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Regulation of Reactive Nitrogen Species (RNS) Metabolism and Resistance Mechanisms in *Haemophilus influenzae*: A Dissertation

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REGULATION OF REACTIVE NITROGEN SPECIES (RNS) METABOLISM AND RESISTANCE MECHANISMS IN *HAEMOPHILUS INFLUENZAE*

A Dissertation Presented

By

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Program in Molecular Genetics and Microbiology

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Abstract

Haemophilus influenzae encounters niches within the human host that are predicted to differ in availability of oxygen and reactive nitrogen species (RNS: nitrite and nitric oxide), which influence the environmental redox state. Previously reported data has indicated that an altered redox condition could serve as a signal recognized by *H. influenzae* to optimize its survival within host microenvironments. To elucidate the role of redox signaling in virulence, we examined regulation by the FNR homolog of *H. influenzae*, whose counterpart in *E. coli* has been reported to be a direct oxygen sensor and a regulator of genes responsible for RNS metabolism and resistance. Many members of the FNR regulon are subject to coordinated transcriptional control by NarP, a regulator in *E. coli* that is activated by cognate sensor NarQ in response to environmental nitrite. To study the regulatory activities of FNR and NarQ-NarP in *H. influenzae*, I targeted a gene predicted to be FNR-regulated, *nrfA*, which encodes nitrite reductase, a periplasmic cytochrome-c involved in anaerobic respiration. The *fnr*, *narP* and *nrfA* mutants were assayed for nitrite reduction, which implicated the roles of FNR, NarP and NrfA in RNS metabolism. Using Western blot detection of an epitope-tagged reporter protein fused to the endogenous *nrf* promoter (Pnrf-HA), I demonstrate that FNR and NarP, but not NarQ, are required for full activation of the *nrf* promoter. Additionally, Pnrf-HA expression increases as oxygen becomes depleted and decreases when exposed to high concentrations of nitrite, implying that the *nrf* promoter is modulated by environmental redox signals.

FNR of *E. coli* has been implicated in regulation of resistance mechanisms to a reactive nitrogen species, nitric oxide (NO), which is produced by innate immune cells during infection as a host defense mechanism. A mutant lacking FNR is more sensitive to NO exposure and killing by activated macrophages than wild type *H. influenzae* after anaerobic pre-growth. Mutants of *nrfA* and *narP* have been tested and initial experiments have shown both mutants have a lesser NO sensitivity phenotype as compared to the *fnr* mutant, suggesting that other factors could be involved in FNR-mediated NO resistance in *H. influenzae*. Upon examination of potential factors that might be involved to this phenotype, we discovered FNR-regulated gene, *ytfE*, which contributes to defense against nitrosative stress. The *fnr* and *ytfE* mutants are more susceptible to killing by activated macrophages indicating that FNR regulation of *ytfE* might be important for *in vivo* infection.

Acknowledgements

I received a piece of advice a few years ago that I attribute to my successes in graduate school: When going through a rough patch in the lab and nothing is working, keep at it. When experiencing a good science day filled with exciting results, work even harder. These are words from my very wise mentor, Dr. Brian Akerley, who has provided excellent scientific training. I feel privileged to have the opportunity to work with such a brilliant individual who has an endless supply of creative approaches to experimental design and amusing analogies to interpret results. I also accredit my science accomplishments to the tremendous amounts of assistance I have received from my fellow lab members: Dr. Sandy Wong, Dr. Jeffrey Gawronski, and Charles Rosadini. Sandy directly assisted in the progress of my project by providing many of the strains used in this thesis. Jeff and Charles have always been constant sources of advice, encouragement and of course, affectionate teasing.

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Table of Contents

List of Tables

List of Figures

CHAPTER II

CHAPTER III

CHAPTER IV

Preface

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CHAPTER I

Introduction

Haemophilus influenzae is Gram negative pathogen found only in the human host and lives primarily asymptomatically in the nasopharynx of healthy individuals. Although a vaccine exists specific to the type B strain, non-typeable *H. influenzae* strains (NTHi) remain a significant health concern for children and immune compromised adults, manifesting as otitis media, meningitis, septicemia, and respiratory infections (Foxwell *et al.*, 1998). NTHi is a significant medical concern for reoccurring bacterial exacerbations seen with patients suffering from chronic obstructive pulmonary disease and cystic fibrosis (Moller *et al.*, 1995; Murphy *et al.*, 2000; Sethi, 2000). How the bacteria can survive in very distinct anatomical locations (lungs, blood, brain, etc.) and cause such diverse diseases is not well understood. The ability of *H. influenzae* to specifically express the appropriate genes that are optimal for survival and virulence under these dramatically different microenvironments is critical for persistence. There is building evidence that modulation of gene expression by global regulators is important for *H. influenzae* pathogenesis and experimental data suggests that redox signaling leads to altered gene regulation *in vivo*.

Oxygen: Environmental Conditions in the Host

Although relative oxygen tension has been measured in human tissue with bacterial infections (Boekstegers *et al.*, 1994; Sair *et al.*, 2001), the oxygen availability

that *H. influenzae* directly encounters remains undefined. Bacteria were isolated from infection sites shared with *H. influenzae,* which indicates that low oxygen environments are likely experienced. Obligate anaerobic bacteria have been isolated from respiratory infections (Brook, 2007a), septicemia (Brook, 2007b), and cerebrospinal fluid (Brook, 2002). *Pseudomonas aeruginosa* requires anaerobic respiration to survive in sputum from a cystic fibrosis patient (Palmer *et al.*, 2007) and oxygen measurements demonstrated that *P. aeruginosa* biofilms are anaerobic (Borriello *et al.*, 2004). Oxygen is not only important for metabolism and signaling of bacteria but also for host cells as oxygen influences metabolism (Simon *et al.*, 1973) and gene regulation (Murdoch *et al.*, 2005) of immune effector cells such as macrophages. Effects of anaerobiosis on bacterial virulence were also examined with *Salmonella typhimurium* and results indicate that bacteria cultured anaerobically were more virulent than aerobic cultures, yielding a lower LD50 with *in vivo* mouse infection and increased survival in murine macrophages (Singh *et al.*, 2000). Oxygen availability also changes host-pathogen interactions demonstrated with the decreased susceptibility of *Candida albicans* to killing by immune effector cells under anaerobic conditions (Thompson and Wilton, 1992).

Reactive oxygen species (ROS) are produced by immune effector cells as an antimicrobial defense mechanism utilized by the host. In response to detection of bacterial infection via TLR signaling, macrophages produce superoxide from NADPH oxidase (Laroux *et al.*, 2005). TLR signaling in macrophages also leads to upregulation of inducible nitric oxide synthase (iNOS), which generates the reactive nitrogen species (RNS), nitric oxide, using arginine as a substrate (Stuehr *et al.*, 1991). Oxygen is

required for this reaction, but under conditions of high oxygen, nitric oxide is spontaneously oxidized to another RNS, nitrite (Lewis and Deen, 1994). The resulting composition of ROS and RNS after production of nitric oxide and superoxide from phagocytes is very complex, contingent on oxygen availability within microenvironments. Therefore, the appropriate expression of resistance genes for nitrosative versus oxidative stress is additionally complex and is believed to shift for relative importance over the course of infection with *Salmonella enterica* (Vazquez-Torres and Fang, 2001).

Consequently, bacteria have evolved diverse defense responses to protect against host-derived oxidative and nitrosative stress and equally elaborate regulation of these mechanisms. Oxygen-modulated regulation of oxidative stress resistance has been demonstrated to be important for *H. influenzae* pathogenesis (Wong *et al.*, 2007); however, RNS signaling and resistance to nitrosative stress have not been previously studied in *H. influenzae*.

Reactive Nitrogen Species: Environmental Conditions in the Host

Nitric oxide ($NO^{\cdot}$) produced from macrophages is highly unstable, spontaneously forming nitrogen intermediates (NO^{+}, NO^{-}); nitric oxide can also react with hydrogen peroxide to form peroxynitrite ($ONOO^{-}$), free thiols (ex. glutathione) to form S-nitrothiols (ex. S-nitroglutatione, GSNO) or oxygen to form nitrite (NO_2^{-}), which is more stable molecule. For this thesis, these compounds are collectively referred as reactive nitrogen species (RNS). Because NO is highly reactive, NO_2^{-} is frequently used to

indirectly measure NO production with in vivo infection. Elevated NO_2^- levels in cerebrospinal fluid of rats (Buster *et al.*, 1995) and human infants (Tsukahara *et al.*, 1996; Tsukahara *et al.*, 1998) with *H. influenzae* meningitis suggest that NO is generated during bacterial infection. NO_2^- is also considered a "storage pool" for NO, also call a NO donor, as it reverts back to NO under acidic conditions (Dejam *et al.*, 2003), which is potentially relevant to *H. influenzae* infection given that mucus in cystic fibrosis patients is acidic (Yoon *et al.*, 2006). Because high concentrations of NO can also be toxic to the host, it is advantageous for nitrosative stress to be specifically targeted to bacteria, such as NO generated from NO_2^- in the acidic phagosome compartment of macrophages (Haggie and Verkman, 2007). Another source of RNS in the human host is by dietary ingestion of nitrate (NO_3^-). NO_3^- is reduced to NO_2^- (Gladwin, 2004), thus changing the concentrations of RNS in tissues and blood, which has been demonstrated in rats (Bryan *et al.*, 2005). Bacteria metabolize NO_3^- and NO_2^- for anaerobic respiration when oxygen is depleted, so these terminal electron acceptors can also serve as signals for gene regulation.

Oxygen: Bacterial Metabolism and Signaling

When oxygen is no longer available, a bacterium must alter gene expression profiles to utilize alternative electron acceptors for respiration. Gene expression is modulated by regulators that respond to changes in environmental redox conditions, including anaerobically-active regulator ArcA and cognate sensor ArcB. Regulation by ArcA is significant for *H. influenzae* infection as the *arcA* mutant is attenuated in vivo

(De Souza-Hart *et al.*, 2003; Wong *et al.*, 2007). Microarray data examining ArcA regulation in *H. influenzae* indicates that that ArcA represses genes involved in aerobic metabolism under low oxygen conditions (Wong *et al.*, 2007). Studies conducted with *S. enterica* and *H. influenzae* demonstrated that the *arcA* mutant is hypersensitive to oxidative stress after challenge with hydrogen peroxide, an intermediate of superoxide, thus assigning another role to ArcA regulation significant for bacterial virulence (Lu *et al.*, 2002; Wong *et al.*, 2007). Increased levels of oxygen radicals, including hydrogen peroxide, have been measured in middle ear fluid of patients with chronic otitis media from *H. influenzae* infection versus healthy individuals (Takoudes and Haddad, 1999), indicating this molecule is generated during *H. influenzae* infection. Further examination of the ArcA regulon led to the identification of *dps* as an ArcA-regulated gene that confers resistance to oxidative stress and expression of *dps* is differentially expressed in response to changes in oxygen availability. ArcA induces *dps* expression in low oxygen conditions, but not high oxygen conditions, to preemptively protect the bacteria from oxidative stress caused by superoxide production from immune effector cells (Wong *et al.*, 2007). Additional experimental evidence further supports that oxygen is important for modulation of virulence factors in *H. influenzae*, specifically the phosphorylcholine (PC) epitope of lipooligosaccharide (LOS) (Wong and Akerley, 2005). The presence of the PC-epitope aids in bacterial attachment; however, the PC-epitope is highly immunogenic so constitutive expression is likely disadvantageous to the bacteria. *H. influenzae* has adapted to exhibit PC-epitope presentation under conditions where oxygen is low, which correlates with reduced expression of *galU,* a gene involved in synthesis of

LOS (Wong and Akerley, 2005). A homolog of FNR, another regulator that has been reported to be oxygen-responsive in other bacteria, is present in *H. influenzae* and has not been previously characterized.

Reactive Nitrogen Species: Bacterial Metabolism and Signaling

Bacteria that can survive in aerobic and anaerobic environments, facultative aerobes like *H. influenzae*, generally employ a repertoire of mechanisms to utilize alternative terminal electron acceptors when oxygen is depleted. Microarray data examining differential gene expression in *H. influenzae* showed that anaerobic respiration genes are up-regulated under microaerobic conditions versus aerobic conditions (Wong and Akerley, 2005). FNR has been identified as a global regulator of anaerobic respiration, controlling an estimated 10% of the *E. coli* genome under low oxygen conditions (Constantinidou *et al.*, 2006), including genes responsible for RNS metabolism and resistance. Unlike the oxygen-sensing mechanism of ArcA, which is modified by ArcB sensing changes in the quinone pool, FNR of *E. coli* is considered a direct oxygen sensor and its activity is modulated by redox state of its iron-sulfur (Fe-S) cluster (Lazazzera *et al.*, 1996). Under low oxygen conditions, FNR is active as a dimer, binding to consensus sequences in promoter regions and inducing transcription of operons involved in anaerobic respiration, including the *nap* and *nrf* operons, encoding nitrite and nitrate reductases, respectively (Constantinidou *et al.*, 2006; Salmon *et al.*, 2003; Shalel-Levanon *et al.*, 2005). These operons are subject to additional transcriptional control by dual two-component systems, NarX-NarL and NarQ-NarP. In

general, two component regulation systems consist of a sensor kinase, NarX or NarQ in this case, that is autophosphorylated in the presence of an inducing signal and a response regulator, NarL or NarP, that modulates gene expression when phosphorylated by the activated sensor kinase (reviewed in(Laub and Goulian, 2007)). Both NarX and NarQ can be activated by either NO_3^- or NO_2^- and each can subsequently phosphorylate NarL and NarP, resulting in regulation of the *nap* and *nrf* operons, which illustrates an example of a complex regulation mechanism in response to environmental cues (Stewart, 1994a). NO_3^- is the most prevalent anaerobic terminal electron acceptor in the human host, concentrations ranging from ~0.1 mM in serum to 2 mM in saliva after digestion of nitrate rich food (Iijima *et al.*, 2002) (Lundberg and Govoni, 2004). NO_3^- appears to be significant electron acceptor for *E. coli* and *S. enterica* given they possess three nitrate reductases (NarG, NarZ and Nap), that can reduce NO_3^- to NO_2^-, which is further metabolized to ammonia by two nitrite reductases (NrfA and NirB) (reviewed in (Simon, 2002; Stolz and Basu, 2002)). The presence of NO_3^- and NO_2^- metabolism pathways is highly varied among bacterial species as are the relative importance of each enzyme in virulence. The genome of *P. aeruginosa* encodes only two nitrate reductases, NarG and NapA; however, NarG is required for survival in sputum from patients with cystic fibrosis under anaerobiosis, but Nap is not required (Palmer *et al.*, 2007). The *napG* mutant in *P. aeruginosa* was attenuated for infection in *Caenorhabditis elegans* and the *narG* mutant was fully virulent (Van Alst *et al.*, 2007). Reduction of NO_3^- or NO_2^- can produce toxic NO in *S. enterica* and *E. coli* so these bacteria possess enzymes such as nitric oxide reductase, NorV (*norV*), that can utilize NO as a terminal electron acceptor

(Corker and Poole, 2003; Gilberthorpe and Poole, 2008). Metabolism of NO is also critical for the bacteria to defend against toxic levels generated from immune cells. In addition to NorV, many RNS resistance mechanisms that detoxify NO have been identified in *E. coli* and *S. enterica,* including flavohemoglobin (*hmp*) and nitrite reductase (*nrfA*) (Mills *et al.*, 2008; Poole *et al.*, 1996; Stevanin *et al.*, 2002; van Wonderen *et al.*, 2008). Other genes have been identified in *E. coli* that confer RNS resistance by repairing damage caused by NO including the di-iron protein, YtfE (*ytfE*), that can restore iron-sulfur clusters (Justino *et al.*, 2005; Justino *et al.*, 2006; Justino *et al.*, 2007). NO oxidizes Fe-S clusters and heme groups, which are present in many enzymes involved in respiration, thus nitrosative stress disrupts respiration. When YtfE is present, it can repair NO-damaged Fe-S clusters, and restore enzymatic activity of proteins involved in respiration (Justino *et al.*, 2007). Additionally some transcriptional regulators possess an Fe-S cluster that is sensitive to nitrosative stress(Crack *et al.*, 2008), such as FNR which regulates genes involved in RNS resistance in *S. enterica, N. gonorrhea* and *E. coli* (Constantinidou *et al.*, 2006; Fink *et al.*, 2007; Whitehead *et al.*, 2007), thus YtfE could also repair NO-damaged regulators, resulting in induction of NO resistance genes.

Regulation of RNS metabolism and resistance is a complex mechanism of coordination between multiple regulators, dependent on environmental cues including availabilities of oxygen and RNS (reviewed in (Spiro, 2007)). Transcription of *norV* is dependent on induction by NO-responsive NorR in *E. coli* (Tucker *et al.*, 2006; Tucker *et al.*, 2008). Oxygen is another signal that can dictate which defense mechanism is

appropriate for the conditions: *hmp* requires oxygen and is repressed by anaerobically-active global regulator FNR when oxygen is low (Poole *et al.*, 1996). Over-expression of *hmp* can be detrimental to the cell so it is also repressed by another regulator, NsrR, which is inactivated by oxidation of Fe-S cluster in the presence of NO(Tucker *et al.*, 2008), thus leading to induction of *hmp* (Bang *et al.*, 2006; Gilberthorpe *et al.*, 2007; Stevanin *et al.*, 2007). The *hmp* promoter is subject to modulation by yet another regulatory mechanism through regulators, NarP or NarL, whose activity is controlled by nitrate/nitrite-responsive cognate sensors, NarX or NarQ (Poole *et al.*, 1996). Regulation of *hmp* promoter is the best characterized transcriptional control of a RNS resistance mechanism; however, *H. influenzae* does not possess a predicted homolog of *hmp*. To investigate the RNS defense mechanisms employed by *H. influenzae*, the sequenced genome was queried to identify homologs of genes predicted to encode regulators involved in RNS resistance. This search yielded regulators, FNR and NarP, and RNS resistance factors, NrfA and YtfE. This thesis identifies mechanisms for metabolism of terminal electron acceptors, NO_3^- and NO_2^-, and RNS resistance and elucidates the role of FNR and NarP regulation of RNS metabolism and resistance.

CHAPTER II

FNR is required for *nrfA* expression and nitrite reductase expression under low oxygen growth conditions.

Summary

Anaerobically-active ArcA regulates an oxidative stress resistance mechanism and is essential for *in vivo H. influenzae* infection, implicating that redox signaling in low oxygen environments is important for virulence. The genome of *H. influenzae* encodes an FNR homolog, which has been identified as an oxygen-responsive regulator of genes required for RNS metabolism and resistance in other bacteria; therefore, I sought to characterize FNR regulation in *H. influenzae.* Based on computational predictions from multiple sources, a comprehensive list of genes in *H. influenzae* strain Rd KW20 with putative FNR binding sites was compiled. Using RT-qPCR, transcript levels in wild type and *fnr* mutant strains that were cultured in depleted oxygen conditions were evaluated for differential abundance of potential FNR targets, *nrfA*, *napA*, *dmsA* and *bisC*, which encode predicted reductases of anaerobic terminal electron acceptors. To elucidate the role of FNR regulation in expression of nitrate and nitrite reductases, wild type, *fnr, nrfA* and *napA* mutant strains were assayed for the ability to reduce NO_3^- and NO_2^-. The *fnr* mutation results in lower *nrfA* transcript levels and impaired ability to reduce NO_2^-. To further characterize FNR regulation, a *nrf* promoter reporter fusion, Pnrf-HA, was constructed. In the *fnr* mutant strain, Pnrf-HA expression was undetectable under microaerobic growth conditions; whereas, a distinct Pnrf-HA band was visible with

immunoblotting in Fnr+ strains cultured microaerobically. Additionally, an increase in Pnrf-HA expression correlates to a decrease in oxygen availability in the media, suggesting that the *nrf* promoter is modulated by oxygen signals.

CHAPTER II

Introduction

H. influenzae, a facultative anaerobe, can survive in the presence or absence of oxygen; however, it must alter its gene expression profile to adapt for respiration appropriate to the aeration conditions. ArcA, an anaerobically-active regulator, modulates gene expression in response to changes in oxygen (Georgellis *et al.*, 2001; Wong *et al.*, 2007). *H. influenzae* posses a predicted homolog of another anaerobically-active regulator, FNR, that controls genes involved in anaerobic respiration in other bacteria. FNR (formate-dependent nitrite reductase regulator) is a conserved protein present in many human pathogens including *E. coli, H. influenzae, Vibrio cholerae, P. aeruginosa, Salmonella enterica, Klebsiella pneumoniae, Yersinia pestis, Pasteurella multocida, Neisseria meningitidis* and *Neisseria gonorrhoeae* (reviewed in (Gerasimova *et al.*, 2001; Spiro, 1994)). Microarray data with *E.coli* wild type and *fnr* mutant strains cultured in aerobic and anaerobic growth conditions implicate approximately 100 operons predicted to be members of the FNR regulon, revealing that FNR acts as a global regulator when the bacteria transition from conditions of high oxygen to low oxygen (Constantinidou *et al.*, 2006; Salmon *et al.*, 2003; Shalel-Levanon *et al.*, 2005). The FNR of *E. coli* is a 250 residue protein composed of domains for dimerization, DNA binding and an iron-sulfur "sensing" cluster (Crack *et al.*, 2004). When oxygen is present, the Fe-S cluster of FNR is oxidized and the protein is monomeric and inactive. When oxygen levels are low, the Fe-S cluster is reduced, causing a conformational change that exposes the dimerization and

DNA binding domains, resulting in the active dimeric form (Crack *et al.*, 2006; Crack *et al.*, 2007; Crack *et al.*, 2008). FNR is primarily monomeric in aerobic cultures and dimeric holoprotein in anaerobic cultures (Dibden and Green, 2005; Sutton *et al.*, 2004). Mutations of the critical cysteine residues that make up the Fe-S cluster (C20, C23, C29, C122) yield forms of FNR that failed to induce transcription of FNR-regulated genes, *dmsA* or *frdA* (Lazazzera *et al.*, 1996; Melville and Gunsalus, 1990). Additional residues, leucine 28 and aspartic acid 154, are involved in oxygen sensing as mutations result in an FNR protein that is active under conditions of high oxygen (Lazazzera *et al.*, 1996). *In vitro* biochemical characterization of oxygen sensing by FNR has been further verified by *in vivo* examination of expression of FNR-dependent promoters in *E. coli* cultured under aerobic and anaerobic growth condition (Dibden and Green, 2005; Jervis and Green, 2007).

Homologs of FNR in *S. enterica* and *N. meningitidis* have been identified as essential virulence factors based on the evidence that *fnr* mutants were attenuated for in vivo infection; fewer viable colonies of the *fnr* mutant were recovered from *in vivo* mouse infection model as compared to the wild type strains (Bartolini *et al.*, 2006; Fink *et al.*, 2007). Evaluation of gene expression profiles with a microarray conducted with cultures of *N. meningitidis* shifted from oxygen-rich to oxygen-limited conditions indicated that 175 genes are differentially expressed with the change of oxygen availability and confirmed that 11 operons are positively regulated anaerobically by FNR, 6 of which are involved in anaerobic metabolism (Bartolini *et al.*, 2006). Microarray data comparing the anaerobic transcriptome of wild type *S. enterica* to the *fnr* mutant listed

311 genes that have altered expression in the *fnr* mutant, including genes important for metabolism, motility, RNS defense and virulence (Fink *et al.*, 2007), which would likely account for the attenuation of the *fnr* mutant.

The role of FNR regulation *H. influenzae* virulence has not been previously characterized. FNR of *H. influenzae* is 79% identical to FNR of *E. coli* and contains the critical residues C20, C23, L28, C29, C122, and D154; therefore, I hypothesize that FNR of *H. influenzae* senses oxygen in a similar fashion as FNR of *E. coli*. Residues found at the DNA binding helix are highly conserved between the two species, suggesting that promoter sequence recognition might be similar (Spiro and Guest, 1990). Computational predictions analyzing the promoter regions in *H. influenzae* of operons shown to be FNR-regulated in *E. coli* support similarity of the FNR binding box between the two species, TTGAT (N_4) ATCAA (Gerasimova *et al.*, 2001; Ravcheev *et al.*, 2007; Tan *et al.*, 2001). Although the lists of genes in the *H. influenzae* putative FNR regulon differ based on the computational criteria used, many of the genes are responsible for anaerobic respiration in other bacteria. Under conditions in which oxygen is no longer available for respiration, FNR is activated and upregulates operons associated with enzymes encoded by *napA, nrfA, frdA, dmsA* and *bisC* that can utilize alternative terminal electron acceptors like nitrate, nitrite, fumarate, dimethyl sulfoxide (DMSO) and triethylamine-N-oxide (TMAO), respectively. Microarray studies examining gene expression in *H. influenzae* cultured under aerobic and microaerobic conditions show that *nrfA* and *dmsA* are up-regulated under low oxygen growth conditions (Wong and Akerley, 2005), which

is consistent with the prediction that genes important for anaerobic respiration are induced when oxygen is depleted.

Many species of γ-Proteobacteria contain redundant pathways that reduce NO_2^- to ammonia including the NrfA and NirB protein complexes of *E. coli* and *S. enterica* (reviewed in (Simon, 2002)). *H. influenzae* is predicted to contain only a single periplasmic nitrite reductase encoded by the *nrfABCD* operon, which I hypothesized to be an essential component of the NO_2^- ammonification pathway, given that the putative enzymes in *H. influenzae* are 68%, 34%, 64%, and 48% similar to NrfA, NrfB, NrfC and NrfD of *E. coli*, respectively (Hussain *et al.*, 1994). The six-electron reduction of NO_2^- by Nrf of *E. coli* is linked to formate dehydrogenase as an electron source via membrane quinones, generating a proton gradient. NrfA, the enzymatically-active subunit, is a penta-heme cytochrome-c and directly receives electrons from membrane-associated NrfB, also a penta-heme cytochrome-c (Bamford *et al.*, 2002). Membrane-bound NrfC has an Fe-S center and is partnered with NrfD, a transmembrane protein, to shuttle electrons from the quinones to NrfB (Hussain *et al.*, 1994). Some human bacterial pathogens also have multiple enzyme systems that reduce nitrate, suggesting the evolutionary importance of utilizing a substrate ubiquitous in the human host (reviewed in (Stolz and Basu, 2002)). *E. coli* possess three such enzymes encoded by operons: *napFDAGHBC*, *narGHJI* and *narZYWV* (reviewed in (Cole, 1996)). NarG and NarZ complexes, both found in the cytoplasm and considered functionally redundant, are not predicted to be present in *H. influenzae*, whose genome does include a homologous *napFDAGHBC* operon. The Nap enzyme complex is localized to the periplasm with

NapA, NapB and NapC, identified as the essential components for nitrate reduction in *E. coli* (Stewart *et al.*, 2002). NapA, the enzymatically active subunit that contains an Fe-S cluster and molybdenum co-factor, reduces NO_3^- to NO_2^- with the transport of two electrons (Jepson *et al.*, 2007). Based on the characterized functions in *E. coli*, the predicted reduction of NO_3^- and NO_2^- by Nap and Nrf enzymes in *H. influenzae* is summarized by this biochemical pathway: $NO_3^- \xrightarrow{Nap} NO_2^- \xrightarrow{Nrf} NH_4^+$

The enzymatic activity of Nap in *H. influenzae* has not been previously examined; however, the regulation of the *H. influenzae napF* promoter ($napF_{Hi}$) was studied in *E. coli*. The regulatory elements in the *napF* promoter of *E. coli* ($napF_{Ec}$) were too complex to clearly define the interactions between FNR, NarL and NarP and the $napF_{Hi}$ promoter appeared to be activated by all three proteins (Stewart and Bledsoe, 2005). The regulation differences observed between the two different *napF* promoters in this one study support that FNR regulation needs to be directly studied in *H. influenzae*.

