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Determining the role of Ap_4A in the stress response of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus causes a variety of infections, such as skin and soft tissue infections (SSTI), infectious endocarditis, bacteremia, osteomyelitis, and pneumonia. The virulence of *S. aureus* can be partially explained by its ability to acquire resistance to a wide range of antibiotics and innate immune effectors. In this study, a second messenger pathway involving the compound diadenosine tetraphosphate (Ap_4A) was disrupted to determine its role in *S. aureus* stress response. Ap_4A exists in both prokaryotes and eukaryotes; however, in bacteria, its primary role is believed to be a second messenger signaling molecule. In *S. aureus*, Ap_4A is synthesized from AMP and ATP when aminoacyl tRNA synthetases lack tRNA substrate, and it is degraded by the enzyme YqeK into two ADP. To determine the role of Ap_4A in *S. aureus*, we deleted the gene *yqeK* to create a strain with excess Ap_4A. We then characterized the phenotype of this mutant during Kanamycin, nitric oxide (NO·), and acid stress. Wild-type *S. aureus*, the deletion mutant, a complemented mutant, and an overexpression mutant were grown in increasing concentrations of Kanamycin, 20 mM of an NO· donor, or acidified (pH 5.5) media for 24-hour periods. The deletion of *yqeK* significantly lowered the minimum inhibitory concentration of Kanamycin and showed a general trend of delayed growth with NO· or acid stress. To investigate the mechanism of how *yqeK* is linked to *S. aureus* stress responses, RNA-Seq was performed on WT and *ΔyqeK* strains during NO· and acid stress. The *yqeK* deletion mutant exhibited reduced expression of genes involved in stress response pathways, which likely contribute to the observed phenotypes. Surprisingly, deletion of *yqeK* also resulted in reduced expression of several genes encoding toxins and virulence factors,

while expression of genes suggesting the initiation of prophage induction was increased. These results suggest that increased levels of Ap_4A in *S. aureus* may be detrimental to cell growth, antibiotic resistance, evasion of the human immune response, and virulence factor production. Future research will involve quantifying the concentration of Ap_4A in wild-type and mutant *S. aureus* when exposed to various stressors and using qRT-PCR to confirm RNA-Seq results.

Introduction

Staphylococcus aureus is a highly virulent bacterium that can cause skin and soft tissue infections (SSTI), bacteremia, infectious endocarditis, sepsis, and death [1,2]. *S. aureus* belongs to a group of pathogens infamous in the clinical world for being highly virulent and antibiotic resistant. The group of bacteria, consisting of six different species, are known as ESKAPE pathogens, named after the first letter of their respective genus. As an ESKAPE pathogen, it is one of six bacteria primarily responsible for nosocomial infections. Additionally, ESKAPE pathogens are becoming increasingly multidrug-resistant, an epidemic that is one of the top three threats to global public health [3]. Methicillin-Resistant *S. aureus* (MRSA) infections have been on the rise since *S. aureus* strains acquired a new gene to resist β-lactam medications by encoding an additional transpeptidase, PBP2a, with reduced affinity for β-lactams [3]. In 2017, there were 120,000 cases of *S. aureus* bloodstream infections, and 20,000 associated deaths in the United States, despite an annually decreasing rate of hospital-acquired MRSA infections [1]. While efforts have been made to reduce the rate of nosocomial infections, hypervirulent community-acquired MRSA (CA-MRSA) strains have appeared that are capable of infecting healthy individuals [4]. The introduction of hypervirulent community-acquired MRSA calls for the exploration of the mechanisms of this pathogen's resistance to the immune system and antibiotic stressors.

S. aureus has been shown to continue replication in high concentrations of nitric oxide (NO·), suggesting that *S. aureus* is able to resist the wide variety of attacks that can be utilized by this radical [6]. NO· resistance is particularly significant due to the great deal of diversity in functions that it carries out in the human immune response. As a radical, nitric oxide is very reactive and interacts with targets such as DNA, proteins, thiols, prosthetic groups, and reactive oxygen species [5]. This allows for a multifaceted approach that the immune system can employ against foreign microbial pathogens. In order to explore what contributed to the innate resistance of this pathogen, a previous study used a transposon screen to identify the genes that were essential for NO· resistance in S. aureus [7]. The study found a total of 168 genes that were required for full S. aureus's NO· resistance. One gene identified in this study was later characterized as *yqeK*, which encodes the diadenosine tetraphosphate (Ap_4A) hydrolase, YqeK [8].

Ap_4A is a dinucleotide that appears in both eukaryotic and prokaryotic cells [9]. Ap_4A is synthesized by an aminoacyl tRNA synthetase from ATP and an amino acid-bound AMP when no cognate tRNA is present, though additional synthesis mechanisms occur in eukaryotic cells [9]. In Gram-negative bacteria, the ApaH family of enzymes consists of Ap_4A hydrolases, which symmetrically cleave Ap_4A into two ADP molecules [9]. Gram-positive bacteria lack ApaH enzymes, and contain the enzyme YqeK instead [8,9]. YqeK similarly cleaves Ap_4A symmetrically into two ADP, and has been shown to reduce in vivo levels of Ap_4A in studies with *B. subtillis* [8,9]. While YqeK has been shown to cleave Ap_4A in vitro, as well as in other bacteria, there are no studies showing in vivo levels of Ap_4A being manipulated by YqeK in *S. aureus*. In human cells, the degradation of Ap_4A is regulated by the tumor suppressor protein, Nudix hydrolase 2 [9].

Studies have shown that across cell types, Ap_4A levels generally increase during stress [9]. Heat stress and stress from reactive oxygen species, from both hydrogen peroxide and Kanamycin, significantly increased the levels of Ap_4A in *S. typhimurium* and *E. coli* [9]. However, in these same two species, the most dramatic increase in Ap_4A level was caused by the induction of oxidative stress [9]. ApaH knockout mutants in E. coli lose the ability to degrade Ap_4A and become significantly more susceptible to kanamycin than the wild type strain [9]. This shows that Ap_4A accumulation is likely detrimental to cell survival. Studies on Ap_4A in Gram-positive bacteria have found similar results, though there is significantly less research with Gram-positive bacteria. In *Streptococcus mutans*, a *yqeK* deletion strain exhibited delayed cell growth, inhibited biofilm formation, and reduced production of water-insoluble extracellular polysaccharides [10]. Ap_4A was found to bind inosine-5'-monophosphate dehydrogenase (IMPDH) in *B. subtillis*, causing a conformation change to the enzyme's inactive form [11]. IMPDH is an essential protein, as it is required for the synthesis of guanine nucleotides necessary for DNA and RNA repair and replication [12]. The involvement of Ap_4A in antibiotic susceptibility, stress responses, biofilm formation, and regulation of essential proteins suggests that this molecule takes on the role of a second messenger. While originally thought to be a potential stress metabolite, the hypothesis that Ap_4A acts as a bona fide second messenger has become increasingly accepted as evidence continues to come out about the detrimental effects its accumulation seemingly has on cells [13].

