THE DIVERSE FUNCTIONS OF NITRIC OXIDE SYNTHASES IN PROKARYOTES

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Nitric Oxide Synthases (NOSs) are highly regulated enzymes that oxidize L-arginine to synthesize the cytotoxin and signaling molecule nitric oxide (NO). NO in mammals is involved in many cellular process ranging from regulation of blood pressure to protection against pathogens; however, the roles of homologous prokaryotic NOSs are just beginning to be revealed. In *Streptomyces turgidiscabies* NOS supplies NO to produce 4-nitro-tryptophan which is a component of thaxtomin, a plant toxin responsible for the potato scab disease. In *Bacillus subtilis*, NO appears to protect against oxidative stress.

In this work I investigated the function of NOS in *Deinococcus radiodurans* (*D. radiodurans*), a bacterium that withstands desiccation, reactive oxygen species, and doses of radiation that would be lethal to most organisms. Deletion of the *nos* gene severely compromises the recovery of *D. radiodurans* from ultraviolet (UV) radiation damage. The Δ*nos* defect can be complemented with recombinant NOS, rescued by exogenous nitric oxide (NO) and mimicked in the wildtype strain with an NO scavenging compound. UV radiation induces the upregulation of the *nos* gene and cellular NO production on similar time scales. NOS-derived NO upregulates transcription of *obgE*, a gene involved in bacterial growth proliferation and stress response. Overexpression of the ObgE GTPase in the Δ*nos* background substantially

alleviates the growth defect after radiation damage. Thus, NO acts as a signal for transcriptional regulation for growth proliferation in *D. radiodurans*.

However, there is evidence of additional roles of NOS in *D. radiodurans.*. It has been shown previously that DrNOS interacts with tryptophanyl-tRNA synthetase II (TrpRS II), and DrNOS selectively nitrates tryptophan at the 4-position and TrpRS II couples this species to $tRNA^{Trp}$. I have studied the effect of Δ*nos* on $tRNA^{Trp}$ pools in *D. radiodurans*. These studies have found that *D. radiodurans* contains at least two forms of $tRNA^{Trp}$, whose presence depend upon the *nos* gene and the growth phase of the bacteria. One of the forms shows acid sensitivity, where as the other does not. Gene knockouts of both *trpRS* I and *trpRS* II in *D. radiodurans* are viable although growth of Δ*trpRS* II is compromised under stress. No link was found between NOS-associated tRNA modification and the ability to withstand radiation damage. Thus, in bacteria as in mammals, NOS appears to have multiple functions in *D. radiodurans*.

BIOGRAPHICAL SKETCH

Bhumit Patel was born to Ashok and Sangita Patel on May 29, 1983 in Anand, India. The three of them moved to United States in 1993, where they eventually settled in Middletown, New York. From 1997-2001 Bhumit attended Pine Bush High School in Pine Bush, NY and then went to State University of New York in Binghamton to study Biochemistry. While an undergraduate, he had the opportunity to conduct research with Dr. Susan Bane working on computational research of breast cancer analogs. Upon receiving his Bachelor's of Science degree from Binghamton University in 2004, Bhumit entered the Biochemistry, Molecular and Cell Biology graduate program at Cornell University. He joined the laboratory of Dr. Brian Crane in the Department of Chemistry and Chemical Biology, where he carried out his Ph. D. research on the diverse roles of nitric oxide synthases in bacteria.

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LIST OF ABBREVIATIONS

ATP: Adenosine triphosphate

B. subtilis: *Bacillus subtilis*

cPTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

CuFL: Copper fluorescein ligand

Dr: *Deinococcus radiodurans*

D. radiodurans: *Deinococcus radiodurans*

DNA: Deoxyribonucleoside

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraaceticacid

EtOH: Ethanol

FAD: Flavin Adenine Dinucleotide

FMN: Flavin Mononucleotide

H_2O_2: Hydrogen Peroxide

H_4B: Tetrahydrobiopterin

HPLC: High pressure liquid chromatography

5-HRP: 5- Hydroxy tryptophan

LB:Luria Broth

LC/MS: Liquid chromatography/Mass spectrometry

IPTG: Isopropyl β-D-1-thiogalactopyranoside

MeOH: Methanol

mNOS: Mammalian Nitric Oxide Synthase

MS: Mass Spectrometry

NADPH: β-Nicotinamide adenine dinucleotide phosphate,

NaOAc: Sodium Acetate

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NOSoxy: Nitric Oxide Synthase oxygenase domain

NOSred: Nitric Oxide Synthase reductase domain

NO: Nitric Oxide

4-NRP/4-NitroTrp: 4-Nitro tryptophan

SNP: Sodium Nitroprusside

TGY: 0.5% Tryptone, 0.1% Glucose, 0.3% Yeast Extract

THF: Tetrahydrofolate

TrpRS II: Tryptophanyl tRNA synthetase

Tris: Tris(hydroxymethyl)aminomethane

UV: Ultraviolet

WT: Wildtype

CHAPTER 1

THE VARIED ROLES OF BACTERIAL NITRIC OXIDE SYNTHASES

1.1 Abstract

Nitric oxide (NO) is produced from the oxidation of L-arginine (L-arg) to L-citrulline and is catalyzed by the heme-containing Nitric Oxide Synthases (NOSs). In mammals, NO is a widespread metabolite, cytotoxic agent and signaling molecule that participates in a large number of processes such as regulation of vascular tension, hormone release and neuronal signaling. Enzymes homologous to mammalian NOS have been found in prokaryotes; however, only a few studies have explored the functional role of bacterial NOSs. In certain *Streptomyces* strains, NOS is involved in nitration of a toxin, which is the causal agent of potato scab disease. In *Bacillus*, NOS plays a protective role against oxidative stress. In *Deinococcus*, NOS associates with an unusual tryptophanyl-tRNA-synthetase and can also nitrate tryptophan. The functional, catalytic, and structural properties of bacterial NOSs can be used to understand the mechanism and the targets of these varied enzymes.

1.2 Mechanism and chemistry of Nitric Oxide Synthases

NOSs are highly regulated enzymes that use L-Arg to synthesize the cytotoxin and signaling molecule nitric oxide (NO) (1-3). In mammals, NOSs are involved in many cellular activities such as regulation of blood pressure, protection against pathogens, and neuronal transmission (4). These enzymes are homodimers made up of an N-terminal heme oxygenase domain (NOS_{ox}) and a C-terminal flavoprotein reductase domain (NOS_{red}). The oxygenase domain binds L-Arg, heme, and the cofactor 6R-tetrahydrobiopterin while the reductase domain provides electrons from

FAD, FMN, and NADPH. A calmodulin binding domain connects the oxygenase and reductase domains and regulates the reduction of NOS_{ox}(1). NOS performs two successive monooxygenase reactions in order to generate nitric oxide. First, two electrons are contributed by NADPH, and a guanidino nitrogen of arginine undergoes a two electron oxidation leading to the intermediate-N-hydroxyl-L-arginine (NOHA) which in the second step gets further oxidized to form L-citrulline and NO (Figure 1.1) (5). Although much is known about the mechanism of NOS, there remain some questions on NOS catalysis, particularly concerning the heme-oxygen intermediates responsible for this oxidation.

Over the last decade, a great deal of work has been invested in understanding the chemistry of NO and reactive nitrogen species (RNS) such as nitrous acid (HNO_2), nitroxyl ion (NO^-), and nitrosonium cation (NO^+) (6). One of the best understood RNS is peroxynitrite ($ONOO^-$) which is formed when NO reacts with O_2^-; peroxynitrite is then used in oxidation (addition of NO) and nitration (addition of NO_2) reactions. One such nitration reaction involving peroxynitrite is the nitration of tyrosine which plays a key role in the pathogenesis of motor neuron disease (7). Another nitration, though enzymatic, is that of tryptophan which will be discussed in detail in two bacterial species, *Streptomyces turgidiscabies* and *Deinococcus radiodurans*.

1.3 Prokaryotic NOS – Structure and Enzymology

Genome sequencing has uncovered truncated homologous bacterial NOS proteins in Gram-Positive genera such as *Deinococcae*, *Streptomyces*, *Bacillus* and *Staphylococcus.* Bacterial NOSs are homologous to mammalian NOSs (mNOS) in the N-terminal heme oxygenase domain, but they lack the reductase domain. Nevertheless bacterial NOSs are dimeric, bind the substrate L-Arginine and produce

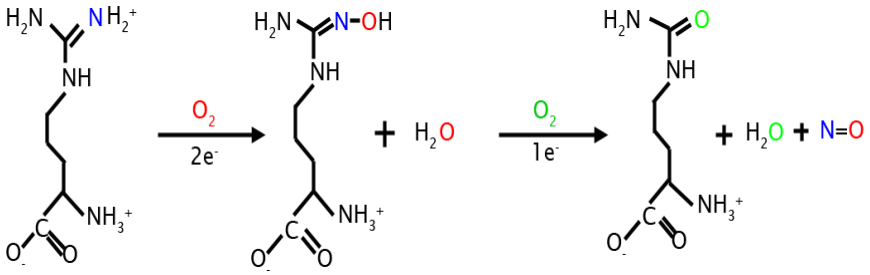

Figure 1.1 Reaction mechanism of NOS. In the first step, NOS converts the substrate L-Arginine into N-hydroxyl-L-Arginine (NOHA) by an oxidation reaction. It further oxidizes NOHA into citrulline and nitric oxide.

NO (8). Additionally, they retain structural and catalytic properties similar to their mNOS counterparts (9-13). The structures of *B. subtilis* NOS (bsNOS) bound with L-Arg and THF(10), *S. aureus* NOS (saNOS) complexed with S-ethyl-isothio-urea and NAD^+(14), and *G. stearothermophilus* NOS (gsNOS) with L-Arg(11) confirmed that the prokaryotic NOS are structurally similar to $mNOS_{ox}$ with the exception of the missing N-terminal region (10).

Despite lack of the reductase domain compared to mNOS, bacterial NOS are capable of accepting electrons from a number of different reductase partners. In *B. subtilis*, YkuN, a flavodoxin, can function as an electron donor for bsNOS and support NO synthesis *in vitro* (15, 16). However, if the *ykuN* gene is deleted, NO activity is still detected (16). Also, bacterial NOSs overexpressed in *E. coli* (which does not contain a NOS like protein) produce NO *in vivo* by utilizing heterologous reductases (17). The overproduction of the NOS protein is not toxic to *E.coli*. Thus, unlike mNOS which have a connected reductase module, bacterial proteins do not contain a dedicated reductase partner (16). On the other hand, *Deinococcus radiodurans* NOS (DrNOS) must produce NO in the absence of a flavodoxin reductase module, as the Dr genome lacks flavodoxin-like proteins. Whether a dedicated reductase is employed by DrNOS or not, a flavodoxin-like protein is not necessary to generate NO. Nonetheless, it is possible that particular functions require specific, yet to be determined, reductase enzymes. Recently, S*orangium (Polyangium) cellulosum*, a Gram-negative bacterium, was found to have a NOS (scNOS) with a covalently attached reductase module (13, 18). Compared to mNOS, the reductase domain of scNOS is N-terminal to the NOS_{ox} rather than C-terminal. Additionally, the flavodoxin module in mNOS is replaced by an FeS cluster, which may be capable of one electron chemistry (13).

Some prokaryotes, like *D. radiodurans* lack the pathway to synthesize the

necessary mNOS cofactor tetrahydrobiopterin (H_4B); however, there is *in vitro* evidence that another reduced pterin tetrahydrofolate (THF), could support NO production (9, 19, 20). Structural studies of NOS have also indicated that there is substantial variation in the region of the bacterial enzymes that recognize the pterin side chain (9).

1.4 The functional roles of bacterial NOSs

The first evidence of biological NO production was found in an anaerobic bacterium, *Pseudomonas perfectomarinus* which undergoes denitrification. This is the process of converting nitrite into NO, through copper or heme-based nitrite reductases (21). Although, NO is an important signal for these bacteria, they do not contain a NOS. However, with the sequencing of genomes, NOS-like proteins have recently been identified in a small subset of gram-positive bacteria, thus the function of these enzymes is limited. NO generated from NOS has been shown *in vivo* for *S. turgidiscabies*, *B. subtilis,* and *B. anthracis*(22-24). While the function of mNOS is well characterized, less is known about its bacterial counterparts. Of what is known, NO in bsNOS, DrNOS and stNOS (*S. turgidiscabies* NOS) play a role in different processes, though parallels seem to be emerging.

1.4.1 Streptomyces NOS (stNOS) is involved in the toxicity of thaxtomin

The first known function of NOS in bacteria is that from the pathogenic species of *Streptomyces* (25) . These organisms produce phytotoxin dipeptides called thaxtomins that contain a 4-nitrotryptophan moiety (26) and interfere with the function of the plant cell wall (Figure 1.2) (27). Thaxtomin is a cyclization of tryptophan and a phenylalanine connected by a dipeptide by nonribosomal peptide synthase genes (28).

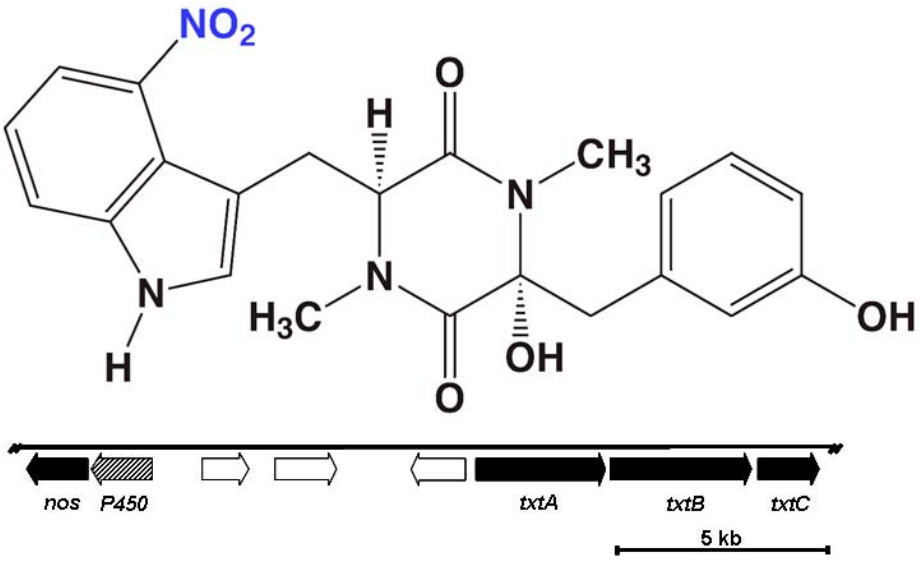

Figure 1.2. Thaxtomin contain a 4-nitrotryptophan moiety. *nos* is found on the pathogenicity island responsible for producing thaxtomin in *Streptomyces turgidiscabies*. NOS is responsible for nitration on the 4-position of tryptophan along with a p450 which is found adjacent to it. *txtA* and *txtB* are non ribosomal peptide synthase genes while *txtC* is another p450 which hydroxylates the phenylalanine.

Many experiments contributed to the discovery of the function of stNOS. The first clue came when the *nos* gene was located on a pathogenicity island that confers thaxtomin's biosynthetic ability. Through an *in vitro* assay that measures nitrite production, stNOS activity and thaxtomin production was shown to be dependant on L-Arginine and to be suppressed by NOS inhibitors. Additionally, thaxtomin production was greatly decreased in a *nos* knockout (Δ*nos*) strain, and was partially restored with *nos* complementation (25). Feeding studies with L-Arg-guanidino $^{15}N_2$-HCl confirmed the result (NOS is the only enzyme that oxidizes the guanidinium group of L-Arg to NO) and showed the radiolabel at the 4-nitro position (8). Taken together, the results definitively show that stNOS is involved in the nitration of thaxtomin. *In vitro,* agents capable of nitrating aromatic amino acids produce various products such as 4-nitro, 5-nitro, 6-nitro, 7-nitro, N'-nitroso, etc. Peroxynitrite preferentially nitrates tryptophan at the indole 6-position; however, stNOS is selective in its nitration and has the enzymatic control to nitrate tryptophan at the 4-position (29). It is known that cytochrome p450, a gene adjacent to the *nos* on the pathogenicity island is involved in the nitration process of thaxtomin; however, difficulties in the expression of soluble proteins (both NOS and p450) has hindered further biochemical characterization. *Deinococcus radiodurans* is another organism where 4-nitrotryptophan is thought to play an important role.

1.4.2 NO in Bacillus subtilis plays a cytoprotective role under oxidative stress

NO has been shown to protect various types of eukaryotic cells from H_2O_2 toxicity (30). Parallel studies in *Bacillus subtilis* utilized endogenous and exogenous NO for protection against oxidative damage from H_2O_2 toxicity. NO increases the survival of *B. subtilis* exposed to a low concentration of H_2O_2 (30 uM) but cannot protect the cells from a lethal dose (10 mM). It has also been shown Δ*nos* mutant

cells are 25 times more sensitive to oxidative stress than wildtype cells (23). H_2O_2 toxicity is attributable to DNA damage that occurs upon interaction with free cellular iron. H_2O_2 forms hydroxyl radicals (•OH) that react with DNA bases causing modification and strand breaks. In order for this to occur, iron must be reduced using reductants such as $FADH_2$ and cysteine to sustain the Fenton Reaction (23). NO suppresses the Fenton Reaction by inhibiting cysteine reduction of thioredoxin/thioredoxin reductase pathway via S-nitrosylation (31) . NO also mediates the cytoprotection by boosting the activity of preexisting H_2O_2 scavenging enzymes such as the iron-heme protein catalase, KatA, which degrades hydrogen peroxide into water and oxygen (23). Similar finding of NO-mediated thiol protection was found in the pathogenic *S. aureus* (23).

Macrophages are known to produce large amounts of nitric oxide and reactive oxygen and nitrogen species as a defense mechanism to infection (1). In *B. anthracis* NOS dependent resistance to oxidative stress from macrophages is thought to originate from NO-mediated activation of a bacterial catalase, like in *B. subtilis* and suppression of damage by the Fenton reaction. Additionally, *B. anthracis* Δ*nos* strain loses its virulence in a mouse model and shows compromised survival in macrophages (24). It is intriguing that NO protects the bacteria from the host and is also produced by the host as a component of its own protective oxidative burst.

1.4.3 The organism – Deinococcus radiodurans (*D. radiodurans*)

The *Deinococcaceae* are a family of bacteria that have an extraordinary ability to tolerate lethal effects of DNA damaging agents (32). Due to this ability, they have been a target of genetic manipulation and a candidate for the bioremediation of radioactive waste sites. Scientific evidence suggests that Deinococci do suffer massive DNA damage following irradiation; however, their efficient DNA repair

allows them to survive the exposure (32). One member of the family, *Deinococcus radiodurans,* is red pigmented, non spore-forming, non motile, gram positive and spherical (33). The genome of *D. radiodurans* consists of 2 chromosomes [2.64 Mbp, 412kbp], one megaplasmid [177kbp], and one plasmid [46kbp], carrying 3195 predicted genes. *D. radiodurans* is also multigenomic with four copies of its genome in stationery phase and 8-10 copies in the exponential phase per cell (34). This complexity may be necessary for quick repair of double stranded breaks. Evidence suggests that *D. radiodurans* does not passively protect its genome from radiation, although there is a group that has shown correlation that the higher the Mn/Fe ratio, the more the radio resistant the organism (35). They have proposed that sensitive bacteria sustain lethal levels of protein damage at radiation doses that elicit relatively little DNA damage, and that extreme resistance in bacteria is dependent on protein protection (36). Rather, it rapidly and accurately repairs DNA damage through inhibition of DNA replication and DNA degradation, and using its genome multiplicity for interchromosomal recombination (37-39). *D. radiodurans* adaptation involves multiple protective mechanisms, including efficient homologous recombination among its 8-10 genome copies, a tight nucleoid organization, and unusually high intracellular Mn/Fe ratio, which can support/participate in protection against oxidative damage (36, 40-42). *D. radiodurans* also has excessive amounts of carotenoids, superoxide dismutase and catalase which play a vital role in the defense mechanism. Despite years of investigation, our knowledge of *D. radiodurans*' extraordinary ability to survive DNA damage is incomplete (32). Additionally, most of the implicated repair genes against radiation are similar to those found in other organisms and the repair mechanisms themselves are not unusual (36, 40-42).

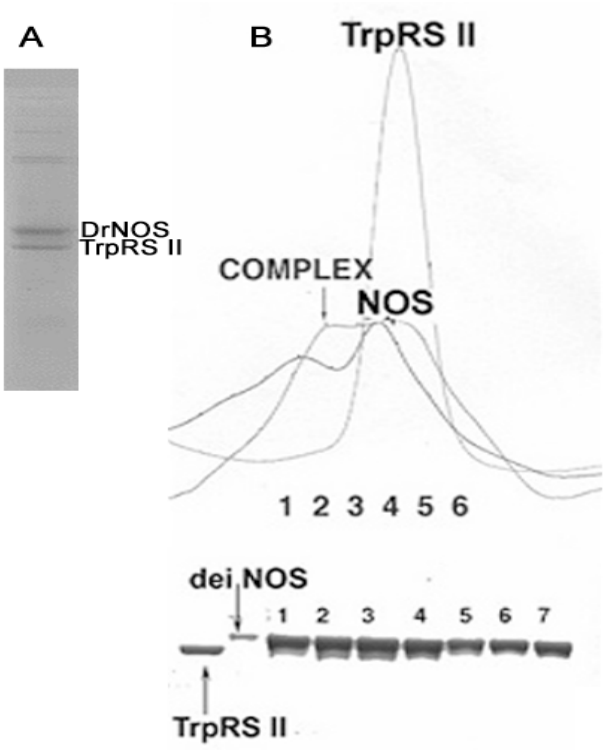

Figure 1.3. DrNOS-DrTrpRS II interaction. A) Co purification of recombinant His_6-tagged deiNOS and untagged drTrpRSII through affinity chromatography. B) Co-migration of DrNOS and DrTrpRS II on size exclusion chromatography. Below is a gel of the corresponding fractions. Samples 2-4 strongly correspond to the complex of NOS-TrpRS II.

