

Inhibition of Staphylococcal Cell-To-Cell Communication and  
Virulence by the Innate Immunity Mediator Nitric Oxide

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A dissertation

submitted in partial fulfillment of the  
requirements for the degree of

Doctor of Philosophy

University of Washington

2017

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Program Authorized to Offer Degree:

Microbiology

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**Abstract**

Disruption of Staphylococcal Cell-To-Cell Communication and  
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Nitric oxide (NO) is a major mediator of host innate immunity with antimicrobial activity against a broad range of pathogens. Specific targeting of protein metal centers, thiols, and other radicals can disrupt microbial metabolism and limit pathogen growth during infection. The opportunistic pathogen *Staphylococcus aureus* is relatively resistant to NO-mediated growth inhibition, yet NO remains important to control infection. A possible mechanism by which host NO is protective, beyond growth inhibition, may be through the direct targeting of systems that regulate the production of virulence factors, such as toxins. In *Staphylococcus aureus*, cell-to-cell communication known as quorum sensing regulates virulence and determines whether interactions with a mammalian host are commensal or pathogenic. Despite the importance of quorum sensing to infection, little is known about how host immunity affects inter-bacterial communication. In this thesis, I show that NO, a bacteriostatic effector of innate immunity, suppresses virulence by targeting the staphylococcal Agr quorum sensing system. Inhibition of

Agr results from the direct modification of cysteine residues C55, C123, and C199 of the AgrA transcription factor. Cysteine modification decreases AgrA promoter occupancy and transcription of the *agr* operon and quorum sensing-activated toxin genes. In a murine model of staphylococcal pneumonia, mice lacking inducible NO synthase developed more severe disease, elicited higher pro-inflammatory cytokine responses, and displayed different histopathology compared to wild-type mice. These findings point toward a novel anti-virulence role for NO.

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## LIST OF ABBREVIATIONS

AIP: auto-inducing peptide	NO <sub>2</sub> : nitrogen dioxide
α-KDH: α-ketoglutarate dehydrogenase	NO <sub>2</sub> <sup>-</sup> : nitrite
bNOS: bacterial nitric oxide synthase	NO <sub>3</sub> <sup>-</sup> : nitrate
DEA/NO: diethylamine NONOate	N <sub>2</sub> O: nitrous oxide
eNOS: endothelial nitric oxide synthase	N <sub>2</sub> O <sub>3</sub> : dinitrogen trioxide (nitrous anhydride)
GFP: Green Fluorescent Protein	OONO <sup>-</sup> : peroxyntirite
IFN-γ: interferon-gamma	PAMP: pathogen-associated molecular pattern
iNOS: inducible nitric oxide synthase	PAPA/NO: propylamine propylamine NONOate
L-NMMA: L-N <sup>G</sup> -monomethyl arginine	PDH: pyruvate dehydrogenase
LPS: lipopolysaccharide	PSMs: phenol soluble modulins
LTA: lipoteichoic acid	QS: quorum sensing
MSCRAMMS: microbial surface component recognizing adhesive matrix molecules	RFP: Red Fluorescent Protein
nNOS: neuronal nitric oxide synthase	
NO: nitric oxide	

## ACKNOWLEDGMENTS

First and foremost, I thank my current mentor Dr. Ferric C. Fang for giving me the opportunity to form part of his laboratory. I greatly appreciate the liberty and trust placed in me to explore my own ideas and train to become an independent scientist. I appreciate the optimistic outlook on my research project, his encouragement, patience, and limitless suggestions. Overall, I admire his drive to be scientifically rigorous and to tackle challenging and exciting biological questions. I also thank Ferric for creating a scientifically stimulating environment and recruiting highly motivated scientists.

I give special thanks to Fang lab members Dr. Steve J. Libby, Dr. Elaine R. Frawley, and Joyce E. Karlinsey for their generosity with their time, selfless technical support, and insightful scientific discussions. Steve, I greatly appreciate your gregarious personality, good sense of humor, and willingness to help others. Equally important was your support outside of the lab, helping me with my automotive needs, dragging me to the gym, and your invitations to sample the wine out in Woodinville. Helen Warheit-Niemi and Bianca Pérez Dunn, thank you for placing your trust in me and allowing me to begin developing my mentoring skills. Your fresh excitement and curiosity reminded me of my own scientific outlook upon starting graduate school and motivated me to push beyond the challenges of science.

Some of my most productive and exciting times in graduate school were made possible through collaborations. I am indebted to Dr. Alexander R. Horswill for hosting me during my visit to University of Iowa, his generosity with reagents, and for providing feedback throughout the years via writing and in person at scientific conferences. I thank Dr. Harry Ischiropoulos and Dr. Paschalis-Thomas Doulias for sharing with us their proteomic tools and knowledge on nitric

oxide biochemistry. I also thank Dr. Denny H. Liggitt for sharing his expertise on histopathology.

I am grateful to my thesis committee for their attention, interest in my work, constructive criticism, and helpful suggestions. Dr. Beth A. Traxler and Dr. Everett P. Greenberg, I am especially thankful for kindly accepting to be part of my reading committee. Pete, your work on quorum sensing inspired me to come to Seattle. Beth, I thank you for going beyond being just a committee member, for your continuous interest in my research project, and for your support as my academic advisor. Dr. Colin Manoil, I appreciated your attentive listening and input at committee meetings. I also thank Dr. Samuel I. Miller for appearing to sleep, but then asking good questions at meetings. Lastly, I thank Dr. Houra Merrikh who I consider an honorary member of my committee for always asking me about my project and offering career advice.

Lastly, I thank the University of Washington Microbiology graduate students for creating a warm, friendly, and supportive environment. Special thanks to Dr. Kyle C. Costa, Dr. Kevin G. Hicks, Dr. Alistair B. Russell, Dr. Mayim E. Wiens, Dr. Merika T. Koday, and Alan J. Bohn for sharing time over brews and creating a fun and welcoming community.

This work was supported by NIH grants T32 AI55396 (Rodolfo Urbano), AI39557 (Ferric C. Fang), and AI123124 (Ferric C. Fang).

## **DEDICATION**

This thesis is in part dedicated to my past mentors, Dr. Victor Nizet, Dr. Nicholas J. Webster, and Dr. Brian Palenik who inspired me to follow this path. Your passion for science motivated me to pursue graduate school and incited my interest in microbiology.

Ultimately, I dedicate my thesis to my parents Mario T. Urbano and Ernestina A. Urbano for their sacrifice to give me the opportunity to pursue a higher education. I greatly value your dedication and hard work to improve our family's condition. I hope one day I can begin to repay you for your effort and support. Please forgive the emotional and physical distance my academic pursuits have created in our family.

# Chapter 1. Introduction to Nitric Oxide

## 1.1 Nitric oxide as an innate immunity effector

### 1.1.1 Integral roles of nitric oxide in human physiology

Nitric oxide (NO) is a reactive nitrogen species with important physiological roles as a signaling molecule and as an antimicrobial effector of innate immunity. The versatility arises from its biochemical properties as an uncharged free radical gas with permeability across biological membranes and reactivity with cellular iron, thiols, and other radicals. Both non-enzymatic and enzymatic processes can produce NO in the body. Non-enzymatic NO is primarily generated in the stomach where it serves antimicrobial functions. The process begins in the mouth where commensal bacteria reduce salivary and dietary nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ) and continues in the acidic stomach environment where nitrite is protonated to nitrous acid ( $\text{HNO}_2$ ), which readily dismutates to NO (Lundberg and Weitzberg, 2009; Lundberg et al., 2008). In the stomach, NO enhances the antimicrobial properties of gastric acid and creates a more hostile environment for pathogens to survive (Björne et al., 2006; Dykhuizen et al., 1996). In contrast, enzymatically-generated NO is not derived from nitrate but instead produced from L-arginine and oxygen by three NO synthase isoforms (neuronal, nNOS; endothelial, eNOS; inflammatory or inducible, iNOS) that are separately encoded and expressed at distinct tissue sites (Marletta, 1994; Nathan and Xie, 1994). Calcium-dependent nNOS and eNOS are constitutively expressed in neurons and endothelial cells where they function in neuronal signaling and modulate vascular tone (Bredt and Snyder, 1992; MacMicking et al., 1997; Palmer et al., 1987). The inducible nitric oxide synthase (iNOS) is not calcium-regulated and is primarily induced at the transcriptional level by cells of the immune system to serve in immunomodulatory and antimicrobial roles. The role of

NO as an antimicrobial molecule has been primarily studied in macrophages, but iNOS is also expressed by other cell types, including neutrophils and nasal epithelial cells (Bogdan, 2015; Fang, 1999; Lundberg et al., 1995; MacMicking et al., 1997). Aside from differences in regulation and tissue source, it is the high cytotoxic concentrations achieved by iNOS that make NO a critical component of host defense. Although much is known about NO in human physiology, its plurifunctional character and versatile biochemistry open the possibility for additional, unappreciated roles.

### **1.1.2 Nitric oxide is a major antimicrobial component of host innate immunity**

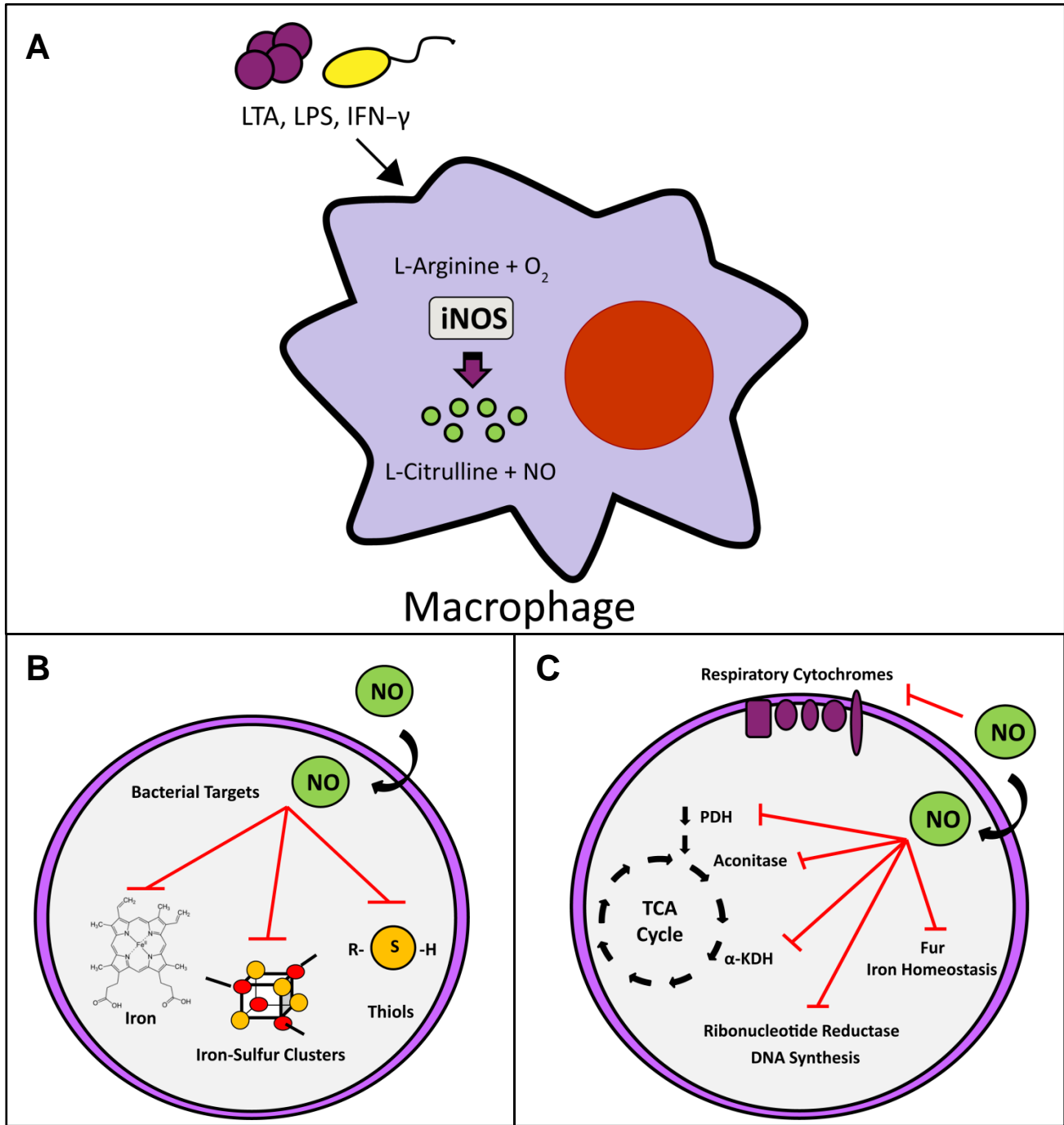
Initial observations showing that activated macrophages accumulate nitrogen oxides in response to microbial products led to the hypothesis that NO could function as an antimicrobial mediator (Hibbs et al., 1987; Stuehr and Marletta, 1985). In the early 1990s, the purification and characterization of iNOS from rat and murine macrophages was concurrently accomplished by three independent groups (Hevel et al., 1991; Stuehr et al., 1991; Yui et al., 1991). Carl Nathan's lab, one of the three research groups, further demonstrated that NO production by murine macrophages was induced by both bacterial lipopolysaccharide (LPS) and the inflammatory cytokine interferon- $\gamma$  (IFN- $\gamma$ ) (Xie et al., 1993). Establishing that NO played a similar antimicrobial role in human macrophages was more complicated and initially met with skepticism when several research groups failed to detect NO in *ex vivo* studies (Denis, 1994). Much of this controversy was fueled by limited knowledge of differences in iNOS regulation across species (Weinberg, 1998). Although iNOS expression can be complicated due to modulation by a variety of cytokines, today there is a clearer picture of the stimuli that lead to NO production. Critical stimuli include pathogen-associated molecular patterns (PAMPs) acting through toll-like receptors and the transcription factor NF $\kappa$ B (Figure 1.1A) (Aktan, 2004; Mizel

et al., 2003; Muhl et al., 2011; Pautz et al., 2010). Equally important is the IFN- $\gamma$  JAK-STAT signaling pathway that is triggered by inflammation (Aktan, 2004; Muhl et al., 2011; Pautz et al., 2010). Apart from regulation and induction pathways, it is now known that iNOS expression, while predominant in macrophages, occurs in neutrophils, epithelial cells, and other cell types — the precise function of NO at these sites remains an open topic of research.

The antimicrobial properties of NO arise from a broad reactivity with numerous microbial targets including iron, thiols, and other radicals. The biological effects are mediated by both direct mechanisms and through complex biochemical reactions involving NO-derived species such as nitrogen dioxide (NO<sub>2</sub>), dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), and peroxynitrite (OONO<sup>-</sup>) (Fang, 2004; MacMicking et al., 1997; McLean et al., 2010; Patel et al., 1999; Radi, 2004). The modification of enzyme metal centers such as heme and iron-sulfur clusters is a major mechanism behind the antimicrobial properties of NO and it occurs directly. However, the reversible modification of protein cysteines and low molecular weight thiols first requires NO reactivity with oxides and metals to promote *S*-nitrosylation (R-SNO); *S*-nitrosothiols can then further react with other sulfhydryl groups in transnitrosylation reactions (Anand and Stamler, 2012; Hess et al., 2005). Because cysteines serve important roles in protein structure and as catalytic residues in enzymes, *S*-nitrosylation can have a profound impact on physiological processes. The combined antimicrobial actions of NO and related reactive nitrogen species are referred to as nitrosative stress and collectively contribute to host defense.

NO functions as an antimicrobial effector in bacterial, fungal, parasitic, and viral infections (Bogdan, 2015; Fang, 1997, 2004; Fang, 1999; MacMicking et al., 1997). Studies using iNOS inhibitors and iNOS deficient animal models have demonstrated decreased host survival and increased morbidity for a variety of pathogens. Nitrosylation of protein thiols and

iron centers, including mononuclear iron, iron-sulfur clusters, and heme can inactivate important metabolic enzymes (Figure 1.1B) (Stamler, 1994). In energy metabolism, enzymes that are inhibited by NO include the dihydrolipoamide E3 component of pyruvate and  $\alpha$ -ketoglutarate dehydrogenases, the tricarboxylic acid cycle enzyme aconitase, and respiratory cytochromes in the electron transport chain (Figure 1.1C) (Fang, 1997; Gardner et al., 1997; Richardson et al., 2011; Stamler, 1994). In many bacteria iron homeostasis is maintained by the Fur transcriptional factor, which contains a mononuclear iron that is sensitive to NO (D'Autreaux et al., 2002). NO can also impede microbial DNA synthesis by inactivating ribonucleotide reductase through the modification of a catalytic tyrosyl radical (Lepoivre et al., 1991; Lepoivre et al., 1994; Roy et al., 1995). Additional inhibitory mechanisms of DNA replication were shown to involve zinc mobilization during nitrosative stress in *Salmonella*, implicating DNA-binding metalloproteins as NO targets (Schapiro et al., 2003). The pleiotropic effects on microbial physiology ultimately result in growth inhibition of many pathogens, making NO a critical component of innate immunity.



**Figure 1.1 Simplified model of nitric oxide production by innate immune cells and common microbial targets**

(A) Macrophage induction of iNOS and nitric oxide production upon sensing pathogen-associated molecular patterns such as lipopolysaccharides (LPS) and lipoteichoic acids (LTA) and inflammatory cytokines such as interferon-gamma (IFN- $\gamma$ ).

(B) Biochemical properties of nitric oxide showing membrane permeability and specific reactivity with iron centers and thiols inside bacteria.

(C) Common targets of nitric oxide that disrupt bacterial physiology include heme in respiratory cytochromes, iron centers in aconitase and the Fur iron regulator, thiols in the dihydrolipoamide E3 component of pyruvate dehydrogenase (PDH) and  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -KDH), and a catalytic tyrosyl radical in ribonucleotide reductase.

## 1.2 Bacterial adaptation to nitrosative stress

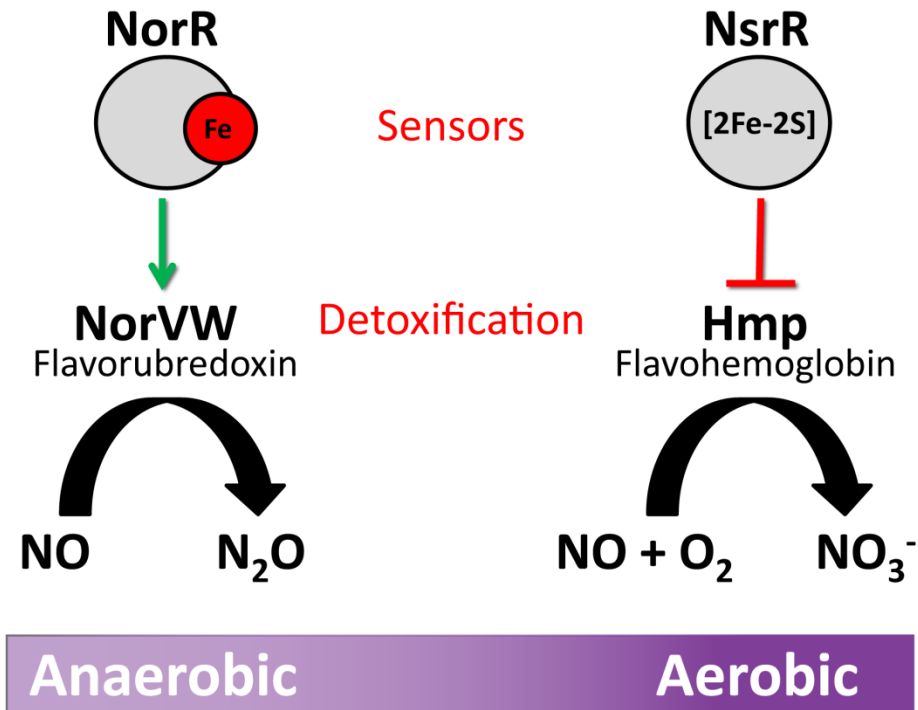
### 1.2.1 Nitric oxide sensing and detoxification

Nitric oxide is not unique to human physiology. Organisms ranging from bacteria to plants and mammals have evolved diverse mechanisms to produce and sense NO at low non-cytotoxic levels where it serves as an important signaling molecule (Crane et al., 2010; Domingos et al., 2015; Förstermann and Sessa, 2012; Plate and Marletta, 2013). Therefore, it is not surprising that some pathogens have evolved mechanisms to resist nitrosative stress and even use NO as part of their own physiology. In nitrogen metabolism, denitrifying and enteric bacteria generate NO as an intermediate and by-product of anaerobic respiration (Corker and Poole, 2003; Spiro, 2007; Zumft, 1997). As in eukaryotes, dedicated enzymatic NO sources also exist in bacteria. A more active role in signaling and regulation has been proposed for NO produced by bacterial NO synthases (bNOS) found in Gram-positive bacteria. Our laboratory showed that the bNOS from *S. aureus* regulates electron transfer by inactivating respiratory cytochrome oxidases, a mechanism that maintains membrane energetics under microaerobic conditions by diverting electron flow to nitrate reductase (Kinkel et al., 2016). In other Gram-positive bacteria (e.g. *Streptomyces* and *Bacillus*) bNOS also participates in the biosynthetic nitration of microbial products, resistance to antibiotics, and protection against oxidative stress (Crane, 2008; Gusarov and Nudler, 2005; Gusarov et al., 2009). Unlike high output iNOS in macrophages, which induces bacteriostasis, bacterial derived NO is thought to function more specifically in signaling and regulation. While much of this research is ongoing, the intrinsic role of NO in multiple microbial processes suggests that adaptation to nitrosative stress is more complex than currently appreciated.

The coevolution of bacteria with the immune system has led pathogens to develop diverse strategies to cope with NO in microbial-host interactions. Studies with enteric bacteria and macrophages have uncovered sophisticated mechanisms employed by pathogens to circumvent cytotoxicity by metabolizing host NO to less toxic nitrogen species. In the late 1990's, Gardner *et al.* and Hausladen *et al.* demonstrated NO-detoxifying activity for the widespread microbial flavohemoglobins (Hmp) (Gardner and Gardner, 2002; Gardner *et al.*, 1998; Hausladen *et al.*, 1998). These studies revealed that *E. coli* Hmp confers protection against nitrosative stress by using oxygen (O<sub>2</sub>) to convert NO to nitrate (NO<sub>3</sub><sup>-</sup>). It was later shown that Hmp also functions anaerobically by converting NO to nitrous oxide (N<sub>2</sub>O) (Hausladen *et al.*, 2001; Justino *et al.*, 2005). For the intracellular pathogen *Salmonella enterica*, Hmp is required for virulence in mice and confers protection against NO produced by macrophages (Bang *et al.*, 2006; Gilberthorpe *et al.*, 2007; Stevanin *et al.*, 2002). Since the characterization of Hmp, additional bacterial enzymes have been found with NO-detoxifying activity; notably *E. coli* flavorubredoxin (NorVW), an enzyme that detoxifies NO to N<sub>2</sub>O in anaerobic environments (Gardner *et al.*, 2002). With Hmp and NorVW, enteric bacteria are well equipped to cope with nitrosative stress over a wide range of oxygen concentrations. Although NO reductases in denitrifying bacteria have long been known to protect against endogenous NO, Hmp and NorVW seem to be specifically adapted to help bacteria cope with high NO concentrations from exogenous sources.

Critical to bacterial NO resistance is the ability to sense NO before it reaches toxic concentrations. In *E. coli*, *S. enterica*, and other microbes, Hmp and NorVW are inducible and require NO sensors to up-regulate their production. Because NO is reactive with iron, and bacteria have many iron-containing proteins, the characterization of authentic NO sensors has

been complicated. Transcription regulators that mediate responses to other known cellular stresses (Fur, IscR, FNR, OxyR, SoxR) are known to be responsive to high NO concentrations (Spiro, 2007; Tucker et al., 2010). Identification of *bona fide* NO sensors has been greatly facilitated by studying the regulation of NO detoxification enzymes. NorR is a  $\sigma^{54}$ -dependent transcription activator that is required for expression of NorVW (Gardner et al., 2003; Hutchings et al., 2002). Importantly, direct nitrosylation of the mononuclear iron center of NorR has been demonstrated (D'Autreaux et al., 2005). Work on *E. coli hmp* regulation identified NsrR as an additional NO sensor (Bodenmiller and Spiro, 2006). NsrR has an iron-sulfur cluster that is susceptible to nitrosylation, an event that results in derepression of NsrR-regulated promoters (Tucker et al., 2008). In contrast to other transcription factors that interact with NO, NorR and NsrR mediate protective responses to physiologically relevant levels of nitrosative stress and are, consequently, authentic NO sensors (Figure 1.2). Undoubtedly, these regulator of nitrosative stress responses are critical for enteric bacteria that encounter high cytotoxic NO levels in the mammalian gastrointestinal tract and within innate immune cells.

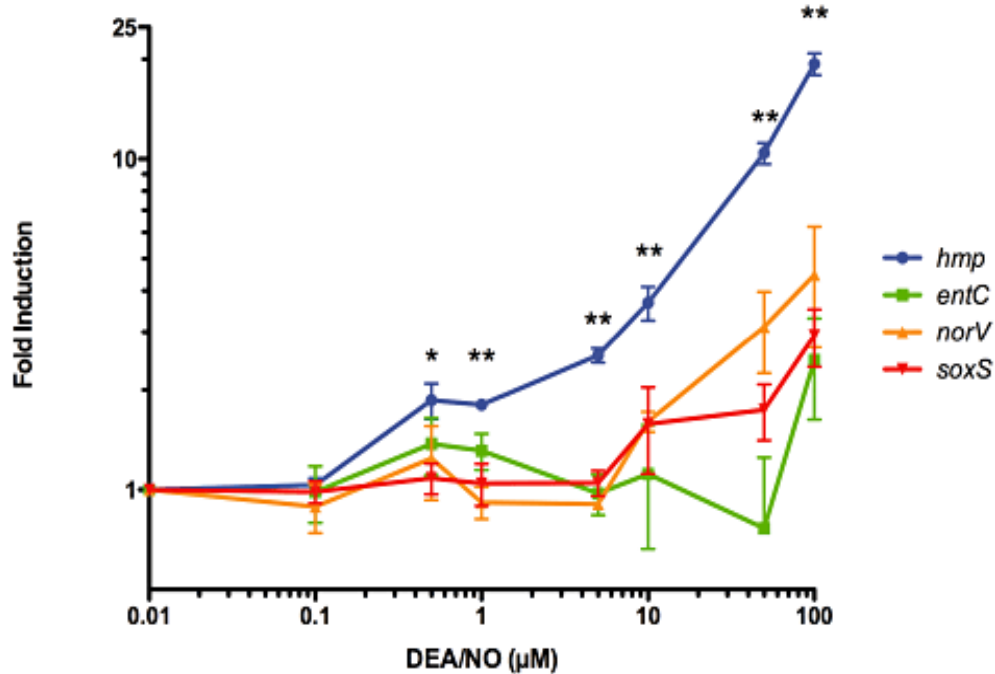


**Figure 1.2 Nitric oxide sensing and detoxification in enteric bacteria**

Bacteria such as *E. coli* and *S. enterica* have exquisitely sensitive sensors that detect nitric oxide directly through iron centers. NorR activates transcription of the flavorubredoxin (NorVW) upon binding nitric oxide through a mononuclear iron center. NsrR is a repressor of flavoheмоglobin (Hmp) in the absence of nitric oxide but derepresses transcription upon inactivation of an iron-sulfur cluster. Flavorubredoxin and flavoheмоglobin are enzymes that detoxify nitric oxide by reducing it to nitrous oxide (N<sub>2</sub>O) or oxidizing it to nitrate (NO<sub>3</sub><sup>-</sup>), respectively. Flavoheмоglobin also functions anaerobically by reducing NO to N<sub>2</sub>O.