Given the role of Ap_4A in other bacteria, and its identification in *S. aureus* as a possible contributor to NO· resistance, exploring the role of this second messenger in *S. aureus* stress could give insight into new pathways and mechanisms responsible for *S. aureus* resistance and virulence. In this study, growth curves were used to determine differences in susceptibility to NO· and Kanamycin stress between wild type and *yqeK* deletion mutants of *S. aureus*. RNA sequencing was performed to analyze how transcriptional pathways are impacted by *yqeK* deletion during normal and stress conditions, including acid and NO·. The results showed that high levels of Ap_4A significantly decrease *S. aureus* viability in kanamycin stress, and a general trend of delayed growth under NO· stress. From RNA-Seq results, it was found that the typical *S. aureus* response to acid and NO· stress involved enrichment of pathways that generally provided relief for oxidative and osmotic stress, while also increasing toxin production. Conversely, increased levels of Ap_4A under these stress conditions led to the enrichment of pathways that suggest the initiation of prophase induction. The lack of certain enriched pathways observed when Ap_4A levels were increased suggested that toxin production was likely reduced in the *yqeK* deletion mutant compared to wild type under NO· and acid stress.

Methods

Cultures

For *S. aureus* growth experiments and RNA-Sequencing, the USA300-LAC strain of CA-MRSA, a dominant source of MRSA infections in North America and a common lab strain, was used [4,14]. The LAC *ΔyqeK* deletion mutant used for these experiments was previously constructed and contains an in-frame deletion of the *yqeK* gene, leaving behind only the start and stop codons to prevent downstream genes in the same operon from experiencing any subsequent effects. Complement and overexpression strains of *yqeK* were created using pRMC2 plasmids where *yqeK* gene expression is controlled by an anhydrotetracycline-inducible promoter. However, expression from this promoter is leaky, and full phenotypic complementation was achieved without induction, so anhydrotetracycline was not used in our experiments. These plasmids use chloramphenicol resistance as a selectable marker. Strains of both wild type (WT) and *ΔyqeK* were also created containing empty pRMC2 plasmids to act as vector controls. Bacterial overnight cultures were grown in glass culture tubes containing 5 mL tryptic soy broth (TSB) for 16-20 hours, or until stationary phase, at 37°C while shaking at 250 rpm. When necessary to maintain a plasmid, the bacteria were grown under the same conditions but with the addition of 10µg/mL chloramphenicol.

Table 1. Strains of USA300-LAC used in the following experiments.

USA300-LAC Strains			
Strain name	Gene deletions	Plasmids	Purpose
WT	none	none	normal function
ΔyqeK	*yqeK*	none	deletion of *yqeK*
WT + VC	none	pRMC2 + CM resistance	normal function with plasmid control
ΔyqeK + VC	*yqeK*	pRMC2 + CM resistance	deletion of *yqeK* with plasmid control
WT + *yqeK*	none	pRMC2 + CM resistance + *yqeK*	overexpression of *yqeK*
ΔyqeK + *yqeK*	*yqeK*	pRMC2 + CM resistance + *yqeK*	*yqeK* complement

Cell Stress Growth Curves

To set up the cell stress growth curves, bacterial overnight cultures were diluted 1:10 using fresh TSB. The dilution was measured for optical density (OD) at 650 nm using a Spectronic Genesys 2 spectrophotometer. After OD was determined, cultures were further diluted to an OD of 0.01. To a 96-well polystyrene plate, 200 µl of the diluted culture was added to each well used in the experiment. The cell stressor would be added to all wells except for the designated controls. The plate was then measured for OD650 every 15 minutes for 24 hours while shaking at 37°C in a Biotek Synergy HTX plate reader.

For kanamycin cell stress, the first two wells in a row received no treatment, while each set of two for the following wells in the row contained a concentration of kanamycin starting at 3.125 µg/mL, and doubling until reaching a concentration of 50 µg/mL in the last two wells of the row. This pattern was followed for every row of wells that contained culture.

For NO· cell stress, half of the wells with culture contained no treatment, while the other half received 20 mM of (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETANO), an NO· donor, dissolved in 0.01N NaOH.

A previous student (Catherine Anderson) performed growth curves to test the effect of acid stress on growth using a similar growth curve set up, but with TSB acidified to a pH of 5.5 with acetic acid. These data are included since they led to one of the RNA-Seq experiments described below.

Significant differences between treatment groups in growth curves were determined by measuring and comparing lag time and Minimum Inhibitory Concentration (MIC). Lag time was defined as the average time it took for cultures to reach an OD of 0.2. MIC was defined as the average minimum concentration of kanamycin where the OD did not exceed 0.15 at 14 hours of growth. Differences between strains undergoing NO· stress and acid stress were determined using lag time, while MIC was used to determine differences under kanamycin stress. These results were analyzed using a two-way ANOVA with a Sidak's multiple comparison test in R-studio

Hemolysis Plates

Sheep's blood agar plates, made with 5% sheep's blood in Tryptic Soy Agar (TSA), were streaked with WT + VC, *ΔyqeK* + VC, *ΔyqeK* + *yqeK*, and WT + *yqeK* strains. Plates were incubated at either 37°C or 43°C for 48 hours. The plates were photographed once at 24 hours to assess hemolytic activity, and again at 48 hours. Hemolytic activity could be determined by observing the zone of hemolysis around streaks of bacteria. A clear or orange color indicates β-hemolysis (complete lysis of red blood cells), while a green color indicates α-hemolysis (partial lysis).

RNA cultures

To extract RNA for RNA-Sequencing, RNA cultures were first grown by diluting overnight bacterial culture 1:100 with fresh TSB. The day of RNA collection, overnight cultures were back-diluted into fresh media in an Erlenmeyer flask and allowed to grow at 37°C while shaking at 250 rpm until they reached mid-exponential phase.

For acid stress cultures, strains were grown in TSB acidified to pH 5.5 with acetic acid. After reaching an OD650 of 0.5, the liquid culture was poured into a sterile 50 mL conical and centrifuged at 4°C for 5 minutes at 10,000x g. The supernatant was disposed of and cells were flash frozen using liquid nitrogen. The frozen cells were stored in a -80°C freezer until RNA extraction.

The cultures grown for NO· stress were grown to an OD of 0.4. After reaching this OD, 10 mM of DETANO was added to the flask and the culture was allowed to continue growing for 1 hour. Then, the cells were centrifuged and stored the same way as for acid stress.

RNA Extraction

This protocol was modified from a previously published research paper [15]. The stored, frozen cells were thawed and resuspended in 100 µL of 8.0 pH TE buffer. The suspension was transferred to a Lysing matrix B tube (MP Biomedicals) and cells were lysed via bead beating with VWR Mini Bead Mill Homogenizer at setting 5 for 30 seconds. The tubes were placed on ice, and 650 µL of RLT buffer containing β-mercaptoethanol (10 µL β-mercaptoethanal: 1 mL RLT) was added to each tube. The tubes were bead beat for another 30 seconds before being returned to the ice. The Lysing tubes were then centrifuged at full speed for 1 minute. After, 600 µL of the supernatant was extracted and mixed with 900 µL of 100% EtOH in a 1.5 mL tube. The lysate mixture was added 600µL at a time to an RNeasy mini spin column (Qiagen) and centrifuged at max speed for 30 seconds. The column was washed with 700 µL of RW1 buffer before the column was transferred to a sterile 2 mL collection tube. The column was washed twice with 500 µL of RPE buffer. The spin column was dried by centrifuging at full speed for 2 minutes. The spin column was placed on a sterile 1.5 mL tube and 53 µL of nuclease-free water was added. The tube was allowed to sit for 1 minute before being centrifuged at full speed for 1 minute. The concentration of RNA was measured using a Nanodrop, and the extracted RNA was diluted with nuclease-free water to a concentration of 200-500 ng/µL, while keeping the total volume at 50 µL.

