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Production of a plasmid to facilitate gene interruption of the nitric oxide synthase gene from Bacillus subtilis

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Production of a Plasmid to Facilitate Gene Interruption of the Nitric Oxide Synthase Gene from *Bacillus subtilis*

Master of Science in Chemistry

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Abstract

A genetic interruption of *Bacillus subtilis* 168's native nitric oxide synthase enzyme (bsNOS) is to be created by inserting a gene for kanamycin resistance (KAN) into the center of the gene. To accomplish this, a plasmid has been constructed (pUC19ΔbsNOS) which includes the bsNOS gene separated into 5' and 3' halves with a KAN gene from p34S between them. The plasmid vector was isolated by digesting *Escheria coli* plasmid pUC19 with *Kpn*I and *Pst*I, and the KAN gene was cut from *Escheria coli* plasmid p34S using *Xba*I. The 5' and 3' halves of the bsNOS gene were amplified using the polymerase chain reaction with custom primers that imparted appropriate digestion sites to the ends of both amplified fragments. The vector and bsNOS fragments were all produced and ligated into a preliminary plasmid form (pNOS20) and amplified. This plasmid was subsequently digested with *Xba*I, and the KAN fragment was ligated into place between the 5' and 3' *Xba*I sites. Knockout colonies were generated by amplifying the desired insert from the pUC19ΔbsNOS plasmid using PCR and then using it for transforming competent wild-type *Bacillus subtilis* via electroporation. Potential *Bacillus subtilis* ΔbsNOS knockout colonies may have been generated but are pending confirmation.

List of Figures

List of Abbreviations

A

AMP, Ampicillin

B

B.sub, Bacillus subtilis

BLAST, Basic Local Alignment Search Tool

BSA, Bovine Serum Albumin

bsNOS, Bacillus subtilis Nitric Oxide Synthase

C

CaM, Calmodulin

cNOS, Constitutive Nitrc Oxide Synthase

D

ΔbsNOS, Knockout bsNOS gene

E

EDTA, Ethylenedinitrilo-tetraacetic acid

eNOS, Endothelial Nitric Oxide Synthase

F

FAD, Flavin Adenine Dinucleotide

FMN, Flavin Mononucleotide

G

H

H_4B, Tetrahydrobiopterin

I

iNOS, Inducible Nitric Oxide Synthase

IPTG, Isopropyl β-D-1-thiogalactopyranoside

J

K

KAN, Kanamycin

KatA, Catalase A

L

LB, Luria-Bertani Broth

M

mNOS, Mammalian Nitric Oxide Synthase

N

NADPH, Nicotinamide Adenine Dinucleotide Phosphate-oxidase

NCBI, National Center for Biotechnology Information

nNOS, Neuronal Nitric Oxide Synthase

NO, Nitric Oxide

NOS, Nitric Oxide Synthase

O

$OD_{(595nm)}$, Optical Density at 595 nm

P

PCR, Polymerase Chain Reaction

Q

R

S

S.O.C, Super Optimal Broth

SDS, Sodium Dodecyl Sulfate

SodA, Superoxide Dismutase A

T

TE Buffer, Tris-EDTA Buffer

Trx, Thioredoxine

TrxRed, Thioredoxine Reductase

U

u, Units (enzymatic activity)

V

W

X

X-gal, 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

Y

Z

Dedication

This work is dedicated to my family and friends, without whom few accomplishments would have been possible and even fewer would have been meaningful.

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Contents

Chapter 1: Background

Nitric Oxide as a Biological Signal

Nitric oxide (NO) was first identified as a eukaryotic trans-cellular signaling molecule in 1987 (1) and was later shown to be generated from L-arginine by an enzyme (Figure 1.1-A) known as nitric oxide synthase (NOS) (2). Mammalian NOS enzymes have been found to perform functions ranging from vasodilatation (3) to acting as a broad-spectrum antimicrobial during immune system responses (4).

Nitric oxide is effective as a broad spectrum antibiotic because of its high reactivity as a free radical species and its lipophilic nature (5). It is a small, membrane-permeable molecule capable of competitively binding precious metals, inhibiting enzymes, and generating oxidizing chemicals such as nitric acid, peroxynitrite, or superoxide in solution which in turn damage DNA (5). In eukaryotes, nitric oxide is generally produced at concentrations high enough to have these effects by inducible NOS (iNOS) during macrophage attack. However, nitric oxide has also been shown to affect bacteria at concentrations too low for toxic or acidifying effects (6), indicating more involved mechanisms at work.

Later efforts revealed prokaryotic NO-dependent pathways associated with denitrification and anaerobic gene expression. Nitric oxide was shown to be an activator of transcription factors ResD and ResE, key signals for anaerobic gene expression in *Bacillus subtilis* (Figure 1.1-B) and other microbes. While they were known at the time to be activated under low-oxygen conditions, it was discovered that full activation also required elevated concentrations of NO (7-9). This mechanism suggested that many bacteria have evolved to detect NO as an indication of impending oxidative stressors, such as those typically experienced during immune system attack. In non-infectious microbes such as *Bacillus subtilis*, NO likely warns of increasing concentrations of nitrosative species produced by soil denitrifiers sharing the microbe's environment. Nitric oxide would have been a likely candidate during the evolution of such a signal as its permittivity to cell membranes makes it one of the earliest species' detectable (10).

Nitric Oxide Synthase in *Bacillus subtilis*

It is perhaps because of NO's common roles as an antimicrobial and exogenous signal molecule that the discovery of nitric oxide synthase analogs in the prokaryotic species *Bacillus subtilis* was considered surprising. In 2002 the completion of the *Bacillus subtilis* genome sequencing project made it possible to perform Basic Local Alignment Search Tool (BLAST) searches on the bacterium's genome (11), revealing a sequence homologous to mammalian NOS. The protein encoded by this gene was isolated and shown to actively manufacture NO when given concentrations of NADPH, L-arginine, and a mammalian reducing domain (12).

Attempts to determine the purpose of this NO production began with comparisons of *Bacillus subtilis'* nitric oxide synthase enzyme (bsNOS) (Figure 1.3) and its mammalian analogs (mNOS). Mammalian NOS enzymes are homodimeric enzymes 135-160 kDa in size, and have been classified into three forms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Inducible NOS produces high concentrations of NO during immune system activation, while eNOS and nNOS (collectively known as constitutive or cNOS) produce lower concentrations of NO for use as second messengers targeting isoforms of soluble guanylyl cyclase. All three classes consist of a single polypeptide with two functional domains. The N-terminal domain binds the redox cofactor tetrahydrobiopterin (H_4B) and also secures a cytochrome P450-like heme group containing the enzyme's reactive iron species (13, 14). The C-terminal strand binds reducing species NADPH, and also flavin adenine dinucleotide and flavin mononucleotide (FAD and FMN, respectively) which serve to shuttle electrons from NADPH to the iron center. Eukaryotic NOS enzymes are dependent upon the Ca^{2+}-sensing protein calmodulin (CaM), which upon binding triggers a conformational change in the NOS dimer that facilitates this electron transfer. The enzyme has no activity outside of its dimeric form, which is stabilized by a zinc-binding domain located on the N-terminal end (13, 14). With regards to activity, mammalian NOS produces NO and citrulline from L-arginine in the presence of CaM, H_4B, and oxygen (15). One exception is seen in iNOS, which has CaM bound permanently and is therefore perpetually active, dependent only on transcription and the

presence of substrates. All forms of NOS have activity modulated by feedback inhibition: increasing concentrations of NO result in lower levels of manufacture.

The bsNOS gene shares 34% overall sequence similarity with cNOS, and the protein shows 52% structural similarity in active site regions (12). It is smaller than eukaryotic NOS by approximately 360 residues and is missing key components of the cNOS species. The dimer-stabilizing N-terminal hook is absent, as is part of the H_4B-binding domain (15), and mNOS's internal reductase domain. Consequently, bsNOS has been shown to scavenge reductase activity from other reductase enzymes (of which there are several in the *Bacillus subtilis* genome) which are not explicitly dedicated to NO production (16).

In 2005, Ivan Gusarov and Evgeny Nudler suggested that native-manufactured nitric oxide functioned as a means of "instant adaptation" when a microbe was exposed to oxidative stress. While exogenous NO would promote a switch to anaerobic metabolism, significantly lower levels of endogenous NO were suggested to perform two additional functions: inhibit the formation of dangerous oxidative chemicals, and activate the oxidative stress response enzyme superoxide dismutase A (SodA, also known as catalase A or KatA), which actively neutralizes oxidative species.

Damage from oxidative chemicals such as hydrogen peroxide is brought about by the Fenton reaction (Figure 1.3), a sequence of steps in which the oxidative species reacts with cytoplasmic Fe^{2+} to generate $OH^{\cdot}$, a radical chemical species capable of damaging DNA. However, the already low cytoplasmic concentration of Fe^{2+} is rapidly consumed as this occurs, and $OH^{\cdot}$ production would stop completely without reducing activity to return the resulting Fe^{3+} back to Fe^{2+}. In microbes like *Bacillus subtilis*, this activity is provided by the cytoplasmic concentrations of the amino acid cystine, a thiol species. This oxidized form of cysteine, known as cystine, must be reduced as well, and this is accomplished by thioredoxin and its counterpart thioredoxin reductase (Trx_{OX} and Trx_{RED}, respectively). Left alone, cells exposed to oxidative chemicals oxidize cytoplasmic iron to produce damaging ·OH species, at which point cytoplasmic cystine would reduce this iron back to Fe^{2+} for another reaction. Cysteine would then be actively reduced by cellular Trx_{OX}/Trx_{RED}, perpetuating the cycle at the expense of cellular energy stores until cell death. Gusarov and Nudler showed that to arrest this cycle, cytoplasmic

NO serves to inhibit the activity of thioredoxine reductase, ultimately limiting the availability of reduced iron with which to perpetuate the reaction. This system acts as a buffer which can provide several minutes of protection which allows a stressed cell to activate a more elaborate transcription-based oxidative stress response. One such condition, the activation of SodA, is also NO dependent; the enzyme is S-nitrosylated by NO to become active, and in turn begins actively scavenging oxidative species (10).

This hypothesis was confirmed four years later, with one addition: NO is also suggested to play a key role in antibiotic resistance. Given that the modes of activity of many bactericidal species ultimately come down to production of oxidative species, NO's activity in minimizing oxidative damage in general may be a subsequent function. It was known that endogenous NO concentrations were generally too low to compete with oxidative species stoichiometrically, though it is suggested that they may still have an impact on incoming antibiotic species. Gusarov and Nudler indicated that while this was an insufficient function on its own, endogenous NO levels did reduce the impact of antibiotic species such as acriflavin and pyocyanin early on by directly modifying them in addition to halting the Fenton cycle they are designed to initiate. The combination of these two functions with NO's activation of oxidant-scavenging SodA makes native NO concentrations an effective means of preempting, detecting, and responding to oxidative stress levels in *Bacillus subtilis*, and may have many analogous defense mechanisms among prokaryotic organisms. Other species have already been shown to employ this tool, including *Staphylococcus aureus* and *Bacillus anthracis*. Indeed, it has been suggested that this mechanism may open a new avenue for improving antibiotic effectiveness: by inhibiting bacterial NO synthesis, it is possible that antibiotic and/or oxidative attack from both medical treatments and immune responses could be bolstered (17), and given the inherent differences between mammalian and prokaryotic NOS enzymes it is plausible that such treatments could be applied chemically with minimal effect on the host.

However, kinetics studies of bsNOS do not indicate that NO concentrations resulting from the enzyme during normal conditions would be sufficient to provide any stoichiometrically relevant level of protection against molecules either oxidative or antibiotic. The rate of dissociation of the Fe(III)-NO complex (the releasing step for NO) is between 10 and 20 times slower than mNOS, and establishes a

limit for how quickly the enzyme can manufacture NO; bsNOS ultimately generates different N-oxides if the rate of Fe(III)-NO complex formation significantly exceeds its rate of dissociation (12). Combined with NO's particularly short half life in biological solutions, this characteristic makes the possibility of bsNOS single-handedly maintaining levels stoichiometrically comparable to sudden oxidative stress unlikely, and so much of the direct neutralization behavior observed may be provided by exogenous rather than endogenous sources.