CHAPTER II

Materials and Methods

Pre-Growth Conditions: For anaerobic pre-growth, strains were cultured at 35°C for 16 hours in brain heart infusion broth (BHI) supplemented with 2.5mM xylose, 10 μg/ml hemin and 10 μg/ml NAD (sBHI), standing in anaerobic chamber (BD Anaerobic GasPak EZ) to an optical density at 600 nm (OD_{600}) of 0.5-0.6. For microaerobic pre-growth, strains were cultured at 35°C for 16 hours in sBHI, exposed to ambient air to optical density at 600 nm (OD_{600}) of 0.7-0.8.

Generation of putative FNR targets list and FNR binding consensus sequence: Sources of computational predictions of potential FNR regulated operons in *H. influenzae* were compared and a complete list, including all possible targets, was assembled (http://www.ccg.unam.mx/Computational_Genomics/tractorDB) (Gerasimova *et al.*, 2001; Tan *et al.*, 2001). Putative FNR binding sites were identified upstream of *nrfA, napA, dmsA* and *ytfE* based on previously reported FNR binding sequences for *H. influenzae*. The resulting sequences were used to generate a binding logo diagram using (http://weblogo.berkeley.edu/logo.cgi/).

Strain construction: Strains Rfnr, RfnrV, RfnrC, RHA, RHAfnr, RHAfnrV, RHAfnrC, and plasmid pXTfnrC were constructed as described previously (Harrington, 2009). Strain RnapA containing a transposon insertion mutation in *napA* was generated by targeted *in vitro* transposon mutagenesis of the *napA* locus with the *Himar1* derivative *magellan1*, followed by identification of the desired mutant by screening for its loss of

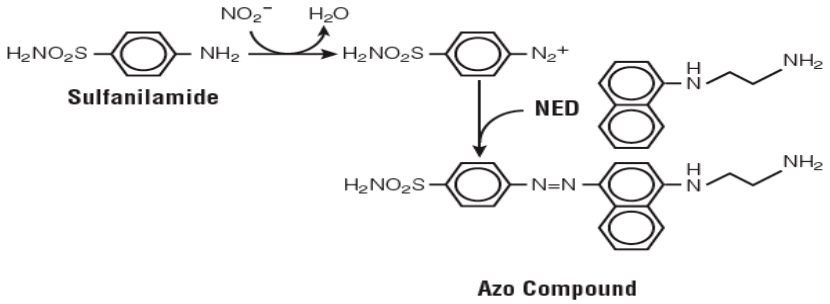
nitrate reductase activity (Akerley and Lampe, 2002). A pool of transformants containing the transposon insertion were cultured on sBHI agar plates for single colony formation and individual isolates were patched a sBHI agar plate and into 200µl sBHI broth containing 1 mM $NaNO_3$ in a 96-well dish and incubated at 35°C for 2 hours. Supernatants were assayed for the presence of NO_2^- with Griess reagents (as described below) and isolates that were impaired in the ability to reduce $NaNO_3$, as indicated by the absence of detectable NO_2^- in the supernatant, were colony purified and verification of the transposon insertion in the *napA* open reading frame was determined by PCR.
To generate strain NTfnr, a fragment consisting of 1.4 kb upstream of the transposon insertion in *fnr* to 1.3 kb downstream of the insertion was amplified by colony PCR using Rfnr as a template with primers 5'FNRupstreamCK (5'-GCGGCAAGAATGGCAGCGTTATCC-3') and 3'FNRdownstreamCK (5'-ACCTGTGCGTCCCACTGTGCC-3'). The resulting product was used to transform strain NT127 resulting in strain NT127. Kanamycin resistant recombinants were selected on sBHI agar and the resulting mutation was verified by PCR.

Real-Time Polymerase Chain Reaction: Anaerobic cultures were used to inoculate 40 mL sBHI with an initial density of OD_{600} 0.01, and were subsequently incubated at 35°C shaking at 120 rpm (ThermoForma Orbital Shaker) in anaerobic chamber (BD Anaerobic GasPak EZ) for 5.5 hours to mid-log growth (OD_{600} 0.3-0.4). Cultures were placed immediately on ice and total RNA was isolated using TRIzol Reagent (Invitrogen). Generation of cDNA and real time cycler conditions were as described previously (Rosadini *et al.*, 2008; Wong and Akerley, 2005). One tenth of the cDNA reactions were

used as a template for qRT-PCR with primers for *nrfA* (HI1069-5' and HI1069-3'), *dmsA* (HI1047-5' and HI1047-3'), *napA* (5'napA-RT and 3'napA-RT), *bisC* (5'bisC-RT and 3'bisC-RT), and *rpoA* (HI1047-5' and HI1047-3'). Approximate transcript values were calculated from a standard curve of each primer set.

NO_2^- Depletion Assays: Microaerobic cultures were used to inoculate 30 mL MIc (Barcak *et al.*, 1991) in a 25 ml flask with an initial density of OD_{600} 0.02, and were subsequently incubated at 35°C shaking at 250 rpm (ThermoForma Orbital Shaker) for 3 hours to mid-log growth (OD_{600} 0.3-0.4). Cultures were normalized to 7.5 OD_{600} units by centrifugation (Eppendorf Centrifuge 5415D) at 5000 x g for 5 minutes and the pellets were resuspended in 24 ml MIc plus 1 ml defined containing 6.25mM $NaNO_2$ or $NaNO_3$ for final concentrations of 0.25mM $NaNO_2$ or $NaNO_3$ and OD_{600} 0.3. Cultures were incubated 35°C shaking at 200 rpm and aliquots (200μl) were taken at indicated time points and stored on ice in a 96-well dish until completion of the enzyme assay. The 96-well dish was centrifuged at 5000xg, 4°C for 5 minutes to pellet cells. The supernatant (50 μl) was assayed for NO_2^- concentrations with Griess reagents: 50 μl of Solution A (5% phosphoric acid, 1% sulfanilamide) & 50 μl of Solution B (0.1% N- N-1-napthylethylenediamine dihydrochloride) and measured on VersaMax spectrophotometer at 540 nm (Misko *et al.*, 1993). When NO_2^- is present, it covalently reacts with sulfanilamide and NED to form a pink Azo compound, allowing for colorimetric detection (see diagram below). For each plate, a standard curve with 0.25, 0.125, 0.0625 & 0.03125 mM NO_2 in MI_c was assayed and NO_2^- concentrations in the supernatants

were calculated from the slope of the standard curve. Summary of Griess Reaction:



Immunoblotting: Microaerobic cultures were used to inoculate 30 mL MIc in 25 ml flask with an initial density of OD_{600} 0.02, and were subsequently incubated at 35°C shaking at 250 rpm (ThermoForma Orbital Shaker) for 3.5 hours to mid-log growth (OD_{600} 0.3-0.4). For varied aeration conditions, RHA was cultured in 5, 10, 20 or 30 ml sBHI in 25 ml flask, with the identical starting densities. Appropriate volumes to yield a pellet of 0.5 OD units were centrifuged for 5 minutes at 15700 x g and resulting pellets were resuspended in 50 µl MIc. Samples (0.4 OD_{600} equivalents per lane) were lysed with SDS-PAGE sample loading buffer, boiled for 5 minutes, centrifuged for 1 minute at 15700 x g, then resolved on SDS-PAGE gels for 14 hours at 12 mAmps (Gibco BRL vertical gel apparatus). Gels were electrotransferred to Immobilon-P (Millipore Corporation, Billerica, MA), blocked with 1% dry milk (Carnation) in 1% TBS – 0.1% Tween, probed with anti-HA1.1 (1:1000; Covance, Berkeley CA) for 60 minutes, washed then probed with secondary antibody goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:5000, Upstate, Lake Placid, NY) for 30 minutes and visualized with West-One Chemiluminescent Solution (Sigma Aldrich).

Table 1: Strains used in this study

Strain	Freezer Vial	Genotypes, Description and/or Relevant Features	Reference
Rd	CH2	Wild type: *H. influenzae* capsule deficient type d	(Wong *et al.*, 2007)
RHA	CH55	Rd *nrfAΔ::helha*; Pnrf-HA reporter strain and *nrfA* deletion mutant carrying Pnrf-HA reporter construct driven by *nrfA* promoter and replacing *nrfA*	(Harrington *et al.*, 2009)
RnapA	CH60	Rd *napA'::nptII*; *napA* mutant strain with Km^r transposon insertion in *napA*	This study
Rfnr	CH123	Rd *fnr'::nptII*; *fnr* mutant with Km^r transposon insertion in *fnr*	(Harrington *et al.*, 2009)
RfnrV	CH105	Rfnr $xylA_{\Delta 4\text{-}804}$*::tetAR*; *fnr* mutant carrying empty Tet^r vector sequence from pXT10	(Harrington *et al.*, 2009)
RfnrC	CH110	Rfnr $xylA_{\Delta 4\text{-}804}$*::fnr*; *fnr* mutant complemented with *fnr* expressed via the *fnr* promoter from pXTfnrC	(Harrington *et al.*, 2009)
RHAfnr	CH92	RHA *fnr'::nptII*; Pnrf-HA reporter strain and *fnr* mutant with Km^r transposon insertion in *fnr*	(Harrington *et al.*, 2009)
RHAfnrV	CH149	RHAfnr $xylA_{\Delta 4\text{-}804}$*::tetAR*; Pnrf-HA reporter strain and *fnr* mutant carrying empty Tet^r vector sequence from pXT10	(Harrington *et al.*, 2009)
RHAfnrC	CH153	RHAfnr $xylA_{\Delta 4\text{-}804}$*::fnr* Pnrf-HA strain and *fnr* mutant complemented with *fnr* expressed via the *fnr* promoter from pXTfnrC	(Harrington *et al.*, 2009)
RnarP	CH67	Rd Δ*narP::aacCI*; *narP* mutant with gentamicin cassette replacing *narP*	This study
RnarPV	CH157	Rd Δ*narP::aacCI*, $xylA_{\Delta 4\text{-}804}$*::tetAR*; *narP* mutant carrying empty Tet^r vector sequence from pXT10	This study
RnarPC	CH161	Rd Δ*narP::aacCI*, $xylA_{\Delta 4\text{-}804}$*::narP*; *narP* mutant complemented with *narP* expressed via *narP* promoter from pXTnarPC	This study
RnarQ	CH70	Rd Δ*narQ::aacCI*; *narQ* mutant with gentamicin cassette replacing *narQ*	This study
RHAnapA	CH63	RHA *napA'::nptII*; Pnrf-HA reporter strain and *napA* mutant with Km^r transposon insertion in *napA*	This study
RHAnarP	CH72	RHA Δ*narP::aacCI*; Pnrf-HA reporter strain and *narP* mutant with gentamicin cassette replacing *narP*	This study
RHAnarPV	CH189	RHAnarP $xylA_{\Delta 4\text{-}804}$*::tetAR*; Pnrf-HA reporter strain and *narP* mutant carrying empty Tet^r vector sequence from pXT10	This study
RHAnarPC	CH193	RHAnarP $xylA_{\Delta 4\text{-}804}$*::narP*; Pnrf-HA reporter strain and *narP* mutant complemented with *narP* expressed via the *narP* promoter from pXTnarPC	This study
RHAnarQ	CH74	RHA Δ*narQ::aacCI*; Pnrf-HA reporter strain and *narQ* mutant with gentamicin cassette replacing *narQ*	This study
RHAnarPV	CH201	RHAnarQ $xylA_{\Delta 4\text{-}804}$*::tetAR*; Pnrf-HA reporter strain and *narQ* mutant carrying empty Tet^r vector sequence from pXT10	This study
RHAnarPC	CH205	RHAnarQ $xylA_{\Delta 4\text{-}804}$*::narQ*; Pnrf-HA reporter strain and *narQ* mutant complemented with *narQ* expressed via the *narQ* promoter from pXTnarQC	This study
RV	CH252	Rd $xylA_{\Delta 4\text{-}804}$*::tetAR;* wild type carrying Tet^r vector sequence from pXT10	(Wong and Akerley, 2005)
RytfeV	CH254	Rd Δ*ytfE::aacCI*, $xylA_{\Delta 4\text{-}804}$*::tetAR*; *ytfE* mutant carrying empty Tet^r vector sequence from pXT10	(Harrington *et al.*, 2009)

Strain	Freezer Vial	Genotypes, Description and/or Relevant Features	Reference
RytfeC	CH259	Rd *ytfE*Δ*::aacCI, xylA*$_{\Delta 4\text{-}804}$*::ytfE*; *ytfE* mutant complemented with *ytfE* expressed via *ytfE* promoter from pXTPytfeC	(Harrington *et al.*, 2009)
NT127	CH83	Non-typeable *H. influenzae* clinical isolate from human cerebrospinal fluid	This study
NTV	CH301	NT127 *xylA*$_{\Delta 4\text{-}804}$*::tetAR*; wild type carrying Tet^r vector sequence from pXT10	(Harrington *et al.*, 2009)
NTfnr	CH101	NT127 *fnr'::nptII*; *fnr* mutant with Km^r transposon insertion in *fnr*	This study
NTnarP	CH84	Rd Δ*narP::aacCI*; *narP* mutant with gentamicin cassette replacing *narP*	This study
NTnarQ	CH88	Rd Δ*narQ::aacCI*; *narQ* mutant with gentamicin cassette replacing *narQ*	This study
Nytfe	CH302	NT127 *ytfE*Δ*::aacCI, xylA*$_{\Delta 4\text{-}804}$*::tetAR*; *ytfE* mutant carrying empty Tet^r vector sequence from pXT10 replacing *xylA*	(Harrington *et al.*, 2009)
NytfeC	CH306	NT127 *ytfE*Δ*::aacCI, xylA*$_{\Delta 4\text{-}804}$*::ytfE*; *ytfE* mutant complemented with *ytfE* expressed via *xyl* promoter from pXTytfeC	(Harrington *et al.*, 2009)
RfnrnarP	CH97	RfnrΔ*narP::aacCI* ; *fnr* mutant strain and *narP* mutant with gentamicin cassette replacing *narP*	This study
RfnrytfeC.1	CH270	Rfnr *fnr'::nptII, xylA*$_{\Delta 4\text{-}804}$*::ytfE*; *fnr* mutant strain with *ytfE* expressed via *xyl* promoter from pXTytfeC	This study
RfnrytfeC.2	CH271	Rfnr *fnr'::nptII, xylA*$_{\Delta 4\text{-}804}$*::ytfE*; *fnr* mutant strain with *ytfE* expressed via *xyl* promoter from pXTytfeC	This study
RfnrytfeC.3	CH272	Rfnr *fnr'::nptII, xylA*$_{\Delta 4\text{-}804}$*::ytfE*; *fnr* mutant strain with *ytfE* expressed via *xyl* promoter from pXTytfeC	This study

Table 2: Plasmids used in this study

Plasmid	Freezer Vial	Description and Relevant Features	References
pXT10	CH122	Delivery vector for chromosomal expression at the xylose locus of *H. influenzae* containing *xylF*, *xylB*, $xylA_{\Delta 4\text{-}804}$, and the *tetAR* tetracycline resistance cassette	(Wong and Akerley, 2003)
pXTfnrC	CH121	pXT10 carrying *fnr* expressed from the *fnr* promoter	(Harrington *et al.*, 2009)
pBNheltag	CH22	pBR322 vector carrying Pnrf-HA reporter construct (*nrf* promoter-*hel* ORF-HA tag-*nrfB* homology)	(Harrington *et al.*, 2009)
pXTnarPC	CH124	pXT10 carrying *narP* expressed from the *narP* promoter	This study
pXTnarQC	CH131	pXT10 carrying *narQ* expressed from the *narQ* promoter	This study
PXTPytfeC	CH250	pXT10 carrying *ytfE* expressed from the *ytfE* promoter	(Harrington *et al.*, 2009)
pXTytfeC	CH251	pXT10 carrying *ytfE* expressed from the *xylA* promoter	(Harrington *et al.*, 2009)

Table 3: Genes with predicted FNR binding sites

Locus Tag	Operon	Predicted Function	Locus Tag	Operon	Predicted Function
HI0001	***gapdH***	glyceraldehyde-3-phosphate dehydrogenase	HI0932	***Eno***	phosphopyruvate hydratase
HI0006	***fdnGHI***	formate dehydrogenase	HI1031	**hypo**	unknown function
HI0017	***yfiD***	Hypothetical	HI1032	**hypo**	unknown function
HI0018	***Ung***	uracil-DNA glycosylase	HI1047	***dmsABC***	DMSO reductase
HI0050	**Hypo**	unknown function	HI1069	***nrfABCD***	nitrite reductase
HI0055	***uxuA***	mannonate dehydratase	HI1076	***cydAB***	cytochrome d oxidase
HI0075	***nrdD***	anaerobic ribonucleoside triphosphate reductase	HI1077	***pyrG***	CTP synthase
HI0146	**Hypo**	unknown function	HI1078	***hypo***	unknown function
HI0164	**Hypo**	unknown function	HI1088	***sodA***	superoxide dismutase
HI0190	***fur***	ferric uptake regulation protein	HI1089	***ccmABCD***	heme exporter protein A
HI0225	***nhaA***	sodium/proton antiporter	HI1104	**hypo**	unknown function
HI0348	***napFDAGHBC***	nitrate reductase	HI1116	***deoCABD***	deoxyribose-phosphate aldolase
HI0390	***fadD***	long-chain fatty acid transport protein	HI1117	***comM***	competence protein
HI0401	***fadL***	long-chain fatty acid transport protein	HI1125	***talB***	Transaldolase
HI0520	**Hypo**	unknown function	HI1209	***argR***	arginine repressor
HI0534	***aspA***	aspartate ammonia-lyase	HI1210	***Mdh***	malate dehydrogenase
HI0605	***glpABC***	glycerol-3-phosphate dehydrogenase	HI1218	***lctP***	L-lactate permease
HI0621	**Hypo**	unknown function	HI1219	***cmkA***	cytidylate kinase
HI0643	***bisC/torYZ***	biotin sulfoxide reductase	HI1348	***pepT***	peptidase T
HI0686	***glpT***	glycerol-3-phosphatase transporter	HI1350	***cdd***	cytidine deaminase
HI0690	***glpFK***	glycerol uptake facilitator protein	HI1356	***malQ-glgBXCAP***	4-alpha-glucanotransferase
HI0726	***narP***	nitrate/nitrite response regulator protein	HI1379	***phoB***	transcriptional regulatory protein PhoB
HI0744	**Hypo**	unknown function	HI1385	***rsgA***	Ferritin
HI0745	***ansB***	L-asparaginase II	HI1398	***fumC***	fumarate hydratase
HI0746	***dcuB***	anaerobic C4-dicarboxylate transporter	HI1425	***fnr***	fumarate/nitrate reduction regulatory protein
HI0747	***Ndh***	NADH dehydrogenase	HI1448	***moeAB***	molybdopterin biosynthesis protein
HI0757	***gpmA***	phosphoglyceromutase	HI1550	***bioD***	dithiobiotin synthetase
HI0809	***pckA***	phosphoenolpyruvate carboxykinase	HI1659	***nrdAB***	ribonucleotide-diphosphate reductase alpha subunit
HI0835	***frdABCD***	fumarate reductase	HI1662	***sucABCD***	2-oxoglutarate dehydrogenase
HI0836	***genX***	lysyl-tRNA synthetase	HI1676	***moaABCD***	molybdenum cofactor biosynthesis protein A
HI0884	***arcA***	aerobic respiration control protein ArcA	HI1677	***ytfE***	NO resistance factor

(http://www.ccg.unam.mx/Computational_Genomics/tractorDB, Gerasimova *et al.*, 2001; Tan *et al.*, 2001)

CHAPTER II

Results

FNR is a positive regulator of genes predicted to metabolize anaerobic terminal electron acceptors under low oxygen growth conditions. In many bacteria, FNR acts as a global regulator of anaerobic respiration and positively regulates genes involved in utilization of alternative electron acceptors, such as NO_3^- and NO_2^-. Previous computational predictions have generated a list of potential FNR regulated targets in *H. influenzae*, based on FNR consensus sequences identified in other bacteria (http://www.ccg.unam.mx/Computational_Genomics/tractorDB, Gerasimova *et al.*, 2001; Tan *et al.*, 2001) A complete list of all the genes with putative FNR binding sites from the three different sources was assembled (Table 3) and 10 of 52 genes on this list have been previously linked to anaerobic respiration. To study the regulatory role of FNR in *H. influenzae*, transcript levels were examined of *napA*, *nrfA*, *dmsA* and *bisC*, which encode nitrate, nitrite, DMSO, and TMAO reductase, respectively. Total RNA was obtained from cultures of parental strain (Rd), the *fnr* mutant with vector sequences in the *xyl* locus (RfnrV), and the *fnr* mutant complemented with the *fnr* gene provided at the *xyl* locus (RfnrC) grown in the oxygen-depleted condition. Real-time PCR was used to measure transcript levels of each gene normalized to the housekeeping gene, *rpoA* (Fig. 2.1). Transcript abundance of each of the candidate FNR regulated genes was decreased in the *fnr* mutant, and restored to wild-type levels in the complemented strain.

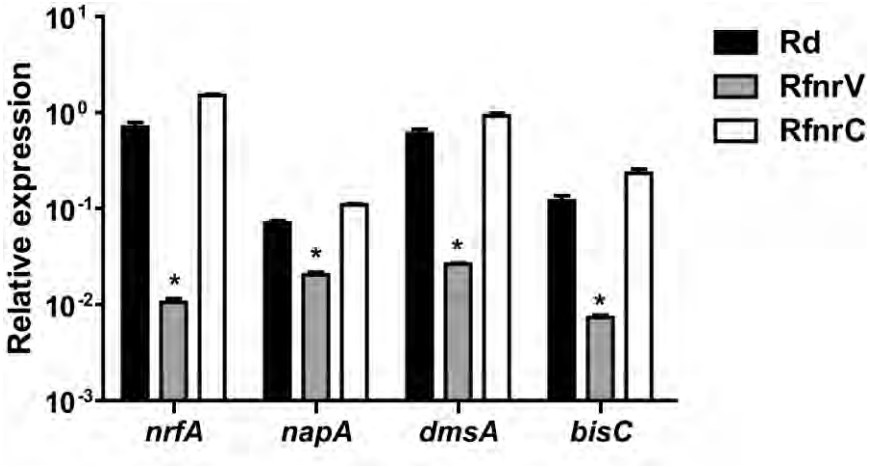


Figure 2.1: Effect of *fnr* mutation on expression of anaerobic reductases.

Total RNA was extracted from Rd, RfnrV (*fnr*-, empty vector) and RfnrC (*fnr* complemented) cultured in triplicate (duplicate for *bisC*) to log phase in an oxygen-depleted condition and expression of *nrfA*, *napA*, *dmsA* and *bisC* was examined with qRT-PCR. All transcripts were normalized to *rpoA* expression. Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test (*$p<0.001$).

A 70-fold decrease in *nrfA* mRNA levels was observed in the *fnr* mutant relative to the parent strain. The *dmsA* and *bisC* transcript levels were similarly affected, exhibiting a decrease of 23-fold and 16-fold in the *fnr* mutant, respectively. The *napA* gene was only moderately influenced, and its expression was decreased by 3-fold in the *fnr* mutant. These data are the first reports of FNR regulation in *H. influenzae* and the results are consistent with the prediction that FNR is an anaerobically-active regulator.

FNR is essential for nitrite reductase expression, but not nitrate reductase expression. Once it was demonstrated that *nrfA* and *napA* transcript levels were lower in RfnrV, I postulated that their respective enzyme expression might also be altered in the *fnr* mutant. To determine whether FNR is required for reduction of nitrate (NO_3^-) or nitrite (NO_2^-), wild type, Rd, Rfnr (*fnr* disruption mutant), RfnrV, and RfnrC were compared for the ability to reduce nitrate and nitrite from culture supernatants over time. NO_2^- was steadily depleted from supernatants of Rd and RfnrC cultures during the first 45 minutes, at which time NO_2^- levels were below the level of detection (Fig. 2.2A). In contrast, concentrations of NO_2^- were not depleted in culture supernatants of the *fnr* mutants (Rfnr and RfnrV), providing evidence that FNR is required for expression of nitrite reductase under these conditions. When the media is supplemented with NO_3^-, formation of NO_2^- can be quantified as an indirect measure of nitrate reductase activity. After the addition of exogenous NO_3^-, supernatants of Rfnr and RfnrV had similar levels of NO_2^- present as compared to Rd and RfnrC between 5 and 20 minutes (Fig. 2.2B). After 25 minutes, NO_2^- was depleted from supernatants of Rd and RfnrC but the levels of

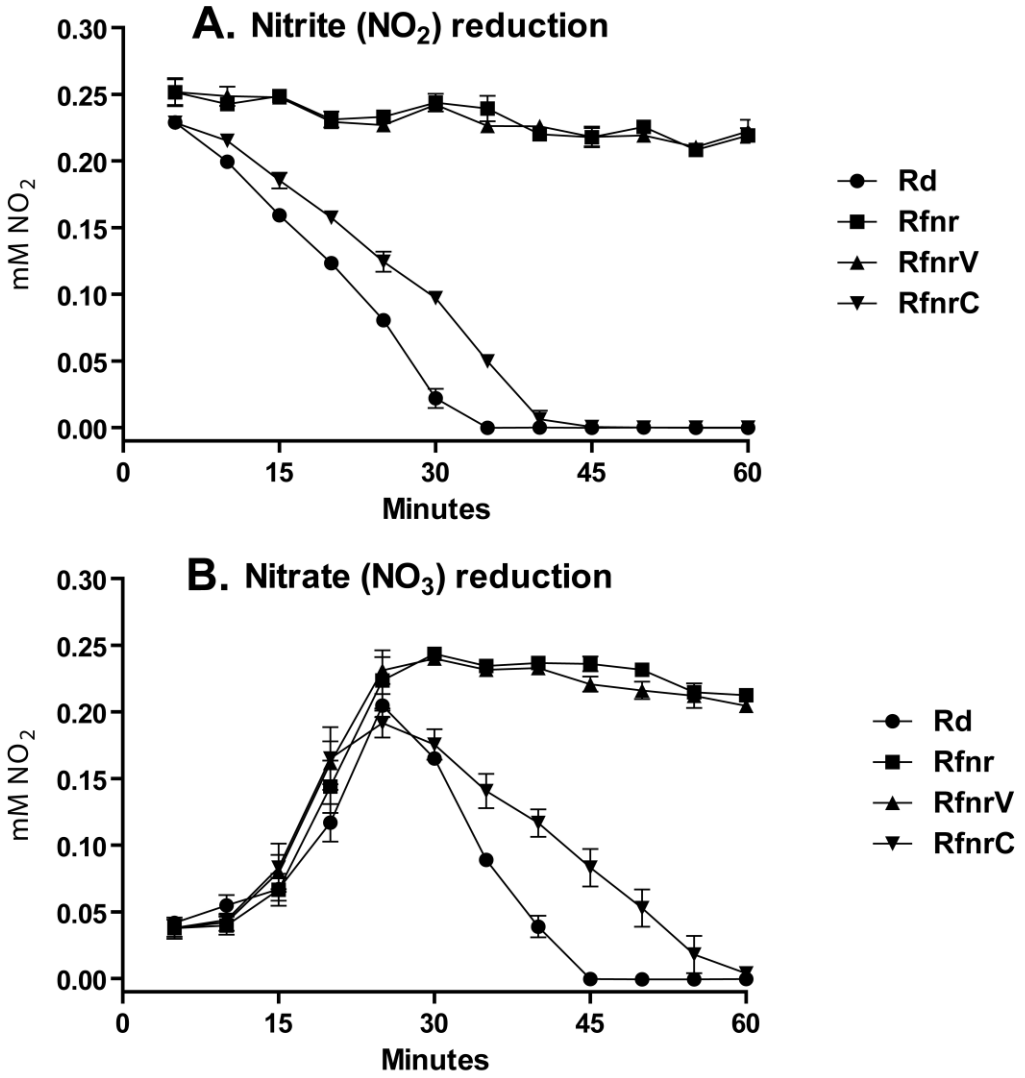


Figure 2.2: Effects of *fnr* mutation on nitrite and nitrate reductase expression.