The diluted extraction was treated with Turbo DNase (Invitrogen) by adding 6 µL of 10x DNase buffer and 1 µL of DNase I. The mixture was incubated at 37°C for 60 minutes. The DNase reaction was stopped by adding 7 µL of DNase deactivation buffer and flicking the tube to mix. The tubes were centrifuged at 10,000 x g for 2.5 minutes. After being centrifuged, 42 µL of supernatant was extracted into a sterile 1.5 mL tube. The RNA concentration was assessed using a Nanodrop, and the quality of the RNA was assessed using gel electrophoresis with an agarose gel. The extracted samples were stored at -80°C.

RNA-Sequencing

RNA samples were sent to SeqCenter (Pittsburgh, PA). Samples were DNAse treated with Invitrogen DNAse (RNAse free). Library preparation was performed using Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit and 10bp unique dual indices (UDI).

Sequencing was done on a NovaSeq X Plus, producing paired end 150bp reads. Demultiplexing, quality control, and adapter trimming was performed with bcl-convert (v4.2.4). No additional trimming or filtering was performed.

Read mapping was performed with HISAT2 to *Staphylococcus aureus subsp. aureus* USA300_FPR3757 genome (NCBI accession number NC_007793.1). Read quantification was performed using Subread's featureCounts2 functionality. Read counts loaded into R were normalized using edgeR's5 Trimmed Mean of M values (TMM) algorithm. Subsequent values were then converted to counts per million (CPM). Differential expression analysis was performed using edgeR's glmQLFTest. Differentially expressed genes were defined as having a $|\log FC| > 1$ and $p < .05$ (log fold change is base-2).

RNA-Seq figures were made using ShinyGo v0.85.1 [16]. Differentially expressed genes with a positive fold change for a given strain under one of the stress conditions (NO· or acidified) were uploaded to the site, and output was given as gene pathways enriched for that strain compared to a baseline of no stress.

Results

In order to explore the role of Ap_4A in *S. aureus* resistance to antibiotics and the immune system, cell growth assays were performed with WT and *ΔyqeK* mutant strains. These assays involved growing strains of *S. aureus* with and without stress, and measuring optical density over a 24-hour period. The stressors chosen to represent immune stress and antibiotic stress were NO· and kanamycin, respectively.

In a previous transposon screen, *yqeK* was implicated as possibly contributing to the resistance of NO· in *S. aureus*, a key factor of the bacteria's virulence and immune evasion [5,6,7]. Here, we tested the ΔyqeK deletion mutant and a complemented version of the mutant to investigate the impact of NO· stress on growth. We observed a trend that the *ΔyqeK* + VC strain grew slower than the other strains, including the complemented mutant, after the addition of DETA/NO (Figure 4). However, there was not a significant difference when comparing growth rates between strains. This may be partially due to the low number of replicates and the high variability in growth phenotypes during NO· stress; more replicates are currently underway.

Because accumulation of Ap_4A impacts antibiotic resistance in other species of bacteria, we next wanted to investigate whether *yqeK* deletion impacts antibiotic resistance in *S. aureus*. Kanamycin belongs to a class of antibiotics called aminoglycosides, which are commonly used to treat staph infections in combination with other drugs, such as beta-lactams or glycopeptides. As a commonly used treatment, strains of MRSA are often found with resistance genes to aminoglycosides, making kanamycin a good representative to show how the deletion of *yqeK* can impact resistance [17].

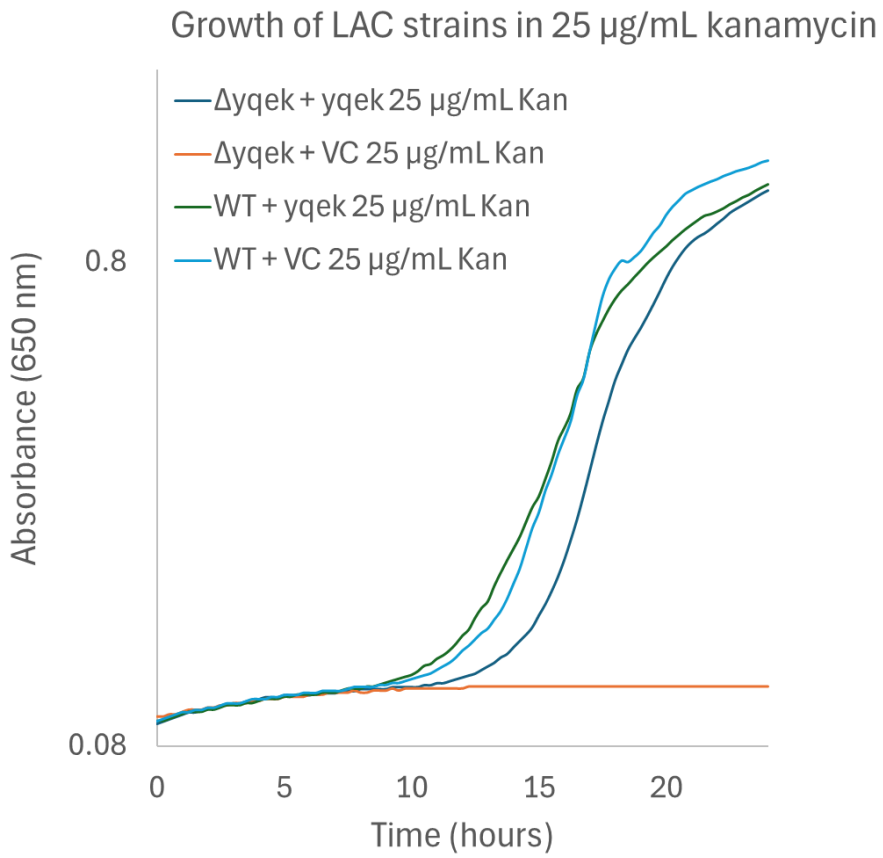

Figure 1. Representative growth curve showing *ΔyqeK*, *ΔyqeK* compliment, *yqeK* overexpression, and WT LAC strains growing in kanamycin.

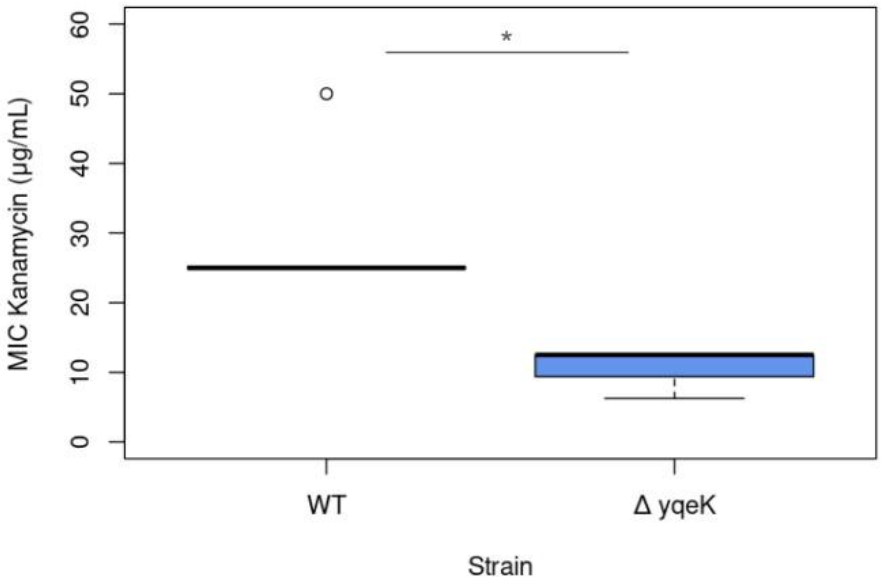

Figure 2. The kanamycin MIC was significantly lower for the LAC *ΔyqeK* strain than for the LAC WT strain (t=5.2271, p<0.001). The MIC was calculated by determining the lowest concentration where absorbance at 650 nm did not exceed 0.15 at 14 hours.