Given that bsNOS is most comparable in activity to eukaryotic cNOS isoforms, it is possible that native NO production in *Bacillus subtilis* and other prokaryotic species is used for chemical signaling. Further, given bsNOS's low output it is likely that the message it is sending is not intended to be effectively transmitted during oxidative attack at all as it would be immediately swamped by high levels of exogenous NO. However, the typical receptor of eukaryotic NO signals -- guanylyl cyclase -- is not present in the *Bacillus subtilis* genome, indicating that if NO's function is as a second messenger, some other receptor(s) must be present (12).

Interest in *Bacillus subtilis* Nitric Oxide Activity

Interest in characterizing this function in *Bacillus subtilis* originates from the species' status as a model bacterium. It is a common non-pathogenic, gram-positive soil bacterium, and has been studied and characterized intensely; to date, its genome coding roughly 4100 genes has been mapped (18). Further, it is cultured and handled easily, and is of close evolutionary relationship to species such as *Staphylococcus aureus*, a pathogen of interest in recent years after the emergence of multiple-resistant strains (19). It is hoped that advances in our understanding of this species can be applied across several cell lines, potentially toward the establishment of new treatment options for infection.

Previous work used chemical inhibition of bsNOS with aminoguanidine to show that inhibition of NO signaling during logarithmic growth (and without significant oxidative stress) resulted in the down-regulation of an MreB-like protein, a nucleoside diphosphate kinase, and a phage shock protein (20). While to date these findings have not pinpointed the nature and function of the pathways involved with

NO, they do indicate that some form of signal transduction may be operating outside of oxidative stress conditions. Our work attempts to supplement this data by producing a genetic knockout of bsNOS and comparing the resulting difference in protein inventories using two-dimensional gel electrophoresis. This should confirm that differences observed that are not a direct result of chemical treatment but of the absence of a native NO signal.

Intended Study

To achieve this, a plasmid was designed (arbitrarily designated pUC19ΔbsNOS) which contained a gene coding kanamycin (KAN) resistance (~1 kbp in length) inserted into the middle of the bsNOS coding gene (Figure 1.5). To construct this plasmid, the sequence of bsNOS was selectively amplified in two halves (5' and 3') using the polymerase chain reaction (PCR). By designing custom primers it was possible to impart digestion sites at both ends of each fragment, allowing them to be selectively cut and ligated to desired sequence fragments simply by matching digestion sites during ligation procedures. The gene for kanamycin resistance was digested out of the plasmid p34S with *xba*I, and the vector species used was made from a pUC19 plasmid linearized with *kpn*I and *pst*I enzymes. The use of a plasmid made possible the amplification of this DNA stock by transforming it into competent DH5α cells via heat shock and then growing colonies selected for kanamycin resistance. The desired plasmid was then collected using mini-prep.

The sequence to be inserted (the bsNOS sequence with the inserted KAN domain) was amplified out of the pUC19ΔbsNOS plasmid using PCR and then inserted into competent wild-type *Bacillus subtilis* cells using electroporation, a method for generating pores in cell membranes via electrical field in order to insert otherwise membrane-impermeable molecules (21). Following electroporation, *B. sub* cells are incubated in LB broth and then grown on kanamycin-laced plates in order to select for colonies with successfully incorporated inserts.

Ideally, cells dividing in the presence of added DNA incorporated the insert by double recombination. In the process of replicating genomic DNA, these cells enzymatically separated the two DNA strands as well as the added DNA insert, and with its two ends matching the sequence of bsNOS the insert was able to match up with its compliment in the genome and allow the formation of a Holliday junction. From here, there were two possibilities: either the Holliday junction could twist to allow one strand with the insert to recombine with one strand of the wild-type genome (single recombination), or it could twist to combine two DNA strands containing the desired insert (double recombination). The latter of these two events should have yielded cells with a full interruption of the bsNOS gene (ΔbsNOS); having both copies of the gene interrupted with the gene for KAN resistance, it would now be impossible for the bacterium to manufacture functional bsNOS and, subsequently, NO. This can be confirmed using PCR to amplify the entire insert sequence and then observing them in an agarose gel. The presence of bands at ~1 kbp and ~2 kbp indicated a single recombination as both a wild type and an insert strand of DNA were present, but the presence of a single ~2 kbp strand would have indicated that all present copies of the gene had received an insert and had thus undergone double recombination. With a suitable knockout colony chosen, it will then be possible to generate 2-dimensional gel electrophoresis images for statistical comparison with chemically-inhibited wild-type *Bacillus subtilis* gels.

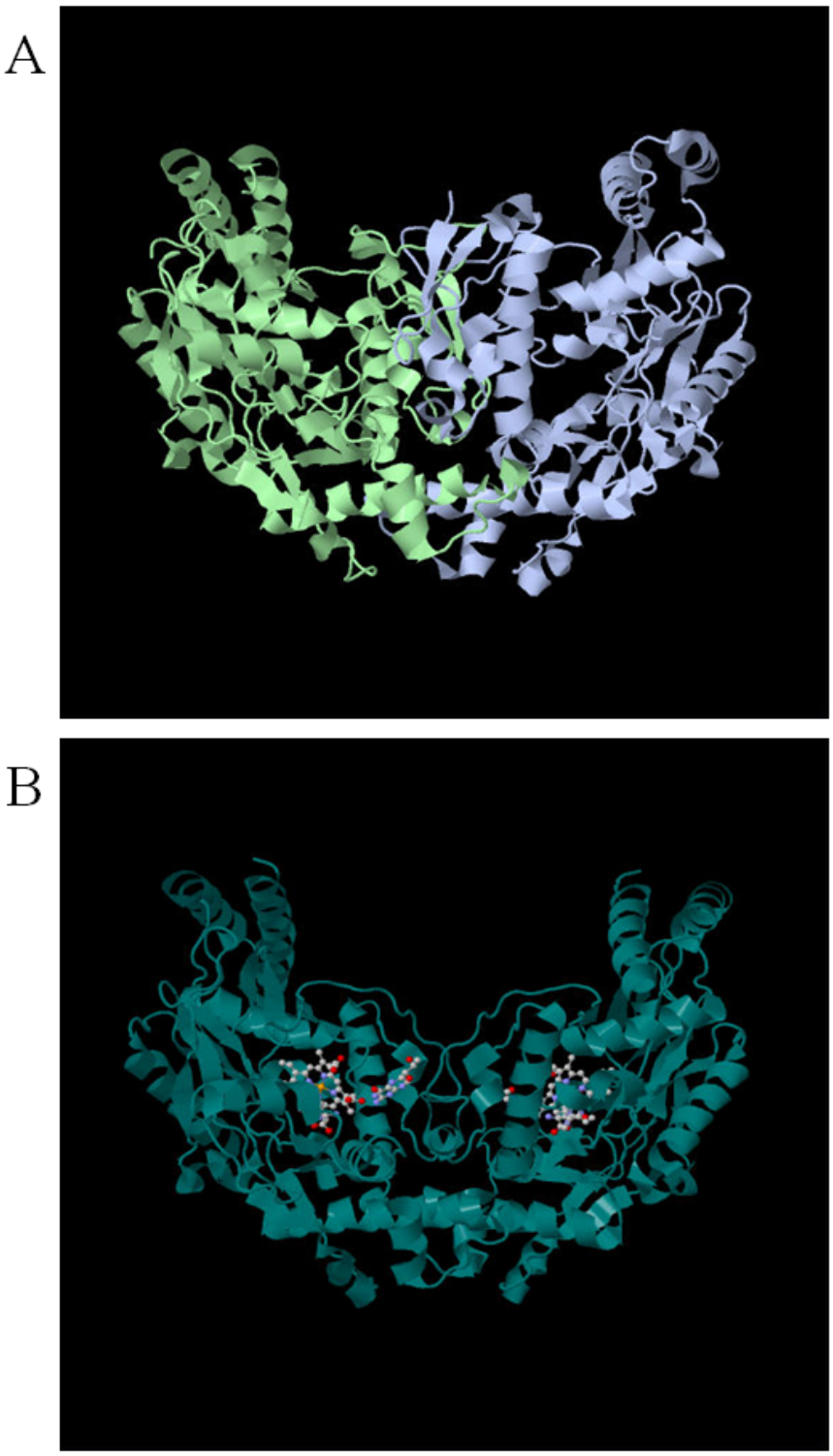

Figure 1.1. Comparison of Mammalian nNOS and bsNOS. (A) Mammalian neuronal nitric oxide synthase (nNOS) in its dimeric form, complexed with N~5~-(2-(methylsulfanyl)ethanimidooyl)-L-ornithine (22).(PDB code 3JT3) (B) *Bacillus subtilis* nitric oxide synthase (bsNOS) with heme groups bound (PDB code 2FBZ **)** (23)**.**

