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Atomic force and super-resolution microscopy support a role for LapA as a cell-surface biofilm adhesin of *Pseudomonas fluorescens*

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Abstract

Pseudomonas fluorescens Pf0-1 requires the large repeat protein LapA for stable surface attachment. This study presents direct evidence that LapA is a cell-surface-localized adhesin. Atomic force microscopy (AFM) revealed a significant twofold reduction in adhesion force for mutants lacking the LapA protein on the cell surface compared to the wild-type strain. Deletion of *lapG*, a gene encoding a periplasmic cysteine protease that functions to release LapA from the cell surface, resulted in a twofold increase in the force of adhesion. Three-dimensional structured illumination microscopy (3D-SIM) revealed the presence of the LapA protein on the cell surface, consistent with its role as an adhesin. The protein is only visualized in the cytoplasm for a mutant of the ABC transporter responsible for translocating LapA to the cell surface. Together, these data highlight the power of combining the use of AFM and 3D-SIM with genetic studies to demonstrate that LapA, a member of a large group of RTX-like repeat proteins, is a cell-surface adhesin.

Keywords

AFM; Adhesin; Biofilm; Weibull analysis; 3D-SIM

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1. Introduction

In natural settings, bacteria are commonly found in multicellular communities known as biofilms (Costerton et al. 1995). The transition between a planktonic state and a biofilm mode of growth is strictly regulated and is often required for pathogenesis or survival in hostile environments (Hall-Stoodley et al. 2004; Stanley and Lazazzera 2004). The initiation of biofilm formation is dependent on environmental factors as well as expression of intact flagella, pili, lipopolysaccharides and production of extracellular DNA, among other molecules (Flemming et al. 1998; O'Toole and Kolter 1998; Pratt 1998; Whitchurch et al. 2002). Protein adhesins necessary for irreversible surface attachment have been identified largely via genetic studies, but there has been little direct experimental support for these surface proteins mediating cell-surface interactions (Cucarella et al. 2001; Hinsä et al. 2003; Latasa et al. 2005; Martínez-Gil et al. 2010; Pratt and Kolter 1998; Syed et al. 2009; Theunissen et al. 2010).

P. fluorescens Pf0-1 requires the large protein LapA for stable surface attachment (Hinsä et al. 2003). Biochemical studies have revealed that the protein is exported to the cell surface by an ABC transporter, encoded by the *lapEBC* genes. LapA is present in the cytoplasm, localizes to the cell surface and is released into the supernatant (Hinsä et al. 2003; Newell et al. 2011). Cells which do not encode a fully functional LapA protein or those in which LapA transport to the cell surface is blocked are unable to initiate biofilm formation and achieve stable, “irreversible” binding to a large variety of surfaces, indicating a role for LapA as a biofilm adhesin (Hinsä et al. 2003).

In conditions unfavorable for biofilm formation (e.g. low P_i levels), the LapA protein is released from the cell surface by the periplasmic cysteine protease LapG, thus preventing attachment to surfaces (Newell et al. 2011). Under conditions favorable for biofilm formation, LapG no longer cleaves LapA and *P. fluorescens* is competent for stable surface attachment. Deletion of the *lapG* gene results in a hyper-adherent biofilm phenotype because LapA is not released from the cell surface, resulting in no detectable levels of LapA in the supernatant and a twofold increase in LapA on the bacterial cell surface (Newell et al. 2011).

The LapA protein has an estimated molecular weight of ~520 kDa and contains an extensive repetitive region consisting of 37 repeats of ~100 amino acids. Bioinformatics tools have predicted an N-terminal transmembrane region and several conserved motifs and domains at the C-terminus of the protein, namely Calx- β , von Willebrand factor type A (vWA), seven repeats-in-toxins (RTX) sequences, and a type I secretion system (T1SS) signal. With the exception of the Calx- β domain, the function of those motifs and domains in LapA has not been experimentally investigated. While in other proteins, the Calx- β domain has demonstrated involvement in calcium binding and regulation (Schwarz and Benzer 1997), deletion of the Calx- β domain in LapA does not impact biofilm formation or LapA localization (Boyd et al. 2012).

