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Investigating the roles of Na^+/H^+ antiporters *nhaC* and *SAUSA300_0617* during *Staphylococcus aureus* nitrosative stress

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA), a Gram-positive bacterium, is one of the most virulent clinical pathogens due to its numerous defense mechanisms against the host innate immune response. Antibiotic-resistant strains have emphasized the need to identify new drug targets. Recent research has examined the role of nitric oxide (NO•), an immune effector. While the presence of NO• inhibits microbial growth, *S. aureus* has unique adaptations to resist nitrosative stress. A pooled transposon screen studying a strain of MRSA (USA300 LAC) identified 168 genes that were likely essential for NO• resistance. Two were genes encoding Na^+/H^+ antiporters, including *nhaC* and *SAUSA300_0617*. An earlier study identified the significance of NhaC during pH and salt stress. This research evaluated the roles of these two antiporter genes during nitrosative stress through creation of two deletion mutants, △*nhaC* and △*0617*. While the transposon screen identified both to be essential for NO• resistance, we found that exposure to NO• slowed but did not stop growth in the △*0617* deletion mutant compared to wild-type. Growth inhibition of the mutant became more pronounced at higher concentrations. This confirms that SAUSA300_0617 aids in *S. aureus* NO• resistance but is not essential for growth and survival during nitrosative stress. Investigation of the Δ*nhaC* mutant is still in progress. Future studies will identify the phenotype of additional single deletion mutants of *S. aureus* that encode Na^+/H^+ antiporters and determine their suitability as potential drug targets.

1. Introduction

Staphylococcus aureus is a major human pathogen with a well-documented record of antibiotic resistance, including multi-drug resistant strains[1]. A growing number of community-associated infections in the United States are due to community acquired methicillin resistant *S. aureus* (CA-MRSA)[2]. These infections can occur due to mutation accumulation and mobile genetic element acquisition, leading to skin and soft tissue infections (SSTIs) and increased risk of disease recurrence[3]. Multi-drug resistant strains of *S. aureus* are both a financial burden and increasingly difficult to treat[1]. Much of the pathogen's success is due to multiple adaptive mechanisms that allow it to succeed even in adverse growth environments. One adaptation includes resistance to high concentrations of nitric oxide (NO·), a major host immune component[4].

NO· is an antimicrobial gas produced by host-activated phagocytes during infection by *S. aureus*. The membrane-permeable gas disrupts further infection by destroying bacterial DNA, lipids, and protein[5]. *S. aureus* frequently encounters respiration-limiting conditions during infection. This includes hypoxia and low iron concentrations, which inhibit bacterial access to electron acceptors to continue growth[4]. However, during anaerobic respiration, it can utilize nitrate as an alternative electron acceptor to continue growth.

Na^+/H^+ antiporters are integral membrane proteins which transport sodium and protons in opposite directions across the plasma membrane. They are responsible for maintaining a sodium gradient as well as intracellular pH. Likewise, respiration maintains the gradient and facilitates proton pumping in *S. aureus*. However, NO· disrupts both by forcing the microbe to utilize alternate pathways to maintain intracellular pH and fitness[5]. This includes the induction of Ldh1, an L-lactate dehydrogenase unique to *S.*

aureus, to maintain redox[6]. Even when NO· inhibits respiration, it does not arrest growth. One strategy to continue growth under nitrosative stress is via NO·-mediated activation of the SrrAB two-component system[7]. Under NO· stress, SrrAB induces Hmp, a flavohemoprotein which detoxifies NO· into nitrate.

To validate the results of a transposon screen, researchers tested an F_1F_0 ATPase mutant (△*atpG*) of *S. aureus* LAC, confirming it was required to maintain intracellular pH[5]. When the membrane potential was compared to wild-type (WT), the mutant had a hyperpolarized and acidified membrane after NO· stress. The authors surmised was due to cation extrusion as an alternative to protons. Moreover, NO·-stressed *S. aureus* requires an alkaline intracellular pH to preserve the function of metabolic enzymes for growth[5]. One type of exchanger that had been screened but not evaluated was Na^+/H^+ antiporters.

