To my family
ACKNOWLEDGMENTS

I thank my parents for always supporting me, and the Gator Masters Swim Team for being my family in Gainesville. I thank my professor, Dr. Rice, for helping me on this project, and my collaborator Dr. Richardson. I thank my undergrad helper, Jenna, for being a pleasure to work with.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................................................... 4
LIST OF TABLES .................................................................................................................................................... 7
LIST OF FIGURES .................................................................................................................................................. 8
ABSTRACT .............................................................................................................................................................. 9

CHAPTER

1 INTRODUCTION .................................................................................................................................................. 11
   Staphylococcus aureus ........................................................................................................................................ 11
      MRSA ......................................................................................................................................................... 12
   S. aureus Virulence Factors .......................................................................................................................... 13
   Regulation of S. aureus Virulence Factors ................................................................................................... 14
   Metabolism .................................................................................................................................................... 15
   Biofilms ......................................................................................................................................................... 16
   S. aureus Biofilms .......................................................................................................................................... 18
      Attachment Phase .................................................................................................................................... 18
      Accumulation Phase ............................................................................................................................... 19
      Maturation and Dispersal Phases ............................................................................................................ 20
   Nitric Oxide .................................................................................................................................................. 20
      Role of Nitric Oxide in Biofilms .............................................................................................................. 21
      Role of NO Metabolism in S. aureus Virulence ...................................................................................... 22
      Potential Routes of Endogenous NO Formation in S. aureus ............................................................... 23
   Nitrate Reductases ..................................................................................................................................... 24
      Regulators of nar ...................................................................................................................................... 25
      NO Production by nar in Other Organisms ............................................................................................. 26
   Hypothesis ..................................................................................................................................................... 27

2 METHODS AND MATERIALS .......................................................................................................................... 29
   Bacterial Strains and Growth Conditions ..................................................................................................... 29
   Creation of Mutants ....................................................................................................................................... 29
   Creation of the Trans Complement Plasmid pCN-nar ................................................................................. 31
   Biofilm Assays for Confocal Microscopy ..................................................................................................... 33
   Measurement of Relative NO Production in Static Biofilms .................................................................... 34
   Treatment Conditions ................................................................................................................................ 35

3 RESULTS .......................................................................................................................................................... 43
   Effect of Nitrate Metabolism on S. aureus Biofilms .................................................................................... 43
   NO Production Varies Between Wild-type and Mutants ............................................................................. 45
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Strains and plasmids used in this study</td>
<td>37</td>
</tr>
<tr>
<td>2-2</td>
<td><em>S. aureus</em> genes investigated in this study</td>
<td>38</td>
</tr>
<tr>
<td>2-3</td>
<td>Primers used in this study</td>
<td>39</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Postulated routes for NO formation and consumption in <em>S. aureus</em></td>
<td>28</td>
</tr>
<tr>
<td>2-1</td>
<td>Diagram representing primer locations</td>
<td>39</td>
</tr>
<tr>
<td>2-2</td>
<td>Confirmation of Δnar mutants in <em>S. aureus</em> UAMS-1</td>
<td>40</td>
</tr>
<tr>
<td>2-3</td>
<td>Confirmation that the <em>nar</em> complement plasmid is present and carries the <em>narGHJI</em> genes in <em>E.coli</em> DH5α.</td>
<td>41</td>
</tr>
<tr>
<td>2-4</td>
<td>Confirmation that the <em>nar</em> complement plasmid is present and carries the <em>narGHJI</em> genes in <em>S. aureus</em> strain UAMS-1</td>
<td>42</td>
</tr>
<tr>
<td>3-1</td>
<td>Effect of the <em>nar</em> mutation on biofilm formation.</td>
<td>50</td>
</tr>
<tr>
<td>3-2</td>
<td>Effect of the <em>nirBD</em> mutation on biofilm formation.</td>
<td>51</td>
</tr>
<tr>
<td>3-3</td>
<td>Effect of the <em>nreC</em> mutation on biofilm formation.</td>
<td>52</td>
</tr>
<tr>
<td>3-4</td>
<td>COMSTAT analysis of wild-type (Newman) and <em>nar, nir, and nre</em> mutant biofilms</td>
<td>53</td>
</tr>
<tr>
<td>3-5</td>
<td>Relative NO production by wild-type (Newman) and <em>nar, nir, nos, hmp, and nre</em> mutants, as measured by DAF-FM diacetate staining</td>
<td>54</td>
</tr>
<tr>
<td>3-6</td>
<td>Relative NO production in nitrate-treated cultures by wild-type (Newman) as measured by DAF-FM diacetate staining</td>
<td>55</td>
</tr>
<tr>
<td>3-7</td>
<td>Effect of the <em>nar</em> mutation on biofilm formation in UAMS-1.</td>
<td>56</td>
</tr>
<tr>
<td>3-8</td>
<td>COMSTAT analysis reveals statistically significant differences between wild-type (UAMS-1) and the <em>nar</em> mutant</td>
<td>57</td>
</tr>
<tr>
<td>3-9</td>
<td>Relative NO production by wild-type (UAMS-1) and <em>nar</em> mutant as measured by DAF-FM diacetate staining.</td>
<td>58</td>
</tr>
</tbody>
</table>
EFFECT OF THE NITRATE REDUCTASE OPERON ON STAPHYLOCOCCUS AUREUS BIOFILM FORMATION

By

Sara Elizabeth Holman

August 2011

Chair: Kelly C. Rice
Major: Microbiology and Cell Science

*Staphylococcus aureus* is thought to produce endogenous nitric oxide (NO) primarily through the action of NO synthase (NOS). It is known that nitrate reductases of plants and *Salmonella typhimurium* can convert nitrite to NO, but whether this pathway exists in *S. aureus* is unknown. NO, a possible signaling molecule, has been shown to inhibit attachment during *S. aureus* biofilm formation. This purpose of this study was to determine if *S. aureus* nitrate reductase has an effect on biofilm phenotype, and if so, whether this phenotype is attributable to altered levels of NO production. *S. aureus* Newman and its corresponding *nar* mutant were grown as biofilms in the presence or absence of sodium nitrate (NaNitrate) or sodium nitrite (NaNitrite). Biofilm differences were observed using Live/Dead staining and confocal microscopy. Untreated Newman biofilms were weakly attached and patchy, but treatment with NaNitrate or NaNitrite significantly increased the biomass and average thickness. The *nar* mutant biofilm did not respond to NaNitrate treatment, but showed significantly more biomass and average thickness when treated with NaNitrite. A *nar* mutant was also created in strain UAMS-1, which contains an NO-reductase gene not present in Newman, possibly altering its response to NO production. Although UAMS-1
produced a more robust biofilm compared to Newman, treatment with NaNitrate caused a similar effect on its biofilm phenotype (increased biomass and average thickness), and its nar mutant showed no change in average thickness when treated with NaNitrate. Relative levels of NO production in wild-type and mutant biofilms were measured using DAF-FM diacetate assays. NaNitrate treatment in both wild-type strains resulted in increased NO production compared to untreated biofilms, whereas NaNitrite treatment had a less pronounced effect. NO levels in the nar mutant in both strains did not increase in response to treatment with NaNitrate. Collectively, these results demonstrate that S. aureus nitrate reductase affects biofilm development when grown in the presence of NaNitrate or NaNitrite, but further study is required to determine if altered NO levels are responsible for these phenotypes. This study has also uncovered a previously-unrecognized role for nitrate reductase in contributing to endogenous NO production in S. aureus.
CHAPTER 1
INTRODUCTION

Staphylococcus aureus

*Staphylococcus aureus* is a bacterium that belongs to Micrococcaceae family, and appears as gram-positive cocci in clusters when observed under a microscope (Lowy, 1998). *S. aureus* can be distinguished from other members of the *Staphylococcus* genus because of its gold pigmentation of colonies, positive coagulase test, mannitol fermentation, and deoxyribonuclease tests (Wilkinson, 1997). The *S. aureus* genome consists of a circular chromosome around 2800 base pairs in length, containing prophages, plasmids, and transposons (Lowy, 1998).

*Staphylococcus aureus* is one of the most successful human pathogens, causing invasive infections in both immunocompromised and immunocompetent hosts (Gaujoux-Viala et al., 2011; Shankar et al., 2009; Minhas et al., 2011; Imataki et al., 2006). Infections by *S. aureus* are notoriously difficult to prevent and treat, as this bacterium has many ways to evade the host's immune response, including resistance to phagocytosis and interference with the influx of neutrophils, which is the host's primary defense against *S. aureus* (Richardson et al., 2006). *S. aureus* can cause skin and soft tissue infections (Decker et al., 1986), bacteremia (Mylotte et al., 1987), abscesses (Cheng et al., 2011), endocarditis (Espersen and Fridmodt-Moller, 1986), toxic shock syndrome (Bohach et al., 1990), sepsis (Bone, 1994), and nosocomial infections (Stamm et al., 1981). However, *S. aureus* is also considered a part of the normal flora, as it asymptptomatically colonizes healthy people (Chambers and DeLeo, 2009). Approximately 30% of humans are nasal carriers and are an important source of the spread of *S. aureus* strains among individuals (Gorwitz et al., 2008; Kluytmans et al.,
Transmission is primarily through direct skin-to-skin contact with a colonized individual (Miller and Diep, 2008). Persons colonized with *S. aureus* are at increased risk for subsequent infection (Wenzel and Perl, 1995). Rates of colonization are high among intravenous drug users, patients with Type I diabetes (Tuazon et al., 1975), surgical patients (Kluytmans et al., 1995), patients with acquired immunodeficiency syndrome (Weinke et al., 1992), and patients with defects in leukocyte function (Waldvogel, 1995).

