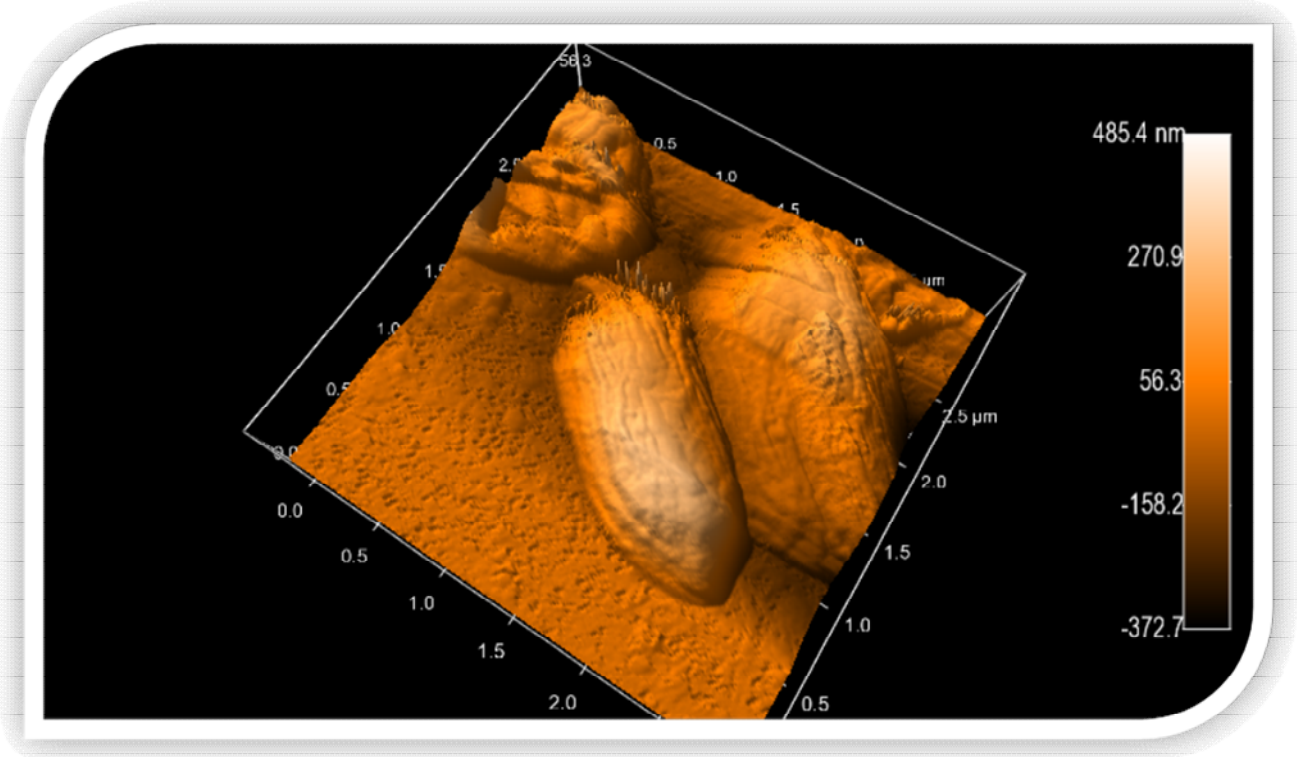
Project work in

"Interactions with surfaces"

LVA Nr. 134.114

"AFM measurements on vegetative and sporulated *Bacillus subtilis*"



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Title image: Height image of a UV resistant *B. subtilis* spore on a poly-L-lysine coated glass slide

Contents

1 Abstract

Atomic force microscopy can yield valuable information concerning changes in material properties of living organisms. *Bacillus subtilis* is a bacterium commonly found in soil. It can sporulate, i.e. reversibly form a tough and protective endospore that allows the organism to tolerate extreme environmental conditions. *B. subtilis* is not harmful to human health and its robust spores may therefore serve as safe model organisms for pathogenic microorganisms in drinking water. Thus, this organism is used to evaluate water disinfection devices that utilize UV radiation.

By inducing adverse environmental conditions to living *B. subtilis* cells while imaging them with the atomic force microscope, the sporulation procedure was successfully recorded over a time span of about 50 hours. Given this promising result, the recording time of material properties had been extended to a week, thereby trying to obtain more detailed information about the sporulation procedure from the vegetative cell to the spore.

Two methods of spore production resulting in different types of spores were included in the investigation. One type of *B. subtilis* spores is highly resistant to UV irradiation, whereas the other type shows a low UV resistance. By means of the atomic force microscopy technique characteristics of the spores surface structure and morphogenesis shall be elucidated.

2 Biological Applications of AFM

Bioimaging with the Atomic Force Microscope has become an ambitious field of research in the last two decades. There are several reasons for this development; the most important is that the AFM is a general purpose instrument for analyzing nonconducting submicron structures. Some of the applications are analysis of DNA and RNA, protein-nucleic acid complexes, chromosomes, cellular membranes, proteins and peptides, molecular crystals, polymers and several biomaterials [9]. Compared to other analytic instruments the AFM and especially the ambient AFM has a variety of advantages. Some of them will be discussed below in more detail.

The main advantage is the ability to analyze non-conducting samples without additional preparation like metallising with gold or similar techniques. This holds especially with vegetative biological samples as for instance the *Bacillus subtilis* which has been studied in this project work. The second big benefit is the little preparation work required for AFM analysis with the ambient AFM. For most applications you simply have to fix the sample on the sample holder or substrate before starting the measurement whereas other imaging techniques need vacuum and often troublesome prerequisites. Further information on sample preparation is given in paragraph 4.

AFM techniques can also be used for manipulating living samples and measuring mechanical properties of cell and bacteria membranes. In most cases it is simply used for imaging the topological properties of the sample of interest. Furthermore it is possible to acquire real time data of the morphogenesis of living structures with sufficient image quality by using the non-damaging Tapping Mode. We used this technique for imaging the morphogenesis of the *Bacillus subtilis*. In this AFM working mode the tip abrasion can be neglected, due to the slight treatment of the sample under test.
Another plus of the above-mentioned Tapping Mode is the possibility of detecting different surface components or properties by taking phase images with the AFM. A detailed description of this imaging technique is given in paragraph 5.4. Besides the possibilities for gathering information about the sample investigated, it is obvious that the Atomic Force Microscopy is only a surface sensitive measurement. Thus it cannot be used for direct analysis of bulk properties.

To complete this introduction about the biological applications of the AFM table 2.1 provides

	conventional optical microscopy	scanning electron microscopy (SEM)	Field Emission In lens Scanning Electron Microscope (FEISEM)	atomic force microscopy (AFM)
microscopic environment	ambient liquid vacuum	vacuum	vacuum	ambient liquid vacuum
field depth	small	high	medium	medium
focus depth	medium	small	very small	small
resolution x,y z	100 nm n/a	5 nm n/a	0,7 nm n/a	0,1 - 1,0 nm 0,01 nm
magnification	$1x - 2*10^3x$	$10x - 10^6x$	$9x-10^5x$	$5*10^2x - 10^8x$
necessary sample preparation	low	critical point drying or freeze-drying, coating	critical point drying if neccesary	low
necessary sample properties	samples do not have to be completely transparent for visible light	samples should not charge and have to be vacuum compatible	vacuum compatibility	samples should do not have excessive changes in height compared to tip geometry

Tab. 2.1: Overview about the possibility of commonly used microscopic techniques for bioimaging [1]

an overview about the possibilities of some microscopic techniques for imaging topological properties of biological samples [1].

3 The *Bacillus subtilis*

3.1 General Information

Bacillus subtilis (lat. *bacillum/bacillus*, stick; *subtilis*, simple) is a chopsticklike, gram-positive bacterium with flagella and the ability to sporulate.
Like every bacterium of this species, also *B. subtilis* is growing in aerobe conditions and it is building endospores. The endospores allow the organism to resist extreme environmental conditions.

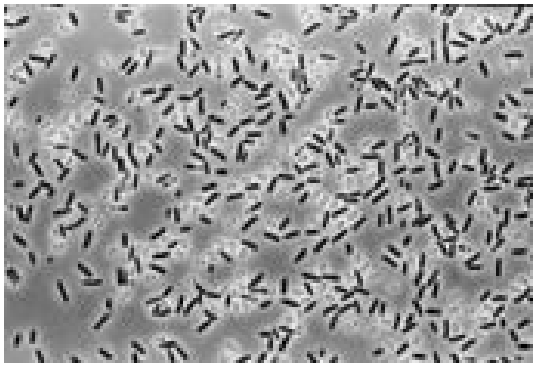


Fig. 3.1: *Bacillus subtilis* in a phase-contrast photomicrograph with oil immersion lens (magnification x100) [2]

Starvation triggers an uneven bisection of the protoplast in between the envelope of the cell, a process like endocytosis which is building the spore. In contrary to most other spores, endospores are no forms of breeding, because every bacillus is only producing one, in rare cases two or three spores and the living bacillus itself is destroyed during sporulation.

B. subtilis was discoverd in 1835 by Christian Gottfried Ehrenberg and described as *Vibrio subtilis* (bent chopstick). Later it was renamed to *Bacillus subtilis*. It is about 2 – 3 µm in length and about 0.6 µm in height. Because the flagella are arranged all round the bacillus' body (peritrich) it has the ability to move fast.
B. subtilis is widespread and can be isolated from earth (compost), water and air. The natural habitats are the superficial layers of the ground. Due to the rapidly changing circumstances in the ground, it is often subject to stress and starvation.
The *B. subtilis* has been proven to be highly amenable to genetic manipulation, and has therefore become widely adopted as a model organism for laboratory studies, especially for sporulation, which is a simplified example of cellular differentiation.
B. subtilis is using nutrients which are produced by other organisms to convert energy for reproduction and for building up cell components. Thus it is an important part of the nutrient circle (rot bacterium).
A fascinating property of this bacterium is that it can absorb extracellular DNA for eating or to expand its own genome.

The B. subtilis is used in several drugs to treat chronic dermatosis and gastrointestinal diseases. In agriculture it is used as biological fungicide (for cotton, vegetables, peanuts and soya) with its maximum effect in presence of glucose. The bacterium inhabits the roots during blastogenesis and as a result it is preventing the occurrence of fungi.
Furthermore B. subtilis is used in medicine for newborn diagnostics. Some blood is put on a nutrient ground with the bacillus. If the aminoacid phenylalanine is pathologic increased the B. subtilis will grow. This would be an evidence for the disease Phenylketonurie [3].

3.2 Morphogenesis

Sporulation is a mechanism of bacteria to adapt to starvation. Unlike most adaptive responses in bacteria, sporulation takes many hours and includes major changes in cellular morphology as well as in biochemistry and physiology. Morphogenesis needs the cooperation of two sister cells, which both are starting with the same genome. The first cell is packaged into a tough resistant coat, while the other cell is contributing most of its sources to this process and then lyses (cell death). This process is also an example for the differentiation of certain cells and for some kind of feedback mechanism between these two specific cells. The genetic interactions can be explained by the action of transcription factors. Sporulation is among the best understood of developmental systems and helps answering basic questions of biology at molecular level.

