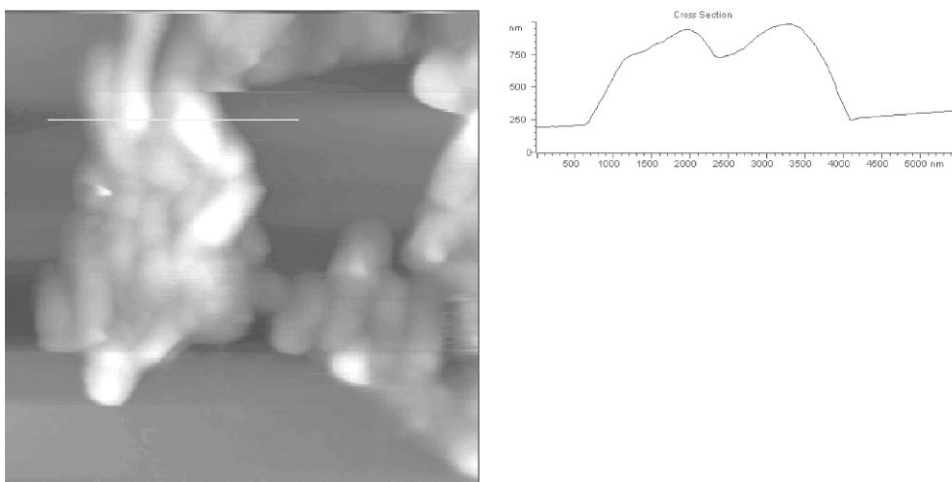


Fig. 6. MAC mode image of *E. coli* bacteria. AFM data were obtained in liquid (bacteria cultivation medium). Image size $10.5 \times 10.5 \mu\text{m}^2$.

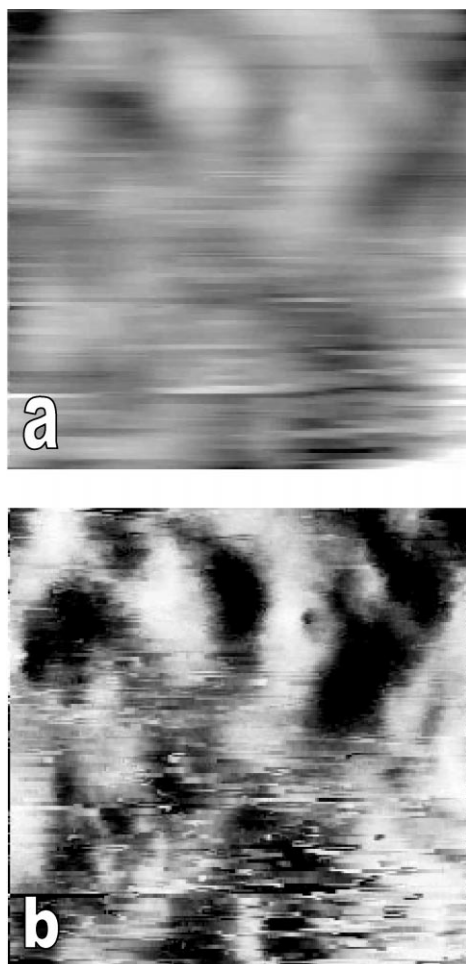


Fig. 7. AFM image of *E. coli* bacteria after lysozyme treatment. Height image (a) and deflection image (b). AFM data were obtained in liquid in contact mode. Image size $6.6 \times 6.6 \mu\text{m}^2$.

contains saccharides and other compounds that cannot penetrate through the cell membrane. Nevertheless, images became blurred, which suggested that the surface of cells became softer. The estimated average ratio of length-to-width for lysozyme-treated cells reduced to 1.3, compared to 2.2 for non-treated ones. The height distribution demonstrates two distinct peaks at $H_1 = 490 \pm 80 \text{ nm}$ and $H_2 = 90 \pm 45 \text{ nm}$. We refer the first peak to those cells whose membranes remained intact and the second one to those whose membranes were punctured (possibly by the probe), which led to the cells' compression in hypertonic medium.

When $2 \times \text{YT}$ medium in the AFM fluid cell was replaced with distilled water lysozyme-treated bacteria (spheroplasts) exploded in hypotonic medium. Remaining low-height fragments observed on mica surface (Fig. 8) have the height (thickness) of 14 nm that probably are debris of the bacteria walls.

4. Conclusions

Appropriate experimental procedure for in situ imaging of bacteria *E. coli* based on polylysine treatment of mica substrate gives good results for the immobilization of bacteria and further AFM measurements. Contact mode and MAC mode provide the best results, image quality being the same for both modes of operation. MAC mode required lower scan rate for successful imaging. Tapping mode imaging in liquid gives underestimated values of bacteria vertical dimensions. Drying of samples led to appearance of two artifacts – decrease of height and width and appearance of surface pattern. Lysozyme treatment led to changes in cells' shape and compression of cells with pierced membranes.

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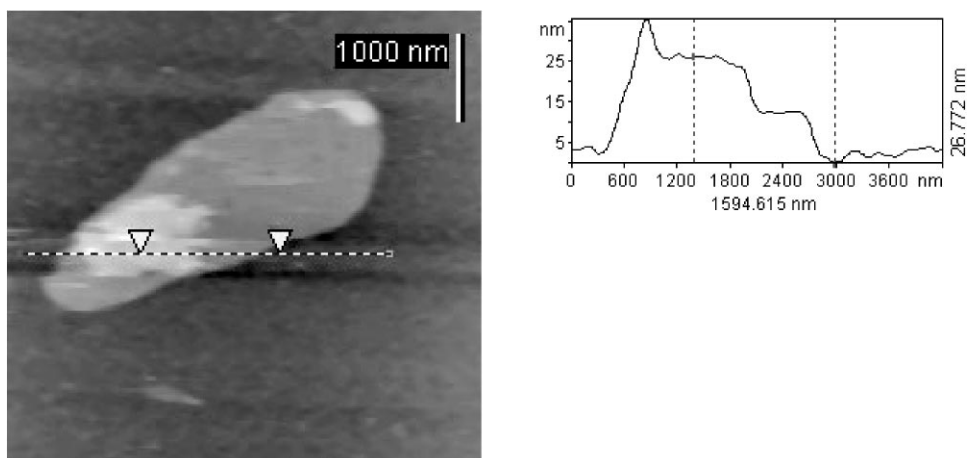


Fig. 8. AFM image of *E. coli* bacteria after lysozyme treatment and rinse in distilled water. AFM data were obtained in liquid in contact mode. Image size $5.1 \times 5.1 \mu\text{m}^2$.

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