# Evaluating Roles of Two $Na^+/H^+$ Antiporters in *Staphylococcus aureus* Nitric Oxide Resistance

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## Abstract

*Staphylococcus aureus*, a Gram-positive pathogen, has been a pervasive public health issue in the form of community-acquired methicillin-resistant *S. aureus* (CA-MRSA; Diep et al. 2006; Klevens et al. 2007). Despite the human immune response having multiple mechanisms to combat pathogens, *S. aureus* has the unique ability to resist nitric oxide (NO·), a major immune component (Grosser et al. 2016; Grosser et al. 2018). This resistance has drawn much scientific interest toward developing drug targets.

A Tn-Seq performed by Grosser et al. (2018) identified two genes that encoded for $Na^+/H^+$ antiporters which impacted *S. aureus* during NO· stress. However, there has been no published scientific work evaluating their impact. Other research has established a compensatory relationship between specific *S. aureus* antiporters and pH and proton regulation during non-NO· stress (Vaish et al. 2019). Thus, it is highly likely that similar relationships exist for additional antiporters when CA-MRSA encounters high salt and acidity from the human immune response. The aims of this research are to develop single and double mutants of two genes, NhaC and USA300_0617 under a modified allelic exchange method (Fuller et al. 2011). After developing a wild-type variant (WT), we will measure growth curves and compare mutants to WT under NO·, low pH, anaerobiosis, and high salt stress. Given our evaluation of antiporters, membrane potential will also be reviewed. To obtain additional characterization data, mutants will be exposed a range of antibacterial compounds.

## Background

*Staphylococcus aureus* is a human pathogen that adapts to a variety of adverse growth environments, but it most commonly causes skin and soft tissue infections (SSTIs). Much of its success comes from its unique resistance to nitric oxide (NO·), a major host immune component, allowing it to be a highly successful pathogen. Consequently, *S. aureus* genes and corresponding proteins that confer NO· resistance would make promising drug targets. In recent years, the USA300 strain of *S. aureus* has been investigated for its virulence and prevalence as community-acquired methicillin-resistant *S. aureus* (CA-MRSA; Seybold et al. 2006; Grosser et al 2018). Its adaptiveness to non-hospital settings, disproportionate rates of infection, and potential to acquire multidrug resistance make the pathogen a significant public health issue (Diep et al. 2006; Klevens et al. 2007).

Grosser et al. (2018) performed deep sequencing of USA300 LAC transposon junctions to obtain a list of genes required for fitness during NO· stress. Under this condition, their Tn-Seq found most genes encoding for $Na^+/H^+$ antiporters to be nonessential, but two impacted fitness: USA300_2250 (NhaC herein) and USA300_0617, an unnamed gene. More interestingly still, deletion of the NhaC gene decreased *S. aureus* fitness under NO·-induced stress, but deletion of USA300_0617 increased its fitness under the same condition (Grosser et al. 2018). Whether the simultaneous deletion of both genes affects *S. aureus* fitness has yet to be evaluated. While their investigation analyzed a mixed pool of mutants, these two genes need individual confirmation to elucidate their mechanisms.

Respiration is a major means of proton pumping for *S. aureus* and thus affects intracellular pH. However, high NO· concentrations inhibit this process, forcing the pathogen to utilize alternate pathways to continue regulating intracellular pH (Grosser et al. 2018**)**. The pathogen functions optimally with a slightly alkaline cytoplasm during NO· stress. Because high levels of NO· inhibit *S. aureus* respiration, it is likely that viability depends on secondary transporters to regulate pH and membrane potential (Grosser et al. 2018; Vaish et al. 2018; Vaish et al. 2019). The work of Vaish and colleagues (2019) found NhaC and CPA1 candidate antiporters, responsible for efflux of $Na^+$ and/or $K^+$, to be synergistic in proton and pH regulation. Indeed, more synergic or compensatory interactions between other proton antiporters likely exist, and NhaC and USA300_0617 are two likely candidates. There is also the possibility that these antiporters contribute to the high salt and acidity tolerance of CA-MRSA, two other conditions encountered on the skin, sometimes simultaneously with NO·.

