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Nitric Oxide Sensing and Response in Vibrio Cholerae

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Abstract
Nitric oxide (NO) is a radical capable of inhibiting bacterial growth. Bacteria in turn have multiple mechanisms of resisting the toxic effects of NO, usually encoded by genes under the control of NO-responsive transcription factors. However, our knowledge of the protein targets of NO is limited, as is the function of many NO-regulated genes. We studied two genes in V. cholerae, hmpA and nnrS, which encode a flavohemoglobin and a protein of unknown function, respectively, both predicted to be under control of the NO-responsive transcription factor NorR. We confirmed that both promoters were regulated by NorR and found that all three genes were important for growth in the presence of NO stress. We then performed a metabolomic study on multiple strains of V. cholerae, finding new potential metabolic targets of NO. In particular we found that substrates of iron-sulfur cluster-containing proteins accumulated in strains lacking nnrS, and that aconitase activity was decreased in cell-free extracts of nnrS mutants. Chelation of ferrous iron reversed the growth defect imposed by nnrS deletion; furthermore, strains lacking nnrS possessed lower ferrous iron concentrations. These data suggest that NnrS, a protein of previously unknown function, protects against the formation of NO-iron complexes. We also found that hmpA and norR are important for survival during colonization of the mouse intestines in response to host-generated NO, whereas nnrS is dispensable.

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I dedicate this dissertation to

my family, Hartley, Dorothy, and Daniel Stern;

my mentors, Jay Zhu, James Coulton, and Benoit Cousineau;

and my English teachers, Murray Wilson and John Hodgson.

ABSTRACT

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Andrew M. Stern

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Nitric oxide (NO) is a radical capable of inhibiting bacterial growth. Bacteria in turn have multiple mechanisms of resisting the toxic effects of NO, usually encoded by genes under the control of NO-responsive transcription factors. However, our knowledge of the protein targets of NO is limited, as is the function of many NO-regulated genes. We studied two genes in *V. cholerae*, *hmpA* and *nnrS*, which encode a flavohemoglobin and a protein of unknown function, respectively, both predicted to be under control of the NO-responsive transcription factor NorR. We confirmed that both promoters were regulated by NorR and found that all three genes were important for growth in the presence of NO stress. We then performed a metabolomic study on multiple strains of *V. cholerae*, finding new potential metabolic targets of NO. In particular we found that substrates of iron-sulfur cluster-containing proteins accumulated in strains lacking *nnrS*, and that aconitase activity was decreased in cell-free extracts of *nnrS* mutants. Chelation of ferrous iron reversed the growth defect imposed by *nnrS* deletion; furthermore, strains lacking *nnrS* possessed lower ferrous iron concentrations. These data suggest that NnrS, a protein of previously unknown function, protects against the formation of NO-iron complexes. We also found that *hmpA* and *norR* are important for survival during colonization of the mouse intestines in response to host-generated NO, whereas *nnrS* is dispensable.

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PREFACE

The second law of thermodynamics states that the entropy of the universe is always increasing. In other words, time is an inexorable march towards a state in which all energy in existence is evenly distributed throughout space. We must currently be near the beginning of this progression, however: in defiance, life on Earth continues to produce an incomprehensible array of complex, ordered systems of interacting molecules. Ultimately, we have the sun to thank for the energy to compensate for this entropy barrier and make life exergonic, but an energy source alone is not sufficient to produce a living organism. Instead, precisely tuned collections of enzymes collaborate efficiently to convert disordered energy – photons, sugars, proteins, hamburgers – into a bacterium, or a tree, or a scientist who discovers the second law of thermodynamics. The word we use to describe this feat is, of course, metabolism. The reductionist definition of metabolism, then, is the assembly of simple, high-enthalpy substances into complex, high-entropy substances called organisms.

We study the metabolism of bacteria for two reasons. First, because they are easy to study. Understanding the basic principles of metabolism is easiest in a reduced setting in which the metabolism is simple, the organism is small and easy to grow, and perturbation of its metabolism though genetics or biochemistry is feasible. Despite their simplicity, many of the lessons learned in bacteria can be used to infer analogous processes in humans or other species, and potentially improve human quality of life through medical or industrial innovation. Second, because many bacteria themselves are the cause of human disease. A common conception of bacterial infection is simply the colonization and exploitation of the human body as a niche for growth – again, an attempt

to use the human host as an energy source to permit the high-entropy production of more bacteria. In this sense, infection could be viewed as a metabolic process, and studying "why" bacteria infect people – that is, how their metabolism changes to their benefit during infection to permit growth – is the ultimate insight into infectious processes. Understanding bacterial metabolism will allow us to inhibit it, and thus treat disease, through pharmacologic or other means.

CHAPTER ONE: Introduction

Throughout their multimillion year evolution, bacteria have had to contend with threats to their metabolism in all environments, including during infection. This dissertation will discuss the particular threat of nitric oxide (NO). NO has been the subject of intense investigation in multiple contexts: as a signaling molecule causing vasodilation in mammals, as an intermediate in the nitrogen cycle, and as an antibacterial toxin. I am primarily interested in the latter aspect of NO biology – how NO affects bacterial metabolism and how bacteria respond to NO toxicity. During my thesis work, I focused on one bacterial species, *Vibrio cholerae*, which causes cholera and remains a large global public health threat.

In this chapter, I will present a review of the literature on nitric oxide biochemistry, its sources, its metabolic targets and bacterial tolerance strategies; a general introduction to the biology and pathogenesis of *V. cholerae*; and a statement of the hypotheses for the experiments performed. Chapter Two presents a study in which factors are described that are important for *V. cholerae* tolerance of NO *in vitro* and during intestinal colonization. Chapter Three probes the effects of NO on *V. cholerae* metabolism and identifies a novel factor, NnrS, important for resisting the effects of NO on iron-containing proteins. Chapter Four contains concluding remarks.

A. Biochemistry of nitric oxide and reactions with bacterial enzymes

Nitric oxide is a diatomic radical gas, composed of one atom each of nitrogen and oxygen with a single unpaired electron. Its chemical symbol is written as NO· to indicate the unpaired electron, but for the purposes of this document will be written as NO. Its chemical composition confers it unusual, and perhaps somewhat unintuitive, chemical

properties. The reactions NO undergoes in living systems have been difficult to tease apart, but Toledo and Augusto[1] make the useful distinction between reactions that occur at low (nanomolar) NO concentrations, thought to be more "physiologic," and those at high (micromolar) concentrations, thought to be more "pathologic." The first category refers primarily to the now classical conception of NO as a signaling molecule, in which capacity NO binds to the heme moiety of soluble guanylate cyclases (sGC), leading to production of cyclic guanidine monophosphage (cGMP) and relaxation of smooth muscle in mammalian blood vessels. This aspect of NO biology will not be discussed further here. Instead, the myriad reactions that NO undergoes at higher concentrations are of particular importance to bacteriology. A summary of some of the important reactions that NO can undergo is displayed in Fig. 1, including some well-studied bacterial enzymes that convert NO to less reactive compounds such as nitrate (NO_3^-), nitrous oxide (N_2O) or ammonia (NH_3) (discussed below); reactions that result in enzyme inhibition; and reactions that occur especially at high NO concentrations and in the presence of reactive oxygen species that generating damaging oxidative chemicals.

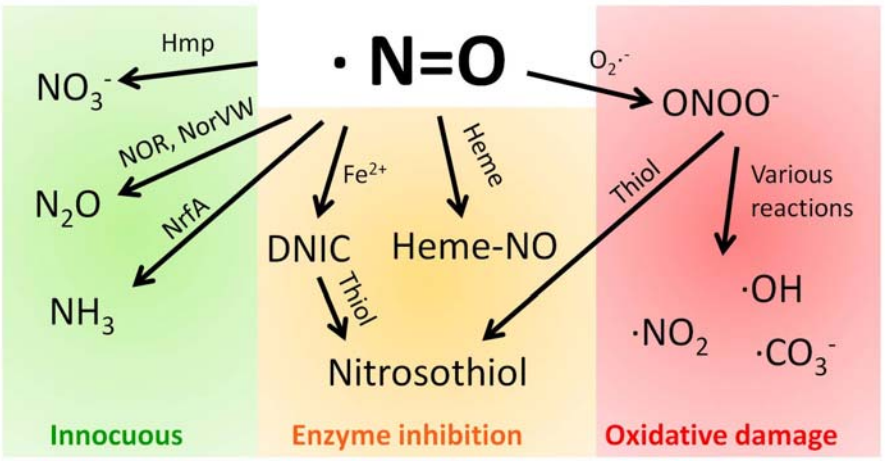

Fig. 1. Summary of biologically relevant reactions of NO. Hmp (flavohemoglobin), NOR (nitric oxide reductase), NorVW (flavorubredoxin), and NrfA (periplasmic cytochrome *c* nitrite reductase) refer to bacterially encoded enzymes that convert NO to less toxic nitrogen species. DNIC: dinitrosyl iron complex.

Although it is a radical, NO is not particularly

susceptible to oxidation or reduction[2]. Instead, it can react with various oxygen species such as superoxide (O_2^-), and molecular oxygen (O_2), to form so-called "reactive nitrogen species" (RNS) like peroxynitrite ($ONOO^-$), nitrogen oxide radical ($NO_2\cdot$), dinitrogen trioxide (N_2O_3), nitrosonium cation (NO^+) or nitroxyl anion (NO^-). In biological systems, these transient species can go on to form other reactive species such as hydroxyl and carbonate radicals ($OH\cdot$ and $CO_3\cdot$, respectively). Some of these species are potent oxidizers and can directly damage DNA through one-electron oxidation. For example, high concentrations of NO can lead to the deamination of cytosine, causing C->T mutations[3] and peroxynitrite can directly oxidize guanosine residues to 8-oxo-2'-doxyguanosine and cause strand breaks[4,5]. Furthermore, the base excision repair system has been shown to be important for preventing DNA damage by host-generated NO in both *S.* Typhimurium and *V. cholerae*[6,7]. In this sense, RNS derived from NO are a form of direct oxidative stress to bacteria.

Besides forming potent oxidants, the other results of the formation of these nitrogen oxides are the stable macromolecule modifications that are unique to RNS: dinitrosyl iron complexes (DNICs), S-nitrosylated cysteines, and nitrated tyrosines. These are capable of influencing protein function, and consequently gene expression and cellular physiology. NO exhibits strong reactivity with transition metals; the most-studied NO-metal reaction is with iron, an abundant element in biology. NO reacts readily with both heme and non-heme iron, with a higher affinity for ferrous than ferric iron. This property is similar to molecular oxygen, because both NO and triplet O_2 possess unpaired electrons able to form coordinate covalent bonds with the *d* orbitals of iron atoms. For example, NO forms a complex with ferrous hemoglobin and terminal oxidases of the

electron transport chain at sites normally bound by molecular oxygen. Both of these reactions are significant to bacteria: the former is used as a defense mechanism because can result in the conversion of NO to nitrate (discussed below), the latter because it results in inhibition of bacterial respiration[8,9]. NO can bind to the cytochrome *bd* or *bo'* complexes, the two terminal oxidases in *Escherichia coli* to inhibit oxygen respiration[8,10]. The mechanism of this inhibition is not perfectly clear but likely involves binding two transition metals: the high-spin heme iron and the copper atom that make up the active site[11], and even results in some reduction of NO to N_2O, though the physiological relevance of this reaction is uncertain. Thus, respiration itself is a target for NO toxicity in bacteria.

NO also binds to non-heme iron. In particular, the formation of dinitrosyl iron complexes (DNICs) has received attention in research recently due to the ability of these complexes to inhibit protein function. DNICs are detectable by electron paramagnetic resonance (EPR) spectroscopy and have been shown to be responsible for the potent inhibition of iron-sulfur cluster-containing proteins, in particular the dehydratase family enzymes aconitase[12,13] and dihydroxyacid dehydratase[14]. These enzymes perform catalysis through direct reaction of their iron-sulfur clusters with their substrates (citrate and 2,3-dihydroxyisovalerate, respectively), meaning the cluster is solvent-exposed and susceptible to binding by NO. The origin of the iron in DNICs was shown to be the "chelatable iron pool" (CIP), which is not spatially defined, but is simply the component of cellular iron that can be bound by chelators[15]. It is unclear whether this implies that NO first reacts with free iron, which is quite limiting in cells due to its toxicity, or if the entire reaction occurs at the site of the iron-sulfur cluster; Landry *et al.*[16] recently

demonstrated that some component of the CIP is actually part of iron-sulfur clusters, and thus the latter mechanism may be more likely. On the other hand, the same lab demonstrated that cysteine and oxygen could decompose protein-bound DNICs[17] and hypothesized a protein-free cysteine-iron-NO intermediate which can be synthesized *in vitro*, implying that there may exist both protein-bound and protein-free DNICs in the cell when exposed to NO.

Not only are DNICs directly inhibitory to enzymes, but they may also mediate the formation of another biologically important NO-dependent protein modification – the nitrosothiol (S-NO). Nitrosothiols form commonly at cysteine residues and may affect protein function. Multiple studies on the S-nitrosoproteome have demonstrated that a considerable number of proteins are S-nitrosylated in human tissue in various settings[18–20], and that many bacterial proteins are S-nitrosylated during cellular exposure to NO or even during respiration on nitrate [21–23]. This modification can have major consequence for protein function and for virulence. Kim *et al* [24] found that OxyR, a transcription factor initially found to mediate responses to oxidative stress, can be S-nitrosylated at a critical cysteine residue, causing it to alter its DNA binding affinity and regulate a gene sets distinct from the genes regulated by OxyR in the presence of reactive oxygen species (as opposed to nitrogen species). OxyR was subsequently shown to exhibit a constitutive level of S-nitrosylation during respiration on nitrate[23], suggesting that this protein modification can even possess a housekeeping role in bacterial physiology. Savidge *et al*. found that *Clostridium difficile* toxin becomes S-nitrosylated when given to mice orally, inhibiting its action[19]. They also found that directly feeding the mice nitrosothiols in the form of S-nitrosoglutathione (GSNO) could mitigate disease. In *Salmonella enterica*

serovar Typhimurium (*S.* Typhimurium), S-nitrosylation of the regulatory protein SsrB changed its affinity for virulence genes, and this NO-dependent switching was important for virulence in a mouse model[25]. Thus, cysteine S-nitrosylation is a widespread mechanism for affecting protein function and gene expression in bacteria and can directly influence disease processes. How nitrosothiols form *in vivo*, however, is somewhat of a mystery because NO does not itself react with thiols in physiologic conditions. Instead, NO must be oxidized to an NO^+ equivalent that can then modify thiols. At least two mechanisms are possible. The first is the reaction of NO with O_2 to form N_2O_3 (after $ONOO^-$ and $NO_2\cdot$ intermediates), which can directly nitrosylate thiols[26]. It is unclear whether this mechanism is the relevant source of nitrosothiols *in vivo* since it requires high NO concentrations and the presence of oxygen. Cells grown in the absence of oxygen still produce nitrosothiols[23,27], suggesting that nitrosothiol formation proceeds through a different mechanism (though they do produce somewhat more in the presence of oxidative stress[27]). It is more likely that formation of nitrosothiols from NO occurs through first forming DNICs. A cysteine-bound DNIC was first found to trans-nitrosate human serum albumin *in vitro*[28], suggesting that trans-nitrosation of thiols *via* DNICs might be a mechanism *in vivo*. Then, Bosworth *et al*.[27] demonstrated that DNICs likely mediate nitrosothiol formation in living cells by performing a critical experiment in which iron chelation prevented nitrosothiol formation, suggesting that chelatable iron – that which is involved in forming DNICs – is also necessary for nitrosothiol formation. Thus, nitrosothiol formation likely occurs through transfer of NO^+ equivalents from nitrosylated metals to thiols, but may also include direct reaction of thiols with higher order nitrogen oxides in certain contexts.

Another protein modification formed in the presence of NO is nitrotyrosine. Again, NO itself does not react with tyrosine, but nitrotyrosine can form readily from peroxynitrite through formation of a tyrosyl radical intermediate[29]. Gene expression and proteomic analysis revealed overlap between the effects of peroxynitrite and oxidative stress, suggesting that the character of peroxynitrite stress lies somewhere between that of stress due to NO and oxidative stress due to hydrogen peroxide or superoxide[30,31]. There are fewer examples of specific bacterial metabolic pathways inhibited by tyrosine nitration than by cysteine S-nitrosation. *In vitro, g*lutamine synthetase[32,33] and ribonucleotide reductase[34] were shown to be inhibited by tyrosine nitration. In addition, human neutrophils generate nitrotyrosine after they engulf bacteria, likely in both neutrophil and bacterial proteins[35,36]. Recent studies from Lindemann *et al.*[30] and McLean *et al.*[31] demonstrated that nitrotyrosine forms *in vivo* in response to peroxynitrite, and thus it appears that nitrotyrosine formation is likely a true factor in nitrosative stress in bacteria; however, it requires the presence oxidative species, where as cysteine S-nitrosation does not, meaning that nitrosothiol formation may occur more commonly. Nitrotyrosine has also been studied more extensively for its role in human physiology, and reviews on this subject can be found elsewhere[29,37].

In summary, NO reacts through two pathways that shape its interaction with living systems: reaction with oxygen or superoxide to generate RNS, and direct nitrosation of transition metals. The former can cause macromolecule modification as well as directly oxidize DNA. The latter leads to stable complexes such as nitrosyl heme and DNICs which can affect protein function in both physiological and pathological ways.

B. Environmental and host sources of nitric oxide

Given that NO is a two-atom combination of the two most abundant elements in the atmosphere, it is perhaps no surprise that bacteria encounter NO frequently. Virtually every bacterium studied has some mechanism for detecting and/or tolerating NO, so it is safe to conclude that NO is present in many different environments.

