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A comparison of the surface nanostructure from two different types of gram-negative cells: *Escherichia coli* and *Rhodobacter sphaeroides*

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ABSTRACT

Bacteria have been studied using different microscopy methods for many years. Recently, the developments of high-speed atomic force microscopy have opened the doors to study bacteria in new ways due to the fact that it uses much less force on the sample while imaging. This makes the high-speed atomic force microscope an indispensable technique for imaging the surface of living bacterial cells because it allows for the high-resolution visualization of surface proteins in their natural condition without disrupting the cell or the activity of the proteins. Previous work examining living cells of *Magnetospirillum magneticum* AMB-1 demonstrated that the surface of these bacteria was covered with a net-like structure that is mainly composed of porin molecules. However, it was unclear whether or not this feature was unique to other living bacteria. In this study we used the high-speed atomic force microscope to examine the surface of living cells of *Escherichia coli* and *Rhodobacter sphaeroides* to compare their structure with that of *M. magneticum*. Our research clearly demonstrated that both of these types of cells have an outer surface that is covered in a network of nanometer-sized holes similar to *M. magneticum*. The diameter of the holes was 8.0 ± 1.5 nm for *E. coli* and 6.6 ± 1.1 nm for *R. sphaeroides*. The results in this paper confirm that this type of outer surface structure exists in other types of bacteria and it is not unique to *Magnetospirillum*.

1. Introduction

In the past, high-resolution imaging of the outer membrane of gram-negative bacteria has relied on fixing cells and using transmission electron microscopy (TEM) (Beveridge, 1999; Matias and Beveridge, 2005). Using this method the membrane could be seen in cross-section, or by using freeze fracturing techniques, the surface of one side of the membrane could be examined. Even though it provides nanometer resolution, an acute shortcoming of electron microscopy is that it relies on examining dead cells, which have inherent artifacts and opens the possibility of whether or not the image is depicting something real or artificial. Fluorescent microscopy imaging is useful for examining living cells and can easily target protein molecules, however, this technique relies on an indirect method to image proteins. On the other hand, the conventional atomic force microscope has the ability to directly image individual living cells in their native state with no labeling (Amro et al., 2000; Schabert and Engel, 1994; Scheuring et al., 2002). However, one drawback to the conventional AFM is the applied force and speed at which it captured images. Capturing a single image requires at least a minute or more to acquire. This means that any molecule moving at a speed faster than the capture speed would be impossible to image. Another problem with the slow scan speed is that the tip dwells on the sample for a longer time which disturbs the sample, particularly soft samples, making it difficult or impossible to image soft samples such as proteins on the surface of cells.

However, the high-speed atomic force microscope (HS-AFM) is a recent innovation that has all the features of a conventional AFM, but applies a smaller force on the surface of cells; therefore it disturbs the sample less but still provides a high-resolution image (Ando et al., 2001, 2008a,b, 2014). Two principal factors contribute to the cantilever's low impact during its interaction with the sample, the small time delays during the processing of the cantilever's position and the small force applied by the cantilever tip. Time delays during the sample interaction are an inherent shortcoming of the conventional AFM, but these have been reduced in the HS-AFM by redesigning the components and the cantilevers (Ando,

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2013). The other advantage is that it has a gentle tapping force (~80 pN), which permits the microscope to image soft molecules. This makes it possible to image bacteria, which can be difficult to image with the conventional AFM because of the larger tapping force.

Previous studies on the outer membrane of gram-negative bacteria showed that it is a multipurpose barrier that directly interacts with the environment by selecting what leaves or enters the cell (Beveridge, 1999; Beveridge and Graham, 1991). The organization of the outer membrane of gram-negative bacteria is believed to be made of an asymmetrical lipid bilayer with the inner layer made of mostly phospholipids, and the outer layer containing many lipopolysaccharides (LPS) (Kamio and Nikaido, 1976). LPS are thought to form a dense, gel-like layer at the surface of bacteria, which help the outer membrane to act as a protective barrier (Takeuchi and Nikaido, 1981). Interspersed into the LPS are transmembrane proteins called porins, which form channels through the membrane (Nakae, 1976). Generally, porins are a trimeric structure consisting of three β-barrel subunits (Eisenberg, 1984). These structures act as a gate, which allow hydrophilic molecules less than 600 Da, such as antibiotics, nutrients, and waste products, to pass through them (Nikaido, 2003; Schulz, 2002). One estimate reports that there are ~10^5 copies of porin molecules per cell (Rosenbusch, 1974) and these molecules form a lattice that covers approximately 70% of the surface of the outer membrane (Jarosławski et al., 2009).

