# Single-Molecule Analysis of *Pseudomonas fluorescens* Footprints

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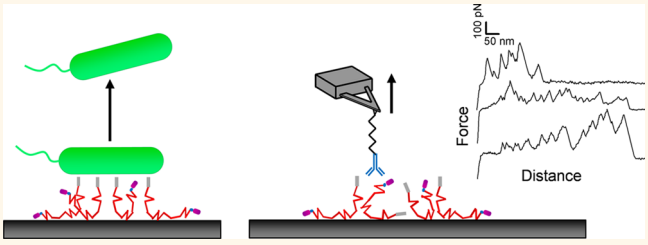
**ABSTRACT** Understanding the molecular mechanisms of bacterial adhesion and biofilm formation is an important topic in current microbiology and a key in nanomedicine for developing new antibacterial strategies. There is growing evidence that the production of extracellular polymeric substances at the cell–substrate interface plays a key role in strengthening bacterial adhesion. Yet, because these adhesive polymers are available in small amounts and are localized at interfaces, they are difficult to study using traditional techniques. Here, we use single-molecule atomic force microscopy (AFM) to functionally analyze the biophysical properties (distribution, adhesion, and extension) of bacterial footprints, that is, adhesive macromolecules left on substrate surfaces after removal of the attached cells. We focus on the large adhesin protein LapA from *Pseudomonas fluorescens*, which mediates cell attachment to a wide diversity of surfaces. Using AFM tips functionalized with specific antibodies, we demonstrate that adhesion of bacteria to hydrophobic substrates leads to the active accumulation of the LapA protein at the cell–substrate interface. We show that single LapA proteins left on the substrate after cell detachment localize into microscale domains corresponding to the bacterial size and exhibit multiple adhesion peaks reflecting the adhesion and extension of adsorbed LapA proteins. The mechanical behavior of LapA-based footprints makes them ideally suited to function as multipurpose bridging polymers, enabling *P. fluorescens* to attach to various surfaces. Our experiments show that single-molecule AFM offers promising prospects for characterizing the biophysics and dynamics of the cell–substrate interface in the context of bacterial adhesion, on a scale that was not accessible before.




**KEYWORDS:** cell adhesion · polymer footprints · AFM · single molecules · LapA adhesin · *Pseudomonas fluorescens* · biofilms

In the natural environment, most bacteria are not singly isolated but are attached to surfaces in microbial communities called biofilms.[1,2] Bacterial adhesion and biofilm formation have important consequences which may be either beneficial, such as in biotechnological and environmental applications, or detrimental, such as in industrial systems and in medicine. The formation of a bacterial biofilm involves several steps: conditioning of the substrate by adsorption of macromolecules, transport of the cells toward the substrate, initial adhesion through molecular interactions at the cell–substrate interface, strengthening of cell adhesion through the production of extracellular polymeric substances (EPS), and multiplication of attached cells. The production of EPS by bacteria has been observed by electron microscopy since the early 1970s.[3,4] In their pioneering work, Marshall and colleagues suggested that the production of adhesive polymers during the initial adhesion stage results in irreversible adhesion, by bridging the cell and substrate across the repulsion barrier.[3] Until now, bacterial polymers have been essentially characterized after isolation from culture supernatants or from the cell surface. Yet, it is unclear whether these macromolecules are directly involved in the establishment of the initial adhesive contacts, or whether they play other roles, such as mediating cell–cell adhesion or contributing to the formation of the biofilm matrix.[5,6]

Bacterial footprints are adhesive biopolymers left on substrate surfaces after removal of the attached cells using shear forces, sonication, or enzyme treatment.[5,7,8] Because they accumulate at the cell–substrate interface and they are available in small amounts, these bridging polymers are very difficult to study. Footprints were first observed by Marshall *et al.* using electron microscopy.[3] Two decades later, footprints left on polystyrene after detachment of marine bacteria were examined by scanning and transmission electron microscopy and characterized

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Received for review November 24, 2013 and accepted January 20, 2014.

**Published online January 23, 2014**
**10.1021/nn4060489**

using lectins combined with a fluorescent protein stain.[5] Along the same line, microbial footprints of *Pseudomonas aeruginosa* cells attached to hydrophobic and hydrophilic substrates were analyzed with fluorescently labeled lectin probes. Footprint composition varied, depending on cell physiology and substratum surface chemistry, suggesting that substratum properties affected the cell surface structures of attached organisms.[9] Although valuable, these studies do not provide information on the biophysical properties and molecular interactions of the adhesive polymers. Hence, there is much interest in complementing traditional assays with new techniques capable of analyzing the interaction forces of microbial footprints at high resolution.

