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Investigating virulence phenotypes of *yqeK* deletion mutant in *Staphylococcus aureus*

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Abstract

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) can result in life threatening skin and soft tissue infections (SSTIs) in healthy individuals. SSTIs can lead to deeper bloodstream infections resulting in bacteremia, which is responsible for high mortality rates ranging from 20-50%. In response to infections, phagocytes from the host immune system produce radical nitric oxide (NO□) to limit bacterial proliferation. The complex biomechanisms of *S. aureus* enable it to evade NO□ immune defenses. With the rising occurrence of *S. aureus* bacteremia in developing countries and MRSA resisting host defenses and prescribed antibiotics, it is important to identify new targets for developing novel antibiotics. A previous student created a deletion mutant in the *yqeK* gene and characterized the phenotype of the *yqeK* mutant in NO□ stress and on sheep's blood agar plates. This resulted in delayed growth, dysregulated expression of genes in stress response, decreased hemolytic toxin production, and a decrease in virulence gene expression. The aim of this study is to investigate the virulence phenotypes of the yqeK deletion mutant in *S. aureus*, using a quantitative hemolysis assay and *Caenorhabditis elegans* as whole animal models. A liquid hemolysis assay in sheep blood was performed and showed a decreased production of hemolytic toxins in the *yqeK* mutant, thus

suggesting a decrease in virulence gene expression. *C. elegans* represents an established model to study *S. aureus* infections since they are infected and killed by the same *S. aureus* virulence factors involved in human SSTIs. A *C. elegans* infection assay was performed and resulted in complete death in all treatments, including the negative control. Future work will focus on increasing replications and optimizing the nematode assays. Expected outcomes for the *yqeK* mutant include increased survival rates in the animal model assay, decreased bacterial gut proliferation, and decreased hemolytic activity.

Introduction

The gram-positive bacterium *Staphylococcus aureus* exists among the skin and mucous membranes as a commensalist (Taylor et al 2025). Roughly one third of the population carries *S. aureus* asymptomatically (Henderson and Nimmo 2018), but colonization and infection of many host organs are also possible. *S. aureus* infections--traditionally acquired in nosocomial settings--may result in life-threatening diseases like pneumonia, bacteremia, and skin and soft tissue infections (Tobin et al 2025). While treated with many antibiotics, methicillin was one of the prescribed beta-lactam class antibiotics for *S. aureus* infections that *S. aureus* eventually evolved to evade. As a result, Methicillin-resistant *Staphylococcus aureus* (MRSA) and MRSA clones developed (Lakhundi and Zhang 2018). Albeit first documented in hospitals, MRSA has transversed into community settings giving rise to community-associated MRSA (CA-MRSA). CA-MRSA may cause life-threatening skin and soft tissue (SSTIs) infections in healthy individuals, those with low socioeconomic status, and disproportionately among African Americans (Henderson and Nimmo 2018, Tobin et al 2025, Lakhundi and Zhang 2018, Kwiecinski and Horswill 2021). Whereas skin infections can be locally limited, deeper bloodstream infections like bacteremia are increasing in developing countries with high mortality rates ranging from 20-50% (Kwiecinski and Horswill 2021). Although the innate immune system is the body's first line of defense, *S. aureus* uses immune evasion proteins to bypass the hosts' general defenses (Jong et al 2019). Therefore, with the rise of CA-MRSA in conjunction with a warming climate, the increase of S. aureus bacteremia cases in developing countries and the immune and antibiotic resistance of S. aureus, it is important to identify new targets for developing novel antibiotics (Henderson and Nimmo 2018).

To identify new targets, there needs to be an understanding of intracellular signaling molecules--such as alarmones--that promote bacterial survival through stress signaling (Ahmad 2025). S. aureus creates an alarmone called diadenosine tetraphosphate (Ap_4A) that may play a role during infection and immune evasion (Vidaud 2025). Ap_4A is produced in all cells when experiencing environmental stress and cleaved into two ADP molecules by ApaH hydrolases to regulate Ap_4A levels. While gram-negative bacteria possess the ApaH

family hydrolase enzyme, gram-positive bacteria lack that enzyme and instead have the largely conserved YqeK hydrolase that also symmetrically cleaves Ap_4A (Minazzato et al 2020; Ferguson et al 2020). The absence of this hydrolase can lead to large quantities of Ap_4A in gram-positive bacteria (Yang et al 2025). Heightened levels of Ap_4A in gram-positive bacteria can interfere with cell division, decrease metabolic abilities, increase sensitivity to oxidative stress, heat and aminoglycoside antibiotics (Yang et al 2025). These findings have isolated and identified the *yqeK* gene as a new target of virulence phenotypes of gram-positive bacteria. In suit, a previous study has supported that knocking out the *yqeK* gene results in elevated Ap_4A levels in *S. aureus* (Vidaud 2025).