**MRSA**

*Staphylococcus aureus* is notorious for its ability to become resistant to antibiotics, which is often acquired by horizontal gene transfer, or in some cases by chromosomal mutation and antibiotic selection (Chambers and DeLeo, 2009). Methicillin-resistant *S. aureus* (MRSA) strains are resistant to practically all β-lactam antibiotics, a class represented by penicillins and cephalosporins (Chambers and Neu, 1995). MRSA was once confined to hospitals and other health care environments (termed health-care associated MRSA or HA-MRSA) along with the patients frequenting these facilities, however since the 1990s there has been an explosion of the number of cases in people lacking the risk factors and exposure to these facilities (Gorak et al., 1999; Herold et al., 1998; Wu et al., 2002). New MRSA strains, referred to as community associated MRSA (CA-MRSA) strains are largely responsible for these cases, and appear to have rapidly disseminated among the general population in most areas of the United States (David and Daum, 2010). While HA-MRSA strains mostly infect older individuals with previous health conditions who have been exposed to the health care setting, CA-MRSA strains tend to infect younger and otherwise healthy individuals (Naimi et al., 2003; Fridkin et
al., 2005). CA-MRSA infections have become commonplace and have created a public health crisis in the U.S. emergency departments and other clinical settings (David and Daum, 2010). There are few antibiotics available to treat MRSA infections (Daum and Seal, 2001), and the development of new classes of antibiotics has slowed (Talbot et al., 2006). Furthermore, there have been reports of isolates resistant to each of the few antibacterial drug classes effective against MRSA, raising the possibility that an untreatable multi-drug resistant strain of *S. aureus* may develop in the future (Dowzicky et al., 2000; Luh et al., 2000; Marty et al., 2006; Meka et al., 2004; Rose and Rybak, 2006).

**S. aureus** Virulence Factors

*S. aureus* possesses a broad array of virulence factors that contribute to its pathogenesis, including colonization/adherence factors, toxins, phagocytosis inhibitors, and immune evasion molecules (Bartlett and Hulten, 2010). The first step in infection is the attachment to host cells or the extracellular matrix by Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMS), which are cell wall anchored proteins secreted by the sec system (Bartlett and Hulten, 2010). These molecules have an exposed binding domain for interaction with the host, a cell-wall spanning domain, and a domain for covalent or non-covalent attachment to bacterial surfaces (Patti et al., 1994). As soon as *S. aureus* gains access to tissues in the body, several chemoattractants are released that produce a concentration gradient that stimulate the migration of neutrophils to the site of infection (Gasque, 2004). However, *S. aureus* is able to synthesize chemotaxis inhibitory proteins (CHIPS) that block receptors for the chemoattractants, which inhibits leukocyte activation and migration (de Haas et al., 2004). In addition, *S. aureus* can produce a number of secreted toxins and exo-
enzymes that can destroy specific host cell and tissue types (Lowy, 1998). *S. aureus* can also exploit the inflammatory response of the host by surviving inside the phagosome if it becomes engulfed by neutrophils (Gresham et al., 2000). Becoming intracellular allows *S. aureus* to escape host immunity until the neutrophils lyse and release DNA, forming extracellular traps that bind and finally kill the bacteria (Brinkmann and Zychlinsky, 2007).

**Regulation of *S. aureus* Virulence Factors**

The first well-characterized regulator of virulence factor expression in *S. aureus* was the accessory gene regulator ( Agr) (Recsei et al., 1986). The *agr* system consists of two divergently transcribed loci controlled by two promoters, P2 and P3, that regulate the transcription of a cell-density-sensing two-component regulator system and a regulatory RNA known as RNAIII (Novick et al., 1995). The P2 promoter controls four genes: *argA, agrB, agrC* and *agrD* (Somerville and Proctor, 2009). When AgrD, an autoinducer peptide, reaches a threshold level outside of the cell due to its accumulation during bacterial growth, it complexes with AgrC (membrane sensor kinase) and activates its kinase domain (Lina et al., 1998). Subsequent transfer of a phosphoryl group from AgrC to AgrA (response regulator) activates this transcription factor, which in turn increases transcription from both the P2 and P3 promoters (Koenig et al., 2004). Transcription from P3 produces the riboregulator RNAIII, which enhances the synthesis of secreted virulence determinants (such as alpha toxin, serine protease) and represses the synthesis of surface-associated proteins (such as protein A) (Recsei et al., 1986). The second major virulence regulator identified in *S. aureus* was the staphylococcal accessory regulator ( SarA) (Cheung et al., 1992). SarA encodes a DNA binding protein which functions in part to regulate the transcription of *agrABCD* and...
RNAIII (Morfeldt et al., 1996). More recently, a whole family of SarA proteins has been identified in *S. aureus*, which comprise a complex network of regulators (Cheung et al., 2004; Cheung et al., 2008). In addition, the *S. aureus* sigma factor $\sigma^B$ is activated under stress conditions (environmental or nutritional), growth transitions, or during morphological changes (Senn et al., 2005). In the absence of environmental stimuli, $\sigma^B$ is bound to an anti-sigma factor, RsbW (Somerville and Proctor, 2009). Stress-inducing stimuli are hypothesized to activate a signal cascade that results in the activation of the anti-anti-sigma factor, RsbV, which binds RsbW in a competitive manner to increase the concentration of free $\sigma^B$ (Palma and Cheung, 2001). This allows for the association of $\sigma^B$ with the core RNA polymerase, which can then bind to promoters to allow for virulence gene transcription (Biscoff et al., 2004).

**Metabolism**

Central to the metabolism of *S. aureus* is the TCA cycle, which supplies biosynthetic intermediates, reducing potential, and ATP (Somerville and Proctor, 2009). Under nutrient-rich conditions and during exponential growth phase, TCA cycle activity is very low since the bacterial demand for intermediates is supplied by via carbohydrate fermentation (Somerville et al., 2003). However, when environmental conditions change and carbohydrates and nutrients are limited during stationary phase, TCA cycle activity is increased and the bacteria begin to catabolize non-preferred carbon sources such as the secreted end products of carbohydrate fermentation (Somerville et al., 2002). Repression of the TCA cycle is mediated primarily by CcpA, CodY, and SrrA, which respond to changes in the availability of carbon, nitrogen, and oxygen, respectively (Seidl et al., 2008; Soneneshein, 2005; Throup et al., 2001). Thus, as the
metabolic status changes, the DNA binding ability of the regulators also changes, and the TCA cycle is activated (Somerville and Proctor, 2009). Numerous studies have implicated the TCA cycle in the regulation of virulence (Bae et al., 2004; Coulter et al., 1998; Mei et al., 1997; Somerville et al., 2003). TCA cycle mutants result in a small-colony phenotype and a slow-growing subpopulation that causes chronic and relapsing infections (Kriegeskorte et al., 2011). A recent study showed that when *S. aureus* TCA cycle mutants were cultured in-vitro with the soft tissue of mice, the tissue cells produced significantly lower amounts of nitric oxide and an inducible nitric oxide synthase compared to cells exposed to wild-type bacteria (Massilamany et al., 2011). It was proposed that this altered metabolism allows the bacteria to evade host immune responses, which enhances their ability to survive within the host (Massilamany et al., 2011).

*S. aureus* is a facultative anaerobic organism, meaning it can grow in either aerobic conditions or anaerobic/low oxygen conditions, however the growth rate is drastically reduced after a shift from aerobic to anaerobic growth (Fuchs et al., 2007). Growth under anaerobic or low oxygen conditions is supported by carbohydrate fermentation or nitrate respiration (Strasters and Winkler, 1963; Burke and Lascelles, 1975). Oxygen plays a role in virulence gene regulation and the ability of the bacteria to persist in the host environment (Chan and Foster, 1998; Pragman et al., 2004; Ross and Onderdonk, 2000; Ohlsen et al., 1997).

**Biofilms**

A biofilm can be defined as a community of microorganisms attached to a surface by extracellular polymeric substances, which are produced by the bacteria themselves (Donlan, 2002). Bacteria in biofilms are physiologically distinct from their corresponding
planktonic bacteria, and they function in a coordinated way that resembles multi-cellular organisms (Davey and O'toole, 2000). Biofilms can consist of a single species of microbe or can be multi-species or even multi-kingdom groupings (Jefferson, 2004). An important aspect of the biofilm is that it forms a sticky matrix that typically contains mostly exopolysaccharides, but can also be compromised of proteins, nucleic acids, lipids, or other polymers (Flemming and Wingender, 2010). This matrix aids in the ability of the biofilm to resist antimicrobials and harmful chemicals (Otto, 2006). In bacteria, biofilm formation is the most common microbial lifestyle in both man-made and natural environments, and occurs on all surface types (Lindsay and Von Holy 2006). While planktonic (free-swimming) cultures have been used in many microbial studies of the past and present, planktonic cell studies do not always reflect the growth of bacteria in nature, and have provided a biased view of microbes living in the environment (Parsek and Fuqua, 2004).

Established biofilms are extremely hard to combat in living hosts because they are up to 1000-fold more resistant to antimicrobials than are planktonic cells (Brooun et al., 2000). This increased resistance can be partially attributed to the reduced growth rate of bacteria within biofilms or the wide variety of metabolic states within the biofilm (Kwon et al., 2008). As a result, many times in clinical settings a bacterial biofilm infection will relapse and it becomes necessary to frequently remove or replace the infected tissue or medical device (Goerke and Wolz, 2010; Reslinksi et al., 2009). In addition to being less susceptible to antibiotics, biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, and disinfectants better than planktonic bacteria (Jefferson, 2004). Some proposed reasons that bacteria form biofilms include: 1) protection from the
harmful conditions in the host environment (Gilbert et al., 2002), 2) division of labor where the bacteria within the biofilm perform different metabolic functions (Shapiro, 1998), 3) biofilms promote the exchange of genetic material (Nguyen et al., 2010), and 4) to remain fixed in a favorable niche, such as a nutrient-rich area (Jefferson, 2004).

