Surface Structure and Nanomechanical Properties of *Shewanella putrefaciens* Bacteria at Two pH values (4 and 10) Determined by Atomic Force Microscopy

Fabien Gaboriaud,* Sidney Bailet, Etienne Dague, and Frédéric Jorand

Laboratoire de Chimie Physique et Microbiologie pour l’Environnement, UMR 7564, CNRS, Université Henri Poincaré, Nancy 1, 405 rue de Vandœuvre, F-54600, Villers-lès-Nancy, France

Received 3 January 2005/Accepted 21 February 2005

The nanomechanical properties of gram-negative bacteria (*Shewanella putrefaciens*) were investigated in situ in aqueous solutions at two pH values, specifically, 4 and 10, by atomic force microscopy (AFM). For both pH values, the approach force curves exhibited subsequent nonlinear and linear regimens that were related to the progressive indentation of the AFM tip in the bacterial cell wall, including a priori polymeric fringe (nonlinear part), while the linear part was ascribed to compression of the plasma membrane. These results indicate the dynamic of surface ultrastructure in response to changes in pH, leading to variations in nanomechanical properties, such as the Young’s modulus and the bacterial spring constant.

Bacterial surfaces are structurally complex, with several components constituting the bacterial cell envelope. In gram-negative bacteria, the envelope is made up of two lipid bilayer membranes: (i) the plasmic membrane, delimiting the inner part of the cell from the external one, and (ii) the outer membrane (OM), the most external membrane. Between these two layers, a thin and strong polymer, murein, or peptidoglycan layer is embedded in a concentrated gel-like matrix, the periplasmic space. The OM contains two types of lipids, lipopolysaccharide (LPS) and phospholipids, as well as a number of characteristic proteins. The LPS is composed of three parts: (i) the lipid A region that anchors the LPS to the OM; (ii) the distal, hydrophilic, O-antigen polysaccharide region that protrudes into the extracellular medium; and (iii) the core oligosaccharide region that connects the two (17). In addition, bacterial cell surfaces can also be studded or covered by extracellular polymeric substances (29) or some specific external structures (pili or fimbriae and flagella) (5).

As the outer surface of the OM directly interacts with the extracellular environment of the bacterial cell, characterizing such surfaces can provide crucial information for understanding processes such as bacterial adhesion, surface recognition, bio-mineralization, and others. As cell surface components are suspected of being very tenuous and sensitive to chemical treatment, cell surface ultrastructure components have been characterized by electron microscopy using a freeze substitution preparation. Such experiments have revealed new features of the cell surfaces, such as the external fringe that had not previously been observed using conventional methods (4). However, cell surfaces are also suspected of being sensitive to dehydration. For this reason, spatially resolved in situ direct observation of the organism in aqueous solutions would represent a significant advance, as a local characterization of the structural properties of bacterium-water interfaces lead to a generalized physicochemical understanding of bacterium-mediated mechanisms implied in many environmental processes.

Although atomic force microscopy (AFM) was originally introduced as a high-resolution imaging device, it can also be used to interact forces exerted on a cantilever in aqueous solutions. Many reviews about the theoretical and experimental backgrounds of such methods have been published and illustrate the numerous parameters that can be measured using force spectroscopy (e.g., references 9 and 12). The investigation of biological cells by AFM provides fundamental insights regarding long-range surface interactions and mechanical properties of cell surfaces from the interaction forces between the AFM probe (classical or modified tip, bacterial probe) and such surfaces (1, 3, 6, 8, 14, 16, 24, 26, 27, 30, 31).

Despite the numerous studies devoted to the microscopic quantification of force curves, the dynamic of the cell envelope in aqueous media is still difficult to elucidate. In this context, the above-described studies were aimed at describing the effects of pH on surface properties of *Shewanella putrefaciens*, probing in situ the AFM force curves on a nanometer scale. This model bacterium is a gram-negative facultative anaerobe and is considered to be one of the most efficient and versatile dissimilatory metal-reducing microorganisms (15). A great deal of research has focused on the ability of microorganisms like *Shewanella* to reductively transform iron oxyhydroxides (7, 11, 18, 21). However, few studies have been conducted on the physicochemical surface properties of *Shewanella* cells to help understand mechanisms at bacterium–aqueous-solution interfaces (7). Therefore, this paper compares force curves of *S. putrefaciens* at two pH values (4 and 10) through the natural adsorption of cells onto flat and noncoated inert polystyrene substrates. In order to closely preserve as much as possible the ultrastructures of the bacterial complex surfaces, the immobilization of bacteria was successfully carried out after the incubation stage under controlled physicochemical conditions to achieve bacterial adhesion. Yet, rod-shaped bacteria were im-

* Corresponding author. Mailing address: Laboratoire de Chimie Physique et Microbiologie pour l’Environnement, UMR 7564, CNRS, Université Henri Poincaré, Nancy 1, 405 rue de Vandœuvre, F-54600, Villers-lès-Nancy, France. Phone: 33 (0)3 83 68 52 39; Fax: 33 (0)3 83 27 54 44. E-mail: gaboriaud@lcpm.cnrs-nancy.fr.
mobilized on polymer-coated substrates that may promote structural rearrangements in the bacterial cell surface structure (25). However, the choice of such extreme pH values, of acidic versus basic media, was initially motivated to assess the repercussions of pH stress on bacterial surface properties (and not on physiological activity) usually addressed in macroscopic surface charge measurements, such as potentiometric titrations or electrophoretic mobility measurements. Nevertheless, the extrapolation of surface properties analyzed from immobilized bacteria to planktonic cells might be considered with caution.

