High-Resolution Imaging of Dried and Living Single Bacterial Cell Surfaces: Artifact or Not?

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Introduction

When studying highly resolved scanning electron microscope images of cell surfaces, the question arises, whether the observed patterns are real or just artifacts of the cell preparation process. The following steps are usually necessary for preparation: fixation, drying, and metal coating. Each step might introduce different artifacts. Clever techniques have been developed to dry cells as gently as possible, for example critical point drying with different organic solvents and CO_2. Instrument manufacturers also have taken account of this issue, for example, through the realization of the environmental scanning electron microscope (ESEM), operating with a low-vacuum environment saturated with water so that samples might stay hydrated. Another approach is the extreme high-resolution scanning electron microscope (XHR SEM), where the electron beam is decelerated shortly before reaching the sample. This technique requires no metal coating of the sample. Cryo-SEM also may be used, where no sample preparation is required beyond freezing in a high-pressure freezer or other cryo-fixation device. Then the cell can be examined in the frozen, hydrated state using a cryostage. However, at least some kind of preparation is necessary for SEM imaging, and we wanted to find out what changes the preparation makes on the cell surface.

Methods and Materials

To elucidate the different possible artifacts of each sample preparation step, complementary techniques were used to image the surface of the model bacterium *Sinorhizobium meliloti* [1]. *S. meliloti* is a gram negative soil bacterium of 0.5 to 3 µm in length, which lives in symbiosis with certain host plants, helping them to reduce and fix nitrogen.

In our study, the following scanning electron microscopy techniques were used: SEM and XHR SEM. They were complemented by atomic force microscopy (AFM) to image dried cells under ambient conditions, as well as viable cells in liquid. This means that conditions ranged from living bacteria in liquids to fixed bacteria in high vacuum. Each step of cell preparation was investigated (fixation, drying, metal coating) by imaging the resulting cell surface patterns. We found that the observed wrinkled protrusions in SEM images were not generated *de novo* but evolved from similar and naturally present structures on the surface of living bacteria [1]. Detailed analysis of AFM images of living bacteria exhibited surface structures of the size of single proteins, emphasizing the usefulness of AFM for high-resolution cell imaging.

Results

Conventional SEM images of *S. meliloti* showed prominent wave-like surface patterns when the cells were fixed, dried, and metal-coated with a 12 nm gold layer in vacuum (see Figure 1).
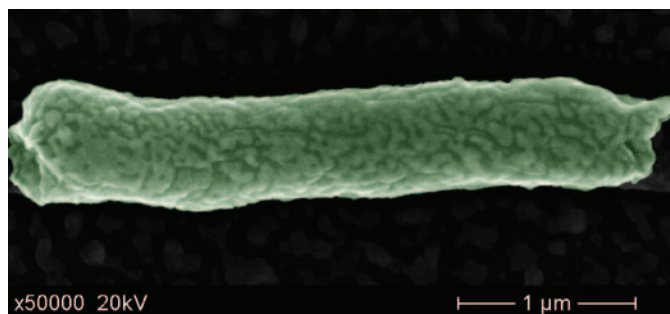

Figure 1: SEM image of fixed, dried, and metal-coated *S. meliloti*. The bacterial surface is decorated with wrinkled protrusions.