Rd, Rfnr (*fnr*-), RfnrV (*fnr*-, empty vector) and RfnrC (*fnr* complemented) were cultured microaerobically (filled 25mls flask) to mid-log growth and standardized to 0.3 OD_{600} in defined. After addition of (A) $NaNO_2$ or (B) $NaNO_3$ (final concentrations 0.25 mM), aliquots of the cultures (200µl) were taken at indicated time points and kept on ice. Upon completion of the assay, samples were centrifuged and nitrite concentrations in supernatants were monitored with Griess reagents.

NO_2^- remained steady in the supernatants of the *fnr* mutants, Rfnr and RfnrV, suggesting that FNR is required for expression of nitrite reductase, but not nitrate reductase. The results of this assay are consistent with the differences observed between Rd and RfnrV with transcript levels of *nrfA* (700-fold) and *napA* (3-fold); indicating that *nrfA* expression is dependent on FNR but *napA* is expressed in the absence of FNR.

To test if nitrite reductase expression is dependent on FNR in a clinical isolate, the *fnr* mutation was moved into a non-typeable *H. influenzae* clinical isolate, NT127, and assayed for nitrite reductase activity (Fig. 2.3). NT127 depleted the supplemented NO_2^- to undetectable levels by 45 minutes. The *fnr* mutant, NTfnr, was impaired in its ability to reduce the exogenous NO_2^-, suggesting that the role of FNR in regulation of NO_2^- metabolism is conserved between *H. influenzae* strains.

The *napA* and *nrfA* genes of *H. influenzae* are required for reduction of nitrate and nitrite, respectively. I initially demonstrate that nitrite assays are a simple, valid assay to examine regulatory effects of FNR on enzymatic activity. I modified this assay as a genetic screening method to isolate mutants of *nrfA* and *napA.* Previous observations that a strain with a transposon insertion in *nrfD* was unable to deplete supplemented NO_2^- from growth media suggested that the *nrf* operon is essential for NO_2^- metabolism in *H. influenzae* (data not shown). Wild type *H. influenzae*, Rd, was transformed with a linear PCR product that resulted in a nonpolar deletion of *nrfA* after homologous recombination. Transformants were plated on sBHI agar with no selection, inoculated in

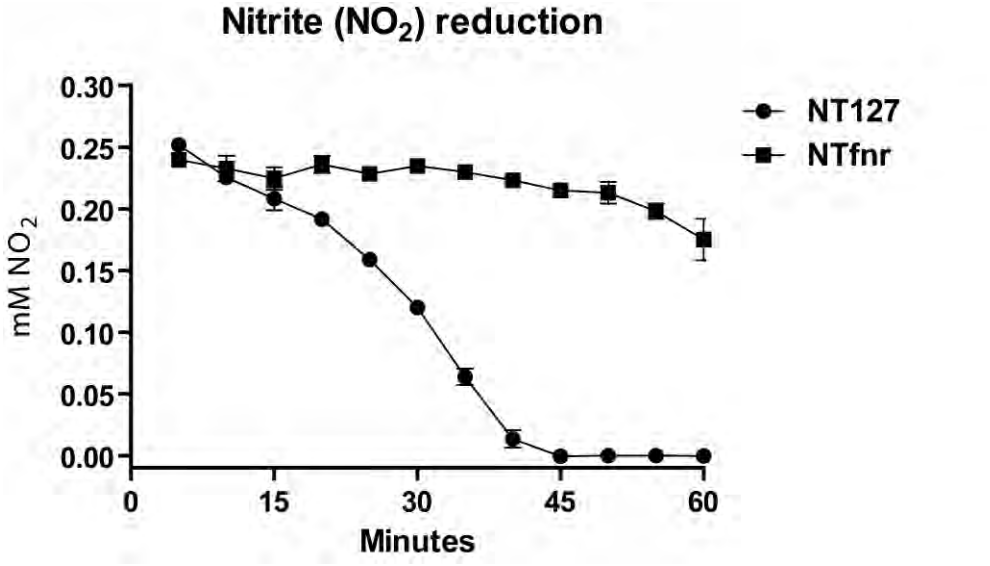


Figure 2.3: Effect of *fnr* mutation on nitrite reductase expression in a non-typeable *H. influenzae* strain.

Wild type, NT127, and *fnr* mutant strain (NTfnr) were cultured microaerobically (filled 25mls flask) to mid-log growth and standardized to 0.3 OD_{600} in MIc. After addition of $NaNO_2$ (final concentration 0.25 mM), aliquots of the cultures (200µl) were taken at indicated time points and kept on ice. Upon completion of the assay, samples were centrifuged and nitrite concentrations in supernatants were monitored with Griess reagents.

sBHI broth containing 1 mM NO_2^- and incubated for 14 hours. The presence of NO_2^- was detected in the cultures using the Griess reagents and the *nrfA* mutant, termed RHA, was isolated based on the loss of ability to deplete exogenous NO_2^-. From this outcome, I conclude that *nrfA* is required for NO_2^- reduction and the nitrite assay is a novel, effective method for nonselective screening for an unmarked mutant. A scan of the genome indicated only one predicted homolog of a nitrate reductase complex *napFDAGHBC*, so I postulated that *napA* is required for NO_3^- reduction and used the NO_2^- assay as a screening tool to isolate a *napA* mutant from a pool of transformants with targeted transposon mutagenesis *Himar1* derivative *magellan1* (Akerley and Lampe, 2002).

After isolating *napA* and *nrfA* mutants, the strains were evaluated for NO_3^- and NO_2^- reduction in the same assay as used to examine the effects of the *fnr* mutation (Fig. 2.4). When cultures were supplemented with 0.25 mM NO_2^-, RHA failed to deplete the exogenous NO_2^- (Fig. 2.4A). When the media was supplemented with NO_3^-, levels of NO_2^- accumulated by 15 minutes and remained steady (Fig. 2.4B), indicating that RHA is able to reduce NO_3^- but not NO_2^-. These data indicate that the *nrf* operon likely encodes the only nitrite reductase of *H. influenzae.* The *napA* mutant, RnapA, was additionally evaluated for its ability to reduce NO_3^- and NO_2^- under the same culture conditions. RnapA was able to deplete exogenous NO_2^- as effectively as Rd (Fig 2.4C). After addition of exogenous NO_3^-, levels of NO_2^- accumulated in the media of Rd cultures and NO_2^- was below the levels of detection in the media of RnapA cultures (Fig. 2.4D), which indicates that RnapA is unable to reduce NO_3^-. I conclude that the nitrate reductase activity of *H. influenzae* is dependent on *napA* expression.

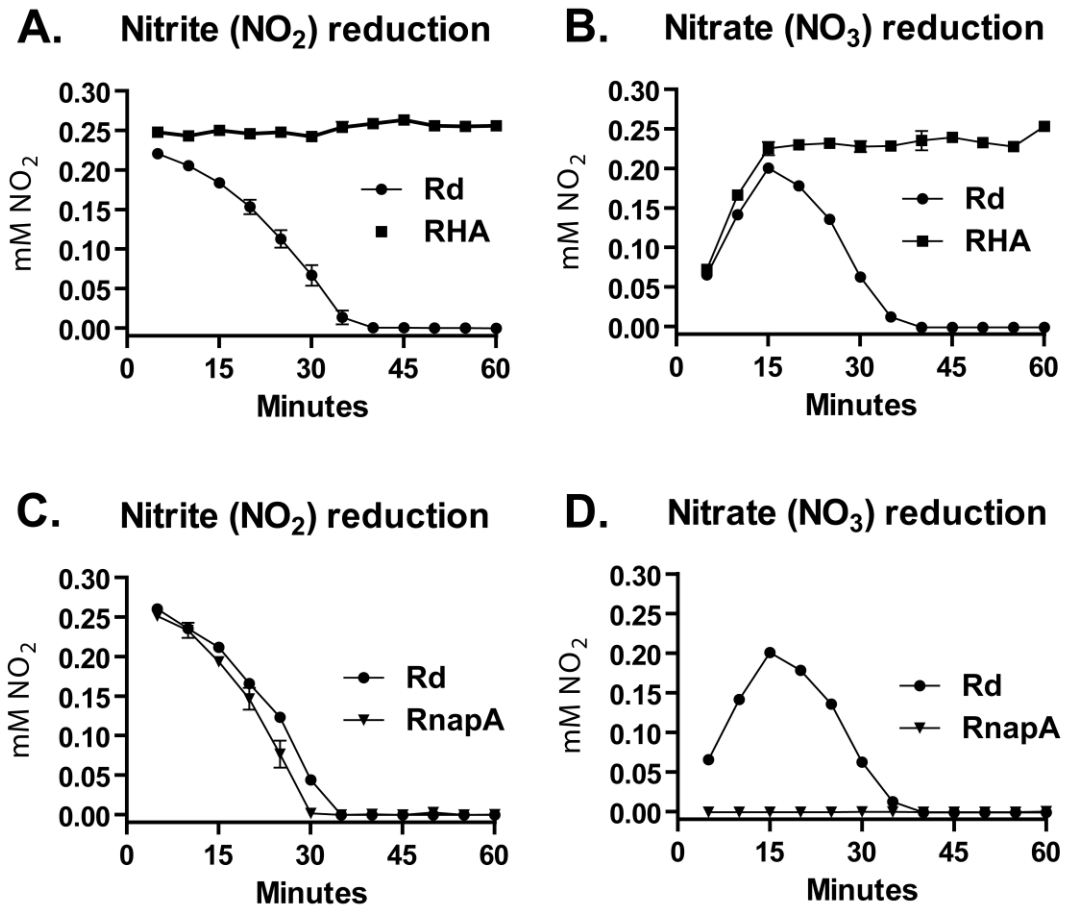


Figure 2.4: Effects of *nrfA* and *napA* mutations on nitrite and nitrate reductase activities.

Rd, RHA (*nrfA*-, A and B) and RnapA (*napA*-, C and D) were cultured microaerobically (filled 25mls flask) to mid-log growth and standardized to 0.3 OD_{600} in MIc. After addition of (A and C) $NaNO_2$ or (B and D) $NaNO_3$ (final concentrations 0.25 mM), aliquots of the cultures (200µl) were taken at indicated time points and kept on ice. Upon completion of the assay, samples were centrifuged and nitrite concentrations in supernatants were monitored with Griess reagents.

FNR is required for expression of *nrf* promoter reporter fusion, *Pnrf-HA*.

Data from the nitrite assays and transcript analysis implicated *nrfA* as a gene that is tightly controlled by FNR; therefore, to further study FNR activity, we characterized regulation of the *nrf* promoter. To facilitate studies of gene expression driven by the *nrf* promoter, we generated a reporter fusion construct, Pnrf-HA, in which the *nrf* promoter, including sequences up to the ATG start codon of *nrfA* open reading frame, was cloned to the coding sequence of the *hel* gene encoding a surface expressed *H. influenzae* protein P4. Sequence encoding an influenza virus hemagglutanin epitope (HA) was also added to the 3' end of the *hel* gene to allow immunological detection of the resulting reporter protein, Pnrf-HA, with monoclonal antibody HA.11. To examine promoter elements controlling *nrfA* expression, the reporter was designed to replace the native *nrfA* by homologous recombination at its endogenous locus. A markerless allelic exchange procedure was used to introduce this construct into *H. influenzae* to create strain, RHA (method described above), thereby avoiding the need for antibiotic resistance genes and facilitating subsequent strain construction.

The reporter strain was used to assess whether FNR is required for expression of the *nrf* promoter reporter fusion, *Pnrf-HA*. The *fnr* insertion mutation, complementation construct, or 'empty vector' sequences were introduced into RHA to generate derivatives containing the *fnr* insertion mutation (RHAfnr), *fnr* mutation and 'empty vector' sequences at the *xyl* locus (RHAfnrV), or *fnr* mutation and a wild-type copy of *fnr* at the *xyl* locus (RHAfnrC). Rd, RHA, RHAfnr, RHAfnrV, and RHAfnrC were then assayed

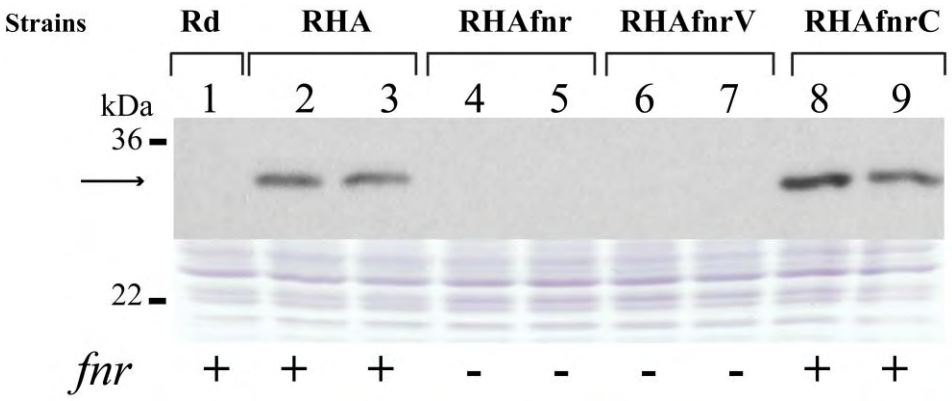


Figure 2.5: Effect of *fnr* mutation on *Pnrf-HA* expression.

Whole cell lysates of Rd (Lane 1), RHA (Lanes 2 and 3), RHAfnr (Lanes 4 and 5), RHAfnrV (Lanes 6 and 7) and RHAfnrC (Lanes 8 and 9), cultured in low oxygen conditions in MIc, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. The arrow indicates 29 kDa band, corresponding to the Pnrf-HA fusion protein.

for levels of the Pnrf-HA fusion protein by immunoblotting after growth under low oxygen conditions where FNR is predicted to be active (Fig. 2.5). Pnrf-HA was not present in lysates of the parental Rd strain, which served as a negative control; however, Pnrf-HA was readily detectable as an ~29kDa band in lysates of RHA and RHAfnrC, consistent with the size of the predicted fusion protein. Lysates of *fnr* mutants, RHAfnr and RHAfnrV, contained no detectable Pnrf-HA, suggesting that *Pnrf-HA* expression requires activation by FNR. Verification that FNR is essential for expression of the fusion protein from the *nrf* promoter supports our hypothesis that expression of fusion reporter is a valid indication of FNR activity.

***Pnrf-HA* expression is modulated in response to different oxygen availabilities.**

The dependence of the *Pnrf-HA* reporter fusion on FNR for its expression suggested that this fusion could be used to obtain information concerning the environmental signals influencing FNR activity in *H. influenzae*. Because FNR activation has been shown to be contingent on low oxygen levels in *E. coli* and other species (Dibden and Green, 2005; Jervis and Green, 2007), *Pnrf-HA* expression was evaluated in response to altered aeration conditions. A range of oxygen conditions was generated by varying the volume of culture media in a series of flasks of the same size. As culture volume increases the liquid/air interface decreases, thus yielding lower rates of oxygen supply (D'Mello *et al.*, 1997). Genomic transcription profiling experiments with *H. influenzae* have shown that altering aeration conditions modulates expression of *nrfA* and other genes predicted to be controlled by FNR (Wong and Akerley, 2005). The effect of these conditions on oxygen levels was verified by measuring dissolved oxygen levels in cultures at the time of

sample harvesting (Fig. 2.6). In parallel, Pnrf-HA levels in lysates from the same cultures were monitored by immunoblotting. As shown in Figure 2.6, increased expression of Pnrf-HA correlated with decreased oxygen availability. Although the intracellular oxygen exposure of FNR is related to a complex function of the oxygen consumption rate and other factors not measured here, the results demonstrate that activation of the *nrf* promoter occurs under a low oxygen condition but induction does not require complete anaerobiosis. These results indicate that FNR of *H. influenzae* increases *nrfA* expression in response to conditions of decreased culture oxygen concentrations.

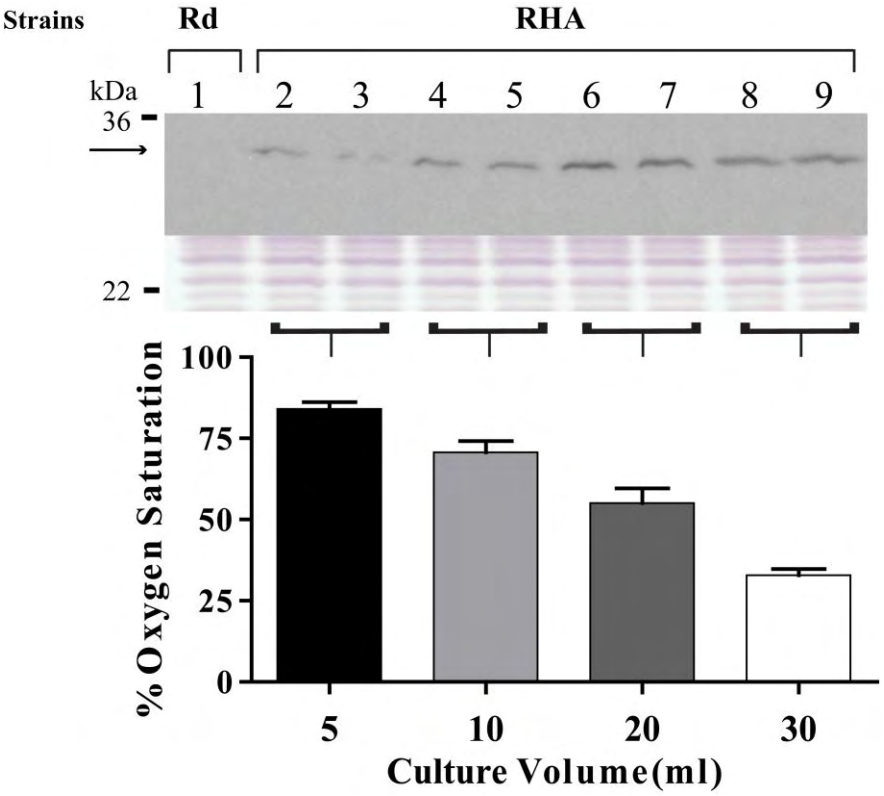


Figure 2.6: Effect of oxygen availability on *Pnrf-HA* expression.

Whole cell lysates of Rd (Lane 1) and RHA (Lanes 2-9), cultured in sBHI in a 25 ml flask with a range of media volumes to equivalent optical density, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. Percent oxygen saturation was determined in parallel with a Clark-type probe (model DO-166, Lazar Research labs). The arrow indicates 29 kDa band, corresponding to the Pnrf-HA fusion protein.

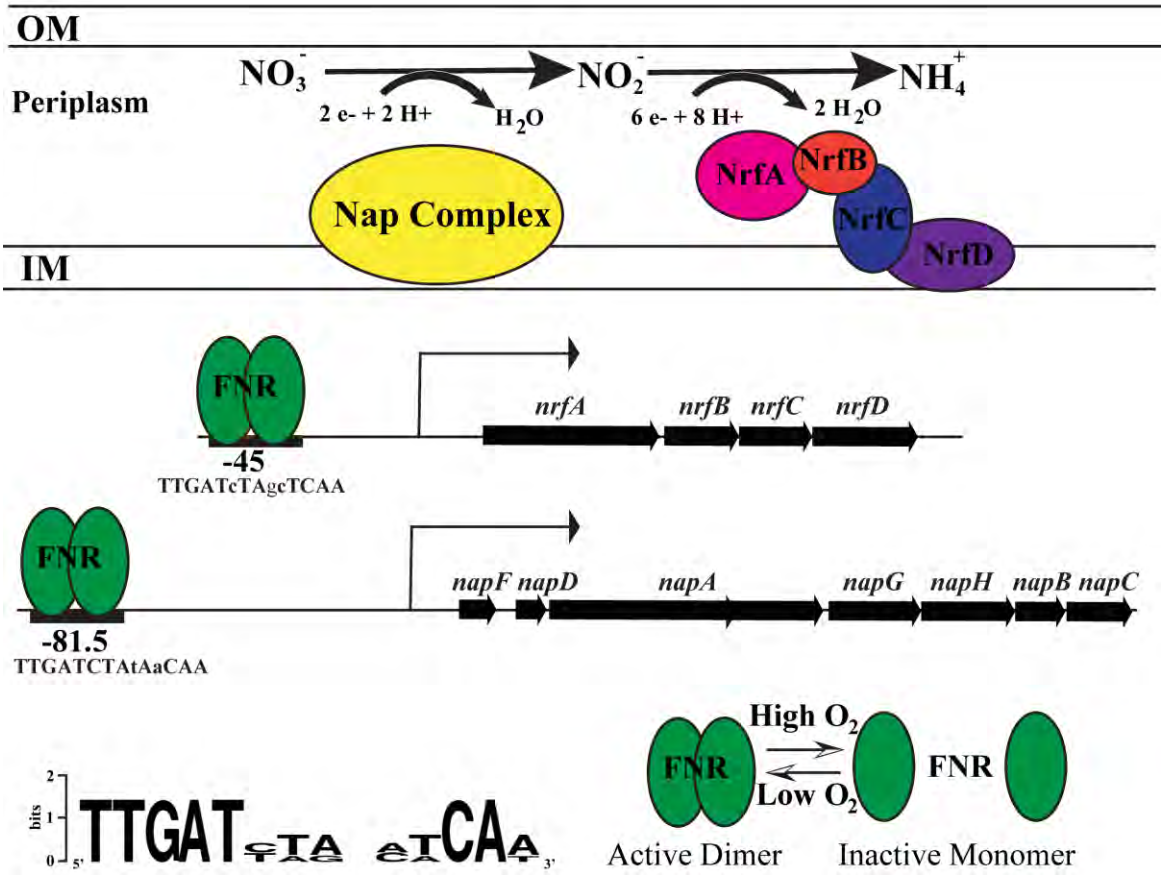


Figure 2.7: **Proposed model of FNR regulation in *H. influenzae*.**

Under aerobic growth conditions, FNR (green circle) is monomeric and inactive; when oxygen is depleted, FNR can dimerize and recognize a binding sequence in promoters. The putative FNR binding sites (numbers denote basepairs relative to the predicted transcriptional start sites) are labeled in the *nrf* and *nap* promoters and conserved residues are denoted with capital letters. Based on homology to Nap and Nrf protein complexes in *E. coli*, the *nap* and *nrf* operons encode periplasmic nitrate and nitrite reductases, which can reduce NO_3^- and NO_2^-, respectively.

CHAPTER II

Discussion

Prior to these experiments, FNR regulation of anaerobic respiration in *H. influenzae* had not been characterized. Studies of NO_2^- reduction have proven to be simple methods to identify genes required for nitrite reduction and to characterize FNR regulation. Nitrite assays are an effective method to 1) measure reduction of NO_2^- and NO_3^-, 2) study the regulation of the nitrate and nitrite reductase activities, and 3) non-selectively screen for genetic mutations. The *fnr* mutant was impaired for nitrite reductase activity in both Rd and non-typeable backgrounds, suggesting that FNR regulation of NO_2^- metabolism is conserved between strains, consistent with observations made in other bacteria. Based on studies using other bacteria and a report on regulation of the *napF* promoter of *H. influenzae* (Stewart and Bledsoe, 2005), I predicted disruption of *fnr* would result in an altered phenotype for nitrate reduction; however, the *fnr* mutant converted NO_3^- to NO_2^- as efficiently as wild type, implying the nitrate reductase expression occurs in the absence of FNR. With this assay, essential components for nitrate and nitrite reduction were identified through a non-selective screen. The *nrfA* mutant was successfully isolated without the use of an antibiotic marker, thus *nrfA* is required for nitrite reduction under the conditions tested.

Results from transcript analysis were consistent with the nitrite assay observations with the *fnr* mutant: FNR is required for *nrfA* expression, but not as important for *napA* expression. Considerably less *nrfA* transcript was measured in the *fnr* mutant as compared to wild type; whereas, only slightly less *napA* transcript was detected in the *fnr* mutant. If expression of the

nap operon is independent of FNR activity, nitrate reductase might be constitutively expressed at low levels when oxygen is present so the bacteria can continue to respire when quickly shifted from conditions of high to low oxygen, now utilizing NO_3^- as an electron acceptor. Upon activation of FNR under microaerobic conditions, the *nrf* operon is induced, leading to the reduction of NO_2^-, the product of nitrate reduction. This sequential utilization of the specific anions corresponds to the relative reduction rates of each anion observed in the nitrite assay.

Previous data showed that *nrfA* of *H. influenzae* is expressed at high levels under microaerobic conditions as compared to aerobic conditions as determined by global expression profiling (Wong and Akerley, 2005). Expression of the *nrf* reporter fusion, *Pnrf-HA*, is dependent on FNR so the effects of oxygen availability on *Pnrf-HA* expression were investigated. High levels of Pnrf-HA were detected in the crude extracts of cultures grown with decreased oxygen availability, indicating that the *nrf* promoter is more strongly induced when oxygen levels are lower. The results of this experiment indicate that absolute anaerobiosis is not required for upregulation of the *nrf* promoter and that expression of the fusion is modulated by oxygen. The differential expression of the *Pnrf-HA* reporter strain cultured in varied aeration conditions is supportive of the model that FNR regulates the *nrf* promoter and that FNR activity is dependent on low oxygen conditions.

From these results, along with previous data from other bacteria, I can formulate a model of regulation of the *nrf* and *nap* operons by FNR in *H. influenzae* (Fig. 2.7). The *napF* promoter has been mapped in the *E. coli* background and the FNR binding box (TTGATCTATAACAA) is centered at basepair -81.5, relative to the transcriptional start site (Stewart and Bledsoe, 2005). The FNR binding consensus is conserved between different bacterial species (Gerasimova *et al.*,

2001), allowing us to generate a predicted FNR binding motif in *H. influenzae*. A putative FNR binding box (TTGATCAACGTCAA) is present in the *nrf* promoter, centered at basepair -45. The proximity of the FNR site to the transcriptional start site might account for the differences of observed with *nrfA* and *napA* transcript levels in the *fnr* mutant and the effects of the *fnr* mutation on nitrate and nitrite reductase activities**.** Because expression of the *nrf* promoter is responsive to changes in oxygen, the expression of the *napF* promoter under different aeration conditions is of interest for future studies. Additionally, the importance of nitrite and nitrite reduction and FNR regulation during *H. influenzae* infection has yet to be determined.

