Probing Large Protein Adhesin Molecules on Pseudomonas fluorescens with Atomic Force Microscopy

Mary Elizabeth Schwartz

Worcester Polytechnic Institute

Follow this and additional works at: https://digitalcommons.wpi.edu/mqp-all

Repository Citation

This Unrestricted is brought to you for free and open access by the Major Qualifying Projects at Digital WPI. It has been accepted for inclusion in Major Qualifying Projects (All Years) by an authorized administrator of Digital WPI. For more information, please contact digitalwpi@wpi.edu.
Probing Large Protein Adhesin Molecules on *Pseudomonas fluorescens* with Atomic Force Microscopy

A Major Qualifying Project submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science

Mary Schwartz

Advised by:
Professor Terri A. Camesano

December 19, 2013
Probing Large Protein Adhesin Molecules on \textit{Pseudomonas fluorescens} with Atomic Force Microscopy

A Major Qualifying Project
Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
Professor Terri A. Camesano, Advisor
by

___________________________
Mary Elizabeth Schwartz

December 19, 2013

Approval:

_______________________
Terri A. Camesano, Advisor
Abstract

Protein adhesins are an important type of surface structure, and are components of some Gram-negative bacterial outer membranes, such as *Pseudomonas fluorescens*. The LapA adhesin is a major surface protein on this bacterium, which is required for irreversible adhesion and the initiation of biofilm formation. Atomic force microscopy (AFM) was used to characterize surface structures of *P. fluorescens* Pf0-1. The wild-type and three genetically modified strains were studied, namely a strain consisting of a single cross-over knockout mutation disrupting the *lapA* gene, a strain consisting of a single cross-over knockout mutation disrupting the *lapB* gene, (LapA is maintained in the cytoplasm and not transported to the cell surface), and *lapG* (LapA is unable to be cleaved from the cell surface). AFM data was modeled using the Alexander - de Gennes (A-dG) relation for steric repulsion. We calculated the equilibrium layer thickness of the surface structures as well as spacing between adhesins. The wild-type strain and the *lapA* and *lapB* mutants all showed similar spacing for surface proteins. The strain lacking LapG had the smallest spacing between molecules. This suggests that the absence of the LapG protease allowed the LapA protein to accumulate, thus decreasing the overall molecular spacing of the protein on the bacterial surface compared to the wild-type strain. We found that the *lapG* mutant strain of *P. fluorescens* behaved like a classical polymer brush, in which the spacing between molecules was very small (3.3 nm), which would allow intermolecular interactions between protein units. Recent work has shown that the *lapG* mutant has greater adhesion and biofilm formation than the wild-type, *lapA*, and *lapB* strains, and exhibits stiffer conformation of LapA due to higher protein density and aggregation. Taken together, our results and these recent studies support the finding that LapA adhesin conformation is related to irreversible bacterial adhesion.
Acknowledgements

I would like to thank my project advisor, Professor Terri A. Camesano, Department of Chemical Engineering at Worcester Polytechnic Institute for providing so much opportunity through research and mentorship as well as supporting me through the writing of a journal article and national conference. I would also like to thank Professor Camesano’s graduate students Rebecca Gaddis, Lindsay Lozeau, Todd Alexander, Kathleen Wang, Elaheh Kamaloo as well as Gawain Thomas for their insight and support. Additionally, this research could not have been done without the collaboration with Professor George A. O’Toole and student Chelsea D. Boyd, and the help of Ivan Ivanov, Samantha O’Connor, and Professor Nancy Burnham.

Authorship

Materials and Methods, Results and Discussion and Conclusions sections of this Major Qualifying Project are taken from a paper in review from the editors of the Journal of Bionanoscience for which I was the primary author. The contents of this report are a representation of the work done by the main author. Contributions to these sections also were made by Ph.D. students Samantha O’Connor and Rebecca Gaddis in the department of physics at Worcester Polytechnic Institute. They were very helpful in the development of the MATLAB program. Dr. George A. O’Toole and Dr. Chelsea D. Boyd of Dartmouth College contributed in the development and characterization of the bacterial strains.
Contents
1.0 Introduction ................................................................................................................................. 1
2.0 Background ................................................................................................................................. 3
  2.1 Bacterial Biofilms ....................................................................................................................... 3
  2.2 *Pseudomonas fluorescens* Pf0-1 ............................................................................................... 5
  2.3 Atomic Force Microscopy .......................................................................................................... 7
  2.4 MATLAB Programming for Data Analysis ............................................................................. 8
  2.5 Steric Modeling ......................................................................................................................... 9
3.0 Materials and Methods .............................................................................................................. 10
  3.1 Bacterial Preparation ............................................................................................................... 10
  3.2 AFM Calibration ....................................................................................................................... 11
  3.3 Steric Modeling ......................................................................................................................... 12
  3.4 Data Analysis and Computation ............................................................................................... 13
4.0 Results and Discussion ............................................................................................................... 15
  4.1 Spacing and Length of Protein Molecules on *P. fluorescens* ................................................. 15
  4.2 Polymer Conformation on the Surface of *P. fluorescens* ...................................................... 17
  4.3 Relationship of Protein Conformation to Bacterial Adhesion ................................................ 20
5.0 Conclusions ............................................................................................................................... 22
References Cited ............................................................................................................................... 23
1.0 Introduction

Bacteria are found in most environments and often form biofilms, or surface-attached communities, which provide a protective environment for the microbes and make infections very difficult to treat (Mulcahy, Isabella, & Lewis, 2013). The process of bacterial attachment and biofilm development are highly regulated (G.A. O'Toole & R. Kolter, 1998). Efforts are underway to study bacterial adhesion and biofilm formation from multiple aspects, and the combination of genetic studies with nanoscale-studies represents a promising approach that is currently being pursued (El-Kirat-Chatel, Beaussart, Boyd, O’Toole, & Dufrêne, 2013; Ivanov et al., 2012; Ivanov et al., 2011).