*Pseudomonas fluorescens*, a prominent example of a model organism used for studying biofilm formation, attaches to a wide diversity of surfaces *via* the large adhesin protein LapA.[10−15] LapA is a ∼520 kDa protein composed of an N-terminal region containing the LapG cleavage site, followed by 37 repeats each of ∼100 amino acids and a C-terminal region composed of a Calx-$\beta$ domain, a von Willebrand factor type A (vWA) domain, six repeats-in-toxins (RTX), and a type 1 secretion system signal (Figure 1a).[14] While the multiple repeats of LapA are believed to mediate cell adhesion,[11,15] little is known about the molecular mechanism underlying this process. LapA at the cell surface is regulated by the LapD−LapG signaling system that allows precise control of cell attachment and subsequent biofilm formation.[16−18] Inorganic phosphate ($P_i$) is a key environmental signal controlling LapA exposure.[13] In high-$P_i$ concentration conditions, LapA is exported from the cytoplasm by the ABC transporter encoded by the *lapEBC* genes and accumulates at the cell surface.[11] By contrast, in low-$P_i$ conditions, LapD-mediated inhibition of the LapG protease is relieved, and the LapG protease cleaves the N-terminus of LapA.[13,17,19,20] The proteolytically processed LapA is then released from the cell surface, leading to cell surface detachment and loss of biofilm.[11,18,20]

Although LapA's role in biofilm formation has been studied in some detail,[11−13] we know little about the molecular mechanism by which this protein mediates cell adhesion. Specifically, the extent to which LapA accumulates actively at the cell−substrate interface to strengthen adhesion is unclear, owing to the paucity of appropriate high-resolution probing techniques. To tackle this issue, bacterial footprints remaining on hydrophobic substrates after adhesion of *P. fluorescens* are analyzed using single-molecule atomic force microscopy (AFM).[21,22] The use of several mutant strains demonstrates that adhesion of *P. fluorescens* leads to the local accumulation of LapA on the substrate surface. Footprint proteins from wild-type (WT) *P. fluorescens* display multiple adhesion peaks with extended rupture lengths that are likely to be critical for strengthening cell adhesion.

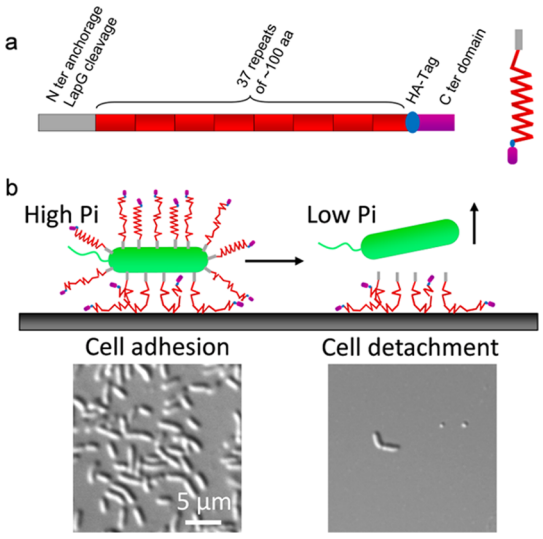


**Figure 1. Phosphate concentration controls *P. fluorescens* surface adhesion. (a) Primary structure of LapA with the N-terminal region containing the LapG protease target sequence, the 37 repeated hydrophobic sequences each of 100 amino acids forming the largest part of the protein, the HA tag for single-molecule detection, and the C-terminal domain. (b) Schematic showing how the $P_i$ concentration controls adhesion and detachment of WT *P. fluorescens* and corresponding DIC optical microscopy images. In high-$P_i$ conditions, LapA accumulates on the cell surface, leading to cell attachment, while under low-$P_i$ conditions, cells detach as a result of LapA cleavage by LapG.**

## RESULTS AND DISCUSSION

**Changes in Environmental Conditions Lead to Cell Detachment.** To analyze LapA footprints, WT *P. fluorescens* was incubated with hydrophobic model substrates for 8 h, and then detachment was stimulated by reducing the $P_i$ concentration. The use of alkanethiol monolayers as a model substrate allowed us to obtain smooth, homogeneous, and chemically defined surfaces with controlled hydrophobicity. In medium with high-$P_i$, LapA accumulates at the cell surface, thereby promoting adhesion, while in low-$P_i$ conditions, the adhesin is released from the cell surface, preventing cell adhesion.[13] Consistent with this behavior, microscopic adhesion assays demonstrated that incubation of the substrates with WT bacteria in high-$P_i$ conditions promoted cell adhesion (surface coverage of ∼25% after 8 h), while further incubation in low-$P_i$ conditions strongly reduced adhesion (surface coverage of ∼1% after 8 h) (Figure 1b). These data confirm that changes in $P_i$ conditions lead to cell detachment, thus providing a physiologically relevant means to generate bacterial footprints.

**AFM Unravels the Biophysical Properties of Bacterial Footprints.** Does *P. fluorescens* leave adhesive footprints on the substrate after cell detachment? To answer this question, substrates to which bacteria had adhered (high-$P_i$) and subsequently detached (low-$P_i$), were analyzed using AFM (Figure 2a,b). Topographic imaging of the substrates with silicon nitride tips revealed

