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# Anti-Infective Mechanism-Based Drug Discovery via Sortase A

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06-07-2019

Bioengineering Senior Thesis

Anti-Infective Mechanism-Based Drug Discovery via Sortase A

Santa Clara University

Department of Bioengineering

I HEREBY STATE THAT THE THESIS WAS PREPARED UNDER MY SUPERVISION  
BY:

Huong Chau, Alice Matsuda, & Leepakshi Johar

ENTITLED:

ANTI-INFECTIVE MECHANISM-BASED DRUG DISCOVERY VIA SORTASE A

THIS THESIS IS TO BE ACCEPTED FOR THE FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF:

**BACHELOR OF SCIENCE  
IN  
BIOENGINEERING**

Zhiwen Zhang      6/07/19  
Thesis Advisor                      Date

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Department Chair                      Date

Anti-Infective Mechanism-Based Drug Discovery via Sortase A

By

Huong Chau, Alice Matsuda, & Leepakshi Johar

Advised by

Dr. Zhiwen Zhang

**SENIOR DESIGN PROJECT THESIS**

Submitted to  
the Department of Bioengineering  
of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements  
for the degree of

Bachelor of Science in Bioengineering

Santa Clara, California  
2018-2019

## **Abstract**

Sortase A is a transmembrane protein prominent in gram-negative bacterial strains. It is a virulence factor that anchors other proteins, which facilitate MRSA infections. In the long term, we plan to utilize this protein to create anti-infective drugs as antibiotic resistance continues to become a global health issue. Starting with five NIH drug candidates, we decided to first study Sortase A with B12 due to its historical past of ancient civilizations using natural sources of B12 to treat infections. Our senior design is further centered around understanding how vitamin B12 interacts with Sortase A in vitro, particularly the binding affinity between the two. The in vitro experiments will verify the previous in vivo studies.

## Acknowledgements

We would first like to acknowledge Santa Clara University for allowing us to utilize Alumni Science and Daly Science laboratories for research purposes. Without the usage of these labs, we would not have been able to perform experiments to complete our design project.

We would also like to thank the SCU School of Engineering for funding our research endeavors. Without Santa Clara University's generosity, we would not have been able to conduct our experiments.

Next, we would like to thank Dr. Zhuang on his expertise on *E. Coli* cell culturing, protein purification, and SDS gel page running. We would also like to thank our lab managers from Alumni Science and Daly Science, Daryn Baker and Owen Gooding, for helping us navigate through using separate research equipment and ordering protocol. Finally, we would like to thank Dr. Zhang's graduate students, Neha and Akhil, for their research-related input.

Above all, we would like to thank our advisor, Dr. Zhiwen Zhang, for allowing us to research in his group. His knowledge of protein structure and bioengineering techniques greatly contributed to our project. We appreciate his guidance, expertise and patience throughout the year-long process. Dr. Zhang further helped us to grow as engineers and challenged us to re-adapt to any problems we faced during our design process.

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## List of Abbreviations

MRSA: Methicillin-resistant *Staphylococcus aureus*

SDS Page: sodium dodecyl sulfate polyacrylamide gel electrophoresis

EHS: Environment, Health and Safety Department

Maldi-Tof: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

FPLC: Fast protein liquid chromatography

HPLC: High Performance Liquid Chromatography

*E. Coli: Escherichia coli*

LB: Luria Broth

DNA: Deoxyribonucleic acid

OD: Optical Density

IPTG: Isopropyl  $\beta$ -D-1-thiogalactopyranoside

Ni-NTA: Nickel-nitrilotriacetic acid

MW: Molecular Weight

PMSF: Phenylmethylsulfonyl fluoride

His-tag: Polyhistidine tag

ITC: Isothermal Titration Calorimetry

# Chapter 1: Introduction

## 1.1 Background and Motivation

The motivation of this project is to provide a more permanent solution to MRSA infections by surpassing the antibiotic resistance that antibiotics bring. The better quality of life and symptom reduction of MRSA patients is the ultimate motivation for this project. The objective of our senior design project is to test the molecular interactions between Vitamin B12 and protein virulence factors present on Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria. Ultimately, the main long-term goal of this project is to develop more effective drugs to treat MRSA.

Overall, our senior design is centered on mechanism-based drug discovery. One of the particular strains that are of potential focus for this project is MRSA, which transitioned from a hospital-acquired infection to the community-acquired and livestock-acquired infections. The hair-like virulence factor on the gram-positive bacteria, specifically cilia or pili, are used for communication between cells. In this particular project, the cell-to-cell communications between gram-positive bacteria and mammalian (human) cells. Narrowing the already identified drug target library will help to identify Sortase A, which is an enzyme that catalyzes the anchoring and interactions of cell surface proteins.

Patients who are suffering from MRSA will greatly benefit from the development of more effective gram-positive anti-infective drugs. This will allow patients to have a better lifestyle and will eventually have fewer restrictions on the family and the patients. Ethical issues in this project will be implemented to address the safety of patients.

