EVALUATING THE CONTRIBUTION OF A METAL INDEPENDENT ALDOLASE TO RESISTING NUTRITIONAL IMMUNITY

BY

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THESIS

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ABSTRACT

The sequestration of essential nutrients from pathogens is a key component in the vertebrate immune response. One aspect of this defense, known as nutritional immunity, is the sequestration of the essential metals manganese and zinc. An important component of this defense is the metal binding host protein calprotectin, which inactivates metal-dependent bacterial enzymes. The ability of *Staphylococcus aureus* to cause infection suggests that it has mechanisms that enable it to minimize the impact of nutrient metal limitation. The consumption of glucose is critical to the ability of *S. aureus* to overcome nitrosative stress. Yet at the same time several of the primary glycolytic enzymes utilized by *S. aureus* are predicted to be dependent on a metal for function, including a putatively zinc-dependent aldolase. *S. aureus* possesses an additional metal-independent aldolase, which is regulated by the two-component regulator ArlRS. This two-component system has previously been linked to the ability of *S. aureus* to resist calprotectin-imposed metal limitation. In light of these observations, we evaluated if the metal-independent aldolase contributes to the ability of *S. aureus* grow in metal restricted environments. These data show that when the metal-independent aldolase is impaired, *S. aureus* shows greater sensitivity to metal limitation. This is alleviated upon addition of manganese, but not zinc. Increasing manganese limitation by ablating manganese transport exacerbates the phenotype of the aldolase mutant. In addition, the availability of an alternative carbon source can reduce calprotectin sensitivity, provided its entry point in glycolysis is below the metal-dependent blockage. This suggests that a blockage at the aldolase step in glycolysis is important for *S. aureus* to resolve, either through the use of a metal-

independent isozyme or other carbon sources, in order to resist calprotectin-induced metal limitation.

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Thank you to my family, friends, advisor, and thesis committee for their unending patience and tolerance throughout this whole process. A special thank you to Ash, whose image provided much-needed comfort and sanity.

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CHAPTER 1: INTRODUCTION

Staphylococcus aureus is a pervasive and virulent global human pathogen. It transiently and asymptomatically colonizes up to 50% of the healthy adult population, with persistent colonization in 10-20% of individuals (19). This is typically localized to the nares and skin; however, *S. aureus* is capable of infecting nearly every tissue in the host if the epithelial barrier is broken (19). Persistently colonized individuals are therefore at greater risk for infection and disease (28). Strains of methicillin-resistant *S. aureus* (MRSA) can arise from both hospital and community sources (6). According to a CDC report, MRSA infections resulted in an estimated 11,000 deaths out of the 80,000 annual infection cases estimated in the United States (5). Both methicillin-resistant and -sensitive strains of *S. aureus* can result in serious diseases including endocarditis, bacteremia, septic shock, and toxic shock syndrome (19). *S. aureus* is able to evade various aspects of the human immune response, including the development of effective immunity to reinfection and the innate immune system's nitric oxide burst (11, 25). Given *S. aureus*'s ubiquitous nature and the seriousness of its epidemiology, it is important to examine its physiology in the context of infection in order to identify possible novel targets for therapeutics.

During infection, the vertebrate immune system will withhold essential nutrients in processes broadly termed "nutritional immunity". A crucial aspect of nutritional immunity is the sequestration of metals such as iron, manganese, and zinc (14). Metals are essential for all life. It is estimated that over 30% of known enzymes require a metal cofactor for proper function (1). In an infection where the pathogen must obtain all

nutrients from the host environment, restricting metal availability is a well-established mechanism of host defense (14). In a staphylococcal abscess, the transition metals manganese and zinc are depleted from the area of infection despite being replete in the surrounding tissue (7). Manganese depletion in the abscess is due in part to the chelating activity of the host protein calprotectin, a heterodimer which binds both manganese and zinc with nanomolar and subnanomolar affinity at two distinct sites (2, 8, 20, 29). A calprotectin-deficient mouse shows an inability to sequester manganese; however, the depletion of zinc is not significantly affected (7). Calprotectin is produced by neutrophils and is found in concentrations in excess of 1 mg/mL in areas of infection (27, 8). The combination of high affinity binding and high concentration of protein contribute to the virtual depletion of manganese and zinc from the area of infection.

There are multiple metal-dependent steps in glycolysis. Therefore, when metals are limited, glycolysis suffers an interruption. This can be seen by the increased sensitivity to calprotectin shown when *S. aureus* is grown on glucose compared to amino acids as the sole carbon source (21). In addition, growth on pyruvate can rescue the metal starvation phenotype, indicating that calprotectin blocks glycolysis but does not significantly impact gluconeogenesis. Utilization of alternative carbon sources, such as amino acids, though typically repressed in the presence of glucose, assists in the resistance of metal starvation (13). Amino acids in particular are a physiologically relevant carbon source in an abscess. The pathways of amino acid catabolism have been well characterized in model organisms ranging from *Escherichia coli* to humans (12, 26). Wildtype *S. aureus* shows decreased sensitivity to calprotectin when grown on amino acids; however. a *ΔarlRS* mutant shows a severe defect in the ability to utilize amino

acids (21). Both these observations hold true even in the presence of glucose, which would normally repress utilization of alternative carbon sources through the regulator CcpA (13).

Another method of bypassing interruptions is the usage of metal-independent variants of metal-dependent enzymes. There are a few known isozymes of metal-dependent enzymes in glycolysis. In particular, there are metal dependent and independent variants of the fructose 1,6-bisphosphate aldolase as well as the phosphoglycerate mutase. The fructose bisphosphate aldolases are FbaA (metal dependent) and FdaB (metal-independent). The metal-independent phosphoglycerate mutase GpmA has been shown to minimize calprotectin sensitivity (22).