CHAPTER III

Two-component regulatory system, NarP-NarQ, modulates RNS metabolism and *nrfA* expression.

Summary

Genes responsible for anaerobic respiration are induced by FNR when oxygen is limited. Many of these genes, including *nrfA*, are differentially expressed in response to changes in concentrations of NO_3^- and NO_2^- in the environment. NO_3^-/NO_2^- signaling is controlled by a two component signal transduction system, composed of a transmembrane sensor, NarQ, and cognate regulator, NarP. Although NarQ-NarP activities have been characterized in *E. coli*, the role of NarQ-NarP regulation of nitrate and nitrite reduction and NO_3^-/NO_2^- signaling have not been previously demonstrated in *H. influenzae*.

To initially characterize NarP regulation of *nrfA*, *nrfA* transcript levels in a *narP* mutant strain were compared to transcripts in a wild type strain and a slight, but not significant, decrease was observed when *narP* was mutated. The *narP* and *narQ* mutants were assessed for the ability to reduce NO_3^- and NO_2^- and the narP mutant was delayed for nitrate and nitrite reduction, as compared to wild type or the *narQ* mutant. Lower transcript levels of *nrfA* and delayed NO_2^- depletion by the *narP* mutant suggest that NarP is a positive regulator of *nrfA*. Additionally, less Pnrf-HA reporter protein was detected in lysates of NarP- strains than in lysates of NarP+ strains, supporting that NarP

positively regulates the *nrf* promoter. *Pnrf-HA* expression was examined in response to altered NO_3^- and NO_2^- concentrations to determine if either of these anions serves as a signal for redox regulation. *Pnrf-HA* expression appeared to be repressed when cultured in the presence of high NO_2^- and this repression was dependent on NarQ. NarQ-NarP is reported to only positively regulate the *nrf* promoter and the results presented in this thesis indicate that regulation by NarQ-NarP of *H. influenzae* is different than regulation by NarQ-NarP of *E. coli*.

CHAPTER III

Introduction

Under anaerobic conditions, FNR upregulates genes encoding enzymes that can utilize alternative electron acceptors like NO_3^-, NO_2^-, fumarate, DMSO, and TMAO. Depending on the availability of each anion bacteria have evolved a second method of regulation to ensure that the most effective enzyme is expressed. The activation state of some regulators, such as FNR, is directly influenced by environmental signals; whereas with two component regulation systems, the activation state of the response regulator is modified by a sensor kinase that responds to environmental signals (reviewed in (Laub and Goulian, 2007)). In addition to regulation by FNR in response to low oxygen, many facultative anaerobic bacteria modulate anaerobic respiration genes in response to changes in NO_3^- and NO_2^- in the environment via the two-component regulation system, NarQ-NarP (Rabin and Stewart, 1993; Stewart, 1994b). As outlined below, regulation in response to NO_3^- and NO_2^- is exquisitely complex in some enteric bacteria such as *E. coli* (summarized in Fig. 3.1) and S. *enterica,* which possess a functionally redundant system, NarX-NarL (reviewed in Stewart, 1993). The NarX-NarL of *E. coli* is the most frequently studied nitrate/nitrite response-regulatory system; however, most γ-proteobacteria human pathogens only possess homologs of NarP and NarQ, including *H. influenzae* (Ravcheev *et al.*, 2005) and signaling by NarQ-NarP in these organisms is poorly characterized. Observations that the *napF* promoter of *H. influenzae* is regulated differently in an *E. coli* cell (activated by NarL) than the *napF* promoter of *E. coli*

(repressed by NarL) suggest that regulation by NO_3^-/NO_2^- signaling differs between species (Stewart and Bledsoe, 2005).

NO_2^- signaling and RNS metabolism has been examined in *N. gonorrhoeae,* a human pathogen that encodes NarQ-NarP, but not NarX-NarL. The *narP* mutant of *N. gonorrhoeae* had a slight growth defect under conditions of low oxygen; *aniA*, was which encodes a nitrite reductase that reduces NO_2^- to NO, was shown to be NarP-regulated (Lissenden *et al.*, 2000). Examination of NO_2^- reduction in *narP* and *narQ* mutants implicated NarP and NarQ as positive regulators of *aniA* as both mutants had decreased nitrite reductase activity (Lissenden *et al.*, 2000), a phenotype also observed in *narQP* double mutant of *N. meningitidis* (Rock *et al.*, 2005). More extensive analysis of NarQ-NarP regulation verified that *aniA* is directly activated by NarP and is upregulated in response to NO_2^-, similar to *E. coli* regulation of *nrfA* (Overton *et al.*, 2006a). Comparisons of regulation by NarQ-NarP in *E. coli* and *N. gonorrhoeae* imply that these bacteria employ different mechanisms of NO_2^- signaling as NarQ of *N. gonorrhoeae* is reported to be insensitive to the NO_2^- signal (Overton *et al.*, 2006b; Whitehead and Cole, 2006).

The predicted homologs of NarP and NarQ of *H. influenzae* are 59% and 38% identical to NarP and NarQ of *E. coli*. NarQ of *H. influenzae* shares all the critical residues that have been demonstrated as essential for NO_2^- and NO_3^- sensing by NarQ in *E. coli* (Stewart, 2003); therefore, the mechanisms of NarQ-NarP and NarX-NarL signaling in *E. coli* were considered (summarized in Fig. 3.1). The anions are sensed via

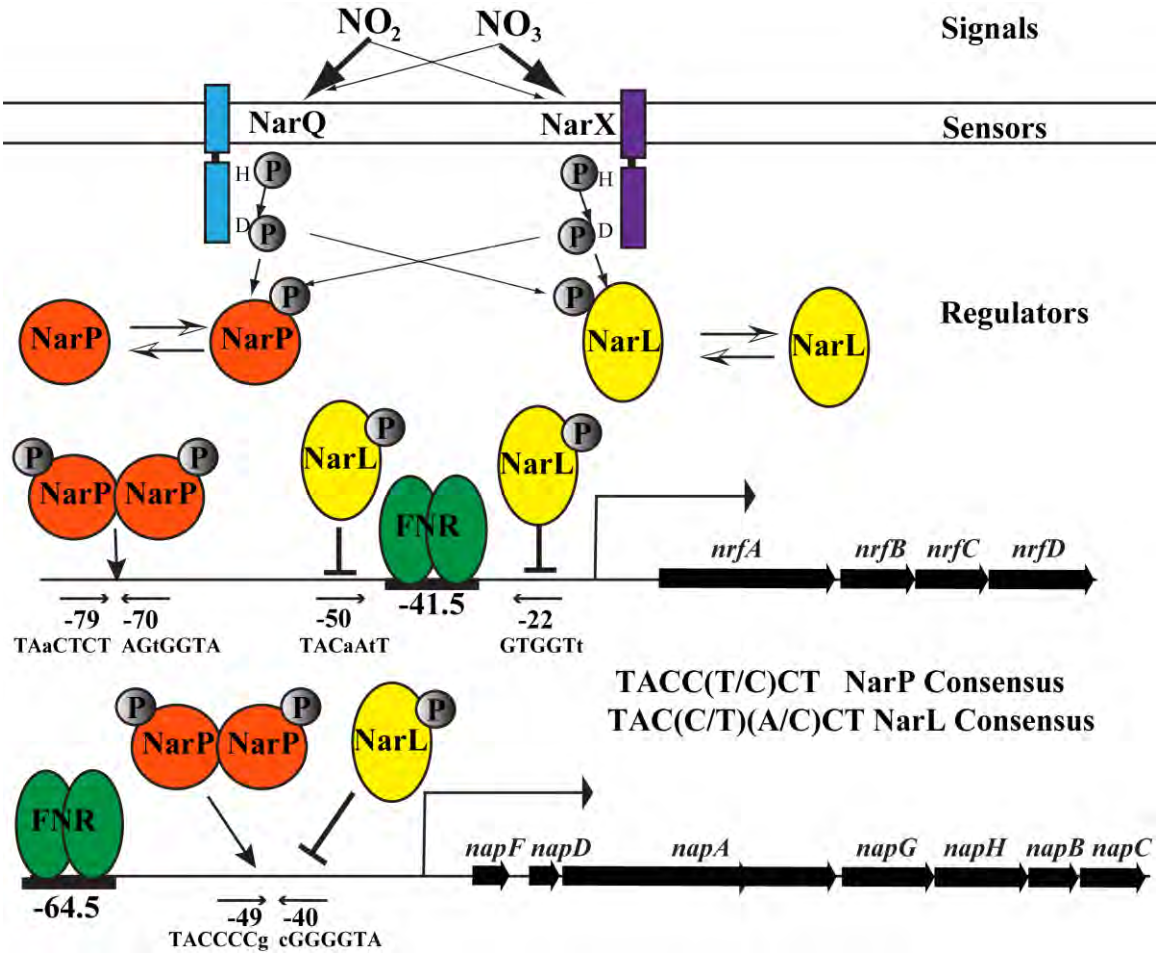


Figure 3.1: Model of NarQ-NarP and NarX-NarL regulation in *E. coli.*

Transmembrane sensor kinases, NarQ (blue square) and NarX (purple square), can detect environmental NO_3^- or NO_2^-, leading to autophosphorylation of a conserved histidine residue (H). The phosphate is transferred to an aspartate residue followed by the cognate regulators, NarP (orange circle) or NarL (yellow oval). NarP and NarL recognize heptameric sites in promoter regions (numbers correspond to the center of the binding site in basepairs relative to transcriptional start site and orientation is denoted with directional arrows). Putative NarP/NarL binding sites are listed below the directional arrows and residues shared with the consensus sequences are denoted with capital letters. Resulting regulation (activation or induction) is determined by interactions with FNR (green circle) and the sequence and locations of the binding sites.

the periplasmic P-box domain of transmembrane sensors of *E. coli*, NarQ and NarX, (Cavicchioli *et al.*, 1996; Chiang *et al.*, 1997), causing autophosphorylation of a conserved histidine in the cytoplasmic domains (Cavicchioli *et al.*, 1995; Noriega *et al.*, 2008; Schroder *et al.*, 1994). Although NarQ and NarX can both sense NO_2^- and NO_3^- and activate either NarP or NarL, unique differences have been observed with the specific signaling pathways. NarX responds to the NO_3^- signal more robustly than to NO_2^- and phosphorylates NarL, which both activates and represses genes (Lee *et al.*, 1999). NarQ targets NarP in response to the NO_2^- signal and NarP primarily acts as a positive regulator (Rabin and Stewart, 1993).

Examination of expression of NarL and NarP regulated genes demonstrate another layer of regulatory complexity as both proteins can act on a single promoter with opposing signals, dictated by the arrangement and relative affinities of the DNA binding sites (Darwin *et al.*, 1997). The *nrfA* promoter of *E. coli*, an example of differential regulation by NarP and NarL, is repressed by NarL and activated by NarP at unique binding sites (Browning *et al.*, 2002; Darwin *et al.*, 1997; Tyson *et al.*, 1994). NarP activates the *nrfA* promoter by binding to divergent sites consisting of 7 nucleotide residues and separated by 2 base pairs (7-2-7), centered at sites -79 and -70 relative to the transcription start site, located 30 base pairs upstream of the FNR binding site (summarized in Fig. 3.1); whereas, NarL can activate the *nrfA* promoter by binding to the divergent sites, but NarL also causes repression when bound to single heptameric sites at base pairs -50 and -22 (Browning *et al.*, 2002). When low levels of NarL are phosphorylated, the response regulator only binds to the heptameric site with high

basepair match to the NarL consensus sequence. Increasing the concentration of phosphor-NarL results in binding to lower affinity sites -50 or -22, which have fewer matches the consensus sequence (Darwin *et al.*, 1997).

Studies conducted with *nrfA* promoter expression in response to altered NO_2^- and NO_3^- signals correlate with the proposed NarP and NarL binding model. *PnrfA-lacZ* expression was highest upon addition of 1 mM NO_2^-, dependent on induction by NarP and *PnrfA-lacZ* expression was lowest in the presence of concentrations of NO_3^- greater than 2 mM, due to repression by NarL (Wang and Gunsalus, 2000). Increased concentrations of the inducing signal lead to activation of the sensor kinase, NarX, and phosphorylation of the response regulator, NarL. High amounts of phosphor-NarL results in repression of the *nrfA* promoter by phospho-NarL binding to the low affinity sites (Darwin *et al.*, 1997). The *nap* operon is activated by NarP and repressed by NarL, depending on the availability of NO_3^- and NO_2^- (Stewart *et al.*, 2002) (see Fig. 3.1). The binding sequences are additionally important for NarL and NarP regulatory activities as the *napF* promoter of *H. influenzae*, which also has divergent heptamer sites at base pairs -49 and -40, was activated by both NarL and NarP when expressed in an *E. coli* cell (Stewart and Bledsoe, 2005). The significant complexities of NarX-NarL and NarQ-NarP regulation in *E. coli* make it difficult to dissect the exact mechanisms for response to environmental NO_2^- and NO_3^-. *H. influenzae* is a good model organism to study this redox responsive regulation because only one sensor, NarQ, and one regulator, NarP, are present.

CHAPTER III

Materials and Methods

Strain construction: A nonpolar, in-frame deletion of *HI0727* (*narP*) was created by replacement of the protein coding sequences with the *aacC1* gentamicin resistance cassette to create strain RnarP by overlap extension PCR as follows: A 1002 bp PCR product containing the 5' flanking region of *HI0727* was amplified from Rd with primers 5'narPKO up (5'atggctcgtaagaaaaaaac) and 3'narPKOup/gent (5' ATTCGAGAATTGACGCGTAATTAGCTCAATAGATTTAATATC). A 1453 bp PCR product containing the 3' flanking region of *HI0727* was amplified from Rd with primers 5'narPKO down/gent (5' CTTCCCGGCCGACGCGTAATTTCTCCTTTAGTGGTTAG) and 3'narPKO down (5' atgaatttcttccaatataaac). A 800 bp fragment containing the *aacC1* gentamicin resistance cassette was amplified with primers 5'pBLZA-G (5' ACGCGTCAATTCTCGAATTGACAT) and 3'pBLZA (5' TTAAGGCCTACGCGTCGGCCGGGAAGCCGATCTC) from pBSL182 (Alexeyev *et al.*, 1995). The 1002 bp, 1453 bp, and 800 bp products were combined in a PCR reaction with primers 5'narPKO up and 3'narPKO down. The resultant 3.2 kb product was introduced into Rd and GmR transformants were selected on sBHI agar containing Gm to create strain RnarP. The mutation was verified by PCR.

RnarPC was generated by cloning the *narP* gene including its native promoter amplified by PCR using Rd as template with primers 5'pnarPC (5'-TTTGCTTCTATGCCATCAATAAACCTACCTCAAATAGTAAG 3') and 3'narPC.II

(5’-TTTGCTCTTCTTTAACGATTTTGTTCAAAAAATAATACCGTTG -3’), which introduce *Sap*I restriction sites flanking *narP* and its predicted promoter region. The resulting 1.4 kb product was digested with *Sap*I and cloned into *Sap*I digested pXT10. The resulting plasmid, pXTnarPC, was linearized with *Apa*LI and transformed into RnarP with selection for TcR.

To generate strain RHAnarP, the *narP* mutation was amplified from template RnarP, using primers 5’narPKOup and 3’narPKOdown and the resulting PCR product was transformed into strain RHA. Transformants were selected on sBHI plates containing Gm. pXT10 and pXTnarPC were linearized with *Apa*LI and transformed into RHAnarP with selection for TcR to create strains RHAnarPV and RHAnarPC, respectively.

To generate strain RHAnapA, the *napA* mutation was amplified from template RnapA, using primers 5’0340-ORF (atgacacaaacttttgcc) and 3’0347-ORF (gtttccgtaacctttcatc) and the resulting PCR product was transformed into strain RHA. Transformants were selected on sBHI plates containing Km.

A nonpolar, in-frame deletion of *HI0267* (*narQ*) was created by replacement of the protein coding sequences with the *aacC1* gentamicin resistance cassette to create strain RnarP PCR as follows: A 1241 bp PCR product containing the 5’ flanking region of *HI0267* was amplified from Rd with primers 5’narQKO up (5’ GTAATAATACTAAAGTGAGTGTT) and 3’narQKO up/gent (5’ ATTCGAGAATTGACGCGTAGGAAACTCCAGTGGAAATTTAG). A 1051 bp PCR product of the 3’ flanking region of *HI0267* was amplified from Rd with 5’narQKO down/gent (5’GCTTCCCGGCCGACGCGTACATCAAAATGCAAAATTTAC) and

3’narQKO down (5’ cgtgataatttgctcactatttac). The 1241 bp and 1051 bp products were combined with the 800 bp *aacC1* gentamicin resistance cassette in a PCR reaction with primers 5’narQKO up and 3’narQKO down. The resultant 3.0 kb product was introduced into Rd and GmR transformants were selected on sBHI agar containing Gm to create strain RnarQ. The mutation was verified by PCR To generate strain RHAnarQ, the *narQ* mutation was amplified from template RnarQ, using primers 5’narQKOup and 3’narQKOdown and the resulting PCR product was transformed into strain RHA. Transformants were selected on sBHI plates containing Gm. Plasmid pXTnarQC was generated by cloning the *narQ* gene including its native promoter amplified by PCR using Rd as template with primers 5’pnarQc (5’-TTTGCTCTTCTATGCATTCAACGCTTATGGCGTGGTCAAG-3’) and 3’narQC.II (5’-TTTGCTCTTCTTTAATATAATGTATGTGGCAAGGTAATTTTG-3’), which introduce *Sap*I restriction sites flanking *narQ* and its predicted promoter region. The resulting 2.5 kb product was digested with *Sap*I and cloned into *Sap*I digested pXT10. pXT10 and pXTnarQC were linearized with *Apa*LI and transformed into RHAnarQ with selection for TcR, resulting in strains RHAnarQV and RHAnarQC, respectively.

Immunoblotting growth conditions: Microaerobic cultures were used to inoculate 7.5 mL sBHI ml supplemented with 0.1, 0.5, 1.0 or 2.0 mM $NaNO_2$ or $NaNO_3$, in 8 mL glass vials with an initial density of OD_{600} 0.02, and were subsequently incubated at 35°C shaking at 250 rpm for 3.5 hours to mid-log growth (OD_{600} 0.3-0.4). For varied aeration conditions, RHA was cultured in 5, 10, 20 or 30 ml sBHI in 25 ml flasks. Immunoblotting procedures were as previously described above in Chapter II.

CHAPTER III

Results

NarP positively regulates *nrfA* expression and contributes to expression of nitrate and nitrite reductases. In *E. coli*, NarP regulates *nrfA* expression through recognition of NarP binding sites in the promoter region (Browning *et al.*, 2006). Predicted NarP-binding motifs are also present in the *nrf* promoter region of *H. influenzae*, thus similar regulation via NarP may influence *nrfA* expression. To address NarP regulation of *nrfA*, transcription profiles and nitrite reductase activities were examined in wild type and *narP* mutant genetic backgrounds. Using qRT-PCR, *nrfA* transcript levels were quantified from cultures grown under oxygen depleted conditions (Fig. 3.2). In comparison to wild type Rd strain, the *narP* mutant strain, RnarPV, had ~20% less *nrfA* transcript. Second, nitrite reductase activities were monitored for Rd, *narP* mutants, RnarP, RnarPV, and the *narP* complemented strain, RnarPC (Fig. 3.3A). RnarP and RnarPV reduced NO_2^- at slower rates. Nevertheless, both strains were able to deplete NO_2^- to below detectable levels after 45 minutes. NarP appears to enhance *nrfA* expression, leading to higher levels of nitrite reductase in the cell.

The ability of RnarP to reduce NO_3^- was compared to that of Rd to determine if NarP influences nitrate reductase expression (Fig. 3.3B). When exogenous NO_3^- was added to the media, lower amounts of NO_2^- had accumulated in the media of RnarP cultures (0.15 mM) than in Rd cultures (0.20 mM) by 15 minutes, implying that less NO_3^- had been reduced by RnarP.

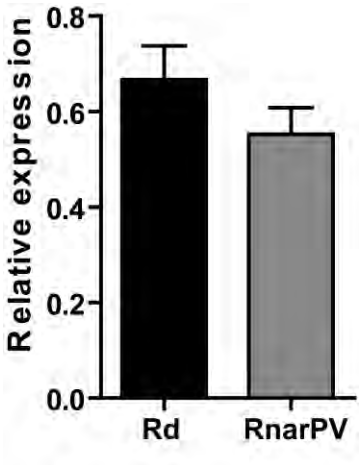


Figure 3.2: Effect of *narP* mutation on *nrfA* transcript levels.

Total RNA was extracted from Rd and RnarPV (*narP* mutant) cultured in triplicate (Rd) or duplicate (RnarPV) to log phase in an oxygen-depleted condition in sBHI and expression of *nrfA* was examined with qRT-PCR. All transcripts were normalized to *rpoA* expression.

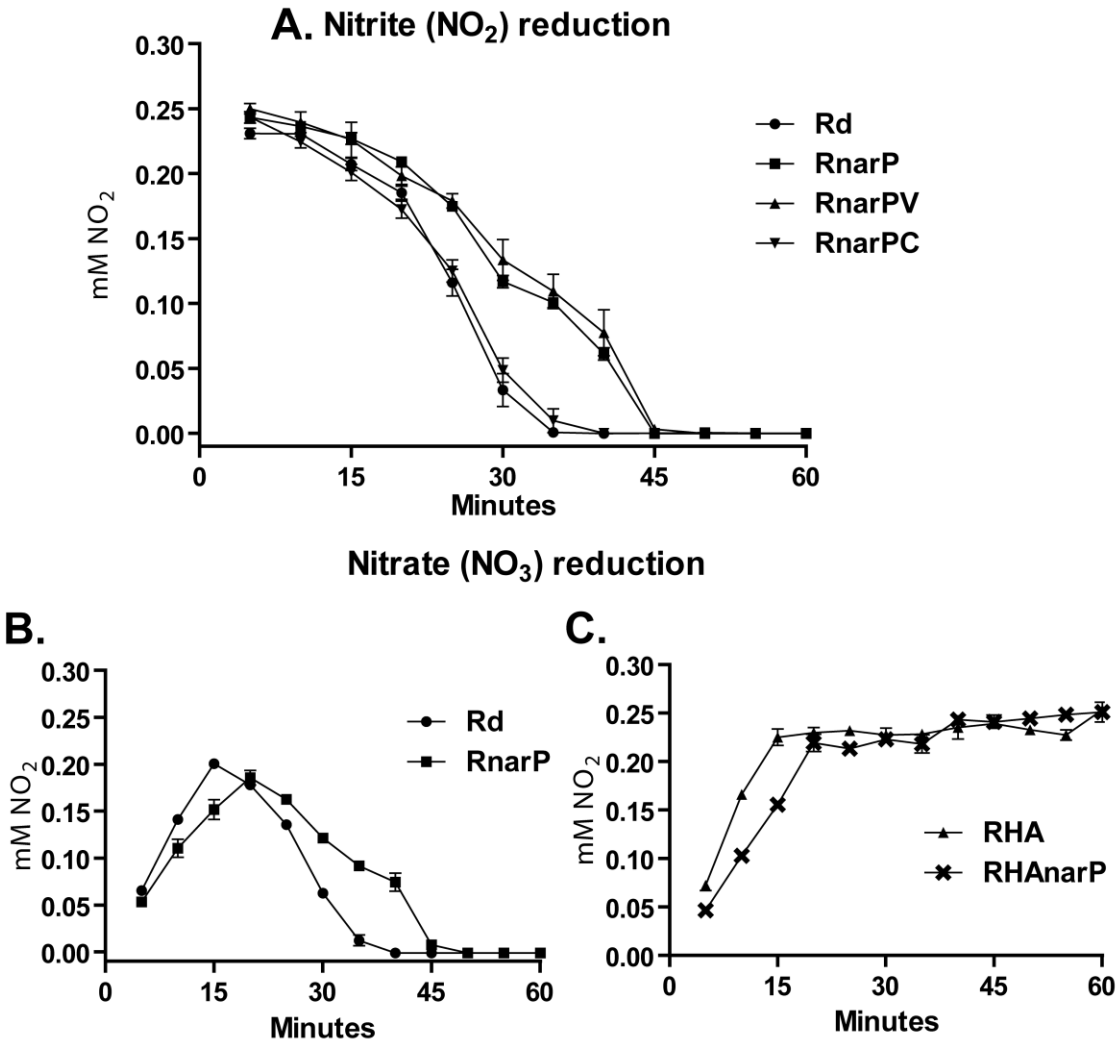


Figure 3.3: Effect of *narP* mutation on nitrate and nitrite reductase expression.

Rd, RnarP (*narP*-), RnarPV (*narP*-, empty vector), RnarPC (*narP* complemented), RHA (*nrfA*-), RHAnarP (*narP-/nrfA*-) were cultured microaerobically (filled 25mls flask) to mid-log growth and standardized to 0.3 OD_{600} in MIc. After addition of (A) $NaNO_2$ or (B and C) $NaNO_3$ (final concentrations 0.25 mM), aliquots of the cultures (200µl) were taken at indicated time points and kept on ice. Upon completion of the assay, samples were centrifuged and nitrite concentrations in supernatants were monitored with Griess reagents (see methods in Chapter II).

To separate the differential activities of NO_3^- and NO_2^- reduction, the *narP* mutation was moved into a strain background that could not reduce NO_2^-, RHA, resulting with the strain, RHAnarP (*nrfA/narP* double mutant) and the two strains were compared for NO_3^- metabolism (Fig. 3.3C). RHAnarP was impaired in its ability to reduce NO_3^-, quantified by less NO_2^- in the supernatants (0.15 mM) than in the RHA cultures (0.22 mM) by 15 minutes, which was very similar to the results observed with nitrite reductase activities of Rd and RnarP. NO_2^- concentrations continued to accumulate in the supernatants until reaching steady levels that were comparable in the two cultures, verifying that RHAnarP is unable to reduce NO_2^-. Under these culture conditions, NarP positively contributes to the nitrate reductase expression.

NarP is a positive regulator of *Pnrf-HA* expression. Observations that NarP positively regulates nitrite reductase activity prompted the investigation of the role of NarP in regulation of the *nrf* promoter, using the *Pnrf-HA* reporter fusion. RHAnarP, used in the NO_2^- assay described above, was transformed with "empty" vector or complemented with *narP* at the *xyl* locus to generate strains RHAnarPV and RHAnarPC, respectively. Strains were examined for relative *Pnrf-HA* expression by detection of Pnrf-HA by immunoblotting (Fig. 3.4). Lysates of RHAnarP and RHAnarPV had significantly less Pnrf-HA present than in lysates of parental strain, RHA; complementation of *narP* in strain, RHAnarPC, restored *Pnrf-HA* expression. Consistent with previous results with transcript analysis and nitrite assays, NarP is required for full induction of the *nrf* promoter in *H. influenzae*.