Biofilm formation begins with the initial bacterial adhesion phase in which bacteria reversibly adhere to a conditioned surface under favorable conditions, followed by irreversible adhesion. Exopolysaccharides, proteins, pili and receptor-specific ligands initiate this latter phase (P. D. Newell, Monds, & O'Toole, 2009; O'Toole, Kaplan, & Kolter, 2000). *P. fluorescens* utilizes these protein adhesins for irreversible surface attachment, as a biological control agent on a variety of crops, by preventing colonization of detrimental bacteria (Hinsa, Espinosa-Urgel, Ramos, & O'Toole, 2003). *P. fluorescens* has a large adhesive protein (LapA) of ~520 kDa, which is present in the cytoplasm and exported to the cell surfaces by an ABC transporter, encoded by the *lapEBC* genes, and is required for irreversible attachment of the bacterium (Peter D. Newell, Boyd, Sondermann, & O'Toole, 2011). *P. fluorescens* Pf0-1 cells in which the *lapG* gene has been deleted accumulate LapA on the surface, and these cells form a very robust biofilm (Boyd, Chatterjee, Sondermann, & O’Toole, 2012; Peter D. Newell et al., 2011).
While the overall function of surface organelles and proteins of many common microbes when assembled in a biofilm are understood macroscopically, research on the molecular and nanoscale properties of the individual organelles and initial adhesion is ongoing (Ivanov et al., 2012; Pranzetti et al., 2013; Tripathi et al., 2013). This information is extremely important because it is the initial adhesion of a few bacteria that leads to biofilm development.

Atomic force microscopy (AFM) is useful for providing more detailed information on bacterial adhesion and surface molecules. With AFM, bacterial surface structures are probed, and intermolecular forces are studied in real time (Dupres, Alsteens, Andre, & Dufrêne, 2010; Joshua Strauss, Nancy A. Burnham, & Terri A. Camesano, 2009). Application of modeling to AFM data allows for the characterization of physiochemical properties of bacterial surface molecules. Previous AFM measurements were reported for *P. fluorescens* Pf0-1 (Ivanov et al., 2012). AFM with a silicon nitride tip was used to describe how different genes affected LapA function and overall adhesion of the bacteria. For the *lapG* mutant strain, adhesion forces measured with an AFM tip were roughly twice the force observed between the wild-type strain and the AFM tip, presumably due to the *lapG* mutant strain expressing more LapA protein on the surface than the wild-type strain (Boyd et al., 2012). A recent AFM study showed that under conditions that induce biofilm formation, including growth in a phosphate-rich medium or when the gene encoding LapG was deleted, adhesion of *P. fluorescens* was greater towards both hydrophobic and hydrophilic substrates (El-Kirat-Chatel et al., 2013).

As an extension of prior work, we applied a model accounting for steric interactions to AFM data. When two surfaces, at least one of which is covered with grafted polymers, are brought into close contact with each other, the interactions between the two plates cause changes in the conformation of the polymers. Steric models of the form developed by Alexander - de Gennes (A-
dG model) have been used to characterize molecules on bacterial surfaces including lipopolysaccharides (LPS), extracellular polymeric substances, and surface polymers (de Gennes, 1987; Oh et al., 2012; Taylor & Lower, 2008). Therefore, the objective of this study was to apply the steric model to AFM data, and characterize the physical characteristics of the LapA protein of P. fluorescens.

2.0 Background

2.1 Bacterial Biofilms

Bacteria are found in environments across the world and may form surface-bound communities, called biofilms, in which the individual bacterium are able to interact. These biofilm formations can be pathogenic or beneficial; able to kill cells or necessary for survival of entire ecosystems (Friedlander et al., 2013; G. A. O'Toole & R. Kolter, 1998). Upon forming a surface-bound biofilm, groups of micro-organisms undergo a physical change from a planktonic (or group-like) state to a multicellular and symbiotic population. This change is essential for quorum sensing, bacterial survival, and ultimately, pathogenesis (Potera, 2012). When Bacteria aggregate they become physically connected through production of an extracellular matrix with many different types of extracellular polymeric substances (EPS) including various proteins and polysaccharides. Bacteria have illustrated an increased resistance to antibiotics when they are in this biofilm formation and can often withstand very high doses of antibiotics which would kill planktonic cells, however the mechanism by which this occurs is not understood completely (Bjarnsholt, 2013; George A. O'Toole & Roberto Kolter, 1998).