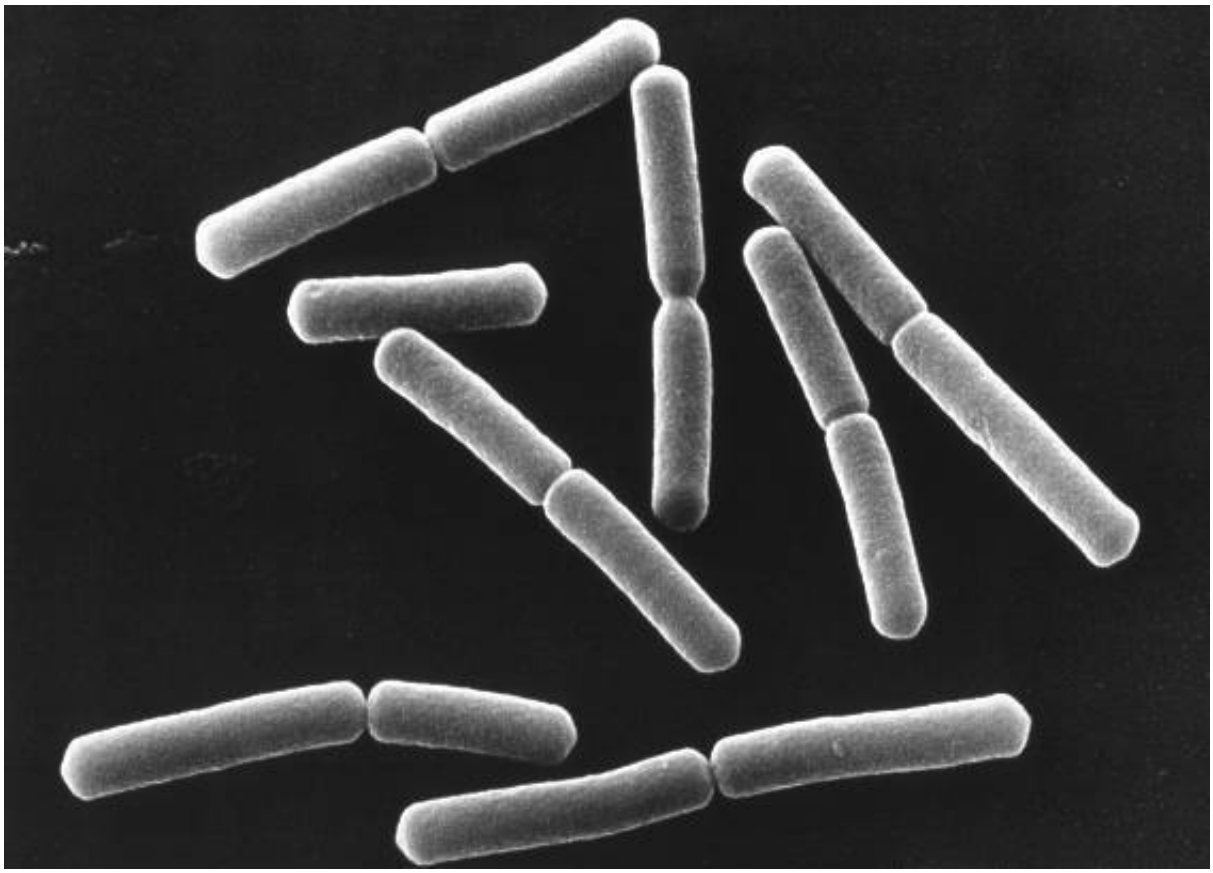

Figure 1.2. Electron Micrograph Image of *Bacillus subtilis* Cells (24)**.**

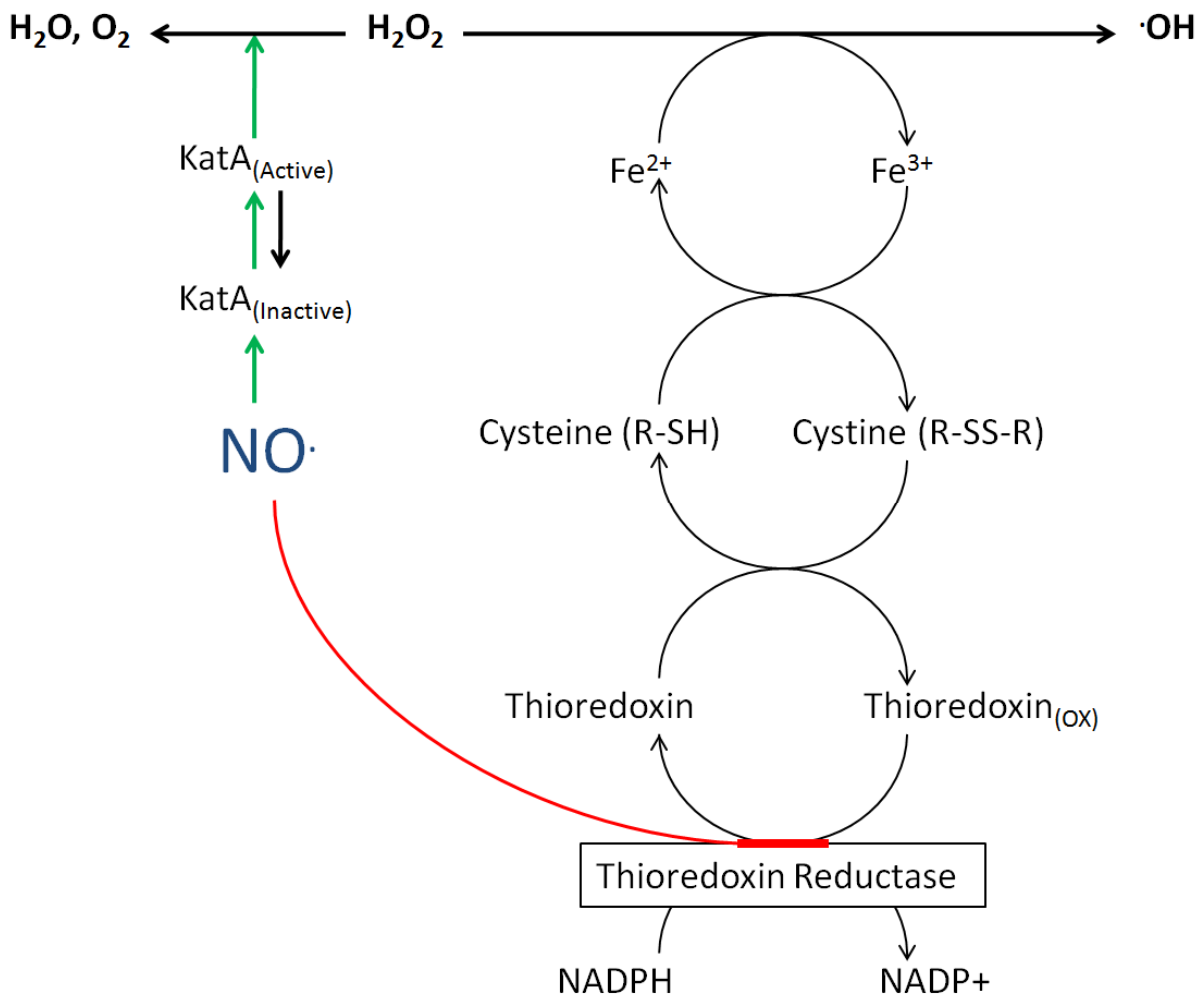

Figure 1.3. Nitric Oxide Interaction with Fenton Reaction, where KatA indicates superoxide reductase A (SodA), and Thioredoxin$_{(OX)}$ indicates oxidized thioredoxin, which is inactive until reduced by thioredoxin reductase. Green arrows indicate activation pathways,while red indicates inhibition. Adapted from Gusarov and Nudler, Figure 5 (10).

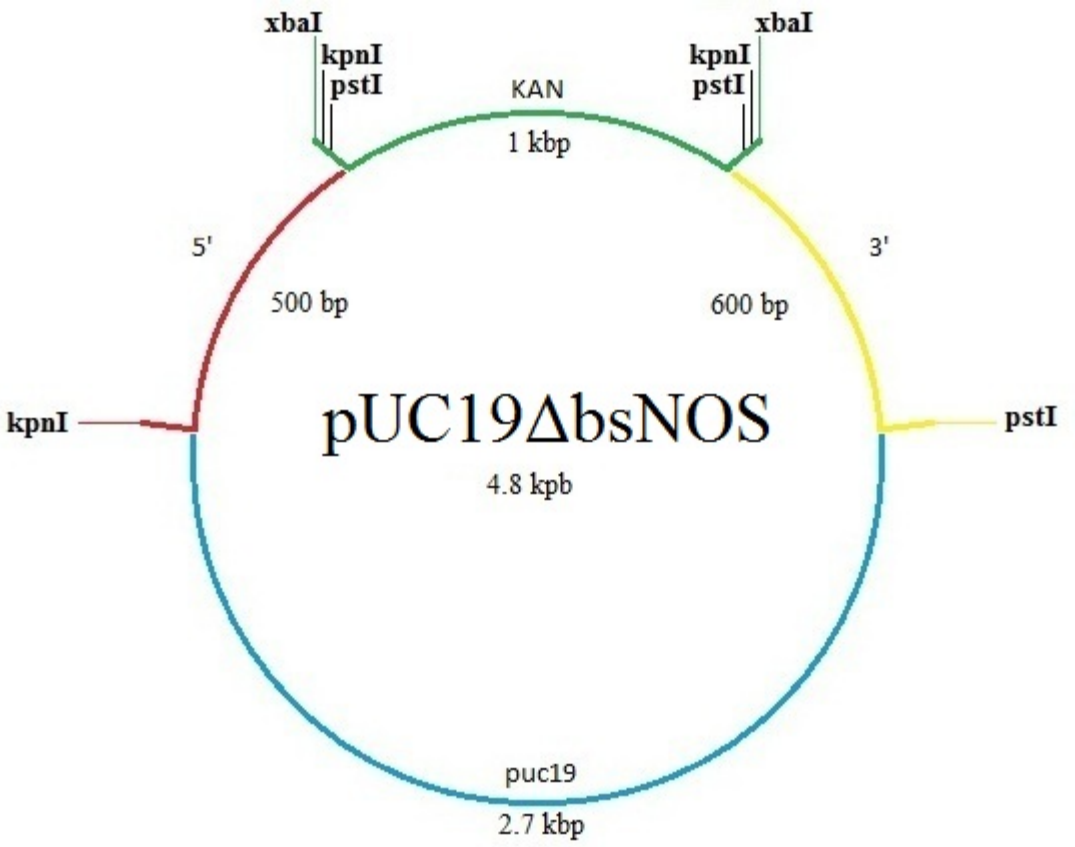

Figure 1.4. pUC19ΔbsNOS Plasmid Map with approximate fragment sizes and relevant restriction sites.

Chapter 2: Methods

Cell Cultures

Bacillus subtilis cell stocks were frozen for long-term storage at -80 °C, and stock cultures were grown by collecting shavings of the frozen pellets with a 1 mL micropipette tip and immersing them in 50 mL sterile Luria-Bertani (LB) broth. Cultures were grown in loosely capped sterile 250 mL polycarbonate Erlenmyer flasks and incubated at 37 °C with vigorous shaking (100-160 rpm) for at least 10 hours prior to use. Growth in a single flask was not allowed to proceed for more than 24 hours in order to limit stresses from nutrient exhaustion or increasing waste concentrations; prior to this time a 1:100 dilution with fresh broth was performed in a new sterile flask. Cultures were not passaged more than four times.

Competent *Bacillus subtilis* cultures were produced for electroporation procedures using fresh, 750 µL stocks of cells frozen once at -80 °C. Stocks were thawed on wet ice for 10 minutes and diluted 1:200 in sterile LB broth in a loosely capped conical tube, then incubated overnight with shaking at 37 °C. The resulting stock broth was then diluted 1:20 in sterile LB then incubated at 37°C with shaking, with optical density readings taken every 30 minutes. Once stocks had reached approximately 0.600 $OD_{(595nm)}$ (~3 hr), they were centrifuged at 4,400 rpm for 2 minutes to pellet and the LB broth was decanted using an aspirator. Pellets were washed three times with deionized water (1/3 the volume of the decanted broth), then twice more with the same volume of sterile 10% glycerol in deionized water. Pellets were then resuspended in sterile 10% glycerol solution (1:40 wash volumes) and diluted with an equal volume of deionized water to give a suspension in 5% glycerol. Stock was then divided up into 50 µL aliquots and frozen on dry ice, then stored at -80°C until used.

Competent *Escheria coli* DH5α stocks were purchased from Invitrogen (Catalog #18258-012) and stored at -80°C. Stocks were thawed, divided into 15µL aliquots, and refrozen on dry ice and stored at -80°C.

Transformation

E. coli DH5α aliquots were removed from -80°C freezer and thawed on ice for 10 minutes, then plasmid samples were added to give 25-50 ng/μL plasmid in each solution. Samples were then mixed by tapping and incubated on ice for 20 minutes. Next, samples were heat shocked for 1 minute in a 42°C water bath and rescued by diluting 1:30 in sterile Super Optimal Broth (S.O.C) (VWR Direct, Catalog # 101446-764) and incubating 2 hours at 37°C with shaking.

Bacillus subtilis 168 aliquots were removed from -80°C storage and thawed on ice for 10 minutes, and then plasmid samples were added to give 25-50 ng/μL plasmid concentrations in each solution. Samples were mixed by tapping and electroporated at settings known to be effective with *Bacillus subtilis* from previous works (25) (25 mF, 200W and 2.5 kV), then rescued in 1:10 dilutions of S.O.C broth incubated 4 hours at 37°C with shaking.

Rescued stocks were removed from incubation and 50-200 μL of each were used to inoculate appropriate antibiotic plates. For lower-yield plasmids (modified pUC19 vectors or ligation samples in particular) stocks were then centrifuged at 13,200 rpm for 1 minute to pellet the cells, then 50-80% of remaining broth was removed and the pellets were resuspended via pipetting. The resulting concentrated suspensions (100-200 μL) were then plated onto appropriate antibiotic plates.

Antibiotic Selection

Selection was performed on 1% LB-agar plates containing 50-100 μg/mL kanamycin (KAN), ampicillin (AMP), or both. Plates were produced in batches using roughly 25 mL of the broth-agar mixture per plate, and prior to use this broth-agar solution was autoclaved at 125°C for 15 minutes (not including heating/cooling time). The solution was allowed to cool to ~65°C based on touch, at which point appropriate volumes of antibiotics were added and mixed by gentle swirling. Plates were then poured by hand and allowed to cool and solidify on the bench top covered at room temperature (~15 min) before being stored at 4°C. Plates were used within 1 week of pouring at all times to avoid antibiotic degradation.

