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A luminescence-based approach to study dinucleotide alarmone regulation in *Staphylococcus aureus*

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Abstract

Diadenosine tetraphosphate (Ap_4A) is an evolutionarily conserved alarmone, or small intracellular signaling molecule that rapidly accumulates in response to stress, reprograming cellular physiology to promote survival over growth. Ap_4A is implicated in the stress response of bacteria, yet its role remains largely uncharacterized in *Staphylococcus aureus*. In *S. aureus*, YqeK encodes a putative Ap_4A hydrolase, implicated in regulating alarmone metabolism and stress responses by controlling intracellular Ap_4A levels. In this study, a luminescence-based assay to quantify Ap_4A accumulation in wild-type, *ΔyqeK*, and a genetically complemented strain of *S. aureus* was developed. Cultures were normalized, lysed, and processed to remove interfering nucleotides, followed by selective enzymatic treatment to distinguish Ap_4A from other adenylated species. Resulting nucleotide pools were analyzed using an ATP-dependent luciferase readout, allowing indirect but sensitive detection of Ap_4A levels. Using this approach, *ΔyqeK* demonstrated an increased level of more than 1000-fold Ap_4A concentration relative to wild-type and complemented strains, supporting a central role for the *yqeK* gene in Ap_4A regulation in *S.*

aureus. Notably, accumulation of Ap_4A was elevated in the *ΔyqeK* strain, indicating that Ap_4A levels are stress-responsive and exacerbated in the absence of functional YqeK. This assay provides a reliable method for probing dinucleotide alarmone metabolism and the evaluation of genetic and chemical perturbations affecting Ap_4A metabolism.

We define a reliable approach to measure Ap_4A in *Staphylococcus aureus* and identify *yqeK* as a key regulator of its levels. Our findings link Ap_4A signaling to adaptation under infection-relevant stress, highlighting YqeK and this pathway as potential vulnerabilities in drug-resistant strains such as MRSA. As an extension of this research, development of an endocardial vegetation model is underway to examine Ap_4A regulation in a disease-relevant context.

Introduction

Dinucleoside polyphosphates, in particular diadenosine tetraphosphate (Ap_4A), are highly conserved, stress-associated nucleotides that accumulate in cells across diverse organisms in response to physiological stress. In bacteria, Ap_4A has been recognized as an alarmone-like molecule that acts as an intracellular signal under stress conditions, impacting transcription, stress tolerance, and survival under adverse conditions [1]. For this reason, cells must closely regulate Ap_4A levels, as dysregulated accumulation has been implicated in impaired growth, altered stress response, and reduced fitness [2].

Ap_4A is primarily generated as a side reaction of aminoacyl-tRNA synthetases, as well as by other nucleotide-metabolizing enzymes, and its production increases under conditions of cellular stress. In many bacteria, Ap_4A homeostasis is maintained by specific hydrolases that degrade Ap_4A to two ADP [3]. In Firmicutes, these enzymes belong to the YqeK family, which remainsuL relatively less characterized than the ApaH family found in Gram-negative bacteria. In Gram-positive bacteria, such as *Bacillus subtilis* and *Streptococcus mutans*, disruption of Ap_4A hydrolases prevents Ap_4A degradation and leads to intracellular accumulation [4]. Elevated Ap_4A may interfere with normal stress-response pathways by altering target protein activity, altering transcription regulation, and impairing adaptation to stress.

In *Staphylococcus aureus*, a major human pathogen and the leading cause of infective endocarditis [5], the role of Ap_4A metabolism in stress adaptation and virulence remains poorly defined. Infective endocarditis is a life-threatening infection of the inner lining of the heart in which bacteria colonize the heart valves and form structured biofilm communities. These biofilms demonstrate enhanced defenses against host immune responses and antimicrobial therapies, contributing to the high morbidity and mortality of the disease [6]. Infective endocarditis is characterized by exposure to fluctuations in oxygen, reactive

nitrogen species, and high shear force within cardiac vegetations [7;8], stressors that demand coordinated bacterial adaptation.

We hypothesize that Ap_4A and its regulation by YqeK are critical factors in the *S. aureus* response to these specific stressors encountered during endocarditis.