Bacterial biofilm formations can be detrimental and fatal causing diseases such as bacterial endocarditis, catheter-related fungal nosocomial bloodstream infection, and cystic
fibrosis lung infections as well as many chronic infections (Parsek & Singh, 2003; Schinabeck et al., 2004). These biofilms are difficult to prevent once colonized, as antibiotics often have little or no effect on these bacteria (Bjarnsholt, 2013; Friedlander et al., 2013). The occurrence of medical complications coming from hospital stays has been increasing, caused in part by antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), and multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) which are becoming more often integrated into these already difficult-to-treat biofilm communities (Ojha et al., 2008). Because of their increasing resistance to antibiotics, treatment options are being severely limited. In 2007 alone there were 99,000 deaths associated with healthcare associated infections and an estimated $5 billion dollars a year spent to combat these infections, a significant amount being biofilm related (Bryers, 2008).

It is known that for initial bacterial attachment, a bacteria reversibly adheres to a conditioned surface in favorable conditions to the particular strain of bacteria. Once there is initial attachment, the bacteria undergo physiological changes to begin irreversible adhesion induced by exopolysaccharides, proteins, pili, and receptor specific ligands (P. D. Newell et al., 2009; G. A. O'Toole & R. Kolter, 1998; O'Toole et al., 2000). The bacteria are then in a maturation stage and grow sub-populations in a 3-D structure, and finally some bacteria dis-attach to form other colonies (Bjarnsholt, 2013; Coppadoro, Thomas, & Berra, 2013). In order to better understand biofilm formation, the original attachment of the bacteria to the surface in the beginning stages of biofilm formation must be better understood.

While the overall function of some surface organelles and proteins assembled in a biofilm are understood macroscopically, the research into the microscopic properties of the individual organelles controlling initial adhesion is only just beginning (Ivanov et al., 2012). A few of these
experiments include Lactic acid bacteria; *Lactobacillus crispatus*, *Lactobacillus helveticus* and *Lactobacillus johnsonii* have been investigated to understand their adhesion to the intestinal epithelium (Schaer-Zammaretti & Ubbink, 2003). *Escherichia coli* adhesion has been tested on different surfaces, such as Teflon and hydrophobic and hydrophilic glass to determine biomaterial surface interactions as well as with cranberry juice to study urinary tract infections (Y. Liu, M. A. Black, L. Caron, & T. A. Camesano, 2006; Ong, Razatos, Georgiou, & Sharma, 1999) *Massilia timonae*, *Pseudomonas aeruginosa* and *Bacillus subtilis* have been studied to determine adhesion differences between gram-negative and gram-positive bacteria and physicochemical properties analyses on the bacterial surfaces (Harimawan, Rajasekar, & Ting, 2011; Yatao Liu, Matthew A. Black, Lizabeth Caron, & Terri A. Camesano, 2006; Ong et al., 1999; Schaer-Zammaretti & Ubbink, 2003).

### 2.2 *Pseudomonas fluorescens* Pf0-1

The *Pseudomonas* genus contains bacteria which aerobic Gram-negative rods which are non-sporulating and found in biofilm or planktonic forms. The genus encompasses a wide variety of organisms from an enormous variety of environments. They can be found in soil, water, plants and animals and are known for their vast metabolic diversity. They are able to metabolize a large number of substrates including aromatic hydrocarbons and in some strains, their metabolites have been shown to stimulate plant growth and inhibit plant pathogens (Moore et al., 2006; Ozen & Ussery, 2012). They are medically and biotechnologically important due to the large variety of niches they reside in and the pathogenic nature of some strains. Most which are pathogenic, are pathogenic to plants, with several pathogenic to animals (Ozen & Ussery, 2012). Strains of *Pseudomonas* are often resistant to antibiotics, disinfectants, detergents, and organic solvents and because of this, have been of great interest in the biomedical field (Moore et al., 2006). As of
2012, there are 202 species in the genus *Pseudomonas*, classified by a combination of 16S rRNA, analysis of cellular fatty acids and differentiating physiological and biochemical tests (Moore et al., 2006; Ozen & Ussery, 2012).

The mechanisms by which bacteria adhere to surfaces and other cells are thought to be the most significant in the quest for understanding transport and viability of bacteria in various environments (Boyd et al., 2012; Rijnaarts, Norde, Bouwer, Lyklema, & Zehnder, 1995). *Pseudomonas fluorescens* PF0-1 for example, has a large adhesive protein (LapA) which is used for irreversible attachment. The bacteria are used as biological control agents for a variety of crops as it colonizes and benefits roots by preventing colonization of detrimental bacteria. Studying the initial adhesion of *Pseudomonas fluorescens* PF0-1, allows the assessment of a fairly large adhesive protein, which is known to irreversibly attach to surfaces and promote the growth of biofilms (Dunne, 2002; O'Toole et al., 2000). This ~520 kD LapA protein is essential for stable surface attachment and irreversible adhesion of *Pseudomonas fluorescens* (Peter D. Newell et al., 2011). Bis-(3′-5′) cyclic dimeric guanosine monophosphate (c-di-GMP) levels within the cell fluctuate with the availability of the essential nutrient, inorganic phosphate and regulate the cellular production of this surface attachment protein. When low inorganic phosphate levels occur, cellular c-di-GMP levels are reduced, which through another pathway utilizing the LapA targeted protease, LapG, allows the lapA protein to be released (Navarro et al., 2011).