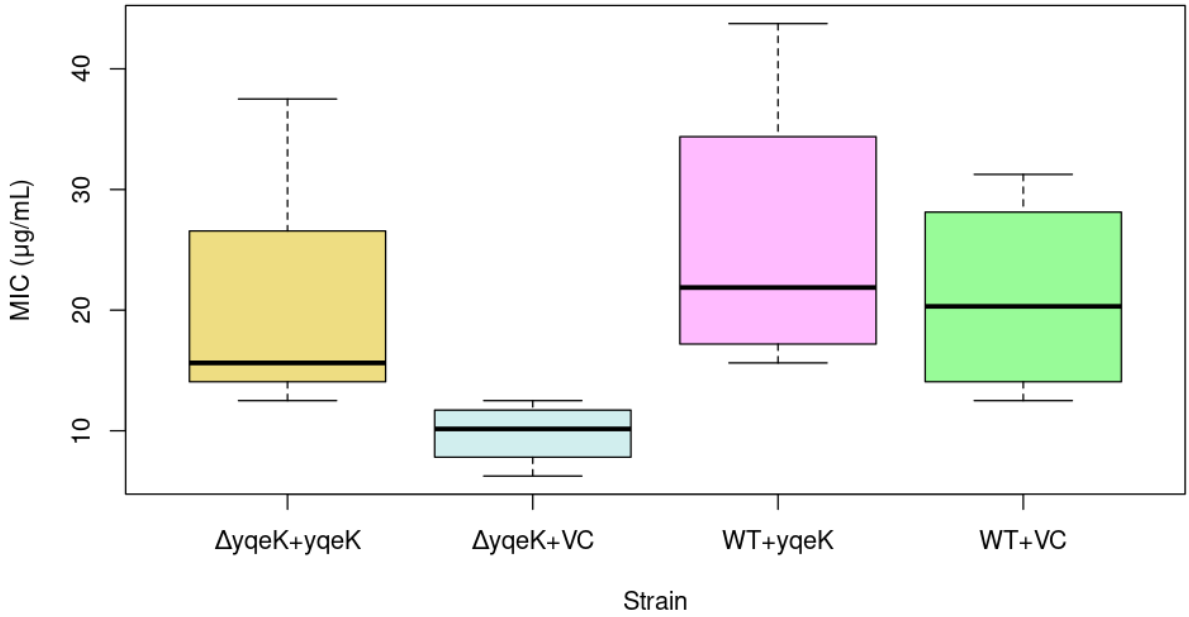

Figure 3. The kanamycin MIC was significantly lower for *ΔyqeK* + VC compared to WT + *yqeK* and WT + VC (p<0.01, p<0.05, respectively). There was no significant difference between *ΔyqeK* + VC and *ΔyqeK* + *yqeK* (p=0.072). There were no significant differences between WT + *yqeK*, *ΔyqeK* + *yqeK*, or WT + VC (p>0.5). The MIC was calculated by determining the lowest concentration where absorbance at 650 nm did not exceed 0.15 at 14 hours.

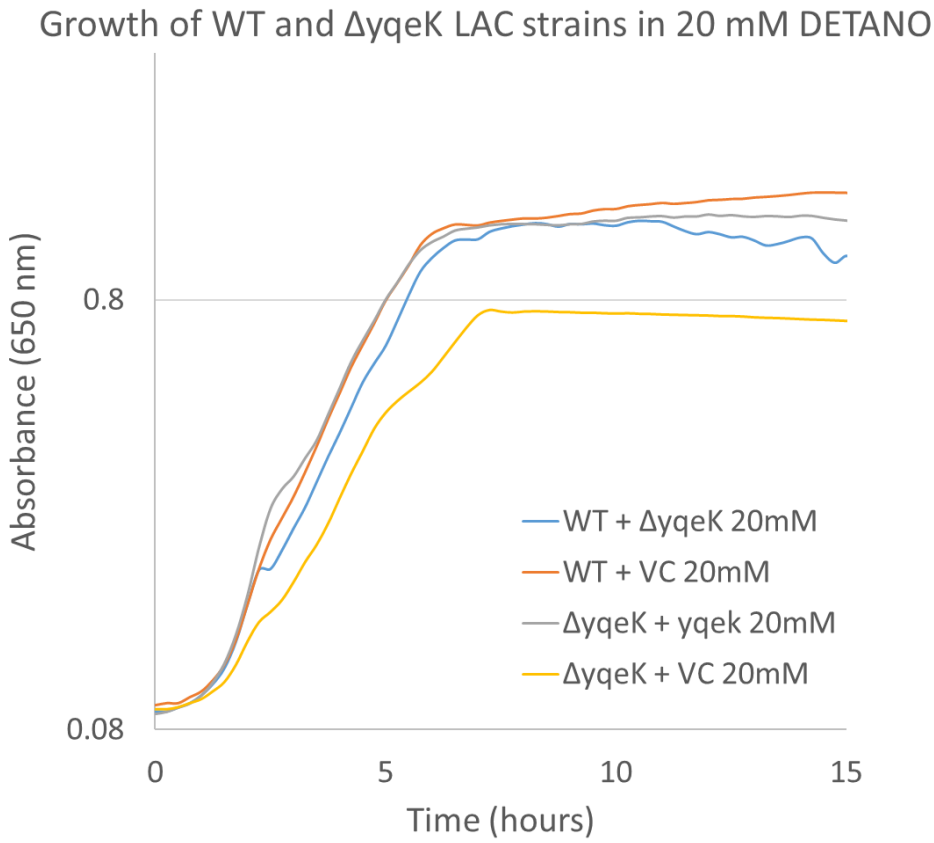

Figure 4. Representative growth curve showing *ΔyqeK* + VC, *ΔyqeK* + *yqeK*, WT + *yqeK*, and WT + VC LAC strains growing in 20 mM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO).

We found that the *ΔyqeK* mutant exhibited increased sensitivity to kanamycin. In Figure 1, the general trend of growth can be observed for strains of *S. aureus* growing in kanamycin. Between all strains, the *ΔyqeK* + VC strain generally grew the slowest in the presence of kanamycin, and failed to grow at all in concentrations of kanamycin that other strains were able to resist. The difference in MIC between the *ΔyqeK* + VC and both the WT + *yqeK* and WT + VC strains were significant ($p<0.01$, $p<0.05$, respectively, Fig. 3), with *ΔyqeK* + VC having a much lower MIC. There was no significant difference found between the *ΔyqeK* + VC and *ΔyqeK* + *yqeK* strains, nor was there any significant difference found between *ΔyqeK* + *yqeK*, WT + *yqeK*, and WT + VC. Strains of WT and *ΔyqeK* without plasmids were also tested, and *ΔyqeK* was found to have a significantly lower MIC compared to WT ($p<0.001$, Fig. 2).