Perhaps the best-studied source of NO, at least in terms of pathogenesis, is inducible nitric oxide synthase (iNOS), a mammalian enzyme that produces NO at high concentrations inside immune cells such as macrophages and neutrophils. To illustrate the importance of iNOS in combating bacterial infection, a role for this enzyme has been demonstrated for host survival or control of bacterial replication in mouse models of *Salmonella* Typhimurium[38,39], *Vibrio cholerae*[7,40], *Staphylococcus aureus*[41,42], *Mycobacterium tuberculosis*[21,43–45], *Coxiella burnetii*[46], *Listeria monocytogenes*[47], *Chlamydia trachomatis*[48–50], *Porphyromonas gingivalis*[51], *Bordetella pertussis*[52], and *Leptospira interrogans*[53]. iNOS is one of three mammalian NOSs, but it is unique for two reasons. First, its expression is inducible, whereas the other two NOSs, neuronal and endothelial NOS, are constitutively expressed. Second, due to its high expression level and activity, it is capable of generating micromolar concentrations of NO at sites of inflammation. As outlined above, NO at micromolar concentrations can undergo substantial autoxidation in the presence of oxygen and superoxide to generate the myriad nitrogen oxides that comprise RNS, causing cellular toxicity through the direct oxidation of DNA. Accelerating the production of reactive nitrogen oxides is the co-expression of iNOS with NADPH oxidase, an enzyme that generates superoxide, causing macrophages

to independently produce peroxynitrite[54]. Thus, the NO generated by iNOS and the superoxide generated by NADPH oxidase collaborate to form a potent cocktail of oxidative species that limit bacterial replication.

iNOS generates NO by catalyzing the reaction of L-arginine, an amino acid, with molecular oxygen, to generate NO and L-citrulline while consuming NADPH[55,56]. The activation of iNOS activity occurs mainly through upregulated transcription of *nos2,* the gene encoding iNOS. Transcription is controlled by numerous transcription factors, reviewed in detail by Pautz *et al*[57]. These transcription factors activate iNOS expression as a result of signaling in response to pathogenic signals such as lipopolysaccharide (LPS) though the production of interferons and other cytokines[58]. The result is that immune cells sense the presence of bacteria and upregulate iNOS, which then presents a strong suppressor of growth with which the invading bacteria must contend.

Although iNOS is a "dedicated" host response to enzymatically generate NO to slow microbial growth, an equally important source of NO for gastrointestinal pathogens is the orogastric nitrate-nitrite-NO system[59]. First, dietary nitrate is reduced to nitrite by anaerobes respiring in the mouth. Nitrite is then swallowed, whereupon it enters the acidic gastric lumen. Here, nitrite is protonated to nitrous acid (HNO_2), which then decomposes spontaneously into $NO_2^{\cdot}$, NO, and water. This is a formidable source of NO and may be considered one reason, beyond the toxicity of the acid itself, why the low pH of the stomach is a barrier to infection for many gastrointestinal pathogens. In the case of *V. cholerae*, for instance, a mutant lacking its main NO-resistance protein, HmpA, is attenuated for colonization at least partially due to stomach NO[7,40]. There is also evidence that NO impairs the ability of *S.* Typhimurium to respond to acid stress[60], suggesting that

NO and acid may work synergistically to prevent gastrointestinal infection. An additional possible source of NO in the gut is the commensal flora. Many bacteria are known to produce NO *in vitro* (discussed below), but it is unknown how much these pathways contribute to NO generation in the mammalian intestines. One study by Sobko *et al.*[61] demonstrated that human fecal flora generated considerable NO in response to added nitrite, suggesting that if some of the nitrite escapes decomposition in the stomach, the intestinal flora may use it to produce yet more NO distally.

Phylogenetic analysis of bacterial NO response networks reveals that many non-pathogens encode NO-detoxification mechanisms[62]. This suggests that although NO is an important component of innate immunity, it is in no way restricted to the setting of infection. Bacteria themselves can generate significant quantities of NO through multiple mechanisms. First, NO is an intermediate in the sequential reduction of nitrate to molecular nitrogen, a process termed denitrification. The reactions proceed through several enzyme complexes generally encoded together in operons, starting with the most oxidized substrate, nitrate (NO_3^-), then nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and finally molecular nitrogen (N_2), generally under anoxic conditions. Of course only the first two reactions are necessary for the generation of NO. In fact, only the first reaction, the conversion of nitrate to nitrite by nitrate reductase, is necessary if the ambient pH is sufficiently low to cause decomposition of nitrite to NO. The amount of NO generated during denitrification varies depending on the strain and culture conditions and is usually in the nanomolar range for most monocultures[63,64], but in at least one case involving *Rhodobacter sphaeroides* reached micromolar levels[65]. In addition to denitrification, a different process, nitrification, involves the conversion of ammonium to

nitrate *via* hydroxylamine (NH_2OH) and nitrite. Although this process does not directly produce NO, there is evidence that nitrifying organisms can often produce NO through a process termed aerobic denitrification, in which the nitrite produced from nitrification is reduced to NO. In addition, if nitrifiers and denitrifiers coexist in the same microenvironment, the nitrate and nitrite produced by nitrifiers from ammonia could be reduced to NO by denitrifiers[66,67]. Thus, one could conclude that the presence of active denitrification and nitrification in a microbial environment would require sensing and tolerance of NO to survive, even for coinhabitants that are not themselves denitrifiers or nitrifiers.

Intriguingly, some bacteria are capable of generating NO through a NOS isoform, termed bacterial NOS (bNOS). This was first discovered in *Nocardia*[68,69], and has been shown to be phylogenetically distributed within several Gram-positive species[70]. bNOS operates through the same mechanism as eukaryotic NOSs but usually lacks the reductase domain of the latter[70], with some exceptions[71]. The study of bNOSs has mostly been limited to its beneficial role in protection against oxidative stress[72,73] and antibiotic resistance[74], and thus the degree to which NO derived from bNOS might inhibit growth of other bacteria in polymicrobial environments has not been established.

Given that bacteria can be potent sources of NO, the concentration of NO in polymicrobial environments has been directly measured. High nanomolar and micromolar amounts of NO are present in soil[75] and in marine sediments[76], suggesting that bacteria must contend with considerable nitrosative stress in these environments. Using an innovative microsensor, Schreiber *et al.*[77] recorded micromolar quantities of NO within a biofilm derived from sewage. Considering these results along with data from

pathogenesis experiments, one can conclude that NO is nearly a ubiquitous stress for bacteria, both pathogens and non-pathogens.

C. Transcriptional responses to nitric oxide

Since NO is ubiquitous and reactive, it is no surprise that bacteria have tailored complex and specific transcriptional responses to it. Rodionov *et al.*[62] performed a useful bioinformatic analysis of all the known dedicated NO-responsive transcription factors and their DNA binding sites throughout the genomes of proteobacteria. They outline several well-described such transcription factors: NorR, NnrR, NsrR, HcpR, and DNR. They also discuss several NO-related enzymes the expression of which these regulators control: the flavohemoglobin Hmp*;* the flavorubredoxin NorVW; the hybrid cluster protein Hcp; denitrification complexes Nir, Nor, and Nos; the iron-sulfur cluster repair protein DnrN; and proteins of unknown function such as NnrS. This does not include all the known NO-responsive proteins in the bacterial kingdom, but presents some interesting conclusions to be made. The first is that the known NO tolerance proteins are phylogenetically widespread – the same mechanisms of NO tolerance appear to be used by many different species of bacteria. The second is that the genetic control of these proteins is also widespread, but that there is considerable "mixing and matching" of regulators with effectors. On the one hand this suggests that some of these regulators are functionally redundant. Indeed, virtually all the NO-sensitive regulators rely on formation of iron-NO complexes to alter protein conformation and regulate gene expression. Most, including FNR, DNR, NsrR, and NnrR, use a [4Fe-4S] cluster, although there are other

regulators, such as NorR, that use a different mechanism of iron-dependent NO sensing. On the other hand, the

coexistence of more than one regulator in the same species suggests that there may be nuances to the biochemical characteristics of these regulators that separate them

Table 1. Summary of NO-responsive regulators

Regulator	**Family**	**Repressor or Activator**	**Regulatory domain**
NorR	EBP	Activator	Non-heme iron
NsrR	CRP/FNR	Repressor	Fe-S cluster ([4Fe-4S] or [2Fe-2S])
NnrR	CRP/FNR	Activator	Unknown
DNR	CRP/FNR	Activator	Heme iron
HcpR	CRP/FNR	Activator	Unknown
NssR	CRP/FNR	Activator	Unknown
FNR	CRP/FNR	Repressor or activator	[4Fe-4S] cluster
Fur	CRP/FNR	Repressor	Non-heme iron

functionally. These detailed comparisons, which might explain the diversity in NO-binding transcription factors, remain to be made. A summary of the NO-responsive regulators discussed in this chapter is shown in Table 1.

One of the first NO-responsive transcriptional regulators to be described was NorR. NorR is a member of the enhancer-binding protein (EBP) family, which activate expression from sigma-54-dependent promoters[78]. This family of promoters is distinct from classical sigma-70-dependent promoters in that formation of an open complex and transcription requires ATP hydrolysis by an EBP such as NorR. EBPs often contain a

domain that regulates ATPase activity through ligand binding; in the case of NorR, this ligand is NO. In its sensing domain, NorR was found to contain a non-heme iron center that, in the presence of NO, forms a unique mononitrosyl iron complex, leading to ATP hydrolysis, binding of sigma-54[79], oligomerization[79], and open complex formation[80]. NorR binds the minimal consensus sequence GT-N_7-AC[81], and requires three copies of this consensus in *E. coli*[82]. However, the absence of a third binding site in other NorR-controlled promoters, such as *hmpA* in *V. cholerae*, suggests that the three-sequence motif is not always a requirement for regulation by NorR[40,62].

NsrR is another well-studied transcription factor that responds to NO and leads to expression of genes involved in NO tolerance. Unlike NorR, NsrR is a repressor, in that its deletion results in the constitutive expression of the genes it regulates. Also unlike NorR, which contains a single non-heme iron, NsrR contains an iron-sulfur cluster in its sensory domain. NsrR was first discovered as a regulator of denitrification genes in *Nitrosomonas europaea*[83]; in that study, nitrite was used as the activating signal, but the pH-dependence of downstream gene transcription suggested that NO might be the true ligand. Subsequently NsrR was identified as the major repressor of several important NO-responsive genes in *E.coli*, such as *hmp*[84], *hcp-hcr*[85], *nrf*[85] and *ytfE*[84] and that NO, not nitrite, is directly responsible for transcriptional activation[84,86,87]. Genome-wide expression studies in *E. coli*[88,89], *N. meningitidis*[90], and *S.* Typhimurium[91] have determined that NsrR could be a considered a "dedicated" NO regulator, in the sense that it always regulates genes involved in NO tolerance. However, ChIP-Chip analysis in *E. coli* revealed many NsrR binding sites in the chromosome in genes from diverse pathways[89,92]. There is also some recent evidence in *Bacillus subtilis* that suggests that

NsrR may control more genes than its canonical NO-response regulon and that this regulation may be insensitive to NO, further suggesting that NsrR may play a minor role in non-NO-dependent gene regulation[87,93].

There is some controversy as to the nature of the NsrR iron-sulfur cluster. The first purified form of NsrR, from *Streptomyces coelicolor*, was found to contain a [2Fe-2S] cluster[94], and subsequently NsrR from *Neisseria gonorrheae* was shown to contain a similar cluster[95]. However, the *Bacillus subtilis* NsrR contained a [4Fe-4S] cluster[96]. Regardless, all the biochemical studies reported a sensitivity of the cluster to NO that affected DNA-binding, suggesting that the mechanism of gene regulation is through cluster disruption, causing detachment from DNA and activation of transcription. NsrR is thus a critical and widespread regulator of NO-related genes.

DNR and NnrR are members of the CRP/FNR family of transcription factors that, like NsrR, transduce an NO signal into gene transcription[97]. NnrR and DNR were discovered first as NO-sensitive regulators in *Rhodobacter sphaeroides*[98,99] and *Bradyrhizobium japonicum*[100] (for NnrR) and *Pseudomonas aeruginosa*[101,102] (for DNR). However, the precise mechanisms of NO binding and signal transduction by NnrR and DNR are less well-defined than for NorR and NsrR. The crystal structures of DNR have only been obtained without prosthetic groups[103,104], but these were consistent with the possibility that the sensing domain of DNR contains a heme moiety rather than an iron-sulfur cluster or non-heme iron. Furthermore, DNR was shown to bind heme *in vitro*[105], and perturbation of the heme synthesis capabilities of the cell reduced the capacity of DNR to activate transcription of the *nor* promoter[106], suggesting that heme is indeed involved in NO sensing by DNR. In the case of NnrR, no specific mechanism of NO

sensing has been proposed. The regulatory targets of NnrR and DNR have thus far been limited almost exclusively to denitrification genes[62,107,108], suggesting that these regulators are specifically involved in activating denitrification. However, there is some evidence that heme synthesis is also in part regulated by NnrR and DNR[108,109]. Combined with the likelihood that heme is the functional prosthetic group of DNR and possibly NnrR, this suggests that these regulators might serve dual roles as NO sensors and heme sensors.

Another member of the CRP/FNR family that regulates NO-response genes is HcpR. It was initially described in an *in silico* analysis of sulfate-reducing bacteria as a likely regulator of the functionally nebulous gene *hcp* (discussed below) and sulfate and nitrate reduction genes[62,110]. Since its principal regulatory target, *hcp*, has been assigned a role in nitrosative stress tolerance, HcpR has thus also been assigned such a role. Furthermore, HcpR was definitively shown to regulate *hcp* expression in *Porphyromonas gingivalis*, a dental pathogen, and strains lacking *hcpR* were hypersensitive to NO[111]. Thus, HcpR has a definite role in responding to NO by upregulation of *hcp*. However, as the precise function of Hcp is unknown (see below), and no ligand for HcpR has been identified, much remains to be discovered about this regulator.

One final member of the CRP/FNR family of regulators is NssR, which has only been studied in *C. jejuni*. It regulates the expression of the globin Cgb which is important for resistance to NO in this organism. Unlike NsrR, NssR is an activator, in that deletion of the *nssR* gene abolishes transcription of its targets[112]. Furthermore, unlike other regulators, NO binding to NssR does not appear to affect DNA binding affinity[113], suggesting a different mechanism of transcriptional regulation for this regulator.

There are also many regulators of NO tolerance that are not "dedicated" NO sensors, in that they also control other aspects of bacterial physiology. Examples of these regulators include FNR and Fur. FNR, as the name indicates, is also a member of the CRP/FNR family of transcriptional regulators, and also contains a [4Fe-4S] cluster[114,115]. It controls the activity of over 100 promoters and is a critical mediator of the switch between anaerobic and aerobic metabolism, regulating genes involved in carbon utilization, alternative electron acceptor utilization, nucleotide synthesis, and transport[116–119]. Several of the genes regulated by FNR, however, include NO-tolerance genes such as *hcp* and *hmp*[120–122]. Since nitrate is a preferred bacterial electron acceptor in the absence of oxygen, and anaerobic respiration of nitrate is likely to generate NO, it is not surprising that FNR might also regulate NO-specific genes. Yet another iron-containing transcription factor that serves a canonically distinct role, yet also regulates the response to NO, is the ferric uptake regulator (Fur). Fur is a widely conserved factor that regulates the promoters of genes involved in iron uptake; when intracellular iron concentrations drop, the non-heme iron in its sensory domain is lost, causing the protein to release from DNA and allow transcription of iron import systems. Interestingly, the *hmp* promoter in *S.* Typhimurium was capable of titrating Fur away from a promoter normally bound by Fur, suggesting that Fur directly regulates *hmp* by binding to its promoter[123]. Moreover, *hmp* and other NO-responsive gene expression is heavily dependent on the presence or absence of Fur or the chelation of iron[124], again suggesting a strong link between the iron status of the cell and its responses to NO. As one might expect with a ferrous iron moiety in its regulatory domain, interaction with NO causes formation of a dinitrosyl iron complex[125,126], causing the Fe-NO-Fur complex to dissociate from DNA.

In summary, there are several different mechanisms used by bacteria for recognizing NO as a signal and causing the expression of NO tolerance genes. The common theme is the exploitation of the reactivity of NO with iron atoms. This is clearly highlighted by the presence of at least one iron atom in the regulatory domain of all these regulators, such as a heme, an iron-sulfur cluster, or a non-heme iron atom. Furthermore, studying the interaction of non-canonically NO-dependent regulators such as FNR and Fur with NO has thus illustrated that NO influences diverse physiologic pathways, such as iron metabolism and anaerobiosis.

D. Bacterial nitric oxide tolerance systems

Bacteria use multiple systems to detoxify NO under various conditions. Many directly scavenge NO, but others do not, and rather serve indirect roles in resisting some of the toxic effects of NO on bacterial physiology. Some of the most well-studied NO detoxification proteins are summarized in Table 2.

The most well-characterized system for NO removal is the flavohemoglobin Hmp. Hmp has been shown to be an important NO-detoxifying mechanism in several pathogenic bacteria, including uropathogenic *E. coli*[127], *S.* Typhimurium[128], *V. cholerae*[7,40], *P. aeruginosa*[129], and *S. aureus*[130], particularly in the presence of oxygen[131]. The principal function of Hmp is to catalyze the conversion of NO to nitrate. To accomplish this reaction, Hmp possesses three domains: a globin domain, which contains the heme active site, an oxidoreductase domain, which gathers electrons from NADH, and an FAD-containing domain, which transfers electrons to the active site heme through an FAD moiety. An interesting exception to this structure is a protein related to Hmp, the nitric

oxide-detoxifying hemoglobin Cgb from *Campylobacter jejuni*. Cgb resembles Hmp but lacks a flavin-containing or oxidoreductase domain. Cgb is still important for NO resistance in *C. jejuni*[132] despite not having a known redox partner to recycle the ferrous heme[133].