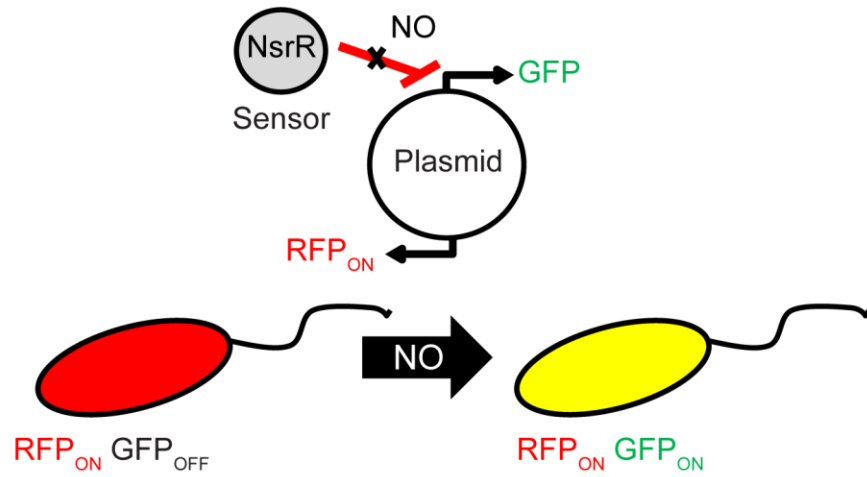
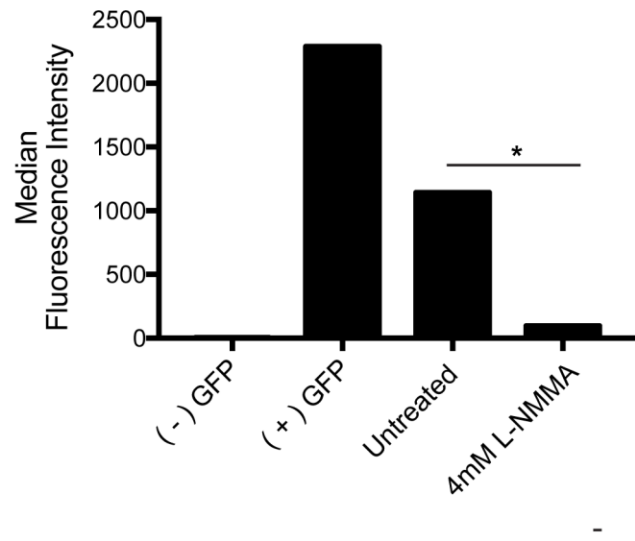
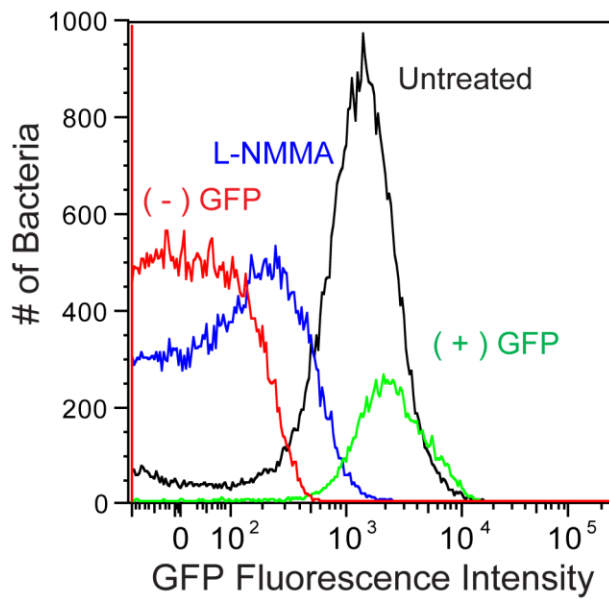
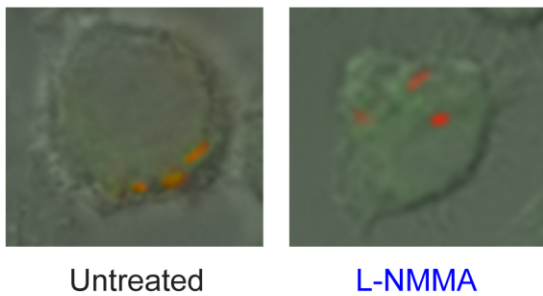
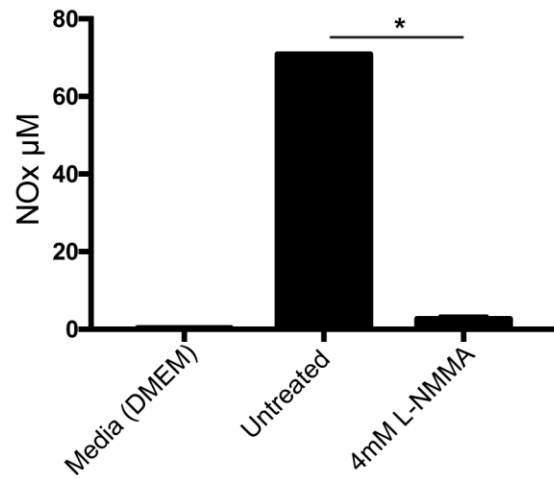
### 1.2.2 The nitrosative stress response of *Salmonella* to macrophage-derived nitric oxide

My work in the Fang laboratory has contributed to the understanding of how *S. enterica* senses and responds to NO produced by macrophages. Since *Salmonella* encodes NsrR, NorR and other NO responsive regulators (Fur, SoxR), it was unclear which sensors function aerobically and respond to NO during growth inside macrophages. In a recent co-authored publication, I demonstrated *in vitro* that NsrR is exquisitely sensitive to low micromolar concentrations of NO (Karlinsky et al., 2012). As determined by RT-qPCR, the NsrR-repressed *hmp* gene was induced at lower concentrations of DEA/NO (NO chemical donor) than genes regulated by NorR, Fur, and SoxR (Figure 1.3). To show that NsrR responds to NO within the macrophage *Salmonella*-containing vacuole, I constructed a Green Fluorescent Protein (GFP) fusion to the NsrR-repressed *stm1808* promoter (Figure 1.4A). Using flow cytometry and epifluorescence microscopy of *Salmonella*-infected RAW-264.7 macrophages, I demonstrated that NsrR indeed responds to NO within macrophages (Figure 1.4B-C). Furthermore, NsrR derepression was prevented by treating with L-NMMA, a NO synthase inhibitor. GFP expression from the NsrR regulated promoter directly correlated with NO production as measured via the Griess assay (Figure 1.4D). Collectively, these findings show that NsrR is bona fide sensor of iNOS-derived NO from macrophages.



**Figure 1.3 The NsrR transcription factor of *Salmonella* that regulates flavohemoglobin (Hmp) is exquisitely sensitive to nitric oxide**

Measurement of the NO responsiveness of iron-containing transcription factors by quantitative RT-PCR. Quantitative RT-PCR was performed on RNA samples isolated from *S. Typhimurium* 14028s cultures grown to early log-phase in LB then treated with increasing concentrations of diethylamine NONOate (DEA/NO) for 15 min (*Experimental procedures*). A representative gene was measured as an indicator of activation of the following transcription factors: NsrR (*hmp*, blue circles), Fur (*entC*, green squares), NorR (*norV*, orange up triangles) and SoxR (*soxS*, red down triangles). *P*-values were calculated using the Wilcoxon Rank Sum test. \**P* = 0.05 *hmp* versus *soxS* fold induction, \*\**P* = 0.05 *hmp* versus *norV*, *entC* or *soxS* fold induction. Karlinsey Joyce E. *et al.* *Molecular Microbiology* 2012; DOI: 10.1111/j.1365-2958.2012.08167.x. Figure and legend reprinted with permission.

**A****B****C****Epifluorescence Microscopy****D****Griess Assay**

**Figure 1.4** *Salmonella* NsrR senses nitric oxide during macrophage infection

(A) Experimental design of a GFP transcriptional fusion with the NsrR-repressed *stm1808* promoter. *Salmonella* constitutively express RFP and derepress GFP upon sensing nitric oxide.

(B) Flow cytometry of *S. enterica* with the plasmid encoded NsrR GFP-reporter 16 h post-infection in RAW-264.7 macrophages. L-NMMA was used to inhibit iNOS and prevent NO-dependent GFP expression. No promoter controls (- GFP) and maximal expression (+ GFP) in *S. enterica*  $\Delta$ *nsrR* are shown for reference. Median GFP fluorescence of the bacterial population is shown on the right.

(C) Epifluorescence microscopy of macrophage and intracellular *Salmonella* expressing an activated or inactivated NsrR-regulated GFP-reporter in untreated or L-NMMA treated infections. Bacteria in nitric oxide-producing macrophages (yellow) show expression of constitutive RFP and NsrR-dependent GFP expression. Bacteria in L-NMMA treated macrophages not producing nitric oxide only show constitutive RFP expression.

(D) Griess assay showing nitrite levels from nitric oxide production during a 16 h *Salmonella*-macrophage infection.

\*  $p < 0.05$

### 1.2.3 *S. aureus* adaptation to host nitric oxide

Transcription factors that sense NO and detoxification enzymes are widespread among bacteria and certainly at the forefront of resistance mechanisms to nitrosative stress, but some organisms have adapted diverse strategies to cope with cytotoxicity and even grow in the presence of NO. The opportunistic pathogen *Staphylococcus aureus* lacks NsrR and NorR to directly detect NO and instead responds to impaired electron flow caused by NO inactivation of respiratory cytochromes. Upon sensing reduced menaquinone, the two-component SrrAB system activates an NO adaptive response that includes Hmp and other nitrosative stress resistance genes (Kinkel et al., 2013; Richardson et al., 2006). In addition to detoxifying NO, *S. aureus* induces a metabolic adaptation response that allows for replication in the presence of NO. This is accomplished through an NO-inducible lactate dehydrogenase that maintains redox homeostasis through fermentation as respiration is impaired by NO (Richardson et al., 2008). As a pathogen, NO detoxification and resistance to growth inhibition by *S. aureus* are required to circumvent innate immunity (Richardson et al., 2006; Richardson et al., 2008; Vitko et al., 2015).

The intricate relationship of *S. aureus* with its human host provides a unique opportunity to study new roles for NO in host-pathogen interactions. As a commensal, *S. aureus* primarily colonizes the anterior nares of humans where NO is produced constitutively by the paranasal epithelia, presumably to serve an antimicrobial role (Lundberg et al., 1995). Over the long coevolution with humans, it is likely that *S. aureus* has fine-tuned its adaptive responses to nitrosative stress allowing it to stably persist in this host environment. Equally important are the pathogenic interactions of *S. aureus* with innate immunity where NO is a major antimicrobial effector. Little is known about the protective roles of host NO beyond bacterial growth

inhibition. A novel antimicrobial mechanism whereby host NO inhibits bacterial virulence is proposed herein.

## **Chapter 2. Inhibition of Staphylococcal Virulence by Nitric Oxide**

### **2.1 *Staphylococcus aureus*: a persistent commensal and formidable pathogen**

#### **2.1.1 Impact on public health**

*Staphylococcus aureus* is primarily a human commensal that colonizes more than two billion people worldwide. It persistently or transiently inhabits the skin and anterior nares of individuals, spreading through human contact with carriers and fomites (Brown et al., 2014; Kooistra-Smid et al., 2009; Otto, 2010; Wertheim et al., 2005). In the skin and nasal environment, carriage is largely asymptomatic, but infection at compromised skin sites and access to soft tissue can result in a variety of local and invasive disease manifestations. *S. aureus* carriage is an important factor for the development of staphylococcal disease, and colonized individuals are at risk for serious infections (Kooistra-Smid et al., 2009; Otto, 2010; Wertheim et al., 2005). Many factors including microbial determinants, host genetics, and interactions with the host microbiota and immune system can influence colonization and the outcome of disease (Brown et al., 2014; Liu et al., 2015; Yan et al., 2013).

Staphylococcal infections may involve many tissues in the body and can have acute or chronic manifestations. The spectrum of staphylococcal disease includes skin and soft tissue infections, bacteremia, endocarditis, osteomyelitis, septic arthritis, pneumonia, toxic-shock syndrome and food poisoning (Thomer et al., 2016; Todd, 2005; Tong et al., 2015). Device-related infections are a serious concern in hospital settings, and the emergence of drug resistant strains in hospitals and the community has further complicated successful treatment and clearance of infection (Tong et al., 2015). Widespread use of antibiotics has now led to an ever growing number of infections with methicillin-resistant *S. aureus* strains (MRSA), and even

some vancomycin-intermediate and vancomycin-resistant strains (VISA/VRSA) (Appelbaum, 2006; David and Daum, 2010; Enright et al., 2002; King et al., 2006; Okuma et al., 2002; Shenoy et al., 2014). With only modest success in the development of new effective antibiotics to combat life-threatening bacterial infections, *S. aureus* poses a serious public health concern.

### **2.1.2 Staphylococcal virulence**

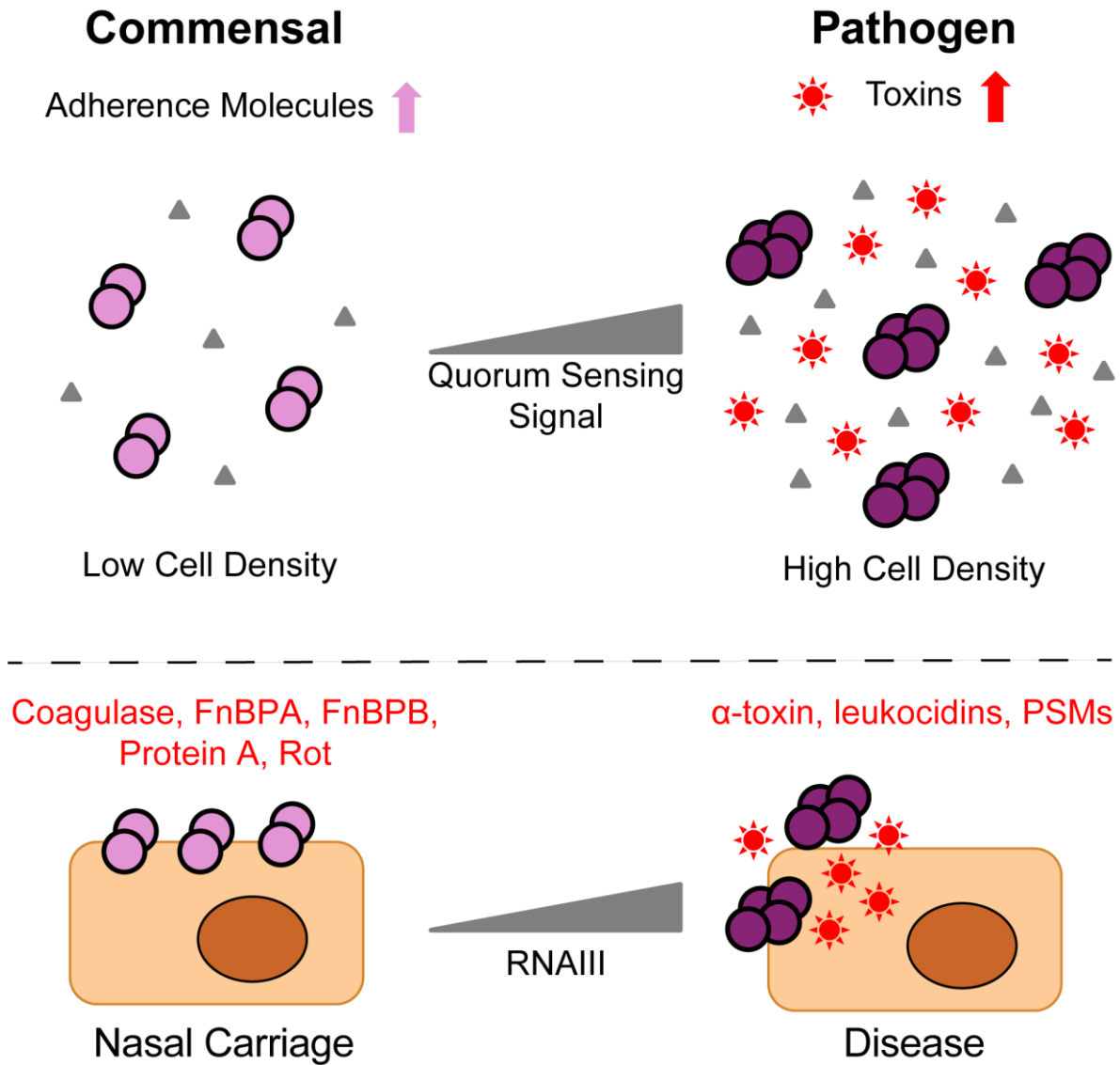
The success of *S. aureus* as a pathogen is partly explained by the expression of a multitude of virulence factors. Coevolution with humans and other mammals has armed staphylococci with surface adhesins, immune evasion proteins and secreted toxins. Microbial surface components that recognize adhesive matrix molecules (MSCRAMMS) are a large class of cell wall-anchored bacterial proteins that promote adherence to host surfaces, invasion of host tissues and evasion of the immune response (Foster et al., 2014). MSCRAMMS include clumping factor A and B, which bind fibrinogen and other host proteins to promote nasal colonization and allow the establishment of invasive infections (Deivanayagam et al., 2002; Josefsson et al., 2001; McDevitt et al., 1997; Ní Eidhin et al., 1998; O'Brien et al., 2002; Schaffer et al., 2006; Weidenmaier et al., 2012). In addition, fibronectin binding protein A and B are involved in adhesion to the host extracellular matrix and also promote invasion of host cells and tissues (Dziewanowska et al., 1999; Schwarz-Linek et al., 2003; Sinha et al., 2000; Sinha et al., 1999). For immune evasion, one of the most prominent strategies employed by *S. aureus* involves the multifunctional IgG-binding Protein A, which interferes with opsonophagocytosis, disrupts adaptive immunity as a B cell superantigen and stimulates host cell signaling to induce inflammation (Forsgren and Quie, 1974; Gómez et al., 2004; Gómez et al., 2006; Kobayashi and DeLeo, 2013). In addition to surface proteins, *S. aureus* secretes a variety of toxins with cytolytic properties that thwart cellular defenses. Alpha-toxin and leukocidins are pore-forming toxins that

target specific cellular receptors to induce cellular death and drive inflammation (Berube and Bubeck Wardenburg, 2013; DuMont and Torres, 2014; Otto, 2014b; Spaan et al., 2017). Moreover, *S. aureus* secretes amphipathic peptides known as phenol-soluble modulins (PSMs) that can drive pro-inflammatory responses and disrupt cellular membranes to allow *S. aureus* escape from the phagosome and induce cell lysis (Cheung et al., 2014; Otto, 2014a; Syed et al., 2015). Collectively, this arsenal of adhesins, immune evasion proteins, secreted toxins, and other virulence factors makes *S. aureus* a formidable pathogen.

### **2.1.3 Quorum sensing and virulence regulation**

The staphylococcal Agr quorum sensing (QS) system is central to virulence regulation. QS determines whether *S. aureus* behaves as a commensal or as a pathogen by maintaining tight control over toxins and colonization factors (Figure 2.1). In the laboratory, low cell density conditions lead to the expression of surface proteins that function as adhesins and promote niche adaptation. Among the surface proteins negatively regulated by Agr are coagulase, FnBPA, FnBPB, and protein A, which are all negatively regulated by the Agr-induced small regulatory RNA, RNAIII (Chevalier et al., 2010; Huntzinger et al., 2005; Saravia-Otten et al., 1997). At high cell numbers, Agr mediates a switch to toxin production and triggers the onset of the invasive phenotype. Agr-dependent toxins are induced by multiple mechanisms including direct transcriptional activation of PSMs and RNAIII post-transcriptional control (Queck et al., 2008). RNAIII directly promotes  $\alpha$ -toxin translation and negatively regulates the Rot (repressor of toxins) transcription factor that controls leukocidin expression (Boisset et al., 2007; Morfeldt et al., 1995; Queck et al., 2008; Spaan et al., 2017). The cell density-dependent activation of the Agr quorum sensing system observed in the laboratory is mirrored in the human host during commensalism and pathogenesis. During asymptomatic nasal carriage, surface proteins such as

FnBPA are highly expressed, while RNAlII,  $\alpha$ -toxin, and PSMs are found at low levels (Burian et al., 2010). In contrast to the human nose where *S. aureus* lives as a commensal, Agr plays a critical role in disease development. Bacterial mutants defective in Agr QS have attenuated virulence in skin and soft tissue infections, pneumonia, endocarditis, osteomyelitis, and septic arthritis (Novick and Geisinger, 2008; Thoendel et al., 2011). Despite the importance of Agr QS for staphylococcal infections, not much is known about the homeostatic conditions and host determinants that modulate Agr activation *in vivo* to maintain commensalism or trigger pathogenesis.



**Figure 2.1 Staphylococcal quorum sensing**

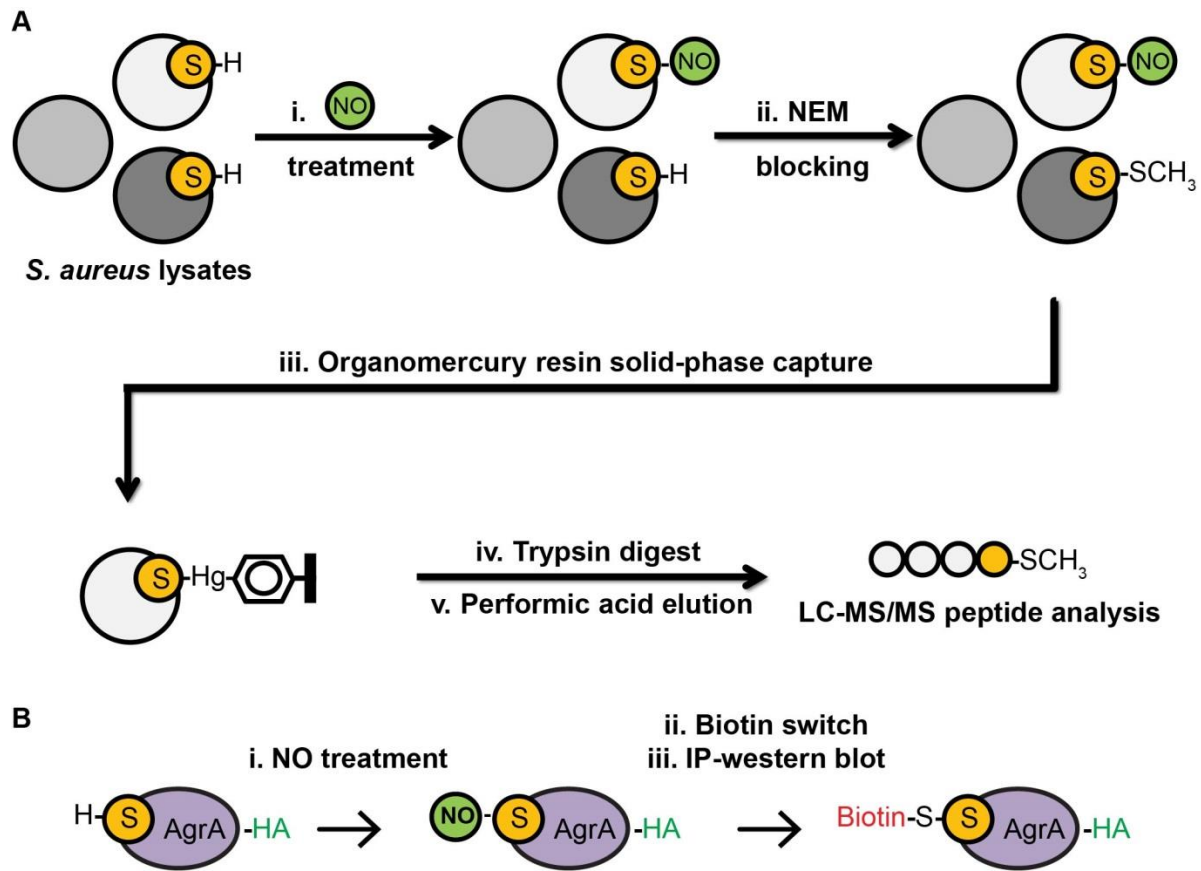
The Agr QS system controls the shift of *S. aureus* from a commensal to a pathogen. At low cell density, bacteria secrete low levels of quorum sensing signal and primarily express surface proteins that serve as adhesins and colonization factors. High cell density ramps up quorum sensing signal production and triggers the secretion of toxins. The Agr-mediated shift from human commensal to pathogen is also observed *in vivo*. In persistent nasal carriers, Agr and secreted toxins are found at low levels, but Agr is activated during the onset of disease.

## 2.2 Quorum sensing inhibition by nitric oxide

### 2.2.1 Proteomic screen to identify staphylococcal proteins targeted by nitric oxide

Enzymatic NO production by innate immune cells is important to control staphylococcal infections even though *S. aureus* is intrinsically resistant to NO-mediated growth inhibition (Li et al., 2014; McInnes et al., 1998; Richardson et al., 2008; Rothfork et al., 2004; Sasaki et al., 1998). Host mechanisms that suppress virulence factors and their regulation may have a substantial impact on outcome of bacterial infections. Although numerous studies have established that high cytotoxic levels of NO produced by iNOS can dramatically alter microbial metabolism and physiology, little is known about the effect of NO on the expression of virulence factors.

To better understand the basis of NO protection and further characterize nitrosative stress responses in *S. aureus*, we employed an unbiased biochemical approach to identify bacterial proteins that are susceptible to NO modification. Novel proteomic tools have recently been developed to identify eukaryotic proteins containing cysteine residues that are susceptible to *S*-nitrosylation, the reversible covalent modification of sulfhydryl groups (Doulias et al., 2010). To identify *S*-nitrosylated proteins in *S. aureus*, we adapted mercury resin-assisted capture (MRC) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 2.2A). In the following sections, I present evidence that the staphylococcal Agr QS system is susceptible to *S*-nitrosylation, identify the AgrA transcription factor as a direct molecular target of NO, and assess the effect of QS inhibition on virulence factor production in bacterial cultures. My findings open the possibility that host NO may function as an anti-virulence mediator in staphylococcal infections.



**Figure 2.2 Biochemical methods to detect *S*-nitrosylation in *S. aureus* lysates**

(A) Experimental strategy to identify *S*-nitrosylated proteins following NO treatment of bacterial lysates by mercury resin-assisted capture of *S*-nitrosopeptides and mass spectrometry-based identification. NEM, N-ethylmaleimide.

(B) Biotin-switch assay of NO-treated bacterial lysates containing hemagglutinin-tagged AgrA with biotinylation of *S*-nitrosylated sulfhydryl groups and detection by immunoprecipitation-western blot. HA, hemagglutinin; IP, immunoprecipitation.

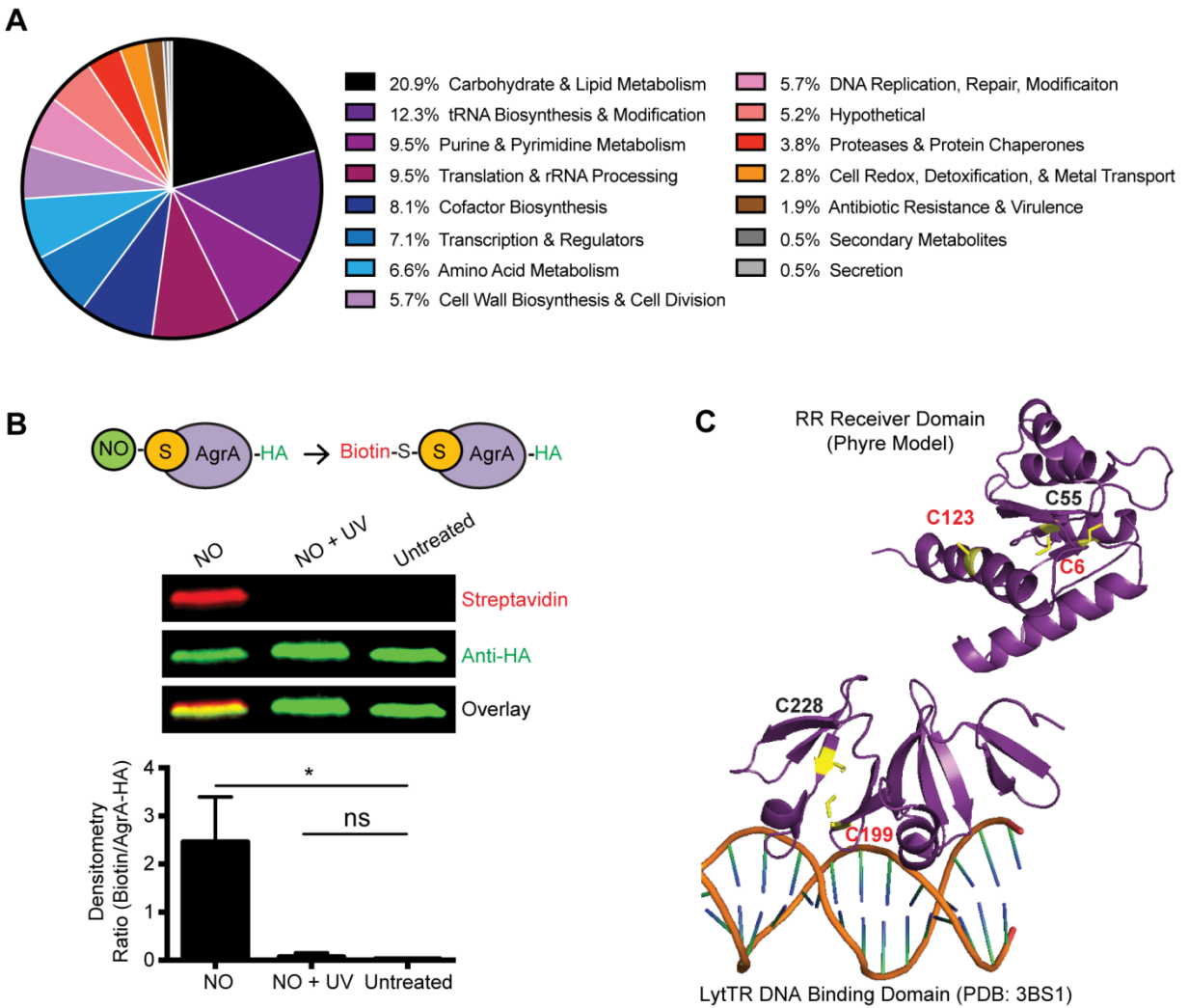
See Methods section for experimental details.