To quantify stress-induced Ap_4A accumulation in *S. aureus*, a luciferase-based nucleotide detection assay was optimized and employed, capable of measuring intracellular Ap_4A levels under defined stress conditions. Using this assay, it was demonstrated that deletion of *yqeK* results in significantly elevated Ap_4A accumulation under normal growth as well as acid and nitric oxide stress, two conditions encountered by *S. aureus* during host infection and immune challenge. These findings support the hypothesis that YqeK contributes to bacterial stress resilience by preventing toxic accumulation of Ap_4A, and suggest that perturbation of this pathway may create metabolic vulnerability.

Materials and Methods

Bacterial Culture Preparation for Acid Stress

Three strains of *Staphylococcus aureus* were used in this study: wild type (WT), *ΔyqeK*, and *ΔyqeK* complemented with pRMC2-*yqeK*. Cultures of each strain were grown overnight under two conditions: half of the samples were cultured in tryptic soy broth (TSB), while the remaining half were cultured in TSB acidified with acetic acid to pH 5.5. The complemented strain culture received 5uL of chloramphenicol to maintain the plasmid. Following overnight incubation, optical density at 650nm (OD650) was measured for all samples. Because acid-grown cultures exhibit growth inhibition, normalization densities differed between conditions; TSB grown samples were normalized to OD 5.0, while acid grown samples were normalized to OD 1.0. Samples that were normalized to OD 1.0 were then pelleted by centrifugation and serially resuspended five times: the first pellet was resuspended in buffer, and this suspension was used to sequentially resuspend each subsequent pellet until all material from each respective sample was combined. This ensured a final density of ~OD 5.0, so that the same number of bacteria were used across conditions in the quantification assay.

Ap_4A Quantification

For stationary phase Ap_4A measurements, normalized cultures were processed as follows: one milliliter of each normalized culture was centrifuged at 21,300 x g for 1 minute, and the supernatant was discarded. The pellet was washed once by resuspension in 1 mL

phosphate buffered saline (PBS, pH 8), centrifuged, and the supernatant removed. The pellet was then resuspended in 1 mL Tris buffer (10mM, pH 8). Each sample then received 5 uL of 12.5 mg/mL concentration lysostaphin, was vortexed, then incubated for 15 minutes at 37C to facilitate enzymatic digestion of the cell wall.

Cells were mechanically lysed by bead-beating at setting 5 for three cycles of 30 seconds each, with 2 minute incubations on ice between cycles. The lysate was centrifuged at 17,700 x g for 5 minutes to pellet beads and cell debris. After, 600 uL of the clarified supernatant was transferred to a clean microtube and boiled at 95C for 10 minutes to inactivate enzymes. Samples were centrifuged again at 21,300 x g for 2 minutes, and the supernatant was transferred to a clean microtube.

To deplete ATP from the lysates, Antarctic phosphatase buffer (66 uL) and Antarctic phosphatase enzyme (5 uL) were added to each lysate sample. Samples were incubated overnight at 37C with shaking at 150 rpm.

Following incubation, the phosphatase was inactivated by heating to 80C for 5 minutes. Each inactivated lysate was divided into two equal fractions. One fraction was treated with 5 uL phosphodiesterase (0.01 mg/mL) for 10 minutes at room temperature to convert the Ap_4A in the sample to ATP and AMP. The other fraction served as untreated control. The resulting ATP was then quantified using the BacTiter-Glo Microbial Cell Viability Assay (Promega) as an indirect measure of Ap_4A levels. Following this, 50 uL of each sample was added to 50 uL BacTiter-Glo reagent in a white-walled 96 well plate in triplicate, with adjacent wells left empty to minimize signal crosstalk. Luminescence was measured immediately following reagent addition (BioTek SYNERGY HTX).

Nitric Oxide Treatment and Ap_4A Quantification During Exponential Growth

For Ap_4A measurements of exponential phase cultures exposed to NO-, two overnight cultures of each strain were grown in TSB, were diluted 1:100 into fresh TSB and incubated at 37C with shaking (250 rpm). Growth was monitored by measuring OD650 every 30 minutes, beginning at 30 minutes post dilution. When cultures reached OD of 0.4, the nitric oxide donor diethylamine NONOate (DETANO) was added to one of each strain sample at a final concentration of 10 mM (100 uL per 5 mL culture), and incubation continued for an additional hour. Following NO treatment, five 1 mL samples of each culture were pelleted at a 21,300 x g for 1 minute with resuspension and combination into one sample, where subsequent lysis, phosphatase treatment, phosphodiesterase treatment, and BacTiter-Glo luminescence detection were performed as described above for the stationary phase Ap_4A assay.

