

In Vivo Imaging of S-Layer Nanoarrays on *Corynebacterium glutamicum*

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Crystalline bacterial cell surface layers (S-layers) are monomolecular arrays of (glyco)proteins that have recently produced a wealth of new opportunities in nanotechnology. Whereas the in vitro imaging of isolated S-layers is well established, their direct imaging on live cells remains very challenging. Here we use atomic force microscopy (AFM) to visualize S-layer nanoarrays on living *Corynebacterium glutamicum* bacteria. We demonstrate the presence of two highly ordered surface layers. The most external layer represents the hexagonal S-layer, and the inner layer displays regular patterns of nanogrooves that could act as a biomolecular template promoting the 2-D assembly of S-layer monomers. These nanoscale analyses open new avenues for understanding the structure of protein monomolecular arrays, which is a crucial challenge in current nanoscience and life science research.

Bacterial surface layers (S-layers) are 2-D crystalline arrays of (glyco)proteins with molecular weight ranging from 40 000 to 200 000 Da, which represent one of the most common cell surface structures in bacteria.^{1,2} They usually exhibit oblique, square, or hexagonal lattice symmetry with spacing of the morphological units in the 3–30 nm range. Because they constitute the frontier between the cells and their environment, S-layers play several key roles, such as protecting the cell from hostile factors and serving as molecular sieves. In addition, a range of S-layer-based technologies have recently emerged, providing new approaches for nanotechnology.^{2,3}

The past years have witnessed tremendous advances in using atomic force microscopy (AFM) to image 2-D assemblies of cellular proteins at nanometer resolution.^{4,5} Examples of such protein arrays that have been visualized with nanometer resolution include *Bacillus* S-layers⁶ and the hexagonally packed intermediate (HPI) layer of *Deinococcus radiodurans*.^{7,8} Because specimens are imaged directly under physiological conditions, the information provided by AFM is complementary to that obtained by electron and X-ray crystallography. Whereas the in vitro imaging of isolated S-layers is well established, their in vivo observation represents a new challenge.

A striking example of highly ordered S-layers is found in *Corynebacterium glutamicum*, a gram-positive bacterium widely used for the production of glutamic acid.⁹ The native monomer,

PS2, has an apparent molecular weight of 63 kDa,¹⁰ with the level of expression depending on the strain and on growth conditions.¹¹ Electron microscopy and AFM studies have revealed that PS2 assembles into a highly ordered hexagonal S-layer.^{10,12,13} Whether AFM can reveal the organization of this S-layer directly on living bacteria is the question that we address here.

Cells were trapped in a porous polymer membrane for noninvasive in vivo imaging. Figure 1 shows height and deflection images of a single *C. glutamicum* bacterium grown in nutrient broth. Because of the large curvature of the cell, the height image has fairly poor resolution (Figure 1a) whereas the deflection image is much more sensitive to the surface relief (Figure 1b). The cell is surrounded by artifactual features resulting from the contact between the AFM tip and the pore edges. As can be seen, the cell surface showed a rough contrast with spongelike structures. Images recorded at higher resolution (Figure 1b, inset) were fuzzy and of poor quality and showed streaks oriented in the scanning direction reflecting strong interactions between the tip and loosely bound material. Presumably, this material is composed of polysaccharides because the analysis of the molecular composition of the outermost cell surface components of corynebacteria species revealed that they mainly consist of neutral macromolecular carbohydrates.¹⁴ Different cells cultured under nutrient broth conditions were investigated, and they never displayed ordered arrays that could reflect S-layer proteins. This means that, under these conditions, S-layer proteins were either lacking or hindered by an outer layer of polysaccharides.

We therefore analyzed bacteria grown under conditions favoring S-layer production.¹² Figure 2 shows deflection (Figure 2a,c) and height (Figure 2b,d–f) images obtained on a *C. glutamicum* cell grown in heart–brain infusion. Unlike cells grown in nutrient broth, the cells showed a rather smooth, well-resolved surface as well as patches of highly ordered material. For two reasons, we

