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# Nanoscale imaging of the growth and division of bacterial cells on planar substrates with the atomic force microscope

M. Van Der Hofstadt [a], M. Hüttener [a,b], A. Juárez [a,b], G. Gomila [a,c,*]

[a] Institut de Bioenginyeria de Catalunya (IBEC), C/ Baldiri i Reixac 11-15, 08028 Barcelona, Spain
[b] Departament de Microbiologia, Universitat de Barcelona, Avinguda Diagonal 645, 08028 Barcelona, Spain
[c] Departament d'Electronica, Universitat de Barcelona, C/ Marti i Franqués 1, 08028 Barcelona, Spain

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ABSTRACT

With the use of the atomic force microscope (AFM), the Nanomicrobiology field has advanced drastically. Due to the complexity of imaging living bacterial processes in their natural growing environments, improvements have come to a standstill. Here we show the in situ nanoscale imaging of the growth and division of single bacterial cells on planar substrates with the atomic force microscope. To achieve this, we minimized the lateral shear forces responsible for the detachment of weakly adsorbed bacteria on planar substrates with the use of the so called dynamic jumping mode with very soft cantilever probes. With this approach, gentle imaging conditions can be maintained for long periods of time, enabling the continuous imaging of the bacterial cell growth and division, even on planar substrates. Present results offer the possibility to observe living processes of untrapped bacteria weakly attached to planar substrates.



## 1. Introduction

Since the first images of dried bacterial cells were obtained with the Atomic Force Microscope (AFM) [1], this technique has significantly contributed to the understanding of the nanoscale structural and physical properties of single bacterial cells [2–6]. Examples include the high resolution imaging of the dynamics of bacterial membrane proteins [7,8], the molecular recognition of cellular membrane proteins [9,10], the visualization of the effects of antibiotics on the cell surface [11,12], and imaging of the extrusion of bacteriophages [13]. In this way, the AFM has decisively contributed to the emerging field of Nanomicrobiology [5].

Imaging living bacterial cells with the Atomic Force Microscope still poses a major challenge. This limitation arises from the relatively reduced adsorption forces of most living bacteria to the standard substrates used for AFM (such as glass or mica). In contraposition, the non-living bacterial cells (i.e dried bacteria) show stronger adhesion forces, making imaging easier and extensively used [14,15].

Two different approaches have been reported to overcome the difficulty of imaging living bacteria. The first approach relies on increasing the strength of the forces that immobilize the bacteria to the substrates. The second approach is focused to reduce the shear forces exerted by the AFM tip on the bacteria and which are responsible for cell detachment during imaging. Among the first approach, we can find the physical entrapment of bacterial cells into polycarbonate filters [8,16] or microwells [17], or the use of specific substrate coatings (such as APTES [11], PEI [18], poly-L-Lysine [19,20], polyphenolic proteins [21] or gelatine [21–23]) or surface chemical binding groups (e.g. cross-linking of $NH_2$ groups via glutaraldehyde [24]). Concerning AFM imaging modes, conventional modes such as contact mode or dynamic mode can only be used when bacteria are relatively strongly attached to the substrates [25]. For weakly attached bacteria (for most coated planar substrates) the use of the intermittent contact mode with magnetically excited probes seems to offer the best performance [17,19,22]. This has been attributed to the fine tuning of the dynamic oscillation in liquid conditions.

Despite these developments, relatively little progress has been made in the nanoscale imaging of living bacterial processes, such as bacterial growth and division [16,17], specially for bacterial cells on planar substrates [19,26]. The use of planar substrates provides a more natural condition to study these bacterial processes. They offer a less constrained space (compared to physical entrapment methods) for bacterial growth and division, together with weak electrostatic adsorption forces. In this way, it mimics the bacterial natural way of adhesion onto several types of substrates, including those present in biofilm formation on natural and synthetic surfaces [27,28]. In this paper, we present the use of an alternative

* Corresponding author at: Institut de Bioenginyeria de Catalunya (IBEC), C/ Baldiri i Reixac 11-15, 08028, Barcelona, Spain.
*E-mail address:* ggomila@ibecbarcelona.eu (G. Gomila).

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AFM imaging mode to study living bacterial cells, the so called dynamic jumping mode. With this method, we have been able to image living bacterial cells weakly absorbed onto planar substrates, following its growth and division. When using dynamic jumping mode, the probe is oscillated at its resonance frequency and approached to the sample until a prefixed oscillation amplitude set point is reached. At this point, the probe is retracted a given distance and laterally displaced out of contact from the sample until the next point. This out of contact lateral displacement, together with the use of the intermittent contact mode and of soft probes, drastically reduces the shear forces exerted onto the weakly absorbed bacterial cells. It should be noted that dynamic jumping mode offers a better performance than its static version [29], which has already been widely used in the imaging of viruses on planar substrates in physiological conditions [30,31].