1.4.4 NOS-TrpRS II complex in D. radiodurans

Since there was evidence from the stNOS system that NOS is involved in nitration of tryptophan, we tested if DrNOS was involved in trp metabolism . It was found that DrNOS interacts with tryptophanyl tRNA synthetase (TrpRS) *in vitro* (Figure 1.3). Aminoacyl tRNA synthetases (AARSs) catalyze the same two step chemical reaction, $AA + ATP \Leftrightarrow AA\text{-}AMP + PP_I + tRNA \Leftrightarrow AA\text{-}tRNA^{AA} + AMP$, where AA represents an amino acid and $tRNA^{AA}$ represents a transfer RNA specific for that amino acid. AARSs play a key role in maintaining integrity and accuracy during translation of the genetic code. To achieve this challenging task, AARSs have to discriminate between amino acids that are closely related not only in structure but also in chemical nature.

In the case of *D. radiodurans* the interaction between NOS-TrpRS II increases the solubility of NOS and the stability of both proteins. More importantly, it allows NOS to nitrate tryptophan at the 4-position, producing the same moiety generated in thaxtomin (43), a link between DrNOS and stNOS that correlates well with them falling into the same subgroup after various phylogenetic comparisons (13) .

An unusual feature of *D. radiodurans* is that it contains two TrpRS genes. TrpRS I has ~40% identity to most single copy bacterial TrpRS, while the second, TrpRS II, has 28% identity (8, 43). Genome searches have found that of the prokaryotes with two TrpRS proteins, the second synthetase has 50% identity with Dr TrpRS II. The complex of TrpRS II and DrNOS also has a higher tryptophan nitration activity as compared to DrNOS alone(8). Investigation of the crystal structure of TrpRS II gave evidence that tryptophan binding pocket can accommodate tryptophan derivatives such as 4-nitrotryptophan (4NRP) and 5-hydroxyltryptophan (44, 45). *In vitro* results have shown that TrpRS II catalyzes the adenylation of Trp and the

charging of *D. radiodurans* $tRNA^{Trp}$. TrpRS II can also adenylate 4NRP and charge *in vitro* transcribed *D. radiodurans* $tRNA^{Trp}$ at about the same efficiency as it does tryptophan. In contrast, TrpRS I is unable to charge these indole derivatives (44). Taken together, these studies suggest a biological function for the association of TrpRS II with NOS that will likely show a difference in the $tRNA^{Trp}$ pools *in vivo*.

1.5 Characteristics and function of transfer RNA

Transfer RNAs, about 75 nucleotides long, are of key importance in the translational machinery. They are involved in ribosome-mediated protein synthesis where they read the information encoded in the mRNA and insert the appropriate amino acid into the growing peptide chain (46). While most transfer RNAs are involved in protein synthesis, a few are involved in the synthesis of secondary metabolites. One such example is that of glutamyl-tRNA which is involved in the biosynthesis of δ-amino-levulinic acid, the sole precursor to porphyrin rings of heme and chlorophyll. It was found in *Synechocystis*, a single $tRNA^{Glu}$ transcribed as monomeric precursor tRNA matures into two $tRNA^{Glu}$ species. One of which participates in protein synthesis, while the second with a modification in the anticodon participates in porphyrin synthesis (47). Features of transfer RNA that contribute to their function are the number, location and type of modifications.

The fact that the modified nucleosides on each tRNA have vastly different chemical structures, occur in various locations, and influence tRNA reactions suggests that each nucleoside may have its own function (48). Typically about 10% of the nucleosides in a particular tRNA are modified. Over 80 of these have been characterized; and many were found to be conserved across all three phylogenetic domains of life (49). The abundance of modified nucleotides in tRNA suggests that they have a pivotal role in the function of a tRNA. Modifications in the anticodon

loop alter codon recognition and aminoacylation properties, and prevent ribosomal frameshifting. Additionally, some modifications provide significant stabilization to the folded tRNA. In eukaryotes, modified nucleosides are thought to play a role in disease. Point mutations in the genes encoding mitochondrial tRNAs are frequently found in diseases such as mitochondrial myopathy and lactic acidosis (50). Modified nucleosides have also been found in the urine of patients with leukemia and lymphoma as a result of turnover and degradation of RNA (51). The modifications on $tRNA^{Lys}$ in humans is used as the primer for reverse transcription by HIV-1 (52). These are just some of the important roles tRNAs play in controlling cellular processes; the function of many tRNA modifications is not yet established.

1.6 Conclusions

With so many diverse lifestyles of prokaryotes, the function of NOS seems to be varied. As more cellular studies unveil new functions of bacterial NOSs, we hope to learn about novel biochemical pathways. In parallel to mNOS, bacterial NOSs appear to be a general source of NO for multiple cellular processes within the same organism. However, to discover novel chemical and biological aspects of NO, we must learn the specifics of NO in these simple, yet unique organisms.

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CHAPTER 2

ENDOGENOUS NITRIC OXIDE ENABLES *D. RADIODURANS* TO RECOVER FROM EXPOSURE TO UV LIGHT

2.1 Abstract

Deinococcus radiodurans (Dr) withstands desiccation, reactive oxygen species, and doses of radiation that would be lethal to most organisms. Deletion of a gene encoding a homolog of mammalian nitric oxide synthase (NOS) severely compromises the recovery of *D. radiodurans* from ultraviolet (UV) radiation damage. The Δ*nos* defect can be complemented with recombinant NOS, rescued by exogenous nitric oxide (NO) and mimicked in the wildtype strain with an NO scavenging compound. UV radiation induces both upregulation of the *nos* gene and cellular NO production on similar time scales. Growth recovery does not depend on NO being present during UV irradiation, but rather can be manifested by NO addition hours after exposure. Surprisingly, *nos* deletion does not increase sensitivity to oxidative damage and hydrogen peroxide does not induce *nos* expression. However, NOS-derived NO upregulates transcription of *obgE*, a gene involved in bacterial growth proliferation and stress response. Overexpression of the ObgE GTPase in the Δ*nos* background substantially alleviates the growth defect after radiation damage. Thus, NO acts as a signal for transcriptional regulation for growth proliferation in *D. radiodurans*.

2.2 Introduction

Nitric oxide (NO) is a widespread metabolite, cytotoxic agent and signaling molecule that reacts directly with a select few biological targets (1, 2). In mammals and other higher organisms, NO participates in a large number of processes, including protection

against pathogens, regulation of vascular tension, hormone release and neuronal signaling (3, 4). In bacteria, NO is a key intermediate in nitrate respiration (denitrification), and has recently been shown to act as a regulatory signal for cell dispersal and nitrosative stress responses (5, 6). In mammals, NO is produced from the oxidation of L-arginine (L-arg) to L-citrulline and is catalyzed by the heme-containing NO synthases (NOSs). Mammalian NOSs (mNOSs) are homodimers that contain two domains: an N-terminal heme oxygenase domain (NOS_{ox}) which binds the substrate L-arg and cofactors heme and tetrahydrobiopterin (H_4B), and a C-terminal reductase domain (NOS_{red}) that binds FAD, FMN, and NADPH (7-9). Proteins with homology to the $mNOS_{ox}$ domain are found in several bacteria mainly from the Gram-positive genera *Streptomyces*, *Bacillus*, *Staphylococcus* and *Deinococcus* (10-13). These proteins lack NOS_{red}, but retain structural and catalytic properties similar to their mNOS counterparts (10, 12-15). Only a few studies have explored the functional role of bacterial NOSs. NOSs from certain *Streptomyces* strains are involved in the nitration of a tryptophanyl moiety of thaxtomin, a dipeptide phytotoxin which interferes with plant cell wall synthesis (11, 16, 17). In contrast, NOS-derived NO appears to protect against oxidative damage in Bacilli and Staphylococci (*18, 19*).

In both mammals and plants, NO production is an important response for exposure to ultraviolet (UV) radiation (20-22). *D. radiodurans* is especially adapted to survive UV radiation, ionizing radiation, desiccation, and oxidative damage (23, 24). *D. radiodurans* adaptation involves multiple protective mechanisms, including efficient homologous recombination among its 8-10 genome copies, a tight nucleoid organization, and unusually high intracellular Mn/Fe ratio, which can support/participate in protection against oxidative damage (23-26). Nevertheless, most of the implicated genes are similar to those found in other organisms and the repair mechanisms themselves are not unusual (23-26).

We have previously demonstrated through biochemical means that the NOS from the radiation-resistant bacterium *D. radiodurans* (DrNOS) interacts with an unusual auxiliary tryptophanyl tRNA-synthetase (TrpRS II) (27); however, the significance of this association remains unclear (*28, 29*). Here, we have undertaken a genetic approach in an attempt to discover functions for DrNOS. These studies have revealed that the NO generated by DrNOS aids in the recovery of *D. radiodurans* from ultraviolet (UV) radiation damage.

Deletion of *nos* (Δ*nos*) renders *D. radiodurans* more susceptible to UV radiation than the wildtype (wt) strain. The mutant can be rescued by genetic complementation, addition of NO donor compounds, and application of exogenous NO gas. Remarkably, the rescue is effective even when the NO is supplemented hours after UV exposure. Furthermore, we observe that the *D. radiodurans nos* gene is induced by UV damage and causes a measurable increase in NO production within the cell. We further show that NO upregulates *obgE*, a gene for an essential GTPase involved in the regulation of many growth-related processes. Overexpression of ObgE in a Δ*nos* background partially alleviates the growth defect observed after UV irradiation.

2.3 Methods

Bacterial strains and growth conditions. Bacterial strain wildtype (wt) *Deinococcus radiodurans* R1 was obtained from the American Type Culture Collection (ATCC 13939). Cells were grown in TGY (0.5% tryptone, 0.3% yeast extract and 0.1% glucose) at 30°C or plated on TGY with 1.5% Bactoagar (Difco).

*UV treatment***.** 2 mL of cells in a 3.5 mL quartz cuvette (optical density at 600 nm (OD_{600}) 0.8-1.0) were exposed to polychromatic UV radiation (200-500 nm, 30

mW/cm^2) from a mercury/xenon lamp for 5 min. Irradiated cells were then diluted 1:100 in fresh TGY and OD_{600} measured as a function of time. Cells were also plated and single colonies were counted at 24 and 48 hrs**.** Overall we found rates of growth as measured by optical density a more robust method for quantifying recovery from UV damage than colony counts. This is largely because it is difficult to completely kill *D. radiodurans* and as such even highly irradiated cultures will eventually produce colonies. To test the effects of NO donors and scavengers, wt cells were preincubated with 100μM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) or 1 mM SNP (sodium nitroprusside) 10 min prior to UV irradiation. To evaluate the effect of NO addition post irradiation, NO to a final concentration of 5μM was bubbled to 1:100 diluted cells already exposed to UV irradiation after various lag times and the cell density measured at 22 hours post exposure. The concentration of NO in solution was determined using the hemoglobin assay (30). For the overexpression experiments, the wt, Δ*nos*, and complementation strains Δ*nos*:pNOS, Δ*nos*:pObgE were grown to OD_{600} 0.2 induced when necessary with 10mM IPTG, and grown for 9 hours or OD_{600} 1.0 before being irradiated for 5min. The cells were diluted 1:100 in TGY and allowed to recover for ~22 hours. At least three, and in some cases more than eight independent experiments were performed for each condition. BacLight (Molecular Probes) was used to assess the percent of viable cells prior by determining cells that had intact membranes or not, prior and following irradiation according to manufacturer's protocol.

*Gene disruption***.** The gene *nos* was disrupted by targeted mutagenesis using techniques previously described (31). Briefly, the streptomycin resistance gene fused to the *D. radiodurans katA* promoter was cloned from the plasmid TNK103 (31). Genomic DNA sequences 1kb upstream and downstream of *nos* (DR2597) were

appended to the drug cassette by overlap extension. This process added HindIII and XhoI sites for cloning into Litmus-28 (New England Biolabs). The resulting plasmid was transformed into *D. radiodurans* R1 by electroporation. Recombinant cells of Δ*nos* were selected on TGY plates containing 8μg/ml streptomycin. The transformants were serially plated, isolated, and re-plated at least 8 times on streptomycin. Disruption was followed by isolation of genomic DNA and PCR analysis for the native *nos* gene and the disruption cassette. Final isolates were tested by RT-PCR to confirm the lack of *nos* transcript. A similar protocol was followed to generate knockout mutants for the *trpRS* I (DR0558) and *trpRS* II (DR1093) genes. Plasmid TNK104 which contains the katA promoter fused to a hygromycin resistance gene was used instead of TNK103 to generate Δ*trpRS* I and Δ*trpRS* II. Double knockouts of Δ*nos*Δ*trpRS* I and Δ*nos*Δ*trpRS* II were constructed by the recombination of individual plasmids made above in the Δ*nos* strain containing the antibiotic resistance sequence and then selecting the clones on both streptomycin and hygromycin background.

*Genetic complementation***.** A plasmid to express recombinant NOS in *D. radiodurans* R1 was constructed from the *E. coli* shuttle vector p11530, which contains a Pspac IPTG-inducible promoter and cam^R antibiotic marker (32). NOS gene fragment was amplified by PCR from pet15_NOS template (the coding sequence of which was initially isolated from genomic DNA) (10) and cloned into p11530 using PdiI and XhoI restriction sites generating pNOS. The plasmid was electroporated into the Δ*nos* strain to form Δ*nos*:pNOS and transformants were selected on TGY plates containing 3μg/ml chloramphenicol. Reintroduction of the *nos* gene was confirmed by PCR. The same protocol and plasmid were used for overexpression of *obgE* in Δ*nos*.

mRNA expression. Cells (10 mL, OD ~ 0.8) were grown at 30º C, UV-irradiated for 5 minutes, and harvested after 30, 60, 180, 360, 540 minutes (Control cells (t = 0) were treated similarly without irradiation). Cells were resuspended in 100µl Tris-EDTA and lysed by vortexing with 25µl glass beads (Sigma). Total RNA was extracted from non-irradiated and irradiated cells using the RNEasy kit (Qiagen). DNA (0.5µg) from each sample was treated with DNase-I (Promega), and converted to cDNA with First Strand Synthesis (Invitrogen) using random hexameric oligonucleotides following the manufacturer's protocol. PCR amplification of ~350bp was carried out using 2 µL of cDNA as template. Fluorescent imager and ImageJ software was used to quantify band intensities. Results are representative of at least three independent experiments. To measure *obgE* expression, the cDNA obtained above was subjected to quantitative real-time PCR (QRT-PCR) by the use of QuantiFast SYBR Green PCR Kit (Invitrogen) following manufacturer's protocol and data acquired using Applied Biosystems 7500 Real-Time PCR System. The following primers for the genes encoding nitric oxide synthase (DR2597) and glyceraldehyde 3-phosphate dehydrogenase (DR1343)**:** nosF 5'ctgctccagcatctcgacgacgcct 3', nosR 5' atgtccaggtgcatgtcgctgatg 3', gapF 5' gagtacgacgaaagcagcctgacg 3' and gapR 5' cgttggtgtagctgtgcacggtggtc3'. Controls were carried out without addition of reverse transcriptase and with Rnase treatment prior to PCR to confirm that amplified bands were not due to contamination by genomic sequences. The PCR reaction (50µl) was carried out using the following cycling conditions: 98ºC for 5 min, 37 cycles of 95ºC for 1 min, 52ºC for 30 sec, and 72ºC for 30 sec. Samples were then electrophoresed on a 1% agarose gel, and stained with ethidium bromide. *nos* message levels were compared to that of *gap*, whose expression is not

altered by UV radiation. PCR reactions were confirmed to be within the linear range of amplification by varying amplification cycles. The forward and reverse primers in the 5' → 3' order for other genes are listed below:

obgE fp: ggtcgaggaattacaacagctt, obgE rp: aactcgggcaacaactggaata

recA fp: gcaaactcgacgtgcaggtcgtcagca, recA rp: gtgctcggcgtcgataaacgcacag

uvrA fp: tcacgcaggttcttcagcgtgccgat, uvrA rp: ggctgaagtttctggtggacgt

uvsE fp: gctgtgctcacgcatctagcgccgca, uvsE rp: agcagattccagggcgtgcgcgccag

*Fluorescence microscopy***.** NO production was detected from cells with the cell permeable NO specific probe Cu(II)Fluorescein ligand (CuFL) (Strem Chemicals Inc.) as described (33). Cu(II)-fluorescein was freshly prepared by mixing the FL ligand (1mM in DMSO) with $CuCl_2$ (1 mM) in a 1:1 ratio. Cells grown to an OD_{600} ~ 3 were washed with phosphate-buffered saline (PBS) to remove TGY. Cells were UV irradiated for 5 min and then incubated with 10μM CuFL for one hour at room temperature. Cells were then washed with 1ml PBS to remove excess CuFL and observed under the microscope. Images were obtained at the PCIC (Plant Cell Imaging Center) supported by TRIAD Foundation (NSF DBI-0618969) using an Olympus SZX-12 stereo fluorescence microscope equipped with a 63x objective lens in water, a I3 wavelength filter [excitation (450-490nm) emission (515nm)], and the Optronics MagnaFire acquisition software.

Microarrays. Ten milliliters of *D. radiodurans* wt and Δ*nos* were harvested 1 hour after exposure to UV irradiation for 5 min. Controls cells without UV irradiation were harvested similarly and RNA was extracted as mentioned previously. RNA (4μg) was annealed to 9μg of random hexamer primers (Invitrogen) in total volume of 18μl at 70° C for 10 min followed by placing them on ice for 2min. The First strand synthesis

mix contained 6µl 5x first strand buffer, 3µl 0.1M DTT, 0.4µl 50x dNTP mixture, 1µl RNAout and 2µl Superscript III RT enzyme for a total of 12.4µl. cDNA was synthesized at 42° C overnight in total of 31µl using SuperScript III Reverse Transcriptase Kit (Invitrogen) with 0.5mM dNTP mix containing amino allyl-dUTP (Sigma). Microarray design and usage were carried out as previously described (34). Genes that showed a 2x difference (wt – Δ*nos,* post irradiation) in 3 experiments were selected for further analysis by RT-PCR.

2.4 Results

2.4.1 Deletion of nos affects growth recovery after UV irradiation

We produced single gene deletions of *nos*, *trpRS* I, and *trpRS* II and double deletions of *nos/trpRS* I and *nos/trpRS* II in *D. radiodurans* strain R1 using allelic replacement. Deletions were confirmed with genomic PCR specific for the target and replacement genes, and with RNA transcript analysis by reverse transcriptase PCR (RT-PCR). The expression levels of the flanking genes do not change significantly under basal and post-irradiation conditions in the Δ*nos* strain compared to the wt (Figure 2.1). Δ*nos* exhibited slightly slower growth compared to wt under rich media conditions (Figure 2.2). Nonetheless, enhanced growth defects or changes in cell morphology were not observed when Δ*nos* was subjected to a variety of stress conditions, including increased temperature, acidity, salinity, DNA damaging agents (methyl methanesulfonate, phleomycin), and oxidative stress (Table 2.1). Furthermore application of hydrogen peroxide (H_2O_2) at concentrations as high as 1M did not distinguish the mutant from wt (Figure 2.3). However, Δ*nos* did display a striking difference in the ability to grow after a 5 min exposure to polychromatic UV irradiation (30 mW/cm^2) (Figure. 2.2). This UV exposure, which is lethal to non-UV resistant bacteria such as *E. coli*, was sufficient to cause extensive DNA shearing as

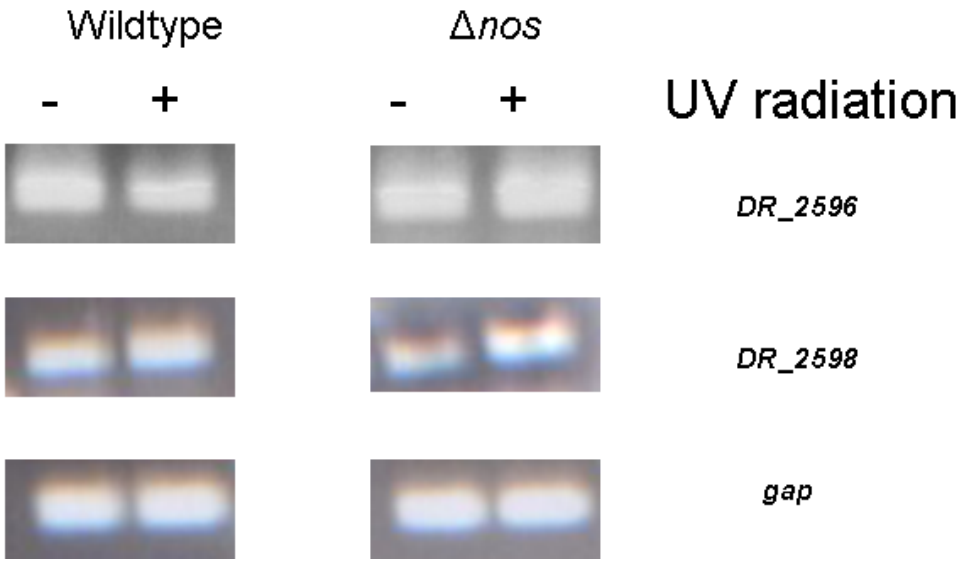

Figure. 2.1. The genes flanking DR_2597 (*nos*): DR_2596 and DR_2598 code for proteins of unknown function. Their expression patterns remain unchanged in the Δ*nos* compared to the wt strain under basal and post-irradiation conditions. DR_2596 is unaffected by radiation but DR_2598 appears to be upregulated. This is not surprising as the open reading frames for *nos* and *DR_2598* are expressed on the same mRNA.