Despite the fact that the host limits the availability of essential nutrients, pathogens like *S. aureus* are still capable of causing infection. One potential route of circumvention is the usage of dedicated metal transporters. *S. aureus* expresses dedicated Mn and Zn transporters able to compete with the host for metals during nutritional stress (4, 17, 10). Despite the presence of these systems, *S. aureus* still loses the struggle for metals (10, 22, 23) Therefore, the bacterium must have other mechanisms of coping with this nutrient limitation. In addition to transporters, *S. aureus* utilizes the global two-component regulator ArlRS to sense and respond to manganese limitation (21). ArlRS indirectly detects decreased intracellular manganese concentrations, activating aspects of the manganese starvation response such as amino acid consumption (21). A *ΔarlRS* mutant strain of *S. aureus* displays significantly greater sensitivity to calprotectin-induced metal limitation; this sensitivity is primarily in response to the sequestration of manganese (21).

The work within sought to investigate a potential role for the metal-independent fructose bisphosphate aldolase FdaB in resisting nutritional immunity. As the presumably main aldolase, the metal-dependent FbaA, is putatively zinc dependent, it was thought that the metal-independent isozyme may be expressed in zinc-limiting conditions (3). FdaB was indeed found to be important in resisting nutritional immunity. However, the results indicate that it is part of the staphylococcal response to manganese limitation. Further investigation into the role of zinc and the precise metal dependency of FbaA will need to be undertaken to clarify why the metal-independent isozyme for a putatively zinc dependent enzyme is involved in the response to manganese limitation.

CHAPTER 2: METHODOLOGY

Ethics statement

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (IACUC license number 15059) and performed according to NIH guidelines, the Animal Welfare Act, and U.S. federal law.

Strains and growth conditions

All strains used were variants of *S. aureus* Newman or USA300 JE2. For overnight cultures, bacteria were grown in 5 mL of either tryptic soy broth (TSB) or Chelex-treated RPMI plus 1% casamino acids (NRPMI) supplemented with 1 mM $MgCl_2$, 100 μM $CaCl_2$, and 1 μM $FeCl_2$ in 15 mL conical tubes at 37°C on a roller drum (17, 21). If the strains contained a plasmid, 10 μg/mL of chloramphenicol was added for plasmid maintenance.

Calprotectin growth assays

CP assays were performed as described previously (16, 8, 21). For assays using 3xNRPMI, overnight cultures grown in TSB were diluted 1:100 in 96-well round-bottom plates containing 100 μL of growth medium (38% 3xNRPMI and 62% calprotectin buffer (20 mM Tris pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$)) in presence of varying concentrations of CP. The growth medium was supplemented with 1 μM $MnCl_2$, 1 μM $FeCl_2$, and 1 μM $ZnSO_4$. For complementation experiments, 10 μg/mL of chloramphenicol was added. For assays using defined growth media (referred to as CDM), overnight cultures were diluted 1:50 in TSB and grown for an additional hour at 37°C on a roller drum before being

diluted 1:100 in 96-well round bottom plates. The wells contained 100 µL of growth medium (62% calprotectin buffer defined above and 38% CDM). The defined medium is based on that developed by Richardson et al. and modified by Radin et al. (24, 21). The defined medium (2.6X) consisted of 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 2.0 g/L KH_2PO_4, 7.0 g/L Na_2HPO_4, 0.228 g/L biotin, 0.228 mg/L nicotinic acid, 0.228 mg/L pyridoxine-HCl, 0.228 mg/L thiamine-HCl, 0.114 mg/L riboflavin, 0.684 mg/L calcium pantothenate, 0.104 g/L phenylalanine. 0.078 g/L lysine, 0.182 g/L methionine, 0.078 g/L histidine, 0.026 g/L tryptophan, 0.234 g/L leucine, 0.234 g/L aspartic acid, 0.182 g/L arginine, 0.078 g/L serine, 0.15 g/L alanine, 0.078 g/L threonine, 0.130 g/L glycine, 0.208 g/L valine and 0.026 g/L proline. The defined medium was then supplemented with 6 mM $MgSO_4$, 1 µM $FeCl_2$, 1 µM $MnCl_2$ and 1 µM $ZnSO_4$. Casamino acids (6.5%), glucose (1.3%) or glycerol (144 mM) were provided as carbon sources where indicated. For all assays, the bacteria were incubated with orbital shaking (180 RPM) at 37°C and growth was measured by assessing optical density (OD_{600}) every 2 hours. Prior to measuring optical density, the 96-well plates were vortexed.

Metal starvation growth assays

For growth assays using Chelex-treated media rather than calprotectin to impose metal limitation, overnight cultures grown in NRPMI were diluted 1:10 in fresh media before being further diluted 1:100 in 96-well round bottom plates containing NRPMI supplemented with 1 mM $MgCl_2$, 100 µM $CaCl_2$, and 1 µM $FeCl_2$. 1 µM $MnCl_2$ and 1 µM $ZnSO_4$ were added as specified. For complementation experiments, 10 µg/mL of chloramphenicol was added. Bacteria were incubated with orbital shaking (180 RPM) at

47°C and growth was measured by assessing optical density (OD600) every 2 hours. Prior to measuring optical density, the 96-well plates were vortexed.

Expression analysis

The expression of *fbaA* and *fdaB* was assessed using a YFP-promoter fusion (excitation and emission wavelengths of 505 nm and 535 nm, respectively) as previously described (9, 10). Bacteria were grown as described above for metal starvation growth assays with the addition of chloramphenicol to maintain the plasmid.

