Journal of Microbiological Methods 93 (2013) 80–84

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Antibiotic-induced modifications of the stiffness of bacterial membranes

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ARTICLE INFO

Article history:
Received 5 December 2012
Received in revised form 31 January 2013
Accepted 31 January 2013
Available online 22 February 2013

Keywords:
AFM
Antibiotics
E. coli
Force volume
Mechanical properties
Nucleoids

ABSTRACT

In the latest years the importance of high resolution analysis of the microbial cell surface has been increasingly recognized. Indeed, in order to better understand bacterial physiology and achieve rapid diagnostic and treatment techniques, a thorough investigation of the surface modifications induced on bacteria by different environmental conditions or drugs is essential.
Several instruments are nowadays available to observe at high resolution specific properties of microscopic samples. Among these, AFM can routinely study single cells in physiological conditions, measuring the mechanical properties of their membrane at a nanometric scale (force volume). Such analyses, coupled with high resolution investigation of their morphological properties, are increasingly used to characterize the state of single cells.
In this work we exploit such technique to characterize bacterial systems. We have performed an analysis of the mechanical properties of bacteria (*Escherichia coli*) exposed to different conditions. Such measurements were performed on living bacteria, by changing in real-time the liquid environment: standard phosphate buffered saline, antibiotic (ampicillin) in PBS and growth medium. In particular we have focused on the determination of the membrane stiffness modifications induced by these solutions, in particular between stationary and replicating phases and what is the effect of the antibiotic on the bacterial structure.

1. Introduction

Infections due to multidrug-resistant bacteria are a major public health problem, causing increasing mortality and morbidity worldwide. One of the most important tasks of the clinical microbiology laboratory is the identification and characterization of pathogens in samples as well as the realization of their antibiogram. As a whole, the majority of the most common characterization techniques are based on the assumption that the tested organisms represent a homogenous population in relation to their physiological and physical properties (Lay, 2001; Poppert et al., 2005; Turnidge and Paterson, 2007). For instance, only few works focus on the determination of the mechanical properties of single bacterial membranes in order to highlight variations occurring on single living bacteria (Alsteens et al., 2009; Dufrêne, 2008). In view of this, modern bacteriology is increasingly demanding a more in-depth study of bacterial characteristics, abandoning the overall population analyses and leaning towards the single-cell level (Brehm-Stecher and Johnson, 2004).

Nowadays, several instruments are available to characterize specific physiologically-related properties of living microscopic samples. Among these, Atomic Force Microscopy (AFM) (Binnig et al., 1986) stands out as the instrument of choice to perform high resolution imaging coupled with a point-by-point characterization of the mechanical and adhesive properties of micro and nanosized samples. Moreover, such measurements can be performed seamlessly in physiological conditions and in presence of different liquid media. This versatility is reflected in the AFM literature which presents with increasing frequency novel results regarding mechanical properties of single cells, bacteria or even single molecules (Berquand et al., 2010; Kasas and Dietler, 2008). At a cellular scale, it has been demonstrated that the mechanical properties of the membrane can reflect the physiological state of the entire system and that these properties are clearly altered in the occurrence of pathological conditions (Cross et al., 2007; Girasole et al., 2010; Lekka et al., 2005). It seems reasonable to extrapolate these observations to bacterial cells, in order to track a possible correlation between their morphological and mechanical characteristics and their state or pathogenic potential (Gaboriaud et al., 2008; Miklossy et al., 2008; Rossetto et al., 2007).

Unfortunately, most of the works currently present in literature exploit only partially the capabilities of the AFM of characterizing living specimens in physiological buffer. In fact, most papers focus only on the morphological modifications induced by antibiotics, imaging in very-high detail the dehydrated membranes of the bacteria (Cui et al., 2012; Dubrovin et al., 2012; Hyldgaard et al., 2012). The few groups that investigate the stiffness of the living bacteria have, up to now, prepared samples using bacteria exposed to different environmental conditions and have only compared the average stiffness values of

Abbreviations: PDMS, polydimethylsiloxane; PBS, phosphate buffered saline; LB, lysogeny broth; APTES, 3-aminopropyltriethoxysilane; FD, force distance; FV, force volume.