Previous AFM measurements were reported for *P. fluorescens* Pf0-1 (Ivanov et al., 2012). AFM with a silicon nitride tip was used to describe how different genes affected LapA function and overall adhesion of the bacteria. For the lapG mutant strain, adhesion forces measured with an AFM tip were roughly twice the force observed between the wild-type strain
and the AFM tip, presumably due to the lapG mutant strain expressing more LapA protein on the surface than the wild-type strain (Boyd et al., 2012). A recent AFM study showed that under conditions that induce biofilm formation, including growth in a phosphate-rich medium or when the gene encoding LapG was deleted, adhesion of P. fluorescens was greater towards both hydrophobic and hydrophilic substrates (El-Kirat-Chatel et al., 2013).

2.3 Atomic Force Microscopy

Atomic force microscopy (AFM) does not use the traditional methods to obtain and analyze images. Instead of using light and series of lenses, the AFM “views” a surface via differences in heights. These differences are measured by a pointed or rounded tip, which is on the micron scale, which is attached to a micro-cantilever. Often, a rounded tip is used for surfaces in which adhesion forces are to be measured, or in cases which resolution is not important. When the imaging quality is important, tips can be used to obtain resolution down to the nanometer scale and obtain images 1nm by 1nm in size. To obtain this image, a laser is centered on this tip and the change in surface morphology results in change in the deflection of the laser on the tip. This deflection is measured by differences of voltages on a photodiode. From these differences, an image is collected which can be used to further study interactive forces between the tip and a surface.

This image can be obtained through several different modes, the two most common of which are contact and tapping mode. In contact mode, the cantilever is brought to the surface, and dragged across the surface. This method allows for topographical data to be collected, but may also damage a sensitive or soft surface. This is typically not used for analysis of biological systems or soft samples. Tapping mode is based on the vibration of the cantilever. The vibration is controlled through electronics using resonance frequency and drive amplitude. After being brought to the surface, the cantilever is vibrated to tap the sample at the bottom of each vibration phase.
This method of imaging allows for less contact between the tip and the surface, and thus less possibility of damage to the surface. The drive amplitude must be monitored, however, to ensure the tip is not contacting the surface too aggressively.

In addition to obtaining images, interactive forces can be obtained to study the adhesion and repulsion between the tip and the sample. In the AFM software, the tip can be centered at any location and from there, move towards said surface. As the tip moves towards the surface, there are steric repulsive forces acted on the tip, which can be measured from the force data. Once on the surface, the tip may dwell at a surface for an allotted time, allowing bonds to form between the tip and the surface, and then retract away from the surface. A series of force verses separation data curves, created by AFM data, are used to collect adhesion, elastic, steric and repulsive force information.

Atomic force microscopy (AFM) is useful for providing more detailed information on bacterial adhesion and surface molecules. With AFM, bacterial surface structures are probed, and intermolecular forces are studied in real time and surfaces can be visualized at the nanometer level (Dupres et al., 2010; Joshua Strauss et al., 2009). Application of modeling to AFM data allows for the characterization of physiochemical properties of bacterial surface molecules. AFM has not only allowed the visualization of the bacterium surface, but also has the ability to record adhesion events. When the bacteria are mutated, characteristics of individual genes can be investigated without the effect of signaling, interactions between bacterium in the biofilm.

2.4 MATLAB Programming for Data Analysis

MATLAB is a fourth generation programming language, often used to analyze or create data. It operates using matrices and allows for matrix manipulations, creation of algorithms, and plotting of functions among other abilities. MATLAB is developed by MathWorks and is utilized
in applications around the world. Users are able to write their own programs to analyze data or upload previously gathered data for manipulation and analysis.

Previously, adhesion data had been analyzed through the use of a MATLAB script which allowed the researcher to manually pick adhesion points. This resulted in a data collection system which varied with the person analyzing the data. Because of this, and in order to automatically apply the steric model to existing data sets in a time-efficient manner, a new MATLAB script has been written to automate the process and achieve higher accuracy in the results.

2.5 Steric Modeling

As an extension of prior work, we applied a model accounting for steric interactions to AFM data. When two surfaces, at least one of which is covered with grafted polymers, are brought into close contact with each other, the interactions between the two plates cause changes in the conformation of the polymers. Steric models of the form developed by Alexander - de Gennes (AdG model) have been used to characterize molecules on bacterial surfaces including lipopolysaccharides (LPS), extracellular polymeric substances, and surface polymers (de Gennes, 1987; Oh et al., 2012; Taylor & Lower, 2008).

The Alexander and de Gennes model determines the force per unit area \( F \) between two plates with grafted polymer brush surfaces by considering a constant monomer density throughout the brush (Alexander, 1977; de Gennes, 1987). The original equation applies to interactions with polymers on both the tip and the surface, but it has been shown that the same equation is applicable to cases where the AFM probe is not coated with polymers. The equation is simply changed to account for the spherical nature of the tip’s contact region instead of the flat planes that the original equation was written for. The non-linear region on the AFM approach curve can be fitted to determine the length and density of the polymers under various conditions. For steric modeling,
the point of zero distance is defined as the position where the polymer brush is compressed so much by the tip that an additional increase of the force does not lead to further compression. The point of zero force is the point where the surface exerts an initial repulsive force on the tip and the region between these two points is approximately the size of the tip radius, and is used along with the Alexander and de Gennes equation to determine three variables; the equilibrium layer thickness of the polymer brush, spacing between molecules and the offset distance (Butt, 1999). Through the application of the steric model to the AFM data, the physical characteristics of a surface may be further understood.