Suspicion that Hmp might be involved in NO stress first arose when its promoter in *E. coli* was found to be strongly upregulated by low concentrations of NO or higher concentrations of nitrite[122]. *Hmp* was subsequently deleted from *E. coli*, which rendered the cells hypersensitive to NO[134]. In that elegant study by Gardner *et al*., Hmp copurified in a fraction that contained an oxygenase activity, in which NO was converted to nitrate. The Hmp protein was then purified and shown to exhibit this activity. Subsequent to this study, some controversy arose as to the precise mechanism of Hmp catalysis. It was at first thought that O_2 bound the heme and that this oxy-heme species reacted with NO to form nitrate[134]. However, it was also realized that NO possessed much higher affinity for the heme than O_2, making it unlikely that the oxy-heme species would form under physiologic conditions. Thus, it was subsequently shown that in fact a nitrosyl heme species forms first, which takes on an nitroxyl (NO^-) character, and this species reacts directly with O_2 to generate nitrate in a reaction termed denitrosylation (as opposed to dioxygenation, in which oxy-heme forms first)[135]. This distinction was also consistent with another study had shown that Hmp could reduce NO to N_2O anaerobically[136]. In that case, it is thought that in the absence of O_2, the NO^- formed in the Hmp active site could dissociate, dimerize, and form N_2O. In both cases (denitrosylation and reduction), the formation of NO^- in the Hmp active site is required. This is enabled by the transfer of an electron from the ferrous heme iron to NO, generating a ferric iron. The ferrous heme is

Table 2. Summary of NO tolerance systems

Enzyme	Gene	Active Site	Substrates	Products	Species[a]
Flavohemoglobin	*hmp*	Heme	NO	NO_3^-, N_2O	*E. coli*, *S.* Typhimurium, *V. cholerae*, *P. aeruginosa*, *S. aureus*
Flavorubredoxin	*norV*	Dinuclear iron center (heme and non-heme iron)	NO	N_2O	*E. coli*, *S.* Typhimurium
Denitrifying nitric oxide reductase	*norB*	Dinuclear non-heme iron center	NO	N_2O	*M. catarrhalis*, *N. meningitidis*
Periplasmic cytochrome *c* nitrite reductase	*nrfA*	Heme	NH_2OH, NO, NO_2^-	NH_4^+	*E. coli*, *C. jejuni*, *W. succinogenes*
Hybrid cluster protein	*hcp*	Unknown (contains [4Fe-4S] and [4Fe-2O-2S] clusters)	Unknown (potentially NH_2OH)	Unknown (potentially NH_3)	*D. vulgaris*, *S.* Typhimurium
Single-domain globin	*cgb*	Heme	NO	NO_3^-	*C. jejuni*
Iron-sulfur cluster repair proteins	*ytfE*, *dnrN*, *scdA*	Dinuclear non-heme iron center	Damaged Fe-S clusters	Repaired Fe-S clusters	*E. coli* and *H. influenzae* (YtfE), *N. gonorrheae* (DnrN), *S. aureus* (ScdA)

[a]For which a definitive role in nitrosative stress resistance has been demonstrated.

restored by transfer of an electron from the oxidoreductase domain of the protein, which transfers an electron from NADH, to FAD, to the ferric heme in the globin domain.

In an attempt to outline broadly the interaction of a bacterial cell with NO, Robinson and Brynildsen[137] constructed a complex model based on the published kinetic values of myriad reactions of NO in *E. coli* physiology. Taking into account the diffusion

kinetics of NO into the cell, reactions with various organic and inorganic targets, and the *E. coli* detoxification machinery, the authors made the interesting conclusion that Hmp is responsible for virtually all the NO detoxification capability in an aerobic *E. coli* culture. In a strain lacking Hmp, the flavorubredoxin NorV (discussed below) played an increased role, but autoxidation was the primary mechanism for removal of NO. Thus, Hmp, in addition to being the most heavily studied NO detoxification enzyme, is probably the most important, at least in oxygenated environments.

Although Hmp is the best-studied mechanism of NO resistance in bacteria, there are others that are also important, particularly because Hmp has decreased activity in low-oxygen conditions. One oxygen-independent NO resistance factor is the flavorubredoxin, encoded in many bacteria by the *norV* gene. Flavorubredoxins are so named because they are composed of an FMN-containing domain and a domain with a non-heme mono-iron site that is homologous to a family of proteins called rubredoxins, but also contains a di-iron site that is its catalytic active site[138]. A link to NO tolerance came when *E. coli*, which lacks a denitrifying NO reductase, was found to possess an NO-inducible NO reductase activity that was both independent of Hmp and sensitive to O_2[139]. The genes responsible were then shown to be *norV* and its operon companion *norW,* which encodes a cognate oxidoreductase that transfers electrons from NADH to NorV *via* an FAD moiety[140]. *E. coli* cells lacking NorVW were attenuated for survival in the presence of human macrophages[141], suggesting a possible role for NorVW in infectious settings. However, although NorV was important for resistance to NO by *S.* Typhimurium *in vitro*[142], no further role has been assigned to NorVW in infectious settings, indicating that it is less important during pathogenesis than Hmp. Given the

sensitivity of NorV to oxygen and oxidative stress, it is possible that the co-occurrence of oxidative species such as hydrogen peroxide and peroxide with NO could reduce the role of NorV during inflammation.

Although structurally and mechanistically distinct from flavorubredoxins, the denitrifying nitric oxide reductases (NORs) also catalyze the reduction of NO to N_2O[143]. Instead of deriving electrons from NADH, NORs derive electrons from cytochrome *c*-containing proteins (in the case of the cNOR type) or quinols (in the case of qNOR). Furthermore, NOR complexes possess a distinct active site, composed of one heme and one non-heme iron[144], as opposed to the entirely non-heme di-iron active site of flavorubredoxin. One might consider the NOR complexes as serving housekeeping roles, as they are often co-transcribed with other members of the denitrification pathway. However, roles for resistance to exogenous nitrosative stress for cNOR has been demonstrated in both *Moraxella catarrhalis*[145] and *Neisseria meningitidis*[146], suggesting a cooption of these "housekeeping" complexes for stress resistance.

A surprising contributor to NO detoxification is the cytochrome *c* periplasmic nitrite reductase NrfA. This enzyme is commonly regarded primarily for its role in converting nitrite to ammonium through a six-electron reduction (a process which, depending on the circumstances, might also result in an indirect decrease in ambient NO by preventing the pH-dependent decomposition of nitrite)[147]. This mechanism is distinct from the copper-containing nitrite reductase Nir found in denitrifiers, which converts nitrite to NO. The NrfA reaction mechanism is thought to proceed through multiple steps involving transfer of electrons from its five hemes to its substrate, with NO and hydroxylamine (NH_2OH) intermediates formed. Consequently, it has been found that

NrfA can directly reduce NO and hydroxylamine to ammonium, thus making NrfA an NO-protective enzyme. In addition, NrfA is located in the periplasm, whereas Nir is embedded in the cytoplasmic membrane, suggesting a spatially different role in NO control. Growth of *E. coli* under anaerobic conditions in the presence of NO is strongly inhibited in the absence of NrfA[142,148] as is reduction of NO[149]. In addition to *E. coli*, a role for NrfA in resistance to NO stress has been demonstrated in the epsilonproteobacteria *C. jejuni* and *Wolinella succinogenes*, though no direct link with pathogenesis has been demonstrated[150,151].

An interesting though poorly characterized agent of resistance to nitrosative stress is the hybrid cluster protein Hcp. This protein was initially named prismane because it was thought to contain a [6Fe-6S] cluster; however, subsequent analysis has shown that it instead contains one [4Fe-4S] cluster and one unusual [4Fe-2O-2S] cluster[152]. Like NorV, Hcp is frequently (though not always) encoded with a cognate oxidoreductase, Hcr, which regenerates the redox state of its active site[152]. Because it is regulated by NO-sensitive transcriptional regulators, it was thought that it might have a role in NO tolerance. In *Desulfovibrio vulgaris*, for instance, a strain lacking *hcp* was hypersensitive to NO, but did not exhibit a defect in NO scavenging[153]. Deletion of *hcp* from a strain of *S.* Typhimurium already lacking *nsrR* and *hmp* resulted in a delay in oxygen and NO scavenging. However, slight growth defects have also been observed for strains of *E. coli*[154] and *Clostridium perfringens*[155] lacking *hcp* in the presence of peroxide stress, suggesting a broader function for Hcp beyond strictly NO stress. More recent evidence has suggested a specific role for Hcp in detoxifying hydroxylamine (NH_2OH), which might be generated during nitrosative stress, perhaps as an intermediate in the reduction

of nitrite or NO to ammonium by NrfA. Indeed, purified Hcp from *E. coli*[156] and *Rhodobacter capsulatus*[157] demonstrated hydroxylamine reductase activity, and overexpression of *R. capsulatus hcp* in *E. coli* promoted growth on hydroxylamine[157]. However, no definitive role for Hcp has been concluded *in vivo* to date, and the true function of this protein remains elusive.

Although the vast majority of genes upregulated in response to NO that have been studied are involved in direct NO or RNS removal, there are also mechanisms for resisting the growth inhibition caused by NO without actually removing it. An example of such a protein is YtfE, which was first found to be important for resisting anaerobic NO stress in *E. coli*[158]. It was then shown that activity of iron-sulfur cluster proteins such as fumarase and aconitase were hypersensitive to disruption of their iron-sulfur clusters in a *ytfE* mutant; furthermore, purified YtfE could restore function to damaged iron sulfur clusters, suggesting a direct role in repair[159]. YtfE contains a non-heme di-iron center but its mechanism of repair is unknown[160]. Its role in resistance to macrophage-derived NO in *H. influenzae* suggests a possible role in virulence[161], but this has not yet been demonstrated by animal infection model. Along with YtfE, two other factors appear to repair NO-damaged iron sulfur clusters: DnrN in *N. gonorrheae* and ScdA in *S. aureus*[162]. In fact, ScdA was sufficient to complement the defect introduced by deleting *ytfE* in *E. coli*, suggesting that repair of iron-sulfur clusters is an important and widespread part of NO tolerance.

To summarize, there are several mechanisms for resisting the deleterious effects of NO on bacterial growth, most of which involve converting NO to less reactive nitrogen oxides, such as nitrate, nitrous oxide, or ammonium. Some proteins resist NO

stress by repairing the damage of NO, such as YtfE. All of these proteins are important for growth *in vitro* and are often important during infection.

E. Enzymatic targets of NO

The inhibition of bacterial growth by NO appears to be mainly through inhibition of metabolic enzymes, as opposed to other essential cellular functions such as macromolecule synthesis or cell division. This fits with the general chemical properties of NO. Transition metals, hemes, and other redox-active centers that react with NO are commonly found within metabolic enzymes, which use redox potentials to interconvert carbon molecules or transfer energy to usable forms like ATP. Defining the precise enzymatic targets within bacterial metabolism is a relatively new endeavor, however; although many interesting targets have been found, there are likely many more that remain to be discovered. Importantly, although many enzymes can be shown to bind NO *in vitro*, only a few have been identified as responsible for causing growth arrest *in vivo*.

In an elegant study in *Salmonella*, Richardson *et al.* discovered several targets of NO[13]. They found that NO caused a methionine and lysine (MK) auxotrophy, suggesting a metabolic cause for growth inhibition, in particular the TCA cycle. The authors identified aconitase as the most NO-sensitive TCA cycle enzyme, as had been reported previously[12]. Surprisingly, however, the growth inhibition and MK auxotrophy caused by NO actually resulted from the inhibition of the enzyme dihydrolipoamide dehydratase, or LpdA. LpdA oxidizes dihydrolipoamide to lipoamide, a cofactor necessary for the function of several TCA cycle enzymes; the MK auxotrophy was because of an inability of lipoamide-dependent TCA cycle enzymes to produce succinate. The growth defect in

the presence of NO could be restored by adding succinate back to the culture. In *S. aureus*, another study by Richardson *et al.*[42] found that not only did NO inhibit respiration, but also identified the fermentative enzymes pyruvate formate lyase (PFL) and pyruvate dehydrogenase (PDH) as targets of NO. Thus, in the absence of respiration and fermentative pathways, there were few options left for the organism to regenerate NADH. The only remaining pathway was lactate dehydrogenase (LDH), an enzyme resistant to NO; they found a second LDH enzyme to be specifically upregulated in the presence of NO in order to meet the additional demand for this pathway under NO stress. The mechanism of inhibition of these enzymes is unknown. In *E. coli*, Hyduke *et al.*[14] noticed that supplementation with isoleucine, leucine, and valine (ILV) completely reversed NO-dependent growth inhibition. This was due to inhibition of dihydroxyacid dehydratase, a dehydratase that is hypersusceptible to NO due to DNIC formation and is required for the synthesis of ILV. Interestingly, however, the same phenotype was not observed in *Salmonella*[13], suggesting that the relevant pathways of inhibition by NO vary from species to species, even within the same family of bacteria.

Other studies have revealed transition metals other than iron as additional targets for NO. In *S.* Typhimurium and *Borrelia bergdorferi*, the causative agent of Lyme disease, treatment of cells with NO resulted in the release of zinc from zinc metalloproteins[163,164]. In *B. bergdorferi*, this resulted in the inhibition of fructose-1,6-bisphosphate aldolase, an enzyme of glycolysis; however, it is uncertain how much inhibition of this particular protein contributed to growth inhibition. In *S.* Typhimurium, zinc release correlated with DNA damage and cell filamentation, suggesting that zinc metalloproteins are an additional target of NO. Djoko *et al*.[165] found that intracellular

copper exacerbates the toxic effects of nitrosative stress in *N. gonorrheae*. The mechanism is not known, but given the fact that copper toxicity targets dehydratases in the same manner that NO does[166], it is possible that shared targets account for this effect. On the other hand, NO can nitrosylate copper[167], it is possible that nitrosyl copper complexes may also play a role.

Thus, although extensive work has characterized *in vitro* biochemistry of NO, relatively few metabolic targets of NO have definitively been proven to result in growth arrest in bacteria. These include enzymes such as dihydrolipoamide dehydratase, pyruvate formate lyase, pyruvate dehydrogenase, and dihydroxyacid dehydratase.

F. Summary

Nitric oxide biology is a relatively new field; even newer is the study of how bacteria respond to NO. Remarkable progress has been made to identify sources, targets, and responses to NO. Some common themes have emerged. First, the direct targets of the NO radical itself appear to be restricted to transition metals in primarily metabolic enzymes; the reaction of the unpaired electron of NO with the *d* orbital of iron and other metals leads to formation of nitrosyl species. In some cases this leads directly to enzyme inhibition, such as for iron-sulfur cluster-containing proteins like the dehydratases aconitase and dihydroxyacid dehydratase, or for heme-iron enzymes such as cytochromes *bo'* and *bd*. These metal nitrosyl complexes can lead downstream to nitrosothiol formation in a variety of metabolic and non-metabolic proteins, such as in transcription factors OxyR and SsrB or virulence effectors such as *C. difficile* toxin. In contrast, the oxidative stressed caused by RNS is not due to NO itself but due to reactive nitrogen

oxides such as peroxynitrite. Second, NO stress is ubiquitous. Although studied primarily in the human host, particularly when derived from iNOS, it has become clear that bacteria themselves are also a considerable source of NO, and that virtually all bacteria have the capacity to respond to it. Third, bacteria respond to the presence of NO through iron-containing transcription factors that, upon binding to NO, lead to the upregulation of genes specifically dedicated to NO removal. Deleting these response systems frequently leads to defects in growth or host colonization.

Responding to NO can thus be considered a critical component of bacterial physiology. However, much remains to be discovered about bacterial NO biology, especially because the effects of NO have only been intensely investigated in a handful of organisms. Furthermore, only a few metabolic targets of NO have been definitively described. And there are many bacterial enzymes upregulated by NO for which we only have a preliminary sense of their function or mechanism. Far more investigation is needed to comprehend this vital and important bacteria-stress interaction.

G. A brief introduction to *Vibrio cholerae* and cholera

Cholera is a diarrheal illness caused by the ingestion of drinking water contaminated with *Vibrio cholerae* organisms. Because modern water filtration technology has been implemented throughout the developed world, cholera is no longer a problem there; however, it remains a large problem in underdeveloped countries, ranging from Bangladesh and India to sub-Saharan Africa to Haiti. The World Health Organization (http://www.who.intl) estimates that there are 3-5 million cases and over 100 000 cholera deaths per year worldwide. Most cases are due to a strain designated El

Tor, named for the town where it was first isolated, and exhibit the O1 serotype. Fewer infections are due to strains of the O139 serotype. Cholera is characterized by a voluminous non-bloody, watery diarrhea[168]. The loss of volume can be so great that the usual cause of death due to cholera is dehydration. Treatment with oral rehydration salts (ORS) is effective at preventing mortality, but does not prevent or cure the diarrhea itself. There are two vaccines in existence, both of which use killed whole cells. Neither has been demonstrated to provide protection for more than six months; in contrast, having had cholera is thought to be protective for years or longer[169].

Infection begins when the organism is ingested from contaminated drinking water, a problem that is particularly common in urban slums and refugee camps. First, as

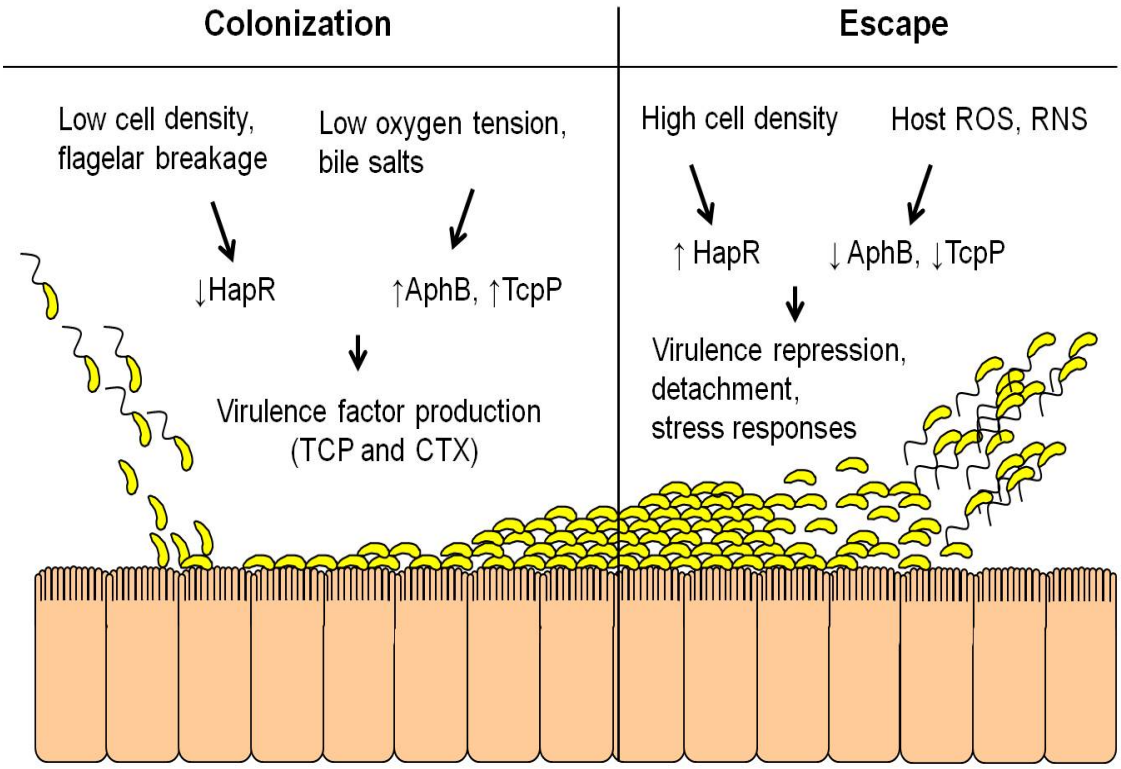

Fig. 2. Summary of the *Vibrio cholerae* infectious cycle.

with all orally transmitted gastrointestinal pathogens, the organisms must survive the acidity of the stomach. *V. cholerae* is surprisingly sensitive to acid[170], and it is thought that aggregation in biofilms aids resistance to acid. After reaching the intestines, the *V. cholerae* infectious cycle can be roughly broken into two phases: colonization and escape (Fig. 2). Colonization involves integrating multiple signals to lead to upregulation of virulence genes. First, only a few organisms survive transit to the small intestines, causing a drop in cell density. This causes the repression of the quorum sensing repressor HapR, allowing expression of virulence genes[171]. Breakage of flagella in the host mucus also leads to HapR repression[172]. In addition, a combination of bile salts and low oxygen tension in the small intestines causes changes to the redox state of virulence gene transcription factors AphB[173] and TcpP[174], leading to production of the two main virulence factors of *V. cholerae*: cholera toxin (CT) and the toxin-coregulated pilus (TCP). Both are required for virulence in humans[175]. The exact function of TCP is not known, but is thought to cause aggregation of bacteria, which is in some way required for growth in the intestines. CT, however, is the component directly responsible for virulence[168]. It is composed of the active A subunit and five carrier B subunits that bind to its receptor GM_1 ganglioside on the small intestinal epithelium. Upon entry into the cell, the A subunit causes ADP-ribosylation of a G_s protein, leading to constitutive cyclic AMP production and chloride excretion, and thus voluminous diarrhea.