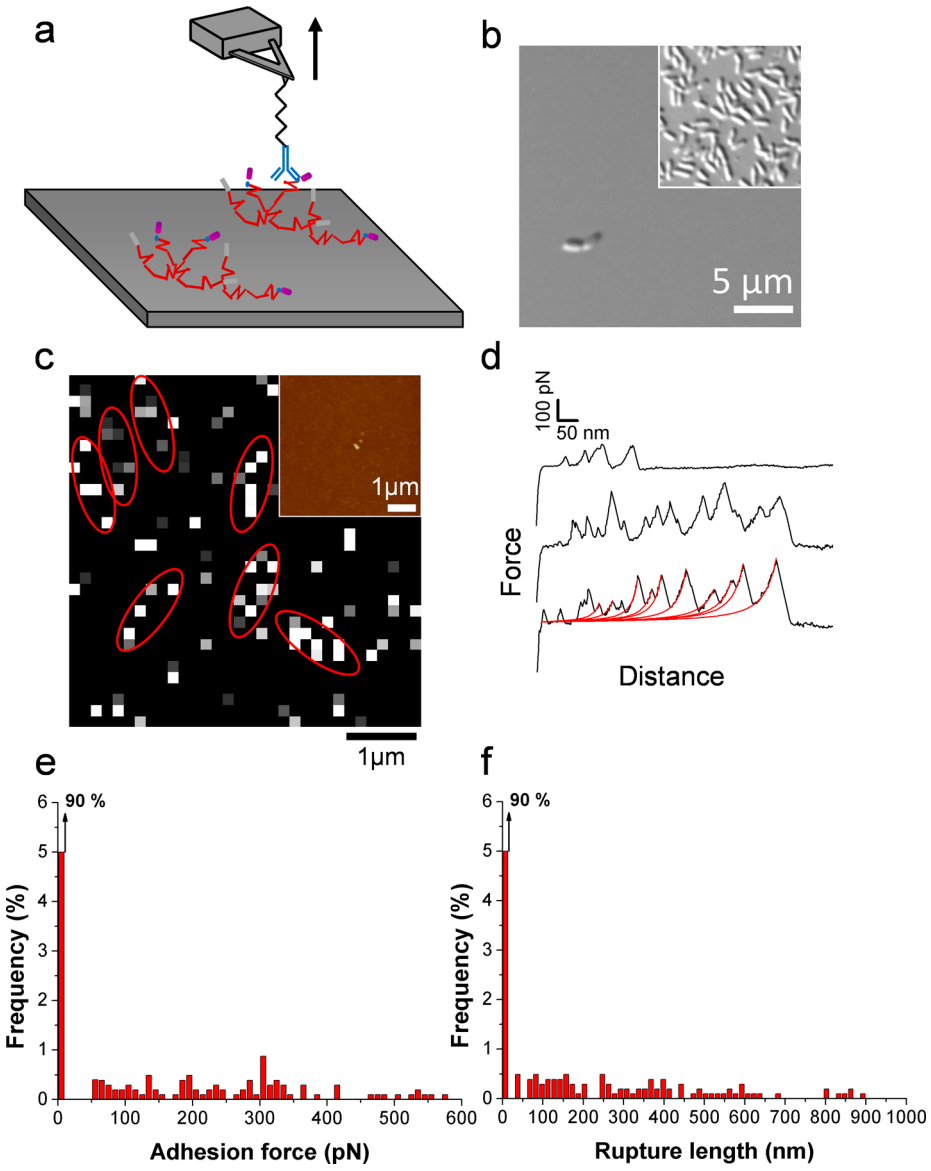


**Figure 2. AFM deciphers the biophysical properties of the LapA-based bacterial footprints. (a) Single-molecule AFM was performed on hydrophobic substrates after *P. fluorescens* WT cells adherence (8 h incubation in high-$P_i$ medium), then subsequent detachment (8 h incubation in low-$P_i$). AFM tips functionalized with anti-HA antibodies were used to detect, localize, and mechanically analyze single HA-tagged LapA adhesins left on substrates after cell detachment. (b) Representative optical microscopy (DIC) image of a substrate following cell detachment (inset: image of a substrate before cell detachment). (c) Adhesion force map (5 $\mu$m $\times$ 5 $\mu$m; *z* range = 300 pN) recorded in buffer between an anti-HA tip and a hydrophobic substrate following detachment of WT *P. fluorescens*. Bright pixels document the detection of single LapA proteins. Red ellipses suggest that the adhesins are organized into microscale domains (we define a microdomain as a group of at least 5 pixels making a domain of at least 1 $\mu$m). The inset is a contact mode deflection image of the substrate surface recorded with a silicon nitride tip. (d–f) Corresponding adhesive force curves (d), adhesion force (e), and rupture length (f) histograms (*n* = 1024). Red lines on the bottom curve correspond to WLC fits (see text for details). Similar data were obtained in multiple experiments using different tips and cell cultures.**

a smooth surface without aggregates or cell debris, meaning that cell detachment *via* low-$P_i$ did not significantly change the substrate surface topography (Figure 2c, inset). AFM tips functionalized with monoclonal anti-HA antibodies were then used to detect LapA proteins containing an HA tag located directly after the repeat regions (Figure 1a). Using spatially resolved single-molecule force spectroscopy (SMFS), we mapped and functionally analyzed single LapA molecules that were putatively left on the substrate surface. Figure 2c–f shows the adhesion force map, the representative force curves, and the histograms of adhesion forces and rupture lengths recorded between the antibody tip and the substrate, respectively. In ~10% of the cases, force curves showed multiple force peaks of 50–600 pN magnitude and 50–900 nm rupture length that we attribute to LapA adhesive interactions (see Figures 3 and 4). The measured ruptures (<900 nm) were shorter than expected for fully stretched LapA proteins (~1875 nm), suggesting

