Ultramicroscopy 110 (2010) 1290–1296

Contents lists available at ScienceDirect

Ultramicroscopy

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

High resolution imaging of surface patterns of single bacterial cells

Dominik Greif [1], Daniel Wesner [1], Jan Regtmeier *, Dario Anselmetti

Experimental Biophysics and Applied Nanoscience, Bielefeld University, Universitätsstraße 25, 33615 Bielefeld, Germany

ARTICLE INFO

Article history:
Received 13 October 2009
Received in revised form
26 April 2010
Accepted 1 June 2010

Keywords:
AFM
SEM
Artefact
Cell surface
Life cell imaging
Sample preparation

ABSTRACT

We systematically studied the origin of surface patterns observed on single *Sinorhizobium meliloti* bacterial cells by comparing the complementary techniques atomic force microscopy (AFM) and scanning electron microscopy (SEM). Conditions ranged from living bacteria in liquid to fixed bacteria in high vacuum. Stepwise, we applied different sample modifications (fixation, drying, metal coating, etc.) and characterized the observed surface patterns. A detailed analysis revealed that the surface structure with wrinkled protrusions in SEM images were not generated *de novo* but most likely evolved from similar and naturally present structures on the surface of living bacteria. The influence of osmotic stress to the surface structure of living cells was evaluated and also the contribution of exopolysaccharide and lipopolysaccharide (LPS) by imaging two mutant strains of the bacterium under native conditions. AFM images of living bacteria in culture medium exhibited surface structures of the size of single proteins emphasizing the usefulness of AFM for high resolution cell imaging.

1. Introduction

In order to gain structural information concerning the bacterial envelope, electron microscopy methods were mostly used. For example, scanning electron microscopy (SEM) can visualise topographic patterns of the bacterial surface, the freeze-fracture technique allows to image replicas of the membrane and its different layers [1] and transmission electron microscopy (TEM) images sections of the envelope [2]. Since samples for conventional SEM are examined in vacuum and often have to be fixed, dried and metal-coated [3], it is necessary to be aware of artefacts that might be introduced by sample preparation. Especially during the drying process, it is crucial to preserve natural structures [4], which can be altered by high surface tension occurring during water evaporation. To avoid these alterations, the medium is exchanged for organic solvents like isopropanol or acetone from which samples can be dried in air or by using critical point drying (CPD).

New developments in electron microscopy opened possibilities to image samples without some of the above mentioned preparative steps: for example, environmental scanning electron microscopy (ESEM) operates in low vacuum saturated with water so that samples might stay hydrated; using extreme high resolution scanning electron microscopy (XHR SEM) the electron beam can be decelerated shortly before reaching the sample to approximately 50–2000 V. This allows imaging of the sample to a certain depth depending on the beam energy. A great advantage of these techniques is that no conductive coating is necessary anymore.

Atomic force microscopy (AFM) offers a similar lateral resolution compared to SEM and additionally gives very precise topological information over distances of several micrometers resulting in 3D topographic images [5]. Since this technique works in air as well as in liquid media AFM is well suited to analyse surface structures of biological material under native conditions [6] and living bacteria [7]. Thus, by using the latter microscopy method, different steps in sample preparation like fixation or drying can be omitted and the consequences regarding surface morphology can be compared.

We have chosen *S. meliloti* as model bacterium to investigate the surface morphology of gram-negative alpha-proteobacteria. The typical length scale of this rod-like bacterium ranges from 0.5 to 3 μm. *S. meliloti* is a symbiotic soil bacterium that is able to induce the formation of nodules on the roots of its leguminous host plants. These specialized plant organs are invaded by *S. meliloti* which can reduce and fix atmospheric nitrogen while being in an endosymbiotic organelle-like bacteroid state within the plant cell [8]. This process involves specific recognition of both bacterial and host cells. Symbiotic bacteria contribute significantly to an ecologically-sound and cost-efficient nitrogen supply. Additionally the non-pathogenic rhizobia are closely related to the important plant, animal and human pathogens such as *Brucella, Burkholderia* and *Ralstonia* allowing transfer of knowledge to these medically relevant bacteria [9]. *S. meliloti* is one of the best studied rhizobia. Its genome was sequenced [10] and a

* Corresponding author. Tel.: +49 521 106 5388; fax: +49 521 106 2959.
E-mail address: jan.regtmeier@physik.uni-bielefeld.de (J. Regtmeier).
[1] Contributed equally.

0304-3991/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ultramic.2010.06.004

number of post genomic approaches have been established for this organism. Therefore a lot of mutants have been established and are available for investigations.

The bacterial surface possesses unique structures that distinguishes it from the eukaryotic cell membrane but also enables a classification between different bacteria. Gram-negative bacteria feature a three-layered structure with inner and outer membranes which enclose the periplasm containing one layer of peptidoglycan [11]. The outer membrane is decorated with exopolysaccharides and lipopolysaccharides (LPS) being important endotoxins. LPS consists of lipid A that anchors the molecule to the membrane followed by core and O-antigen structures both based on carbohydrates [12]. This O-antigen is important, e.g. for the symbiotic interaction of some *Rhizobium*-strains and legumes [13,14]. Additionally some bacteria, but not *S. meliloti*, produce stiff capsules consisting of exopolysaccharides.