**S. aureus Biofilms**

*S. aureus* biofilm formation plays an important role in many diseases such as native valve endocarditis (Chambers et al., 1983), osteomyelitis (Priest and Peacock, 2005), chronic wound infections (Siddiqui and Berstein, 2010), and chronic lung infections in cystic fibrosis patients (Rajan and Saiman, 2002). In clinical settings, biofilms of *S. aureus* can also form on the surfaces of medical devices and cause persistent infections (Zimmerli, 2006). In general, biofilm formation can be divided into four distinct phases: attachment, accumulation, maturation, and dispersal (Christensen et al., 1994).

**Attachment Phase**

The attachment phase is induced by environmental signals and may take only seconds to activate (Aparna and Yadav, 2008). These environmental signals vary by organism and include nutrient changes, pH, temperature, oxygen concentration, hydrodynamics, osmolarity, the presence of specific ions, and factors derived from the biotic environment (Goller and Romeo, 2008). Attachment is reversible, as some cells may detach from the substrata, which is crucial for the dissemination of bacteria to other colonization sites (Otto, 2008). The primary determinants of attachment in *S. aureus* are a group of surface associated proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that bind to human matrix proteins such as fibrinogen or fibronectin (Patti et al., 1994). Studies have shown that the
autolysin class of MSCRAMMs play a direct role in mediating the attachment of bacteria to plastic surfaces and human matrix proteins, which serves as a pre-requisite for biofilm formation (Heilmann et al., 1997, Heilman et al., 2003). Cell lysis and subsequent release of genomic DNA (eDNA) has also been shown to be an important mediator of attachment in S. aureus (Rice et al., 2007, Mann et al., 2009).

**Accumulation Phase**

In the accumulation phase, cells irreversibly bind and begin to multiply, causing cell aggregates become thicker and more layered (Aparna and Yadav, 2008). The mechanisms responsible for exopolysaccharide (EPS) production are also activated during this phase, allowing the growing biofilm to trap nutrients and encapsulate planktonic bacteria (Aparna and Yadav, 2008). In general, the biofilm matrix not only consists of exopolysaccharides, but a vast array of proteins, adhesins, and extracellular DNA (eDNA), which all contribute to the structural integrity of the biofilm (Branda et al., 2005). The nature of the biofilm matrix greatly depends on the growth conditions, medium, and substrates, as well as the species present within the biofilm (Lopez et al., 2010). The accumulation phase of S. aureus biofilm development also relies upon polysaccharide adhesins that promote the adhesive interactions between cells (Gotz, 2002). The polysaccharide intracellular adhesin (PIA), encoded by the icaABCD operon, is thought to be the main determinant of accumulation in S. aureus (Heilman et al., 1996). However, PIA production does not seem to be of universal importance for S. aureus biofilm formation, as PIA-independent biofilms have been demonstrated in certain strains of S. aureus (Rohde et al., 2007) and in Staphylococcus epidermidis (Arciola et al., 2006; Rohde et al., 2005). An example of PIA-independent accumulation
in *S. aureus* comes from hip and knee joint infection isolates of *S. aureus*, where it was found that the adhesive protein Aap substitutes for PIA (Rohde et al., 2007).

**Maturation and Dispersal Phases**

As cells continue to grow and divide, eventually a mature biofilm is developed that has a complex architecture consisting of bacterial microcolonies interspersed with less dense regions of the matrix that include water channels that carry nutrients and waste products (Stoodley et al., 1994; Costerton et al., 1994). At the end of the maturation phase, the biofilm reaches its maximal thickness (Aparna and Yadav, 2008). In the final phase, cell detachment or dispersal is observed, in which some of the bacteria develop the planktonic phenotype and leave the biofilm (Aparna and Yadav, 2008). Detachment can be caused by many factors, including external perturbations such as increased fluid shear, internal biofilm processes such as endogenous enzymatic degradations, or by the release of EPS or surface-binding proteins (Hall-Stoodley et al., 2004). Controlled detachment maintains a certain biofilm thickness and governs a specific rate of biofilm dissemination, which in *S. aureus* is regulated by the quorum-sensing system *agr* (Boles and Horswill, 2008). The Agr system is, to date, the most well-characterized regulator of *S. aureus* biofilm detachment.

**Nitric Oxide**

Nitric oxide (NO) is a small, hydrophobic molecule that can easily pass through membranes, and regulates a wide range of biological functions via the post-translational modification of proteins (Blaise et al., 2005). NO is an important bioregulatory molecule in the nervous, immune and cardiovascular systems of eukaryotes, and participates in the regulation of daily activities as well as cytotoxic events (Blaise et al., 2005). NO is formed by the oxidation of the amino acid L-arginine to give NO and citruline, in a
process catalyzed by nitric oxide synthase (NOS) (Marletta et al., 1998). NO undergoes reactions with oxygen, superoxide ions, and reducing agents to produce products that themselves are reactive toward particular targets, sometimes resulting in toxic events such as nitrosative stress (Hughes, 2008). Nitrosative stress occurs when the production of NO or other reactive nitrogen intermediates overwhelms the ability of the cell to remove them, resulting in damage to DNA, lipids and proteins (Hughes, 2008).

**Role of Nitric Oxide in Biofilms**

In a recent study, bacterial-derived NO has been shown to promote cell death and dispersal of *Pseudomonas aeruginosa* biofilms at low, sublethal concentrations (Barraud et al., 2006). In this study, two mutants were analyzed: a nitrite reductase mutant, $\Delta nirS$, which lacks the only known enzyme capable of NO production through anaerobic respiration, and an NO reductase mutant, $norCB$, which is incapable NO reduction to $N_2O$ (Barraud et al., 2006). The wild-type and mutant strains all exhibited normal biofilm development at 2 days growth. After 6 days of maturation however, significant differences between the mutants and wild-type were observed. The $\Delta nirS$ mutants showed much thicker biofilms that were confluent over the entire surface and showed no dispersal or cell death compared to the wild-type strain. Conversely, the $\Delta norCB$ mutant biofilms showed enhanced dispersal of cells, displayed hollow voids within the biofilm, and exhibited enhanced cell death compared to the wild-type (Barraud et al., 2006). This study showed that NO plays a role in biofilm development and dispersal in *P. aeruginosa*. More recent studies have shown that this holds true for other species as well, such as *Neisseria gonorrhoeae* (Falsetta et al., 2010). In *S. aureus*, it has been shown that acidified nitrite inhibits of biofilm formation, and that the
addition of NO scavengers abrogates this effect, suggesting that NO is either directly or indirectly involved (Schlag et al., 2007).

**Role of NO Metabolism in *S. aureus* Virulence**

Generation of NO by the host inducible NO synthase (iNOS) is an important mechanism of host resistance to pathogens (Fang, 2004). The presence of *S. aureus* stimulates the production of NO by human phagocytes (Shay et al., 2003), which has been observed in people with staphylococcal infections (Choi et al., 1998). However, *S. aureus* is able to resist the antimicrobial action of NO via its nitrosative stress response, which involves the transition into hypoxic/anaerobic metabolism (Richardson et al., 2006; Richardson et al., 2008). This ability of *S. aureus* to replicate when NO is present distinguishes it from many other pathogenic species (Richardson et al., 2008).

Aerobic respiration in *S. aureus* is inhibited by NO as it competitively binds the cytochrome hemes of the terminal oxidases (Richardson et al., 2008). As *S. aureus* transitions to anaerobic metabolism under nitrosative stress conditions, an increase in lactate dehydrogenase activity can be seen (Richardson et al., 2008). This increase in lactate dehydrogenase activity due to NO is relatively specific for *S. aureus*, and was not seen in similar pathogenic species such as *S. epidermidis* and *S. saprophyticus* (Richardson et al., 2008). After NO exposure, aerobic and fermenting *S. aureus* bacteria produced almost exclusively L-lactate (Richardson et al., 2008). This increase in L-lactate suggests that the major glucose metabolic pathways are being restricted by nitrosative stress, and this reflects the increase in lactate dehydrogenase production (Richardson et al., 2008). L-lactate dehydrogenase, encoded *ldh1*, is divergently transcribed from the NO-scavenging flavohemoglobin encoded by *hmp* (Richardson et al., 2008). The *hmp-ldh1* locus thus encodes not only L-lactate dehydrogenase, but a
also a detoxification system for host derived NO that prevents the detrimental consequences of NO on bacterial metabolism (Richardson et al., 2008). Production of the flavohaemoprotein, Hmp, by *S. aureus* is an effective adaptive response to nitrosative stress and neutralizes host-derived NO (Richardson et al., 2006). Hmp is responsible for about 90% of measurable NO consumption in *S. aureus* and is therefore critical for NO detoxification (Richardson et al., 2006). In addition to the increase in Ldh1 and Hmp, many other proteins have been shown to be upregulated in response to nitrosative stress. These include the DNA response regulator SrrA, which upregulates anaerobic gene expression, pyruvate formate lyase (PflB), and several other fermentation pathway enzymes (Hochgrafe et al., 2008). This indicates that the ability to switch to anaerobic metabolism during NO stress is critical for the resistance of *S. aureus* to this condition (Hochgrafe et al., 2008).

