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Klebsiella pneumoniae antibiotic resistance identified by atomic force microscopy

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In the last decade the detection of the resistance of bacteria to antibiotics treatment, developed by different kind of bacteria, is becoming a huge problem. We hereby present a different approach to the current problem of detection of bacteria resistance to antibiotics. Our aims were to use the atomic force microscopy (AFM) to investigate bacteria morphological changes in response to antibiotics treatment and explore the possibility of reducing the time required to obtain information on their resistance. In particular, we studied *Klebsiella pneumoniae* bacteria provided by the Lavagna Hospital ASL4 Liguria (Italy), where there are cases linked with antibiotics resistance of the *Klebsiella pneumoniae*. By comparing AFM images of bacteria strains treated with different antibiotics is possible to identify unambiguously the *Klebsiella pneumoniae* strains resistant to antibiotics. In fact, the analysis of the AFM images of the antibiotic-sensitive bacteria shows clearly the presence of morphological alterations in the cell wall. While in the case of the antibiotic-resistant bacteria morphological alterations are not present. This approach is based on an easy and potentially rapid AFM analysis.

Keywords. AFM; Antibiotic resistance; Bacteria images; *Klebsiella pneumoniae*

1. Introduction

Klebsiella pneumoniae is a Gram-negative bacterium that can cause a number of different infections, and it is often found in the human intestinal tract, where they are normally harmless. However, when the immune system of a person is compromised and is exposed to this especially virulent form of *Klebsiella pneumoniae*, the consequences can be deadly (Decré *et al.* 2011). Klebsiella bacteria have developed antimicrobial resistance, most recently to the classes of β-Lactam antibiotics. The resistance of *Klebsiella pneumoniae* to the β-lactam antibiotics is due to the bacterium production of special enzymes such as carbapenemase, and New Delhi Metallo-beta-lactamase (Kumarasamy *et al.* 2010). These enzymes provide antibiotic resistance by breaking the chemical structure of the antibiotics deactivating the antibacterial properties of the antibiotics (Yigit *et al.* 2001; Vrioni *et al.* 2012). The β-lactam antibiotics are the most widely used group of antibiotics, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. Being the outermost and primary component of the cell wall, the peptidoglycan layer is crucial for the integrity of the cell wall structure, especially in Gram-positive organisms. By interfering with peptidoglycan synthesis, the β-lactam antibiotics lead to the death of the bacterial cell as a consequence of the osmotic instability or autolysis, which cause the alteration of the surface morphology and structure of the bacteria (Rice and Bayles 2008; Epand *et al.* 2016).

Moreover, the rapid increase in the prevalence of Gram-negative pathogens resistant to β-lactams, including Carbapenems, and Cephalosporins, has prompted to reconsider Polymyxin antibiotics as a promising therapeutic option. The Polymyxin antibiotics (also known as Colistin), are antimicrobial peptides with long, hydrophobic tails that target Gram-negative bacteria. This specificity is based on their binding to the cell membrane, and the antibacterial activity is conferred by the hydrophobic chain, which disrupts the cell membrane

(Adams *et al.* 2009; Lim *et al.* 2010; Blair *et al.* 2015). Colistin has become widely used in the treatment of infections caused by multidrug-resistant Enterobacteriaceae and, as a consequence, also Colistin resistance has arisen. The antibiotics resistance is often associated with modifications of the cell membrane that reduce binding of the drug to the cell membrane itself. This is a common resistance mechanism in *Klebsiella pneumoniae* (Cannatelli *et al.* 2014). Evidences concerning the actions of antibiotics have been obtained not only biochemically (Doumith *et al.* 2009; Beceiro *et al.* 2014; Liu *et al.* 2015), but also by means of the direct observation of morphological alterations with optical microscopy and scanning probe microscopy (Braga and Ricci 1998; Soon *et al.* 2009; Formosa *et al.* 2015). In the last decade there have been several applications of Atomic Force Microscopy (AFM) to characterize the morphology of several types of cells (Ierardi *et al.* 2008; Allison *et al.* 2011; Lekka *et al.* 2012; van Helvert and Friedl 2016). On the back of its high resolution capability, the AFM can analyse the cell structure, at a nanometric level, and is thus a very useful tool for investigating morphological alterations of the bacterium surface induced by antimicrobial agents (Fantner *et al.* 2010; Li *et al.* 2007; Longo *et al*. 2013).

Once a bacterial culture is well established, the standard procedure to test the sensitivity of an isolated bacterial strain to different antibiotics relies on antibiogram profiling. Antibiograms should be carried out in the shortest possible time, and the time for reading the results ranges from 36 to 48 hours in the case of the classic antibiograms, and from 16 to 18 hours for the more rapid version of well-established laboratory techniques (Cleven *et al.* 2006; Wiegand *et al.* 2008; Horvat 2010; Tissari *et al.* 2010; Waldeisen *et al.* 2011; Zhang *et al.* 2011). Therefore, rapid tools to assess the susceptibility of bacteria to drugs are desirable to promptly address infections.

This work is based on our idea of using AFM to investigate the resistance of bacteria to antibiotics and to explore the possibility to reduce the time required to obtain information on the bacteria resistance to antibiotics. As matter of fact, thanks to the AFM capability to investigate structures of the size of nanometers, our approach does not depend on the number of bacteria, therefore in principle, it does not depend on a full growth bacterial culture, but it needs only a few colony-forming units (CFU) in order to obtain information on the bacteria resistance to a particular antibiotic treatment.

Our method consists in the AFM analysis of the plasma membrane and cell wall of *Klebsiella pneumoniae* bacteria strains treated with two β-Lactam antibiotics (Cephalosporin and Carbapenem) and with Colistin. In particular, we have investigated strains with and without resistance to these antibiotics. We tested the *Klebsiella pneumoniae* antibiotic resistance in conditions as close as possible to a real clinical situation, by using commercially available antibiotics in the same concentration and preparation as those used in clinical treatments.

2. Material and methods

The investigations of the interaction between bacteria and antibiotics has been performed by means of Atomic Force Microscope Dimension 3100 equipped with a hybrid XYZ scanning probe microscope head by Veeco®. The images were collected in tapping mode at 1 Hz, with a resolution of 256×256 and 512×512 pixels, using an Olympus OMCL-AC160TS tip probe with a nominal apical radius of 7 nm. The acquisition time is around 5 min for the images with lower resolution and around 10 min for the others.

2.1 *Bacterial samples treatments*

Tests were performed on samples taken from rectal swabs, urine or blood cultures. The sample culture was seeded on plates of chromogenic agar, Agar Carba by bioMerieux selective for the growth of enterobacteria resistant to carbapenems, and aerobically incubated for 24 h in a thermostat at 35°C. Subsequently, a further isolation of the grown colonies was performed by using chocolate agar, PolyViteX plate by bioMerieux. The antibiograms of the bacterial strains with related MIC were then collected

Figure 1. Hodge test used to reveal carbapenemase production. The presence of a distorted inhibition zone due to growth of the indicator strain toward the Carbapenem disc is interpreted as a positive result. This occurs due to production of carbapenemase.

