Over Expression of the RhoGAP Domain to Assess Inhibition of

*Staphylococcus aureus* Infection

Senior Honors Thesis (HONRS 499)

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ABSTRACT

*Staphylococcus aureus* is a Gram- positive bacterium that can cause serious infections, often leading to sepsis, which can be fatal. By focusing on the infection process of *S. aureus*, we hope to characterize the mechanism by which the bacterium is entering the cell. Our hypothesis is that the protein, p85, is integral in the infection process. To investigate the role of a domain of p85, known as the RhoGAP domain, we will over-express this domain in the host cell and assess its effect on *S. aureus* infection.
INTRODUCTION

Severe sepsis is a common, expensive, and frequently fatal condition, with as many deaths annually as those from acute myocardial infarction (Angus, 2001). The pathophysiological process of sepsis is a disease continuum from infection, signs and symptoms of infection, sepsis, severe sepsis, and multi-organ dysfunction (Gao, 2008). *Staphylococcus aureus* has long been recognized as a major human pathogen responsible for a wide range of infections, from mild skin infections to wound infections and bacteraemia (Hardy, 2004). *Staphylococcus aureus* is characterized as a Gram-positive bacterium. Signs and symptoms of infection that may result in sepsis include temperature $>38.3^\circ\text{C}$ or $<36.0^\circ\text{C}$, heart rate below 90 beats per minute, and hyperglycemia in the absence of diabetes (Gao, 2008). Sepsis is most often treated with various antibiotics, but depending on the severity of the case, even antibiotics may not counteract the infection caused by the bacterial invasion. Numerous trials have been conducted of agents that block the inflammatory cascade that causes sepsis, including corticosteroids, anti-endotoxin antibodies, tumor necrosis factor (TNF) antagonists, interleukin-1-receptor antagonists, as well as several other agents (Hotchkiss 2003). However, over time *S. aureus* has developed drug-resistance, especially to certain antibiotics. One of the most common strains of the bacteria is known as MRSA, or Methicillin Resistant *Staphylococcus aureus*. As of 2001, MRSA accounted for greater than 40% of total *S. aureus* cases, which is an increase from $\sim2\%$ in the early 90’s (Hardy, 2004). Intensive care patients are at great risk of infection from MRSA. They have increased risk factors,
including extended stays in hospitals, high antibiotic consumption and numerous i.v. devices inserted. The severity of their underlying disease also means that the consequences of infection with MRSA cause significant morbidity or mortality. One reason for the failure of anti-inflammatory strategies in patients with sepsis may be a change in the syndrome over time. Initially, sepsis may be characterized by increases in inflammatory mediators; but as sepsis persists, there is a shift toward an anti-inflammatory immunosuppressive state (Hotchkiss 2003). An immunosuppressive state is a state in which the immune response by the body is lowered. Due to the lowered immune response, it is much harder to treat sepsis, especially with antibiotics or other drugs. A class of drugs that has been shown to have a positive effect on the outcome of patients with sepsis is statins. The statins are a class of lipid-lowering drugs which inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Gao, 2008). HMG-CoA reductase catalyses the conversion of hydroxymethylglutaryl-CoA to mevalonate, an early rate-limiting step in cholesterol biosynthesis, leading to cholesterol-lowering effects. HMG-CoA reductase inhibitors (statins) have been shown to exhibit important biological effects independent of lipid lowering (Merx, et al. 2005). The clinical studies that have described effects of statins in sepsis have either addressed the effects of statins in reducing sepsis incidence/severity or retrospectively looked at mortality in those taking statins who developed sepsis (Gao, 2008). Since those studies, there has been a big emphasis studying statins, especially simvastatin. Basic research with statins has focused on blocking steps within the inflammatory cascade associated with sepsis. In the lab, we have focused on the cholesterol biosynthesis pathway and the way in which simvastatin is involved. We hope to imitate the effects of the drug in vitro and better
understand the mechanism by which the bacteria seem to exploit the host cell for invasion. By altering different aspects of the endocytic pathway, we hope to uncover a mechanism for preventing *S. aureus* from entering the cell.

The mechanism by which statins modulate the immune response is complex, but is often regarded as lipid independent as they are not related to lowering LDL cholesterol (Gao, 2008). These effects primarily involve the inhibition of isoprenoid lipid production and subsequent protein prenylation (Greenwood, 2006). Examples of the isoprenoid intermediates include geranylgeranyl pyrophosphate (GGpp) and farnesylpyrophosphate (Fpp). Once this was established, each component of the pathway was studied individually to determine which component of the pathway was indeed affecting the ability of *S. aureus* to invade the cells.

The first step in narrowing it down to the specific component that was affecting bacterial invasion was to start by adding certain intermediaries of the pathway back to cells that had been pre-treated with simvastatin to determine where bacterial invasion was being blocked by simvastatin. By introducing cholesterol, an end product of the pathway, back into the cells, invasion was not restored (Horn, 2008). Therefore, it was determined that cholesterol is not necessary for bacterial invasion. Other intermediaries such as GGpp and Fpp were also added back into the cells, and it was observed that they were able to completely or partially restore invasion into the cells. Therefore, we were able to conclude that intermediaries within the cholesterol biosynthesis pathway, such as GGpp and Fpp are necessary for bacterial invasion, but the end product, cholesterol is not.

Through understanding of cellular invasion by other types of bacteria, it is believed that molecular cell signaling pathways and cytoskeletal rearrangements are very
important in invasion. *S. aureus* is able to invade eukaryotic cells by indirectly engaging β₁ integrin-containing host receptors (Agerer, 2003). We began to study the molecular signaling that enables the components of the cytoskeleton to rearrange to allow for bacteria to invade the cell. There are several parts of the signaling process that we believe to be necessary for bacteria, specifically *S. aureus*, to enter the cell. At the cell surface, there are several receptors and binding proteins that enable certain substances to cross the cell membrane. The first is fibronectin, which is an extracellular matrix protein that is often found on the cell surface. Internalization of *S. aureus* involves an interaction between fibronectin binding protein (FnBp) and the host cell, resulting in signal transduction, tyrosine kinase activity, and cytoskeletal rearrangement (Dziewanowska, 1999). In order to gain entry to the cell, *S. aureus* has proteins on its surface, collectively named, MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) (Alexander, 2001). Host cells also contain surface receptors that recognize and bind to fibronectin, most notably, the integrin α₅β₁. Due to this receptor, *S. aureus* is often able to bind to the cell surface and almost directly come into contact with the α₅β₁ integrin (Sinha, 1999). This interaction initiates the process by which *S. aureus* is able to be internalized by the cells through cytoskeletal rearrangements. Integrins transmit important extracellular information such as cell attachment, matrix composition, and rigidity, into the cell. Once these integrin-protein complexes have been made, several other types of protein and protein complexes start to form on the inner side of the cellular membrane.