Sporulation (see Fig. 3.2) is generally induced by starvation. In a good growth medium they would double in length and divide centrally to produce two identical daughter cells. But at the beginning of sporulation there is an asymmetric division, producing sister cells differing

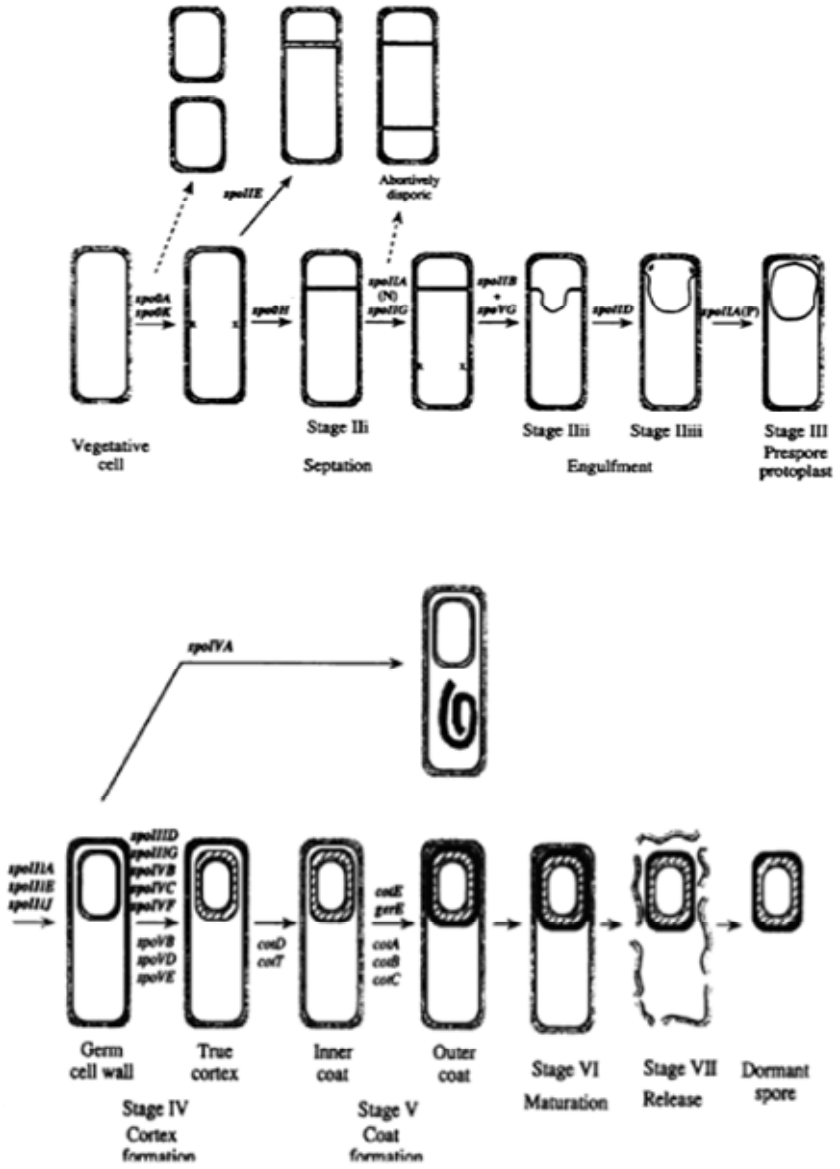


Fig. 3.2: Morphological events of *B. subtilis* sporulation

in size. Completion of the specialized spore septum was defined as stage II. Engulfment of the smaller prespore by the mother cell is defined as stage III. The distinct ovoid shape of the mature spore begins to become apparent as a layer known as the cortex, a modified form of cell wall is synthesized between the prespore membranes (stage IV). About at the same time a proteinaceous spore coat is being deposited on the outside of the cell (stage V). Maturation of the spore is defined as stage VI and finally the release of the mature spore by the lysis of the mother cell is defined as stage VII [4]

Initiation of sporulation

The initiation of sporulation is complex, but there are at least three types of input signals which are integrated by the cell before sporulation begins.

1. Nutritional signal: Starvation for sources of carbon, nitrogen, or phosphorus can induce sporulation; good carbon sources, such as glucose, repress it.

2. Population density: sporulation could not be induced efficiently in cells maintained at a low population density – it seems as vegetative cells grown to a relatively high density produce a substance, possibly an oligopeptide that is necessary for efficient sporulation. How the cell could sense the rate of oligopeptide uptake, or interpret this information, is unknown.

3. Cell cycle: the sporulation cycle can occur only at a specific point in the cell division cycle.

Because of the fact that their coat spores are very resistant against heat, draught and other unfavorable conditions, *B. subtilis* will remain as spore until conditions are better again before the spore retransforms to a vegetative bacillus.

4 Sample Preparation

4.1 Biological Sample Preparation

In this paragraph we try to give an idea of the most commonly used preparation techniques for AFM measurements of biological samples and in particular of the *B. subtilis*.

Generally we have to distinguish between the preparation of vegetative *B. subtilis*, which have flagella for movement and the preparation of *B. subtilis* spores which have no more flagella and hence are immovable. There are many different preparation techniques for both, spores and vegetative bacteria, but they all have some basic steps in common:

- Synthesizing the samples (spores or vegetative bacteria)
- Choosing an adequate substrate serving as sample holder and
- Selecting a proper method for fixing the samples on the substrate

4.1.1 Synthesizing the samples

The vegetative and sporulated *B. subtilis* samples were kindly supplied by the Institute for Hygiene of the Vienna Medical University (Dr. R. Sommer, Department for Water Hygiene).

We obtained the vegetative bacteria suspended in a nutrient solution with sugar and other nutrient additives. This suspension was sifted to make sure that nothing larger than the bacilli remains in the liquid sample. The *B. subtilis* spores were supplied in a liquid suspension, like the vegetative bacteria and additionally in form of a powder of dried spores.

4.1.2 Choosing an adequate substrate

The appropriate substrate has to provide a sufficient mechanical fixing of the samples and it should be easy to handle. Searching the literature one can find a variety of substrates you can choose for the preparation of biological samples, but as we will see, most of them are very similar. If one wants to do AFM imaging on particles smaller than 100nm one additionally has to consider that the substrate roughness has to be less than the particle size.

Some of the substrates which are principally used for the investigation of biological samples with the ambient AFM are:

- Glass slides
- Poly-L-lysine slides
- Gel slides
- Mica
- HOPG
- Different kinds of membranes

Besides this, one can also perform investigations in fluid using a fluid cell, i.e. the nutrient solution is surrounding the samples during the measurement. In this case the mechanical fixation of the samples is much more difficult but for some vegetative samples the investigation in fluid cell is necessary.

Glass slides: They are cheap and easy to handle but they are not adhesive. For some applications, like the analysis of immovable samples, glass slides can be a proper substrate. For a better mechanical fixation one can obtain glass slides with different adhesive coatings as described in the following.

Poly-L-lysine slides: These are glass slides with a poly-L-lysine coating on the surface working as an adhesive. Poly-L-lysine is often used in microscopy and is available in liquid form. There are established protocols on how to coat glass slides with poly-L-lysine.

Gel slides: These are glass slides with a special gel coating providing an improved attraction of the dry samples like the spore powder but the samples are not completely immobilized. Thus it can happen that the AFM tip changes the position of the spores which leads to artefacts in the AFM image. The reason for this bad mechanical fixing is that the gel coating stays in a non-rigid state even after drying the sample.

Mica: There are different types of mica and grades (according to the ASTM [1]grading

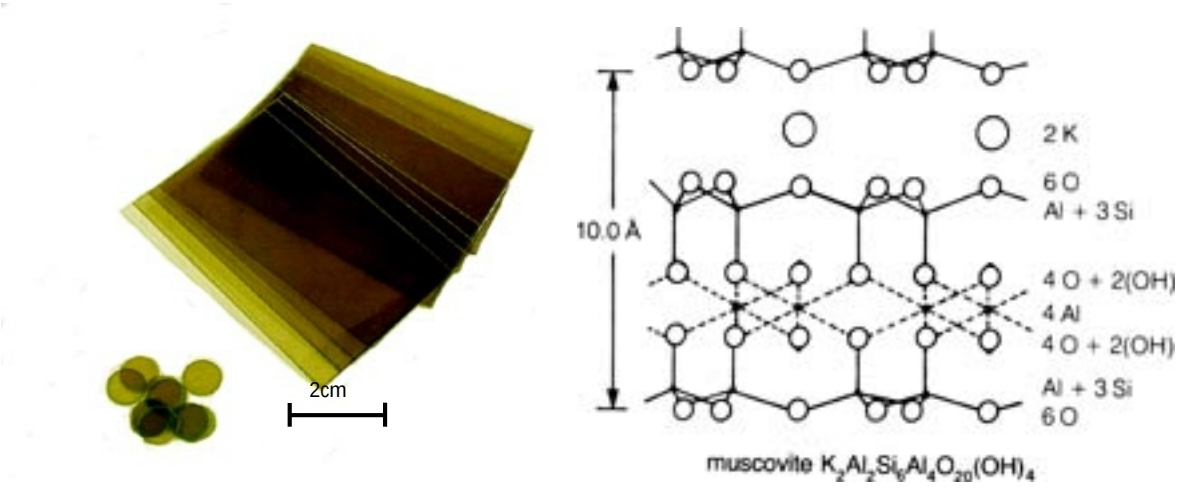


Fig. 4.1 Samples of mica substrate for AFM imaging and chemical structure of muscovite mica [5]

scheme) of quality classifying it. The most commonly used is the muscovite form. Mica exhibits a layered structure and can therefore easily be cleaved. The quality grade is defined by the number of steps per unit area on freshly cleaved mica. The price of this substrate material strongly depends on the grade which is required for the measurement but it is still cheaper than HOPG when used for the same purpose. Mica is a polar material, so it is mostly used when the polarity does not matter or if it is needed for measurement. Finally Mica with a high grade of quality can be used for AFM calibration studies [5].

[1] ASTM is a standardisation scheme and stands for American Society for Testing and Materials

HOPG - Highly Orientated Pyrolytic Graphite: HOPG is a relatively new type of high purity carbon. It shows a high chemical inertness and it is available with a very low roughness down to 0.2 – 0.3nm. Therefore it is used for analysis of escharotic[2] chemical samples and for elemental analysis, because of the uniform background of the carbon atoms. Due to its layered structure HOPG cleaves similar to mica but is completely non polar and can therefore be used if a hydrophobic substrate surface is required. For example if a water

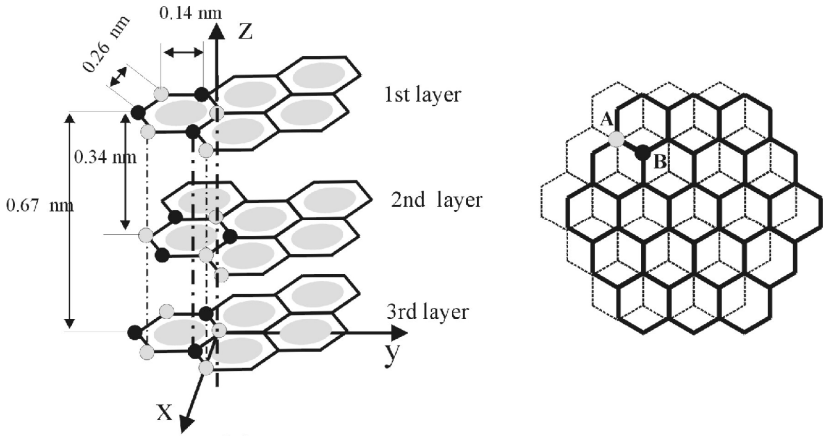


Fig. 4.2 Structure of HOPG – Highly Orientated Pyrolytic Graphite [10]

monolayer in ambient AFM should be avoided. The simplest method to cleave a layer is to take a double sided tape, press it on the HOPG and pull it of again [5].

Membranes: Filter membranes are mainly used for the AFM imaging of vegetative and moveable biological samples. They provide sufficient immobilization of the living samples, if they get stuck in the membrane pores. Therefore the suspension which contains the samples

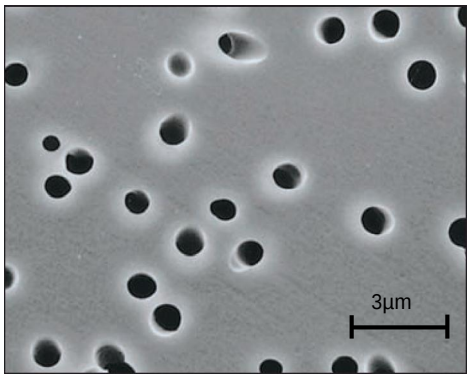


Fig. 4.3: Magnification of a Polycarbonate filter membrane [6]

[2] A caustic or corrosive substance

of interest, like in our case the *B. subtilis* bacteria is sucked through such a filter membrane with a pore size similar to the sample size. If the AFM measurements are done in air, the membranes are not essential, but if the measurements are done in liquid, this immobilization method is preferable.