During nitrosative stress that inhibits respiration, the cytoplasm of *S. aureus* exhibits a slightly alkaline pH[5]. An alkaline intracellular pH is required for proper function of all three lactate dehydrogenases in *S. aureus*[8]. This includes Ldh1, whose induction is a unique NO resistance strategy to the species[6]. Below a pH of 7.3, Ldh1 activity is nearly undetectable. Some Na^+/H^+ antiporters, like the CPA3 family, require even more strongly alkaline conditions to function. Mnh1 has an optimum pH of 7.5 for antiporter activity and Mnh2 has an optimum pH of 9.0[9]. Of the two, only Mnh1 antiporter activity is present during slightly acidic conditions[9]. Therefore, the microbe likely induces expression of secondary transporters that are not dependent on respiratory elements or reverses the direction of its ATP synthase into ATP-hydrolysis mode.

A pooled transposon screen of the *S. aureus* CA-MRSA strain USA300 LAC identified 168 genes that were essential to confer full NO· resistance[5]. While most Na+/H+ antiporters did not contribute to fitness during NO· stress, *NhaC* (*SAUSA300_2250*) and a previously unnamed gene (*SAUSA300_0617*; herein *0617*) impacted fitness. Moreover, under NO· stress, transposon disruption of NhaC decreased fitness while disruption of 0617 increased it in a pooled competition assay.

The aims of this project were to characterize the phenotypes of two essential Na^+/H^+ antiporters from *S. aureus* LAC and compare growth patterns of pure culture deletion mutants with and without NO·. We accomplished this by constructing knockout plasmids within *E. coli* to allow for targeted chromosomal deletion of USA300 LAC, using *S. aureus* RN4220 as an intermediate.

2. Methods

2.1. Knockout Plasmid Construction for Targeted Chromosomal Deletion

An IBI High-speed Plasmid Mini Kit was used to extract the pBTK plasmid from *E. coli* with total DNA concentration measured via a BioTek plate reader (ng/µl). The gDNA of *S. aureus* LAC was purified with a Lucigen Gram Positive DNA Purification Kit. To amplify the approximately 1000-base pair flanking regions upstream and downstream of SAUSA300_2250 (*NhaC*) and SAUSA300_0617 (0617), polymerase chain reaction (PCR) was performed using NEB Q5 High-Fidelity DNA Polymerase and constructed primers (Table 1).

Table 1. Primer list for the project

Primers	Sequence
USA300_0617-5'FWD	5'-CACTAGAATTCCGTAATGTCAATTGTGGGTG-3'
USA300_0617-5'REV	5'-CACTAGAATTCTAAATTGAGTTAGCGACCAC-3'
USA300_0617-3'FWD	5'CACTAGGATCCGAAGGGCTTTTCATCAATCC-3'
USA300_0617-3'REV	5'-CACTAGGATCCACTGTCGTTCCCTTCATCTC-3'
USA300_2250-5'FWD	5'-CACTAGAATTCAAACGCAATCGAAAAGGGCG-3'
USA300_2250-5'REV	5'-CACTAGAATTCCCCCTTCGCTTATTGATGAG-3'
USA300_2250-3'FWD	5'-CACTAGGATCCGTAGCGGAACATGTTGATAT-3'
USA300_2250-3'REV	5'-CACTAGGATCCCACATGGAAAGGTAACTGGC-3'

2.2. Deletion of *nhaC* and *SAUSA300_0617* from the *S. aureus* LAC Chromosome

After verification of complete KO plasmids, extraction of *pBTK_0617* occurred to transform into *S. aureus* RN4220. This strain is restriction-deficient, making it a suitable intermediate to uptake and methylate *E. coli* DNA before introducing the plasmid to *S. aureus* LAC. Electrocompetent RN4220 cells were electroporated following an established procedure. A temperature-sensitive origin of replication in pBTK required incubation at permissive temperature (30°C) for 48 hours. Individual colonies were then grown and shaken overnight in 5-µl TSB cultures containing 10 µg/ml chloramphenicol (Cm10). O/N cultures were miniprepped following a modified IBI miniprep protocol, with the addition of 2500 µg/ml lysostaphin after PD1 resuspension. This addition promoted *S. aureus* cell wall lysing to aid transformation.