### 1.1.1 MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is caused by a type of *Staphylococcus aureus* gram-positive, round shaped bacteria that has become resistant to many of the antibiotics used to treat ordinary *Staphylococcus aureus* infections, including methicillin. (Siddiqui) Methicillin is an antibiotic  $\beta$ -lactam antibiotic of the penicillin class, which inhibits cell wall synthesis in bacteria. MRSA is resistant to many antibiotics and is therefore called a “superbug”. MRSA is the result of years of often unnecessary antibiotic use. Even when antibiotics are used appropriately, they contribute drug-resistant bacteria

because they do not destroy every bacterium they target. Bacteria live on an evolutionary fast track, so germs that survive treatment with one antibiotic soon learn to resist others. As a result, when one bacterium is resistant to an antibiotic it survives and divides rapidly and therefore, there is a dense population of bacteria that are resistant to antibiotics. These bacteria can also transfer the antibiotic resistant gene to other bacterial strains, which increases resistance to that antibiotic.

MRSA is a bacterium that causes infection in different parts of the body. Usually, it causes a mild infection under the skin such as boils and sores. It can also cause serious skin infections and can cause infection in surgical wounds, bloodstream, lungs, or the urinary tract. Even though most infections of MRSA are not serious, some of these infections can be life threatening. The common *Staphylococcus aureus*, like MRSA, start as swollen and painful red bumps that are possibly warm to the touch, have pus or other drainage, and accompanied by fever. Over time, they can turn into painful abscesses that require surgery to relieve the fluid and pain. If these infections are not on the surface of the skin, they can also cause infections in bone, joints, surgical wounds, bloodstream, heart valves, and the lungs. (Siddiqui, WebMD)

Most MRSA infections occur in individuals who have been in hospitals or other health care settings. When it occurs in these settings, it's known as health care-associated MRSA (HA-MRSA). HA-MRSA infections are typically acquired from invasive procedures or devices, such as surgeries, intravenous tubing or artificial joints. With time, MRSA infections have occurred in the community beyond hospitals. This is known as community-associated MRSA (CA-MRSA), which often begins as a painful skin boil. It is spread by skin-to-skin contact. (Zhang, Siddiqui)

## 1.2 Existing Technologies

Currently, anti-infectives is a new field that was started by Dr. Zhiwen Zhang, and there are no current anti-infective drugs on the market or under research. As of now, only antibiotics are available, and that is part of the problem due to antibiotic resistance.

## 1.3 Literature Review

Staphylococcus aureus Sortase A Exists as a Dimeric Protein In Vitro, Changsheng Lu, Jie Zhu, Yun Wang, Aiko Umeda, Roshani B. Cowmeadow, Eric Lai, Gabrielle N.

Moreno, Maria D. Person, and, and Zhiwen Zhang\*, *Biochemistry* 2007 46 (32), 9346-9354 DOI: 10.1021/bi700519w

Simon G. Patching, Surface plasmon resonance spectroscopy for characterisation of membrane protein–ligand interactions and its potential for drug discovery, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, Volume 1838, Issue 1, Part A, 2014,, Pages 43-55, ISSN 0005-2736, <https://doi.org/10.1016/j.bbamem.2013.04.028>.

## **1.4 Project Goals**

The goals of this project included was to complete our research pipeline for developing the mechanism-based discovery assay. We initially started off with having a thorough understanding the basis of this project through literature review. Since, the project pertains to five potential NIH drug candidates that were tested in vivo with our protein target, it was important to understand which drug candidate we will be using to test the molecular interactions between Sortase A and our drug candidate. From, there we worked on planning how we would obtain Sortase A through protein purification. Ideally, we wanted to obtain a high-grade purified protein in order to reduce any false positive and false negatives that contaminants can produce when molecular interactions are tested. We also wanted to research about what instruments can be used for testing the molecular interactions between our Sortase A protein and our drug analyte.

## **1.5 Alternative Solutions**

In order to prevent the spread of infection, wounds on the skin should be kept clean and covered with sterile, dry bandages. Washing hands after contact with an infected wound, dirty clothes, changing bandages is also recommended. Also, sharing personal items such as towels, washcloths, razors, and clothing can increase the chances of infection. Along with these preventative measures, there are ways of removing bacteria from the skin. Antibacterial body wash or powder for the skin (chlorhexidine baths), cream for inside the nose (if the infection is present there), antibacterial shampoo for the scalp (chlorhexidine soap shower/bath procedure), germ-killing soap and ointments can potentially remove bacteria from areas where they are in contact with the body.