Cell cultures to be plated were added to the top of solidified plates in volumes of between 100 and 250 μL and were spread using sterile technique. Plates were then incubated overnight at 37°C. Following incubation, colonies were picked from plates by scraping with sterile pipette tips and briefly immersing and pumping them in sterile LB broth laced with antibiotic concentrations equal to those of the corresponding plate. These broth solutions were incubated overnight at 37°C with shaking. Broth volumes of 50 mL were inoculated in order to maximize miniprep yields.

Blue/White Screening

Blue/white screening was used to select colonies for the pCR 2.1 TOPO plasmids containing the two bsNOS fragments. Plates for this selection were poured identically to 100 μg/mL ampicillin plates with the exception that following sterilization 0.05% 5-bromo-4-chloro-3-indolyl-**β**-D-galactopyranoside (x-gal) and 0.005% Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. Colonies growing with a blue coloration were expected to have taken in a plasmid without an insert and were not selected for further study. Selected colonies were then amplified in LB broth containing 100 μg/mL ampicillin and kanamycin and incubated at 37°C overnight.

Plasmid Isolation

Plasmids were isolated using E. Z. N. A. Plasmid Mini Kit I (D6942-02, 200 preps) according to the manufacturer's standard protocol, including two washes with DNA wash buffer. Stock culture volumes processed were increased to between 25 and 50 mL each (as opposed to the kit's recommended 1-5 mL) to increase yield at the expense of efficiency; doing so showed total plasmid yields to increase up to 20 fold for some plasmids. Additionally, once plasmid samples were loaded onto the microfuge columns they were allowed to sit 5 minutes prior to centrifugation.

Separation of Plasmid Fragments

Electrophoresis was performed on 100mL, 1% agarose gels containing 1.5 μM ethidium bromide. A stock solution of 50X TAE buffer (2 M Tris, 1 M acetic acid, 0.05 M EDTA, pH 8.0) was diluted to 1X. Agar-agar was added (1 g) to 100 mL of 1X TAE and then dissolved using a microwave with periodic stirring. The clear solution was then allowed to cool to 65°C, and then 6 μL of 10 mg/mL ethidium bromide was added and mixed by stirring. The solution was allowed to cool to 55°C and then poured into a horizontal gel caster (BioRad Wide-Mini Sub Cell, No. 18S/7864) to solidify at room temperature for one hour. The gel was removed from the caster, placed into a horizontal gel box, and immersed in 1X TAE buffer. Samples without included loading dyes (ladders and PCR mixtures) were diluted to 80% in 5X loading dye (0.015% bromophenol blue, 40% [w/v] glycerol in deionized water) and added to gel wells by micropipette. Gels were then run at 80 V for up to 1.5 hours and quickly placed in a UV light box (UVP BioDoc-It Imaging System) for imaging. Gels from which samples were to be extracted had desired bands removed using a clean razor blade and then stored in a clear 1.5 mL microfuge tube for extraction.

Restriction of Plasmid DNA

All digested samples were digested with one or more of the restriction enzymes *Xba*I, *Kpn*I, or *Pst*I. Mixtures consisted of 0.05 to 0.2 μg/μL of DNA or plasmid sample, depending on the purpose of the gel; higher DNA concentrations were used for digestions intended for excision and extraction of bands after electrophoresis, while mapping digestions and others in which no collection would be used were given lower concentrations of DNA. Additional components of digestion mixtures included 0.04 u/μL each of appropriate restriction enzymes, 0.1 mg/mL sterile bovine serum albumin (BSA), 1X multicore buffer solution, and deionized water. Digestion mixtures were incubated at 37°C for between 1 and 4 hours, and were immediately mixed with loading dye and resolved through electrophoresis. Gels were imaged using a UV light box, and those with bands intended for extraction were excised.

DNA and Plasmid Ligations

Ligations were performed using vector/insert ratios specific to each step, which were determined by trial and error. DNA fragment solutions were combined and mixed by gentle tapping, and were heated to 50°C on a heat block for 5 minutes and then removed from heat and incubated on ice for 20 minutes to allow digested ends to combine. Next, 0.3 u/μL DNA ligase, 1X ligase buffer, and deionized water were added to volume, and the solution was left at room temperature for 1 hour and then incubated for between 24 and 72 hours at 4°C. Prior to transformation, ligase was deactivated by heating ligation samples to 70°C for 5 minutes.

Purification of DNA Fragments from Agarose Gels

Excised gel bands were extracted using the QIAquick Gel Extraction Kit (Quiagen, Cat. # 28704) with a few modifications to the kits standard protocol. Gel fractions were massed and then dissolved in QG buffer at volumes of 2 μL/mg of gel (as opposed to the 3 μL/mg in the kit protocol), then incubated at 50°C for 10 minutes with periodic vortexing. Next, isopropanol was added at 1/3 the added volume of QG buffer and mixed by inversion. Samples were then transferred into microfuge columns and allowed to sit for five minutes before being centrifuged, washed, and eluted according to the official protocol.

Polymerase Chain Reaction

The sequence of the gene coding for *Bacillus subtilis*' NOS enzyme was obtained from the National Center for Biotechnology Information (NCBI) website (26) and was divided roughly in half: the first 500 bp were chosen as the 5' end of the gene, and the remaining 562 bp were chosen as the 3' end. Primers were chosen to compliment the first ~35 base pairs of each half in both directions (5' to 3' and 3' to 5') as well as desired restriction sites and are identified as 5F (5' Forward; sequence gggggggtaccatggaagaaaaagaaatactctgga), 5R (5' Reverse; sequence ggggtctagaaaatccgttcgctctccgcgccagc), 3F (3' Forward; sequence ggggtctagatgacctgctgccgctcatttttcgc), and 3R (3' Reverse; sequence ggggctgcagttactcataaggcttatcttgataa). Primer 5F complimented the

sequence for a *Kpn*I restriction site from the 5' end of the bsNOS gene, and the 3R primer complimented the sequence for a *Pst*I restriction site from the 3' end. Both the 5R and 3F primers complimented the sequence for an *Xba*I restriction site from the 3' and 5' ends of their respective halves. These four primer sequences were purchased from Eurofins MWG Operon and were mixed with deionized water to give a 100 μM solution of each, which were divided into 20 μL aliquots and stored at -20°C.

The PCR solution was mixed to contain 50% by volume of the GoTaq Green Mastermix polymerase solution (Promega Cat. # M7122), 42 ng of the isolated B.sub 168 DNA, and 10 μM concentrations of each desired primer. The mixture was then diluted to volume with deionized water and placed in a thermocycler. The thermocycler program was set for an initial temperature of 94°C for two minutes, followed by 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, before remaining at the annealing temperature of 72°C for 7 minutes and then being cooled to 4°C until removal. PCR samples were then stored at -20°C.

Quantification of DNA Samples

Measurements of isolated DNA or plasmid sample concentrations were performed using 1μL of the measured sample on an ND-1000 spectrophotometer (nanodrop). After initialization with water appropriate solvents were used to zero the device; for plasmid samples isolated using the E.Z.N.A miniprep kit, 1 μL of the kit's elution buffer was used. For plasmid or DNA samples extracted from an electrophoresis gel using the QIAQuick gel extraction kit, 1 μL of the kit's elution buffer was used. DNA concentrations were recorded as well the ratio of absorbance at 260nm and 280nm. Given the tendency of DNA to absorb at 260 and proteins to absorb at 280nm, a higher value for the ratio of absorbance at these two wavelengths (260/280) indicates a solution with fewer protein contaminants. Samples with 260/280 ratios of 2.0 to 1.6 were considered clean enough for use; others were discarded.

Bacterial gDNA Isolation by Isopropanol Precipitation

Stock cultures of *Bacillus subtilis* were grown to $OD_{(595nm)}$ = 0.600 and centrifuged at 13,200 rpm for 2 minutes at room temperature to pellet the cells, and the supernatant broth was decanted using a micropipette and the tube drained. Pellets were resuspended in sterile Tris-EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH brought to 8.0 using 1M HCl) with 0.6% by volume of sodium dodecyl sulfate (SDS) and 120 μg/mL proteinase K. The solution was mixed and allowed to incubate for 1 hour at 37°C.

Next, an equal volume of a 1:1 mixture of phenol and chloroform was added and inverted until phases were mixed, and the vial was centrifuged at 13,200 rpm for 2 minutes at room temperature and the top aqueous layer was transferred into a new container. An equal volume of 1:1 phenol-chloroform was added and again mixed via inversion until the phases had mixed, and the vial was again centrifuged. The aqueous phase was transferred into a clean tube and 0.6 volumes of isopropanol was added and mixed by inversion until a DNA precipitate formed. The vial was centrifuged at 13,200 rpm to pellet the DNA precipitate and the supernatant was decanted. The DNA pellet was then washed with 70% ethanol, centrifuged to re-pellet the precipitate, decanted, and resuspended in sterile TE buffer (pH 8.0). DNA stocks were then stored at -20°C.

Production of 5’ and 3’ bsNOS DNA Fragments

A starter culture of *Bacillus subtilis* was grown overnight and then the genome was isolated by isopropanol precipitation. Next, PCR was performed for both the 5’ and 3’ fragments of the bsNOS gene. For the 5’ sample, 5F and 5R primers were added. For the 3’ sample, 3F and 3R primers were added. The PCR mixture was then cleaned using electrophoresis on a 1% agarose gel with 100 bp and 1 kb ladders added as controls and imaged. Bands identified at 500 bp (5’ fragment) and 600 bp (3’ fragment) were excised and extracted. A diagram depicting the PCR primer setup and amplification products is shown in Figure 2.1.

Amplification and digestion of bsNOS DNA fragments was achieved using a pCR 2.1 TOPO plasmid kit, with 1:8 volumes each of plasmid, DNA, and salt solutions added and diluted with deionized water. These samples were incubated 30 minutes at room temperature and then transformed into DH5α

stocks and selected using 100μg/mL ampicillin and kanamycin blue/white plates. White colonies were amplified in 50 mL LB with 100 ng/μL ampicillin and kanamycin and incubated overnight at 37°C with vigorous shaking. Plasmids were isolated by miniprep and resulting plasmid concentrations were identified using nanodrop. Stocks of pCR TOPO plasmids with the 5' or 3' insert were kept for future transformations.

DNA inserts within 5' TOPO plasmids were released by digestion using *Xba*I and *Kpn*I digestive enzymes, while 3' TOPO plasmids were digested with *Xba*I and *Pst*I. The resulting digests were cleaned using electrophoresis and the resulting 500 and 600 bp bands (5' and 3' samples, respectively) were excised, extracted, and stored at -20°C.

Production of p34S KAN Insert

The p34S plasmid coding for kanamycin resistance was received from Dr. Andre Hudson's inventory stock. Cell culture shavings from this strain were introduced to a 50 mL volume of sterile LB broth using a pipette tip and incubated with shaking at 37°C overnight. Next, sterile streaking technique was used to plate the culture on an LB-agar plate with 50 μg/mL kanamycin sulfate, and this plate was incubated at 37°C overnight. After incubation, colonies were taken from the kanamycin plate using sterile technique and introduced to 15 mL solutions of sterile LB broth laced with 50 μg/mL kanamycin sulfate and incubated at 37°C with shaking overnight. The following day 14 mL of this culture was transferred to a 15 mL conical tube and centrifuged to pellet the cells. The broth was decanted and disposed of, plasmids were extracted from cell pellets using miniprep, and the resulting plasmid solution was stored at -20°C.

The p34S plasmid was amplified by transformation into DH5α, followed by selection on a 100ng/μL kanamycin plate and amplification in 50 mL of 100ng/μL kanamycin LB broth. Plasmids were isolated using miniprep and stored at -20°C.

To extract the KAN resistance gene insert, the p34S plasmid was digested with *Xba*I for 2 hours and then run on a 1% agarose electrophoresis gel using 2 μg undigested p34S plasmid, along with 100 bp,

and 1 kbp VWR DNA ladders as controls. The gel was then imaged and the band corresponding to ~1 kbp fragment size was excised, extracted, and stored at -20°C.