Animal experiments

All animal infections were performed as previously described (21, 9, 10). Nine-week-old female C57BL/6 or C57BL/6 S100A9-/- ($CP^{-/-}$) mice were retro-orbitally infected with approximately $1x10^7$ CFU suspended in 100uL of sterile PBS. The infection was allowed to proceed for 4 days, after which the mice were sacrificed. The liver, heart, and kidneys were removed, organs were homogenized, and the bacterial burdens were determined by plating serial dilutions.

CHAPTER 3: RESULTS

Deletion of the metal-independent aldolase FdaB increases the sensitivity of *S. aureus* to manganese limitation

As previously stated, *S. aureus* encodes two fructose bisphosphate aldolases: the metal-dependent FbaA and the metal-independent FdaB. To determine whether FdaB acts to relieve calprotectin-induced metal limitation, the sensitivities of wildtype *S. aureus* and *ΔfdaB* to metal limitation were measured. The strains used were Newman, a methicillin-sensitive strain, and USA300 JE2, a community-acquired isolate of MRSA obtained in the United States. These isolates have some metabolic differences which are not completely understood. Therefore, the purpose for testing both is to see broadly whether the trends observed in a methicillin-sensitive strain are consistent in a methicillin-resistant isolate. *S. aureus* wildtype and *ΔfdaB* were challenged with increasing levels of calprotectin in Chelex-treated tissue culture medium (Fig. 1). In these conditions, both Newman and USA300 *ΔfdaB* have significant growth defects relative to their parent strain. This phenotype can be reversed through complementation (Fig. 1C).

The metal-independent aldolase is necessary to resist manganese limitation

Calprotectin has two binding sites: site 1, capable of binding either manganese or zinc; and site 2, capable of binding only zinc (7, 8). To probe whether manganese or zinc sequestration was responsible for the observed phenotype, Newman and USA300 JE2 wildtype and *ΔfdaB* were challenged with variants of calprotectin deficient in either the Mn/Zn or Zn binding sites (Fig. 1B). The growth defect of *ΔfdaB* was reversed upon

challenge with ΔMn/Zn calprotectin but not ΔZn, indicating that the growth defect is dependent on the sequestration of manganese rather than zinc.

To verify whether the increased calprotectin sensitivity of *ΔfdaB* is due to manganese limitation, it was necessary to impose more specific metal starvation. For this, an NRPMI assay using a chelex-treated tissue culture medium was performed as described by Grim et al. (10). Iron, calcium, and magnesium were added to the medium so the bacteria were only restricted in manganese and zinc. In both Newman and USA300 JE2, the *ΔfdaB* mutants show a significant growth defect compared to the wildtype (Fig. 2A, 2B). This growth defect is reversed when manganese is present. This suggests that these strains are more sensitive to manganese limitation. This starvation phenotype can be reversed through complementation (Fig. 2C).

Increasing manganese limitation worsens the growth defect of a strain lacking the metal-independent aldolase

S. aureus possesses two manganese transporters MntABC and MntH and their loss results in a defect in manganese import (15, 17, 21, 23). The *ΔfdaB* mutation was moved into a *ΔmntCH* background. The combined mutant showed significantly increased sensitivity to calprotectin compared to both single mutants (Fig. 3). This provides further evidence that the metal dependent aldolase is promoting resistance to manganese starvation.

The metal independent aldolase promotes retention of glycolysis when *S. aureus* is metal starved

To determine whether the growth defect of strains lacking the metal-independent aldolase is due solely to an interruption in glycolysis, Newman and USA300 JE2

wildtype and *ΔfdaB* were assayed in defined media with glucose, glycerol, or amino acids as the sole carbon source as described by Radin et al. (Fig. 4) (21). Amino acids circumvent glycolysis entirely, and have already been shown to decrease calprotectin sensitivity of wildtype *S. aureus* (21). Glycerol enters the glycolytic pathway just after the aldolase step; if the decreased growth of the *ΔfdaB* mutant is due to a blockage at the aldolase step, its growth on glycerol should be comparable to wildtype. Indeed, amino acids are able to not only decrease the calprotectin sensitivity of *S. aureus* overall, but the growth defect of *ΔfdaB* relative to wildtype is also ablated. Glycerol does not reduce the calprotectin sensitivity of wildtype *S. aureus*—as expected, since there are further steps in glycolysis that are metal-dependent. However it is able to rescue the growth defect of the *ΔfdaB* mutant at higher levels of calprotectin. Taken together, these results indicate that under metal starvation, FdaB is necessary for the continuation of glycolysis, however gluconeogenesis is not significantly impacted by its loss.

ArlRS-regulated factors in addition to the metal-independent aldolase are important for resisting metal limitation

As the metal-independent aldolase FdaB may be partially positively controlled by ArlRS, the calprotectin sensitivity of a *ΔarlRSΔfdaB* mutant was assayed (Fig. 5). As previously observed, the *ΔarlRS* mutant showed a significant growth defect relative to wildtype *S. aureus* in these conditions when challenged with calprotectin (21). However, the *ΔarlRSΔfdaB* was not significantly impaired compared to the *ΔarlRS* mutant. This suggests that these mutations impact the same pathway for resisting manganese. In addition, these data show that ArlRS-regulated factors beyond the aldolase are important

for resisting manganese limitation, as the *ΔarlRS* mutant does not phenocopy the *ΔfdaB* mutant.