With the use of the dynamic jumping mode we have been able to image living single bacterial cells belonging to two different *Escherichia coli* strains, the MG1655 and the enteroaggregative (EAEC) 042, both being weakly adsorbed onto planar gelatine coated substrates. In addition, we have been able to monitor the growth and division of *E. coli* 042 in its native state over long periods of time.

## 2. Materials and methods

### 2.1. Cell types and cultures

*E. coli* strain MG1655 is well known to be the common non-pathogenic laboratory *E. coli* strain for biological research [32], while strain 042 is the archetype of the EAEC pathotype [33–35]. EAEC strains display a characteristic aggregative or "stacked-brick" pattern of adherence to intestinal epithelial cells [36]. When grown at initial stages of biofilm, bacteria secrete less extracellular polymeric substance (EPS) [37].

Stock samples of the common laboratory strain *E. coli* MG1655 and the EAEC *E. coli* 042, were kept on Luria broth (LB) (Laboratiorios Conda, S.A.) agar plates at 4 °C.

### 2.2. Preparation of substrates for AFM imaging

Three types of substrates, namely, glass, gold and mica were used, in all cases coated with gelatine. Three different substrates have been used to show the generality of the approach presented and to evaluate any eventual effect of substrate roughness. Glass coverslips (No. 26024 Ted Pella, INC.) and gold substrates (Arrandee) were rinsed following a sequential sonication washing with acetone, iso-propanol and milli-Q water. Drying was performed with a nitrogen flow. The mica substrate (No. 52–6 Ted Pella, INC.) was freshly cleaved. The coating of the three substrates with gelatine was done with an adaptation of the protocol described in Ref. [22]. Briefly, the gelatine solution was prepared by dissolving 0.5 g of gelatine (Sigma-Aldrich, G6144) and 10 mg of Chromium (III) potassium sulfate (Sigma-Aldrich, 243361) in 100 ml milli-Q water. The resulting solution was heated up to 90 °C and left to cool down to 60 °C. The substrates were vertically dipped into the solution and allowed to air dry overnight inside a cabinet.

### 2.3. Sample preparation

For topographic imaging of bacterial cells, samples were prepared by using two different protocols. Protocol 1 used early stationary phase bacterial cells, obtained after an overnight cell culture. This is a standard microbiology protocol that ensures that bacterial cells have only small differences in growing times, collecting bacteria at the same growth phase. In this approach, the sterile loop was used to scrap a small quantity of bacteria grown on an agar plate into 10 ml of LB, which was left at 37 °C at 250 rpm for 15 h (overnight culture). 600 μl were then transferred into a micro-centrifuge tube and centrifuged at 3000 rpm for 3 min. The pellet was re-suspended in 600 μl milli-Q water. To attach cells onto the gelatine substrate, from the aliquot prepared, 40 μl of the milli-Q bacterial suspension were pipetted onto the gelatine substrate and spread using the help of the pipette tip. For dried samples, the sample was left until its complete dryness. For semi-dried samples, the sample was left to dry until the drop of water was not appreciable (but bacteria were not completely dry). For fully hydrated samples, the bacteria were allowed to deposit from the droplet of the solution for 30 min in a humid environment. The samples were rinsed in a soft stream of either 10 mM HEPES buffer solution at pH 8 (imaging in liquid conditions) or with milli-Q water (imaging in dry conditions). Samples for liquid imaging were left in the buffer solution, while samples for dry imaging were left in dry conditions and imaged under nitrogen ambient flow (~0% Relative Humidity).

Protocol 2 used the *E. coli* 042 strain in early biofilm forming stage. Cells were directly grown on the imaging substrate. To achieve this, *E. coli* 042 strain was grown overnight in LB broth at 37 °C and 16 μl were pipetted into a 12 well cell culture plate with a gelatine coated mica substrate at the bottom. The well contained 2 ml of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen 11966025) supplemented with 0.45% of glucose. The culture plate was left to stand at 37 °C for 4 h. Substrates holding the bacterial growth were softly rinsed with fresh growth medium (DMEM plus glucose) and either covered in it, or rinsed with milli-Q water and left to dry (in the case of the dried samples).

### 2.4. AFM imaging of bacterial cells

AFM topographic images in air were recorded in dynamic mode using Tap150Al-G probe (BudgetSensors) with a spring constant of 2.7 N/m under nitrogen ambient flow (~0% Relative Humidity). The Cervantes microscope (Nanotec Electronica S.L.) was used at a scan speed of 0.7 Hz and 256 pixels per line.