In our lab, we believe that phosphoinositide 3-kinase is necessary for host cell invasion by *S. aureus*. Previous studies in the lab have shown that inhibition of PI3K
decreases actin stress fiber disassembly and *S. aureus* invasiveness (Horn, 2008). This was determined by using the PI3K inhibitor, LY294002. In a dose-dependent manner, the inhibitor, LY294002, inhibited *S. aureus* host cell invasion.

PI3K is a heterodimer commonly composed of one of three catalytic subunits (p110α, p110β, p110δ) and one of five regulatory subunits (p85α, p85β, p85γ, p55α, p50α) (Geering, 2007). In order to broaden our understanding of PI3K, we have done several projects aimed at understanding the role of PI3K in endocytosis. Mammalian cells take up extracellular material by a variety of different mechanisms that are collectively termed endocytosis (Mukherjee, 1997).

The endocytosis process is of critical importance for a variety of cellular life functions (Qualmann, 2000). Endocytic mechanisms serve many important cellular functions including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, maintenance of cell polarity, and antigen presentation. Endocytic pathways are also utilized by viruses, toxins, and symbiotic microorganisms to gain entry into cells.

The extracellular matrix controls cytoskeletal organization through several distinct signal transduction pathways regulated by different members of the Rho family of GTPases (Clark, 1998). More specifically, actin reorganization is dependent on both Cdc42 and Rac. It has been suggested that Rho GTPases are involved in endocytosis, however the connection between the two activities is not completely understood (Fernandez-Borja, 2005). Therefore, our lab is interested in the role that these small GTPases play in the endocytosis of *S. aureus*. The small GTPases are known to interact with PI3K via a binding domain of the p85α isoform (Zheng, 1994). The RhoGAP
domain is a 200-residue conserved segment, which has been found in a large group of proteins, including p85 (Musacchio, 1996). The ability of the PI3K RhoGAP domain to interact with small GTPases, Rac1 and Cdc42 may be important for translocating PI3K to membranes and for stimulating its catalytic activity. For the next step of the endocytic pathway to occur, the prenylated small GTPase bound to PI3K must be anchored near the cell membrane. By doing so, this brings the catalytic domain of PI3K, p110, close to phosphoinositide.

Phosphoinositide (PI), normally found to be dually phosphorylated, (PI 4,5), becomes phosphorylated by an interaction with PI3K (Vanhaesebroeck, 2001). Phosphoinositide is normally found in the cytosol. When the catalytic domain of PI3K, p110, is brought into the proximity of PI, it can then phosphorylate the substrates phosphoinositide (PI), PI-4-phosphate (PIP), and PI-4,5-phosphate (PIP2). PIP2 appears to be most readily used by the Class IA PI3Ks. Therefore, when PIP2 becomes phosphorylated by PI3K, it becomes PI-3,4,5-phosphate (PIP3).

α-Actinin is a 100 kDa actin binding protein (Lee, 2004). We believe that α-actinin may play a role in integrin-mediated internalization of bacterial pathogens. Localization of this protein is key in determining its function. Studies suggest that α-actinin interacts with phosphoinositides, which regulate its activity within the cell (Fraley, 2005). More specifically, it interacts with the phosphates at the 4th and 5th position of the inositol head group. Phosphoinositide binding of α-actinin inhibits it from interacting with actin filaments, by blocking its actin-binding domain (Fraley, 2003). Since α-actinin becomes bound to PIP3, and its actin-binding domain is blocked, actin stress fibers are believed to disassemble due to the loss of contact with α-actinin. Actin
stress fibers enable the cell membrane to maintain its shape and motility (Chant, 1995). However, since the actin stress fibers are removed, the cell membrane loses its rigidity thus allowing the bacteria to enter the cell.

We believe that simvastatin plays an important role in the endocytic mechanism that S. aureus uses to gain entry into the cell. Given that we do not know the exact function that simvastatin plays in this process, several members in the lab have worked on different projects aimed at determining its precise role. We believe that simvastatin reduces bacterial invasion by forcing Cdc42 coupled to PI3K to accumulate in the cytosol. By sequestering PI3K in the cytosol, Cdc42 is restricted from interacting with PI at the membrane. Therefore, since PIP2 is not phosphorylated, α-actinin is allowed to remain intact with the actin stress fibers. Because the stress fibers are allowed to remain intact, the forces necessary for endocytosis to occur are removed, thus keeping S. aureus on the outside of the cell.

The specific project that I worked on focused mainly on the role of the RhoGAP domain in the endocytic process of S. aureus. The hypothesis is that the over expression of the RhoGAP domain of p85 will compete with endogenous p85 for binding to small GTPases, limiting the recruitment of endogenous p85 to the cell membrane. Therefore, the endogenous p85 cannot interact with the small GTPase Cdc42 and therefore cannot translocate to the cell membrane. If PI3K and the small GTPase cannot localize to the cell membrane, the endocytic pathway that the bacteria uses will be broken. If our hypothesis is correct, S. aureus will not be able to enter the cell and infection will not occur.
MATERIALS AND METHODS

Amplification of RhoGAP Domain from p85 WT DNA

By amplifying the RhoGAP domain from p85 WT DNA, we will be able to over express this domain in host cells, in order to assess the inhibition of *S.aureus*. p85 WT DNA was obtained from Origene (#SC115320; Rockville, MD). Primers were designed in order to amplify the specific DNA region of interest, the RhoGAP domain. A forward primer was designed to be 27 base pairs long, and the reverse primer was designed to be a 28 base pair reverse complement to the forward primer. The base pair sequences were as follows:

RhoGAP Forward Primer: CAC CGC AGA TGT TGA ACA ACA AGC TTG
RhoGAP Reverse Primer: CTA CTG TCG TTC ATT CCA TTC AGT TGA G

An Accuprime Pfx SuperMix (Invitrogen #12344-040) was used. This mix includes the polymerase, which is important in the polymerase chain reaction or PCR reaction. The primers, along with this polymerase were combined with p85 WT DNA and were run on the following cycle:

1 cycle: 95° C for 2.5 min.
35 cycles: 95° C for 15 sec
60° C for 1 min
72° C for 1 min
Remains at 4° C after above cycles are complete.
The polymerase along with the primers will amplify the DNA going forward and backward, so that only the fragment of interest remains, the RhoGAP domain of p85. In order to determine if the correct fragment was obtained, the PCR sample was run on a 1% agarose gel at 80 V for 1 hour. The expected size of the RhoGAP domain fragment is 550 b.p.