Addressing the biomechanisms of *S. aureus* genes responsible for its pathogenicity, virulence, and cell growth in response to antibiotic and immune defense is important to restrict its spread in community settings amongst healthy individuals. To investigate the role of Ap_4A and YqeK in *S. aureus*, a previous student created a deletion mutant (*ΔyqeK*) in the *yqeK* gene to generate excess Ap_4A production. Another student (Catherine Anderson) performed growth curves on *ΔyqeK* to test the effects of acid stress on growth and found that *ΔyqeK* is more sensitive to acid stress than wild-type S. aureus. The following student, Jenna Vidaud, characterized the phenotype of the *ΔyqeK* in Kanamycin (Km), NO and acid stress and found a low Minimum Inhibitory Concentration (MIC) of Km, and delayed growth in the presence of acid and NO stress (Vidaud 2025). Through RNA-Sequencing, they also found dysregulated expression of genes involved in stress response pathways and a downregulation in toxin gene expression because of *yqeK* deletion (Figure 1).

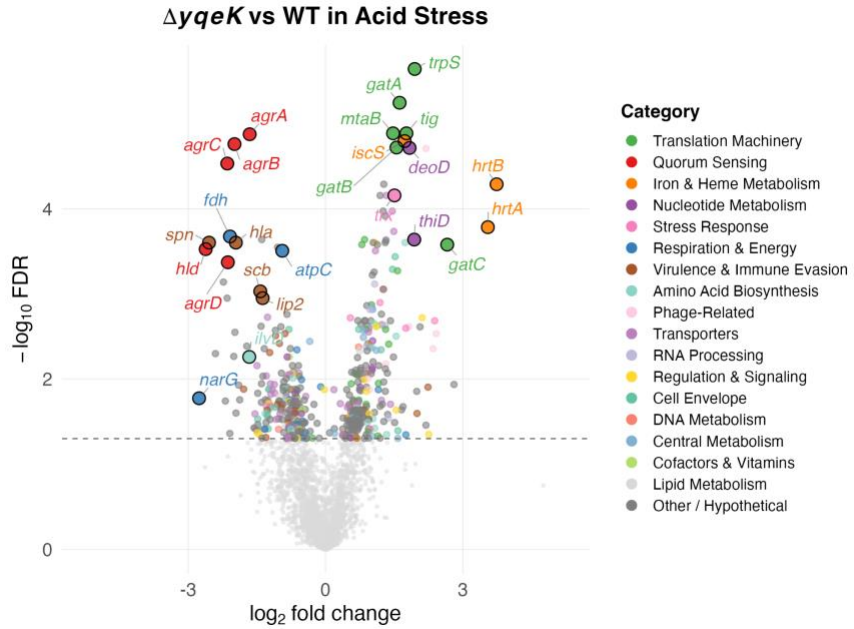

Figure 1. RNA-Sequencing of the *yqeK* deletion mutant revealed the downregulation of the genes encoding delta toxin (hld), alpha toxin (hla) and the quorum sensing system (*agrC*, *agrA*, *agrB*, *agrD*) on the left and the upregulation of other genes on the right.

The downregulation of toxin gene expression in *ΔyqeK* included the pore-forming alpha-toxin and delta-toxin, suggesting decreased virulence towards host cells (Vidaud 2025). The alpha-toxin and delta-toxin are membrane damaging (cytolytic) hemolysins (lyse RBCs) that can lead to cell death (Otto 2015). A previous study (Vidaud 2025) performed a qualitative plate assay on hemolytic activity of *S. aureus* strains (LAC) and found a decrease in hemolysis for the *yqeK* mutant (Figure 2, Figure 3). Bacteremia is a common infection correlated with *S. aureus* infections, and understanding *S. aureus* secreted hemolytic toxins is important for novel target identifications. If deletion of the *yqeK* gene leads to the downregulation of virulent toxins and decreased hemolysis, it is important to determine if *ΔyqeK* also phenotypically and quantitatively exhibits reduced virulence. In this study, we looked at virulence in two ways: a quantitative liquid hemolysis assay in sheep's blood and infection of a *Caenorhabditis elegans* animal model.