4.1.3 Selecting a proper method for sample fixing

In the previous paragraph we have already mentioned some means for the fixation of the samples, like the use of self-adhesive slides which are mostly glass slides with an adhesive coating. For the other substrates like glass slides without coating, HOPG and mica the use of liquid poly-L-lysine is a proper means for mechanical fixation. In the following paragraphs we will see that in some cases simply drying the sample on the substrate is effective enough. With filter membranes the mechanical fixation is done by the membrane pores which we already discussed before.
Another possible method for fixing and at the same time stiffening the biological samples is to cool them down, e.g. with liquid nitrogen. Compared to the other methods this one is rather laborious and of course not suited for vegetative samples. Thus you will only choose this possibility if the other methods are not sufficient.

Out of this variety of possible preparation techniques we tried to find an adequate method for the AFM imaging of *B. subtilis*:

4.2 Preparation of the *Bacillus subtilis*

4.2.1 Preparation of B. subtilis spores in aqueous solution

The *B. subtilis* spore solution was sifted to remove everything larger than the spores. About 1ml of this solution was carefully dropped on poly-L-lysine coated and uncoated glass slides. After fifteen minutes drying in air, some of the samples were washed with PBS (phosphate buffered solution) in order to reduce the spore density on the substrate. According to the inclined position of the slides during the washing and the amount of PBS used more or less *B. subtilis* remained attached to the slide. The sample slides were allowed to dry in air for about forty minutes before the first measurements.

4.2.2 Preparation of dry *B. subtilis* spores (powder)

The powder (supplied by the Institute for Hygiene of the Vienna Medical University) was dissolved with PBS and dropped on the poly-L-lysine coated and uncoated glass slides. For the uncoated slides we used liquid poly-L-lysine for mechanical fixation. The further procedure of the preparation of dry *B. subtilis* spores is the same as for the spores in aqueous solution (see chapter 4.2.1).
Generally, the immobilization for *B. subtilis* spores is not crucial due to their inability to move on their own. Nevertheless they have to be fixed on the substrate, otherwise they could get displaced by the AFM tip.

4.2.3 Preparation of vegetative B.subtilis

Due to the limited lifetime of the vegetative samples and their ability to move by using their flagella the immobilization is an important aspect. Therefore the use of either adhesively coated substrates or filter membranes is recommended. It turned out to be sufficient to use the coated substrates for mechanical fixing for single and continuous imaging. In the literature the use of membranes is a common method for measurements in liquids.

The preparation is the same like for the *B. Subtilis* spores in aqueous solution, but in this case only with adhesive poly-L-lysine coated substrates (see figure 4.5) for a proper immobilization and the use of a PBS buffer solution for diluting instead of distilled water. Otherwise the distilled water would effect the bacteria membrane to burst because of the osmotic pressure.

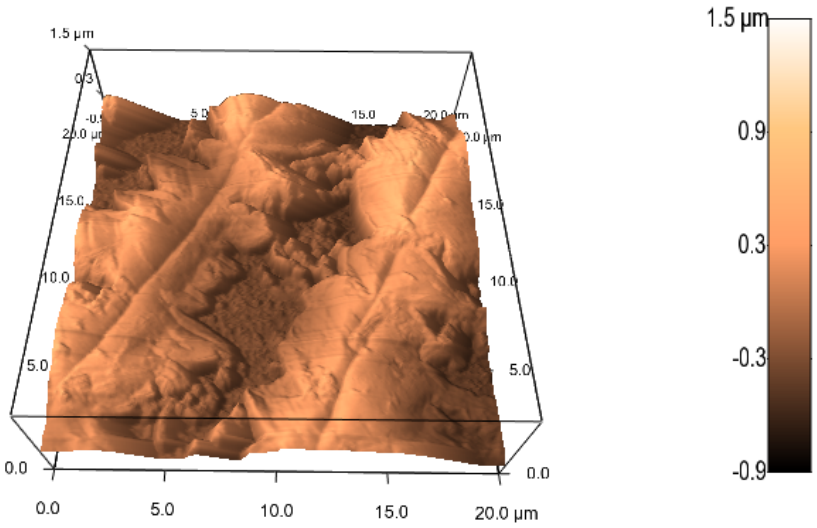


Fig 4.4: AFM height image of parasitic structures due to the nutrients in the nutrient solution

The vegetative B.subtilis are usually dissolved in a nutrient solution. Nutrients can cause parasitic structures on the substrate surface (see Fig. 4.4).

To prevent acquiring unintentional structures, a proper method is to smear off the excess solution with another slide. Due to the absence of water after drying the sample you have to consider that the bacteria will possibly start the morphogenesis, which can be intended in some cases e.g. for real-time imaging of changes in the bacterial membrane. To assure the

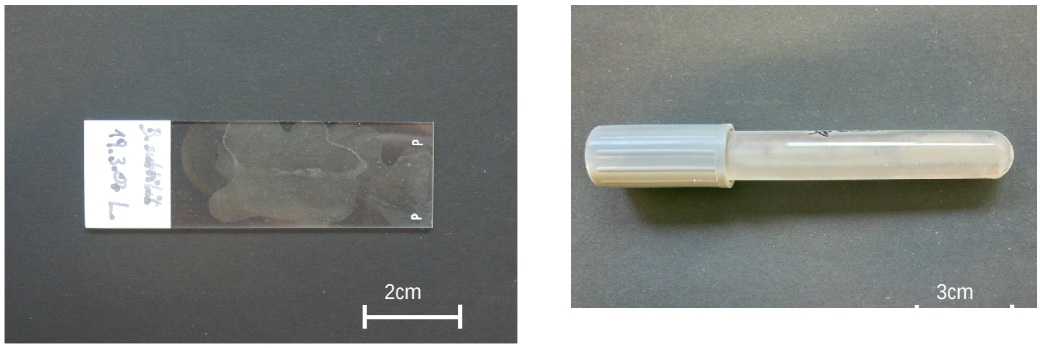


Fig. 4.5: left: sample with vegetative B.subtilis on Poly-L-Lysine coated glass slide
right: vegetative B. subtilis supplied by the Institute for Hygienic of the Medical University of Vienna

morphogenesis will start, the drying process must not take place too fast because a little amount of water is required for initiating the conversion from the living bacteria to the spore. Otherwise the majority of the bacteria would die off before any morphological changes can happen.

The use of HOPG and mica is not absolute necessary for our AFM images because the samples of interest, the bacteria and their spores, have dimensions of about 2µm to 3µm in length and about 0.6µm in width. Thus the substrate surface of the glass slides and the adhesive glass slides are enough even for good topological contrast.

5 Atomic Force Microscopy (AFM)

The Atomic Force Microscope (AFM) was invented by Binning, Quate and Gerber in 1986. It is a very high resolution type of scanning probe microscope for imaging, measuring and also manipulating matter.

The main parts of the AFM are the cantilever with a sharp tip at its end, which is used to scan the sample surface, a UV-laser, which is aligned with the cantilever, a deflection sensor, which is a photodiode detecting the position of the cantilever and an optical microscope. The measuring principle is based on the fact that there are very small forces (<1nN) between

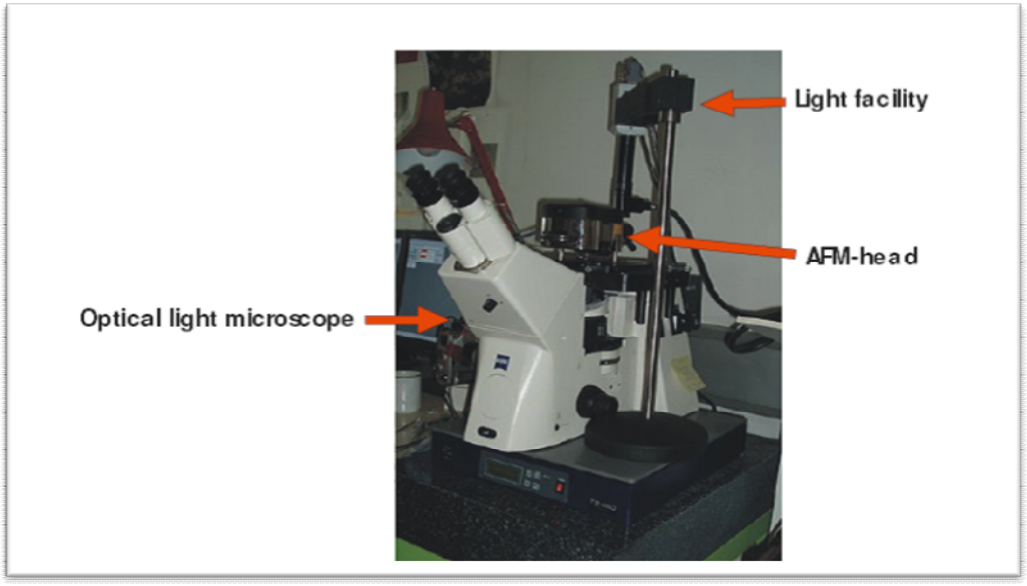


Fig. 5.1: Ambient Atomic Force Microscope

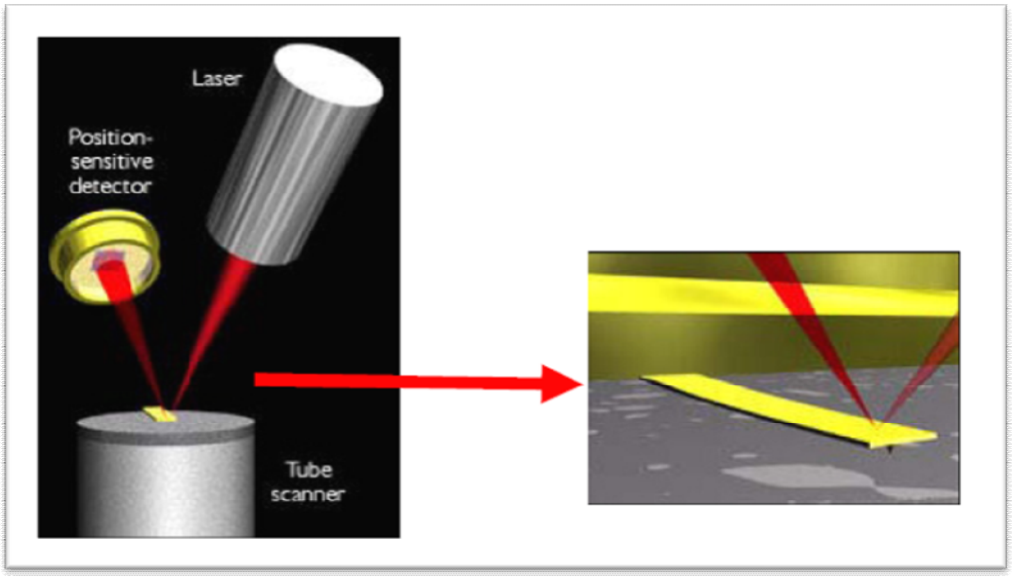


Fig. 5.2: Detection of the cantilever position with laser beam and a photodetector

the AFM tip and the sample surface.

In the case of our AFM the prepared sample is mounted on a piezoelectric tube, which makes it possible to scan the sample in x- and y- direction. There are two possibilities of doing measurements with the AFM, the contact mode (DC mode) and the dynamic mode (tapping, AC mode) [7].

5.1 Contact mode

In contact mode the cantilever tip is in close contact with the surface of the sample. During the first contact, the atoms of the cantilever tip sense a repulsive force, which is caused by the overlap of the electronic orbitals of the atoms of the sample surface. To scan the surface of the sample the tip has to be in contact with it. This causes a movement of the cantilever in z-direction and so changes of the deflection signal. The deflection signal is permanently sensed and compared in a DC feedback amplifier to a preset value of deflection, the set point. If the deflection value is different from the preset value, the feedback amplifier applies a voltage to the piezo to raise or lower the cantilever tip relative to the sample surface in order to restore the preset value of deflection. So the voltage to the piezo is directly correlated to the cantilever movement and the height of the topography of the sample surface is given [7].