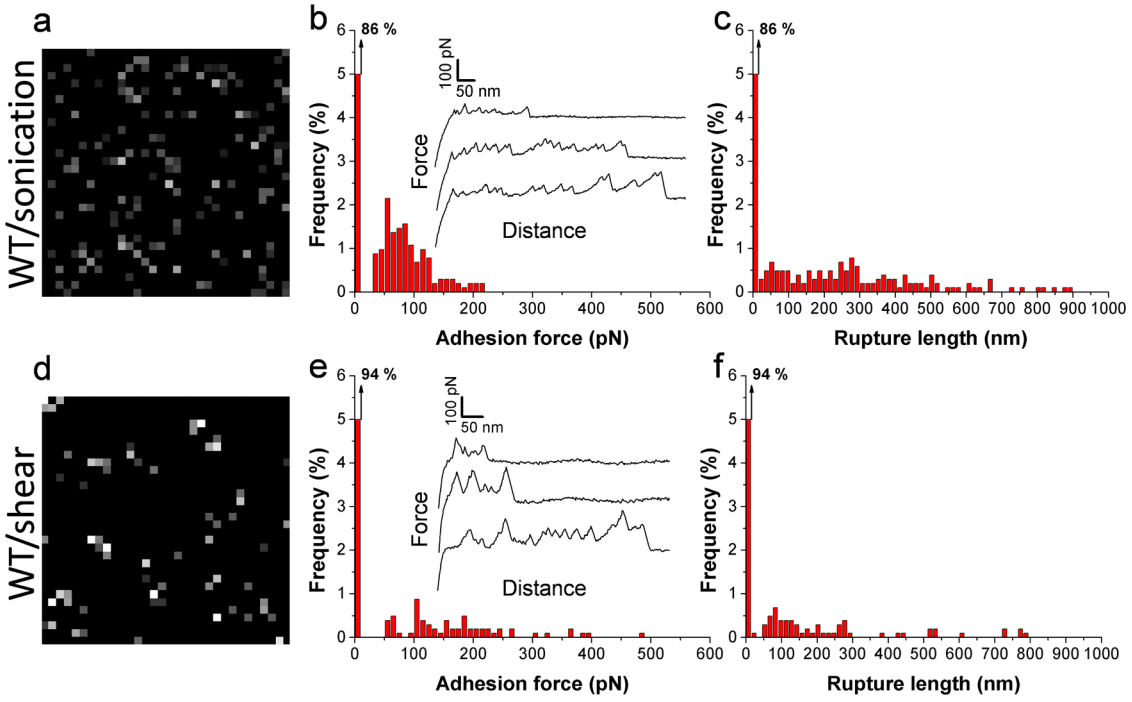


**Figure 3. Influence of sonication and mechanical shear on the biophysical properties of LapA-based footprints. (a,d) Adhesion force maps (5 $\mu$m $\times$ 5 $\mu$m; *z* range = 300 pN), (b,e) corresponding adhesion force histograms together with representative force curves, and (c,f) rupture length histograms recorded between an anti-HA tip and hydrophobic substrates following adhesion (8 h in high-$P_i$) then detachment by sonication of WT *P. fluorescens* (a−c), or following adhesion (8 h in high-$P_i$), detachment (8 h in low-$P_i$), and mechanical shear of WT *P. fluorescens* (d−f).**

that all repeats were not unfolded and/or that the proteins did not desorb completely upon pulling. Interestingly, ∼5% of the curves showed well-defined force peaks of 200−350 pN magnitude and 300−600 nm rupture lengths that were well-fitted by the worm-like-chain (WLC) model (Figure 2d, red lines[23,24]) using a persistence length $l_p$ of 0.4 nm: $F(x) = k_bT/l_p[0.25(1 - x/L_c)^{-2} + x/L_c - 0.25]$, where $L_c$ is the contour length of the molecule, $k_b$ is the Boltzmann constant, and $T$ the absolute temperature. As these signatures were never observed for mutant strains altered in LapA secretion or release (see below), we believe they represent the stretching of LapA proteins. Although we believe most signatures reflect single-molecule interactions, we cannot exclude that some of them are due to multiple molecular interactions. The contour length increments, $\Delta L_c$, were found to vary from 20 to 100 nm (from $n > 100$ peaks), thus without showing equally spaced force peaks. This lack of regular unfolding pattern is in apparent contrast with the multimodular nature of LapA (37 repeats each of ∼100 amino acids), therefore suggesting strongly that LapA proteins that are adsorbed on the substrate are denatured. Notably, the adhesion maps revealed heterogeneous distributions of adhesive events (Figure 2c, red ellipses). A substantial fraction of adsorbed LapA proteins seemed to be concentrated into elongated microdomains with an average length of 0.85 ± 0.2 $\mu$m ($n$ = 40 from five different maps obtained using three independent cultures), which is slightly smaller than the bacterial cell length (1.7 ± 0.4 $\mu$m, $n$ = 50). This analysis strongly suggests that the adhesin has accumulated at the specific locations of the attached cells, thus yielding polymeric footprints after cell detachment. To support this view, substrates were analyzed after detaching the cells by sonication rather than by environmental changes (Figure 3a−c). After sonication, ∼14% of the force curves showed adhesion events that featured multiple force peaks and extended ruptures and were organized into microscale domains. On close examination, force profiles exhibited force peaks that were weaker and less-defined than after low-$P_i$, suggesting that protein conformations are further altered upon sonication. Accordingly, these results demonstrate that, upon cell detachment, *P. fluorescens* leaves strongly adhesive bacterial footprints on the substrate, which are composed of LapA proteins.