Colonization of the host with *V. cholerae* is self-limiting, so long as the dehydration is not fatal. Late during infection, after virulence genes have been expressed and the bacteria have replicated, the second phase of infection begins when *V. cholerae* undergoes a process termed the “mucosal escape response.” Virulence genes are

repressed and stress resistance genes are upregulated as the bacteria prepare to exit into the environment[176]. It is unclear how this process is regulated, but involves the stationary phase sigma factor RpoS[177]. It may also involve an increase in cell density, causing activation of HapR, as well as the simple oxidation of the same redox-sensitive transcription factors that were reduced upon entry into the intestines. The re-oxidation could occur as a result of an increase in oxidative species produced by the host's immune system during infection. Although it was previously thought that there was no inflammation involved in cholera, and thus no reactive oxygen and nitrogen species, recent studies have indicated that there is a low-level immune infiltrate in the intestines of cholera patients[178–182]. Furthermore, upregulation of iNOS in duodenal tissue and an increase in serum and urine nitrite has been observed in patients with cholera[183,184], suggesting that nitrosative stress is indeed encountered by *V. cholerae* not only during transit through the stomach (as discussed earlier) but as a result of the host immune system. The reactive nitrogen species produced by the host could both trigger the activation of stress response genes in *V. cholerae* such as *hmpA* but also the mucosal escape response.

V. cholerae possesses the remarkable property of being an intestinal pathogen despite not being a part of any normal intestinal flora. This is rare: most other well-known intestinal bacterial pathogens such as *E. coli*, *S. enterica, S. flexneri, C. jejuni, C. difficile,* and *B. cereus* are all found to naturally inhabit human or animal gastrointestinal tracts. In contrast, *V. cholerae* inhabits various aquatic ecosystems, including marine, brackish, and freshwater environments, and is thought to normally reside in biofilms on the surface of microscopic crustaceans called copepods[185,186]. Obviously, this is a rather

different microenvironment from the human gut. Most crustacean exoskeletons are composed of a polysaccharide of N-acetylglucosamine called chitin, and there are otherwise likely to be few carbon sources available to *V. cholerae*. In the gut, there is turnover from ingested food and an enormous amount of metabolic activity from the resident flora – potentially a far more complex environment. And yet *V. cholerae*, when ingested by an unfortunate human, can colonize the small intestines, replicate, and cause one of the most severe dehydrating diarrheal syndromes known to man. This dramatic shift in environments must require great metabolic flexibility, which is not well-understood.

H. Statement of hypotheses

Vibrio cholerae encodes a limited repertoire of NO-response genes. These encode the regulatory protein NorR, the flavohemoglobin Hmp (renamed HmpA in the case of *V. cholerae*) and the protein of unknown function NnrS. There are putative NorR-binding sites upstream of both *hmpA* and *nnrS*[62]. Thus, I hypothesized that in *V. cholerae*, NorR regulates the expression of *hmpA* and *nnrS* in response to NO. Given that NO is generated by the host during cholera infection[183], I further hypothesized that at least HmpA and NorR, and perhaps NnrS, might be important for resistance to NO and survival in the host intestines. These hypotheses were addressed in the experiments in Chapter Two. Given the handful of *in vitro* and *in vivo* studies on the effects of NO on bacterial metabolism in such organisms as *E. coli* and *S.* Typhimurium, I hypothesized that NO would have wide-ranging effects on metabolism in *V. cholerae,* and that there may be as-yet undiscovered targets of NO in bacterial metabolism. I also hypothesized

that NnrS might have a role in specific situations in response to NO. These hypotheses were addressed in the experiments in Chapter Three.

CHAPTER TWO: The NorR regulon is critical for resistance to nitric oxide and sustained colonization of the intestines

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A. Introduction

Vibrio cholerae causes the disease cholera and represents a large global health problem in impoverished countries. Cholera continues to cause epidemics and has the ability to spread to new locations, having caused over 4,500 deaths in Haiti since the earthquake in 2010 [187]. Cholera is characterized by profuse dehydrating diarrhea and can be treated with vigorous oral rehydration and supplementary antibiotics. Despite these interventions, cholera remains a source of considerable worldwide morbidity and mortality. Cholera toxin, which directly causes secretory diarrhea, and its transcriptional regulation are well-understood [188,189]. However, the bacteria that cause cholera, or any intestinal infection, encounter chemical and physical barriers during the establishment and maintenance of colonization. The host-derived stresses that *V. cholerae* encounters while infecting a host are not well-characterized, and even less well-understood is how *V. cholerae* senses these stresses.

One of the toxic chemical species elaborated by the host during bacterial infection is nitric oxide (NO). NO is a toxic radical, disrupting the function of proteins containing cysteine residues, enzymes catalyzing iron-dependent reactions, and members of the

electron transport chain [190]. Furthermore, NO reacts with other small molecules produced by the immune system to form other toxic reactive nitrogen species (RNS) such as nitroxyl and peroxynitrite [191,192]. In the host, NO is generated by acidified nitrite in the stomach and by enzymes of the nitric oxide synthase (NOS) family, which derive NO from arginine [57]. There are three isoforms of NOS, and the form associated with the immune system is inducible NOS (iNOS), which is capable of generating large quantities of NO in an inflammatory setting. Epithelial cells are known to express iNOS, as are immune cells such as macrophages and dendritic cells [57,193–195]. Clinical studies have demonstrated that patients with cholera have increased NO metabolites in serum and urine, as well as an increase in the expression of iNOS in their small intestines during cholera infection, suggesting that *V. cholerae* encounters NO during infection of humans [183,184,196]. To cope with NO produced during infection, many pathogenic bacteria have evolved mechanisms to convert NO into other, less toxic, nitrogen oxides [190]. The only enzyme predicted to have this activity in *V. cholerae* is HmpA (VCA0183), a member of the flavohemoglobin family of enzymes that is well-characterized in other bacteria such as *Escherichia coli* [62]. In low-oxygen conditions such as one might find in the gut, HmpA catalyzes the conversion of NO to N_2O or NO_3^-, both of which are less toxic to the bacterium [197]. Within HmpA is an iron-heme moiety that directly catalyzes the reaction as well as a flavin group and NADPH oxidase domain that mediate transfer of the electrons to and from NO [197]. HmpA homologs are important for detoxification of NO during infection of other bacterial pathogens such as *E. coli, Yersinia pestis, Staphylococcus aureus,* and *Salmonella enterica,* as well as *V. fischeri* colonization of its squid host [128,130,198–200]. In *V. cholerae*, *hmpA* emerged as a gene expressed in both infant mice and

in rabbits in two different *in vivo* screens [201,202]. A recent study demonstrated that in the infant mouse model of cholera infection, HmpA was important for resisting NO generated in the stomach from acidified nitrite [7]. However, since the suckling mouse model of cholera is limited to 24-hour studies, it is unknown whether NO might be generated later during infection and present a second NO barrier to *V. cholerae* infection beyond the stomach. Furthermore, it remains unknown how the expression of *hmpA* is regulated. Here we demonstrate that *hmpA* expression is controlled by the NO sensor NorR (VCA0182), a predicted σ^{54}-dependent transcriptional regulator [80]. A previous bioinformatic study predicted a NorR-binding site upstream of *hmpA,* and also upstream of one other gene, *nnrS* (*vc2330*). The function of NnrS, a membrane protein, is previously unknown but may have a role in metabolism of nitrogen oxides [203]. We also demonstrate that expression of *nnrS* is controlled by NorR, and that *nnrS* is important for resisting NO *in vitro* when *hmpA* is deleted. In addition, we show that *hmpA* and *norR* are critical for long-term colonization of the adult mouse intestines.

B. Materials and methods

Bacterial Strains and Plasmids. The parent strain used in this study was *V. cholerae* O1 El Tor C6706. Sucrose counterselection [204] was used to generate all clean deletions. Promoter-*lacZ* transcriptional fusions were generated by cloning the approximately 500 bp proximal to the ATG start codon upstream of a promoterless *lacZ* gene in a low-copy plasmid [205]. Strains were propagated in LB containing appropriate antibiotics at 37°C, unless otherwise noted.

Gene Expression Studies. For *in vitro* gene expression studies in microaerobic conditions, saturated overnight cultures in LB were inoculated 1:100 into minimal medium containing 79 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.65 mM $MgSO_4$, 0.07 mM $CaCl_2$, 0.018 mM $FeSO_4$, 0.013 mM $MnSO_4$, and 0.2% glucose (w/v) in filled, sealed glass vials. After four hours of growth, 50 μM DEA-NONOate (from a 50 mM stock in DMSO) (Cayman Chemical) was added to the cultures. Diethylamine was used as a negative control. Two hours later, the OD_{600} of the cultures was measured and a Miller assay [206] was used to measure LacZ production. For experiments in LB, bacteria were inoculated 1:1000, with 2.5 hours of growth prior to NO addition and 1.5 hours growth thereafter.

Growth Curves. To measure *in vitro* growth, strains were inoculated from saturated LB cultures 1:100 into minimal media (described above) in 0.25 mL in a 96-well plate. Plates were sealed with an optically clear film and incubated at 37ºC while the OD_{600} was measured every ten minutes by an automated plate reader (Bio-Tek Synergy HT). To

measure the effect of NO on growth, 10 μM DETA-NONOate (Cayman Chemical) was included.

***In vivo* Mouse Colonization Studies.** Mouse colonization competition studies were performed using a protocol modified from [207]. Six week-old C57bl/6 or C57bl/6 *iNOS*$^{-/-}$ (Strain B6.129P2-*Nos2*^{tm1Lau}/J) mice were obtained from the Jackson Laboratory. Two days before inoculation, 0.5% (w/v) streptomycin and 0.5% (w/v) glucose was added to the drinking water; this treatment was maintained throughout this experiment, with regular replacement every 2-3 days. One day before inoculation, food was removed from the cages. On the day of inoculation, stomach acid was neutralized with 0.05 mL 10% (w/v) $NaHCO_3$ by oral gavage. Twenty minutes later, 0.4 mL of saturated cultures of each of the two strains were mixed with 0.2 mL 10% (w/v) $NaHCO_3$, and 0.1 mL of this mixture was administered to each mouse by oral gavage. The size of the inoculum was enumerated by serial dilution and plating on LB plates containing 0.1 mg/mL streptomycin and 0.04 mg/mL 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal). Food was replaced two hours after inoculation. On days 3, 5, and 7 post-inoculation, 2-3 fecal pellets were collected from each mouse, resuspended in LB, then serial diluted and plated on plates containing streptomycin and X-gal. The competitive index was calculated as the ratio of mutant to wild-type colonies normalized to the ratio contained in the inoculum. At the end of the experiment, mice were sacrificed and competitive indices calculated from homogenates of their small intestines.

Statistical Analyses. For all experiments, a two-tailed student's t-test was performed to determine statistical significance. Data points below the limit of detection were considered at the limit of detection for statistical analyses. A difference in means was considered statistically significant if $p < 0.05$.

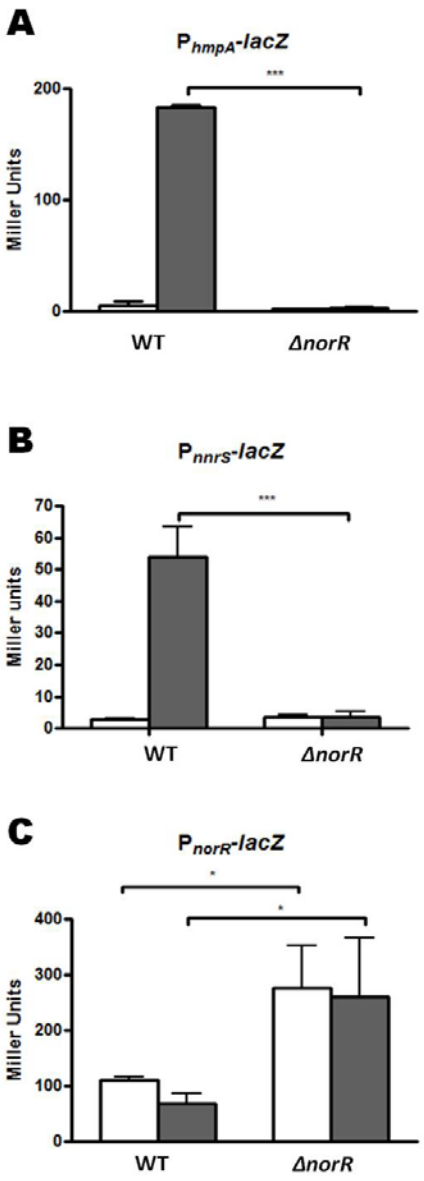

Fig. 3. The effect of NO and NorR on expression of NO detoxification genes. Strains containing a promoter-*lacZ* reporter were grown in minimal medium in microaerobic conditions. Activity of the *hmpA* (A), *nnrS* (B), or *norR* (C) promoter was measured after addition of 50 μM control compound diethylamine (white bars) or NO donor DEA-NONOate (gray bars) and reported as Miller units. Experiments were performed in wild-type strain C6706 or a strain containing a clean deletion of the entire *norR* ORF. Error bars represent standard deviation. * indicates P<0.05 and *** indicates P<0.001 for experiments performed in triplicate.

C. Results

NorR is required for NO-inducible expression of *hmpA* and *nnrS* and represses its own expression.

The regulatory networks controlling NO detoxification vary widely between bacterial species [62]. *V. cholerae* has a limited repertoire of NO-related genes that includes *hmpA*, encoding a flavohemoglobin, *nnrS*, a widely conserved gene of previously unknown function, and *norR*, encoding a NO-responsive DNA-binding regulatory protein [80,124,208]. A computational study predicted that NorR would control expression of *hmpA* and *nnrS* [62]. This is different from enteric species and other *Vibrio* species, in which NorR controls or is predicted to control expression of the NO reductase *norVW*. There is no *norVW* homolog present in the *V. cholerae* chromosome. To determine the effect of NO on expression of *hmpA*, *nnrS*, and *norR*, we constructed transcriptional reporter plasmids containing the promoters of these

genes fused to the *lacZ* gene. Strains grown in minimal medium had low background transcription of *hmpA* and *nnrS*, but the addition of 50 μM of the NO donor DEA-NONOate resulted in a dramatic upregulation of both these promoters (Fig. 3A and 3B). DEA-NONOate releases NO over a short period of time. In aerobic conditions, there was no upregulation of either *hmpA* or *nnrS* promoters (data not shown), likely because the NO diffused out of the system or reacted with O_2. In the closed-tube microaerobic conditions of this experiment, 50 μM DEA-NONOate did not inhibit growth of any of the strains. These experiments were performed in minimal media because the background activity of the *hmpA* and *nnrS* promoters was low. However, performing the experiments in LB media in microaerobic conditions still resulted in >10-fold upregulation of both promoters (Fig. S1). In a *norR* deletion background, however, virtually no upregulation of *hmpA* or *nnrS* reporters was observed (Fig. 3A and 3B), suggesting that NorR is absolutely required for the activation of both these promoters. Taken together, these data suggest that NorR controls the NO-inducible upregulation of both *hmpA* and *nnrS.* We further investigated how *norR* is regulated by comparing *norR-lacZ* expression in wild-type and *norR* mutants with or without NO. The activity of the *norR* promoter was not altered by the addition of NO (Fig. 3C). Interestingly, the *norR* promoter activity was significantly increased in the *norR* background (Fig. 3C). These data suggest that NorR represses its own expression independent of NO.