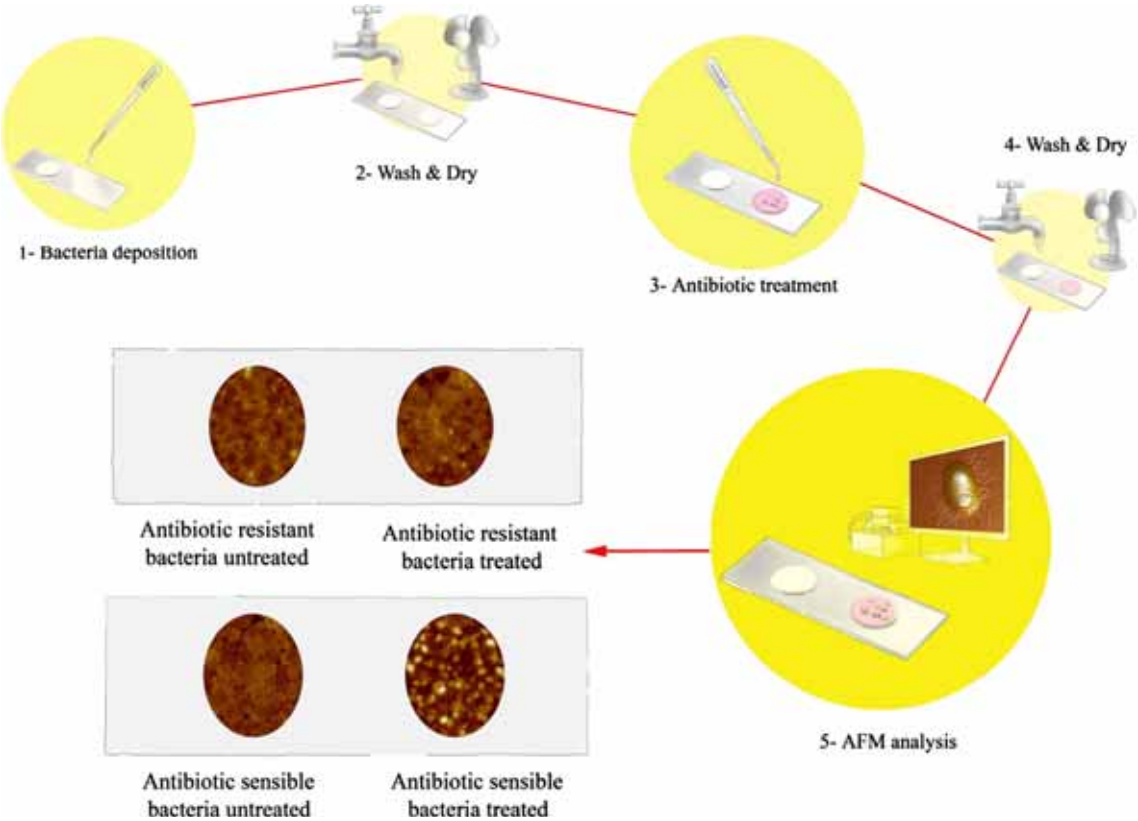

Figure 2. Specimens preparation scheme.

by using a VITEK 2 system by bioMerieux. The confirmation of the strains resistance to the carbapenems has been performed by means of phenotypic and genotypic tests. The samples of bacteria were screened for the ESBL and carbapenemase producing phenotypes by using a standard double disc synergy test and a combination of the modified Hodge test plus the EDTA synergy test. Figure 1 displays an example of a positive Hodge tests performed on resistant *Klebsiella pneumoniae* strain. The detection of β-lactamase genes was performed by PCR, as described in (Marchese *et al.* 2010).

2.2 *Samples preparation for AFM investigation*

The suspensions of the bacterial strains were prepared from bacterial cultures following the procedure described below. An aliquot of 3–4 CFU of bacteria per ml was washed in saline solution of phosphate buffered at pH 7.4 by Sigma® and gently centrifuged at 2000 rpm for 15 min. The final pellet was re-suspended in 1 mL of saline solution of phosphate buffered at pH 7.4. For each bacteria strain we prepared specimens using two aliquots of 250 μL of the suspension, which were deposited onto two microscope slides coated with poly-D-lysine, in two different spots for each slide and dried in air at RT. Then, the slides were washed gently in H_2O MilliQ for five times, in order to rid it of the phosphate salt, and dried in air at RT. Following the washing procedure one of the two spots previously deposited onto the slide was treated with 200 μL of Ceftazidime in one case and Meropenem in the other. The second spot was instead treated with 200 μL of

Table 1. Bacteria resistance

Sample	$MIC_{(Ceftazidime)}$	$MIC_{(Meropenem)}$
1	≥ 64 R	≥ 16 R
2	≥ 64 R	≥ 16 R
3	≥ 64 R	≥ 16 R
4	≥ 64 R	– R
5	≥ 64 R	– R
6	≥ 64 R	≥ 16 R
7	≤ 1 S	≤ 0.25 S
8	≤ 1 S	≤ 0.25 S
9	≤ 1 S	≤ 0.25 S
10	≤ 1 S	≤ 0.25 S
11	≤ 1 S	≤ 0.25 S
12	≤ 1 S	≤ 0.25 S
13	≥ 16 R	≤ 0.25 S

R = Resistant; S = Sensitive.

MIC = minimum inhibitory concentration in μg/mL.

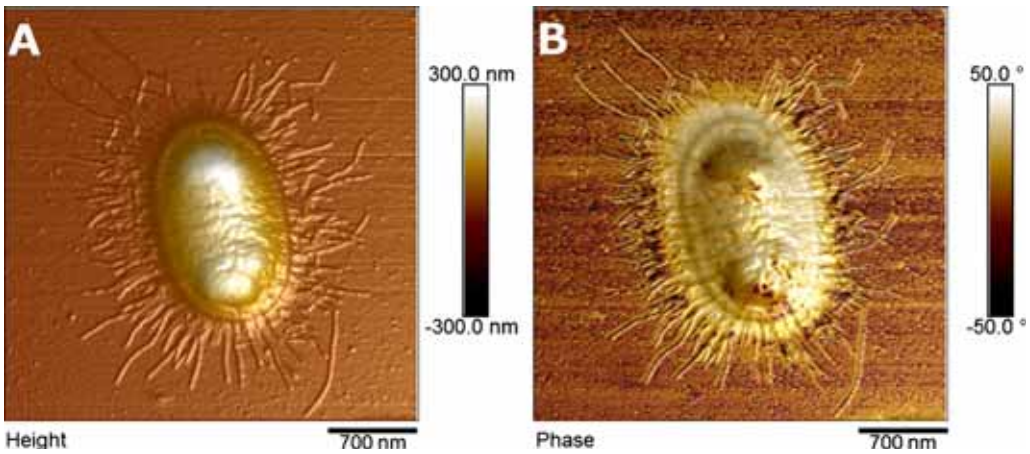

Figure 3. Tapping mode AFM images of *Klebsiella pneumoniae* not treated. (**A**) Topography image. (**B**) Phase image.

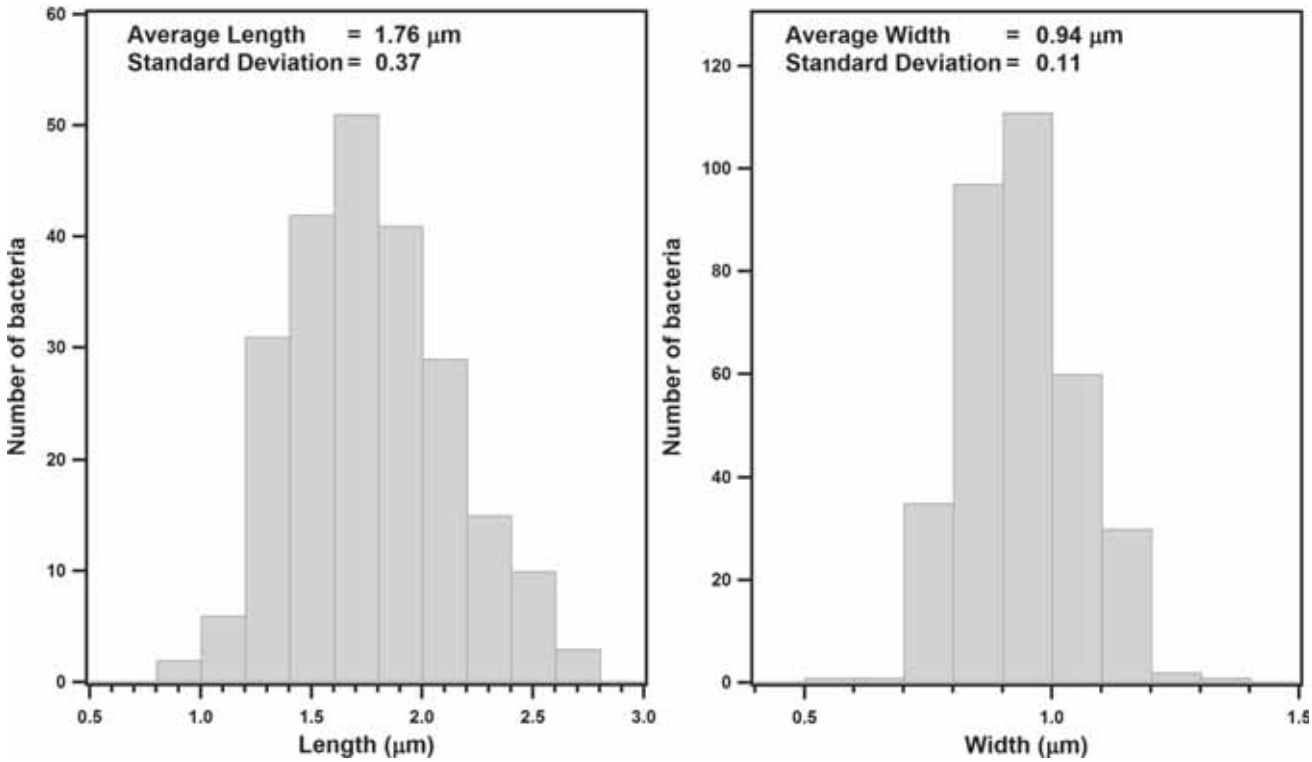

Figure 4. Histograms of the bacteria lengths (left), and bacteria width (right).