### 2.2.2 Staphylococcal metabolic enzymes and Agr QS are susceptible to S-nitrosylation

Our comprehensive proteomic analysis identified 210 unique S-nitrosocysteine residues in 5.8% of the total proteins encoded in the *S. aureus* genome (Figure 2.2A and Table S1). The dataset included constituents of carbohydrate and lipid metabolism (20.9%) as well as cell wall (5.7%), nucleotide (9.5%), tRNA (12.3%), cofactor (8.1%) and amino acid (6.6%) biosynthetic pathways (Figure 2.3A). Proteins with roles in protein translation (9.5%), transcription (7.1%), DNA replication and DNA repair (5.7%) were also detected. A small percentage of the dataset consisted of proteins of unknown function (5.2%), and less represented pathways included proteins involved in protein stability (3.8%), cell redox (2.8%), antibiotic resistance and virulence (1.9%), secondary metabolites biosynthesis (0.7%), and secretion (0.7%). The predominance of metabolic enzymes and proteins important for bacterial physiology is consistent with the general growth inhibitory effects of NO on bacteria (Fang, 1999; Richardson et al., 2006; Richardson et al., 2008; Richardson et al., 2011).

Although virulence factors and regulators comprised only a small proportion of this dataset, AgrA, a central component of staphylococcal QS and major activator of virulence genes was found to be S-nitrosylated at cysteine residues C6, C123, and C199 within the response regulator (RR) receiver and DNA-binding domains (Figure 2.3 and Table S1). Modification of other AgrA cysteine residues was not detected (C55, C228). To further confirm AgrA as an NO target, the biotin-switch method in which NO-modified thiols are replaced with a stable biotin label was employed (Jaffrey and Snyder, 2001) (Figure 2.2B). In agreement with the proteomic analysis, biotin-switch showed S-nitrosylation of AgrA in lysates treated with NO, but not in untreated samples or in samples where S-nitrosylation was reversed with UV light prior to

biotinylation (Figure 2.3B). Together these results show that AgrA cysteines are susceptible to chemical modification by NO.



**Figure 2.3 Mass spectrometry-based proteomic analysis identifies the AgrA virulence regulator as a target of S-nitrosylation**

(A) Summary of identified NO-modified proteins categorized by biological pathway.

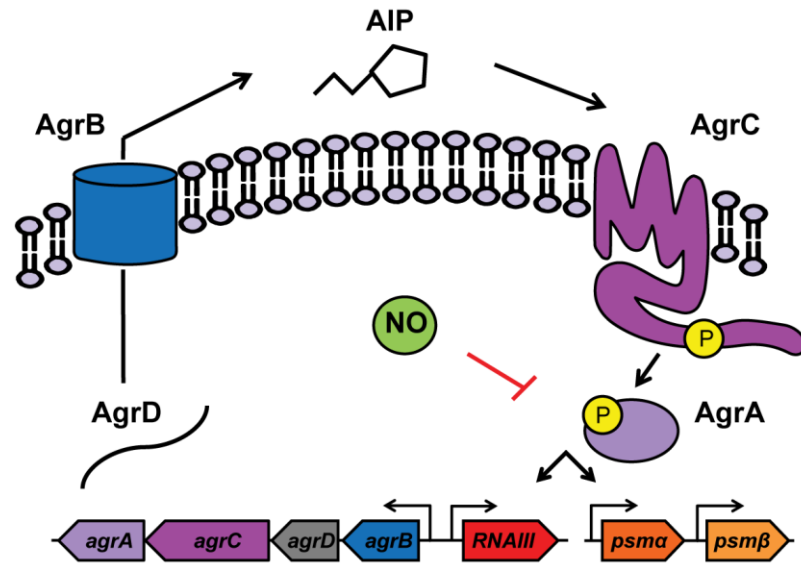
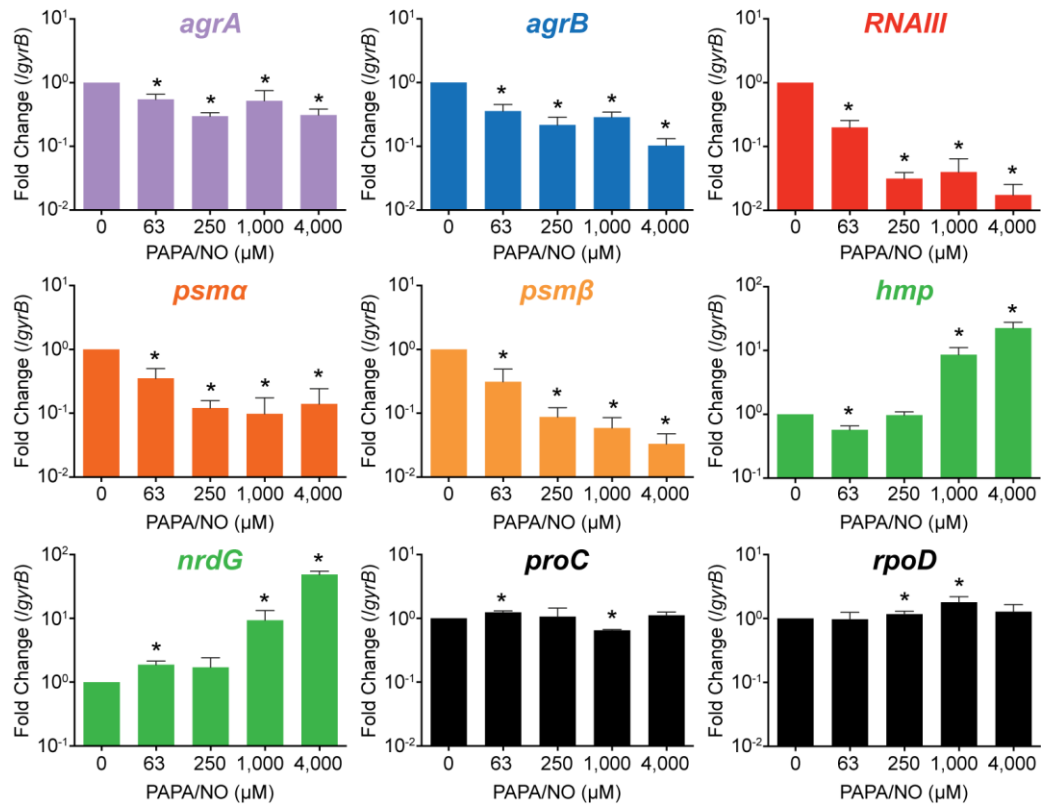
(B) Biotin-switch validation of AgrA cysteines as NO targets. A representative immunoprecipitation-western blot (top, n=3) probed with streptavidin and anti-HA antibody to detect biotinylation and HA-tagged AgrA, respectively is shown. Densitometry of three independent experiments (bottom) quantified the ratio of biotin to AgrA. UV, ultraviolet light. Student's t test, \*  $p < 0.05$ ; ns, not significant.

(C) AgrA cysteine residues in the response regulator (RR) receiver domain (top) and LytTR DNA-binding domain (bottom). S-nitrosylation of C6, C123, and C199 (red) was detected by mass spectrometry.

See also Table S1.

### 2.2.3 Nitric oxide inhibits transcription of the *agr* operon and Agr-dependent toxin genes

An earlier gene expression study from our laboratory had suggested possible NO inhibition of QS when the *hld* gene coding for  $\delta$ -toxin was found to be downregulated in *S. aureus* cultures treated with NO (Richardson et al., 2006). The  $\delta$ -toxin transcript is part of RNAIII, a small regulatory RNA and a hallmark of *S. aureus* QS (Novick and Geisinger, 2008). In staphylococci, QS is mediated by a multicomponent system encoded by the *agr* operon (Figure 2.4A). The QS signal, known as the auto-inducing peptide (AIP), is formed when the AgrD propeptide is cleaved and exported by the membrane-bound AgrB endopeptidase. As bacterial cell density increases, AIP accumulates extracellularly and binds the AgrC receptor. Upon binding, AgrC is auto-phosphorylated and activates the AgrA response regulator. AgrA phosphorylation results in positive auto-regulation via the *agrPII* promoter and the induction of additional promoters that trigger toxin production. Activation of *agrPIII* induces RNAIII, which post-transcriptionally stimulates expression of the cytolytic  $\alpha$ -toxin and blocks translation of the Rot repressor of toxins (Boisset et al., 2007; Morfeldt et al., 1995). Additionally, AgrA directly drives the expression of other virulence factors such as the phenol soluble modulins (PSMs) (Queck et al., 2008). To determine whether S-nitrosylation disrupts the ability of AgrA to activate transcription, transcript levels from four independent Agr-regulated promoters were measured by RT-qPCR, and all transcripts (*agrA*, *agrB*, RNAIII, *psmA* and *psm $\beta$* ) showed dose-dependent inhibition with increasing NO concentrations (Figure 2.4B). Transcription of the *proC* and *rpoD* housekeeping genes was unaffected, and the control NO-responsive flavohemoglobin (*hmp*) and *nrdG* genes were induced as expected (Figure 2B). These experiments show that inhibition of QS results in a specific downregulation of virulence genes controlled by AgrA and that the decrease in transcript levels is not the result of a global impairment of transcription.

**A****B**

**Figure 2.4 Nitric oxide inhibits transcription of the *agr* quorum sensing operon and AgrA-regulated virulence factors**

(A) Model of Agr quorum sensing and proposed mechanism of NO inhibition.

(B) RT-qPCR from *S. aureus* cultures (n=4) treated with increasing concentrations of the NO donor PAPA/NO. Agr-regulated genes include *agrA*, *agrB*, *RNAIII*, *psmA* and *psm $\beta$* ; nitrosative stress-induced genes (green) include *hmp* and *nrdG*; control genes (black) include housekeeping genes *rpoD* and *proC*. Student's t test, \*  $p < 0.05$ .

### 2.3 Discussion

Our proteomic analysis identified a large number of staphylococcal proteins susceptible to *S*-nitrosylation, many of which are conserved in other bacteria. The large number of targets is consistent with the known pleiotropic properties of NO and its diverse effects on bacterial physiology. As NO is able to inhibit growth of many organisms, it was not surprising to find that NO is able to modify many metabolic enzymes and other proteins responsible for basic physiological processes. Previous studies in *S. aureus* have also identified a high numbers of cysteine residues susceptible to oxidation by H<sub>2</sub>O<sub>2</sub>, some of which were also found in our study (Deng et al. 2013).

The presence of several proteins previously identified as NO targets or associated with nitrosative stress is worth discussing. Interestingly, we identified the staphylococcal bNOS as subject to NO modification. It is known that *S*-nitrosylation inhibits eNOS and is involved in regulation of NO production (Erwin et al., 2005; Ravi et al., 2004; Tummala et al., 2008). However, it has not been reported that bNOS is subject to regulation by a similar mechanism and it is unclear whether this could occur through auto-targeting or in response to exogenous NO sources. In carbon metabolism, we found proteins such as isocitrate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. Both are known to be inhibited through *S*-nitrosylation (Molina y Vedia et al., 1992; Yang et al., 2002). The Fur regulator of iron homeostasis has an iron center that is directly modified by NO (D'Autreaux et al., 2002). We did not identify Fur, likely because NO-targeting does not occur through *S*-nitrosylation. Interestingly we did find Zur, a Fur homologue that is involved in zinc homeostasis (Lindsay and Foster, 2001). The specific cysteine modified in Zur is not directly involved in Zn<sup>2+</sup> coordination but is proximal to four other Zn<sup>2+</sup>-coordinating cysteine residues (Gilston et al., 2014),

suggesting metal catalyzed mechanism of *S*-nitrosylation. Several proteins involved in redox stress were also identified, including catalase, coenzyme A disulfide reductase, a putative heme dependent-peroxidase, and ferredoxin-NADP reductase. Among these, catalase is inhibited by NO via its heme group and is also transcriptionally down-regulated in *S. aureus* by NO (Brown, 1995; Richardson et al., 2006). Since heme can catalyze *S*-nitrosylation of proximal thiols, this could be a mechanism of catalase *S*-nitrosylation (Weichsel et al., 2005). Transition metal based catalysis is a major mechanism of *S*-nitrosylation, and the identification of many metal-containing proteins was expected.

We were interested to discover that NO modifies AgrA, the response regulator of the Agr QS system which plays a central role in staphylococcal pathogenesis. My observations conclusively demonstrate that NO is a potent inhibitor of staphylococcal QS in bacterial cultures. Four operons under direct control by AgrA are downregulated by NO in a dose-dependent manner. Furthermore, this downregulation occurs at or below NO concentrations that induce genes involved in the staphylococcal nitrosative stress response. Unaffected levels of housekeeping genes show that NO-mediated downregulation is not the result of a general inhibition of bacterial transcription. Multiple molecular mechanisms exist by which bacteria can sense NO and elicit a physiological response. As our laboratory has previously demonstrated, *S. aureus* can indirectly sense NO by monitoring metabolic stress through SrrAB activation in response to impaired respiration (Kinkel et al., 2013). There are at least twelve transcription regulators that have been shown to positively or negatively modulate the Agr system in response to metabolic and environmental cues (Thoendel et al., 2011). In preliminary data (not shown), multiple mutant strains lacking some of these regulators retained Agr sensitivity to NO, suggesting that these regulators are not involved in QS inhibition during nitrosative stress. Our

observations that AgrA can be directly modified by NO and its presence as the sole Agr component in our proteomic study suggests that *S*-nitrosylation of AgrA is responsible for the inhibition of staphylococcal QS by NO.

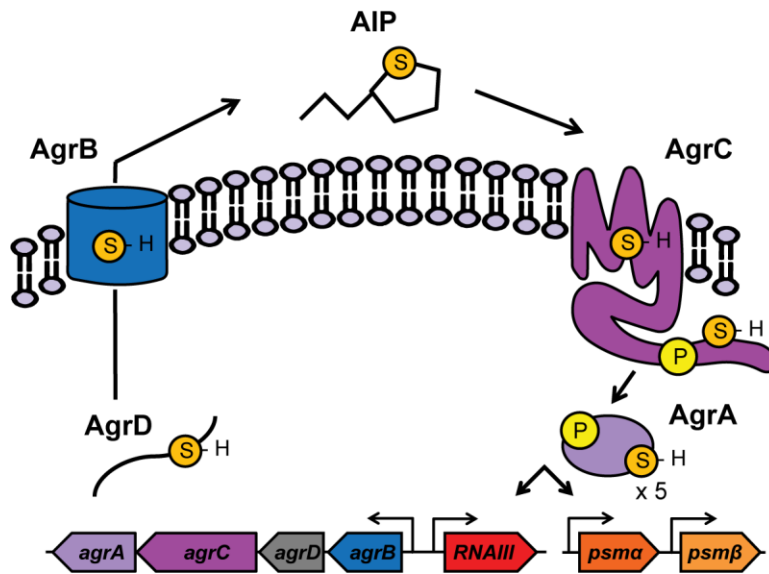
## Chapter 3. Molecular Mechanism of Agr Inhibition by Nitric Oxide

### 3.1 Putative targets of nitric oxide in Agr quorum sensing

Direct NO-modification of proteins in the Agr system is a plausible mechanism for inhibition of QS. The membrane permeability of NO makes both intracellular and extracellular Agr components accessible to direct NO modification. Although the Agr system lacks any iron centers or radicals that could serve as direct NO targets, all Agr components have cysteine residues that could be susceptible to *S*-nitrosylation (Figure 3.1). Interestingly, many of the cysteines in Agr proteins serve important functional roles. The AgrB membrane endopeptidase uses a catalytic cysteine to cleave the AgrD pro-peptide during export (Qiu et al., 2005; Thoendel and Horswill, 2009; Thoendel and Horswill, 2013), and AgrD has a sole cysteine that is involved in the formation of the thiolactone ring in AIP (Ji et al., 1995; Novick and Geisinger, 2008). The quorum sensory apparatus also has multiple cysteines that could impair AIP sensing or downstream signal transduction. The AgrC sensor kinase has one cysteine in a transmembrane loop and a second cysteine near an ATP-binding pocket that is involved in auto-phosphorylation. Lastly, the AgrA transcription factor has five cysteines: two located in the DNA-binding domain and three in the response-regulator receiver domain. *S*-nitrosylation of one or multiple cysteines within any Agr component(s) might disrupt Agr function.

Our proteomic screen and biotin switch assay showed that AgrA is susceptible to *S*-nitrosylation, making it a primary candidate in the mechanism of QS inhibition. AgrA is central to staphylococcal QS by regulating expression of the *agr* operon through auto-regulation and also by driving transcriptional responses downstream of Agr through direct transcription

activation of RNAIII and the *psm* operons. Thus, direct NO targeting of AgrA is a plausible and potentially effective mechanism to inhibit staphylococcal QS and shut down toxin production.



**Figure 3.1** Cysteine residues in the staphylococcal Agr system

Phosphorylation sites and sulfhydryl groups in Agr cysteines are indicated. The AgrB membrane endopeptidase requires a catalytic cysteine for cleavage of AgrD. The cysteine in AgrD forms a thiolactone ring in the AIP quorum sensing signal molecule. The AgrC sensor kinase has two cysteines located in a transmembrane and a cytosolic domain. The AgrA response regulator has five cysteines, three located in the response regulator receiver domain and two in the DNA-binding domain.

## 3.2 *S*-nitrosylation of AgrA as a mechanism of quorum sensing inhibition

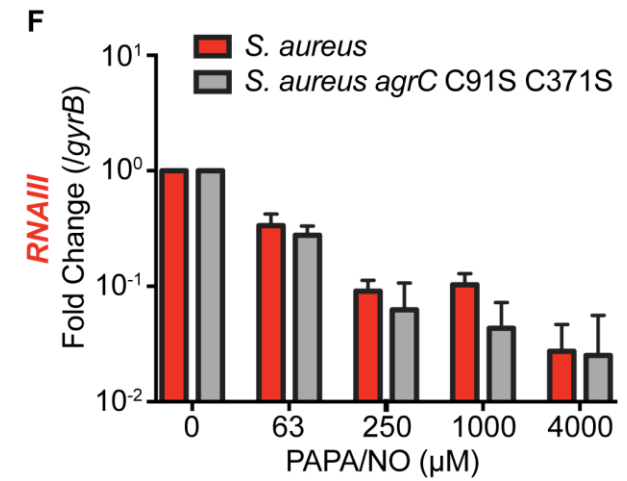
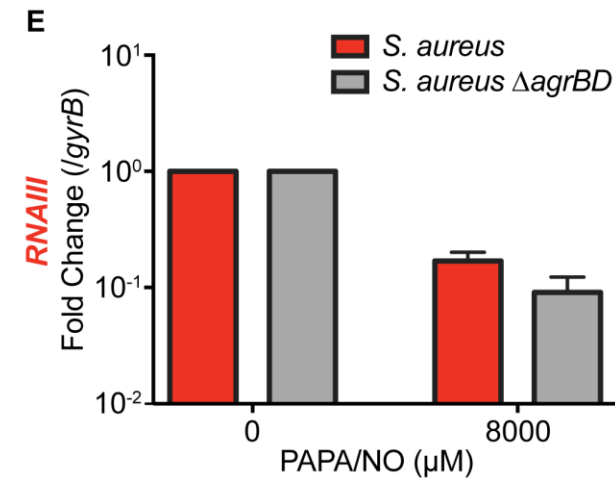
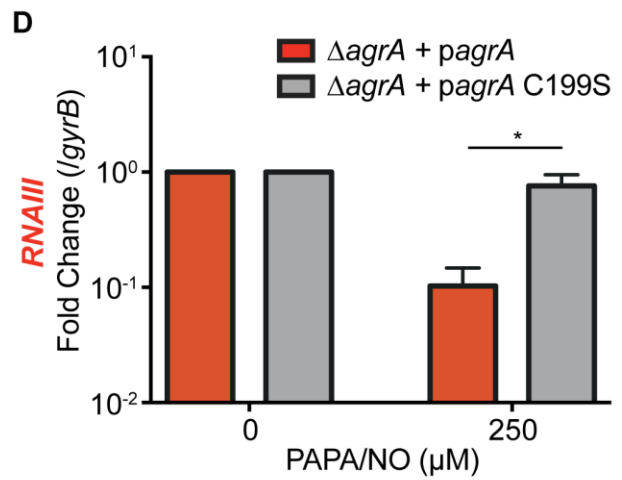
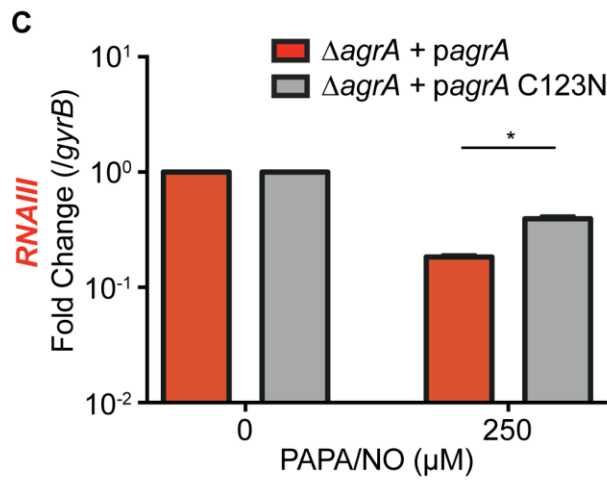
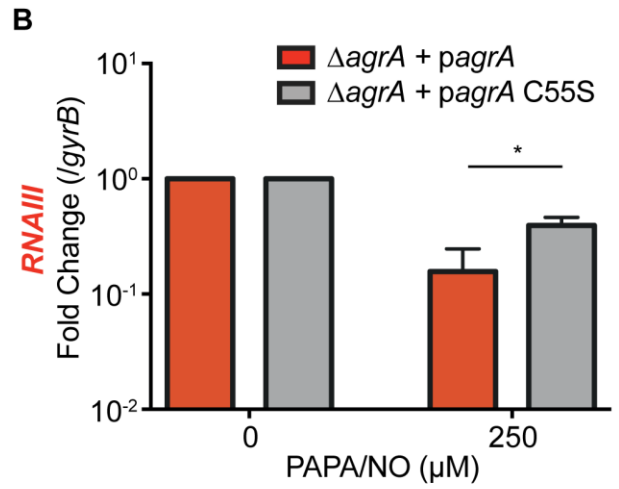
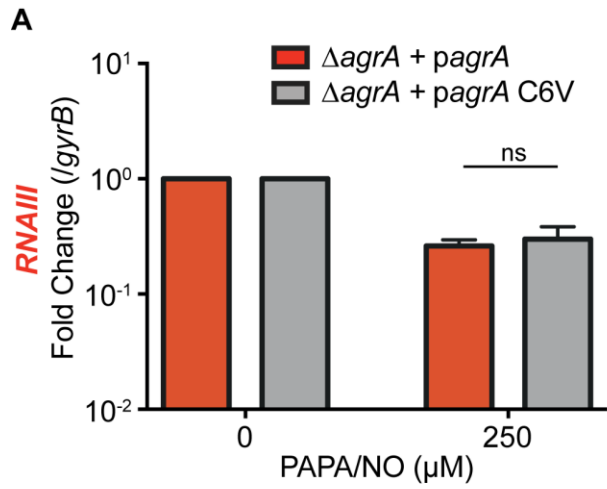
### 3.2.1 AgrA cysteines mediate inhibition by nitric oxide

The susceptibility of cysteine residues to *S*-nitrosylation and the stability of this modification are dependent on their biochemical context within a protein (*e.g.* solvent accessibility, hydrophobicity and electrostatics). It is expected that some unstable modifications might not be detected by our proteomic approach. It is also important to note that not all cysteine modifications will necessarily affect protein function and mediate a biological effect. To determine the AgrA cysteine residues required for NO to inhibit QS, we performed site-specific mutagenesis to replace cysteines with NO-insensitive amino acids. When expressed ectopically, all AgrA cysteine mutants with the exception of C228 maintained functionality with similar activity to the wild-type allele. In our proteomic study, C6, C123, and C199 were each shown to be *S*-nitrosylated. However, site-specific mutagenesis showed that only a C199S mutation conferred a dramatic reduction in NO sensitivity (Figure 3.2D). C55S and C123N mutants displayed a modest reduction in NO-sensitivity, and a C6V mutant was as sensitive to NO as wild-type AgrA (Figure 3.2A-C).

As multiple AgrA cysteines are *S*-nitrosylated and site-specific mutations exhibited varying effects on QS inhibition, it is possible that *S*-nitrosylation of multiple cysteines have distinctive effects on AgrA protein function. Nevertheless, mutant C199S showed the greatest resistance to NO and given its location within the DNA-binding domain, disruption of DNA-binding is likely to be the primary mechanism by which NO disrupts AgrA protein function. AgrA belongs to the LytTR family of regulators which are found widely distributed among bacteria as regulators of virulence and other diverse microbial processes. Intriguingly, many

LytTR regulators show conservation of the NO-reactive cysteines identified in our study (Figure 3.3), suggesting that NO might impact such processes in other bacterial species.

In addition to AgrA, other Agr components with cysteine residues potentially susceptible to *S*-nitrosylation include AgrB, AgrC, and AgrD. An AIP biosynthesis mutant (*S. aureus*  $\Delta agrBD$ ) stimulated with spent medium from wild-type cultures retained NO-sensitivity, indicating that Agr is inhibited independent of AIP production (Figure 3E). Similarly, a mutant strain lacking AgrC cysteines (*S. aureus agrC* C91S C371S) also remained sensitive to NO (Figure 3F). Together these results indicate that NO inhibits the expression of QS-regulated genes by *S*-nitrosylation of AgrA.



**Figure 3.2 Nitric oxide inhibition of quorum sensing is mediated by AgrA C55, C123, and C199**

(A-D) RT-qPCR of *RNAIII* in PAPA/NO-treated *S. aureus* cultures (n=3) ectopically expressing AgrA alleles with single site-specific mutations (C6V, C55S, C123N, or C199S). Student's t test, \*  $p < 0.05$ ; ns, not significant.

(E) RT-qPCR of *RNAIII* in an AIP biosynthesis mutant lacking *agrBD* after stimulation with 20% spent medium from wild-type *S. aureus* and treatment with PAPA/NO (n=3).

(F) RT-qPCR of *RNAIII* in PAPA/NO-treated *S. aureus* cultures (n=3) chromosomally expressing an AgrC allele with two site-specific mutations (C91S and C371S).