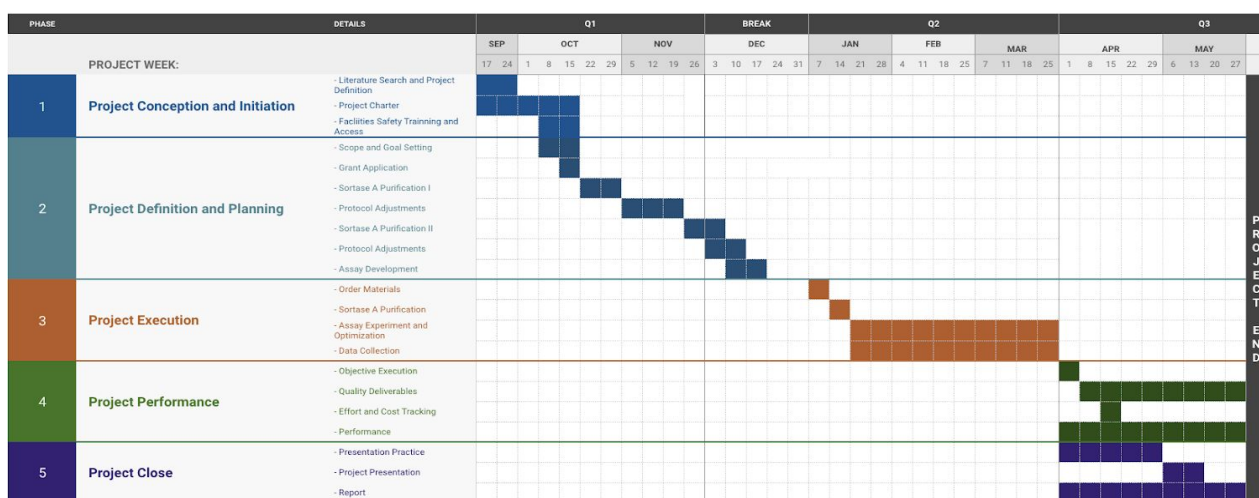
## **1.6 Team Management and Operations**

We started our project in September 2019 and will be concluding our experiments at the end of June 2019. We budgeted our time in Fall Quarter 2019 as well as Winter Quarter

2019 to optimizing our His-tag protein purification protocol. We then spent the bulk of Spring Quarter 2019 analyzing our protein samples via mass spectrometry and biacore. The majority of our budget was spent on various protein purification materials such as Ni-NTA beads, purification buffers and Qiagen reagents. We also used a quarter of our budget on SDS page gels and protein ladders in order to obtain better resolution in our gel results, which detailed the presence of our protein monomer and dimer.

## 1.6.1 Senior Design Timeline

Figure 1. Timeline and deadlines for Senior Design Project 2018-2019.



## 1.6.2 Senior Design Budget

Table 1. Project Budget.

<u>Materials/Equipment</u>	<u>Cost</u>
Bacterial Cells and Cell Solutions (LB media, etc.)	\$123.00
Chemicals and Reagents	\$269.00
Protein Purification Materials (Ni Bead, Purification Kits)	\$650.00
SDS PAGE and Gel Electrophoresis (Thermo Fisher: 3*10 gels/box)	\$458.00
<b>Total Cost</b>	<b>\$1500.00</b>

## **Chapter 2: Collaborative Research**

We claim that there has been no collaborative research and would like to acknowledge as well as thank Santa Clara University for allowing us to research in the Alumni Science, Daly Science and engineering facilities.

## Chapter 3: Project Outline and Design

### 3.1 Cell Culture

To make sure that we had an optimum amount of Sortase A protein to purify, we had to practice and become proficient in cell culture techniques. The cell culture protocol is completed in the span of three days. The first step is to transform *E.coli* cells with the pET-2b plasmid that contains the sequence for Sortase A with the 6-histidine tag. After the transformation is complete, the cells are grown on Agar plate with LB media for 48 hours. A small 5 ml culture was created by growing these *E.coli* cells in the incubator shaker for 12 hours. After the small culture reached an OD of approximately 0.600, the small culture was transferred to 1500 ml of LB with Kanamycin to grow a larger culture. In this we also added IPTG to initiate induction, or the expression of Sortase A in the *E.coli* cells. After 6-8 hours of induction, the cell culture in the LB/Kanamycin solutions were placed in pre-weighed bottles and spun down in the centrifuge to filter out and get the cell pellets. The cell pellets plus bottles were weighed and stored in -80°C.

### 3.2 Protein Purification

#### 3.2.1 Initial Native Protein Purification Protocol

After our cell culture protocol, we needed to extract and further purify our protein. We first purified via Ni-NTA beads with buffers (wash, elution, lysis) that we had prepared. This protein purification method was centrifuge-based and needed to be optimized to ensure maximum protein yield. Throughout our optimization process, we needed to alter the time in which the cell solution was sonicated, the pH of the lysis buffer, wash buffer, as well as the elution buffer, in addition to the amount of time each consecutive solution was centrifuged to avoid protein denaturation. To further prevent protein degradation, PMSF was later added in small increments to our buffers.

#### 3.2.2 Qiagen Fast-Start Purification

As a cross-comparison, we used the Qiagen Ni-NTA Native Protein Fast-Start Purification Kit to test for maximum protein yield. It should be noted that as opposed to our original protein purification procedure, the Qiagen kit provided us with spin columns and pre-made lysis buffers, elution buffers and wash buffers along with solutes that needed to be dissolved. To prevent protein degradation, we also added PMSF to each of the pre-made buffers.