The *lapABCE* genes appear to be conserved among many environmental pseudomonads (*P. fluorescens*, *P. putida*, *P. chlororaphis*, *P. entomophila*), but are absent from pathogenic pseudomonads such as *P. aeruginosa* and *P. syringae* (Hinsä et al. 2003). The vast majority of these LapA proteins contain conserved domains, like Calx- β , as well as domains with a variable number of amino acid repeat sequences. Interestingly, large repeat surface proteins are not only constrained to the pseudomonads, but are relatively widespread in the microbial domain. Yousef and Espinosa-Urgel (2007) have classified these proteins into seven families based on phylogenetic similarities with the most prominent member of the group, namely AidA in *Magnetospirillum magneticum*, Bap in *Staphylococcus aureus* and *Staphylococcus epidermidis*, Bsp in *Bacillus* spp., Ebh in *S. aureus*, FhaL in *Bordetella*

pertussis, FhaB in *Xanthomonas axonopodis* pv. *citri*, and LapA in *P. fluorescens*. Common characteristics among most of these proteins are the presence of vWA, calcium binding, cadherin, RGD, hemagglutinin or leucine zipper domains and involvement in cellular adhesion and tissue colonization. Other widely studied large adhesins with characteristic amino acid repeat stretches include LapF, a protein expressed by *P. putida* that is required for late stages of biofilm formation (Martínez-Gil et al. 2010), SiiE in *Salmonella enterica*, which functions as an adhesin for epithelial cells (Gerlach et al. 2007), FrhA in *Vibrio cholerae*, which is associated with hemagglutination, adherence to epithelial cells, biofilm formation and chitin binding (Syed et al. 2009), the biofilm-promoting factor BpfA in *Shewanella oneidensis* (Theunissen et al. 2010) and RtxA in *Legionella pneumophila*, which has demonstrated involvement in adhesion and entry into macrophages and amoebae (Cirillo et al. 2002).

The function of specific domains and amino acid repeats in adhesion proteins, however, has often not been experimentally studied and their role as adhesins has only been inferred indirectly from genetic studies and biofilm assays (O'Toole et al. 1999). Atomic force microscopy (AFM) presents a sensitive tool to study bacterial adhesion molecules and the process of bacteria adhesion at the single cell level (Dufrêne 2002). AFM is increasingly used in biology with recent applications in differentiating normal from cancerous cells, studying pathogen-host interactions or correlating force of adhesion to bacterial virulence (Ivanov et al. 2011; Iyer et al. 2009; Ovchinnikova et al. 2012; Park et al. 2009). In this study, we utilized AFM, together with super-resolution 3D structured illumination microscopy (3D-SIM), to support the role of the LapA protein as a cell surface adhesin.

2. Materials and methods

2.1. Bacterial culture and harvesting

Bacterial strains were cultured overnight in 50 mL of lysogeny broth (LB) broth at 30°C and with shaking (250 rpm). Overnight cultures were diluted 1:100 in K10T-1 growth medium (50 mM Tris-HCl pH 7.4, 0.2% wt/vol tryptone, 0.15% vol/vol glycerol, 0.6 mM MgSO₄, and 1 mM K₂HPO₄) and incubated for an additional 6 h until reaching the exponential growth phase. Bacterial cells were harvested by centrifugation at 7,000 rpm for 10 min and washed once with saline (0.85% wt/vol NaCl in water). The wild-type strain analyzed here, designated SMC4798, has three HA epitopes inserted after residue 4093 of LapA, and as reported, the strain carrying this variant behaves like the parent, non-HA-tagged strain (Monds et al. 2007). All other strains studied here are a derivative of SMC4798. The other strains analyzed here have been reported: SMC5145 (*lapA*::pKO, Monds et al. 2007), SMC5164 (*lapB*::pMQ89, Monds et al. 2007), SMC5207 (Δ *lapG*, Newell et al. 2011) and SMC5678 (Δ CalX- β , Boyd et al. 2012).