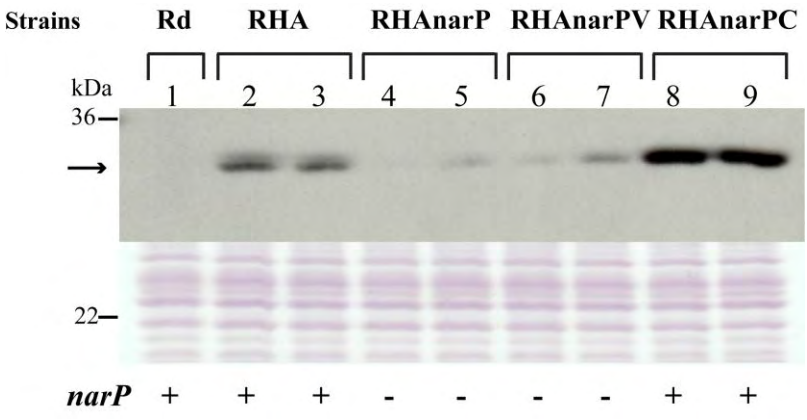


Figure 3.4: Effect of *narP* mutation on *Pnrf-HA* expression.

Whole cell lysates of Rd (Lane 1), RHA (Lanes 2 and 3), RHAnarP (Lanes 4 and 5), RHAnarPV (Lanes 6 and 7) and RHAnarPC (Lanes 8 and 9), cultured in low oxygen conditions (filled 25 ml flask) in MIc, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. The arrow indicates 29 kDa band, corresponding to the Pnrf-HA fusion protein.

NarQ exerts an inhibitory effect on nitrate reductase expression, but not nitrite reductase expression. Previous studies conducted with NarP and NarQ in *E. coli* propose the model that NarP activation is dependent on phosphorylation via NarQ (Cavicchioli *et al.*, 1995; Chiang *et al.*, 1997). In contrast to the results observed with NO_2^- reductase activity of RnarP, the *narQ* mutant, RnarQ, reduced NO_2^- as efficiently as Rd (Fig. 3.5A). Strains were pre-cultured in MIc, which does not contain anions known to stimulate NarQ activity. NarQ is not required for full nitrite reductase activity under these growth conditions.

The *narQ* mutant, RnarQ, was additionally assayed for NO_3^- reductase activity (Fig. 3.5B). When supernatants were assayed for NO_2^- concentrations at 10 minutes, 40% more NO_2^- was present in the media of RnarQ cultures than in the media of Rd cultures, which indicates that RnarQ reduced NO_2^- more rapidly than Rd. he specific influences of the *narQ* mutation on NO_3^- reduction cannot be determined when NO_2^- is simultaneously reduced. The *narQ* mutation was transformed into the RHA strain background, generating strain RHAnarQ (*nrfA/narQ* double mutant). When the two strains were compared for NO_3^- reduction, ~20% more NO_2^- had accumulated by 10 minutes in the supernatants of RHAnarQ versus RHA (Fig. 3.5C). The *narQ* mutant strains, RnarQ and RHAnarQ, are able to reduce NO_3^- more efficiently than strains with wild-type *narQ* allele, Rd and RHA, thus the presence of NarQ results in an inhibitory effect on nitrate reductase expression.

To ascertain whether NarQ and NarP regulate nitrite reductase expression in a

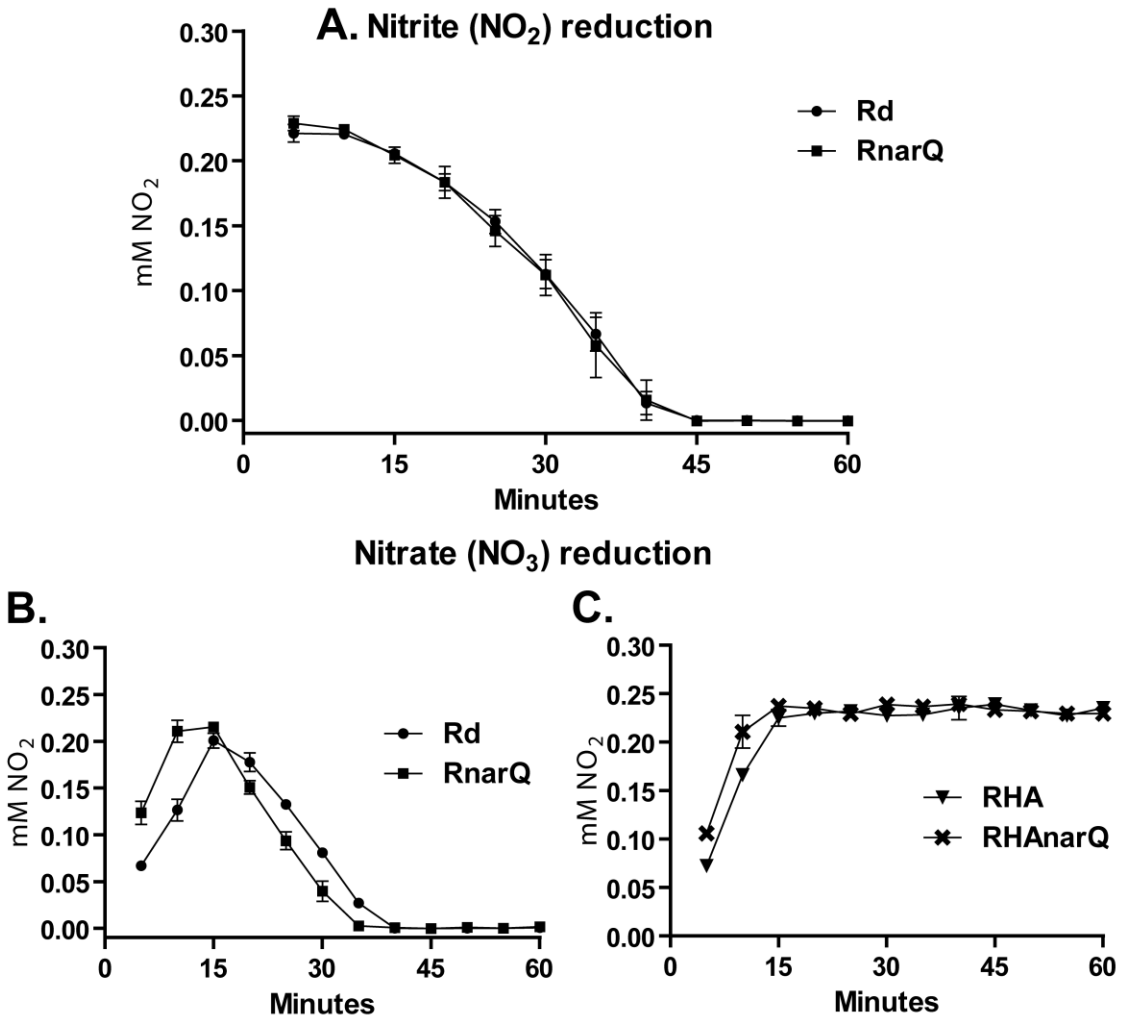


Figure 3.5: Effect of *narQ* mutation on nitrate and nitrite reductase expression.

Rd, RnarQ (*narQ*-), RHA (*nrfA*-) and RHAnarQ (*narQ*-/*nrfA-)* were cultured microaerobically (filled 25mls flask) to mid-log growth and standardized to 0.3 OD_{600} in MI_C. . After addition of (A) $NaNO_2$ or (B and C) $NaNO_3$ (final concentrations 0.25 mM), aliquots of the cultures (200µl) were taken at indicated time points and kept on ice. Upon completion of the assay, samples were centrifuged and nitrite concentrations in supernatants were monitored with Griess reagents.

non-typeable strain, *narP* and *narQ* were deleted in the NT127 background and the resulting strains, NTnarP and NTnarQ, were assayed for NO_2^- reduction (Fig. 3.6). Wild type, NT127, and NTnarQ reduced exogenous NO_2^- with comparable efficiencies and NTnarP was delayed in its ability to reduce NO_2^-. These results indicate that the functions of NarP and NarQ, in regard to nitrite reductase activity, are conserved between *Haemophilus* strains.

***Pnrf-HA* expression is modulated by NO_2^- availability in the media.** NarQ of *E. coli* recognizes environmental nitrite and nitrate and triggers activation of NarP (Schroder *et al.*, 1994). Given the observation that NarP induces *Pnrf-HA* expression, the roles of NO_2^- and NO_3^- were investigated as potential signals for regulation of *Pnrf-HA* expression in RHA. The inability of RHA to reduce NO_2^- is beneficial for examining the regulatory impact of NO_2^-, without the complication of changes in concentrations due to reduction of NO_2^-. However, when cultures of RHA are supplemented with NO_3^-, the strain is able to reduce NO_3^- to NO_2^- and any changes in *Pnrf-HA* expression might be due to the exogenously added NO_3^- or the endogenously generated NO_2^-. To circumvent this issue, the *napA* mutation from RnapA was moved into the RHA strain background to generate RHAnapA (*nrfA/napA* double mutant), which is unable to reduce either NO_2^- or NO_3^-. *Pnrf-HA* expression was examined in RHA and RHAnapA under conditions with increasing concentrations of NO_2^- or NO_3^-.

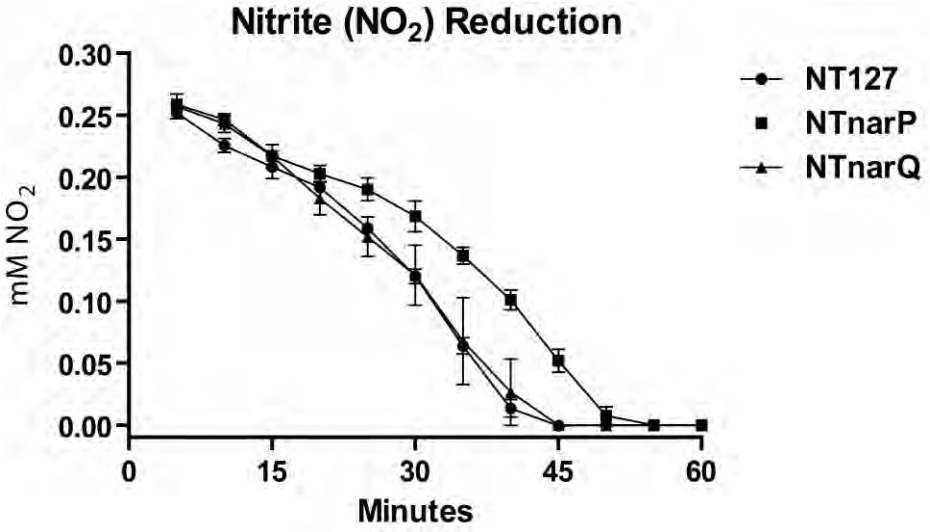


Figure 3.6: Effects of *narP* and *narQ* mutations on nitrite reductase activity in a non-typeable *H. influenzae* strain.

NT127, NTnarP (*narP*-) and NTnarQ (*narQ*-) were cultured microaerobically (filled 25mls flask) to mid-log growth and standardized to 0.3 OD_{600} in MIc. After addition of $NaNO_2$ (final concentration 0.25 mM), aliquots of the cultures (200µl) were taken at indicated time points and kept on ice. Upon completion of the assay, samples were centrifuged and nitrite concentrations in supernatants were monitored with Griess reagents.

Based on previous results examining conditions that induce *Pnrf-HA* expression (Fig. 2.6), RHA and RHnapA were cultured in sBHI under a low oxygen condition to stimulate FNR-dependent transcription of the *nrf* promoter and the media was supplemented with different NO_2^- concentrations. The concentrations of NO_2^- were selected based on a previous study done with the *nrf* promoter of *E. coli*, which reports that expression is highest with 1 mM exogenous NO_2^- under microaerobic growth conditions (Wang and Gunsalus, 2000). Visualized by immnoblotting, the Pnrf-HA band intensities were similar in lysates of RHA and RHAnapA cultured in sBHI without supplemented NO_2^-, suggesting that the *napA* mutation does not affect activation of the *nrf* promoter under these conditions (Fig. 3.7). Additionally, the expression of Pnrf-HA in lysates of RHA and RHAnapA exposed to 0.1 mM NO_2^- were comparable to Pnrf-HA levels observed in lysates of cultures without additional NO_2^-. However, as NO_2^- concentrations were increased to above 0.5 mM, less Pnrf-HA was detected in lysates of RHA. Decreased Pnrf-HA band intensities were also observed in lysates of RHAnapA cultured with 0.5 mM and 1.0 mM NO_2^- versus without supplemented NO_2^-. These results implicate NO_2^- as a modulatory signal for regulation of the *nrf* promoter at concentrations greater than 0.5 mM.

To determine if NO_3^- is an additional signal for modulation of the *nrfA* promoter, cultures of RHA and RHAnapA were supplemented with increasing concentrations of NO_3^- (Fig. 3.8). At low concentrations (0.1 mM), NO_3^- did not noticeably effect *Pnrf-HA* expression; Pnrf-HA band intensities were comparable in lysates of RHA and RHAnapA, similar to Pnrf-HA detected in cultures without NO_3^- supplementation.

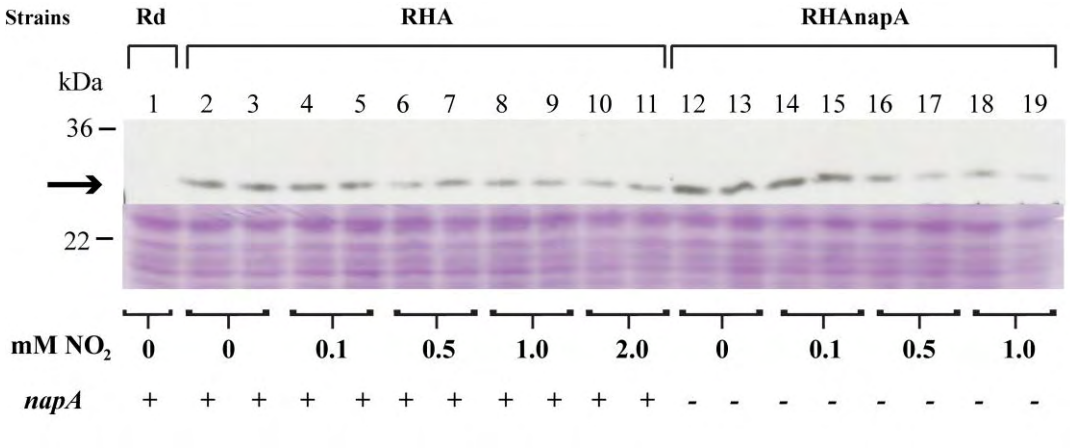


Figure 3.7: Effects of *napA* mutation and NO_2^- availability on *Pnrf-HA* expression.

Whole cell lysates of Rd (Lane 1), RHA (*nrfA*-, Lanes 2-11), and RHAnapA (*nrfA-/napA*, Lanes 12-19) cultured in low oxygen condition (7.5 ml in 8 ml sealed glass vial) with sBHI supplemented with varying concentrations of NO_2^- (0.1 mM lanes 4, 5, 14, 15; 0.5 mM lanes 6, 7, 16, 17; 1.0 mM lanes 8, 9, 18, 19; 2.0 mM lanes 10, 11) to equivalent optical density, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. The arrow indicates 29 kDa band, corresponding to the Pnrf-HA fusion protein.

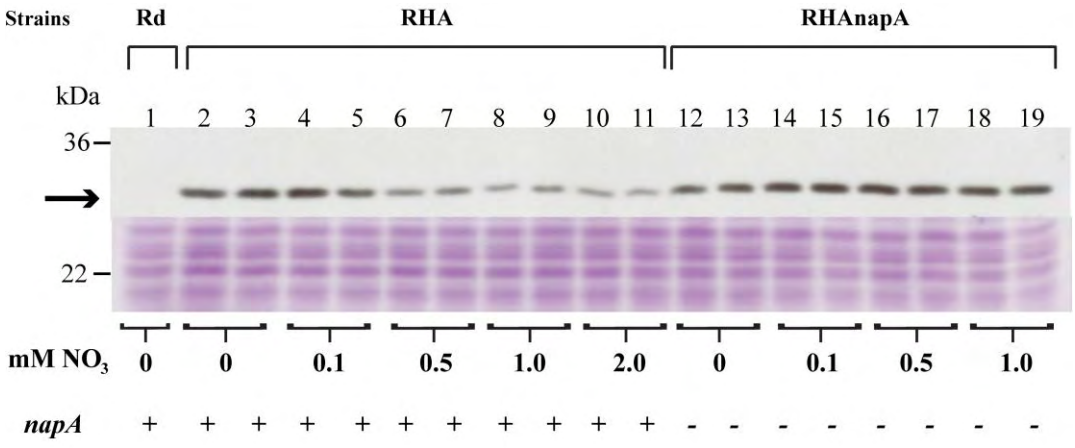


Figure 3.8: Effects of *napA* mutation and NO_3^- availability on *Pnrf-HA* expression.

Whole cell lysates of Rd (Lane 1), RHA (*nrfA*-, Lanes 2-11) and RHAnapA (*nrfA-/napA*-, Lanes 12-19) cultured in a low oxygen condition (7.5 ml in 8 ml sealed glass vial) with sBHI supplemented with varying concentrations of NO_3^- (0.1 mM lanes 4, 5, 14, 15; 0.5 mM lanes 6, 7, 16, 17; 1.0 mM lanes 8, 9, 18, 19; 2.0 mM lanes 10, 11) to equivalent optical densities, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. The arrow indicates 29 kDa band, corresponding to the Pnrf-HA fusion protein.

In lysates of RHA cultured in the presence NO_3^- greater than 0.5 mM, less Pnrf-HA was detected than in lysates of cultures without exogenous NO_3^-, which was analogous to the *Pnrf-HA* expression pattern observed in RHA with NO_2^- supplementation. In contrast, regardless of the concentrations of NO_3^- present, relative amounts of Pnrf-HA in lysates of RHAnapA were indistinguishable. Supernatants of the immunoblotted lysates were assayed for NO_2^- concentrations prior to collection of lysates (data not shown). In cultures of RHA, NO_2^- concentrations were equal to the initial amount of NO_3^- added, verifying that RHA had reduced NO_3^- to NO_2^-; whereas, NO_2^- levels were below the limit of detection in supernatants of RHAnapA, as this strain is unable to reduce NO_3^-. Nitrate reductase activity is likely to account for the differences seen with *Pnrf-HA* expression in RHA and RHAnapA with NO_3^- addition. Because repression of *Pnrf-HA* expression was observed in RHA cultures when NO_2^- is present in the media (from exogenous sources or reduction of NO_3^-) and no repression was observed in RHAnapA cultures supplemented with NO_3^-, it is likely that high concentrations of NO_2^- modulates expression of the *nrf* promoter under the conditions tested.

Repression of the *nrf* promoter by NO_2^- is dependent on NarP and NarQ.

The observations that the *nrf* promoter is regulated by NarP and is differentially expressed in response to altered NO_3^- and NO_2^- concentrations led to the investigation of the effects of the *narQ* and *narP* mutations on *Pnrf-hel* expression in the presence of the two anions. The band intensities were less in lysates of RHA cultured in the presence of 2 mM NO_2^- or 2 mM NO_3^- than in the lysates of RHA cultured in the absence of either anion (Fig 3.9, lanes 2-7). In comparison to RHA cultures, the levels of Pnrf-HA were

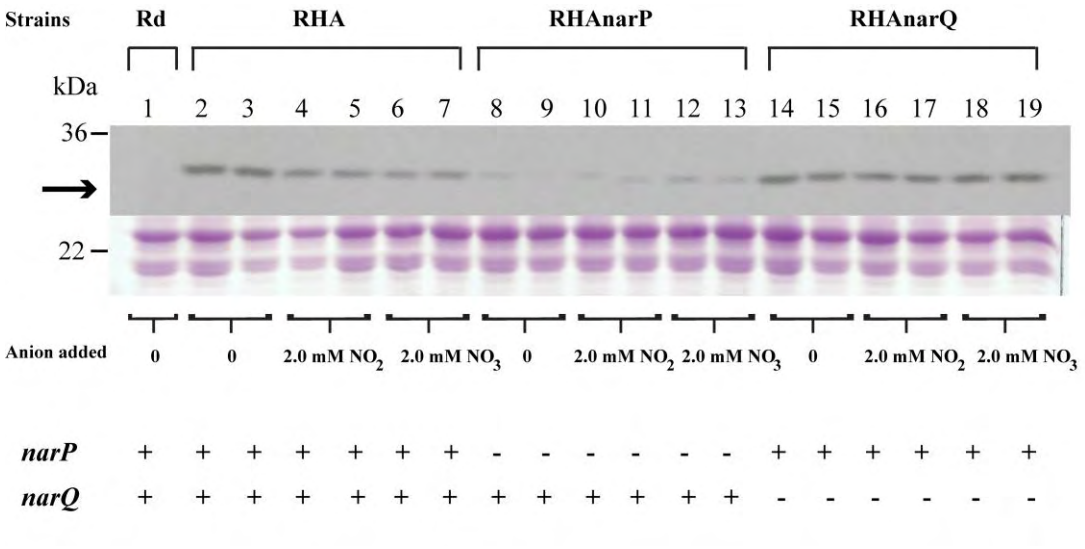


Figure 3.9: Effects of *narP* and *narQ* mutations on *Pnrf-HA* expression in the presence of exogenous NO_2^- and NO_3^-.

Whole cell lysates of Rd (Lane 1), RHA (*nrfA*-, Lanes 2-7), RHAnarP (*nrfA-/narP*-, Lanes 8-13) and RHAnarQ (*nrfA-/narQ*-, Lanes 14-19) cultured a low oxygen condition (7.5 ml in 8 ml sealed glass vial) with sBHI supplemented with 2 mM NO_2^- (Lanes 4, 5, 10, 11, 16, 17) or 2 mM NO_3^- (Lanes 6, 7, 12, 13, 18, 19) to equivalent optical densities, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. The arrow indicates 29 kDa band corresponding to the Pnrf-HA fusion protein.

less in lysates of all RHAnarP cultures, regardless of the presence of either anion (Fig. 3.9, lanes 8-13), which was previously demonstrated in Figure 3.4. Relative levels of Pnrf-HA from lysates of RHAnarP cultured with NO_2^- (Fig. 3.9, lanes 10 and 11) or NO_3^- (Fig. 3.9, lanes 12 and 13) were similar to levels of Pnrf-HA from lysates of RHAnarP cultured without either anion (Fig. 3.9, lanes 8 and 9), indicating that *Pnrf-HA* expression was not differentially expressed in response to environmental signals when NarP is not present. I additionally tested effects of the *narQ* mutation on *Pnrf-HA* expression to address the hypothesis that nitrite-responsive signaling occurs through NarQ. In contrast to the effects of the *narP* mutation, the Pnrf-HA band intensities in RHAnarQ cultures (Fig. 3.9, lanes 14 and 15) were comparable to those observed in RHA cultures with no anion added (Fig. 3.9, lanes 2 and 3), which indicates that the *nrf* operon does not require NarQ for expression, consistent with the results obtained with the NO_2^- reduction assays.. Furthermore, no differences were apparent in Pnrf-HA band intensities in lysates of RHAnarQ cultured with NO_2^- (Fig. 3.9, lanes 16 and 17) or NO_3^- (Fig. 3.9, lanes 18 and 19) which were comparable to RHAnarQ cultured without either anion (Fig. 3.9, lanes 14 and 15), suggesting that modulation of the *nrf* promoter expression in response to the NO_2^- signal is dependent on NarP and NarQ. Complementation of *narQ* verified that NarQ mediates repression of the *nrf* promoter in response to high concentrations of NO_2^- (Fig. 3.10). In the absence of NO_2^-, the band intensities of Pnrf-HA were comparable in lysates of the NarQ- strains, RHAnarQ and RHAnarQV (Fig. 3.10, lanes 6 and 7, lanes 10 and 11), and in lysates of the NarQ+ strains, RHA and RHAnarQC (Fig. 3.10, lanes 2 and 3, lanes 14 and 15). However, in the presence of 2 mM NO_2^-, *Pnrf-HA* expression

was significantly lower in cultures of RHA (Fig. 3.10, lanes 4 and 5) and RHAnarQC (Fig. 3.10, lanes 16 and 17), but not in cultures of RHAnarQ (Fig. 3.10, lane 9) and RHAnarQV (Fig. 3.10, lanes 12 and 13), indicating that NarQ does influence expression of the *nrf* operon in the presence of the regulatory signal, NO_2^-.

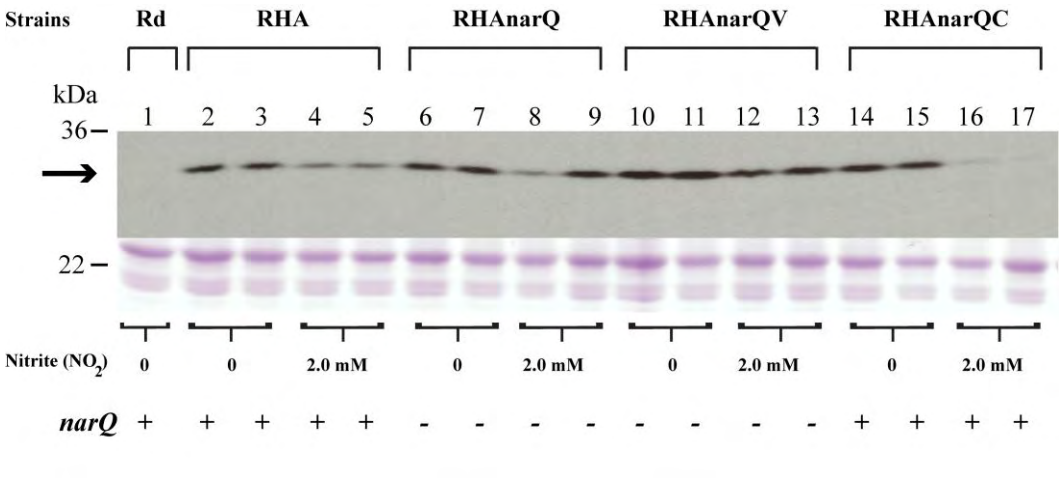


Figure 3.10: Effects of *narQ* mutation and 2 mM NO_2^- on *Pnrf-HA* expression.

Whole cell lysates of Rd (Lane 1), RHA (*nrfA*-, Lanes 2-5), RHAnarQ (*nrfA-/narQ*-, Lanes 6-9), RHAnarQV (*nrfA-/narQ*-, empty vector, Lanes 10-13) and RHAnarQC (*nrfA-/narQ*-, *narQ* complemented, Lanes 14-17) cultured in a low oxygen condition (filled 25 ml flask) with sBHI supplemented with 2 mM nitrite (Lanes 4, 5, 8, 9, 12, 13, 16, 17) to equivalent optical density, were resolved with SDS-PAGE and analyzed by αHA immunoblotting. The arrow indicates 29 kDa band, corresponding to the Pnrf-HA fusion protein.