Production of pUC19 Vector Fragment

A solution of pUC19 plasmid received with DH5α stock cultures (Invitrogen, ~ 10 pg/μL) was used to transform a DH5α aliquot, and was selected on 100 ng/μL ampicillin plates. Amplification was done in 50 mL of LB with 100 ng/μL ampicillin. Plasmids were isolated using miniprep and stored at -20°C. The pUC19 stock was then digested with *Kpn*I + *Pst*I for 2 hours at 37°C in a water bath and then run on a 1% agarose electrophoresis gel using 2 μg undigested pUC19 plasmid, 100 bp, and 1 kbp ladders as standards. The gel was then imaged and the ~2.7 kbp band was excised, extracted, and stored at -20°C.

Production of pNOS20 Phase I Plasmid

The phase I plasmid, pNOS20, was created by ligating together the pUC19 vector fragment and the 5' and 3' bsNOS fragments in a 1:10:10 ratio, respectively (Dr. Andre Hudson). The ligation procedure was followed and the mixture incubated for 72 hours, deactivated, and then transformed into a DH5α aliquot and selected using 100 ng/μL AMP plates. Resulting colonies were amplified using 50 mL of LB broth with 100 ng/μL AMP and incubated overnight. Samples were then miniprepped, concentrations were measured by A_{260}/A_{280} using a Nanodrop spectrophotometer, and the plasmid was map digested with *Kpn*I + *Xba*I and *Pst*I + *Xba*I to observe the presence of 5', 3', and pUC19 vector fragments. Plasmid stock was stored at -20°C.

Production of pUC19ΔbsNOS Phase II Plasmid

The phase II plasmid, pUC19ΔbsNOS, was created by first linearizing pNOS20 with *Xba*I and then ligating it together with the digested p34S KAN insert at a 1:10 ratio, respectively. The ligation procedure was followed and incubated at 4°C for 24 hours, deactivated, and then transformed into a DH5α aliquot and selected for using 100 ng/μL AMP + KAN plates. Resulting colonies were amplified

using 50 mL LB broth with 100 ng/μL AMP + KAN incubated overnight. Plasmids were extracted using a miniprep kit and concentrations were measured by UV absorbance on a Nanodrop spectrophotometer, and the plasmid was map digested with *Pst*I + *Kpn*I to observe the presence of 5', 3', KAN insert, and pUC19 vector fragments. Plasmid stock was stored at -20°C.

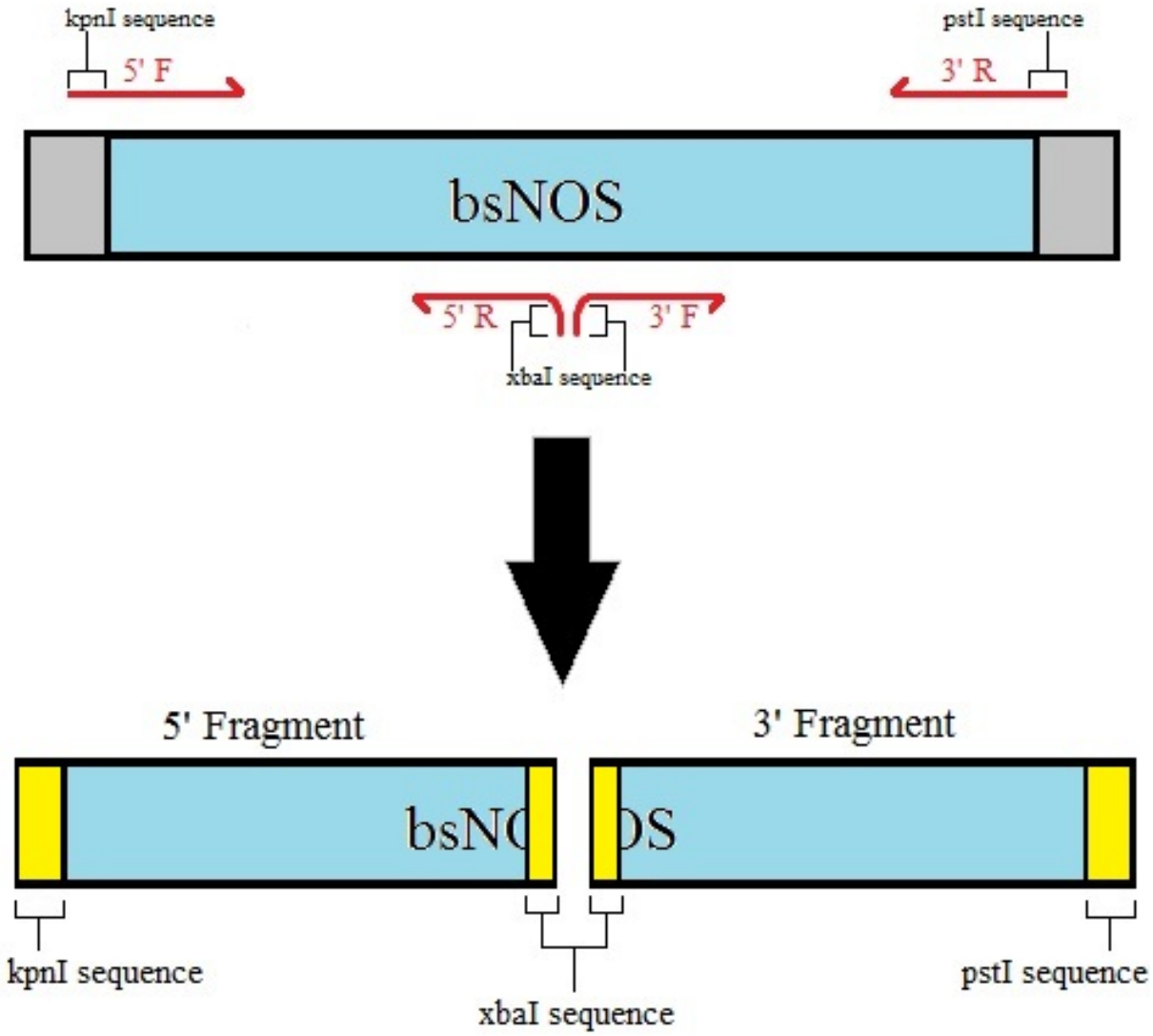

Figure 2.1. Schematic of PCR Amplification of bsNOS Gene from genomic DNA to give 5' and 3' half-fragments of the bsNOS gene with respective restriction sites. Primers are shown as red arrows, bsNOS-coding DNA is shown in blue, restriction-site DNA is yellow, and non-bsNOS coding DNA is shown in gray.

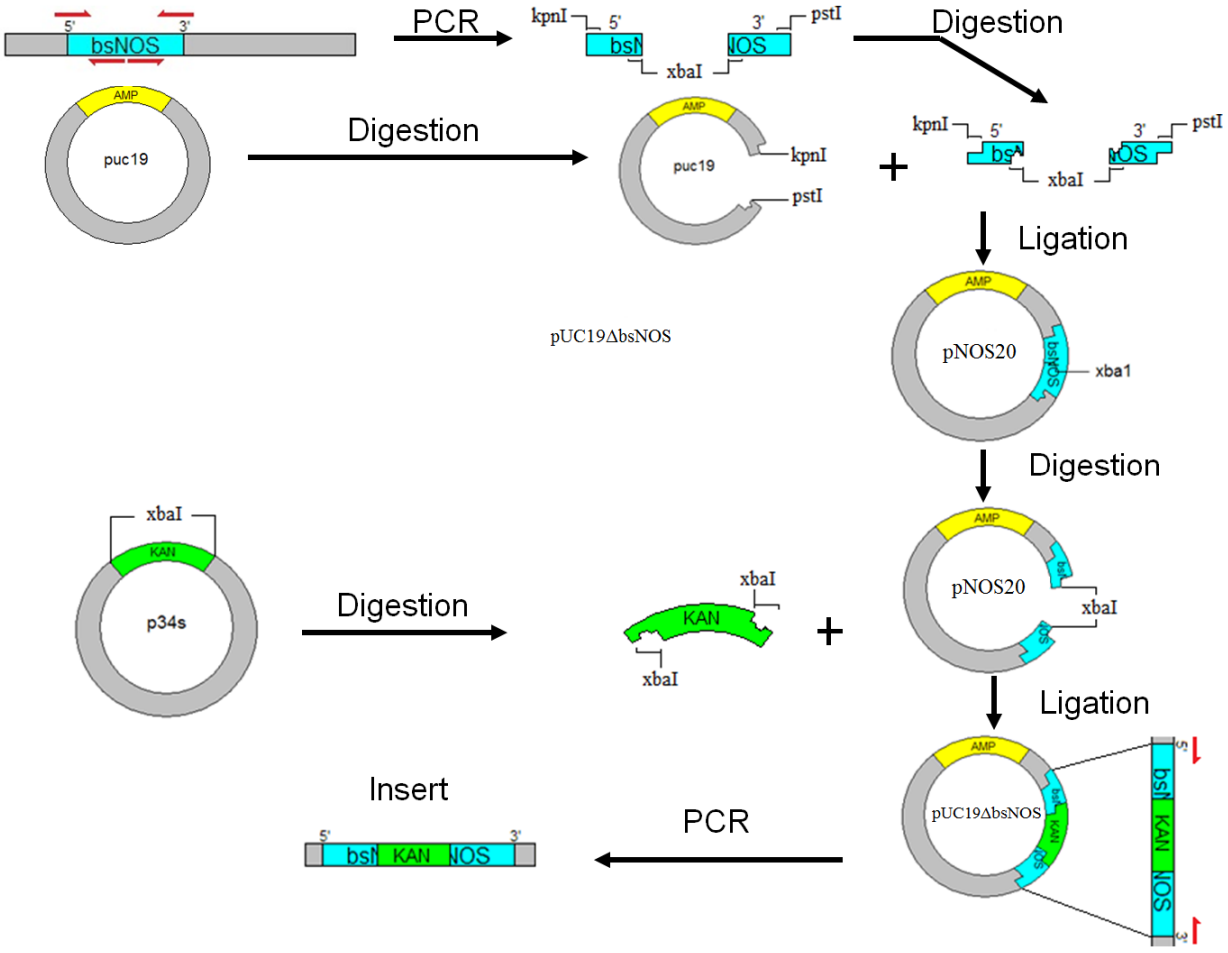

Figure 2.2. Schematic of pUC19ΔbsNOS Production from starting components to insert sequence.

Chapter 3: Results

Following the isolation of *Bacillus subtilis 168's* genomic DNA, ~15 ng of DNA was loaded into PCR mixtures for 5' (500 bp) and 3' (600 bp) fragments. The gene fragments were amplified with custom primers and separated using gel electrophoresis, and the resulting gel is shown in Figure 3.1. All six bands were excised and extracted separately to give different undigested 5' and 3' DNA samples. The sizes of these bands with respect to each other and to the DNA ladders suggest that the amplification was successful, though the different relative intensities of these bands may indicate slight differences in the reaction's efficiency or more likely may simply be the result of shorter chain lengths containing fewer numbers of ethidium bromide-binding nucleotides.

After extracting these bands, both 5' and 3' fragment stocks were ligated into pCR TOPO 2.1 and the resulting plasmid was used to transform cultures of *Escheria coli* strain DH5α, selected for using ampicillin and kanamycin at 50 μg/mL. After amplifying and miniprepping the colonies, 12 μg of the 5' and 3' TOPO plasmids were digested with *Kpn*I and *Pst*I, respectively, along with *Xba*I in 50 μL mixtures. The digestion mix was then run through an electrophoresis gel, pictured in Figure 3.2, and the bands corresponding to the 5' and 3' inserts (500 and 600 bp, respectively) were excised. Fragments cut from pCR TOPO plasmids are considered fully digested as removal from the plasmid necessitates digestion, and so the 5' and 3' bsNOS fragments were now extracted and stored prior to ligation.