**LapA-Based Footprints *versus* Cell Surface LapA.** We next asked whether WT LapA proteins that accumulate in the footprints and on the cell surface feature similar mechanical properties. Optical microscopy images showed that a few cells (∼1% coverage) remained attached after incubation in low-$P_i$ conditions (Figure 2b). We therefore probed these attached cells with single-molecule AFM to determine the biophysical properties of cell surface LapA (Figure 4a,b). Figure 4b−d shows the adhesion force map, the adhesion force histogram, and rupture length histogram with

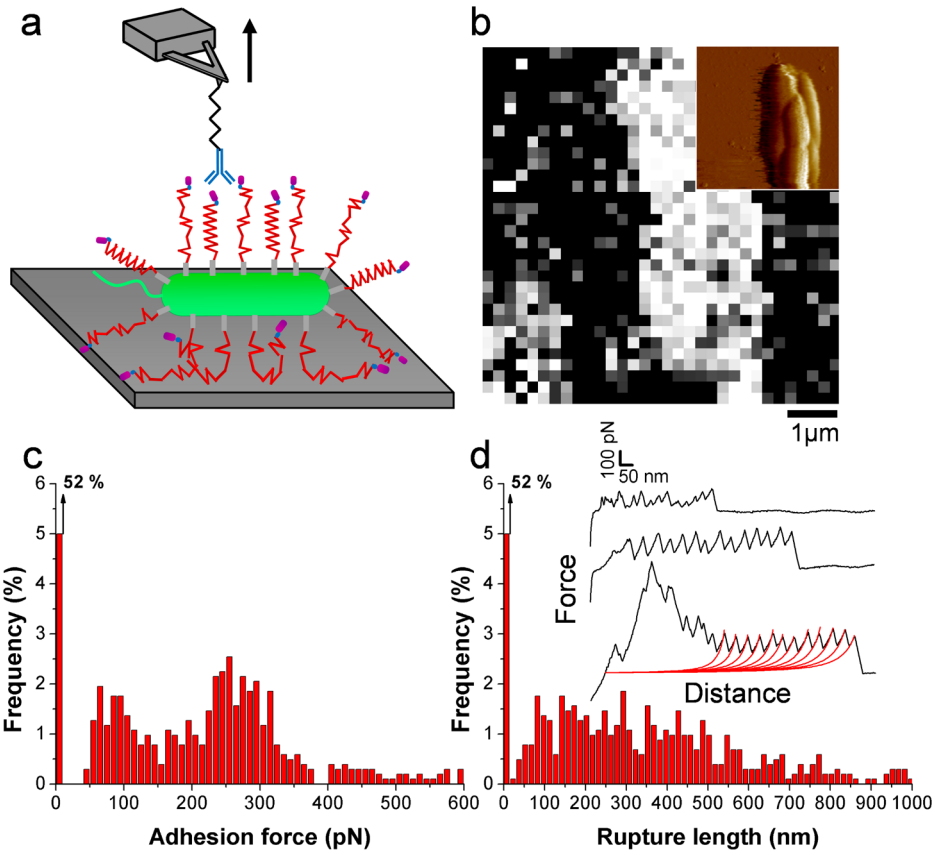


**Figure 4. AFM analysis of LapA on adhered bacteria. (a) Anti-HA tips were used to map and functionally analyze HA-tagged LapA produced on the surface of adhering WT *P. fluorescens* bacteria, after incubation 8 h in high-$P_i$ and 8 h in low-$P_i$. (b) Adhesion force map (7 $\mu$m × 7 $\mu$m; *z* range = 300 pN) recorded in buffer between an anti-HA tip and a small aggregate of WT *P. fluorescens* adhering on a hydrophobic substrate. The inset shows the corresponding deflection image. Bright pixels document the detection of LapA proteins on the cell surface. Corresponding adhesion force (c) and rupture length (d) histograms ($n$ = 1024), together with representative force curves. Similar data were obtained in multiple experiments using different tips and cell cultures.**

representative force curves, respectively, obtained between an anti-HA tip and a small aggregate of WT cells adhering on a hydrophobic substrate. Most curves recorded on the cells showed multiple force peaks of 50−350 pN magnitude and 50−800 nm rupture length, indicating that LapA was exposed on the cell surface even in low-$P_i$ conditions. A substantial fraction of the curves (~30%) showed sawtooth patterns with well-defined, equally spaced force peaks of 300−400 pN magnitude and 400−800 nm rupture lengths. Force peak values were in the range of unfolding forces reported for $\beta$-fold domains, such as Ig and fibronectin domains in titin,[24] and were well-described by the WLC model (Figure 4d, red lines). These observations support the notion that sawtooth signatures originate from the sequential unfolding of the multiple LapA repeats. Yet, the change in contour length $\Delta L_c$ was 55.9 ± 8.9 nm (from $n$ > 100 peaks), which is larger than that expected for the unfolding of a single LapA repeat. Indeed, as an amino acid contributes 0.36 nm to the contour length of a fully extended polypeptide, we expect the full extension of a single repeat of ~100 amino acids to be ~36 nm. Such repeatable ~55 nm peak-to-peak distances were never observed on the footprint proteins, a finding that we attribute to the fact that adsorption of the adhesins on hydrophobic substrates leads to the partial unfolding of their hydrophobic domains.[15] Alternatively, association of LapA with the cell surface may help stabilize protein structure.