***norR, hmpA,* and *nnrS* are critical for NO resistance *in vitro*.** A recent study by Davies *et al.* [7] implicated *hmpA* as an important gene for resistance to NO under aerobic conditions in the presence of high (millimolar) concentrations of NO donors. To examine

whether the NorR regulon, including *hmpA,* is important for resistance to NO in a microaerobic environment more similar to what bacteria are likely to encounter during infection of the small intestines, we performed growth curve assays in sealed 96-well plates. We added 10 µM DETA-NONOate, which continuously releases NO with a half-life of 20 hours to the cultures and measured the OD_{600} every ten minutes at 37ºC in a plate reader. The *hmpA*, *norR*, and *nnrS* single mutant, as well as *hmpA/nnrS* double mutant strains were examined. None of these mutations conferred a growth defect in the absence of NO (Fig. 4A). Similar to the results of Davies *et al*. where bacteria were grown aerobically, deletion of *hmpA* resulted in a growth defect in the presence of NO (Fig. 4B). Deletion of *norR* resulted in a more severe defect, and interestingly, deletion of both *hmpA* and *nnrS* resulted in the most severe phenotype. The *nnrS* single mutation, however, did not result in an NO-sensitive defect. These data suggest that the NorR regulon, containing *hmpA* and *nnrS*, is critical for resistance to NO. Similar but less dramatic results were obtained

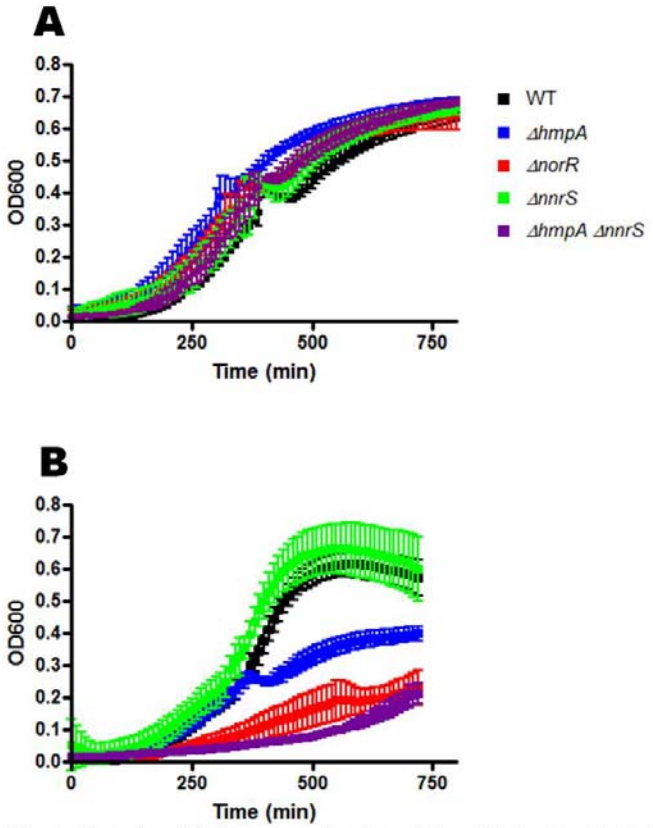

Fig. 4. The role of the NorR regulon in resisting NO *in vitro*. To test the effect of NO on growth of mutant strains, strains were inoculated in triplicate in a sealed 96-well plate in minimal media at 37ºC in the absence (A) or presence (B) of 10 µM DETA-NONOate. Growth was measured by OD_{600} every ten minutes. Strains used were black – wild-type; blue – *ΔhmpA*; green – *ΔnnrS*; red – *ΔnorR*; purple - *ΔhmpA ΔnnrS*. Error bars indicate standard deviation.

using 10 μM spermine-NONOate, which releases NO with a half-life of approximately 39 minutes (data not shown). Almost no defect could be detected with micromolar concentrations of DEA-NONOate, which releases NO with a half-life of approximately two minutes. This suggests that during continuous exposure to NO, such as might be found during infection, physiologically relevant concentrations of NO [7,209] are sufficient to affect growth of *V. cholerae.* The importance of *nnrS* is revealed only in an *hmpA* mutant background, suggesting that it may serve a redundant role in NO detoxification. Alternatively, NnrS may catalyze the detoxification of a related reactive nitrogen species. We tested whether *nnrS* mutants were more sensitive to peroxynitrite ($ONOO^-$), Angeli's salt (a donor of nitroxyl anion, NO^-), and nitrite (NO_2^-) but found no difference from wildtype (data not shown), suggesting that the role of *nnrS* in resistance to RNS is important but subtle. The function of *nnrS* is a subject of ongoing research.

***prxA* expression is induced by H_2O_2 and not by NO, and is not important for NO resistance in microaerobic conditions.** Davies *et al.* recently found that deletion of *prxA*, a gene encoding a putative peroxiredoxin, resulted in sensitivity to NO [7]. We examined the NO sensitivity of a strain in which *prxA* and the adjacent gene *vc2638* from the same operon were deleted, in microaerobic conditions in minimal media. In their study, a high concentration of DEA-NONOate (1 mM) was used. This results in the full release of 2 mM NO over a period of approximately 10 minutes in aerobic conditions. However, in microaerobic conditions in minimal media containing 10 μM DETA-NONOate, conditions which significantly inhibited growth of strains lacking *hmpA* or *norR* (Fig. 2B), there was no detectable growth defect in *prxA* mutant (Fig. 5A and 5B).

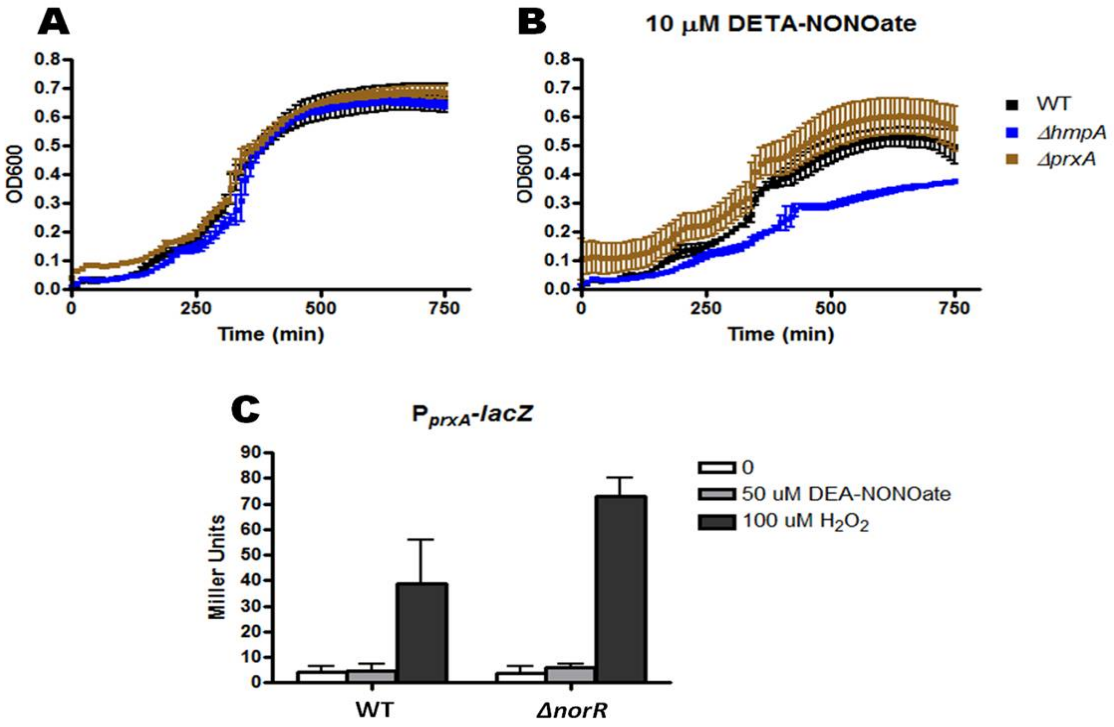

Fig. 5. The importance of *prxA* in response to NO and H_2O_2 in microaerobic conditions. The growth of a strain of *V. cholerae* lacking *prxA* and its adjacent gene *vc2638* was compared to wild-type and *ΔhmpA* in the absence (A) or presence (B) of 10 μM DETA-NONOate in microaerobic conditions in minimal medium. In (C), strains of wild-type or *ΔnorR V. cholerae* containing a reporter plasmid encoding *lacZ* fused to the *prxA* promoter were grown in minimal media. After addition of 50 μM DEA-NONOate (gray bars), 100 μM H_2O_2 (black bars), or nothing (white bars), activity of the *prxA* promoter was measured by Miller assay.

To study whether *prxA* expression could be induced by NO, we constructed a reporter consisting of the *prxA* promoter fused to *lacZ*. The addition of 50 μM DEA-NONOate, which caused dramatic upregulation of *hmpA* and *nnrS* (Fig. 3), did not result in activation of the *prxA* promoter (Fig. 5C). However, addition of 100 μM H_2O_2 did result in upregulation of the *prxA* promoter in both wild-type bacteria and a strain lacking *norR*. The *prxA* gene is located divergent from the *oxyR* gene, which has been shown in other bacteria to mediate responses to oxidative stress [210]. We speculate that the results of Davies *et al*. resulted not directly from NO but from other species generated in aerobic conditions during a burst of millimolar concentrations of NO from a short-lived NO donor.

***norR and hmpA* are critical for sustained colonization of the adult mouse intestines.** Previous experiments [7] tested the effect of *hmpA* deletion in an infant mouse model and demonstrated a moderate defect (competitive index = 0.13) that was partially dependent on the presence of acidified nitrite in the mouse stomach. We repeated these experiments and found a similar competitive index of 0.40 ± 0.01, confirming these results. However, the infant mouse model only allows for a 24-hour experiment and is not suitable for studying extended survival of a bacterial strain in the intestines. The incubation time of cholera infection is typically 2-3 days and symptoms can last for long after this time [187], suggesting that *V. cholerae* may be exposed to challenges such as RNS for prolonged periods of time during infection. Furthermore, the majority of people inoculated with cholera do not develop symptoms but continue to shed vibrios in their stool for days, a time when RNS may still be generated in the host [168,207]. To determine the importance of *hmpA* as well as *norR* and *nnrS* in the setting of long-term colonization, we employed an adult mouse model [207] during which we could monitor colonization levels by collecting fecal pellets.

We used a competition assay in our mouse studies. After treatment with streptomycin and neutralization of stomach acid, mice were coinoculated with a wild-type strain and a mutant strain. Either the mutant or the wild-type strain lacked the *lacZ* gene, allowing differentiation on plates containing X-gal. At the end of each experiment, the small intestines of each mouse were homogenized, and competitive indices calculated from the homogenates. In each experiment performed, the competitive indices from

intestinal homogenates were always virtually identical to those from the fecal samples (data not shown).

Interestingly, deletion of *hmpA* resulted in a colonization defect at 3 days post-inoculation that worsened to nearly undetectable levels by 7 days, suggesting that HmpA is important for sustained colonization of the intestines (Fig. 6A). A competitive index was considered below the limit of detection (denoted by a dotted line in Fig. 6A) if there were zero *hmpA* mutant colonies detected. The *norR* mutant displayed a more moderate but significant defect as well. As in the *in vitro* studies, the *nnrS* single deletion mutant displayed no colonization defect and perhaps even a slight advantage over wild-type bacteria in wild-type mice (Fig. 7A). We hypothesized that, similar to our *in vitro* data, this phenotype might be reversed in an *hmpA* mutant background and that the *hmpA/nnrS* double mutant have an even more severe defect than the *hmpA* single mutant. However, competition of the *hmpA/nnrS* double mutant against wild-type bacteria displayed a defect similarly profound to the *hmpA* single mutant (Fig. 7A). To determine if a smaller *nnrS*-mediated defect might be masked by the larger defect due to *hmpA* mutation, we competed the *hmpA/nnrS* double mutant against the *hmpA* single mutant. We were surprised to find, however, that the double mutant did not fare significantly worse or better than the single *hmpA* mutant (Fig. 7B).

To assess the contribution of iNOS to the observed colonization defects, we repeated the experiments in *iNOS*$^{-/-}$ mice. By day 7 post-inoculation, the severe colonization defect of the *hmpA* mutant was attenuated more than ten-fold in *iNOS*$^{-/-}$ mice (Fig. 6A), suggesting that *iNOS* presents a long-term challenge for *V. cholerae* that is dealt with by *hmpA*. The *norR* mutant displayed a similar effect, in which the defect observed in wild-type was completely attenuated in *iNOS*$^{-/-}$ mice (Fig. 6B). This again suggests that on the time-scales that occur during a cholera infection, *iNOS*-generated RNS present a significant challenge for *V. cholerae* to overcome. Unexpectedly, however, the *nnrS* single mutant displayed a small but significant *defect* in *iNOS*$^{-/-}$ mice (Fig. 6C). Furthermore, the competition defect of the *hmpA/nnrS* double mutant was not mitigated in *iNOS*$^{-/-}$ mice as it was for the *hmpA* single mutant at seven days post-inoculation (Fig. 7A). In wild-type mice, the competitive index at day 7 for the *hmpA nnrS* double mutant was significantly higher

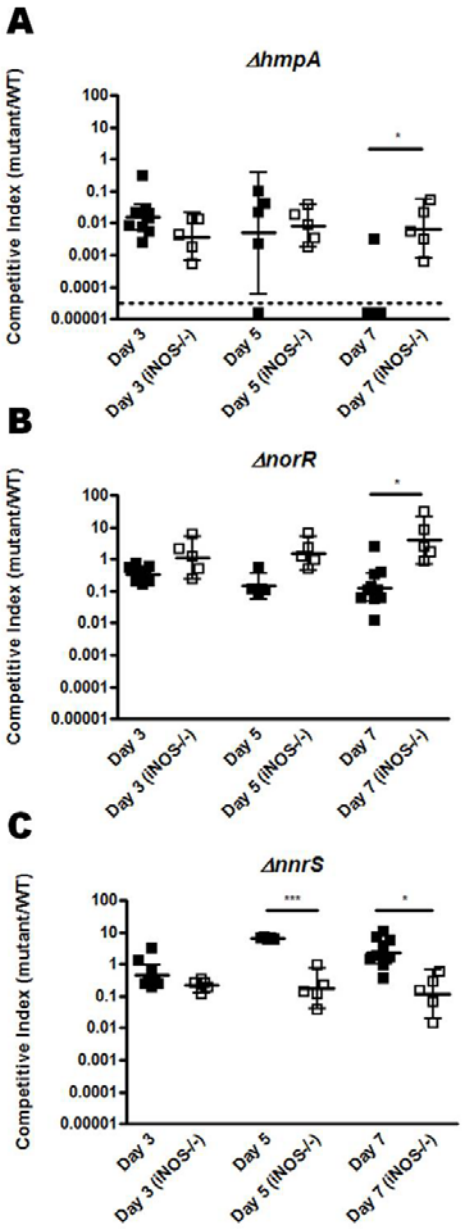

Fig 6. The importance of *hmpA* and *norR* for sustained colonization of the adult mouse. Six week-old C57bl/6 (black squares) or C57bl/6 *iNOS*$^{-/-}$ (white squares) mice were coinfected with wild-type *V. cholerae* and either a *ΔhmpA* (A) or *ΔnorR* (B) mutant strain. Fecal pellets were collected on days 3, 5, and 7 post-inoculation and plated on differential media. The competitive index was calculated as the ratio of mutant to wild-type colonies normalized to the input ratio. Error bars indicate 95% confidence interval. Data points below dotted line indicate that there were no mutant colonies detected. * indicates $P<0.05$.

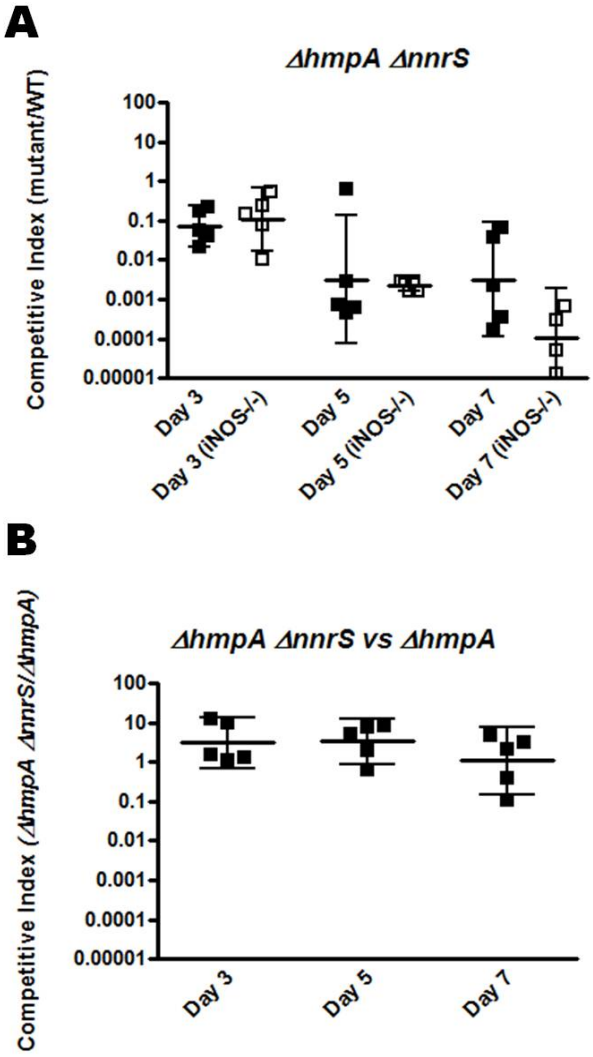

Fig. 7. The effect of deletion of *nnrS* on colonization of wild-type and *iNOS*$^{-/-}$ mice. As in Fig. 6, mice were inoculated with a mixture of wild-type *V. cholerae* and *ΔnnrS* (A) or *ΔhmpA ΔnnrS* (B). In (C), mice were inoculated with a mixture of *V. cholerae ΔhmpA* and *ΔhmpA ΔnnrS*, and the competitive index is reported as the ratio of *ΔhmpA ΔnnrS* to *ΔhmpA* colonies normalized to the input. Error bars indicate 95% confidence interval. * indicates $P<0.05$. *** indicates $P<0.001$.

than for the *nnrS* single mutant ($p = 0.0397$). These data suggest that in our long-term colonization model, *nnrS* may actually be detrimental to detoxification of iNOS-derived stresses. The exact mechanism behind this requires further investigation.

D. Discussion

Despite a wealth of research on the virulence factors that allow *V. cholerae* to cause disease, relatively little is known about the challenges that *V. cholerae* encounters during infection of the intestines and how it senses and overcomes them. In this study, we have identified how *V. cholerae* senses and responds to nitric oxide, a common challenge to intestinal pathogens. We have further demonstrated that one of the NO detoxification genes *hmpA* and its transcriptional activator NorR, are critical for sustained colonization of the intestines of mice.

Previous bioinformatic analysis led to the identification of a remarkably limited repertoire of nitric oxide-related genes encoded in the *V. cholerae* genome, even when compared to highly related *Vibrio* species [62]. Using reporter assays, we demonstrated that the expression of two of these genes, *nnrS* and the flavohemoglobin-encoding gene *hmpA*, is highly inducible by the addition of NO to microaerobically-growing cells. This upregulation was dependent on the σ^{54}-dependent transcriptional regulator NorR [80,124,208]. Growth curve analysis demonstrated that these genes are essential for resisting NO *in vitro*. Intriguingly, a strain of *V. cholerae* lacking both *hmpA* and *nnrS* was the most attenuated for growth in the presence of NO; concomitantly, deletion of *norR* resulted in a nearly equivalent growth defect in the presence of NO. These data demonstrated that HmpA is the principal detoxifier of NO, but that NnrS may serve an auxiliary role. The only study published to date on NnrS identified it as a heme- and copper-containing membrane protein in *Rhodobacter sphaeroides* [203]. However, *nnrS* homologues are encoded in the genomes of human pathogens such as *Pseudomonas*, *Brucella, Burkholderia, Bordetella,* and *Neisseria*, suggesting that it may serve NO-detoxification

roles in a variety of infectious settings. The exact function of NnrS is an area of current investigation in our laboratory.