Growth in metal restricted NRPMI has a minor but statistically significant impact on the expression of both the metal-dependent and -independent aldolases

Transcriptional reporters were used to evaluate expression of the aldolase isozymes in the presence and absence of either manganese or zinc (Fig. 6). Expression of each aldolase does shift in response to metal availability in a manner consistent with previous results; expression of the metal-dependent aldolase increases in the presence of manganese but decreases in the presence of zinc, while expression of the metal-independent aldolase decreased slightly in the presence of zinc with a further decrease in the presence of manganese. These changes were found to be statistically significant.

Strains lacking in either the metal-dependent or -independent aldolase have a virulence defect in a murine model of infection

In order to determine the importance of the aldolase isozymes in infection, C57BL/6 mice were retro-orbitally infected with wildtype *S. aureus* Newman, a *ΔfdaB* mutant, and a *ΔfbaA* mutant to establish a systemic infection. *ΔfdaB* and *ΔfbaA* showed significant decreases in bacterial load in the heart and liver compared to wildtype *S. aureus* (Fig. 7). The kidney showed no significant differences in bacterial burdens. These data suggest that both aldolases are important for full virulence of *S. aureus* in this model. To test whether the defect of *ΔfdaB* is due to metal sequestration, $CP^{-/-}$ mice were also challenged with wildtype, *ΔfdaB*, and *ΔfbaA S. aureus* Newman. Bacterial burdens in the liver were examined, as this is the organ which displays a metal associated phenotype during infection (7, 17). In the CP-deficient mice, the virulence defect of the metal-

independent aldolase mutant was ablated. Importantly, $CP^{-/-}$ mice are deficient in manganese but not zinc sequestration, reinforcing the manganese dependency of the *ΔfdaB* phenotype (7).

CHAPTER 4: DISCUSSION

Staphylococcus aureus faces a variety of challenges during infection. One major player in the host immune arsenal is calprotectin, which sequesters the essential metals zinc and manganese, rendering abscesses virtually depleted of these metals (18, 7, 8). *S. aureus* must cope with this restriction either by competing with calprotectin for metals or by reducing its own cellular demand for these metals. The former is tackled via the use of high affinity metal transporters such as the Cnt and MntABC systems (17, 10, 23). The latter can be accomplished by the use of alternative enzymes with different or absent metal requirements. This can be seen in the shift from manganese-dependent SodA to the cambialistic SodM, or in the switch from the metal-dependent phosphoglycerate mutase GpmI to the metal-independent isozyme GpmA (9, 22). The fructose bisphosphate aldolase step in glycolysis has been shown through these results to be an example of the latter. FbaA is the metal-dependent aldolase (putatively zinc-dependent) and FdaB is the metal-independent aldolase.

FdaB is broadly important for resisting metal limitation. When treated with calprotectin, a strain lacking the metal-independent aldolase has a significantly increased sensitivity to calprotectin. A the *ΔfdaB* mutant also has a significant growth defect compared to wildtype *S. aureus* in metal-deplete media, and a virulence defect in a murine model of infection. FdaB is important for maintaining glycolysis when *S. aureus* is metal restricted but is not necessary for gluconeogenesis. When grown on a non-glycolytic carbon source or a carbon source which enters glycolysis after the aldolase

step, the *ΔfdaB* mutant no longer has a difference in calprotectin sensitivity compared to wildtype.

The metal-independent aldolase FdaB is specifically important for responding to manganese limitation. The metal dependency of the *ΔfdaB* phenotype was tested in three different ways: through the use of mutant variants of calprotectin lacking either the Zn or Mn/Zn binding site, through selective addition of either manganese or zinc to metal-deplete media, and through deletion of manganese transport in an *ΔfdaB* mutant background. Manganese was found to be crucial for restoring growth of the *ΔfdaB* mutant; addition of zinc did not rescue growth. Inactivation of the Mn/Zn binding site of calprotectin also relieved the increased sensitivity of the *ΔfdaB* mutant, showing the requirement for manganese sequestration. Finally, increasing manganese limitation by ablating manganese transport significantly worsened the calprotectin sensitivity of the *ΔfdaB* mutant. It is also important to note that the virulence defect of the *ΔfdaB* mutant disappeared in the $CP^{-/-}$ mice, which have been shown to be deficient in manganese but not zinc sequestration (7).

Although the phenotype of a *ΔfdaB* mutant has been found to be manganese-dependent, there are still important questions to be answered before concluding that it is not responding to zinc. The possible effects of ablating zinc transport on a *ΔfdaB* mutant should be examined. Although there is some preliminary data on expression of the aldolase isozymes in response to metal availability, the question of the extent of ArlRS involvement remains. The manganese dependency of the *ΔfdaB* phenotype is especially interesting in light of the putative zinc dependence of its counterpart, FbaA (3). The metal dependency of FbaA has not been specifically tested; elucidating the identity of the metal

utilized by FbaA is crucial to understanding the role of the metal-independent aldolase FdaB in resisting nutritional immunity.

These data show the importance of maintaining glycolysis as part of the staphylococcal defense against nutritional immunity. The growth defect of a strain lacking the metal-independent aldolase shows that nutritional immunity—specifically the sequestration of manganese—interrupts glycolysis. The metal-independent aldolase FdaB is important for circumventing this blockage and allowing glycolysis to continue.