The observed wavy structures had a width of 29 ± 12 nm and a length of approximately 92 ± 31 nm (mean ± SD). To figure out if these patterns were caused by metal coating, uncoated bacteria were examined with XHR SEM (fixed, dried, in vacuum). In Figure 2 elongated surface protrusions can be seen with a width of 18 ± 5 nm and a length of 80 ± 27 nm. Additionally, the XHR SEM images allowed the detection of distinct round protrusions with a diameter of 21 ± 4 nm. The interpretation of these images is not trivial because structures several nanometers below the surface might also be visible, producing fainter contrast on the surface.
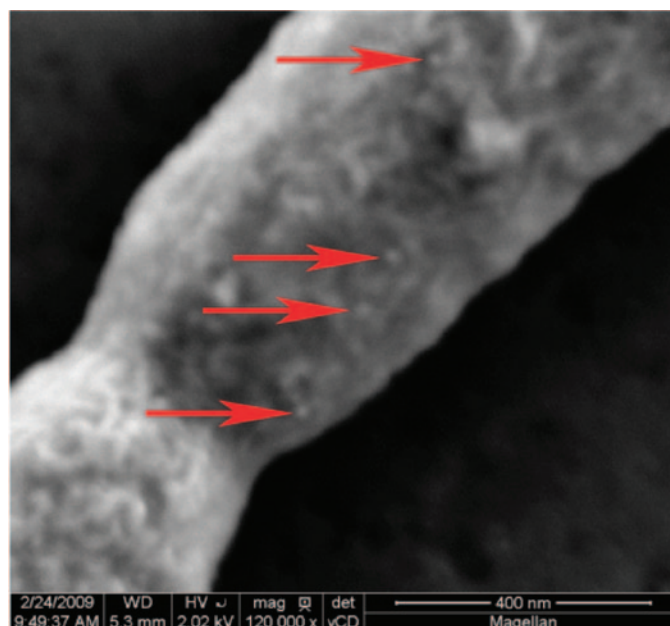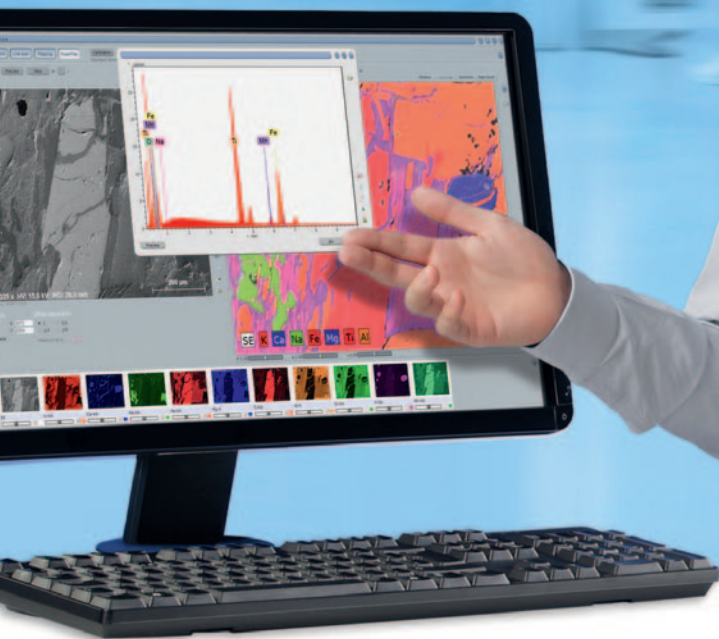

Figure 2: XHR SEM image of *S. meliloti* fixed, dried, and imaged in vacuum without metal coating. Elongated wrinkles cover most of the bacterium. In addition, several single dots (some examples marked by arrows) are distributed over the surface.
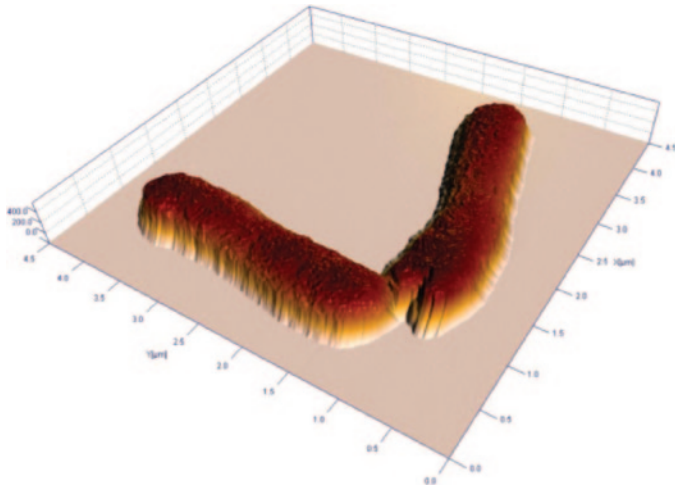
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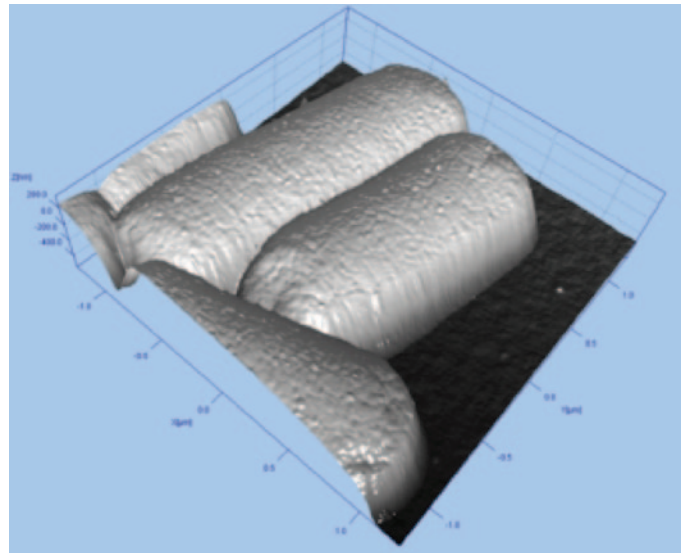
Figure 3: AFM 3-D height image of the *S. meliloti* wild-type-strain Rm2011, fixed, dried, and imaged in air. The whole bacterial surface is covered with protrusions that show an elongated and wrinkled structure closely connected. Scan size = 4.5 µm × 4.5 µm.


