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Review

Morphological and nanostructural surface changes in *Escherichia coli* over time, monitored by atomic force microscopy

Ibtissem Gammoudi[a,*], Marion Mathelie-guinlet[a], Fabien Morote[a], Laure Beven[b], Daniel Moynet[c], Christine Grauby-heywang[a], Touria Cohen-bouhacina[a,*]

[a] Université de Bordeaux, LOMA, CNRS UMR 5798, 351 Cours de la Libération, 33405 Talence, France
[b] Université de Bordeaux, INRA, CNRS UMR 1332 BFP, 71 Avenue Edouard Bourlaux, 33882 Villenave d'Ornon Cedex, France
[c] Université de Bordeaux, INSERM U1035, Bordeaux 33706, France

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ABSTRACT

The present study aims at evaluating intrinsic changes in *Escherichia coli* (*E. coli*) surface over time, by Atomic Force Microscopy (AFM). For that purpose, bacteria were immobilized on mica or on mica previously functionalized by the deposition of a polyelectrolyte multilayer cushion. AFM images reveal that *E. coli* population goes through different stages. Firstly, after a week, the number of healthy bacteria decreases resulting in a release of cellular components which likely become, in turn, a nutrition source for increasing the healthy population after around two weeks. Finally, after one month, most of the bacteria is dead. Our study shows a transition of a healthy rod-shaped bacterium to a dead collapsed one. Most importantly, along with the morphological evolution of bacteria, are the structure changes and the mechanical properties of their outer membrane, emphasized by AFM phase images with very high resolution. Indeed, the surface of healthy bacteria is characterized by a phase separation pattern, thereafter mentioned as "ripples". Bacterial ageing goes along with the loss of this organized structure, turning into circular areas with irregular boundaries. These changes are likely caused by a re-organization, due to external stress, of mainly lipopolysaccharides (LPS) present in the outer membrane of *E. coli*.

Contents

* Corresponding authors.
E-mail addresses: ibtissem.gammoudi@u-bordeaux.fr (I. Gammoudi), touria.cohen-bouhacina@u-bordeaux.fr (T. Cohen-bouhacina).

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1. Introduction

Bacterial adhesion and biofilm formation have a great interest both in natural environments and in industrial processes, biofilms being the predominant life form for bacteria to multiply and to protect themselves from environmental stresses such as UV radiation, osmotic shock and anti-microbial agents [1–4]. Bacterial biofilms undergo several stages of development from the initial attachment of bacteria to detachment and colonization of new interfaces [5].

To better understand mechanisms of such biofilm formation and bacterial metabolism inside over time, the detailed study of the biological envelop, a vital component of bacteria, might be helpful [6]. Gram negative bacteria, like *Escherichia coli* (*E. coli*), possess, along with a peptidoglycan cell wall, an outer membrane, this particular one being absent in Gram positive bacteria. This outer membrane consists of an asymmetric lipid bilayer and lipopolysaccharide (LPS) molecules as the outermost constituent, overlying a gel-like periplasm and a thin peptidoglycan layer [7]. It serves, especially LPS, as a selective permeability barrier and a protection (for instance, mutants without LPS are more permeable to mutagens) while proteins embedded or anchored to this membrane allow specific transmembrane exchanges [8]. LPS possess (i) an endotoxic part enchored to the membrane, the lipid moiety called lipid A, (ii) a long polycaccharide chain, (iii) sometimes ended by a third part pointing outwards the membrane, the *O*-chain, responsible for the bacterial antigenic specificity [9]. These extracellular sugar extensions create mutual attraction forces that curve the membrane. Part of Gram-negative bacteria's danger lies in LPS. In bacterial infections, LPS fragments from damaged bits of the bacterial walls are released locally, triggering an immune response in human organism [7,10].

Consequently, investigating the structure and properties of bacterial surfaces with high resolution is of great importance to understand how biofilms behave. The ultrastructure of microbial surfaces has been extensively studied and their chemical composition and physicochemical properties have been probed, for instance, by X-ray photoelectron spectroscopy and infrared spectroscopy [11,12]. However, none of these techniques can provide direct information with a nanometer scale lateral resolution. Because of its high spatial resolution and its ability to image in real time under physiological conditions, Atomic Force Microscopy (AFM) has been used more and more frequently in biological field [13]. Indeed, AFM, as a scanning probe technique, is ideally suited to study surface properties of biological systems, such as bacteria, from topographical to nanomechanical properties, including composition and adhesion [13,14]. The non intrusive tapping mode is particularly interesting for those fragile systems as phase imaging gives access to physico-chemical properties of sample surfaces at the local scale to hundreds of μm^2.