The complete KO plasmid was transformed into electrocompetent *S. aureus* LAC, following the aforementioned electroporation method and plated on TSA + Cm10 at permissive temperature. Individual colonies were subsequently diluted 1:1000 and grown overnight at non-permissive temperature (43°C) on TSA + Cm10. This condition would force a recombination event whereby the plasmid inserts into the LAC chromosome at either the homologous *0617* 5' or 3' region. Successful colonies were grown at 30°C with 10 µg/ml kanamycin (Kan10) but without Cm10 to promote a second recombination event to cure the plasmid. During the second event, the plasmid would be removed from the chromosome, with a 50% chance of *0617* chromosomal deletion and a 50% chance of WT regeneration. A 3-day serial passage at 30°C with Kan allowed for replicated cells to be either mutants or regenerated WT that had not yet been cured. A successful mutant would be kanamycin resistant and chloramphenicol-sensitive, as evident by growth on media containing Kan and no growth on media containing Cm.

2.3. NO· Growth Curve Assays

We prepared 24-hour growth curves in 96-well plates containing TSB media with different NO· concentrations via DETANO, a NO· donor. We then compared mutant △*0617* growth phenotypes to those of wild-type (Figure 3). Replicates are underway.

3. Results

3.1. Confirmation of Chromosomal Deletion Mutants △*0617* and △*nhaC*

A single-deletion mutant, △*0617,* was constructed using targeted mutagenesis so that its phenotype could be identified during NO· stress. To develop the knockout plasmids, *E. coli* constructs consisted of the kanamycin resistance gene *aph*-A3 (KmR), flanked by the corresponding nucleotide sequences of *S. aureus nhaC* or *0617*. Complete KO plasmids were extracted and purified before undergoing electroporation in RN4220, a restriction-deficient strain of *S. aureus.* This strain was required for the successful transformation of *E. coli*-cloned KO plasmids into electrocompetent *S. aureus* LAC. Under non-permissive growth temperatures, homologous recombination events occurred between target gene flanking regions and the KO plasmid. This involved integration of the plasmid into the chromosome of *S. aureus* LAC, followed by further recombination that removed the plasmid from the chromosome. Removal of the KO at the end of recombination allowed for a single replacement of *nhaC* or *0617* with KmR. Chloramphenicol-resistant plasmids were cured via serial passage, after which we selected for chloramphenicol sensitivity and kanamycin resistance. Colony growth on kanamycin-enriched but not chloramphenicol-enriched media indicated successful curing of the chloramphenicol-resistant plasmid plus allelic replacement of the target gene with kanamycin resistance (Figure 1). Mutants were further verified using Q5 and OneTaq PCR (Figure 2). The KO plasmid for *nhaC* has been constructed. Completion and confirmation of the full △*nhaC* mutant continues.

