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Testing the Effectiveness of Filastatin using 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
In Chemical Engineering
By
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Submitted and Approved on
By
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Abstract

Fungal infections caused by yeasts of the genus *Candida* are the fourth most common cause of healthcare-associated bloodstream infections in the United States. Previous research has identified a small molecule, named Filastatin, which reduces the adhesion ability of *Candida* to surfaces and prevents its ability to form biofilms. The ultimate goal of this project was to develop biophysical techniques to study fungal attachment and how this may be altered pharmacologically using drugs such as Filastatin or genetically using mutants. Atomic force microscopy was used as an experimental technique to determine single-cell adhesion forces of *Candida albicans*. The experiments conducted will help determine the effectiveness of Filastatin in reducing the adhesion of *Candida* to medical surfaces.
Acknowledgements

We would like to thank our project advisors, Professor Reeta Rao and Professor Terri Camesano, for their help throughout our project. We would also like to thank the members of the Rao lab group; including Cat Harwood for her help with culturing the *Candida* strains and Diego Vargas for his help in preparing the Filastatin surfaces. We would also like to give a special acknowledgment to Gawain Thomas for all of his help with the AFM, and his advisor, Professor Qi Wen, for his feedback on our experiments.
Introduction

Emergence of antimicrobial resistant microbes is a serious public health threat. Infections from resistant microbes are common primarily because of overuse or improper use of antimicrobial drugs. This problem is compounded by the fact that the antimicrobial drug pipeline for major pharmaceutical companies is dry. Together this undermines the ability of healthcare workers to fight and manage infectious diseases. An additional problem in the treatment of fungal diseases is that they are eukaryotic, a shared characteristic with human cells. Therefore an antifungal drug that kills the *C. albicans* is also harmful to the host (Mitchel & Nobile, 2006).

*Candida albicans & Biofilms*

*Candida* is the fourth most common cause of healthcare-associated bloodstream infections in the United States. Infections are typically caused by yeasts of the genus *Candida*. There are more than 20 species of *Candida* yeasts that can cause infection in humans, the most common of which is *Candida albicans* followed by followed by *C. tropicalis, C. parapsilosis, C. dubliniensis,* and *C. glabrata.* (Trofa, Gácser, Nosanchuk, 2008). *Candida* yeasts are commensal and normally live on the skin and mucous membranes without causing infection. However, overgrowth of these microorganisms can cause serious and sometimes deadly disease. Symptoms of candidiasis vary depending on the area of the body that is infected. Superficial infections may be chronic while blood stream infections often result in death. (Trofa, Gácser, Nosanchuk, 2008).
Candida infections tend to occur in the sickest of patients. They most often infect humans through intravascular catheter infections, which account for 200,000 infections in the United States annually (Saint, 2015). CDC estimates that each case of *Candida* infection results in 3–13 days of additional hospitalization, and a total of $6,000–$29,000 in direct healthcare costs. Roughly 30% of patients with bloodstream infections die during their hospitalization. Based on these estimates, the CDC calculates resistant *Candida* infections may add millions of dollars in excess costs to U.S. healthcare expenditures each year (Saint, 2015).

*C. albicans* has the ability to form biofilms (Kuhn DM & Ghannoum MA, 2004), a persistent problem in the healthcare industry. Biofilms allow *C. albicans* to cluster together and form stronger layers on surfaces. Cells in the context of a biofilm are particularly resistant to antimicrobial therapy. As a result, they have the potential to create a source of persistent infections. In fact, biofilms are the most common form of bacterial growth in nature. They are also an important aspect with clinical infections.