3.0 Materials and Methods

3.1 Bacterial Preparation

The wild-type bacterial strain used here, and the construction of the derivative strains, has been reported previously. The consequence of each mutation on biofilm formation and LapA localization is described in Table 1 (Ivanov et al., 2012; Peter D. Newell et al., 2011).

Bacterial strains were cultured overnight in 50 ml of Lysogeny broth (LB) at 30°C with shaking at 250 rpm. Cultures were diluted 1:100 in K10T-1 growth medium (50mM Tris-HCL pH 7.4, 0.2% wt/vol tryptone, 0.15% vol/vol glycerol, 0.6 mM MgSO₄ and 1mM K₂HPO₄) and incubated for 6 hours until reaching the late exponential growth phase. Bacteria was harvested by centrifugation at 1284 x g for 10 min and washed once with saline (0.85% wt/vol NaCl in water).
Glass slides were cleaned with 2% vol/vol RBS-35 (Thermo Fisher Scientific, Rockford, IL) for 10 minutes followed by rinsing with copious amounts of ultrapure water. Cleaned slides were rinsed with 100% methanol and soaked with 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. After centrifugation, bacterial cells were re-suspended in saline with an addition of 300 μL of 100 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 hours to promote bacterial attachment. These slides were equilibrated with 0.5 ml of phosphate buffered saline (PBS) for AFM measurements.

3.2 AFM Calibration

An MFP-3D Bio AFM (Asylum Research, Santa Barbara, CA) was calibrated in order to reduce the effect of noise and to measure the cantilever spring constant. The silicon nitride probe (DNP, Veeco Instruments Inc., Santa Barbara, CA) with resonance frequency of ~24 kHz had a spring constant of approximately 0.12-0.15 N/m, which was calibrated before each experiment.
Optical sensitivity was determined in PBS on glass before testing the bacterium. At least 25 measurements per bacterium were obtained by probing the center top of the cell and each strain was tested thrice. During experiments, there was a constant trigger threshold of 5 nN and a surface dwell of 0.99 s. Deflection and z-sensor measurements obtained through AFM analysis were used to calculate force and separation values needed to fit the A-dG model. The separation is equal to deflection minus z-sensor values and force is equal to the spring constant of the cantilever multiplied by the deflection.

3.3 Steric Modeling

The Alexander -de Gennes model determines the force $F$, as a function of separation distance, $D$, between two surfaces, one of which contains a grafted polymer brush with a constant monomer density throughout the brush (Alexander, 1977; de Gennes, 1987). When one surface is a flat plate and the other is a spherical AFM probe, the force is given as

$$F(D) = \frac{8kTn_L}{35s^3} \left[ 7 \left( \frac{L}{D} \right)^{5/4} + 5 \left( \frac{D}{L} \right)^{7/4} - 12 \right]$$

where $k$ is the Boltzmann constant, $T$ the absolute temperature, $s$ the spacing between molecules, $L$ the equilibrium layer thickness of the polymer brush, and $D$ is the separation distance. This value, $D$, was offset to account for the layer thickness at maximum applied force (Chang, Abu-Lail, Guilak, Jay, & Zauscher, 2008).

The non-linear region on the AFM approach curve can be fitted using Equation 1 to determine the layer thickness of the polymers in a brush form as well as the spacing of the polymers under various conditions. AFM data were calibrated and processed with a MATLAB script, and the steric model was applied to calculate $L$ and $s$. The calculated $L$ and $s$ values were only used if the function which fit the data resulted in a least-squares mean fit of 0.76 or greater. The fit produced average least-squares mean fits of 0.97.
In order to compare data, whisker and box plots were created and statistical tests were performed. \( L \) and \( s \) values of bacteria strains were compared using \( P \leq 0.050 \) to determine statistically significant distances with the Kruskal-Wallis one-way analysis of variance on ranks (Tukey’s Test for all pairwise multiple comparisons) in SigmaPlot 12.5 (Systat Software Inc., Chicago, IL).

### 3.4 Data Analysis and Computation

To obtain as much information as possible from the AdG model, AFM data needs to be calibrated and processed in several ways. To do this, a MATLAB script was written to allow to calibrate and crop and then fit the data curves to the curved function of the AdG model. The methods followed by this script are illustrated in Figure 1. Igor software controls the AFM and also stores the data. Through this program, curves can be viewed to see overall shape. Curves which did not have a sharp jump to

---

**Figure 1:** Flow diagram for the methods by which data is collected and manipulated for analysis. First curves are obtained through the IGOR AFM software and selected based on known characteristics of the bacterial surface. This data is then modified to obtain force vs separation data and further analyzed to obtain adhesion data and fit the Alexander and de Gennes model.
contact transition, and without sporadic adhesion forces were selected for data analysis. Z-sensor and deflection values for both extension and retraction curves were copied into excel files for use in the MATLAB script. These excel files were imported in the MATLAB script, and the smallest x-values and largest y-values were set to zero for comparability. The spring constant was put into the script by the user and force was calculated by deflection times spring constant for each set of data points. Separation was then calculated by deflection minus z-sensor for each set of data points. After this calculation is completed, the forces caused by either steric repulsion as the tip comes near the sample, and the forces needed to break bonds created between the tip and sample can be determined and analyzed. The horizontal approach region of the curve was first set to a force of zero in order to have a starting value to which the change can be compared to. This allowed multiple curves to be analyzed at once and all of the curves to be comparable to one another. The curves were then smoothed to reduce noise and the slope of the horizontal area was calculated and applied to the entire curve so none of the curves were tilted and they could again be compared more accurately. This allowed the correct region of the curve to be analyzed for the AdG model and for the adhesion forces to be relative to one another.