The role of NO detoxification genes in *V. cholerae* pathogenesis has been examined in an infant mouse model, in which bacteria are allowed to colonize intestines for 24 hours [7]. After this brief period, there was a moderate colonization defect for the *hmpA* mutant attributed to the low pH of the stomach. We were interested in whether nitric oxide resistance could be important to colonization of the intestines over a time period resembling that of a human infection. Interestingly, we found that the importance of HmpA was much greater than previously thought; there were virtually no *hmpA* mutants recovered from fecal samples or small intestinal homogenates after seven days. This defect was partially due to iNOS-derived stress, as the colonization defect was partially mitigated in *iNOS*$^{-/-}$ mice at seven days. The remaining defect is unlikely due to stomach acidity because the mice were administered bicarbonate prior to inoculation. Mice and humans possess two other NOS isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS) [211], which may also account for some of the defect that persists in *iNOS*$^{-/-}$ mice.

We were surprised to discover the effects of the *nnrS* mutation on colonization. Although the *hmpA /nnrS* double mutant was severely inhibited *in vitro*, this mutant fared no better in *iNOS*$^{-/-}$ mice than wild-type. Furthermore, the single *nnrS* mutant slightly outcompeted wild-type *V. cholerae* in wild-type mice, but was attenuated in *iNOS*$^{-/-}$ mice. It is difficult to interpret these data given the unknown function of NnrS, but we hypothesize that the complex metabolism of reactive nitrogen species results in the buildup of detrimental chemical products in some contexts. Furthermore, an

acknowledged disadvantage of competition studies is that a defect for the *nnrS* mutant may be complemented *in trans* by the wild-type coinoculated strain. Future studies may address this possibility. Given the *in vitro* importance of NnrS, however, we speculate that there are infectious settings in which NnrS is critical to survival of *V. cholerae*. In addition, we were surprised to find that the *hmpA/nnrS* double mutant had a far more severe colonization defect than the *norR* mutant in wild-type mice (Fig. 6), since NorR is absolutely required for the upregulation of *hmpA* and *nnrS* in response to NO (Fig. 3). One possible explanation for the discrepancy between the colonization defects is that baseline transcription of *hmpA* and *nnrS* in the *norR* deletion mutant, however low, is sufficient to detoxify a significant proportion of the NO stress found *in vivo*. Alternatively, signals other than NO, and thus regulators other than NorR, might cause the upregulation of *hmpA* and *nnrS in vivo.* This could allow better colonization efficiency than when *hmpA* and *nnrS* are deleted entirely. Our laboratory is currently working to find these alternative signals and regulators of *hmpA* and *nnrS*.

Davies *et al*. [7] recently demonstrated a growth defect for a strain of *V. cholerae* lacking the *prxA* gene, which encodes a putative peroxireductase. The authors used a large, short-lived bolus of NO in aerobic conditions and found that the strain exhibited a delayed log phase. In the presence of a low dose of continuously released NO, a strain lacking *prxA* exhibited no defect compared to wild-type. Furthermore, expression of *prxA* was not increased in the presence of NO but was dramatically increased in the presence of H_2O_2. We suspect that PrxA is important for resistance to reactive oxygen species that may have been generated in aerobic conditions in the presence of large amounts of NO, but we conclude that it serves no role directly related to NO detoxification.

In summary, we have demonstrated the importance of the NorR regulon in sensing and resisting the toxicity of NO. Furthermore, we identified the importance of NO detoxification genes during extended colonization of the mouse intestines. Our work highlights the role of resistance to chemical stresses to successful survival of *V. cholerae* during infection, and ultimately its ability to cause disease.

E. Acknowledgments

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CHAPTER THREE: A novel protein protects bacterial iron-dependent metabolism from nitric oxide

Additional contributors to this work: Binbin Liu (Norwegian University of Life Sciences), Lars R. Bakken (Norwegian University of Life Sciences), James P. Shapleigh (Cornell University)

This work is currently in press at the Journal of Bacteriology.

A. Introduction

Vibrio cholerae causes cholera, a severe watery diarrhea responsible for millions of cases and thousands of deaths each year (Centers for Disease Control and Prevention, http://www.cdc.gov). It is not, however, a member of the Enterobacteriaceae - its natural habitat is aquatic. It is thought that during most of its life cycle, when not infecting humans, *V. cholerae* resides in association with zooplankton, forming biofilms on the chitinous surfaces of crustaceans[185,186]. Thus, *V. cholerae* must display metabolic flexibility in order to thrive in these two different environments and respond to the different metabolic challenges therein.

A commonly encountered metabolic stress to pathogenic and non-pathogenic bacteria is the presence of reactive nitrogen species (RNS), in particular the well-studied molecule nitric oxide (NO). NO is formed as a byproduct of nitrogen metabolism for many bacteria as an intermediate in denitrification[212], as well as from dedicated nitric oxide synthases (NOSs) in both bacteria and eukaryotes[70,211], and is present in micromolar concentrations in some bacterial biofilms[77]. NO can also be formed by chemical decomposition of nitrite in acid environments such as the human stomach[59]. NO

is also a prominent component of the mammalian innate immune system, part of a battery of reactive oxygen (ROS) and nitrogen (RNS) species produced by phagocytes when they encounter bacteria[59].

The mechanisms whereby NO inhibits bacterial growth are diverse, but one of the most important properties of NO is its ability to bind iron and form dinitrosyl iron complexes (DNICs) bound to iron-sulfur cluster proteins, inhibiting their function[15,16]. DNICs have also been shown to mediate the formation of nitrosothiols, another form of nitrosative stress that inhibits thiol-containing proteins[27]. Through this mechanism and others, NO has been shown to inhibit a handful of enzymes *in vitro*, including such central metabolic enzymes as aconitase[12], dihydroxyacid dehydratase[14], alpha-ketoglutarate dehydrogenase[13], fructose-1,6-bisphosphate aldolase[163], argininosuccinate synthase[213], and components of the respiratory chain[214,215]. However, these enzymes have largely been studied in isolation and there has not been any comprehensive study on the effects of NO on bacterial metabolism.

Bacteria possess several strategies for coping with nitrosative stress. The most obvious strategy is to directly remove the NO, and there are several enzymes known to convert NO into less toxic nitrogen oxides such as nitrate (NO_3^-) or nitrous oxide (N_2O). Another strategy is to alter carbon flux to bypass blockades and maintain redox homeostasis, a method used by *Staphylococcus aureus* by up-regulating lactate dehydrogenase[42]**.** The genes required for these responses are usually under control of a NO-responsive transcription factor[62]. One gene that is conserved throughout many Gram negative bacteria, including such pathogens as *Pseudomonas, Neisseria,* and *Brucella,* and is also usually found under control of one of these transcriptional regulators, is *nnrS*.

NnrS was initially described in *Rhodobacter* as a heme- and copper-containing transmembrane protein[203]. Although the function of NnrS is unknown, we have previously shown that it contributes to nitrosative stress tolerance in *V. cholerae* [40].

In this study, we performed a metabolomic screen with two goals: to identify more fully the effects of NO on bacterial metabolism by surveying the relative concentrations of metabolites from many different pathways in *V. cholerae*, and to use these data to determine the function of NnrS. We found drastic changes in metabolic pathways in response to NO, suggesting that nitrosative stress forces bacteria to adapt dramatically. We also find that NnrS does not directly remove NO but instead protects the cellular iron pool from the formation of DNICs, thus protecting critical metabolic pathways from inhibition.

B. Materials and methods

Bacterial strains, plasmids, and growth conditions. All strains of *V. cholerae* in this study were derived from O1 El Tor strain C6706. In-frame deletion strains were generated by sucrose counterselection as described previously [216]. Minimal media used contained 79 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.65 mM $MgSO_4$, 0.07 mM $CaCl_2$, 0.018 mM $FeSO_4$, and 0.013 mM $MnSO_4$ with carbon sources added as indicated. For growth curves involving 2,2'-dipyridyl, $FeSO_4$ was omitted and 0.2 mM 2,2'-dipyridyl was added. Yeast extract was added when indicated at a concentration of 0.5% (w/v). The plasmid used to complement the *nnrS* deletion was derived from pMal-c2x (New England Biolabs), in which the *malE* gene was replaced at the NdeI and SalI sites with

nnrS tagged with six histidine codons at its 3' end. Expression was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the growth media.

Growth curves. To monitor growth continuously, overnight saturated cultures were washed in PBS, and 2 μL of washed culture were inoculated into 200 μL of relevant growth medium in a 96-well plate in triplicate. (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate, Cayman Chemical) was included at a range of concentrations. The plate was covered with a transparent film, and growth was monitored every four minutes using the absorbance at 600 nm, after shaking for two minutes at each time point, using an automated plate reader (BioTek Synergy HT). Anaerobic growth curves were performed by inoculating 30 μL of washed, saturated culture into 3 mL minimal media in individual test tubes, then placing the cultures in an anaerobic chamber (Coy Laboratories) equipped with a 37 ºC standing incubator, and periodically withdrawing 200 μL and measuring the absorbance at 600 nm. For aerobic growth curves, 10 mM glucose was used as a sole carbon source, and for anaerobic growth, 25 mM glucose was used, unless otherwise indicated.

Measurement of NO consumption. Strains were inoculated into 120 mL serum flasks with 50 mL minimal medium containing 0.25% glucose (w/v), Teflon magnetic bars, and crimp-sealed butyl septa. Prior to inoculation, the headspace atmosphere was replaced with helium by evacuation and re-filling six times, then supplied with pure oxygen to reach 15 mL L^{-1} and pure NO to 300-350 ppm (equivalent to ~493-575 nM in the liquid). The flasks were then placed in a 37 ºC water bath. Initial pressure was adjusted to

1 atmosphere by releasing the over-pressure through a 0.5 mm (ID) cannula. The flasks were inoculated with 1 mL culture containing ~$3*10^8$ cells and stirred while monitoring the oxygen and NO concentrations in the headspaces, which were then used to calculate the concentrations in the liquid. This incubation and measurement system has been described in detail previously[217].

Metabolomic study. Overnight cultures of the three strains were inoculated at a ratio of 1:1000 into 440 mL of LB in centrifuge bottles filled to the top and closed tightly. Twenty μM DETA-NONOate was added to the samples, or 20 μM diethylamine triamine (DETA) to the control samples. All samples were incubated for seven hours at 37 ºC, after which 200 mL were discarded and the remainder centrigured at 6000 rpm in an SLA-3000 rotor for ten minutes. Pellets were resuspended in 1 mL of PBS, then centrifuged again in a Nalgene cryovial for five minutes at 13,000 rpm in a tabletop centrifuge. Pellets were then flash-frozen in an ethanol/dry ice bath and stored at -80C. This experiment was carried out five times on separate days, then analyzed in conjunction with Metabolon, Inc. (Durham, NC, USA). A detailed description of the extraction protocol, instrument settings, data processing, and quality control has been described previously [218,219]. In brief, samples were extracted and analyzed with ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) and gas chromatography/mass spectrometry (GC/MS). Metabolites present in samples were identified by matching chromatographic and mass spectral data to an in-house library of chemical standards, and relative abundances of metabolites were determined by area under peak analysis. Data were normalized to the protein concentration of the sample and

further normalized such that the median concentration of each metabolite across all samples was 1.

Measurement of aconitase activity. One mL of saturated overnight cultures were inoculated into 200-mL volumes of LB in 500-mL flasks with shaking at 37 ºC in the presence or absence of 100 μM DETA-NONOate, which had been freshly dissolved in 10 mM NaOH. After three hours of growth, bacteria were centrifuged and resuspended in 0.5-1 mL 50 mM Tris-HCl pH 7.4, 0.6 mM $MnCl_2$. Three hundred μL of resuspended cells were lysed quickly by sonication at 400W and spun in a tabletop centrifuge to remove cell debris. Thirty μL of supernatant were immediately aliquotted in triplicate to a 48-well plate. Using a multichannel pipette, the aconitase reaction was started by adding 1 mL of reaction mix (50 mM Tris-HCl pH 7.4, 0.6 mM $MnCl_2$, 0.2 mM NADP+, 1 U/mL porcine heart isocitrate dehydrogenase, 5 mM trisodium citrate) to the extracts. The citrate was added to the reaction mix immediately before initiation. Activity was then calculated by monitoring the rate of formation of NADPH for approximately ten minutes every fifteen seconds in an automated plate reader (BioTek Synergy HT), using an extinction coefficient at 340 nm of 6,220 $M^{-1}cm^{-1}$.

Measurement of ferrous iron. One mL of saturated overnight cultures were inoculated into 200-mL volumes of LB broth in 500-mL flasks with shaking at 37 ºC in the presence or absence of 100 μM DETA-NONOate, which had been freshly dissolved in 10 mM NaOH. Cultures were centrifuged, then washed twice and resuspended in 50 mM Tris-HCl pH 7.4. A 300-μL sample was sonicated briefly at 400W. To avoid oxidation by

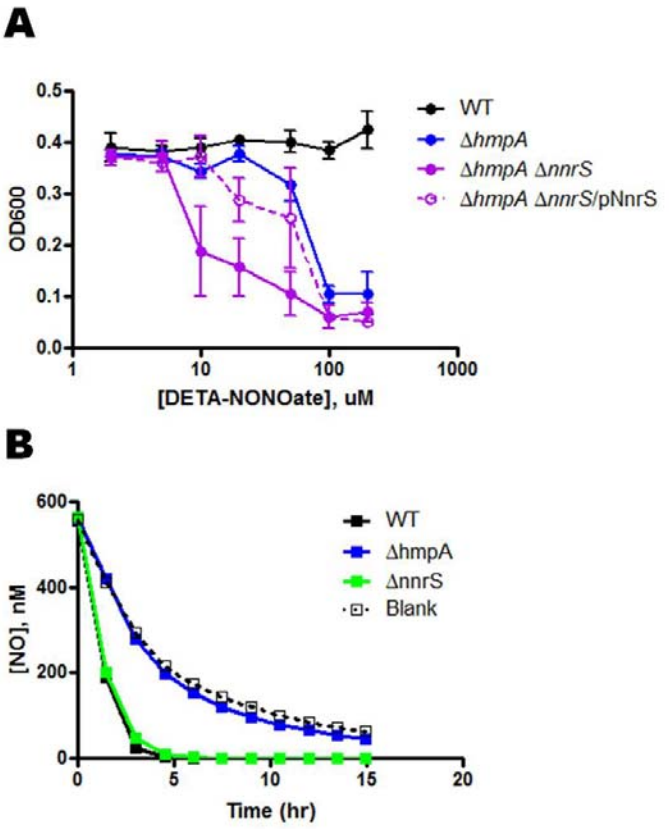

Fig. 8. NnrS protects the cell from NO but does not remove it. (a) Strains of *V. cholerae* were inoculated into minimal media with 10 mM glucose as the sole carbon source in the presence of increasing concentrations of NO donor DETA-NONOate, and the OD600 was recorded over time to generate growth curves for each strain at each concentration. Represented on the graph is the OD600 of each strain six hours after inoculation, at the approximate time when the wildtype (WT) strain reached stationary phase. Error bars represented standard deviation. Data representative of three independent experiments. (b) Strains of *V. cholerae* were inoculated into minimal media and the consumption of NO, which was added as a bolus of authentic NO gas into the headspace, was measured. "Blank" refers to a flask of media without bacteria. Shown in this figure is an experiment done in single replicates, representative of at least three identical experiments. The concentration of NO represented on the graph is the concentration calculated in the liquid based on measurement of the concentration in the headspace.

oxygen, 100 µL were added within seconds to 10 µL of FerroZine reagent (3-[2-pyridyl]-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid disodium salt, 10 mM dissolved in 100 mM ammonium acetate). The samples were then centrifuged, and the absorbance at 562 nm was recorded then compared to a freshly prepared ferrous ammonium sulfate standard and normalized to protein concentration.

C. Results

NnrS is important for resistance to NO but does not remove NO.

We previously reported that a strain of *V. cholerae* lacking the flavohemoglobin HmpA (Δ*hmpA*), which removes NO by conversion to nitrate, or lacking the transcriptional regulator NorR, was hypersusceptible to growth inhibition by NO and was defective in colonizing the mouse gastrointestinal tract[40]. Although a strain lacking only NnrS

(Δ*nnrS*) displayed comparable NO resistance to wildtype, the deletion of *nnrS* in a Δ*hmpA* background (Δ*hmpA* Δ*nnrS*) resulted in severe sensitivity to NO compared to Δ*hmpA*[40]. Thus, although HmpA is likely the dominant NO-resistance protein of *V. cholerae*, NnrS plays an auxiliary role and may be important in environments in which HmpA is non-functional (such as strictly anaerobic conditions, discussed below). To expand on our previous findings and begin to search for the function of NnrS, we performed growth curves over a range of concentrations of the NO donor DETA-NONOate and found that the Δ*hmpA* Δ*nnrS* strain was approximately one log more sensitive than the Δ*hmpA* strain (Fig. 8A). This phenotype could be complemented by expressing NnrS from a plasmid (Fig. 8A, Δ*hmpA* Δ*nnrS*/pNnrS).

We next determined whether NnrS might remove NO directly. However, we were unable to detect any difference in the rate of NO consumption between the wildtype strain and Δ*nnrS* (Fig. 8B). In addition, we were unable to detect any metabolism of NO in the Δ*hmpA* strain above background autooxidation[220]. These data suggest that HmpA is responsible for the removal of NO in *V. cholerae* and that NnrS protects against NO through a different mechanism.

A metabolomic study to identify pathways inhibited by NO.

Although several enzymes are known to be inhibited by NO, to date, no comprehensive study of central metabolism has been conducted to determine the breadth of its effects. By identifying molecules that increase or decrease in concentration in the cell, we reasoned that such a study could identify new enzymes inhibited by NO through the accumulation of intermediates upstream (and decrease downstream) of an NO-inhibited enzyme.