Abiotic prosthetic materials, such as catheters and IV tubes, offer the perfect substrate and conditions for *C. albicans* biofilms (Chandra, 2001). This process is a multi-step regime that begins when the *C. albicans* cells first adhere to a surface. As yeast and bacterial cells begin to prosper in the biofilm, the fungus continues to grow. Because the *Candida albicans* are being formed in the biofilm, it makes them particularly resistant to antifungal treatments (Chandra, 2001). *Candida albicans* biofilm development can be seen in Figure 1.
**Figure 1:** Biofilm Development: Diagram illustrating the phases of *C. albicans* biofilm development. (Chandra, 2001).
When first-line and then second-line antimicrobial treatment options, such asazole drugs, are limited by resistance or are unavailable, healthcare providers are forced to use therapies, such as Amphotericin B, that may be more toxic to the patient and frequently more expensive and less effective. Even when alternative treatments exist, research has shown that patients with resistant infections are often much more likely to die, and survivors have significantly longer hospital stays, delayed recuperation, and long-term disability. **Previous research has performed a functional** high-throughput phenotypic screen of 30,000 small molecules and identified, Filastatin. (Boyd, Cabral, 2014).

*Filastatin*

A small molecule has previously been discovered that inhibits adhesion of *C. albicans* to polystyrene surfaces, surgical silicone mesh, and cultured human epithelial cells. This molecule, called Filastatin, has the potential to be used as a coating material to protect medical devices from *C. albicans* biofilm attachment. Tests have previously been conducted that show that Filastatin reduces the number of *Candida* cells to polystyrene surfaces (Fazly, et.al, 2013). It was observed that wild-type *Candida* was unable to form biofilms in the presence of Filastatin. Figure 2 shows the chemical structure of Filastatin.
A possible hypothesis for how Filastatin prevents biofilm formation is a disruption of iron metabolism pathways. Figure 3 shows a possible explanation for these observations. Iron plays a key role Candida’s ability to survive and become pathogenic (Weissman, Kornitzer, 2004). By disrupting this pathway, Filastatin blocks the fungi’s ability to survive and form biofilms in low iron conditions. It disrupts this pathway by blocking PGA10 surface iron receptors. By blocking these receptors, the fungi are unable to uptake hemoglobin iron and form biofilms. Filastatin has also been shown to have a strong inhibition of filamentation.
**Figure 3:** Wild-type SC5314 strain with Pga10 and Pga7 mutant strains with and without the presence of Filastatin.

This project was designed to further test the effectiveness of Filastatin against *C. albicans*. Atomic force microscopy was used to test adhesion forces of wild-type *Candida*, various mutant strains, and in the presence of Filastatin. By conducting these experiments, more information can be gathered about the effectiveness of Filastatin against *C. albicans* to be used as a coating on medical devices.

Filastatin is a small molecule that:

- **Inhibits** fungal adhesion to abiotic surfaces like catheters and also to cultured human epithelial cells. This is an important finding because infection begins with adhesion to host cells.
• **Inhibits** formation of **biofilms**, which are difficult to treat with existing therapeutics. Once fungi attach to abiotic surfaces they form biofilms, the complex, multicellular networks that are often formed by fungal pathogens on implanted medical devices.

• **Alters** fungal biofilm **morphology** on mouse tissue and **inhibits** the ability of fungi to adopt an elongated **hyphal morphology** that is often correlated with pathogenesis.

• **Protects** the **host from killing** during an active fungal infection suggesting the drug is not toxic to the host.

These findings suggest this compound has the potential to be widely useful as a reagent to coat dressings and prosthetics used in war wounds.

The **goal of our research** was to develop quantitative tools to:

• Measure the force of adhesion between *C. albicans* and abiotic surfaces.

• Test the hypothesis that non-adherent *C. albicans* mutants show decreased adhesion forces to surfaces.

• Test the hypothesis that surfaces coated with Filastatin show decreased adhesion forces of *C. albicans* as compared to untreated surfaces.

The **results of our study allowed us to help evaluate whether** various **surgical and prosthetic materials, dressings** and other surfaces can be coated with the drug to **inhibit fungal adhesion**.
The state of *Candida albicans* can be analyzed by understanding the death process. A similar study was performed by treating the *Candida albicans* with antifungal agents flucytosine and amphotericin B (Kim, Kim, Han, Kim, Jung, Park, 2011). After the cells were treated with the drugs the *Candida albicans* were physically altered, showing that they had been perforated, deformed, and shrunken, as seen in Figure 4. An unexpected finding was that the cells’ viability decreased by 90% when exposed to the drugs for a short time, t=1, but then increased in viability when exposed for double the time. It was found that this can be attributed to the cells culturing and creating new cells that were unaffected by the drugs, i.e. emergence of drug resistance. The physical properties of the cells were observed utilizing AFM, which allowed effects of the drugs to be seen quantitatively (Kim, Kim, Han, Kim, Jung, Park, 2011).