H_2O MilliQ, both of them for 40 min. Thus for each studied strain, we analyse a reference sample, which is prepared in the same way, except for the antibiotic treatment. The two slides were then washed twice in H_2O MilliQ and dried at RT. The same procedure was repeated in the case of samples treated with Colistin. The total time for the preparation of the samples is around 1 h. Subsequently AFM images were collected for each strain. In figure 2 it is possible to see the scheme of the preparation procedure of the specimens.

3. Results

We have analysed the effects of the antibiotics in bacteria obtained from patients of the Lavagna Hospital and tested two different commercial preparations of β-Lactam antibiotics, and one of Colistin. The first β-Lactam antibiotic contains Ceftazidime, a third-generation of cephalosporin, and it differs from earlier generation for the presence of a $C{=}N\text{-}OCH_3$ group in the chemical structure that provides better stability against beta-lactamase enzymes produced by

some bacteria. These bacterial enzymes rapidly destroy earlier-generation cephalosporins by breaking the β-lactam ring, leading to antibiotic resistance. The second one contains Meropenem, which belongs to the subgroup of carbapenem. It is highly resistant to degradation by β-lactamases or cephalosporinases. The Colistin used is in form of colistimethate sodium with 34 mg of Colistin Base Activity (CBA) in 4 mL.

The Lavagna Hospital microbiology lab provided us with seventeen specimens of bacteria: thirteen with different resistance/sensibility to the Ceftazidime and to the Meropenem (table 1); and four with different resistance/sensibility to the Colistin, i.e. two strains resistant to the Colistin with Minimum Inhibitory Concentration (MIC) > 16 μg/mL, and two strains sensitive to the Colistin with MIC ≤ 0.5 μg/mL.

Figure 3 shows the normal morphology of *Klebsiella pneumoniae* not treated with antibiotics collected by AFM in air environment. The different appearance between the AFM images in figure 3 and the others AFM images is due to different superficial bacteria density. Indeed, only one bacterium is shown in figure 3, while in the others figures a layer of bacteria is visible. The AFM analysis of approximately 300 untreated bacteria shows an average length of 1.76 μm with a standard deviation of 0.37 μm, while the average width is 0.94 μm with a standard deviation of 0.11 μm (figure 4). Lengths and widths of the bacteria do not show evident changes due to the antibiotics treatment. On the contrary, in the case of the antibiotic-sensitive bacteria strains the heights show changes as a result of the antibiotic treatment that produces clear surface alterations. In addition, for longer antibiotic treatment time (>> 40 min), we have observed an almost complete lysis of the bacteria structure.

Only antibiotic-sensitive strains show a change in their morphology following the treatment. The AFM analysis shows clear *bubble-like* alterations in sensitive bacteria strains. The alterations in the cell wall are likely to be the first step of cell wall lysis.

Figure 5 shows the effects of the Ceftazidime on the sensitive bacteria strains. The *bubble-like* alterations of the bacteria surface after the treatment have a diameter ranging from 80 to 200 nm. On the contrary, following the antibiotic treatment, the surface of the resistant bacteria strains does not show morphological alteration. Figure 6 shows an example of the Ceftazidime-resistant bacteria treated and untreated with the antibiotic.

Similar results were observed in the case of bacteria strains resistant and sensitive to Meropenem. Indeed, the AFM analysis of the sensitive bacteria strains treated with Meropenem shows also clear *bubble-like* alterations, while in the case of the resistant ones there are not present evident alterations (figures 7 and 8).

In the case of the bacteria treated with Colistin, the effect of the antibiotic treatment is even more evident, as the AFM analysis of the samples has shown that the membranes of the sensitive strains are completely damaged (figure 14). On the other hand, such damage does not arise in the resistant bacteria strains, as shown in figure 16.

Therefore, by comparing AFM images of treated and untreated specimens we can obtain a criterion to unambiguously distinguish antibiotic-resistant bacteria from antibiotic-sensitive bacteria.

4. Discussion and conclusion

In order to exclude the presence of morphological alterations caused by undesired phenomena, we have treated all specimens in the same way, i.e. they were washed and dried out the same number of times. Furthermore, being surrounded by cellular wall, bacteria have a surface much more rigid than that of animal cells, which simplifies AFM study. Dried

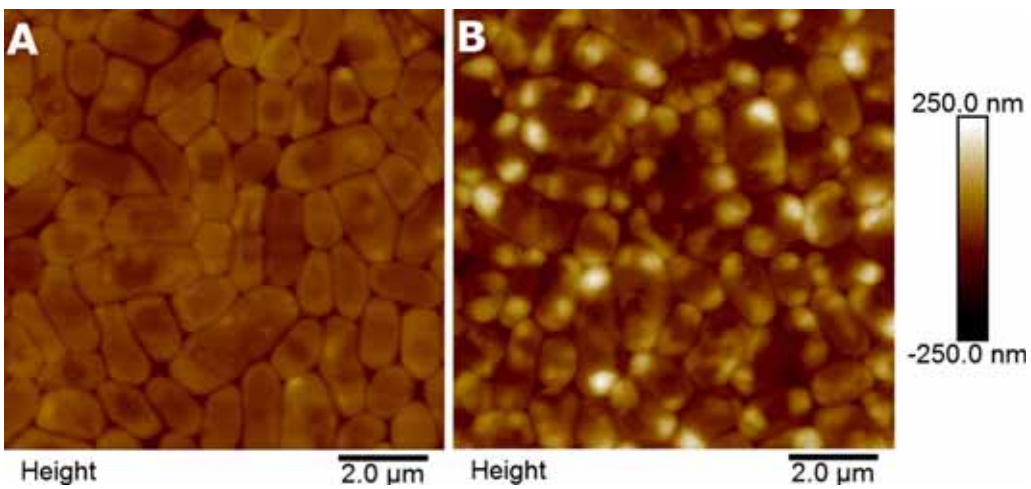

Figure 5. Tapping mode AFM image of the Ceftazidime-sensitive bacteria: untreated (**A**) and treated (**B**).

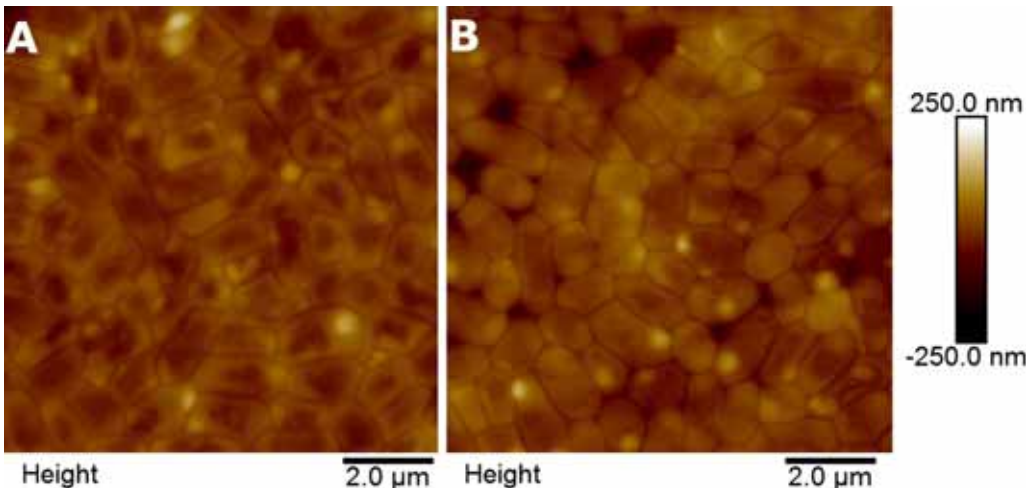

Figure 6. Tapping mode AFM image of the Ceftazidime-resistant bacteria: untreated (**A**) and treated (**B**).