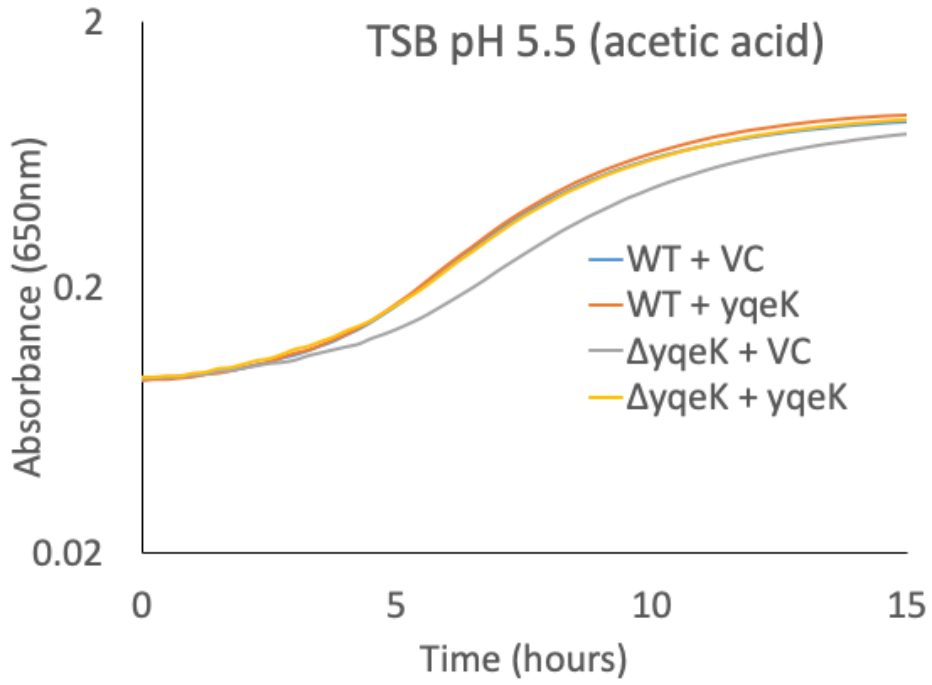

Figure 5. Representative growth curve showing LAC WT versus LAC *ΔyqeK* when grown at pH 5.5. The pH of the media was altered using acetic acid. This experiment was performed by a previous student in the lab.

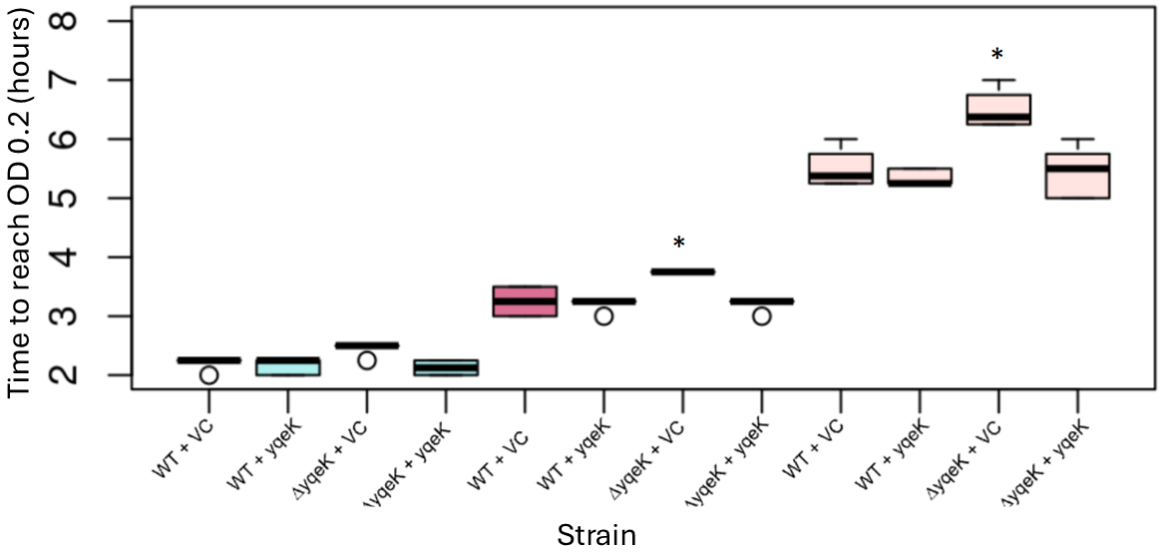

Figure 6. The strain containing no expression of *yqeK* had the longest lag time under acidic conditions. The *ΔyqeK* LAC strain complemented with a *yqeK* plasmid performed the same as WT LAC. From left to right, each grouping of colors represents lag time in pH 7.3, pH 6.0, and pH 5.5, respectively. The pH of the media was altered using acetic acid. Lag time was determined by the time to reach an OD650 of 0.2. This experiment was performed by a previous student in the lab.

To continue exploring the role of Ap_4A in *S. aureus*'s stress response, a previous student in the lab explored the effect of acid stress on cell growth between WT and *ΔyqeK* mutants. Acid stress, along with NO·, is another antimicrobial factor employed by macrophages in the immune response [16]. It was found that there was a significant increase in lag time, or the time taken to grow to an OD of 0.2, between *ΔyqeK* + VC and *ΔyqeK* + *yqeK*, WT + *yqeK*, and WT + VC at a pH of both 6 and 5.5 (Fig. 6).

Having established that *yqeK* deletion is detrimental to *S. aureus* fitness during multiple types of stress, we next sought to investigate a potential mechanism for the increased sensitivity to stress by performing transcriptomics. RNA-Seq was performed on WT and *ΔyqeK* strains under normal growth conditions (TSB only) and two stress conditions: organic acid (pH 5.5 with acetic acid) and NO· stress (10mM DETANO). This would unveil the transcriptional pathways directly and indirectly affected by the deletion of *yqeK* and a presumably increased level of Ap_4A.

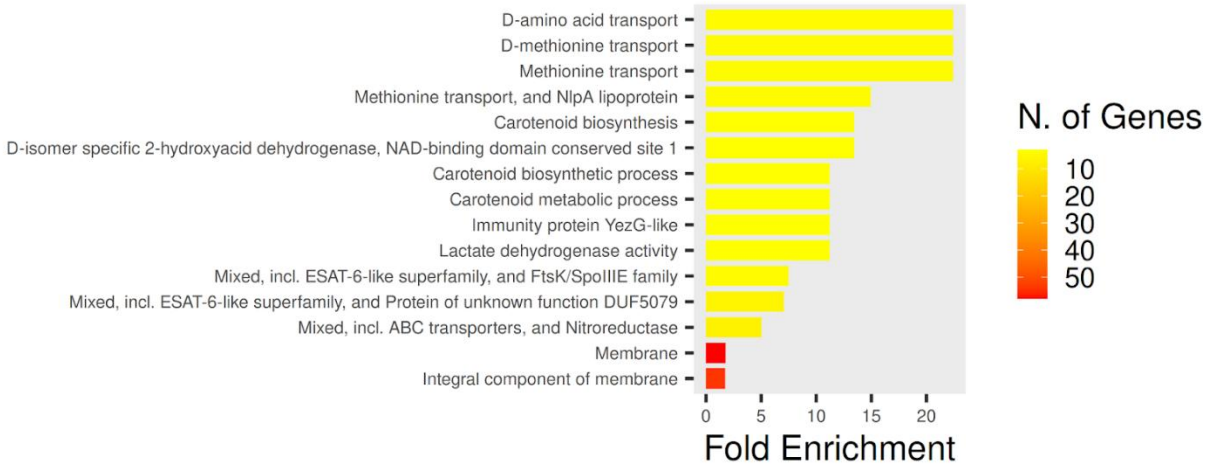

Figure 7. Gene pathways enriched in WT when grown in acid stress.