**Figure 4: Cell death phase (CDP) of Candida albicans induced by antifungal drugs:** According to AFM images, CDP of *Candida albicans* can be categorized into four steps. The first step is CDP₀, which the cells are not visibly affected by the drug and show very clear, intact, and defined shapes. The next step is CDP₁, in which the cell shape is partially deformed, depending on the type of antifungal drug used. The third step is CDP₂, in which the cells lose their original shapes. The final step is CDP₃, in which the cells are shrunken and completely destroyed.
**AFM**

Atomic force microscopy (AFM) was used as an experimental technique to study *Candida* adhesion forces. AFM is a form of microscopy that can obtain 3-dimensional profiles of nanoscale surfaces. These profiles are obtained by measuring the forces between a sharp probing device and the surface being observed at distances between 0.2 and 10 nanometers (Wilson and Bullen, 2006). The probing tip is usually less than 10 nanometers in length and is attached to the end of a cantilever. The cantilever acts as a spring, with a defined spring constant that is used to find force measurements, as defined by Hooke’s Law:

\[ F = -k \times \Delta \delta \]

In Hooke’s Law, F is the force measured, k is the spring constant, and \( \delta \) is the cantilever deflection. The cantilever bends when force is applied and deflection measurements are recorded. The material of the probes (usually silicon nitride or silicon), along with the cantilever length, shape, and material allow for variations in measurements taken (Benoit and Hermann, 2002).

A semiconductor diode laser is aimed at the back of the cantilever, which sends a signal to a photodiode detector. A feedback loop and piezoelectronic scanner translates this data into measurements. Force curves are created from these measurements and 3-dimensional images can be obtained. The AFM used in this report is an Asylum Research MFP-3D machine. This specific machine uses a superluminescent diode (SLD) to create a reflection off of the back of the cantilever to a position sensitive diode (PSD) (Fuierer, 2009). An optical lever detector that monitors the SLD light source measures the deflection of the cantilever. This laser
spot on the back of the cantilever is held constant in the same XYZ position, so that all force measurements are directly comparable to each other. Figure 5 shows a depiction of the AFM process for obtaining force measurements.

**Figure 5: Diagram of AFM Process:** A close up depiction of the AFM probe tip and the sample. (Benoit and Hermann, 2002).

There are many different modes for imaging in AFM. The three most common are contact mode, intermittent mode, and non-contact mode. Contact mode involves bringing the probing tip in full contact with a surface in an XY pattern and keeping a positive deflection on the cantilever. Contact mode is used to created images of surfaces, mainly for rough samples, as this mode can damage soft samples. Intermittent mode, also referred to as AC mode, involves oscillating the cantilever and tapping the surface of a sample. This mode creates high-resolution images and is much less damaging to the sample. Non-contact mode involves oscillating the
probe above the surface and monitors changes in the amplitude. This mode exerts a very low force on the sample, but creates low-resolution images. (Wilson and Bullen, 2006)

A less widely used mode with AFM involves single-cell adhesion force measurements. Adhesion measurements can be taken between cell to cell interactions or cell to material interactions. This method has three steps to obtain measurements. The first step is called contact formation, where a charged coating on the probing tip, usually poly-L-lysine or concanavalin-A, creates attachment to the cell in study (Sen et al., 2005). The next step is adhesion, where a period of dwell is usually allowed so that formation is completed between the tip and the cell. Once adhesion is achieved, force measurements are taken to observe the interaction between cells or between the cell and a material. Force measurements create rupture between the two surfaces and a dwell period repeats to allow reattachment so that more measurements can be taken. Figure 6 shows an image of a probing tip attempting to create adhesion to a single cell of Candida albicans.
**Mutants Analysis**