**Potential Routes of Endogenous NO Formation in *S. aureus***

Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) can be used as terminal electron acceptors under anaerobic conditions (Schlag et al., 2007). All bacterial nitrate reductases catalyze the reduction of nitrate to nitrite (Moreno-vivian et al., 1999). Nitrite produced by the *S. aureus* NarGHJI nitrate reductase can be further reduced to ammonia by a cytoplasmic NADH-dependent nitrite reductase encoded by the *S. aureus nirBD* genes, which are also referred to in the literature as *nasDE* (Schlag et al., 2008). Nitrite dissimilation mediated by NirBD does not generate a proton motive force and is not a respiratory pathway (Schlag et al., 2007). Rather, it serves to detoxify accumulating nitrite in nitrate-respiring cells and acts as an electron sink to regenerate NAD$^+$ (Schlag et al., 2007). All *S. aureus* strains sequenced to date contain genes encoding a bacterial NOS (saNOS) enzyme and Hmp, which play a role in the formation and consumption of NO.
in *S. aureus*, respectively, as depicted in Figure 1-1. Like their eukaryotic counterparts, bacterial NOS produces NO and citruline through the enzymatic oxidation of L-arginine (Marletta et al., 1998). As discussed above, Hmp is the major system used by *S. aureus* to detoxify NO, and requires microaerobic (or low oxygen) conditions in order to properly function (Nobre et al., 2008). NO can also be reduced to nitrous oxide (N\textsubscript{2}O) by NO reductase (Nor) (Hendricks et al., 2000). The *nor* gene is so-far only known to exist in the sequenced strain MRSA252 and the related methicillin-susceptible clinical strain UAMS-1 (Holden et al., 2004, unpublished data). The *nor* gene is not present in the sequenced genome of *S. aureus* strain Newman (Baba et al., 2008). The postulated pathways of NO production via acidified nitrite and nitrite reduction via NarGHJI depicted in Figure 1-1 will be further discussed below.

**Nitrate Reductases**

Both eukaryotic and bacterial nitrate reductases contain a pterin-based molybdenum cofactor at their active sites, and all bacterial nitrate reductases contain an iron-sulfur cluster (Moreno-Vivian et al., 1999). There are three types of bacterial nitrate reductases: 1) cytoplasmic assimilatory (Nas) 2) membrane-bound respiratory (Nar), and 3) periplasmic dissimilatory (Nap) nitrate reductases (Moreno-Vivian et al., 1999). The *narGHJI* genes of *S. aureus* encode a membrane-bound respiratory nitrate reductase (Moreno-Vivian et al., 1999). Nar-type nitrate reductases are dissimilatory, and generate a transmembrane proton motive force by using nitrate as electron acceptor in anaerobic conditions, allowing the synthesis of ATP (Moreno-Vivian et al., 1999). The Nar system is induced by nitrate and repressed by oxygen (Moreno-Vivian et al., 1999). NarG is a catalytic alpha subunit with an MGD cofactor (Blasco et al., 2001), NarH is a beta subunit with four iron-sulfur centers (Guigliarelli et al., 1996), and
NarI is a quinol-oxidizing gamma subunit (Sodergren et al., 1988). NarJ is not part of the final enzyme, but participates in the assembly of the alpha-beta complex (Dubourdieu and DeMoss, 1992)

**Regulators of nar**

There are currently two known regulators of the *S. aureus* narGHJI operon. NreABC is a very specific two-component regulatory system that in response to oxygen depletion activates the genes involved in dissimilatory nitrate reduction and transport of nitrate and nitrite (Schlag et al., 2008). Deletion of NreABC results in severe impairment of dissimilatory nitrate and nitrite reduction (Schlag et al., 2008). The promoters upstream of *nirR, narG*, and *narK* have been shown to be under the positive control of NreABC (Schlag et al., 2008). YhcSR is a two-component signal transduction system that is essential for survival of *S. aureus* (Yan et al., 2011). YhcSR regulates expression of both narGHJI and nreABC operons to positively modulate the nitrate respiratory pathway in anaerobic conditions (Yan et al., 2011). YhcR directly binds to the promoter regions of *narG* and *nreABC* to positively regulate their expression (Yan et al., 2011). In addition, under anaerobic conditions, addition of nitrate to the media dramatically increased expression of yhcSR, whereas the addition of nitrite had no effect (Yan et al., 2011).

Biofilm growth conditions also regulate the expression of the nar operon. In a *S. aureus* biofilm mode of growth the *narG, narH, and narI* genes of the nitrate reductase operon were found to be upregulated at least two-fold compared to planktonic cultures (Beenken et al., 2004). Anaerobic conditions also upregulate transcription of the nar operon as well as the nir operon (Fuchs et al., 2007). Given that many areas of mature *S. aureus* biofilm are low-oxygen or anaerobic microenvironments, it is possible that nar
expression is also upregulated in these areas of the biofilm. Although the studies mentioned above verify the importance of nitrate reductase during anaerobic and biofilm growth, little attention has been paid to studying the full effect of nitrate reductase on *S. aureus* biofilm development, nor has its role in endogenous NO production been investigated. In this respect, it has been shown that during *S. aureus* static biofilm growth, NO appeared to be produced in the presence of acidified nitrite, which was directly or indirectly involved in the inhibition of biofilm formation (Schlag et al., 2007). In this study by Schlag et al., preformed static biofilms were eradicated by the addition of either nitrate or nitrite, and furthermore the inhibition of biofilm formation by nitrite was abrogated by the addition of NO scavengers, suggesting that NO is involved (Schlag et al., 2007). Interestingly, Schlag et al. (2007) also demonstrated that a *S. aureus narG* mutant strain did not exhibit nitrate-responsive biofilm inhibition, but did display inhibition of biofilm formation in response to nitrite.

**NO Production by nar in Other Organisms**

Studies on NarGHJI nitrate reductases in organisms other than *S. aureus* provide valuable insight into the possible mechanisms of action of endogenous NO production by the *S. aureus* nitrate reductase. In *Salmonella typhimurium*, NarGHI mutants are unable to produce NO, which indicates that the enzyme is somehow responsible for NO production (Gilberthorpe and Pool, 2008). In addition, production of NO in the wild-type *Salmonella* strain only occurred in the absence of nitrate (Gilberthorpe and Pool, 2008). Nitrate reductases in plants have been known to generate NO from nitrite since the early 1980s (Harper, 1981; Dean and Harper, 1988). This occurs when the plant accumulates NO$_3^-$ or NO$_2^-$ under stress conditions such as anaerobic conditions, fungus infestation, or when photosynthetic activity is inhibited (del Rio et al., 2004). In
plants, the mechanism of NO generation from nitrite by nitrate reductase involves NADH as an electron donor and the molybendum cofactor likely serves as the catalysis site (Yamasaki et al., 1999; Rockel et al., 2002). The plant nitrate reductase also produces peroxynitrite simultaneously with NO (Yamasaki and Sakihama, 2000). The nitrate reductase of *P. aeruginosa* is also known to produce NO (Wharton and Weintraub, 1980). Therefore, it is possible that *S. aureus* nitrate reductase may contribute to endogenous NO production in one of two ways (Figure 1-1): 1) Direct enzymatic conversion of nitrite to NO or 2) indirectly by reducing nitrate to nitrite, a portion of which in turn is chemically converted to NO under low pH conditions.

**Hypothesis**

Based on the studies discussed above, we hypothesize that the *S. aureus* nitrate reductase is important for biofilm formation, possibly by contributing to endogenous NO production. To this end, this study will pursue two specific aims: 1) to determine if mutation of the *narGHJI* gene cluster in *S. aureus* strains Newman and UAMS-1 has an effect on biofilm phenotype and 2) to determine if nitrate reductase contributes to endogenous NO production. Studying both of these *S. aureus* strains will allow a comparison of the role of nitrate reductase in biofilm formation and NO production between a strain possessing two known NO-detoxifying genes (*nor* and *hmp*, in strain UAMS-1) versus a strain that only has one known NO-detoxifying system (*hmp* only, in strain Newman). Since we hypothesize that *nar*-dependent differences in biofilm formation could be due to altered NO levels, these two strains could have two very different responses to the *nar* mutation due to the presence or absence of the *nor* gene.
Figure 1-1. Postulated routes for NO formation and consumption in *S. aureus*. The potential routes for NO metabolism depicted above are based on the presence of these genes in the *S. aureus* MRSA252 sequenced genome (a closely-related strain to UAMS-1) (Holden et al., 2004), in combination with published data from *S. aureus* and/or other NO-producing bacteria. The potential routes of endogenous NO production are depicted in blue. The *nor* gene (depicted in red) is not conserved throughout all published *S. aureus* genomes, however it is present in strain UAMS-1 and in the published genome of MRSA252.
CHAPTER 2
METHODS AND MATERIALS

Bacterial Strains and Growth Conditions

*Staphylococcus aureus* strains and plasmids used in this study are listed in Table 2-1. Planktonic cultures of *S. aureus* were grown in tryptic soy broth (TSB) or biofilm media (TSB-NaGlc: TSB supplemented with 3% (wt/vol) NaCl and 0.5% (wt/vol) glucose). Where indicated, the following antibiotics where added: erythromycin (Erm), 2 μg/ml or 10 μg/ml, chloramphenicol (Cm), 10 μg/ml, and spectinomycin (Spec), 1000 μg/ml. *Escherichia coli* was grown in Luria-Bertani (LB) broth with 50 μg/ml ampicillin (Amp) or 50 μg/ml kanamycin (Km). Glycerol stocks were prepared by mixing an equal volume of overnight culture with 50% glycerol (vol/vol) in cryogenic tubes. Stocks were maintained at –80°C. For each experiment, fresh *S. aureus* culture was obtained from the frozen glycerol stocks and streaked onto tryptic soy agar (TSA) containing the appropriate selective antibiotic as listed in Table 2-1.

Creation of Mutants

Plasmid pBT2-nar and the nos, hmp, nre, nir, and nar mutants in *S. aureus* strain Newman were created by Dr. Anthony Richardson (University of North Carolina at Chapel Hill), while plasmid pCN51-nar and allele replacement mutagenesis of the nar operon (using pBT2-nar) in *S. aureus* strain UAMS-1 was performed in this study. Mutations used in this study and gene functions are listed in Table 2-2.

