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Mounting of *Escherichia coli* spheroplasts for AFM imaging

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Abstract

The cytoplasmic membrane of *Escherichia coli* (*E. coli*) is the location of numerous, chemically specific transporters and recognition elements. Investigation of this membrane in vivo by atomic force microscopy (AFM) requires removal of the cell wall and stable immobilization of the spheroplast. AFM images demonstrate that spheroplasts can be secured with warm gelatin applied to the mica substrate just before the addition of a spheroplast suspension. The resulting preparation can be repeatedly imaged by AFM over the course of several hours. Confocal fluorescence imaging confirms the association of the spheroplasts with the gelatin layer. Gelatin molecules are known to reorder into a network after heating. Entrapment within this gelatin network is believed to be responsible for the immobilization of spheroplasts on mica.
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1. Introduction

Ongoing genomics and post-genomics-based studies are providing complete inventories of the molecular components that constitute biological systems. Deducing function, however, is more complex and requires complementary techniques that identify and characterize these systems at the molecular and cellular level. One significant challenge is characterizing the large fraction of the proteome (on the order of 25–30%) that is at or near the cell membrane. The cell membrane serves as both the container for the cell and as an interface to the outside environment. It is also the location of numerous, chemically specific transporters and

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recognition elements. Characterization of this surface is possible by atomic force microscopy (AFM), which allows molecular scale resolution of biological materials in physiologically relevant conditions (i.e. in liquid and without vacuum).

AFM has been used for investigation of adherent eukaryotic cells [1–4] and immobilized microbes [5–9]. Imaging of bacterial cells, particularly the investigation of their membrane proteins and receptors, presents unique challenges when compared to eukaryotic cells. In addition to their small size, bacteria contain a cell wall that restricts access to the cytoplasmic membrane [10]. The cytoplasmic membrane is the selective barrier for the cell and as such, is the location of the major transport proteins. To access these proteins, the cell wall must be removed prior to AFM analysis. This is typically accomplished by treatment with a combination of lysozyme and ethylenediaminetetraacetate (EDTA) [11]. Lysozyme degrades the peptidoglycan matrix of the periplasm while EDTA chelates the ions in the outer cell wall, leading to its degradation [12,13]. Following enzymatic digestion of the cell wall, the osmotically sensitive cells, termed spheroplasts, can be maintained in sucrose containing buffers to prevent rupture. Immobilization of spheroplasts is necessary for AFM-based imaging studies that characterize membrane transporters and other cytoplasmic membrane constituents (e.g. phospholipids, lipid rafts, membrane associated proteins). Further, physical access to the cytoplasmic membrane of immobilized spheroplasts facilitates molecular and charge recognition experiments that can help determine protein location, charge contributions and interactions with periplasmic proteins/enzymes or other probe molecules tethered to the AFM probe [14–19]. In this paper the immobilization of spheroplasts in appropriate buffer conditions for study by AFM is reported.

2. Materials and methods

2.1. Cell cultures

E. coli strain BL21-AI (Invitrogen, Carlsbad, CA) carrying a pBBR1MCS-5 plasmid [20] which has been modified for Gateway insertional cloning (Invitrogen, Carlsbad, CA) and contains the gene sequence for green fluorescent protein (GFP) was used in these experiments. Cultures were grown overnight at 37 °C in a shaking water bath in Luria Broth (LB) supplemented with 5 μg/ml gentamicin to maintain plasmid selection.

2.2. Spheroplasting

The spheroplasting procedure used is an adaptation of the one published by Birdsell and Cota-Robles [11]. Briefly, 1 ml of stationary phase *E. coli* bacteria from an overnight culture was collected by centrifugation and washed in 1 ml 0.01 M phosphate buffer, pH 7.0 (PB). The cells were then pelleted by centrifugation at 3000 *g* and resuspended in 500 μl of a 0.5 M sucrose solution made in PB (SPB1) to induce plasmolysis. Lysozyme (Sigma, St. Louis, MO) was added to the cell suspension at a final concentration of 50 μg/ml. After incubation in a 37 °C water bath for 1–2 h, the suspension was diluted 1:1 with PB and EDTA was added to a final concentration of 10 mM. The suspension was returned to the 37 °C water bath for an additional 10 min. After this incubation, transition of the rod-shaped bacteria into spheres was determined by light microscopy and used to measure progression of the spheroplasting reaction. When ~80% of the cells were spheroplasted, the reaction was stopped by pelleting the cells at 500 *g* for 15 min. The spheroplasts were then washed in 0.25 M sucrose in PB (SPB2), pelleted at 500 *g* for 15 min and resuspended in SPB2.