### **3.3 SDS Page Gel**

#### **3.3.1 Changes and Specifications**

Initially, we used an SDS gel apparatus and gels from Bio-rad (Hercules, CA) to qualitatively test our protein purity. After multiple runs, we came to the conclusion that the Bio-rad products that we used did not reach the resolution that we desired in a gel image. Consequently, we decided to move on to use Invitrogen gels and an invitrogen apparatus (Carlsbad, CA), which enabled us to have a separation range of 10 kDa to 40 kDa. This allowed us to clearly see whether we had acquired the Sortase A monomer, which is located at the 17kDa mark, and the Sortase A dimer, which is located at the 34kDa mark.



## **Chapter 4: Materials**

### **4.1 Cell Culture Materials**

The the kanamycin was obtained from VWR (Radnor, PA) and IPTG was obtained from US Biologicals. The rest of the materials such as the bottles and centrifuges were from the Bioengineering labs on campus.

### **4.2 Protein Purification Materials**

Initially, we had started using the Qiagen protocol (Germantown, MD) that consisted of protein purification in the span of three days. This protein purification protocol was not as accurate when tested through the and therefore we switched to Qiagen's Fast-Start Purification (Germantown, MD), which took 90 minutes. The Ni-NTA beads used for anchoring the Sortase A protein is from Qiagen. The PMSF used in the lysis buffer is also from Qiagen (Germantown, MD).

### **4.3 SDS Page Gel Materials**

The protein ladder was obtained from invitrogen (Carlsbad, CA). This was used to calibrate and make sure that we can identify the protein qualitatively. Initially, we had used Bio-Rad (Hercules, CA) apparatus to run the gel, but due to compatibility issues with the protein ladder, samples, and gel, we transitioned to the Invitrogen apparatus (Carlsbad, CA). The running buffer and SDS Page Gel were also from Invitrogen to make sure that there was consistency in the data (Carlsbad, CA).

### **4.4 Mass Spectrometry Materials**

Mass spectrometry, also known as MALDI-TOF, utilized materials (buffers, solutes) and kits provided directly from GE Healthcare (Chicago, IL).

# Chapter 5: Methods

## 5.1 LB & Agar Plate Preparation

We first needed to autoclave 2000 mL Erlenmeyer flask under gravity (solid) conditions. Next, we made 500 mL of an LB and agar mixture by weighing out 7.5 g of Agar and 12.5 g of LB. We poured the weighted LB and Agar into our autoclaved Erlenmeyer flask. We filled the Erlenmeyer flask with 500 mL of di-water and Mixed solution until the LB and Agar completely dissolved. Afterwards, we autoclaved the LB + Agar solution under liquid conditions for about 45 minutes and allowed it to cool once finished. An addition of 250 uL of Kanamycin was finally added to the solution for plasmid selection. For safety purposes, we then mixed the solution in a sterile environment by wiping down surface and pipets with 70% ethanol and lighting an alcohol burner. After lighting the alcohol burner, we poured 10-15 mL of solution into each lab plate. We allowed the plates to solidify for 1 hour in sterile environment with the lid half-way open and placed the lid over the plates and let the plates sit in sterile environment upside-down for 1 hour. It is important to parafilm each plate and store it in the 4 degrees Celsius fridge upside-down.

## 5.2 Transformation

We thawed the DNA vector and the Invitrogen - One shot Top10 Chemical Competent *E.coli* kit on ice. We added 5 uL of DNA vector sample into competent cells. We made sure not to pipet mix the DNA vector sample into competent cell and gently tapped the tube to mix. We heat-shocked the sample by placing them in a water bath set at 42 degrees Celsius for 30 seconds. The sample is then placed on ice for another 30 seconds. Then, 250 uL of prewarmed Super Optimal Broth with Catabolite Repression (SOC) media at 37 degrees Celsius to the sample and store them in the 4 degrees Celsius fridge.

## 5.3 Plating Transformed Cells

Starting in a sterile environment, we pipetted 50 uL of transformation solution onto the center of the LB + Agar plate. We then used a sterile pipet tip to spread the sample around the LB + Agar plate while keeping the plate close to the alcohol burner. The

sample plate was then covered with a lid and stored upside-down in an incubator set at 37 degrees celsius. The colonies were allowed to grow for roughly 48 hours on average. After the growth period, the sample plates were stored in a four degree refrigerator.