5.2 Dynamic mode

In dynamic mode (tapping mode, AC mode) there is only intermediate contact between the tip of the cantilever and the sample surface because in tapping mode the cantilever is oscillating at or close to its resonance frequency. This technique is used to avoid damaging

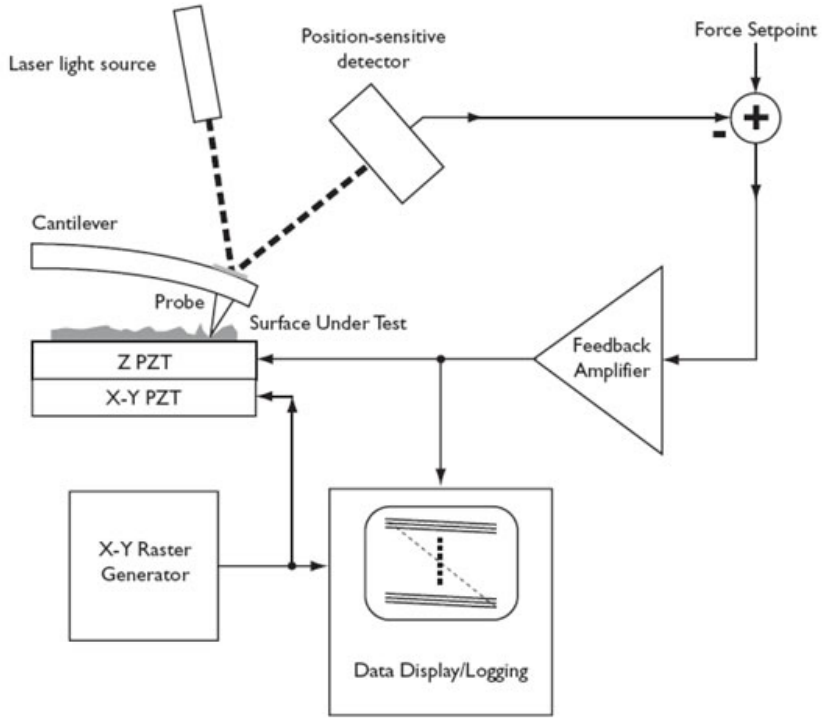


Fig. 5.3: Feedback control system of the AFM

the sample by scratching over it. The amplitude, the phase and the frequency are interacting by tip-sample interaction forces. These modifications provide information about the sample's characteristics. In contrast to contact mode, the cantilever oscillation amplitude is kept constant. Therefore the amplitude is measured permanently and a feed back loop adjusts the value of the cantilever z-value due to the separation between the tip and the sample surface which is defined by the set-point amplitude. Through this process the topography of the sample surface is obtained [7].

5.3 Phase Images in Dynamic Mode

As mentioned above, in Tapping Mode the cantilever is oscillating at or close to its resonance frequency. The amplitude, the phase and the frequency are interacting by tip-sample interaction forces. The obtained height and amplitude images are usually used for topographical AFM imaging. Phase images on the other hand are not appropriate to receive a topographic contrast but they provide some additional information about the surface, more precisely the phase images can give us information about the viscoelastic properties of the sample surface. Thus it can be seen as an alternative method to force imaging without damaging the sample especially in the case of vegetative samples, like the B.subtilis.

To get an idea of the relation between the samples surface elasticity and the phase of the oscillating cantilever tip we use the damped driven harmonic oscillator approach [8],

$$m \cdot \ddot{z}(t) = -\alpha \cdot \dot{z}(t) - k \cdot z(t) + k \cdot z_0(t) \qquad (1)$$

where the cantilever spring is supposed to obey Hooke's law with the spring constant k. The attenuation due to the viscoelastic behavior of the tip-surface interaction is represented by the damping coefficient α, the driving oscillation is $z_0(t)$ and the tip position is z(t) with

$$z_0(t) = A_0 \cdot \cos(\omega \cdot t). \qquad (2)$$

With some simple transformation we get

$$z(t) + \frac{\alpha}{m} \cdot z(t) + \omega_0^2 \cdot z(t) = A_0 \cdot \omega_0^2 \cdot \cos(\omega \cdot t) \quad (3)$$

where we defined $\omega_0 = k/m$.

If we assume a measurement period long enough to reach a steady state, which is satisfied for low scan rates the solution of the equation can be approached by:

$$z_s(t) = A_s \cdot \cos(\omega t + \varphi) \qquad (4)$$

Using (4) leads to the final expression for the phase φ, used for the phase images in Dynamic Mode AFM:

$$\varphi = \arctan\left(\frac{\alpha}{m} \cdot \frac{\omega}{\omega_0{}^2 - \omega^2}\right) \qquad (5)$$

This expression shows, that the phase shift depends on the damping coefficient and thus on the viscoelastic properties of the sample surface [8]. In this simplified approach of the damped driven harmonic oscillator we may consider two cases:

- a stiff surface and
- an elastic surface

In the first case the damping coefficient is rather low and therefore the phase shift is small. The second case will show a relatively high attenuation which results in a rather high phase shift. Therefore the phase imaging technique can be used for a distinction between different components of the cellular membrane of a biological sample. Fig. 5.6 shows a sample phase image of a small part out of the surface of a *B. subtilis* spore. This image does not necessarily have to correspond to topological properties. In fact we can see something like the viscoelastic map of the surface. This specific phase image for example reveals a

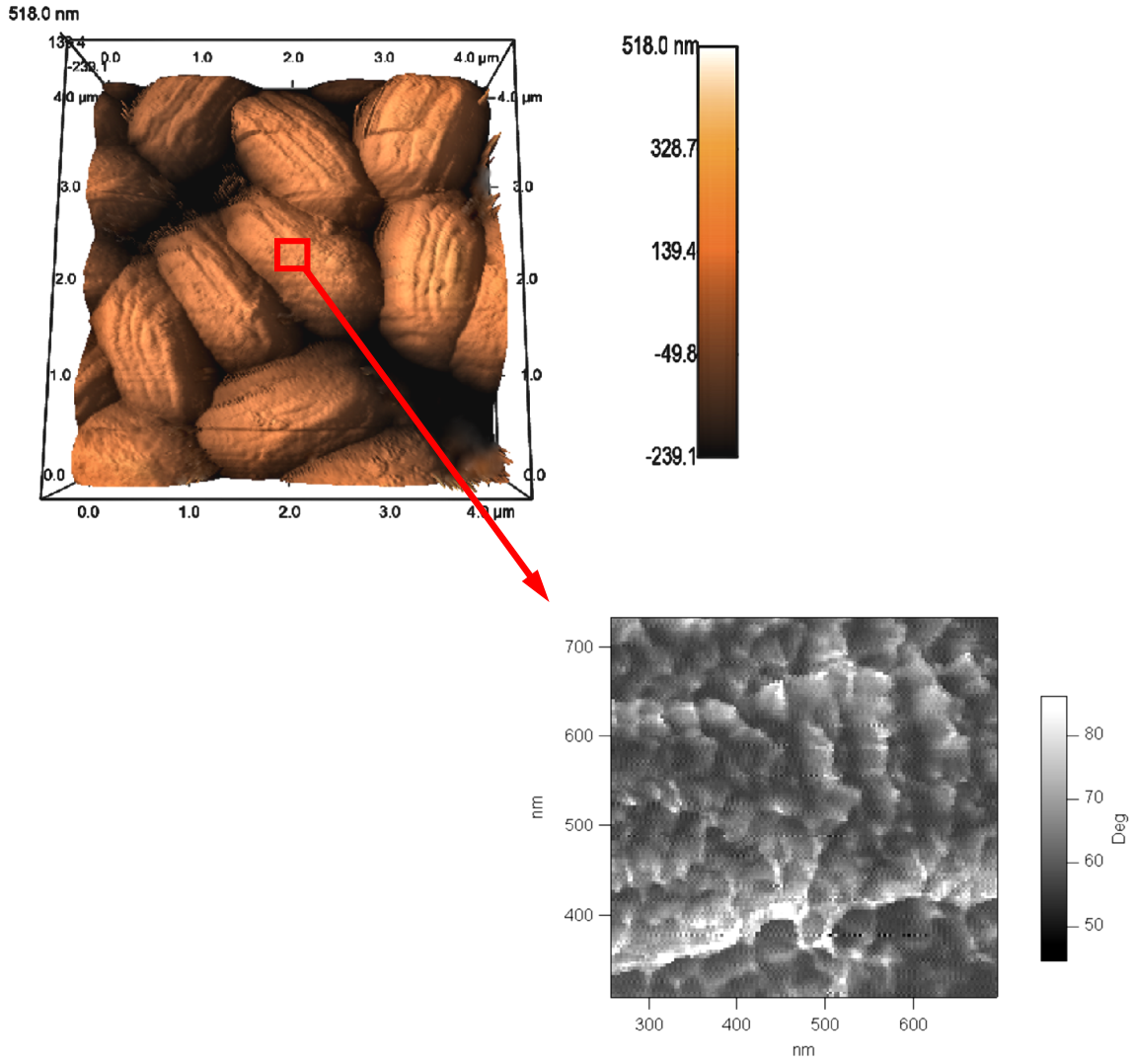


Fig. 5.6: Agglomeration of *B. subtilis* spores (UV resistant) and phase image of a small part of the spore surface. You can see the viscoelastic properties of the substructure.

substructure of the spore coating which can't be seen in amplitude or height images.

Obviously, the phase image technique gives us a non-damaging method for analyzing intrinsic properties of coatings or membranes, especially the viscoelastic properties. However with this technique one can not measure absolute values but only relative

deviations because of the absence of an appropriate viscoelastic standard. Therefore it is better used for the fast visualization of intrinsic surface structures. To achieve absolute results at certain areas Force Mapping would be a proper method.

6 Imaging of *B. subtilis* Spores

To achieve lifelike images with marginal damages of the *B. subtilis* spore surface it is recommended to work with the AFM in Tapping Mode. Some measurements were also done in Contact Mode but this led to damages and some additional problems, like e.g. with the mechanical fixation of the sample on the substrate. Different tip driving frequencies were used for imaging the spores. The best images were obtained using 70kHz cantilevers[3]. The prepared samples were analyzed at different positions with an investigation area of 20x20µm². The parts of interest were then magnified.