The pBT2-nar plasmid was generated by Dr. Richardson as follows: PCR amplification of the narGHJI genes from *S. aureus* strain COL was carried out using the nar-specific primers listed in Table 2-3. This fragment was cloned into vector pCR-BluntII, followed by transformed into *E. coli* strain W3110 Δdam using standard methods.
(Inoue et al., 1990) so that internal Clal sites would not be methylated, which would inactivate the cut sites. The plasmid was cut with Clal liberating an internal 3.5 kilo-base long nar fragment, and the remaining vector was then blunted by Klenow DNA polymerase and ligated with a blunted 1.4 kilo-base EcoRI fragment from plasmid pLZ-Sp12 containing a spectinomycin resistance (SpR) cassette. A 4.8 kilo-base fragment containing this nar::SpR allele with PvuII cut sites at each end was then cloned into the EcoRV of plasmid pBT2, creating the final plasmid pBT2-nar.

Plasmid pBT2-nar was subsequently used for the creation of a nar mutation in S. aureus strains Newman and UAMS-1, using the following allele replacement mutagenesis procedure: the pBT2-nar plasmid was first transformed into S. aureus strain RN4220 (a chemically-mutated S. aureus strain that more readily accepts foreign DNA) by electroporation using standard methods (Schenk and Laddaga, 1992). The RN4220 strain containing the pBT2-nar plasmid (grown at 30°C to allow plasmid replication) was then used to produce a phage lysate for the purpose of transferring the plasmid to strain UAMS-1. This was done using standard methods with a ϕ11 phage added to achieve a multiplicity of infection (MOI) of 0.1 (Novick, 1991). The plasmid was then phage transduced into UAMS-1 using standard methods and an MOI of 0.1 with growth at 30°C (Shafer and Iandolo, 1979). The integration of pBT2-nar into the nar gene on the UAMS-1 chromosome was achieved as follows: UAMS-1 was grown at 43°C (non-permissive temperature for plasmid replication) in the presence of spectinomycin to promote integration of the plasmid into the chromosome via homologous recombination at the nar gene locus. To promote a second recombination event, a single colony was used to inoculate TSB (no antibiotic) and grown at for five
days at 30°C. An aliquot of the culture was diluted 1000-fold into fresh TSB (no antibiotic) every 24 hours. On days 3-5, the culture was serially-diluted and spread on TSA containing spectinomycin. Isolated colonies were screened for spectinomycin resistant and chloramphenicol-sensitive phenotypes by picking and patching colonies onto TSA-Spec$^{1000}$ and TSA-Cm$^5$ plates. Verification that the potential knockout strains were correct was carried out using PCR amplification with nar complement primers (Figure 2-2).

**Creation of the Trans Complement Plasmid pCN-nar**

Complementation of the nar mutation in *S. aureus* strains Newman and UAMS-1 was carried out as follows: PCR amplification of the narGHJI operon from *S. aureus* strain Newman was performed using the nar-specific complement primers listed in Table 2-3, with *Sphl* and *BamHI* restriction sites engineered into the forward and reverse primer sites, respectively. The resulting 7.5 kilobase fragment was purified using a DNA Clean and Concentrator kit (Zymo Research). The nar PCR fragment and pCN51 plasmid vector (Charpentier et al., 2004) were both digested with *Sphl* and *BamHI* restriction enzymes. The pCN51 vector was then gel purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research) to liberate the native promoter. This was necessary so that the native nar promoter would be the sole promoter for controlling gene expression of the narGHJI operon to be inserted into the vector. The remaining pCN51 vector fragment was then ligated to the nar PCR product. The ligation was transformed into *E. coli* DH5α via heat shock using standard methods (Inoue et al., 1990) and plated on Luria-Bertani (LB) broth with 50 µg/ml ampicillin
(Amp). PCR was used to check transformants for the nar insert using nar internal primers (Figure 2-3).

To account for any effects the vector may have, a vector-only control plasmid previously created in Dr. Rice’s lab was used (University of Florida). In brief, this plasmid was constructed by digestion of the pCN51 vector with BamH1 and Sph1 restriction enzymes in order to separate the Pcad promoter from the rest of the vector. The purified vector minus the promoter was treated with Klenow enzyme to create blunt ends, followed by a self-ligation to regenerate a circular plasmid, which was then transformed into E. coli DH5α, creating plasmid pCN-klenow. The loss of the Sph1 restriction site was confirmed by enzymatic digestion with Sph1.

Plasmids pCN-nar and pCN-klenow were subsequently electroporated into S. aureus strain RN4220 using standard methods (Schenk and Laddaga, 1992). Electroporations were plated onto TSB with 2 μg/ml erythromycin (Erm) to select for the vector. Restriction digests of the plasmids using Sph1 enzyme were performed in order to assure that the insert was still present in pCN-nar (data not shown). A positive clone was selected for preparation of a phage lysate using the φ11 phage added to an MOI of 0.1 using standard methods (Novick, 1991). This lysate was then used for a phage transduction of pCN-nar and pCN-Klenow each into S. aureus Newman and UAMS-1 using standard methods with an MOI of 0.1 and growth at 37°C (Shafer and Iandolo, 1979). Resultant strains were named Newman nar pCN51-nar, Newman nar pCNKlenow, UAMS-1 nar pCN51-nar, and UAMS-1 nar pCNKlenow. When plasmids purified from these strains were checked by PCR with the nar internal primers, all appeared to contain the nar operon (Figure 2-4A). Subsequent restriction digestion of
these plasmids with SphI (which should linearize into a 12-kb fragment) revealed the predicted sized fragment in the UAMS-1 nar mutants (Figure 2-4B), however digestion of pCN-nar from the Newman nar mutant revealed the presence of multiple bands, possibly indicating the rearrangement of the plasmid in this strain (data not shown). Therefore, subsequent analysis of these complement strains solely focused on the UAMS-1 nar mutant.

**Biofilm Assays for Confocal Microscopy**

To determine phenotypic differences between the *S. aureus* Newman wild-type strain and the nreC, nir, and nar mutant strains, static biofilm assays were performed in conjunction with confocal microscopy. Wells of an optically-clear bottom 96-well tissue culture plate (Costar 3614) were pre-coated for 24 hours with 200 µl of 20% (vol/vol) human plasma (Sigma) in bicarbonate buffer (Sigma Carbonate-Bicarbonate capsules, 1 capsule dissolved per 100 ml of water). After coating, the plasma was removed from the wells and 200 µl of overnight culture of *S. aureus* in TSB-NaGlc diluted to an OD600 of 0.05 was added to each well. Cultures were grown for 24 hours at 37°C. Culture supernatants were then removed and LIVE/DEAD stain (Invitrogen) was added (1.5 µl/ml propidium iodide and 0.5 µl/ml Syto9 in 0.85% vol/vol NaCl). The stain was allowed to absorb at room temperature for 20-30 minutes under aluminum foil to preserve light-sensitivity. Stain was then removed and 200 µl of 0.85% (vol/vol) NaCl was added to each well. Static biofilm analysis of UAMS-1, its isogenic nar mutant, and complement strain was performed using the assay protocol described above.

Biofilm wells were subjected to imaging using a Zeiss Pascal LSM5 Confocal Laser Scanning Axiovert 200 Microscope using an Argon-laser and a 40x water
immersion lens. Two wells were inoculated for each biological replicate, and two representative images were taken of each well. At least three biological replicates were imaged per strain and condition, for a total of at least 10 acquired z-stacks. The z-stacks were taken at 1.0 μm z-slice intervals and a scanning speed of 8, on frame mode. The images were processed using the LSM Browser software (Zeiss) and biofilm characteristics were quantified using COMSTAT software for MatLab (Heydorn 2000). Sigma Plot was used to organize data and perform statistical analyses. A one-way Analysis of Variance (ANOVA) as well as a normality test and an equal variance test were performed on all data. Holm-Sidak test was performed on sets of data that passed the normality and equal variance tests. For comparisons of data that did not pass these tests, a Dunn’s test was performed to determine statistical significance. In all cases, a p-value of p<0.05 indicates statistical significance.

Measurement of Relative NO Production in Static Biofilms

To determine the different levels of NO produced by the S. aureus wild-type, mutant, and complement strains, biofilm assays were performed followed by DAF-FM diacetate staining for the quantification of NO. DAF-FM diacetate is a non-fluorescent molecule that can permeate the cell membrane, where it is then cleaved by intracellular esterases to release DAF-FM, which can then react with NO to produce a highly fluorescent Benzoltriazole derivative (Kojima et al., 1998). Biofilm assays were performed as previously described, except that 24-well plates were pre-coated with 350 μl of 20% (v/v) human plasma. After coating for 24 hours, the plasma was removed from the wells and 1 ml of overnight culture of S. aureus in TSB-NaGlc was added after being diluted to an OD₆₀₀ of 0.05. Cultures were grown for 8 hours at 37°C. The total well
biomass (adherent biofilm + supernatant) was resuspended by scraping off the surface of the well and pipetting to mix before being aliquoted into 1.5 ml microcentrifuge tubes. Suspensions were centrifuged at 13 RPM for 5 minutes, and supernatants were carefully removed by pipetting. Cell pellets were resuspended in 1 ml of 2.5 μM of DAF-FM diacetate in 0.85% NaCl. This was done in minimal lighting to preserve light sensitivity. 200 μl of each suspension were added in duplicate to a 96-well plate for fluorescence readings from a Biotek Synergy HT plate reader. The plate reader was set to read fluorescence from the bottom of the plate, and readings were taken using the Gen5 version 1.09 which took a RFU reading with filter settings of 495/20 excitation and 515/20 emission and sensitivity of 75. Although the density of cells in each well were similar, a corresponding OD₆₀₀ reading was also taken for each and used to calculate the RFU/OD₆₀₀ to account for slight variations in OD. Levels of DAF-FM fluorescence (proportional to the amount of intracellular NO) were recorded after 1 hour at 37°C. Data was collected for at least three biological replicates, where one biological replicate equals two wells, for each strain and condition. Sigma Plot was used to organize data and perform statistical analyses. A one-way Analysis of Variance (ANOVA) as well as a normality test and an equal variance test were performed on all data. Student-Newman-Keuls Tests were performed on sets of data that passed the normality and equal variance tests. For comparisons of uneven sample sizes that did not pass one or either of these tests, a Dunn’s test was performed to determine statistical significance. In all cases, a p-value of p<0.05 indicates statistical significance.