2.3. Sample preparation

A 20 mm hole punch (Ralmikes TOOL-A-RAMA, South Plainfield, NJ) was used to punch circular mica disks out of a flat mica sheet. The disks were then cleaved several times to obtain thin individual disks that were freshly cleaved on both sides. A gelatin solution was prepared by dissolving 0.5 g gelatin (Sigma #G6144) and 10 mg chromium ammonium sulfate in nanopure distilled water at 60 °C. After cooling to 40 °C, surfaces for immobilizing and imaging intact bacteria were

prepared by vertically dipping the freshly cleaved mica into the solution and permitting it to air dry overnight by standing on edge on a paper towel.

For imaging stationary phase *E. coli* bacteria, 1 ml of an overnight culture was pelleted, washed in the same volume of PB, pelleted again and resuspended in 500 μl nanopure deionized water. This cell suspension (20 μl) was applied onto a gelatin-treated mica disk and spread with the pipette tip to a diameter of roughly 5–7 mm. The sample was allowed to stand for 10 min before it was rinsed in distilled water.

For imaging *E. coli* spheroplasts, 4μl of warm gelatin (50–55 °C) was applied onto the freshly cleaved mica and spread with the pipette tip. Approximately 20 μl of the spheroplast suspension was then immediately added using a large bore pipette tip (i.e. 1 ml pipette tip) to avoid lysing the fragile spheroplasts. After allowing the sample to stand for 30 min, the sample was rinsed with SPB2 to remove loosely bound cells.

2.4. Atomic force microscopy

Samples of intact or spheroplasted cells were loaded in the liquid cell of a Pico Plus atomic force microscope (Molecular Imaging Inc., Tempe, AZ) and imaged using a 100 μm scanning head. The instrument was operated in MACMode at 256 or 512 pixels per line scan at speeds ranging from 0.6 to 2 Hz. Intact bacterial cells were imaged in nanopure deionized water or PB using silicon cantilevers (Type 1 Maclevers, Molecular Imaging, Tempe, AZ) with a nominal spring constant of 0.6 N/m and a resonant frequency in air of 75 kHz. Spheroplasts were imaged in SPB2 using silicon nitride cantilevers (Molecular Imaging, Tempe, AZ) with a spring constant of 0.1 N/m and a resonant frequency in air of 38 kHz. All of the images presented are first-order flattened.

2.5. Fluorescence microscopy

A Leica TCS SP2 confocal laser scanning microscope system (Leica Microsystems Inc., Exton, PA) was used for fluorescence imaging. *E. coli* bacteria were spheroplasted and mounted as described in Sections 2.2 and 2.3 except that rhodamine 6G (Aldrich Chemical, Milwaukee, WI) was added to the gelatin solution at a final concentration of 3 ng/ml. This was done to aid in localizing the spheroplasts within the gelatin layer. A drop of SPB2 was added to the mica disc which was then mounted between a slide and coverslip and sealed with a warmed 1:1:1 mixture of petroleum jelly, lanolin and paraffin (VALAP) to prevent drying. Images were processed using Leica confocal software version 2.5.

3. Results and discussion

AFM is emerging as a useful research tool for studying bacteria, due in part to the fact that it overcomes limitations in spatial resolution associated with optical microscopy [21]. Although high-resolution imaging of bacteria is possible with electron microscopy (EM), cells must be fixed with cross-linking agents and imaged under a vacuum, conditions that prevent imaging of live cells. In contrast, live cells can be imaged under physiological conditions with nanometer resolution using AFM. In addition to recording cell morphologies, surfaces can be probed by the AFM stylus to reveal structural and physicochemical properties not accessible via other microscopy techniques. Such data have yielded important insights concerning turgor pressure in magnetotactic bacteria and surface proteins in lactic acid bacteria. [8,9]. One essential preparatory step common to these AFM-based investigations is immobilization of the microbes. Here, a technique for reliably immobilizing spheroplasts, enabling access to the cytoplasmic membrane, is presented.