Bacterial imaging in liquid media was performed using dynamic jumping mode plus (Nanotec Electronica S.L.) using Biolevers (BL-RC150VB-C1, Olympus) with a nominal spring constant of 0.03 N/m. This innovative mode follows the jumping mode in liquid [38] with the modifications described in [29], and with the advantage of the acoustic oscillation [39]. Briefly, the probe performs a force vs. distance curve at each point of the sample surface in dynamic mode until the prefixed oscillation amplitude set point is achieved. Due to the less invasive properties of the dynamic mode and the use of soft cantilevers, forces of $< 0.2$ nN can be applied as set point, what turned out to be crucial when imaging weakly adhered living bacteria. Once reached the set point, the tip retracts a given distance to perform the raster scan of the tip at maximum tip-sample separation, avoiding shear forces when imaging but maintain high control of the forces applied when imaging [40]. Scan speed was of 0.5 Hz at 256 or 128 pixels per line scan, being independent of the scan size. Images were obtained at room temperature. A simple flatten was done to all images using WSxM 5.0 Develop 6.5 [41]

### 2.5. Viability assays

To assess the viability of bacterial cells, the commercial viability test Live/Dead BacLight from Invitrogen was used. This kit allows the labeling of nucleic acids, which is dependent on the membrane's permeability. A disrupted membrane means a dead bacterium, being this shown by a red fluorescent stain. An intact membrane is a living bacterium, which is shown by a green

fluorescent stain. Viability tests were performed on the sample prepared in exactly the same way as for AFM imaging, with the only difference that after preparation, the freshly prepared viability test solution was added to cover the sample and left incubating for 15 min in the dark. Fluorescence images were done using a Leica inverted microscope DMIRBE. SYTO® 9 presents an excitation wavelength 480 nm and emission 530 nm, while propidium iodide an excitation of 485 nm and emission of 630 nm. We calculated the survival % by using image J. The plugin "analyze particles" was used to count the number of present dead and alive bacteria, independently, and then the % of living cells was calculated.

## 3. Results

### 3.1. Imaging bacterial cells on planar substrates in buffer solution

For further reference, we started the analysis by analyzing the *E. coli* 042 strain grown according to protocol 1 in both dry and re-hydrated conditions. Fig. 1A shows an image obtained under nitrogen ambient flow (~0% Relative Humidity) of a dried (and hence dead) bacterial cell on a gelatinized gold substrate. Dried cells presented a rod-shaped structure ~2 μm long and ~1 μm wide and with a maximum height ~261 ± 6 nm ($N=13$), as obtained from cross-sectional profiles taken along the main bacterial axis (Fig. 1B blue line).

When adding HEPES buffer solution at pH 8 to the dried dead bacteria, bacteria re-hydrated (Fig. 1C). These bacteria still preserved the rod shape under buffer solution, presenting similar length and width, but a sensibly larger height (920 ± 21 nm, $N=13$) (Fig. 1B green line). The viability test done on these dried re-hydrated bacteria indicated that all bacteria were dead (Fig. 1D).

The semi-dried bacteria sample imaged in buffer solution (Fig. 1E), show a similar appearance to the dried re-hydrated sample. The topographic cross-section (Fig. 1B purple line) shows that the bacteria height (920 ± 26 nm, $N=13$) was similar to the one of dried re-hydrated bacteria. The viability test of the semi-dried bacteria (Fig. 1F) illustrated a slight increase in the viability of this sample preparation, up to 30% of living bacterial cells are present as indicated by the green staining. Further on, we note that in both cases flagella can be observed as shown by the insets (Fig. 1C and E for re-hydrated and semi-dried bacteria respectively), where the *Z*-scale has been reduced to favor visualization. Similar results have been obtained on gelatinized mica substrates and with the other strain considered in this study (data not shown).

In contrast to the samples described above, where some sort of drying process was involved, the observations of fully hydrated (living) bacterial cells could not be achieved with conventional imaging modes. Such images could only be reproducibly obtained with the use of the dynamic jumping mode with very soft cantilevers, since this method showed to exert very weak shear forces on the bacterial samples. In Fig. 1G a topographic image of fully hydrated individual *E. coli* 042 bacteria on a gelatine coated gold substrate in HEPES buffer solution at pH 8 is shown. The rod shape of bacteria has been naturally preserved, with a height of 1084 ± 32 nm ($N=13$) (Fig. 1B red line). The height is slightly greater than the re-hydrated or the semi-dried bacteria. The viability test (Fig. 1H) shows that more than 95% of the cells are viable. It should be noted that even if the bacteria are observed in HEPES buffer medium, which is depleted from nutrients, bacteria