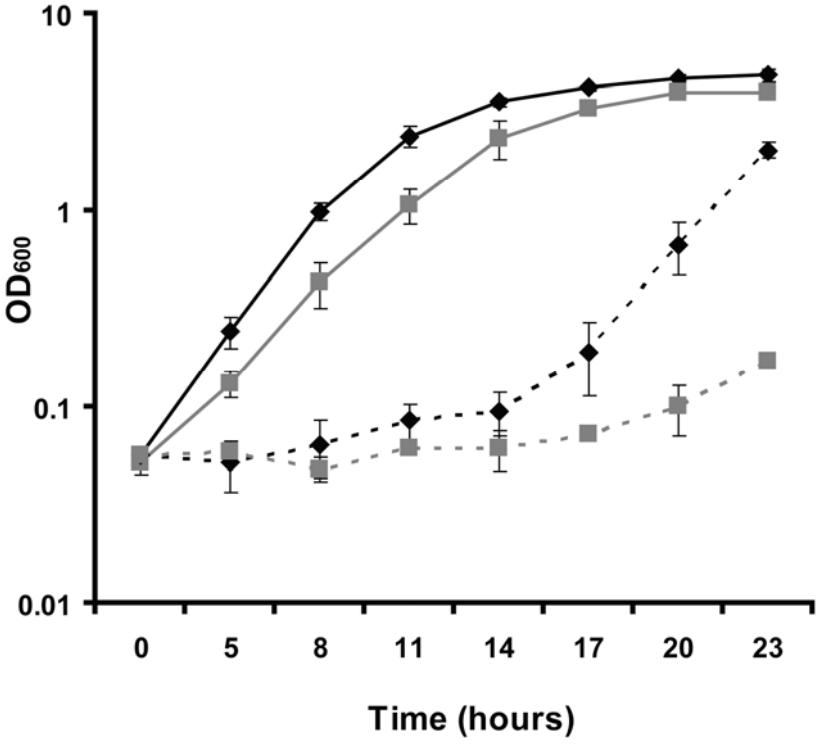

Figure 2.2. Δ*nos* is more susceptible to UV radiation than wt. Growth curves (monitored by OD_{600}) are represented as (—♦—) non-irradiated wt, (—■—) non-irradiated Δ*nos*, (--♦--) irradiated wt and (--■--) irradiated Δ*nos*. Irradiated cultures (OD ~0.8) were exposed to polychromatic UV radiation (30mW/cm^2) prior to 1:100 dilution into TGY media. Data are plotted as mean ± s.d. of three independent experiments.

Table 2.1 Comparison of wt and Δ*nos* growth under various stress conditions. Growth was evaluated by measuring the growth recovery where cells were taken in log phase and diluted into liquid cultures containing the appropriate stress condition, plating cells after stress exposure and counting the colony formation units (CFU). Zone of inhibition (ZOI) assay is where the stress condition was placed on a filter disk which was placed on a lawn of cells.

Condition tested	**Growth assessed by**	**Growth affect on wt and Δ*nos***
Temperature - 48°C	Growth recovery	50% growth of normal levels for both
Acidity – pH 3.7	ZOI assay	Little growth on plates for both
High salt – 1.2M NaCl	ZOI assay	Little growth on plates for both
H_2O_2 – 100µM, 10mM, 100mM, 1M	CFU Growth recovery ZOI assay	Figure 2.3
Ethanol – 3%, 10%	CFU Growth recovery ZOI assay	No change detected between wt and Δ*nos* in either liquid or plated cultures. 3% - 20% reduced growth 10% -50% reduced growth
MMS - 0.01%, 0.05%, 0.001%	CFU Growth recovery ZOI assay	No change in liquid culture recovery. Hard to score plates as smaller colonies would appear.
Phleomycin 5ng/µl, 1ng/µl	CFU Growth recovery ZOI assay	No change detected between wt and Δ*nos* in either liquid or plated cultures.

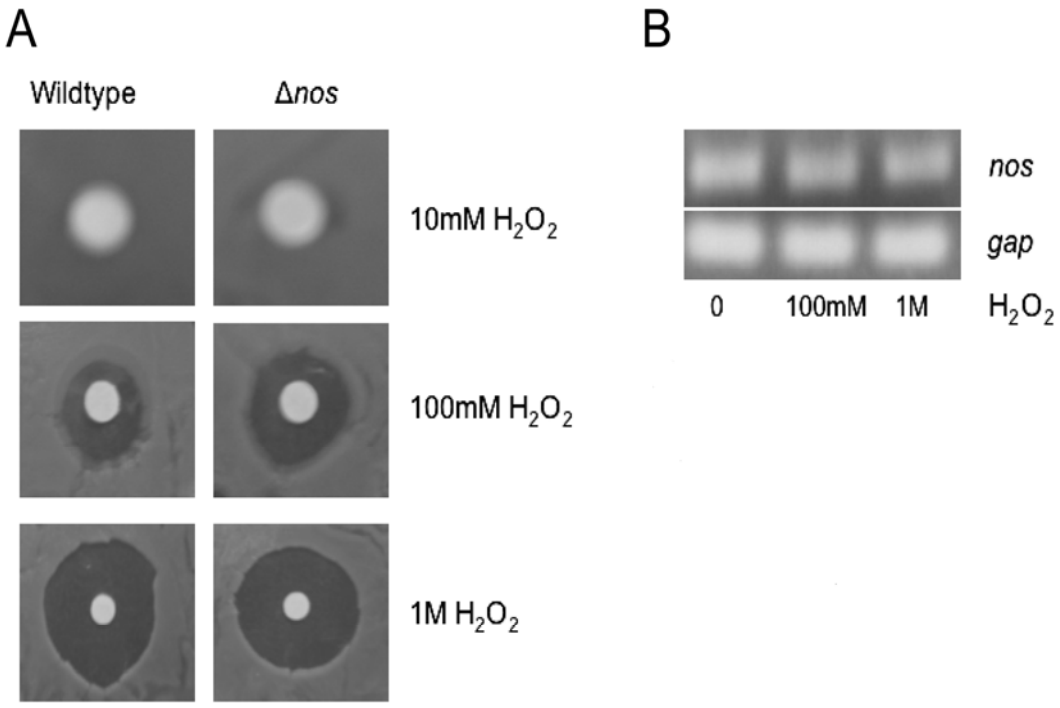

Figure 2.3. Δ*nos* does not harbor a growth defect in the presence of peroxide. (A) WT and Δ*nos* were grown to OD_{600} at 30°C. The cells were then plated on TGY agar plates, and a filter disk, saturated in the appropriate concentration of H_2O_2, was placed on top of the lawn. The plates were allowed to grow for two days at 30°C. Δ*nos* (right panel) is not affected by any concentration of H_2O_2 compared to the wildtype (left panel), ruling against *nos* playing a protective a role against peroxide in Dr. The same result is obtained if cells are grown in the presence of H_2O_2 in liquid cultures. Dr shows high tolerance to peroxide, as cell growth is unaffected by 10mM H_2O_2. (B) Additionally, the *nos* mRNA levels remain unchanged after exposure to as much as 1M H_2O_2 for one hour (more DNA was loaded at concentrations comparable to the UV irradiation experiment in **Figure 2.8** to clearly show the presence of the *nos* gene, which is not highly expressed under these conditions).

resolved by DNA gel analysis and promoted upregulation of key DNA repair genes *recA*, *uvrA*, and *uvsE* (Figure 2.4). Compared to wt, Δ*nos* cell density measured by optical density (OD_{600nm}) was reduced by over 95% and required 6 more hours (which corresponds to ~ 4 doubling times under rich media conditions) to reach its exponential growth phase after UV radiation (Figure. 2.2). We quantified the growth recovery of the wt and Δ*nos* strains in terms of colony forming units (CFUs) by serial dilution of culture suspensions onto TGY/agar plates immediately after UV exposure. This analysis showed that Δ*nos* produces 10^3-10^4 fewer CFUs visible to the naked eye, than wt two days after plating. Assays for cell viability indicated that irradiation does kill a substantial number of cells (~40%) in both cases but the number of lysed cells prior to and following irradiation was the same for wt and Δ*nos* (Figure 2.5). The lysed cells do not have intact membranes; therefore, are assumed to be dead. These results show that deletion of the *nos* gene does not lead to more cell death immediately following radiation but result in slower growth recovery.

To ensure that the growth defect of Δ*nos* subsequent to UV exposure was mediated by *nos,* a chloramphenicol resistant expression plasmid under the control of an IPTG inducible promoter was introduced in the Δ*nos* strain to form Δ*nos*:pNOS. The expression level of *nos* from induced Δ*nos*:pNOS is higher than that of wt without UV exposure, but lower than that of wt with UV exposure (Figure 2.5B). However, we were able to induce the expression of the *nos* gene in *trans* and rescue the growth phenotype of Δ*nos* to levels 55% of wt (Figure 2.6). Expression of recombinant proteins in *D. radiodurans* has only been achieved in a few cases due to the difficulty of maintaining exogenous plasmids in the bacterium (35, 36). It should also be noted that selection with antibiotics following irradiation retards growth of uninduced *D. radiodurans* Δ*nos*:pNOS compared Δ*nos*. The Δ*nos*:pNOS strain was grown in the presence of antibiotics in order to keep the plasmid from being lost; however,

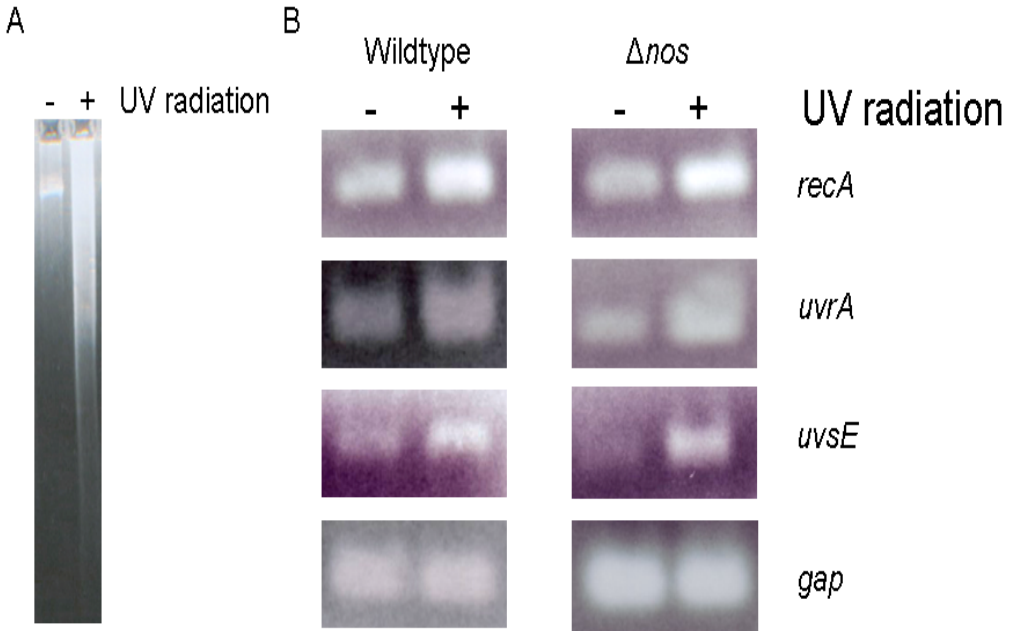

Figure 2.4. NOS does not affect the induction of key UV DNA repair genes. (A) The exposure of cells to UV radiation reveals smaller fragments of DNA. (B) mRNA expression levels were evaluated for *recA*, *uvrA*, and *uvsE* with the same procedure used to measure *nos* mRNA levels. In the wt, the mRNA levels of these genes increase after exposure to radiation. However, Δ*nos* also shows a similar increase in mRNA levels. Thus, *nos* does not appear to be required for the induction of these repair genes.

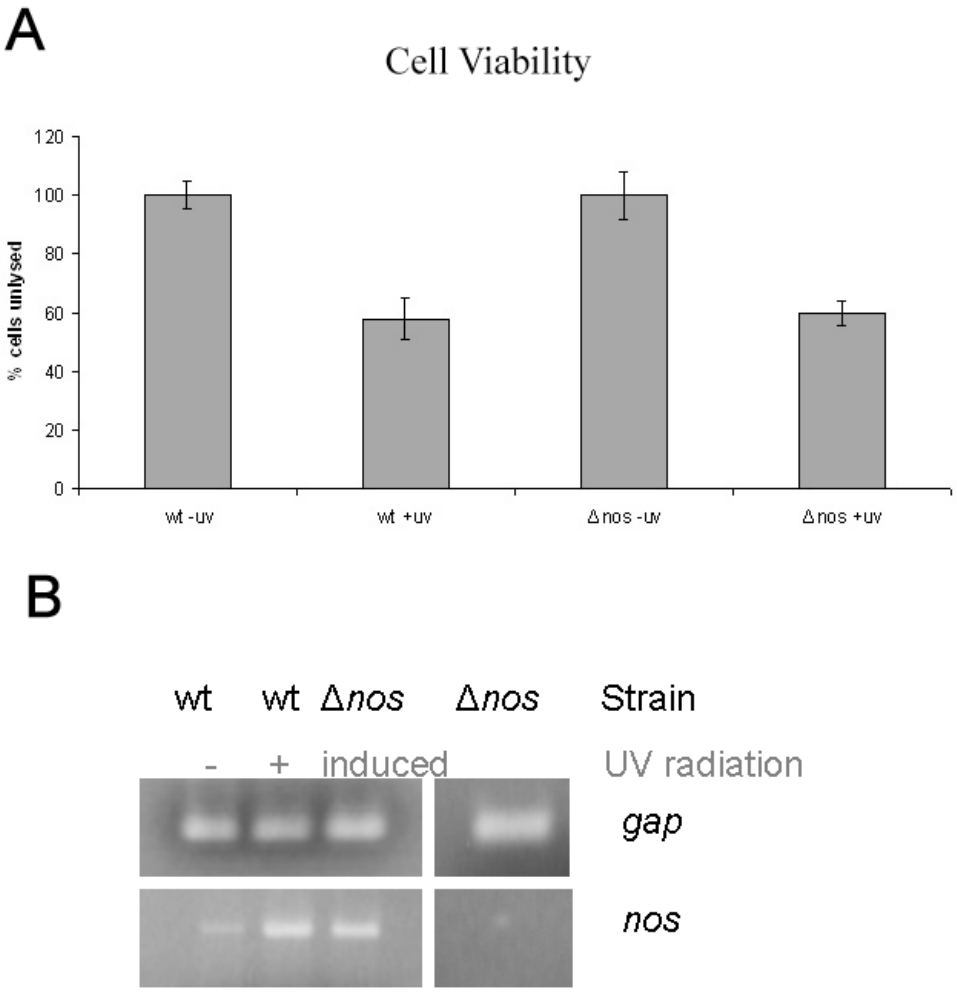

Figure 2.5. **Cell viability and *nos* expression**. (A) The percent of input CFU viable following irradiation of wt and Δ*nos* is the same using the LIVE/DEAD® BacLight Bacterial Viability Kit (Molecular Probes). The assay has two fluorescent probes which can distinguish cells with intact membranes from those with damaged membranes. The experiment was carried out by following the manufacturer's protocol. (B) *nos* gene expression in the complemented strain. mRNA was extracted and converted to cDNA using protocols described in the Methods. The expression levels of *nos* from the complementation (Δ*nos* :pNOS) is higher than the levels of wildtype without UV exposure; however, the levels are lower than wildtype with UV exposure. Δ*nos* does not shown any *nos* mRNA.

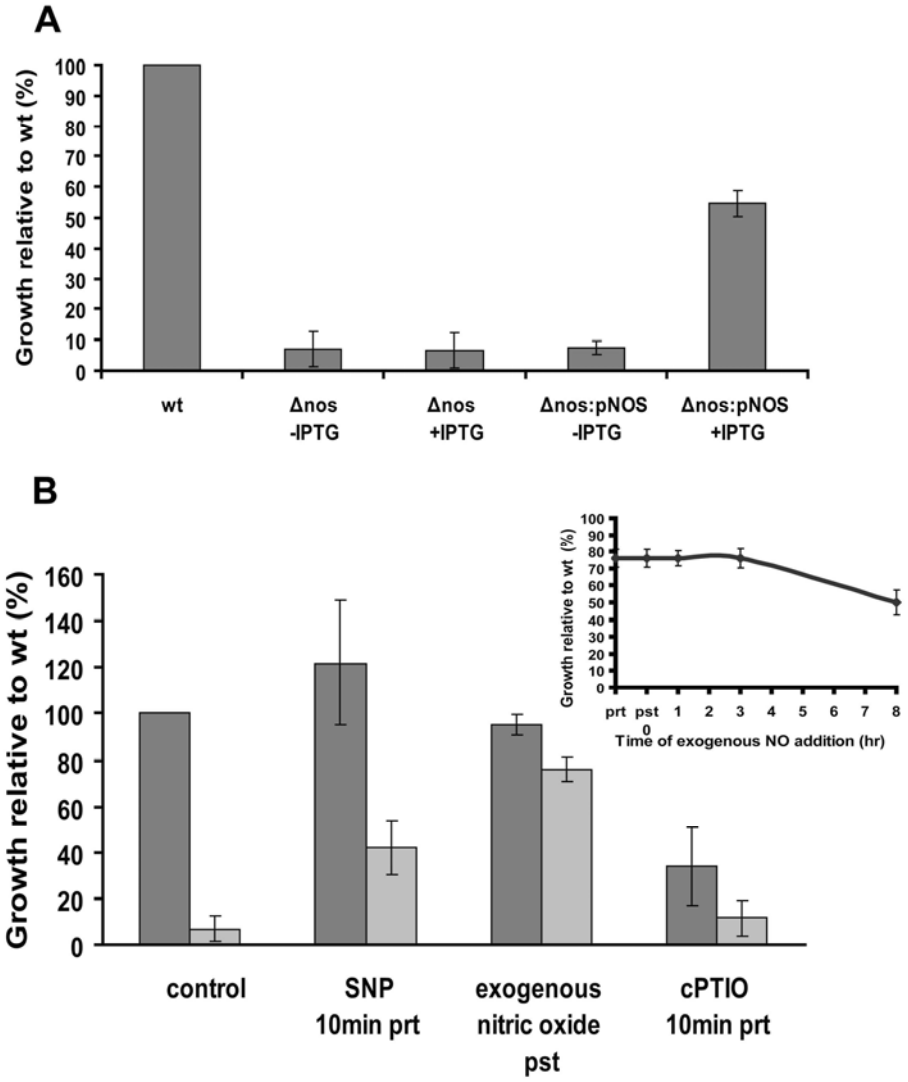

Figure. 2.6. Nitric oxide plays a critical role in the recovery of Dr growth after UV radiation as monitored by OD_{600}. (A) The complementation strain Δ*nos*:pNOS was generated by introducing an IPTG inducible recombinant *nos* gene on expression plasmid p11530. Cells were grown to OD ~ 0.2 and IPTG was added to induce the *nos* gene until the cell density reached OD ~ 1.0 prior to irradiation. Uninduced Δ*nos* and Δ*nos*:pNOS served as controls. The cell growth (assessed by OD_{600}) is shown as mean ± s.d. relative to irradiated wt cells at 22 hours after exposure. The complemented strain grows more slowly than Δ*nos* due to the added pressure of antibiotic selection. (B) Both wt (dark gray bars) and Δ*nos* (light gray bars) cells were grown for 22 hours with pre-incubation (prt) or post-incubation (pst) of NO donors (SNP and exogenous NO) and NO scavenger (cPTIO) after 5 min of UV radiation. Control cells were grown in the absence of additive compounds. The relative growth at 22 hours after UV was compared to that of wt cells, which were given a value of 100. Inset: Degree of NO rescue by the addition of exogenous NO at various times post irradiation. These values are set relative to the growth of wt cells post irradiation. The data represents an average of three independent experiments ± s.d.

following irradiation the cells were diluted in media with no antibiotics. It is possible that Δ*nos*:pNOS grown in the presence of antibiotics prior to UV grows a bit slower than one grown without the addition of antibiotics. A proper control of wt:pNOS grown in a similar way as Δ*nos*:pNOS would answer this question. Additionally, the 55% growth recovery we observe for Δ*nos*:pNOS may be more than reported. With these considerations, the complementation results suggest that loss of *nos* is likely the main reason for the growth defect of the mutant.