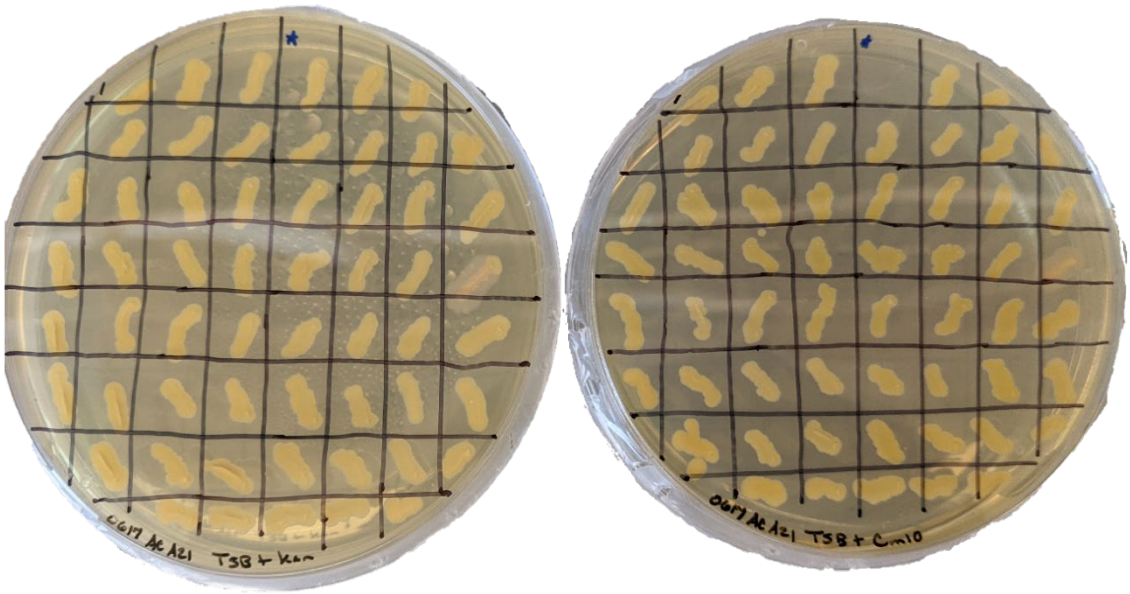

Figure 1. *⊿0617*-2 colonies grown on KmR and Cm10 plates

Successively patched *⊿0617*-2 colonies (1-57) from TSB + KmR and TSB + Cm10 (left and right, respectively). The starred patch (4) on both plates is indicative of an *⊿0617* mutant that underwent recombination, eliminating its chloramphenicol-resistant KO plasmid for the kanamycin resistance gene.

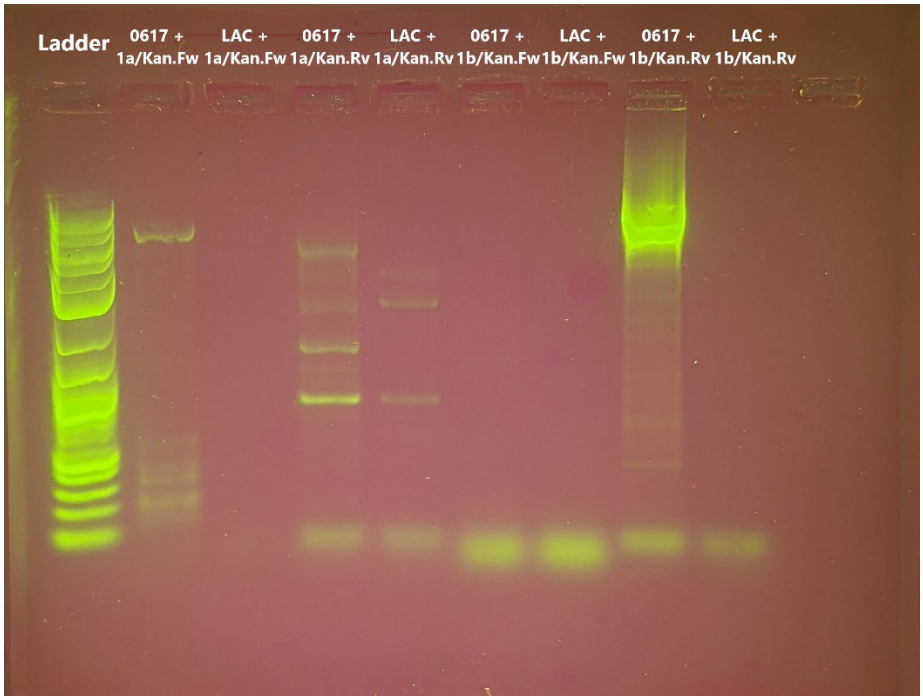

Figure 2. OneTaq confirmation PCR of *⊿0617-2.*

Figure 2 The OneTaq touchdown PCR paired a primer internal to the kan cassette (1a or 1b) with a primer that flanked the chromosomal DNA of *0617.* A successful product (notably, second from right) indicated the presence of KmR and correct positioning within the chromosome. Since wild-type gDNA (LAC) lacks the KmR cassette, its products are not observed.