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http://dx.doi.org/10.1016/j.mimet.2013.01.022

the different samples (Formosa et al., 2012; Su et al., 2012). This is somewhat limiting, since the investigation of mean stiffness values does not allow characterizing more complex mechanical modifications induced by the antibiotic on the bacterial membrane. Moreover, since AFM is principally a single-cell technique, in order to exploit this strength it should be used to study both the morphology and the mechanical properties of one single living microorganism with a high spatial resolution and while changing its environmental conditions. Indeed, in this work we present an AFM-based morphological and mechanical investigation of bacteria (specifically the Gram-negative bacilli *Escherichia coli*) during their exposure to different environmental conditions. In particular we will highlight the modifications that arise when these microorganisms are treated with an antibiotic (ampicillin).

2. Materials and methods

2.1. AFM and liquid cell

The mechanical properties were investigated using a Bioscope I AFM (Bruker AXS), mounted on top of an inverted microscope. The microscope is equipped with a custom polydimethylsiloxane (PDMS) sample holder, capable to maintain the sample under investigation in a controlled environment. This device is coupled with a liquid changing system capable of replacing the incubation medium during the measurements, with virtually no effect on the AFM acquisition (Fig. 1) (Kasas et al., 2010).

All the images were collected using Bruker DNP-10 cantilevers, choosing the tip with a nominal spring constant of 0.06 N/m. The mechanical investigations have been performed by indenting the AFM tip into the sample and by measuring the cantilever deflection during the process. The resulting curve, called force distance (FD) curve, delivers high-resolution information regarding the stiffness and adhesion of the analyzed area. Importantly, the AFM can perform such measurements in virtually any kind of environment, and in particular in different liquid media, allowing the observation of biological processes in nearly physiological conditions.

The images presented in this work were collected in force volume (FV) mode, a setup in which the AFM tip is periodically indented into the sample and the deformation of the cantilever is recorded to reconstruct an image formed by a large number of FD curves. All the presented images contain 32×32 pixels and, for every pixel, 256 points per force curve were collected. The cantilever speed for all the presented images was of 0.1 lines per second, i.e. around 5 min per image. For each curve, the tip–sample interaction was limited to a deflection maximum of 30 nm (~2 nN).

The FV data files were recorded on several bacteria (more than 50 individual cells were imaged) and analyzed assuming that the cantilever behaves accordingly to the Hook law (i.e. the deflection of the cantilever is directly proportional to the vertical component of the force applied onto the tip). In this case the FD curves collected on the sample can be subtracted from FD curves obtained onto a hard surface. The resulting curves are called indentation curves, indicating the force needed to push the tip for a defined length into the sample (i.e. to indent it). The shape of each indentation curve is then used to calculate the mechanical properties of the sample (specifically the stiffness) (Cerf et al., 2009; Polyakov et al., 2011; Rossetto et al., 2007). The data analysis was performed in a fully automated fashion by using *OpenFovea*, a free data processing software that uses the Hertz model to reconstruct stiffness maps of the entire image (Roduit et al., 2012). For the calculations we assumed that the tip is spherical with an apical radius of 20 nm and that the sample's Poisson ratio is 0.5. Moreover, some of the FD curves have been processed using the software in stiffness tomography mode, an innovative feature that allows revealing the mechanical differences that the AFM tip encounters during its indentation path, delivering cross section images of the sample's mechanical properties (Roduit et al., 2009).