Figure 4: AFM 3-D images of the topography of the *S. meliloti* wild-type-strain Rm2011, fixed and imaged in PBS buffer. The bacterial surface is completely decorated with round protrusions. Scan size = 2.5 µm × 2.5 µm.

Switching from SEM to AFM, similar surface patterns became visible using AFM under ambient conditions. In this nanomechanical probing technique, a tiny tip scans the topology of the surface. This provides surface imaging of fixed and dried bacterial cells without metal coating or vacuum. In the AFM image shown in Figure 3, we found that the bacterial surface is completely decorated with a dense network of surface protrusions (12.1 ± 4.9 nm heights, 26 ± 6 nm lateral diameters and 72 ± 28 nm lengths).

A quantitative comparison of the observed patterns from XHR SEM and AFM is difficult because SEM suffers from beam penetration and interaction volume effects, and AFM can suffer from tip-convolution effects. However, the estimated values determined by AFM are in good agreement with the XHR SEM data. So the AFM results confirm that the structures are actually present on the cell surface and not part of sub-surface structures.

In order to evaluate possible drying artifacts, AFM images were taken of fixed *S. meliloti* bacteria in PBS buffer (see Figure 4). Under these conditions the protrusions appear mostly round-shaped with a height of 4.7 ± 2.7 nm and a lateral diameter of 33 ± 8 nm.

Next, the fixation was omitted to analyze living bacteria. The bacteria exhibited protrusions of a height of 3.9 ± 1.7 nm and a width of 28 ± 6 nm (see Figures 5 and 6). The dimensions are similar to chemically fixed bacteria. In consequence, the observed structures are not artifacts caused by the fixation

procedure but naturally occurring features on the outer bacterial envelope.

Discussion

No obvious change in surface morphology could be observed comparing bacteria imaged with AFM in liquid with and without fixation (Figures 4 and 5). Drying, however, changes the morphology, although no patterns are generated *de novo*. On dried bacteria, the observed patterns are more wave-like instead of round protrusions found on cell surfaces imaged by AFM in liquid (see AFM in air, Figure 3; XHR SEM, Figure 2; and SEM Figure 1). There are several possible mechanisms for wrinkle formation. One possibility is the wrinkling of the outer membrane during drying. A second possibility is the formation of elongated wrinkles by the
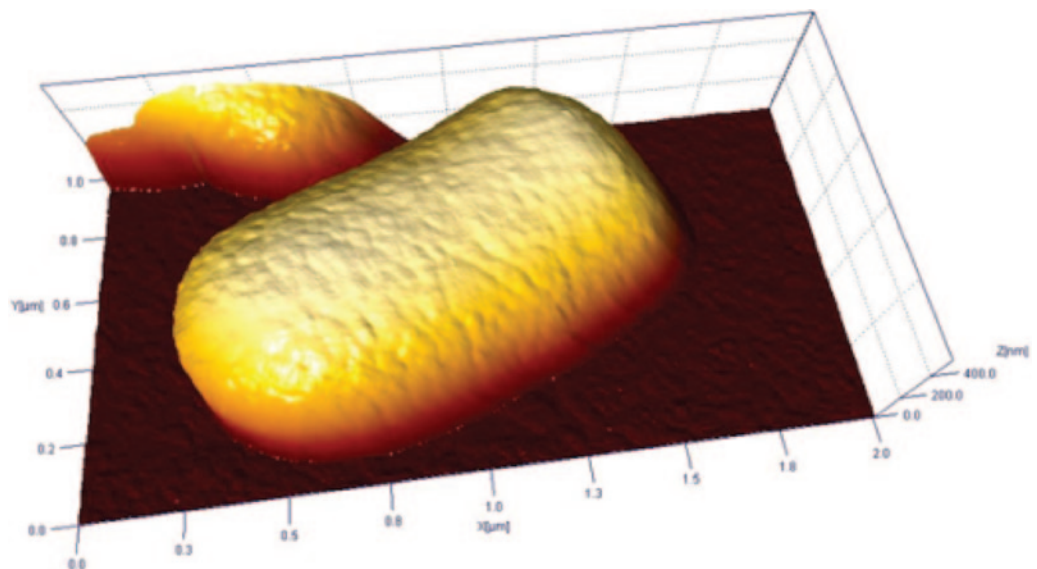
Figure 5: AFM 3-D height image of the *S. meliloti* wild-type-strain Rm2011, living bacterium in culture medium. Protrusions can be recognized, positioned closely together on the bacterial surface. Scan size = 1 µm × 2 µm.
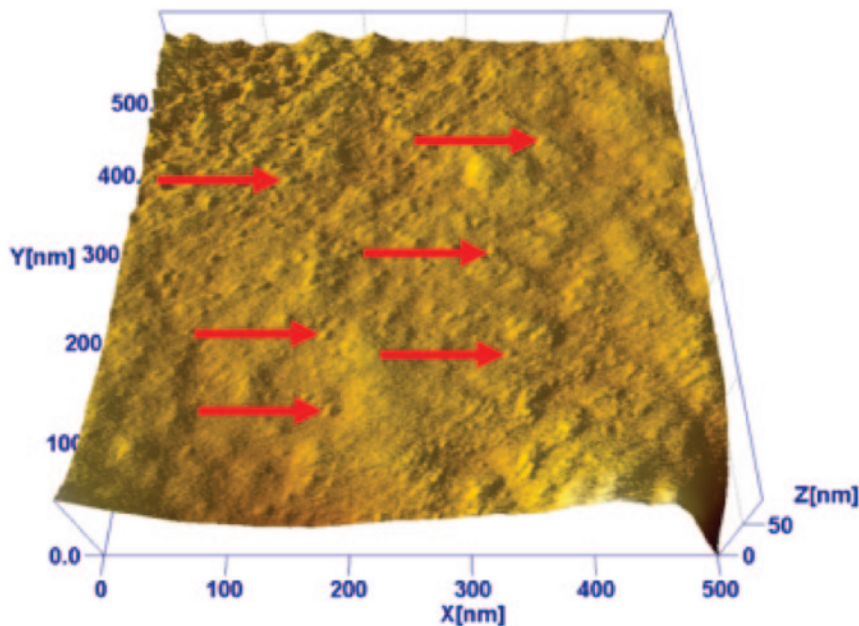
Figure 6: High-resolution AFM 3-D height image of the *S. meliloti* wild-type strain, living bacteria imaged in culture medium (data 2. order flattened). Small protrusions (some examples marked by arrows) on the surface might represent the extracellular part of single proteins (for example, outer membrane proteins [Omp]).