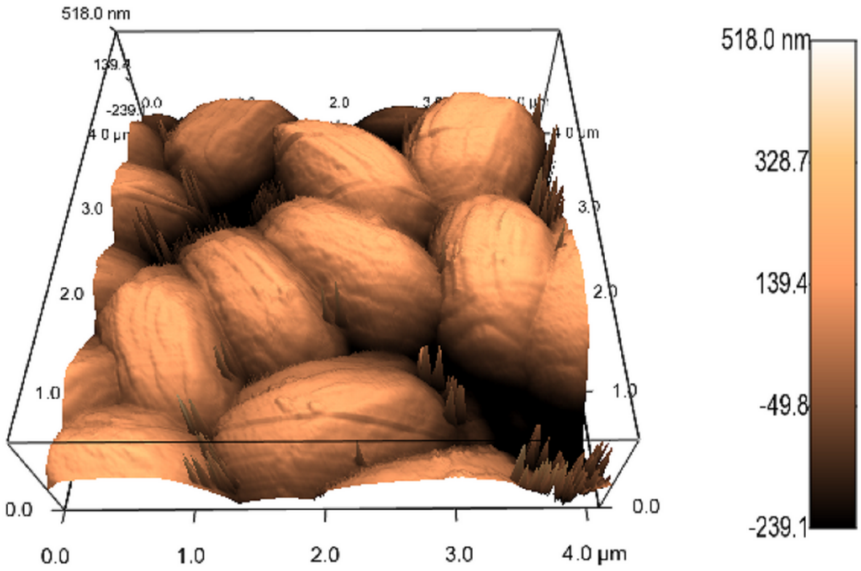


Fig. 6.1: Agglomeration of *B. subtilis* spores (UV resistant) on a glass slide

[3] Cantilever type: Olympus AC 240 TS, f: 61,1-83,1kHz, C: 1,2-3,0N/m

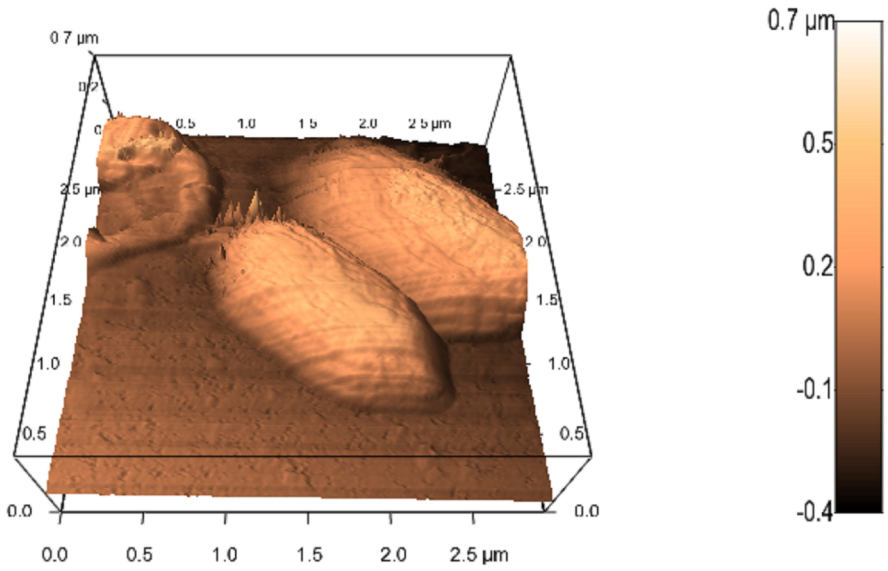


Fig. 6.2: *B. subtilis* spores (UV resistant) on a Poly-L-Lysine slide

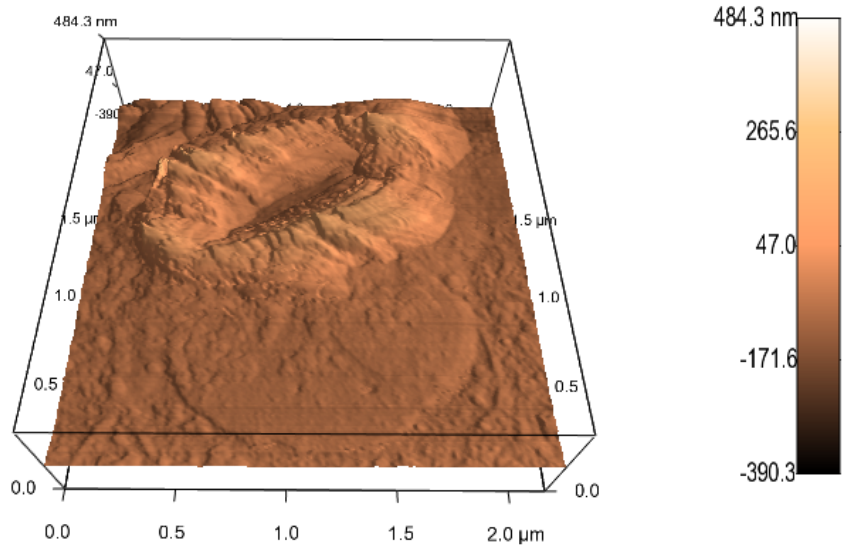


Fig. 6.3: Damaged *B. subtilis* spore (non UV resistant) which is surrounded by its intracellular liquid

Only a few problems with artefacts occurred at the edges of the spores, which are caused by the remaining nutrient solution and the additives for mechanical fixation of the sample on the substrate. This kind of artefacts could be seen more often in the images of vegetative *B. subtilis*, because of their flagella where the parasitic substances can accumulate much better than on the surface of the spores. Fig. 6.1 and 6.2 show height images of *B. subtilis* spores.

The substrates in these images were glass slides and poly-L-lysine slides and it is obvious that the smoothness of the used substrates is adequate for our measurements. Another aspect referring to the sample preparation is visualized in Fig. 6.3 where you can see a spore damaged during the preparation process, surrounded by its intracellular liquid.

7 Viscoelastic Properties of the Spore Surface

A rather theoretical view on the use of the phase image technique for gathering information about the viscoelastic properties of a certain sample surface has already been given in a

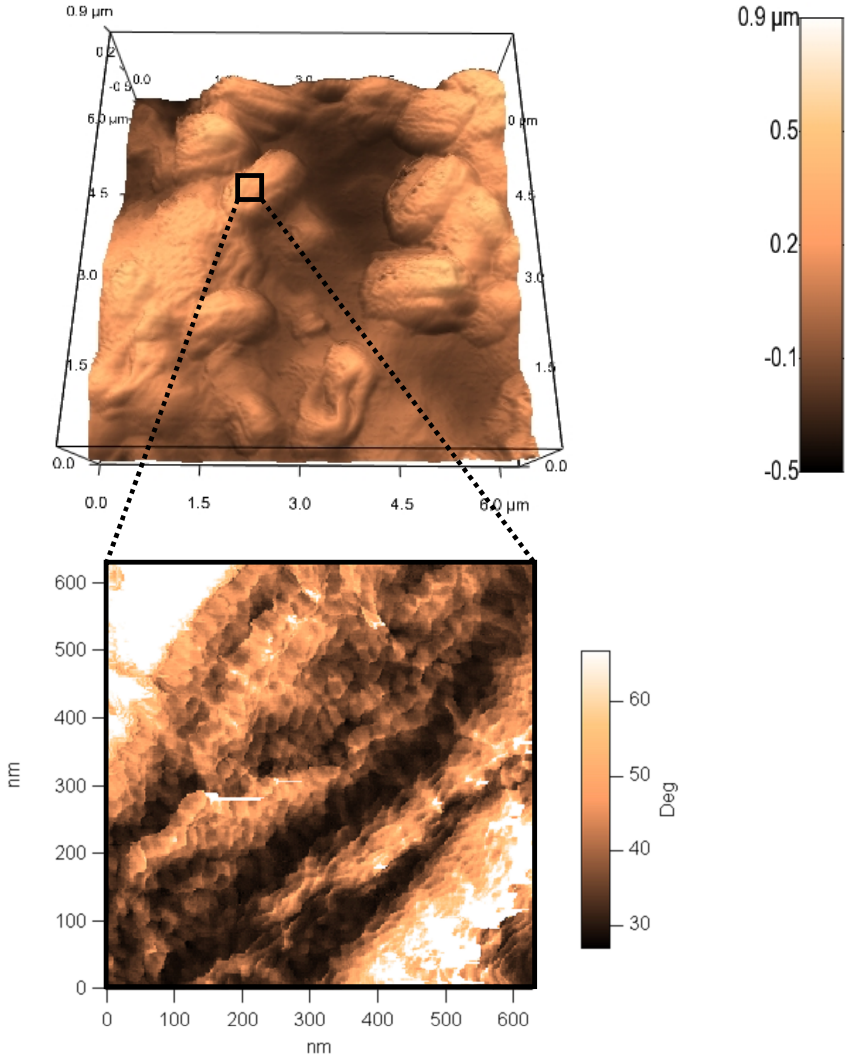


Fig. 7.1: Phase image of a small part on a UV resistant *B. subtilis* spore surface

previous chapter. Now we want to have a closer look on the viscoelastic properties of the *B. subtilis* surface.

Before we do this, it has to be mentioned that there are two different types of *B. subtilis* spores. On the one hand there are spores that are relatively resistant against irradiation with ultraviolet radiation (UV) at 253,7nm and on the other hand one can find spores that are much less resistant to UV irradiation. It depends on the growth medium used for growing a specific *B. subtilis* culture which type of spore is formed. This medium can be either liquid or

solid where ferric salts are used in the liquid growing medium, which results in rather UV sensitive spores.

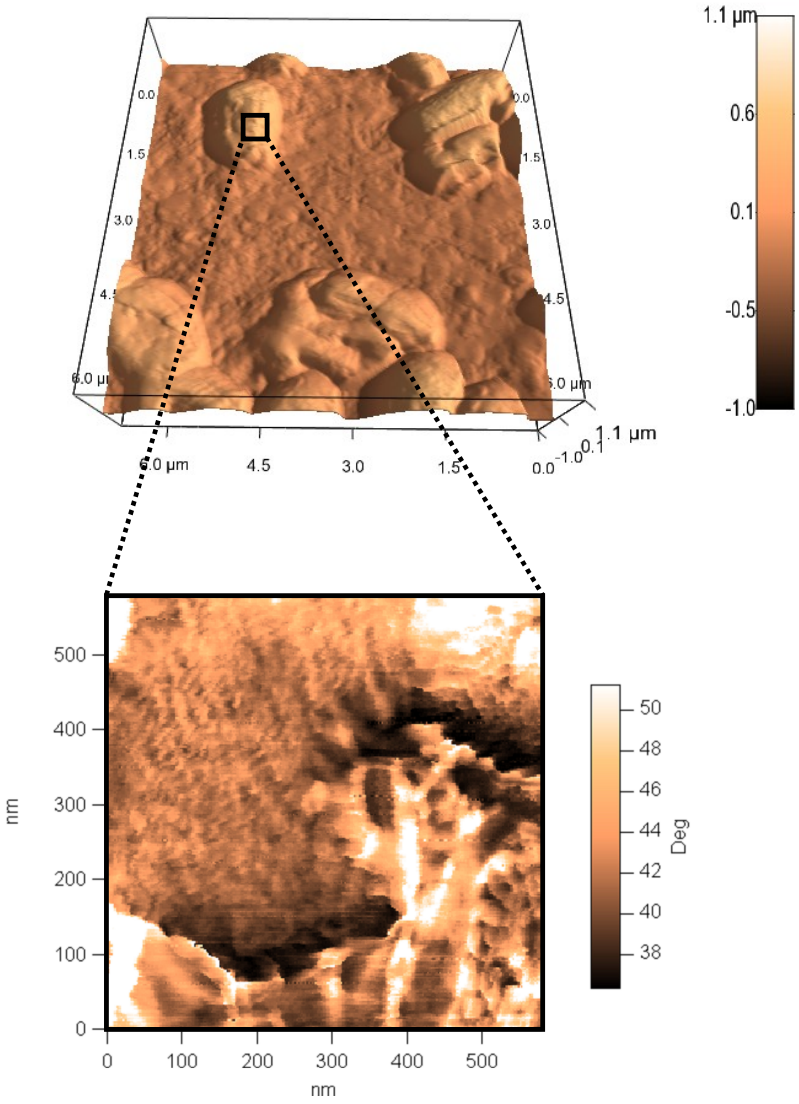


Fig. 7.2: Phase image of a small part out of a non UV resistant *B. subtilis* spore surface

Since the *B. subtilis* spores are used as a model system for monitoring the efficiency of radiant sterilization systems for drinking water it is important to have a good knowledge about their UV resistance. The viscoelastic properties of the spore coating can therefore provide some information about the structure of the coating and, which would be relevant for the UV resistance, about the thickness of the coating. Furthermore they could reveal indications about different chemical components in the coating of the two spore types, which would result in other viscoelastic values. The usually analyzed height or amplitude images could not reveal differences between the two spore types. Both have the same shape and dimensions. But the phase image technique could give us some additional information about the structure of the coating.

As can be seen in Fig. 5.6 a nanoscopic area of the *B. subtilis* spore surface was analyzed in tapping mode. With the acquired phase images we obtained the viscoelastic map of the surface region investigated. This procedure was performed for several spores of the two types with the same force set point for the cantilever tip.