2.2. Bacteria preparation

The bacteria investigated in this work are *E. coli*, Gram-negative rod-like shaped bacteria (typically 500 nm wide and several microns long). More precisely, we chose to study *E. coli* strain DH5α because it is a well-known laboratory strain that is susceptible to ampicillin. Frozen stocks of *E. coli* were kept at −80 °C in LB media supplemented with glycerol. For the experiments, bacteria from stock were streaked on LB agar media. Then, a few bacterial colonies were collected from the agar plate and incubated overnight at 37 °C in LB. After incubation, the bacteria were washed three times for 10 min at 5000 rpm, resuspended in phosphate buffer saline (PBS, pH 7.4) and deposited on a 12 mm PDMS substrate for AFM investigation. To ensure a firm adhesion of the living bacteria on the substrate, the substrates were preliminarily coated with a linker molecule, (3-aminopropyl) triethoxysilane (APTES), a highly effective silane coupling agent used on a wide series of substrates to enhance cell adhesion. First the clean substrates were exposed for 1.5 min to 0.2% APTES and rinsed in ultrapure water. Next, the bacteria were left to incubate on the coated substrates for 15 min before thorough rinsing to remove loosely adhering cells. The samples were finally placed in 0.5 ml PBS in the liquid cell holder for AFM investigation. During the investigation, and without interrupting the measurements, the medium was changed,

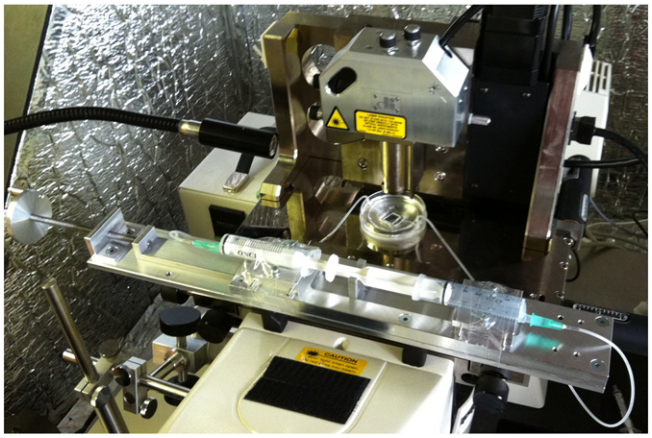

Fig. 1. The Bruker Bioscope I equipped with the custom PDMS sample holder and coupled with the liquid changing system.

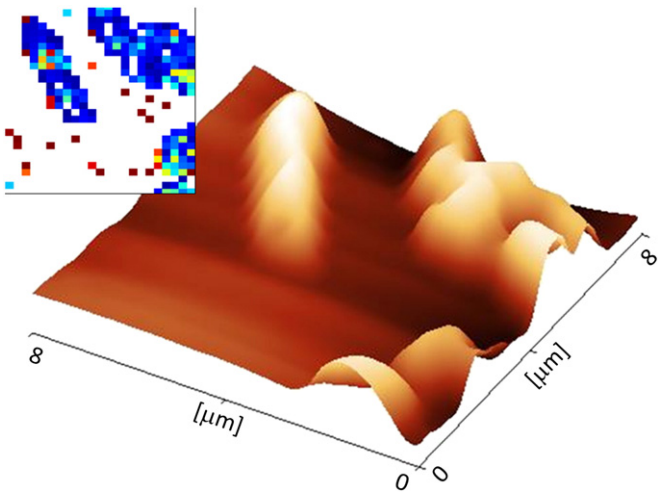

Fig. 2. 8×8 µm, 32×32 pixels, 3D reconstruction of the topography of *E. coli* bacteria immobilized on the substrate and imaged in PBS. Inset: force information of the very same area, showing how the different bacteria appear to have uniform stiffness. Well-defined stiffer structures can be seen on all the bacteria.

first with 0.5 ml of 4.5 mg/ml ampicillin in PBS and, in a second step, with 0.5 ml LB.

3. Results and discussion

As said, the experiment was divided in three steps. First, the bacteria were imaged in PBS, a buffer medium without nutrients. This induces a stationary state reducing the bacterial movement and the ability to replicate (Tortora et al., 2009). Secondly, the liquid changer system was used to introduce ampicillin diluted in PBS. This is an antibiotic of the β-lactam class that interferes with the formation of peptidoglycan, which is an essential component of the bacterial cell wall and whose degradation ultimately causes cell lysis and bacterial death (Rodionov and Ishiguro, 1995). Finally the liquid medium was changed with LB to verify if the membrane damage could be recovered by the bacteria.