Previous efforts have shown that intact bacteria can be immobilized on gelatin-coated mica for high-resolution AFM imaging in liquid [22]. Intact bacteria mounted by this method were imaged in water using AFM or confocal fluorescence microscropy (Fig. 1a–c). No apparent morphological abnormalities are observed in these cells except that some *E. coli* cells expressing GFP are slightly elongated, compared to cells without the plasmid (Fig. 1a, b and data not shown). In these images, bacterial cells appear to be hydrated and measure within the expected height and length ranges. Over

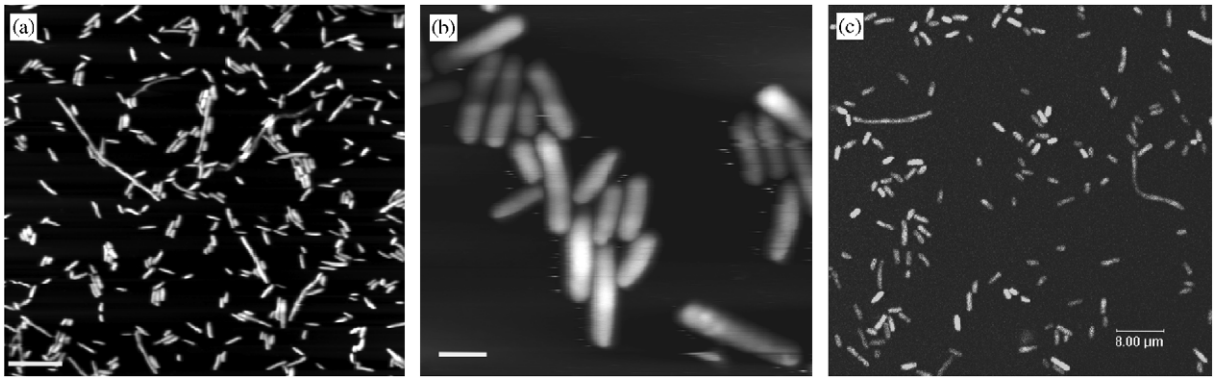

Fig. 1. Intact *E. coli* on gelatin-coated mica imaged in water. Shown are (a) large area AFM scan, 512 pixels per line, topographic, MacMode images taken at 0.6 Hz, bar = 10 μm, (b) a small area AFM scan, 512 pixels per line, topographic, MacMode images taken at 1.1 Hz, bar = 2 μm and (c) a confocal fluorescence image, bar = 8 μm. Bacteria appear hydrated and are within expected height and width ranges with the exception of a few elongated cells.

350 bacteria are seen in the 75 μm × 75 μm scan area of the AFM image (Fig. 1a). Several bacterial species, including *E. coli*, bind collagen and gelatin (denatured collagen) via specific binding sites on the intact bacterial surface [23,24]. It is likely that these binding sites, along with electrostatic and hydrophobic interactions, contribute to retaining bacteria on gelatin-coated substrates.

The major transport proteins in bacteria localize to the cytoplasmic membrane, a site normally inaccessible to the scanning probe microscope tip due to the presence of the outer cell wall. Therefore, characterization of proteins in this membrane by AFM requires removal of the outer wall using a spheroplasting procedure that renders the cells osmotically sensitive [11]. When these spheroplasts were immobilized using the protocol designed for intact bacteria, stable imaging was not possible (Fig. 2). Instead, the spheroplasts appeared to move from their original position and change morphology after multiple scans due to force interactions with the cantilever tip. These findings are similar to behavior described for *Deinococcus geothermalis* in which a firm but slippery attachment was found to exist when these microbes were attached to stainless steel and imaged in water [25]. The observation that spheroplasts are not securely immobilized using this method is likely due to different, less stable interactions between gelatin-coated mica and the cytoplasmic membrane which lacks the surface proteins, lipopolysaccharides and pili thought to contribute to immobilization of intact bacteria. For this reason, it was necessary to find an alternative method for immobilizing spheroplasts for AFM studies.