2.2. Bacterial immobilization on glass slides

Glass slides were cleaned by sonication in 2% vol/vol RBS-35 (Thermo Fisher Scientific, Rockford, IL) for 10 min followed by rinsing with copious amounts of ultrapure water. Cleaned slides were rinsed with 100% methanol and immersed in 30% vol/vol 3-aminopropyltrimethoxysilane (Sigma-Aldrich, St. Louis, MO) in methanol for 20 min. Functionalized slides were rinsed with copious amounts of methanol and ultrapure water. Washed bacterial cells were resuspended in saline, supplemented with 3 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (Thermo Fisher Scientific, Rockford, IL) and 2.4 mM *N*-hydroxysulfosuccinimide (Thermo Fisher Scientific, Rockford, IL), and poured over the functionalized glass slides. The slides were agitated at 70 rpm for 2 h to promote bacterial attachment.

2.3. Atomic force microscopy

An MFP-3D Bio atomic force microscope (Asylum Research, Santa Barbara, CA) was used to measure the adhesive forces of the bacterial strains. Silicon nitride probes (DNP, Veeco Instruments Inc., Santa Barbara, CA) with resonance frequency of ~24 kHz and spring constant of ~0.12 N/m were calibrated before every experiment, and AFM optical sensitivity was determined in PBS on glass. Areas of 100 μm^2 of the glass slides were imaged at 0.5 Hz in the alternating contact mode under PBS to locate bacterial cells. On the average, 50 force curves were acquired on the center top of the cell from at least 10 bacterial cells from at least two independent cultures per strain examined. During force curve acquisition, a constant trigger threshold of 5 nN and surface delay of 1 s were used. The magnitude of all adhesion events was recorded and used for construction of a box plot. For Weibull analysis (van der Mei et al. 2010), only the maximum adhesion force from all recorded force curves was included in the analysis.

2.4. Super resolution microscopy

Bacterial cells in the mid-exponential growth phase were incubated with 5 $\mu\text{g/mL}$ FM4-64FX lipophilic styryl dye (Life Technologies, Grand Island, NY) for an additional 30 min. The cells were fixed in 4% (w/v) paraformaldehyde and permeabilized using 0.1% (v/v) Triton X-100 in PBS. Cells were blocked in 0.1% (w/v) BSA/0.1% Triton X-100 solution, incubated with rabbit anti-HA (1:5000, Abcam, Cambridge, MA) and goat anti-rabbit IgG Alexafluor-488-labelled antibodies (1:2000, Life Technologies, Grand Island, NY) and mounted on poly-L-lysine-coated Fluorodishes (World Precision Instruments, Sarasota, FL) with Vectashield (Vector Labs, Burlingame, CA) for microscopy.

Imaging was performed using a DeltaVision® OMX V3 3D-SIM System fitted with a Blaze SIM module (Applied Precision Inc, Issaquah, WA) as previously described (Strauss et al. 2012). Raw 3-phase images were reconstructed as previously described (Gustafsson et al. 2008; Schermelleh et al. 2008). Reconstructed images were rendered in 3D, with interpolation, using IMARIS v. 7.5 (Bitplane Scientific, Zurich, Switzerland).

2.5. Statistical analysis

Statistically significant differences ($P = 0.001$) between adhesion force measurements were determined using the Kruskal-Wallis one-way analysis of variance on ranks in SigmaPlot (Systat Software Inc., Chicago, IL). The two-sample Kolmogorov-Smirnov test with power $\alpha = 0.001$ was used to determine statistically significant differences between probability distributions.