In this work, we probed the surface structure of *E. coli* using AFM in tapping mode. Bacterial populations were followed over a month to determine their viability. More precisely, morphological and mechanical properties of *E. coli* bacteria, especially the outer membrane ones, were investigated with high resolution, revealing fine details of its structure. We also showed that this specific organization underwent major changes with time, corresponding to modifications of the intrinsic behavior of LPS molecules. These changes allow for a rapid and accurate quantitative discrimination between viable and dead bacteria. Moreover, we proceeded with AFM, for the first time, in a complete study on *E.coli* bacteria: different cell divisions patterns, bacteria population organizations, morphology and rheology evolution were observed in real time.

2. Materials and methods

2.1. Materials

Gram negative *E. coli* bacteria (MRE 162 strain) were a kind gift from the *Centre d'Etudes du Bouchet*, *DGA* (Direction Générale de l'Armement, France). Bacteria were grown on a solid Luria medium and incubated for 16 h at 37 °C. After scraping, bacteria were suspended in purified water (pH 5.5, resistivity > 18.2 MΩ cm) and placed in 1.5 mL microtubes for a centrifugation at 3000 g for 20 min. Cells were then washed twice with purified water and adjusted to 10^8 cells/mL.

Mica, purchased from Electron Microscopy Sciences, was used for all AFM measurements and images. With an average roughness of 0.3 nm/μm^2, it is one of the best substrate to work with if one wants to get free from the substrate roughness.

2.2. Bacteria imaged on nude mica

Freshly cleaved mica is appropriate when imaging in air, as bacteria will not be submitted to a fluid flow. As mentioned above, mica has also a homogeneous surface which allows discriminating any topographic contrast. A 5 μL droplet of bacteria suspension was directly applied on the mica and left to dry under a desiccator. To avoid aggregation and the presence of non-fixed bacteria, the sample was then rinsed with ultrapure water before imaging (Fig. 1a). Consequently, this process led to more frequent isolated bacteria and, thus, was preferred when a detailed study of the bacterium was required. For over-time experiments, we kept samples either under the AFM setup, protected from dust, to follow the same population at each step, or under a desiccator to check their general behavior over time.

2.3. Bacteria immobilization on a polyelectrolyte multilayer surface

To image in liquid medium or to study the morphological and rheological changes over time, we needed to immobilize bacteria on the substrate. To do so, alternative polycationic/anionic treatment of the substrate was previously used successfully [15]. Indeed, Layer by layer (LbL) self-assembly is one of the most versatile techniques to make multilayer composite films. It consists in sequential depositions of oppositely charged solutions of polyelectrolytes (PE) to form, in an inexpensive and easy way, a self-assembled molecular multilayer [16]. Biological applications are numerous; among them, worth mentioning is the immobilization of biological systems (such as DNA, proteins, enzymes or cells) and the production of multifunctional nano-scaled structures and materials [17]. This method was successfully applied in one of our previous AFM study [15].

PE considered in this work are cationic poly(allylamine hydrochlorure) (PAH, Mw = 56000), and anionic poly (styrene sulfonate) (PSS, Mw = 70000). They were purchased from Sigma Aldrich. PAH and PSS were dissolved in ultrapure water at a concentration of 0.5 g/L. Each PE layer was deposited through the LbL method resulting in self-assembled molecular multilayers PAH-$(PSS\text{-}PAH)_n$ on the mica substrate. Typically, PAH and PSS solutions were alternatively deposited during twenty minutes, adsorption and rinsing steps being repeated to obtain the desired number of PE layers, forming then polyelectrolytes multilayers (PEM, Fig. 1b). In this work the last layer was made of positively charged PAH to guarantee the fixation of negatively charged *E. coli* bacteria (Fig. 1b). As for the direct drop-off, 5 μL of bacteria suspension was deposited on the functionalized mica and left to dry under a desiccator before imaging. Consequently, we applied this PEM technique to immobilize bacteria on the substrate in order to prevent their potential shift