**Functional Analysis of Bacterial Footprints.** Next, we further investigated the functional role of bacterial footprints by studying the impact of mutant strains involved in LapA cell surface localization on the biophysical properties of the adhesive proteins (Figure 5). In the LapA- mutant strain, a single crossover knockout mutation disrupts the *lapB* gene, encoding the inner membrane component of the ABC transporter, resulting in a strain that produces the LapA adhesin, but the mutant is unable to transport LapA to the cell surface.[13] By contrast, the LapA*s* mutant is deleted for the *lapD* gene, resulting in the continuous export and release of the LapA protein as opposed to the adhesin remaining associated with the cell surface.[17] Consistent with earlier studies,[11,13,17,20] incubation of LapA- and LapA*s* cells in high-$P_i$ conditions led to very poor levels of cell adhesion, thus further incubation in low-$P_i$ conditions

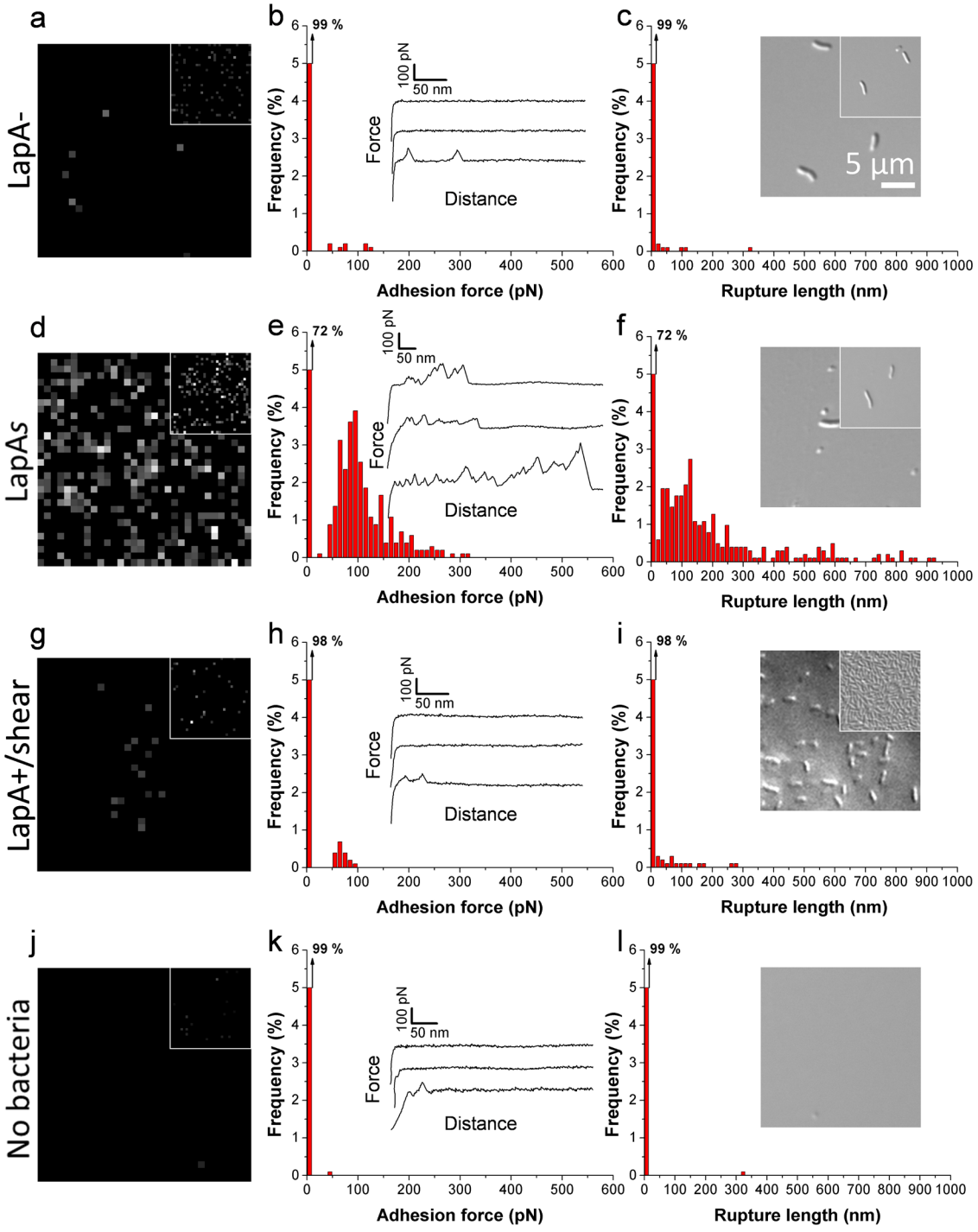


**Figure 5. Functional analysis of LapA-based footprints using bacterial mutants. (a,d,g) Adhesion force maps (5 $\mu$m × 5 $\mu$m; *z* range = 300 pN) recorded in buffer between an anti-HA tip and hydrophobic substrates following adhesion (8 h in high-$P_i$) and subsequent detachment (8 h in low-$P_i$ for a and d, and shear for g) of the *P. fluorescens* mutant strains LapA- (a), LapA*s* (d), and LapA+ (g). Insets correspond to maps recorded in independent experiments. Corresponding adhesion force histograms (*n* = 1024) with representative force curves (b,e,h) and rupture length histograms (*n* = 1024) together with optical images prior to (inset image) and after (main image) cell detachment (c,f,i). (j−l) Force data of a control experiment in which substrates where incubated with high-$P_i$ then low-$P_i$ media in the absence of bacteria. Similar data were obtained in multiple experiments using different tips and cell cultures.**