★ C6

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S. aureus AgrA      1  -----MKTFTICEDDPKQRENMTIINKNYIMIEEKPMETALATDNP 40
C. perfringens VirR 1  -----MFSIALCEDNSLQREELKNNLSKVLDEIGVEYKLLTFETGE 41
C. difficile CdtR/AgrA1 1  -----MDILIFDNDVCFGIKLEKINNLIKEGFNDVIRLYYNA 40
C. difficile AgrA2 1  MNRNFIYLLKIKYKKGCLRIVISIGICDDELHYRIKIKDILSELSSYPINYNIYEFSSGE 60
L. monocytogenes AgrA 1  -----MLPVIICEDNRMQREERLTKYIEDYIMVEHFDMKLELSTGDP 41
E. faecalis DR75 1828 1  -----MISIIICEDDWRCBQMLETYVKNYIMMESLDMELVFSTGNP 41
S. pyogenes FasA 1  -----MMNFIILEDDFIQQTRIESIVVGIKETRIPCNCLEVFSTP 41
S. pneumoniae BlpR 1  -----MRIFVLIEDDFSQQTRIEETLEKLLKAHHIIPSSFVFGKP 40
K. pneumoniae YehT 1  -----MLRVLIVDDEPLARENLR-----LLETQRDIIVGECGNA 36
E. coli YehT 1  -----MIKVLIVDDEPLARENLR-----VFLQEQSDIIVGECGNA 36
K. pneumoniae KPHS38090 1  -----MSGEKMKVIVIEDEFLLAQQELS-----WLINTHSQMEIVGSFDDG 40
E. coli YpdB 1  -----MKVIVIEDEFLLAQQELS-----WLIKESQMEIVGTFDDG 35

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★ C55

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S. aureus AgrA      41  YEVLECAKMNND-IGCYFLDIQL-----STDINGIKLGSERKHDPVGNIFVTSHSELT 94
C. perfringens VirR 42  ---DLLEREYPENLDMLFLDIDM-----GELTGMETARKVRYDDKVEIIFITALWDYI 91
C. difficile CdtR/AgrA1 41  NLLKELTEKNK-VKIYFIVDAKIKYKISNELCDGLWIAKIRESDYISPIIFLTNHIEM 99
C. difficile AgrA2 61  ---ELNNYPKDLIDILIMDIQM-----KTINGMDIARKIREFDHKLKLEIFVTSFVEM 110
L. monocytogenes AgrA 42  FELVSRMPHQG-MGLYFLDIDLG-----QPDMNGFELAQEIRKFDPRGFIIFITTHAELS 96
E. faecalis DR75 1828 42  LEVLEFVKQSRIIGLYFLDIDL-----QHMSGTLAAKIRDYDNLGKIVFVTHGELS 96
S. pyogenes FasA 42  QKLFESIQERGD-HQLYFLDIEIG-----EYTRCGLELAAAIRQKOPNAVIVFVTHSEFA 96
S. pneumoniae BlpR 41  DCLLAEVHEKGA-HQLFFLDIEIR-----NEEMKGLEVARKLRDRPYALIVFVTHSEFM 95
K. pneumoniae YehT 37  VEAIGAVHKLRP--DVLFLDIQM-----PRISGLEMVG-MLDPEHFRPYIVFLTADEYA 87
E. coli YehT 37  VEGIGAVHKLRP--DVLFLDIQM-----PRISGLEMVG-MLDPEHFRPYIVFLTADEYA 87
K. pneumoniae KPHS38090 41  LDVLFKFLQHNKV--DAIFLDINI-----PSLDGVLAAQNISQFAKPFIVFITAWKEHA 92
E. coli YpdB 36  LDVLFKFLQHNRV--DAIFLDINI-----PSLDGVLAAQNISQFAKPFIVFITAWKEHA 87

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★ C123

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S. aureus AgrA      95  YLTFVYKVAAMDFIFKID-DPAELRTRIIDOLETAHTRL---QLLSKDNSVETIELKGRGS- 149
C. perfringens VirR 92  QKGL--YEVRAFRYLKPKVKFKELQEQVTAOVENILHKR---YTYIT-----LKDKN- 137
C. difficile CdtR/AgrA1 100  LGIFDYRLEVMDFILKH-DMEIAESKIKAIKRY---VKEYNRSNFFTYSDS 153
C. difficile AgrA2 111  QEG--YEVKAYRYILKPINKEKISKVLPONEMMKR---NNYIT-----INVKN- 156
L. monocytogenes AgrA 97  YMTFTYKVEALDYIKD-DIDLHDDRVLACMKQAEERI---SNDQ--DMQKYFTFKVSDK 150
E. faecalis DR75 1828 97  YLTFYKVEALDYIKD-NKEHLHQKICENTQLAHERI---TSERG-DTKKLFKFDGD- 150
S. pyogenes FasA 97  PISFKYKVSALDFIDKAGGQKQFKEQIEECIRYTYDMM---SSRES---KDMFLFETPQ- 149
S. pneumoniae BlpR 96  PLSFRYQVSALDYIDKALSAEEFESRIETALLYANSQD---SKSLA---EDCFYFKSKF- 148
K. pneumoniae YehT 88  VKA--FEEHAFDYLLKPIEAARLEKTLARLRQERNLQD---VSLLDQAQQLKYIPCTGH 142
E. coli YehT 88  IKA--FEEHAFDYLLKPIEAARLEKTLARLRQERSKQD---VSLLPENQALKFIPCTGH 142
K. pneumoniae KPHS38090 93  VEA--FELEAFDYILKPYQESRIINMLQKLTAWQQQNNAASGLASAAPRENDTINLIKD 150
E. coli YpdB 88  VEA--FELEAFDYILKPYQESRITGMLQKLEAAWQQQN-TSSTPAATVTRENDTINLVKD 144

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★ C199

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S. aureus AgrA      150  NSVYVQYDDIMFFESSTKSHRLIAHLDNRCIEFYGNLKELSQLDDR---FFRCHNSFVVNR 207
C. perfringens VirR 138  NVLKIIRTEDILFLETF--ERKVIHTNSQDYIVKMSMNKLEKELNNKGFFRCHTSYIVN- 194
C. difficile CdtR/AgrA1 154  SLWKISFDEVYFETSAIPHKIKLWTTTSRIEFYKSLRSLSDLDAC---FIRVHKSFVVK 211
C. difficile AgrA2 157  YVDRKIDSITVLETDA--RPNILIYTHDDMYITKMSISKIEKILNEYGFFRCHNSYIVN- 213
L. monocytogenes AgrA 151  KIHHELLDILFFETAPTIHKVILHGKNRQVEFYGKLNIEKMLDES--FYRCHRSYIVNK 209
E. faecalis DR75 1828 151  TIRSDMAIIFFESSVSHKIVLHLENGEIEFYGSLKEIEEQSND--FYRCHRSYLINR 208
S. pyogenes FasA 150  TRKLPYKIDILYFATAATPHKVCWLTQTERLEFYGNLSEIQAVAPK--LFLCHRSYLVNL 207
S. pneumoniae BlpR 149  AQFQYPFKVEVYLETSPRAHRVILYTKDRLEFTASLEEVFKQEP--LLRCHRSFLINP 206
K. pneumoniae YehT 143  SRWLLQMEDVAFVSSRMSGIYVTDREGKGFTELTLRLESRT--LLRCHRSYLVNM 199
E. coli YehT 143  SRIYLLQMKDVAFVSSRMSGVYVTSHEGKEGFTTELTLRLESRT--LLRCHRSYLVNL 199
K. pneumoniae KPHS38090 151  ERIIVTSHDIYYAEAHEKMTFVYTR-ESFVMPMNIIEFCSKLPDAHFRCHRSYCVNL 209
E. coli YpdB 145  ERIIVTPIINDIYYAEAHEKMTFVYTR-ESYVMPMNIIEFCSKLPDPHFRCHRSYCVNL 203

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★ C228

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S. aureus AgrA      208  HNIESTDSKER---IVYFKNKEHCYASVRNVKKTIL----- 238
C. perfringens VirR 195  --LIKIEEIKK---DYLLINKFTLPVSKHRMKNLKLRLTSLIGLIG 236
C. difficile CdtR/AgrA1 212  YHIVSLDLKKN---NIKMSNGHICRISNTYRNILKNIKTI--- 248
C. difficile AgrA2 214  --LKLIVESMSG---STVIDGKSIPIKRYRVKGLKLAITNIGLIG 255
L. monocytogenes AgrA 210  KNIHELDTTKG---VVKMSNGENCYASSKLIKSLSL----- 242
E. faecalis DR75 1828 209  KHISKVLIKSER---IVEMSNGERCLVSVRAMKNL----- 239
S. pyogenes FasA 208  DKVVRIKSKQ---LLYFENGDSMVSRLKMKCLFERWEALR----- 246
S. pneumoniae BlpR 207  ANVHLDKKEK---LLFFPNGGSLIARYKIVREMSAATNKLIH----- 245
K. pneumoniae YehT 200  AHLKEIRLEENGQAELLMR-AGQTVPVSRRYLKSLEAIGL----- 239
E. coli YehT 200  AHLQEIIRLEDNGQAELILR-NGLTPVSRRYLKSLEAIGL----- 239
K. pneumoniae KPHS38090 210  SKIREIEPWFNNTYILRLRDLDFQVPVSRSKIVKEFRQLMNL----- 250
E. coli YpdB 204  NKIREIEPWFNNTYILRLKDLDFEVPVSRSKIVKEFRQLMHL----- 244

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**Figure 3.3 Amino acid sequence alignment of AgrA with LytTR transcription regulators**

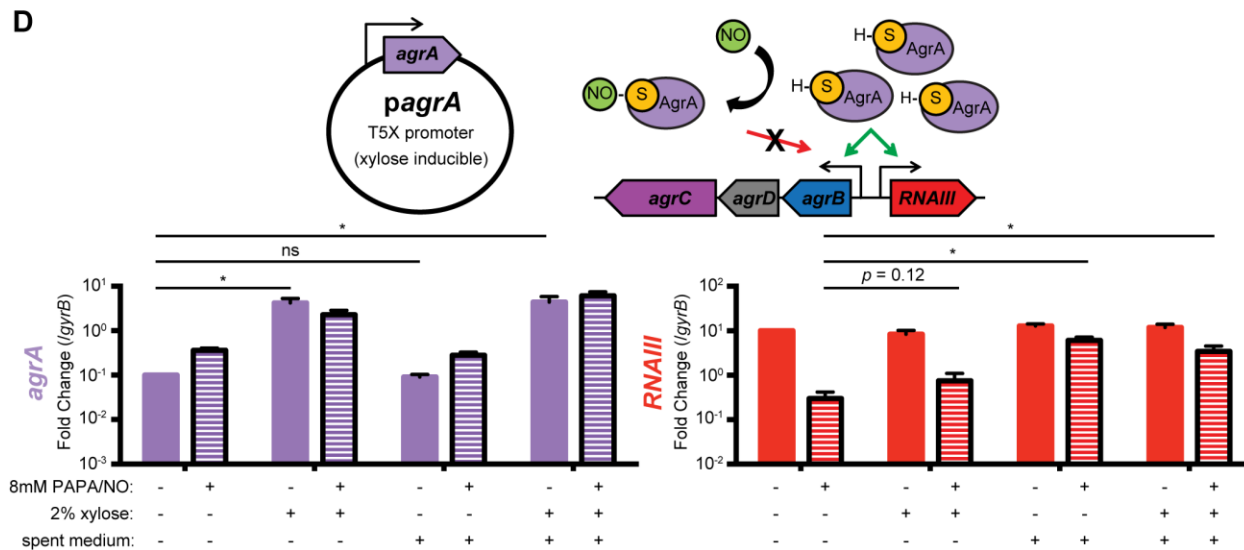
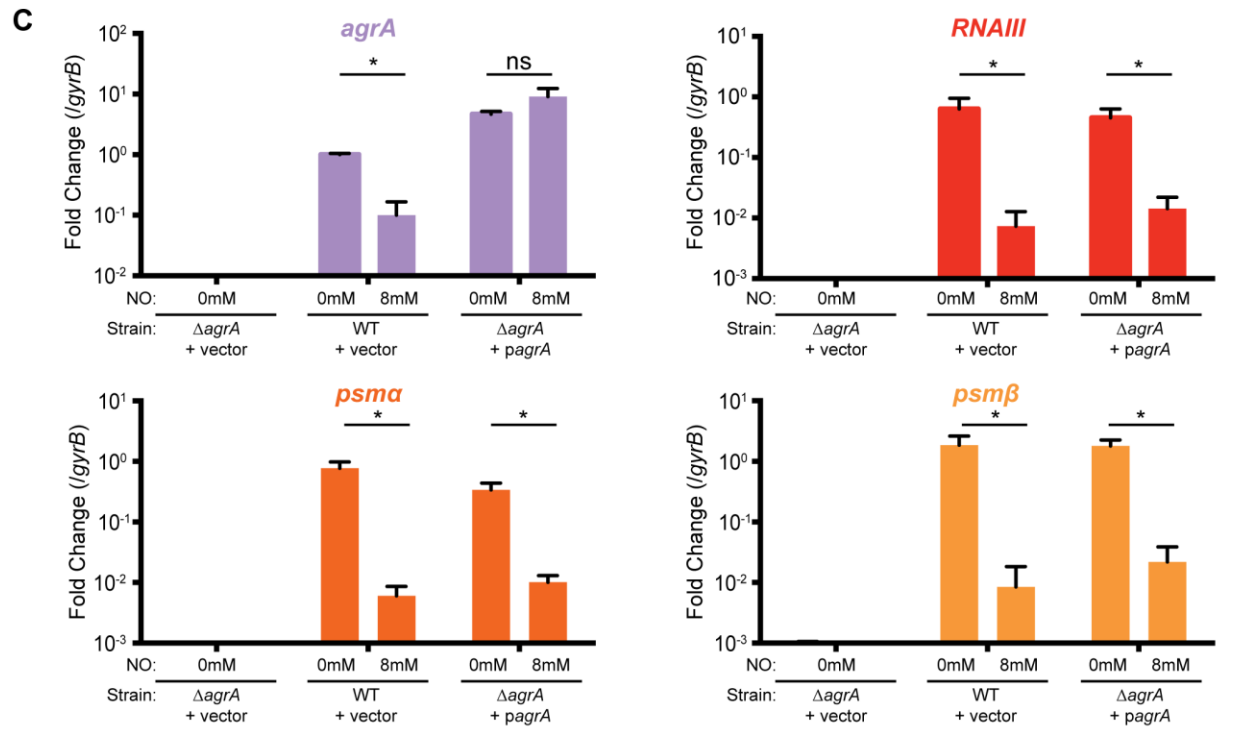
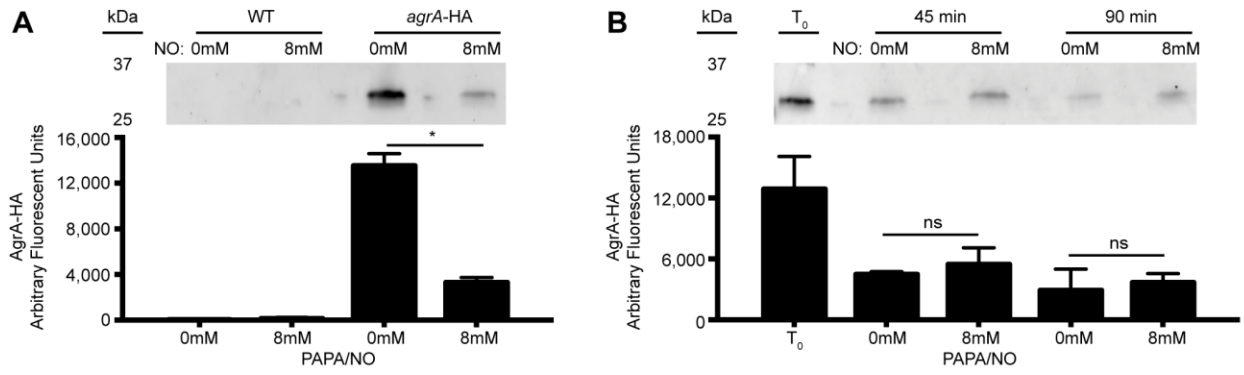
Amino acid similarities and identities are shaded gray; stars show the position of cysteine residues also present in *S. aureus* AgrA; red stars indicate cysteines shown to mediate NO inhibition in this study.

### 3.2.2 Nitric oxide disrupts AgrA occupancy of target promoters

To gain further insight into the mechanism of QS inhibition, the effects of NO on AgrA protein stability and function were determined. As AgrA is auto-regulated, it was unsurprising that NO lowered *agrA* transcript (Figure 2.4B and Figure 3.4C) as well as protein levels (Figure 3.4A). Protein stability assays showed no impact on AgrA abundance if NO treatment occurred after protein translation was inhibited with tetracycline (James et al., 2013) (Figure 3.4B), thereby excluding the possibility that NO enhances the rate of AgrA degradation. Instead, low transcript and protein levels result from impaired auto-activation of the *agrP<sub>II</sub>* promoter. We reasoned that if native AgrA auto-regulation were eliminated by promoter replacement, AgrA transcript levels would become NO-insensitive while AgrA-activated genes would remain inhibited. This was confirmed using a strain carrying *agrA* on a plasmid driven by the T5X promoter, which exhibited low levels of RNAIII, *psma* and *psm $\beta$*  transcripts following NO treatment despite preserved *agrA* transcript levels (Figure 3.4C). It was further predicted that increasing bacterial levels of activated AgrA would also protect against NO inhibition. Accordingly, AgrA activation with AIP-containing spent medium rescued inhibition of RNAIII (Figure 3.4D), but induction of AgrA was unable to restore RNAIII expression in the absence of spent medium.

Modification of AgrA cysteines, in particular C199 within the DNA-binding domain, is predicted to impair binding to AgrA-regulated promoters (Sun et al., 2012). To test whether NO decreases AgrA occupancy of the *agrP<sub>II</sub>* and *agrP<sub>III</sub>* promoters, chromatin immunoprecipitation-qPCR (ChIP-qPCR) was performed. DNA proximal to AgrA binding sites, within the *agr* intergenic and RNAIII coding regions, showed specific ChIP-enrichment compared to control DNA regions (*gyrB*, *gmk*, *rpoD*, and ChIP<sub>c</sub>) (Figure 3.5). Importantly, AgrA promoter occupancy significantly decreased following NO treatment. Decreased promoter

occupancy was not the result of inhibited auto-regulation, as AgrA was expressed from the T5X promoter in these experiments. We conclude that NO inhibits staphylococcal QS by impairing AgrA-mediated transcriptional activation of target promoters.



**Figure 3.4 S-nitrosylation of AgrA impacts AgrA protein abundance by disrupting Agr auto-activation**

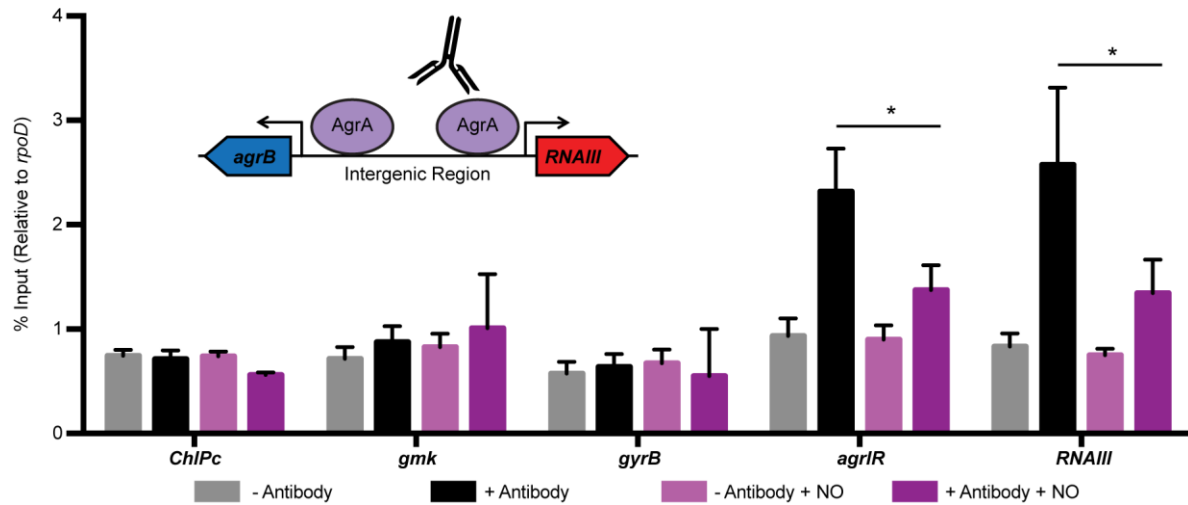
(A) Western blot of HA-tagged AgrA from *S. aureus* cultures treated with NO (n=3).

(B) Western blot of HA-tagged AgrA from *S. aureus* cultures treated with NO after inhibition of protein synthesis with tetracycline (n=2).

(C) RT-qPCR of AgrA-regulated genes after NO treatment of *S. aureus*  $\Delta agrA$  *pagrA* cultures expressing AgrA from the T5X non-native promoter (n=3).

(D) RT-qPCR of *agrA* and RNAPIII from cultures ectopically expressing AgrA and activated with spent medium preceding NO treatment (n=3).

Student's t test, \*  $p < 0.05$ ; ns, not significant.



**Figure 3.5 Nitric oxide decreases AgrA promoter occupancy at target promoters**

ChIP-qPCR of NO treated *S. aureus* cultures (n=4). DNA regions proximal to AgrA binding sites include *agrIR* and *RNAIII*; control regions include *ChIPc*, *gmK*, and *gyrB*. All chromosomal regions are normalized to a control region of the *rpoD* gene. Student's t test, \*  $p < 0.05$ ; ns, not significant.

### 3.3 Discussion

My observations demonstrate that *S*-nitrosylation of AgrA impairs the transcription of QS-regulated virulence factors. The inhibitory effect of NO on the Agr QS system is largely dependent on a single cysteine target in AgrA (C199). Although the Agr system has multi-components with several critical cysteines residues that are required for function, I did not observe a requirement for QS inhibition of components other than AgrA. Inhibition of QS occurred in the absence of AIP biosynthesis, showing AgrB and AgrD are not required and that inhibition occurs at or downstream of AIP sensing. Inactivation of the AIP molecule by NO is not supported by its biochemistry given the lack of sulfhydryl groups. Highly oxidative species such as hypochlorite and peroxynitrite have been shown to inactivate QS by oxidizing the methionine residue of AIP (Rothfork et al., 2004), but NO reactivity in the absence of other strong oxidants (*e.g.* superoxide) is limited to metal centers, thiols, and other radicals. The cysteines in the AgrC sensor kinase have not been implicated in Agr function but in principle could have mediated NO inhibition by preventing signal transduction upon AIP binding. However, we found no evidence in our proteomic study that AgrC cysteines are susceptible to NO modification, and AgrC site-specific mutants lacking cysteine residues remain sensitive to NO. Multiple cysteine thiols in AgrA are subject to *S*-nitrosylation, and the AgrA C199S mutation confers resistance to NO *in vitro*. The mechanism of QS inhibition highlights the importance of molecular context for protein cysteines to undergo *S*-nitrosylation and to elicit a physiological response. The sensitivity of AgrA C199 to NO demonstrated in our study, along with the susceptibility of this residue to oxidation by H<sub>2</sub>O<sub>2</sub> (Sun et al., 2012), suggest that this residue has evolved to integrate redox signaling with QS.

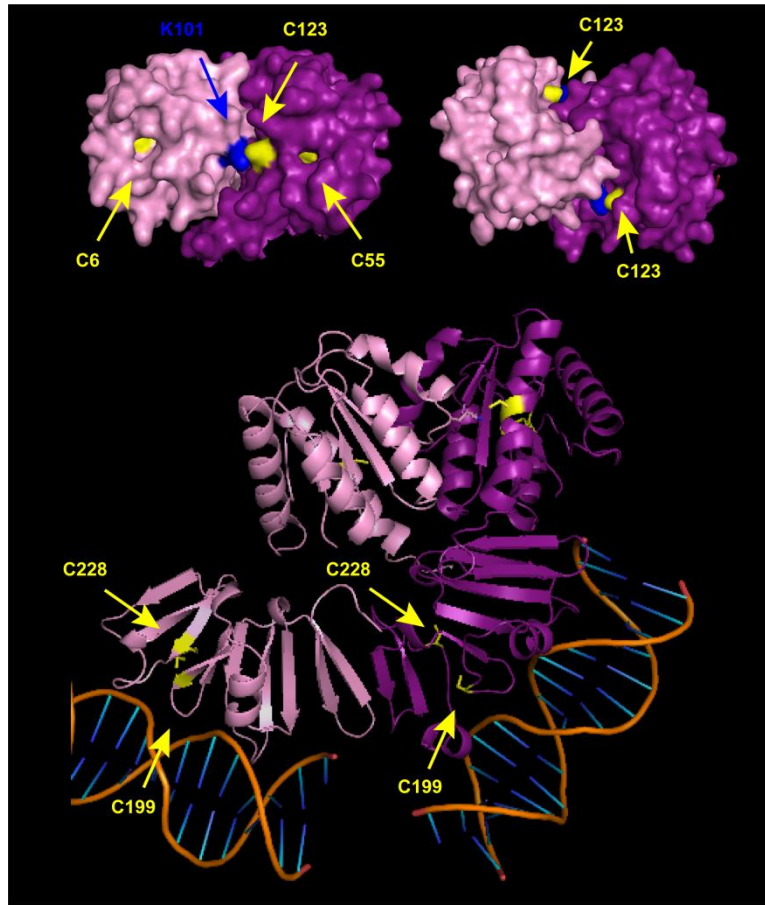
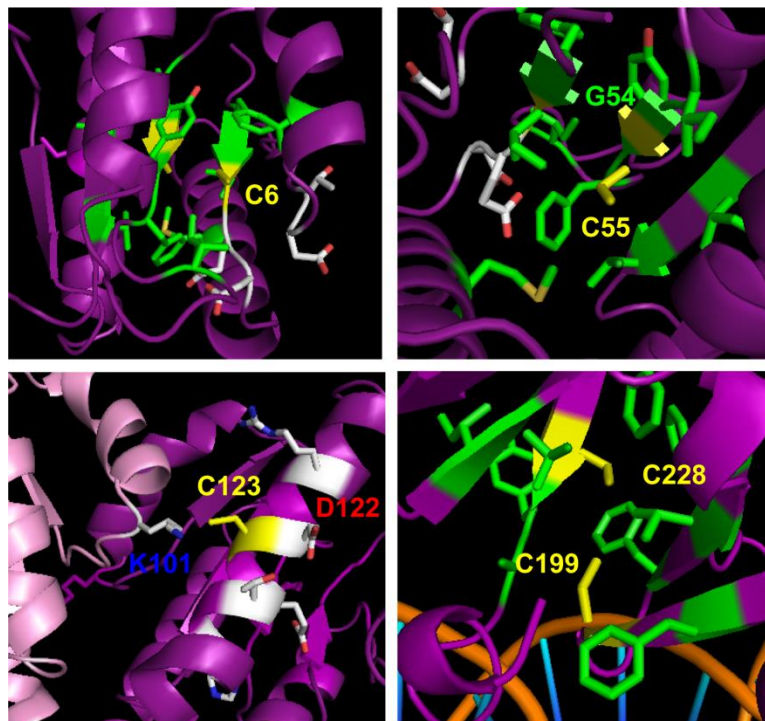
The biochemical basis for *S*-nitrosylation is not completely understood, and multiple mechanisms have been proposed to explain protein selectivity. Thiyl radicals can directly interact with NO to form *S*-nitrosylthiols. However, direct reactivity of NO with cysteine sulfhydryl groups (thiols) does not occur but rather requires the one-electron oxidation of NO by transition metals or molecular oxygen prior to nitrosylation (Smith and Marletta, 2012; Stamler, 1994). Another potential mechanism involves the oxidation of NO to N<sub>2</sub>O<sub>3</sub>, which can directly react with thiolate groups to form nitrosylthiols (Smith and Marletta, 2012). Lastly, transnitrosylation reactions can occur in a protein-protein manner or between protein thiols and low molecular weight thiols such as *S*-nitrosoglutathione (GSNO) (Smith and Marletta, 2012). The protein context in which a thiol is located also plays a role in the formation and stability of *S*-nitrosylthiols, and the multiple protein environments in which cysteine residues are found are important determinants of their reactivity. Both hydrophobic and hydrophilic environments can harbor cysteines that are prone to *S*-nitrosylation (Doulias et al., 2010). The favored formation of N<sub>2</sub>O<sub>3</sub> has been proposed to explain *S*-nitrosylthiols in hydrophobic environments, whereas charged residues in surface-accessible areas may favor transnitrosylation reactions (Doulias et al., 2010; Mitchell et al., 2007; Nedospasov et al., 2000; Smith and Marletta, 2012).