**Treatment Conditions**

Stocks of sodium nitrate (NaNitrate) and sodium nitrite (NaNitrite) were prepared by dissolving 5.10 grams of NaNitrate and 4.14 grams of NaNitrite each in 200ml of
deionized H$_2$O to achieve a final concentration of 0.3M, followed by filter-sterilization. All additions of nitrate or nitrite to biofilms were taken from these stocks. Biofilms were either left untreated or supplemented with 2, 4, 6, 8, or 10 mM NaNitrate or 10 mM NaNitrite at the time of inoculation. Where indicated, cultures were also supplemented with 1 mM of the NO scavenger carboxy-PTIO (cPTIO; Sigma) at the time of inoculation.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Host strain for construction of recombinant plasmids</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN4220</td>
<td>Easily transformable restriction deficient strain</td>
<td>Kreiswirth et al., 1983</td>
</tr>
<tr>
<td>Newman</td>
<td>Lab strain</td>
<td>Duthie and Lorenz, 1952</td>
</tr>
<tr>
<td>AR0495</td>
<td>Newman ΔnarGHJI:Spec&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Obtained from A. Richardson, UNC Chapel Hill</td>
</tr>
<tr>
<td>AR0100</td>
<td>Newman nos::Erm</td>
<td>Obtained from A. Richardson, UNC Chapel Hill</td>
</tr>
<tr>
<td>AR0094</td>
<td>Newman hmp::Erm</td>
<td>Richardson 2006</td>
</tr>
<tr>
<td>AR0570</td>
<td>Newman nirBD::Km</td>
<td>Obtained from A. Richardson, UNC Chapel Hill</td>
</tr>
<tr>
<td>AR0328</td>
<td>Newman nreC::Spec</td>
<td>Obtained from A. Richardson, UNC Chapel Hill</td>
</tr>
<tr>
<td>Newman nar pCN51-nar</td>
<td>Newman ΔnarGHJI + narGHJI gene complement</td>
<td>This work</td>
</tr>
<tr>
<td>Newman nar pCNKlenow</td>
<td>Newman ΔnarGHJI + vector control</td>
<td>This work</td>
</tr>
<tr>
<td>UAMS-1</td>
<td>Osteomyelitis clinical isolate</td>
<td>Gillaspy et al., 1995</td>
</tr>
<tr>
<td>UAMS-1 nar</td>
<td>UAMS-1 ΔnarGHJI:Spec&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>UAMS-1 nar pCN51-nar</td>
<td>UAMS-1 ΔnarGHJI + narGHJI gene complement</td>
<td>This work</td>
</tr>
<tr>
<td>UAMS-1 nar pCNKlenow</td>
<td>UAMS-1 ΔnarGHJI + vector control</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCN51</td>
<td><em>E. coli-S. aureus</em> shuttle vector with origin of replication for <em>S. aureus</em>; Amp&lt;sup&gt;R&lt;/sup&gt;, Erm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Charpentier et al., 2004</td>
</tr>
<tr>
<td>pBT2</td>
<td><em>E. coli-S. aureus</em> shuttle vector with thermosensitive origin of rep. for <em>S. aureus</em>; Amp&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bruckner, 1997</td>
</tr>
<tr>
<td>pBT2-nar</td>
<td>ΔnarGHJI homologous recombination vector; Spec&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Obtained from A. Richardson, UNC Chapel Hill</td>
</tr>
<tr>
<td>pCN51-nar</td>
<td>narGHJI gene complement in the pCN51 vector</td>
<td>This work</td>
</tr>
<tr>
<td>pCN Klenow</td>
<td>pCN51 vector without original Pcad promoter</td>
<td>Obtained from the lab of K.C. Rice, University of Florida</td>
</tr>
<tr>
<td>Gene/operon</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>narGHJI</em></td>
<td>membrane-bound respiratory nitrate reductase</td>
<td>Moreno-Vivian et al., 1999</td>
</tr>
<tr>
<td><em>nreABC</em></td>
<td>Transcriptional activator of genes involved in reduction and transport of nitrate and nitrite</td>
<td>Schlag et al., 2008</td>
</tr>
<tr>
<td><em>nirBD</em></td>
<td>reduces nitrite to ammonia</td>
<td>Neubauer et al., 1999</td>
</tr>
<tr>
<td><em>hmp</em></td>
<td>detoxifies NO to nitrate</td>
<td>Poole, 2005</td>
</tr>
<tr>
<td><em>nos</em></td>
<td>synthesizes NO from L-arginine</td>
<td>Hughes, 2008</td>
</tr>
</tbody>
</table>
Table 2-3. Primers used in this study

<table>
<thead>
<tr>
<th>Primer role</th>
<th>Forward/Reverse</th>
<th>Oligonucleotide sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>nar complement</strong></td>
<td>Forward</td>
<td>CCCGCATGCTGAAATTGTACCTGGTGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCGGATCCATTGCTTCTGGTGTAANATC</td>
</tr>
<tr>
<td><strong>nar locus for the creation of pBT-nar</strong></td>
<td>Forward</td>
<td>AATTTAATGGGAAATTGGTGCGATCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCTTTACCTCTTTATGGCTTACAC</td>
</tr>
<tr>
<td><strong>nar internal primers</strong></td>
<td>Forward</td>
<td>CGCCATTCTGCCACTTGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGCAAGTGAATGGAAACC</td>
</tr>
</tbody>
</table>

Figure 2-1. Diagram representing primer locations for the *nar* complementation primers (orange), *nar* internal primers (pink), and *nar* locus primers used in the creation of pBT-*nar* (green).
Figure 2-2. Confirmation of Δnar mutants in *S. aureus* UAMS-1. PCR reactions of *nar* mutants were performed with *nar* complement primers.
Figure 2-3. Confirmation that the *nar* complement plasmid is present and carries the *narGHJI* genes in *E.coli* DH5α. The presence of the *narGHJI* genes in the plasmid was confirmed by PCR reactions of *nar* complementation plasmid and *S. aureus* UAMS-1 DNA with *nar* internal primers.
Figure 2-4. Confirmation that the *nar* complement plasmid is present and carries the *nar*GHJI genes in *S. aureus* strain UAMS-1. A) PCR reactions of *nar* complementation plasmids and UAMS-1 DNA were performed with *nar* internal primers. B) Restriction digestion of *nar* complement plasmids with *SphI* enzyme, producing a linear 12-kb fragment.
CHAPTER 3
RESULTS

Effect of Nitrate Metabolism on S. aureus Biofilms

To determine if the nar, nir, or nreC mutations in S. aureus strain Newman have an effect on its biofilm phenotype, wild-type and nar mutant biofilms were grown as untreated static biofilms or in media supplemented with either 10 mM NaNitrate or 10 mM NaNitrite. The LIVE/DEAD stain used in this study utilizes a green (Syto 9) and red (propidium iodide) dye to differentiate between live (green) and dead (red) cells. The Syto 9 labels both live and dead bacteria, however the propidium iodide penetrates only dead or damaged bacteria, with the red stain fluorescence dominating the green fluorescence. Using this method of biofilm visualization, confocal microscopy after 24 hours of biofilm growth revealed qualitative differences in the overall structure and thickness of the nar, nir, and nre mutant biofilms compared to wild-type (Figures 3-1, 3-2, and 3-3). To analyze the data in quantitative terms, COMSTAT and Holm-Sidak statistical analyses were performed on all the confocal biofilm data to measure differences in the average thickness and biomass of the biofilms (Figure 3-4A and B). Specifically, biofilms of wild-type Newman treated with nitrate and nitrite had significantly more total biomass and average thickness than its respective untreated biofilm. Interestingly, the nar mutant biofilm had significantly increased biomass under untreated growth conditions compared to the wild-type strain. Furthermore, treatment of the nar mutant biofilm with nitrate did not alter its biomass or average thickness, but treatment with nitrite did increase these parameters. These results suggest that nitrate reduction has a significant effect on biofilm morphology under these in vitro growth conditions.
A nitrite reductase (nir) mutant was also analyzed by confocal microscopy to test if the effect of nitrate on the biofilms is due to increased production of ammonia (produced by Nir from nitrite). Additionally, an nre (positive regulator of nar and nir expression) mutant was also analyzed to see if perturbing the regulation of nar and nir expression has a similar phenotypic effect as the nar and/or nir mutants. The nir mutant biofilm displayed increased biomass and average thickness in the untreated condition compared to wild-type, however the nre mutant biofilm was similar to the wild-type strain under untreated conditions (Figure 3-4A and B). In addition, nitrate treatment had no real effect on nre, whereas nitrate treatment caused an increase in biomass and average thickness in nir compared to the untreated condition. Furthermore, nitrite treatment had no effect on nir, but resulted in increased biomass and average thickness in nre compared to the untreated condition, which was also seen in the nar mutant biofilm. Confocal images of 24-hour biofilms are in agreement with these COMSTAT analyses (Figures 3-2 and 3-3). This suggests that Nre may not be playing a large role in regulating nir transcription under these conditions, however is likely very involved in nar regulation. Overall, these results demonstrate that perturbations in the nitrate metabolism pathway in the form of nar, nir, and nre mutations results in an altered phenotype when nitrate or nitrite is added to the biofilm. Furthermore, since the nir mutant biofilm responded to nitrate treatment in a manner similar to the wild-type strain (Figure 3-4A and B), this suggests that the observed effects of nitrate on biofilm morphology are due to accumulation of nitrite and not due to increased production of ammonia.
NO Production Varies Between Wild-type and Mutants