2.4.2 Nitric oxide rescues growth of irradiated Δnos

To establish a link between the presence of the *nos* gene and NO production in the susceptibility to UV radiation, we provided NO to Δ*nos* and scavenged it in the wt strain. Preincubation with the NO donor 1mM SNP (sodium nitroprusside) or addition of exogenous NO gas (5μM final concentration in solution) rescued the growth of Δ*nos* to 42% and 76% of wt levels, respectively (Figure 2.6B). Diazeniumdiolate-based NO donors such as NOC-7 also enhanced growth of Δ*nos* following UV, but showed some detrimental effects on growth in the absence of UV, as did application of NO at concentrations exceeding 10uM. Full recovery by chemical complementation could be hampered by a number of variables related to the kinetics of NO released by these compounds at their sites of action. Addition of L-citrulline, the other product of NOS activity, ferrous/ferric cyanide (a side product of SNP) and spermine (polyamine) did not enhance the growth of Δ*nos* (Figure 2.7). Interestingly, NO also rescued the modest growth phenotype of non-irradiated Δ*nos* to within 76% of wt. Thus, the minor defect in Δ*nos* under non-stress conditions is also significant. Conversely, addition of 100μM NO scavenger cPTIO, (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) sensitized wt *D. radiodurans* to UV radiation

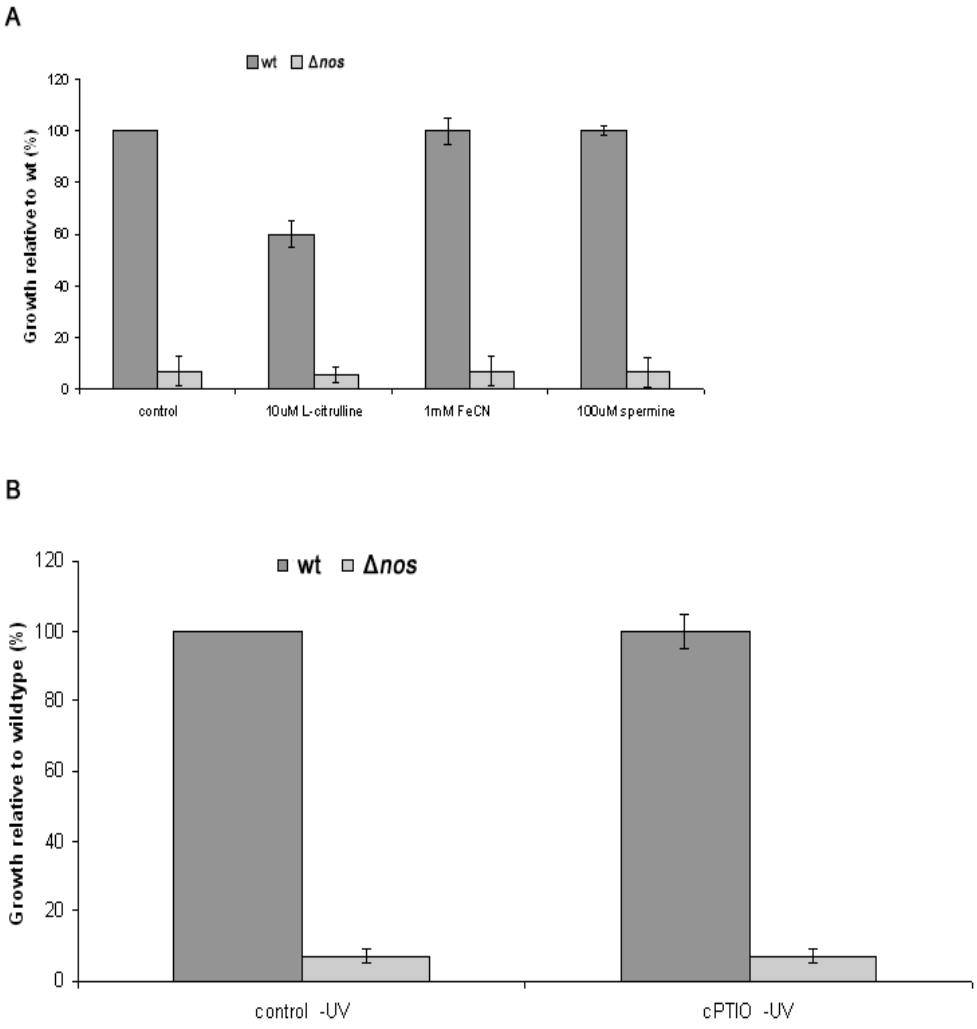

Figure 2.7. Effects of secondary products on UV recovery. (A) Both wt (dark gray bars) and Δ*nos* (light gray bars) cells were grown for 22 hours with pre-incubation (prt) of 10uM L-citrulline, 1mM ferrous cyanide and 100uM spermine after 5 min of UV radiation. Control cells were grown in the absence of supplemented chemicals. The relative growth of wt cells exposed to UV radiation was given a value of 100. There was no change in the growth of the strains in the presence of these compounds without UV irradiation. (B) 100µM cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was added to wt and Δ*nos* for 10 min and has no effect on either WT or Δ*nos* without UV irradiation. The same protocol for assaying growth with NO donor compounds and other additives was followed here.

by reducing growth 34% after UV exposure (Figure 2.6) and had no effect on growth of Δ*nos* or non-irradiated cells (Figure 2.7). This is probably due to the fact that NO is not present in the Δ*nos* and wt cells prior to UV exposure (see below). Remarkably, exogenous NO rescued growth of Δ*nos* whether it was added before, during, or up to 8 hours after irradiation (Figure 2.6B, inset). In contrast, UV-induced oxygen radicals were scavenged within seconds of exposure (Table 2.2). These results suggest that the rescue by NO does not involve its reaction with, or effect on, unstable chemical species generated during UV exposure (e.g. reactive oxygen radicals (ROS)). Additionally, this is consistent with the observation that the same numbers of wt and Δ*nos* cells were viable after UV and hence the inability of Δ*nos* to proliferate is not caused by UV-induced cell death, but rather a delay in resuming growth post-irradiation.

2.4.3 nos is upregulated by UV irradiation

Considering the importance of the *nos* gene to recover from UV radiation, we looked at its expression pattern following UV treatment. The amount of *nos* transcript as determined by RT-PCR, substantially increased within one hour after irradiation (relative to non-irradiated cultures) and remained roughly constant until decreasing nine hours post irradiation at a time that slightly precedes the onset of log phase growth (Figure 2.8). Upregulation of *nos* expression was evident as early as 30 min after UV exposure. This pattern of *nos* expression is similar to that of genes involved in DNA repair during damage responses. For example, when exposed to ionizing radiation, the *recA* recombinase mRNA level increases 30 min after UV exposure, is highest at 1.5 hours and diminishes after 12 hours (37). These results strengthen the hypothesis that the cell regulates *nos* levels in response to damage and requires its

Table 2.2. Presence of reactive oxygen species (ROS) during and following UV irradiation. ROS generated during irradiation reacts with added N-hydroxyl-L-Arginine (NOHA) and lead to formation of nitrite, which can be detected by the Griess Assay. NOHA in anaerobic Phosphate Buffered Saline (PBS) does not yield any ROS on irradiation as detected by this method, but aerobic PBS gives a strong nitrite signal. Likewise, if NOHA is added to cells and irradiated, large amounts of nitrite are detected. However, if NOHA is added, even seconds after irradiation is ceased, no nitrite production is observed. Thus, ROS are rapidly depleted after exposure ends.

Condition Tested	Reactive Oxygen Species (ROS) Detected
PBS + UV then NOHA	-
PBS + NOHA + UV	+
Anaerobic PBS + NOHA + UV	-
Dr cells + UV then NOHA	-
Dr cells + NOHA – UV	-
Dr cells + NOHA + UV	+

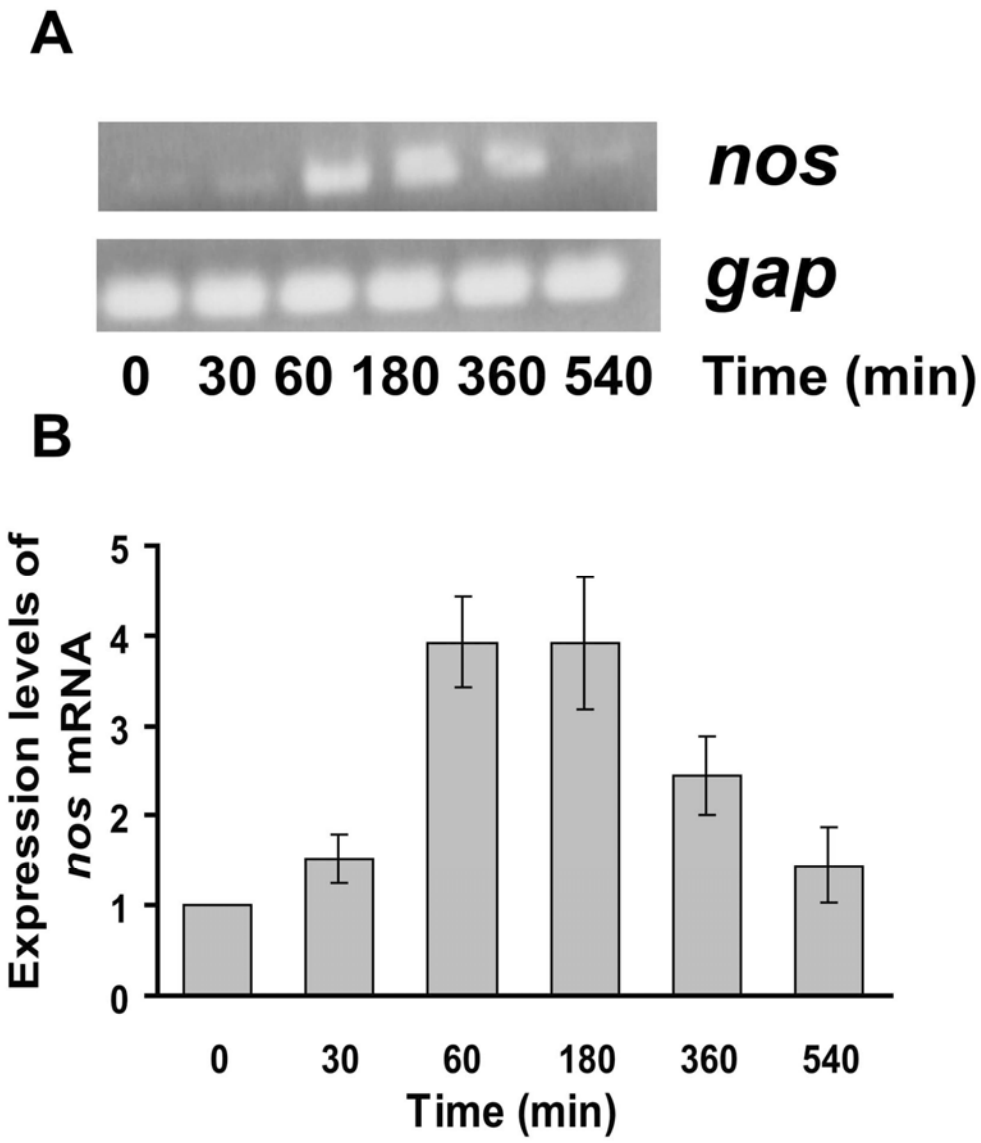

Figure 2.8. *nos* mRNA levels increase after UV irradiation. (A) RNA was extracted from non-irradiated cells (time = 0) and cells that were harvested 30, 60, 180, 360, 540 min after exposure to UV. RNA was converted to cDNA prior to PCR with random primers. The top panel shows mRNA from the *nos* gene, and bottom is from a control gene, *gap*, glyceraldehyde 3-phosphate dehydrogenase, the expression of which is unaffected by UV and hence serves as a loading control. (B) The *nos* mRNA expression levels were quantified using image J software and plotted as average ± s.d. relative to non-irradiated cells, whose mRNA levels were set to 1.

product NO in the repair or growth process. Notably, NO availability does not appear to regulate some obvious DNA repair genes associated with UV protection, as we found no major differences between Δ*nos* and wt in the induction of *recA*, *uvrA*, or *uvsE* after UV irradiation (Figure 2.4).

2.4.4 UV irradiation induces nitric oxide production

Nitric oxide production was detected in *D. radiodurans* cells using the intracellular highly NO-specific copper fluorescein probe (CuFL) (33). Neither wt (Figure 2.9A) nor Δ*nos* cells (Figure 2.9B) showed significant CuFL fluorescence in the absence of UV irradiation. However, after irradiation a strong fluorescence from CuFL was detected in the wt background (Figure 2.9C) compared to Δ*nos* (Figure 2.9D). Moreover, the timing of NO production was consistent with the expression profile of the *nos* gene, peaking at about 3 hours post irradiation and diminishing approximately 8 hours post irradiation. The low background fluorescence observed in Δ*nos* could be attributed to non-specific reactivity of CuFL with products generated during UV treatment. In control experiments we did find that the fluorescence of CuFL increases slightly in the presence of H_2O_2 + UV + $FeSO_4$. Much greater background effects were seen with the DAF fluorophore, which is sensitive to oxygen radicals, and like CuFL, is fluorescein based. Overall, the CuFL experiments show that DrNOS produces NO in response to UV radiation.

2.4.5 NO induces the gene for ObgE, a GTPase that regulates growth

In an attempt to elucidate the mechanism by which DrNOS confers protection, we compared the transcription profiles of wt and Δ*nos* cells with and without UV irradiation using microarrays (34). A number of candidate genes, whose expression levels were significantly increased in wt compared to Δ*nos* after UV exposure were

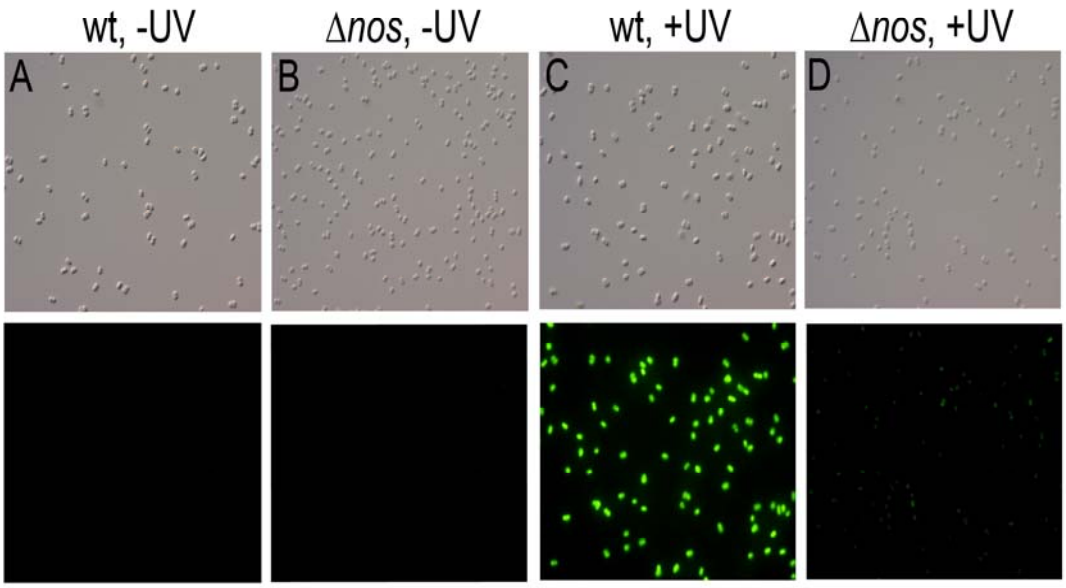

Figure. 2.9. NO production by *D. radiodurans* after UV radiation requires *nos*. The cell permeable NO specific probe CuFL was used to detect nitric oxide in unexposed and UV-exposed wt and Δ*nos* cells. Cells were irradiated, washed with PBS, incubated with 10μM CuFL and photographed after one hour. The top panel shows the differential interference contrast (DIC) images and the bottom panel the fluorescent images of wt without irradiation (A), Δ*nos* without irradiation (B), wt with irradiation (C), and Δ*nos* with irradiation (D). The low level of fluorescence seen in Δ*nos* with irradiation may be due to the radicals that react non-specifically with CuFL. We did find that the fluorescence of CuFL increases slightly in the presence of H_2O_2 + UV + $FeSO_4$.

further investigated with quantitative real time PCR. In particular, *obgE*, which codes for an essential GTP binding protein in many bacteria was found to be elevated in irradiated wt cells, but not irradiated Δ*nos* cells (Figure 2.10). The ratio of expression of *obgE* to the control gene, *gap,* was 4.7x higher in the irradiated wt compared to non-radiated cells, whereas no significant changes in expression were observed for Δ*nos* after identical exposures (Figure 2.10). Furthermore, treatment of the Δ*nos* with exogenous NO upregulated the *obgE* gene, although not to the same extent as that observed in the wt with UV irradiation (Figure 2.11). Bacterial *obgE* genes are often essential and the GTPases they code for play important roles in growth regulation and cell proliferation (*38, 39*). Introduction of an inducible *obgE* gene in the Δ*nos* background showed an improvement in bacteria growth recovery following UV treatment (Figure 2.10B). This effect was dependent on the extent and level of *obgE* induction. If *obgE* expression is induced prior to UV irradiation the Δ*nos* cells only recover to 20% of wt levels whereas up to 40% recovery was achieved when *obgE* expression was continuously induced. Additionally, a control experiment with wt:pObgE may show that this 40% increase is actually more as it is grown in the presence of antibiotics prior to UV irradiation. Thus, NO generated from NOS induces the *obgE* gene and production of the derived GTPase promotes cell growth.

2.5 Discussion

This study demonstrates that DrNOS produces NO in its cognate organism *D. radiodurans*. Both *Streptomyces* NOS (17) and *Bacillus anthracis* NOS (40) have been shown to produce NO *in vivo*. However, unlike *Bacilli*, *D. radiodurans* does not appear to contain the biosynthetic enzymes necessary to produce the mammalian NOS cofactor H_4B (*10, 41*). *In vitro*, DrNOS (as with other bacterial NOSs) can utilize the alternative reduced pterin tetrahydrofolate (THF), a ubiquitous cofactor that can be

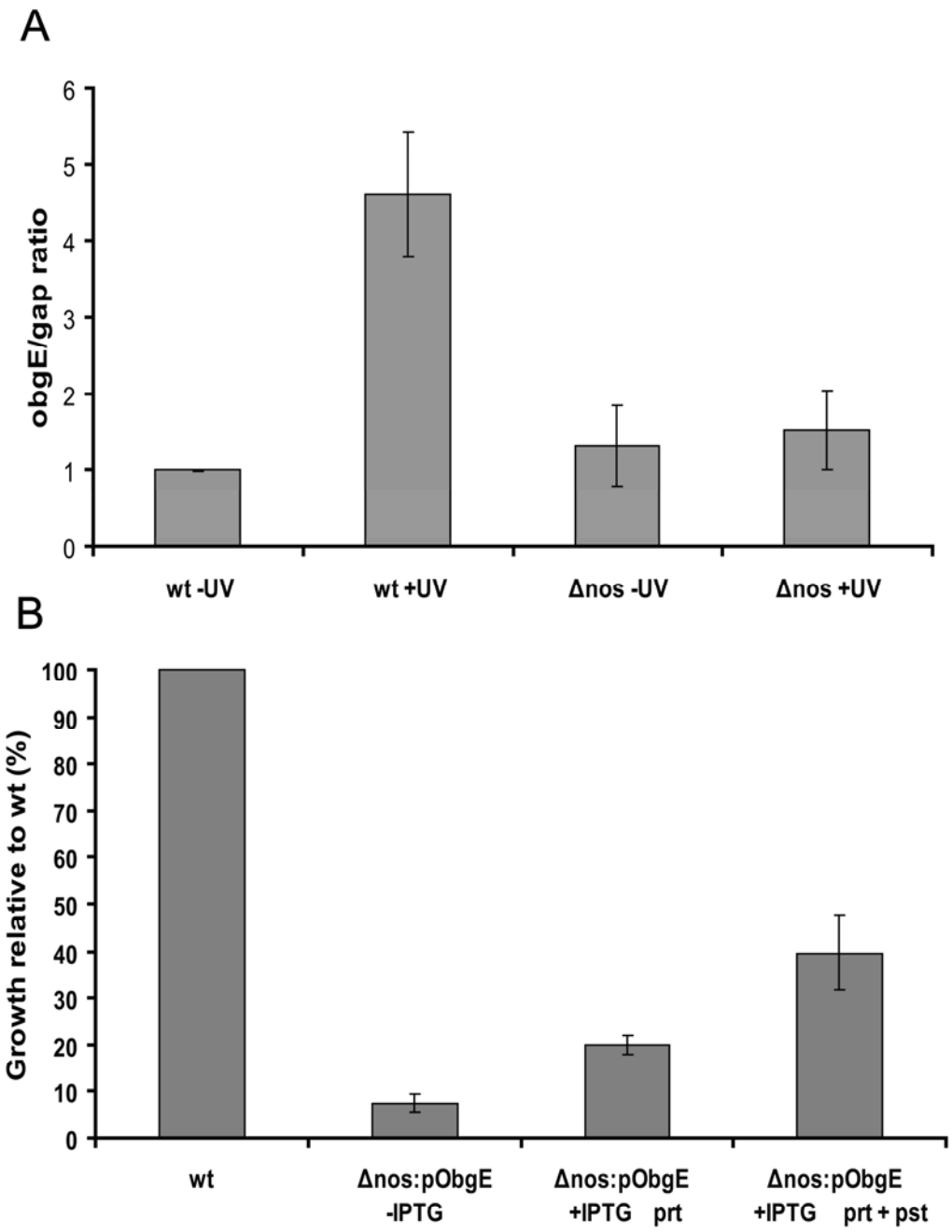

Figure 2.10. *obgE* mRNA expression levels increase after UV irradiation and rescues Δ*nos*. (A) Quantitative Real-Time PCR was used to determine the ratios of *obgE*/*gap* in wildtype and Δ*nos* cells prior to and following irradiation. The average ± s.e.m is shown for three independent experiments. (B) The complementation strain Δ*nos*:pObgE was generated by introducing an IPTG inducible recombinant *obgE* gene on expression plasmid p11530. Δ*nos*:pObgE cells were grown to OD ~ 1.0 and either no IPTG, IPTG only prior to irradiation or IPTG prior and post irradiation (throughout growth) was added to induce the o*bgE* gene. Cells were evaluated at 22 hours after UV exposure.

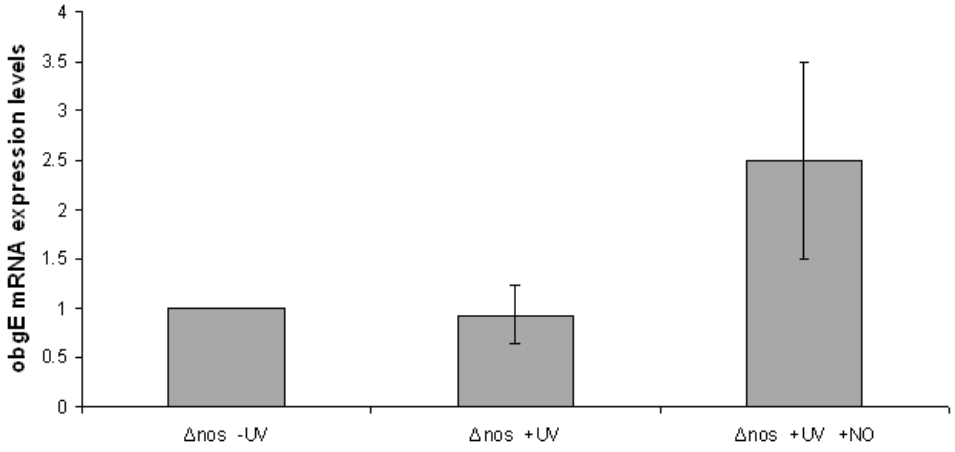

Figure. 2.11. *obgE* mRNA levels in Δ*nos*. The mRNA levels of the *obgE* gene from Δ*nos* cells without UV irradiation, with irradiation, and with irradiation on the addition of exogenous NO were measured using a similar protocol to that employed to detect expression of the *nos* gene. The level of *obgE* increases in the irradiated Δ*nos* cells upon treatment with NO, although not to the same extent as that observed in the wt with UV irradiation (Figure. 2.10).

generated by *D. radiodurans* (*10, 29*). Consistent with the binding of an alternative cofactor by bacterial NOSs, structural studies indicate that there is substantial variation in the region of the bacterial enzymes that recognize the pterin side chain (10). DrNOS must also produce NO in the absence of a flavodoxin reductase module, as the *D. radiodurans* genome lacks flavodoxin-like proteins. Work with *B. subtilis* proteins has demonstrated that a flavodoxin (YkuN), which is similar to the mNOS FMN domain, effectively donates electrons to *B. subtilis* NOS (42). However, deletion mutants of YkuN were not sensitive to oxidative stress, an assay used to monitor NOS activity, and *B. subtilis* NOS expressed recombinantly in *E. coli* still produces NO (19). These data suggest that bacterial NOSs do not require a specific reductase to produce NO. Whether a dedicated reductase is employed by DrNOS or not, a flavodoxin-like protein is not necessary to generate NO.