The five cysteine residues in AgrA are found in distinctive protein environments that include hydrophobic regions and surface-accessible sites. In the AgrA DNA-binding domain, the sulfhydryl side chains of C199 and C228 point toward each other and are surrounded by hydrophobic residues (Figure 3.6). Modification of C199 may occur via the formation of N<sub>2</sub>O<sub>3</sub> in this hydrophobic environment. In addition, the proximity of C199 with C228 could allow transnitrosylation reactions. We did not identify C228 in our proteomic study, but this does not rule out the possibility that this residue is also modified. C228 is thought to be required for

proper protein folding, and substitution mutations to multiple amino acid residues at this site disrupt AgrA function (Sun et al., 2012). Although the exact biochemical mechanisms by which C199 is *S*-nitrosylated will require further biochemical studies, its proximity to bound DNA and the decreased occupancy of AgrA at its target promoters strongly suggest that NO disrupts AgrA binding to DNA. C6 and C55 are in distinct but similar hydrophobic regions within the response regulator receiver domain (Figure 3.6). Interestingly, these are found in deep, yet surface-accessible pockets with proximal charged residues. However, it is not evident how *S*-nitrosylation could disrupt protein function. With a glycine preceding the modified cysteine and a largely hydrophobic context, C55 fits a major primary sequence motif identified in a previous study of *S*-nitrosylthiols (Doulias et al., 2010). The most distinctive protein context is that of C123, which is found in a surface-exposed region and fits another primary sequence motif characterized by an aspartic acid at position -1 (Doulias et al., 2010). This sulfhydryl is located on an  $\alpha$ -helix near the AgrA dimer interface, and interestingly, a lysine residue from the opposing monomer seems to make electrostatic interactions with C123. The presence of a lysine or other basic residues can favor thiol deprotonation and formation of a thiolate group, whereas proximal acidic residues promote NO<sup>+</sup> donation from nitrosylthiols; together the acid-base environment in the surface-exposed context of C123 suggests a transnitrosylation mechanism (Hess et al., 2005; Pérez-Mato et al., 1999; Stamler et al., 2001). *S*-nitrosylation of this residue could disrupt the stability of the AgrA dimer, explaining the partial NO-resistance of the C123S mutant.

AgrA sensitivity to NO is dependent on multiple cysteines but most strongly attributed to C199. These observations may reflect the propensity of this thiol to react with NO. However, the location of C199 within a DNA-binding region may also facilitate a physiological response

through disruption of protein function. This is in contrast to C6, which is susceptible to *S*-nitrosylation but does not mediate Agr inhibition by NO. The contribution of C55 and C123 may be additive and could have evolved in subsequent steps over evolutionary time. Interestingly, we found that a triple AgrA mutant (C55S, C123N, C199S) is severely functionally compromised (not shown), despite functionality of the respective single mutants. This suggests that multiple *S*-nitrosylation events may lead to a greater inhibitory effect. Collectively, my site-specific mutagenesis studies, along with the ChIP-qPCR experiments probing AgrA promoter occupancy, provide a strong molecular basis for Agr inhibition by NO.

**A****B**

### Figure 3.6 Protein contexts of AgrA cysteines

(A) Model of the AgrA dimer based on the structures of the AgrA DNA-binding domain (PDB 3BS1) and ComE dimer (PDB 4CBV). AgrA monomers are shown in pink and purple. Top images show the response regulator receiver domain with solvent-exposed C6, C55, and C123 (left). Possible interactions between K101 and C123 in opposing monomers are indicated (left) and displayed at both sides of the AgrA dimer (right). Bottom image shows C199 and C228 in the DNA-binding domain of the two monomers.

(B) Amino acid residues within 6Å of AgrA cysteines (hydrophobic side chains, green; charged amino acids, white). C6, C55, C199, and C228 show a protein context dominated by hydrophobic interactions, whereas the context of C123 is dominated by interactions with positively- and negatively-charged residues. G54 near C55 shows a characteristic glycine of a primary sequence motif associated with *S*-nitrosylthiol formation. Adjacent to C123, K101 and D122 show a possible acid-base catalytic motif typical of thiols involved in transnitrosylation reactions.

## **Chapter 4. Nitric Oxide Protects the Host against Staphylococcal**

### **Infection**

#### **4.1 Agr QS in murine pneumonia**

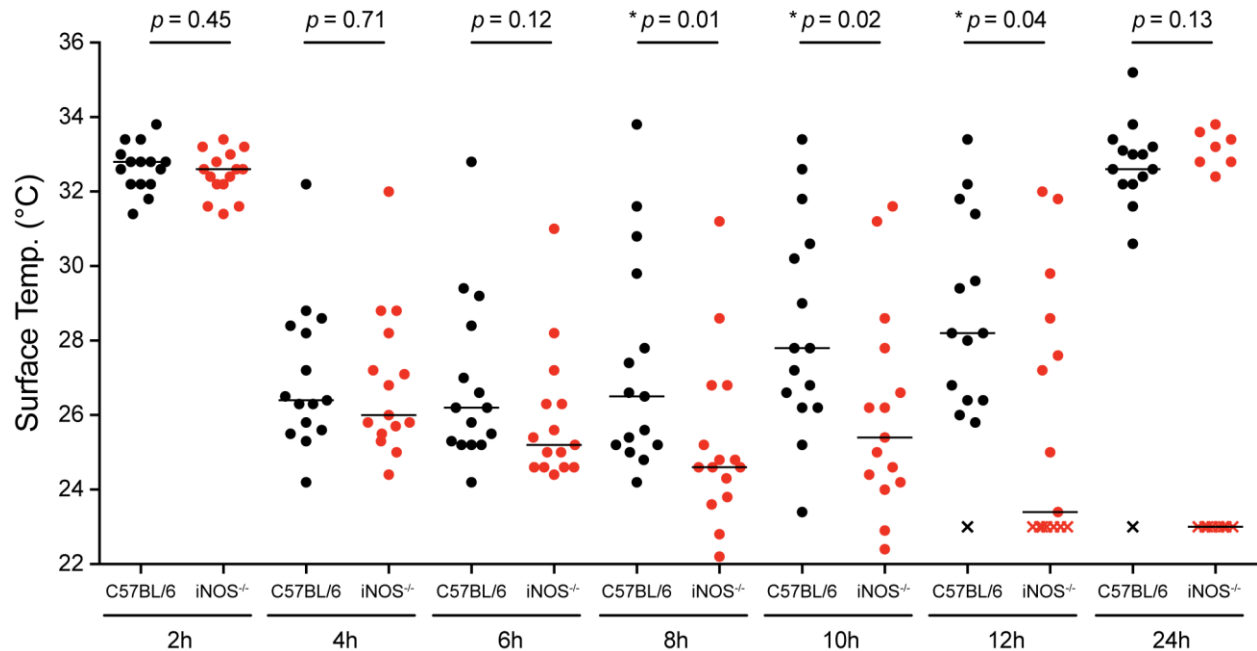
Virulence in *S. aureus* is largely dependent on Agr QS. Mutants defective in Agr function are attenuated in multiple models of infection including endocarditis, osteomyelitis, septic arthritis, skin abscess, and pneumonia (Novick and Geisinger, 2008; Thoendel et al., 2011). In the murine model of staphylococcal pneumonia, the Agr-regulated  $\alpha$ -toxin mediates lung damage, increased inflammation and mortality (Bartlett et al., 2008; Bubeck Wardenburg et al., 2007a; Bubeck Wardenburg et al., 2007b). Mutant strains lacking Agr,  $\alpha$ -toxin, or PSMs display reduced cytotoxicity toward human and murine immune cells (Kitur et al., 2015). Additionally, PSM $\alpha$ 4-stimulated release of heparin-binding protein by neutrophils increases lung vascular leakage and contributes to pathogenesis (Li et al., 2016). The availability of C57BL/6 and congenic iNOS-deficient mice, along with a strong dependence on Agr-regulated toxins for pathogenesis, makes the murine pneumonia model suitable for testing the contribution of NO to host defenses.

#### **4.2 Host production of nitric oxide is protective against staphylococcal infection**

##### **4.2.1 Mouse mortality increases and more severe disease develops in the absence of iNOS**

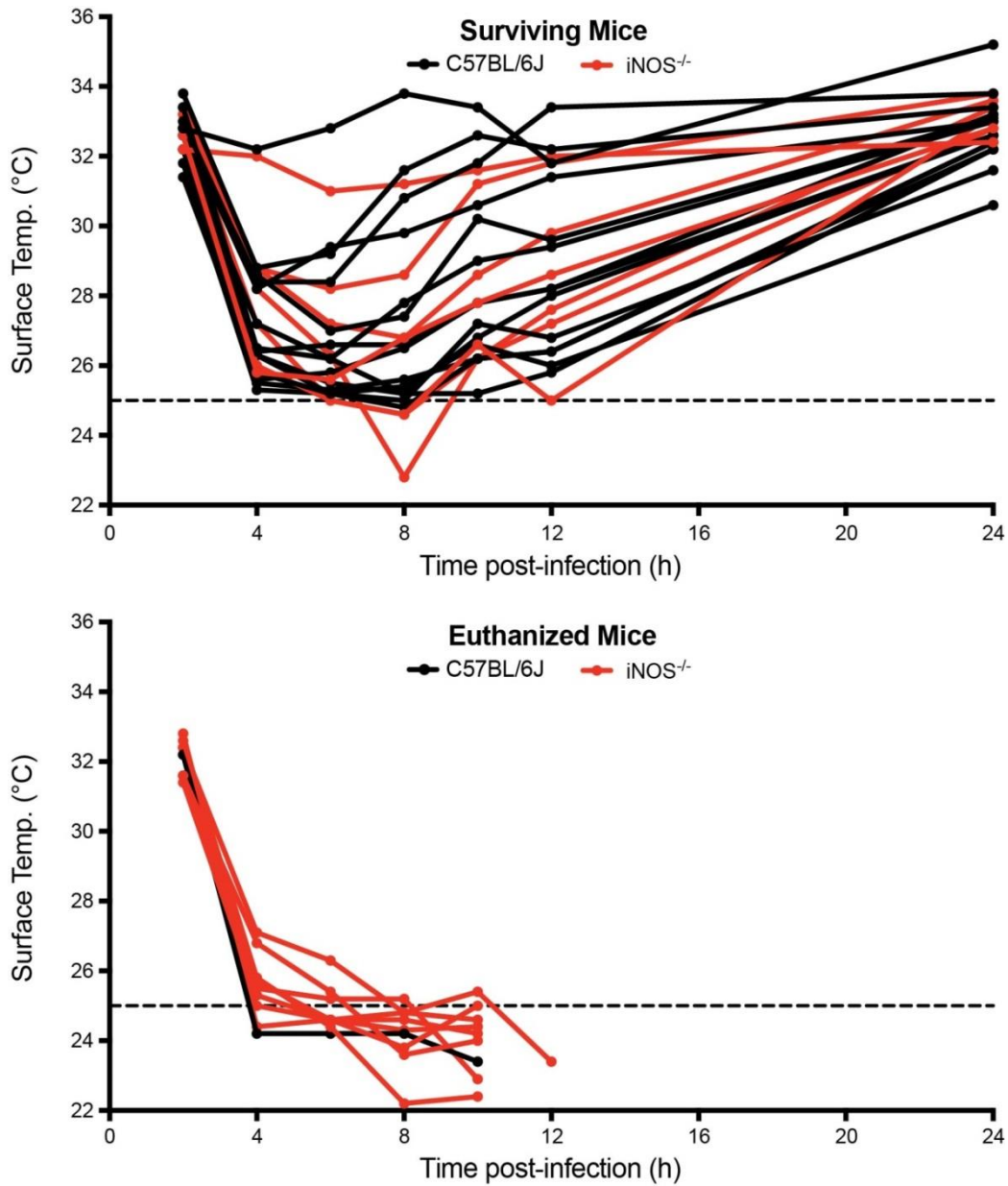
To determine whether host-derived NO has a protective role *in vivo*, C57BL/6 mice and congenic iNOS knockout mice were challenged with wild-type *S. aureus* via an intranasal route. As mice become moribund and succumb to infection, they experience a fall in body surface temperature, a sign associated with impending mortality (Bast et al., 2004). We monitored body surface temperature over 24 h post-infection and euthanized mice that became moribund.

Strikingly, 53% of iNOS knockout mice (8 of 15) became moribund compared to 7% of C57BL/6 (1 of 15 mice), and statistically-significant differences in body surface temperature were observed from 8-12 h post-infection (Figure 4.1A). Euthanized mice displayed signs of increased disease severity including reduced mobility, hunched posture, and labored breathing. In addition to the visual criteria used to euthanize mice, body surface temperature was notable for multiple measurements below 25°C in mice that became moribund, while surviving mice approached 25°C before rebounding (Figure 4.2). Continuous monitoring of mouse body surface temperature thus provided an objective approach to assess disease severity and showed that iNOS knockout mice develop more severe staphylococcal pneumonia compared to C57BL/6 mice.



**Figure 4.1 Nitric oxide is protective in staphylococcal pneumonia**

Surface temperature of C57BL/6 (black circles, n=15) and congenic iNOS<sup>-/-</sup> (red circles, n=15) mice at indicated time points post-infection with wild-type *S. aureus*. X= mice euthanized due to a moribund state. Mann-Whitney U test, \*  $p < 0.05$ .



**Figure 4.2** Body surface temperature of surviving and euthanized mice infected with *S. aureus*

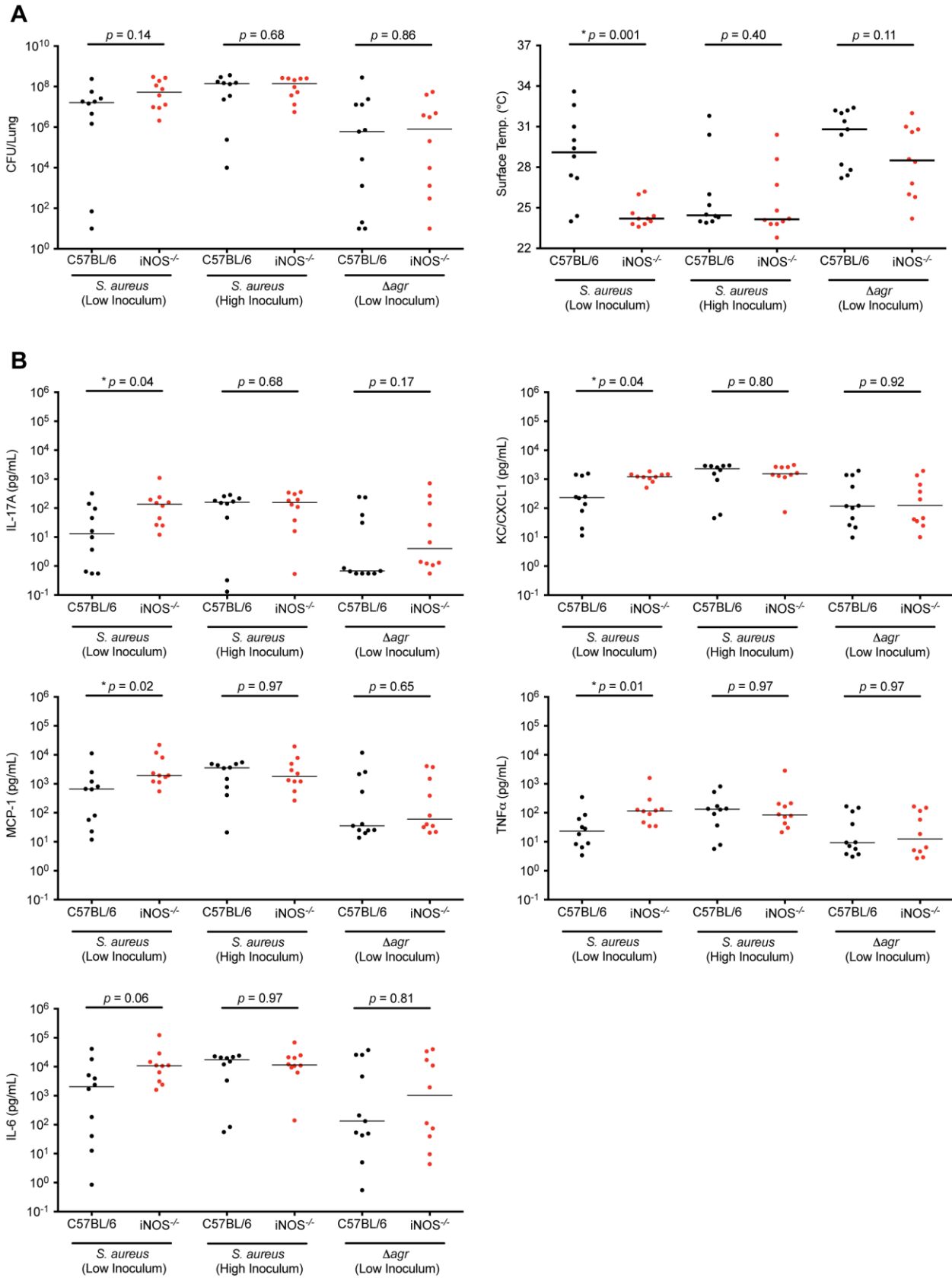
Measurements of surface temperature in C57BL/6 (black lines, n=15) and congenic iNOS<sup>-/-</sup> (red lines, n=15) mice at indicated time points post-infection. Dashed line at  $y=25^{\circ}\text{C}$  marks one criterion used for euthanasia. Other euthanasia criteria included low mobility, labored breathing and a moribund state.

#### 4.2.2 iNOS-deficient mice elicit a high inflammatory cytokine response

As NO can slow staphylococcal growth (Richardson et al., 2008), we determined whether the superior clinical status of infected C57BL/6 mice could be attributed to lower bacterial burdens. The lungs of iNOS knockout mice did not contain significantly different bacterial burdens at 8 h post-infection despite their lower body surface temperature (Figure 4.3A) and higher levels of pro-inflammatory cytokines (Figure 4.3B). In contrast to C57BL/6 mice, iNOS knockout mice did not show a statistically significant relationship between bacterial burden and body surface temperature, which is a predictor of mortality (Figure 4.4). Similar bacterial burdens in C57BL/6 and iNOS knockout mice, along with the lack of an association between CFU and lower body surface temperature in iNOS mice, suggest that the protective effect of NO in staphylococcal pneumonia cannot be attributed to the bacteriostatic properties of NO.

Rather, the protective effects of NO in C57BL/6 mice may result from the inhibition of Agr QS and reduced production of virulence factors, as observed *in vitro*. As the Agr-regulated  $\alpha$ -toxin plays a critical role in lung damage and inflammation, serum cytokine levels in wild-type and iNOS-deficient mice were compared. Serum levels of multiple pro-inflammatory cytokines including KC, MCP-1, IL-17A, and TNF $\alpha$  were significantly higher in the iNOS knockout mice than in congenic C57BL/6 controls (Figure 4.3B). A higher trend for IL-6 ( $p = 0.06$ ) was also observed (Figure 4.3B). In previous studies, KC and Th17 cytokine responses were found to be stimulated by  $\alpha$ -toxin and associated with poor outcomes in staphylococcal pneumonia (Bartlett et al., 2008; Frank et al., 2012; Martin et al., 2011). Similarly, higher levels of IL-6, MCP-1, and TNF $\alpha$  have correlated with worsened clinical outcomes (Kitur et al., 2015; Parker et al., 2015; Robertson et al., 2008; van den Berg et al., 2013).

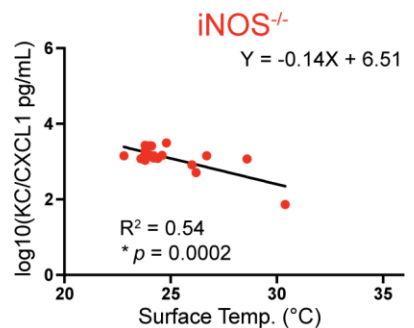
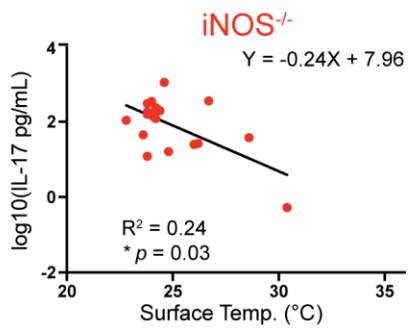
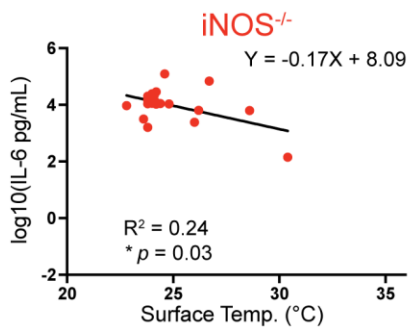
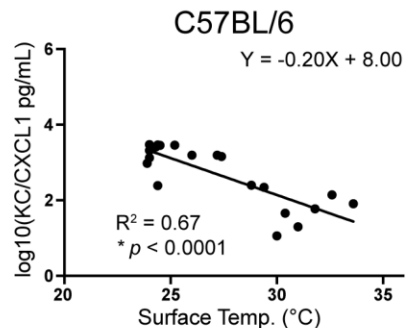
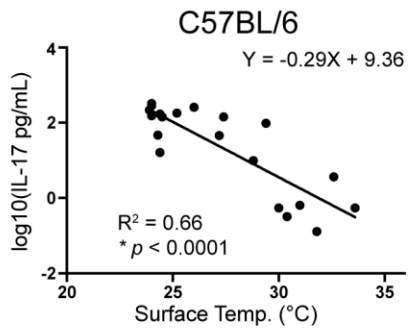
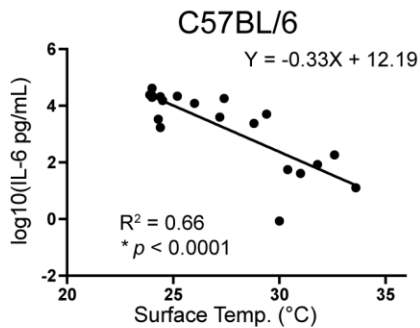
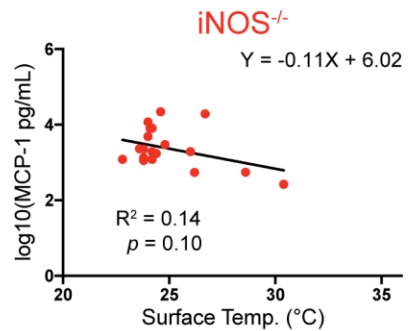
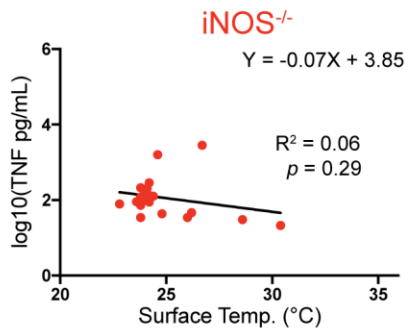
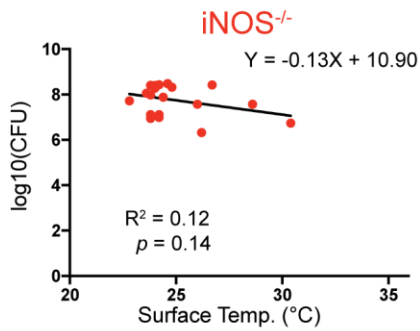
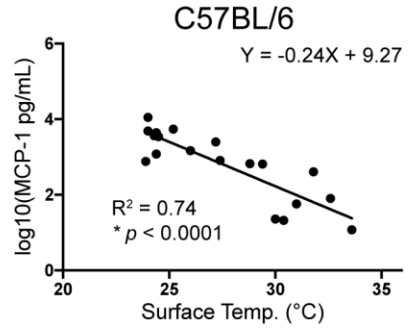
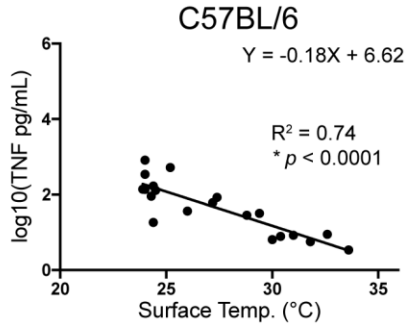
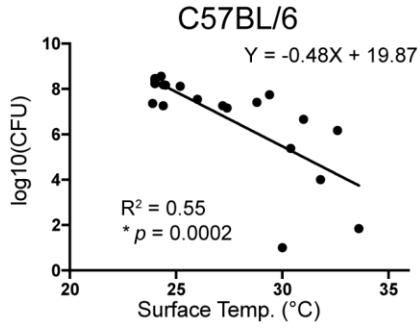
The administration of increased bacterial inocula abrogated NO-dependent differences in disease severity (as indicated by body surface temperature) and pro-inflammatory cytokine responses (Figure 4.3), indicating that the protective effect of NO can be overwhelmed by high bacterial burdens. Nevertheless, the analysis of individual mice showed that disease severity correlates with bacterial burden only in wild-type but not in iNOS-deficient mice after both low and high bacterial inocula (Figure 4.4), whereas linear regression analysis revealed a negative relationship between disease severity and serum levels of pro-inflammatory cytokines IL-6, IL-17A, and KC in all mice, consistent with the importance of differential  $\alpha$ -toxin production in the protective actions of NO during staphylococcal pneumonia. Collectively, these results suggest that NO protects the host by inhibiting the expression of bacterial virulence factors that promote inflammatory responses.



**Figure 4.3 iNOS deficiency is associated with high serum levels of inflammatory cytokines**

(A) Bacterial burden (CFU per lung), surface temperature, and (B) pro-inflammatory cytokines levels of C57BL/6 (black circles, n=10) and congenic iNOS<sup>-/-</sup> (red circles, n=10) mice measured at 8 h post-infection. Two inocula are indicated for infections with wild-type *S. aureus* (~5 x 10<sup>7</sup> or ~1 X 10<sup>8</sup> CFU) and one inoculum for mice infected with an *agr* mutant (~5 x 10<sup>7</sup> CFU).

Mann-Whitney U test, \*  $p < 0.05$ .



**Figure 4.4 Linear regression analysis of body surface temperature against bacterial burden and serum cytokines**

Relationship of mouse body surface temperature with log<sub>10</sub> transformed bacterial burden (CFU/Lung) and cytokine serum levels (pg/mL) in C57BL/6 (black) and congenic iNOS knockout mice (red).

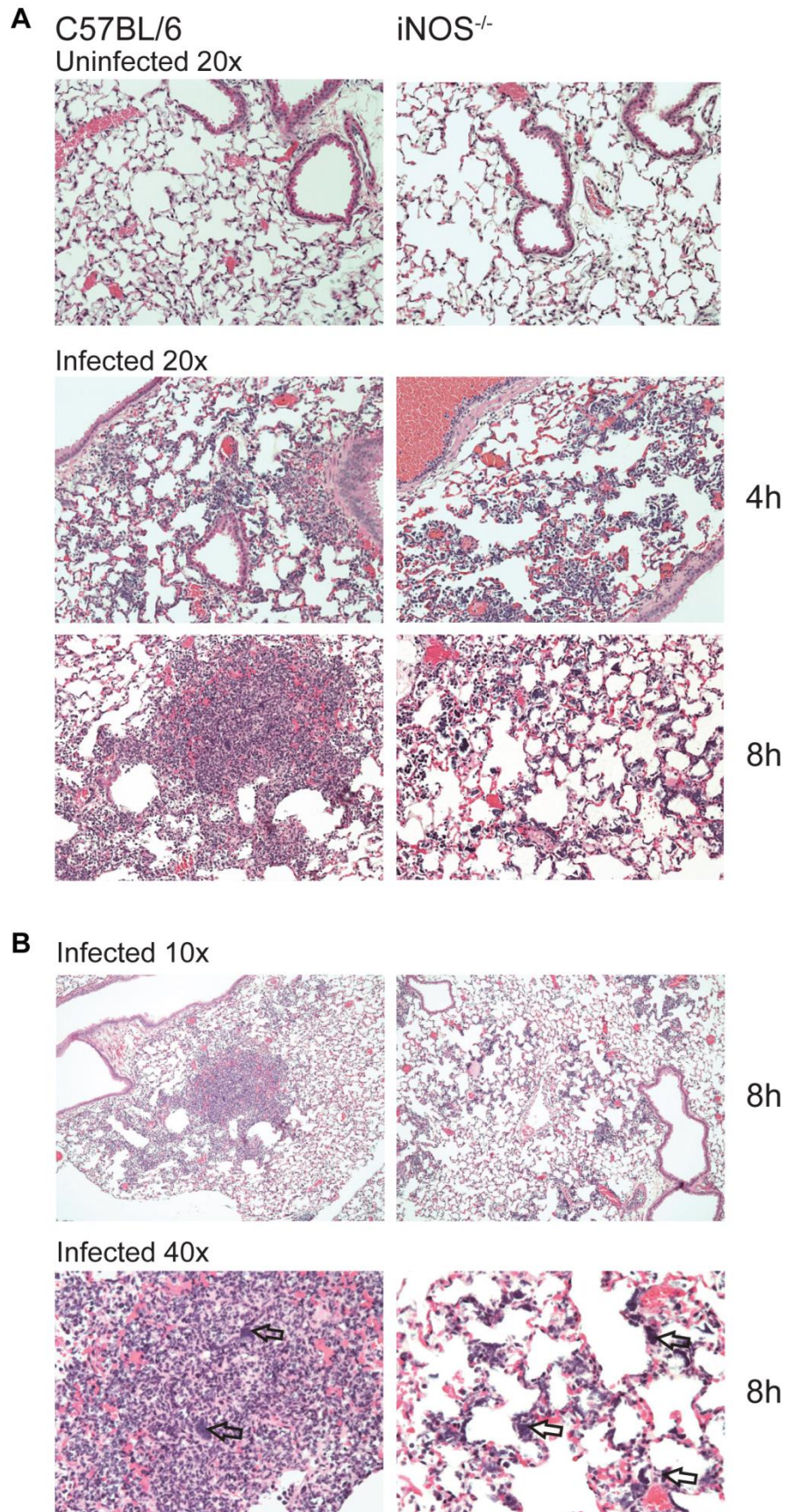
R<sup>2</sup> indicates goodness of fit; \* p < 0.05 indicates slope is different from zero, n=20.