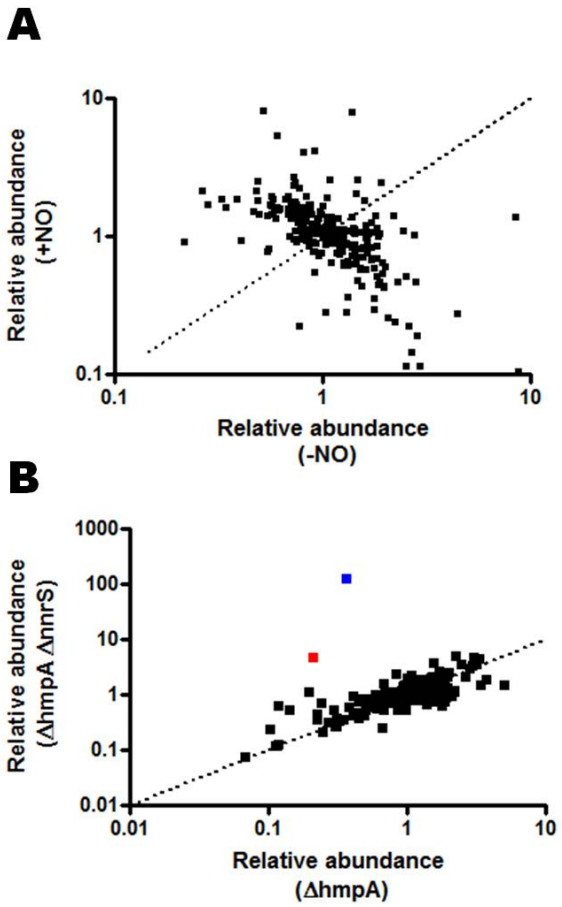

Fig. 9. Scatter plots of metabolomic analyses of the effects of NO on *V. cholerae*. The relative concentrations of hundreds of metabolites were recorded for wildtype, Δ*hmpA*, and Δ*hmpA* Δ*nrrS* strains of *V. cholerae*. (a) Scatter plot of the relative abundance of metabolites in wildtype *V. cholerae* in the absence (x-axis) and presence (y-axis) of NO donor DETA-NONOate. Area above the dotted line indicates relative accumulation in the presence of NO; area below indicates relative accumulation in the absence of NO. (b) Scatter plot of the relative abundance of metabolites in the presence of NO for the Δ*hmpA* strain (x-axis) compared to the Δ*hmpA* Δ*nrrS* strain (y-axis). Area above the dotted line indicates accumulation in the Δ*hmpA* Δ*nrrS* strain; area below the dotted line indicates accumulation in the Δ*hmpA* strain. Blue dot represents citrate, and red dot represents *cis*-aconitate.

We thus grew *V. cholerae* in the presence of DETA-NONOate or the control compound DETA and subjected bacterial pellets to analysis by mass spectrometry to identify the relative content of a broad array of metabolites. We employed three strains: wildtype (WT), Δ*hmpA*, and Δ*hmpA* Δ*nnrS*. Intermediates from glycolysis, the tricarboxylic acid

(TCA) cycle, amino acid synthesis, nucleotide synthesis, lipid metabolism, and various other pathways were quantified. The complete data set is available in the supplementary information to be published online. A large variety of these metabolites differed significantly between the NO-treated and untreated samples (Fig. 9A). Several enzymes previously shown to be inhibited by NO could be identified by the buildup of their substrates or upstream intermediates. For instance, fructose-1,6-bisphosphate aldolase is a zinc-dependent glycolytic enzyme demonstrated to be NO-sensitive in *Borrelia bergdorferi*[163]. In our study, upstream glycolytic intermediates such as glucose, glucose-6-phosphate, and fructose-1,6-bisphosphate all accumulated in the presence of NO, whereas downstream metabolites such as 2-phosphoglycerate and 3-phosphoglycerate decreased (Fig. S2), thus confirming that this enzyme is likely inhibited in *V. cholerae,* too. Argininosuccinate synthetase, which converts citrulline to argininosuccinate in order to produce arginine, has been shown to be inhibited by NO in mitochondria[213]. The fifteen-fold accumulation of citrulline in the presence of 20 μM DETA-NONOate in our study (Fig. S3) suggests that this enzyme may be inhibited in bacteria as well.

Comparative metabolomics reveals a role for NnrS. Knowing that NnrS is important for resistance to NO but that it does not remove NO, we hypothesized that there might be specific metabolic pathways protected by NnrS from nitrosative stress. Thus, we compared the results of the metabolomic study between the Δ*hmpA* and Δ*hmpA* Δ*nnrS* strains (Fig. 9B). We found that in the presence of NO, the Δ*hmpA* Δ*nnrS* strain accumulated more than 200-fold more citrate and 23-fold more *cis*-aconitate than the Δ*hmpA* strain (Fig. 9B, 10A,B). In addition, 2,3-dihydroxyisovalerate accumulated more

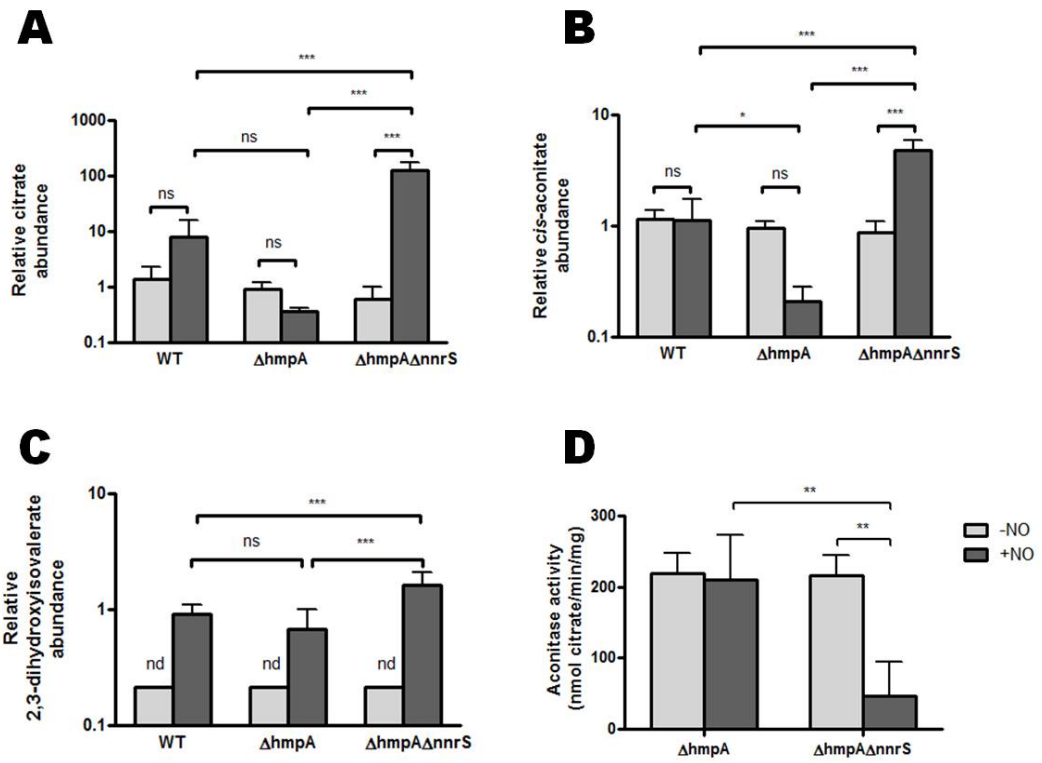

Fig. 10. NnrS protects dehydratases from NO. Relative concentrations of citrate (a), *cis*-aconitate (b), and 2,3-dihydroxyisovalerate (c) in the three strains from the metabolomic study in the presence (+NO) or absence (-NO) of DETA-NONOate. (d) Aconitase activity in cell-free extracts of *V. cholerae* grown in the presence (+NO) or absence (-NO) of DETA-NONOate. Error bars represent the standard deviation of the mean of three independent experiments. Statistical significance was determined by two-way ANOVA with Bonferroni's post-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant; nd, not detectable.

in the Δ*hmpA* Δ*nnrS* strain than the Δ*hmpA* strain, though only by 2.4-fold (Fig. 10C). Citrate and *cis*-aconitate are substrates of aconitase, and 2,3-dihydroxyisovalerate is a substrate of dihyrdoxyacid dehydratase, both enzymes of the dehydratase family known to be sensitive to NO[12,14]. The dehydratase family of enzymes is a unique family in which the iron-sulfur cluster reacts directly with its substrate. Dehydratases are thus exquisitely sensitive to NO due to the solvent-exposed nature of their iron-sulfur clusters: NO binds and forms dinitrosyl iron complexes at these sites, inactivating the enzyme [16]. On the other hand, substrates of non-dehydratase enzymes such as citrulline and 1,6-fructose bisphosphate that accumulated in all three strains did not accumulate any further in the

absence of *nnrS* (Fig. S2, S3). This suggested to us that NnrS, although not removing NO directly, might serve some role specifically in protecting dehydratases or other iron-sulfur cluster-containing proteins from inhibition by NO. To test this hypothesis, we measured the aconitase activity in cell-free extracts of Δ*hmpA* and Δ*hmpA* Δ*nnrS* strains (Fig. 10D). We found that in the absence of NO, both strains had similar activity, but upon the addition of NO, aconitase activity in the Δ*hmpA* Δ*nnrS* strain dropped to approximately 25% of the Δ*hmpA* strain. This suggests that a decrease in the activity of dehydratases such as aconitase due to NO is prevented by NnrS.

NnrS protects the cellular iron pool from NO. The inhibition of dehydratases by DNIC formation occurs through the reaction of NO with the "chelatable iron pool" (CIP), which is not a chemically defined mixture but is thought to be the cellular iron that is loosely coordinated and can thus be bound by chelators[15]. It is thought that the chelatable iron pool is composed of both free and protein-bound iron[15,16]. Other groups have shown that chelation of iron with 2,2'-dipyridyl prevents the formation of DNICs[16]. Thus, we hypothesized that chelation of iron might complement the NO-dependent toxic effect of the deletion of *nnrS* by depleting the free iron available to react with NO. When we added both yeast extract and 2,2'-dipyridyl to minimal media with no added iron, severely restricting cellular iron, there was no growth defect in the Δ*hmpA* Δ*nnrS* strain compared to Δ*hmpA* (Fig. 11A). The addition of yeast extract alone only partially complemented the defect (compare Fig. 11A to Fig. 8A). This latter result is to be expected, since many of the pathways dependent on iron-sulfur clusters are biosynthetic, and thus their inhibition might be overcome by supplementation with yeast extract. To

further test the hypothesis that NnrS protects against iron-NO complex formation, we measured the chelatable ferrous iron content of cells treated with NO. It has been demonstrated that addition of NO to cellular systems depletes chelatable iron by causing it to form macromolecule-bound DNICs[15]. In addition, the decomposition of DNICs by oxygen and L-cysteine causes the release of ferrous iron[17]. Thus, a cell with an increased number of DNICs, or one defective in decomposing them, would have a lower ferrous iron concentration detectable by reagents such as FerroZine. Indeed, we found the he Δ*hmpA* Δ*nnrS* strain had lower chelatable ferrous iron content than the Δ*hmpA* strain (Fig. 11B), again supporting the hypothesis that NnrS prevents the formation of iron-NO complexes.

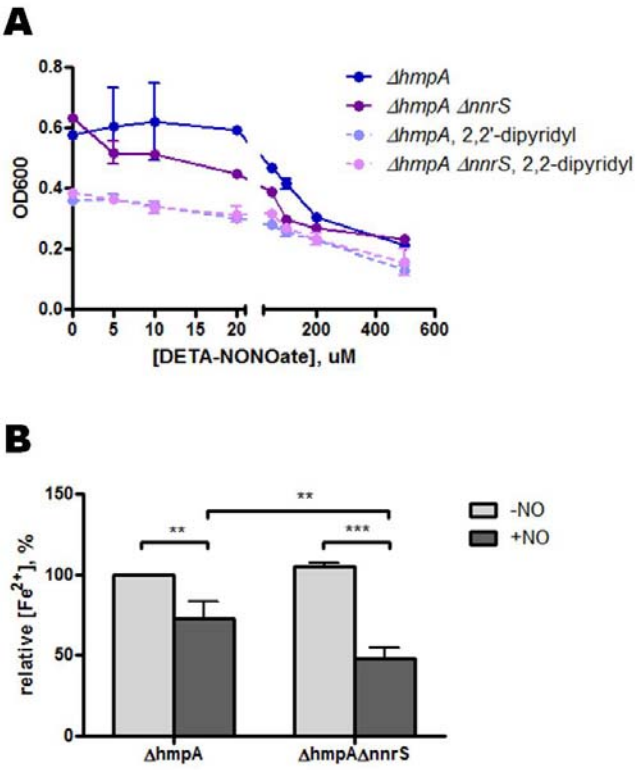

Fig. 11 NnrS protects the cellular iron pool from NO. (a) *V. cholerae* strains Δ*hmpA* and Δ*hmpA* Δ*nnrS* were inoculated into minimal media with glucose and yeast extract in the presence of increasing concentrations of DETA-NONOate with (dotted lines) or without (solid lines) the iron chelator 2,2'-dipyridyl. Represented on this graph is the OD600 at ten hours of growth, representative of two independent experiments. (b) Relative ferrous iron content of Δ*hmpA* and Δ*hmpA* Δ*nnrS* strains in the presence (+NO) or absence (-NO) of DETA-NONOate. Error bars represent the standard deviation of the mean of three independent experiments. Statistical significance was determined by two-way ANOVA with Bonferroni's post-test to compare individual groups. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

NnrS is important during anaerobic nitrosative stress. To this point, all the effects of deleting *nnrS* were examined only in the genetic background lacking *hmpA*. To determine

the physiological relevance of NnrS, we sought to find a condition in which the single deletion of *nnrS* might have effects on resistance to NO. Previous work has shown that the primary mechanism of action of flavohemoglobins such as HmpA is though dinitrosylation of NO, a reaction that is dependent on O_2[134]. Hmp of *E. coli* also possesses an O_2-independent NO reductase activity *in vitro*[136], but the activity is slow and its physiological relevance is uncertain[131]. Thus we hypothesized that in anaerobic conditions, the effect of HmpA in *V. cholerae* might be less dominant, and NnrS might become more important.

We found that in a strictly anoxic environment, even wild-type *V. cholerae* was highly sensitive to NO: growth was inhibited at micromolar concentrations of DETA-NONOate in anaerobic conditions (Fig. 12), whereas millimolar concentrations had no effect in the presence of oxygen (Fig. 8A). This heightened sensitivity is probably due to multiple factors, including the absence of non-enzymatic clearance of NO by O_2, but may in part be explained by the oxygen-dependence of HmpA. Interestingly, the Δ*nnrS* strain was more sensitive than wild-type in anoxic conditions (Fig. 12). We observed this phenotype during fermentation (Fig. 12) and during anaerobic respiration on fumarate (Fig. S6). This suggests that NnrS may serve an important role in anaerobic environments.

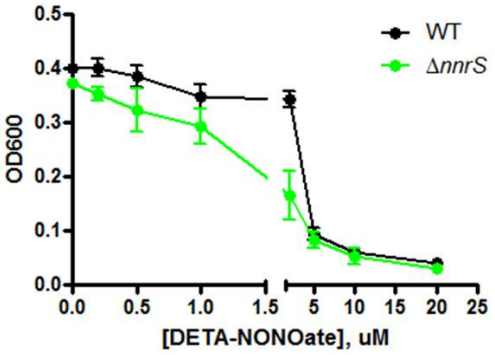

Fig. 12. NnrS is important during nitrosative stress in anaerobic conditions. Growth of wildtype and Δ*nnrS* strains in the presence of increasing concentrations of DETA-NONOate in an anaerobic environment in minimal media with 25 mM glucose as the sole carbon source. This graph represents the OD600 at twelve hours of growth. Error bars represents standard deviation. Data representative of three independent experiments.

D. Discussion

Nitrosative stress, derived from reactive nitrogen species such as NO, is a ubiquitous challenge for bacteria. During infection, pathogenic bacteria encounter high concentrations of NO released by phagocytes[54]. NO is also formed inorganically when nitrite from the mouth reaches the low pH of the stomach[59]. NO can also be generated by other bacteria through denitrification or by nitric oxide synthase[65,70]. Furthermore, NO has been found to reach high concentrations in polymicrobial biofilms[77]. In other words, bacteria are constantly encountering NO and must adapt metabolically. To date, there had been no study detailing the scope of these metabolic effects, so we performed a metabolomic study on NO-treated and –untreated cells. We found a wide breadth of effects most pronounced in central carbon metabolism: an accumulation of upstream glycolytic intermediates pointed to a block in glycolysis at the fructose-1,6-bisphosphate aldolase step, and an accumulation of citrate indicated a block in the TCA cycle. In addition, high citrulline concentrations implied a defect in arginine synthesis, all validating studies from various other prokaryotic and eukaryotic systems that identified these pathways as targets of NO. We also found that the concentrations of polyamines 1,3-aminodipropane (DAP) and spermidine were increased nine- and three-fold, respectively (Fig. S3). In uropathogenic *E. coli*, nitrosative stress has been shown to increase polyamine production, which was linked to RNS resistance[221], suggesting that perhaps such a mechanism exists in *V. cholerae* too. Polyamines have also been linked to biofilm production in *V. cholerae*[222]. Furthermore, NO sensing has been shown to influence biofilm formation in other bacterial species through H-NOX domain proteins

and cyclic-di-GMP production[223–225]. The increase in polyamines thus suggests an additional possible link between NO and biofilms, which we are currently investigating.

This study also identified some metabolic pathways that may be affected by NO but have not been described as such before. We observed an accumulation of cysteine and glycine, as well as a decrease in both oxidized and reduced glutathione concentrations (Fig. S5). Taken together, this may indicate a block in glutathione synthesis, which occurs from the ligation of cysteine, glutamate, and glycine. Glutathione is a critical molecule in maintaining the proper functional redox state of many intracellular enzymes by regenerating the active form of thiol-dependent active sites. Glutathione is formed by two enzymes, gamma-glutamylcysteine synthetase and glutathione synthetase; we are currently investigating the inhibition of these enzymes by NO. We did not observe an increase in glutamate as one might expect (in fact there was a slight decrease). However, glutamate is a critical branch point for many pathways in central carbon metabolism, so the interruption of glutathione synthesis may not necessarily result in a detectable accumulation of glutamate.