has essentially no effect (Figure 5c,f). However, the force signatures of the substrates after cell adhesion were markedly different for the two mutants. After adhesion of LapA- cells, adhesion events were rarely detected (<2%), consistent with the notion that LapA- cells do not expose LapA adhesins on their surface (Figure 5a−c). By contrast, incubation of the substrates with LapA*s* cells yielded force curves with numerous LapA signatures (~30%), that is, adhesive curves with multiple peaks and extended ruptures (Figure 5d−f).

As the *lapD* deletion causes the constitutive release of LapA, this behavior may be attributed to the adsorption of free LapA proteins from the solution. Yet, we note that LapA*s* and WT LapA force profiles differed, as LapA*s* force profiles result in weaker adhesions (50−150 pN) and shorter ruptures (50−300 nm). These data suggest that the mechanical behavior and conformational properties of LapA differ depending on whether LapA makes contact with the substrate while still attached to the cell surface or contacts the substrate after being released from the cell surface. We also note that LapA*s* force profiles obtained after incubation in high-$P_i$ conditions were very similar to those in low-$P_i$ conditions, confirming that low-$P_i$ treatment is not required for LapA release in this mutant.

The LapA+ mutant is a strain in which the *lapG* gene has been deleted, resulting in the accumulation of LapA on the cell surface and to a hyper-adherent biofilm phenotype.[11,25] In agreement with the increased amount of LapA on the cell surface, LapA+ cells showed a very strong adhesion phenotype in both high- and low-$P_i$ conditions (surface coverage of 90 and 60%, respectively). Therefore, to analyze the substrate surface after cell adhesion, the cells were removed by mechanical shearing (surface coverage of 5%, Figure 5i). In these conditions, adhesion events were rarely detected (<2%), demonstrating that LapA had not been released (Figure 5g−i), a finding consistent with the lack of the LapG protease in this strain, which is required for the proteolytic cleavage of LapA. Could mechanical shear induce the detachment of footprint material, that is, of adsorbed LapA proteins? To rule out this possibility, we showed that mechanical shear did not substantially influence the force signatures recorded on substrates that were incubated with WT cells in high- and low-$P_i$ conditions (Figure 3d−f). Finally, to further confirm the specificity of LapA detection, substrates were analyzed after incubation in high- and low-$P_i$ conditions in the absence of bacteria. As shown in Figure 5j−l, very few (∼1%) force curves presented adhesive events, confirming the specificity of the measurements. Together, these experiments demonstrate that the force signatures we recorded after adhesion of WT bacteria are indeed associated with the active accumulation of LapA adhesins at the cell−substrate interface. Hence, they represent true LapA-based bacterial footprints and do not originate from the direct contact with other cell surface proteins or from the passive adsorption of LapA from solution. We anticipate that the modular properties of LapA are of biological relevance as they are expected to strengthen cell adhesion by increasing the lifetime and energy of the cell−substrate adhesive bonds. While our data are consistent with the idea that we are assessing the properties of single LapA molecules in our analyses, we do acknowledge the possibility that LapA might be functioning as a multimer, although we currently have no evidence supporting such a model. Finally, an interesting question to address in future work is whether the biophysical properties of LapA are the same when bound to other substrates showing various degrees of hydrophobicity.

## CONCLUSIONS

The development of methods capable of analyzing the biophysical properties and molecular interactions of the cell−substrate interface is critical to our understanding of cell adhesion processes. We have shown that single-molecule AFM provides direct and quantitative information on the adhesive and mechanical properties of bacterial footprints, thereby complementing traditional approaches (electron microscopy and fluorescence imaging). Application of the method to *P. fluorescens* footprints demonstrates that (i) adhesion of the bacteria to hydrophobic substrates involves the local accumulation of LapA adhesins at the cell−substrate interface; (ii) LapA proteins that are left on the substrate after cell detachment concentrate into microscale domains corresponding roughly to the bacterial size; (iii) while regular force peaks are observed if LapA is unfolded directly from the bacterial surface, random force peaks are detected if LapA is unfolded from the footprints on the substrate, probably due to denaturation of the protein or an alteration in structure upon release from the cell; and (iv) control experiments with different mutant strains demonstrate that the force signatures of footprint proteins are associated with the active accumulation of LapA adhesins at the cell−substrate interface, rather than from passive adsorption phenomena. We suggest that the production of adhesive proteins at the interface will play an important role in strengthening *P. fluorescens* adhesion and biofilm formation. The multimodular nature of LapA-based footprints provides a molecular basis for the ability of *P. fluorescens* to attach to a variety of surfaces in the natural environment. Finally, LapA-like proteins and the systems that regulate the cell surface localization of these adhesins are conserved in a number of bacterial pathogens, including *Vibrio cholerae*, *Legionella pneumophila*, and *Bordetella pertussis*,[16] thus the approaches outlined here can be applied to the study of these important disease-causing microbes on the diversity of surfaces (biotic and abiotic) with which they interact.