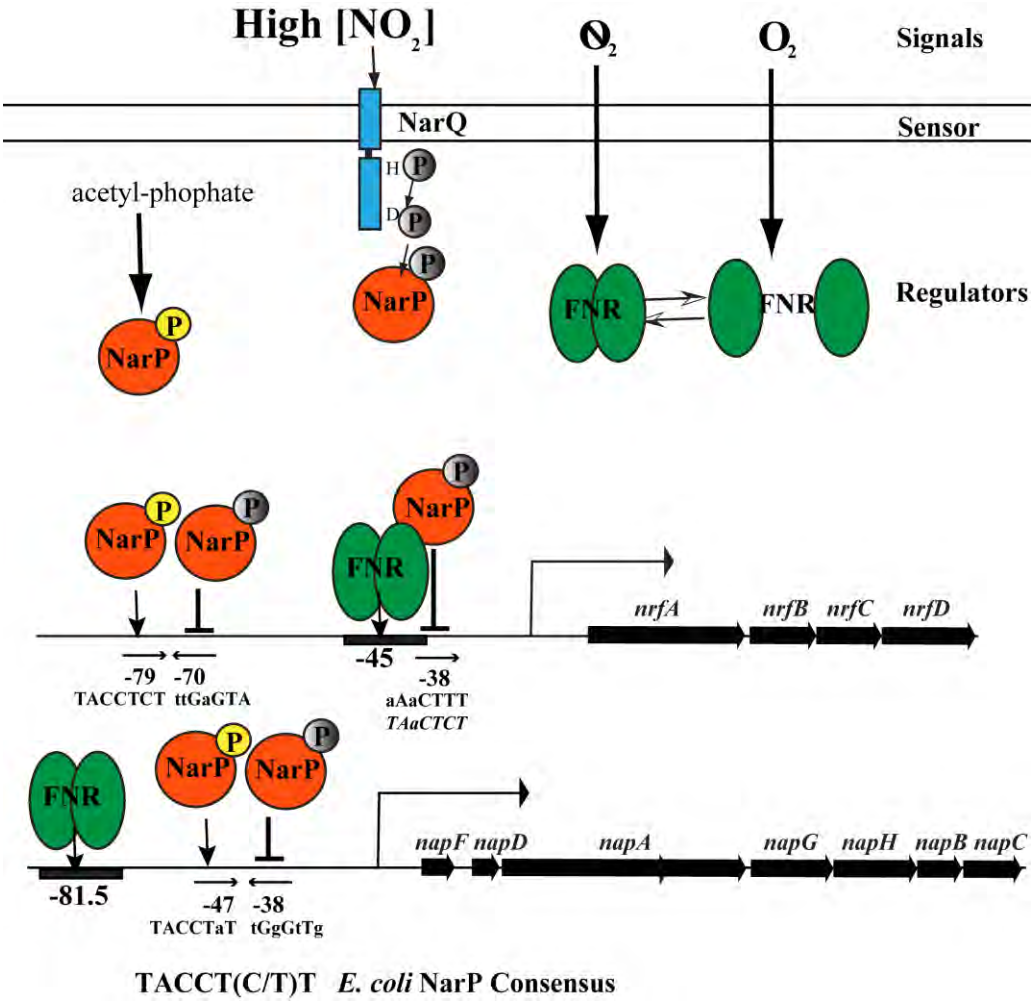


Figure 3.11: Model of NarQ-NarP regulation in *H. influenzae.*

When NO_2^- is absent from the media, acetyl-phosphate can phosphorylate low amounts of NarP (orange circle with yellow phosphate, which denotes phosphorylation from acetyl-phosphate), resulting in induction of gene expression by phospho-NarP binding to sites with high sequence matches to the consensus sequence. Transmembrane kinase sensors, NarQ (blue square) can detect environmental NO_2^- and phosphorylate cognate regulator, NarP (orange circle with grey phosphate, which denotes phosphorylation from NarQ). High amounts of phospho-NarP can recognize heptamer sites in the *nrf* and *nap* promoter with fewer matches to consensus sequence, leading to repression. Putative NarP binding sites are listed below the directional arrows (bp relative to the predicted transcriptional start site) and residues shared with the *E. coli* NarP consensus sequence are denoted with capital letters. The sequences of the -38 binding site of the *nrf* promoter is compared to the -79 NarP binding site (in italics) from the *nrf* promoter of *E. coli.*

Discussion

The results of these studies are the first demonstration of NarQ-NarP regulation and NO_2^- signaling in *H. influenzae*, which appears to be different than NarQ-NarP regulation in *E. coli*. Characterization of NO_3^-/NO_2^- signaling in *E. coli* is complicated by redundancy of three nitrate reductases, two nitrite reductases, two nitrate/nitrite-responsive sensors and regulators (Stewart, 1994b). The relative simplicity of the RNS metabolism in *H. influenzae* makes it a desirable organism to study NO_3^- and NO_2^- signaling and metabolism, given that only one nitrite reductase, nitrate reductase, nitrate/nitrite sensor and regulator are present in *H. influenzae*. Based on the results with nitrite assays, RT-qPCR and immunoblotting, NarP contributes to induction of *nrfA* expression and increased nitrite reductase activity, which is consistent with previous reports of NarP regulation of *nrfA* in *E. coli* (Browning *et al.*, 2002; Wang and Gunsalus, 2000). If the activity of NarP was reliant on signaling by NarQ, a *narQ* mutant would likely have the same phenotype as a *narP* mutant, resulting in delayed reduction of NO_2^-. To the contrary, the *narQ* mutant strain was not impaired for nitrite reductase activity. In the absence of a cognate sensor, a response regulator can be phosphorylated non-specifically by acetyl-phosphate (reviewed in (Wolfe, 2005)), which might be a possible mechanism of NarP activation, independent of NarQ. The observation that the *narQ* mutant strain reduced NO_3^- more rapidly than the wild type strain implicated that NarQ does play a role in regulation of genes involved in RNS metabolism, through a mechanism different than that reported in *E. coli*.

Examination of regulation of the *nrf* promoter using the *Pnrf-hel* reporter strain cultured with different anion concentrations further supported the hypothesis of NarQ-NarP signaling. Comparisons of Pnrf-HA expression after supplementation with increasing anion concentrations indicate that NO_2^- can be an inhibitory signal leading to repression of the *nrf* promoter and that NO_2^- repression is dependent on NarQ. Because NarQ-dependent repression of the *nrf* promoter requires high concentrations of NO_2^-, the *narQ* mutation would have no effect on *nrf* expression in cultures used in the NO_2^- assays, which were pre-incubated in MIc. To determine if NarQ can inhibit nitrite reductase activity, the assay must be repeated with sBHI supplemented with NO_2^-.

Using the information on NarP-NarQ and NarX-NarL signaling mechanisms in *E. coli*, a model of NarP-NarQ regulation in *H. influenzae* can be postulated (summarized in Fig. 3.11). A possible mechanism of nitrite-response regulation is that NarQ-NarP signaling is a "toggle" switch for activation and repression of the *napF* and *nrfA* promoters. The *napF* promoter of *H. influenzae* has been mapped and two divergent NarP sites, centered around basepairs -47 and -38 relative to the transcription start site, downstream of the FNR binding site (Stewart and Bledsoe, 2005). The relative affinities of each site can be speculated based on conserved residues when compared to the *E. coli* NarP consensus sequence, TACC(C/T)CT (Darwin *et al.*, 1997). The NarP site at -47 shares 6 of the 7 basepairs; whereas, the divergent site at -38 only has 4 of the 7 conserved basepairs**.** In *H. influenzae*, NarP may not require NarQ phosphorylation and is phosphorylated by acetyl-phosphate at basal levels when NarQ is absent or inactive. When low concentrations of phosphor-NarP are present in the cell, NarP only binds to

highly conserved sites with lower affinity to induce expression of the *nap* promoter and NarP positively regulates nitrate reductase activity. When NarQ is present, it can phosphorylate more NarP. When concentrations of phospho-NarP are increased in the cell, NarP can bind to sites with sequences that have fewer sites shared with the binding consensus, leading to repression of the *nap* operon, which would explain why NO_3^- metabolism is accelerated in the *narQ* mutants.

Even though the *nrf* promoter has not been mapped, the results presented in this thesis provide more information regarding NarP and NarQ regulation. Three putative NarP binding sites were identified in the *nrf* promoter region based on the NarP consensus sequences and a comparison of the *nrf* promoter of *E. coli* (Darwin *et al.*, 1997). Two divergent heptameric sites are located at -79 and -70, relative to the predicted transcriptional start site, which is the same positioning of the NarP binding sites in the *nrf* promoter of *E. coli.* The -79 site is an exact match to the NarP consensus sequence and the -70 site has 5 out of 7 matching basepairs. When the -79 and -70 NarP binding sites in the *nrf* promoter of *E. coli* were compared to sequences in the *H. influenzae* promoter, a third putative NarP site was identified, centered at basepair -38, which overlaps the FNR binding site. The -38 binding site has 5 matches to the consensus sequence and it highly resembles the -79 NarP site *E. coli*. Full induction of the *nrf* promoter requires NarP, deduced from transcript analysis, NO_2^- assays and Pnrf-HA expression, which might be mediated by NarP binding to the sequence that shares the greatest match to the consensus sequence when NarP is phosphorylated at low levels. Repression of the *nrf* promoter appears to be dependent on NarQ and high concentrations

of NO_2^-, implying that NarQ senses NO_2^- and possibly phosphorylates more NarP, thus higher concentrations of phosphor-NarP present in cell leads to bind to lower affinity sites with fewer sequence matches with the consensus sequence. In *E. coli*, repression of the *nrf* promoter occurs at high concentrations of NO_3^- and NO_2^- while the *nirB* promoter is induced (Wang and Gunsalus, 2000) so the cell can metabolize greater amounts of NO_2^- by the cytoplasmic nitrite reductase, NirB. Because *H. influenzae* does not possess a homologue of NirB, it is unclear why NarQ would repress the *nrf* promoter under conditions with high amounts of NO_2^-. In *E. coli*, there is suggestive evidence that nitric oxide, which is toxic to bacteria, is a product of nitrite reduction by NrfA (Corker and Poole, 2003). If NrfA of *H. influenzae* also produces nitric oxide, repression of the *nrf* promoter when high concentrations of NO_2^- were encountered in the environment would likely protect the cell from the toxic effects of endogenously made nitric oxide. The demonstration that NarP and NarQ influence regulation of the *nap* and *nrf* operons likely implicates that *H. influenzae* senses environmental signals like NO_2^- to alter gene expression; however further characterization of the *nrf* and *nap* promoters is required to develop this model. Additionally, the mechanism of NarP activation in the absence of NarQ remains undefined and warrants more extensive investigation of the phophorylation of the predicted amino acid residues of NarP.

CHAPTER IV

FNR and YtfE are required for resistance to nitrosative stress via anaerobic induction of *ytfE* by FNR.

Summary

During infection, *H. influenzae* likely encounters macrophages, which generate oxidative and nitrosative stress by production of ROS and RNS when activated by inflammatory cytokines. FNR has been identified as a regulator of genes involved in RNS defense in other pathogenic bacteria but its potential role in regulation of RNS resistance mechanisms in *H. influenzae* has not been previously characterized. The results presented in this thesis show that the *fnr* mutant is significantly more sensitive than wild type to nitrosative stress when exposed to ASN or GSNO *in vitro*, suggesting that regulation is important for RNS resistance. The *narP* mutant was additionally tested for sensitivity to *in vitro* NO donors and displayed a hypersensitive phenotype, but more resistant to challenge with the NO donors than the *fnr* mutant. After evaluation of the list of predicted FNR regulated genes, *nrfA* and *ytfE* were identified as a likely targets as they has been previously identified as RNS defense mechanisms. Enumeration of viable colonies recovered after challenge with ASN and GSNO verified that *nrfA* and *ytfE* is required for resistance to *in vitro* nitrosative stress from NO donors. Comparison of survival rates of the *fnr* and *ytfE* mutants to the wild type strain cultured with activated macrophages led to the conclusion that FNR and YtfE are important for resistance to *in vivo* host-derived nitrosative stress.

CHAPTER IV

Introduction

The results presented in this thesis demonstrate that FNR and NarP both exert regulation of *nrfA* in response to environmental signals. Regulation of nitrite reduction is important for anaerobic respiration; this regulation has also been shown to be involved in protection against nitrosative stress in other pathogenic bacteria. Another consideration when exploring RNS resistance in *H. influenzae* is the different microenvironments encountered during infection as it is likely that RNS concentrations are varied in the upper respiratory tract, lung, blood and brain. Microarray studies in other species indicate that genes important for defense against reactive nitrogen species (RNS) are differentially regulated in response to changes in oxygen, nitrate and nitrite (Constantinidou *et al.*, 2006; Overton *et al.*, 2006a). It has been reported that under low oxygen conditions, FNR of *E. coli* upregulates RNS defense mechanisms, *nrfA* and *hcp*, yet represses other genes *hmp* and *ytfE* that confer resistance to nitrosative stress (Constantinidou *et al.*, 2006). Exposure to NO oxidizes the Fe-S cluster in FNR, inactivating the regulator thus leading to upregulation of *hmp*. Many RNS defense genes are induced by addition of nitrate and nitrite including *hmp, ytfE, hcp, nrfA* and *norV* (Constantinidou *et al.*, 2006), which implicates control by nitrate/nitrite responsive regulators NarP or NarL. NarL regulation of one NO defense mechanism, hybrid cluster protein of *E. coli*, has been verified by gel retardation assay with NarL binding to the *hcp* promoter (Filenko *et al.*, 2007). NarL is not present in *H. influenzae;* however, a predicted homolog of NarP has been identified in the *H. influenzae* genome and likely

regulates *nrfA,* which has been previously been identified as an NO detoxification mechanism in other bacteria (Mills *et al.*, 2008; Poock *et al.*, 2002). Therefore, the role of NarP in RNS resistance was additionally investigated.

The conserved biological role of NrfA in RNS defense is unclear as results are conflicting in different organisms. One study reports that *nrfA* mutant of *E. coli* is hypersensitive to NO gas and nitric oxide donor, SNAP (Poock *et al.*, 2002); whereas, the *nrfA* mutant of *S. enterica* displayed the same growth rates as wild type when exposed to NO unless *norV* was additionally mutated (Mills *et al.*, 2008). However, a *nrfA* mutant of *S. enterica* was slightly attenuated for in vivo infection in the mouse model (Bang *et al.*, 2006). NrfA is proposed to contribute to RNS resistance in *E. coli* by detoxification of NO as the purified enzyme is able to reduce NO in vitro (Clarke *et al.*, 2008; van Wonderen *et al.*, 2008). Conversely, another study reports that wild type *E. coli* produces NO as a product of nitrite reduction by NrfA and the *nrfA* mutant failed to produce NO after nitrite addition (Corker and Poole, 2003). In *S. enterica*, a *nrfA* mutant produces as much NO as wild type grown in media supplemented with nitrite (Gilberthorpe and Poole, 2008), but the *nrfA* mutant displayed impaired NO consumption as compared to wild type (Mills *et al.*, 2008). The significance of *nrfA* in NO resistance is likely dependent on conditions and coordination with other NO defense mechanisms.

When screening for RNS defense mechanisms in *E. coli* with microarray analysis, *ytfE* was highly up-regulated after nitric oxide exposure and the *ytfE* mutant strain displayed a growth defect when cultured anaerobically in the presence of NO gas (Justino *et al.*, 2005). The *E. coli ytfE* mutant is also defective for growth when cultured

anaerobically, but not aerobically, in MIc supplemented with nitrate, nitrite, DMSO or fumarate as terminal electron acceptors, which correlates with the *ytfE* mutant impaired reductase activity for these anions (Justino *et al.*, 2007). Nitrate, nitrite, DMSO and fumarate reductases contain Fe-S clusters, which led to the hypothesis that YtfE is involved in maintenance of Fe-S formation. A comparison of wild type *E. coli* and the *ytfE* mutant with over-expressed fumarase A and aconitase B, both enzymes that contain Fe-S clusters, showed that after NO stress, the *ytfE* mutant had impaired enzyme activity and an altered electron paramagnetic resonance (EPR) signal (Justino *et al.*, 2007). Proteins with reduced Fe-S clusters $[4Fe\text{-}4S]^{2+}$ do not produce an EPR signal; however, proteins oxidized Fe-S clusters $[3Fe\text{-}3S]^{1+}$ generate an EPR signal of g~2.02. After exposure to oxidative stress, cells with overexpressed fumarase A and aconitase B yielded an g~2.02 EPR signal, which disappeared after 30 minutes in the wild type cell, but not in the *ytfE* mutant cell, indicating that the *ytfE* mutant failed to repair the Fe-S clusters (Justino *et al.*, 2007). Enzyme activity of fumarase A and aconitase B were restored in the *ytfE* mutant when cultures were supplemented with purified YtfE (Justino *et al.*, 2007). Characterization of the YtfE protein of *E. coli* identified a non-heme di-iron center that is sensitive to oxidation by oxygen or nitric oxide, implicating a mechanism of Fe-S cluster repair (Todorovic *et al.*, 2008). Studies conducted with *ytfE* homologs (*ytfE* of *S. enterica*, *dnrN* of *N. gonorrhoeae*, *scdA* of *S. aureus*) suggest that its role in defense against nitrosative stress is conserved (Gilberthorpe *et al.*, 2007; Overton *et al.*, 2008). When cells are exposed to NO anaerobically, *ytfE* is one of the most highly expressed genes in *E. coli* (Pullan *et al.*, 2007). Similar to *hmp*, *ytfE* is predicted to be

repressed by FNR as increased transcript levels are observed in the *fnr* mutant (Constantinidou *et al.*, 2006; Justino *et al.*, 2006; Overton *et al.*, 2006a). Microarrays comparing wild type and *fnr* mutant of *N. gonorrhoeae* showed that more *dnrN* (*ytfE* homolog) transcript was observed in *fnr* mutant (Whitehead *et al.*, 2007). In addition to FNR regulation, a NarL binding site is predicted in the *ytfE* promoter, indicating that expression of this gene is modulated by nitrate/nitrite responsive regulation (Bodenmiller and Spiro, 2006). The specific growth conditions also dramatically impact the regulation of the RNS resistance mechanisms. Using microarray analysis, cultures exposed to NO donor, GSNO, displayed an altered global transcription profile than cultures exposed to another NO donor, NOC-5 (Flatley *et al.*, 2005; Pullan *et al.*, 2007). Because *H. influenzae* does not have many of the regulators (NsrR, NorR) and enzymes (Hmp, HCP, NorV) that contribute to RNS resistance in *E. coli*, *H. influenzae* likely employs alternative patterns of regulation to protect itself from host-derived nitrosative stress**.**

CHAPTER IV

Materials and Methods

Strain construction: Strains RV, RytfeV, RytfeC, NTV, NytfeV and NytfeC and plasmids pXTPytfeC, pXTytfeC were constructed as described previously (Harrington, 2009).

NO-donor sensitivity assays: Anaerobic cultures were used to inoculate 5 mL sBHI with an initial density of OD_{600} 0.02, and were subsequently incubated at 35°C shaking at 120 rpm (ThermoForma Orbital Shaker) in an anaerobic chamber (BD Anaerobic GasPak EZ) for 5.5 hours to mid-log growth (OD_{600} 0.4-0.5). To determine sensitivity to nitric oxide donors, $5x10^5$ cells in 50 μl sBHI were transferred to a 96-well dish containing 130 μl sBHI/pH6.5 and 20 μl PBS +/- 125 mM $NaNO_2$ or 130 μl sBHI/pH7.5 and 20 μl PBS +/- 50 mM GSNO (S-nitrosoglutathione, Sigma). Cultures were sealed in anaerobic BD GasPak EZ & grown for 14 hours at 35°C. Overnight cultures were diluted into sBHI pH7.5 and plated for viable bacterial counts. For transient exposure to ASN, anaerobic cultures were standardized to a culture density of 0.01 OD_{600} in sBHI pH5.5 and 180 μl of each culture in triplicate was transferred to 96-well dishes containing 150 mM $NaNO_2$ in 20 μl PBS. The 96-well dishes were either sealed in individual anaerobic BD GasPak EZ or exposed to ambient air and incubated at 35°C for 30, 60, 90 or 120 minutes. Cultures were diluted into sBHI pH7.5 and plated for viable bacterial counts.

Macrophage bactericidal assay: Bone-marrow derived macrophages (BMM) were generated by differentiating C57BL/6J bone marrow cells in a complete BM medium (DMEM, 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and

10% L929 fibroblast-conditioned medium as a source of M-CSF) for 6 days in 10-cm Petri dishes. Prior to exposure to bacteria, BMM were washed in antibiotic free BM medium and incubated with or without 100 U/mL IFN-γ for 24 hours, 100 μM N_{ω}9 - nitro-L-arginine methyl ester HCL (L-NAME) for 24 hours, or 100 ng/ml LPS for 2 hours. Anaerobic bacteria cultures were used to inoculate 5 mL sBHI with an initial density of OD_{600} 0.02, and were subsequently incubated at 35°C shaking at 120 rpm (ThermoForma Orbital Shaker) in anaerobic chamber (BD Anaerobic GasPak EZ) for 5.5 hours to mid-log growth (OD_{600} 0.4-0.5). Cultures were serially diluted to $2.5x10^5$ cells in 50 ul DMEM and added to wells containing adherent $5x10^4$ bone-marrow derived macrophages (BMM) from C57BL/6J mice. The 96 well dish was centrifuged for 5 minutes at 200 x g, 4°C then sealed in a BD BBL Anaerobic GasPak and incubated for 30 minutes at 36°C, 5% CO_2. 250uL of 0.12% saponin in DMEM was added to the wells and vigorously pipetted to lyse macrophages & release bacteria. Dilutions of the samples were plated on sBHI plates & grown overnight at 35° to determine bacterial survival.

CHAPTER IV

Results

FNR confers resistance to *in vitro* nitric oxide donors. To investigate the potential role of FNR in RNS resistance in *H. influenzae*, the sensitivity of the *fnr* mutant was evaluated to two NO donors that are present in the human host, S-nitrosoglutathione (GSNO) and nitrite (Lu *et al.*, 2002; Rassaf *et al.*, 2004). GSNO, acts by slow release of NO causing bacterial damage or indirect damage by nitric oxide through transnitrosation, a covalent transfer of a NO group to free thiol groups of bacterial proteins (Singh *et al.*, 1996). When conditions are acidic, nitrite becomes a nitric oxide donor, commonly called ASN acidified sodium nitrite (Dejam *et al.*, 2003). Strains Rd, RfnrV and RfnrC were compared for survival after challenge with GSNO in an anaerobic condition for 14 hours (Fig. 4.1A). Recovery of CFU for the *fnr* mutant strain, RfnrV, was decreased by 600-fold relative to that of parental strain, Rd, and complementation with *fnr* in strain, RfnrC, restored resistance to that of Rd. Treatment of cultures with GSNO in the presence of oxygen resulted in no measurable growth inhibition of any of the strains (data not shown), consistent with the instability of nitric oxide in the presence of oxygen (Singh *et al.*, 1996). Exposure to ASN for 14 hours yielded results comparable to those obtained with GSNO (Fig. 4.1B). The *fnr* mutant, RfnrV, exhibited a 40-fold decrease in recovered CFU compared to that of Rd, and complementation with the wild-type *fnr* in strain RfnrC fully restored resistance to parental levels.

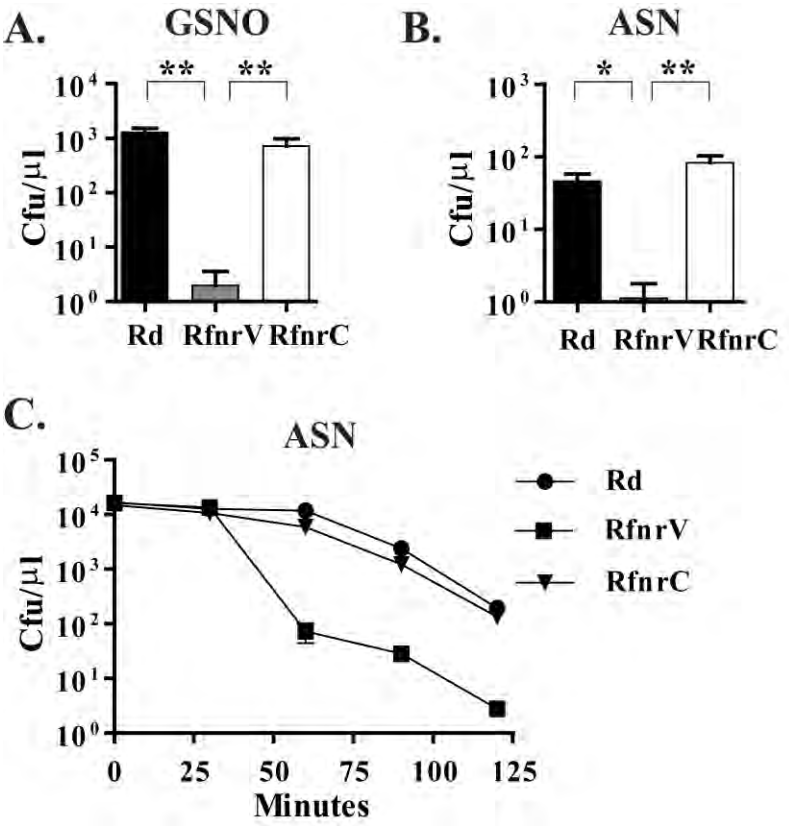


Figure 4.1: Effects of *fnr* mutation on susceptibility of *H. influenzae* to nitric oxide donors, GSNO and ASN.

A. and B. 5.0 x 10^4 cells of Rd, RfnrV and RfnrC, cultured in triplicate in a depleted oxygen condition, were treated with 5 mM GSNO in sBHI, pH7.5 (A) or 10 mM $NaNO_2$ in sBHI, pH6.5 (B) for 14 hours. Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test (*p<0.01 or **p<0.001).

C**.** 5 x 10^5 cells of Rd, RfnrV and RfnrC, cultured in triplicate in a depleted oxygen condition, were treated with 15 mM $NaNO_2$ in sBHI, pH5.5, sealed in BD GasPak™ EZ Anaerobic Chamber and viable colony counts were enumerated at indicated time points.

To accelerate the rate of NO production from ASN, the pH of the growth medium was lowered to 5.5, allowing assessment of decreased in CFU counts over a relatively short period of time (Samouilov *et al.*, 1998). Cultures of strains Rd, RfnrV, and RfnrC were incubated in acidified medium under oxygen-depleted conditions in the presence or absence of ASN and monitored for recoverable CFU for 120 minutes (Fig. 4.1C). Cultures incubated in the absence of ASN exhibited no growth and no loss of viability over the course of the assay (data not shown), suggesting that decreases in recoverable CFU in the assay reflect loss of viability, and that strain differences are not the result of pH sensitivity. The *fnr* mutant exhibited a dramatic decrease in CFU by 60 min, dropping by 161 or 81-fold relative to that of the parental or complemented strains (Fig. 4.1C), which were only marginally affected at this time (1.4 or 2.5 fold less than starting CFU). By 90 minutes, the parental and complemented strains began to exhibit sensitivity, whereas RfnrV remained 69 and 28-fold more sensitive than these strains. Similar to GSNO exposure, cultures exposed to ASN in an oxygen rich condition exhibited no appreciable decreases in CFU, consistent with the rapid oxidation of NO to nitrite expected to occur under this condition (Wink *et al.*, 1993). Together, these results indicate that FNR is required to promote resistance of *H. influenzae* to RNS under low oxygen conditions.