All these data suggest that the effects of NO on bacterial physiology are quite broad. It is no wonder, then, that bacteria have evolved multiple mechanisms to cope with this stress. One obvious strategy is to simply remove the NO itself directly. There are multiple enzymes known to perform this task, including nitric oxide reductase (NOR), flavorubredoxin (NorV), and flavohemoglobin (Hmp), as well as the hybrid cluster protein (Hcp) thought to remove the related compound hydroxylamine[190]. These compounds are nearly always under control of an NO-responsive transcriptional regulator, such as NnrR, NsrR, NorR, or HcpR[62], all of which bind NO and alter gene

expression. In many gamma-proteobacteria, however, there is another gene, *nnrS*, that is also under control of one of these regulators but the function of which was previously unknown[203]. Unlike most of the other factors under control of these regulators, NnrS does not appear to remove NO directly. Instead, we found that it relieves a major stress caused by NO: formation of iron-NO complexes. Mutants lacking *nnrS* were significantly inhibited for growth in the presence of NO, mainly due to the sequestration of the cellular iron pool by NO. One of the most toxic effects of NO is the formation of protein-bound dinitrosyl iron complexes (DNICs), which are directly inhibitory to iron-sulfur cluster proteins[15]. We found that NnrS protects against this effect, allowing critical enzymes such as aconitase to function in the presence of NO.

We noticed similarities between our findings regarding NnrS and another protein involved in NO tolerance, YtfE. In *E. coli*, *ytfE* is under control of the regulator NsrR and has been shown to protect iron-sulfur cluster-containing proteins such as aconitase and fumarase from damage due to NO or hydrogen peroxide[159,162,226]. YtfE is a member of a putative family of non-heme di-iron proteins that includes ScdA from *Staphyolococcus aureus* and DnrN from *Neisseria gonorrheae*[162]. Interestingly, we noticed that this family of proteins (Pfam family PF04405, http://pfam.sanger.ac.uk) is distributed primarily among the order Enterobacteriales and absent from the Vibrionales, whereas NnrS (Pfam family PF05940) is absent from the Enterobacteriales but found widely within the Vibrionales. Although both are present within the Alteromonadales, particularly within the genus *Shewanella*, the phylogenetic distribution suggests perhaps some convergent evolution between these two different proteins fulfilling a similar function. On the other hand, parallels between NnrS and YtfE are not perfect. The growth defect in the *ytfE*

deletion mutant of *E. coli* was found to worsen in the presence of 2,2'-dipyridyl[226], whereas it improved growth of the *nnrS* deletion strain (Fig.11A). Further work on both NnrS and YtfE will hopefully shed light on how these proteins protect iron-sulfur clusters from NO.

V. cholerae is an aquatic organism and lives frequently on the molts of microscopic crustaceans[185], where the carbon sources are likely more limited. We have previously shown[40] that NnrS probably does not play a significant role during growth in the mammalian intestines, where carbon sources are likely more diverse than on a crustacean molt, which are made primarily of chitin, a polymer of the amino sugar N-acetylglucosamine. In fact, *V. cholerae* can use chitin as its sole carbon source[227,228], a situation resembling minimal media. Thus we suspect that in "minimal media-like" environments such as chitinous surfaces, NnrS might play a more prominent role in resistance to NO, as demonstrated by the more pronounced growth defect in minimal media (Fig. 8A) compared to rich media (Fig. 11A). Interestingly, there is one bacterial species, *Saccharophagus degradans*, which has been described to possess *nnrS* as its only gene under control of a dedicated NO-responsive transcription factor[62]. This species of bacteria is found in a habitat in which its only carbon source is agar, which is another sugar polymer[229]. Thus, the phylogenetic distribution of NnrS as well as the data in this study support the conclusion that NnrS is important in resisting nitrosative stress, particularly in environments with low carbon diversity, abundant iron, or low oxygen, in order to protect the cell against inhibition of iron-containing proteins by NO.

In summary, this work employed metabolomics for the first time to identify new targets of NO, a common source of metabolic stress for bacteria. We also found that one

of the most important targets of NO, the cellular iron pool and iron-sulfur cluster enzymes, is protected from damage by NnrS, an NO-regulated protein of previously unknown function.

E. Acknowledgments

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CHAPTER FOUR: Conclusions and Future Directions

Several hypotheses have been addressed in this work. As has been shown in *E. coli,* the flavohemoglobin Hmp is critically important for resisting NO stress in *V. cholerae*. Deleting *hmpA* resulted in both hypersensitivity to NO (Figs. 4B and 8A) and the inability to scavenge NO (Fig. 8B). The *hmpA* deletion mutant also had a severe defect in colonizing the streptomycin-treated mouse (Fig. 6A). This was likely due to at least two sources of nitrosative stress: iNOS, as demonstrated by a partial restoration of the defect, and gastric acid-generated NO, which cannot be corrected by deleting *iNOS*. Of course there are other possible sources of NO for the non-iNOS-dependent defect, but given that stomach acid neutralization corrected the defect of *hmpA* in infant mice[7] and the non-iNOS-dependent defect appeared early in colonization, gastric NO is a reasonably likely cause of the defect. Recently, the surprising finding was made that streptomycin itself causes a mild inflammation in mouse intestines that causes upregulation of iNOS and production of NO[230]. This may account for the discrepancy in the magnitude of the colonization defect for the *hmpA* mutant between streptomycin-treated adult mice and infant mice (which are not treated with streptomycin), in which the infant mice only displayed a colonization index for *hmpA* of ~0.4. The length of the experiment may also amplify the defect in adult mice compared to infants. Nevertheless, we cannot predict which model is a more accurate depiction of the importance of Hmp in an actual human infection. The truth may lie somewhere in between the two mouse models.

We also found, as predicted computationally[62], that NorR regulates *hmpA* and *nnrS* expression (Fig. 3). We were surprised to find, however, that phenotypes of the

norR mutant do not completely recapitulate the *hmpA nnrS* double mutant. *In vitro*, both strains displayed similar growth inhibition in response to NO (Fig. 4B), and neither strain could scavenge NO (data not shown). However, the *norR* mutant only displayed a mild colonization defect in adult mice (Fig. 6B). As outlined in the Discussion of Chapter Two, it is likely that there are other signals activating *hmpA* expression. Preliminary experiments in our lab have indicated that *hmpA* may in fact be regulated by the virulence regulator AphB – thus the signals that regulate virulence through AphB (low oxygen tension, host digestive molecules) may also activate *hmpA* expression in the mouse, and thus account for the differences in colonization.

After observing that deleting *nnrS* exacerbated the hypersensitivity of the *hmpA* mutant (Figs. 4B and 8A), and that *nnrS* was regulated by NorR (Fig. 1B), we hypothesized that NnrS serves some role in tolerance to NO. Through metabolomics, we deduced that NnrS plays a particular role in protecting iron-sulfur proteins such as aconitase. However, much remains unknown about NnrS – i.e. the mechanism by which it protects the cell. Given that its homologue in *R. sphaeroides* contains heme and copper[203], it likely performs a role in reduction or oxidation of some species that forms in the presence of NO. A likely candidate is DNICs, which are hypothesized to exist at least transiently in a non-protein-bound state[17]. Further experiments will address this possibility. It could also simply react with chelatable ferrous iron in some way so as to prevent formation of DNICs. It is unlikely, however, that it simply removes NO, since cultures lacking NnrS do not display any defect in NO scavenging (Fig. 8B).

The role of NnrS in the *V. cholerae* life cycle also remains to be explored. We showed that it does not appear to affect colonization in the mouse (Fig. 6C, 7B).

However, as mentioned in the Discussion of Chapter Three, it may serve roles in other environments. NnrS appears to be important for NO resistance in strictly anoxic environments (Fig. 12); furthermore, its utility increases in minimal media containing only glucose compared to complex media such as LB. Interestingly, polymicrobial biofilms appear to exhibit this type of structure. In the deeper portions of a biofilm, NO concentrations increase and O_2 concentrations decrease[77]. Furthermore, carbon diversity may be quite limited on, for example, the chitinous exoskeleton of a copepod, which is composed primarily of only one polysaccharide. Thus an important set of future experiments will address whether NnrS might be useful in this environmental niche.

Last, this work began to address some important gaps in knowledge about the general effect of NO on bacterial metabolism. Numerous pathways were identified as being affected in the presence of NO: glycolysis, arginine synthesis, glutathione synthesis, polyamine synthesis, and the TCA cycle. There may be still more to be identified through detailed pathway analysis of the metabolomics dataset. This will hopefully be a starting point for a more in-depth understanding of the broad effects of NO on bacterial growth.

In conclusion, this work has analyzed the response of an important human pathogen, *V. cholerae*, to an important bacterial stressor, NO, and found that NO plays an important role in *V. cholerae* physiology both inside and outside the host. NorR, HmpA, and NnrS were found to be the critical mediators of this response, preventing damage to metabolic pathways that would otherwise result in growth arrest. This work has illuminated one critical aspect of bacterial physiology and virulence and will hopefully lead to further advances in the field.

APPENDIX: Supplementary Figures

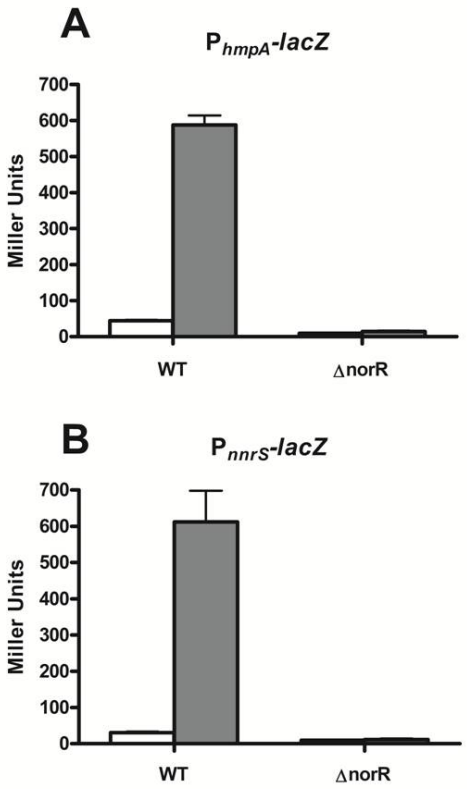

Fig. S1. The effect of NO and NorR on expression of *hmpA* and *nnrS* in LB medium. Strains containing a promoter-*lacZ* reporter were grown in LB in microaerobic conditions. Activity of the *hmpA* (A) or *nnrS* (B) promoter was measured after addition of 50 μM control compound diethylamine (white bars) or NO donor DEA-NONOate (gray bars) and reported as Miller units (15). Experiments were performed in wild-type strain C6706 or a strain containing a clean deletion of the entire *norR* ORF. Error bars represent standard deviation for experiments performed in triplicate.

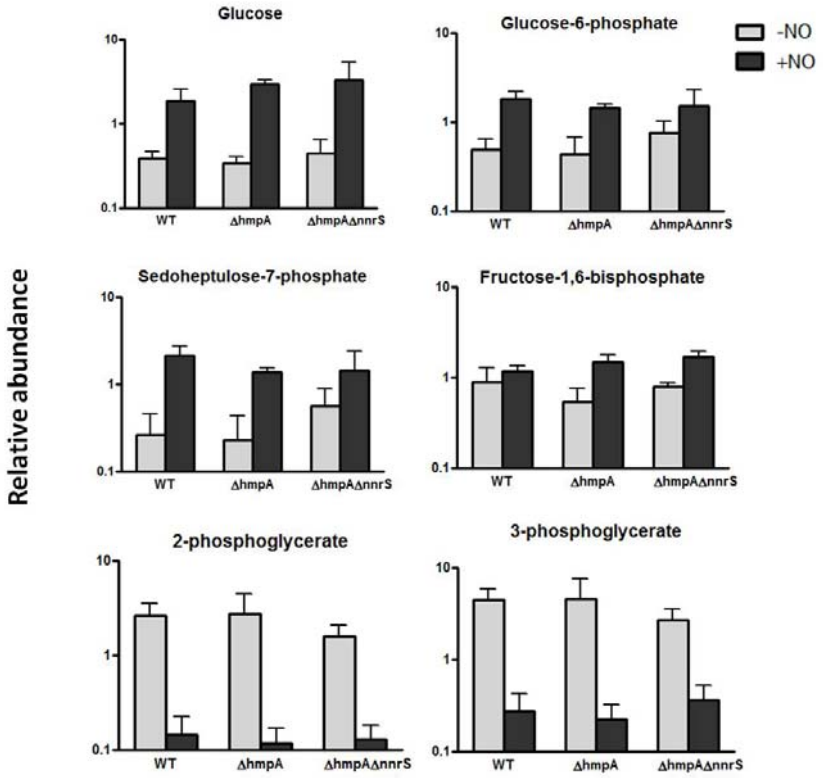

Fig. S2. Nitric oxide inhibits glycolysis. Relative concentrations of glycolytic intermediates in the three strains from the metabolomic study in the presence (+NO) or absence (-NO) of DETA-NONOate. Error bars represent standard deviation. Statistical significance was determined by two-way ANOVA, and for all metabolites, there was a significant effect of NO treatment on the variance across all samples ($P<0.001$).

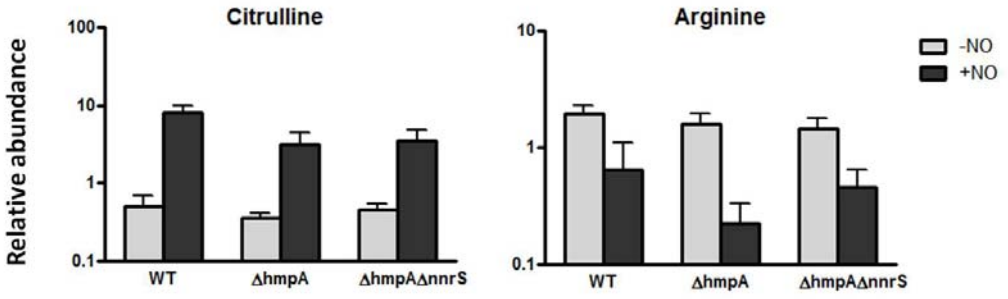

Fig. S3. Nitric oxide inhibits arginine synthesis. Relative concentrations of arginine synthesis intermediates in the three strains from the metabolomic study in the presence (+NO) or absence (-NO) of DETA-NONOate. Error bars represent standard deviation. Statistical significance was determined by two-way ANOVA, and for all metabolites, there was a statistically significant effect of NO treatment on the variance across all samples ($P<0.001$).

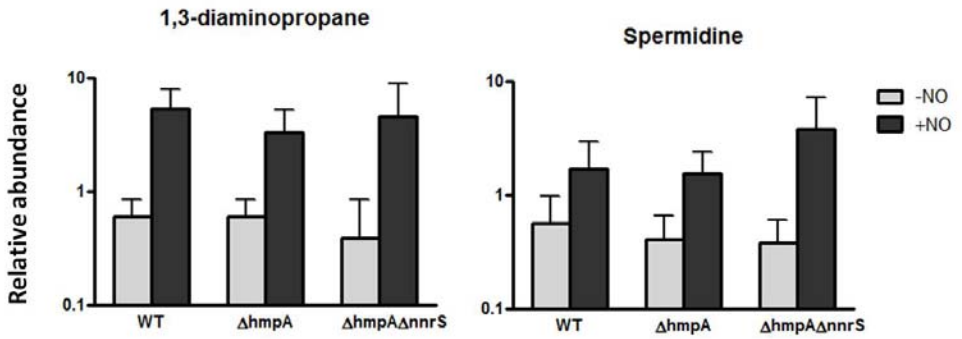

Fig. S4. Nitric oxide causes an increase in polyamine synthesis. Relative concentrations of polyamines in the three strains from the metabolomic study in the presence (+NO) or absence (-NO) of DETA-NONOate. Error bars represent standard deviation. Statistical significance was determined by two-way ANOVA, and for all metabolites, there was a statistically significant effect of NO treatment on the variance across all samples ($P<0.001$).

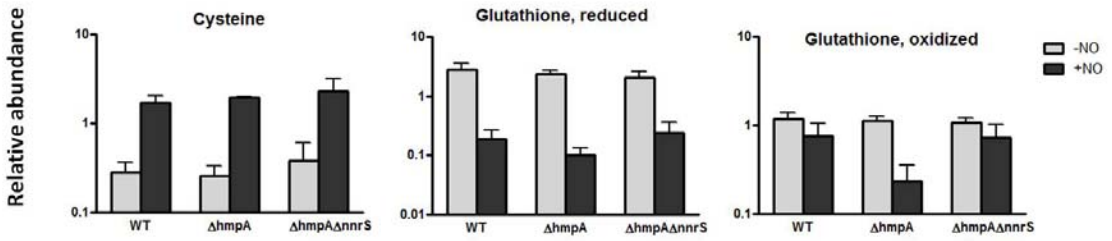

Fig. S5. Nitric oxide inhibits glutathione synthesis. Relative concentrations of glutathione and cysteine in the three strains from the metabolomic study in the presence (+NO) or absence (-NO) of DETA-NONOate. Error bars represent standard deviation. Statistical significance was determined by two-way ANOVA, and for all metabolites, there was a statistically significant effect of NO treatment on the variance across all samples ($P<0.001$).

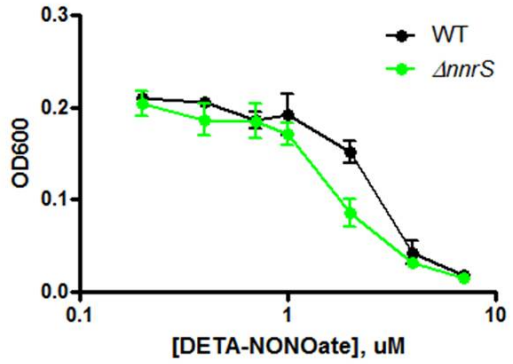

Fig. S6. NnrS is important for growth under nitrosative stress during anaerobic respiration. *V. cholerae* wildtype and *ΔnnrS* strains were inoculated into minimal media containing 0.2% (w/v) glycerol and 40 mM fumarate and placed in an anaerobic chamber. Growth of wildtype and Δ*nnrS* strains was monitored in the presence of increasing concentrations of DETA-NONOate. This graph represents the OD600 at 40 hours of growth.

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