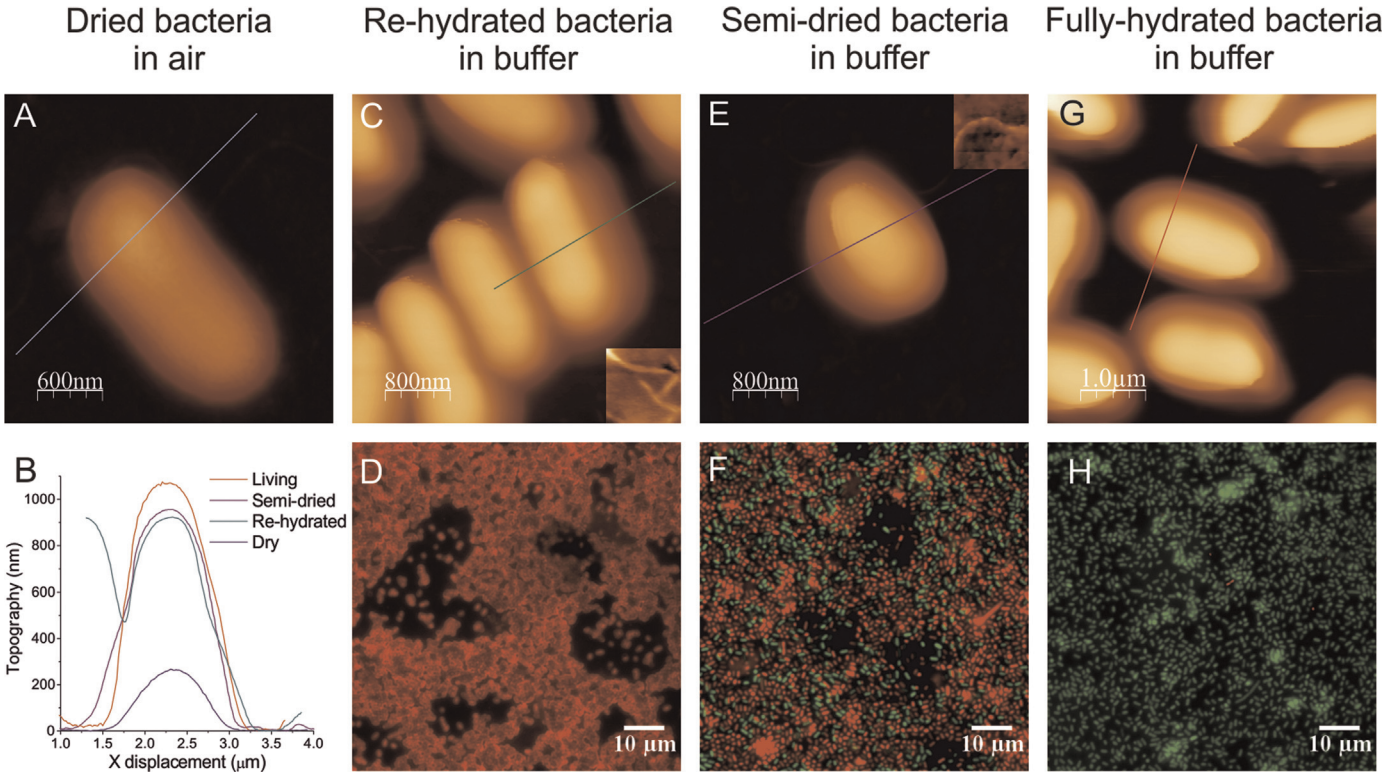


**Fig. 1.** AFM images of individual *E. coli* 042 bacterial cells dried and imaged in dry conditions (A) and of re-hydrated cells (C), semi-dried bacteria (D), and fully hydrated bacteria (E) in HEPES buffer solution at pH 8. Insets show the presence of flagella. (B) A comparison of the cross-sections taken along the lines indicated in the images: dried bacteria (blue line), re-hydrated bacteria (green line), semi-dried bacteria (purple line), and fully hydrated bacteria (red line). Fluorescence images from the viability kit stain for dried bacteria (D), semi-dried bacteria (F) and living bacteria (H), where green illustrates living bacteria and red dead bacteria. All bacterial cells are on a gelatinized gold substrate. Image in A was acquired in conventional dynamic mode and has a *Z* scale bar of 0.5 μm. Images in figures C, E and G were acquired in dynamic jumping mode and have a *Z* scale bar of 1.5 μm. In the insets the *Z* scale bar is of 100 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

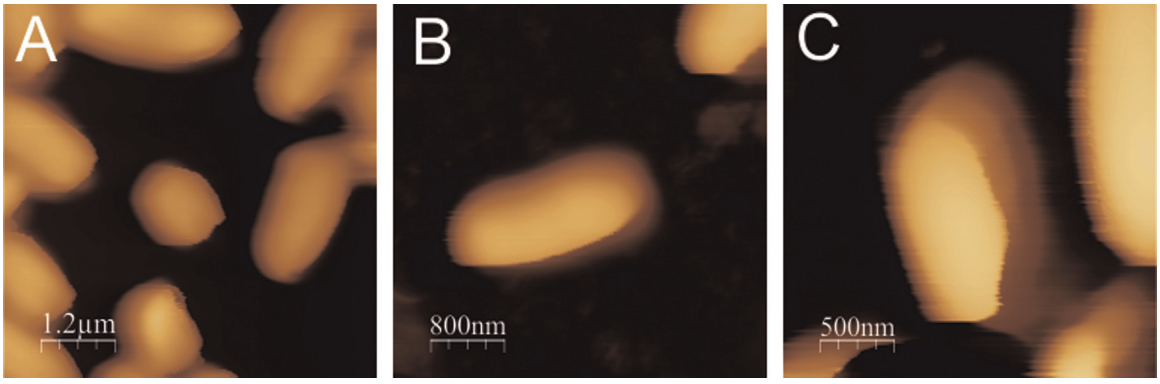


**Fig. 2.** AFM images obtained using dynamic jumping mode of living individual EAEC 042 cells resuspended in HEPES buffer solution and deposited on gelatinized coatings of glass (A), and mica (B); and living individual MG1655 cells deposited on a gelatinized coating of gold. *Z* scale bar of 1.5 μm.

still remain alive for long periods of time. This is due to bacterial ability to survive under starvation conditions in its stationary phase.