FIGURES

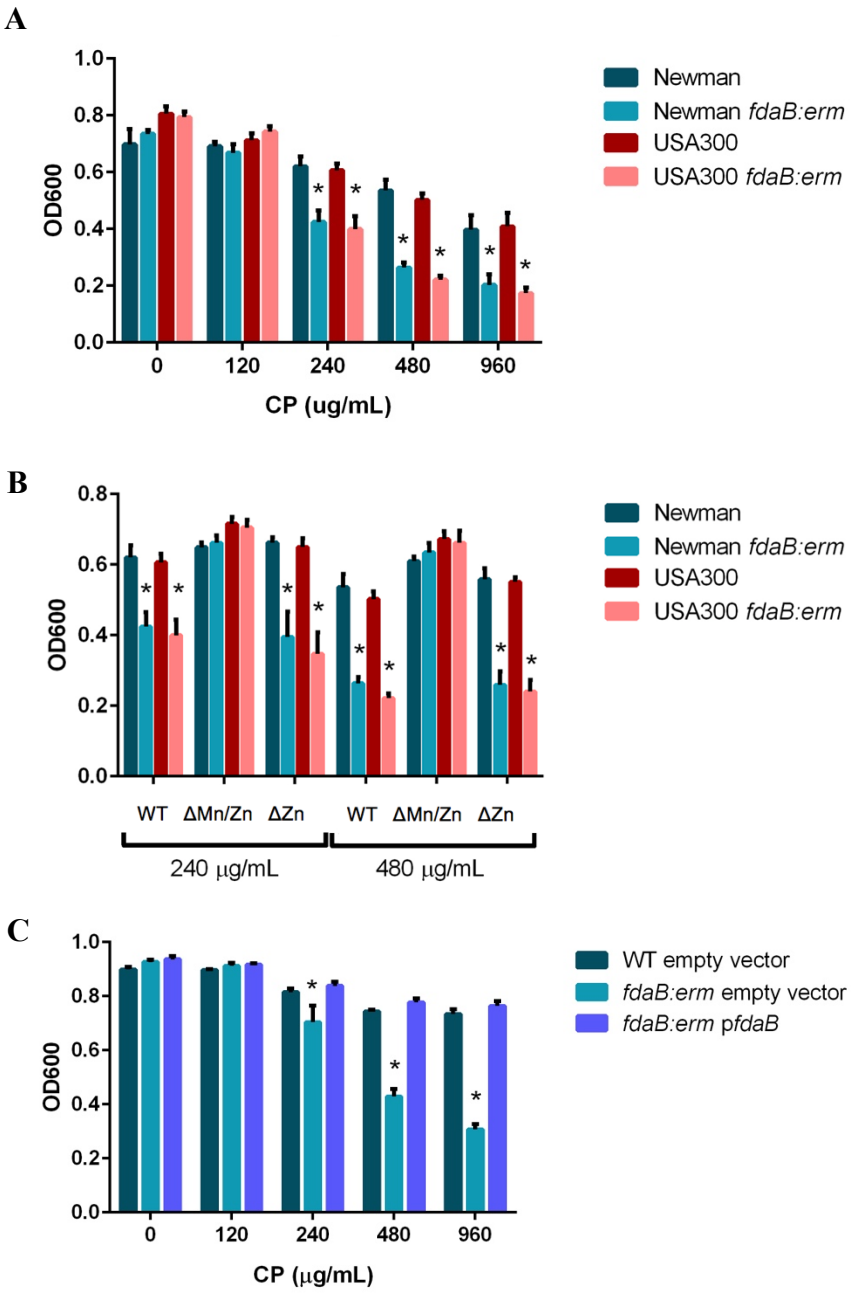

Figure 1: *S. aureus* Newman and USA300 JE2 were grown in 3xNRPMI containing 1μM zinc and 1μM manganese and challenged with increasing concentrations of calprotectin. A) Dose response of Newman and USA300 JE2 with wildtype calprotectin; B) growth inhibition by site mutant variants of calprotectin; C) complementation in Newman using wildtype calprotectin. Growth was assessed at 6 (A/B) or 8 (C) hours. Statistical analysis was performed via a two-way ANOVA with multiple comparisons. (*$p<0.05$ compared to parent WT) n=3 Error bars indicate SEM.