## **5.4 Creating Small Scale Culture**

A 2000 mL Erlenmeyer flask was autoclaved under gravity (solid) conditions. We created 500 mL of LB by weighing out 12.5 g of LB. The measured LB was poured into the autoclaved Erlenmeyer flask. The Erlenmeyer flask was filled with 500 mL of di-water and mixed in solution until the LB completely dissolve. The LB solution was autoclaved under liquid conditions. We waited until the the LB solution cooled down to room-temperature and then added 1:100 to 1:50 ratio of Kanamycin. We mixed the LB solution, so that Kanamycin is distributed throughout the solution. We pipetted 5 mL from the LB solution into a separate cell culture tube. Then, we collected the LB + Agar plate with colonies from 4 degrees Celsius fridge and used a sterile loop or pipet tip to pick a colony and inoculated it in the 5 mL in a sterile environment. We placed the 5 mL small scale culture in a shaking incubator at 225 rpm at 37 degrees Celsius. The 5 mL small scale culture grow for 26 hours. The big scale LB solution in the Erlenmeyer flask was parafilmmed and store in 4 degrees Celsius fridge. The OD of the 5 mL small scale culture was monitored on the spectrophotometer. We used plain LB without kanamycin as a blank. We wanted to make sure the OD is close 1 (the stationary stage of a bacteria life cycle).

## **5.5 Large-Scale Culture**

When the OD for the small scale culture was neat 1, we added the culture to a 1500 mL kanamycin and LB solution. The large scale culture was then left in the shaking incubator at 225 rpm at 37 degrees celsius for roughly 8 hours. Once the OD of the culture reached 0.6, we collected one milliliter of the cell solution as a negative control for SDS page steps in the future. Next, we added IPTG at a final concentration of 0.1 mM to induce cell growth. From this point, our large-scale culture was incubated at 225 rpm at 37 degrees celsius for 15-20 hours.

## **5.6 Qiagen Native Protein Purification Kit**

Before starting the experient, the native Lysis Buffer must be prepared. The lysis buffer must consist of lysozyme and Benzonase Nuclease. The contents of the lysozyme vial

are dissolved in 600  $\mu\text{l}$  of native Lysis Buffer. To create a 10 ml aliquot of native Lysis Buffer, 100  $\mu\text{l}$  of the lysozyme solution is added. If not used immediately, the remaining lysozyme solution is stored at  $-20^{\circ}\text{C}$ . The vial containing Benzonase® Nuclease solution is thawed and 10  $\mu\text{l}$  is added to the 10 ml aliquot of native Lysis Buffer. The cell pellet is thawed for 15 min on ice and resuspended in 10 ml of native Lysis Buffer. They are incubated on ice for 30 minutes and mixed 2–3 times by gently swirling the cell suspension. The lysate is centrifuged at  $14,000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$  to pellet the cellular debris. The cell lysate supernatant is retained. The supernatant now contains the soluble fraction of the recombinant protein. The resin is gently resuspended in a Fast Start Column by inverting it several times. The seal was broken at the outlet of the column and the screw cap was opened, allowing the storage buffer to drain out. The outlet seal must be broken before the screw cap is removed. The cell lysate supernatant is applied to the column. The flow-through fractions are collected. The column was washed twice with 4 ml of native Wash Buffer. Both washes were collected. The bound 6xHis-tagged protein was eluted with two 1 ml aliquots of Native Elution Buffer. Each elution fraction was collected in a separate tube. All fractions collected were stored in  $-20$  degrees Celsius and analyzed by SDS page Gel Electrophoresis.

## 5.7 SDS Page Gel Electrophoresis

First, we started by collecting our stored supernatants from a  $-20$  degrees celsius and placing them all on ice to thaw out slowly. Once they were ready, we used a nanodrop to find the concentrations of each of the different supernatants, which included two washes and two elutions. For the nanodrop, wash buffer was used as a blank for the Flow Through and the six washes. The Elution Buffer was used as a blank for the two Elutions. The 26616 Protein MW Ladder and 5X dye was used. Separate samples to run through SDS-Page gel were loaded on the gel. Each sample consisted of 48  $\mu\text{L}$  of specific sample and 12  $\mu\text{L}$  of 5X dye, with the total volume of 60  $\mu\text{L}$ . One liter of Running Buffer was made by mixing the Running Buffer Powder in 1 L of di-water. We set up the SDS-Page apparatus and poured the running buffer to overfill the gel and make sure the running buffer is filled up to the 2 gel mark. The prepped samples were loaded on the gel. The ladder and samples, 7  $\mu\text{L}$  and 60  $\mu\text{L}$  respectively, were loaded. The running buffer was pipetted back into the gel if needed. The SDS-Page apparatus was connected to the Power Box and we ran it at 110 V for 50 minutes. After running the gel, the Power Box was turned off Power Box and we disconnected the SDS-Page apparatus. The running buffer was poured in a container and stored for later use —it can be used a total of 3 times. The plastic sheets around the gel were removed the gel was rinsed with di-water. The gel was placed in a container of di-water and microwaved for 20-30

seconds. The di-water was poured out and the container was filled with LabSafe Gel Blue. The gel was stained for 1 hour under the incubator shaker. After 1 hour, the LabSafe Gel Blue was poured in an Erlenmeyer flask and parafilmmed. The LabSafe Gel Blue can be used a total of 2 times. The gel container was filled with di-water and destained overnight under the incubator shaker. The next day, the gel was screened in the LAS 500 imager. The images were saved and analyzed and the gel was stored in di-water in 4 degrees Celsius .