An 8×8 μm, 3D reconstruction of the topography of the specimen, obtained by performing force volume imaging of the sample immersed in PBS, is depicted in Fig. 2. Several bacteria can be seen attached to the substrate. The corresponding stiffness is shown in the inset of Fig. 2, showing the soft (blue) bacteria attached to a much stiffer (white = no indentation) substrate. Such more wide images were collected to confirm that all the bacteria in the area had uniform stiffness values. The uniformity of the results was verified by investigating minimally 3 areas for each sample and by comparing the results obtained in 10 different and independent preparations. The total number of bacteria analyzed in this lower resolution exceeded 50 cells.

In order to increase the obtainable resolution, for each sample preparation we concentrated on a single *E. coli* cell, in this way we were able to increase the number of force curves collected on the bacterial membrane and the time needed to perform the measurements. We followed the evolution of the topography and membrane stiffness of these particular microorganisms while changing the media. The presented results were consistent in all the tested samples and the presented results well-represent all the experimental evidence.

Fig. 3A depicts the topography of the chosen bacterium in PBS environment (5×5 μm, Fig. 3A), while Fig. 3B shows the respective stiffness

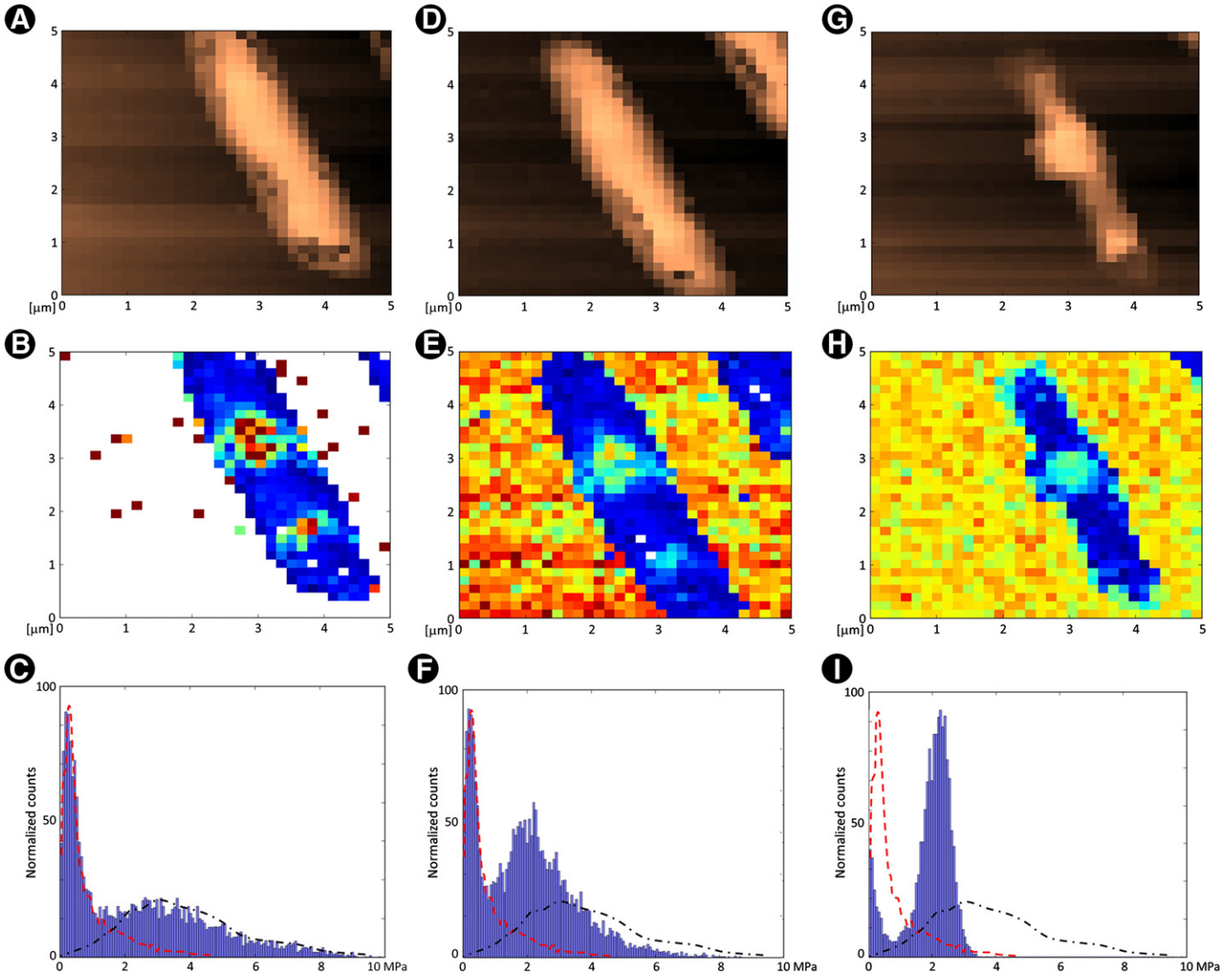

Fig. 3. Panel A: 5×5 μm, 32×32 pixel topography image of a single *E. coli* immobilized on the substrate and imaged in PBS. Panel B: corresponding stiffness image evidencing that the mechanical properties are uniform throughout most of the bacterial membrane, except two well-defined stiffer areas. Panel C: histogram of the stiffness values obtained from several images collected on the very same area. Two curves are evidenced: in red the membrane contribution and in black the peak of the substrate stiffness. Panel D: 5×5 μm, 32×32 pixel topography image of the very same *E. coli* imaged after introduction of ampicillin in PBS. Panel E: corresponding stiffness image, showing a reduction of the overall stiffness of the two stiffer areas and of the substrate. Panel F: histogram of the stiffness values obtained from several images collected on the very same area in the same conditions. The substrate peak has moved towards lower stiffness values. Panel G: 5×5 μm, 32×32 pixel topography image of