The porin molecules of *Escherichia coli*, OmpF, OmpC, and PhoE, and the porins from *Rhodobacter capsulatus* and *Rhodobacter blasticus* have been extensively studied (Kreusch and Schulz, 1994; Schirmer, 1998; Weiss et al., 1991). Their structures have been imaged in vitro using the TEM and AFM (Casuso et al., 2012; Sass et al., 1989; Schabert et al., 1995). However, so far the studies of porin molecules with the AFM involved isolating the membranes from cells or studying porin molecules attached to artificial surfaces (Müller and Engel, 1999). The surface of *E. coli* was examined using the HS-AFM, however the scan-size was large and the details of the ultrastructure were not visualized (Fantner et al., 2010). More recently, the HS-AFM was used to probe the porin molecules of living cells of *Magnetospirillum magneticum* AMB-1, a type of magnetotactic bacteria (Yamashita et al., 2012). They found that the dominant feature on the outer surface was a net-like structure. Using the HS-AFM, they performed a nano-dissection on isolated outer membranes and also performed a proteomic analysis of isolated outer membranes. These two methods revealed that the major component of this net-like structure was porin proteins.

The goal of the research presented here is to determine whether or not the outer surface structure found in *M. magneticum* AMB-1 can be found in other organisms such as *R. sphaeroides* and *E. coli*, which are useful in molecular genetics. To do this we used the HS-AFM to image the outer surface of *E. coli* and *R. sphaeroides*. The HS-AFM proved to be a beneficial technique because of its ability to gently scan the surface of living bacteria in their native state, allowing the surface to be imaged at very high resolution and give a direct image of the surface. This work established that the HS-AFM is a useful technique to observe the surface of living bacteria because it can reveal new insight into the structure of their outer surface.

2. Materials and methods

2.1. Culture conditions

Two types of bacteria were used in this study, *E. coli* strain MG1655 and *R. sphaeroides* NBRC 12203. *E. coli* cells were cultured in glass tubes using M9 media with 20% glucose and grown at 37 °C with shaking until they reached late log phase (Sambrook and Russell, 2001). *R. sphaeroides* cells were cultured in glass tubes in the dark at 30 °C, without shaking, for 40–45 h using media 802 from the NBRC catalog. The bacteria used for imaging were removed directly from the culture tube without concentrating the cells to help avoid damaging the bacteria before imaging.

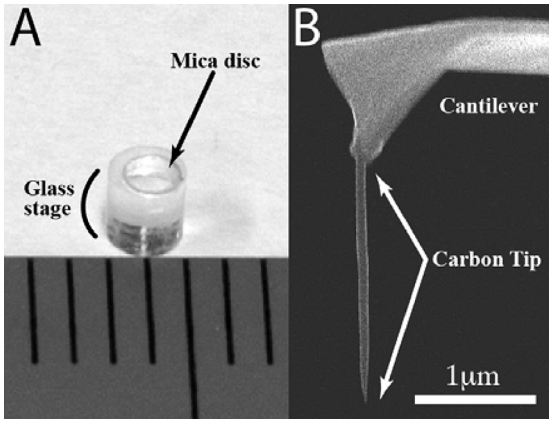

Fig. 1. Examples of the stage and cantilever tip used for imaging in the HS-AFM. (A) A mica disc is glued to a small glass stage using epoxy. The mica disc has many layers, which can be peeled off with tape so that there is a clean, flat surface for the sample to adhere to. (B) A carbon tip is electron beam deposited onto each Olympus cantilever; carbon has the advantage of being longer and sharper than the natural cantilever.

2.2. High-speed atomic force microscope sample preparation

Imaging samples on the HS-AFM requires a hard, flat surface, for this we used mica discs 1.5 mm in diameter (Furuuchi Chemical, Japan) glued to a small glass stage (2.5 mm high and 2 mm in diameter) with epoxy (Fig. 1A). The mica discs are composed of many layers of mica sheets that can be peeled off with tape prior to use, such that for each experiment there is a fresh surface, so there is no contamination from prior experiments. Before the cells are put on the mica, it is prepared in a way that will help the cells attach to the surface, but without killing or sacrificing the integrity of the cell. We used the method previously described by Yamashita et al. (2012), where we place 3 μL of 0.1% poly-L-lysine onto the mica and placed them in an oven and allowed it to dry for 3 h at 60 °C. The mica/glass stages are stored in a closed box until they are ready to be used.

The bacterial samples are prepared just prior to imaging. First, 3 μL of 0.02% glutaraldehyde is incubated on the poly-L-lysine coated mica/glass stages for 15 min, then washed 3 times using 20 μL of milli-Q water. While keeping the stages wet, the bacteria sample was applied to the mica by placing several microliters on the stage, then wicking it off and repeating this for 20 μL worth of bacterial suspension. The stage is then incubated for 1 h in a moist chamber to allow the bacteria to attach to the mica, and then rinsed with the same media that was used to culture the cells, M9 for *E. coli* and media 802 for *R. sphaeroides*. This ensures that the cells are kept in as near a native state as possible while imaging. The stages were rinsed by applying several microliters of media, then wicking it off, and repeating this for 60 μL of media. This gentle rinsing is necessary to prevent the cells from detaching from the surface of the mica. During the last rinse, a drop of media is left on the stage so that the cells remain in liquid and are never given a chance to dry out. Finally, the glass stage containing the mica and bacterial sample is adhered to the HS-AFM scanner using nail polish. This creates a strong bond between the stage and the scanner, but it can be easily removed using acetone. Each sample is prepared just prior to imaging ensuring that the cells are fresh for each round of imaging.