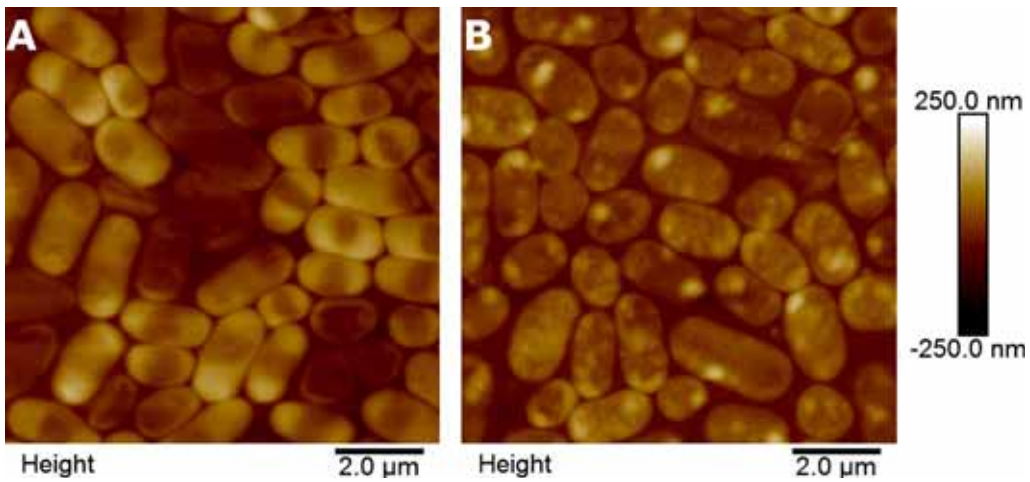

Figure 7. Tapping mode AFM image of the Meropenem-sensitive bacteria: untreated (**A**) and treated (**B**).

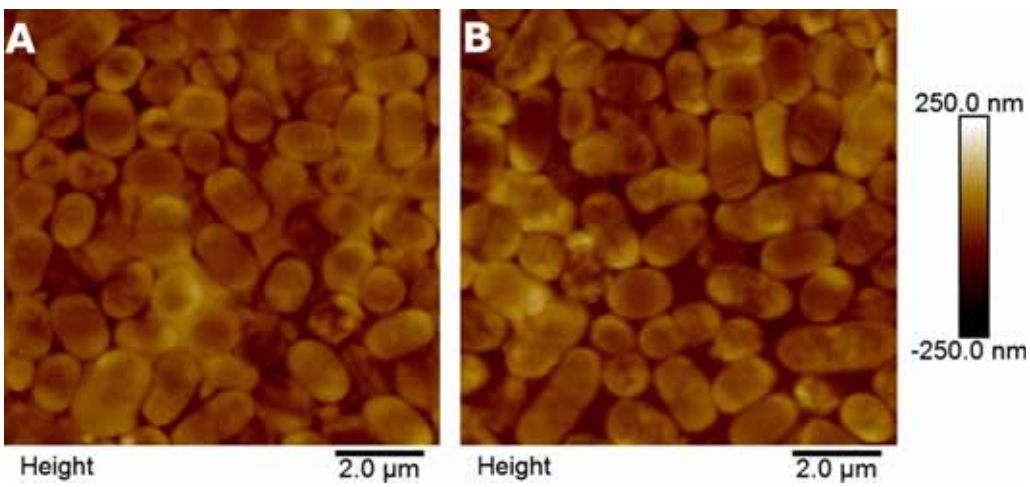

Figure 8. Tapping mode AFM image of the Meropenem-resistant bacteria: untreated (**A**) and treated (**B**).

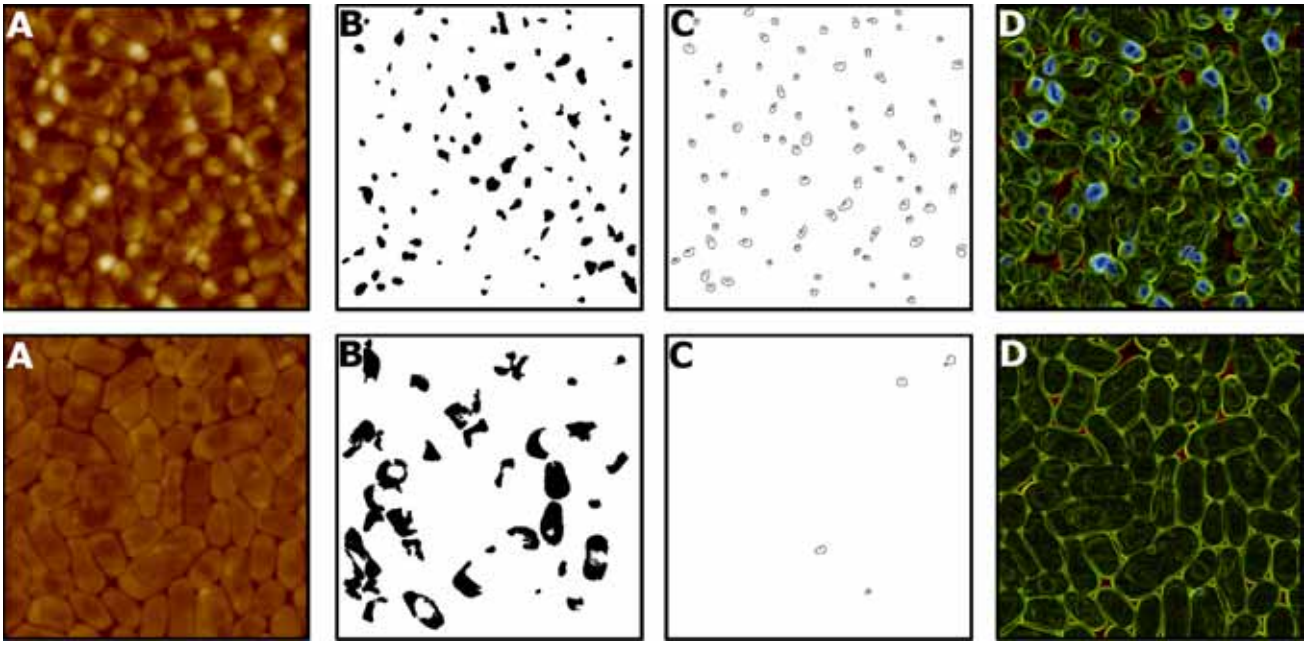

Figure 9. An example of how the AFM images are processed using the software *ImageJ*. In the top images is shown the case of a sensitive bacteria strain, while in the bottom images is shown the case of a resistant strain. Both samples are treated with antibiotics. The size of the AFM images is 10 μm.