To determine adhesion strength, a fixed minimum point was determined to prevent points from being counted which were not adhesion events. This minimum value was determined to be \(7 \times 10^{-11}\) N. Secondly, a threshold was set to determine what would be considered an adhesion event. The adhesion events look like sharp peaks on the curve, and for MATLAB to be able to recognize this region, the program must find a difference between two adjacent points on a graph, with this difference being the threshold. This threshold was set to be \(5 \times 10^{-13}\) meters and was found by observing which adhesion events were recorded from multiple data sets. Some
curves had many adhesion events and some had none, all of which was recorded in a new excel spreadsheet with all of the results.

The force and separation data require precise cropping and fitting parameters to fit the steric model. The force curve must be cropped in order to obtain the region from where AFM tip first comes in contact with the surface to the point where the tip begins to indent the bacteria. This was estimated to be the length of the tip radius and is fit with the AdG function. Due to the difficulty of finding the exact region to crop every one of the many curves, functions which gave protein length values on the micron scale were discarded, as this would be one half the length of the bacterium itself, or greater. Values were only used if they had least mean squares fit values of .75 or greater, with average values of .95 or greater. The full procedure is represented in the image below.

4.0 Results and Discussion

4.1 Spacing and Length of Protein Molecules on *P. fluorescens*

*P. fluorescens* Pf0-1 has evolved methods to adapt to changes in nutrient availability, which in turn affects the bacterium’s ability to form biofilms. A critical protein for this process is the large adhesin protein, LapA. An ABC transporter encoded by the *lapEBC* genes exports the LapA adhesin to the cell surface and several other proteins are involved in the maintenance of LapA on the cell surface. Most relevant to this study is the LapG protease, which promotes the release of the LapA protein from the cell surface in conditions unfavorable for biofilm formation (Boyd et al., 2012).

AFM force measurements were performed on individual bacterial cells in PBS. At least 75 measurements were made per bacterial strain, and the steric model was applied to each force
curve. The fitted parameters from the model were tabulated and statistical analyses were performed.

The key parameters obtained from the steric model are the layer thickness, $L$, and the spacing between proteins, $s$. When the equilibrium layer thickness values were calculated, only the value for the lapG mutant varied from the other strains, with the median $L$ for the lapG mutant was found to be 279 nm (Figure 2). This value was found to be statistically different from the other three strains. The $L$ values of the lapA and lapB mutants and wild type were 152 nm, 134 nm, and 134 nm, respectively, all of which were found to be statistically similar. This indicates that elongated molecules were present on the surface of the lapG mutant compared to the other three strains.

A difference in the lapG mutant was also demonstrated in the spacing values, $s$, between the surface molecules when compared to the wild-type and lapA and lapB mutant strains. A-dG calculations showed that $s$ was 3.3 nm for the lapG mutant, which was statistically different than the $s$ values for the other three strains. The $s$ values were 34.8 nm for the lapA mutant, 35.8 nm for the lapB mutant, and 34.5 nm for the wild-type strain, and these latter three values were statistically indistinguishable, according to the Kruskal-Wallis

![Figure 2: Box and whisker plots to demonstrate the $L$ values calculated via the A-dG model for the different strains of *P. fluorescens*. The star indicates statistically similar values as obtained by the Kruskal-Wallis one way analysis of variance on ranks (Tukey’s test for all pairwise multiple comparisons, P<0.001). Solid lines inside the boxes show the median of the data set. The median L values were 279 nm for the lapG mutant, 152 nm for the lapA mutant, 134 nm for the lapB mutant, and 134 nm for the wild-type strain.](image)
one-way analysis of variance on ranks (Tukey’s test for all pairwise multiple comparisons; Figure 3).

4.2 Polymer Conformation on the Surface of *P. fluorescens*

Variations of the A-dG model have been used to describe the molecules on bacterial surfaces in a number of different systems. In the original form of the scaling laws that were developed, when the surface polymer density was low, polymer height was independent of the spacing between molecules, and this refers to a “mushroom” like regime for surface coverage (Alexander, 1977; Dumont, Belmas, & Hess, 2013). However, at higher coverage, the layer thickness of the polymer begins to scale with the third root of the grafting density (inversely related to *s* in Equation 1), and the polymers behave like a “brush”. This transition from a mushroom to a brush conformation of surface biopolymers can be observed experimentally, as Wu et al. presented for grafted polyacrylamides (Wu, Efimenko, & Genzer, 2002). When the density of polymers on the surface is great enough, individual molecules may start to interact with one another, and elongation of the polymer occurs to form a brush layer.

For a given spacing *s* between polymers, one can calculate the radius of gyration (*R_{GT}* of a polymer that would have a mushroom conformation (which will be identical to that of an isolated non-interacting polymer). This radius of gyration is calculated by equating *s*² to the cross-sectional
area \( \pi R_{GT}^2 \). Such a polymer would have a height equal to \( 2R_{GT} \). If a polymer has an \( L \) greater than \( 2R_{GT} \), then such a polymer will not be in a mushroom conformation, but will be stretched due to interactions with neighboring polymers and be in a brush conformation. Therefore, for a given \( s \), the calculated \( R_{GT} \) is the radius of gyration of a polymer in the transition state between a mushroom and brush conformation.