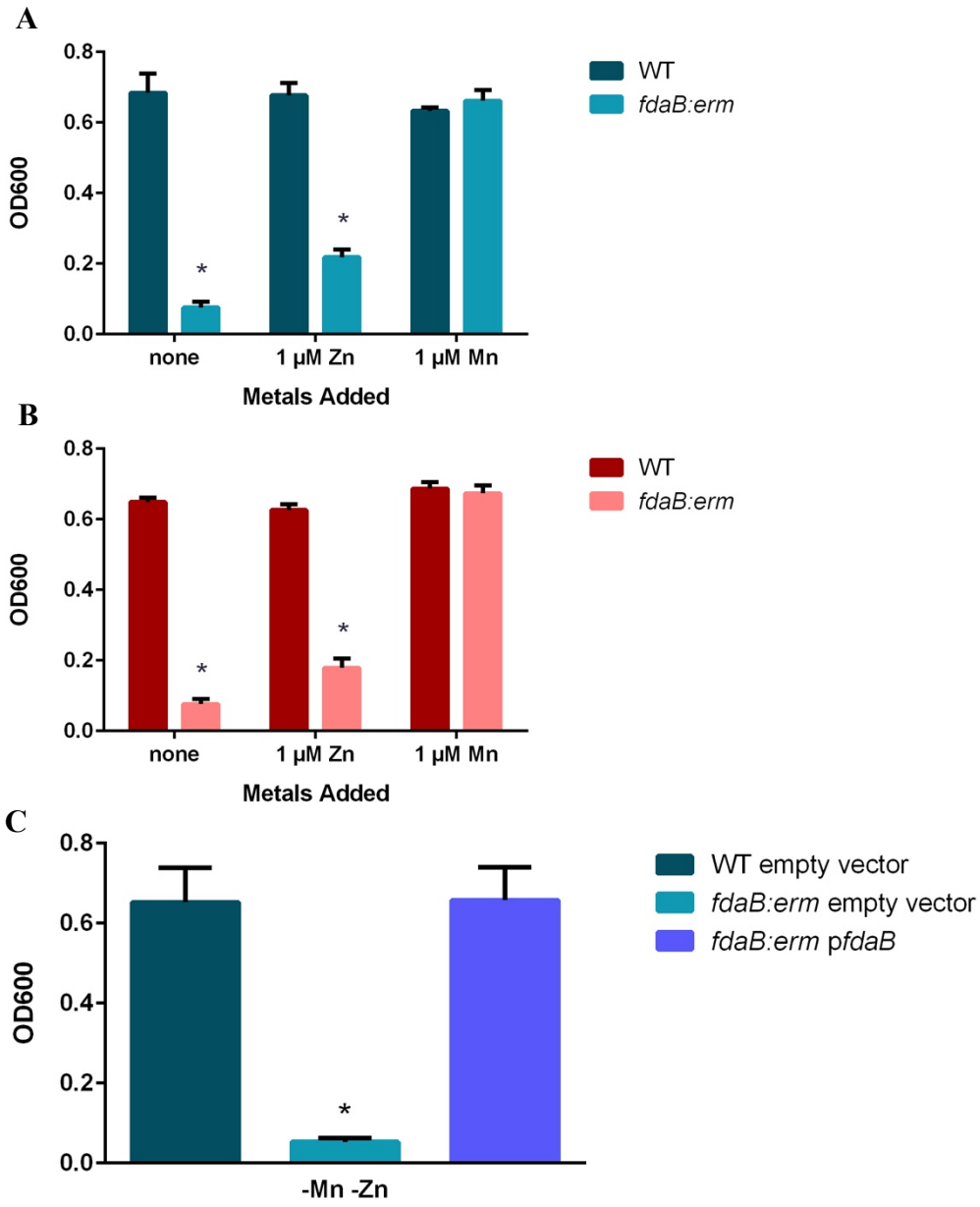

Figure 2: *S. aureus* Newman (A) and USA300 JE2 (B) were grown in manganese- and zinc-depleted NRPMI. For the assay, either zinc, manganese, or neither were added. Growth was assessed at 8 hours. (C) *S. aureus* Newman was grown in manganese- and zinc-depleted NRPMI. Growth was assessed over 8 hours. Statistical analysis was performed via a two-way ANOVA with multiple comparisons. (*$p<0.5$ compared to parent WT) n=3 Error bars indicate SEM.

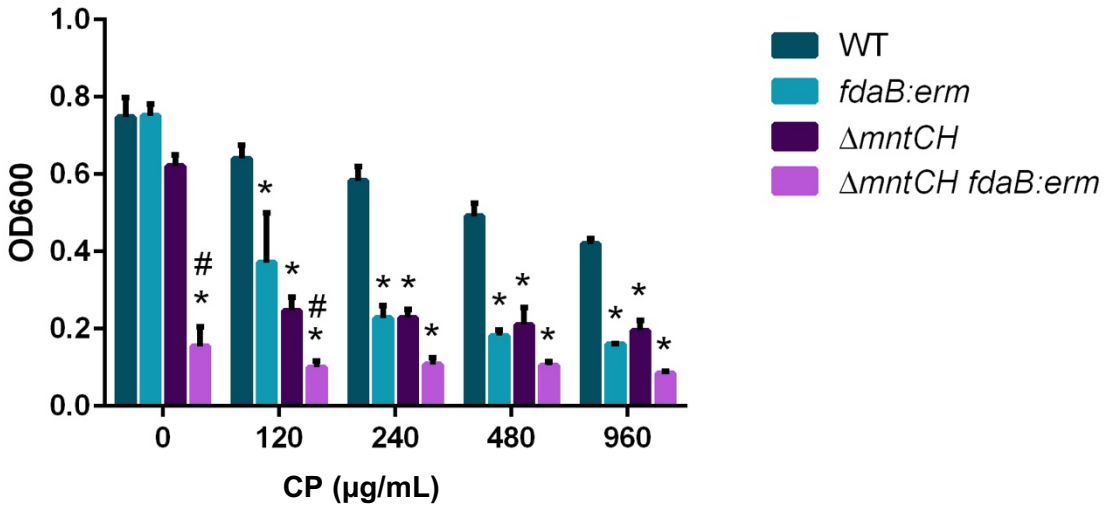

Figure 3: *S. aureus* Newman was grown in 3xNRPMI containing 1µM zinc and 1µM manganese and challenged with increasing concentrations of calprotectin. Growth was assessed at 6 hours. Statistical analysis was performed via a two-way ANOVA with multiple comparisons. (*$p<0.05$ compared to parent WT; #$p<0.05$ compared to *fdaB:erm*) n=3 Error bars indicate SEM.