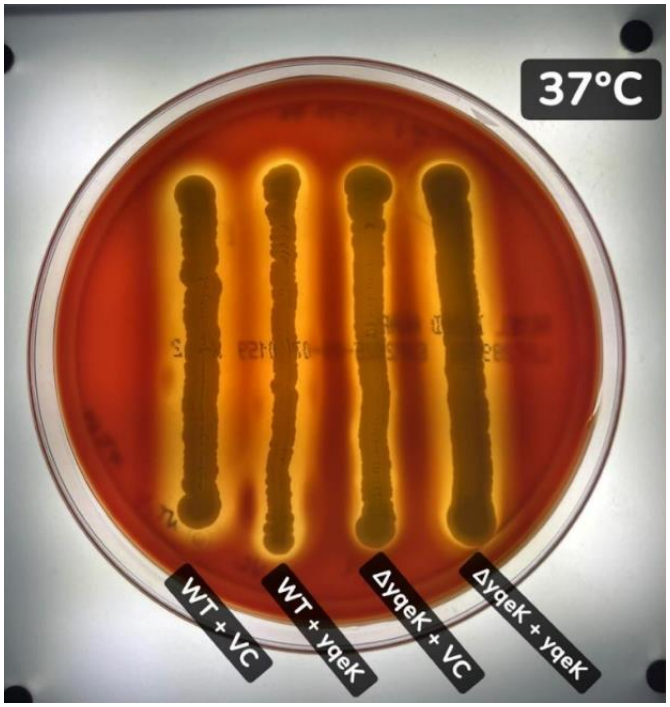

Figure 2. Qualitative sheep's blood agar plate assay with zones of hemolysis formed by the different LAC strains after 48 hours of growth at 37℃. The yellow coloration is evidence of complete blood cell lysis. The image and assay were conducted by Jenna Vidaud (2025).

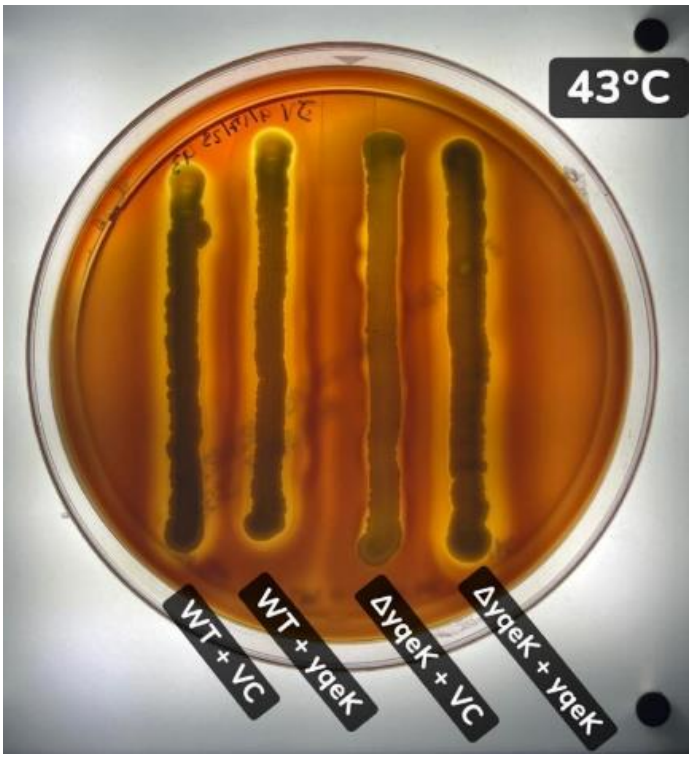

Figure 3. Qualitative sheep's blood agar plate assay with zones of hemolysis formed by the different LAC strains after 48 hours of growth at 43℃. The yellow coloration is evidence of complete blood cell lysis. The image and assay were conducted by Jenna Vidaud (2025).

First, a quantitative assay was performed to understand the hemolytic capabilities of toxins produced by each strain. With *ΔyqeK*, we predict that there will be an increase in Ap_4A production and therefore decreased hemolytic activity. The wild type and complement strain will retain the YqeK hydrolase and therefore have the ability to cleave Ap_4A and decrease sensitivity to environmental stressors. The wild type and complement strains are expected to have the highest trend of hemolytic activity.