**Cloning into pENTR/D-TOPO Vector**

The amplified fragment, RhoGAP domain of p85, was cloned into the pENTR/D-TOPO vector using a TOPO Cloning Kit (Invitrogen #45-0218) according to manufacturer’s instructions. The reaction was then transformed into OneShot Top10 Competent cells (Invitrogen #44-0301). A control sample of pENTR DNA was also transformed into the OneShot cells. A 1 μL PCR sample was added to components of the kit, including 1 μL of the pENTR/D-TOPO vector to reach a final volume of 6 μL. 2 μL of the TOPO Cloning reaction was introduced into the OneShot Top 10 cells; 1 μL of the pENTR control DNA was introduced into a separate tube of OneShot Top 10 cells. The cells were incubated on ice for 15 minutes, and then heat shocked in a 42° C water bath for 30 sec. RT SOC medium was then added at a volume of 250 μL to the tubes and were then placed in shaking incubator at 37° C for 1 hour. Cells from each tube were then spread on kanamyacin plates in three separate volumes (50, 100, 200 μL), in order to vary the amount of cells on each plate. Plates were then inverted in bacterial incubator ON. Five colonies were chosen from the experimental plates and grown at 37° C overnight in L-broth (Q Biogene #3001-221) containing 50 mg/mL kanamycin.
Extraction of DNA from Bacterial Cells

A QIAPrep Spin Mini Kit (Qiagen #27104) was used to extract the DNA from the bacterial overnight cultures of RhoGAP/pENTR/D-TOPO #1-5. The samples were first centrifuged for 1 min at 14,000 rpm in order to pellet the bacterial cells. This step was repeated in order to obtain a larger amount of cells. The cells were resuspended in a combination of buffers in order to lyse open the cells. The plasmids were isolated by centrifugation and the supernatant was transferred to the QIAPrep column. The samples were subjected to a series of washes followed by centrifugation in order to isolate the plasmid. The column is designed to bind the plasmid, so the plasmid can be eluted by water after purification. A portion of the sample was separated to be used for restriction digest analysis and the remainder was saved in the case of the correct clone being obtained.

Restriction Digest to Assess Clones

Restriction enzymes were identified that could be used to confirm if cloning was successful. For each sample, 8 μL of DNA was combined with 2 μL React 2 buffer and 1 μL Hind III enzyme for a final volume of 10 μL. An undigested sample was also run using one of the samples, which consisted of 8 μL of DNA and 2 μL of water. The samples were digested at 37° C for 1 hour. After digestion, 2 μL 10X Blue Juice (Invitrogen #10816-015) was added to each of the samples. The samples were run on a 1% agarose gel at 80V for 1 hour. The gel was imaged to determine if the correct construct was obtained. If cloning was successful, the expected band sizes were ~2900
and 341 b.p. In order to further isolate the correct mutant, a separate restriction digest was performed on four of the six samples. For this digest, 17 μL of the samples RhoGAP/pENTR/D-TOPO #’s 1,3,5,6 were digested with 2 μL NEB Buffer #2 and 1 μL of Nhe I. An undigested sample was also run using 17 μL of sample #1 and 3 μL of water. Samples were digested at 37° C for 1 hour. After digestion, 2 μL of 10X Blue Juice was added to each of the samples. The samples were then run on a 1% agarose gel at 80V for 1 hour. The gel was then imaged in order to determine if the correct construct was obtained. For this digest, expected band sizes were again ~2900 and 341 b.p. if the cloning was successful.

**Large Scale Plasmid Prep of RhoGAP Construct**

Bacteria from each of the overnight cultures used in the mini preps were used to start an 8-hour sample. The bacteria were grown in 5 mL of L-broth containing 50 mg/mL kanamycin. From these 8-hour samples, overnight cultures were grown in 100 mL of L-broth containing 50 mg/mL kanamycin in 37° C shaking incubator. DNA was then extracted from each of the two samples using an Endofree Plasmid Maxi Kit (Qiagen #12362) according to manufacturer’s instructions. Overnight culture samples were centrifuged in order to pellet the cells. Cells were then lysed open to release the DNA using a combination of buffers. The cellular components were then separated from the plasmid DNA during centrifugation. This supernatant was added to the QIAPrep column, which binds the plasmid DNA, which can then be eluted using water. The absorbance of the constructs was measured at 260 nm using a spectrophotometer. From this reading, the concentration of the two samples was calculated. A restriction digest of these samples was run in order to verify that the correct clone was obtained. Both samples
were digested with Nhe I and Hind III restriction enzymes separately at 37°C for one hour. Digests were prepared with Blue Juice loading buffer and run on a 1% agarose gel at 80 V for one hour. Expected band sizes were 2900 and 341 b.p. if the correct clone was obtained.

**Recombining into pcDNA3.1nV5/DEST Vector**

Once large scale preps were performed on the two RhoGAP/pENTR/D-TOPO samples #3 and #5, transformation into a new vector began. An LR recombination reaction was performed for each of these samples. 3 μL of each RhoGAP/pENTR/D-TOPO sample was added to 2 μL of the destination vector, pcDNA3.1nV5/DEST (Invitrogen #12290-010), 4 μL of 5X LR Clonase Reaction Buffer, and TE Buffer: pH 8.0 to a final volume of 16 μL. The LR Clonase enzyme was then added to the reaction at a volume of 4 μL, and mixed well by vortexing briefly. Reactions were incubated at 25°C overnight. Proteinase K was then added at a volume of 2 μL the next day to terminate the reaction. The samples were vortexed briefly and then incubated at 37°C for 10 minutes. The resulting sample, RhoGAP/pcDNA3.1nV5/DEST was transformed into One Shot Top 10 competent cells as described previously. The DNA was isolated using a QIAPrep Spin Mini Kit and the isolated DNA samples were analyzed by a restriction digest, using the enzymes Pst I and Bam HI separately. The plasmid was digested at 37°C for 1 hour and then run on a 1% agarose gel at 80V for 1 hour. For the digest with Pst I, if the plasmid construct was in the correct orientation in the destination vector, expected band sizes would be ~4111 and ~2000 b.p. For the digest with Bam HI, if the construct was in the correct orientation in the vector, expected band size would be ~6200 b.p.
Large Scale Plasmid Prep of RhoGAP/pcDNA3.1nV5/DEST #5D