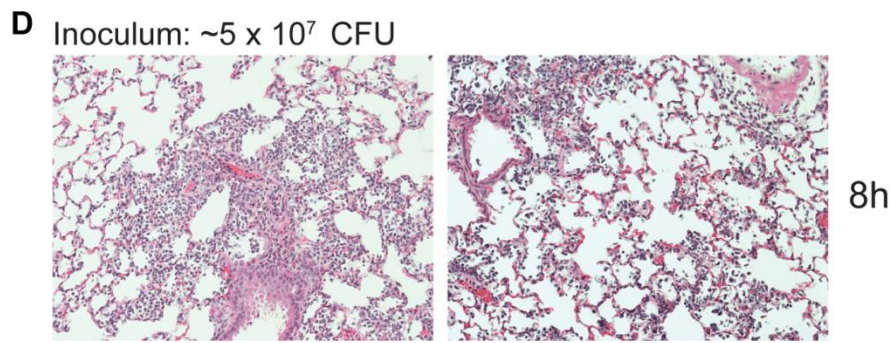
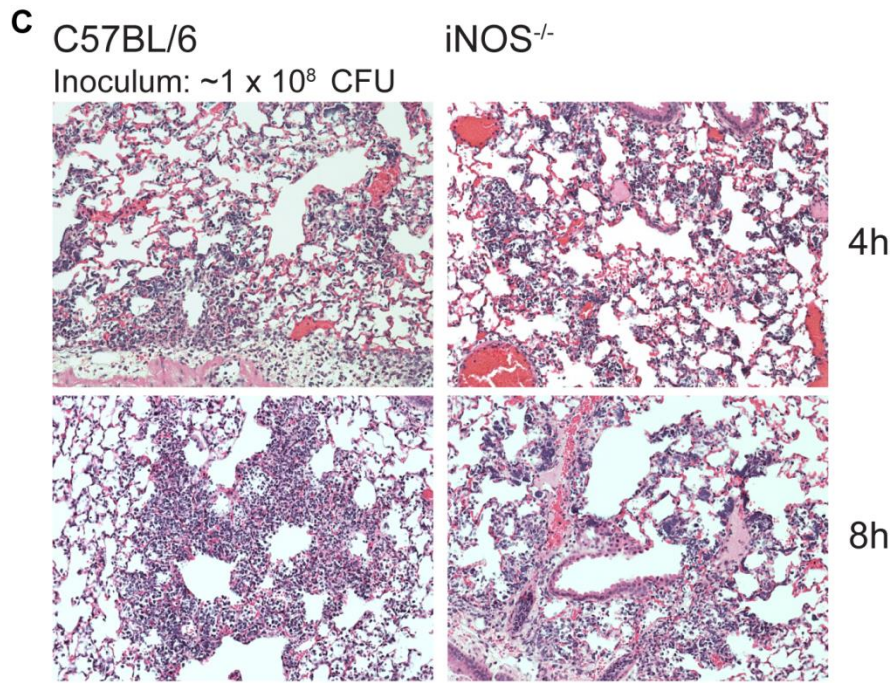
### **4.2.3 C57BL/6 and iNOS-deficient mice display distinctive lung histopathology**

Increased virulence factor production in iNOS-deficient mice during staphylococcal pneumonia is expected to result in different lung inflammatory responses. At 4 h post-infection, histologic changes between C57BL/6 and iNOS knockout mice were subtle. However, by 8 h post-infection distinctive morphologic patterns distinguishing infected C57BL/6 and iNOS-deficient mice became evident (Table 4.1). Lungs from C57BL/6 mice displayed numerous highly cellular, focal-to-confluent aggregations of densely packed neutrophils with few mononuclear cells (Figure 4.5). These foci, scattered throughout the lung sections, surrounded blood vessels and small bronchioles, variably involving adjacent alveolar walls (Figure 4.5B). Clusters of bacteria and necrotic debris were evident particularly within the centers of aggregates (Figure 4.5B). At 8 h post-infection, these inflammatory cell aggregates were morphologically consistent with early-stage abscess formation. In contrast, lungs from iNOS-deficient mice exhibited more diffuse infiltration of inflammatory cells with many bacteria and an interstitial distribution that was predominately localized along alveolar walls (Figure 4.5 and Table 4.1). Some gender-dependent differences were noted, with definition of lesions tending to be less distinct in female mice and inflammatory cell accumulations less prominent than was observed in males (Figure 4.5C and Table 4.1). At the lower inoculum, female mice showed a further reduction in inflammation and pattern definition, although the basic trends were still evident (Figure 4.5D). The distinctive histopathological differences observed between C57BL/6 and congenic iNOS knockout mice are consistent with a different immune response and ability to control infection resulting from differences in virulence factor production, further highlighting the protective role of host NO in staphylococcal pneumonia.

**Table 4.1 Histology scores for mouse lung sections**

<b>Mouse Group (n=mice per group)</b>	<b>Predominant Lesion Pattern</b>	<b>Mean Inflammatory Cell Accumulation (scale: 1-4)</b>	<b>Mean Bacterial Load (scale: 1-4)</b>
4 h post-infection Inoculum: $\sim 1 \times 10^8$ CFU			
C57BL/6 Males (n=6)	Alveolar	1.2	2.2
C57BL/6 Females (n=5)	Alveolar	1.2	2.4
iNOS <sup>-/-</sup> Males (n=5)	Mixed	1	2
iNOS <sup>-/-</sup> Females (n=6)	Mixed	1.3	1.8
8 h post-infection Inoculum: $\sim 1 \times 10^8$ CFU			
C57BL/6 Males (n=7)	Focal Aggregation	3.9	3
C57BL/6 Females (n=8)	Focal Aggregation	2.4	2.5
iNOS <sup>-/-</sup> Males (n=6)	Alveolar	1.8	3.7
iNOS <sup>-/-</sup> Females (n=8)	Alveolar	2.1	2.5
8 h post-infection Inoculum: $\sim 5 \times 10^7$ CFU			
C57BL/6 Females (n=7)	Mixed	2.1	1.3
iNOS <sup>-/-</sup> Females (n=5)	Alveolar	1	1.8





**Figure 4.5 Deficiency in host nitric oxide production alters the histopathological pattern of staphylococcal pneumonia**

(A) Representative H&E stains of lung section at 20x magnification from C57BL/6 and iNOS knockout male mice either uninfected (top panels) or infected with  $\sim 1 \times 10^8$  CFU at 4 h (middle panel) and 8 h (bottom panel) post-infection.

(B) Representative H&E stains of lung sections at 10x (top panel) and 40x (bottom) magnification from C57BL/6 and iNOS knockout male mice at 8 h post-infection. Arrows indicate bacterial clusters.

(C) Representative H&E stains of lung sections at 20x magnification from C57BL/6 and iNOS knockout female mice infected with  $\sim 1 \times 10^8$  CFU at 4 h (top panels) and 8 h (bottom panels) post-infection.

(D) Representative H&E stains of lung sections at 20x magnification from C57BL/6 and iNOS knockout female mice infected with  $\sim 5 \times 10^7$  CFU at 8 h post-infection.

### 4.3 Discussion

The protective actions of iNOS during staphylococcal infection despite the intrinsic resistance of *S. aureus* to NO-growth inhibition are most likely attributable to the repression of virulence gene expression by NO. Consistent with this hypothesis, iNOS-deficient mice showed no significant difference in bacterial burden at 8 h post-infection despite a dramatic difference in disease severity as indicated by hypothermia, higher inflammatory cytokines responses, and differential lung histopathology. The inhibition of *S. aureus* virulence gene expression by NO *in vitro*, along with the increased expression of cytokines/chemokines known to be dependent on the Agr-regulated  $\alpha$ -toxin, further support a role of NO in repressing bacterial virulence. Alveolar and interstitial macrophages are appropriately situated to detect lung pathogens and regulate inflammatory responses (Hussell and Bell, 2014; Kopf et al., 2015). In the murine pneumonia model, lung macrophages represent a possible source of NO, although a contribution from airway epithelial cells cannot be excluded.

A protective role for NO in bacterial pneumonia has also been demonstrated for *Streptococcus pneumoniae* and other lung pathogens (Yang et al., 2014). Interestingly, Yang et al. showed the role for eNOS (endothelial NO synthase, NOS3) in pneumococcal pneumonia to be gender-specific. Overall, we did not find that the protective role of iNOS in staphylococcal pneumonia is gender-dependent, although some differences were observed histologically. Our findings contrast somewhat with an earlier study that failed to observe an effect of iNOS on mouse survival in staphylococcal pneumonia (Köhler et al., 2011). However, we note differences in the inoculum size and bacterial culture conditions between the two studies, and found that a modest difference in inoculum has a substantial impact on clinical outcomes (Figure 4.3).

As a commensal and opportunistic human pathogen, *S. aureus* is well adapted to the selective pressures imposed on bacteria by host immunity. Inhibition of QS by NO may have evolved to maintain a stable relationship between *S. aureus* and the human host. Other scenarios have been described in which staphylococcal immune evasion factors appear to have evolved to directly interact with immune molecules (Thammavongsa et al., 2015). The ability to coordinate virulence factor production in response to host immune responses may provide an evolutionary advantage for *S. aureus* when it encounters the high NO concentrations present in the human nose (Lundberg et al., 1995), where downregulation of exotoxins might help to maintain the bacteria in a commensal state. Indeed, humans with persistent asymptomatic *S. aureus* nasal carriage have been shown to exhibit low expression levels of Agr-activated genes (Burian et al., 2010), although the molecular basis for this observation remains to be determined. A dual role as a commensal and a pathogen might explain why staphylococci maintain a QS system that is sensitive to NO.

## Chapter 5. Conclusions and Future Directions

### 5.1 A new antimicrobial function for nitric oxide as an anti-virulence molecule

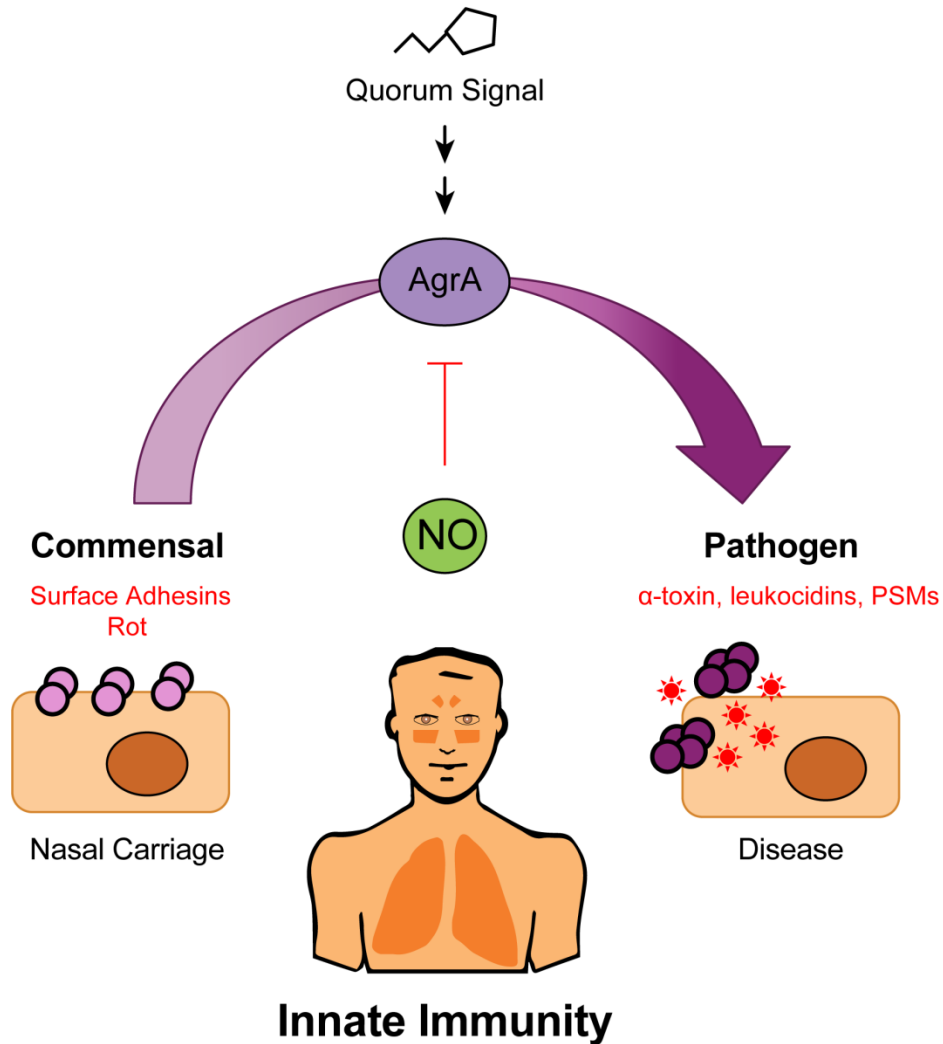
NO is a versatile molecule that plays an important role as an antimicrobial effector in host defense. The ability of iNOS to produce high cytotoxic concentrations of NO confers innate immune cells an effective antimicrobial mechanism to limit pathogen growth during infection. The antimicrobial actions of NO are generally attributed to the bacteriostatic properties that emerge from its pleiotropic effects on microbial metabolism and physiology. Certainly, for many microbial pathogens, growth inhibition by NO is the primary mechanism by which immune cells resist infection. However, the complex biochemistry of NO also allows other less appreciated antimicrobial functions. Microorganisms such as *S. aureus* are well adapted to resist NO cytotoxicity and thrive under conditions of nitrosative stress. Nevertheless, NO is able to protect the host in a variety of staphylococcal infections including pneumonia. My thesis work was undertaken to address this apparent paradox and to better understand the mechanisms of NO-mediated protection during bacterial infections.

I have demonstrated that NO suppresses the expression of staphylococcal virulence genes and characterized the mechanism of inhibition in bacterial cultures. This *in vitro* observation opens the possibility that NO protects the host by additional mechanisms beyond the inhibition of bacterial growth. Through biochemical studies, I identified the Agr QS system as a primary target of NO. Agr is of fundamental importance in inter-bacterial communication and staphylococcal pathogenesis. The activation status of Agr has fundamental consequences for the lifestyle of *S. aureus* that directly affect its relationship with its human host. By suppressing colonization factors and inducing toxin expression, Agr controls the transition of *S. aureus* from

a commensal to a pathogen. I have characterized the molecular mechanism by which NO inactivates QS and inhibits production of downstream virulence factors. My biochemical studies show that the AgrA transcription factor is directly modified at multiple cysteine residues through S-nitrosylation. AgrA modification by NO at C55, C123, and C199 disrupts protein function and blocks transcriptional activation of QS-regulated toxins likely by inhibiting DNA-binding, and perhaps by other mechanisms as well.

Importantly, I provide evidence suggesting that NO inhibition of Agr may occur *in vivo*. My findings in the staphylococcal murine pneumonia model unequivocally show that iNOS protects mice from the most severe outcomes of infection. Decreased mortality, lower inflammatory cytokine responses, and a histopathological pattern that suggest bacterial containment highlight the protective role of NO in staphylococcal disease. The absence of NO-dependent differences in the bacterial burden of infected mice show that canonical NO antimicrobial mechanisms are not the basis for iNOS protection. Collectively my *in vitro* and *in vivo* observations support that NO functions as an anti-virulence molecule by targeting *S. aureus* QS and suppressing virulence factor production (Figure 5.1). This allows QS to modulate *S. aureus* virulence and its interaction with the human host. The innate immune system protects the host from staphylococcal infections by producing NO, which directly targets AgrA and prevents toxin production. The anti-virulence mechanism of protection benefits the host during staphylococcal infection by circumventing toxin tissue damage and may also be beneficial to *S. aureus* by promoting commensalism in the high NO environment of the human nose.

# Staphylococcal Quorum Sensing



**Figure 5.1** NO anti-virulence model of innate immune protection against *S. aureus*

As a commensal bacterium, Agr QS is inactivated and *S. aureus* primarily expresses surface-anchored colonization factor, along with the expression of negative regulators of toxins (e.g. Rot). Upon disruption of NO homeostasis or infection at peripheral sites such as compromised skin or soft tissues, the absence of NO and an increase in bacterial growth triggers pathogenesis and the expression of bacterial toxins (e.g.  $\alpha$ -toxin, leukocidins and PSMs). The innate immune system responds to infection by inducing iNOS expression. NO production directly targets AgrA to suppress virulence and promote bacterial clearance.

## 5.2 Future directions

### 5.2.1 Demonstration of nitric oxide anti-virulence role *in vivo*

My findings show that iNOS has a protective role in murine pneumonia and suggest that NO is beneficial to the host by functioning as an anti-virulence defense mechanism. Although there is substantial evidence supporting the ability of NO to inhibit QS *in vitro*, there is presently no direct evidence that the same mechanism occurs *in vivo*. This gap in my body of work is largely due to technical challenges in attempting to directly link NO and Agr in the murine pneumonia model.

One possible experimental approach to demonstrate Agr inhibition by iNOS *in vivo* would be a transcriptional analysis of bacteria extracted from infected mouse lungs. It is expected that iNOS-deficient mice would show a greater expression of Agr-dependent transcripts compared to congenic C57BL/6 mice. However, one technical issue with this approach is that the number of bacteria that are recoverable from lungs is low, and the RNA yield from a single mouse is insufficient to perform RT-qPCR. In preliminary work, I found that bacteria from at least 4-5 mice must be pooled per sample. Given that multiple mouse groups, multiple time points, and experimental replicates would be needed, this would quickly lead to a large number of animals used. As in many mouse experiments, a further complication arises from the heterogeneous response of different mice within a group. Pooling mice has a risk of obscuring differences in infection that are manifested only in individual subjects. Furthermore, the bacteria present in the mouse lung are pooled from heterogeneous microenvironments. Unlike a bacterial culture, in which most of the population is synchronously exposed to NO, in a mouse infection it is very likely that only some bacterial populations encounter NO (e.g. those inside a macrophage). Spatial differences in iNOS expression are also possible if sepsis has a significant

contribution to the overall disease outcome of the mice. Lastly, temporal differences in bacterial NO exposure are also likely to occur. Without a full understanding of the sources of NO in the lung and the inability to isolate bacterial subpopulations, demonstration of a direct link between Agr and iNOS will be difficult to achieve with the current technical limitations.

An alternative approach to directly link NO to Agr inhibition would be to compare disease outcome between wild-type *S. aureus* and a mutant that is resistant to NO inhibition. Although we have shown *in vitro* that substituting AgrA cysteine residues with NO-insensitive amino acids partially abrogates Agr inhibition, we have been unable to construct a functional mutant that lacks all of the cysteines that mediate inhibition of QS. A triple mutant (AgrA C55S C123S C199S) has no activity and therefore cannot be used directly link NO to Agr *in vivo*. A double mutant (AgrA C55S C199S), although functional, is still somewhat sensitive to NO *in vitro*, and preliminary experiments suggest that this mutant could be attenuated *in vivo* in an NO-independent manner. One possible explanation for the attenuated virulence is the enhanced sensitivity to reactive oxygen species of the C199 mutant, which has been shown *in vitro* (Sun et al., 2012). Without functionally identical Agr mutants that differ only in NO-sensitivity, it is not possible to interpret the *in vivo* behavior of these mutants with confidence.

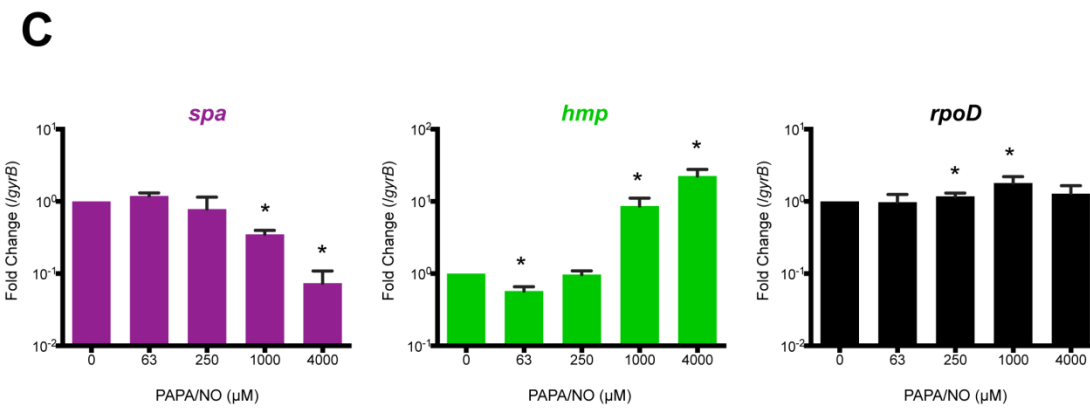
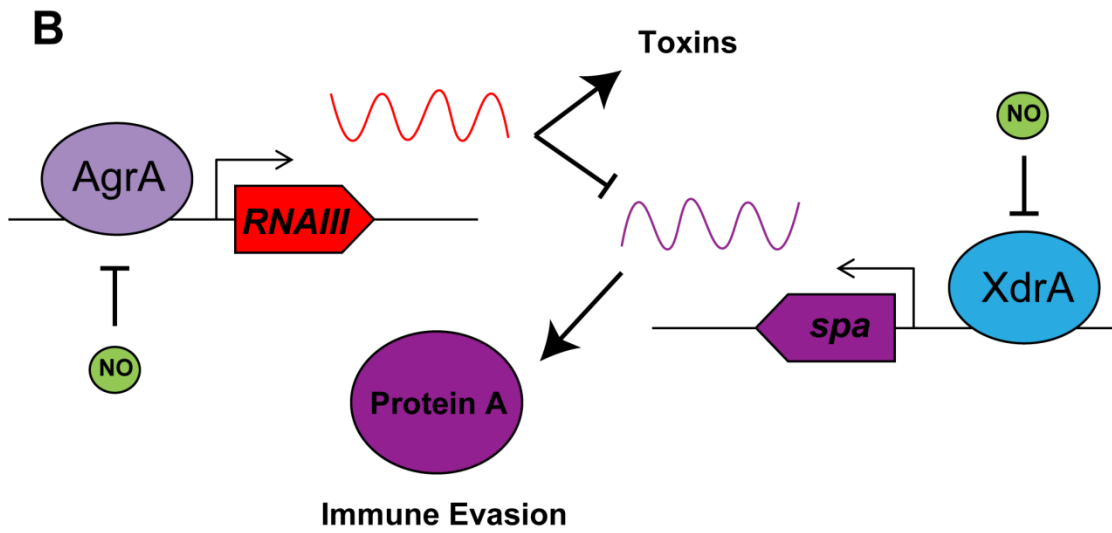
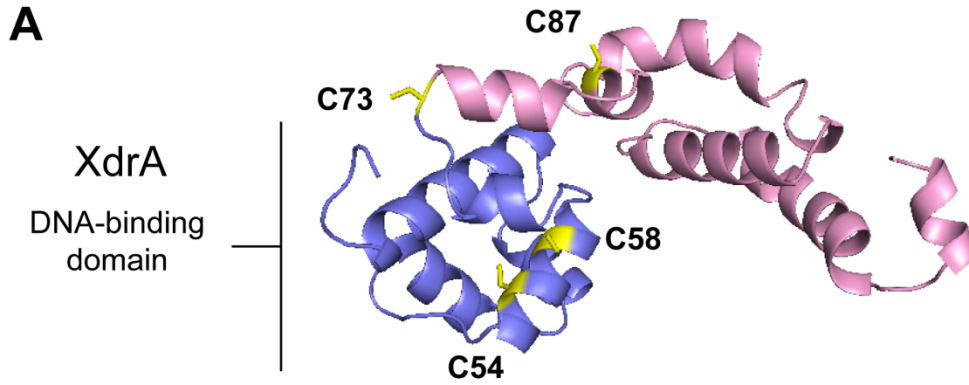
Future efforts to demonstrate that NO directly inhibits Agr QS *in vivo* will have to overcome these hurdles. As more sensitive techniques are developed to measure *in vivo* bacterial gene expression or toxins at a single cell level, it may be possible to definitively demonstrate the anti-virulence role of NO in murine pneumonia.

### **5.2.1 Nitric oxide may also confer protection against staphylococcal protein A**

As I explored the sensitivity of Agr QS to NO, I inadvertently came upon the observation that NO can also inhibit expression of the immune evasion factor, protein A. Agr QS is known to

regulate the expression of staphylococcal surface proteins through RNAIII, which negatively regulates the *spa* transcript to prevent protein A expression. Because of the regulatory link with Agr, it was expected that *spa* transcripts would increase under conditions of nitrosative stress. To my initial surprise, *spa* transcript levels decreased upon NO treatment. Upon further literature review, it became apparent that multiple other regulators modulate expression of protein A. Among these regulators is XdrA, which is required for protein A expression under our conditions. XdrA also contains four cysteine residues that may be targets of S-nitrosylation. If correct, a mechanism analogous to what I observed in AgrA may account for the inhibition of this immune evasion factor by NO.

Inhibition of protein A by NO would have important implications for staphylococcal-host interactions. Protein A protects *S. aureus* against phagocytosis, modulates inflammatory responses, and interferes with proper development of adaptive immune responses. Curiously, Agr activation in the bloodstream is inhibited by serum, and Agr mutants are associated with cases of bacteremia (Painter et al., 2014). Additionally, it has been shown that the adhesion properties of protein A contribute to bacterial dissemination from the bloodstream (Edwards et al., 2012). In this and other contexts where expression of immune evasion factors is promoted, NO inhibition of protein A might be beneficial to the host. Although Agr plays a critical role in pneumonia, protein A also stimulates lung inflammatory responses and contributes to mouse mortality (Bubeck Wardenburg et al., 2007b; Gómez et al., 2004; Gómez et al., 2006). Concomitant NO suppression of Agr-dependent toxins and protein A complicates discerning the two mechanisms by which NO may be protective to the host *in vivo*. Nevertheless, both toxin inhibition and immune evasion factor suppression would be consistent with an anti-virulence role of NO.