NOS-derived NO enables *D. radiodurans* to better survive UV radiation, but it does not appear to provide protection from the other physiological stresses we have tested. *D. radiodurans* differs from *B. subtilis* as exposure of cells to NO prior to H_2O_2 treatment substantially increased resistance to oxidative damage in the latter (18). This protective effect is thought to result from inhibition of thiol reduction by cysteine S-nitrosation. Free reduced thiols fuel Fenton chemistry, which converts H_2O_2 into damaging hydroxyl radicals. Blocking free thiols with S-nitrosation may mitigate the Fenton reaction. In addition, NO activates a major *B. subtilis* catalase which further protects the cell against oxidative stress (18). Furthermore, NOS-derived NO was shown to protect the human pathogen *B. anthracis* from oxidative damage induced by macrophages (40). Given these observations, it was unexpected that *D. radiodurans* Δ*nos* was not more susceptible to H_2O_2 than wt and that peroxide treatment did not induce *nos* gene expression (Fig. S2). Also, the fact that applications of NO up to 8 hours post irradiation, when oxygen radicals are no longer present in

cells, reinforced the fact that NO is more than a general protector against oxidative stress in *D. radiodurans*. Our data suggest that in *D. radiodurans,* NO serves to initiate recovery or remove some impediment to growth in latent cells. As *D. radiodurans* has high intracellular Mn/Fe levels and highly active superoxide dismutases which protect the cells from oxidative damage (*23, 43*), NO may simply not be needed in this capacity.

UV radiation damages DNA by directly cross-linking pyrimidine bases and by generating radical species (often oxygen based) that can participate in a plethora of reactions, including DNA strand cleavage (44). In *D. radiodurans*, NO production does not upregulate the recombinase *recA* gene, the nucleotide-excision repair *uvrA* gene or the UV damage endonuclease *uvsE* gene, which all appear to be induced normally in the Δ*nos* mutant. Why then is NOS-derived NO induced during UV exposure? And how does it aid in growth recovery? Although many mechanisms may be ultimately involved, we show here that NOS derived NO does upregulate the *obgE* gene. The functions of the ObgE GTPases are not well understood, but where investigated, they have been shown to impact a number of processes affecting growth. For example, in *B. subtilis*, ObgE participates in the regulation of DNA replication, the activation of the stress-response transcription factor σ^B, the monitoring of intracellular GTP levels and proper ribosome function (38). In *E. coli* ObgE acts as a checkpoint control for chromosome segregation and subsequent cell cycle processes (39). In *C. crescentus*, the ObgE homolog, CgtA, is essential for cell viability and its gene expression is enhanced after UV irradiation of cells (38). In human, the expression of the ObgE homolog Gbp45, which is found in the mitochondria, correlates with cell proliferation (45). In *D. radiodurans*, not only is *obgE* upregulated by UV irradiation through NOS activity, but overexpression of ObgE substantially overcomes the growth defect caused by the Δ*nos* mutant. Unlike complementation with *nos*, where induction

prior to UV exposure is sufficient to maximize the effect on recovery, induction of *obgE* is required throughout the recovery and growth period to achieve the greatest benefit. Thus, NO may act to ultimately regulate gene expression important to damage recovery and cell proliferation in *D. radiodurans*, at least partially through the growth regulator ObgE. Notably, many NO-responsive transcriptional regulators and sensor kinase systems have now been characterized in other bacteria (46, 47) and thus it is a strong possibility that NO could act as a regulatory signal in *D. radiodurans*. *D. radiodurans* has seven transcriptional regulators of the MerR class, (possibly an ortholog of SoxR), and two members of the LysR family, (possibly an ortholog of OxyR), paralogs of which are NO responsive in other organisms (48).

It is likely that ObgE expression is not the only response induced by NO production. In addition to reacting with metallo-cofactors of transcription factors and other proteins, NO causes S-nitrosation of cysteine residues. This latter mechanism is known to regulate mammalian phosphatases, kinases and transcription factors such as HIF-1 and NFκB (49). Through a similar mechanism NO could affect the activity of proteins involved in cellular repair. In mammals, UV irradiation increases inducible NOS (*iNOS*) levels in macrophages. NO released by iNOS S-nitrosates a specific cysteine residue on HIF-1α, which plays a key role in various inflammatory diseases and wound healing (20). Additionally, the mRNA expression of *iNOS* increases after UV-A radiation in human skin endothelial cells in the absence of cytokines (21). It may be more than coincidence that UV radiation elevates NO in both mammals and *D. radiodurans* through NOS induction.

Despite its importance in UV radiation recovery, NO may fulfill other functions in *D. radiodurans*. The *nos* gene is expressed during normal growth and the NOS protein is produced (27). Furthermore, the Δ*nos* mutant shows slightly reduced growth in rich media and this defect is rescued by NO. Thus, NO confers a growth

advantage to *D. radiodurans* under normal conditions through a process that may also include regulation of ObgE. A continual benefit from NO would provide a constant selective pressure to maintain the *nos* gene.

So far the known functions of bacterial NOSs appear quite diverse, not unlike the varied roles played by the animal NOSs. In certain *Streptomyces* strains NOS participates in the nitration of a tryptophanyl moiety of the thaxtomin phytotoxins (11). However, NOS produces NO in excess of that needed for plant toxin synthesis and the excess NO diffuses from the cell (17). This feature of NO production may assist pathogenesis because NO is also a plant signaling molecule that plays a role in the growth of new root shoots, which are prime sites for bacterial infection (17, 50). Although NO protection against oxidative stress in Bacilli and Staphyloccoi involves changes to reduced thiol availability (18), it may also involve other factors, such as upregulation of stress-response factors or growth regulators. Further investigations into the NO-mediated survival mechanism of *D. radiodurans* may yet reveal commonalities in the above mechanisms as well as provide insight into UV radiation responses by other organisms.

We decided to further investigate if the mechanism of NO and ObgE (which serves in stress response) would be significant in other bacteria. In *B. subtilis*, we know that the NO plays a protective role in hydrogen peroxide so; we decided to check the parallels to the *D. radiodurans* system. Preliminary results suggest that like *D. radiodurans*, the Δ*nos* strain grows a bit slower in rich media compared to the wt. Additionally, there does not seem to be gross difference between the growth of wt and Δ*nos* under conditions of UV radiation. It is possible that even a short exposure (10s) is too much for *B. subtilis*. Conversely, we do not see the upregulation of *nos* mRNA in *D. radiodurans* upon H_2O_2 stress. The *B. subtilis* wt and the Δ*nos* strains were treated with 100uM hydrogen peroxide or 10s exposure to UV light and recovered for

10 min. Preliminary analysis suggests that the *nos* mRNA increases upon exposure to hydrogen peroxide; however, the *obgE* does not. Additionally, the levels of *nos* and *obgE* do not change dramatically upon exposure to UV radiation. It is possible that each organism adapted its unique way to utilize NO to protect itself from various stress.

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CHAPTER 3

NOS INFLUENCES $tRNA^{Trp}$ POOLS IN *D. RADIODURANS*

3.1 Abstract

In the previous chapter, we showed that *Deinococcus radiodurans* NOS (DrNOS) plays a protective role against UV radiation damage. However, this might not be the only role that NOS plays in *D. radiodurans* as we have *in vitro* evidence that DrNOS interacts with tryptophanyl-tRNA synthetase II (TrpRS II), and DrNOS selectively nitrates tryptophan at the 4-position and TrpRS II couples it to $tRNA^{Trp}$ (1, 2). Additionally, *in vivo* studies have found that NOS-dependent modified $tRNA^{Trp}$ species ($tRNA^{Trp}$*) exist in tRNA pools of *D. radiodurans*. The method for purification of tRNA has been determined where the addition of glass beads greatly increases the yield of tRNA from *D. radiodurans*. The total tRNA pools differ depending on the growth phase of the bacteria and acid sensitivity as detected by acid-urea polyacrylamide gel. Additionally, two acylated forms of $tRNA^{Trp}$ are detected when the *trpRS* I is absence. In order to identify the modified species, the purification method of specific tRNAs has been created. Additionally, TrpRS II can substitute for TrpRS I *in vivo* as there is no difference in growth detected under wt conditions; although growth of Δ*trpRS* II is compromised under stress. No link was found between NOS-associated tRNA modification and the ability to withstand radiation damage.

3.2 Introduction

Bacterial nitric oxide synthases (NOSs) are involved in specific biosynthetic nitration reactions as certain pathogenic *Streptomyces* produce an unusual phytotoxin

dipeptides called thaxtomin (3), which is the causal agent of potato scab disease. These compounds are made up of a tryptophan and phenylalanine connected by a dipeptide. The *nos* gene is found on a large transferable pathogenicity island that contains the genes responsible for the biosynthesis of thaxtomin. Feeding studies with L-Arg-guanidino $^{15}N_2$- HCl found that the nitric oxide synthesized by NOS is involved in the nitration of tryptophan specifically at the 4-position (4). We have found another link between tryptophan and bacterial NOS in DrNOS as it interacts with an unusual tryptophanyl-tRNA synthetase (TrpRS II) (1, 2). Interestingly, *D. radiodurans* contains genes for two different TrpRSs. TrpRS I has ~40% sequence identity to typical TrpRSs that catalyze the formation of tryptophanyl-tRNA, whereas TrpRS II shows ~28% sequence identity(1). The sequence alignment of TrpRS I and TrpRS II proteins revealed that regions involved in binding of tryptophan and ATP are highly conserved; the putative tRNA binding sites however are less conserved in type II proteins. TrpRS II interacts with DrNOS *in vitro* and demonstrates the capability to aminoacylate $tRNA^{Trp}$, improves the solubility of DrNOS, increases the affinity of DrNOS for its substrates, and stimulates the activity of DrNOS (1). Similarly, the complex between DrNOS and TrpRS II catalyzes the formation of 4-nitro-tryptophan. Although DrNOS can catalyze this reaction without TrpRS II, the reaction is greatly enhanced in the presence of TrpRS II and ATP (1, 2). The crystal structure of TrpRS II revealed that the active site pocket was different than TrpRS I and that it can accommodate various tryptophan analogs with substitutions on the indole ring (5). Additionally, TrpRS II can recognize and couple Trp, 4-nitro-Trp or 5-hydroxy-Trp with nearly equal efficiency *in vitro* to $tRNA^{Trp}$; however, TrpRS I can only charge tryptophan (5, 6).

Due to the similarities with the *Streptomyces* system (4), it is possible *D. radiodurans* does not incorporate 4-nitro-tryptophan into proteins, but uses it in the

biosynthesis of metabolites similar to thaxtomins. The *in vitro* results support the model as shown (Figure 1). To elucidate the role of TrpRS II in more detail, *D. radiodurans* knockout strains were generated lacking a functional copy of NOS (Δ*nos*), TrpRS II (Δ*trpRS* II) or both NOS/ TrpRS II(Δ*nos*Δ*trpRS* II). In addition, knockout strains without a functional copy of TrpRS I (Δ*trpRS* I) or NOS/TrpRS I (Δ*nos*Δ*trpRS* I) were constructed. The knockout strains displayed no significant phenotypic differences under wildtype conditions.

3.3 Methods

Bacterial strains and growth conditions: Bacterial strain wildtype (wt) *Deinococcus radiodurans* R1 was obtained from the American Type Culture Collection (ATCC 13939). Cells were grown in 2xTGY (1.0% tryptone, 0.6% yeast extract and 0.2% glucose) at 30°C or plated on 2xTGY with 1.5% Bactoagar (Difco).

Small scale purification of total tRNA: D. radiodurans cultures were grown to log phase (OD_{600} ~ 1.5) or stationery phase (OD_{600} ~ 5) and total tRNAs were isolated through an extraction procedure that used acid-phenol, glass beads, Trizol (Invitrogen), and ethanol precipitation (7, 8). This stringent procedure was necessary as the robust cell wall of *D. radiodurans* makes it difficult to obtain high amounts of tRNA. It is also important to maintain acidic conditions to preserve the linkage between the amino acid and tRNA (9). RNA extraction from *D. radiodurans* was facilitated by the addition of glass beads (approx. 100 µl). Samples were vortexed at top speed for 15 seconds, and immediately placed on ice for 45 seconds. This cycle was repeated 8 – 10 times. Samples were centrifuged for 15 min, the aqueous layer was removed and the phenol phase was re-extracted with 100 µl of 0.1 M sodium acetate pH5. The aqueous layers of both centrifugation steps were combined and

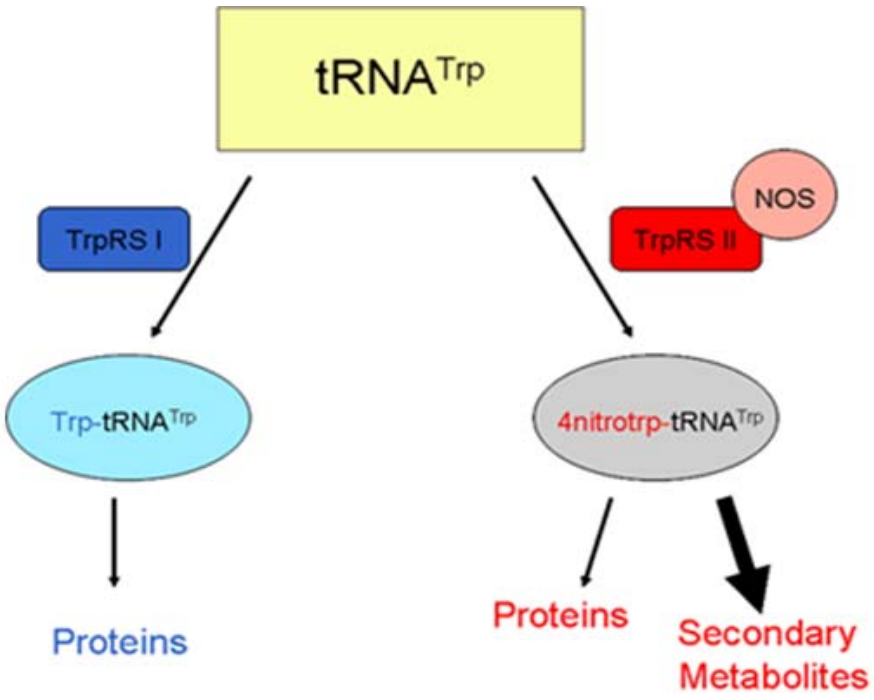

Figure 3.1. Model based on the *in vitro* results. *D. radiodurans* contains TrpRS I, the canonical TrpRS, which is responsible for charging $tRNA^{Trp}$ with Trp. However, the active site of TrpRS II can accommodate modified Trp such as 4-nitro-Trp and charge $tRNA^{Trp}$. This pathway only occurs when TrpRS II-NOS interaction is present. The modified Trp is likely to be involved in the synthesis of secondary metabolites such as thaxtomin, as seen in the *Streptomyces* system.

precipitated with ethanol. Total tRNA isolated under acidic condition was stored in 10 mM sodium acetate pH5 at -80°C.

Acid urea PAGE/Northern analysis of tRNA: Total tRNAs were then analyzed by acid urea polyacrylamide gel electrophoresis (PAGE) followed by a Northern Blot(7). The tRNAs were then transferred onto Hybond-N membrane (Amersham). The ^{32}P-labeled probes used were oligonucleotides directed against the anti-codon loop region of *D. radiodurans* $tRNA^{Trp}$ - 5'-GACCTACGGTTTTGGAG-3' and *D. radiodurans* $tRNA^{Phe}$ - 5'-ACTTGAACCGGCGACCCAACGATTTTCAG-3'to visualize the Northern hybridization. Oligonucleotides (Operon) were 5'-end labeled with γ-[32P]-ATP (3000 Ci/mmol) (Perkin Elmer) using T4-PNK (New England Biolabs) at a molar ratio of oligonucleotide:γ-[32P]-ATP of 3:1. Hybridization solutions were filtered prior to addition to the membranes. Northern blots were analyzed by autoradiography followed by PhosphoImaging using Imagequant software. Standard hybridization protocol for detection of tRNAs from *D. radiodurans*: membranes were prehybridized at 42°C in 6x SSC, 10x Denhardt's solution, and 0.5% SDS for approx. 12 - 18 hours. Hybridization was performed at 42°C for 12 - 18 hours in 6x SSC, 0.1% SDS and 0.25 – 0.5 nM of a ^{32}P-labeled probe. After hybridization, membranes were subjected to a single wash in 6x SSC for 5 min at RT.

Large scale purification of total tRNA: Large scales were grown in the following way. First, 1 ml overnight was grown of wt and/or Δ*nos*. The overnight was diluted into another overnight (500µl into 50ml). This culture was finally diluted 1:100 into 1L 2xTGY media. 4L of total cells were grown for ~8hours until the OD ~ 1.7. 500ml of cells were mixed with 500ml of 10% (w/v) trichloroacetic acid and harvested(9). The cells were resuspended in 20ml 0.3M Sodium Acetate (NaOAc) pH 4.5 and

frozen at -80°C. Total tRNA was extracted using "medium scale prep" as it gave the optimal concentration and maximum amount of tRNA needed for the purification of specific tRNA. Four times the small scale amounts (0.8ml of resuspended cells, 0.5ml of Trizol, and 0.1ml chloroform) were used and added to a 15ml centrifuge tube to give (3.2ml of resuspended cells, 2.0ml of Trizol, and 0.4ml chloroform). More than 50 mixtures were extracted to give 6mg total tRNA. The mixture was kept cold and glass beads added to them. Samples were vortexed at top speed for 15 seconds, and immediately placed on ice for 45 seconds. This cycle was repeated 10 times. Samples were then centrifuged for 25 min at 13,000g and the aqueous layer was removed. The aqueous layers of the centrifugation steps were combined and precipitated with 2.5x ethanol. Ethanol was removed, the samples air dried and resuspended in 700µL CCC Binding Buffer [1.2M NaCl, 30mM Hepes-KOH pH 7.5, 15mM EDTA] and total tRNA isolated under acidic condition was stored at -80°C. The concentration of each sample was about 400ng/µl for a total of 280µg. All the extracted tRNA samples were pooled to give 6mg total tRNA in 20ml volume. This was done for both wt and Δ*nos* samples.

Isolation of specific tRNA: The following procedure was adapted as described in (10-12). Streptavidin agarose (Novagen) was divided into four tubes of 800µl 50% mixed slurry (400µl resin). The agarose beads were washed with 100mM Tris-HCl pH 8.0. Since 100µl resin binds 100µg of biotinylated oligonucleotide (5'-CCTCGAGACCTACGGTTTTGGAGACCGCCGCT /3'BIO and *D. radiodurans* $tRNA^{Phe}$ - 5'-ACTTGAACCGGCGACCCAACGATTTTCAGT-/3'BIO), 140µg oligonucleotide was used to equilibrate the resin. Therefore, 560µg of the appropriate oligonucleotide was mixed with 400µl agarose. 1ml of Tris pH 8.0 was added to the agarose and resin mixture for better equilibration and shaken for 1.5hrs at RT to give

the following samples wt_trp, wt_phe, Δ*nos*_trp, and Δ*nos*_phe. The resin was spun down and washed to remove unbound oligonucleotide. The resin was then equilibrated with CCC Binding Buffer. 20ml of total tRNA isolated (6mg) was split into two fractions. To each 200µl of equilibrated resin with the wt_trp oligo added to the wt total tRNA. The samples were put on a rocker which was in an incubator at 65°C for 40min, and the tRNA and biotinylated-oligo-resin mixed. The incubator was turned off and samples were shaken until the temperature inside the incubator reached 40°C by natural heat dissipation over 30min. The mixture was then poured into a column, resin settled, and the unbound fraction collected. The resin was washed three times (~4ml) with 3x CCC Wash Buffer [0.6M NaCl, 15mM Hepes-KOH pH 7.5, 7.5mM EDTA] followed by 1x CCC Wash Buffer [0.6M NaCl, 15mM Hepes-KOH pH 7.5, 7.5mM EDTA] and finally washed with 0.1x CCC Elution Buffer [20mM NaCl, 0.5mM Hepes-KOH pH 7.5, 0.25mM EDTA] (12). Lastly, the column was eluted with 1ml CCC Elution Buffer placed in an incubator at 65°C. The column was eluted twice having a five minute incubation to increase tRNA recovery. The unbound fraction was then subjected to the biotinylated oligonucleotide for $tRNA^{Phe}$ and the same procedure was followed for $tRNA^{Phe}$ purification. The above protocol was used to purify the specific tRNAs: $tRNA^{Trp}$, $tRNA^{Trp*}$ and/or $tRNA^{Phe}$ from both wt and Δ*nos* strains. Finally, all four elutions were concentrated using Amicon-Ultra-4k centrifugation devices at 1mg total tRNA and 1µg specific tRNAs. The samples were sent to Caroline Koehrer to confirm the presence of $tRNA^{Trp}$ and $tRNA^{Trp*}$ and $tRNA^{Phe}$ by Northern hybridization as described above. The samples were then sent to University of Rochester to Eric Phizicky's Lab to Melanie Baker for HPLC-MS analysis for difference in modified nucleosides corresponding to $tRNA^{Trp*}$. Specific tRNA samples were treated with 5–10 µg of P1 nuclease at 37°C for 16 h, and then with 8 U of calf intestinal alkaline phosphatase at 37°C for 3 h. Nucleosides were

resolved by HPLC (Waters Alliance Model 2690, equipped with Waters 996 photodiode array detector) on a reverse phase C18 column and modified nucleosides were quantified as described (13).