Even though the results above have suggested that nitrate metabolism has an effect on biofilm development, the reason for this is still unclear. One possibility that was discussed in Chapter 1 is that Nar may produce NO, which in turn could impact biofilm development. NO production by Nar is known to occur in a variety of different organisms, such as *Salmonella* (Gilberthorpe and Pool, 2008), *Pseudomonas* (Wharton and Weintraub, 1980), and in plants (Harper, 1981), but whether or not this occurs in *S. aureus* is currently unknown. Therefore, to ascertain the relative levels of NO production by wild-type Newman and *nar, nir, nos, hmp*, and *nre* mutant strains, DAF-FM diacetate assays of 8-hour biofilms, either untreated or grown in the presence of 10mM NaNitrate or NaNitrite were performed (Figure 3-5). Untreated biofilms of all the strains were similar with regards to DAF-FM fluorescence (about 2000-2500 RFU/OD), indicating that relative NO levels did not differ between Newman and its isogenic mutant strains, or that differences were too subtle to be detected by this method. However, a drastic difference in the relative levels of NO produced in the *nar* mutant and wild-type biofilms was observed when grown in the presence of nitrate or nitrite. The *nar* mutant displayed significantly less DAF-FM fluorescence than the wild-type in both treatment conditions, indicating that much less NO is being produced under these growth conditions. Interestingly, the addition of nitrate to the wild-type biofilm resulted in higher levels of NO produced than the addition of nitrite to the biofilm. The *nre* mutant resulted in similarly low NO levels as the *nar* mutant under both treatment conditions. The *hmp* (detoxifies NO to nitrate) mutant served as a control in this experiment, as the mutant should accumulate higher levels of NO than the wild-type strain. As expected, the *hmp* mutant did indeed result in the highest levels of NO when treated with either nitrate or
nitrile. The *nir* (reduces nitrite to ammonia) mutant resulted in slightly more NO produced in the nitrate condition than the wild-type, however the wild-type and *nir* mutant showed no difference in the nitrite condition. The *nos* mutant resulted in no significant difference compared to wild-type in either treatment condition. These results demonstrate that perturbations in the nitrate metabolism pathway in the form of *nar, nir, hmp*, and *nre* mutations results in an different levels of relative NO production when nitrate or nitrite is added to the biofilm. Given these results and the previous COMSTAT results, it can be gathered that these mutations result in both a phenotypic change in the biofilm and an altered level of NO production in the biofilm in comparison to the wild-type.

**Nitrate Levels Affect NO Production**

To determine if the amount of nitrate added to the biofilms is proportionally related to increased production of NO, DAF-FM diacetate assays were performed on 8-hour wild-type biofilms that were grown in the presence of increasing levels (2, 4, 6, and 8mM concentrations) of NaNitrate at the time of inoculation (Figure 3-6). The levels of NO (as indicated by DAF-FM staining) increased in comparison to the untreated condition starting at 4 mM nitrate, with increasing levels observed as the amount of nitrate increased. As a control, parallel cultures were incubated in the presence of carboxy-PTIO, an NO scavenger, which was added at the time of inoculation (Figure 3-6). Surprisingly, cPTIO was only able to partially abrogate the levels of NO (as indicated by DAF-FM fluorescence) at nitrate concentrations above 4mM.

**Analysis of the *nar* Mutation in UAMS-1**

To assess the effects of the *nar* mutation in an *S. aureus* strain with the ability to detoxify NO using two pathways: an Hmp pathway (also present in Newman) and a Nor
pathway (not present in Newman), a knockout of the nar gene locus was created in the S. aureus strain UAMS-1. Wild-type and nar mutant biofilms were grown in the presence and absence of 10mM NaNitrate for 24 hours before being treated with LIVE/DEAD staining. Qualitative differences were observed between the wild-type and the nar mutant in the presence of NaNitrate (Figure 3-7D and E), however the untreated biofilms were very similar in appearance (Figure 3-7A and B). When NaNitrate was added to the biofilm, cell density and thickness increased in the wild-type biofilm, while the nar mutant did not show this effect. To further analyze the data in quantitative terms, COMSTAT and statistical analysis using a Student-Newman-Keuls test were performed on the biofilm data for the wild-type and nar mutant (Figure 3-8). These results confirmed that there was no significant difference in the average thickness or biomass between the wild-type and nar mutant in the untreated condition. However, when 10mM of NaNitrate was added to the biofilms, the wild-type biofilm had significantly more biomass and average thickness compared to its untreated control. Nitrate treatment had no effect on the nar mutant biofilm in terms of average thickness, and surprisingly, biomass was slightly less than the untreated nar mutant biofilm.

Additionally, DAF-FM diacetate assays were performed on 8-hour biofilms of the UAMS-1 wild-type and nar mutant to determine the relative levels of NO produced (Figure 3-9). This experiment showed that the trend of NO production in UAMS-1 and the nar mutant was very similar to that seen in the Newman wild-type and nar mutant, where mutant NO levels were significantly lower than wild-type (Dunn’s test) when grown in the presence of nitrate. Levels of NO (indicated by fluorescence) in the untreated conditions of both wild-type and nar are extremely low compared to the large
RFU/OD value of the wild-type strain treated with nitrate. Interestingly, nitrite treatment resulted in only a slight increase in DAF-FM fluorescence in UAMS-1 compared to its untreated control, which was much more subtle compared to the results observed when Newman was treated with nitrite (Figure 3-5). As was observed with the Newman nar mutant, the addition of nitrate or nitrite to the UAMS-1 nar mutant biofilm did not result in increased NO production. Nitrite addition actually resulted in statistically lower levels of relative NO production in the nar mutant. We can conclude from these results that the nar mutation has different effects on biofilm phenotype and NO production in S. aureus strains Newman and UAMS-1. Key similarities do exist, however, such as the failure of the mutants in both strains to produce the large amounts of NO that are seen in each wild-type when nitrate is added. Also, while both wild-type strains showed an increase in biomass and average thickness in nitrate-treated biofilms, mutation of the nar in both strains abolished this response to nitrate treatment.