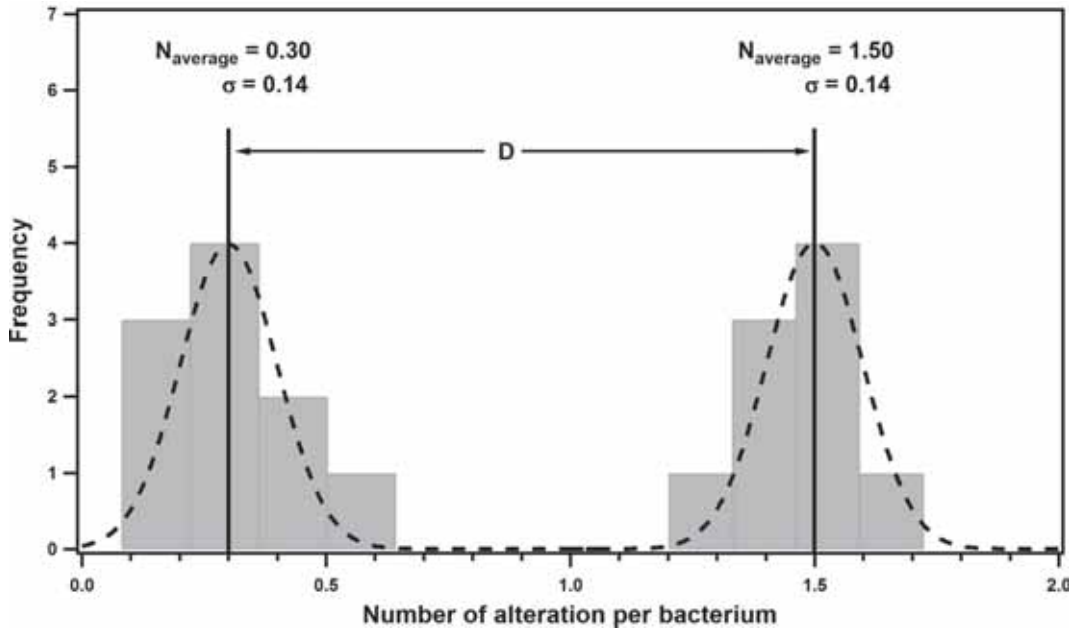

Figure 10. Histograms of the number of alterations per bacterium **(N)** of the Ceftazidime-sensitive bacteria strain. untreated (left) and treated (right) specimens. The dashed gaussian curves are obtained by using $N_{average}$ and standard deviation s calculated from the AFM data, D is the distance between the two histograms.

at ambient conditions bacteria remain alive and returned to a culture medium can continue their life cycle (Bolshakova *et al.* 2001). We have collected twenty AFM images for each sample: ten each for both treated and untreated specimens.

In order to perform a quantitative analysis of the morphological alterations caused by the antibiotic treatment it is possible to define the number of alterations per bacterium (N) by dividing the number of the *bubble-like*

structures per the number of bacteria shown in the AFM images. The alterations as well as the number of bacteria are identified and counted using *ImageJ* (Schneider *et al.* 2012), which is an open source image processing program designed for scientific multidimensional images. The procedure followed in processing the AFM images is reported in figure 9. The first step of this procedure consists in the identification of the peaks present in the AFM image (figures 9A) using the function 'Find Maxima' of ImageJ, which determines the local maxima in an image and creates a binary (mask-like) image of the same size with the maxima (figures 9B). Subsequently, we processed these images using the command 'Analyze Particles', that counts and measures objects in binary images. This command allows us to select the maximum size of the particles, i.e. particles with size (area) outside the range specified in this field are ignored. In this analysis we have considered particles with the size between 0 and 1.5×10^4 nm^2, which correspond to *bubble-like* structures with a maximum diameter of 200 nm (clearly evident in figures 9C). Eventually, the number of bacteria per images is determined using the command "Find Edges", which uses a Sobel edge detector to highlight sharp changes in intensity in the image (figure 9D). The data obtained from this analysis is used in building the histograms shown in the other figures.

In case of Ceftazidime-sensitive bacteria strains N is equal to 0.30 with the standard deviation of $\sigma = 0.14$ for the ten untreated specimens and N = 1.50 with standard deviation of $\sigma = 0.14$ for the ten treated ones (figure 10), while in the case of the resistant bacteria N is equal to 0.25 with $\sigma = 0.05$ for the untreated specimens and N = 0.33 with $\sigma = 0.07$ for the treated ones (see figure 11). The analysis shows that there is a clear increase in the number of *bubbles-like* structures in the antibiotics-sensitive bacteria, but there are no significant morphological alterations in the resistant samples, thus allowing for an unambiguous identification of the resistant bacteria strain.

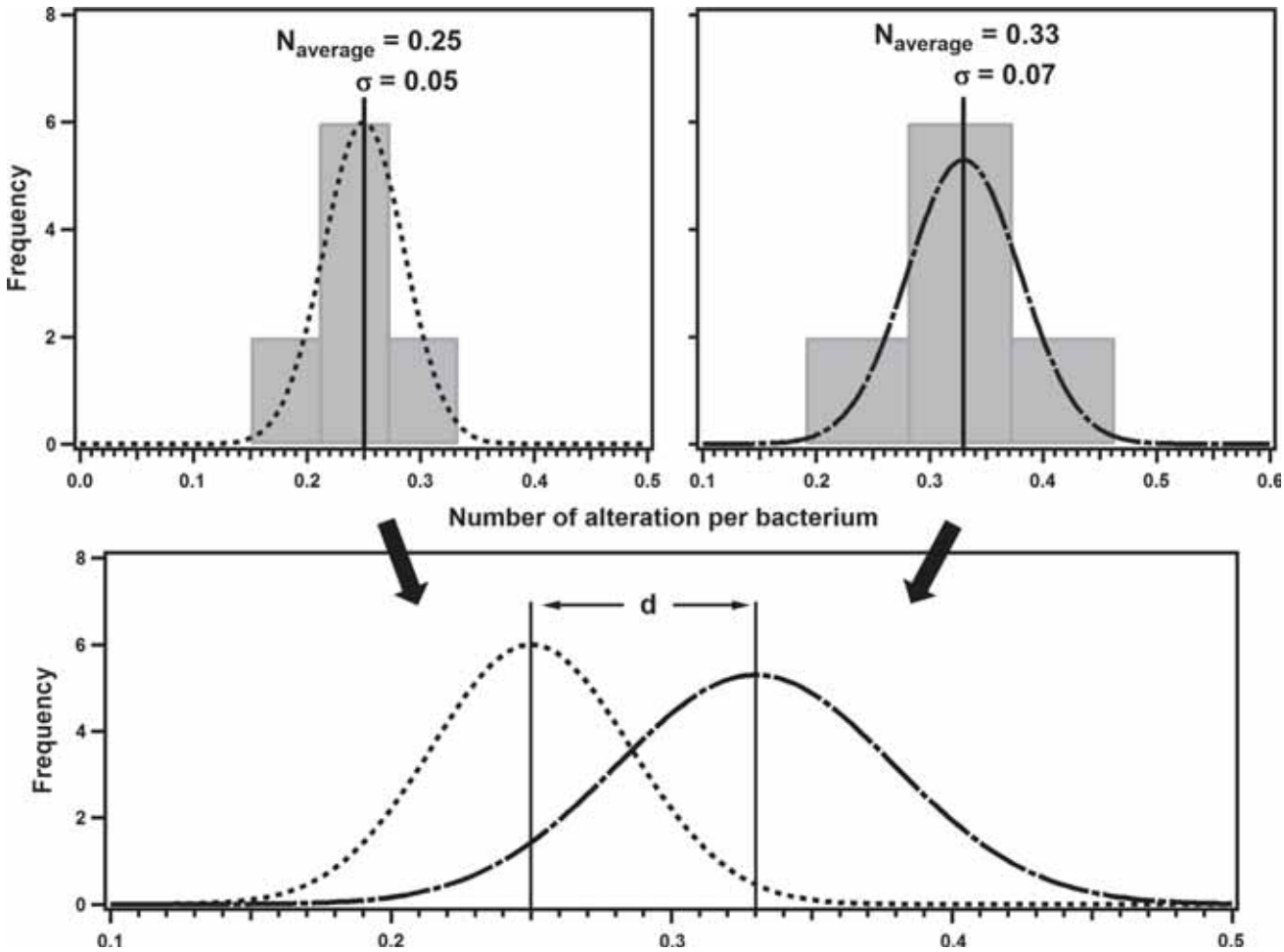

Figure 11. Histograms of the number of alterations per bacterium (N) of the Ceftazidime-resistant bacteria strain. untreated (top left) and treated (top right) specimens. The dashed gaussian curves are obtained by using $N_{average}$ and standard deviation s calculated from the AFM data, d is the distance between the two histograms (bottom).