## Chapter 6: Results and Discussion

We were able to verify the presence of Sortase A via SDS page gel and MALDI-TOF. This in turn verified that we were able to successfully purify Sortase A. Prior to the verification process with SDS page gel and MALDI-TOF, we sequenced our purified cell plasmid to verify that our cells were capable of expressing the gene for Sortase A production. We translated our DNA sequence to a protein sequence, and later found portions of the Sortase A sequence within our own protein sequence. This sequence, and the subsequent weight of Sortase A, is shown in Figure X. As shown below in Figure X, we were then able to verify that the 17 kDa band, associated with the monomer for Sortase A, and the 34 kDa band, associated with the dimer for Sortase A, were both present throughout the gel. Lastly, in Figure X, we were able to observe a peak for Sortase A based on its weight. Overall, our results were significant as we were able to successful isolate and purify Sortase A in order to test its interaction with B12, and later the four other NIH drug candidates.

### Theoretical pI/Mw (average) for the user-entered sequence:

```
    10      20      30      40      50      60
MGQAKPPIPK DKSKVAGYIE IPHADITQPV YPGPATPEQL NRGVSFAEEN ESLDDQNISI
    70      80      90      100     110     120
AGHTFIDRPN YQFTNLKAAK KGSMVYFKVG NETRKYKMTC IRDVKPTDVE VLDEQKGKDK
    130     140     150
QLTLITCDDY NEKTGVWEKR KIFVATEVKL EHHHHHH
```