The result was that substructures can be seen in the phase images of the spore surface, which are not visible in the according height and amplitude images. Fig. 7.1 and Fig. 7.2 show the phase images for the UV resistant and non UV resistant spore and Fig. 7.3 additionally shows the according height image for the latter. These first measurements on

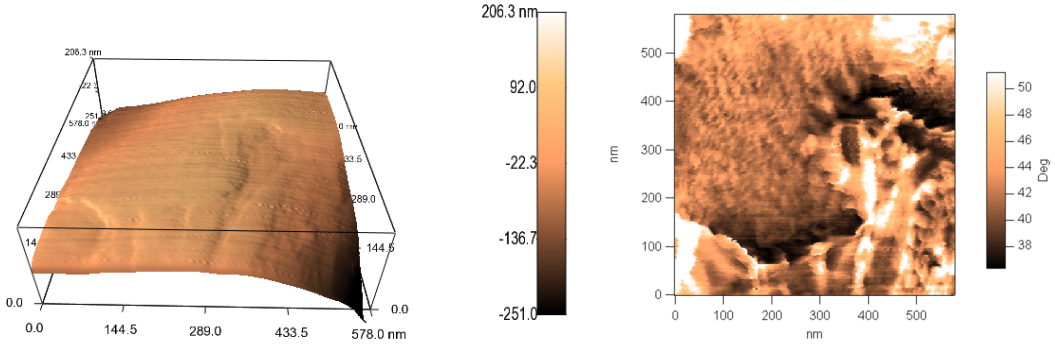


Fig. 7.3: Height image (left) and phase image (right) of a non UV resistant *B. subtilis* spore surface according to Fig. 7.2

the viscoelastic properties of the spore coating are already promising. Therefore it is reasonable to do further measurements e.g. with the Force Mapping Mode of the AFM [7] to obtain also quantitative information, which can't be provided by the phase imaging technique.

8 Imaging of vegetative *B. subtilis*

At the imaging of vegetative *B. subtilis* as well as at imaging its spores, it is necessary for achieving lifelike images with marginal damages of the *B. subtilis* surface, to work with the AFM in Tapping Mode. Best imaging results we achieved with 70kHz cantilevers[4]. The prepared samples have been analyzed at different positions with an investigation area of 20x20µm². The parts of interest were then magnified.

In some literature and also according to Dr. R. Sommer from the Hygiene Institut of the Medical University Vienna, a specialist in causa Bacilli, *B. subtilis* should not appear in chains. On the other hand there exists also some literature where the *B. subtilis* is described to build chains. In our investigations, nearly every time we measured *B. subtilis*, we found them in chains of about two to five cells. Fig. 8.1 shows such a series of four bacilli.

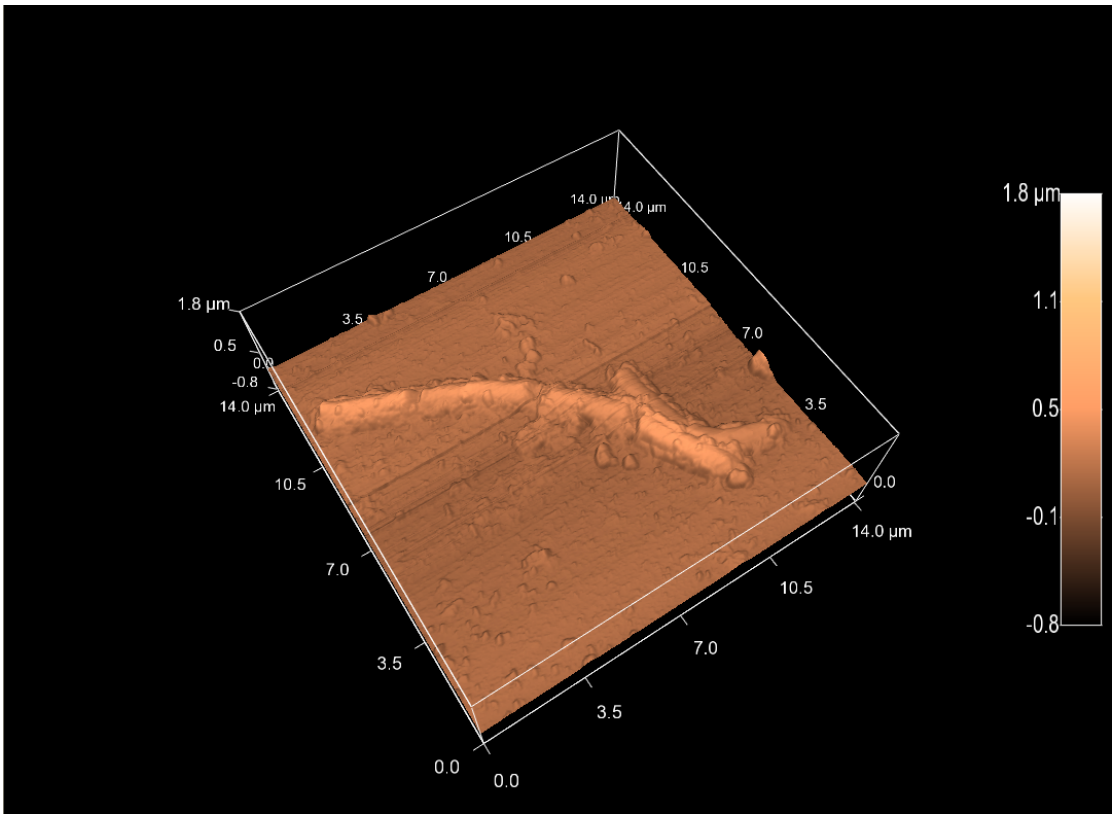
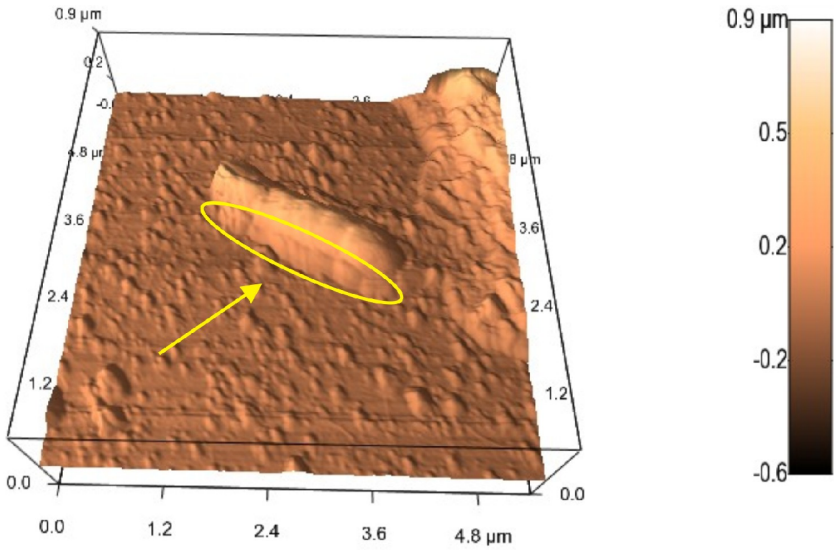


Fig. 8.1: Chain of vegetative *B. subtilis*

[4] Cantilever type: Olympus AC 240 TS, f: 61,1-83,1kHz, C: 1,2-3,0N/m

The feature at the boundary of *B. subtilis* are apparently ingredients of the nutrient solution, in which *B. subtilis* were collected. These particles, like some crystalline sugar, are attached at the *B. subtilis*' flagella (see Fig. 8.2).

Fig. 8.2: Particles of the nutrient solution attached to the bacillus' flagella



We could record many images, where the living *B. subtilis* already had discharged their flagella. This is known to be one of the first acts before *B. subtilis* is initiating the mechanism of sporulation. Fig. 8.3 shows several flagella on a poly-L-lysine glass slide.

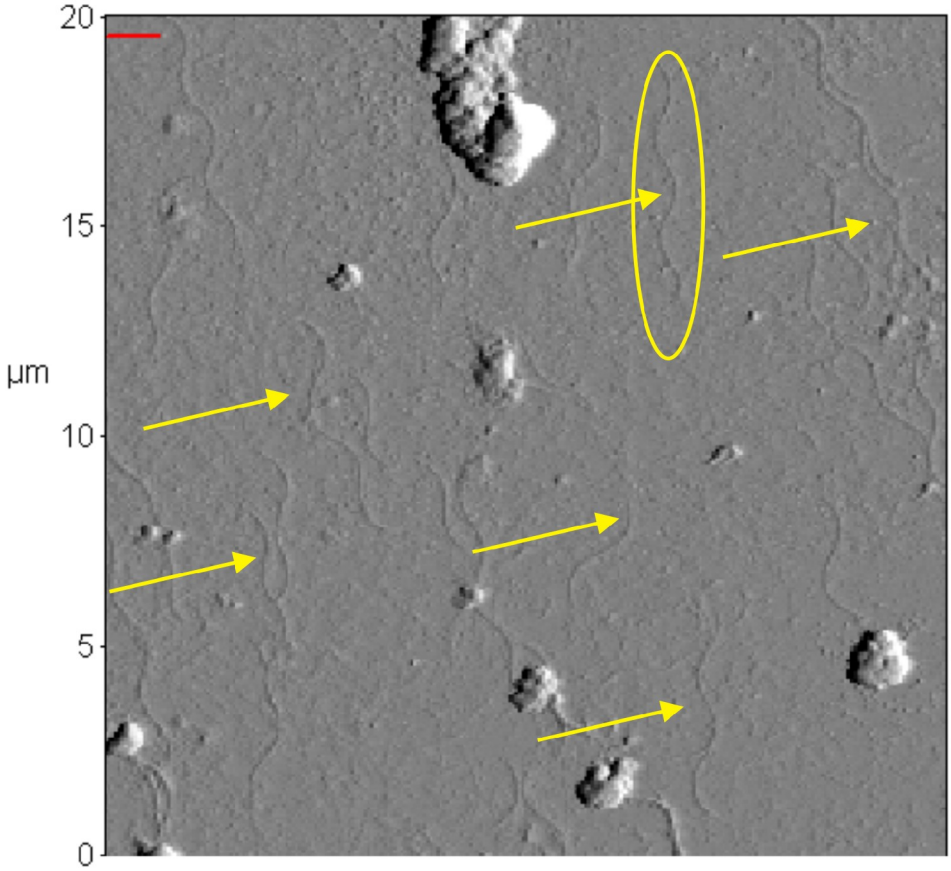
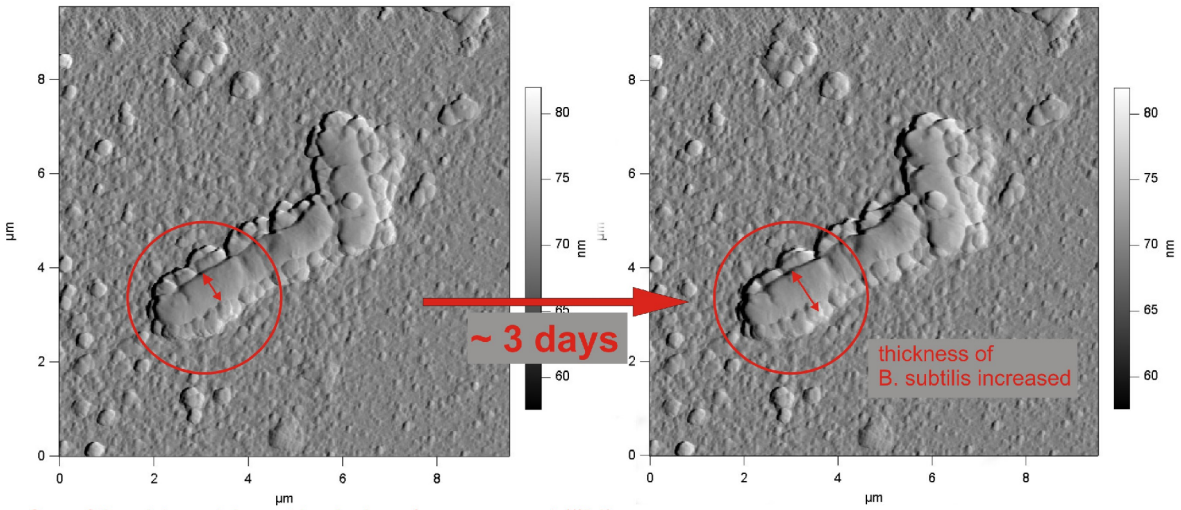


Fig. 8.3: Some flagella of *B. subtilis* on poly-L-lysine glass slides

9 Realtime Morphogenesis

As described in previous chapters the AFM provides the ability to perform measurements on living cells. But only a few pictures of different *B. subtilis* cannot give enough information about the mechanism of sporulation. Thus we performed a long term experiment with several live *B. subtilis* cells in an area of about ten times ten squaremicrometers. Sporulation, as already described above, lasts several days, so we performed the measurements for three and for six days and acquired an image every fifteen minutes, to get a video of the sporulation mechanism.