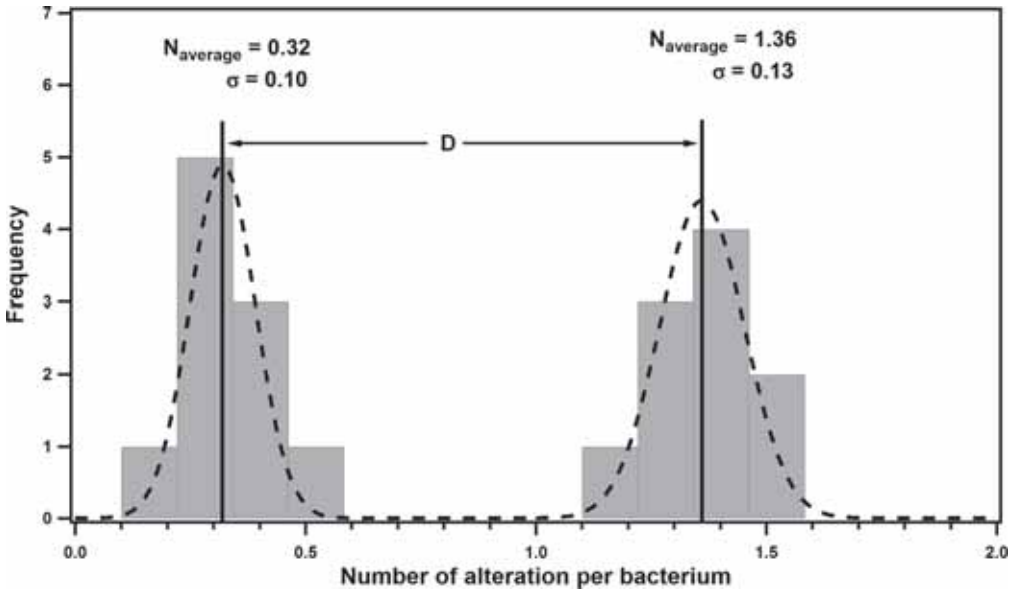

Figure 12. Histograms of the number of alterations per bacterium (N) of the Meropenem-sensitive bacteria. untreated (left) and treated (right) specimens. The dashed Gaussian curves are obtained by using $N_{average}$ and standard deviation s calculated from the AFM data, D is the distance between the two histograms.

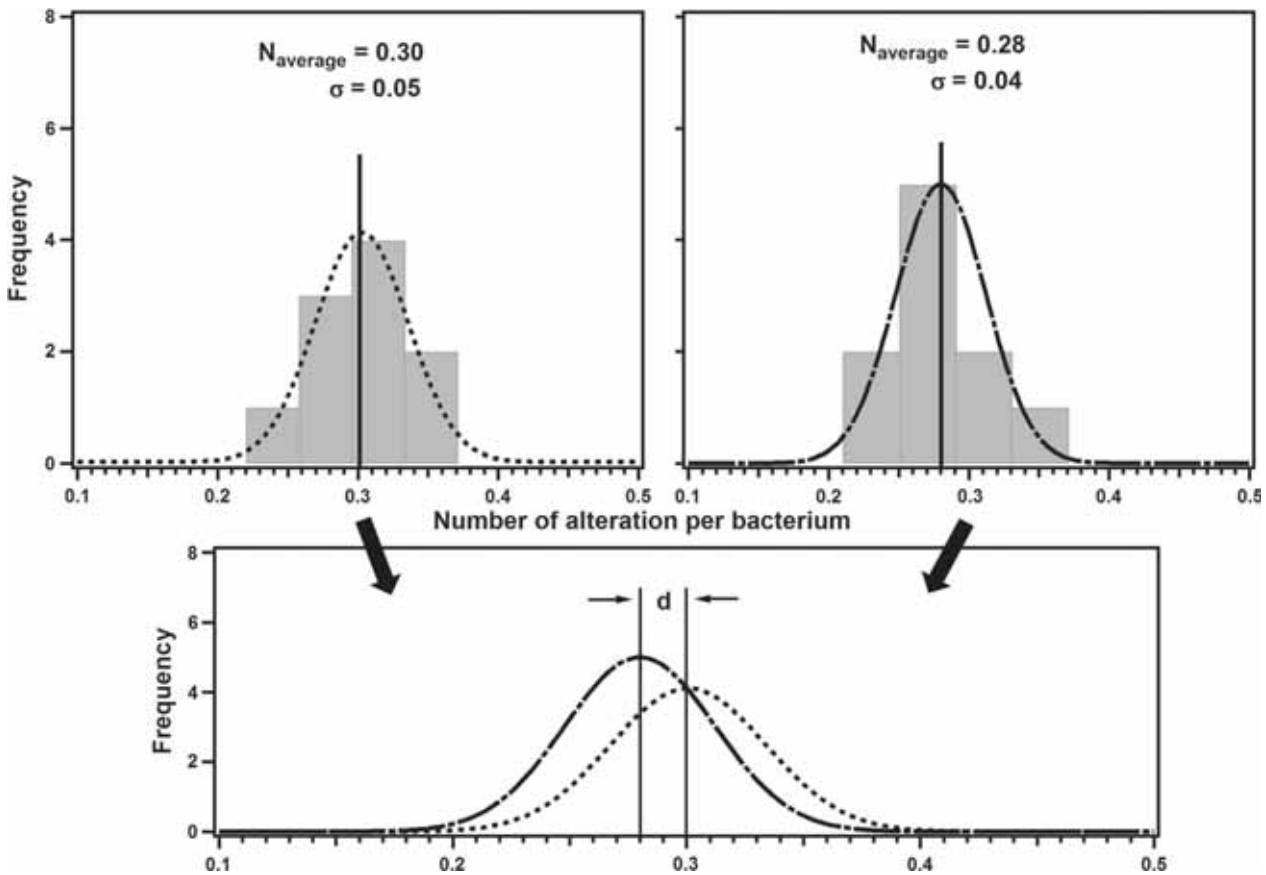

Figure 13. Histograms of the number of alterations per bacterium (N) of the Meropenem-resistant bacteria. untreated (top left) and treated (top right) specimens. The dashed Gaussian curves are obtained by using $N_{average}$ and standard deviation s calculated from the AFM data, d is the distance between the two histograms (bottom).

Figure 10 reports histograms of N for the Ceftazidime-sensitive treated and untreated bacteria strains. In this case the two histograms do not overlap, in fact the distance D between the average value of N for the treated and untreated specimens is about 9 times the standard deviation of the data. In this case, the value of D guarantees that ten AFM images are more than enough to distinguish between the treated and untreated specimen. Indeed, in order to distinguish between the two cases, five AFM images may be sufficient. On the contrary, in the case of the treated and untreated specimens of the resistant bacteria, the histograms of the two data population overlap, as it is displayed in figure 11. The distance d between the average value of N for treated and untreated specimens does not allow distinguishing between treated and untreated specimens. Similar quantitative results are obtained in the case of the bacteria strains treated with