Bacteria from an overnight sample of mini-prep #5D was used to start an 8-hour sample. The bacteria were grown in 5 mL of L-broth containing ampicillin. From these 8-hour samples, overnight cultures were then grown in 100 mL of L-broth containing ampicillin at 37°C shaking incubator. DNA was then extracted from the sample using an Endofree Plasmid Maxi Kit according to manufacturer’s instructions as described previously. The absorbance of the constructs was measured at 260 nm using a spectrophotometer. From this reading, the concentration of the sample was calculated. A restriction digest of the sample was run in order to verify that the correct clone was obtained. A restriction digest was run on #5D using the restriction enzymes Pst I and Bam HI at 37°C for one hour. Digests were then run on a 1% agarose gel at 80 V for one hour. Expected band sizes for digest with Pst I were 4111 and 2000 b.p. if correct. Expected band size for digest with Bam HI was 6200 b.p. if correct.

Analysis of Mutant by DNA Sequencing

Once it appeared that the correct mutant had been obtained, the DNA was sent away for sequence confirmation. One sample of the DNA was sent to a sequencing facility at the University of California, Davis. Primers were designed according the predicted fragment size and sequence. The primers were designed in VectorNTI and sent along with the mutant DNA to the sequencing facility. Primers were designed to be at least 18 b.p. in length, with a G/C content between 50-55%, and a melting temperature between 50-70°C. Sequences obtained from UC Davis were then analyzed using the program
“Chromas.” The sequence will be sent in different lengths depending on the number of primers used. A contig was then created from these sequences. This contig was aligned with the DNA sequence originally designed for the mutant in order to determine if the correct clone was obtained.

**Cloning into pENTR/D-TOPO Vector**

A new cloning reaction was performed using the RhoGAP PCR product. The TOPO cloning reaction was performed using a TOPO Cloning Kit and was done according to manufacturer’s instructions. The new RhoGAP/pENTR/D-TOPO bacterial cultures, as well as original RhoGAP/pENTR/D-TOPO Mini Prep #6 bacterial cultures, were transformed using OneShot Top 10 competent cells as described previously. The plasmid DNA was isolated from the cells using a Qiaprep Spin Mini Kit according to manufacturer’s instructions. A restriction digest was performed on the plasmid DNA samples. The samples were digested using both Hind III and Eco RV at 37°C for 1 hour. Digests were run on a 1% agarose gel at 80V for 1 hour. If the correct construct orientation is achieved, expected band sizes would be big, 374 and 341 b.p.

**Large Scale Plasmid Prep of RhoGAP/pENTR/D-TOPO #14**

Bacteria from an overnight culture was used to start an 8-hour culture, which was then used to start an overnight culture. DNA was extracted using an EndoFree Plasmid Maxi Kit according to manufacturer’s instructions. The absorbance was read using a spectrophotometer, and the concentration was then calculated. A restriction digest was performed on the sample using EcoRV and Hind III at 37°C for 1 hour. The digests were run on a 1% agarose gel at 80V for 1 hour. Expected band sizes if the correct construct was obtained are big, 374 and 341 b.p.
Analysis of Mutant by DNA Sequencing

Once it appeared that the correct mutant had been obtained, the DNA was sent away for sequence confirmation. One sample of the DNA was sent to a sequencing facility at the University of California, Davis. Primers were designed according to the predicted fragment size and sequence. The primers were designed in VectorNTI and sent along with the mutant DNA to the sequencing facility. Primers were designed to be at least 18 b.p. in length, with a G/C content between 50-55%, and a melting temperature between 50-70° C. Sequences obtained from UC Davis were then analyzed using the program "Chromas." The sequence will be sent in different lengths depending on the number of primers used. A contig was then created from these sequences. This contig was then aligned with the DNA sequence originally designed for the mutant in order to determine if the correct clone was obtained.

Recombining into pcDNA3.1nV5/DEST Vector

An LR recombination reaction was performed using the RhoGAP/pENTR/D-TOPO #14 sample. 3 µL of the RhoGAP/pENTR/D-TOPO sample was added to 2 µL of the destination vector, pcDNA3.1nV5/DEST, 4 µL of 5X LR Clonase Reaction Buffer, and TE Buffer: pH 8.0 to a final volume of 16 µL. The LR Clonase enzyme was then added to the reaction at a volume of 4 µL and mixed well by vortexing briefly. Reactions were incubated at 25° C overnight. Proteinase K was then added at a volume of 2 µL the next day to terminate the reaction. The samples were vortexed briefly and then incubated at 37°C for 10 minutes. The resulting sample, RhoGAP.pcDNA3.1nV5/DEST #14 was transformed into One Shot Top 10 competent cells as described previously. The DNA was then isolated using a QIAPrep Spin Mini Kit and the isolated DNA samples were
analyzed by a restriction digest using the enzyme Bam HI. The plasmid was digested at 37°C for 1 hour and then run on a 1% agarose gel at 80V for 1 hour. If the correct construct was obtained and in the correct orientation, the expected band size would be 6000 b.p. If the construct was in the incorrect orientation, the band sizes obtained would be 6000, 700 and 200 b.p.

**Large Scale Plasmid Prep of RhoGAP/pcDNA3.1nV5/DEST #14-J**

Bacteria from an overnight culture were used to start an 8-hour culture, which was then used to start an overnight culture. DNA was extracted using an EndoFree Plasmid Maxi Kit according to manufacturer’s instructions. The absorbance was read using a spectrophotometer, and the concentration was then calculated. A restriction digest was performed on the sample using Bam HI at 37°C for 1 hour. The digests were run on a 1% agarose gel at 80V for 1 hour. Expected band size if the correct construct is obtained would be 6000 b.p. If the correct construct is not obtained, the expected band sizes would be 200, 700, and 6000 b.p.