Atomic force microscopy was used to study the adhesion forces of the wild-type strains SC5314 and SN250. SN250 is the isogenic control for the mutants analyzed in this study. The mutant strains studied were derived from the SN250 strain, from which certain genes were removed. This strain acts as a control for the mutant strains studied. The other control studied was the strain *edt1*. This strain lacks adhesion proteins on the cell wall that are required for *C. albicans* to attach to surfaces (Wheeler, et. al, 2008). With no ability to attach to surfaces, it would be expected to be unable to be measure adhesion forces using AFM. This strain was investigated as a control to compare to all other measurements taken with the AFM.

In addition, four *Candida* mutant strains were studied. These mutants were chosen because they were identified in a reverse genetic screen for genes that might be putative targets of Filastatin. These mutants were *hap43, pga10, rbt5, and pga7*. 

**Figure 6: AFM Probing Tip:** A probing tip attempting to create adhesion to a single cell of *Candida albicans*
The location of Pga10, RBT5 and Pga7 are shown in figure 7. The *edt1* mutant was used a control for our studies. Studying these mutants helped investigate how *Candida* cells attach to surfaces and what role Filastatin plays in preventing adhesion.

The first two mutant strains studied were *rbt5* and *pga10*. These two mutant strains lack surface proteins that aid in the formation of biofilms and attachment of these biofilms to surfaces. Iron plays a crucial role in the spread of pathogens, leading host cells to resist microbial infections by maintaining a low level of free iron to restrict the growth of invading pathogens (Hsu, Yang, Lan, 2010). *C. albicans* must overcome iron-deprived environments to cause infections. The regulator HAP43, is responsible for the repression of genes that encode iron-dependent proteins including *PGA10* and *RBT5*. The *hap43* mutant is less virulent when compared to wild-type strains presumably due to its decreased ability to form biofilms and attach to surfaces (Hsu, Yang, Lan, 2010).

The *pga10* mutant is critical for *C. albicans* to acquire hemin- and hemoglobin-iron. *PGA10* is regulated by HAP43 and plays a crucial role in iron acquisition and metabolism (Weissman, Kornitzer, 2004). The mutant strain, without *pga10*, forms thinner and more fragile biofilms. In the *Candida* wild-type strain, *pga10* receptors acquire hemoglobin iron and attach to other cells, allowing for the formation of biofilms. The *pga10* mutant strain lacks these receptors that acquire hemoglobin. This mutant strain is weakened in its ability to attach to other
cells and form biofilms. Like Pga10, Rbt5 is also cell-surface protein and is important in cell adhesion.

Finally the *pga7* mutant was also included in our studies. Unlike Pga10 and Rbt5, Pga7 is not located in the cell surface, instead it is in the cell membrane and thought to play a role in the transfer hemin- into the cell. Therefore the *pga7* mutant strain was also included in our studies. Pga10, Hap43, and Rbt5 are important for cell wall adhesion (Perez, Ramage, et. al, 2010). With AFM adhesion measurements it would be expected to find lower force readings with these three mutant strains than with the wild-type strain, as these cells ability to attach are reduced.

**Figure 7:** Wild-type SC5314 *Candida* Cell
**Project Goal/Problem Statement**

The ultimate goal of this project was to develop biophysical techniques to study fungal attachment and how this may be altered pharmacologically using drugs such as Filastatin or genetically using mutants. Our project was focused around testing *C. albicans* adhesion forces to glass surfaces with atomic force microscopy (AFM). Experiments studied the adhesion forces of the wild-type SC5314 *Candida* strain to glass surfaces with and without the introduction of Filastatin. In addition, various mutant strains were studied to help understand how *Candida* cells attach to surfaces and how Filastatin affects adhesion. Overall, the goal of this project was to use AFM as an experimental technique to understand adhesion forces involved with *Candida albicans* and to investigate Filastatin as a coating material to protect medical devices.
Methodology

Media and growth conditions

An autoclaved glass vial was filled with 2.5 mL of SC media. Then, a single Candida albicans colony was taken from a glass dish via a toothpick. The sample was then smeared into the glass vial and slightly stirred to ensure that the sample mixes into the media. A control was also set up following the same process but with no Candida albicans added. The samples were then transported over to the rotary drum, which they remained in for 18 hours at 30°C. After the time elapsed, they were removed from the rotary drum, and the solution was then dumped into plastic vials. The vials were then placed into a centrifuge for 2 minutes at 2400 rpm at 25°C. After this, the cells were separated and at the bottom of the vial, so the media can now easily be dumped out. After the media was removed 2.5 mL of PBS buffer was added. The samples were now ready to be observed under AFM.