The reference strain *Shewanella putrefaciens* CIP 8040 (Collection Institut Pasteur, Paris, France) corresponding to ATCC 8071 was primarily isolated from the surface of tinted butter (28). Cells were aerobically grown on a solid medium (plate count agar; BioMérieux 51019) for a 48-h incubation period at 30°C. Nutritive broth (100 ml of Trypticase soy broth; BioMérieux 51072) was seeded with a 5-ml bacterial suspension (absorbance = 0.500 ± 0.025; L = 600 nm). These precultures were stopped after 7 h, at the end of the middle of the exponential growth phase. Cultures were then grown out in 1.5-liter batch reactors initiated with 10 ml of the preculture at an optical density of 0.25 ± 0.10 at 600 nm. The 1-liter cultures were mixed at 150 rpm and 30 ± 0.5°C. The cells were harvested after 24 h of growth by centrifugation (10 min at 10,000 × g). Two washes were performed with KNO₃ solution (10⁻¹ M, pH 7) by centrifugation at 10,000 × g for 10 min. The washed cell pellets were then dispensed in a solution at a given pH in KNO₃ (0.1 M) and incubated at 20 ± 2°C in a polystyrene dish (30-mm diameter, reference no. 306; Caubere Inc., France) overnight (14 h). Since bacterial adhesion increases as a function of time and regardless of a pH range between 3 and 11, the choice of 14 h was a good compromise for sufficient retraction to be obtained from zero deflection on the approach curve. This assumption implies negligible long-range surface forces. Under our experimental conditions, the high ionic strength (0.1 M) decreases commensurately the electrostatic contributions of surface charges. In fact, complementary electrophoresis experiments of bacterial suspensions under the two pH conditions employed here demonstrated low surface charges and no significant pH dependence (zeta potential < −20 mV). Moreover, the retraction curves showed no adhesion between tip and bacteria, indicating the absence of large fibrous polymers on the bacterial surfaces (not shown).

Thus, the force curves, recorded at different pH values (Fig. 1), essentially may correspond to nano-indentation of the AFM tips into bacterial envelopes. Independent of pH, such curves exhibit two common features, specifically, a nonlinear domain at high loading forces (S > 0.5 nN) and a practically linear domain at low loading forces (S > 0.5 nN). The increase in pH significantly changes the nanometric range of these two mechanical regimes, while the loading forces that correspond to the transition between the two regimes were similar for the two pH values: 0.58 nN (pH 4.0) and 0.54 nN (pH 10.0). This suggests that the mechanical transition between the two regimes is probably associated with the same structural compression features. To extract mechanical properties, the local spring constant of bacteria was determined from the slope of the linear portion of force curves using equation 1, yielding to bacterial spring constants of 0.05 N/m and 0.02 N/m for pH 4.0 and 10.0, respectively. These values are in the same order of magnitude as one reported in previous studies conducted with *Escherichia coli* strains (27) (kₛ = 0.04 N/m) and close to the value obtained by Yao et al. (31), who examined *Pseudomonas aeruginosa* in growth medium (kₛ = 0.02 N/m). Furthermore, Arnoldi et al. (2, 3) theoretically demonstrated that the spring constant of bacteria increases with its turgor pressure. The mechanical behavior of bacteria under high loading forces, typically 0.5 nN, could then be correlated with their turgor pressure from the spring constant. Therefore, the increase in the calculated bacterial spring constants at higher pHs suggests that the turgor pressure is about two times higher at pH 4 than at pH 10.

Notice that such measurement of the bacterial spring con-
stant considers the two mechanical regimens as two independent processes, and the eventual contribution of the nonlinear regimen to the linear part is not taken into account. In order to improve this description, force curves were converted into nano-indentation curves from the difference at constant force loading between respective piezo displacement forces measured on a stiff surface and on the deformable bacterial surface, (for details, see references 20 and 24). Figure 2a and b depict the loading force versus indentation depth of bacteria for the two pH values. These curves are fit very well with the combination of the Hertz model for the nonlinear part and a Hook’s law spring. From these analyses, Young’s modulus of the external layers of bacteria at the two pH values clearly showed the increase of stiffness by decreasing pH (0.21 MPa for pH 4 and 0.037 MPa for pH 10). Such a result indicates that the nonlinear regimen at pH 4 is about five times stiffer than at pH 10. Concerning the linear part, the bacterial spring constant demonstrated the same trend with regard to pH dependence as was previously determined with force curves using equation 1 but with values two times lower. This difference in the bacterial spring constant’s values following the fitting procedure (equation 1 or 2) is fully in line with the obvious contribution of both mechanical regimens at a high loading force (up 0.5 nN).