A number of techniques for immobilizing spherical yeast cells have been published which involve stabilizing cells in agarose or trapping yeast cells in the pores of a filter membrane before imaging [26,27]. Although yeast cells and bacterial spheroplasts are both round, yeast cells are larger, have a rigid cell wall and are significantly less fragile than spheroplasts. Use of these techniques with bacterial spheroplasts has not been reported. Initial attempts to trap bacterial spheroplasts in the pores of membranes were unsuccessful and this approach was not pursued further in this study (data not shown). Rather, a method was developed in which a small volume of warm gelatin is applied to a freshly cleaved mica surface followed immediately by addition of the spheroplast suspension. After a brief period of incubation, the spheroplast sample can be stably imaged for several hours in a 0.25 M sucrose solution which prevents rupture of the osmotically sensitive spheroplasts. Unlike the rod-shaped intact *E. coli* cells, the more than 125 spheroplasts in the 75 μm × 75 μm scan area are clearly round and measure 1–3 μm in diameter (Fig. 3a), consistent with published measurements [11,13]. To address

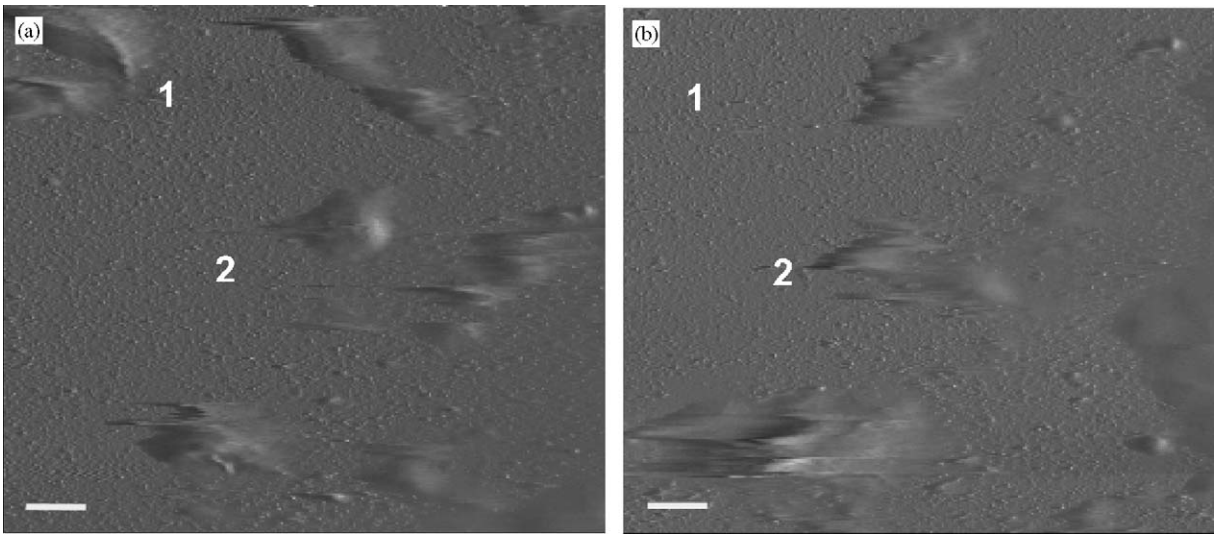

Fig. 2. Spheroplasts immobilized on gelatin-coated mica, scanned at 1.5 Hz and with 256 pixels per line. (a) Amplitude image after one scan, bar = 2 μm; (b) amplitude image of the same area after four scans taken within 20 min of 2a, bar = 2 μm. Note that spheroplasts are present but appear to be repositioned (1) and distorted (2) by the tip as a result of scanning.

whether this method resulted in stable immobilized cells, multiple sample preparations were scanned repeatedly without any apparent movement of spheroplasts. The outcome of such an experiment is given in Figs. 3b–e. Repeated scans of two spheroplasts, made over a period of 3 h, result in no detectable changes in spheroplast morphology or height measurement, indicating the usefulness of this technique for stabilizing spheroplasts for AFM imaging.