NarP contributes to resistance to challenge by *in vitro* nitrosative stress. Previous data obtained with nitrite assays and Pnrf-HA expression indicates that at least one gene in *H. influenzae* is dually regulated by NarP and FNR. Because NarP has been reported to regulate RNS defense mechanisms in other organisms and these genes are highly

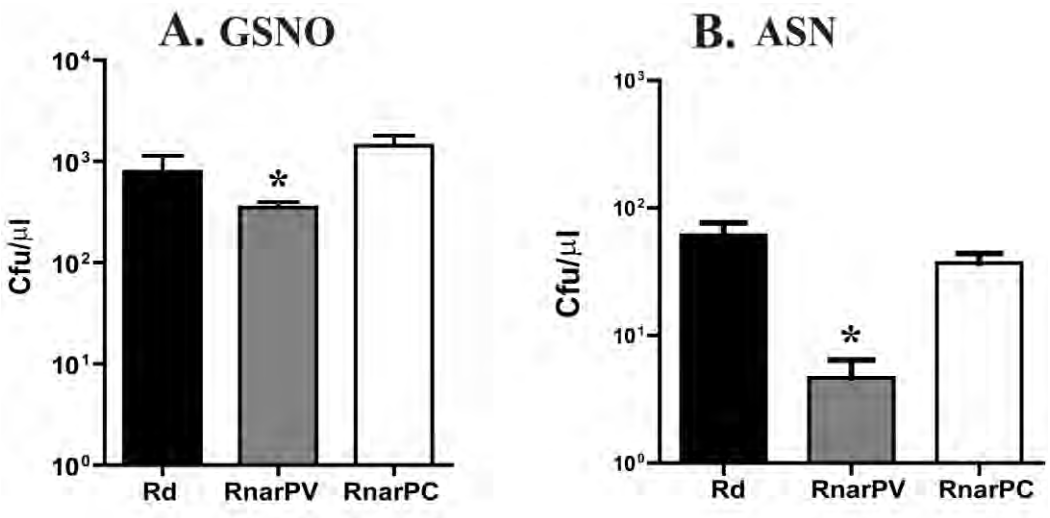


Figure 4.2: Effects of *narP* mutation on susceptibility of *H. influenzae* to nitric oxide donors, GSNO and ASN.

Rd, RnarPV (*narP*- plus empty vector) and RnarPC (*narP* complemented) were cultured in triplicate in a depleted-oxygen condition. A. 5.0 x 10^4 cells from each culture were treated with 5 mM GSNO for 14 hours. B. 5.0 x 10^5 cells were treated with 15 mM $NaNO_2$ in sBHI, pH5.5, sealed in BD GasPak™ EZ Anaerobic Chamber and enumerated for viability at indicated time points. Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test (*p<0.01).

up-regulated when exposed to nitrate or nitrite, Rd, RnarPV (*narP*-) and RnarPC (*narP* complemented) were challenged with nitric oxide donors, ASN or GSNO, then assayed for survival (Fig. 4.2A and 4.2B). The previous conditions used for the assay with RfnrV were repeated to test for the effects of the *narP* mutation on nitrosative stress. RnarPV was only slightly more sensitive to GSNO as compared to Rd or RnarPC by 3.7-fold or 4-fold, respectively (Fig. 4.2A). The differences between NarP+ and NarP- strains are statistically significant but the *narP* mutant did not display as dramatic a phenotype as RfnrV, which had 600-fold fewer colonies recovered than Rd. Similar results were obtained when strains were cultured for 14 hours with ASN; there were 13.0-fold and 7.9-fold fewer viable cells of RnarPV in comparison to Rd and RnarPC (Fig. 4.2B).

NrfA plays a role for RNS resistance *in vitro*. Purified NrfA of *E. coli* can reduce NO (van Wonderen *et al.*, 2008) and a *nrfA* mutant of *E. coli* has a growth defect when cultured in the presence of NO (Poock *et al.*, 2002), suggesting that *nrfA* can act as an RNS defense mechanism. FNR is required for *nrfA* expression and nitrite reductase expression so I hypothesized that *nrfA* is an FNR target that accounts for the hypersensitivity of the *fnr* mutant to nitrosative stress. The *nrfA* mutant, RHA, was compared to wild-type strain, Rd, for resistance to NO generated by ASN or GSNO (Fig. 4.3). After exposure to GSNO or ASN, recovery of CFU for the *nrfA* mutant strain was decreased relative to wild-type by 5.8-fold and 4.2-fold, respectively, which was only a moderate difference, in contrast to the dramatic phenotype of the *fnr* mutant. Although *nrfA* potentially contributes to the FNR-mediated resistance to RNS, it likely is not the only defense mechanism responsible.

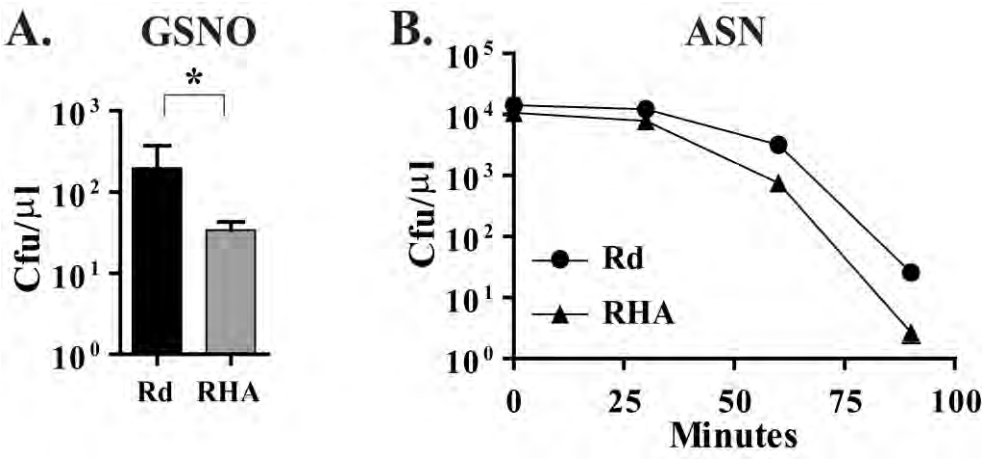


Figure 4.3: Effects of *nrfA* mutation on susceptibility of *H. influenzae* to nitric oxide donors, GSNO and ASN.

Rd and RHA (*nrfA*-) were cultured in triplicate in a depleted-oxygen condition. A. 5.0 x 10^4 cells from each culture were treated with 5 mM GSNO for 14 hours. B. 5.0 x 10^5 cells were treated with 15 mM $NaNO_2$ in sBHI, pH5.5, sealed in BD GasPak^{TM} EZ Anaerobic Chamber and enumerated for viability at indicated time points. Statistics were calculated using *t* test (*p<0.05).

FNR and NarP are positive regulators of *ytfE* under low oxygen conditions. The *ytfE* of *E. coli* encodes a di-iron protein that repairs nitrosative damage (Justino *et al.*, 2006). HI1677, a putative homolog of *ytfE* in *H. influenzae*, encodes a predicted protein with 57% amino acid identity to that of the *E. coli* YtfE. The presence of a potential FNR binding site in the promoter region of *ytfE* in *H. influenzae* suggested that it could play a role in FNR-mediated RNS resistance. Transcript levels of *ytfE* were examined in Rd, RfnrV and RfnrC after anaerobic growth to determine if FNR regulates *ytfE* (Fig. 4.4A). In contrast to the *E. coli ytfE*, which is negatively regulated by FNR (Justino *et al.*, 2006), *H. influenzae ytfE* mRNA levels decreased by 8.8 fold in the *fnr* mutant relative to wild-type, indicating that FNR is required for positive control of *ytfE* under low oxygen conditions in this species. Putative NarP binding sites are present in the *ytfE* promoter regions, so *ytfE* transcripts were quantified in RnarPV and were slightly reduced in the *narP* mutant (Fig. 4.4B).

YtfE is required for resistance to in vitro nitric oxide donors, ASN and GSNO. The FNR dependent transcription of *ytfE* that was detected in *H. influenzae*, together with previously reported roles in defense against nitric oxide of *ytfE* in *E. coli* (Justino *et al.*, 2005), suggested that this gene is likely to be involved in FNR-mediated resistance to RNS in *H. influenzae*. The *ytfE* deletion mutant, RytfeV, exhibited a 25-fold decrease relative to parental strain RV after exposure to GSNO, and complementation restored its resistance to parental levels (Fig. 4.5A). Similarly, after 60 minutes of exposure to ASN,

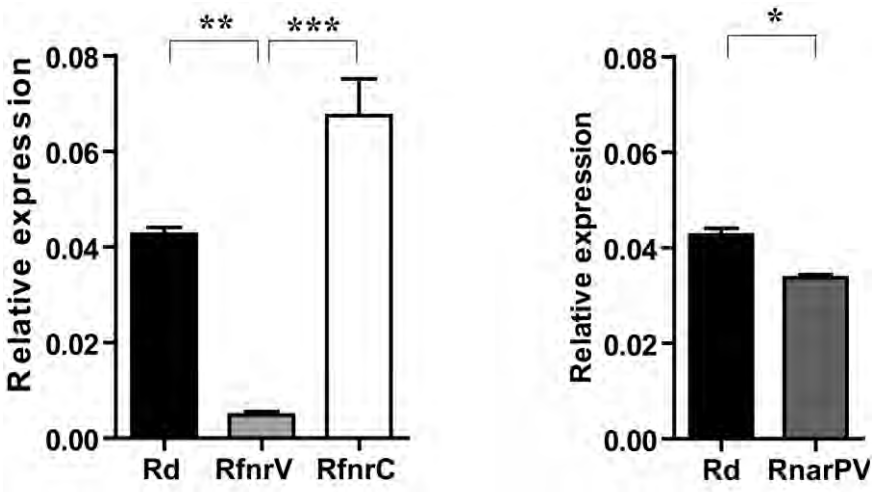


Figure 4.4: Effect of *fnr* and *narP* mutations on *ytfE* transcript levels.

Total RNA was extracted from Rd, RfnrV (*fnr*-, empty vector), RfnrC (*fnr* complemented) and RnarPV (*narP*-, empty vector) cultured in triplicate (or duplicate for RnarPV) to log phase in an oxygen-depleted condition and expression of *ytfE* was examined with qRT-PCR. All transcripts were normalized to *rpoA* expression. Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test (**p<0.01, ***p<0.001) or t-test (*p<0.02).

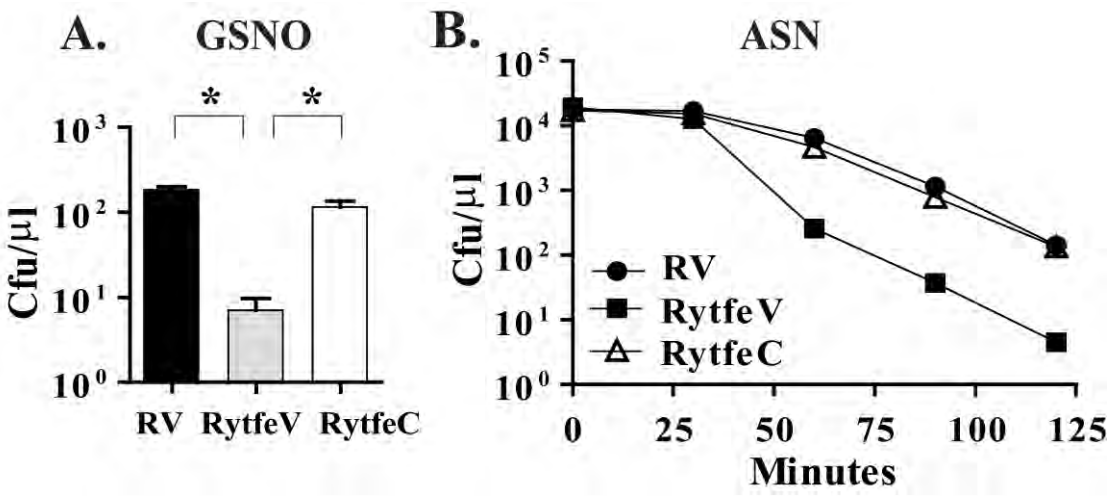


Figure 4.5: Effects of *ytfE* mutation on susceptibility of *H. influenzae* to nitric oxide donors, GSNO and ASN.

Parental strain (RV), RytfeV (*ytfE-*, empty vector) and RytfeC (*ytfE* complemented) were cultured in triplicate in a depleted-oxygen condition. A. 5.0 x 10^4 cells from each culture were treated with 5 mM GSNO for 14 hours. B. 5.0 x 10^5 cells were treated with 15 mM $NaNO_2$ in sBHI, pH5.5, sealed in BD GasPak™ EZ Anaerobic Chamber and monitored for viability at indicated time points. Statistics were calculated using one-way ANOVA with Tukey's multiple comparison test (*p<0.001).

25-fold fewer CFU were recovered from cultures of RytfeV relative to the wild type strain, RV, and complementation restored resistance (Fig. 4.5). Under low oxygen conditions, FNR is required for *ytfE* expression and the *ytfE* deletion mutant exhibits hypersensitivity to RNS exposure. The sensitivity to nitric oxide donors of the *nrfA* mutant RHA was less striking than that of RytfeV, indicating that although *nrfA* may contribute to RNS resistance, the requirement for *ytfE* is greater under these conditions.

FNR and YtfE are required for resistance to killing by activated macrophages.

These results indicate that *fnr* and *ytfE* are important for in vitro nitric oxide (NO) resistance during conditions when oxygen is limiting. Previous reports suggest that *H. influenzae* may encounter low oxygen conditions during infection where macrophages and other immune effector cells are sources of NO. To examine if FNR is important for defense against RNS produced by macrophages, strains were exposed to bone marrow-derived macrophages (BMM) from C57BL/6J mice after growth in low oxygen conditions where FNR is most active. The wild type strain, RV, and the *fnr* mutant, RfnrV, exhibited 70% survival when incubated with BMMs for 30 minutes, however no significant difference in survival was observed between these two strains (Fig. 4.6). Exposure to BMMs pre-stimulated with IFNγ or LPS, both previously shown to increase NO production in BMMs (Lorsbach and Russell, 1992), resulted in significant decrease in survival for both strains. The percent survival for RfnrV was 1.3 and 1.4 fold lower than RV when exposed to IFNγ or LPS stimulated macrophages. Previous data suggests

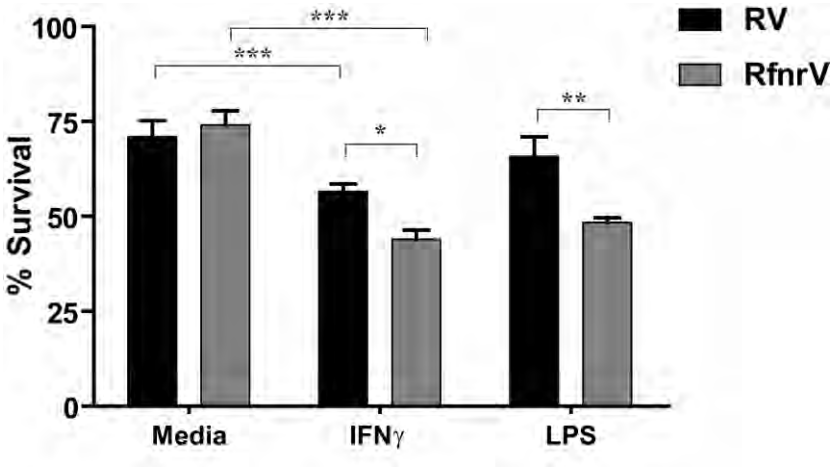


Figure 4.6: Effect of *fnr* mutation on survival by co-infection with activated macrophages.

A. Wild type RV and RfnrV were cultured to mid-log growth in oxygen-depleted condition, then added at an MOI of 5:1 to bone-marrow derived macrophages from C57BL/6J mice pretreated with media alone, 100 U/ml IFNγ for 24 hrs or 100 ng/ml of purified LPS for 2 hrs. Plates were incubated with rocking at 37°C for 30 min. in an anaerobic chamber. Macrophages were lysed with 0.1% saponin, a concentration that does not influence bacterial viability, and surviving bacteria enumerated by plating dilutions for CFU. Percent survival represents the ratio of CFU recovered in the presence of macrophages versus incubation in medium alone. Statistics were calculated from ANOVA with Tukey's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

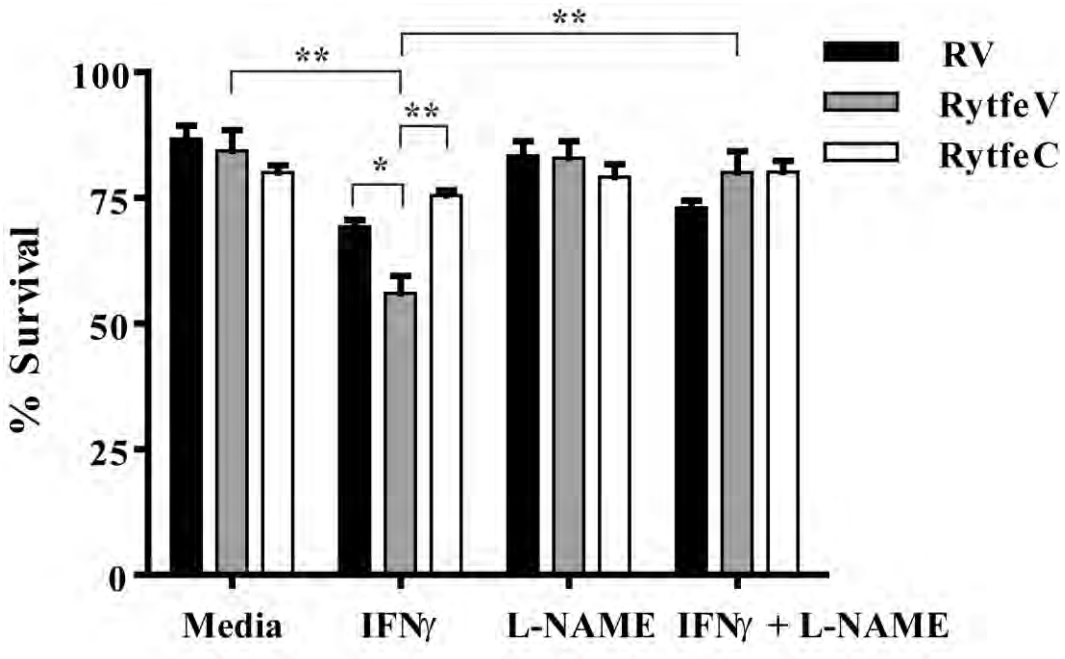


Figure 4.7: Effects of *ytfE* mutation and L-NAME on survival by co-infection with activated macrophages.

Parental strain, RV, RytfeV (*ytfE*-, empty vector) and RytfeC (*ytfE* complemented) were cultured to mid-log growth in oxygen-depleted condition, then added at an MOI of 5:1 to bone-marrow derived macrophages from C57BL/6J mice cultured with media alone, 100 U/ml IFNγ, 100 μM L-NAME or IFNγ plus L-NAME for 24 hrs. Plates were incubated with rocking at 37°C for 30 min. in an anaerobic chamber. Macrophages were lysed with 0.1% saponin, a concentration that does not influence bacterial viability, and surviving bacteria enumerated by plating dilutions for CFU. Percent survival represents the ratio of CFU recovered in the presence of macrophages versus incubation in medium alone. Statistics were calculated from ANOVA with Tukey's multiple comparison test (*p<0.01, **p<0.001).

that up-regulation of *ytfE* is essential for NO resistance in low oxygen so the *ytfE* mutant strain was also examined. RytfeV (*ytfE* mutant) and RytfeC (*ytfE* complemented) were co-cultured with macrophages from different treatment conditions (Fig. 4.7). As with the previous experiment, un-stimulated BMMs reduced survival of all *H. influenzae* strains to approximately 73-80%. However, after exposure to IFNγ stimulated macrophages, percent survival of RytfeV was 1.2 and 1.3 fold lower compared to RV and RytfeC, respectively. To establish that NO specifically participates in killing of *H. influenzae* in this assay, bacteria were co-cultured with IFNγ stimulated and un-stimulated BMMs pre-treated with L-NAME, a specific inhibitor of iNOS (Tsai *et al.*, 1997). Un-stimulated BMMs treated with or without L-NAME killed all strains with similar efficiency suggesting that L-NAME has no apparent effect on viability of *H. influenzae* in this assay. Stimulation of BMMs with IFNγ plus L-NAME yielded similar results as un-stimulated macrophages, indicating that increased killing of *H. influenzae* strains was likely mediated through a nitrosative-dependent mechanism. The increased susceptibility of the *fnr* and *ytfE* mutants to killing by activated macrophages compared to wild type *H. influenzae* is consistent with previous data showing these mutants are sensitive to in vitro generated NO.

The role of YtfE in resistance to activated macrophages is conserved in a clinical isolated non-typeable *H. influenzae* strain, NT127. Results with Rd strains indicate that up-regulation of *ytfE* is an important defense mechanism for *H. influenzae* upon encountering macrophages. Therefore, I investigated if this mechanism is conserved in pathogenic non-typeable *H. influenzae* strains. To do this, the *ytfE* mutation and

complementing construct and empty vector were moved into a clinical strain, NT127, resulting in strains, NytfeV, NytfeC and NTV, which were tested for differential susceptibility to un-stimulated or stimulated macrophages (Fig. 4.8). Wild type and *ytfE* complemented strains, NTV and NytfeC were resistant to killing by all macrophages in this assay. However, the *ytfE* mutant strain, NytfeV, exhibited 22% to 17% lower survival compared to the WT strain and complemented strains when exposed to un-stimulated BMMs, respectively. When co-cultured with BMM that were pre-activated with IFNγ, NytfeV was dramatically more sensitive to macrophages killing, 50% and 52% more than NTV and NytfeC. This effect was abolished when BMMs were treated with L-NAME in addition to IFNγ, indicating that NO was likely responsible for the increased killing of the *ytfE* mutant.

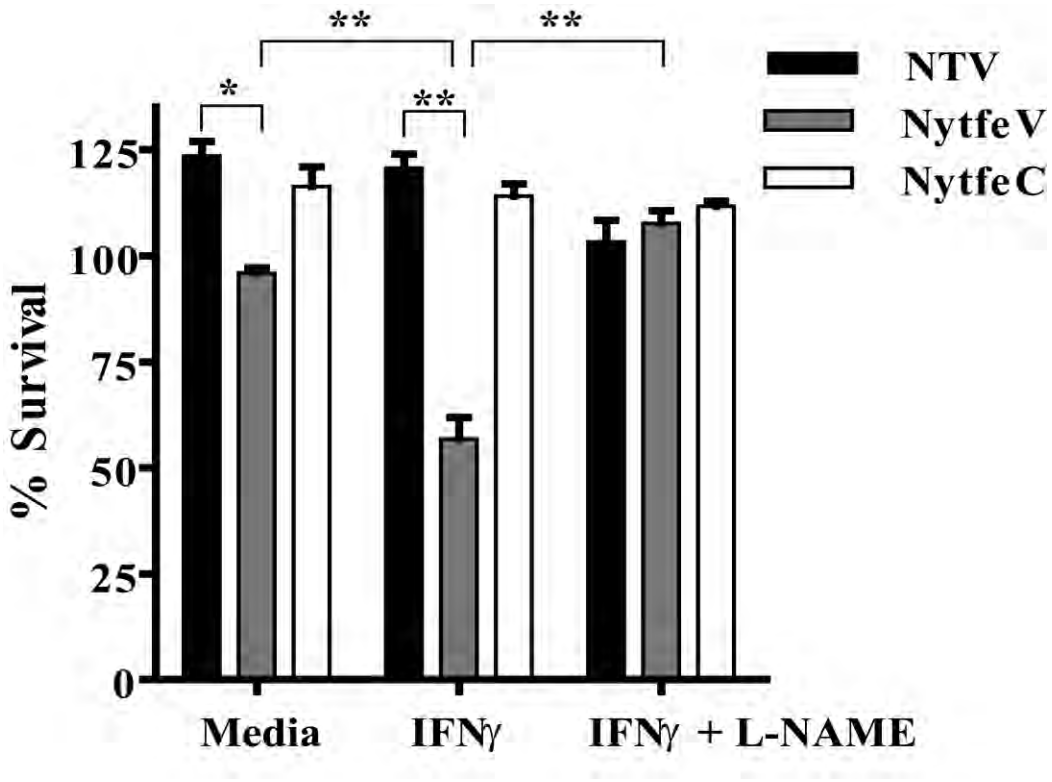


Figure 4.8: Effects of *ytfE* mutation in non-typeable *H. influenzae* strain and nitric oxide inhibitor, L-NAME, on killing by IFNγ activated macrophages.

NTV, NytfeV (*ytfE*-, empty vector) and NytfeC (*ytfE* complemented) were cultured to mid-log growth in oxygen-depleted condition and added at an MOI of 2:1 to bone-marrow derived murine macrophages pretreated with media alone, 100 U/ml IFNγ or IFNγ plus L-NAME for 24 hrs. Macrophages were lysed with 0.1% saponin and surviving bacteria enumerated by plating dilutions for CFU. Percent survival represents the ratio of CFU recovered in the presence of macrophages versus incubation in medium alone. Statistics were calculated from ANOVA with Tukey's multiple comparison test (*p<0.01, **p<0.001).

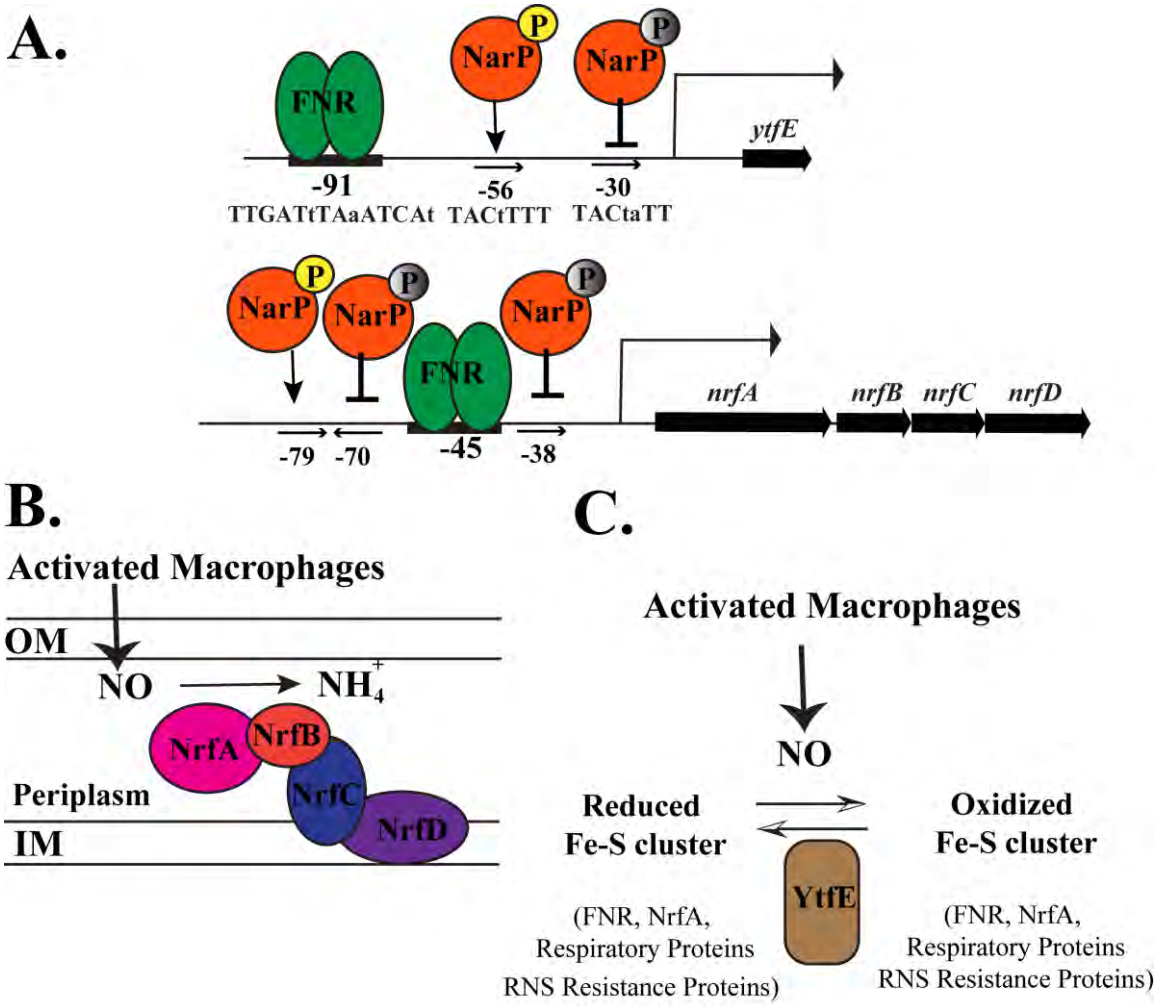


Figure 4.9: Proposed roles of FNR, NarP, NrfA and YtfE for RNS resistance in *H. influenzae*.