3. Results and discussion

3.1. AFM supports a role for LapA as an adhesin

Previous genetic studies suggested that the LapA protein acts as an adhesin (Hinsa et al. 2003; Newell et al. 2011). Here we used AFM to directly measure the force of adhesion between the bacterial cell surface and the AFM probe. In AFM, a sharp (~10 nm in radius) tip is used to softly tap on the sample in order to obtain a topographical image of the surface. When the AFM tip is brought into contact with the sample, repulsive interactions generally arise between the tip and the sample, which are due to steric repulsion caused by bacterial molecules. However, once contact has been made between the bacterium and the tip, some of the bacterial molecules will stay adsorbed to the AFM tip due to intermolecular attractions. Thus, as the AFM tip is retracted from the surface, a force is required to separate the tip and the sample, and its magnitude is used to infer the force of adhesion of the bacterium to this abiotic substratum.

We first examined the adhesive properties of the wild-type strain grown under conditions that support biofilm formation. For cells grown under these conditions, the wild-type strain exhibited a median adhesion force to the silicon nitride AFM probe of 0.60 nN (Fig. 1A). We next tested two mutant strains lacking LapA on the cell surface. A strain with a single cross-over knockout mutation disrupting the *lapA* gene reduced the median force of adhesion approximately 2-fold compared to the wild-type strain, resulting in an adhesive force of 0.29 nN. Similarly, a single cross-over knockout mutation disrupting the *lapB* gene, resulting in a strain that maintains LapA in the cytoplasm but is unable to transport this protein to the cell surface (Hinsa et al. 2003), also reduced the median force of adhesion to a similar extent as mutating the *lapA* gene (~2-fold) to 0.34 nN (Fig. 1A). The reduction in adhesive force between the wild-type and either of these two mutants was significant; however no statistical difference was found in adhesion forces between the *lapA* and *lapB* mutants. These data suggest that production and transport to the surface of the LapA protein increases the adhesive properties of *P. fluorescence* Pf0-1.

We next assessed the effects of increasing surface-localized LapA levels on cell adhesion. We exploited our previous observation that deletion of the *lapG* gene, which codes for a LapA-targeted protease, results in a ~2-fold increase in cell-surface LapA and ~2-fold increase in biofilm biomass compared to the wild-type strain (Newell et al. 2011). AFM analysis of the $\Delta lapG$ m revealed a significant increase of the median adhesion force by approximately 2- fold to 1.32 nN (Fig. 1A). These results indicate that the adhesive force of *P. fluorescence* Pf0-1 is dependent on the levels of cell-surface-localized LapA and suggest a correlation between adhesion force, adhesin levels and biofilm biomass production.

Finally, as an additional control, we examined a LapA variant lacking the Calx- β domain ($\Delta Calx-\beta$). We recently reported that a strain deleted for the Calx- β domain showed no change in biofilm formation using our standard 96-well biofilm assay (Boyd et al. 2012). Consistent with this published finding, compared to a strain expressing the wild-type LapA protein, deletion of the Calx- β domain from the LapA protein led to a reduction in the force of adhesion from 0.60 to 0.47 nN, a difference that is not statistically significant (Fig. 1A). This corroborates our observations that the Calx- β domain in the LapA protein is not important for biofilm formation in our assay conditions.

AFM adhesion data inherently possesses a very large range and the data set is not necessarily well represented only by its median (Mei et al. 2009; Postollec et al. 2006). Instead, an alternative approach to present and compare adhesion results is by fitting a Weibull distribution to the data (van der Mei et al. 2010). Since this analysis requires that the measurements come from independent trials, only the maximum adhesion force from each acquired force curve is used for the analysis. A Weibull plot is then constructed and the data set can be discussed in terms of the scaling parameter, F_0 , and shaping parameter, m , of the distribution. The shaping parameter indicates whether the adhesion bond failure rate decreases ($m < 1$) or increases ($m > 1$) with the applied force and the scaling parameter determines the location of the peak in the probability density function for the distribution along the abscissa. The mean of the distribution, F_{mean} , which is related to F_0 through the shaping parameter and the gamma function may also be used for the comparison. This approach has been successfully used to analyze the reliability of bonded structures, joint fracture surface energy and defect distribution in ceramics and silicon wafers (Jonsson et al. 2001; Köhler et al. 2000).