AFM images of weakly absorbed *E. coli* 042 bacterial cells were also observed over other gelatinized substrates. Substrates used were common laboratory materials, for instance glass and mica (Fig. 2A and B, respectively). When compared to those obtained on the gelatinized gold substrate shown in Fig. 1, images did not show any apparent structural differences. Images of the common laboratory strain *E. coli* MG1655 were also achievable on gelatinized gold substrates (Fig. 2C). No apparent structural differences between the living MG1655 and *E. coli* 042 bacterial cells were observed.

These results demonstrate the capability of dynamic jumping mode to image living bacterial cells weakly attached onto planar substrates.

### *3.2. Imaging living bacterial cells in growth medium on planar substrates*

The addition of nutrients to the HEPES buffer solution caused the irreversible detachment of both MG1655 and *E. coli* 042 cells. Bacteria grown following sample preparation protocol 1 could not be imaged with the presence of nutrients in the solution, being a handicap for the in situ observation of growth and division processes. To overcome this, the property of the *E. coli* 042 strain to form biofilm was then exploited. Biofilm grown samples seem to show a slightly stronger attachment to the substrates since they could be imaged even in the presence of a liquid solution rich in nutrients (sample preparation protocol 2).

As before, and for further comparison, these biofilm samples were firstly observed in dried conditions (~0% Relative Humidity) (Fig. 3A). It was observed that they presented similar properties to