**Analysis of Mutant by DNA Sequencing**

One sample of the DNA was sent to a sequencing facility at the University of California, Davis. Sequences obtained from UC Davis were then analyzed using the program “Chromas.” The sequence will be sent in different lengths depending on the number of primers used. A contig was then created from these sequences. This contig was aligned with the DNA sequence originally designed for the mutant in order to determine if the correct clone was obtained.
**RhoGAP/V5 Stable Line Transfection and Selection**

A stable line was attempted with our construct in order to decrease variability in future experiments in which this construct may be considered useful. Another member of the lab transfected RhoGAP/V5 plasmid DNA into HEK293 cells. HEK293 cells are known for their ability to readily transfect and are easy to work with. FuGENE HD was used to transfect the cells. Once the cells were transfected, selection was done with geneticin. Selection works by means of killing off cells that do not have the vector of interest, in this case our RhoGAP/V5 construct. Eventually, only the cells that contain our vector will remain on the plates. Selection is performed in order to ensure that each colony of cells is genetically identical. Once individual colonies have been isolated, they are allowed to grow and proliferate. After cells have been allowed to grow, they will be lysed, in order to analyze the protein being expressed in the cells.

**Analysis of RhoGAPV5 Stable Line**

Upon completion of selection, cells were lysed/assessed for construct expression. Cells were washed with a wash buffer composed of 20mM Trizma (Sigma # TI378)/137mM NaCl (Fischer # MSX04251)/1mM MgCl2-6H2O (VWR Scientific # EM-5980)/1mM CaCl2 dihydrate (VWR Scientific # EM-3000). The cells were then lysed open using a lysis buffer containing the same components as the wash buffer, with the addition of 10% glycerol (Invitrogen #15514-001)/1.0% NP40 (Sigma #74385). On the day of the experiment, 1 Mini-tab (Roche 1836153) was added to the lysis buffer to inhibit proteases. This entire experiment was done on ice in order to further inhibit any protease activity. Cells were scraped from the plate using a cold cell scraper and then transferred
to a cold microcentrifuge tube. Cells were rocked at 4°C for 20 min. Cells are then centrifuged for 10 min at 10000xg at 4°C. Supernatants transferred to pre-chilled tubes.

Protein concentration was then determined. The lysates were combined with blue dye. The dye should bind to the protein, which is expressed at different levels depending on the protein expression in the sample. The samples were allowed to incubate for 5 min, in order to let the dye completely bind to the protein. The absorbance will be read using a Microtiter plate reader and it will be read at 595 nm.

A standard curve was established using samples of known protein concentration. Serial dilutions were made of the unknown samples. The first sample was left undiluted, then diluted at 1:5 and 1:10. Samples were then placed in the plate reader, which not only reads the samples, but also thoroughly mixes the samples before the reading. The plate reader creates a set of data reporting the standard curve, the unknown sample concentrations, and absorbance values.

**Western Blot Analysis**

A PAGE gel was run with samples A-E. There was also a V5 control used. The gel was run at 200 V for 30 minutes. The gel was then carefully placed into an XCell II Blot Apparatus. This enables the protein from each of the samples on the PAGE gel to transfer onto a blot paper, in order to proceed with a Western Blot. The first step was to block. The PVDF membranes were re-wetted in 100% methanol. The membrane was then rinsed twice in distilled water and once in 1X PBS. The membrane is then placed directly into blocking buffer for 1 hour, shaking. The next step is to probe. The primary antibody must be diluted in a solution composed of the following 40 mL LI-COR blocking buffer (927-400000)/40 µL Tween 20. After mixed, 8 µL anti-V5 (Invitrogen
R960-25) was added. The mixture is poured on the blot and rocked, covered at 4°C ON or for several days. Membranes were washed in 1X PBS +0.1% Tween-20 at RT; once briefly, twice for 5 minutes each. The membranes were probed with IRDye secondary antibodies raised against species of the primary antibodies. In order to dilute the secondary antibody, a mixture of 40 mL LI-COR blocking buffer and 40 μL Tween 20 was used. After mixed, 1 μL anti-mouse 800 (Rockland Immunochemicals) was added. The mixture was poured onto the blot and rocked at RT for no longer than 1 hour. Protection from light is important for the remainder of the Western Blot. The blot was then washed in 1X PBS +0.1% Tween 20 at RT; once briefly, twice for 5 minutes each. The blot was then rinsed in PBS to remove residual Tween 20. The membrane was then scanned using the LI-COR odyssey imager.

FAILED EXPERIMENTS

Amplification of RhoGAP Domain from WT p85 DNA

Several times while attempting to amplify and isolate the RhoGAP domain of p85, different sets of PCR conditions had to be utilized in order to determine the optimal conditions necessary for PCR to work properly. The annealing temperature was especially important in successfully amplifying the RhoGAP domain. The optimal annealing temperature should be 5-10°C lower than the melting temperature of the primers used. The typical range is 55-65°C. The annealing temperature was changed several times before the correct temperature was finally established.

Infection to Assess Inhibition of Invasion

The correct construct was not obtained before summer break commenced, therefore another member of the lab picked up my work for the summer. The correct construct was
finally obtained as RhoGAP/pcDNA3.1nV5/DEST. Cell culture work began with this construct. The plasmid DNA was transfected into HEK 293 cells for infection studies. Once the construct was introduced into these cells, several attempts were made to obtain accurate and statistically significant data on the inhibition of invasion of *S. aureus* following infection of the cells. Unfortunately, the results of the experiments were inconsistent, therefore any further attempts to utilize RhoGAP/pcDNA3.1nV5/DEST to assess inhibition of *S. aureus* invasion was aborted.

RESULTS

**Amplification of RhoGAP Domain**

The RhoGAP domain of WT p85 was amplified using a PCR reaction. This allowed us to over express the RhoGAP domain, which was useful in determining the effect of over expression on *S. aureus* invasion. PCR was performed using WT p85 and a set of primers designed to specifically isolate our fragment of interest. After the PCR reaction, the reaction product was run on a 1% agarose gel in order to determine if the correct product was obtained. The gel was run at 80V for 1 hour and a band was expected at approximately 550 b.p. After several unsuccessful trials of our PCR reaction, the amplified RhoGAP domain was finally obtained (Figure 1) with the following PCR cycle conditions:

1 cycle : 95° C 2.5 min

35 cycles: 95° C 15 sec

60° C 1 min

72° C 1 min

4° C Eternity
**Cloning into pENTR/D-TOPO Vector**

Once the amplified fragment, RhoGAP, was obtained, the process of cloning our fragment into the pENTR/D-TOPO vector was started. Once the reaction was complete, the reaction mixture was transformed into OneShot Top10 competent Cells. A sample of pENTR control DNA was also transformed into competent cells. The cells were then allowed to shake at 37°C for 1 hour. The samples were spread on kanamyacin plates and incubated ON. Overnight cultures were started of each sample. DNA was extracted from the cells using a Qiaprep Spin Mini kit. Cells were suspended in a combination of buffers in order to lyse open the cells, followed by a series of washes in order to wash away any contamination. The plasmid was then isolated and eluted with water. A restriction digest was performed in order to verify that cloning into the vector was successful. The samples were digested with Hind III and the expected band sizes were 2900 b.p. and 341 b.p. The expected bands were observed for samples #1,3,5,6 (Figure 2). In order to further eliminate samples for the rest of the project, another restriction digest was done with the enzyme Nhe I. The expected band sizes were 2900 b.p. and 341 b.p. This digest was able to narrow it down to samples #3 and 5, in which the correct band sizes were observed (Figure 3). Once the correct construct was obtained, a large scale plasmid prep was performed in order to obtain more plasmid DNA of sample #’s 3 and 5. Each of the samples were digested with Nhe I and Hind III separately and expected band sizes were 2900 b.p. and 341 b.p. if the correct construct was obtained. Bands at 2900 b.p. and 341 b.p. were observed for both samples 3 and 5 (Figure 4). Therefore, these samples were used for the continuation of the project.
Recombining into pcDNA3.1nV5/DEST Vector

Once the correct mutant, RhoGAP/pENTR/D-TOPO, was obtained, recombination into a new vector started. LR recombination was used to recombine RhoGAP into the pcDNA3.1nV5/DEST vector. The samples were then transformed into OneShot Top10 competent cells and overnight cultures were made. The DNA was isolated using a QiaPrep Spin Mini Kit and was analyzed using restriction digest. The samples were digested using Pst I and Bam HI separately. The digest was then run on an agarose gel. The expected band sizes if the construct was in the correct orientation when digested with Pst I were ~4111 b.p. and ~2000 b.p. The expected band size if the construct was in the correct orientation when digested with Bam HI would be ~6200 b.p. The expected band sizes were observed in samples 5D and 5F (Figure 5), however, sample #5D was used for the continuation of the experiment. A large scale prep was done in order to obtain more plasmid DNA of #5D. A restriction digest was again performed on the sample to make sure that we still had the correct construct. Two samples of #5D were digested, one with Pst I and one with Bam HI. The expected band sizes were ~4111 b.p. and ~2000 b.p. for the digest with Pst I and ~6200 b.p. for the digest with Bam HI. The gel was initially run for 1 hour, however, further distinction between bands was necessary, so it run for another 45 minutes. After 45 minutes, it was determined that the correct construct was again obtained with #5D.

Analysis of Mutant by DNA Sequencing

The sample RhoGAP/pcDNA3.1nV5/DEST #5D was sent away for sequence confirmation at the University of California-Davis Sequencing Facility. Primers were used to span the length of the RhoGAP fragment plus a small length of vector on each
side. By sequencing, it can be determined if the correct fragment was cloned into the vector, as well as if the fragment was in the correct orientation in the vector. The sequences from the sequencing facility were analyzed using Chromas and Vector NTI. A contig was created from the separate fragment sequences. This contig was then aligned with the DNA sequence originally designed for the mutant to determine if the correct sequence was obtained. Unfortunately, the mutant construct was in the wrong orientation in our vector, therefore, it was necessary to start a new cloning reaction to determine where the construct went into the vector backwards.

**Cloning into pENTR/D-TOPO Vector Trial #2**

A new cloning reaction was performed in order to correctly insert the mutant construct into the RhoGAP/pENTR/D-TOPO vector. The original RhoGAP PCR product was used for this cloning reaction. The new RhoGAP/pENTR/D-TOPO and original RhoGAP/pENTR/D-TOPO Mini Prep #6 bacterial cultures were used for transformation into cells. The samples were transformed into OneShot Top10 competent cells and overnight cultures were obtained from each of the samples. DNA was then extracted from each of the samples using a QiaPrep Spin Mini Kit. This plasmid DNA was then digested with both Hind III and EcoRV. The expected band sizes were big, 374 b.p., and 341 b.p. if the construct is in the correct orientation. Sample #14 has the expected bands at big, 374 b.p., and 341 b.p. which are the expected sizes for the mutant construct (Figure 6). If the construct was in the incorrect orientation in our vector, there would be a band present at 150 b.p. as well as the other bands, and that band is not observed in the image. A large scale prep of the RhoGAP/pENTR/D-TOPO #14 sample was performed in order to obtain more plasmid DNA. A restriction digest was again performed to ensure
that the construct was still in the correct orientation in the vector. The sample was
digested with Hind III and EcoRV and the expected band sizes were big, 374 b.p., and
341 b.p. The observed band sizes were consistent with the expected results (Figure 7),
therefore RhoGAP/pENTR/D-TOPO #14 was used for the continuation of the
experiment.

**Analysis of Mutant by DNA Sequencing**

Once the correct construct was obtained, a sample of the DNA was sent away for
sequence confirmation at UC Davis Sequencing Facility. The sequences sent back from
the facility were analyzed using Chromas and Vector NTI. A contig was created using
the fragment sequences and the contig was aligned with the sequence originally designed
for the mutant. The sequence of RhoGAP/pENTR/D-TOPO #14 shares 100% identity
with the originally designed RhoGAP/pENTR/D-TOPO. Therefore, the RhoGAP
domain has been successfully cloned into pENTR/D-TOPO, the orientation is correct,
and the domain can be cloned into destination vectors.

**Recombining into pcDNA3.1nV5/DEST Vector**

Once the RhoGAP domain was successfully created and in the correct orientation in the
pENTR/D-TOPO vector, recombination into a destination vector was started. An LR
recombination reaction was performed. The samples were then transformed into
OneShot Top10 competent cells. DNA was extracted from the cells using a QiaPrep Spin
Mini Kit and the plasmid samples were then digested using Hind III. The expected band
sizes if the transformation was correct are 341 b.p., 166 b.p., and big. If the construct is
in the incorrect orientation, the expected band sizes would be 341 b.p., 386 b.p., and big.
The observed bands did not match the expected bands, therefore, the LR recombination
and mini plasmid prep were repeated. The new samples were digested with a new enzyme as well. Instead of using Hind III, the enzyme used was Bam HI. If the correct construct is obtained, the expected band size would be ~6000 b.p. If the construct was not correct, the expected band sizes would be 200 b.p., 700 b.p., and 6000 b.p. The expected band at ~6000 b.p. was observed in all six samples, therefore the experiment was continued with RhoGAP/pcDNA3.1nV5/DEST #14J. A large scale plasmid prep of #14J was performed in order to obtain more plasmid DNA. It was later determined that this construct was not correct via analysis by DNA sequencing (Data not shown).