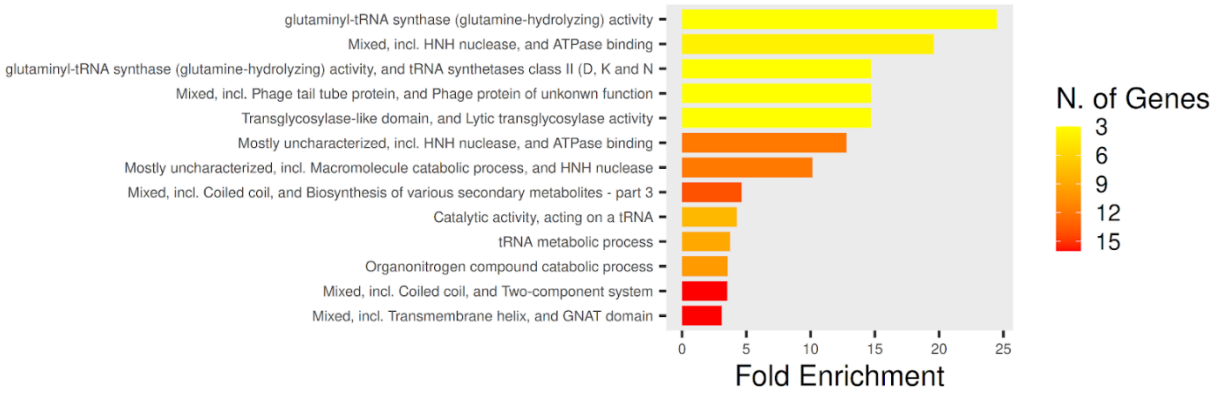

Figure 8. Gene pathways enriched in *ΔyqeK* when grown in acid stress.

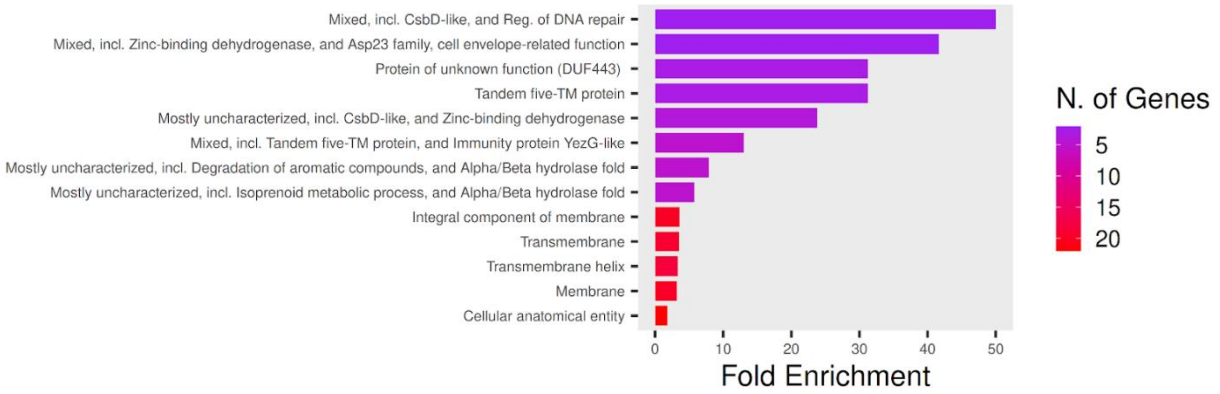

Figure 9. Gene pathways enriched in WT when grown in NO· stress.

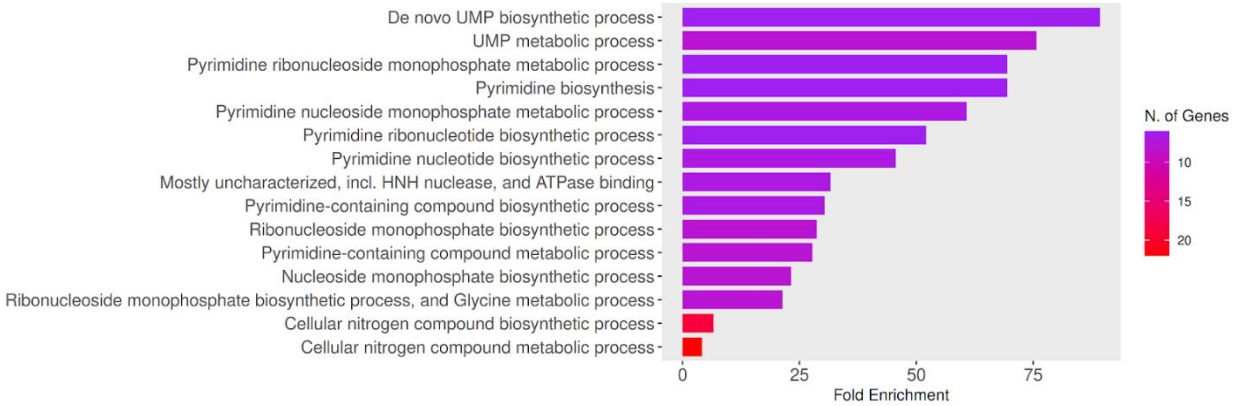

Figure 10. Gene pathways enriched in *ΔyqeK* when grown in NO· stress.

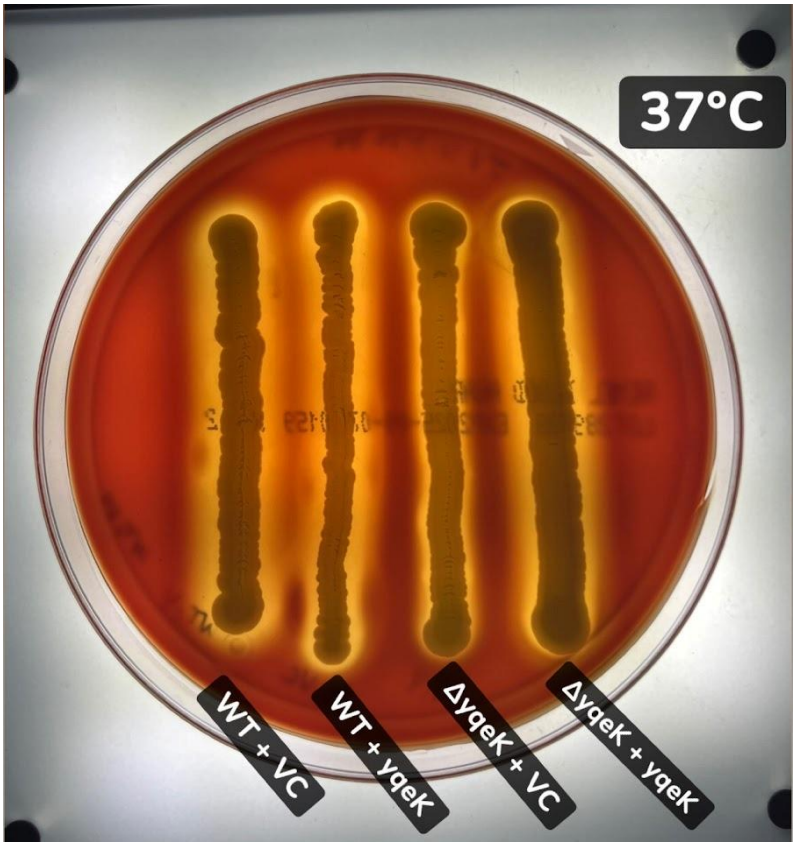

Figure 11. Image of the hemolysis zone formed by the different LAC strains after 48 hours of growth at 37°C. Strains were grown on sheep's blood agar plates. Yellow coloration indicates β-hemolysis (complete blood cell lysis).