A. Under low oxygen condition, dimerized FNR (green circle) binds to putative FNR binding sites in the *nrf* and *ytfE* promoters centered at base pairs -45 and -91, respectively. These are additionally regulated by NarP (orange circle). Residues that match the predicted FNR and NarP consensus sequences are denoted with capital letters. Sites are relative to predicted transcriptional start sites (numbers represent basepairs). B. NrfA enzyme complex potentially contributes to RNS resistance by direct detoxification of nitric oxide (NO) in the periplasm. C. When *H. influenzae* encounters activated macrophages, nitric oxide (NO) can oxidize iron-sulfur clusters (Fe-S) found in FNR, NrfA, respiratory proteins and potentially other unidentified RNS resistance proteins. Di-iron protein, YtfE, repairs Fe-S clusters, thus restoring the activities of FNR, NrfA and respiratory proteins.

CHAPTER IV

Discussion

FNR of *E. coli* and *S. enterica* regulate many RNS defense mechanisms in coordination with NO-responsive regulator NsrR (Filenko *et al.*, 2007; Gilberthorpe *et al.*, 2007; Pullan *et al.*, 2007). Unlike *nsrR* mutants, *fnr* mutants have not been reported to have NO hypersensitivity phenotypes, implicating that FNR regulation is not as critical as regulation by NsrR for NO resistance in these organisms. In contrast, *H. influenzae* does not possess a homolog of NsrR and the *fnr* mutant is more sensitive to nitric oxide donors, ASN and GSNO. The increased killing of *fnr* mutant by activated, but not un-stimulated, macrophages as compared to wild type suggests that FNR might be important for regulation of RNS resistance in the human host. Based on the observed effects of the *narP* mutation on resistance to nitrosative stress, NarP potentially assists in regulation of these genes, though it appears to be less critical than FNR.

The results presented in Chapter II of this thesis indicate that that FNR regulates *nrfA*, a gene predicted to aid in NO detoxification. Although the *nrfA* mutant was slightly more sensitive to NO donors, the phenotype was not as distinct as that observed in the *fnr* mutant. This observation suggests that although *nrfA* might contribute to RNS defense, another FNR-dependent gene is likely responsible for the observed FNR mediate NO resistance, possibly independently or cooperatively with *nrfA* (Summarized in Fig. 4.9). An example of combinatory roles in RNS resistance is evident with *nrfA* mutant in *S.*

enterica, which only displayed a growth defect after exposure to NO when *norV* was mutated (Mills *et al.*, 2008).

The observation that *nrfA* mutant was not as sensitive to nitrosative stress as the *fnr* mutant led to scan of the predicted FNR regulon for an additional RNS resistance gene, which prompted investigation of *ytfE*. Significantly fewer bacteria were recovered from *ytfE* mutants than wild type cells challenged with GSNO and ASN, thus I conclude that *ytfE* of *H. influenzae* confers resistance to RNS. Additionally, *ytfE* mutants co-incubated with activated macrophages, which produce more nitric oxide than un-stimulated macrophages (Lorsbach and Russell, 1992), were more susceptibility to killing, most dramatically when *ytfE* is mutated in the non-typeable *H. influenzae* strain. Analysis of *ytfE* transcripts in FNR+ and FNR- strains cultured with depleted oxygen verifies that FNR regulates *ytfE* under these growth conditions. Less *ytfE* transcript was present in NarP- strain as compared to NarP+ strain, suggesting that NarP regulates *ytfE*, thought the fold differences was not as great as the FNR- strain. The observation that the *narP* mutant is more sensitive to in vitro nitrosative stress than wild type implicates that NarP contributes to RNS resistance, potentially through its regulation of *ytfE* or *nrfA*. The transcript levels of *nrfA* and *ytfE* were considerable lower in the *fnr* mutant, in comparison to the *narP* mutant, which might explain why the *fnr* mutant displayed a more dramatic sensitivity to *in vitro* nitrosative stress.

Given that both *ytfE* and *fnr* mutants are hypersensitive to nitrosative stress and killing by activated macrophages, FNR regulation of *ytfE* plays a significant role in RNS

defense. Preliminary results with an epistasis experiment support this hypothesis as over-expression of *ytfE* from a promoter independent of FNR regulation restores resistance to ASN in the *fnr* mutant (see Appendix). There is additional evidence that *ytfE* regulation is subject to modulation by changes in oxygen availability. The *ytfE* deletion construct consists of gentamicin-resistance cassette driven by the *ytfE* promoter and transformants containing the *ytfE* mutation could only be isolated under anaerobic growth conditions. Anaerobic induction of *ytfE* is a novel regulation model and paradoxical to the model proposed for FNR regulation of *ytfE* and *hmp* in *E. coli* and *S. enterica*. FNR is reported to repress *ytfE* under low oxygen conditions (Constantinidou *et al.*, 2006; Justino *et al.*, 2006; Overton *et al.*, 2006a). When the cell is exposed to nitrosative stress, the Fe-S cluster of FNR is oxidized by NO, inactivating the regulator and resulting in induction of *hmp* and *ytfE,* genes responsible for NO detoxification and repair of damage caused by NO (Cruz-Ramos *et al.*, 2002; Justino *et al.*, 2006). YtfE repairs Fe-S clusters, thus restoring FNR activity and repression of *hmp* and *ytfE*. In many of the reported studies on RNS defense mechanisms, the regulatory activities of FNR examined were in response to the nitric oxide signal (Corker and Poole, 2003; Pullan *et al.*, 2007). I propose that under conditions of low oxygen, FNR up-regulates genes critical for RNS resistance, such as *ytfE,* preemptively prior to exposure to NO, which would account for induction of *ytfE* by FNR opposed to repression. Additionally, the *ytfE* mutant is reported to have impaired nitrite reductase activity and YtfE is proposed to play a role in Fe-S maintenance in NrfA (Justino *et al.*, 2007). If NrfA of *H. influenzae* does detoxify NO, then deletion of *ytfE* would result in a failure to repair the Fe-S in NrfA under nitrosative stress and contribute

to the hypersensitivity phenotype to NO. Because NarQ-NarP likely responds to NO_2^-, if any changes in NrfA activity would alter the regulatory activities of NarQ-NarP because of changes in concentrations of the activating signal. Any modification in NarQ-NarP regulation possibly results in changes in *ytfE* and *nrfA* expression, thus NrfA indirectly impacts *ytfE* expression through NarQ-NarP. If YtfE is involved in Fe-S cluster maintenance and repair, then expression of *ytfE* directly affects the activity of NrfA and FNR. The proposed interactions between these factors are fairly complex and warrant further investigation of how these interactions might contribute to RNS resistance. Further experimentation with double knockouts and epistatic constructs with exposure to nitrosative stress would potentially provide information on the interactions between the pathways. Additionally, YtfE likely repairs Fe-S clusters in other proteins that are members of the FNR regulon, which are involved in respiration or potentially involved in RNS resistance. Disruption of FNR or YtfE affects the activity of many proteins, either through gene expression from FNR activity or through restoration of Fe-S clusters, after exposure to NO. I conclude that genes that are subject to redox-modulated regulation play a significant role in defense against RNS.

CHAPTER V

Discussion and Perspectives

H. influenzae primarily resides in the human nasopharynx asymptomatically with up to 80% of the population (Kuklinska and Kilian, 1984). Physiologically, one would speculate that the microenvironments encountered in the lungs (cystic fibrosis) and the brain (meningitis) or in the nasopharynx of a healthy individual versus an individual with a respiratory infection are drastically different in regards to oxygen availability. The results presented in this thesis validate the hypothesis that *H. influenzae* modulates its gene expression profile in response environmental cues in accordance with observations with ArcA regulation (Wong *et al.*, 2007). The presence of two global regulators that are active during anaerobiosis is also indicative that oxygen is a redox signal that is encountered during infection; however, the significance of signaling in response to environmental RNS during infection has not been studied. FNR is a stronger activator than NarP for *nrfA* expression based on nitrite reductase activity and Western blot results; whereas, *fnr* mutant were able to efficiently reduce NO_3^- and *narP* mutants were impaired for NO_3^- reduction, suggesting that *nap* operon is less dependent on FNR than NarP for expression. Additionally, the *narQ* mutants were able to convert NO_3^- to NO_2^- at an accelerated rate as compared to wild type, *narP* mutants and *fnr* mutants without a previously reported inducer, NO_2^-, in the pre-growth media to activate NarQ. The relative positioning of the putative binding sites might account for the differences in transcriptional control by FNR and NarQ-NarP, which leads to the question of the

biological relevance in vivo. Enteric bacteria, such as *E. coli* and *S. enterica* are exposed to high concentrations of NO_3^- (2-5 mM) in the gut, dependent on dietary intake of the host (reviewed (Dejam *et al.*, 2004; Gladwin, 2004)). Functionally similar reductases are present in the bacteria to reduce NO_3^- and NO_2^- and regulation of the genes is coordinated so the appropriate enzyme is expressed, dependent on the substrate availability. At lower NO_2^- levels, periplasmic NrfA is expressed and it uses NO_2^- as terminal electron acceptor for respiration. When NO_2^- concentrations are increased, approaching toxic levels, the cytoplasmic NirB is expressed to detoxify the substrate (Wang and Gunsalus, 2000). NO_3^- and NO_2^- are present at different concentrations in the host, depending on the anatomical site and state of bacterial infection, but concentrations are sub-micromolar (Gladwin *et al.*, 2000). Repression of the *nrf* promoter was only observed with concentrations of NO_2^- greater than 0.5 mM. Without a secondary nitrite reductase present, what is the advantage of repressing transcription of an enzyme when the substrate levels are high? Differential expression of *Pnrf-HA* with NO_2^- supplementation was only observed in sBHI growth media, not in MIc. Another redox signal present in sBHI might be contributing to NarQ-dependent repression of the *nrf* promoter and this factor might be present in the human host, resulting in regulation in response to much lower NO_2^- concentrations. Observations that NarP is an activator of *nrf* expression and nitrate reductase activity, yet NarQ yields an inhibitory effect, illustrate the differences of NarQ-NarP signaling in *H. influenzae* in comparison to *E. coli*. NarQ-NarP of *E. coli* primarily induces gene transcription and NarX-NarL is responsible for repression (Stewart, 2003). Maintenance of anion homeostasis is a possible physiological basis for

this complex regulation to optimize respiration of alternative electron acceptor. It is of interest to further investigate the role of NarQ-NarP regulation during *H. influenzae* infection.

Regulation of NO_2^- metabolism is also potentially relevant to RNS resistance given that NO_2^- can generate NO in acidic conditions and is considered a storage pool in the human host (Dejam *et al.*, 2004). NrfA of *E. coli* has been identified as a NO reductase and is required for RNS resistance in *E. coli* and *S. enterica*. Although a minor hypersensitivity phenotype of the *nrfA* mutant was observed when cultured with ASN or GSNO, *nrfA* might contribute to NO detoxification but is not the major FNR-regulated mechanism of RNS resistance. In *S. enterica*, the *nrfA* mutant was only dramatically more sensitive when *norV* (nitric oxide reductase) was additionally mutated so it is possible that *nrfA* confers RNS resistance in combination with another factor. The genome of *H. influenzae* does not encode any other gene predicted to be a nitric oxide reductase or any other gene that has been identified as means of NO detoxification, such *hmp* or *hcp*. *H. influenzae* does not possess a homolog of NO-response regulator, NsrR, which is present in many human pathogens. Other γ-proteobacteria species that have NsrR-like regulators and Nor-like proteins also have enzymes that generate NO as a product, including NirB, NarG, and AniA. These enzymes are not present in *H. influenzae*, thus an absence of bacteria-derived NO might modify the need for NO signaling or a secondary NO reductase. Once again, the environmental conditions encountered might also account for this evolutionary difference. Enteric bacteria will experience extensive nitrosative stress when ingested NO_2^- is acidified to NO in the gut

and intracellular bacteria are subject to high concentrations of NO in the acidic phagocytes, which would justify the need for many RNS resistance mechanisms. *H. influenzae* can efficiently persist by evasion of immune clearance so it is possible the bacteria will only encounter relatively low levels of NO that can be detoxified by NrfA alone.

This speculation leads to discussion of the differences observed with that of FNR regulation of *ytfE*. FNR is reported to be a repressor of *ytfE* of *E. coli* (Constantinidou *et al.*, 2006; Justino *et al.*, 2006; Overton *et al.*, 2006a) and *dnrN* (*ytfE* homolog) of *N. gonorrhoeae* (Whitehead *et al.*, 2007). De-repression of *ytfE* occurs after the Fe-S of FNR is oxidized by NO, so regulation occurs in response to nitrosative stress. The results presented in this thesis show that FNR positively regulates *ytfE* under low oxygen conditions, in the absence of nitrosative stress. Both genes are required for resistance to in vitro NO donors or stimulated macrophages. When the bacteria are in conditions of low oxygen in the host, FNR may induce genes involved in RNS resistance under low oxygen conditions, as preemptive protection. When the bacteria encounter activated immune cells that generate nitrosative stress, the bacteria can immediately repair damage to Fe-S cluster by YtfE. The results with dramatic susceptibility to killing by activated macrophages of the *ytfE* mutant strain in the non-typeable *H. influenzae* background suggest that YtfE is a significant mechanism of NO resistance. NT127 was isolated from the cerebrospinal fluid of a patient with meningitis, meaning that this particular strain originated in the nasopharynx, traversed the epithelial layer to the blood and successfully crossed the blood-brain barrier. The concentration of NO encountered in these drastically

different microenvironments is unknown and the significance of RNS resistance mechanisms for *H. influenzae* infection has yet to be determined. Based on the observations that ArcA is required for in vivo infection and resistance to oxidative stress, we speculate that FNR is additionally important for in vivo infection. The regulatory mechanisms employed by *H. influenzae* for RNS resistance warrant further investigation. In conclusion, *H. influenzae* modulates its gene expression in response to environmental redox signals, likely optimizing its survival and virulence in the human host.

Appendix

Title: ASN hypersensitivity screen

Purpose: With the intention of isolating an *ytfE* mutant, Km^r transformants from *Himar1* derivative *magellan1* transposon mutagenesis pools were screened for mutants that are hypersensitive to ASN.

Materials and Methods: Freezer stocks of two GAMBIT pools, 342-352d (CH58) and 447-448 (CH43), were diluted and plated on nonselective sBHI agar plates. Individual colonies were sequentially patched onto new sBHI agar plates and 200 µl sBHI broth (pH7.5) in a 96 well dish. Broth cultures were sealed in an anaerobic baggie and incubated at 35°C for 5 hours to reach mid-logarithmic growth. Aliquots (20 µl) of each culture were seeded into a new plate containing 180 µl sBHI broth (pH6.5) supplemented with $NaNO_2$ at final concentrations of 15mM , 17.5mM or 20mM. Acidic media with PBS alone was used as a positive control for bacterial growth and strain RfnrV was used as a positive control for ASN hypersensitivity. Cultures were sealed in an anaerobic baggie and incubated at 35°C for ~16 hours. Aliquots (20 µl) of each culture were transferred into "recovery media", 180 µl sBHI broth (pH7.5). After incubation at 35°C for ~20 hours exposed to ambient air, cultures were assayed for growth using spectrophotometer, OD_{600}. Transformants that failed to grow in recovery media after previous exposure to ASN were colony purified from parallel growth on sBHI agar plates. The approximate location of the transposon insertion was mapped in the resulting isolates using the MAROUT primer and GAMBIT 342 primer for isolates from pool 342-352d or GAMBIT 447 primer for isolates from pool 447-488.

Data: One transformant from pool 342-352d and 8 transformants from pool 447-448 did not grow in recovery media after prior exposure to ASN at concentrations of 15mM , 17.5mM and 20mM, as indicated by OD_{600} readings less than 0.01. Cultures were not inhibited for growth in recovery media after prior growth in acidic media with no ASN present. Transposon insertions mapped to *HI0344* (*napA*) in the1 transformant from pool 342-352d and *HI1676* (*moaA*) the 8 transformants from pool 447-488.

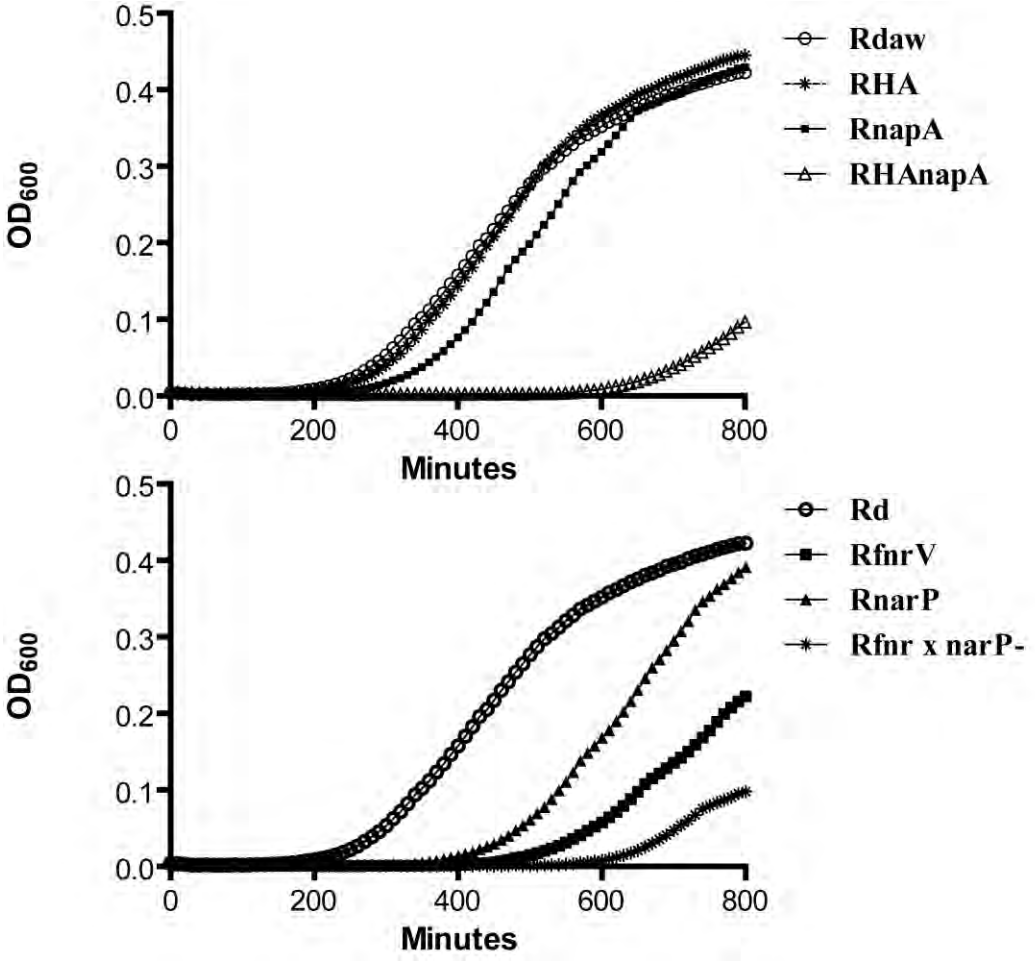
Conclusion: The results from this screen suggest that *napA*, encoding nitrate reductase, contributes to resistance to nitrosative stress generated from ASN. *moaA* encodes a predicted molybdenum cofactor biosynthesis protein and NapA of *E. coli* requires a molybdenum cofactor. The hypersensitivity of *moaA* mutants may be a result of impaired nitrate reductase function.

Title: **GSNO sensitivity assay: *fnr/narP* and *napA/nrfA* double mutants**

Purpose: The sensitivity of single mutants (*fnr*, *narP*, *napA* and *nrfA*) to GSNO exposure was compared to double mutants (*fnr/narP* and *napA/nrfA*) to determine if RNS resistance mechanisms are related or independent.

Materials and Methods: To generate strain RfnrnarP, the *narP* mutation was amplified from template RnarP, using primers 5'narPKOup (5' GTAATAATACTAAAGTGAGTGTT) and 3'narPKOdown (5' atgaatttcttccaatataaac) and the resulting PCR product was transformed into strain Rfnr. Transformants were selected on sBHI agar plates containing Gm. Strains were treated as previously described for NO-donor sensitivity assays in Chapter IV with the following modifications: 1) 2 mM GSNO was used, opposed to 5 mM GSNO, 2) after exposure to GSNO, 20 µl aliquots were transferred to 180 µl sBHI broth and monitored for growth using spectrophotometer.

Data:



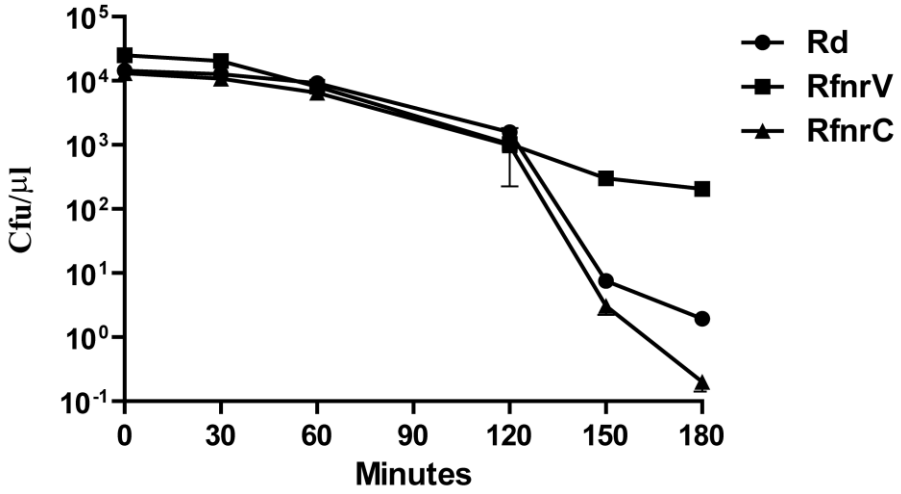
Conclusion: Fewer cells were present in the recovery media with cultures from *fnr/narP* and *napA/nrfA* double mutants after prior exposure to GSNO as compared to the single mutants. FNR and NarP both regulate *nrfA*, *napA* and *ytfE* so mutating both regulators likely results in significantly less expression of all three of these genes than in a single *fnr* or *narP* mutants.

Title: ASN sensitivity assay: *fnr* mutant and ASN under microaerobiosis

Purpose: To determine if the hypersensitivity of *fnr* mutant was limited to anaerobic growth conditions, Rd, RfnrV and RfnrC were cultured with microaerobic growth conditions when challenged with ASN.

Materials and Methods: Overnight anaerobic cultures were used to inoculate 30 mL sBHI (pH5.5) supplemented with $NaNO_2$ (final concentration 15 mM) with an initial density of OD_{600} 0.02 in a 25 mL flask, and were subsequently incubated at 35°C shaking at 250 rpm. Aliquots were taken at indicated time points and viable bacteria were enumerated with plating.

Data:



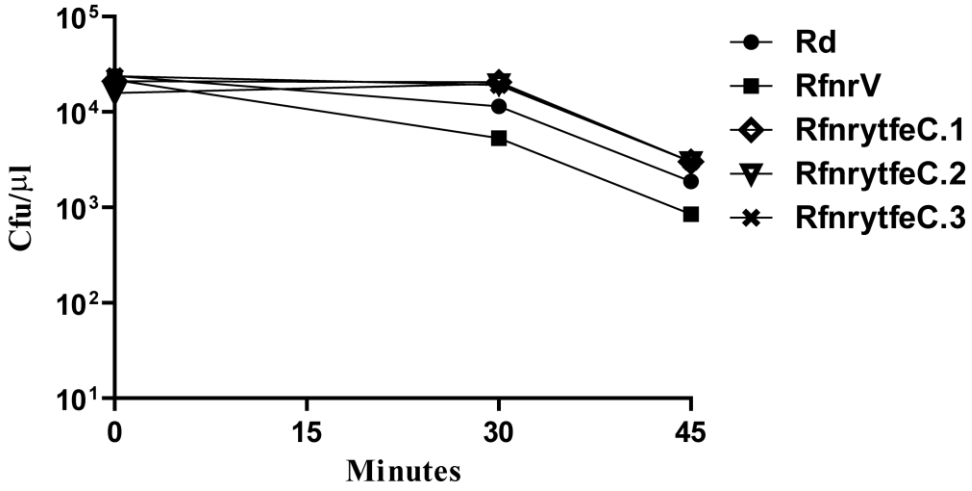
Conclusion: Although *fnr* mutant is more sensitive to ASN challenge under anaerobic conditions, the *fnr* mutant was less sensitive to ASN challenge when the assay was conducted under microaerobic conditions, as indicated by more colonies recovered from RfnrV at 150 and 180 minutes than from Rd and RfnrC. FNR might be repressing a RNS resistance gene during microaerobiosis, which would be expressed at higher levels in RfnrV.

Title: **ASN sensitivity assay: *fnr* mutant with copy of *ytfE* driven by *xylA* promoter**

Purpose: To determine if the hypersensitivity of the *fnr* mutant is due to lack of *ytfE* expression, the *fnr* mutant was transformed with copy of *ytfE* driven by the *xylA* promoter from plasmid pXTytfEC and the resulting strains were challenged with ASN.

Materials and Methods: To generate strains RfnrytfeC.1-3, plasmid pXTytfeC was digested with *ApaL*I, gel purified and transformed into strain Rfnr. Transformants were selected on sBHI agar plates containing Tet. Strains were treated as previously described for NO-donor sensitivity assays in Chapter IV. The listed numbers after strains RfnrytfE denote individual isolates from transformation of Rfnr with *ytfE* in the *xyl* locus.

Data:



Conclusion: Over-expression of *ytfE* in the *fnr* mutant strain background, Rfnr, restored resistance to ASN to that of the wild type strain, Rd, which implicates that decreased expression of *ytfE* results in the ASN hypersensitivity phenotype of *fnr* mutant.

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