Height measurements varied considerably between individual spheroplasts, ranging from 10 to 250 nm (Fig. 3d, e and data not shown). These differences could be explained by either dehydration or by entrapment of spheroplasts at different depths in the gelatin layer. To confirm that the spheroplasts are not lysed in the gelatin, *z*-series optical sections were collected from cells expressing GFP using confocal fluorescence microscopy (Fig. 4a and b). This experiment confirms that the spheroplasts are indeed spherical (Fig. 4b) and are arranged at varying heights within the gelatin layer (Fig. 4b). Thus, apparently only a portion of the spheroplast surface is accessible for characterization using AFM. A model describing the process by which gelatin associates from individual single-stranded coils into a fibrous network and its dependence on concentration and thermal history has been published [28]. Further, AFM-based investigations produced images of intermediate steps throughout this gelation process [29]. Although the time scale in which these experiments were performed precludes visualization of a fibrous gelatin network, it is likely that an under-developed network is involved in the immobilization of spheroplasts via an entrapment mechanism.

4. Conclusion

Adhesion proteins associated with the outer surface and pili of *E. coli* are likely contributors to the immobilization of the intact bacteria on gelatin-coated mica. In order to study bacterial cytoplasmic membrane proteins in vivo by AFM, direct access to the cytoplasmic membrane can be achieved by removing the outer cell wall from the bacteria. However, simple adhesion of spheroplasts to a gelatin surface is not suitable for stable imaging. Entrapment of spheroplasts by applying a warm layer of gelatin is shown to be a suitable