In the present study, we calculated the values for \( R_{GT} \) that are summarized in Table 2. All of the strains show behavior consistent with polymer brushes, since in all cases, the spacing is much lower than twice the radius of gyration. However, some differences could be noted in comparing the strains. Measured values of \( L \) for the wild-type, \( lapA \) and \( lapB \) mutants (~120 nm) were about 1.5 times greater than the calculated values for \( 2R_{GT} \). However, the measured values of \( L \) for the \( lapG \) mutant strain are much larger and the spacing is an order of magnitude smaller compared to the other strains. Therefore, while all of the strains behave as brushes, the \( lapG \) mutant strain has the most elongated polymer brush. The small spacing between molecules of 3.3 nm allows for a high degree of intermolecular interactions.

<table>
<thead>
<tr>
<th>Bacterium and Conditions</th>
<th>( L ) (nm)</th>
<th>( s ) (nm)</th>
<th>( R_{GT} ) (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E. coli ) fimbriae, control</td>
<td>147</td>
<td>10.4</td>
<td>5.87</td>
<td>(Yatao Liu et al., 2006)</td>
</tr>
<tr>
<td>( E. coli ) fimbriae, 5% CJC(^2)</td>
<td>53</td>
<td>11.2</td>
<td>8.32</td>
<td>(Yatao Liu et al., 2006)</td>
</tr>
<tr>
<td>( E. coli ) fimbriae, 10% CJC</td>
<td>48</td>
<td>7.8</td>
<td>4.4</td>
<td>(Yatao Liu et al., 2006)</td>
</tr>
<tr>
<td>( E. coli ) fimbriae, 20% CJC</td>
<td>48</td>
<td>6.6</td>
<td>3.84</td>
<td>(Yatao Liu et al., 2006)</td>
</tr>
<tr>
<td>( P. aeruginosa ) wild type</td>
<td>171</td>
<td>5.7</td>
<td>3.21</td>
<td>(Ivanov et al., 2011)</td>
</tr>
<tr>
<td>( P. aeruginosa ) ( \Delta wzz1)</td>
<td>212</td>
<td>8.4</td>
<td>4.74</td>
<td>(Ivanov et al., 2011)</td>
</tr>
<tr>
<td>( P. aeruginosa ) ( \Delta wzz2)</td>
<td>108</td>
<td>6.6</td>
<td>3.72</td>
<td>(Ivanov et al., 2011)</td>
</tr>
<tr>
<td>( P. aeruginosa ) ( wzy: GM )</td>
<td>34.6</td>
<td>10.3</td>
<td>5.81</td>
<td>(Ivanov et al., 2011)</td>
</tr>
<tr>
<td>( P. aeruginosa ) ( DAM)(^4)</td>
<td>37.7</td>
<td>6.4</td>
<td>3.61</td>
<td>(Ivanov et al., 2011)</td>
</tr>
</tbody>
</table>

\( \text{\( s \) was assumed to be equal to } \sqrt{\pi R_{GT}^2}, R_{GT} \text{ is the radius of gyration of a polymer that will be in the transition state between mushroom and brush conformations, corresponding to the measured } s. \text{ If the measured } L \text{ is greater than } 2R_{GT}, \text{ then the polymer is in a brush conformation. If the measured } L \text{ is equal to or smaller than } 2R_{GT}, \text{ then the polymer is in a mushroom conformation.} \)

\(^2\text{CJC = cranberry juice cocktail}\)

\(^3\text{All the mutants presented here have altered LPS.}\)

\(^4\text{This double mutant is missing the wzz1 and wzz2 gene.}\)
Some molecules are pushed upwards (away from the cell membrane), as they have no space to expand on the bacterial surface. A recent study estimated that the LapA protein accumulated on the \( \text{lapG} \) mutant surface at a density of approximately 450 sites/\( \mu \text{m}^2 \) (El-Kirat-Chatel et al., 2013). This seems consistent with our finding that accumulation of LapA decreased the spacing \( (s) \) values between adjacent molecules, and allowed the molecules to be close enough to interact (Figure 4).

In comparison with previous reports, the spacing of molecules of the \( \text{lapG} \) mutant was consistent with other types of bacterial molecules, but the measured values of \( s \) for the wild type (~35 nm) were much larger than has been observed in other bacterial surfaces studied (~5-10 nm, Table 2). For example, the \( s \) values for the fimbriae of \( E. \text{coli} \) and the LPS of \( P. \text{aeruginosa} \) were not very different from one another, regardless of the specific bacterial strain or chemistry of the surrounding solution (Table 2). The \( s \) values for the wild-type strain and \( \text{lapA} \) and \( \text{lapB} \) mutants of \( P. \text{fluorescens} \), investigated here, are larger than other reported values for either proteins or LPS on bacteria.