Second, in-vivo infection assays were initiated on *Caenorhabditis elegans* animal models to compare the virulence phenotypes of *S. aureus* strains. *C. elegans* are microscopic nematodes with a rapid life cycle (3 days), hermaphroditic ability, bacterivorous diet and easily analyzed and manipulated genetically (Riddle 1997). *S. aureus* uses virulence factors involved in human diseases to infect and kill *C. elegans* making this strain of nematodes an established model to study *S. aureus* infections (Sifri et al. 2003, Irazoqui et al 2010). Additionally, a previous study conducted by Torres et al (2025) found that nearly 80% of human genes have a homolog in C. elegans. As *C. elegans* age, the pharynx and the intestines are frequently distended and crowded with bacterial cells. This bacterial proliferation is correlated with worm aging and decline due to decreased capacity to control intestinal bacterial growth (Portal-Celhay et al 2012).

Therefore, *C. elegans* are effective for early and direct evaluation of in-vivo pathogenesis efficacy (Wu et al 2010; Kong et al 2014). *C. elegans* that consume *S. aureus*, die over the course of several days because of the accumulation of bacteria in their digestive tract (Sifri et al. 2003). With *ΔyqeK* we expect a lower rate of death in *C. elegans* and increased death with the wild type and complement strains.

Traditionally, infection assays involve separating a certain number of worms into multiple wells of a 96-well flat plate. This process requires manual labor that is time consuming and prone to human error. After seven years of development, the Smart Soil Organism Detector (Smart SOD) was recently released by the SoilTech startup located at UNC Asheville. The Smart SOD instrument allows the observation of soil organisms through machine learning and the use of lasers to separate, identify, and organize developmental stages, classes of size, and more. With this instrument, more organisms are readily available for further analysis and will be used to carry out infection assays in this study (Filgueiras et al 2023).

Methods

Bacterial Strains and Culture Conditions

Plasmids are circular DNA molecules in bacteria that separate and replicate independently of chromosomal DNA and contribute to the spread of antibiotic-resistant genes. They are ubiquitous in nature with no essential genes and are often modified by researchers to express genetic manipulation in bacterial strains. Artificial plasmids are often referred to as vectors. To conduct our assays, we use the pRMC2 plasmid in the bacterial strains as the vector control (VC). The LAC mutant (*ΔyqeK*) contains a knock-down plasmid that targets the *yqeK* gene.

The strains used in this study are WT + VC (Wild type), *ΔyqeK* + VC (*yqeK* mutant or *ΔyqeK*) and *ΔyqeK* + *yqeK* (complement strain). All strains were incubated in 5mL of TSB with 5µl of chloramphenicol (cm10) at 37℃ shaking at a speed of 250 rpm for around 16-18 hours.

Nematode Age Synchronization

N2 wild isolate *C. elegans* strains were received from the *Caenorhabditis* Genetics Center. The worms were transferred to seeded nematode growth medium (NGM) 6cm plates and chunked every 7-20 days. An alkaline bleach solution was created with 5% KOH. Plates containing eggs and gravid adults were washed with 1mL of M9 buffer and transferred into a 15 mL centrifuge tube. Around 2-3mL total of M9 was used to wash up to

2 plates. The tubes were table-top centrifuged for 1 minute at 1500 rpm to create a worm pellet. The M9 supernatant was discarded as much as possible without disturbing the loose worm pellet. After removal of the supernatant, 5 mL of Worm Bleach Solution was added to the worm pellet. The tube was immediately capped and then vigorously shaken for three minutes without stopping to ensure the worms were all sufficiently dissolved. After the bleach incubation, 10 mL of M9 was immediately added to stop the bleaching. The tubes were centrifuged again for 1 minute at 1500 rpm. The bleach and M9 supernatant were discarded, leaving the worm debris behind. The worm debris was washed with 10 mL of M9 buffer and resuspended using inversion. The tube was then centrifuged again for 1 minute at 1500 rpm. This step was repeated one additional time. The egg pellet was then resuspended with 1mL of M9 buffer. The tubes were placed in the incubator at 20℃ for 20-24 hours, rocking gently at 75 rpm. The eggs will hatch at the end of the incubation and arrest at the L1 stage. After 24 hours, the quantity of worms per 5µl of M9 buffer was calculated to ensure no more than 500 worms were plated per 6cm plate in *E. coli* OP50 seeded NGM media.