3.2. △*nhaC G*rowth Inhibition is Attenuated but not Eliminated During NO· Stress

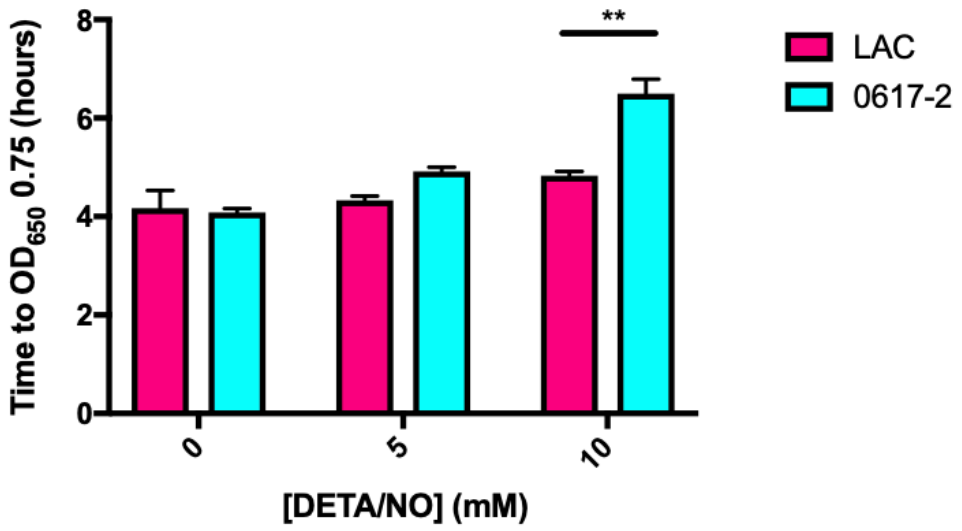

Figure 3. Average time to exponential growth (OD_{650} 0.75) of WT and *⊿0617.*

Figure 3 Exponential growth was recorded for WT (LAC, red) and the second *⊿0617* strain (blue) after exposure to 0, 5, or 10 mM of DETA/NO, an NO· donor (n=1 biological replicate, performed on the same day, with 3 technical replicates were completed per condition and day). A two-way analysis of variance (ANOVA) with *post hoc* Tukey's test revealed *⊿0617-2* and WT growth to be significantly different at 10 mM DETA/NO exposure (**, $p < 0.01$).

4. Discussion

S. aureus has multiple adaptations that make it highly virulent and antibiotic resistant. This includes the production of unique virulence factors that allow it to resist elements of the innate immune response like nitric oxide[5]. One important adaptation to preserve growth and virulence under NO· stress is maintaining intracellular pH balance. To date, there has not been a study that observed the role of Na+/H+ antiporters in cytoplasmic pH homeostasis during NO· resistance. However, better understanding their roles could identify them as potential targets for disrupting pH and cellular function with antimicrobial drugs during bacterial infection. Here, we report on the successful deletion mutant construction of a gene encoding a Na+/H+ antiporter, SAUSA300_*0617*. We evaluated the impact of nitrosative stress on *△0617* and found that high NO· levels extended lag time compared to WT. This suggests that Na+/H+ pumping is necessary for *S. aureus* to reach optimal growth during NO· stress. Notably, this finding deviates from a previous study that characterized *0617* fitness. While the *in vivo* screen found the transposon mutant of *0617* to increase fitness, our clean deletion mutant resulted in reduced fitness during exponential growth. One possible explanation for this discrepancy is that the transposon mutant was only tested in a pooled competition assay, whereas in the current study, pure cultures of mutant and WT were evaluated for differential growth. The transposon mutant was also not a deletion mutant but rather an insertion mutant, so it is possible that the mutation caused altered antiporter function rather than loss of function.

Future directions of this work include PCR confirmation of the *△nhaC* mutant and further characterization of both *△0617* and *△nhaC* phenotypes. This includes measuring membrane potential in WT and mutants with a BacLight Bacterial Membrane Potential Kit and intracellular pH through a pHrodo Red AM Intracellular pH Indicator Kit [5]. Using those values as a baseline, we could then quantify growth of WT and mutants in 96-well plates containing a range of NO· concentrations and pH to test for acid stress with and without NO·. This would identify an optimal pH for growth to persist with and without functional antiporters. Establishing the roles of these antiporters in infection is a first step toward potentially targeting them with drugs to reduce *S. aureus* growth and survival in a host.

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