the same *E. coli*, deflated after introduction of LB. Panel H: corresponding stiffness image, showing the disappearance of one of the two stiff areas and a reduction in stiffness of the other. Panel I: histogram of the stiffness values obtained from several images collected in the same conditions on the very same area. The membrane peak indicated a dramatic reduction of the stiffness of the entire membrane.

values collected for each point. Several subsequent images, collected over a 4 h period (for instance, Fig. 4A shows the same bacterium in the same environmental conditions 1 h later), indicate that the membrane stiffness is stable in time, with only small stiffness variations, probably due to the internal metabolism. To better elaborate and compare the stiffness information from all the collected images, all the data was collected in a histogram, shown in Fig. 3C. This allowed discriminating two well-defined stiffness peaks, one centered at 3 MPa that can be attributed to the typical stiffness of the substrate and the second at 300 ± 70 kPa, associated with the stiffness of the *E. coli* membrane. Indeed, such values are in good agreement with those obtained by similar studies on the same kind of bacteria, confirming the attribution (Chen et al., 2009). To better compare these results with the ones collected in different environmental conditions we have highlighted these two peaks in red (bacteria) and in black (substrate).

On the bacterial surface, two stiffer areas could be identified (red and yellow ovals marked B and D in Fig. 4A). These features were present on all the analyzed bacteria, and their complex structure is evidenced in the higher resolution, 1×1 μm, images (Fig. 4B and D, zooms of the higher and lower structures respectively). A complete characterization of such structures is still underway, but the most probable origin of such features is the presence of agglomerates of stiffer internal cell components (mainly DNA), inside the cytoplasm (nucleoids) (Frenkiel-Krispin et al., 2004; Longo et al., 2012). To better characterize such areas, force tomography analyses were performed. Several force tomography sections were calculated from the data collected in Fig. 4B (section C corresponding to Fig. 4C) and d (section E corresponding to Fig. 4E), showing that in these areas not only the membrane is stiffer than the rest of the bacteria, but that stiffer features were present also inside the bacterial body.