**Western Blot Analysis of RhoGAP/V5 Stable Line**

The RhoGAP/pcDNA3.1nV5/DEST samples were transfected into HEK293 cells to attempt to start a stable line expressing our mutant construct. Once the cells had been transfected and selection had taken place, the cells were lysed open so that the protein being expressed could be analyzed. The protein concentrations of each of the samples were determined using microtiter well plates and compared against a standard curve with proteins of known concentrations. A PAGE gel was ran on the samples A-E and the V5 control. The membrane was then blocked and probed with primary antibody (anti-V5) and secondary antibody (anti-mouse 800). The membrane was imaged with the LI-COR Odyssey imager. A band is expected at about 30 kD if the clone is being stably expressed. The V5 positive control would produce a band at approximately 20 kD. From the image, bands were observed at ~30 kD in samples C and D. Therefore, we believe that samples C and D were stably expressing our mutant (Data not shown).
Figure 1: PCR product is generated. The sample was run on a 1% agarose gel at 80V for 1 hour. A low mass ladder was loaded into Lane 1, which represented band sizes between 2000 and 400 b.p. The sample was loaded into Lane 2. The expected band size was 550 b.p. The PCR product appeared to have the correct band size. Therefore, this product was chosen for use in continuation of the experiment.
Figure 2: Samples #1, 3, 5, 6 appear to be the correct construct. The plasmid DNA was digested with Hind III for 1 hour at 37°C. The samples were then run on a 1% agarose gel at 80V for 1 hour. A low mass ladder was loaded into Lane 1, representing band sizes between 2000 and 100 b.p. An undigested sample was loaded into Lane 2 as a control. The expected band sizes if the correct construct was obtained were 2900 b.p. and 341 b.p. Samples #1,3,5,6 appear to be the correct construct.
**Figure 3:** Samples #3,5 appear to have the correct construct. Samples were digested with Nhe I at 37°C for 1 hour. The samples were then run on a 1% agarose gel at 80V for 1 hour. A low mass ladder was run in lane 1 representing band sizes from 2000 to 100 b.p. An undigested sample was run in lane 3 as a control. The expected band sizes if the correct construct was obtained are 2900 b.p. and 341 b.p. Samples #3 and 5 appeared to yield the correct clone. Therefore, the experiment will utilize these samples.
Figure 4: Restriction digest of RhoGAP/pENTR/D-TOPO #3, 5 large scale prep indicates that sample #5 appears to have the correct construct. Each sample was digested with Nhe I and Hind III separately at 37°C for 1 hour. The samples were then run on a 1% agarose gel at 80V for 1 hour. A low mass ladder was loaded into Lane 1 representing band sizes from 2000 to 100 b.p. An undigested sample was loaded into Lane 2 as a control. The expected band sizes were 2900 and 341 b.p. if the correct construct is obtained. Samples in Lanes 3 and 5 appeared to be correct, therefore, we believe that Samples #3 and 5 are the correct clones.
Figure 5: Sample #5 appears to be correctly cloned into RhoGAP/pcDNA3.1nV5/DEST. The samples were digested with both PstI (Top gel) and Bam HI (Bottom gel) separately at 37°C for 1 hour. The samples were then run on 1% agarose gels at 80V for 1 hour. A high mass ladder was loaded in lane 1 of both gels representing band sizes from 10000 to 1000 b.p. An undigested sample was run in the last lane of each gel. The expected band sizes for digest with PstI was 4111 and 2000 b.p. if the recombination worked correctly. The expected band size for the digest with Bam HI was 6200 b.p. if the recombination worked correctly. Samples #5D and 5F both appeared to be correct, however, sample #5D was chosen for the continuation of the experiment.
Figure 6: Sample #14 was successfully cloned into pENTR/D-TOPO. The samples were digested with Hind III and Eco RV simultaneously at 37°C for 1 hour. The samples were then run on a 1% agarose gel at 80V for 1 hour. A low mass ladder was loaded into lane 1 representing band sizes between 2000 and 200 b.p. The expected band sizes if the cloning reaction was successful and in the correct orientation were big, 374 b.p., and 341 b.p. Sample #14 in lane 5 appears to have underwent cloning successfully.
Figure 7: Large scale prep of sample #14 indicated that it was successfully cloned. The sample was digested with Hind III and Eco RV at 37°C for 1 hour. The gel was then run on a 1% agarose gel at 80V for 1 hour. A low mass ladder was loaded into lane 1 representing band sizes from 2000 b.p. to 200 b.p. An undigested sample was loaded into lane 2. The expected band sizes if the cloning reaction was successful is big, 374 b.p., and 341 b.p. Sample #14 appears to have been cloned correctly.
DISCUSSION

*S. aureus* infection is becoming a serious problem in the health care industry today (Lowy, 1998). This type of infection is becoming a cause for concern due to the antibiotic resistance that several strains of the bacteria have developed. With this growing problem, several hospitals and healthcare communities have started to cycle antibiotics (Lipsitch, 2000). Cycling occurs when one class of antibiotics are used as primary treatment of infections for a period of time, and then as resistance to this class climbs, they switch to emphasize use of a second class of antibiotics to which resistance is rare or absent. Not only have several strains of bacteria started to develop antibiotic resistance, the bacteria themselves are able to evade the host immune system in a different fashion. *S. aureus* secretes proteins that lyse neutrophils, neutralize antimicrobial defensin peptides, and its overall cell surface is modified to reduce effectiveness of the host cell’s defense mechanisms (Foster, 2005). This resistance both by the bacteria, as well as to antibiotic treatment, increases the necessity for an alternate treatment for bacterial-induced sepsis.