3.4 Results

3.4.1 tRNA pools in vivo differ in wt and Δnos

Since *in vitro* experiments showed that the NOS and TrpRS II complex nitrates tryptophan and couples it to the $tRNA^{Trp}$ (2), we decided to confirm the results *in vivo*. To elucidate the role of TrpRS II, NOS and TrpRS I in detail, knockout strains of *D. radiodurans* were made whereby the gene of interest was replaced by an antibiotic cassette. Due to *D. radiodurans* having more four to eight copies of its genome (14), clones were passaged several times to eliminate any false-knockouts. The knockouts were confirmed using Reverse Transcription-PCR. RT-PCR was also used to determine that the genes of interest were expressed under the condition the cells were grown in. The strains displayed no significant phenotypic differences under wt growth conditions proving that TrpRS II can substitute for the function of TrpRS I in cells without significant disadvantages. Using the knockout strains, tRNA pools were analyzed in wt and in Δ*nos*, as it would give insight into whether 4-nitrotryptophan is formed and that whether its synthesis depended on NOS.

In collaboration with Caroline Koehrer at MIT, the *in vivo* aminoacylation state of $tRNA^{Trp}$ was examined in wt, Δ*nos* and Δ*trpRS* II. tRNAs were isolated under acidic conditions to keep the amino acid onto the tRNA. The original yield of total tRNA after standard extraction using acid phenol (7) or Trizol (8) was very low probably due to the robust cell wall typical for *D. radiodurans* (data not shown). To improve the yield, the extraction protocol was modified by including glass beads as described in detail in the Methods section. Total tRNA was isolated from a cell pellet

of 20 ml of culture harvested during mid-to-late log phase (OD~1.5). The quality of the tRNA preparation was assessed by agarose gel electrophoresis to confirm the absence of ribosomal RNA and other higher molecular weight material. Total tRNAs were subsequently subjected to acid urea PAGE followed by Northern hybridization. This type of gel separates tRNA based on size, charge and conformation. Membranes were first probed with an oligonucleotide directed against the anticodon loop-region of *D. radiodurans* $tRNA^{Trp}$ (Figure 3.2). Comparison of the different strains showed that all samples are quantitatively aminoacylated and do not show significant difference in the mobility of $tRNA^{Trp}$. However, after base treatment (0.2 M Tris-HCl pH9.5; 37°C, 45 min), which results in the deacylation of the tRNA, the wild type sample migrates as a single species, whereas both knockout strains show two distinct bands one of which co-migrates with the wild type species (Figure 3.2). These double bands often occur due to differences in tRNA base modification and/or conformation. The second species of $tRNA^{Trp}$ that appears in the Δ*nos* and Δ*trpRS* II knockouts is termed $tRNA^{Trp*}$. The same blot was stripped and re-hybridized with an oligonucleotide directed against *D. radiodurans* $tRNA^{Phe}$ (Figure 3.2, bottom) showing no differences between the samples.

3.4.2 Trichloroacetic acid and growth phase affect tRNA pools

In purifying tRNA, a discovery was made at the harvesting step. In order to keep the tRNA pools in an acidic environment when the cells are harvested, they are spun down with an equal volume of 10% w/v trichloroacetic acid (TCA) (9). If the TCA is not added there is a single species of deacylated $tRNA^{Trp}$ in both wt and the knockouts; however, with the addition of TCA the second species of $tRNA^{Trp}$ becomes apparent (Figure 3.3A). Based on these results we believe that in order for the modification to be evident, TCA is protecting the modification or further modifying it.

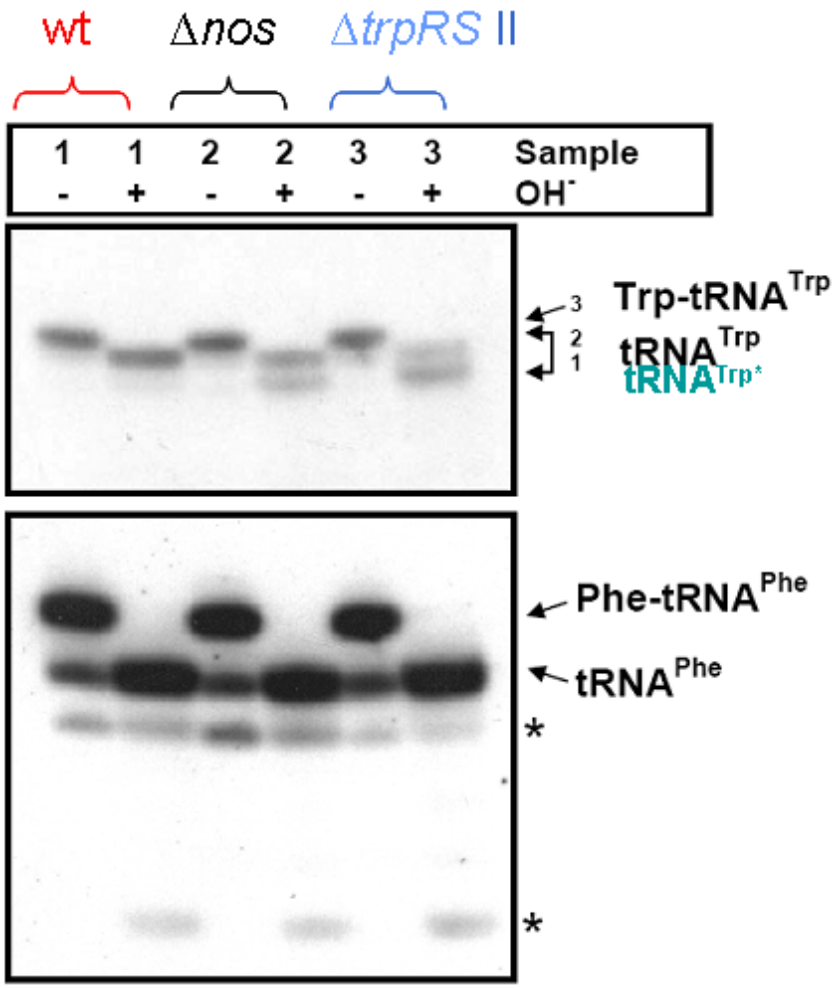

Figure 3.2: Analysis of total tRNA isolated from *D. radiodurans* on an acid urea polyacrylamide gel. tRNAs were visualized by a Northern hybridization using a ^{32}P-labeled DNA probe specific for *D. radiodurans* tRNA^{Trp} (top) and *D. radiodurans* tRNA^{Phe} (bottom). Sample 1, wild type; sample 2, Δ*nos*; Sample 3, Δ*trpRS* II. The (-) are the acylated species without base treatment while the (+) indicate OH- treated deacylated samples. The Phe-tRNA^{Phe} was used as a control and shows no difference between the three knockouts.

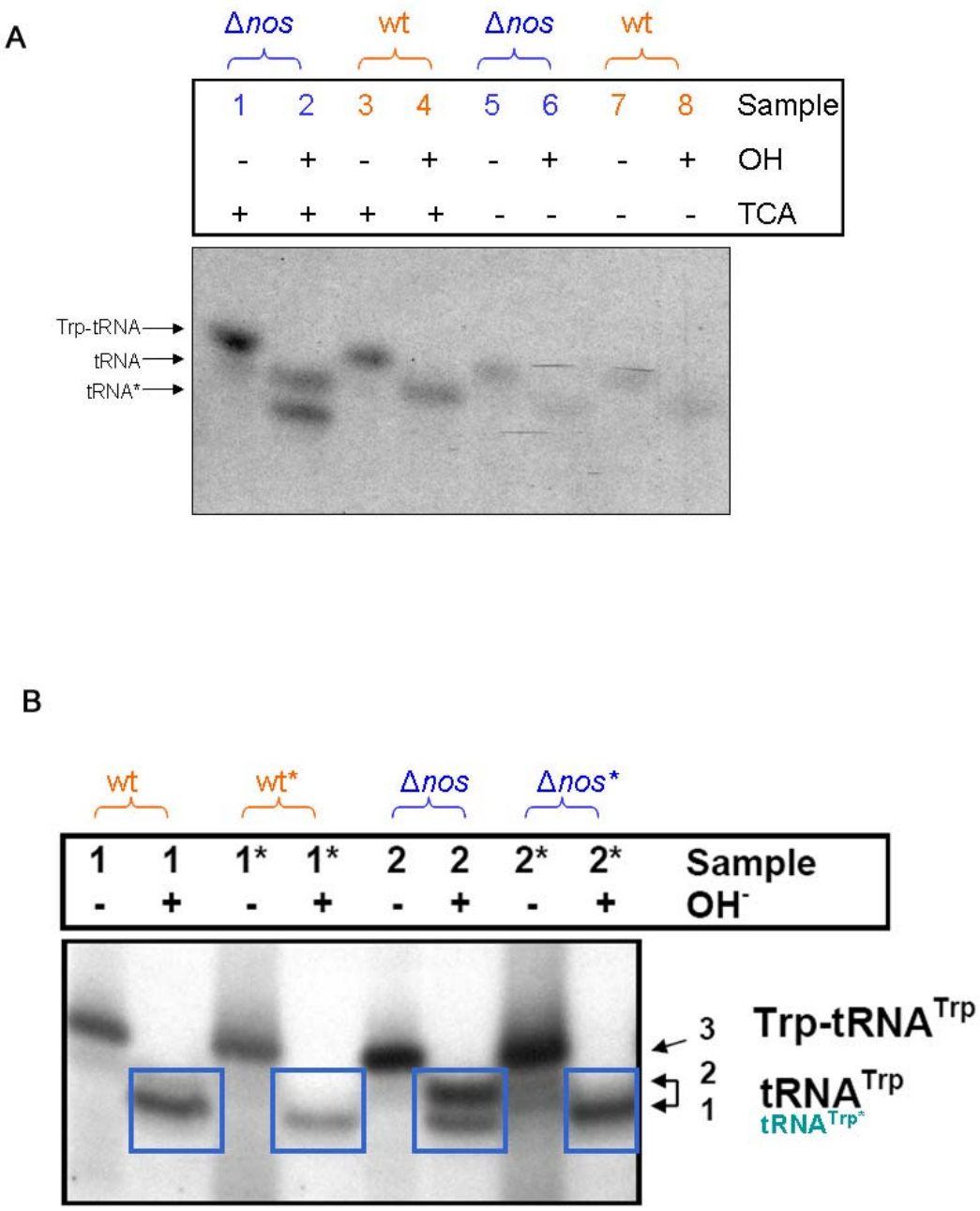

Figure 3.3. The tRNA^{Trp} pattern differs based on (A) **addition of TCA and** (B) **time cells were collected.** **(**A) Stabilization of the tRNA^{Trp} species with trichloroacetic acid leads to two species being present in only the Δ*nos*. The double band pattern of tRNA^{Trp} disppeares when TCA is not present (sample 6). (B) The tRNA^{Trp} pattern is also dependent on the growth phase of the bacteria. The double bands are only seen in the Δ*nos* in the log phase while in stationary phase (indicated by a *) wt * and Δ*nos** both show one tRNA^{Trp} species.

Treatment with strong acid followed by strong base in the deacylation reaction might make the $tRNA^{Trp}$ species different enough to be visible on the gel. Another batch of cells were analyzed to confirm the presence of the double bands in the Δ*nos*. This time cells grown to log phase and stationery phase were collected. We found that the $tRNA^{Trp}$ pools differ depending on the growth of the bacteria at the time the cells were harvested (Figure 3.3B). Consistent with the gel pattern obtained in Figure 3.2, the Δ*nos* shows a distinct double band after deacylation. However, in stationery phase (indicated by a *) this pattern is not present as the double band in question disappears completely, likely due to different ongoing cellular process at various time points in the growth of *D. radiodurans* (Figure 3.3B). Although the nature of these double bands and difference in mobility remain unclear, they seem to correlate with the physiological state of the cells at the time of harvest in addition to the *nos* genotype.

3.*4.3 Two species of tRNA in the acylated pools of* Δ*trpRS I*

The results *in vivo* indicate that the different species of $tRNA^{Trp}$ observed is dependent on the presence of the NOS/TrpRS II complex. To further study the tRNA pattern, we examined the tRNA pools of Δ*trprRS* I. Two bands in the acylated tRNA pools are found in the Δ*trprRS* I where only TrpRS II is the active TrpRS. It is possible that in this case, TrpRS II is charging $tRNA^{Trp}$ with tryptophan and a modified tryptophan (4-nitrotryptophan or 5-hydroxytryptophan). Furthermore, this suggests that TrpRS II can compensate for the canonical TrpRS I protein as $tRNA^{Trp}$ appears to be aminoacylated in both Δ*trpRS* I and Δ*nos*Δ*trpRS* I strains. However, once the TrpRS II/NOS interaction is eliminated TrpRS II can only acylate tryptophan onto the $tRNA^{Trp}$ as seen in the Δ*nos*Δ*trprRS* I (Figure 3.4).

Although the outcome is unexpected in comparison to the *in vitro* results, a reasonable model (Figure 3.5) can be proposed. According to the *in vitro* results that

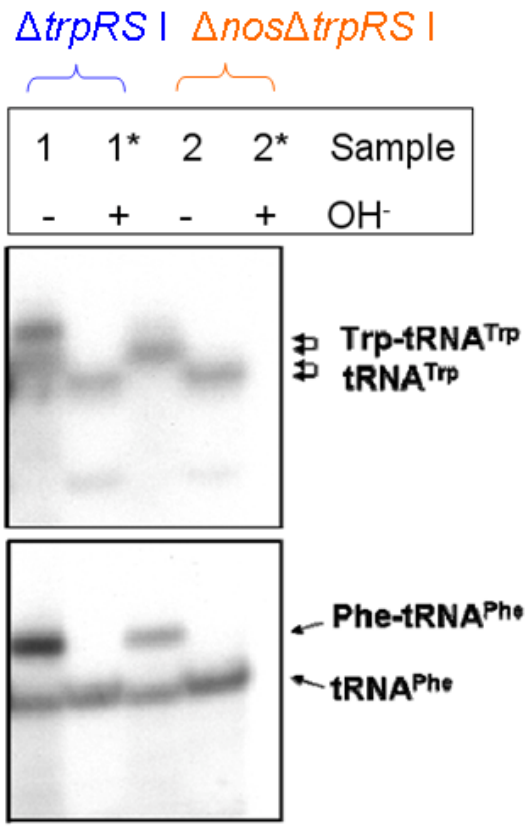

Figure 3.4. Two acylated forms exists in Δ*trpRS* I. Unlike the pattern observed in Figure 3.2, where changes are in the deacylates species, here we see the presence of two acylated forms when TrpRS II is the only TrpRS in cell. The two forms are likely to be Trp-tRNA^{Trp}, and a modified tryptophan-tRNA^{Trp}. When the TrpRS II/NOS interaction is eliminated, TrpRS II can only acylate tryptophan onto the tRNA^{Trp} as seen in the Δ*nos*Δ*trprRS* I.

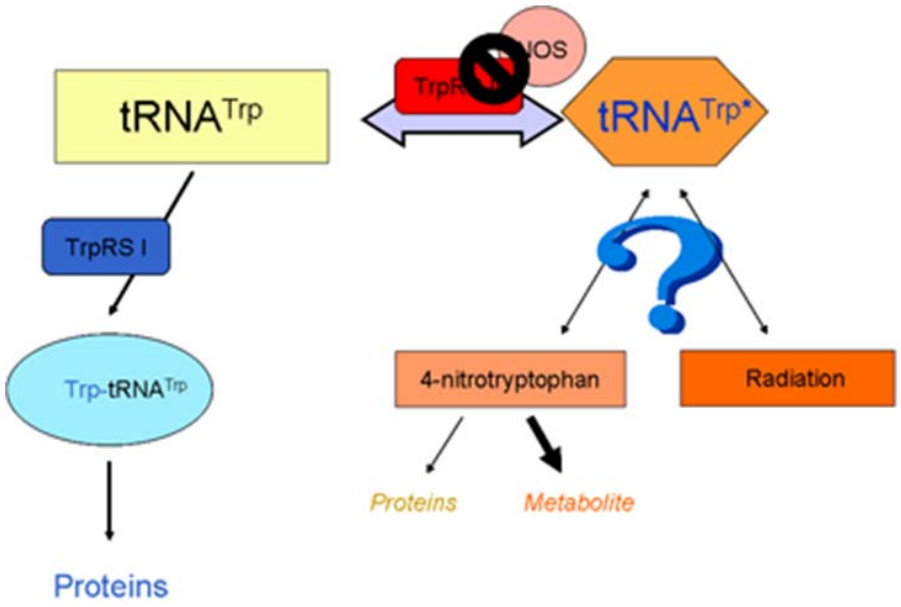

Figure 3.5. Model based on the *in vivo* data. It is possible there are two acylated species in the wt, but the modification is beyond the detection limit of the gel or the amount of the modified acylated tRNATrp* is minimal. When a block is introduced in the normal NOS/TrpRS II pathway, there is an accumulation of the modified $tRNA^{Trp*}$ in the deacylated sample. The modified $tRNA^{Trp}$ may be involved in the synthesis of a secondary metabolite; however, this may not be the case.

NOS can nitrate tryptophan and TrpRS II can charge it to $tRNA^{Trp}$(6), the acylated tRNA pools should have two bands; however, only one band appears in both Δ*nos* and Δ*trpRS* I *in vivo*. One possibility is that there are actually two species even in the acylated form as seen in Δ*trpRS* I, but the modification is beyond the detection limit of the gel or the amount of the modified acylated $tRNA^{Trp}$* is minimal. In addition, when a block is introduced in the secondary pathway (NOS-TrpRS II) there is an accumulation of $tRNA^{Trp}$* in the deacylated species. The modified $tRNA^{Trp}$ is likely to be 4-nitrotryptophan involved in the synthesis of a secondary metabolite similar to the *Streptomyces* system; however, it is possible that 4-nitrotryptophan is used as an amino acid, or there is no 4-nitrotryptophan present as we have yet to detect it.

3.4.4 NOS-TrpRS II interaction does not provide protection against UV light

Additionally, it is possible that NOS-TrpRS II and the metabolite (if produced) play a role in protection against Ultraviolet (UV) radiation damage. In the previous chapter the data showed that Δ*nos* is susceptible to UV radiation damage. Additionally, we know that that DrNOS interacts with TrpRS II (1). TrpRS II also contains an N-terminal extension similar to those found in proteins involved in stress response, in particular stress survival protein E, a class of phosphatases, found in eubacteria and archaea (15). Analysis of *D. radiodurans* transcriptome after radiation exposure reveals large-scale induction of genes involved in chromosome restructuring (such as *recA*, *recG*, *recQ*, *ssb*, *gyrAB, uvrABCD* etc) (16, 17). The expression levels of *trpRS* II increases 2.2-fold, 5 hours after radiation exposure and then return to near baseline 5 hours later. To test whether the Δ*nos* effect is independent of TrpRS II and $tRNA^{Trp*}$ to protect the organism from UV radiation, the susceptibility of *D. radiodurans* knockout for both TrpRS isoforms (Δ*trpRS* I and Δ*trpRS* II) to UV radiation was examined. There was no difference in the growth of Δ*trpRS* II after

irradiation while Δ*trpRS* I showed 40% growth compared to that of wt (Figure. 3.6). This is not surprising as the catalytic parameters and sequence of TrpRS I indicate that it is the primary TrpRS in *D. radiodurans* (1). However, the viability of the *trpRS* I knockout did indicate that TrpRS II could act as a functional TrpRS. The double knockout Δ*trpRS* IΔ*trpRS* II could not be obtained and was assumed to be lethal. The double knockouts Δ*nos*Δ*trpRS* II and Δ*nos*Δ*trpRS* I showed the same growth impairment as Δ*nos* alone (Figure. 3.6). This data suggest that Δ*nos* acts independently of TrpRS II and $tRNA^{Trp*}$ and the biological role of the NOS-TrpRS II interaction remains unclear. It is possible that UV protection is not the main role of *nos* per se, and that it really is protecting against similar damage caused by desiccation. Also, NOS-TrpRS II interaction may have an alternate function in the absence of UV; however, NOS acts on ObgE in the presence of UV in order to promote cell proliferation.

3.4.5 Identification of $tRNA^{Trp*}$

Therefore, in order to further understand what the $tRNA^{Trp}$ pools mean and what role they play, the $tRNA^{Trp*}$ species must be purified and identified. Large scale isolation of total tRNA (6mg) was carried out from both wt and Δ*nos* strains. These were then subjected to a streptavidin agarose-biotinylated oligonucleotide column to purify specific tRNAs (Figure 3.7). Northern hybridization on the elution fractions confirmed that they were indeed $tRNA^{Trp}$, $tRNA^{Trp*}$, $tRNA^{Phe}$. The samples were then concentrated to 1mg total tRNA and 1µg specific tRNAs, treated with Nuclease P1, followed by alkaline phosphatase and analyzed for modification by HPLC. The analysis is underway and the modification found on $tRNA^{Trp*}$ should be determined.