**Figure 5.2 Inhibition of protein A expression by nitric oxide**

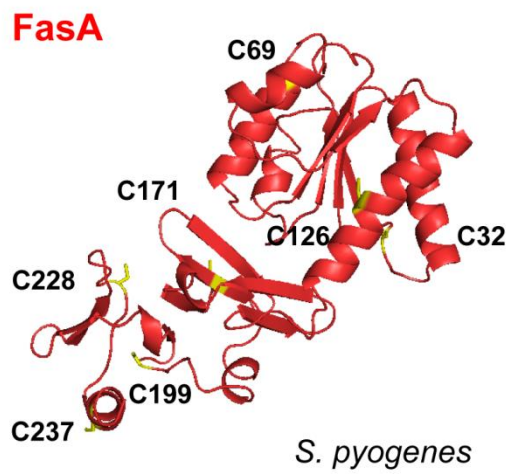
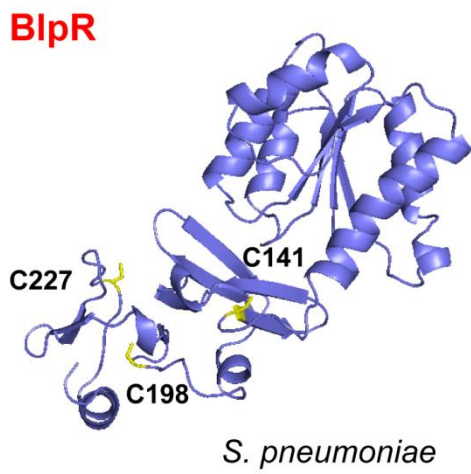
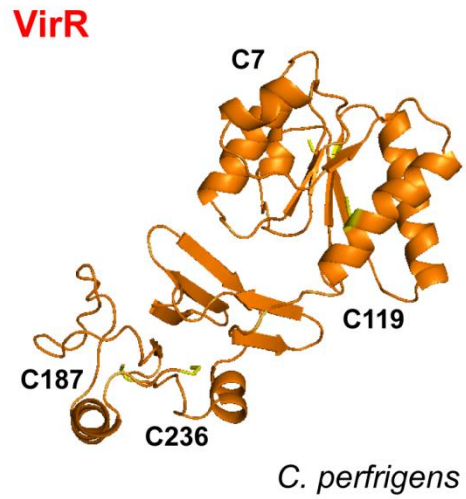
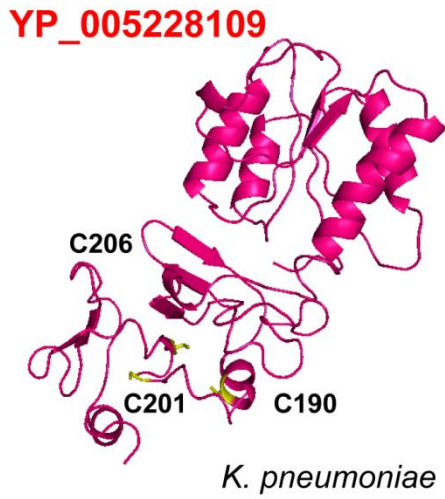
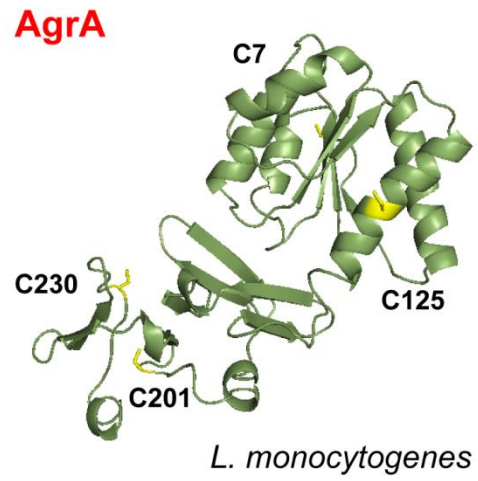
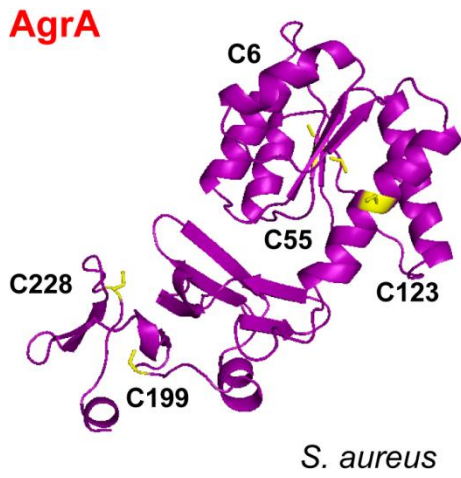
(A) Protein structure model of XdrA indicating putative sites of *S*-nitrosylation. The predicted DNA-binding domain is shown in blue and the dimerization domain in pink.

(B) Partial regulatory model of protein A and proposed mechanism of inhibition by nitric oxide.

(C) RT-qPCR of *S. aureus* bacterial cultures treated with indicated concentration of the NO donor PAPA/NO. The *spa* transcript is shown in purple, the nitrosative stress gene *hmp* is shown in green, and the housekeeping gene *rpoD* is shown in black.

### 5.2.3 Nitric oxide: beyond an anti-virulence role in staphylococcal infections

This dissertation is focused on the interactions of a single transcription factor with one biological molecule. However, my finding with regard to cysteine and transcription factors may be applicable in a broader biological context. Regulators similar to AgrA, belonging to the LytTR family, control diverse bacterial processes and are widely distributed in other clinically important pathogenic bacteria (*e.g. Clostridium, Enterococcus, Listeria, Streptococcus, Klebsiella*) (Galperin, 2008). Protein structure-based models suggest that cysteine residues in other LytTR transcription factors may have analogous roles to those in AgrA (Figure 5.3). Curiously, many of these regulator have characterized primary functions that are not redox sensing. The VirR and FasA proteins of *C. perfringens* and *S. pyogenes* regulate virulence factors similarly to AgrA (BaThein et al., 1996; Klenk et al., 2005; Ohtani et al., 2010; Ramirez-Peña et al., 2010). The *S. pneumoniae* regulator BlpR regulates bacteriocins that are involved in intraspecies competition (Dawid et al., 2007). This suggests that the modulation of protein activity by redox-active molecules may have arisen as a secondary function, perhaps to render bacterial physiological processes responsive to specific environmental conditions. Modulation of QS, virulence, and other bacterial responses by NO or other redox-active species may prove to be a widespread phenomenon in nature.



**Figure 5.3** LytTR regulators with putative redox active cysteines

### 5.3 Concluding remarks

The body of work presented in this thesis provides evidence for how a host-derived mediator can ameliorate the consequences of infection by interfering with cell-to-cell communication in *S. aureus*. There is an increasing appreciation for the importance of inter-bacterial communication in the complex bacterial communities that are found in the environment and those that inhabit the human body. As exemplified in *S. aureus*, quorum sensing can have profound consequences on the commensal or pathogenic behavior of microorganisms. Bacterial communities are not found in isolation, and as they adapt, they benefit from mechanisms that integrate cues from the environment. For a bacterium that successfully colonizes over two billion humans, it is not surprising that its behavior can respond to a fundamental immunological mediator.

Stepping away from bacterial virulence and host immunity, this dissertation may be viewed from an even broader perspective. These observations have provided new insights into how a regulatory protein has evolved to sense and respond to redox signals.

## Chapter 6. Materials and Methods

### 6.1 Contact for reagents and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ferric C. Fang (fcfang@u.washington.edu).

### 6.2 Experimental model details

#### 6.2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 6.1. *S. enterica* serovar Typhimurium 14028s was used for *in vitro* transcriptional studies and macrophage infections. *S. Typhimurium* and *E. coli* strains were grown in Luria-Bertani broth (LB) at 37°C with shaking at 250 rpm. Bacterial cultures were supplemented with 100 µg ml<sup>-1</sup> ampicillin where appropriate. *S. aureus* HG003 (Herbert et al., 2010) was used for *in vitro* experiments and *S. aureus* Newman for animal studies. Unless otherwise indicated, *S. aureus* cultures were grown in tryptic soy broth (TSB) at 37°C with shaking at 250 rpm. For plasmid retention, 10 µg ml<sup>-1</sup> chloramphenicol was used when appropriate. To induce AgrA expression from the T5X promoter, TSB was supplemented with 2% xylose.

Spent medium used in experiments to stimulate Agr activity was prepared from *S. aureus* HG003 cultures of an optical density (OD<sub>600nm</sub>) of 5.0. Bacteria were pelleted by centrifugation and supernatants filter-sterilized with a 0.2 µm polyethersulfone syringe filter (ThermoFisher Scientific).

### **6.2.2 Cell culture**

RAW 264.7 macrophages (ATCC TIB-71) were maintained in RPMI 1640 media (Fisher Scientific) supplemented with 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator. Cells were used within twenty passages of purchase from ATCC.

### **6.2.3 Experimental animals**

Animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee (Protocol #3373-01). Investigators were not blinded and animal studies were non-randomized. No statistical method was used to predetermine sample size. C57BL/6 and congenic iNOS knockout mice were purchased from Jackson Laboratories (Bar Harbor, ME), then bred and maintained at the University of Washington Animal Care and Research Facility. Animal studies were gender-matched and mice were 11-15 weeks old.

## **6.3 *Salmonella* method details**

### **6.3.1 Construction of plasmids for *Salmonella* studies**

Plasmids and primers used in this study are listed in Table 6.1 and Table 6.2, respectively. To construct the pRU001 promoterless-GFP plasmid with constitutive mCherry expression, the *PrspM*-mCherry cassette was excised from pFPV-mCherry with HindIII and BamHI and ligated into pJK682 (unpublished) digested with BamHI and AvaI. Plasmid pJK682 contains a pBR322 origin of replication, encodes a  $\beta$ -lactamase for antibiotic selection, and a promoterless *gfp3*. NsrR reporter *pstm1808*-GFP was constructed by ligating the PCR-amplified *stm1808* promoter region of *S. Typhimurium* 14028s into EcoRI restriction sites in pRU001. Primer pairs RU035-RU036 were used for PCR-amplification.

### **6.3.2 Reverse transcription quantitative real-time PCR in *Salmonella***

Overnight *S. Typhimurium* 14028s cultures were diluted 1:10<sup>2</sup> in 30 ml LB and grown to an OD<sub>600nm</sub> of 0.6. Cultures were treated with indicated concentrations of DEA/NO for 15 min with shaking. After NO treatment, 0.4 ml samples were immediately placed in RNAprotect Bacterial Reagent (QIAGEN) and incubated for 5 min at room temperature. Bacteria were pelleted and saved overnight at -20°C. Cells were lysed with 10 mg ml<sup>-1</sup> lysozyme (Research Products International Corp) by incubating for 10 min at 37°C. RNA was extracted using the RNeasy Mini Kit (QIAGEN) and treated with RNase-Free DNase (QIAGEN) according to the manufacturers recommendations. cDNA was synthesized using 500 ng RNA with the QuantiTect Reverse Transcription Kit (QIAGEN). The Quantifast SYBR Green PCR Kit (QIAGEN) and a CFX96 Real Time System (Bio-Rad) were used to perform qPCR. Data were analyzed using the comparative C<sub>T</sub> method ( $2^{-\Delta\Delta C_T}$ ) (Schmittgen and Livak, 2008) with the housekeeping gene *rpoD* as an internal control and normalized to an untreated sample. Primers used for qPCR are listed in Table 6.2.

### **6.3.3 *Salmonella*-macrophage infections, flow cytometry, epifluorescence microscopy**

Prior to infection, RAW 264.7 macrophage were seeded overnight (~12 h) in DMEM media (Gibco) containing 10% fetal bovine serum, 4.5 g/L D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate. For flow cytometry studies, macrophages were seeded at 5 x 10<sup>6</sup> cells per well in 6-well plates or at 1 x 10<sup>6</sup> cells per well in 24-well plates, over round glass coverslips, for microscopy studies. To inhibit NO production, 4 mM L-NMMA (A. G. Scientific) was added to wells during seeding and maintained during infection. Serum-opsonized *S. Typhimurium* 14028s from overnight cultures was used to infect macrophages at a multiplicity of infection of ~10. Plates were spun for 10 min at 1000 rpm to synchronize phagocytosis and then incubated for 15

min at 37°C in a 5% CO<sub>2</sub> incubator. Four washes with 12 µg mL<sup>-1</sup> gentamicin-containing DMEM media were performed to remove extracellular bacteria. For flow cytometry, macrophages were lysed 16 h post-infection and bacteria were separated by centrifugation. Bacteria were fixed with 2.5% paraformaldehyde and GFP fluorescence intensity measured at the University of Washington Pathology Flow Cytometer Core Facility using an LSRII flow cytometer (BD Biosciences). For epifluorescence microscopy, macrophages were fixed with 2.5% paraformaldehyde 16 h post-infection. Coverslips were removed from wells and mounted on glass-slides with ProLong Gold anti-fade reagent (Molecular Probes). A Nikon Eclipse TE200 was used to acquire images using GFP and RFP filters.

#### **6.3.4 Determination of nitric oxide production**

Culture supernatants collected 16 h post-infections were treated with nitrate reductase from *Aspergillus niger* (Sigma-Aldrich) and nitrite levels measured with the Griess reaction (Vazquez-Torres et al., 2000).

### **6.4 *S. aureus* method details**

#### **6.4.1 Construction of plasmids for studies in *S. aureus***

Plasmids and primers used in this study are listed in Table 6.1 and Table 6.2, respectively. Inducible AgrA expression systems with untagged and HA-tagged C-termini were constructed by PCR-amplification and restriction enzyme-based cloning. PCR amplicons of *agrA* with its native ribosomal binding site were digested with BamHI and KpnI (Fermentas) and ligated into pEPSA5 (Forsyth et al., 2002). Primer pairs RU106-RU107 and RU106-RU116 were used to construct *pagrA* and *pagrA-HA*, respectively. Mutant plasmid derivatives of *pagrA* were

generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) with primer pairs indicated in Table 6.2.

Allelic exchange mutagenesis plasmids were constructed by SOE-PCR as previously described (Monk et al., 2012). For pIMAY $\Delta$ *agrA*, ~1 kbp regions were PCR-amplified upstream of the *agrA* start site and downstream of the stop site with RU044-RU045B and RU048B-RU049, respectively. To join the amplicons, SOE-PCR was carried out with RU044A-RU049AA yielding an *agrA* deletion cassette. The deletion cassette was cloned into pIMAY at the KpnI and NotI restriction sites. pIMAY $\Delta$ *agrC* was constructed similarly with RU076-RU077 to amplify the upstream region and RU078-RU079 the downstream region. Primer pair RU076-RU079 was used for SOE-PCR.

To generate pIMAY*agrA*-HA for allelic replacement, a PCR fragment containing *agrA* plus its ~1 kbp upstream region was amplified with RU044-RU138. The downstream region was amplified with RU139-RU049. Primer pair RU044-RU049 was used for SOE-PCR.

To generate pIMAY*agrC* C91S C371S a PCR amplicon was generated using primer pair RU076-RU079 and cloned into pIMAY at KpnI and NotI. The QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used to introduce site-specific mutations with primer pairs RU080-RU081 and RU082-RU083.

All plasmid constructs were initially electroporated into *E. coli* DH10B ElectroMAX (Invitrogen), isolated with the QIAprep Spin Miniprep Kit (QIAGEN) and electroporated into the restriction-deficient cloning host *S. aureus* RN4220 (Kreiswirth et al., 1983). Plasmids were then transduced via  $\Phi$ -11 into experimental strains as previously described (Novick, 1991).

### 6.4.2 Construction of *S. aureus* mutants

Allelic exchange mutagenesis was performed as previously described (Monk et al., 2012). RU111 was generated from *S. aureus* HG003 with pIMAY $\Delta$ *agrA* and was subsequently used to generate RU226 with pIMAY*agrA*-HA. RU141 was generated from *S. aureus* HG003 with pIMAY $\Delta$ *agrC* and subsequently used to generate RU161 with pIMAY*agrCC91SC371S*. Spontaneous mutant RU371 was selected for streptomycin resistance in tryptic soy agar plates containing 250  $\mu$ g ml<sup>-1</sup> streptomycin and used to generate RU373 with pIMAY $\Delta$ *agrC*.

### 6.4.3 Mercury resin assisted capture and mass spectrometry

Overnight *S. aureus* HG003 cultures were diluted 1:10<sup>2</sup> in 250 ml TSB and grown to an OD<sub>600nm</sub> of 2.0. Bacteria were pelleted and washed once with 50 ml PBS. Bacteria were resuspended in 6 ml lysis buffer (250 mM HEPES pH 7.7, 1 mM DTPA, 0.1 mM neocuproine, 1% Triton X-100), incubated with 100  $\mu$ g ml<sup>-1</sup> lysostaphin (AMBI Products) for 15 min at 37°C and then placed on ice. The cOmplete Protease Inhibitor Cocktail (Roche) was added and bacteria were French-pressed twice at 20,000 psi. Cell debris was removed by centrifugation at 10,000 rcf for 15 min at 4°C. To remove low molecular weight thiols, lysates were dialyzed twice overnight with a 3,500 MWCO Slide-A-Lyzer dialysis cassette (ThermoFisher Scientific) in 1 L of lysis buffer. Ten ml of 0.8 mg ml<sup>-1</sup> protein were S-nitrosylated with 150  $\mu$ M diethylamine NONOate (DEA/NO) (Cayman Chemical) for 10 min at 37°C. A control sample was treated in parallel with 150  $\mu$ M diethylamine (DEA) (Sigma-Aldrich). NO treatment was stopped by precipitating protein in ice-cold 75% acetone and incubating for 20 min at -20°C. Samples were washed once with 75% acetone and resuspended in 10 ml blocking buffer (250 mM HEPES pH 7.7, 1 mM DTPA, 0.1 mM neocuproine, 2.5% SDS). Unmodified protein cysteines were chemically blocked with 50 mM N-ethylmaleimide (NEM) (Sigma-Aldrich) for 60 min at 50°C. Samples

were vortexed every 5 min. NEM blocking was stopped by precipitating in ice-cold 75% acetone and incubating for 20 min at  $-20^{\circ}\text{C}$ . Samples were washed four times with 75% acetone and resuspended in 4 ml loading buffer (250 mM MES pH 6.0, 1 mM DTPA, 1% SDS).

Mercury resin assisted capture was performed as previously described (Doulias et al., 2010). Foiled wrapped Econo-Pac chromatography columns (Bio-Rad Laboratories, inc) were filled with a 4 ml bed of mercury resin and 40 ml of isopropanol passed through the column, followed by 80 ml deionized  $\text{H}_2\text{O}$ , and 80 ml of 0.1 M  $\text{NaHCO}_3$  (pH 8.8). To equilibrate, 80 ml equilibration buffer (50 mM MES pH 6.0, 50 mM NaCl, 1 mM DTPA) was passed through the column. Samples were added by gravity flow and incubated for 1 h. Columns were washed with 200 ml wash buffer (50 mM Tris-HCl pH 7.5, 0.3 M NaCl) containing 0.5% SDS, 200 ml wash buffer containing 0.05% SDS, 200 ml wash buffer containing 1% Triton X-100 plus 1 M urea, 200 ml wash buffer containing 1% Triton X-100 plus 0.1 M urea, and then washed with 800 ml deionized  $\text{H}_2\text{O}$ . Trypsin digest was performed overnight for  $\sim 16$  h with 5 mL of 1  $\mu\text{g}/\text{mL}$  Trypsin Gold (Promega Corporation) in 0.1 M  $\text{NH}_4\text{HCO}_3$ . Columns were then washed with 160 ml of 1 M  $\text{NH}_4\text{HCO}_3$  containing 0.3 M NaCl, 160 ml of 1 M  $\text{NH}_4\text{HCO}_3$ , 160 ml of 0.1 M  $\text{NH}_4\text{HCO}_3$ , and 800 ml deionized  $\text{H}_2\text{O}$ . To elute peptides, 6 ml performic acid were added and columns were incubated for 45 min before 6 ml of deionized  $\text{H}_2\text{O}$  were added. Eluted samples were lyophilized and sent for LC-MS/MS analysis at the Protein Core in Children's Hospital of Philadelphia Research Institute (Philadelphia, PA). Tryptic peptide digests were analyzed by hybrid-Orbitrap Elite mass spectrometer (Thermo Electron, San Jose, CA). A schematic of this protocol is shown in Figure 2.2.

#### **6.4.4 Generation and evaluation of SEQUEST peptide assignments**

The post mass spectrometry analysis has been previously described in detail (Doulias et al., 2010). In brief, the raw files, which contain the MS/MS spectra were submitted to Sorcerer Sequest (Sage-N Research, Inc) for database search and generation of potential sequence-to-spectrum peptide assignments. A *S. aureus* database comprising 10,982 entries (canonical and isoform sequences) was used. Database search included cysteine trioxidation (+48Da), methionine dioxidation (+32Da) and alkylation of cysteine by N-ethylmaleimide (+125Da) as differential modifications. Scaffold Version 4. 0. 4 (Proteome Software) was used to validate protein identifications and perform manual inspection of MS/MS spectra. Empirically defined  $X_{\text{corr}}$  thresholds were applied to filter cysteic-acid and non-modified peptides independently so the false discovery rate (FDR) was  $\leq 1\%$ . Finally, all cysteine-containing spectra were manually inspected based on previously defined criteria (Doulias et al., 2010).

#### **6.4.5 Biotin-switch of HA-tagged AgrA**

The biotin-switch method was adapted and modified from a previous study (Jaffrey and Snyder, 2001). Overnight RU198 cultures were diluted 1:10<sup>2</sup> in 100 ml TSB and grown to an OD<sub>600nm</sub> of 0.6. Cultures were induced with 2% xylose until cultures reached an OD<sub>600nm</sub> of 5.0. Bacteria were pelleted and washed once with 50 ml PBS. Bacteria were resuspended in 8 ml lysis buffer (250 mM HEPES pH 7.7, 1 mM DTPA, 0.1 mM neocuproine, 1% Triton X-100) and incubated with 100  $\mu\text{g ml}^{-1}$  lysostaphin (AMBI Products) for 30 min at 37°C. The cOmplete Protease Inhibitor Cocktail (Roche) was added and bacteria were sonicated with a Microson Ultrasonic Cell Disruptor XL (Misonix). Cell debris was removed by centrifugation at 10,000 rcf for 15 min at 4°C. Low molecular weight thiols were removed with Econo-Pac 10DG desalting columns (BD Biosciences) following manufacturers recommendations. Total protein concentration was

determined using the Coomassie Protein Assay Kit (ThermoFisher Scientific). Two 3 ml samples (1 mg ml<sup>-1</sup>) were treated in dark conical tubes with 1 mM DEA/NO (Cayman Chemical) for 10 min at 37°C. A control sample was treated in parallel with 1 mM DEA (Sigma-Aldrich). To stop *S*-nitrosylation, samples were precipitated in ice-cold 75% acetone and incubated for 20 min at -20°C. Samples were washed once with 75% acetone and resuspended in 3 ml blocking buffer (250 mM HEPES pH 7.7, 1 mM DTPA, 0.1 mM neocuproine, 2.5% SDS). As an additional negative control, one of the two DEA/NO-treated samples was exposed to ultraviolet light for 25 min to denitrosylate proteins. Samples were blocked with 40 mM MMTS for 90 min at 50°C with vortexing every 5 min. MMTS blocking was stopped by precipitating in ice-cold 75% acetone and incubating for 20 min at -20°C. Samples were washed 4 times with 75% acetone and resuspended in 1 ml labeling buffer (250 mM HEPES pH 7.7, 1 mM DTPA, 0.1 mM neocuproine, 1% SDS). To label *S*-nitrosylated proteins, samples were incubated for 90 min with 20 mM sodium L-ascorbate (Sigma-Aldrich) and 400 µM EZ-Link HPDP-Biotin (ThermoFisher Scientific). To stop biotinylation, samples were precipitated in ice-cold 75% acetone and incubated for 20 min at 20°C. Samples were washed once in 75% acetone and resuspended in 1 ml resuspension buffer (250 mM HEPES pH 7.7, 1 mM DTPA, 0.1 mM neocuproine, 0.1% SDS). Protein concentration was determined and 75 µg of biotin-labeled protein immunoprecipitated with Pierce Anti-HA Magnetic Beads (ThermoFisher Scientific) following manufacturer's recommendations. AgrA-HA was eluted using SDS-PAGE sample buffer, subjected to SDS-PAGE and transferred onto a low-fluorescence PVDF transfer membrane (ThermoFisher Scientific). Western blots were probed with Alexa 488 anti-hemagglutinin mouse monoclonal 16B12 antibody (Molecular Probes) and Alexa 647 streptavidin conjugate (Molecular Probes). A FluorChem Q imaging system (Alpha Innotech) was used to visualize

western blots and ImageJ Version 1.47 Software (Wayne Rasband, U. S. National Institutes of Health) used for densitometry. A schematic of this protocol is shown in Figure 2.2.

#### **6.4.6 Reverse transcription quantitative real-time PCR in *S. aureus***

Overnight *S. aureus* HG003 cultures were diluted 1:10<sup>2</sup> in 30 ml TSB and grown to an OD<sub>600nm</sub> of 0.6. Cultures were treated with propylamine propylamine NONOate (PAPA/NO) (Cayman Chemical) for 45 min in 3 ml volumes. After NO treatment, 0.4 ml samples were immediately placed in RNAProtect Bacterial Reagent (QIAGEN) and incubated for 5 min at room temperature. Bacteria were pelleted and saved overnight at -20°C. Cells were lysed with 100 µg ml<sup>-1</sup> lysostaphin (AMBI Products) by incubating for 30 min at 37°C. RNA was extracted using the RNeasy Mini Kit (QIAGEN) and treated with RNase-Free DNase (QIAGEN) according to the manufacturers recommendations. cDNA was synthesized using 500 ng RNA with the QuantiTect Reverse Transcription Kit (QIAGEN). The Quantifast SYBR Green PCR Kit (QIAGEN) and a CFX96 Real Time System (Bio-Rad) were used to perform qPCR. Data were analyzed using the comparative C<sub>T</sub> method ( $2^{-\Delta\Delta C_T}$ ) (Schmittgen and Livak, 2008) with the housekeeping gene *gyrB* as an internal control and normalized to an untreated sample. Primers used for qPCR are listed in Table 6.2. Primers for housekeeping genes and nitric oxide induced genes were based on previous studies (Goerke et al., 2000; Hirschhausen et al., 2012; Richardson et al., 2006; Theis et al., 2007).

#### **6.4.7 Western blots and protein stability assays**

Overnight RU083 and RU226 cultures were diluted 1:10<sup>2</sup> in 26 ml TSB and grown to an OD<sub>600nm</sub> of 0.6. Cultures were treated with 8 mM PAPA/NO (Cayman Chemical) for 45 min. Fifteen min after adding PAPA/NO, cultures were stimulated with 20% spent medium. Bacteria were pelleted and washed once with 30 ml PBS. Bacteria were resuspended in 1 ml lysis buffer (Tris

HCl pH 7.5, 100 mM NaCl) and incubated with 100  $\mu\text{g ml}^{-1}$  lysostaphin (AMBI Products) for 30 min at 37°C. Cell debris was removed by centrifugation at 18,000 rcf and protein concentration was determined using the Coomassie Protein Assay Kit (ThermoFisher Scientific). To improve the western blot signal, 1 mg total protein was immunoprecipitated with Pierce Anti-HA Magnetic Beads (ThermoFisher Scientific) as recommended by the manufacturer. Protein binding to beads was done overnight at 4°C. AgrA-HA was eluted with SDS-PAGE sample buffer by incubating at 95°C for 10 min. Immunoprecipitated samples were subjected to SDS-PAGE and transferred onto a low-fluorescence PVDF transfer membrane (ThermoFisher Scientific). SuperSignal Western Blot Enhancer (ThermoFisher Scientific) was used to improve the western blot signal. Western blots were probed with Alexa 488 anti-hemagglutinin mouse monoclonal 16B12 antibody (Molecular Probes). A FluorChem Q imaging system (Alpha Innotech) was used to visualize western blots and ImageJ Version 1.47 Software (Wayne Rasband, U. S. National Institutes of Health) used for densitometry.

Protein stability assays were performed similarly, except that total protein translation was inhibited with 10  $\mu\text{g ml}^{-1}$  tetracycline 15 min prior to NO treatment. Samples were collected from cultures immediately before adding tetracycline and at 45 min and 90 min after adding PAPA/NO.

#### **6.4.8 Chromatin immunoprecipitation-qPCR**

Overnight RU198 cultures were diluted 1:10<sup>2</sup> in 80 ml TSB and grown to an OD<sub>600nm</sub> of 0.6. The Agr system was stimulated with 10% spent medium and cultures were immediately treated with 6 mM PAPA/NO for 45 min. DNA was cross-linked with 1.5% formaldehyde (Macron Fine Chemicals) for 30 min at 30°C with shaking at 100 rpm. Cultures were quenched with 300 mM glycine (Sigma-Aldrich) for 5 min at 30°C with shaking at 100 rpm. Bacteria were pelleted and

washed once with 40 ml PBS. Bacteria were resuspended in 1 ml lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate), then incubated with 100  $\mu\text{g ml}^{-1}$  lysostaphin (AMBI Products) for 30 min at 37°C. The cOmplete Protease Inhibitor Cocktail (Roche) was added and samples placed on ice for 10 min. FastProtein Blue Matrix (MP Biomedicals) was used to bead-beat samples three times for 30 sec at maximal speed with a Mini-Beadbeater (BioSpec Products). Cell debris was removed by centrifugation and DNA sheared by sonication with a Microson Ultrasonic Cell Disruptor XL (Misonix) for 8 rounds of fifteen 1 sec pulses.