Theoretical pI/Mw: 7.09 / 17901.28

Figure 2. DNA to Protein Sequence Verification

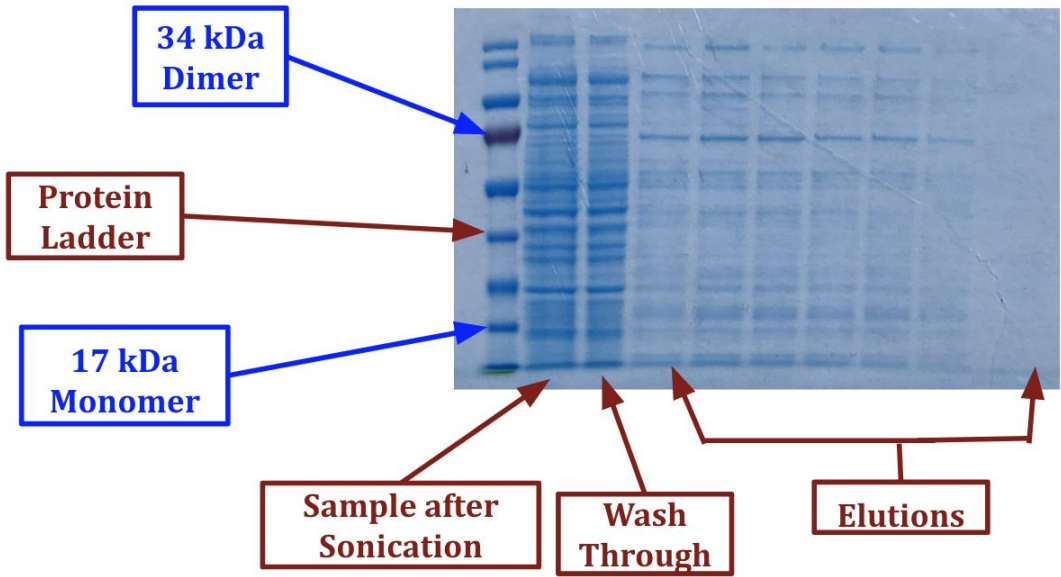


Figure 3: SDS Page Gel Final Result

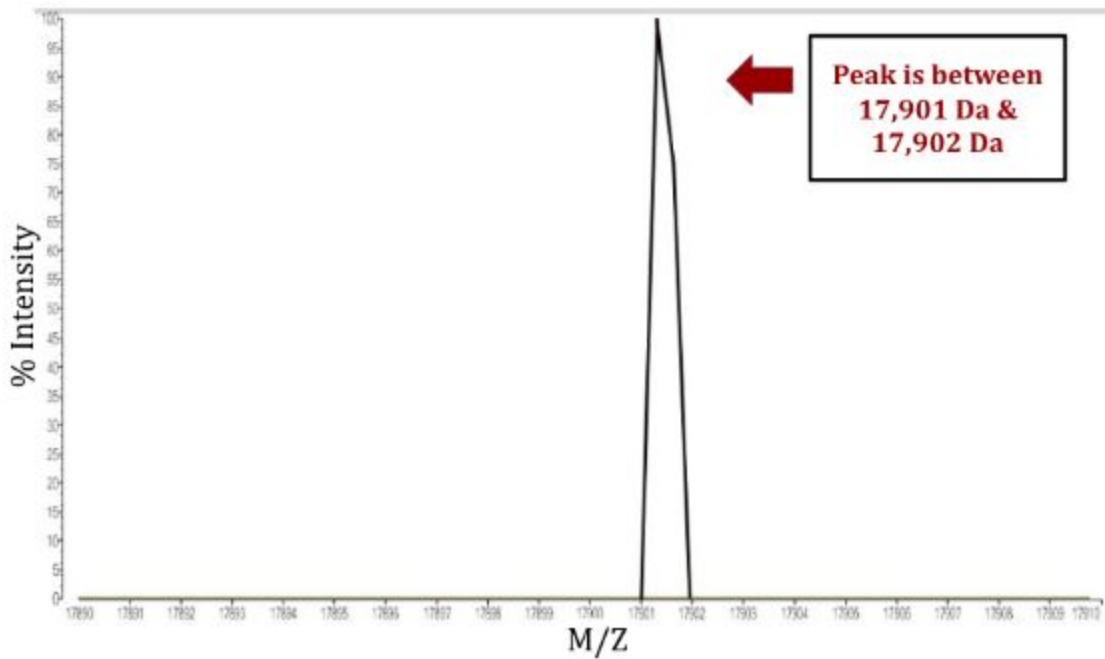


Figure 4: Mass Spectrometry Isolated Peak

# Chapter 7: Project Challenges

## 7.1 Purification Process

The most challenging step of our project was the purification of Sortase A protein. We dedicated the majority of our time in lab purifying Sortase A and with our advisor troubleshooting the protein purification procedure and materials. We encountered challenges working with delicate instruments in lab that required specialists and lab managers to train us and calibrate the instruments. In order to improve the purification process and find the specific step that needed to be optimized, we troubleshooted the procedure into three main steps: Cell growth and protein expression, protein purification and analysis.

## 7.2 Qiagen protocol

The Qiagen Protocol 9. Preparation of cleared *E. coli* lysates under native conditions [cite] was used for our protein purification process as the starting point. There were also previous students purification protocol notes that we used. Since we did not obtain meaningful results on the first purification experiment, we decided discussed possible variations on materials and methods to achieve optimum results. This troubleshooting process was repeated several times during this project.

### 7.2.1 *E. coli* cells and cell growth

In order to identify transformed cells, we used competent *E. coli* cells transformed with the plasmid pET2b. Since there were no information if the cells were expressing Sortase A and which plates were growing cells expressing (C) His6 tag or (N) His6 tag, we decided to sequence the cell via a purified plamid. Initially, our goal was to use the (N) His6 tag growing cells but we were only able to confirm (C) His6 tag expressing cells.

#### 7.2.1.1: Sequencing

In order to confirm we had the two variations of cells, we used Sequetech sequencing services. We were able to confirm the number two which is a C end his6 tag after analysing the amino acid sequence. Having this confirmation gave us confidence that we were growing the right cells and that they were expressing Sortase A. In order to find the location of His6



tag, we used exPasy and located the string of six histidine amino acids, confirming that they were on the (C) end. Once we confirmed the cells, we had and therefore which plasmid had been used to transform these cells, we were also able to confirm the promoter which had been induced with the correct reagent, IPTG.

## **7.2.1.2 Cell growth and protein expression**

### **7.2.1.2.1 Kanamycin**

We used the diluted and aliquoted kanamycin that was available in our lab. Because the eppendorf tubes of aliquoted kanamycin had no dates or information about the antibiotic, we purchase kanamycin in solid form and reconstituted to the concentration we needed. We suspected that the kanamycin we had used from the lab could have been expired. No improvements were noted.

### **7.2.1.2.2 IPTG**

Similarly, we suspected that the IPTG we were using in lab from previous projects were expired and no longer effective. We purchased new IPTG.

### **7.2.1.2.3 PMSF**

In order to prevent Sortase A degradation during the sonication process, PMSF was added to the lysate.

### **7.2.1.2.4 Sonication**

Sonication is an important step to help lyse the cells in order to obtain the Sortase A which is embedded in the cell membrane. The recommended instructions that we obtained from previous experiments were to set the sonicator to alternate from 10 seconds of sonication and 15 seconds of resting period for 20 cycles with amplitude of 30%. Our advisor suggested that we sonicated for a longer period of time (1 hr), by giving a longer period of rest (three minutes) to allow the lysate to cool since the heat generated from

sonication can degrade proteins. We found that 30 cycles of 15 seconds of sonication and 50 seconds of rest period worked best. Color difference in the lysate was noted as the lysate that sonicated for a longer period of time appeared to have a more yellow color than the previous lysates that were more clear color.

### **7.2.1.3 Purification Steps**

#### **7.2.1.3.1 Imidazole Concentration**

The purpose of imidazole is to increase the purity by either promoting binding of Sortase A or minimizing binding of unwanted proteins depending on its concentration in each of the three buffers. Imidazole has similar structure to histidine and excessive concentrations can denature proteins. Initially, imidazole concentration for lysis buffer was 10mM, for wash buffer was 20mM and for elution buffer was 250mM according to Qiagen. We first adjusted to 10mM for the elution buffer and no imidazole on washing and lysis buffer. No improvement on the results were noted. We also tried increasing the lysis buffer imidazole concentration from 10mM to 15mM, but no difference was observed. therefore the initial Qiagen protocol concentrations were maintained.