**Analysis of nar Complementation in UAMS-1**

The S. aureus UAMS-1 nar mutant was transformed with a plasmid containing the nar operon with its native promoter cloned upstream, and assessed for complementation of its biofilm phenotype and relative NO production. Surprisingly, this strain did not show complementation of the nar mutant biofilm phenotype or relative NO production when subjected to confocal microscopy and DAF-FM diacetate experiments (data not shown). One possibility accounting for this was that since these experiments were carried out in the absence of antibiotic selection (both overnight culture growth and subsequent biofilm growth), the plasmid may have been lost. Further investigation pertaining to the stability of the plasmid was carried out, whereby the complementation strain was streaked for isolation on a TSA/Erm\(^2\) agar plate and incubated at 37°C.
overnight. The next day, one colony was used to inoculate a culture tube containing 3mL TSB without antibiotic and incubated at 37°C shaking at 250 RPM overnight. The next day this step was repeated with subculturing into a new tube. On the final day, the culture was serially diluted and plated onto both TSA plain and TSA/Erm² agar plates. The total colonies on each plate were counted and used to calculate the CFU/ml of each plate. The results showed a significant decrease in the amount of colonies on the TSA/Erm² plate (1.04 x 10⁸ CFU/ml) compared to the TSA plate (6.1 x 10⁹ CFU/ml). This indicated that the plasmid was lost in 98.3% of the bacterial cells when antibiotic selection was not used in the growth medium. To remedy this, a preliminary biofilm confocal microscopy experiment was performed whereby antibiotic selection was used in all steps of the protocol. Confocal microscopy results of this preliminary experiment suggested that the nar complementation strain restored the biofilm phenotype back to the wild-type phenotype, since NaNitrate addition resulted in a biofilm with more cell density and thickness than the untreated condition in the complement strain, thus resembling the wild-type treated with NaNitrate (Figure 3-7C and F). Future work will entail repeating the confocal microscopy and DAF-FM studies on the complement strain in the presence of antibiotic selection.
Figure 3-1. Effect of the *nar* mutation on biofilm formation. Depicted are representative orthogonal views of 24 hour static biofilms. The large square in each image represents the top down view whereas the side panels are orthogonal (side) views. The cells are stained with LIVE/DEAD stain whereby red cells are dead or damaged and the green cells are live. A) Newman, B) Newman + NaNitrate, C) Newman + NaNitrite, D) Newman *nar*, E) Newman *nar* + NaNitrate, F) Newman *nar* + NaNitrite. Images were acquired at 400x magnification by confocal microscopy, and are each representative of 18 random fields of view acquired in 3 independent experiments.
Figure 3-2. Effect of the nirBD mutation on biofilm formation. Depicted are representative orthogonal views of 24 hour static biofilms. The large square in each image represents the top down view whereas the side panels are orthogonal (side) views. The cells are stained with LIVE/DEAD stain whereby the red cells are dead or damaged and the green cells are live. A) Newman, B) Newman + NaNitrate, C) Newman + NaNitrite D) Newman nir, E) Newman nir + NaNitrate, F) Newman nir + NaNitrite. Images were acquired at 400x magnification by confocal microscopy, and are each representative of 10-18 random fields of view acquired in 3 independent experiments.
Figure 3-3. Effect of the nreC mutation on biofilm formation. Depicted are representative orthogonal views of 24 hour static biofilms. The large square in each image represents the top down view whereas the side panels are orthogonal (side) views. The cells are stained with LIVE/DEAD stain whereby the red cells are dead or damaged and the green cells are live. A) Newman, B) Newman + NaNitrate, C) Newman + NaNitrite D) Newman nre, E) Newman nre + NaNitrate, F) Newman nre + NaNitrite. Images were acquired at 400x magnification by confocal microscopy, and are each representative of 10-18 random fields of view acquired in 3 independent experiments.
Figure 3-4. COMSTAT analysis of wild-type (Newman) and nar, nir, and nre mutant biofilms. A) Biomass, B) Average thickness. Data represents average ± standard error (SEM) of at least 10 z-stack measurements acquired in at least 3 independent experiments. One asterisk (*) represents statistical significance of the nar, nir, and nre mutant biofilms compared to Newman wild-type under the untreated condition. Two asterisks (**) represent statistical significance of nitrate and nitrite treatment compared to the untreated control of each respective strain. A Holm-Sidak test was used with a P-value of p<0.05 indicating statistical significance.
Figure 3-5. Relative NO production by wild-type (Newman) and *nar, nir, nos, hmp*, and *nre* mutants, as measured by DAF-FM diacetate staining. DAF-FM fluorescence was measured as relative fluorescence units per OD\textsubscript{600} of each sample (RFU/OD). 10 mM of Na-nitrate or Na-nitrite were added as indicated on the graph. Results represent the average ± SEM of 3 independent experiments. Asterisks (*) represent statistically significant measurements when compared to the corresponding Newman measurements under the same condition. A Student-Newman-Keuls test was used with a P-value of p<0.05 indicating statistical significance.
Figure 3-6. Relative NO production in nitrate-treated cultures by wild-type (Newman) as measured by DAF-FM diacetate staining. Fluorescent data is reported as the average RFU/OD ± SEM. Varying amounts of NaNitrate with and without 1 mM cPTIO (an NO scavenger) were added as indicated on the graph. Results represent the average of 3 biological replicates. A Student-Newman-Keuls test was used with a P-value of p<0.05 indicating statistical significance. Asterisks (*) represent statistically significant measurements when compared to the corresponding nitrate + cPTIO treatment measurements.
Figure 3-7. Effect of the nar mutation on biofilm formation in UAMS-1. Depicted are representative orthogonal views of 24 hour static biofilms. The large square in each image represents the top down view whereas the side panels are orthogonal (side) views. The cells are stained with LIVE/DEAD stain whereby the red cells are dead or damaged and the green cells are live. A) UAMS-1, B) nar mutant, C) nar complement D) UAMS-1 + NaNitrate, E) nar + NaNitrate, F) nar complement + NaNitrate. Images were acquired at 400x magnification by confocal microscopy, and are each representative of 12 random fields in 3 biological replicates, with the exception of the complement (panels C and F), which represent 4 random fields of view and 1 biological replicate.
Figure 3-8. COMSTAT analysis reveals statistically significant differences between wild-type (UAMS-1) and the nar mutant. A) Biomass, B) Average thickness. Data represents average ± SEM of 12 z-stack measurements acquired from 3 biological replicates. Asterisks (*) represent statistically significant measurements when compared to the corresponding untreated condition. A Student-Newman-Keuls test was used with a P-value of $p<0.05$ indicating statistical significance.
Figure 3-9. Relative NO production by wild-type (UAMS-1) and nar mutant as measured by DAF-FM diacetate staining. DAF-FM fluorescence was measured as relative fluorescence units per OD$_{600}$ of each sample (RFU/OD). 10 mM of Na-nitrate or Na-nitrite were added as indicated on the graph. Results represent the average ± SEM of 3 independent experiments. A Dunn’s test was used with a P-value of p<0.05 indicating statistical significance. Asterisks (*) represent statistically significant measurements when compared to the corresponding untreated measurements.
CHAPTER 4
DISCUSSION

The wild-type *S. aureus* Newman strain, when treated with NaNitrate or NaNitrite, produced biofilms with significantly more biomass and average thickness, as did the *S. aureus* UAMS-1 strain when treated with NaNitrate. This suggests that nitrate and nitrite metabolism have a positive effect on biofilm formation. The *nar* mutation in *S. aureus* strains Newman and UAMS-1 resulted in an altered phenotype compared to the wild-type in NaNitrate-treated biofilms, indicating that nitrate reductase does have an effect on biofilm formation in *S. aureus*. Although preliminary, the complementation of the *narGHJI* genes in the UAMS-1 *nar* mutant shows that the replacement of these genes back into the *nar* knockout strain restores wild-type phenotype, suggesting that it is the *nar* genes that are producing the effect, and not some other factor.

Analysis of NO production in the Newman and UAMS-1 wild-type strains and their respective *nar* mutants showed that the *nar* mutants produce much less NO than wild-type when treated with either NaNitrate or NaNitrite. This suggests that the nitrate reductase plays a role in endogenous NO production in *S. aureus* under conditions where high levels of nitrate and nitrite are present, and represents a previously unrecognized function of this enzyme in *S. aureus*. Furthermore, the *nre* mutant (regulator of *nar*) in the Newman strain also showed drastically decreased NO levels compared to wild-type, which is what would be expected since *nar* expression in the presence of nitrate is dependent on *nreABC* (Schlag et al., 2008). It was expected that the *hmp* mutant would have a very high level of NO accumulation, since Hmp is a detoxifier of NO, which is exactly what was observed in this study. The *nir* mutant cannot reduce nitrite to ammonia, so it would be expected that higher levels of NO
would be seen in this strain since nitrite would accumulate. Surprisingly, increased relative NO levels were observed in the nir mutant strains in the presence of added nitrate compared to added nitrite. This suggests that exogenous nitrite may not be efficiently taken up by cells under these growth conditions, or alternatively, exogenous nitrite may not be stimulating high-level expression of narGHJI. Both of these possibilities would explain why levels of NO were higher when these biofilms were grown with nitrate as opposed to nitrite. To verify these possibilities, future work will focus on examining nar and nir transcription as well as measuring intracellular nitrite levels under the growth conditions used in this study.

The nos mutant has lost the ability to produce NO through via NO synthase, however the NO levels in the nos mutant were similar compared to wild-type. This could be because other pathways (i.e. nitrate reductase) are greater contributors to NO production compared to NOS under the growth conditions used in this study. Alternatively, NO production by NOS may be at too low or transient levels to be detected by reaction with DAF-FM, or may be occurring at a phase of biofilm development (i.e. earlier or later than 8 hours growth) that was not investigated in these studies. In addition, NO production in the wild-type shows that addition of NaNitrate results in significantly more NO produced than NaNitrite. As mentioned above, this could be due to the fact that nar expression is induced at a higher level in the presence of nitrate compared to nitrite, thus resulting in increased NO production in the presence of nitrate. Indeed, we see that when varying levels of NaNitrate were added to the biofilms, each increase in NaNitrate resulted in a corresponding increase in NO production. Also, the addition of an NO scavenger (carboxy-PTIO) reduced the levels of
NO detected, suggesting that it is specifically NO that is being detected in the experiment. That said, not all of the DAF-FM fluorescence detected was diminished by the scavenger, which could be due to other reactive nitrogen species such as N$_2$O$_3$ possibly reacting with DAF-FM, as this has been shown to be the case with the related fluorescent probe DAF-F2 (McQuade and Lippard, 2010).

While the NarGHI nitrate reductase of *Salmonella typhimurium* produces NO only in the absence of nitrate, our study suggests that the nitrate reductase of *S. aureus* functions on the opposite manner, with NO produced in the presence of nitrate. This is likely due to the regulators of the *S. aureus* narGHJI, NreABC and YhcSR, which both are induced in anaerobic conditions in the presence of nitrate (Schlag et al., 2008; Yan et al., 2011). It is possible that efficient NO production via *S. aureus* nitrate reductase occurs in a sequential fashion, whereby nitrate is first reduced to nitrite, and once nitrate is depleted, the accumulated intracellular nitrite is then reduced to NO via Nar. To investigate this, future experiments will include a time-course study to carefully monitor NO production, nitrate, and nitrite levels, during *S. aureus* biofilm growth. In addition, all current knowledge thus far has lead to the belief that NO promotes cell death and dispersal of biofilms, such as in *P. aeruginosa* (Barraud et al., 2006) and previous studies on *S. aureus* (Schlag et al., 2007). Our current study found that in the wild-type strains Newman and UAMS-1, nitrate addition to the biofilm results in an increase in NO production, and surprisingly, also an increase in biomass and average thickness. This suggests that under the biofilm growth conditions tested here, NO is having the opposite effect than what the previous literature would predict. In the case of *S. aureus*, these differences in results may be due to the fact that Schlag et al. (2007) performed their
studies with a *S. aureus* strain that is dependent on PIA for biofilm formation, whereas the strains used in this thesis study do not require PIA for biofilm formation *in vitro* (Fluckiger et al., 2005; Beenken et al., 2004). Future work to directly implicate NO as the effector of the nitrate and/or nitrite-dependent biofilm phenotypes observed in the studies presented in this thesis should include an experiment that would introduce NO exogenously to the biofilms. If the results of this study can be reproduced by artificially introducing NO to the cell, then this would provide direct evidence that NO is responsible for the biofilm phenotype observed between the wild-type and the *nar* mutation.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Sara Elizabeth Holman grew up in Bloomington, MN, and graduated from Bloomington’s John F. Kennedy Senior High School in 2005. Upon graduation, she attended the University of South Dakota, where she competed for the swim team while pursuing a Bachelor of Science in biology, which she completed in 2009. She went on to attend graduate school at the University of Florida and achieved a Master of Science in microbiology and cell science in August of 2011. Sara plans on becoming an Officer in the United States Air Force with a specialty in scientific research.