merging of round protrusions. These could fuse because of the mechanical stress generated during the drying process, especially considering the similarity of the diameters of the round protrusions (28 ± 6 nm, Figures 5 and 6) in comparison to the width of the elongated patterns (26 ± 6 nm, Figure 3).

It is also interesting to compare SEM and XHR SEM images (see Figures 1 and 2). In the XHR SEM images, smaller structures can be resolved than in conventional SEM images. In SEM images, small protrusions might be covered by the metal layer and could appear as broader protrusion.

An important methodical improvement is the use of hybrid AFM cantilevers used in liquids consisting of a silicon nitride lever and a silicon tip. Usually silicon nitride cantilevers are used in liquid because of the lower spring constant, but they have inferior tip radii and aspect ratios. Hybrid cantilevers overcome these limitations with steeper side walls of the tip, offering superior resolution comparable to AFM images taken under ambient conditions. Another method improvement is the functionalization of the glass slides with PEI (polyethylenimine), a simple and less expensive variant compared to the often-used polylysine. PEI coating enables stable images of good resolution, which is sometimes difficult to achieve in aqueous media. Long-time observations for the collection of time lapse fluorescence imaging (TLFI) data of different *S. meliloti* strains immobilized with PEI in a microfluidic chip revealed a high viability of the cells, supported by doubling times close to the doubling time in liquid cultures [2].

Regarding the origin of the round protrusions found under most physiological conditions using AFM imaging in liquids, we speculate that one possibility is a pushing of proteins or complexes against the membrane from the inside. This might be possible, as it is known that large numbers of proteins can be found between the flexible inner and outer membrane with diameters of 6–10 nm. This might explain the increased height of the patterns on dried bacteria as the membranes collapse. Another possibility could be the formation of membrane vesicles and precursors of outer membrane budding [3, 4]. A third option is that lipopolysaccharides (LPS) may decorate the outer membrane [3, 5]. These LPS molecules are 6–45 nm long and flexible, so that the AFM tip in liquid could easily push them aside. This implies that only the core region of the LPS would be imaged with dimensions comparable to the height observed in AFM images (4–6 nm [3, 5]).

Closer inspection of the AFM images taken in liquid revealed small dots present on the bacterial surface (height 2.0 ± 0.6 nm, diameter 12 ± 3 nm, see Figure 6). Recognizing the small size and tip-convolution effects, these might be proteins associated with the outer membrane. These proteins have typical diameters of 1.4–4 nm [6] and a height of about 1 nm [7], which is in good agreement with the reported heights.

Conclusion

Complementary techniques ranging from SEM in vacuum to AFM in liquids were used to determine whether cell preparation procedures change the observed surface patterns. The conditions included fixed, dried, and metal-coated cells in vacuum to live cells in liquid. For living cells, round surface protrusions were observed on the native, unchanged cell surface. Drying led to the generation of elongated wrinkles, and it is argued that these may evolve from the former round protrusions.

Acknowledgments

References

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