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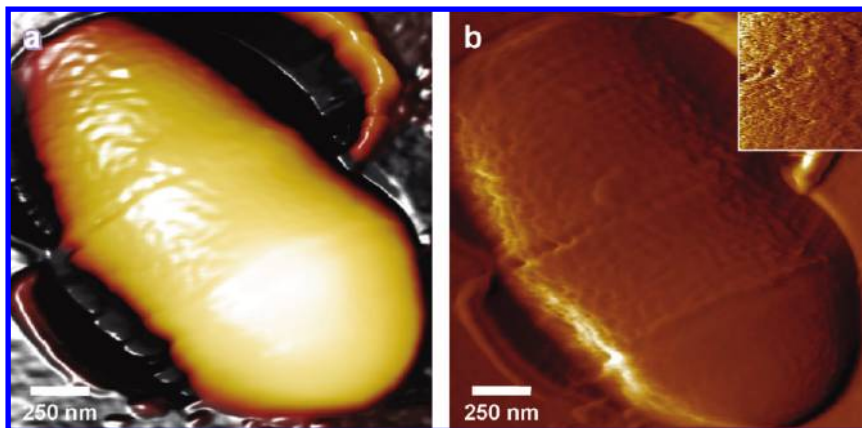


Figure 1. Bacteria grown in nutrient broth do not display ordered arrays on their surface. AFM height (a) and deflection (b) images of a *C. glutamicum* cell grown in nutrient broth. The inset in b shows a high-resolution deflection image of the surface (420 nm × 420 nm).

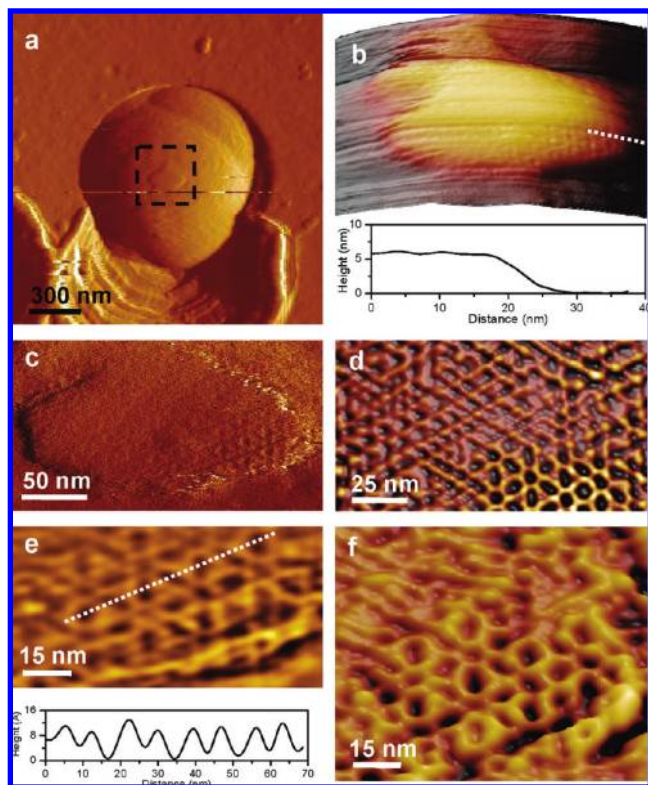


Figure 2. Bacteria grown in a heart–brain infusion show patches of ordered S layers. AFM deflection (a, c) and height (b, d–f) images of a *C. glutamicum* cell grown in a heart–brain infusion. The image in (b) corresponds to the square region shown in (a). Vertical cross sections in (b) and (e) were taken along the dotted lines.

believe that these patches correspond to the *C. glutamicum* S-layer. First, high-resolution imaging revealed that they consisted of regular nanoarrays displaying hexagonal lattice symmetry with 16 nm dimensions (Figure 2c–f), which is similar to earlier observations made by electron microscopy on freeze-fractured preparations^{10,12} and by AFM on isolated S-layer sheets.¹³ Second, the patch thickness was ~6 nm (Figure 2b), which is in the range of values expected for the thickness of the *C. glutamicum* S-layer.¹³

Notably, we observed another highly ordered layer surrounding the S-layer patches (Figure 3). Height (Figure 3a) and deflection (Figure 3b–d) images revealed that this second layer