![Figure 4: Schematic of protein brush layers on bacterial surfaces.](image)
Most of the prior applications of the A-dG model to bacterial surface data interpretation have focused on flexible polymers, such as extracellular polysaccharides and LPS (Camesano & Logan, 2000; J. Strauss, N. A. Burnham, & T. A. Camesano, 2009; Taylor & Lower, 2008; Vadillo-Rodriguez et al., 2004). A few cases have examined proteins on bacterial surfaces, such as the amyloid fibrils on *Escherichia coli*, known as curli. Curli fiber equilibrium lengths on *E. coli* were reported to be 137 - 373 nm (Oh et al., 2012). The equilibrium length of proteinaceous fimbriae on *E. coli* ranged from 125 – 272 nm (Yatao Liu et al., 2006) - comparable to the values measured for the wild-type strain of *P. fluorescens*, and the *lapA* and *lapB* mutants (~120 nm). Some additional examples are reviewed in Table 2.

Interpretation of *L* and *s* values can be described in terms of physicochemical phenomena. *L* can change because of extension or compression. Polymer conformational changes from rigid to flexible molecules were observed in other studies. For example, when the same bacterium was exposed to solutions with different ionic strengths, pH, or other changes in solution chemistry, *L* varied (Camesano & Logan, 2000; Yatao Liu et al., 2006). Our study did not make use of any chemical differences, and the electrostatic interactions are expected to be similar among all studied strains. However, hydrophobic interactions are likely to change because the small spacing of 3.3 nm for the *lapG* mutant strain, along with the large *L* value suggest that the protein is packed much more closely and further extended on the surface. We suggest that when the tertiary structure of the protein begins to disentangle, more hydrophobic regions are likely to be exposed, consistent with another recent study that showed evidence of LapA unfolding in the *lapG* mutant strain, based on AFM force measurements (El-Kirat-Chatel et al., 2013).

4.3 Relationship of Protein Conformation to Bacterial Adhesion
The LapA protein plays a role in bacterial adhesion via fostering surface interactions, a critical first step in formation of a mature biofilm. Genetic studies first suggested that LapA acts as an adhesin (Hinsa et al., 2003; Peter D. Newell et al., 2011). In our prior work, we used AFM to demonstrate that the lapG mutant strain exhibited two-fold greater adhesion than the wild type as measured by a hydrophilic silicon nitride AFM tip, thus providing supporting evidence of the role of LapA as a protein adhesin (Ivanov et al., 2011). In the prior study, the mean adhesion forces were largest for the lapG mutant strain (1.268 nN), and were lower for the wild type as well as the lapA and lapB mutant strains. The mean adhesion forces for the lapA and lapB strains were nearly identical, at 0.204 nN and 0.203 nN, respectively. The mean adhesion force of the wild-type strain (0.272 nN) was significantly higher than for the lapA and lapB strains, but was much lower than that of the strain lacking the LapG protease.

Recently, adhesion of P. fluorescens Pf0-1 wild-type and lapG mutant strains were studied using single-molecule force spectroscopy with antibody-specific probes (El-Kirat-Chatel et al., 2013). Adhesion of the lapG mutant strain was greater than the wild type, regardless of whether a hydrophobic or hydrophilic AFM tip was used. This finding was attributed to multiple domains of LapA, which have both hydrophilic and hydrophobic properties. Furthermore, bacteria over-expressing LapA, and presumably allowing increased interactions among LapA molecules, also showed a surface that was stiffer and with more elongated polymers compared to the wild type (El-Kirat-Chatel et al., 2013). We speculate that the very small spacing observed between LapA molecules in the lapG mutant strain results in the tertiary structure of the protein to unfold (El-Kirat-Chatel et al., 2013). When this unfolding occurs, hydrophobic domains begin to be exposed, perhaps fostering adhesin-adhesin interaction. These interactions may expose hydrophobic domains or facilitate the formation of supramolecular adhesion complexes, and thus
the result is greater adhesion and biofilm formation. Taken together, these results support the strong role of LapA expression in influencing bacterial polymer conformation, bacterial adhesion, and biofilm formation.

We expected a higher adhesion force for the wild-type strain than for the lapA and lapB mutants, because the wild type is still capable of producing LapA. Fluorescence and super-resolution imaging of the LapA protein, along with AFM studies, clearly demonstrated that there is LapA on the cell surface, however it is also apparent that there is significantly less LapA on the cell surface of the wild-type strain when compared to that of the lapG mutant (El-Kirat-Chatel et al., 2013; Ivanov et al., 2012). Perhaps for the wild-type strain, the contribution of LapA on surface properties in masked by LPS and other surface proteins, such as porins and secretins. It is only when LapA is present at high levels, for example in the lapG mutant or when the bacteria are forming robust biofilms, that the full contribution of LapA to cell surface properties can be detected.

5.0 Conclusions

AFM experimental results, combined with application of the A-dG steric model, showed that the LapA protein accumulates and becomes elongated on the surface of P. fluorescens. All strains behave like classical polymer brushes, while the molecules on the lapG mutant strain are the most elongated from the bacterial surface. The significantly small spacing observed for the lapG mutant strain may be due to the partial unfolding of the tertiary conformation of the protein LapA, exposing its hydrophobic domains and this could possibly be the origin of the observed high adhesive and strong biofilm-forming characteristics of the lapG mutant strain.
References Cited


Dupres, Vincent, Alsteens, David, Andre, Guillaume, & Dufrêne, Yves F. (2010). Microbial nanoscopy: a closer look at microbial cell surfaces. *Trends in Microbiology*, 18(9), 397-405. doi: [http://dx.doi.org/10.1016/j.tim.2010.06.004](http://dx.doi.org/10.1016/j.tim.2010.06.004)