Assay Development for Force Measurement using Atomic Force Microscopy

AFM cantilever tips were coated with Con-A for 30 minutes and then rinsed with deionized water. After this, the cantilever was calibrated; knowledge on how the calibration was done can be found in the journal article “Spring constant calibration of atomic force microscope cantilevers” (Sadler, Sanelli, Adamson, Monty, Wei, 2012). The spring constant and optical sensitivity are then determined. Upon this being completed, the AFM machine was now ready to accept samples. The cell samples of Candida albicans were suspended in PBS solution on a glass substrate. Utilizing the AFM’s optical microscope, a healthy cell was selected and the cantilever
was used to pick it up. Force measurements were taken by dwelling the cell onto
the glass substrate for five seconds prior to measurements being taken. If the force
varied by more than approximately 0.5 nN, it could be seen that more than one cell
has adhered to the cantilever tip and the data may be skewed (Ovchinnikova, 2012).
The same procedure was followed for the wild-type SC5314 and SN250 strains, and
all of the mutant strains.

The same AFM procedure was followed when testing Filastatin. The strain
SN250 was chosen to test the effectiveness of Filastatin. The adhesion force of
SN250 was tested on glass slides as a control and on glass slides that were coated
with Filastatin. The two experiments were compared to determine if Filastatin
reduced the adhesion force of the SN250 strain to the glass slides.

MATLAB

A computer program, MATLAB, was used to determine the number of
adhesion points per trial and the adhesions forces. The MATLAB program is able to
analyze the jumps recorded and determine adhesion forces. From this data, overall
average adhesion forces were able to be calculated. Finally, Microsoft Excel was
used to find the standard deviation of the data. Images of the MATLAB force curve
program can be seen in Figure 8. This figure shows how MATLAB calculates
adhesion forces. Figure A shows the force curves generated from AFM. From these
curves, the user selects the adhesion jumps, as seen in figure B. From this, MATLAB
is able to calculate the adhesion forces by making a fitted curve, as seen in figure C.
**Figure 8:** MATLAB Force Curve Program Images: A) force curve generated from AFM, B) user selected jumps, C) MATLAB calculations for adhesion forces

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**Statistical analysis using T-Test**

A t-test is a statistical test to determine if two sets of data are significantly different from each other. This test determines the probability of the differences in the data occurring by chance. This probability is called the p-value and the smaller this value is, the less likely it would be to find a difference in the data by chance. This helps decide whether or not to accept the data. A p-value of 0.05 or 0.01 is usually the significance level used to determine if the data should be accepted (Swingler, 2006). Conversion tables can be used to convert the determined p-values into percent confidence.

Excel was used to determine the p-values for the mutant data to determine if the mutant data was significantly different from the control wild-type SC5314 strain data and if the Filastatin coated slides were statistically different from the glass coated control slides. These p-values are converted into percent confidence using the following table. If the percent confidence was greater than 95%, then the data was recognized as statistically significant.
Results

Optimizing media and conditions for AFM measurements

The first media used to grow *C. albicans* was YPD (Yeast Extract-Peptone Dextrose). Most heterotrophic microorganisms are able to grow in this media. This media was originally used to optimize the growth of *C. albicans* before testing. Four trials were completed with growth in YPD media. Figure 9 shows the results gathered from the four trial dates. An average adhesion force of 2.56E-10 N was found for the SC5314 wild-type strain grown in YPD media.