This study aims to create deletion mutants of two $Na^+/H^+$ antiporter genes, ΔNhaC and ΔUSA300_0617 as well as a double mutant. Given the lack of functional data in the scientific literature, we will characterize ΔNhaC and ΔUSA300_0617 before examining the fitness of all three *S. aureus* mutants under NO·, low pH, and high salt stress. Furthermore, these mutants will be exposed a range of antibacterial compounds to test for growth inhibition when compared to wild-type *S. aureus*. NO· is present at levels high enough to inhibit respiration during early inflammatory phases of skin infection, but *S. aureus* also experiences anaerobiosis within abscesses later during skin infection. These two antiporters may allow CA-MRSA to continue regulation of intracellular pH when respiration is inhibited due to a lack of oxygen, as well as by NO·. Investigating this issue will supplement the Grosser Lab's current research on *S. aureus* infection in abscesses.

**Methods**

Using the USA300 *S. aureus* strain, we will create mutants (ΔNhaC, ΔUSA300_0617, and ΔNhaCUSA300_0617) using targeted mutagenesis under a modified allelic exchange method, where we clone flanking DNA sequences on either side of selected markers in pBTE, the *S. aureus*/*E. coli* shuttle vector, via PCR as described by Fuller and colleagues (2011). We will use EcoRI, BamHI, Phusion Hi-Fidelity DNA polymerase, and T4 DNA ligase for mutant creation. The knockout plasmids will then be verified via gel electrophoresis before developing the wild-type (WT) variant. Antibiotic selection in *S. aureus* mutants and wild type will be performed using chloramphenicol, erythromycin, and cycloserine as performed by Fuller et al. (2011). To verify clean deletion, each strain will have growth curves using 96-well plates to account for growth rate and maximum growth level.

*S. aureus* mutants and WT will be grown in media with different pH levels in Biolog Phenotype Microarray pH plates. We will measure cytoplasmic pH to obtain more individual characterization data as described by Grosser et al. (2018). We will then expose strains to NO·. or to anaerobiosis. Completing this procedure would determine whether the groups show differences at regulating intracellular pH. To obtain more complete mutant data, they will be evaluated for antibacterial sensitivity, recorded via their minimum inhibitory concentrations (MICs). The subsequent step would determine whether these differences affect NO· resistance or ability to grow without oxygen. Since these experiments involve proton antiporters, an additional test would measure membrane potential between mutants and wild-type *S. aureus.*

The USA300 strain of S. aureus has been shown to have high salt tolerance, so our mutants and wild-type will be grown in Biolog Phenotype Microarray osmolyte plates containing

increasing salt conditions to determine if either antiporter plays a role in salt tolerance (Vaish et al. 2019). To make the *in vitro* experiment more closely imitate *S. aureus* exposure to human skin and abscesses, all strains will be exposed to NO· and salt conditions simultaneously.

**Budget**

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| Stipend for 12 weeks of work | $1500.00 |
| Biolog Phenotype MicroArrays, inoculating fluid, and redox dye mix | $248.00 |
| Enzymes, antibiotics, and culture media for mutant creation | $505.00 |
| NO· donor (DETA/NO·; Millipore Sigma) | $42.00 |
| QuickLyse Miniprep Kit (Qiagen) | $205.00 |
| **Total** | **$2500.00** |

**Budget Justification**

I request a stipend of $1500.00 to support myself while completing this research project. The Biolog Phenotype MicroArrays will allow us to test *S. aureus* growth under many different conditions while maximizing our efficient use of lab materials. Enzymes used for the experiment will be necessary to create plasmids that produce copies of the pathogen. Antibiotics will be necessary for mutant selection and to test growth. DETA/NO· will be used as a source of NO· for experiments involving NO·-induced stress. The Miniprep Kit is necessary for preparation of mutant and wild-type strains using small volumes of solutions.

**Timeline of Research Activities**

May 2020 (campus reopening pending): Create and verify *S. aureus* mutants and wild type. Due to UNC Asheville's COVID-19 precautions in place, we have contingency plans to complete primer design remotely.

Jun-Aug 2020: Perform phenotypic microarrays of ΔNhaC and ΔUSA300_0617 compared to wild-type *S. aureus*. Test mutant and WT growth rates under anaerobic conditions and anaerobiosis. Evaluate mutant and WT antibacterial sensitivity for growth inhibition. This part of the timeline can be completed in the fall pending current COVID-19 mitigation strategies.

Fall 2020: *S. aureus* mutant, WT data analyses.

Spring 2021: Finalize data analyses. Prepare work for publication in the UNC Asheville Journal of Undergraduate Research. Present at Spring 2020 Undergraduate Research Symposium and other regional conferences.

**References**

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