The probability of bond failure between the bacterial cell and the AFM tip increases as a function of applied force for all strains (Fig. 1B). The probability of breaking bonds between

the *lapA* or *lapB* mutants and the AFM tip is high at low forces, while bonds with the $\Delta lapG$ mutant are most resilient among the strains studied.

The probability distribution functions for all data sets exhibit similar values for the shaping parameter of the distribution, $m \approx 1.1$ (Table 1). This value is relatively low compared to macroscopic fracture mechanics measurements (Khalili and Kromp 1991) and AFM measurements on abiotic surfaces (van der Mei et al. 2010) ($m = 5-20$), which indicates that the distributions have heavy tails, as expected. The similarity in the values for the shaping parameter and the gamma function ($\Gamma \approx 0.6$) between our measurements allows for comparison between the data sets based on the values of the scaling parameter, F_0 , and the mean of the distribution, F_{mean} . The *lapA* and *lapB* mutants exhibited the lowest F_0 values, at 0.183 and 0.190 nN, respectively, while the $\Delta lapG$ mutant had the highest recorded F_0 value of 1.303 nN (Table 1). These data indicate that greater applied force is required to break the adhesive bonds between the $\Delta lapG$ mutant and the AFM tip than bonds with either the *lapA* or *lapB* mutants. The $\Delta Calx-\beta$ mutant and the wild-type strain showed very similar F_0 values of 0.245 and 0.243 nN, respectively.

The means follow a similar pattern compared to the scaling parameter of the distribution and the median adhesion forces discussed earlier: the *lapA* and *lapB* mutant strains are least adhesive, as they do not possess LapA protein on the cell surface, while the $\Delta lapG$ mutant was more adhesive than the wild-type strain due to its increased LapA levels on the cell surface. Furthermore, the two-sample Kolmogorov-Smirnov test indicated no statistically significant difference between the *lapA* and *lapB* mutants, nor between the wild-type and the $\Delta Calx-\beta$ mutant, as was also confirmed in this and earlier studies (Boyd et al. 2012; Hinsä et al. 2003). Thus these results further demonstrate that the Weibull distribution describes the AFM adhesion data well (high goodness of fit values were obtained for most data sets) and the Weibull analysis can be used as a more comprehensive and robust means to report and compare these AFM data.

3.2. LapA is cell-surface-localized

The AFM results from above, and our previous fractionation studies (Newell et al. 2011), support the conclusion that in the wild-type strain LapA localizes to the cell surface, as would be expected for an adhesin. In order to directly visualize LapA at the cell surface, we employed a super resolution fluorescence microscopy technique, 3D-SIM. 3D-SIM uses multiple interfering bands of light that allow for better resolution than conventional fluorescence microscopy. Multiple cross-sections of the sample are imaged and reconstructed to reveal high-resolution images (Schermerle et al. 2008). Unless otherwise indicated, for the strains studied here, LapA contains internal HA tags (Monds et al. 2007, see Materials and methods for details), which are detected via an HA-specific antibody with an Alexafluor-488 conjugated secondary antibody (shown as green in Fig. 2). In the wild-type strain, LapA is observed in the cytoplasm and on the cell surface in both the center slice, an image through the middle of the cell, and in the maximum projection, a series of image sections stacked into one aggregated image (Fig. 1 top, left and right panels). No LapA is visualized by 3D-SIM in a control strain lacking HA tags within LapA (Fig. 1 bottom, left and right panels).

We next visualized the effects of increasing LapA levels on the cell surface. The 3D-SIM analysis reveals LapA on the cell surface in a $\Delta lapG$ mutant background, as was observed for the wild-type strain (Fig. 2, second from the top, left and right panels). We also noticed little or no LapA present in the cytoplasm of this strain using this method, but at this time we do not understand the significance of this finding. As we reported previously using western blot analysis, we can detect LapA in the cytoplasmic fraction of the $\Delta lapG$ mutant at levels similar to those observed for the wild-type strain (Newell et al. 2011).