To account for the difference in biomass of the samples, these data were normalized by multiplying the final OD of each sample by five, then dividing the RLU of each respective sample by this number.

Results

Accumulation of Ap_4A Between Strains Under Normal Growth Conditions

We first investigated whether the *ΔyqeK* mutant accumulates increased Ap_4A relative to WT *S. aureus* under standard growth conditions. To do this, we optimized a luminescence-based assay in which cells were lysed, endogenous ATP was depleted, Ap_4A was converted to ATP, and ATP levels were then measured using a luciferase-based readout. Under standard TSB growth conditions at stationary phase, *ΔyqeK* exhibited substantially higher luminescence than both WT and complemented strains, with the complemented strain restoring signal to near WT levels. The strain-dependent differences were highly statistically significant (ANOVA, p=5.13*10^-12; Figure 1).

Accumulation of Ap_4A Under Acid Stress

Under acid stress, *ΔyqeK* luminescence increased approximately 1.87-fold relative to the TSB control, rising from 63,750 RLU to 115,416 RLU (paired t-test, $p=9.47*10^{-5}$; Figure 2). In contrast, WT and complemented strains showed no significant changes in luminescence under acidic conditions.

Accumulation of Ap_4A Under Nitrosative Stress

Under NO stress, *ΔyqeK* luminescence remained notably elevated compared to WT and complemented strains (~45,000-47,000 RLU), and again, strain effects were highly significant (ANOVA, $p=4.06*10^{-9}$; Figure 3). However, NO treatment did not significantly alter luminescence within any strain.

Genetic Complementation Restores Wild-type Ap_4A Levels

Across both stress conditions, introduction of the complementing plasmid pRMC2-*yqeK* in to the *ΔyqeK* background restored luminescence to levels comparable to WT.