The vector plasmid pUC19 was now amplified through DH5α and selected for using 50 μg/mL ampicillin plates. After growth and isolation, 200 ng of each were added to a 20 μL digestion mixture containing restriction enzymes *Kpn*I and *Pst*I. The mixtures were then incubated and run through an electrophoresis gel, pictured in Figure 3.3. Some additional fragments were seen in undigested plasmid samples, though these were likely a consequence of supercoiling or linearization due to random degradation in the undigested plasmids. Excised bands from pUC19 and p34S plasmids were extracted to give solutions of digested pUC19 and KAN fragments, respectively.

A ligation mixture was produced containing a 1:10 ratio of digested pUC19 vector to digested 5' and 3' inserts and incubated for 48 hours at 4°C. Following ligation the mixture was deactivated by

incubation at 70°C for five minutes and then was used to transform samples of DH5α. These samples were plated on agar with 50 μg/mL ampicillin and then nine colonies were selected for amplification, after which plasmid content was isolated via miniprep as pNOS20 candidate samples (A through I).

To test for the desired inserts a mapping digest was performed in which roughly 0.1 μg of each plasmid sample was added to a 10 μL digestion mixture containing the restriction enzymes *Xba*I and *Kpn*I in order to cut out the 5' fragment from each plasmid. After incubation these digestion mixtures were loaded into a 30-lane agarose gel to check for the desired band patterns. The desired plasmid should give a 500 bp fragment under these digestion conditions, and all but one sample (sample D) showed bands of this size, as seen in Figure 3.4. All samples with the exception of D were then tested for a 3' fragment insert in the same fashion; roughly 0.1 μg of each was digested in a 10 μL digestion mixture containing *Xba*I and *Pst*I restriction enzymes. The correct plasmid ligation should give a 600 bp fragment under these conditions. An agarose gel was poured and the samples were run through it to check for the desired fragment; the resulting gel is shown in Figure 3.5. All samples were shown to have the 3' insert as well, and were thus confirmed to be the desired pNOS20 plasmid product.

Three of the confirmed pNOS20 samples (B, C, and F) were selected to be linearized with *Xba*I. Three 20 μL digestion mixtures were prepared containing 4 μg of each respective plasmid sample and the *Xba*I restriction enzyme. An additional digest was performed using *Xba*I on a sample containing p34S plasmid in order to provide an additional control sample as well as a fresh sample of the digested KAN fragment for extraction. After incubation the samples were run through an agarose gel with 1kbp and 100bp VWR ladders as controls. This gel is shown in Figure 3.6. The digested bands from each sample as well as the 1.1 kbp p34S digest band were excised and extracted to give solutions of linearized pNOS20 as well as the digested KAN gene, respectively.

Following excision a ligation mixture was produced containing a ratio of 1:10 pNOS20 to KAN insert, with a total of approximately 100 ng of DNA in 25 μL ligation mixtures. The ligation mixture was incubated 24 hours and then deactivated before being used to transform samples of DH5α. After rescue, transformed samples were grown on plates laced with 50 μg/mL kanamycin and ampicillin and four

colonies were selected for amplification (A, B, C, and D). After amplification, plasmids were extracted via miniprep to yield solutions of pUC19ΔbsNOS candidates. These plasmid solutions were then digestion mapped to test for the presence of 5', 3', and KAN inserts. To do this, 20μL digestion mixtures were made containing ~0.2 μg of plasmid along with *Pst*I and *Kpn*I restriction enzymes. Given the presence of all three restriction sites at the ends of the KAN insert, this mixture would be expected to liberate all four fragments from the plasmid, yielding a fragmentation pattern with bands at 500 bp, 600 bp, 1.1 kbp, and ~2.7 kbp. Following incubation the digestion mixtures were run through a 15-well agarose gel alongside undigested samples and a 1 kbp and ladder as controls. This gel is shown in Figure 3.7. Based on the resulting digestion patterns, all five selected colonies yielded pUC19ΔbsNOS plasmids.

With digestion mapping results which suggested that the goal of generating pUC19ΔbsNOS had been accomplished, the final confirmation needed was to sequence the generated plasmid's insert through the University of Rochester's sequencing lab (27). While the total pUC19ΔbsNOS plasmid was available, the pNOS20 plasmid was submitted for sequencing as the KAN gene sequence is already known and would have doubled the required sequence to be amplified. As such, samples from pNOS20 candidate F were produced and submitted to the University of Rochester to be sequenced. When run through the National Center for Biotechnology Information's online BLAST, the sequence returned appears identical to that of bsNOS: among the four samples submitted, total sequence similarity and maximum alignment scores average out to be 97.8% and 1810, respectively. The minimum sequence similarity seen from any of the samples was 97%, and the lowest maximum alignment score was 1753. Overall, it appears definitive that the pNOS20 sample selected was correctly identified, and from this it can be extrapolated that its ligation with digested KAN should have formed the pUC19ΔbsNOS plasmid, again confirming mapping digest data (11). An example of the sequence alignment data given by BLAST search is shown in Figure 3.8.

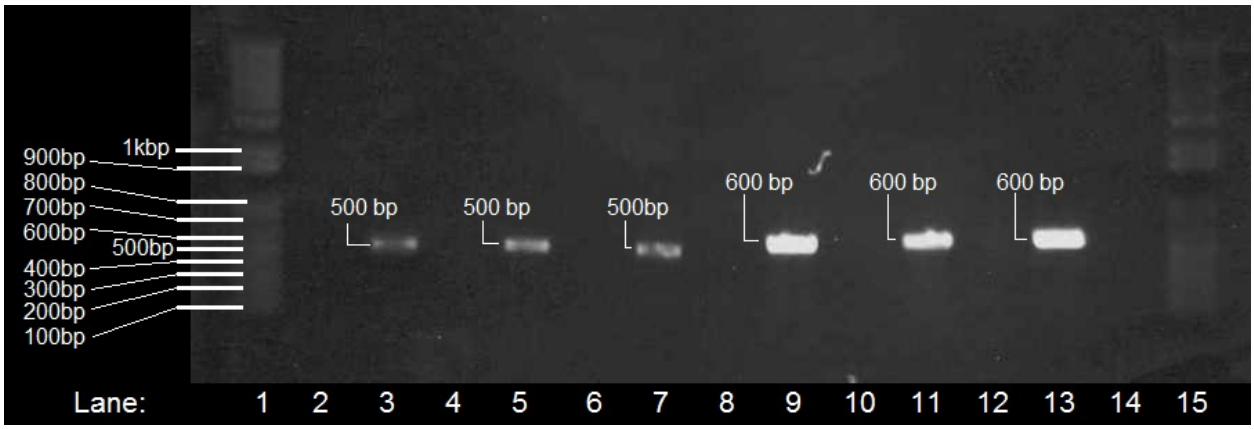

Figure 3.1. PCR-Amplified bsNOS Fragment Gel, showing 5' and 3' fragments resolved on an electrophoresis gel. The 5' fragments were all excised to give 5' fragment samples, and the 3' fragment bands were all excised to give 3' fragment samples. Lanes 1 and 15 contain a mixture of VWR 1kb and 100 bp ladders; lanes 3, 5, and 7contain 5' PCR mixtures, and lanes 9, 11, and 13 contain 3' PCR mixtures.

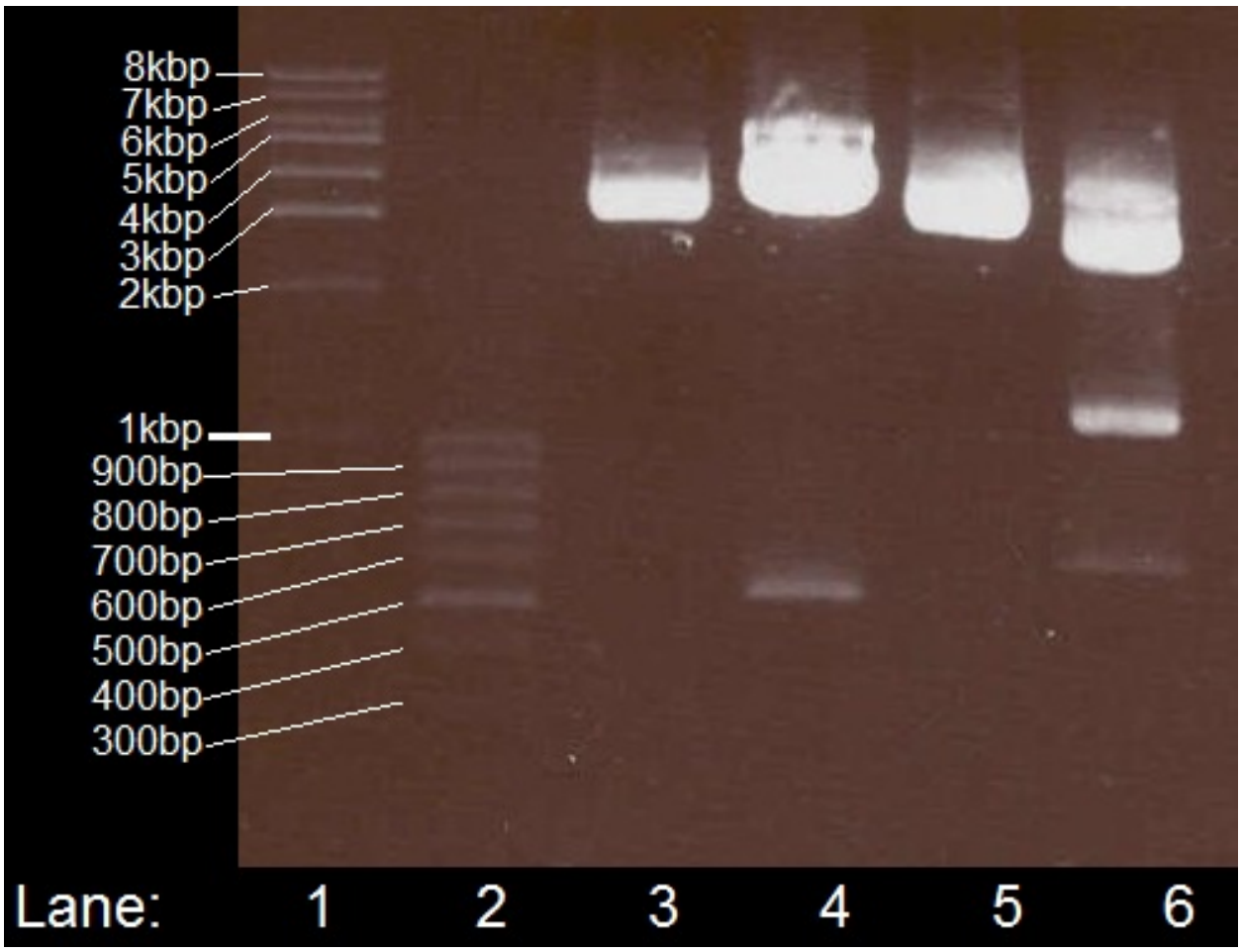

Figure 3.2. 5' and 3' TOPO Plasmid Digests, before and after their digestion. The 500 bp fragment from the 5' TOPO digestion was excised for extraction to give the digested 5' fragment solution. The 600 bp fragment from the 3' TOPO digestion was excised for extraction to give the digested 3' fragment solution. Lanes 1 and 2 contain VWR 1kbp and 100 bp ladders, respectively. Lane 3 contains undigested 5' pCR TOPO plasmid, and lane 4 contains digested 5' pCR TOPO plasmid. Lane 5 contains undigested 3' pCR TOPO plasmid, and lane 6 contains digested 3' pCR TOPO plasmid.