One of the pictures taken at beginning of measurement (#24)

One of the final pictures (#269)

Image # 24 (height profile - marked with the arrow)

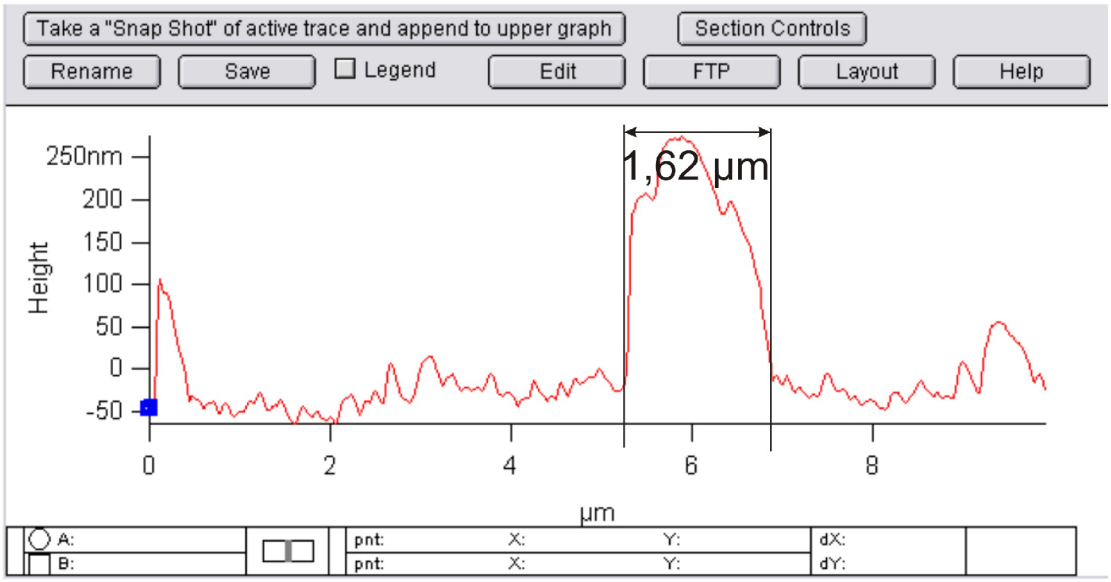


Image # 269 (height profile - marked with the arrow)

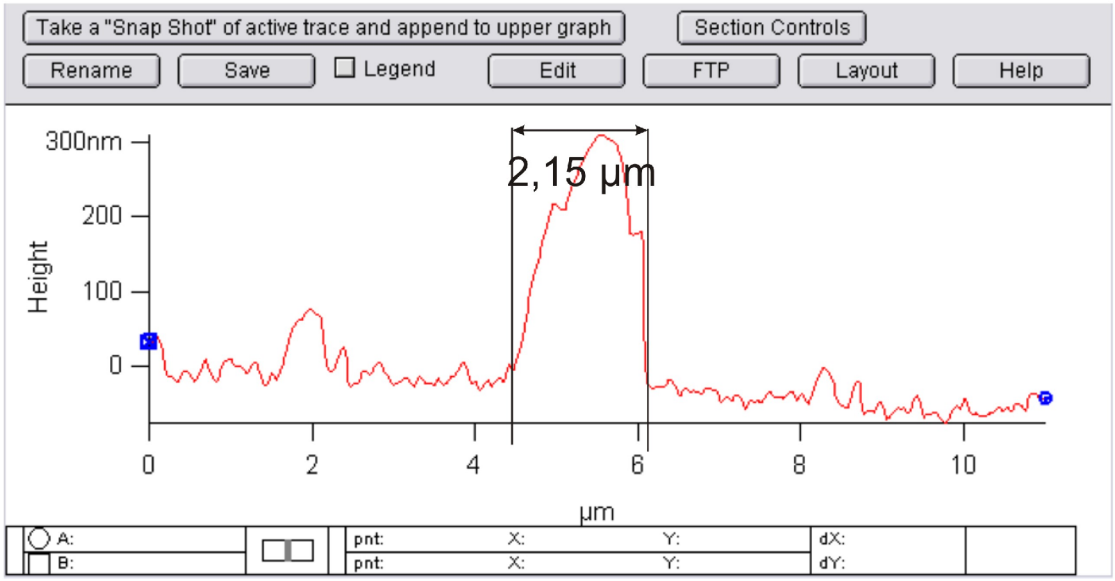


Fig. 9.1: „Real-time“ measurements of the sporulation of *B. subtilis*

Fig. 9.1 shows one of the first (left) and one of the last (right) pictures of a chain of *B. subtilis* cells. At first sight there is no difference visible, but if the two pictures are superimposed – as in Fig. 9. 2 it becomes noticeable that the thickness of the *B. subtilis* increased. The real "growing" of the *B. subtilis* becomes visible by creating a video file or an animated *.gif of the pictures (the CD attached to this project work contains a plentitude of images and also the video files of the sporulation).

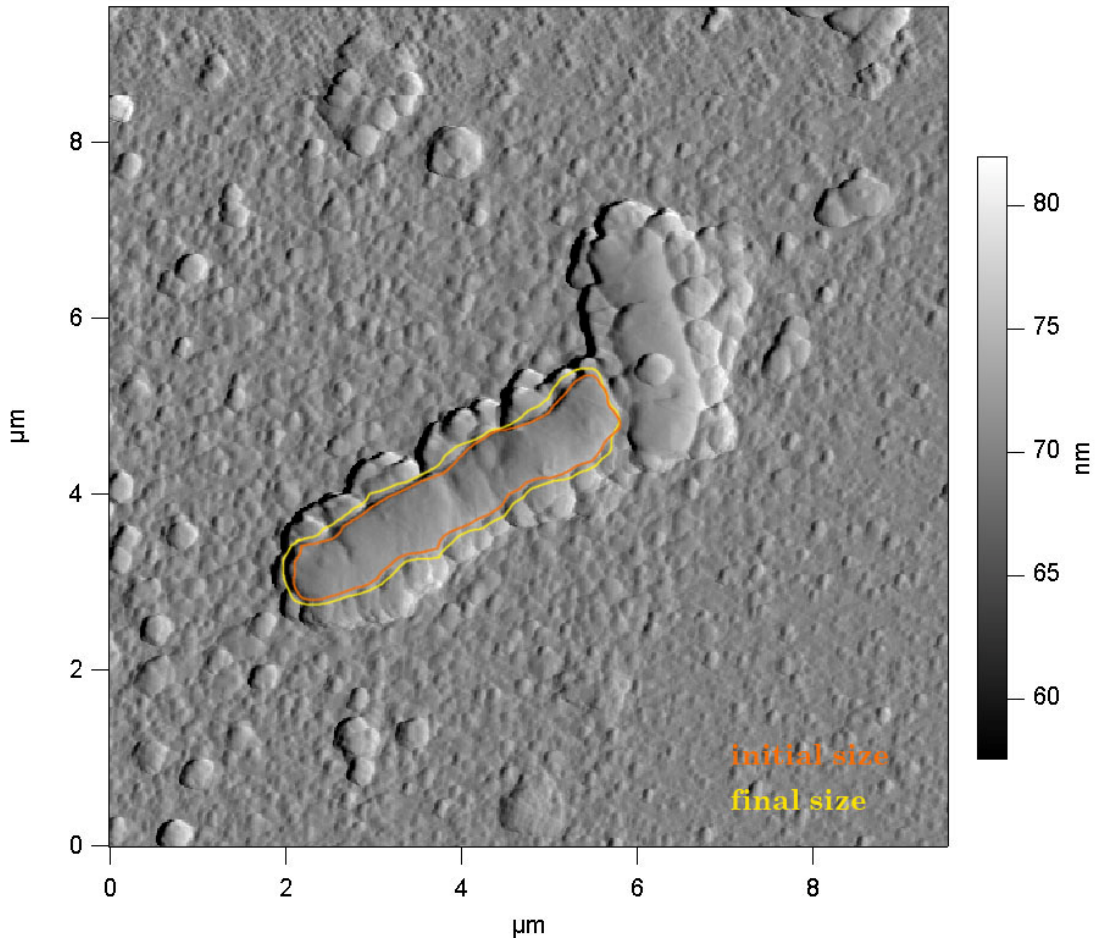


Fig. 9.2: The initial image of the *B. subtilis* chain – the original size in orange and the shape after growing in yellow

One problem we found working on these live images was that the deflection signal (see above) drifted, according to external effects such as the room temperature, up and down. And with the deflection signal also the whole picture "moved" a little bit up and down. By building up the movie we had to carefully position the *B. subtilis* on top of each other. By doing this, we lost some information on the edges of the scanning area.

Another problem of performing measurements on living cells is that one actually does not know whether the organism is still alive or not. As recently has come to our notice, there exists a viability marker for *B. subtilis*. This would reduce the risk of measuring dead bacteria. At our second long-time measurement (about one week) we had bad luck and apparently selected a string of dead *B. subtilis*. We couldn't measure sporulation, but the thickness of the string slightly increased and decreased again (Fig. 9.3, also note the attached movie).