Sheep anti-mouse IgG Dynabeads M-280 (Novex) were incubated in 750  $\mu\text{l}$  blocking buffer (5 mg  $\text{ml}^{-1}$  BSA in PBS) for 30 min at 4°C. 1.5  $\mu\text{g}$  of HA-7 mouse monoclonal anti-HA antibody (Sigma-Aldrich) was bound to beads for 3 h at 4°C in 750  $\mu\text{l}$  blocking buffer. Beads were washed twice with 1 ml ice-cold washing buffer (100 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA), resuspended in 60  $\mu\text{l}$  blocking buffer, and 12.5  $\mu\text{g}$  of total protein were added. Volumes were adjusted to 750  $\mu\text{l}$  with lysis buffer and samples incubated for 3 h at 4°C. Samples were washed twice for 5 min at 4°C with 600  $\mu\text{l}$  lysis buffer, twice with 600  $\mu\text{l}$  wash buffer, and once with 600  $\mu\text{l}$  TE buffer pH 8.0 (Ambion). Samples were eluted in 120  $\mu\text{l}$  TE buffer pH 8.0 (Ambion) with 1% SDS by incubating for 15 min at 65°C.

Cross-links were reversed by adding 130  $\mu\text{l}$  TE buffer pH 8.0 (Ambion) with 1% SDS and incubating at 65°C for 15 h. Samples were diluted with 240  $\mu\text{l}$  TE buffer pH 8.0, 20  $\mu\text{l}$  of 10 mg  $\text{ml}^{-1}$  Proteinase K (Ambion) was added, and samples incubated at 45°C for 2 h. Fifty  $\mu\text{l}$  of 5 mM LiCl were added prior to extracting DNA with 400  $\mu\text{l}$  UltraPure Phenol:Chloroform:Isoamyl Alcohol (Invitrogen). Two  $\mu\text{l}$  of 20 mg  $\text{ml}^{-1}$  glycogen (ThermoFisher Scientific) were added to extracted DNA and samples precipitated with 0.7 volumes of isopropanol at -80°C for 30 min.

DNA was pelleted and washed once with 70% ethanol before DNA was air-dried and resuspended in 200  $\mu$ l H<sub>2</sub>O.

qPCR was performed with the Quantifast SYBR Green PCR Kit (QIAGEN) and a CFX96 Real Time System (Bio-Rad). Enrichment of DNA regions was calculated by the % input method relative to a control region in *rpoD* (test DNA in ChIP sample/test DNA in input sample)/(*rpoD* in ChIP sample/*rpoD* in input sample) (Merrikh et al., 2015). Primers used for qPCR are listed in Table 6.2.

#### **6.4.9 Bioinformatics**

AgrA protein graphics were generated with PyMOL Molecular Graphics System Version 1.8 (Schrödinger, LLC). The structure of the DNA binding domain was downloaded from PDB (3BS1) and the response regulator (RR) receiver domain was modeled using Phyre2 (Kelley et al., 2015; Sidote et al., 2008). Protein structures models of other LytTR regulators were also generated using Phyre2. The AgrA amino acid sequence alignment was generated with Clustal Omega (Sievers et al., 2011) and the graphic rendered with MacVector Version 12.0.3 (MacVector, Inc).

#### **6.4.10 Mouse infections and cytokine measurements**

Mice were infected based on a previously developed murine pneumonia model (Bubeck Wardenburg et al., 2007b). Overnight RU371 and RU373 cultures were diluted 1:10<sup>2</sup> in 100 ml TSB and grown to an OD<sub>600nm</sub> of 0.6. Bacteria were pelleted, washed once with 1 ml PBS and resuspended in 750  $\mu$ l (cytokine study) or 1500  $\mu$ l PBS (24 h surface temperature study). Mice were anesthetized with 3% isoflurane prior to delivering 30  $\mu$ l of a bacterial inoculum (~1 x 10<sup>8</sup> or ~5 x 10<sup>7</sup> CFU, cytokine study; 5 x 10<sup>7</sup> CFU for 24 h surface temperature study) into the left nostril. Mice were held in the upright position for 1 min to allow aspiration. Body surface

temperature was measured over the lower abdomen using a MiniTemp MT6 scanning infrared thermometer (Raytek). Moribund mice displaying low mobility, hunched posture, difficulty breathing, and had surface temperature below 25°C were euthanized using CO<sub>2</sub>.

For the cytokine study, blood was collected by cardiac puncture immediately after CO<sub>2</sub> euthanasia. Blood was allowed to clot at room temperature and serum separated by centrifugation at 18,000 rcf for 5 min. Serum was collected and stored at -80°C until needed for cytokine measurements. Lungs were surgically removed, placed in 1 ml PBS on ice, and homogenized using a PowerGen 125 homogenizer (Fisher Scientific). To determine lung bacterial burden, homogenate dilutions were plated onto TSA plates with 250 µg ml<sup>-1</sup> streptomycin.

To measure cytokines, serum was thawed on ice and cytokine levels determined with the Cytometric Bead Array Mouse Kit (BD Biosciences) and Mouse Enhanced Sensitivity Kit (BD Biosciences). Data was collected at the University of Washington Pathology Flow Cytometer Core Facility using an LSRII flow cytometer (BD Biosciences) and analyzed using the FCAP Array Software Version 3.0 (BD Biosciences).

#### **6.4.11 Histopathology**

Mice were infected as described with an inoculum size of  $\sim 5 \times 10^7$  or  $\sim 1 \times 10^8$  CFU. At 4 h and 8 h post-infection mice were euthanized with 3% isoflurane, and lungs were immediately inflated *in situ* with 1 ml of 10% buffered formalin (ThermoFisher Scientific) through a tracheal incision. Formalin-filled lungs were then tied at the trachea with a suture, excised, and stored in 25 ml of 10% formalin (ThermoFisher Scientific). Lungs were embedded in paraffin, sectioned, and stained with H&E by the University of Washington Histology and Imaging Core (Seattle, WA). Histopathological interpretation and scoring was performed by a veterinary pathologist who was

blinded to mouse genetic background. Determination of the predominant lesion pattern was made following masked review of all sections. Three types of lesions were identified including: a) focal aggregation characterized by multiple distinct and highly cellular inflammatory foci, b) alveolar pattern with bacterial clusters distributed along alveolar walls, and c) mixed with both patterns represented equally. The degree of inflammatory cell and bacterial accumulations were judged semi-quantitatively and assigned an all-inclusive ranking of severity from 1 (mild) to 4 (severe).

## **6.5 Quantification and statistical analysis**

Statistical method and sample size (n) for experiments are indicated in the corresponding figure legend. For all *in vitro* studies, n indicates the number of independent experiments. For *in vivo* studies, n indicates the number of mice per group. Statistical analysis was performed using Prism 6 Software (GraphPad) on the indicated biological replicates. A two-tailed Student's t test was performed on the means of parametric data and the Mann-Whitney U-test performed on the medians of non-parametric data. Statistical significance was defined as  $p < 0.05$ . Error bars on figures show standard deviation.

**Table 6.1 Resources**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-HA mouse IgG1, clone 16B12, Alexa Fluor 488 Conjugate	Molecular Probes	Cat#A21287
Streptavidin, Alexa Fluor 647 Conjugate	Molecular Probes	Cat#S32357
Anti-HA mouse antibody, monoclonal HA-7	Sigma-Aldrich	Cat#H3663
<b>Bacterial Strains</b>		
<i>Salmonella enterica</i> serovar Typhimurium 14028s	ATCC	RU001
14028S + pempty-GFP	This paper	RU043
14028S + <i>pstm1808</i> -GFP	This paper	RU070
14028s $\Delta$ <i>nsrR</i>	Bang et al., 2006	RU002
14028s $\Delta$ <i>nsrR</i> + <i>pstm1808</i> -GFP	This paper	RU075
<i>Staphylococcus aureus</i> HG003; NCTC8325 derivative	Herbert et al., 2010	RU083
HG003 $\Delta$ <i>agrA</i>	This paper	RU111
HG003 $\Delta$ <i>agrC</i>	This paper	RU141
HG003 <i>agrC</i> C91S C371S	This paper	RU161
HG003 + pEPSA5	This paper	RU170
HG003 $\Delta$ <i>agrA</i> + pEPSA5	This paper	RU171
HG003 $\Delta$ <i>agrA</i> + <i>pagrA</i>	This paper	RU174
HG003 $\Delta$ <i>agrA</i> + <i>pagrA</i> C6V	This paper	RU487
HG003 $\Delta$ <i>agrA</i> + <i>pagrA</i> C55S	This paper	RU497
HG003 $\Delta$ <i>agrA</i> + <i>pagrA</i> C123N	This paper	RU494
HG003 $\Delta$ <i>agrA</i> + <i>pagrA</i> C199S	This paper	RU475
HG003 $\Delta$ <i>agrA</i> + <i>pagrA</i> -HA	This paper	RU198
HG003 C-terminus HA-tagged <i>agrA</i> (chromosomal)	This paper	RU226
HG003 $\Delta$ <i>agrBD</i>	This paper	RU299
<i>Staphylococcus aureus</i> Newman Strep <sup>R</sup>	This paper	RU371
Newman $\Delta$ <i>agrC</i> Strep <sup>R</sup>	This paper	RU373
<i>S. aureus</i> RN4220; NCTC8325 derivative, restriction deficient cloning host	Kreiswirth et al., 1983	RU062
<i>E. coli</i> ElectroMAX DH10B; <i>FmcrA</i> $\Delta$ ( <i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i> ) $\Phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> $\Delta$ ( <i>ara</i> , <i>leu</i> )7697 <i>galU</i> <i>galK</i> $\lambda$ <i>rpsL</i> <i>nupG</i>	Invitrogen	Cat#18290015
DH10B + pIMAY	Monk et al., 2012	RU097
DH10B + pIMAY $\Delta$ <i>agrA</i>	This paper	RU127
DH10B + pIMAY $\Delta$ <i>agrC</i>	This paper	RU146
DH10B + pIMAY $\Delta$ <i>agrA</i> -HA	This paper	RU218
<b>Chemicals, Peptides, and Recombinant Proteins</b>		

DEA NONOate	Cayman Chemicals	Cat#82100
L-NG-Monomethyl-L-Arginine (L-NMMA)	AG Scientific	Cat#M-1014
Lysostaphin	AMBI Products	Cat#LSPN-50
Lysozyme	Fisher Scientific	Cat#50-488-779
PAPA NONOate	Cayman Chemicals	Cat#82140
Critical Commercial Assays		
QuikChange Lightning Site-Directed Mutagenesis Kit	Agilent Technologies	Cat#210518-5
Dynabeads M-280 Sheep anti-Mouse IgG	Novex	Cat#11201D
Pierce Anti-HA Magnetic Beads	ThermoFisher Scientific	Cat#88836
EZ-Link HPDP-Biotin	ThermoFisher Scientific	Cat#21341
BD Cytometric Bead Array Mouse/Rat Soluble Protein Master Buffer Kit	BD Biosciences	Cat#558266
BD Cytometric Bead Array Mouse Enhanced Sensitivity Master Buffer Kit	BD Biosciences	Cat#562246
Experimental Models: Organisms/Strains		
C57BL/6J SPF mice	Jackson Laboratory	Cat#000664
B6.129P2- <i>Nos2<sup>mlLau</sup></i> /J SPF mice	Jackson Laboratory	Cat#002609
Oligonucleotides		
Primers for cloning, mutagenesis, and qPCR, See Table 6.2		
Recombinant DNA		
Pempty-GFP	This paper	N/A
<i>pstm1808</i> -GFP	This paper	N/A
pIMAY	Monk et al., 2012	N/A
pIMAY $\Delta$ <i>agrA</i>	This paper	N/A
pIMAY $\Delta$ <i>agrC</i>	This paper	N/A
pIMAY <i>agrC</i> C91S C371S	This paper	N/A
pIMAY <i>agrA</i> -HA	This paper	N/A
pEPSA5	Forsyth et al., 2002	N/A
<i>pagrA</i>	This paper	N/A
<i>pagrA</i> C6V	This paper	N/A
<i>pagrA</i> C55S	This paper	N/A
<i>pagrA</i> C123N	This paper	N/A
<i>pagrA</i> C199S	This paper	N/A
<i>pagrA</i> -HA	This paper	N/A
Software and Algorithms		

Clustal Omega Multiple Sequence Alignment	Sievers et al., 2011	<a href="http://www.ebi.ac.uk/Tools/msa/clustalo/">http://www.ebi.ac.uk/Tools/msa/clustalo/</a>
FCAP Array Software Version 3.0	BD Bioscience	Cat#652099
ImageJ Version 1.47 Software	Wayne Rasband, U. S. National Institutes of Health	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>
MacVector Version 12. 0. 3	MacVector, Inc	<a href="http://macvector.com/index.html">http://macvector.com/index.html</a>
Phyre2	Kelley et al., 2015	<a href="http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index">http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index</a>
PyMOL Molecular Graphics System Version 1.8	S Schrödinger, LLC	<a href="https://www.pymol.org/">https://www.pymol.org/</a>
Prism 6 Software	GraphPad	<a href="https://www.graphpad.com/">https://www.graphpad.com/</a>
Scaffold Version 4. 0. 4	Proteome Software	<a href="http://www.proteomesoftware.com/">http://www.proteomesoftware.com/</a>
Sorcerer Sequest	Sage-N Research, Inc	<a href="https://www.sagenresearch.com/">https://www.sagenresearch.com/</a>

**Table 6.2 Primers in this study**

<b>Primer</b>	<b>Sequence 5'-3'</b>	<b>Reference</b>
<i>Salmonella</i> Cloning primers		
RU035	AACCGAATTCCCCTTGATACACACGCTCC	This paper
RU036	AACCGAATTCCAGTGCCGCCGGAACATTC	This paper
<i>Staphylococcus</i> Cloning primers		
RU044	ATATGGTACCCTCGGATGAAGCTAAAGTAATAAG	This paper
RU045B	GACTTTATTATCTTATTACATACATTCACATCCTT ATGGCTAG	This paper
RU048B	GGATGTGAATGTATGTAATAAGATAATAAAGTCA GTTAACGGC	This paper
RU049	ATATGCGGCCGCCGTAAGCCCTCTGCTGATATG	This paper
RU044A	ATATGGTACCCTGCGCAAGTTCGTCATGATTAT GTC	This paper
RU049AA	ATATGCGGCCGCTCATTTATACGAAGGGAGCAGA TG	This paper
pIMAY $\Delta$ <i>agrC</i>		
RU076	ATATGGTACCCGTATAATGACAGTGAGGAG	This paper
RU077	CACATCCTTATGGCTACATTTGAGTTAATACGAA TAAAC	This paper
RU078	CGTATTAACTCAAATGTAGCCATAAGGATGTGAA TGTATG	This paper
RU079	ATATGCGGCCGCTCACCGATGCATAGCAGTG	This paper
pIMAY <i>agrA</i> -HA		
RU138	TTAAGCATAATCTGGAACATCATATGGATATATT TTTTTAACGTTTCTCACCGATGCATAGC	This paper
RU139	TATCCATATGATGTTCCAGATTATGCTTAATAAG ATAATAAAGTCAGTTAACGGCG	This paper
<i>pagrA</i>		
RU106	ATATGGTACCATAAGGATGTGAATGTATG	This paper
RU107	ATATGGATCCAACAAGATTTACAATTGAATACGC	This paper
<i>pagrA</i> -HA		
RU116	AGCATAATCTGGAACATCATATGGATATATTTTT TTAACGTTTCTCACCGATGCATAGCAGTGTTT	This paper
Site-directed mutagenesis		
<i>pagrA</i> C6V		
RU094	GGATGTGAATGTATGAAAATTTTCATTGTCTGAAG ACGATCCAAAACAAAGAGAAAACATG	This paper
RU095	CATGTTTTCTCTTTGTTTTGGATCGTCTTCGACAA TGAAAATTTTCATACATTCACATCC	This paper

<i>pagrA</i> C55S		
RU070	GCAAGCTAAAAATATGAATGACATAGGCTCTTAC TTTTTAGATATTCAACTTTC	This paper
RU071	GCAAGCTAAAAATATGAATGACATAGGCTCTTAC TTTTTAGATATTCAACTTTC	This paper
<i>pagrA</i> C123N		
RU352	GCTGAATTAAGAACTCGAATTATAGACAATTTA GAAACTGCACATACACGCTTAC	This paper
RU353	GTAAGCGTGTATGTGCAGTTTCTAAATTGTCTAT AATTCGAGTTCTTAATTCAGC	This paper
<i>pagrA</i> C199S		
RU050	GAGTCAATTAGATGATCGTTTCTTTAGATCTCATA ATAGCTTTGTCGTC AATCGC	This paper
RU051	GCGATTGACGACAAAGCTATTATGAGATCTAAAG AAACGATCATCTAATTGACTC	This paper
pIMAYagrC C91S C371S		
RU080	GTAACGTTTATTGTTATGAATAAATCCGCTGATG ATATACCACGCATTCATG	This paper
RU081	CATGAATGCGTGGTATATCATCAGCGGATTTATT CATAACAATAAACGTTAC	This paper
RU082	GACTTCGCAGATTATTCTATACTCTGCTAACTACA TGTATATAG	This paper
RU083	CTATATACATGTAGTTAGCAGAGTATAGAATAAT CTGCGAAGTC	This paper
<i>Salmonella</i> qPCR primers		
entC_F	GGTTTAGTTCGCTGCCGTTA	Karlinsey et al., 2012
entC_R	GATCAACTGCGGAGAGGAAG	Karlinsey et al., 2012
hmp_F	CTTGACGCACAAACCATCGCT	Karlinsey et al., 2012
hmp_R	CGGGTTATGCGTAAACATACG	Karlinsey et al., 2012
norV_F	ACGCAAATCGTGGAAGTGT	Karlinsey et al., 2012
norV_R	GGTCCACTTCGTTGATACCC	Karlinsey et al., 2012
rpoD_F	GTGAAATGGGCACTGTTGAACTG	Karlinsey et al., 2012
rpoD_R	TTCCAGCAGATAGGTAATGGCTTC	Karlinsey et al., 2012
soxS_F	AACATTGATGTGGTGGCAAA	Karlinsey et al., 2012
soxS_R	CTGCGATACATAGCCCAGGT	Karlinsey et al.,

		2012
<i>Staphylococcus</i> qPCR primers		
agrA_F	GAAGACGATCCAAAACAAAGAG	This paper
agrA_R	GTCATTCATATTTTTAGCTTGCTC	This paper
agrB_F	CCAGTTTGCCACGTATCTTC	This paper
agrB_R	GCACCATGTGCATGTCTTC	This paper
agrIR_F	GAAGATACGTGGCAAACACTGGTC	This paper
agrIR_R	TCCTACAGTTAGGCAATATAATG	This paper
ChIP <sub>c</sub> _F	CAAATGCACTGTATAGCTGGC	This paper
ChIP <sub>c</sub> _R	CAAATAAGATTCACGGAGTAGG	This paper
gmk_F	AAGGTGCAAAGCAAGTTAGAA	(Hirschhausen et al., 2012)
gmk_R	CTTTACGCGCTTCGTTAATAC	(Hirschhausen et al., 2012)
gyrB_F	TTATGGTGCTGGGCAAATACA	(Goerke et al., 2000)
gyrB_R	CACCATGTAAACCACCAGATA	(Goerke et al., 2000)
hmp_F	CGTTTAAACGCCAAAAGTTAAATGG	(Richardson et al., 2006)
hmp_R	TGACTTTAGTGAATTTACACCAGG	(Richardson et al., 2006)
nrdG_F	CAGTGTTTATGTATCAGGATGTCC	(Richardson et al., 2006)
nrdG_R	GTTCGCCACCTAATAGACTTAGCC	(Richardson et al., 2006)
proC_F	GGCAGGTATTCCGATTGA	(Theis et al., 2007)
proC_R	CCAGTAACAGAGTGTCCAAC	(Theis et al., 2007)
psm $\alpha$ _F	CATCGCTGGCATCATTAAAG	This paper
psm $\alpha$ _R	5-ACCAGTGAATTTCTCAATTAATCC	This paper
psm $\beta$ _F	CGCAATTAAGATACCGTAACTG	This paper
psm $\beta$ _R	CGATTGCTTCTGCTAGTCCAG	This paper
RNAIII_F	GAAGGAGTGATTTCAATGG	(Goerke et al., 2000)
RNAIII_R	TAAGAAAATACATAGCACTGAG	(Goerke et al., 2000)
rpoD_F	AACTGAATCCAAGTGATCTTAGTG	(Richardson et al., 2006)
rpoD_R	TCATCACCTTGTTCAATACGTTTG	(Richardson et al., 2006)

## **CONTRIBUTIONS**

Rodolfo Urbano performed western blots, RT-qPCR, ChIP-qPCR, biotin-switch assays, tissue culture experiments, flow cytometry, animal infections, cytokine measurements, and assisted Joyce E. Karlinsey with mercury resin-assisted capture sample preparation. Stephen J. Libby contributed to animal experiments and collected mouse serum. Paschalis-Thomas Doulias and Harry Ischiropoulos prepared mercury resin and performed LC-MS/MS peptide analysis. Helen I. Warheit-Niemi contributed to RT-qPCR measurements. Denny H. Liggitt performed histopathological scoring. Alexander R. Horswill assisted with study design and provided reagents. Rodolfo Urbano and Ferric C. Fang designed experiments.

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# CURRICULUM VITAE

## Rodolfo Urbano

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### Education

- 2010- Ph.D. Microbiology  
University of Washington, Seattle, WA, USA.
- 2005-2009 B.S. Human Biology  
University of California, San Diego, CA, USA.

### Research Experience

- 2010- *Ph. D Graduate Student*  
University of Washington, Seattle, WA  
Laboratory of Ferric C. Fang, Department of Microbiology  
Duties: Investigating the role of transcription regulators in bacterial adaptive and pathological responses to host nitric oxide. Projects involve animal infection models, genetic, biochemical, and molecular biology techniques to study the effects of nitric oxide on virulence and microbial physiology.
- 07/09-10/09 *Undergraduate Researcher*  
UCSD, La Jolla, CA  
Laboratory of Victor Nizet  
Duties: Investigating the role of Group A Streptococcus virulence factors in pathogenesis and invasive disease. Projects involved techniques such as cloning, allelic exchange mutagenesis, and assays to test for virulence enhancement and attenuation.
- 07/08-07/09 *Undergraduate Researcher (IMSD Program)*  
UCSD, La Jolla, CA  
Laboratory of Nicholas Webster  
Duties: Investigating alternative splicing regulation of the human insulin receptor; project involved working with tissue cultures, conducting pharmacological treatments, and implementing other techniques such as western blotting and RT-PCR.
- 09/06-07/08 *Laboratory Assistant*  
UCSD, La Jolla, CA  
Laboratory of Brian Palenik, Scripps Institute of Oceanography  
Duties: Conducting phylogenetic studies of coastal bioaerosols; the project involved building 16S and 18S rRNA gene libraries, sequencing, constructing alignments, and generating phylogenetic trees. Other duties included general lab

maintenance, seawater sampling, and media preparation.

## **Publications**

**Urbano R**, Karlinsey JE, Libby SJ, Paschalis-Thomas D, Ischiropoulos H, Warheit-Niemi H, Horswill AR, Liggitt, HD, Fang FC. Host antimicrobial nitric oxide disrupts microbial cell-to-cell communication to inhibit staphylococcal virulence. Manuscript submitted to Cell Host & Microbe.

Lin AE, Beasley FC, Keller N, Hollands A, **Urbano R**, Troemel ER, Hoffman HM, Nizet V. A group A *Streptococcus* ADP-ribosyltransferase toxin stimulates a protective interleukin 1 $\beta$ -dependent macrophage immune response. *mBio* 6(2), 2015.

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Talukdar I, Sen S, **Urbano R**, Thompson J, Yates JR III, Webster NJG. *hnRNP A1 and hnRNP F Modulate the Alternative Splicing of Exon 11 of the Insulin Receptor Gene. PLoS ONE* 6(11), 2011.

**Urbano R**, Palenik B, Gaston CJ, Prather KA. *Detection and phylogenetic analysis of coastal bioaerosols using culture dependent and independent techniques. Biogeosciences*, 8, 301–309, 2011.

## **Research Talks & Poster Presentations**

- 06/2016 American Society for Microbiology General Meeting research talk: Antimicrobial nitric oxide targets staphylococcal quorum sensing to suppress virulence. Boston, Massachusetts.
- 01/2016 University of Washington Work in Progress Meeting (inter-departmental): Antimicrobial nitric oxide targets staphylococcal quorum sensing to suppress virulence. Seattle, Washington.
- 11/2015 American Society for Microbiology Northwest Branch Meeting research talk: Antimicrobial nitric oxide targets staphylococcal quorum sensing to suppress virulence. Seattle, Washington.

- 06/2015 University of Washington Department of Microbiology Retreat poster presentation: Antimicrobial nitric oxide targets AgrA of the *Staphylococcus aureus* Agr system to inhibit quorum sensing and suppress virulence. Urbano R, Karlinsey JE, Doulias PT, Ischiropoulos H, Horswill AR, Fang F. Leavenworth, Washington.
- 06/2015 Wind River Conference on Prokaryotic Biology poster presentation: Antimicrobial nitric oxide targets AgrA of the *Staphylococcus aureus* Agr system to inhibit quorum sensing and suppress virulence. Urbano R, Karlinsey JE, Doulias PT, Ischiropoulos H, Horswill AR, Fang F. Estes Park, Colorado.

### Teaching Experience

- 02/2016 Guest lecturer: Introduction to recombinant DNA techniques (University of Washington Microm 431) Mutations and *in vitro* mutagenesis.
- 03/2015 Guest lecturer: Introduction to recombinant DNA techniques (University of Washington Microm 431). Research in the biological sciences.
- 01/12-03/12 Teaching assistant for Undergraduate Recombinant DNA Techniques Lab (University of Washington Microm 431). Laboratory sessions involved lecturing and demonstrating laboratory techniques (Gel Electrophoresis, Cloning, Mutagenesis, Southern Blot, Western Blots). Other duties included working individually with students, writing and grading exams.
- 09/11-12/11 Teaching assistant for Undergraduate Microbiology Lab (University of Washington Microm 302). Laboratory sessions involve lecturing, demonstrating laboratory techniques, and working individually with students. Other duties include writing and grading exams, holding review sessions and office hours.

### Mentoring

- 06/16-09/16 Mentored visiting undergraduate student Bianca Dunn (David Low laboratory, University of California at Santa Barbara). Ongoing project involved screening clinically relevant antimicrobial compounds for synergy and antagonism with nitric oxide and determining the molecular mechanism of identified interactions.
- 03/15-09/17 Mentored UW undergraduate student Helen I. Warheit-Niemi. Project involved studying nitric oxide-dependent inhibition of the staphylococcal immune evasion Protein A and determining relevance to bacterial pathogenesis.

### Volunteering & Outreach

- 2015 Graduate student representative for UW Microbiology assistant professor faculty search. Attended candidates' seminars and lunch events; gathered graduate students' evaluations and reported to the faculty search committee.
- 2011-2016 SACNAS conference exhibitor for University of Washington Microbiology

Organized and managed the UW Microbiology booth during the annual SACNAS conference. Provided resources and information to undergraduate students interested in graduate school in the biological sciences. Other duties included listening to poster presentations and providing feedback.

2012, 2013 UW Microbiology student retreat committee member and coordinator. Organized and scheduled events for the annual student retreat at Friday Harbor, Washington.

2010, 2011 Panelist (University of Washington Initiative to Maximize Student Diversity)  
Participated in annual graduate student panels for incoming freshman interested in science.

### **Organizations**

2008-2009 Initiative to Maximize Student Diversity (IMSD)

2009 Stipends for Training Aspiring Researchers (STAR)

### **Awards**

2016 American Society for Microbiology Microbe Graduate Student Travel Award

2015 Wind River Graduate Student Travel Award

2013 University of Washington Microbiology Stanley Falkow Graduate Student Award

2012 University of Washington Bacterial Pathogenesis Training Grant

2010 University of Washington Graduate Top Scholar Award

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