Upon exposure to ampicillin, while the overall topographical structure appeared unaffected (Fig. 3D), the force volume images showed several modifications of the mechanical properties (Fig. 3E). While the softer areas of the membrane were unchanged, the two nucleoid areas evidenced a reduction of their stiffness. At the same time the entire substrate appeared to be coated with softer material, indicated by the reduction of its overall stiffness. This was confirmed

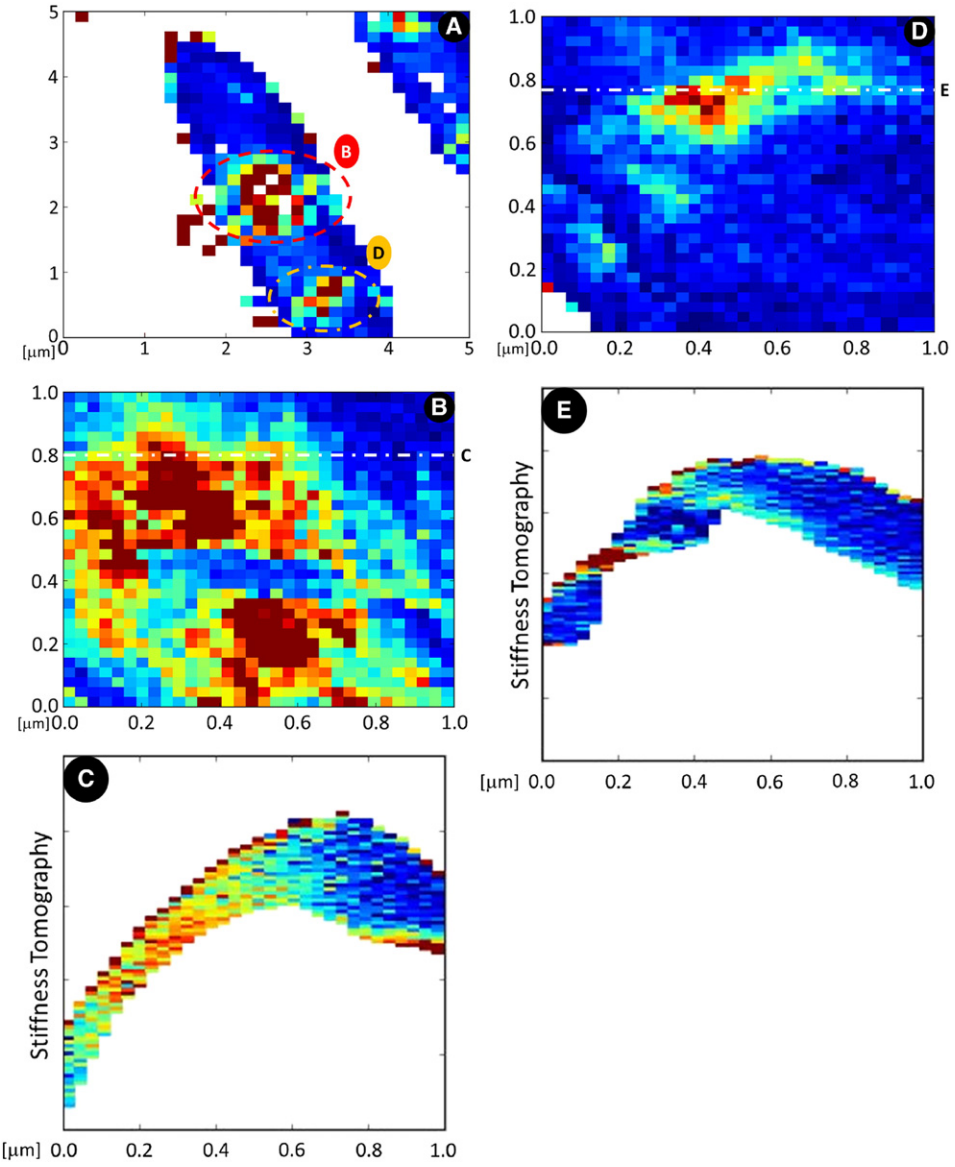

Fig. 4. Panel A: 5×5 μm, 32×32 pixel stiffness image of the same *E. coli* shown in Fig. 3. Two circles (B and D) highlight the two stiffer areas. Panel B: 1×1 μm FV zoom-in image of the nucleoid labeled B in Fig. 4A. A complex structure can be seen. Panel C: Force tomography section obtained over the line marked C in Fig. 4B. Stiffer structures can be seen under the membrane level. Panel D: 1×1 μm FV zoom-in image of the nucleoid labeled D in Fig. 4A. Panel E: Force tomography section obtained over the line marked E in Fig. 4D.

by comparing the histograms of the stiffness values shown in Fig. 3C and f: the peak associated with the membrane stiffness did not move significantly, while the peak of the substrate fell to values around 2.3 MPa. The most plausible interpretation of these data is that the ampicillin, interfering with the formation of peptidoglycan, indirectly caused the formation of membrane lyses and a consequent leak of some of the internal soft bacterial content on the substrate. Since the effect of the ampicillin is maximized when associated with a nutrient medium, this effect was greatly reduced in PBS environment.

Indeed, replacing the medium with LB caused, in a timespan much smaller than that of a typical image, much more dramatic changes: the topography image showed that the bacterium deflated (Fig. 3G) while the corresponding stiffness image (Fig. 3F) indicated that both the bacterial membrane and the material present on the substrate had become much softer. This is in agreement with the interpretation of the phenomena, indicating that most of the material present inside the bacteria had probably flowed out, probably due to osmotic pressure, through the lysis of the membrane (Fig. 3F). Also the two nucleoid areas underwent major changes: while the smaller one had completely disappeared (probably the stiffer material had entirely leaked on the substrate), the other had reached stiffness values similar to those of the rest of the membrane. These dramatic changes are evidenced in the histogram shown in Fig. 3I. Both peaks appear to have moved towards lower stiffness values: the membrane peak is centered at values below 100 kPa while the substrate component is still centered at 2.3 MPa but with a much smaller spread.