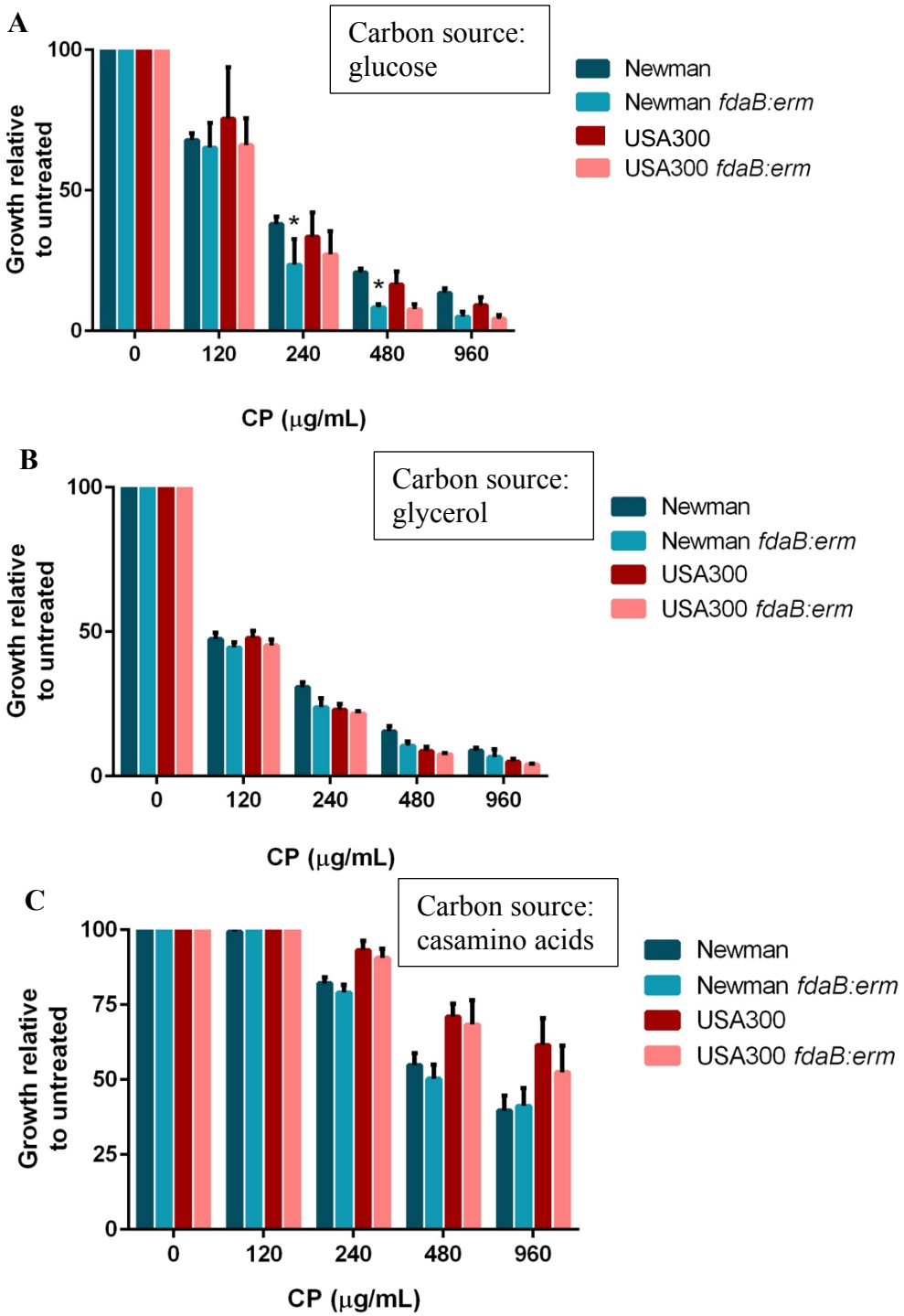

Figure 4: *S. aureus* Newman and USA300 JE2 were grown in defined media containing either glucose (A), glycerol (B), or amino acids (C) as the sole carbon source. These strains were challenged with increasing levels of calprotectin and their growth was followed for 8 hours. Statistical analysis was performed via a two-way ANOVA with multiple comparisons. (*$p<0.05$ compared to parent WT) n=4 Error bars indicate SEM.

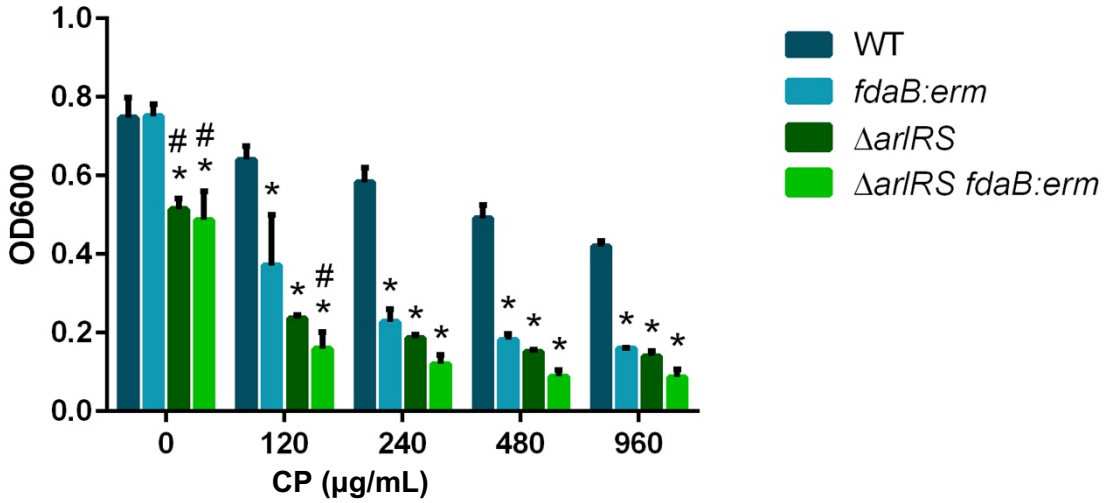

Figure 5: *S. aureus* Newman was grown in 3xNRPMI containing 1µM zinc and 1µM manganese and challenged with increasing concentrations of calprotectin. Growth was assessed at 6 hours. Statistical analysis was performed via a two-way ANOVA with multiple comparisons. (*$p<0.05$ compared to parent WT; #$p<0.05$ compared to fdaB:erm) n=3 Error bars indicate SEM.