#### **7.2.1.3.2 pH adjustment**

The pH also plays a role in the binding affinity of proteins. Values of pH above 10 or below 5.8 are not ideal. According to Qiagen protocol, optimum pH value is 8.0 for lysis, wash and elution buffers. We tested by changing pH to 8.5 for lysis buffer to help lysing the cells. Without observing any changes in the results, we kept the Qiagen recommended pH 8.0 for all three buffers.

#### **7.2.1.3.3 Ni-NTA Beads Binding Time**

We varied the binding time from two hours to four hours and also tried binding overnight (eight to ten hours). For longer binding periods, we also increase the time for the rotator in the washing

step. For the overnight binding, the last wash (wash 6) was adjusted for one hour in the rotator before centrifuge step.

#### **7.2.1.3.4 Qiagen Fast-Start Purification Kit**

After several experiments following the Qiagen protocol and preparing our own buffers, we decided to try a purification kit from Qiagen to save us time and allow us to obtain a high yield of Sortase A for our experiments. The kit was certainly an improvement and a quicker purification process. Although it produced better results, the yield was not very significant.

#### **7.2.1.3.5 Coomassie Blue**

In order to get a clearer contrast on the SDS PAGE gel imaging and improve the quality of the gel staining, we chose to use coomassie Blue staining instead of common blue dye. The blue dye was further more easily contaminated and it was not as sensitive in terms of staining. As we have later found, Coomassie blue staining methods resulted in clearer resolution and greater band visibility.

#### **7.2.1.3.6 Maldi-TOF calibration**

In our second experiment analysing our purified protein, we had no meaningful results from the Maldi-TOF mass spec analysis. The specialist was there at the time and he warned us that the equipment needed to be calibrated, and therefore these results were maybe incorrect. The Maldi-TOF was calibrated and also needed some maintenance before we could rerun our analysis. It took about a week until we were able to use it again.

## **Chapter 8: Engineering Standards**

### **8.1 Economic**

We wanted to make sure that the resources and materials were used efficiently in order to ensure that we use our budget effectively. We kept a track of the materials we used by recording the cost of each material and when it was purchased.

### **8.2 Health and Safety**

The engineering standards for this project were followed based on Santa Clara University's guidelines. Health and safety in the bioengineering labs at Santa Clara University were implemented at all times. This was achieved through maintaining laboratory standards and following protocols to maintain sterility in the lab to make sure that there was no cross contamination. Public health considerations were also taken into account by making sure that different types of wastes were disposed using the correct protocols. Laboratory safety protocols, training for laboratory instruments, and environmental health and safety protocols (EHS) were implemented in the labs.

### **8.3 Environment**

In order to ensure that we are sustainable in what we use, we made sure that we used efficient instruments that did not use extra power. Moreover, we followed the biohazard waste and disposal protocols and laboratory protocols to make sure that we reduce the potential harm to the environment caused by improper waste disposal.

# Chapter 9: Ethical Considerations

## 9.1 Team Ethics

Having a high standard of work ethics was the first priority. As a team, the work was distributed to increase efficiency and everyone had a clear communication with each other. There was honesty, sincerity, and hardwork in this project. Moreover, as a team, we made sure to be cognizant about how the resources were allocated for this project in order to stay within the budget of the grant. The two main priorities were working effectively and ensuring the well-being and development of the team over time. This included listening to each other, being respectful, and making sure that we communicated effectively.

## 9.2 Biosafety

We also used *E.coli* bacterial strains instead of MRSA strains to avoid any contamination and unintended infections. Moreover, we wanted to take precautionary measures to maintain safety in the bioengineering labs by sterilizing all of the glassware and lab benches before use. Cleaning methods were implemented to avoid contamination when other experiments are performed in the lab with those materials. In this way, we wanted to reduce the amount of cross contamination and wanted to develop a more streamlined process.

# Chapter 10: Conclusion

## 10.1 Discussion

While our contribution to the anti-infective development was not the noteworthy, purifying and isolating Sortase A was the one of the most difficult portions of the process as there were constant challenges such as protein degradation, cell culture instability, and cell line mutations. Having a purified Sortase A protein reduces the false positives when testing for the interactions between Sortase A and the Vitamin B12 drug candidate.

## 10.2: Next Steps

### 10.2.1 Fast Protein Liquid Chromatography (FPLC)

Fast Protein Liquid Chromatography is a type of liquid chromatography to analyze a heterogenous mixture of protein samples. Our Sortase A protein can be purified further in order to get more accurate results and better interactions with our Vitamin B12 drug candidate.

### 10.2.2 Biacore

Biacore is a surface plasmon technology (SPR) that monitors molecular binding events in real time. It is useful to measure the binding affinities, thermodynamics, and kinetic rate constants. Once the Sortase A protein is purified further, pur next steps is to test how our protein interacts with the vitamin b12 drug candidate.

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