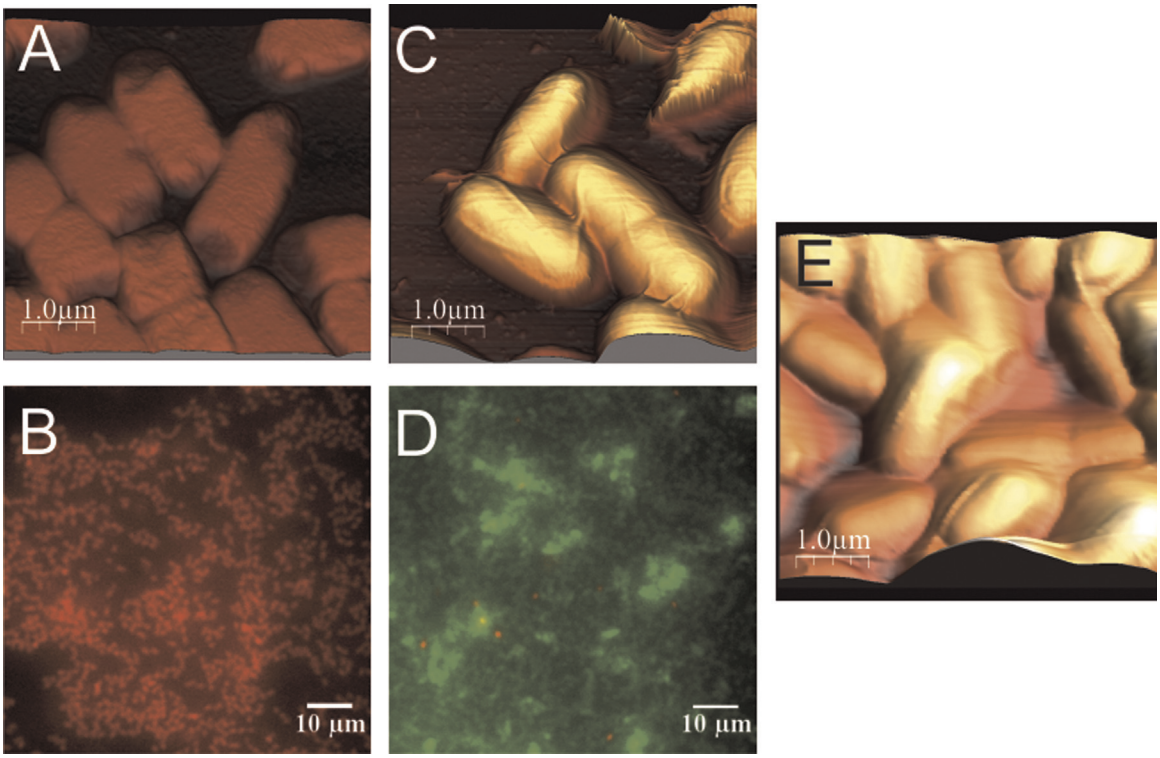


**Fig. 3.** 3D representation images of AFM images of dried *E. coli* 042 aggregation in dry conditions (A), of living EAEC 042 aggregation in HEPES buffer solution (C) and in growing medium (E) on gelatinized coatings of mica. Fluorescence images from the viability kit stain for the biofilm growth of a dried sample (B), and maintained under HEPES buffer solution (D), where green illustrates living bacteria while red dead bacteria. Imaging mode of A was done with conventional tapping mode while figure C and E were done in dynamic jumping mode, with all having a *Z* scale bar of 1.5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the previously shown individual 042 cells, but forming aggregates of several bacteria. The viability test (Fig. 3B) showed that, as in the previous experiments with sample preparation protocol 1, bacteria died during the drying process. The observation of the fully hydrated aggregate in HEPES buffer solution at pH 8 was not much of an impediment (Fig. 3C), where as in the corresponding cases shown before, bacteria remain alive due to its stationary transition capability (Fig. 3D). A stronger adhesion between bacteria was observed on the biofilm growing on the gelatine-coated mica substrate since bacteria appeared close together and were less easily displaced. This increased the adhesion to the substrate and enabled higher resolution images to be taken, which clearly revealed the presence of a flagellum net interconnecting bacteria (Fig. 3D). Finally, images of viable *E. coli* 042 bacterial cells in nutrient medium (DMEM+0.45% glucose) could also be obtained (Fig. 3E). A slightly smaller quality was obtained due to the smaller adhesion of the bacteria to the substrates in this medium, and eventually, due to an increased bacterial cell motility (which was confirmed from optical microscope videos, data not shown).

### 3.3. Imaging bacterial growth and division on planar substrates

Images capturing the growth and division of *E. coli* 042 bacterial cells were obtained by continuously imaging the sample for long periods of time (up to 3 h) in the nutrient medium (DMEM supplemented with 0.45% glucose). During this period of time, single bacterial cells' growth and division could be clearly observed and identified. In Fig. 4 we present a sequence of error images showing the growth of two independent bacterial cells. It can be observed how the bacterium on the left of the images grows from an initial length of 1.8 μm up to 2.7 μm in ~84 min. The septum formation can be observed in the last two sequence images, as indicated by the black arrow. This bacterium seems to extend its growth towards the bottom of the image, as it can be seen from the reference given by the green dashed line, delimiting bacterial initial position and size (Fig. 4). On the other hand, the bacterium on the right of the images starts with a larger initial longitudinal size of 2.1 μm, and grows up to 3.6 μm after ~79 min, where the septum has slightly formed. On the last image of this sequence, the new formed upper cell arisen after the septum formation was blurred. This has arisen from the moving capability of living bacteria, which makes difficult the imaging of bacteria which are not adhered to the substrate. On the ~94 min image, the cell on the top had disappeared and the lower right bacteria was still present (image not shown).

A detailed septum formation for another pair of bacterial cells is shown in Fig. 5. It was observed that from the ~5 min image to the ~20 min image, the septum had formed; giving a time of ~15 min for septum formation under these conditions (room temperature around 24 °C). On the following images of this sequence, it can be observed how the septum was still present. The movement of living bacteria can also be appreciated in Fig. 5, where the cell located at the right of the sequence of images had moved from a vertical position to a diagonal position, attracting itself to the other bacterium.

## 4. Discussion

We have shown that the dynamic jumping mode implemented with soft cantilevers enables the nanoscale AFM imaging of viable and metabolically active bacteria on planar substrates. The use of weak forces (lower than 0.2 nN), together with the lateral displacement of the probe far away from the sample (which drastically reduces lateral shear forces) are at the basis of this capability. Based on the results obtained, this mode can be considered as an alternative to other existing AFM imaging modes for living bacterial studies (e.g. magnetically excited dynamic modes). The main advantage of the mode used here with respect to magnetically excited dynamic modes is that its implementation does not require of any hardware modification nor of any special AFM probe (as long as its spring constant is soft enough, typically below 0.05 N/m). This makes this technique be potentially implemented in almost any AFM system. Moreover, since imaging has been possible with both the *E. coli* 042 strain, and the common laboratory bacterial strain *E. coli* MG1655, we predict that it is quite likely that imaging can be achieved with many other bacterial types.

With the capability to image intact viable bacteria we have verified, once more, that aggressive preparation methods, such as drying processes, substantially alters the structure and viability of bacterial cells [42]. For instance, the dimensions of viable bacterial cells are almost identical to those expected for *E. coli* cells [43,44], while those of re-hydrated or semi-dried bacteria are smaller in height. Moreover, cell viability was strongly compromised when drying was involved (below 30% in the best-case scenario). We note that even when bacterial morphology remains almost intact, this is not a guarantee for viable cell imaging. Therefore, viability tests as complement to AFM imaging are required to confirm viable cell conditions, as also has been recently pointed out by others [19].