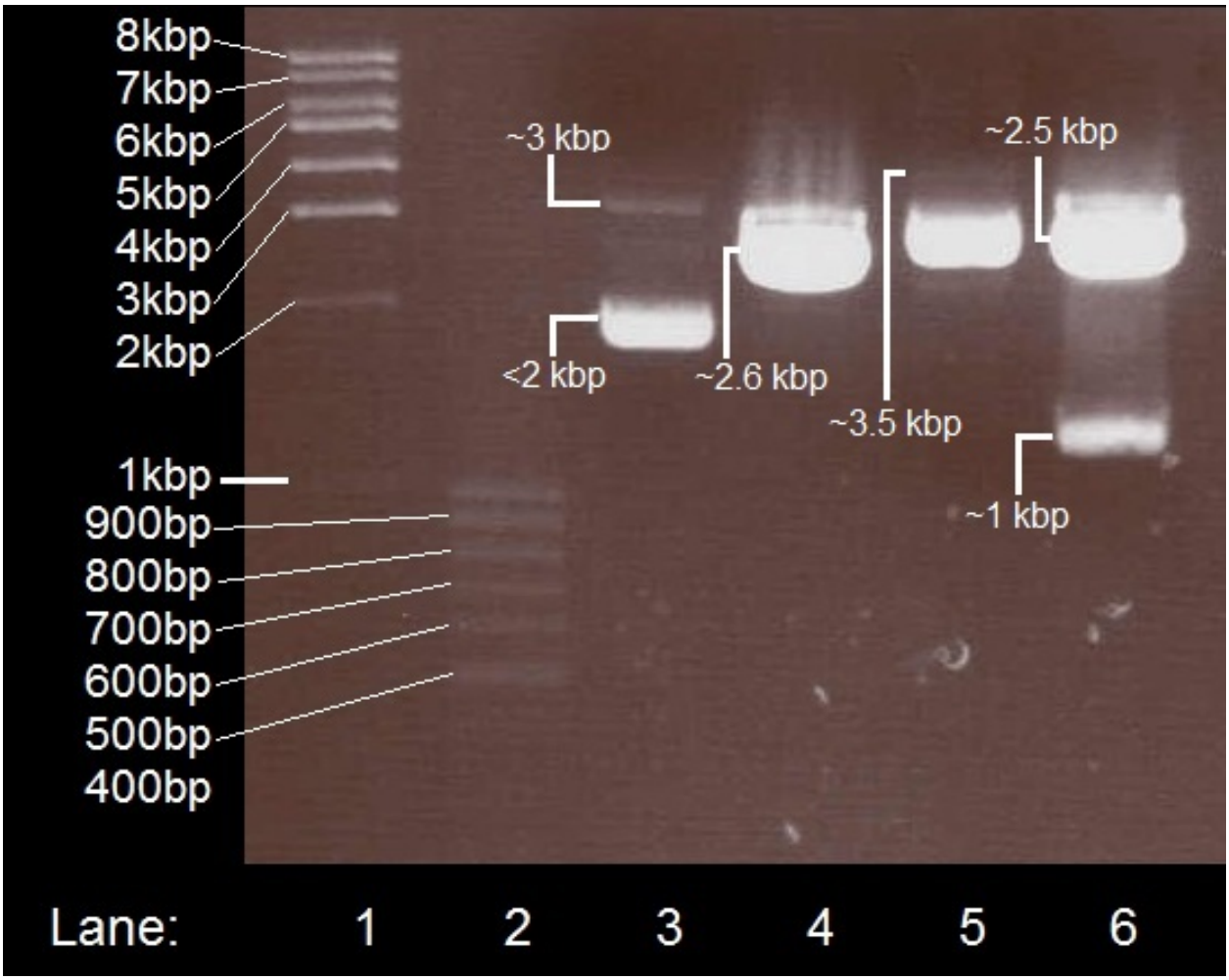

Figure 3.3. Digestion of pUC19 and p34S plasmids. For pUC19 the second fragment removed through digestion is too small (<100 bp) to be identified on a 1% gel with given concentrations of ethidium bromide; thus the 2.6 kbp band was the only one observed after digestion and was subsequently excised for extraction. The p34S digestion yielded two fragments: one at ~1 kbp and another at ~2.5 kbp. The 1 kbp fragment correlated with the desired KAN resistance gene, and so was excised for extraction. Undigested plasmid sizes (lanes 3 and 5) were measured from the faint lines seen above the larger spots as the larger spots likely represent plasmid sizes after supercoiling and therefore do not accurately represent the undigested plasmid's size. Lanes 1 and 2 contain VWR 1kbp and 100 bp ladders, respectively. Lanes 3 and 5 contain undigested pUC19 and p34S plasmids, respectively, while lanes 4 and 6 contain digested pUC19 and p34S, respectively.

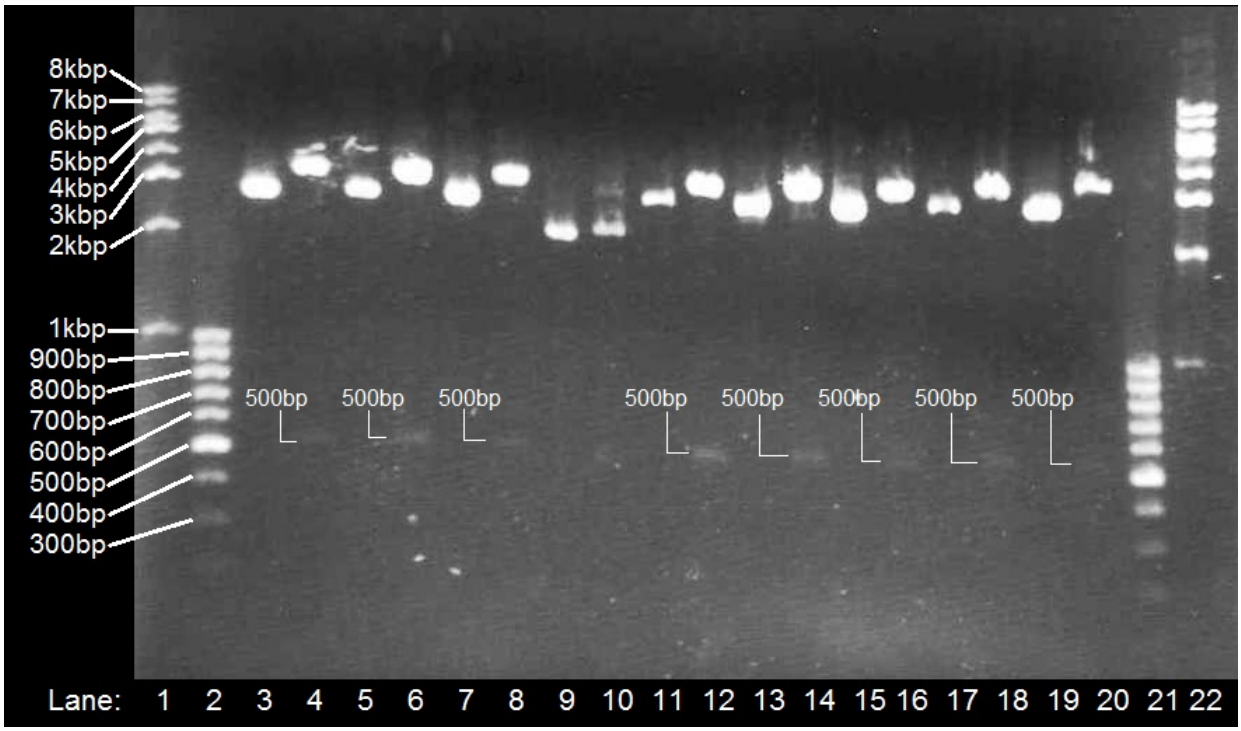

Figure 3.4. 5' Digestion Mapping Gel of pNOS20 ligation samples to test samples A through I for the presence of the 5' bsNOS fragment insert. Samples both digested and undigested were run, with 1 kbp and 100 bp ladders for control. All but sample D exhibited the desired 500 bp fragment. Lanes 1 and 22 contain a VWR 1 kbp ladder, and lanes 2 and 21 contain a VWR 100 bp ladder. Odd numbered lanes from 3 to 19 contain undigested samples A through I (left to right), and even numbered lanes from 4 to 20 contain digested samples A through I (left to right).

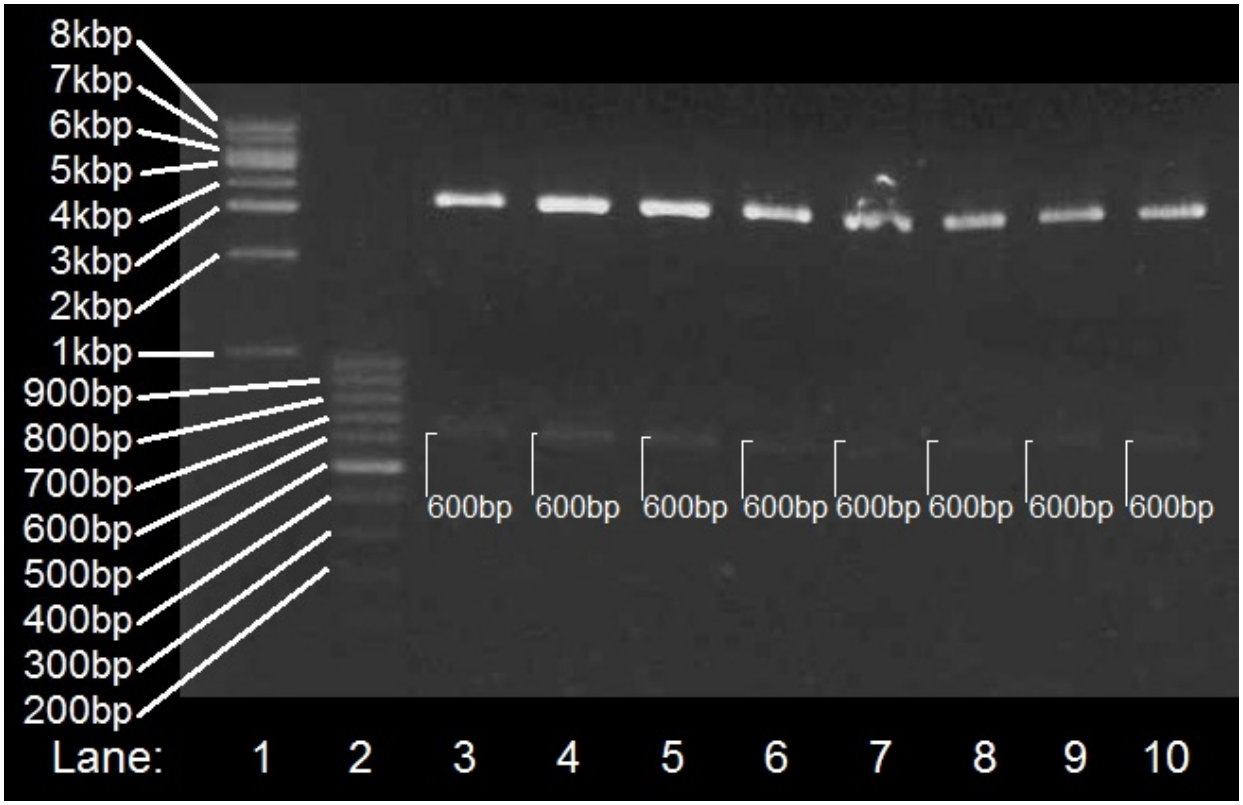

Figure 3.5. 3' Digestion Mapping Gel of pNOS20. Testing of samples A through I (sans D) for the presence of the 3' bsNOS fragment insert. All samples exhibited the desired 600 bp fragment. Lanes 1 and 2 contain VWR 1 kbp and 100 bp ladders for control. Lanes 3 through 10 contain digested samples A through I (left to right, sample D not processed).