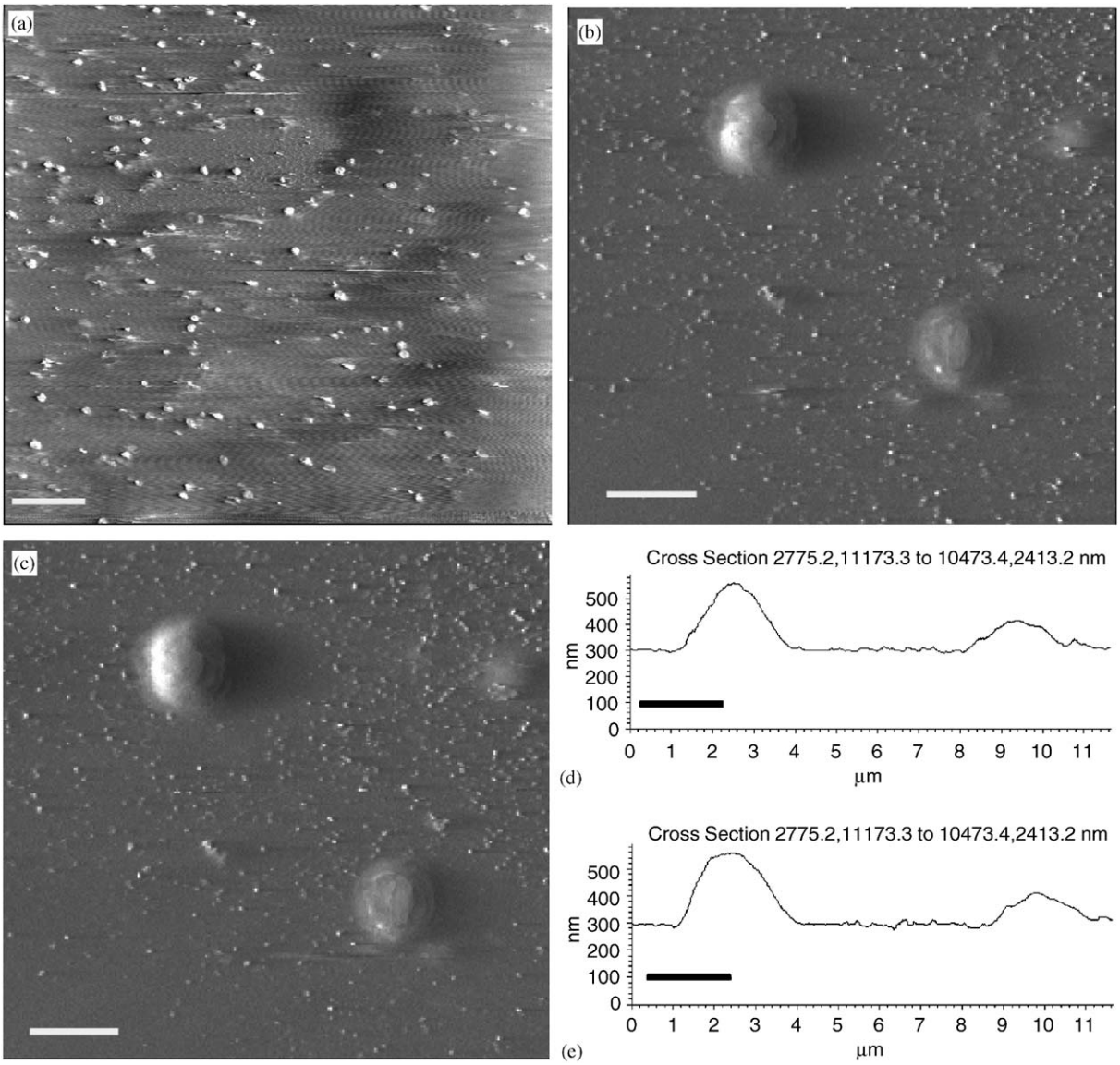

Fig. 3. Amplitude images of spheroplasts immobilized by applying warm gelatin to freshly cleaved mica just prior to adding a spheroplast suspension. After 30 min, the sample is rinsed and imaged in an isotonic buffer. (a) Representative 75 µm scan taken at 512 pixels per line and at a speed of 2 Hz, bar = 10 µm. (b) To demonstrate the stability of the preparation technique, two spheroplasts were imaged after four scans at 1.3 Hz and compared with the image of the same spheroplasts, bar = 2 µm. (c) Imaged after 20 scans at 1.3 Hz over a 3 h period, bar = 2 µm. (d) and (e) are cross sections from the topograph images of (b) and (c) respectively, bars = 2 µm. Note the similarity in the profiles, indicating that the spheroplasts were stably imaged without the changes noted in Fig. 2.

immobilization technique. Upon cooling, gelatin molecules reassociate in an effort to reform collagen fibers, and these gelation intermediates are believed to entrap the spheroplasts, stabilizing them for AFM imaging. This immobilization technique will facilitate AFM-based investigations of the cytoplasmic membrane and its constituents.

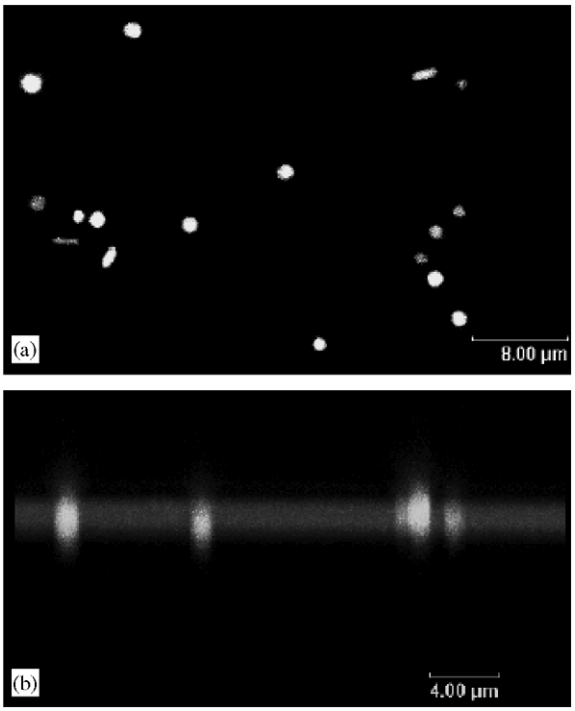

Fig. 4. Spheroplasts immobilized using warm gelatin doped with rhodamine 6G. Appropriate filters for GFP and rhodamine 6G were used in each case to generate a series of confocal fluorescence images compressed into a single image. (a) GFP channel only *xyz* scan, bar = 8 μm. (b) Emission detected on the GFP and rhodamine 6G channels are merged to show that the spheroplasts are spherical and are in proximity of the gelatin surface. The horizontal band results from light reflection and indicates the surface of the gelatin, bar = 4 μm.

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