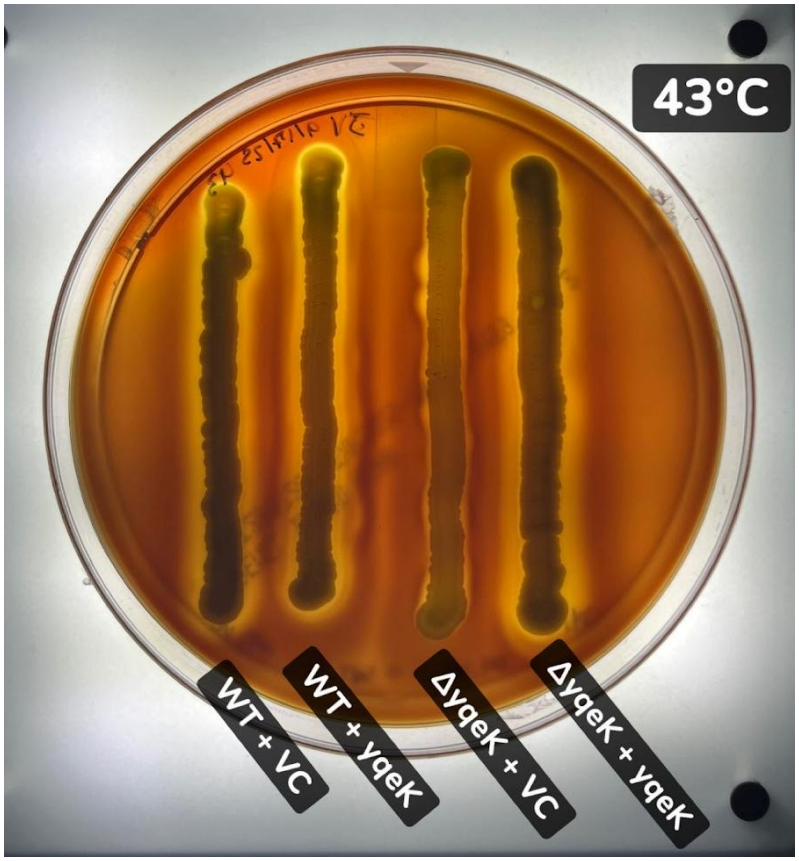

Figure 12. Image of the hemolysis zone formed by the different LAC strains after 48 hours of growth at 43°C. Strains were grown on sheep's blood agar plates. Yellow coloration indicates β-hemolysis (complete blood cell lysis).

During normal growth, there were 101 genes differentially expressed between WT and *ΔyqeK*. There were 171 genes differentially expressed between WT and *yqeK* during NO· stress, and 300 genes differentially expressed during acid stress. Altogether, this indicates that there is a substantial transcriptional response to *yqeK* deletion.

From the RNA-Seq data, it is seen that under acid stress, WT strains upregulate pathways involved in methionine transport, carotenoid biosynthesis, dehydrogenase activity, and immunity protein synthesis to the greatest extent (Fig. 7). Conversely, under the same acid stress condition, *ΔyqeK* strains see the most enrichment in pathways that regulate glutamine hydrolyzing activity, HNH nuclease activity, phage tail tube protein synthesis, and lytic transglycosylase activity (Fig. 8). Compared to WT, the *yqeK* mutant failed to induce SAUSA300_RS14620 (HdeD family acid resistance protein) during acid stress, which may contribute to its growth defect.

Under NO· stress, RNA-Seq data show that heavily enriched pathways in WT *S. aureus* included DNA repair, dehydrogenase activity, tandem five transmembrane protein activity, immunity protein synthesis, aromatic degradation, and isoprenoid synthesis (Fig. 9). For *ΔyqeK* strains under NO· stress, the bacteria focused most on upregulating Uridine monophosphate biosynthesis, pyrimidine metabolism, and HNH nuclease activity pathways (Fig. 10). The divergence in regulation priority seen between WT and the *ΔyqeK* mutant implicates Ap_4A as an active factor contributing to the stress response of *S. aureus*.

Under both stress conditions, the *yqeK* mutant exhibited major downregulation relative to WT of quorum sensing and pathogenesis genes, including the genes encoding alpha-toxin and delta-toxin, which form pores and lyse host cells. The reduction in toxin gene expression suggests that *ΔyqeK* may have reduced virulence towards host cells. To test whether the differences in transcription result in a functional reduction of pore-forming toxins, we examined hemolysis of the *ΔyqeK* strain on sheep's blood agar. Because the difference in toxin gene expression was exacerbated by stress in our RNA-Seq analysis, we performed this assay under both normal growth temperatures and under heat stress. The heat shock response of *S. aureus* is closely intertwined with its response to oxidative and metabolic stress seen in acidic conditions [9,19,20]. To inquire about the effects of deleting *yqeK* on *S. aureus*'s heat stress response, strains of WT and *ΔyqeK S. aureus* were grown on sheep's blood agar plates. One plate was grown at 37°C as a control, and another plate was grown at 43°C for 48 hours. The control plate (Fig. 11) shows visible β-hemolysis as a zone of yellow surrounding the streaks of each strain. The hemolysis zone around *ΔyqeK* + VC is visibly reduced compared to the other strains. The heat stress plate (Fig. 12) similarly shows zones of β-hemolysis (complete lysis) surrounding the streaks of each strain, however, minimal hemolysis occurs surrounding the *ΔyqeK* + VC streak.

Discussion

With the emergence of hypervirulent strains of CA-MRSA, it has become increasingly important to understand the mechanisms behind *S aureus*'s highly adaptable stress response [3,4]. The discovery of Ap_4A as a potential second messenger involved in the stress response of Gram-positive and Gram-negative bacteria, along with the identification of *yqeK* as a potential contributor to *S. aureus*'s NO· resistance, implicate Ap_4A metabolism as a novel target to manipulate for uncovering unknown aspects of *S. aureus*'s stress response [7,9,10,11]. In this study, we see a general trend of *S. aureus* exhibiting decreased survival and virulence when *yqeK* is deleted and Ap_4A levels are increased.

There was a general trend of the *ΔyqeK* mutant strain growing slower than the wild-type and complemented strains in each type of stress that we tested (Fig. 1, Fig. 4, Fig. 5). The *ΔyqeK* mutant has a significantly decreased kanamycin MIC compared to WT (Fig. 2), suggesting that increased levels of Ap_4A negatively impact the cell's antibiotic resistance. Although not all of the *ΔyqeK* deletion mutant phenotypes significantly differed from WT with the current low number of replicates, the phenotype trends are consistent, so we are currently performing additional replicates to better clarify these results.