Nematode Infection Assay

Seeded NGM plates with L1-L4 adult worms were run through the Smart SOD in collaboration with the SoilTech startup and NEMA Lab. The Smart SOD instrument (SOIL Tech) is a flow cytometer that uses lasers to separate and count organisms. This instrument was used to identify and organize *C. elegans* into their developmental stages. With the Smart SOD instrument, 20-40 L1-L4 worms were placed into each well. All worms were incubated with 200µl of liquid media containing 1.5 x 10^5 *S. aureus* (80% M9, 20% TSB, 10µg/mL cholesterol, and 7.5µg/mL nalidixic acid) in each well of the 48-well flat plate. The following strains were tested WT + VC, *yqeK* + VC, and *ΔyqeK* + *yqeK*. E. coli OP50 served as the negative control. The plate was incubated at 20°C and survival was monitored every 24 hours for 72 hours total (Lai 2024). A dissecting microscope from the Clark-Hachtel lab was used to check the survival of the nematodes.

Quantitative Hemolysis Assay in Sheep Blood

To prepare the red blood cells (RBCs) 1mL of sheep blood was centrifuged at 1000xg for 5 minutes at 4℃. After centrifuging, the supernatant was discarded and the pellet resuspended in 1mL of cold isotonic Phosphate Buffered Saline (PBS) using gentle inversion. Resuspension through vortexing and pipetting was not used to avoid mechanical lysis of RBCs. The resuspended sheep blood was centrifuged again at 1000xg for 5 minutes at 4℃. The PBS wash was repeated for a total of three times to remove plasma proteins and isolate RBCs. After the final wash, the isolated RBCs were weighed in units of mg (1mg

=1ul). A 2% suspension was created by dividing the volume of RBCs by 0.02 to get the total volume needed by adding PBS to the RBC volume. The suspension was mixed by gentle inversion and kept on ice until used the same day.

To prepare the bacterial cells, a 1:10 ratio of culture to TSB was created from each overnight (O/N) around the 16–18-hour mark of incubation. Optical density (OD) was measured using the mass spectrophotometer at 650nm. If the O/N had an OD around equal to or greater than ~6, the protocol continued. All ODs were diluted down to the same concentration using the $C_1V_1 = C_2V_2$ formula with a final volume of 1mL. The diluted cultures were then centrifuged at max speed for 1 minute to pellet bacterial cells only to be left with the secreted toxins. The supernatant was transferred to a new 1.5 mL microcentrifuge tube cautiously so as not to disturb the pellet. The media was filter sterilized into a 1.5 mL microcentrifuge tube using a 1cc syringe and a 0.45um filter 13mm in diameter and kept on ice for same day use.

To begin the hemolysis assay, 250µL of the WT + VC, *yqeK* + VC, and the *ΔyqeK* + *yqeK* supernatants were added into 4 separate microcentrifuge tubes. The negative control was PBS and the positive control was water; both were also used at a volume of 250µL. All six tubes had a corresponding 250µL of the 2% RBC suspension added. The tubes were incubated at 37°C for 1 hour without shaking, to avoid mechanical lysis. After incubation, all tubes were centrifuged at 1000xg at 4°C for 5 minutes. Immediately after, 200µL of the liquid supernatant was extracted from each tube to prevent the pellet from dissolving and added into a 96-well plate. Each well was read a total of three times at an absorbance of 540nm to assess the amount of hemoglobin released by lysed RBCs. The percent hemolysis was determined by the formula %hemolysis = $\frac{(sample - negative\ control)}{(positive - negative\ control)} \times 100$

Results

Quantitative Hemolysis Assay

In a previous qualitative hemolysis assay, *ΔyqeK* was documented to have downregulation of genes responsible for coding the pore-forming toxins (Vidaud 2025), leading to decreased hemolytic activity. In this study, we performed a quantitative hemolysis assay to precisely measure the concentration of hemoglobin in the toxin supernatant of the LAC strains.

The quantitative hemolysis assay was performed with WT + VC, *ΔyqeK* + VC and *ΔyqeK* + *yqeK*. After the incubation for one hour at 37℃, absorbances were read and data was collected to perform statistical analysis.