It is relevant to point out here that imaging of bacterial cells

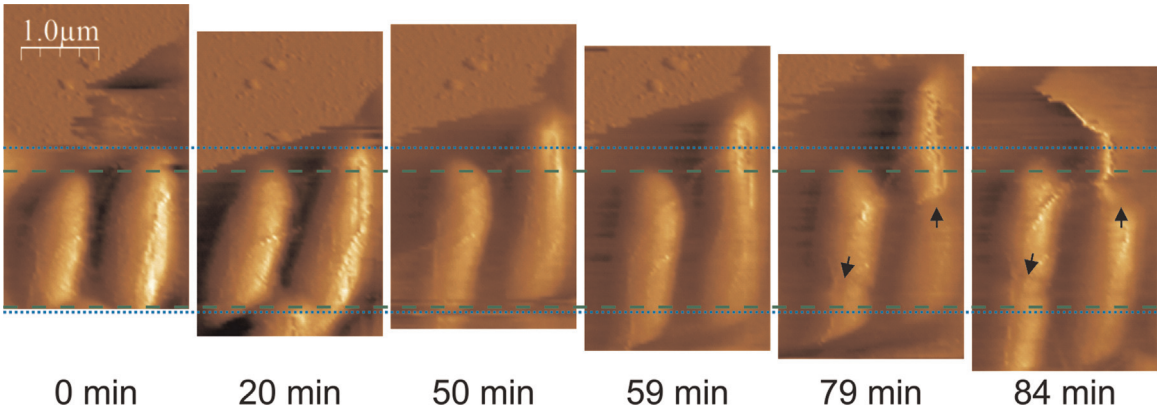


**Fig. 4.** Time sequence AFM error images of growing *E. coli* 042 cells in nutrient medium (DMEM+0.45% glucose) on gelatinized coatings of mica. The growth and the formation of the septum of two bacterial cells can be observed. The green dashed line delimitates the original size of the left bacterium, while the blue dotted line delimitates the original size of the right bacterium. Images have been moved vertically to correct drift error suffered during imaging in liquid. Arrows show septum formation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

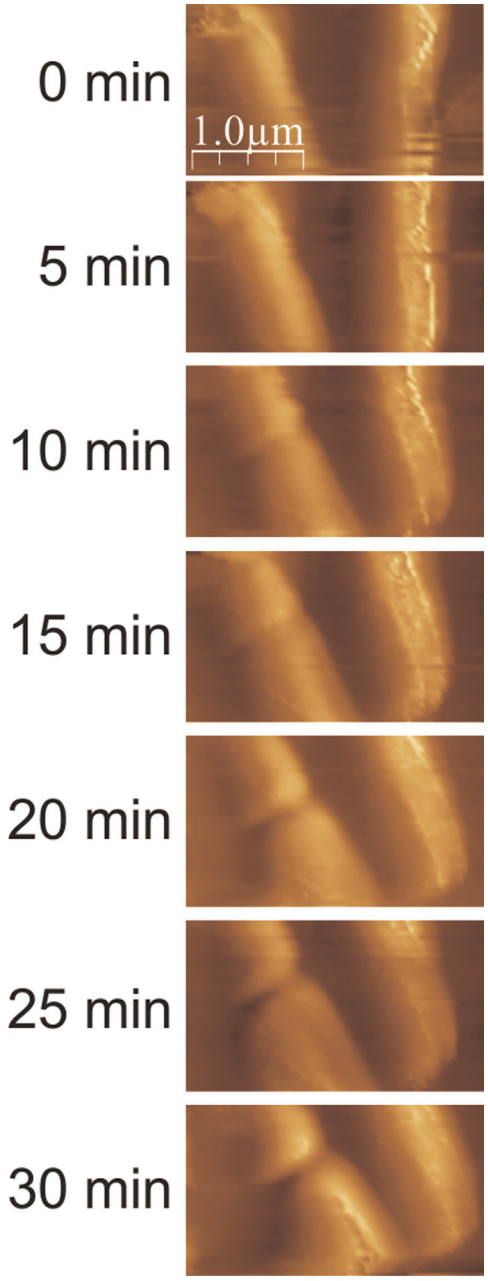


**Fig. 5.** Time sequence AFM error images of growing EAEC 042 bacteria in its natural growing medium (DMEM+0.45% glucose) for its aggregation growth on gelatinized coatings of mica. The formation of the septum in ~15 min can be observed on the left bacteria, while the right bacteria shows the movement bacteria can express (from a vertical position to a diagonal one).

attached to a gelatine coated substrate under growing medium (i.e. actively growing bacteria) is much more difficult than under buffer solution (i.e. non-proliferating bacteria), as also reported elsewhere [19,21]. We hypothesize that the main reason for this difficulty has to be traced back to the motility properties of cells, whereas the reduced bacterial adhesion onto gelatine coated substrates by salts would play a smaller effect. In buffer media, the absence of nutrients drastically reduces both bacterial motility and growth, rendering stationary phase cells. Therefore, under these conditions, once attached to the substrate the bacteria remain immobile on it, facilitating its AFM imaging. Instead, in nutrient rich media the motility and growth resume, introducing additional "forces". These "forces", in addition to the force made by the AFM tip, can favour the detachment of the cells. This hypothesis has been supported from our optical microscopy observations of the samples in both media, where, when compared to buffer solutions, higher motility of the attached bacteria was observed in nutrient growth media (data not shown).