**Figure 9:** YPD results with wild-type SC 5314 strain

However, YPD is an undefined media so we decided to use synthetic complete (SC) media for all subsequent studies. This media contains all required nutrients for cell growth, but was determined to allow for more controlled growth.
of the *C. albicans*. A total of eight trials were completed with the SC5314 wild-type strain grown in SC media. An average adhesion force of 3.05E-10 N was found. Figure 10 shows the results for the SC trials.

**Figure 10:** SC results for wild-type SC5314 strain

These results show that there is a slight difference between the two growth media. The SC5314 wild-type strain led to an average force of 2.56E-10 N in the YPD media and an average force of 3.05E-10 N in the SC media. These results are summarized in Figure 11. SC media was used throughout the remainder of trials because it allowed for more controlled growth of *C. albicans*. The remainder of trials completed with the SC5314 wild-type and mutant strains were grown in SC media.
Future experiments could look into different medias to determine the effect of the selected growth media on the adhesion forces of the cells.

**Figure 11:** Overall YPD vs SC media results

![Adhesion Force (N) Graph](image)

**Mutant Trials**

Four mutants were studied to compare to the wild-type strain, along with two control strains. These mutants, as described earlier were hap43, pga10, pga7, and rbt5, and the controls were SN250 and edt1. The hap43 mutant strain led to an average force of 2.17E-10 N, the pga10 mutant strain led to an average force of 1.63E-10 N, the pga7 mutant strain led to an average of 3.08E-10 N, and the rbt5 mutant strain led to an average force of 1.58E-10 N. Figures of all of these trials can be found in the appendix of this report. Three trials were completed for each mutant strain, except for the pga10 mutant. For the pga10 mutant strain, there was an error during one trial, during which data was unable to be obtained. The control
strain SN250 yielded an average force of 3.47E-10 N and the *edt1* strain yielded an average force of 1.93E-11 N. Two trials were completed for both of these control strains, and these figures can also be found in the appendix of this report. Figure 12 summarizes all of these mutant strain results.

*Figure 12:* Summary of mutant results compared to wild-type SC5314 strain and the two control strains

**Mutant Results**

<table>
<thead>
<tr>
<th>Mutant Results</th>
<th>SC5314</th>
<th>SN250</th>
<th>HAP43</th>
<th>PGA10</th>
<th>PGA7</th>
<th>RBT5</th>
<th>EDT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion Force (N)</td>
<td><img src="image" alt="Graph" /></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

* p > .05, not statistically different from SC5314
** p ≤ .05, statistically different from SC5314

**Filastatin Trials**

Due to time constraints, only preliminary testing was able to be conducted using Filastatin. The SN250 strain was the only strain that was used for testing. The same AFM technique was used for testing SN250 on a control glass slide and with a glass slide coated with the target drug, Filastatin. Two trials were completed for both the control and Filastatin-coated slides. The control slides yielded an average
force of 3.33E-10 N, which was close to the average force of 3.57E-10 N for SN250 trials completed earlier for this project. The Filastatin slides yielded an average force of 1.38E-10 N, which was considerably less than the average force for the control. Figure 13 summarizes these results for the Filastatin trials using SN250. Future experiments will test the SC5314 strain and all of the mutant strains in the same procedure.

**Figure 13**: Comparison of SN250 strain tested on control glass slides and on Filastatin-coated glass slides

SN250 Filastatin Results

![Graph showing adhesion force comparison](image)

*Statistical T-Test*

Statistical T-Tests were completed to determine if the mutant strain data were statistically different from the SC5314 wild-type strain and if the Filastatin coated slides were statistically different from the glass control slides. Excel was used to determine the p-values for the two sets of data. A p-value of 0.05 was the...
significance level used to determine if the data should be accepted. All of these trials for the mutants, except for one, met this significance level. The only trial that did not meet this requirement was the second trial for hap43. With p-values less than 0.05 there is a 95% confidence that the data did not occur by chance. This means that the difference between the adhesion forces obtained for the wild-type strain and the mutant strains can be considered statistically different. The results for the Filastatin coated slides showed that data was statistically different from the glass control slides.
Discussion