4. Conclusions

We have investigated the mechanical properties of the membrane stiffness of *E. coli* exposed to different environmental conditions. Such measurements were performed by force volume investigation delivering, with an unprecedented high resolution, a map of the mechanical properties of the bacterial membrane. We monitored the stiffness values of a single bacterial cell when exposed to different environmental conditions, including the exposure to antibiotics.

Such analyses have evidenced the presence on the bacterial surface of two stiffer areas. By employing an innovative technique capable of revealing stiffer structures below the surface level, such areas have been associated with the presence of features inside the bacterial body. It can be speculated that these areas correspond to nucleoids, i.e. areas where DNA or other proteins agglomerate.

The AFM measurements in liquid were performed using a custom liquid cell that allows changing the growth medium during the imaging with no effect on the measurements. This very stable system has been exploited in order to change the incubation liquid, introducing antibiotics and other substances, while following the evolution of the bacterial topography and stiffness. This new versatile setup opens the way to an all new set of AFM investigations regarding the response of living systems to environmental stimuli.

In conclusion we have demonstrated how force volume imaging can be an extremely powerful tool to monitor the surface stiffness of living systems when exposed to different growing media. We have shown that surface modifications occur on *E. coli* when passing from a stationary to an active state and we have revealed the effect of the exposure to ampicillin on the bacterial structure. This procedure to analyze the mechanical properties of living systems in presence of different media can lead to novel techniques capable to rapidly determine the effect of external stimuli on key cell properties.

Acknowledgments

This work has been supported by Swiss ISJRP 122941 and FN-CR 32I3-130676 grants.

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