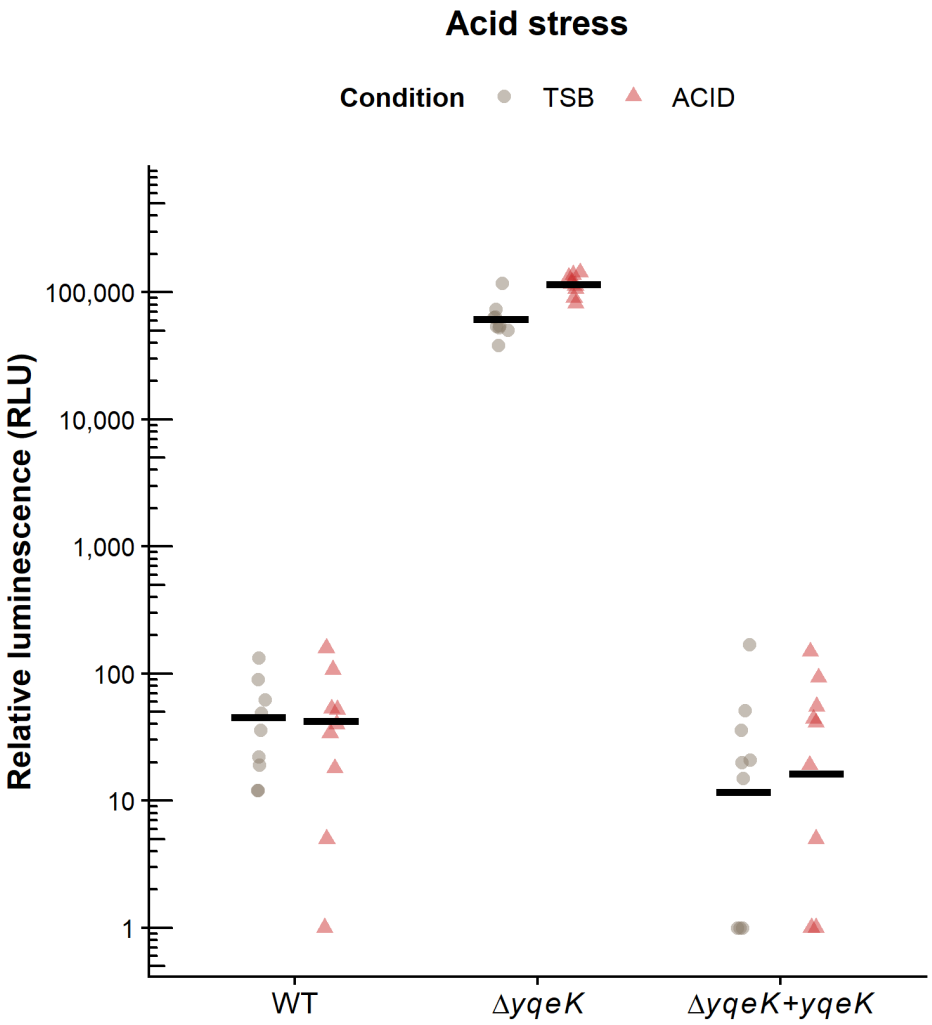

Figure 1. Ap_4A accumulation in *S. aureus* under acid stress. Biological replicate means (n=3) of maximum luminescence (RLU) are shown for each strain under TSB and acid stress conditions. *ΔyqeK* showed significantly higher luminescence than both WT (p=0.000058) and the complement (p = 0.000093), while WT and the complement were not significantly different from each other (p=0.973). One-way ANOVA on log-transformed biological replicate means: $F(2,5) = 139.8$, $p = 4.09 \times 10^{-5}$.

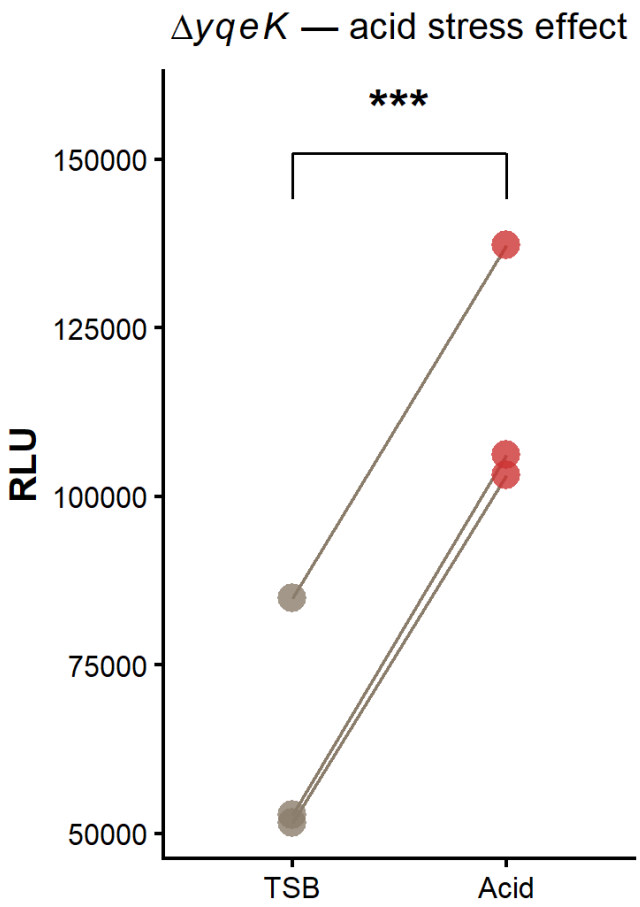

Figure 2. Acid stress *within* *ΔyqeK*. Biological replicate means (n=3) of maximum luminescence (RLU) for *ΔyqeK* under TSB (mean = 63,084 ± 18,864 SD) and acid stress (mean = 115,462 ± 18,868 SD) conditions. Each data point represents the mean of three technical replicates from an independent biological replicate. Lines connect paired biological replicates across conditions. Paired t-test: t = 102.77, p = 9.47×10^{-5}.