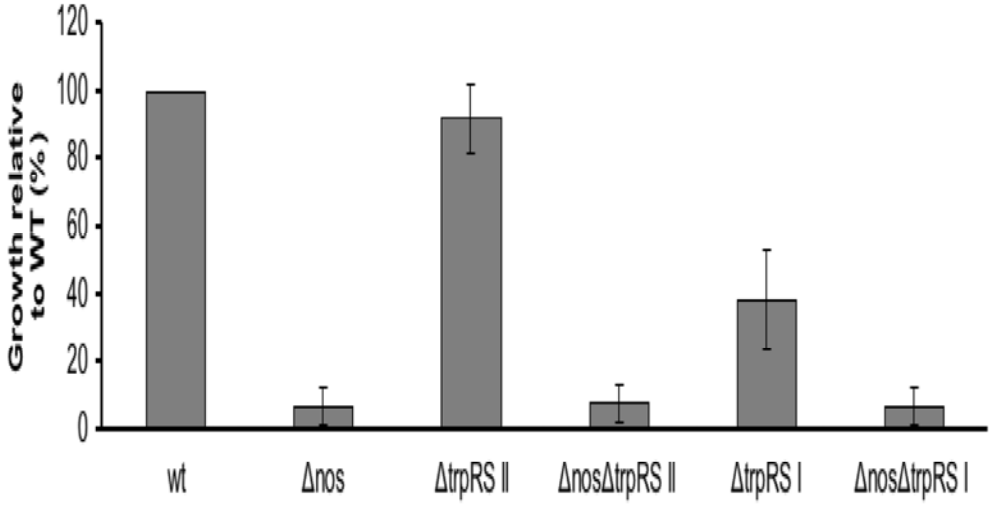

Figure 3.6. *trpRS* I and *trpRS* II unaffected by UV radiation. The Δ*nos* strains show the largest growth defects following UV irradiation. Δ*trpRS* II is unaffected by UV, while Δ*trpRS* I grows 40% to that of wt because TrpRS I is the primary TrpRS in *D. radiodurans*. Both double knockouts Δ*nos*Δ*trpRS* II an Δ*nos*Δ*trpRS* I show growth patterns similar to the single mutant Δ*nos*. Notably, growth of the Δ*trpRS* I and Δ*trpRS* II strains are not differentiated in the absence of UV exposure. Thus, NOS-TrpRS II interaction and $tRNA^{Trp*}$ pools do not seem to play a role in UV damage protection. The data represents an average of three independent experiments ± SD.

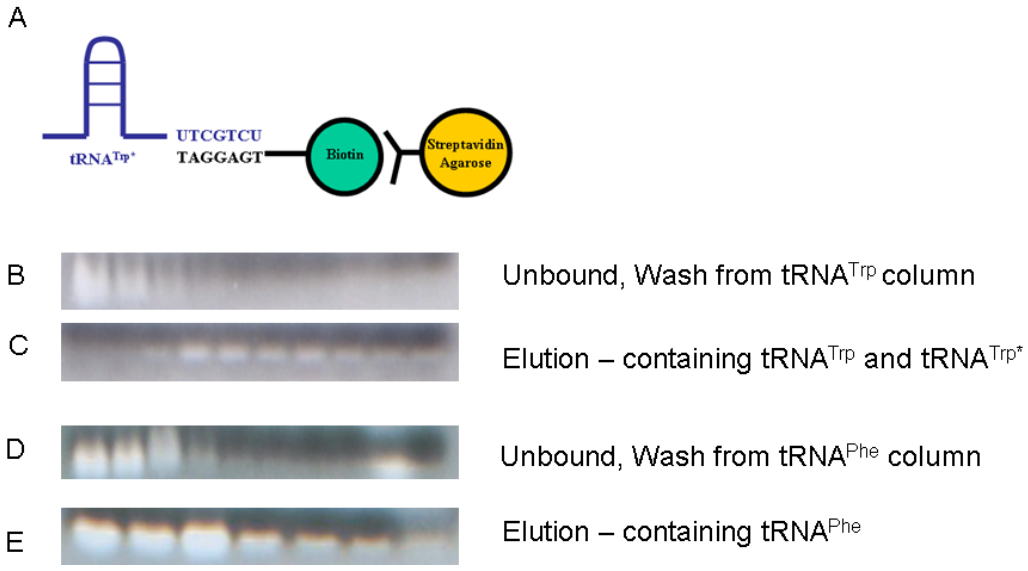

Figure 3.7. Isolation of specific tRNAs. Total tRNA was incubated with streptavidin agarose attached to a biotinylated oligonucleotide specific for either $tRNA^{Trp}$ or $tRNA^{Phe}$ on the 3' end (A). The unbound, wash and elution samples were collected for $tRNA^{Trp}$ (B, C). The unbound fraction was then applies to a column containing $tRNA^{Phe}$ and samples collected (D, E). The elution samples were concentrated and analyzed by HPLC.

3.5 Discussion

Despite sequence homology to stress related proteins, TrpRS II does not seem to play a role in protecting *D. radiodurans* from UV radiation. It suggests that TrpRS II may not always associate with NOS and NOS may have multiple functions in *D. radiodurans*. Additionally, it suggests that TrpRS II can choose to charge tRNA with tryptophan or other modified tryptophan species only under certain conditions (as seen under various growth conditions). Also, it is possible that the NOS-TrpRS II interaction is specific to *D. radiodurans* as it is the only organism which has both TrpRS II and NOS. The others organisms with TrpRS II do have nitrite reductases that can produce nitric oxide. It seems that the ability of *D. radiodurans* TrpRS II to accommodate various substrates has been exploited by its association with *D. radiodurans* NOS- an enzyme capable of producing 4-nitrotryptophan *in vitro*.

The results *in vivo* are intriguing as a different species of $tRNA^{Trp}$ observed are dependent on the presence of the NOS/TrpRS II complex. Based on the results of the modification on the $tRNA^{Trp*}$ it is possible that $tRNA^{Trp*}$ participates in the synthesis of secondary metabolites. The promiscuous TrpRS II can charge $tRNA^{Trp*}$ with modified tryptophan while TrpRS I can charge $tRNA^{Trp}$ with tryptophan, where the modified tryptophan is ultimately involved in the synthesis of thaxtomin like compounds as seen from the *Streptomyce*s system which is known to contain a 4-nitrotryptophan species. While most transfer RNAs are involved in protein synthesis, there are examples of those involved in the synthesis of secondary metabolites. One such case is that of glutamyl-tRNA which is involved in the biosynthesis of δ-amino-levulinic acid, the sole precursor to porphyrin rings of heme and chlorophyll. In the organism *Synechocystis,* a single $tRNA^{Glu}$ transcribed as monomeric precursor tRNA matures into two $tRNA^{Glu}$ species. One species of $tRNA^{Glu}$ participates in protein synthesis, while the second with a modification in the anticodon participates in

porphyrin synthesis (18). The ester linkage between the amino acid and tRNA can serve for specific chemical reactions. It is likely that in *D. radiodurans*, rather than having one gene maturing into two different tRNAs, there are two different synthetases that charge two different $tRNA^{Trp}$ with two different amino acids. By comparing the $tRNA^{Trp}$ pools of wt and Δ*nos*, we should be able to find the nature of the $tRNA^{Trp*}$ species. It will be exciting to see where on the $tRNA^{Trp*}$ species the modification occurs. The modification may be a nitration; however, this type of modification has never been seen on a transfer RNA. Additionally it is more likely that the modification on the $tRNA^{Trp*}$ selectively gets shuttled to the secondary pathway one where the NOS-TrpRS II complex nitrates the tryptophan.

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CHAPTER 4

MAJOR CAROTENOID DIFFERENT IN WT COMPARED TO Δ*NOS*

4.1 Introduction

Carotenoids are naturally occurring pigments that are known to scavenge reactive oxygen species (ROS)(1). The carotenoid biosynthesis starts with the synthesis of phytoene by the condensation of gernaylgenrayl diphosphate. Next, a desaturase (CrtI) results in the synthesis of lycopene which further goes various modification reactions such as cyclization, ketolation, or hydroxylation which leads to the formation of different carotenoid products (2). *D. radiodurans* is a red-pigmented, non photosynthetic bacterium which is best known for its ability to withstand radiation, oxidants and desiccation (3). This characteristic led to many studies on carotenoids and how they protect *D. radiodurans* from such stress. The main pigment found in *D. radiodurans* is a C_{40}-carotenoid called deinoxanthin. According to the genome sequence of *D. radiodurans*, many of the carotenoid biosynthetic genes are encoded (4); however, the biosynthetic pathway of deinoxanthin is not well characterized (5). It is known in a *ctrB* or *ctrI* knockouts, two genes upstream the carotenoid biosynthetic pathway (Figure 4.1) which lead to a colorless strain, increases its sensitivity to oxidative DNA-damaging agents, likely due to its ability to scavenge radicals (6).

4.2 Methods

Metabolite analysis

Metabolite extraction was prepared by growing wt, Δ*nos*, and Δ*nos*:pNOS strains until the OD reached ~1.0. Cold 80% methanol:20% H_2O mixture was stored at -80º C for

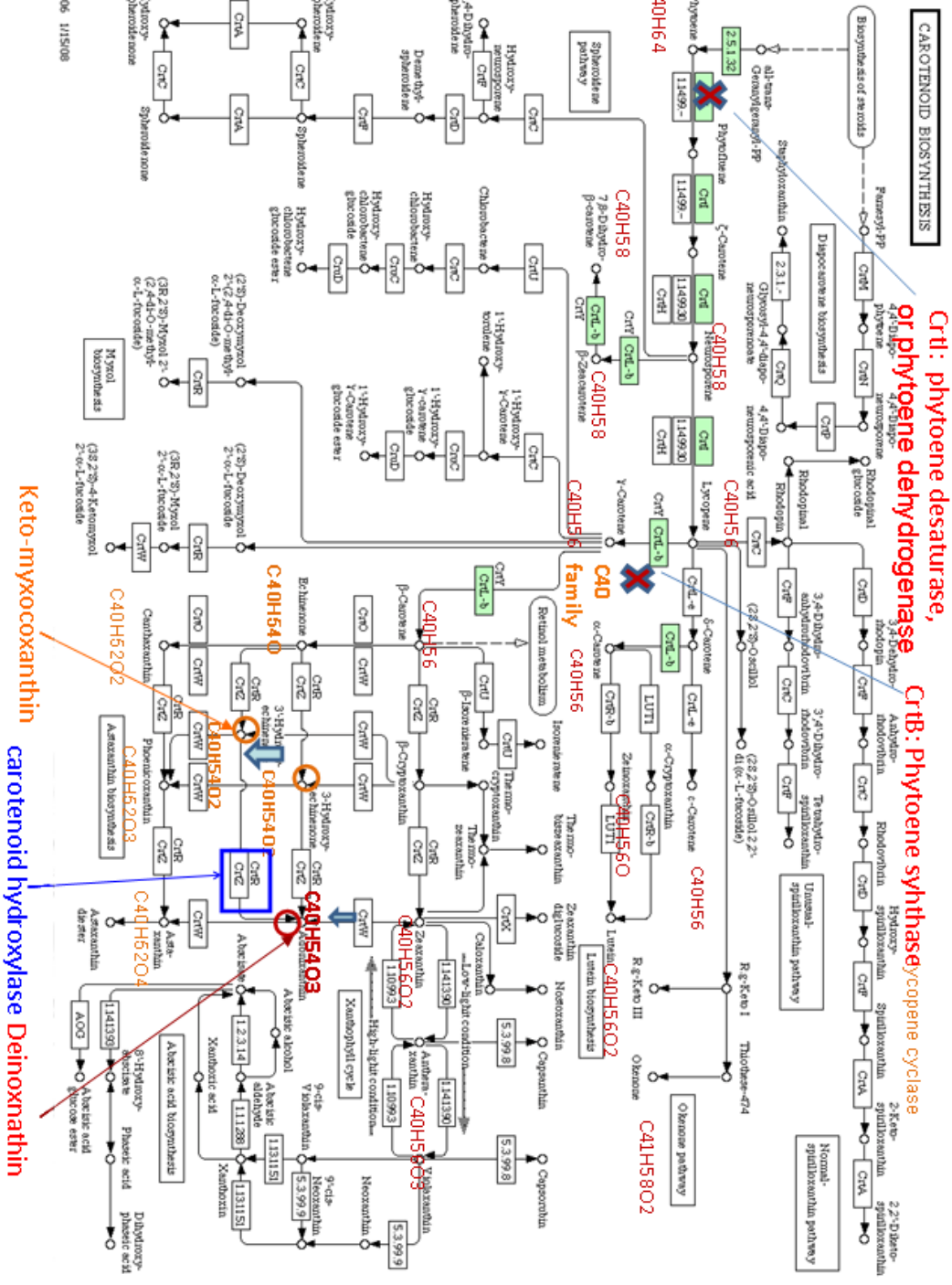

Figure 4.1 *D. radiodurans* carotenoid biosynthetic pathway. The carotenoid that appears in abundance in the wildtype is the keto-myxoxoxnathin ($C_{40}H_{54}O_2$), whereby in the Δ*nos* is the deinoxanthin ($C_{40}H_{54}O_3$). The enzyme that is responsible for this reaction is CrtZ – a carotenoid hydroxylase.

1-2 hours before the extraction process. The grown cultures were harvested and each sample was transferred to 5 prechilled conical tubes (5 replicates of 10ml cells). The spun down cells were then washed twice with cold 1x Phosphate Buffered Saline (PBS) and the supernatant was removed. 300µL of cold 80% methanol was added to the cell pellet, vortexed and transferred to a 1.5ml prechilled microcentrifuge tube. Prechilled glass beads (~100 µL) were added to the methanol mixture. The samples were vortexed at the highest speed for 20s, followed by 5 min incubation on dry ice. The vortex and incubation steps were repeated twice more by extracting them with 300µL methanol. The mixture was then spun down at 4º C and methanol extract transferred to new tubes. Finally, the sample was split into two tubes and SpeedVac was used to dry down them down. They were sent for analysis to Qiuying Chen in Steven Gross' Lab at Cornell Weil Medical School. The mass spectrometry analysis was carried out there.

4.3 Results and Conclusions

In chapter 2, we showed that Δ*nos* plays a protective role against UV damage, so we decided to explore the mechanism using metabolite screening. Besides the activation of *obgE*, a GTPase involved in stress response, it is possible that DrNOS affects other genes/proteins, for example through S-nitrosylation. We compared the wt and Δ*nos* to see if there was a difference in the metabolite pools (Table 4.1). Some of the candidates that were found differently in wt and Δ*nos* are deoxyguanosine 5' monophosphate (dGMP), guanidinesuccininc acid and proline. However, the compound that showed the largest difference was a carotenoid called deinoxanthin. Carotenoids are known to protect the organism against reactive oxygen species that are formed from the irradiation process. Contrary to published reports, we have found keto-myxocoxanthin to be the main carotenoid in the wt *D. radiodurans* R1 strain.

Table 4.1: Analysis of metabolites. These are some of the metabolites that were found to be higher in the Δ*nos* compared to the wt (KO > WT) or vice versa. Deinoxanthin is the carotenoid that was found in abundance in the Δ*nos*.

Mass Name	Value	RT	mass	Name	Formula	
206	1.137	8.079	385.1175	Phosphatidylserine	C13H24NO10P	
1905	1.137	8.14	422.1252	Trp Asp Cys	C18H22N4O6S	
2080	1.137	8.105	428.126	Phenytoin-N-glucuronide	C21H20N2O8	KO>WT
1877	1.137	8.24	442.1417	12-Dehydrotetracycline	C22H22N2O8	
168	1.137	7.858	523.2884	GPSer(18:1(9Z)/0:0)	C24H46NO9P	
761	1.137	1.696	566.4912	1-hexadecanoyl-2-(9Z-hexadecenoyl)-sn-glycerol	C35H66O5	
392	1.137	1.07	582.406	Deinoxanthin	C40H54O3	
1974	1.137	8.128	886.2411			
2075	1.137	8.126	954.7625	TG(20:2(11Z,14Z)/20:4(5Z,8Z,11Z,14Z)/20:4(5Z,8Z,11Z,14Z))[iso 3]	C63H102O6	
668	1.135	1.423	219.1109	Pantothenic Acid	C9H17NO5	
123	1.134	1.482	687.3589			
28	1.133	1.436	275.1009	Ethenodeoxyadenosine	C12H13N5O3	
363	1.132	13.287	132.091	Ornithine	C5H12N2O2	
770	0.882	1.611	338.2822			
259	0.881	13.293	174.1124	Arginine	C6H14N4O2	
436	0.88	1.382	324.2666	11S-HEDE	C20H36O3	
1390	0.88	1.339	338.2826			
256	0.88	1.112	355.3072	N-Hexadecyl-L-hydroxyproline	C21H41NO3	KO<WT
1143	0.88	1.348	725.5432	Cerebroside A	C41H75NO9	

However, in the Δ*nos*, deinoxanthin is the major carotenoid. The difference between keto-myxocoxanthin ($C_{40}H_{54}O_2$) and deinoxanthin ($C_{40}H_{54}O_3$) is a hydroxylation/oxidation. Although the steps leading to these products are the same in the pathway, (Figure 4.1) deinoxanthin requires a CRTZ, a p450-like carotenoid hydroxylase. These results have been repeated three or more times. Comparison of the $\log_2$(Δ*nos*/wt) showed the highest score for deinoxanthin. Conversely, Deoxyflexixanthin/ Keto-myxocoxanthin showed the lowest score. Similarly, experiments with cells exposed to UV radiation showed the presence of highest score for deinoxanthin; however, the absence of keto-myxocoxanthin was not as great as in the non-irradiated sample. It is possible that in the wildtype, NOS produces low levels of nitric oxide which are enough to inhibit the *crtZ* gene, as p450's are known to bind nitric oxide strongly (7). This may be by design as carotenoids have different levels of oxidative protection (6). However, it may be that CRTZ, a heme protein has a strong affinity for NO. Although, this may not be the definitive reason, it is one scenario that may explain how the hydroxylation/oxidation of keto-myxocoxanthin is prevented in the wt strain (Figure 4.2).

This difference in the carotenoids present could affect the ability of the strains to withstand radiation. More experiments need to be performed, such as addition of endogenous NO to the Δ*nos* strain followed by analysis of the metabolites to reconfirm the accumulation of keto-myxocoxanthin. Also, generating a knockout of *ctrZ* and checking for its ability to scavenge ROS might give us insight into the carotenoid pathway. Proteomic analysis (auto MS/MS) from in-solution trypsin digestion of wt and Δ*nos* showed that acetyl-Coa-acetlytransferase, hypothetical protein DR_0888, and phosphoglycerate kinase were differentially expressed. These results are preliminary and need to be repeated. Also, the CRTZ gene can be overexpressed and purified in *E. coli* and the mechanistic properties of NO binding,

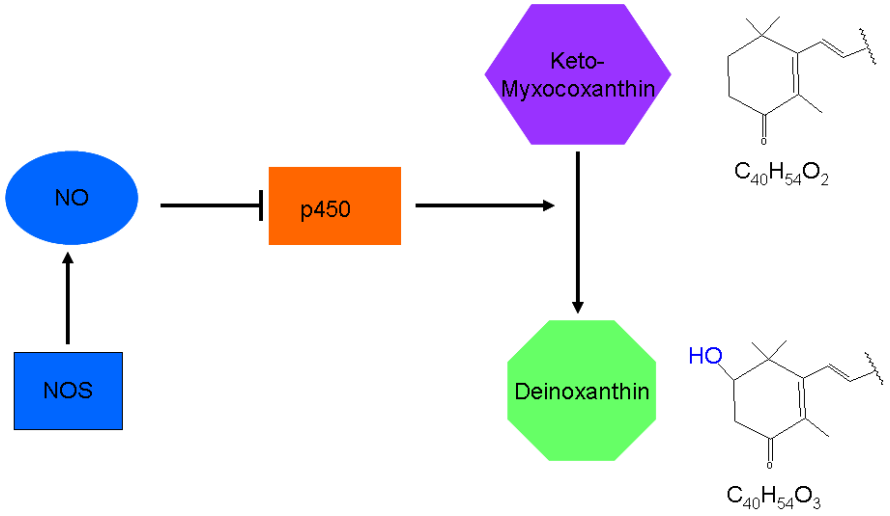

Figure 4.2 Proposed model on how NOS affects carotenoid production. The reason why we see the production of keto-myxocoxanthin in the wt may be due to the inhibition of CRTZ by NO. In Δ*nos*, p450 can hydroxylate keto-myxocoxanthin leading to the formation of deinoxanthin.

and substrate hydroxylation studied. However, the carotenoids are generally insoluble and harder to study.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

We know that *Streptomyces* NOS is involved in the nitration of tryptophan, which is the building block for thaxtomin. Additionally, we know in *Bacilli*, NO protects the organism from oxidative stress. Prior studies in *Deinococcus* showed the interaction between NOS-TrpRS II nitrated tryptophan *in vitro*. The data presented here has identified yet another role of NOS in *D. radiodurans*, as it plays a protective role specific to UV radiation. Also, the NOS-TrpRS II interaction also affects the *in vivo* tRNA pools as a modified form of $tRNA^{Trp}$ has been found. Further investigations into the NO-mediated survival mechanism of *D. radiodurans* may yet reveal commonalities in the above mechanisms as well as provide insight into UV radiation responses by other organisms. As studies on NOS in these and other organisms continue, and as more biochemical pathways are identified we hope to understand their catalytic and functional properties further. One trend is emerging as NO plays various signaling roles similar to mammalian NOS in these simple, yet unique organisms. Although, in these three known cases, it seems that the organisms are using nitric oxide for their own distinct advantage.

5.2 Endogenous NOS protects *D. radiodurans* from UV light

By taking a genetic approach, we tested a variety of stress conditions to find a pathway that NOS could be involved in. We found that Δ*nos* is severely compromised in its growth recovery following irradiation. This defect can be rescued by addition of endogenous NO and addition of chemical donors such as SNP. Genetically we were

able to complement the Δ*nos* as growth recovery was rescued 55% to that of wildtype levels. The mRNA levels of *nos* are relatively low initially; however, they increase four-fold upon irradiation. Mechanistically, the expression profile of *nos* resembles that of other genes involved in the repair process in *D. radiodurans* such as *recA* and *ligT* (1). Additionally, endogenous NO can be supplied prior to, during or after irradiation which also suggests that it is acting as a signaling molecule upregulating genes or activating proteins involved in the growth recovery process. Using microarrays we were able to find that one of the targets of NOS is a GTPase, o*bgE*, a gene involved in bacterial growth proliferation and stress response. The mRNA levels of *obgE* increase upon UV radiation and we can complement the Δ*nos* by overexpression of ObgE to 40% to that of wildtype. It is likely that there are other mechanisms and targets by which NOS can protect *D. radiodurans* from UV light. There are other possible functions of DrNOS that may or may not play a role in UV protection. NO can inhibit CTRZ, a cytochrome p450 which plays a role in the production of an important carotenoid. Lastly, NOS can be involved in the synthesis of modified tryptophan analogs as it interacts with TrpRS II *in vitro* and the $tRNA^{Trp}$ pools differ *in vivo*.