The purpose of the project was to over express the RhoGAP domain of p85, the regulatory subunit of PI3K, which is necessary for binding to small GTPases such as Cdc42. By over expressing this domain, we hope to see an inhibition of cellular invasion by *S. aureus*. It has been previously shown that simvastatin is able to inhibit invasion dependent on a mechanism that operates through the cholesterol biosynthesis pathway (Horn, 2008). If cells are pre-treated with simvastatin, the production of cholesterol, as well as several other important intermediaries, is blocked. From these findings, it was
determined that the depletion of the isoprenoid intermediates, Ggpp and Fpp, was a main reason for the decrease in *S. aureus* invasion. This can be concluded due to the fact that replenishment of each restored invasion and inhibition of Ggpp and Fpp decreased invasiveness (Horn, 2008). These isoprenoid intermediates are important in localization of proteins within the cell, notably those proteins that can become anchored at the cell membrane. In the process of post-translational prenylation, Ggpp or Fpp are added at the cysteine residue of the carboxyl-terminal sequence, or Caax motif (Maltese, 1990). This prenylation-interaction is important for Caax-containing proteins, such as Rac and Cdc42, due to the fact that this prenylation anchors the proteins to the cell membrane.

As previously discussed, protein-protein interaction at the cell membrane is believed to play a large part in the endocytic pathway used by *S. aureus* to invade the host cell. Class IA PI3Ks are heterodimers of p110 catalytic and p85 regulatory subunits that mediate a variety of cellular responses to growth and differentiation factors (Brachmann, 2005). The p85 regulatory subunit is of particular interest in this project, due to the presence of the RhoGAP domain. The RhoGAP domain was first observed by Zheng et al. (1994) to be a 50 kDa protein segment found on the regulatory subunit, p85, which is known to interact with members of the Rho Family of small GTPases, including Cdc42. Upon interaction of the RhoGAP domain of p85 with a small GTPase, it is believed that PI3K will be able to translocate to the cell membrane and become activated. Activation of PI3K catalyzes the production of PIP$_3$ at cell membranes, thus resulting in the recruitment and activation of various signaling components to the cell membrane, some of which have been implicated in the regulation of the cytoskeleton (Chen, 2004).
PI, PIP, and PIP₂ are substrates known to be phosphorylated by its interaction with phosphoinositide 3-kinase. Phosphoinositide (PI) is normally found at the cell membrane. When PI3K is able to interact with small GTPases, PI3K may be brought to the cell membrane, where its catalytic subunit, p110, is able to interact with PI. When this occurs, PI, PIP, and PIP₂ are phosphorylated. PIP₂ appears to be most readily used by Class IA PI3Ks, therefore, PIP₃ is most often formed at the cell membrane.

When PIP₃ is produced, an important actin-bundling and adhesion protein, α-actinin is recruited to the site of PIP₃ formation. It has been suggested that α-actinin interacts with phosphates of PIP₃ (Fraley, 2003). As α-actinin interacts with PIP₃, its actin binding domain becomes altered, which interferes with its actin bundling activity. When this bundling activity is impaired, actin stress fibers are able to disassemble from the cell membrane. It is our hypothesis that when the actin stress fibers disassemble, the cell is not able to maintain its shape and rigidity, thereby allowing endocytosis to occur. Overall, it is believed that simvastatin blocks invasion of *S. aureus* by inhibiting the anchoring of PI3K-bound small GTPases to the cell membrane, via prenylation of isoprenoid intermediates. Therefore, in my project we have attempted to recreate the same effect by altering the RhoGAP domain of p85. By over expressing this domain, we hoped to compete with endogenous p85 for binding to small GTPases anchored at the cell membrane. If endogenous p85 cannot reach the cell membrane to interact with small GTPases, then PI3K will be sequestered in the cytosol. The interaction of PI3K is necessary for the production of PIP₃; if PIP₃ is not produced, then α-actinin remains bound to actin stress fibers, and endocytosis cannot occur.
Amplification of the RhoGAP domain from WT p85 was successful by means of a polymerase chain reaction. Once the RhoGAP domain was isolated and amplified, it was cloned into the pENTR/D-TOPO vector, which is an entry clone useful for recombination into destination vectors. Upon completion of several rounds of cloning experiments, the RhoGAP/pENTR/D-TOPO plasmid underwent recombination into the destination vector, pcDNA3.1nV5/DEST. Destination vectors are important for transfection into cell lines. As soon as the correct mutant was obtained, the sample was sent away for DNA sequencing. This step was important to ensure that the amplified RhoGAP domain was being expressed and in the correct orientation in the destination vector. RhoGAP/pcDNA3.1nV5/DEST was transfected into HEK293 cells for further analysis of the mutant construct. Following transfection of our mutant construct, infection studies commenced. However, the infection studies were not able to produce consistent results regarding its effect on host cell invasion. Although consistent results were not produced in response to infection of cells with our mutant construct, a stable line was attempted with the construct for possible use in future experiments.

RhoGAP/V5 stable lines were produced in HEK 293 cells. These stable lines are composed of cells that underwent selection from one individual colony, therefore, all cells within the line have identical DNA containing the mutant construct.

Over expression of the RhoGAP domain of p85 was one side of an experiment involving the RhoGAP domain. The flip side of my experiment was the deletion of the RhoGAP domain, a project performed by another member of the lab. ΔRhoGAP was the mutant construct produced by deleting the RhoGAP domain of WT p85. This mutant construct essentially has the same effect as over expression of the RhoGAP domain.
When the RhoGAP domain of p85 is deleted, PI3K cannot interact with the small GTPases anchored at the cell membrane. Because PI3K is not able to localize to the cell membrane, it is proposed that PIP₃ is not produced. Because PIP₃ is not produced, α-actinin could maintain its interaction with actin stress fibers, thus maintaining the shape and rigidity of the cell. If the actin stress fibers remain intact, the usual mechanism for *S. aureus* invasion is not possible. ΔRhoGAP was transfected into HEK 293 cells, through which infection studies could be done. Once the mutant DNA was transfected into the cells, the cells underwent a series of infections with *S. aureus*. Upon analysis of infection in the cells, it was determined that there was reduced bacterial invasion in the cells containing the mutant construct (Master’s Thesis, Haaning 2008). Following these studies, it was necessary to clone the ΔRhoGAP construct into an alternate vector for use in further experiments involving actin stress fibers. For this part of the experiment, I became involved in order to initiate the cloning and recombination reactions for this construct.

The bacteria *S. aureus* is the most common cause of infection (Lowy, 1998) leading to sepsis, and due to growing antibiotic resistance, an alternate solution to this problem is necessary. By over expressing the RhoGAP domain of WT p85, we believed that bacterial infection by *S. aureus* would be inhibited or reduced. However, infection studies yielded inconsistent data. Therefore, we were not able to conclude that over expression of the RhoGAP domain will inhibit *S. aureus* invasion. The deletion of the RhoGAP domain however, has produced significant results with respect to bacterial invasion.
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