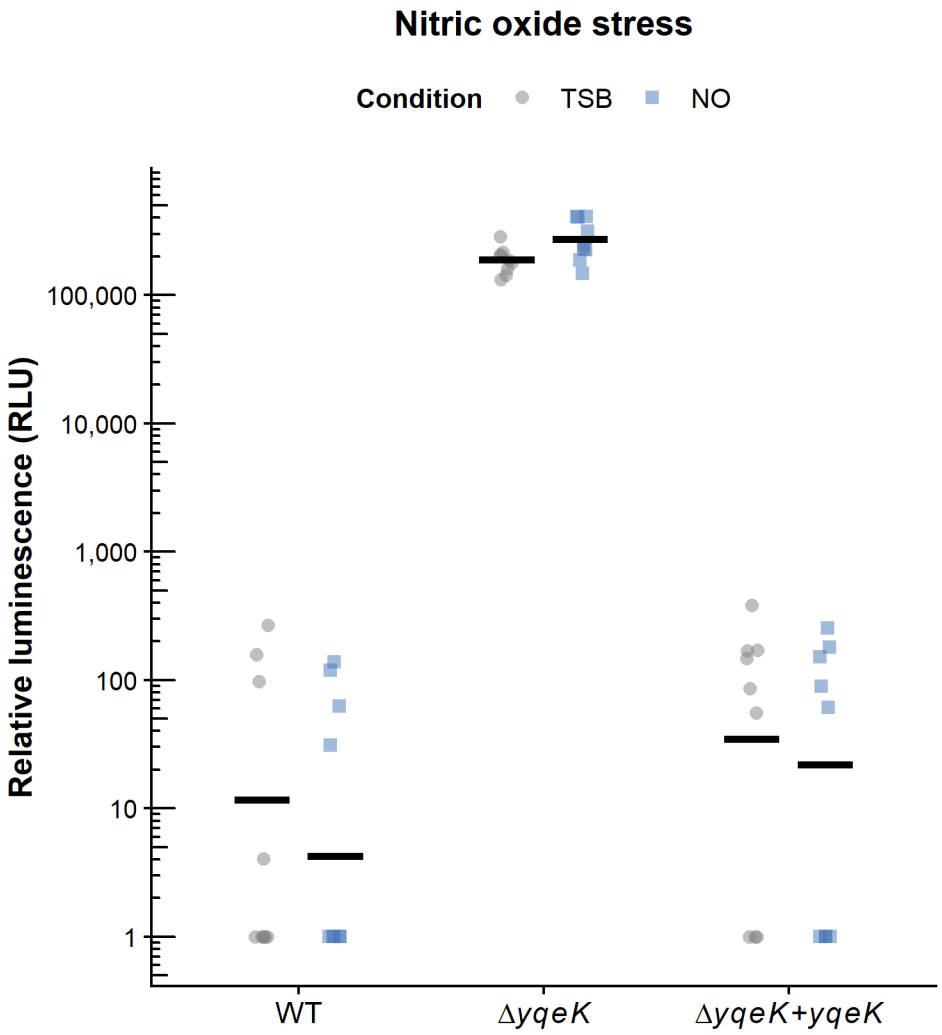

Figure 3. Ap_4A accumulation in *S. aureus* under nitric oxide stress. Intracellular Ap_4A levels (RLU) were measured in WT, Δ*yqeK*, and complemented strains grown in TSB (n=3 biological replicates; crossbars indicate mean). Δ*yqeK* accumulated significantly more Ap_4A than both WT and complemented strains (one-way ANOVA, $p=1.94*10^{-4}$). NO stress did not significantly alter Ap_4A levels within any individual strain (paired t-test, p=0.288 for *ΔyqeK*).

Discussion

The results of this study support the hypothesis that Ap_4A and YqeK play critical roles in the stress response of *S. aureus*. Using a luciferase-based nucleotide detection assay, we demonstrated that deletion of *yqeK* results in substantially elevated Ap_4A accumulation under standard growth conditions, and that this accumulation is further exacerbated by acid and nitrosative stress. Genetic complementation restored Ap_4A levels to near WT,

confirming that the phenotype is specifically attributable to loss of YqeK function. Together, these findings establish YqeK as a central regulator of Ap_4A homeostasis in *S. aureus* and suggest that dysregulation of this pathway may impair bacterial stress adaptation during infection.

The elevation of Ap_4A in the *ΔyqeK* strain under standard growth conditions is consistent with the established role of YqeK-family hydrolases in Gram-positive bacteria. In *B. subtilis* and *S. mutans*, disruption of homologous Ap_4A hydrolases similarly result in intracellular Ap_4A accumulation, impaired growth, and altered stress responses [4]. The conservation of this phenotype across Gram-positive species supports the idea that tight regulation of Ap_4A levels is broadly important for bacterial fitness, and that YqeK serves a non-redundant enzymatic function in *S. aureus* that cannot be compensated by other nucleotide-metabolizing enzymes.