Thus, such fits on the whole indentation curves allowed us to quantify the nanomechanical properties of the two regimens but also their ranges. In fact, the onset of the linear contribution to the loading force could be considered a specific distance in relation to the structural features of the bacterial envelope. These distances were estimated in Fig. 2 close to 60 nm (pH 4) and 155 nm (pH 10). Such a significant increase in the nanometric range by raising the pH was also clearly observed using the first approach (linear portion of force curves) (Fig. 1). In this case, the contribution of the cantilever deflection to the distance that corresponded to the piezo displacement has to be subtracted from values measured on force curves. Such calculation yielded to 62 nm (pH 4) and 136 nm (pH 10) (Fig. 1).

Interestingly, both fitting approaches provided similar nanometric ranges for the transition between the two regimens at the two pH values, while the bacterial spring constants were largely overestimated using the slope of force curves (equation 1) mainly due to the added contribution of nonlinear behavior.

With respect to the structure of the surface layers of gram-negative bacteria, these two mechanical regimens could be correlated to the ultrastructure of the bacterial envelope. As previously demonstrated in the literature (2, 3), the linear regimen is induced by progressive compression of the plasmic membrane, maintained by bacterial turgor pressure. Concerning the nonlinear regimen, the mechanical deformation is thus associated to the progressive compression of the cell wall structure. Therefore, this interpretation indicates that the thickness of the cell wall increased from ~60 nm to ~140 nm and softened up as the pH rose from 4 to 10. It is important to point out that if we considered the nonnegligible contribution of the bacterial turgor pressure to the nonlinear regimen, the differences in thickness should be accentuated due to higher bacterial turgor pressure at pH 4 compared to that at pH 10. The constant transition loading force (~0.5 nN) observed for the two pH values is in favor of a negligible contribution...
because, otherwise, such a value should be dependent on the pH also.

As the retraction curves did not present characteristic negative adhesion signatures of polymeric brush chains as observed previously (8, 22), a change of LPS conformations due to intermolecular repulsive electrostatic forces could not be related to the increase in thickness of the cell wall. In fact, previous investigations of several Shewanella species demonstrated the presence of polymeric fringe structures ranging from 20 to 130 nm in thickness depending on the species (13). Thus, the nonlinear behavior observed on force curves could be interpreted in terms of the presence of such a specific polymeric structure. In this case, the increase in the nanometric range was probably associated with the swelling of the polymeric fringe due to higher intermolecular repulsive forces (increasing void spaces) and/or the cytoplasm to the periplasmic space caused by higher electrostatic repulsive forces between the inner part of the outer membrane and the outer part of the cytoplasmic membrane.

In summary, the results of this study suggest that AFM force curves can be used to probe the influence of environmental parameters, such as pH, on the mechanical surface properties of bacteria at a nanometric scale. The different mechanical regimens identified on average force curves demonstrated that the external layers of the bacteria contribute mainly to nonlinear forces and that the turgor pressure of the cytoplasm corresponds to linear forces.

We thank Guy Jeannesson and Christine Gérard for their tireless efforts to optimize physicochemical conditions to observe bacterial adhesion. We also thank Raz Jelinek and Laurent Michaud for their critical readings of the manuscript. We thank Henri Poincaré University (Nancy, France) for providing the BQR 2001 grant and the RIESE 2000 grant. Furthermore, we thank Jean-Claude Block and anonymous reviewers for critical review of the manuscript and helpful comments.

**REFERENCES**


---

**FIG. 2.** Mean nano-indentation curves obtained under the two pH conditions, pH 4 (a) and pH 10 (b), for S. putrefaciens cells at 0.1 M KNO3. The curves were fitted by summing the contributions of the Hertz model (dashed line) and linear behavior with the bacterial spring constant (dotted line) to reproduce the complete indentation curves as the solid line (equation 2). Best fitting was achieved for an $E$ of 0.21 MPa and a $k_E$ of 0.022 N/m (a) and for an $E$ of 0.037 MPa and a $k_E$ of 0.01 N/m (b). The onset of the linear regime (dotted line) corresponds to a loading force of 0.48 nN at an indentation of 60 nm for pH 4 and of 0.53 nN at an indentation of 155 nm for pH 10.

**FIG. 3.** Schematic view of the dynamic of the cell envelope in response to a change in pH from 4 to 10 (not drawn to scale). OM, outer membrane; IM, inner membrane.