The first part of this project was spent learning the proper technique to test for adhesion forces on fungi cells. Originally, Candida was grown in YPD media, as previously discussed. This early stage of the project yielded fairly inconsistent data. This can mainly be attributed to human error involved with learning the proper technique. There was a wide range of data collected for the YPD data, yielding an average force of 2.56E-10 N with strain SC5314. This data was compared to Candida grown in SC media, as it was seen as a more controlled growth media. Throughout the project, an average force of 3.05E-10 N was found for Candida grown in SC media with strain SC5314. The data for the SC media was more precise than the YPD media, though both media yielded similar adhesion forces. SC media is a more controlled growth media and our results from the AFM were becoming more precise. For the rest of the project, Candida was grown in SC media to maintain more precise readings. Future experiments could determine the effect that growth media has on adhesion forces. For this project, the results for the wild-type SC5314 strain grown in SC media was used to compare to other values gained from other experiments.

The next part of this project dealt with Candida mutant strains. Four mutant strains were tested using the same technique as the wild-type SC5314 strain, along with two control strains. These four mutant strains were hap43, pga10, pga7, and rtb5, and the two control strains were SN250 and edt1. The first mutant strain, hap43, led to an average adhesion force of 2.17E-10 N. The hap43/- null mutant
strain produced a lower adhesion force than the wild-type strain. *Hap43* is a transcription factor that regulates iron-dependent proteins. The *hap43* mutant strain lacks this transcription factor that represses genes that encode for iron-dependent proteins. The *hap43* /- null mutant strain, should be less virulent, as compared to wild-type strains, since its ability to attach to surfaces is reduced. The results obtained from the AFM experiments supports this hypothesis, as the *hap43* /- null mutant strain yielded a lower adhesion force than the wild-type strain.

The two other mutant strains studied were *pga10* /- and *rbt5* /-. These genes are also regulated by *hap43* and play a crucial role in iron acquisition and metabolism. These proteins are thought to act as receptors for hemin- and hemoglobin-iron. This iron is crucial for *Candida* to become virulent by overcoming environments with low levels of free iron. These receptors are also crucial in the ability for *Candida* to attach to surfaces and form biofilms. The *pga10* mutant strain and the *rbt5* mutant strain showed lower adhesion than the wild-type SC5314 strain. The *pga10* /- null mutant strain yielded an average adhesion force of 1.63E-10 N and the *rbt5* /- null mutant strain yielded an average of 1.58E-10N. These results supported the hypothesis that without these receptors, the ability for *Candida* to attach to surfaces is greatly reduced. These two genes play a crucial role in the ability for *Candida* to become virulent and form biofilms on surfaces.
The \textit{pga7 -/-} null mutant strain was the final mutant studied for this project. This mutant strain plays a role in the iron-metabolism pathway. However, unlike the \textit{pga10} and \textit{rbt5} mutant strains, the \textit{pga7} receptor is not located in the cell wall of the fungi. It is located within the cell plasma wall, which does not come in direct contact with outside surfaces. The results from the AFM trials showed an average adhesion force of 3.08E-10 N, which is roughly the same adhesion force obtained for the wild-type \textit{SC5314} strain. These results show that this strain’s ability to attach to surfaces is not reduced due to the removal of the \textit{pga7} gene. This also shows that genes encoding for proteins within the cell wall play a more crucial role in the fungi’s ability to attach to surfaces than proteins that are located within the cell. Further experiments should look into more mutant strains that affect both the cell wall and within the cell. These results would better determine which genes directly affect the fungi’s ability to attach to surfaces.

Two controls were also tested to be compared to the data obtained for the mutant strains. These controls were \textit{SN250} and \textit{edt1 -/-}. \textit{SN250} is the strain that these mutants were derived from and should have yielded a similar force to the \textit{SC5314} wild-type strain. \textit{SN250} yielded an average force of 3.47E-10 N, compared to an average force of 3.05E-10 N for \textit{SC5314}. The \textit{edt1 -/-} null mutant strain lacks adhesion proteins on the cell wall that are required for attachment. It was expected to find little to no adhesion of this strain in the AFM testing. This was determined to be the case, as the \textit{edt -/-} strain yielded an average force of 1.93E-11 N. These two
strains acted as controls to be compared to the four mutant strains that were investigated.