In this study we analyse the surface of *S. meliloti* by using different high resolution scanning microscopic techniques, namely SEM, XHR SEM and AFM in the dry and liquid phases. We compare surface patterns observed by these complementary methods under diverse environmental and preparative conditions. This experimental approach enables us to track the changes of native surface patterns introduced by every single step in preparation, starting from the most artificial conditions for SEM (fixed, dried, metal coated and high vacuum (HV)) to only softly immobilized living bacteria for AFM in the liquid phase. In order to clarify the origin of the surface patterns observed in the latter case, the impact of the osmolarity and two mutants of the wild type strain are studied. One strain lacks the ability to produce exopolysaccharides [15], the other has a defect in the LPS [16], the most dominant surface molecule of *S. meliloti*. Additionally we report on small protrusions present on the bacterial surface under native conditions that might represent single proteins of the outer membrane.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains used in this study are listed in Table 1. *S. meliloti* strains, unless otherwise indicated, were grown at 28 °C in liquid cultures with Vincent minimal medium (VMM) [18] (1 mM $MgSO_4$, 250 μM $CaCl_2$, 10 mg/L $FeCl_3$, 1 mg/L biotin, 2 mM K_2HPO_4, 48.5 nM H_3BO_3, 10 nM $MnSO_4$, 1 nM $ZnSO_4$, 0.55 nM $NaMoO_4$, 0.27 nM $CoCl_2$, 10 g/L MOPS, 10 g/L mannitol, 3.55 g/L Na glutamate)

2.2. Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Germany) including phosphate buffered saline (PBS) with 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate, pH = 7.4. All solutions were prepared with deionised water from a Milli-Q biocel (Millipore, USA). Poly(ethyleneimine) (PEI) 50% (w/v) in H_2O ($M_n \sim 60000$, M_w 750000) was utilised. Paraformaldehyde was depolymerised in water at 60 °C and diluted in PBS before glutaraldehyde was added to the solution. Parafilm (Pechiney Plastic Packaging) was purchased from VWR (Germany). Microscope and glass slides were purchased from Menzel (Germany).

Table 1
Strains used in this study.

S. meliloti strains	Description	Reference or source
Rm2011	Wild-type strain	[17]
Rm0540	exoY$^-$	[15]
Rm6963	LPS defect ⇒ short LPS	[14]

2.3. SEM-imaging

For all experiments the glass was cleaned in an ultrasonic bath (Elma T 490DH), consecutively in acetone, ethanol and in MilliQ-H_2O for 20 s each and afterwards dried with nitrogen.

1 mL of the bacteria from the overnight cultures was washed twice with PBS buffer. Therefore the bacteria were centrifuged 5 min at 1500 rpm (Eppendorf centrifuge S417C), the supernatant was wasted and the pellet resuspended in 500 μL buffer. Washing was followed by fixation of the cells in 1.5% paraformaldehyde and 1.5% glutaraldehyde in PBS for 1 h at room temperature (RT). Afterwards the cells were washed again and resuspended in Milli-Q H_2O. For dehydration the water in the sample was slowly exchanged over at least 48 h against ethanol. Then after centrifugation (5 min at 1500 rpm) the ethanol was wasted and the pellet resuspended in 500 μL isopropanol. For drying 10 and 20 μL of each sample were dropped onto 8 by 8 mm^2 glass slides and dried in an isopropanol saturated atmosphere over at least 48 h at RT. For imaging the bacteria samples were sputtered with a 12 nm gold-layer (BAL TEC MED 020 coating system) and transferred into the scanning electron microscope (JEOL JSM-880).

2.4. XHR SEM imaging

For imaging with a XHR SEM (Magellan from FEI, Eindhoven, The Netherlands) the bacteria were treated as previously described for the SEM but without the slow drying procedure and metal coating.

2.5. AFM ambient imaging

AFM imaging in air was performed with a Multimode IIIa AFM (Veeco, USA) with a J-scanner operating in tapping mode. The silicon cantilevers Tap300Al (Budget Sensors, Bulgaria) with a nominal spring constant of 40 N/m were used. Samples were treated as described for SEM with the exception that no metal coating was applied.

2.6. AFM liquid phase imaging

For AFM imaging in liquid environment a MFP-3D (Asylum Research, USA) on an Olympus IX71 optical inverted microscope was used. Microbiolever AC40TS (Olympus, Japan), with a nominal spring constant of 0.1 N/m and a resonance frequency of 30 kHz in liquid, were used for tapping mode imaging. The scan rate was adjusted between 2 and 4.5 μm/s.

The microscope slides were functionalized with PEI as follows. First the glass slides were covered with Parafilm except the measurement region of about 6 cm^2. Then the Parafilm was firmly bond onto the glass at a temperature of ~50 °C for a few seconds on a hot plate. This results in a hydrophobic barrier preventing liquids to leave the measurement region during the coating process and the AFM imaging in the liquid phase. Then the inner region was coated using 1 mL of a 0.1% PEI solution in MilliQ-H_2O and incubated at RT for at least 30 min. The slide was thoroughly washed using MilliQ-H_20 and dried with nitrogen.

1 mL of the bacteria solution from the overnight cultures or after fixation with paraformaldehyde and glutaraldehyde as described for the SEM imaging was washed. Therefore the bacteria were centrifuged 5 min at 1500 rpm (Eppendorf centrifuge S417C), the supernatant was wasted and the pellet