Both the gentle forces exerted by the dynamic jumping mode, and the ability of the *E. coli* 042 strain to generate confluent growth has helped to overcome the challenges of imaging bacteria in liquid solutions containing nutrients. In dry and buffer imaging conditions, we clearly observed the confluent growth and initial biofilm formation of 042 cells, with bacteria being in close contact (Fig. 3C). Under these conditions, flagella were imaged, indicating that flagella are static. The absence of flagella movement could indicate that motility of bacteria can be considerably reduced when cells tend to aggregate. We note that bacterial motility is not fully suppressed under these conditions, as we have noted in Fig. 5. This must be the bacterial natural behavior since we are using a medium which mimics its natural ambient medium (human intestinal gut), and using a charged surface for its adherence (as reported previously [34]).

The ability of strain 042 cells to adhere to the flat surface made it possible to observe bacterial division (Figs. 4 and 5) following a natural adhesion process onto a substrate, contrary to other methods observed up to now [16,19]. It was possible to obtain a sequence of several images showing a bacterial division, without drastically compromising image resolution (~40 nm). Increasing the resolution would have implied ~18 min per image, thus preventing a real continuous monitoring of the cell growth and division. In the present conditions a spatial resolution below ~20 nm could be achieved, which compares favorably with the theoretical prediction of ~10 nm achievable with amplitude modulated imaging modes in liquid and in soft samples under ideal conditions [45]. The sequence of error images of two independent adjacent bacteria growing and forming a septum for future division has been achieved as shown in Fig. 4. It has been well established that the growing rate of bacteria is dependent on various factors, where for each bacterial cell the dividing rate could be different. This makes the extraction of the dividing rate difficult to normalize when predicted from AFM images on individual bacteria.

Since bacterial biofilms grow three-dimensionally [34,46], the newly formed bacteria are not exclusively restricted to a two-dimensional growth on the substrate. This phenomenon can be observed with the top bacteria formed in the division of the cell located to the right side in Fig. 4. In this case, the newly formed top cell was not as firmly attached to the substrate as the cells located in the lower part of the image. The smudgy image observed had arisen from the metabolically activity of the bacterium, which makes it motile. The detachment of this newly generated cell on the top clearly indicated that the division process had finished (image not shown).

The septum formation in Fig. 5 corresponds to a duration of ~15 min, which corresponds to an average $T$ period ( the time difference between the time required for the initiation of envelope constriction and the generation time) as reported by other authors using other methodologies [47]. This reconfirms that the weak applied forces of the dynamic jumping mode allows the observation of the division process in its almost native state, enabling single cell analysis of bacterial growth and division.

Previous reported AFM studies on bacterial cell division required either the use of trapping pores [8,16,17]) or of the poly-L-lysine [19,20] with magnetically excited probes. Both attachments may compromise membrane integrity, and hence influence cellular physiology and division rate. We have tested static AFM imaging modes with a similar principle of making the raster scan out of contact, such as the static version of the jumping mode, without succeeding in obtaining good images in the case of living

bacteria in a nutrient rich environment. Therefore, we conclude that the use of a dynamic mode is the key in obtaining the results reported. The methodology presented here enables observation of division in cells that are actively growing and not subjected to relevant torsion or attachment forces. As shown in Figs. 4 and 5, a continuous sequence of cell elongation and septum formation can be obtained for individual cells. This methodology can therefore be used in the immediate future for single cell physiological studies about bacterial cell division.

## 5. Conclusions

We have shown that dynamic jumping mode AFM constitutes a powerful technique for the observation of physiological processes of viable bacteria that are weakly attached to biocompatible gelatinous coated planar substrates. Images of intact and viable bacterial cells have been obtained for cells suspended in buffer solution for two different *E. coli* bacterial strains on different substrates, thus predicting a wide applicability of this imaging method. We have observed that when imaging in nutrient media with bacterial cells on planar substrates is additionally challenging due to the inherent bacterial motility associated to the bacterial growth. These forces tend to detach bacteria from the substrates. We have circumvented these difficulties with the dynamic jumping AFM mode in the case of the *E. coli* 042 strain at the initial phase of biofilm formation. This method has made it possible to observe the bacterial growth and division, an event which has not been shown up to date with biocompatible gelatine coated substrates. These results open new possibilities in the in-situ observation of living bacterial processes at the single cell and nanoscale levels.

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