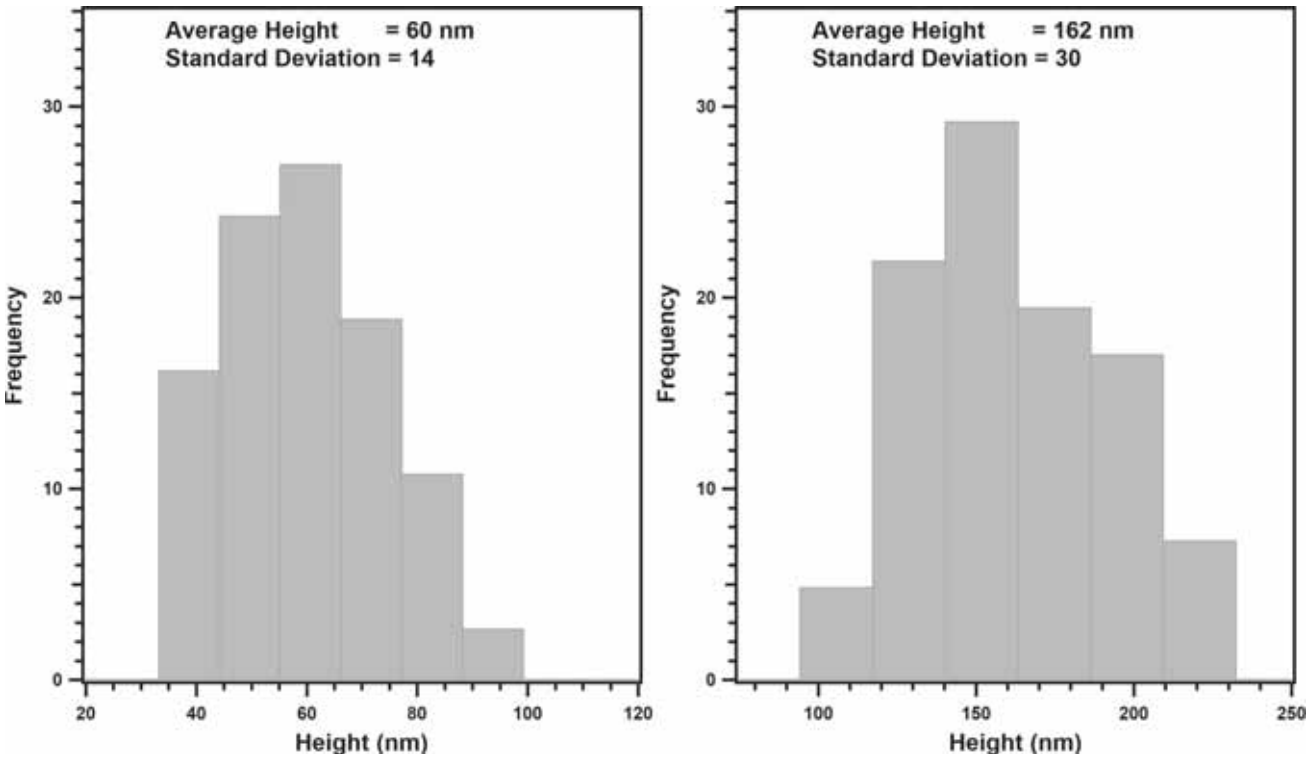

Figure 14. Histograms of the heights of the bubble-like structure in the case of the sensitive bacteria strains before (left) and after (right) the treatment with antibiotics.

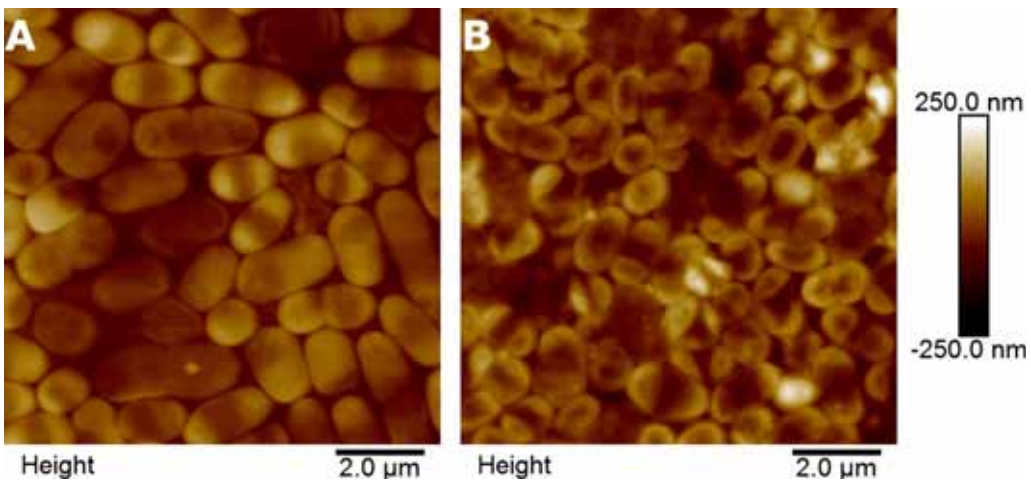

Figure 15. Tapping mode AFM image of Colistin-sensitive bacteria strain: untreated (**A**) and treated (**B**).

Meropenem. The quantitative analysis is displayed in figure 12 and figure 13.

Furthermore, our findings are confirmed also by the fact that in the case of the-sensitive bacteria strains there is a clear difference not only in the number of the *bubble-like* structures but also in their heights. In fact, for the sensitive bacteria strains we found that after the treatment with antibiotics the average height of the *bubble-like* alterations increases from 60 nm (with a standard deviation $\sigma = 14$) for untreated specimens to 162 nm (with a standard deviation $\sigma = 30$) for the treated ones. This difference, which is about 3 times the higher standard deviation (i.e. $\sigma = 30$), allows us to tell apart the sensitive stains from the resistant ones. While in the case of the resistant bacteria strains is not possible to distinguish between the treated and untreated specimens since the average heights for both cases fall, within one standard deviation, around the same value. In figure 14 is possible to see the histograms of the heights of the bubble like structures for the sensitive bacteria strains untreated and treated.

The effects of the Colistin on the sensitive bacteria cell membrane, highlighted in the comparison of the AFM images (figure 15), are different from the effects of the β-Lactams antibiotics (figure 5 and figure 7). This difference is very likely due to the different action mechanism of Colistin and β-Lactams. In fact, the antibacterial activity of the Colistin against gram negative bacteria has been explained as an increase in the permeability of the cell membrane with the resultant leakage of the cell contents (Beceiro *et al.* 2014; Cannatelli *et al.* 2014; Blair *et al.* 2015). This is consistent with the effects on the morphology of the bacteria shown by the AFM analysis on the sensitive bacteria strains treated with Colistin. Indeed, the AFM images reported in figure 15B, specimens treated with Colistin, show that the bacterium structure is collapsed and there are not *bubble-like* structures, as for the samples treated with β-Lactams. While, as it is possible to see in figure 16, the treatment with Colistin has not evident effects on the resistant bacteria strains.

The analysis of the AFM images of the bacterial samples results simpler, i.e. it is enough to count the collapsed bacteria to identify the sensitive strain. Figure 17 reports the histograms of collapsed bacteria per AFM image taken over 10 different images of Colistin-resistant bacteria treated and untreated. The average percentage of collapsed bacteria per AFM image is 6.7% with a standard deviation of 1.1% for untreated samples, which rises to 62.9% with a standard deviation of 4.3% for treat samples.

In the case of the Colistin-resistant strain the average percentage of collapsed bacteria per AFM image is 6.8% with a standard deviation of 1.2% for the untreated samples, and 9.8% with a standard deviation of 1.4% for treated samples (figure 18). Therefore, in this case of the resistant strains the analysis shows that the average percentage of collapsed bacteria does not change with the Colistin treatment.

Despite the difference between the effects of the Colistin and the β-Lactams antibiotics, due to the different antibiotic action mechanisms, it is also possible to clearly distinguish between the resistant and the sensitive strains.

These findings provide a solid ground for future developments of this method in association with microfluidic techniques allowing bacteria separation or bacteria concentration in a rapid way (Hanson *et al.* 2016; Matsumoto *et al.* 2016; Ohlsson *et al.* 2016; Zhihua *et al.* 2016). In fact, the classic method used to obtain information on the effects of the antibiotics on bacteria strains is based on recording antibiograms by means of the diffusion method. In general, the diffusion method requires several steps: the collection of the bacteria from the patients; the culture of the bacteria; the isolation of the bacteria; and eventually the collection of the antibiograms. In order to complete the whole procedure with

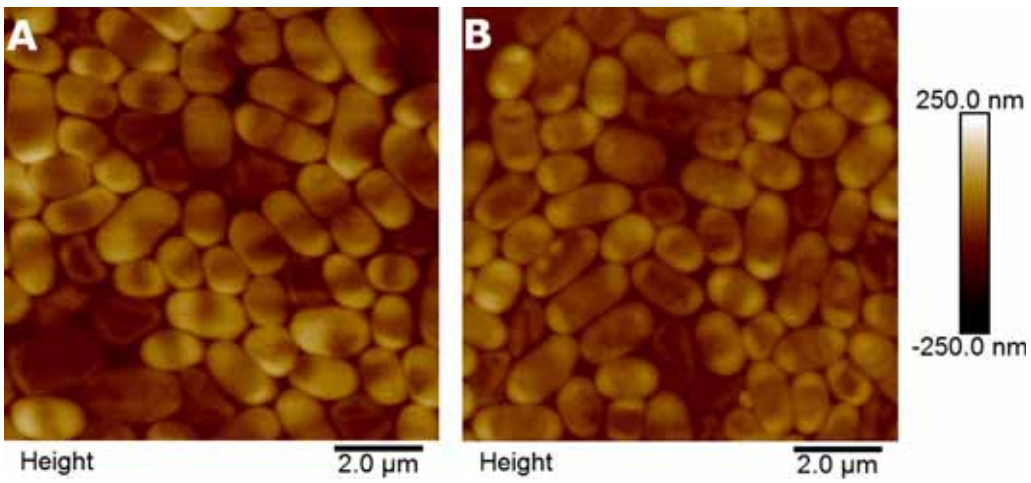

Figure 16. Tapping mode AFM image of Colistin-resistant bacteria strain: untreated (**A**) and treated (**B**).