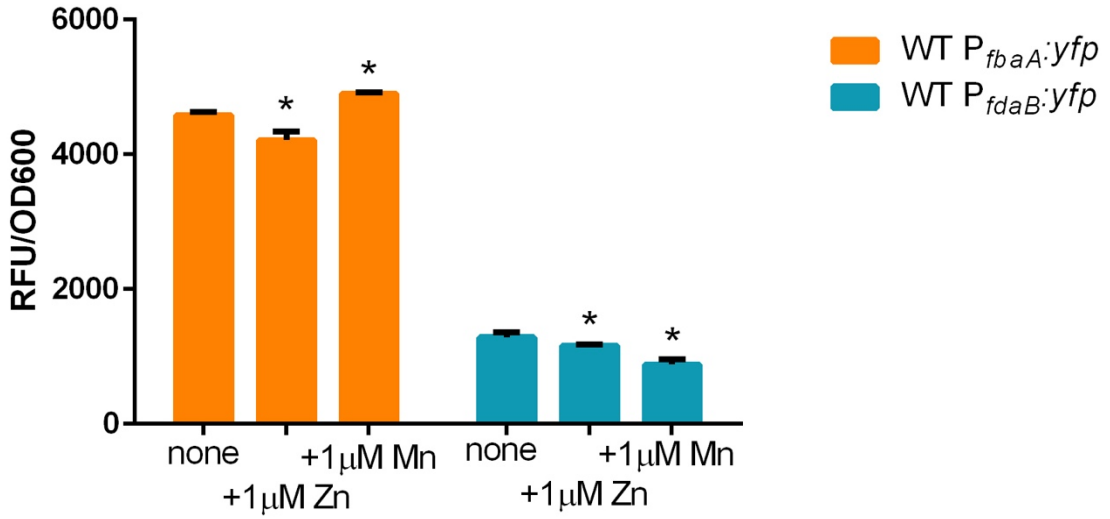

Figure 6: Reporter fusions were created for the aldolase isozymes and expressed in *S. aureus* Newman. They were grown in manganese- and zinc-depleted NRPMI. For the assay, either Zn, Mn, or neither were added. Growth was assessed at 8 hours and expression was measured through fluorescence. Statistical analysis was performed via a two-way ANOVA with multiple comparisons. (*$p<0.05$ compared to no metals added) n=3 Error bars indicate SEM.

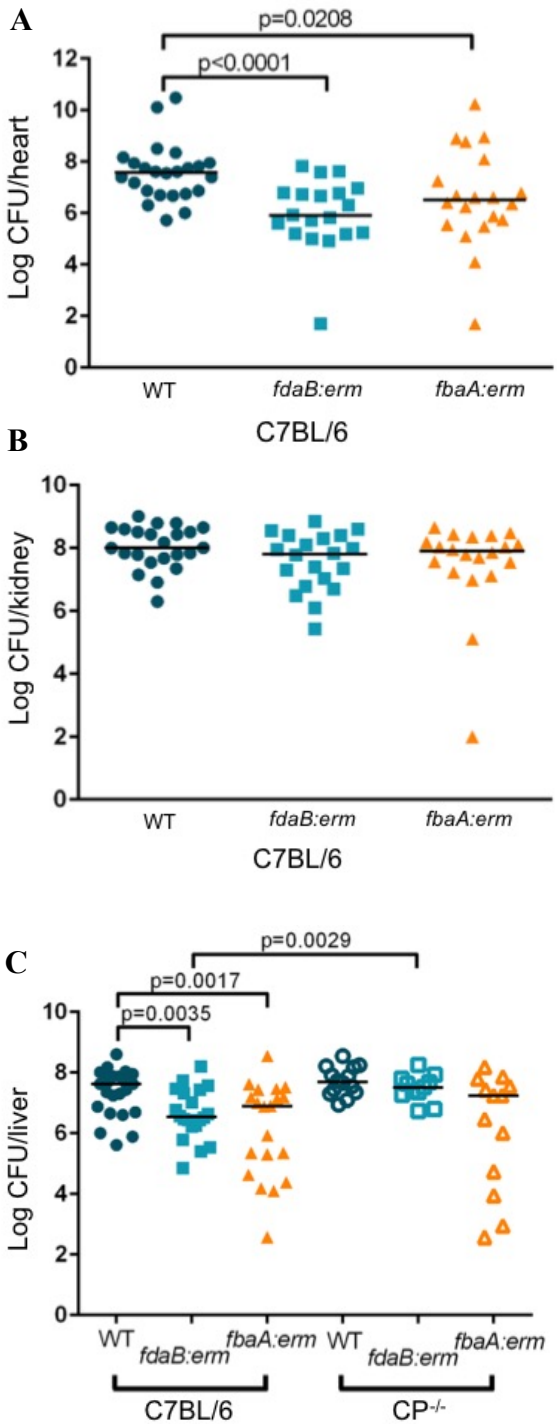

Figure 7: 10-week-old mice were retro-orbitally infected with $1x10^7$ of *S. aureus* Newman wildtype or aldolase mutants. After 4 days they were sacrificed and bacterial burdens in the heart (A), kidney (B), and liver (C) were assessed by CFU counts. Statistical significance was evaluated via Mann-Whitney U test and is noted where $p<0.05$. Bars indicate median.

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