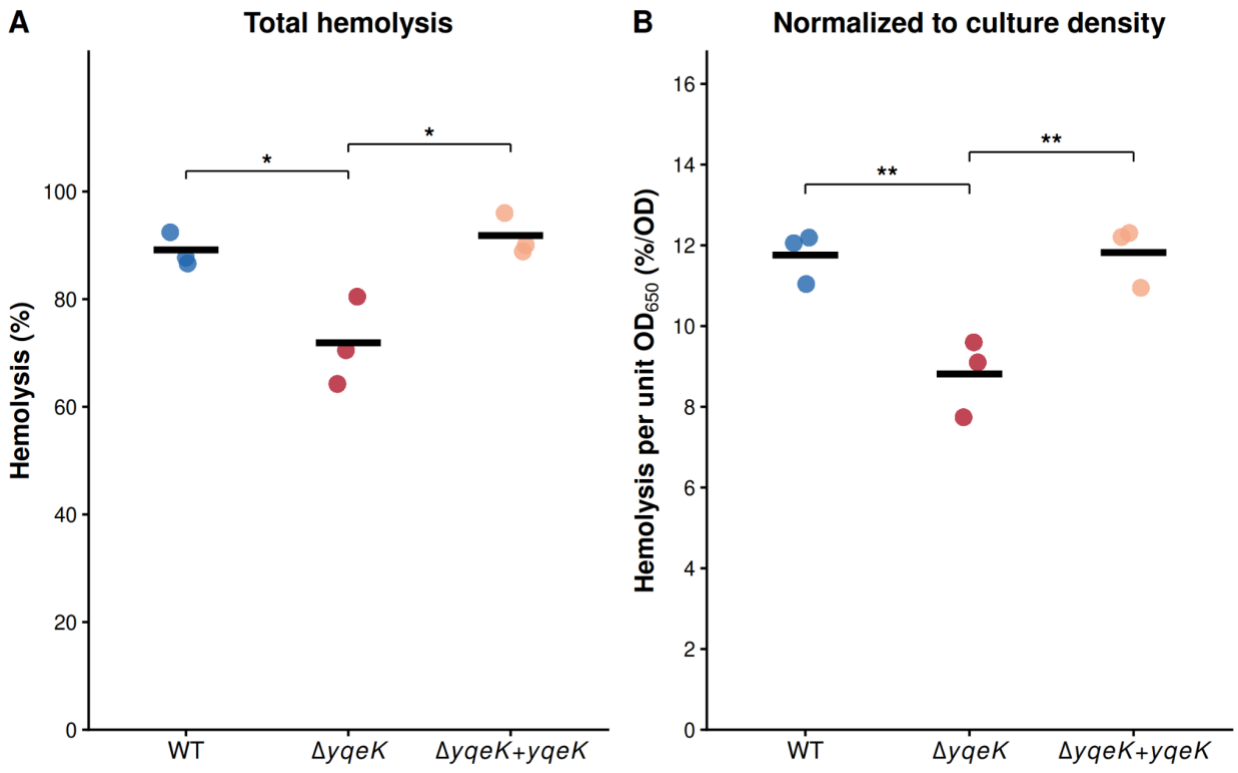

Figure 4. A one-way ANOVA performed on percent hemolysis on the left and percent hemolysis normalized per OD on the right revealed a statistically significant difference in LAC WT strain and LAC *ΔyqeK* strain (Total hemolysis; $F_{(2,6)}=11.5$, $p<0.05$) and LAC *ΔyqeK* strain and LAC *ΔyqeK+yqeK* strain (OD; $F_{(2,6)}=14.1$, $p<0.05$). LAC WT and *ΔyqeK+yqeK* strains were not significantly different, showing full restoration of toxin productivity in the complemented strain ($p>0.8$).

The one-way Anova showed significantly decreased hemolytic activity in *ΔyqeK* + VC in comparison to WT + VC and *ΔyqeK* + *yqeK* (Total hemolysis; $F_{(2,6)} =11.5$, $p<0.05$, Figure 4). There was also around a 15% reduction of total hemolysis of the *yqeK* strain (Figure 4). WT + VC and *ΔyqeK* + *yqeK* had no significant differences in hemolytic activity ($p>0.8$, Figure 4). This reinforces that complementing the *yqeK* deletion with a functional copy of *yqeK* on a plasmid fully restores toxin production. No significant difference was observed in the wild type and complement strains, therefore suggesting that the WT + VC and *ΔyqeK* + *yqeK* produce toxins that result in near complete hemolysis (Figure 4, Figure 5).