The RNA-Seq data revealed transcriptional pathways directly or indirectly impacted by the deletion of *yqeK*. From the data, there is a clear divergence in the gene expression profiles of the WT and mutant bacteria. The pathways that were most upregulated in WT after acid stress were linked to protection against oxidative stress. Methionine transport has been shown to increase acid resistance in *Lactiplantibacillus plantarum* by helping to alleviate the buildup of toxic homocysteine and contributing to cell membrane stability [21]. Carotenoids give *S. aureus* its characteristic golden yellow coloration; however, they are also powerful antioxidants that can help the cell with oxidative stress, osmotic stress, and detoxification [22]. Osmotic stress aid is important during acid stress as the increase of protons in the environment can trigger osmotic dysregulation in the cell. Additionally, D-lactate dehydrogenase is a member of the D-isomer specific 2-hydroxyacid dehydrogenase family [23]. D-lactate dehydrogenase may contribute to acid resistance due to the enzyme's essential role in anaerobic respiration, given that acidic conditions often downregulate aerobic respiration [24,25]. The final pathway that was highly enriched in WT during acid stress was for an immunity protein similar to YezG. YezG, a protein in *B. subtilis*, gives immunity to a toxin, YeeF, secreted by the bacteria. The genes that encode the immunity protein and the toxin are always transcribed and translated together, to ensure the bacteria are not harmed by their own toxin [26]. This indicates that WT *S. aureus* prioritizes upregulation of acid resistance and toxin-producing genes in response to acid stress, which gives insight into how the pathogen may resist and attack responses from the immune system.

The *ΔyqeK* strain substantially differs from WT in its transcriptional response to acid stress, and fails to upregulate the majority of these pathways. Instead, it only enriches a few pathways that may be beneficial for acid resistance, all revolving around the conversion of glutamine to glutamate. Glutamine can be hydrolyzed and converted into glutamate, and in this process, ammonia is released [27,28]. Ammonia can act as a weak base and help balance the pH level to an extent [28]. One additional pathway that may be beneficial is the enrichment of transpeptidases. Transpeptidases can synthesize, degrade, and alter bacterial cell walls [29]. Increased transpeptidase activity may help provide stability for the bacteria by regulating cell wall damage.

The other pathways enriched in *ΔyqeK* are linked to prophage induction. A previous study found that acidic conditions from acetic acid initiated prophage induction in *Lactobacillus reuteri* through a mechanism that likely involved activation of the DNA damage response [30]. The HNH nuclease proteins upregulated in *ΔyqeK* are known to play a key role in terminase packaging reactions for the reproduction of bacteriophages [31]. Additionally, the enrichment of pathways relating to phage tail tube proteins, which are responsible for degradation of the bacterial cell wall, alludes to the creation of new bacteriophages for completion of the lytic cycle [32,33]. The existence of the prophage is mutually beneficial for both *S. aureus* and the virus, as the virus lives on through the bacteria and the bacteria will often gain antibiotic resistance or toxin genes [33]. This suggests that high levels of Ap_4A cause the cell to experience increasingly detrimental effects from stress, to the point of potentially initiating prophage induction and the eventual death of the bacterium from lysis. Therefore, while WT *S. aureus* is able to resist the detrimental cell effects from acid through multiple pathways and simultaneously produce toxins, the *ΔyqeK* mutant becomes stressed to the extent of possible prophage induction.

A similar trend is observed from the NO· stress RNA-Seq data. Under NO· stress, WT *S. aureus* enriches pathways involved in repairing DNA, detoxifying harmful reactive species, ensuring membrane stability, replenishing damaged enzymes, and producing toxins. The focus on DNA repair as the most enriched pathway offers protection for an important target of NO· and ensures that minimal damage or mutations will occur in the genome [5]. The upregulation of zinc-binding dehydrogenase enzymes may occur as a result of zinc metalloproteins being a common target of reactive nitrogen species (RNS) [34]. Enrichment of this pathway may offer resistance in the form of replacing damaged enzymes. Tandem five transmembrane proteins have many functions in bacteria, and can contribute to tethering, movement, and transportation [35]. Enrichment of this pathway could be a way for the bacteria to obtain essential nutrients; however, the benefits of enriching this pathway and the zinc-binding dehydrogenase pathway are less clear. Pathways involving the degradation of aromatic compounds and the synthesis of

isoprenoids were also enriched in response to NO· stress. One study suggests that certain enzymes in the pathway for aromatic degradation can be inhibited by RNS, causing the pathway to reprogram and shift to producing NADPH, which can detoxify reactive species [36]. It has also been shown that isoprenoids make up a diverse set of molecules in bacteria, including cholesterol, carotenoids, and ubiquinones [37]. Therefore, increased isoprenoid synthesis would likely lead to increased levels of these molecules, which act to stabilize the cell membrane, detoxify reactive species, and aid in electron transport [37]. In addition to these pathways, the pathway for the production of the immunity protein was also enriched, which was similarly seen in the response to acid stress. While it is less clear how some of these pathways benefit *S. aureus* under NO· stress, enriched pathways for WT are mostly made up of resistance mechanisms and toxin-producing mechanisms.

In the *ΔyqeK* response to NO· stress, it was observed that almost all of the highly enriched pathways had to do with the synthesis of uridine monophosphate and its derivatives. Uridine, synthesized from uridine monophosphate, has many cellular functions while also being a building block for other pyrimidines [38]. Uridine itself can detoxify reactive species, modulate enzymes to be more efficient antioxidants, and induce the production of NADPH and NADH. The derivatives of uridine also possess antioxidant properties [38]. The other enriched pathway seen in *ΔyqeK S. aureus*'s response to NO· stress is for HNH nuclease, suggesting that prophase induction likely also occurs under NO· stress in the mutant. This follows a similar pattern to that seen in acid stress, where, under NO· stress, the WT strain enriched pathways that allowed for both resistance and toxin production, while the *ΔyqeK* strain enriched pathways that offered protection, but also seemed to initiate prophase induction, which is lethal to the cell. Altogether, this leads to the implication that Ap_4A is detrimental to *S. aureus* survival and virulence under immune stress.

From the hemolysis assay, it was observed that the *ΔyqeK* + VC strain had the most reduced zone of hemolysis compared to the other strains on both the control plate and the heat stress plate (Fig. 7, Fig. 8). Additionally, the zone seemed to be further reduced from the *ΔyqeK* + VC streak on the control plate to the streak of the same strain on the heat stress plate. This observation suggests that increased levels of Ap_4A may suppress toxin production and secretion, and that heat stress exacerbates this effect.

This research shows how deleting the gene *yqeK* in *S. aureus* leads to a significant reduction in antibiotic resistance to kanamycin, decreased hemolytic toxin production, a loss of key enriched resistance pathways under acid and NO· stress, and a likely initiation of prophage induction under acid and NO· stress. There seemed to be a general trend that growth rate was slower and MIC decreased in *ΔyqeK* compared to other strains under NO· stress and kanamycin stress, respectively. However, additional replicates of the growth assays for kanamycin stress and NO· stress will need to be performed in order to draw

accurate conclusions. Ongoing research has involved using luminescence assays to quantify the levels of Ap_4A in all strains used to determine significant changes in the level of Ap_4A between strains and various stress conditions. Future research will involve using qRT-PCR to confirm RNA-Seq results, investigating how Ap_4A regulates pathways through protein binding assays, and using immune cells to determine how Ap_4A levels might impact virulence during simulated infections.

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