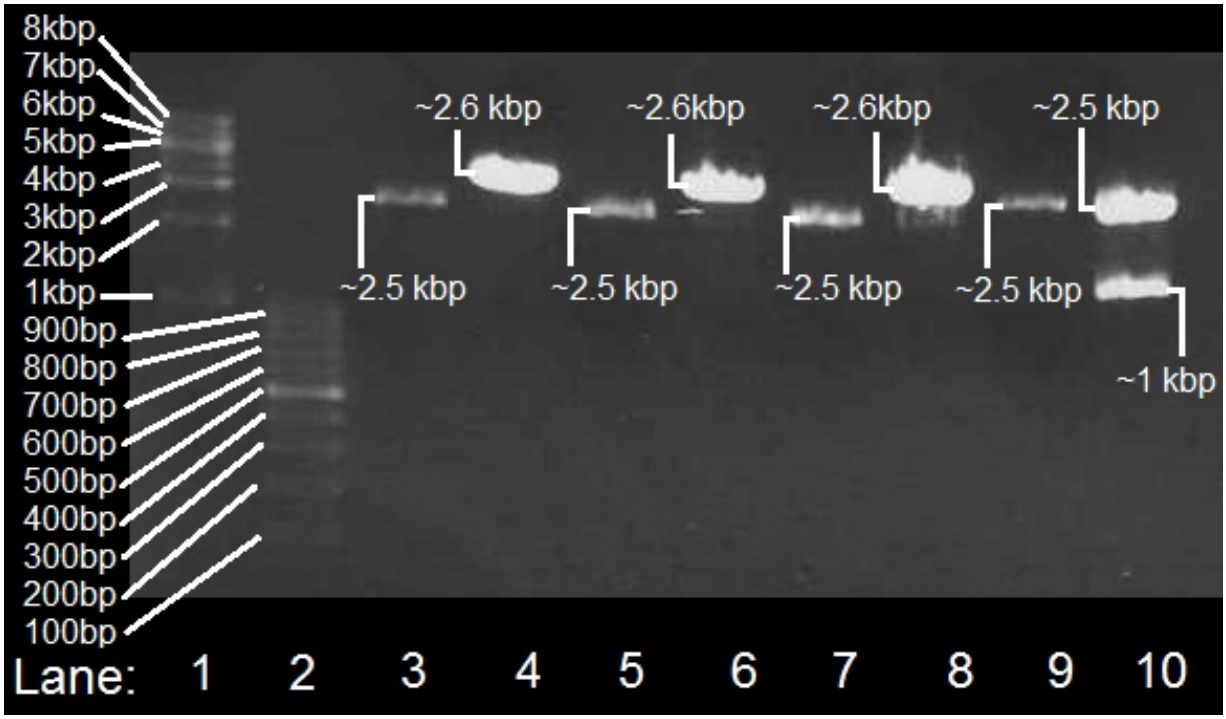

Figure 3.6. pNOS20 Linearization Gel. Ligation samples B, C, and F linearized with *Xba*I. Undigested plasmids traveled slightly further due to supercoiling and so comparisons between digested and undigested plasmid confirm that digestion did occur in each case. Bands of linearized samples were excised for extraction. Plasmid p34S was also digested with *Xba*I and run alongside an undigested control; the ~1 kbp fragment band of this digest was extracted as a source of fresh digested KAN gene sequence. Lanes 1 and 2 contain VWR 1 kbp and 100 bp ladders, respectively. Lanes 3, 5, and 7 contain undigested plasmid samples B, C, and F, respectively, and lanes 4, 6, and 8 contain their digested counterparts, respectively. Lane 9 contains undigested p34S (after supercoiling), and lane 10 contains digested p34S.

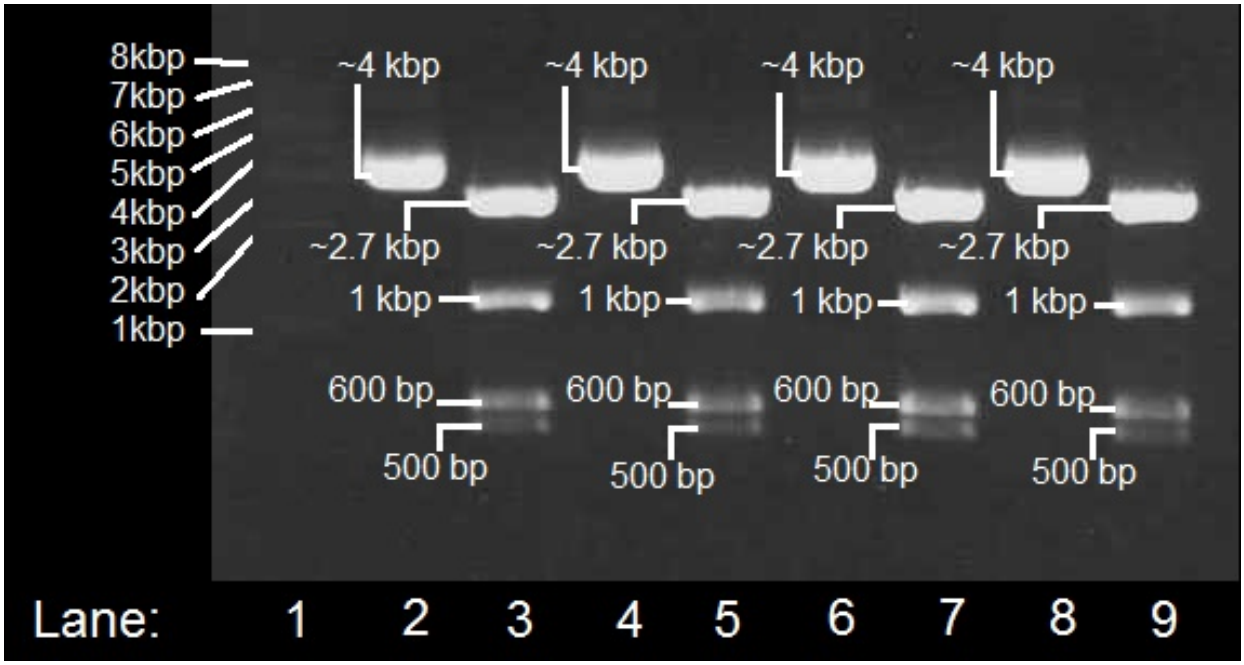

Figure 3.7. Digestion Mapping Gel of pUC19ΔbsNOS Candidates. Samples A-D were digested with *Pst*I and *Kpn*I, and were run alongside undigested samples. All four samples appear to include all four component fragments, suggesting that the pUC19ΔbsNOS plasmid has been successfully constructed in each case. Lane 1 contains a VWR 1 kbp ladder. Lanes 2, 4, 6, and 8 contain undigested samples A through D (after supercoiling), and lanes 3, 5, 7, and 9 contain samples A through D after digestion.

Figure 3.8. Sequence Alignment of the pNOS20 Insert as compared by NCBI BLAST search to the wild-type bsNOS gene. The upper line of each row represents the plasmid sequence submitted, and the lower sequence is taken from the bsNOS genome. Vertical lines represent identical bases, while missing lines represent mismatches. (11).

Chapter 4: Discussion

Based on the sequence data returned by the University of Rochester and the mapping digests performed, it can be concluded that the pUC19ΔbsNOS plasmid has been successfully constructed and should be easily amplified and/or used for transformation of *Bacillus subtilis*. The use of pUC19 as a vector makes amplification easy through transformation and selection of DH5α, though given that PCR is to be used to generate the desired fragments from the pUC19ΔbsNOS only very small concentrations of this plasmid should be needed.

The next step in this method will be the transformation of wild-type *Bacillus subtilis* 168 with the insert. Ideally, treatments should be possible simply by electroporating the entire pUC19ΔbsNOS plasmid into competent *B.sub* stocks, but to date this method has not worked. It is possible that even after unraveling the extra 2.7 kbp of non-insert plasmid regions are making encounters between the insert strand and its compliment difficult; in any case it was decided that the insert needed to be removed from the plasmid before the transformation could work efficiently.

Removal of the insert sequence (roughly 2.1 kbp in length) was originally going to be accomplished using the *Kpn*I and *Pst*I restriction sites, but the presence of both sites on either end of the KAN gene of the p34S plasmid make it impossible to sever the insert as a whole using only the restriction enzymes in our stock. It could be possible to remove this strand using different restriction enzymes, but prior to this it was decided that using PCR to amplify our desired strand would be most efficient. First, it should allow the sequence to be generated in its entirety without any additional non-coding regions of the plasmid being brought along (as would be necessary if new restriction sites down-stream of the insert were to be targeted). Second, the use of PCR means that very low concentrations of pUC19ΔbsNOS plasmid would be needed for a transformation procedure, and that if needed a much higher number of insert copies could be generated than may be possible using digested and cleaned plasmid minipreps.

PCR amplification of the insert from the pUC19ΔbsNOS plasmid has already been performed and indeed has appeared successful. *Bacillus subtilis* colonies were seen on 40 μg/mL kanamycin plates, and to date over a dozen colonies have been selected, grown, and their DNA isolated by isopropyl

precipitation. Once this DNA is purified it should be possible to use PCR to amplify the insert from the genomic DNA; a successful double-recombination should give only one band at approximately 2.1 kbp, while a single recombination should show two bands: one at 2.1 kbp and another at 1 kbp. Unfortunately, complications have arisen in the PCR process. For reasons yet unknown, amplification of the bsNOS site has proven difficult.

While our described methods were (and still are) fully capable of amplifying the 5' and 3' halves of the gene independently, the 1 kbp wild type sequence has been problematic, giving very low yields based on band luminosity and suggesting that some variable in amplification efficiency has yet to be identified. As of this writing all colonies isolated have been tested, but only one or two (wild-type or treated) have shown definitive 1 kbp bands (indicating at best a single recombination product),
and none have shown the 2.1 kbp band associated with a complete insert. Given that all colonies have been selected from kanamycin plates and that a full plasmid was not inserted it can be assumed that the KAN gene has inserted into the genome at some locus, and it is statistically unlikely that this occurred at a random region of the genome (although this possibility is not being ignored). Further, all colonies isolated give strong 500 or 600 bp bands when the 5' or 3' halves are amplified, indicating that all primers are finding targets and that the colonies isolated are all in fact *Bacillus subtilis.* If a complication in the PCR procedure is preventing the 1 kbp strand from being amplified, it is very likely that the same complication is making the 2.1 kbp insert even more difficult. Consequently, the lack of a 2.1 kbp band cannot yet be attributed to a lack of an insert, and so these issues with PCR products must be overcome before definitive testing of the potential knockout mutants can begin. Variables to be changed so far include timing programs for the thermocycler used in the PCR process as well as changes in DNA or primer concentrations.

While definitive PCR data is still lacking, the colonies themselves do appear promising. Wild-type *Bacillus subtilis* does not grow on kanamycin plates, and yet the treated colonies are showing up as small, interspersed "pin-prick" colonies, a pattern typical of such transformation procedures. Additionally, wild-type *Bacillus subtilis*, even when selected from culture plates, grows rapidly and with high viability;

colonies selected can normally be incubated in LB broth overnight and consistently give turbid cultures. Colonies selected from the kanamycin plates, however, grow very slowly, and with a roughly 70% success rate. Incubation of selected colonies in even 10 ng/μL kanamycin (25% of the concentration used in the selection plate) took between 48 and 72 hours to reach even low turbidity, and 30% of colonies selected apparently fail to take hold. This low viability is likely due in part to the presence of kanamycin, but it is also consistent with the findings of other works in which bsNOS knockouts showed lower cell viability in the presence of antibiotics (17).

Chapter 5: Conclusions

Current work has strongly suggested that nitric oxide is manufactured in *Bacillus subtilis* at levels far below those necessary for recently observed oxidative stress mechanisms even at times of non-stressed, logarithmic growth. This may indicate a native pathway in which NO is a key signal, and to date work is underway to identify just what this signal promotes. A simple means of identification is to inhibit production of this molecule and observe consequent changes proteomically, and while previous work allowed native bsNOS to be inhibited chemically it is also necessary to account for the presence of the added inhibitory chemical species. This will be accomplished using a genetic knockout of bsNOS. An antibiotic resistance gene inserted into the center of the bsNOS gene should inhibit the bacterium from producing native NO, and thus any signals carried by this molecule will be silenced. With the pUC19ΔbsNOS plasmid complete, the only steps that remain in generating this knockout species include selecting a successful knockout colony and confirming its status as a complete knockout.

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