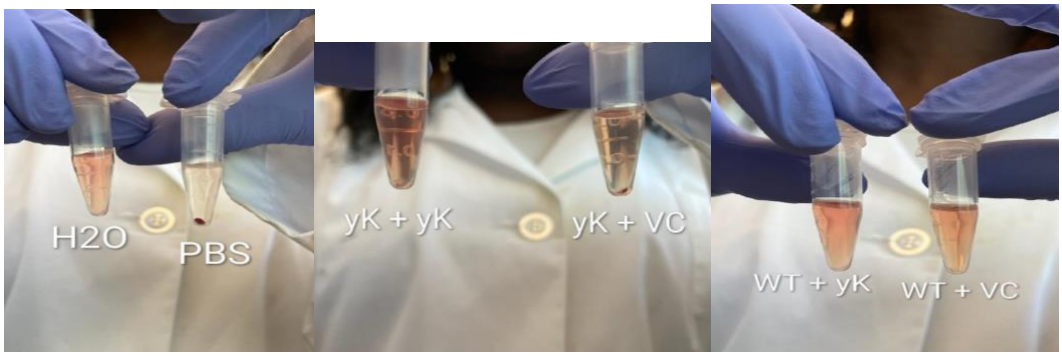

Figure 5. Image of hemoglobin released into the supernatant after hemolysis from the different LAC strains and the controls after 1 hour of incubation at 37℃. All strains and controls were mixed with a 2% PBS suspension of sheep blood. Darker coloration indicated higher levels of hemolytic activity and lower coloration (with a larger pellet of RBCs at the bottom of the tube) indicated lower levels of hemolytic activity.

Infection Assay

To understand the effects of LAC strains in a complex system, we used *C. elegans* nematodes as a whole animal model to conduct an infection assay. *S. aureus* uses virulence factors involved in human disease to infect and kill *C. elegans*. When *C. elegans* consume pathogenic bacteria such as *S. aureus*, their ability to clear their digestive tract of bacteria decreases due to virulence effects of the bacteria therefore resulting in worm death. To perform early and direct evaluation of in-vivo pathogenesis efficacy, it is necessary to start the worms at Day 0 of their cycle beginning at the L4 stage. To successfully sort and use L4 staged worms, we performed an age synchronization protocol in order to run the age synchronized worms through the Smart SOD instrument for accurate sorting and counting. To conduct age synchronization, we used an alkaline bleach solution that aims to isolate nematode eggs by dissolving the worms. If the eggs are isolated, they are grown in M9 media at 20°C or 25°C for at least 24 hours to arrest at the L1 stage and further plated on solid NGM media to synchronously grow into the sequential developmental stages until the L4 stage has been reached. After we performed age synchronization using this protocol, the eggs were dissolved along with the worms and the process was unsuccessful after several attempts. Currently, the age synchronization protocol is being optimized as we have run into chemical concentration issues, improper storage and improper use.

Due to the inconsistencies of the chemical concentration for age synchronization, we collaborated with the SoilTech startup and hand-sorted worms of any developmental stage we had. The worms were separated into 20-40 worms per well in a 48-well flat plate as a

preliminary test. After sorting, the worms were incubated with treatments at 20°C with survival being monitored every 24 hours for 72 hours total. This preliminary assay resulted in a 0% survival rate for all treatments including the negative control. Currently, optimization of the bacterial concentration, incubation, and sterilization of the media are being performed for better results.

Discussion

As CA-MRSA persists in deadly infections in healthy individuals and contributes to increasing levels of bacteremia in developing countries, with a warming climate, it is important to identify new genetic targets for developing novel antibiotics (Henderson and Nimmo 2018). Discovery of the *yqeK* gene in gram-positive bacteria and its role in Ap_4A production makes it a new target to assess phylogenetic virulence in *S. aureus*. Secreted toxins produced by *S. aureus* that damage cell membranes and RBCs are factors in *S. aureus* virulence (Otto 2015). The pore-forming alpha-toxin and delta-toxin responsible for hemolysis and cell death were previously found by Vidaud (2025) to be regulated by the *yqeK* gene product. After creating a *ΔyqeK* deletion mutant, there was a significant downregulation of both the alpha-toxin and delta–toxin suggesting decreased toxin regulation and virulence (Figure 1). While a qualitative hemolysis assay provided observational data showing a decrease in hemolysis, the same trend was observed in the decreased hemolytic activity for the quantitative hemolysis assay performed in this study (Figure 2; Figure 3; Figure 4; Figure 5).