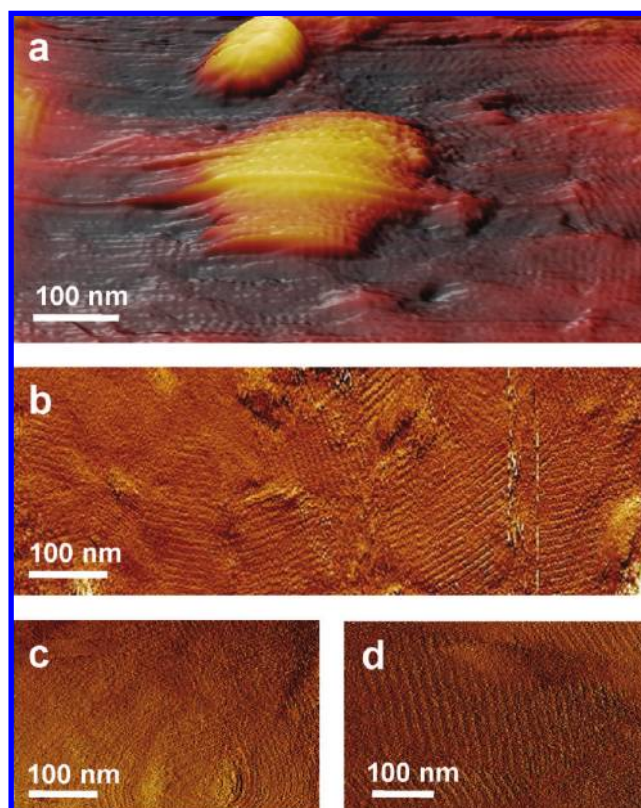


Figure 3. AFM reveals a new inner layer of highly ordered material. AFM height (a) and deflection (b–d) images of *C. glutamicum* cells grown in a heart–brain infusion.

was composed of regular patterns of nanogrooves several hundred nanometers in length displaying a periodicity of 11 ± 2 nm. Changing the scanning angle by 90° resulted in the rotation of the groove orientation accordingly, demonstrating that the observed patterns are due to real surface relief. This finding is consistent with earlier structural and biochemical data^{10,12} suggesting that the *C. glutamicum* surface is composed of two layers of ordered molecules (i.e., the most external layer composed of PS2 and representing the S-layer and a second inner layer interacting closely with PS2). Although the nature of this anchoring layer is unclear, there is evidence supporting the notion that it is composed of hydrophobic mycolic acids. Indeed, Chami et al.¹⁰ showed that the hydrophobic C-terminal sequence of PS2 is strongly associated to the cell wall by hydrophobic interactions,

indicating that PS2 interacts with a hydrophobic layer in the cell wall, most likely mycolic acids.¹⁵ The occurrence of a hydrophobic layer in close contact with the S-layer would explain why the fracture plane propagates close to the cell surface in *C. glutamicum* freeze–fracture experiments.¹⁰

According to these arguments, we suggest that the observed nanogrooves represent a highly organized layer of hydrophobic mycolic acids, which could function as a molecular template promoting the 2-D assembly and crystallization of the PS2 monomers. The proposed mechanism, which fits earlier models,¹⁰ would imply that protein monomers arriving on the cell surface are retained by their C-terminal hydrophobic sequence, diffuse on the cell surface until reaching and interacting with other monomers to assemble into hexameric complexes within a hexagonal lattice. Our model also fits the X-ray diffraction data revealing that mycolic acids in corynebacteria are arranged in a nearly crystalline packing of very low fluidity whereas typical lipid membranes are mostly in a disordered state.¹⁵ Accordingly, the unusual occurrence of two nanostructured layers in *C. glutamicum* could be related to the very specific structural organization of its cell wall.

In summary, our results represent the first direct observation of crystalline S-layers on live bacteria. We observed hexagonal unit cells with dimensions similar to those reported earlier for isolated S-layer sheets. Most interestingly, we also revealed a new inner layer composed of periodic nanogrooves, presumably reflecting the unique specificity of the *C. glutamicum* cell wall. We suggest that this second nanostructured layer could function as a biomolecular template promoting the 2-D assembly and crystallization of S-layer monomers.

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Materials and Methods

The *C. glutamicum* strain used in this study was ATCC 14752 (ATCC, VA). Cells were cultured either in nutrient broth medium (Oxoid, Basingstoke, England) or in Bacto brain heart infusion (HBI) medium (Becton-Dickinson, MD). They were incubated on a shaker at 170 rpm at 37 °C, harvested in the early stationary growth phase (i.e., after 12 or 36 h for HBI and nutrient broth, respectively), and centrifuged (9000 rpm for 10 min) and washed twice by resuspension in buffered solutions (10 mM Tris-HCl, pH 7.5, 250 mM KCl) and centrifugation.

AFM measurements were performed in Tris-HCl buffered solutions using a Nanoscope IV multimode AFM (Veeco Metrology Group, Santa Barbara, CA) and oxide-sharpened microfabricated Si₃N₄ cantilevers (Microlevers, Veeco Metrology Group). Cells were immobilized by mechanical trapping in porous polycarbonate membranes (Millipore). After filtering a concentrated cell suspension, we gently rinsed the filter with buffer, which was carefully cut (1 cm × 1 cm) and attached to a steel sample puck (Veeco Metrology Group) using a small piece of double-face adhesive tape, and the mounted sample was transferred to the AFM liquid cell while avoiding dewetting. Height images were flattened with a 12th-order polynomial and filtered through fast Fourier transforms using the Gwyddion open source program.

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