The increase in Ap_4A accumulation observed in the *ΔyqeK* strain under acid stress is particularly notable. Acid stress is a physiologically relevant challenge encountered by *S. aureus* during host infection, including within phagolysosomes and at sites of tissue inflammation [10]. The fact that WT and complemented strains showed no significant change in Ap_4A levels under the same acidic conditions suggests that functional YqeK is sufficient to buffer stress-induced Ap_4A production under these conditions. The failure of the *ΔyqeK* strain to do so implies that acid stress drives increased Ap_4A synthesis, likely through elevated aminoacyl-tRNA synthetase activity or other stress coupled nucleotide metabolism, and that YqeK-mediated hydrolysis is the primary mechanism by which this accumulation is resolved [1;2].

In contrast, NO- stress did not significantly alter Ap_4A luminescence within any individual strain, despite the *ΔyqeK* strain maintaining substantially elevated Ap_4A relative to WT and complemented controls. While this could be due to the shortened exposure period of 1 hour, compared to the 18+ hours of the acid exposure, this could suggest that while NO exposure does not drive additional Ap_4A synthesis above the already elevated baseline in the *ΔyqeK* strain, the inability to degrade Ap_4A renders the mutant constitutively stressed with respect to this signaling molecule. This interpretation is consistent with Ap_4A functioning as a standing indicator of metabolic status rather than an acutely NO responsive signal in *S. aureus*, though further work is needed to dissect the precise relationship between nitrosative stress and Ap_4A metabolism. The relevance of nitrosative stress in this context is well supported: *S. aureus* encounters reactive nitrogen species produced by host iNOS during infection, and the nitrosative stress response is known to be critical for *S. aureus* virulence and survival within the host [11].

The findings presented here have meaningful implications for understanding *S. aureus* pathogenesis in the context of infective endocarditis. IE is a biofilm-mediated disease [9] characterized by exposure to hostile and dynamic microenvironments including

fluctuations in oxygen availability, reactive nitrogen species, and high shear stress within cardiac vegetations [7;8]. The ability of *S. aureus* to persist within this environment requires coordinated stress adaptation and our data suggests that Ap_4A signaling through YqeK may be one component of this adaptive capacity. Elevated Ap_4A in the absence of YqeK could plausibly interfere with normal stress response pathways, reduce metabolic flexibility, and impair the transition to biofilm -associated growth modes that are characteristic of established IE vegetations [1;2].

A limitation of the current study is that all experiments were conducted in planktonic culture, which does not fully recapitulate the biofilm-associated growth state relevant to IE. Planktonic bacteria are generally more metabolically active and antibiotic-susceptible than biofilm-resident cells, which exhibit reduced metabolic activity and increased tolerance to antibiotics and host defenses [12]. To address this, an endocardial vegetation model is currently under development, which will enable assessment of Ap_4A dynamics and the impact of *yqeK* deletion under conditions that more closely simulate the cardiac infection environment. This is an important next step, as the relationship between alarmone signaling and biofilm tolerance may differ between planktonic and vegetation-associated bacteria.

It is also worth noting that the luciferase-Ap_4A assay developed here relied on an indirect readout; phosphodiesterase treatment liberates ATP (and AMP) from Ap_4A, which is subsequently detected via ATP dependent luminescence after phosphorylation. While this approach is sensitive and operationally tractable, it is susceptible to interference from other adenylated nucleotide species present in the lysate. The phosphatase pretreatment step was designed to address this by dephosphorylating free nucleotides prior to phosphodiesterase treatment, and the consistent restoration of signal to WT levels in the complemented strain provides strong internal validation of the assay's specificity. Importantly, the magnitude of signal separation between the mutant and WT strains, spanning several orders of magnitude, represents a level of dynamic range that is exceptional for this type of biochemical assay and speaks to the robust nature of this approach. This sensitivity, combined with the operational simplicity of a luminescence-based readout, suggests that this assay has strong potential for broader applications in future studies of Ap_4A signaling across a range of bacterial and potentially eukaryotic cell types. Nevertheless, future work using orthogonal methods such as mass spectrometry-based nucleotide quantification would strengthen confidence in the absolute Ap_4A measurements reported here [4].

Collectively, this study establishes YqeK as a functionally important regulator of Ap_4A homeostasis in *S. aureus* and demonstrates that loss of this hydrolase creates measurable metabolic dysregulation under stress conditions encountered during host infection. The stress-responsive accumulation of Ap_4A in the *yqeK* deletion mutant, combined with the

disease relevance of the IE context, positions YqeK and Ap_4A signaling as worthy targets for future investigation into *S. aureus* stress resilience and persistence.

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