From the quantitative hemolysis assay the hemolytic activity in *ΔyqeK* + VC was significantly lower than the wild type and complement strain ($p<0.05$, Figure 4). The hemolytic activity normalized by OD showed the same trend of significant decrease in hemolytic activity for the *ΔyqeK* mutant in comparison to the other LAC strains (Figure 4). This supports the finding that *ΔyqeK* shows reduced virulence towards host cells. Additionally, the complement strain fully restores toxin production showing similar trends in hemolytic activity in comparison to the WT + VC strain (Figure 4, Figure 5).

To further test the virulence phenotype of *S. aureus* in an animal model, we conducted an infection assay on *Caenorhabditis elegans*. The N2 Wild Type Strain of *C. elegans* was selected due to *S. aureus* using similar virulence factors involved in human diseases to infect and kill *C. elegans* (Sifri et al. 2003, Irazoqui et al 2010). Additionally, nearly 80% of human genes have a homolog in C. elegans (Torres et al 2025).

To perform the infection assay the L4 developmental stage is necessary due to that stage counting as Day 0 of the nematode's lifespan. In order to isolate the L4 stage, traditionally age synchronization is performed. It requires a bleach solution that must be created and used within a month and stored away from light to prevent deterioration of the

solution. For this study, we performed age synchronization to isolate all stages of *C. elegans* for the Smart SOD instrument to learn the different developmental stages for more accurate sorting and counting of developmental stages. Using the Smart SOD instrument can cut down preparation time and costs drastically by sorting out L4 staged worms to begin assays at Day 0 of the animal lifespan and avoid the use and purchase of chemicals for age synchronization.

After the worms were bleached, eggs should be isolated and incubated at 20℃ or 25℃ for at least 24 hours to halt the worms at the L1 stage. Isolation of the eggs was not successful due to a variety of reasons. First, the protocol we used was adapted from an MIT molecular genetics protocol that specifically used KOH as the base for the worm bleach solution. However, after further research, many other protocols used NaOH as the base for the worm bleach solution for its cost effectiveness, ease of preparation at higher molarities and consistency in worm bleach solutions. Second, the bleach solution was supposed to be stored away from light and discarded after a month of creation. The solution was stored improperly and exposed to light and was consistently used two months after creation. Current optimizations are on the way to ensure successful age synchronization of worms by bypassing the use of a bleach solution and instead teaching the machine to identify *C. elegans* eggs. This will further speed up the process by placing eggs within well plates with M9 media to halt it at the L1 stage after incubation at 20℃ for 24 hours.

Although the infection assay was not successful due to adjustable factors, the quantitative hemolysis assay provided results that lead us to hypothesize that *C. elegans* infected with the *yqeK* mutant will have a higher survival rate as a result of the downregulation of toxins observed in the RNA-seq by Vidaud (2025) (Figure 1). Not only was there downregulation of alpha-toxin and delta-toxin, there was also downregulation of the entire quorum-sensing system. Quorum-sensing systems facilitate cell-to-cell communication and regulation of virulence factors. In *S. aureus* there is the staphylococcal accessory gene regulator (agr) quorum-sensing system to increase the expression of virulence factors (Yarwood and Schlievert 2003). The RNA-seq showed the downregulations of the toxins and the entire quorum sensing system meaning the entire virulence system is down. Many infection models have also found agr participation in *S. aureus* pathogenesis (Yarwood and Schlievert 2003). This suggests that when we treat *C. elegans* with the *ΔyqeK* in the infection assay there may be decreased virulence and higher rates of survival.

Future research will work on optimizing the age synchronization and infection assay to support the machine learning of the Smart SOD instrument and to observe general trends of virulence in all LAC strains starting the animal models at day 0. Bacterial proliferation in the intestines of *C. elegans* is correlated with worm aging and decline due to decreased

capacity to control intestinal bacterial growth (Portal-Celhay et al 2012). Therefore, after the infection assay, a gut proliferation/bacterial colonization assay will be performed to assess the bacterial colony forming units (CFU) present in the intestinal tracts of the worms for each treatment to assess how well the bacteria are able to survive during a worm infection and immune response. Further directions for this project will focus on a potential pilot drug screening for YqeK inhibitors to explore *yqeK* as an anti-virulence target by identifying the first small molecule inhibitors.

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