Finally we visualized LapA in the *lapB* mutant strain, a strain that still produces cytoplasmic LapA, but is unable to transport this protein to the cell surface. LapA is only visualized in the cytoplasm (Fig. 2, third from the top, left and right panels), which correlates with the low adhesion forces observed for this strain.

In conclusion, AFM is routinely used to investigate the effect of environmental conditions (e.g. ionic strength and pH), bacterial surface structures (e.g. lipopolysaccharides, pili, capsular polysaccharides) and cell surface physicochemical properties upon bacterial adhesion (Abu-Lail and Camesano 2003; Sheng et al. 2008; Touhami et al. 2006; Vadillo-Rodríguez et al. 2005). AFM has been used to both image and measure adhesive forces of bacterial biofilms (as reviewed by Wright et al. 2010) and AFM studies have been used to analyze the detachment force and the size of the adhesive holdfast in *Caulobacter crescentus*, which functions to anchor cells to abiotic and biotic surfaces (Li et al. 2005; Tsang et al. 2006). The Weibull analysis presented here, however, while common in macroscopic fracture studies, is a novel way to present nanoscopic AFM adhesion data. 3D-SIM allows fluorescence imaging with at least a twofold improvement in resolution in all three dimensions, enabling localization of structures in bacteria in unprecedented detail. However, while 3D-SIM has been used to image the vaccinia virus (Horsington et al.), actin from *Plasmodium berghei* (Angrisano et al. 2012), a vertebrate mitotic scaffold protein (Green et al. 2012), and chromatin, nuclear lamina and the nuclear pore complex within the mammalian nucleus (Schermelleh et al. 2008), it has rarely been used to visualize bacterial proteins and structure.

To our knowledge, this study is the first that combines genetic approaches with advanced microscopy for direct evaluation of the role of a biofilm adhesion protein. Visualization of LapA on the cell surface by 3D-SIM correlates to adhesion force measured by AFM and our previous fractionation data, revealing that LapA is a bona fide cell surface adhesin required for biofilm formation. The tools presented in this study may be used to investigate the role of other putative adhesion proteins in order to gain a better understanding of bacterial attachment processes.

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Fig. 1.

AFM analysis of adhesion. A. Box plot of adhesion data obtained from AFM experiments. Boxes span the data from the first quartile to the third quartile with the median of the data shown as a horizontal line through the box; ns, indicates results with no statistical difference; *, indicates results significantly different from the wild-type at $P = 0.001$ by Kruskal-Wallis one-way analysis of variance. B. Weibull probability of bond failure as a function of applied force in AFM. The probability, P_F , is given by $P_F = \frac{n}{N+1}$, where n is the rank number of the data point in ascending order and N is the total number of data points. The solid black lines represent the Weibull fit through the data points for each strain. No statistically significant difference in the probability distributions was found between the *lapA* and *lapB* mutants, nor between $\Delta\text{Calx-}\beta$ and the wild-type strain based on a two-sample Kolmogorov-Smirnov test with power $\alpha = 0.001$.

Fig. 2. Localization of LapA by 3D-SIM. Shown is the localization of LapA-HA using an HA-specific antibody, with Alexafluor-488 conjugated secondary antibody (green). The center slice is longitudinally through a cell (left) and maximum projection is also shown for each image (right). As a control, the wild-type strain lacking an HA-tagged LapA (bottom panels) is shown and only the FM464-FX dye (red) that stains the membrane can be seen. Only bound LapA-HA is visualized. Scale bar, 1 μm .

Table 1

Weibull distribution parameters for the strains in this study.

	<i>lapA</i>	<i>lapB</i>	$\Delta\text{Calx-}\beta$	Wild-type	ΔlapG
F_{mean} (nN)	0.204	0.203	0.256	0.272	1.268
F_0 (nN)	0.183	0.190	0.245	0.243	1.303
Γ	0.605	0.591	0.564	0.589	0.492
m	1.176	1.235	1.176	1.109	1.021
R^2	0.75	0.90	0.97	0.89	0.97