## METHODS

**Microorganisms and Growth Conditions.** The following *P. fluorescens* strains all expressing LapA containing three HA epitope tags after the 4093rd amino acid residue were used in this study: wild-type (WT) strain (SMC4798),[13] LapA+ mutant (SMC5207, Δ*lapG*) which overexpresses cell surface LapA due to loss of the

LapG protease,[25] LapA- mutant (SMC5164, *lapB*::pMQ89) which does not secrete the adhesin due to loss of function of the LapBCE transporter,[13] and LapA*s* mutant (SMC5152, △*lapD*) in which LapA is constitutively secreted due to loss of the LapG protease inhibitor, LapD.[17] Strains were cultivated overnight in lysogeny broth (LB) at 30 °C and shaken at 200 rpm. Gentamycin (30 $\mu$g mL$^{-1}$, Sigma) was added for LapA- cultures.

**Substrate Preparation.** For preparing hydrophobic substrates, glass coverslips coated with a thin layer of gold were immersed overnight in a solution of 1 mM 1-dodecanethiol (Sigma) then rinsed with ethanol and dried under $N_2$.

**Biofilm Assays and Optical Microscopy.** Hydrophobic surfaces were immersed in 24-well plates containing 2 mL of K10T1 (high-$P_i$ medium).[26] For cell adhesion and biofilm formation, 25 $\mu$L of bacteria from overnight LB culture (30 °C, 200 rpm) was added to 2 mL of K10T1 medium (high-$P_i$) in each well, and the plate was incubated 8 h at 30 °C at 200 rpm. After 8 h, surfaces were gently transferred in plates containing 2 mL of K10T$\pi$ medium (low-$P_i$) and incubated 8 h at 30 °C at 200 rpm to promote cell detachment.[26] For optical images, surfaces were rinsed by three consecutive baths of fresh medium before or after incubation in K10T$\pi$ (low-$P_i$ medium) and directly imaged using an inverted optical microscope (Zeiss Axio Observer Z1) equipped with a Hamamatsu camera C10600. For LapA+ mutant, cells were detached after incubation in K10T$\pi$ (low-$P_i$ medium) by mechanical shearing using a 1 mL pipet. For sonication experiments, substrates were transferred in vials containing fresh medium after incubation in high-$P_i$ conditions and submitted to 30 s of sonication in a water bath sonicator (Branson Ultrasonic water bath 1510).

**Atomic Force Microscopy.** AFM measurements were performed at room temperature (20 °C) in low-$P_i$ medium using a Nanoscope VIII Multimode AFM (Bruker Corporation, Santa Barbara, CA) and oxide sharpened microfabricated $Si_3Ni_4$ cantilevers with a nominal spring constant of ~0.01 N m$^{-1}$ (Microlevers, Bruker Corporation). The spring constants of the cantilevers were measured using the thermal noise method (Picoforce, Bruker). After cell detachment, substrates were rinsed by three consecutive baths of fresh K10T$\pi$ medium (low-$P_i$ medium) and attached on sample pucks while avoiding dewetting. Substrates were first imaged in contact mode using bare tips and applying low forces (~100 pN). For single-molecule imaging, AFM tips were functionalized with monoclonal anti-HA antibodies (200 $\mu$g mL$^{-1}$, HA.11 clone 16B12, Covance) using PEG-benzaldehyde linkers[27] and following the previously described protocol.[28] Prior to functionalization, cantilevers were washed with chloroform and ethanol, placed in an UV-ozone cleaner for 30 min, immersed overnight into an ethanolamine solution (3.3 g of ethanolamine into 6 mL of DMSO), then washed three times with DMSO and two times with ethanol and dried with $N_2$. The ethanolamine-coated cantilevers were immersed for 2 h in a solution prepared by mixing 1 mg of acetal-PEG-NHS dissolved in 0.5 mL of chloroform with 10 $\mu$L of triethylamine, then washed with chloroform and dried with $N_2$. Cantilevers were further immersed for 5 min in a 1% citric acid solution, washed in Milli-Q water, and then covered with a 200 $\mu$L droplet of PBS solution containing the probing proteins (0.2 mg mL$^{-1}$) to which 2 $\mu$L of a 1 M $NaCNBH_3$ solution was added. After 50 min, cantilevers were incubated with 5 $\mu$L of a 1 M ethanolamine solution in order to passivate unreacted aldehyde groups and then washed with and stored in buffer. Using an anti-HA-functionalized tip, adhesion maps were obtained by recording 32 × 32 force−distance curves on areas of 5 × 5 $\mu$m or 7 × 7 $\mu$m, calculating the adhesion force for each force curve and displaying the adhesive events as gray pixels. All force curves were recorded with a maximum applied force of 250 pN, using a constant approach and retraction speed of 1 $\mu$m s$^{-1}$.

*Conflict of Interest:* The authors declare no competing financial interest.

*Acknowledgment.* Work at the Université catholique de Louvain was supported by the National Fund for Scientific Research (FNRS), the Université catholique de Louvain (Fonds Spéciaux de Recherche), the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Programme), and the Research Department of the Communauté française de Belgique (Concerted Research Action). Y.D.is Research Director of the FNRS. This work was also supported by the National Science Foundation through grant MCB 9984521 to G.O.

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