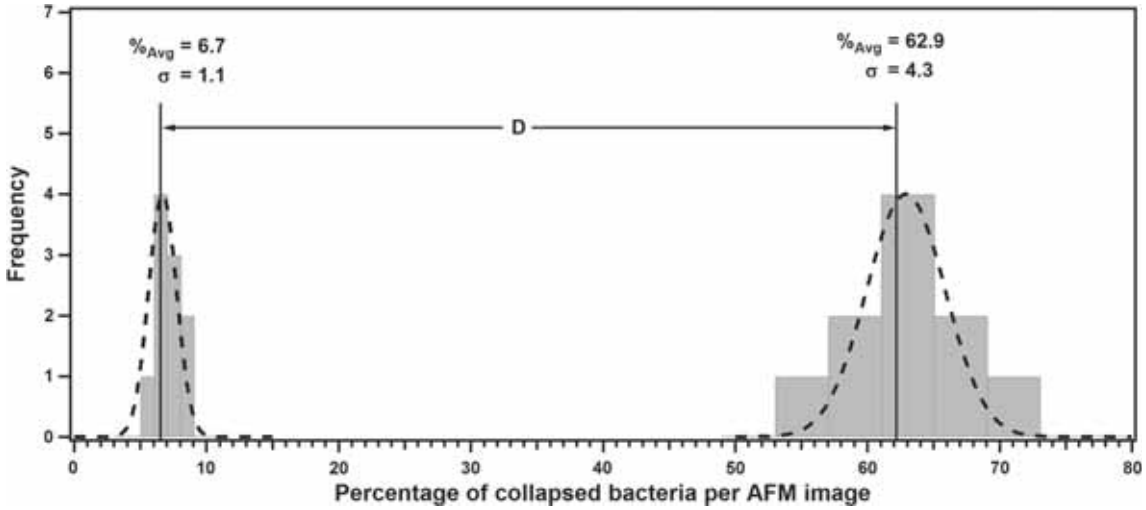

Figure 17. Histograms of the percentage of collapsed bacteria per AFM image of the Colistin-sensitive bacteria strain: untreated (left) and treated (right) specimens. The dashed Gaussian curves are obtained by using average value of the percentage of collapsed bacteria per AFM image and the related standard deviation s, D is the distance between the two histograms.

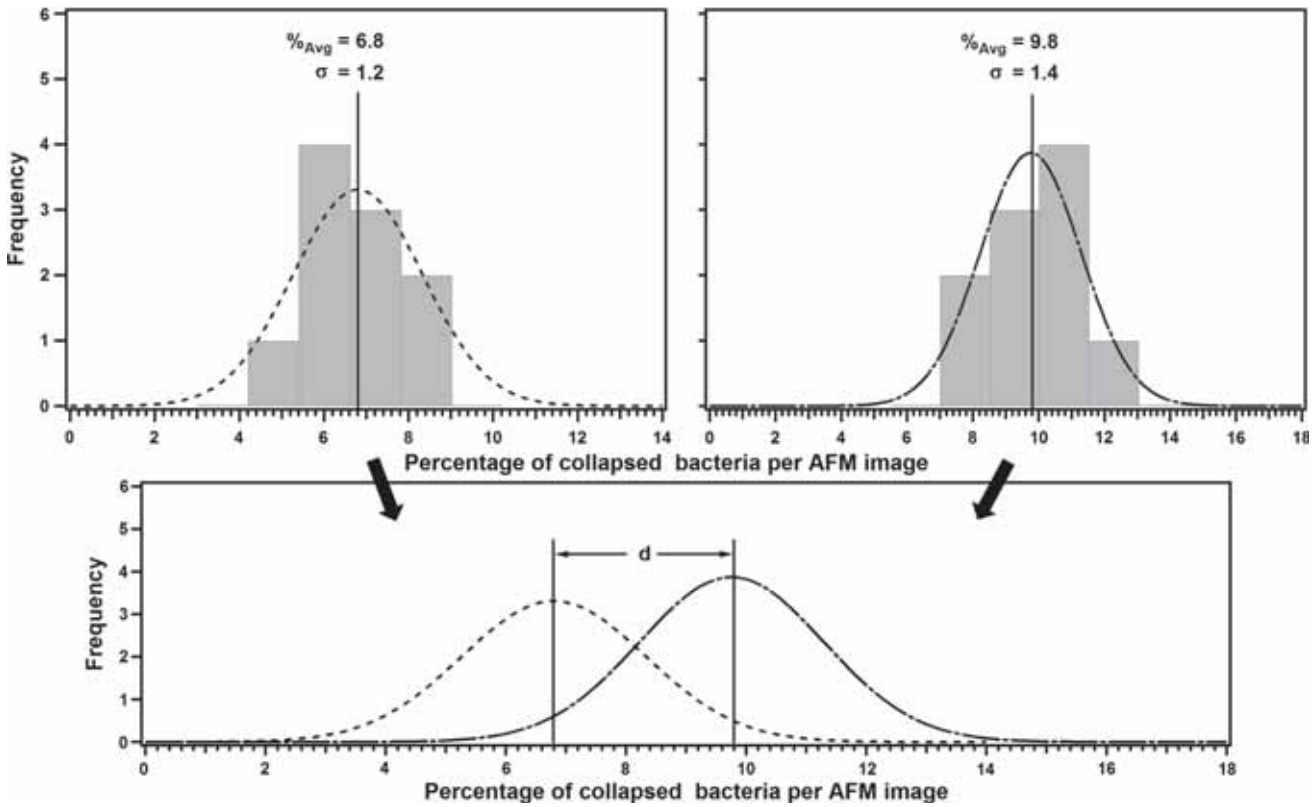

Figure 18. Histograms of the percentage of collapsed bacteria per AFM image of the Colistin-resistant bacteria strain: untreated (top left) and treated (top right) specimens. The dashed Gaussian curves are obtained by using average percentage of collapsed bacteria per AFM image and standard deviation s calculated from the AFM data, d is the distance between the two histograms (bottom).

standard laboratory method 3–4 days are necessary. In our approach only the first three steps are required, because in order to perform the AFM analysis we can select a few CFU at the beginning of the isolation step. Even though at the present stage our approach does not allow obtaining rapid information on the Minimum Inhibitory Concentration

(MIC), the time required to complete the entire analysis for one bacteria strain is around 4–5 hours, in this way it is possible to obtain information on the antibiotic resistance earlier and with a clear indication of the advantages that could be achieved compared to the conventional antibiogram diffusion method. Therefore, as future development association with microfluidic techniques of bacteria concentration/separation that rapidly can provide a small amount of bacteria, skipping the classic phases of culture and separation, this method has a high potential for diagnostic applications.

In conclusion, this study reports an innovative approach to distinguishing between sensitive and resistant *Klebsiella pneumoniae* strains and considers possibilities for a rapid detection of bacteria antibiotics resistance by AFM. We have treated several bacteria strains, which are either antibiotics-resistant or antibiotics sensitive, with two different classes of antibiotics (β-Lactam and Polymyxin) and analysed treated and untreated sample of bacteria by AFM. In all samples analysed were possible to clearly distinguish the resistant bacteria strains from the sensitive bacteria strains by counting the number of deformed bacteria. The procedure evidences unambiguously the strains resistant to antibiotics, which opens a promising way to future application for a rapid identification of the bacteria resistance to a particular antibiotic treatment.

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