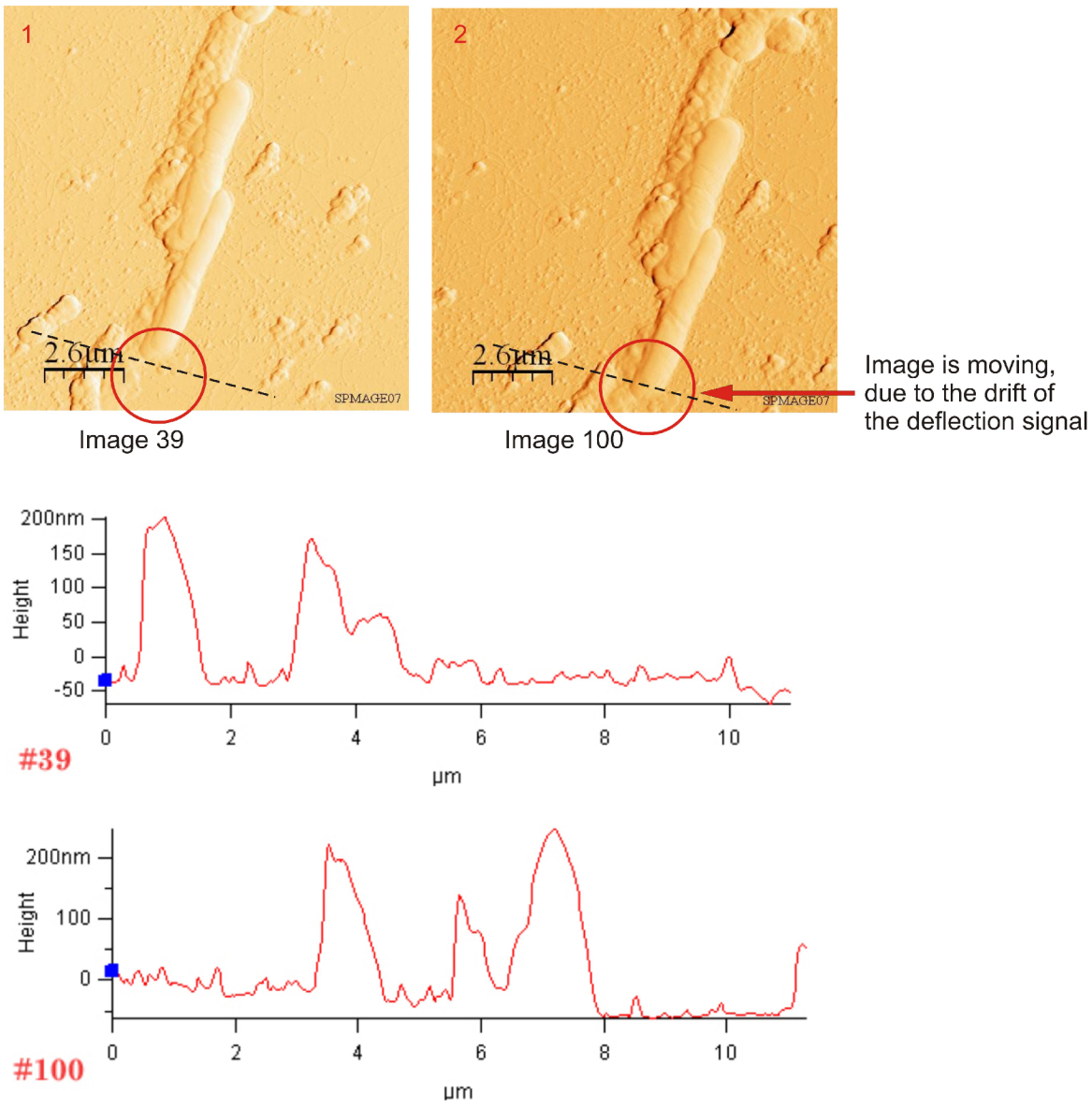


Fig. 9. 3a: "Real-time" measurement of a B. subtilis chain for 6 days

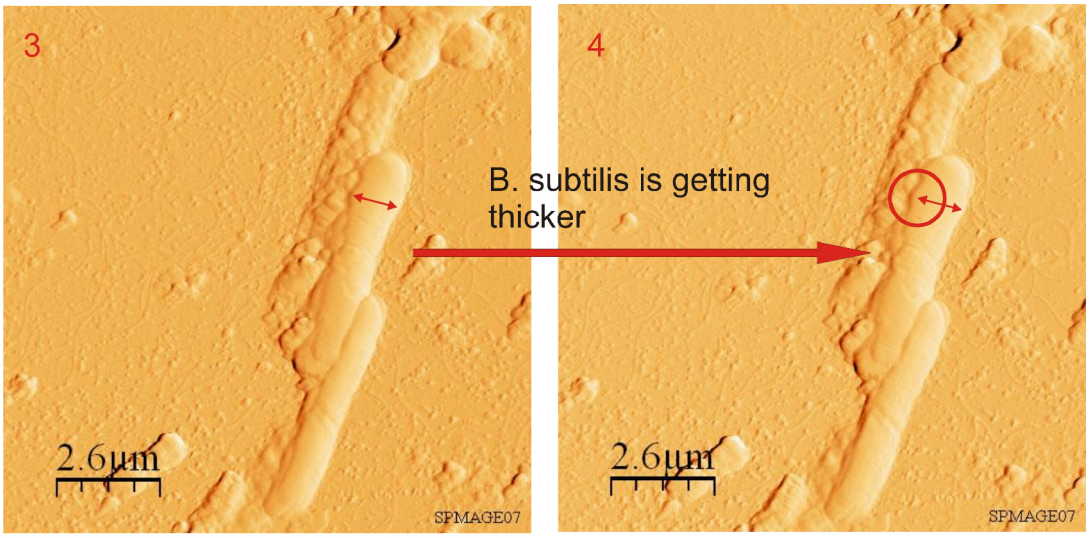
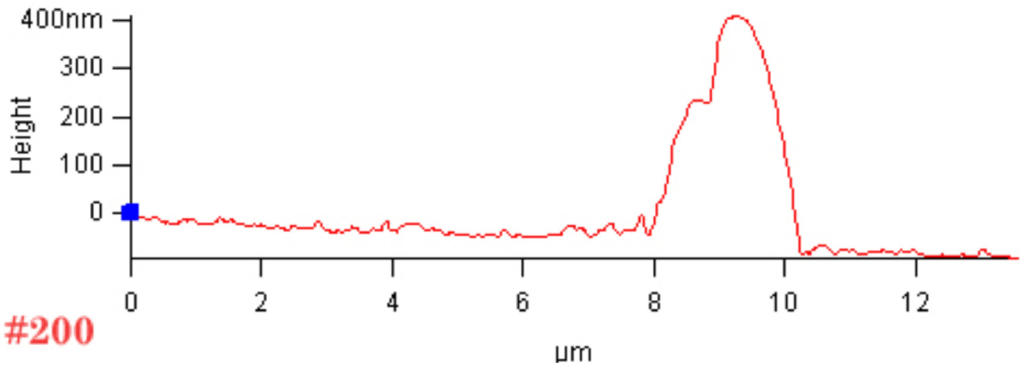


Image 200 Image 300



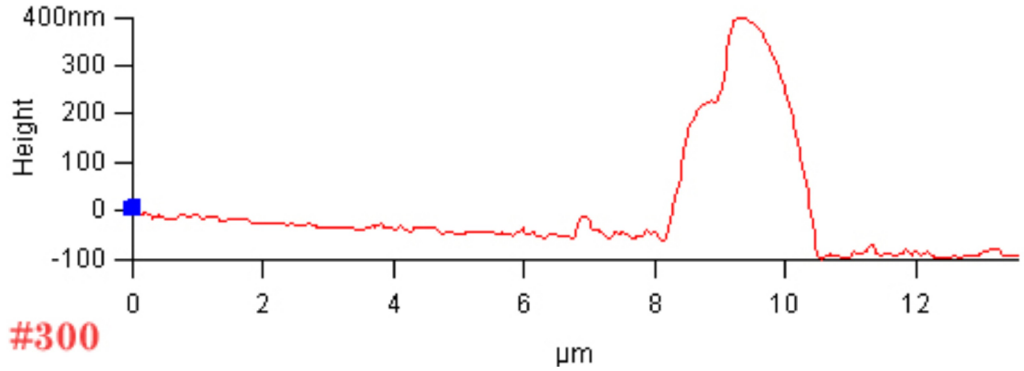


Fig. 9.3b: „Real-time“ measurement of a *B. subtilis* chain for 6 days

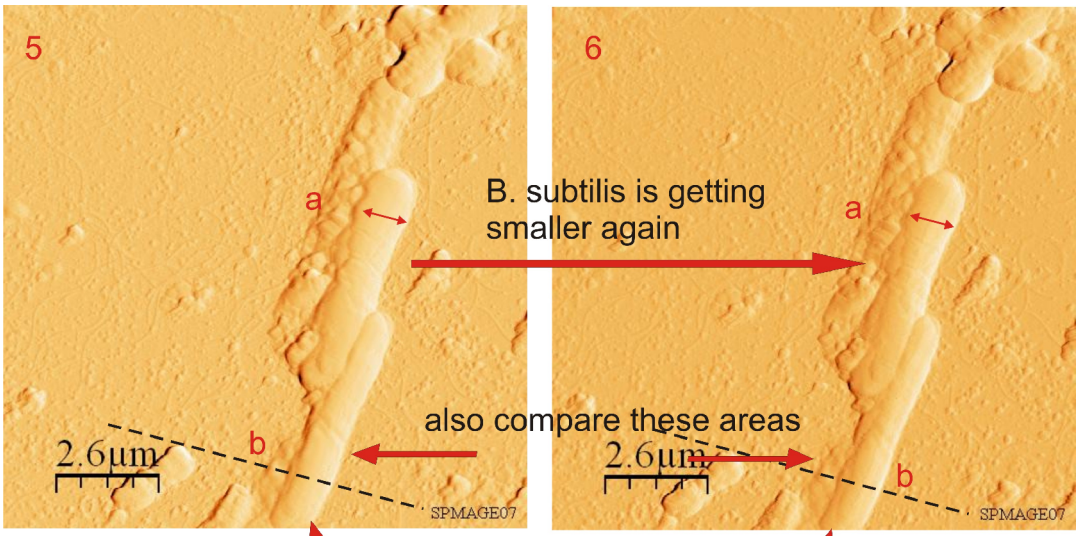
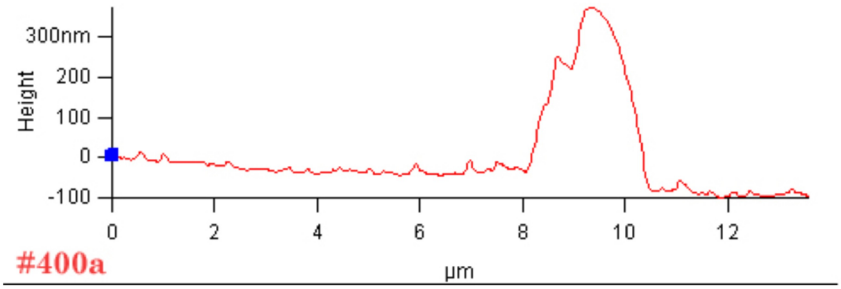


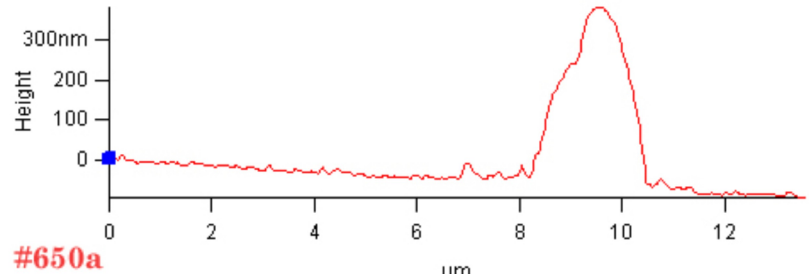
Image 400

Image 650

deflection drift
is smaller than
from 1 to 2



#400a



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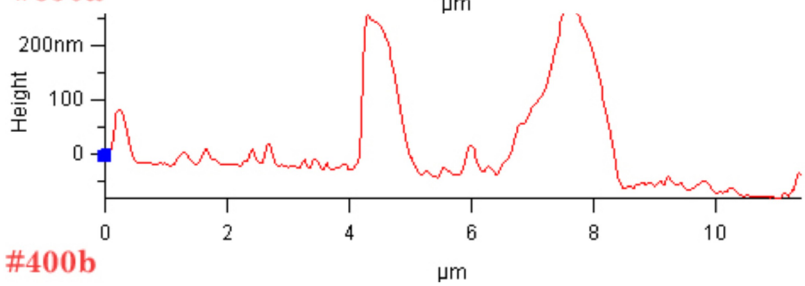

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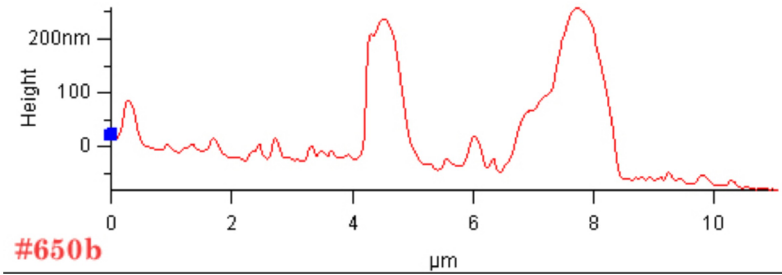

#650b

10 Discussion and Outlook

It was demonstrated that real time imaging of living organisms using the Atomic Force Microscope can yield valuable information on biologic processes. In this project work the mechanism of sporulation of the *B. subtilis* was observed. It was possible to measure a thickening of the bacillus as shown in previous chapters. The method of doing real time measurement on living cells is a promising way to explore the diversity of so far invisible processes in micro- and nanobiology, and it holds a huge potential for researches in medicine.

Regarding to *B. subtilis* the AFM brought an idea of how sporulation works. In future it will be investigated how some specific *B. subtilis* spores become more UV resistant than others by doing force measurements on the coating of the spores by means of the Force Mapping technique of the Atomic Force Microscope [7]. It shall be shown how UV resistance and elasticity of the bacillus' coat correlate.

11 Acknowledgements

We would like to thank Dr. Ille Gebeshuber for giving us the chance to work with the AFM and for the guidance through this project.

Many thanks to Dr. Regina Sommer (Hygienic Institute, Medical University of Vienna) for providing us with *B. subtilis*, her knowledge on bacilli and her helpful advice.

Finally we want to thank (our fellow student) Oliver Hekele, whose experience in the field of AFM analysis was a great support for this project work. furthermore we would like to thank him for proofreading it.

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