5.3 Possible mechanisms of NO

It is know that NO reacts with metallo-cofactors of transcription factors and other proteins. Additionally, NO can S-nitrosate cysteine residues on proteins which can activate the protein. This latter mechanism is known to regulate mammalian phosphatases, kinases and transcription factors such as HIF-1 and NFκB (2). It is very likely that *obgE* is activated by NO in one of these ways. A parallel finding to our study was found in mammals whereby UV irradiation increases inducible NOS (*iNOS*) levels in macrophages. NO released by iNOS S-nitrosates a specific cysteine residue

on HIF-1α, which plays a key role in various inflammatory diseases and wound healing (3). Also, many NO-responsive transcriptional regulators and sensor kinase systems have now been characterized in other bacteria (4, 5) and thus it is a strong possibility that NO could act as a regulatory signal in *D. radiodurans*. There are seven transcriptional regulators of the MerR class, (possibly an ortholog of SoxR), and two members of the LysR family, (possibly an ortholog of OxyR) in *D. radiodurans*, paralogs of which are NO responsive in other organisms (6).

There are many ways we can answer the questions about the mechanism of NO protection in *D. radiodurans*. We can first determine if there are other genes that are elevated in wt *D. radiodurans* compared to Δ*nos* by doing more microarrays (this is already in progress). We have added endogenous NO to the Δ*nos* strain to compare the genes that can be complemented to wt levels. This may give us a clue into genes that may be involved under the control of *obgE* or other independent pathways such as transcription factor σ^B, which we know is activated by ObgE in *B. subtilis*. Besides microarrays, we can do a proteomic study where by proteins from wt and Δ*nos* prior to and after irradiation are run on a 2D gel. Comparison between the samples could give candidates that are different, most likely due to S-nitrosylation. Also, to determine exactly what ObgE targets, a knockout strain can be made and possible candidates examined, although this may be difficult as *obgE* is known to play various roles in many pathways such as chromosome segregation, cell proliferation and ribosome maturation. The interesting detail about the carotenoid production in Chapter 4 which differ in wt and Δ*nos* may give some insight into another way of protection by NO.

It may be that UV protection was not the original intent of NO production. NO may target ObgE even under normal conditions as Δ*nos* does have a slight delay in growth compared to wt strain. However, NOS produces excess NO to activate ObgE,

since it is more crucial in the growth regulation of cells under conditions of stress. It is possible NO plays a signaling role under desiccation condition as a close relationship between bacterial desiccation tolerance and ionizing radiation (IR) resistance has been established. The role of NOS in bacteria appears divergent as *B. subtilis* NOS does not protect the organism from UV stress but it does from H_2O_2. NOS in bacteria appear to have specialized function unique to the organism.

So far our understanding on whether NO targets genes/proteins that play a role in protection against DNA damage or protein damage is limited. ObgE has dual functions as it is regulator of cell growth and chromosome portioning; however, it is also involved in ribosome maturation/biogenesis. Additionally, our data suggests that the probable repair genes (*recA, uvrA, uvsE*) are also upregulated in the Δ*nos*. It may be that we have been unable to identify a gene/protein involved in DNA repair or that NO protects against UV-induced protein oxidation. Regardless, it is necessary to narrow down the protective mechanism of NO by monitoring the growth of wt and Δ*nos* strains after cells have been exposed to a specific wavelength of UV light. Since we have used a polychromatic UV radiation exposure of 200nm-500nm we have caused damage to DNA and protein. Further investigation by the use of a cut-off filter, or a single pass bandwidth filter, or a 254nm crosslinker will give us more information on what kind of damage NO aids in, thus narrowing down the targets.

5.4 NOS-TrpRS II interaction affects $tRNA^{Trp}$ pools

In vivo, we have found that the $tRNA^{Trp}$ pools differ when the NOS-TrpRS II interaction is absent. Surprisingly, we see an accumulation in the deacylated pools of a modified $tRNA^{Trp*}$ species. The pattern is present only when cells are harvested under acidic condition and during log-phase growth. Also, the function of the interaction remains unclear, as it does not seem to have an affect on UV protection.

Hopefully, the on-going studies on the identification of $tRNA^{Trp*}$ species will provide insight into the biological role of NOS, TrpRS II and $tRNA^{Trp*}$, although the model of NOS' nitration of tryptophan to form 4-nitrotryptophan which can be incorporated in to proteins or secondary metabolites remains valid.

5.5 To determine the modified $tRNA^{Trp}$ and/or amino acid

Since the NOS-TrpRS II interaction does not seem to be related to UV protection, in order to elucidate the role of NOS-TrpRS II the modified $tRNA^{Trp}$ must be identified. Based on the results of the HPLC analysis of tRNA nucleosides, if the modification is on the anti-codon region then we can adenylate the *in vivo* purified $tRNA^{Trp*}$ with tryptophan and/or modified tryptophan. This can be done by using a radiolabeled amino acid or by monitoring the release of the pyrophosphate. In either case, a mixture containing the amino acid, TrpRS II and $tRNA^{Trp}$ will be incubated for the reaction to occur. The reaction can be stopped, $tRNA^{Trp}$ precipitated, and the reaction mixture quantified. Using a radiolabeled amino acid, the aminoacylation rate can be measured using a phosphoimager or a scintillation counter. The rate of adenyl-Trp formation can be calculated by measuring the absorbance at 355nm using the EnzCheck PP assay which monitors the release of pyrophosphate (7).

Prior studies to evaluate whether the amino acids were different in wt and Δ*nos* were inconclusive as we were unable to separate the amino acids. If $tRNA^{Trp*}$ analysis is inconclusive, we should try to purify the amino acylated versions of $tRNA^{Trp}$ from the Δ*trpRS* I since, it showed two bands (acylated-$tRNA^{Trp}$). Despite all the attempts, if the results are still inconclusive and the question of 4-nitrotryptophan still exists, feeding studies of radiolabeled amino acid can be supplied to the growth media and the compound tracked. The compounds/proteins can then be extracted, analyzed by HPLC and NMR. Another way to track the amino acid (likely 4nitrotryptophan) is to

raise antibodies against the compound in rabbits and then use the antibodies in immunoprecipitation experiments. Proteins found to bind to the antibody will be identified through peptide mass fingerprinting. Such an experiment has been performed previously to isolate proteins that contain 3-nitrotyrosine (8).

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APPENDIX

PROGRESS TOWARD STRUCTURAL CHARACTERIZATION OF THE THAXTOMIN NITRATION REACTION

A.1 Introduction

Streptomyces are bacterial species known for their scab-like lesions they form on the tuber surface and the epidermis of potatoes**.** Three species of Strepotomyces (*S. scabies, S. turgidiscabies* and *S. acidiscabies*) infect host plants by the synthesis of a nitrated dipeptide phytotoxin called thaxtomin A (1, 2). Thaxtomin is made up of a tryptophan and a phenylalanine connected by a dipeptide by nonribosomal peptide synthase genes (3, 4). The bacteria contain a large mobilizable pathogenicity island to the host plants that include the biosynthetic genes for thaxtomin A such as the non-ribosomal peptide synthase, NOS and p450, the latter two being responsible for nitration and ultimately the toxicity of thaxtomin. Studying these two proteins can give some insight into the mechanism of nitration.

Bacterial NOSs lack the reductase domain, and do not contain a dedicated reductase partner (5). Cytochrome p450s are monooxygenases that can play an analogous function as a reductase partner and provide the oxygenase domain with electrons necessary for catalysis of L-arginine to nitric oxide (6). Additionally, we know that the *p450* (*txtE*) gene is directly next to the *nos* gene and they may be expressed together. It has been found that inhibition of the cytochrome p450 and NOS prevent the nitration at the 4-position of the tryptophanyl moiety in thaxtomin. While reactive nitrogen species such as peroxynitrite (ONOO-) could easily react with the aromatic indole ring in tryptophan, this primarily results in nitration at the 6-position (7). A precise enzymatic control is necessary by p450 and NOS to nitrate tryptophan

at the 4-position. Additionally, feeding studies with L-Arg-guanidino $^{15}N_2$- HCl confirmed the result (NOS is the only enzyme that oxidizes the guanidinium group of L-Arg to NO) and showed the radiolabel at the 4-position. With all the *in vivo* data, it was important to discover whether p450 and NOS nitrate tryptophan prior to forming the peptide bond with phenylalanine or it nitrates thaxtomin. To test the more likely scenario that the nitrated 4-nitrotryptophanyl species combines with phenylalanine to form thaxtomin, we need to obtain soluble protein of both p450 and NOS.

A.2 Methods

The vector that gave soluble *S. scabies* p450 protein was pet151_D-TOPO provided by Sarah Barry. Cells were first transformed with p450 and FC (which was in PACYC_Duet Vector). Using ampicillin and chloramphenicol, large scale cultures (8L) were grown to an OD ~ 0.6 at 37º C, at which time the incubator was turned down to 15 º C. After 30 min the cells were induced with Isopropyl-beta-D-thiogalactopyranoside (IPTG) and 20mg/L of δ-ALA was supplied to the growth media. Cells were grown overnight and purified using common His-tag affinity chromatography techniques. The protein was then passed through a size exclusion chromatography column to remove any contaminants and exchange buffer to 20mM Tris pH 7.5, 100mM NaCl, and 5mM DTT. Various concentration of protein was used to set up initial Hampton Screen conditions by the hanging drop vapor diffusion method.

A.3 Results and Conclusions

Based on the *in vivo* data, understanding the nitration process can lead to new insights on the mechanism of p450 and NOS. Much work has been investigated to get soluble protein such as different vectors (His-tag, maltose binding protein,

sumolyation binding protein), various constructs (Δ18 p450, Δ28 p450, PA167DR p450, p450, NOS, p450 and NOS), various temperatures (15°C, 25°C, 37°C), different expression systems (*E. coli*, *S. turgidiscabies*, *S. lividans*), co-expression and many combinations of these approaches. Additionally, mutations were made within the protein based on homology of known structures of p450. However, none of these techniques gave soluble protein. The protein was either insoluble, not expressed well or even degraded in some cases.

The proteins (NOS and p450) in *S. turgidiscabies* are surprisingly insoluble although their sequence homology is very similar to other bacterial proteins. Also, NOS from *B. subtilis*, *D. radiodurans*, *G. stearothermophilis*, *S. aureus* have been successfully expressed (8). The p450 is extremely interesting in terms of catalysis and possibly because it binds tryptophan for nitration. Unfortunately, without soluble protein the work was halted. Recently, the p450 adjacent to the NOS in *S. scabies* was expressed, found to be soluble and given to us by Sarah Barry in Greg Challis' Lab at University of Warwick for crystallization. Although the homology between S. *turgidiscabies* and *S. scabies* is very strong, there are a few differences that might have let us obtain soluble *S. scabies* p450.

The cytochrome p450 is an interesting protein, as its active site is significantly different compared to other p450s. An alignment from *Saccaropolyspora ertherea* and *B. subtilis* show unique residues on the *S. scabies* p450 in the region of the I-helix (Figure A.1). The I-helix, which sits atop of the heme ring, contains three proline residues unlike the alanines found in other p450s, which must substantially alter the heme-binding site. The protein was purified at a high concentration and crystallization attempts were made with initial Hampton Screen conditions. Unfortunately, no notable hits or crystals were obtained. At this step, an important discovery was made

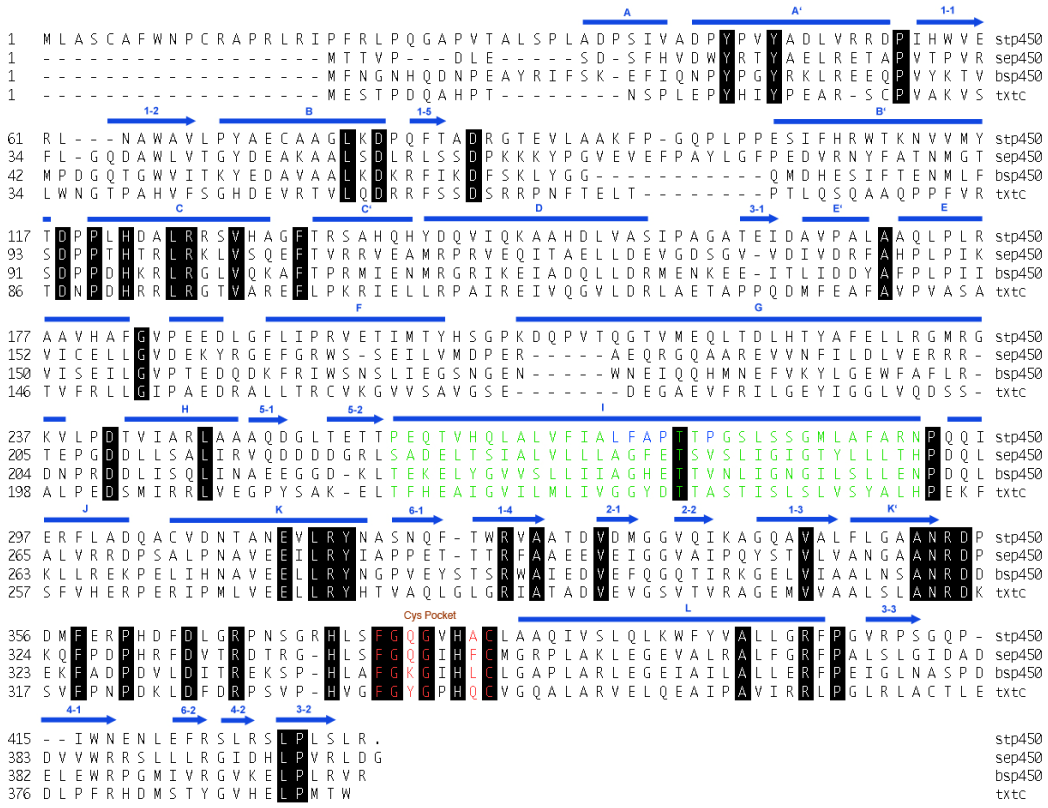

Figure A.1 Sequence alignment of various cytochrome P450s: *Streptomyces turgidiscabies*(stp450), *Saccaropolyspora ertherea* (sep450), *Bacillus subtilis* (bsp450),and *Streptomyces turgidiscabies* txtC (txtc). Homologous residues are in black. The red is the "Cys pocket" with cysteine being the axial ligand of the heme. The I-helix is shown in green and most residues are similar across the four sequences. However, the blue residues found in the stP450 are unique as there is a phenylalanine and two prolines that are in close proximity to the heme.

as the protein ran as two bands on a gel (Figure A.2). Prior work with heme protein led us to believe that the two species of p450 are likely to be the native p450 with heme incorporated into protein and p450 with protoporphyrin IX (free-base porphyrine, heme without Fe inserted) bound instead of heme.

Incomplete heme incorporation into recombinant overexpressed protein is a problem as it may affect biochemical studies and having two species of p450 might hinder the crystallization process(9). Several techniques have been found in the literature to overcome this problem(10). One way is slowing the protein expression rate to not exceed the heme biosynthesis. This will increase the heme content in the overexpressed heme protein, at the expense of protein yield. Also, addition of δ-aminolevulinic acid (δ-ALA), which is a precursor to the heme biosynthesis pathway, to cells just prior to induction also increases the rates of heme production and incorporation (11). Despite these strategies, we were unable to get a large percentage of the protein with heme incorporated. Our lab has discovered another method where overexpression of the protein of interest with ferrochelatase (FC) in the presence of δ-ALA. FC is an enzyme that is responsible for coordinating the iron to the protoporphyrin IX(12). Overexpression of p450 with FC alleviated the incomplete heme incorporation as seen in (Figure A.2). In the p450 prior to expression of FC, there are two bands, which shift upon His-tag cleavage to a lower molecular weight. However, there is only one band with the overexpression of FC. Additionally, spectroscopically the ratio of the Soret peak (417nm) to total protein peak (280nm) is much higher in the sample with FC.

We attempted to crystallize the p450 again with most, if not all the protein incorporated with heme. This time, we used the Phoenix Liquid Handling Robot at Chris Fromme's Lab that automates the process of crystallization. The advantages are that this technique uses very little protein and is very fast. Unfortunately, it does not

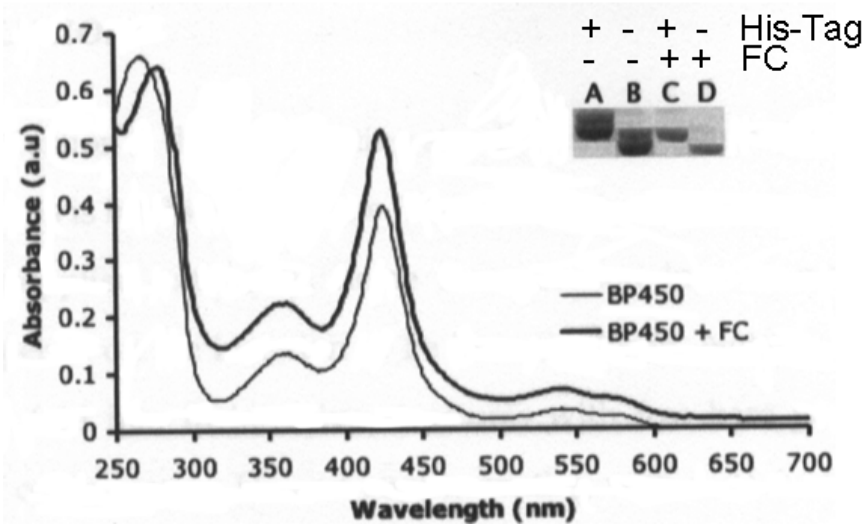

Figure A.2 Coexpression of FC and p450. The spectrum of p450 expressed with and without Ferrochelatase. The co-expression results show a substantial increase in heme incorporation (Abs_{soret}/Abs_{280}). Additionally, the gel shows the presence of two bands without expression of FC; however, there is only 1 species with FC which both shift on His-tag cleavage.

work as well with higher concentrations of proteins and the hits obtained are not as reproducible. So, we set up Hampton Screens (I and II) again at various concentrations and temperature. However, we were unable to acquire any hits or crystals.

We decided to try a different method to crystallize that had success in the lab. Surface Entropy Reduction (SER) or site-specific mutagenesis is a common technique used to alter the surface residues of proteins by replacing certain amino acids with others that are thought to be more conducive to crystallization (13). The SER server (http://nihserver.mbi.ucla.edu/SER/) was used to analyze p450 for potential loop regions that contain a series of residues that have long, hydrophilic side chains. We decided to choose three regions in different part of the protein and changed them to alanine residues. These variants were created and Hampton Screens set up again. This time although we did not get crystals, we did obtain a hit (0.1M NaCl, 100mM Hepes pH 7.5, 1.6M Ammonium Sulfate). Unfortunately, screening for pH, salt, different concentrations of protein, and temperature did not improve the initial observation.

We are now in the process of making more SER mutants, and also trying them in combination which might perturb the protein enough to make it crystallize. Additionally, an observation was made as only the protein that contained 1mM tryptophan gave the hits. This suggests that p450 is binding the tryptophan and is the likely substrate as protein without tryptophan did not give the hit in the same condition. Also, the protein can bind the DTT (when purified on a size exclusion column in the absence of tryptophan and presence of 5mM DTT) as a low-spin-split soret peak is seen (Figure A.3) (14). By removing the DTT the p450 shows the commonly observed single Soret peak. In this case it is likely that DTT is ligating to the heme, as no tryptophan is present. However, with the addition of tryptophan in the

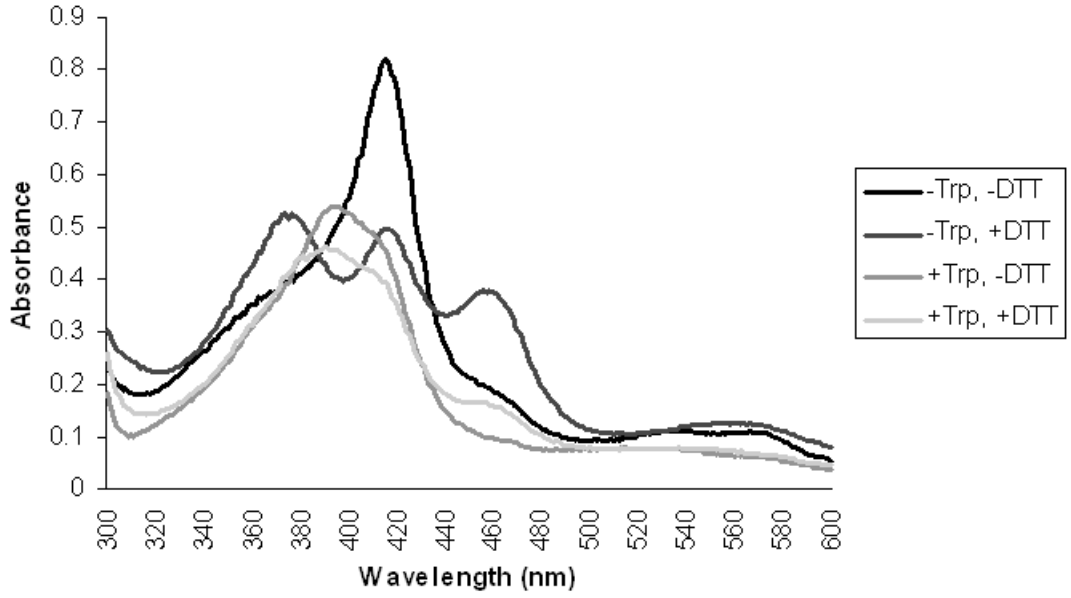

Figure A.3 Spectroscopic features of p450. A single soret peak is absorbed in the absence of Trp and DTT. However, a split soret peak is found in the presence of DTT as DTT binds the p450. However, when Trp is added to p450, the spectra changes as it confirms the binding of tryptophan directly above the heme, regardless of DTT.

presence or absence of DTT, you get a high spin species where tryptophan binds directly on top of p450, kicking off the water. Further work with more SER mutants might lead us to the structure of this unique p450.

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