The final stage of this project involved testing with Filastatin. Due to time constraints, only preliminary testing could be completed. Only the SN250 strain could be tested with Filastatin. The same AFM technique was used to test SN250 on control glass slides and on Filastatin-coated glass slides. The results showed an average adhesion force of 3.33E-10 N for the control glass slides and an average adhesion force of 1.38E-10 N for the Filastatin-coated slides. These experiments show that Filastatin significantly reduces the adhesion force of the SN250 strain to a glass surface, which was the expected outcome. These experiments are the first step in determining the effectiveness of Filastatin. Future experiments will be conducted in the same manner with the SC5314 wild-type strain and all of the mutant strains studied in this report. Studying all of these different strains will determine the ability of Filastatin to be used as a coating device on medical surfaces.

Throughout this project, the results showed high standard deviations. The standard deviations were consistently high; roughly 50% of the average. High standard deviations are the norm with this type of AFM procedure. This experimental technique is not widely used and requires a certain amount of experience to be able to properly test and analyze results for single cells. A better way to evaluate the experimental data is to utilize the standard error of the mean. This was calculated by dividing the standard deviation by the square root of the
number of trials. As previously discussed, statistical T-Tests were completed to see the significance of the results that were obtained. The results for the wild-type SC5314 strain were compared to the mutant data to see if they were significantly different. T-test were also performed to compare the Filastatin coated slides to the control slides to determine if they were statistically different. The analysis showed the results could be considered significantly different.

As mentioned throughout this report, there are a number of different experiments that can be conducted to further explore these results. Further testing should be completed to explore the role that growth media has in the attachment to surfaces. Also, more surfaces should be studied than glass to investigate the role that the surface has on adhesion forces, such as plastics. More mutant strains should be tested and compared with the wild-type strain. Testing more mutants within and inside the cell wall will help understand the role that certain genes play in the attachment of these fungi cells to surfaces. All of these mutant strains should be tested in the presence of Filastatin to help determine how Filastatin affects these mutant strains ability to attach to surfaces. Furthermore, due to time constraint limits for this project, a limited number of trials were conducted for each strain. More trials will yield more data to better support these results.

The purpose of this project was to determine the adhesion ability of Candida albicans to surfaces using atomic force microscopy with and without the presence of Filastatin. The goal was to further support the findings that Filastatin can be used as
a coating material to protect medical devices from *Candida* biofilm attachment. Due to time constraints, only preliminary testing could be conducted. However, these experiments showed that Filastatin significantly lowers the adhesion ability of the SN250 *Candida* strain to glass surfaces. Future experiments will investigate the SC5314 wild-type strain and all of the mutant strains that were studied in this report with Filastatin. As part of this project, various mutant strains were also studied and yielded interesting results about the fungi’s ability to attach to surfaces. This experimental technique of testing for adhesion forces of single cells is one step in determining the effectiveness of Filastatin to be used as a coating material. Future experiments should build off of the work completed for this project in hopes of further determining the effectiveness of Filastatin to protect against *Candida* infections.
References


Hsu, PC, CY, Yang, CY, Lan. Candida albicans Hap43 is a repressor induced under low-iron conditions and is essential for iron-responsive transcriptional regulation and virulence. December 3, 2010.


Appendix

AFM Results

YPD

<table>
<thead>
<tr>
<th></th>
<th>26-Sep</th>
<th>2-Oct</th>
<th>10-Oct</th>
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SC5314

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<table>
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hap43

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pga10

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pga7

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### SN250

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### Edt1

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### Mutant Results

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### SN250 Glass Control

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### SN250 Filastatin

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AFM Trial Data

HAP43 Results

PGA10 Results
PGA7 Results

RBT5 Results
SN250 Filastatin Results

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<tr>
<td>Mean</td>
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- Trial 1: 1.00E-10
- Trial 2: 2.00E-10
- Mean: 3.00E-10