Elucidating the functional role of nitric-oxide in Bacillus subtilis by proteomic analysis

Erin R. Treece
Elucidating the functional role of nitric-oxide in *Bacillus subtilis* by proteomic analysis

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May 2008

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry

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ABSTRACT

A Nitric-oxide synthase-like (NOS) protein has been found to be present in several Gram-positive bacteria, including *Bacillus subtilis*. NOS generates nitric-oxide from the amino acid L-arginine via the stable intermediate N-hydroxy-L-arginine. The function of NO production in Gram-positive bacteria has not yet been elucidated, but studies indicate a function in signal transduction. In this study, a proteomic approach is used to examine the physiological role of NO in the Gram-positive model bacteria, *B. subtilis*. Protein profiles obtained from two-dimensional gel electrophoresis of cells grown in the presence of aminoguanidine, a known NOS inhibitor, revealed the down-regulation of three proteins via NO inhibition over a pI range of 4 – 7 when compared to an uninhibited sample. These three proteins were identified by LC-MS/MS as a nucleoside diphosphate kinase, an MreB-like protein, and a phage shock A protein homolog. The down-regulation of these proteins via NOS inhibition provides preliminary evidence that NO plays a signal transduction role in Gram-positive bacteria.
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LIST OF ABBREVIATIONS

A
AG, aminoguanidine

B
B. anthracis, Bacillus anthracis
BH₄, 6R-tetrahydrobiopterin
BSA, bovine albumin serum
bsNOS, Bacillus subtilis nitric oxide synthase
B. subtilis, Bacillus subtilis

C
CBB, Coomassie Brillant Blue
cGMP, cyclic guanidine monophosphate
CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID, collision induced dissociation
cm, centimeter
cNOS, constitutive nitric oxide synthase

D
Da, Daltons
DTT, D,L-dithiohreitol

E
EDTA, ethylenediaminetetraacetic acid
G
GPC, green-protein complex

H
HCl, hydrochloric acid
HPLC, high performance liquid chromatography
hr, hour

I
IEF, isoelectric focusing
iNOS, inducible nitric oxide synthase
IPG, immobilized pH gradient

J

K
K\textsubscript{D}, dissociation constant, describes how tightly a ligand binds to a protein
kDa, kilodalton
K\textsubscript{M}, Michaelis constant, substrate concentration at which an enzyme shows one-half its maximum velocity

L
LB, Luria-Bertani Broth
LC, liquid chromatography
LC-MS/MS, liquid chromatography-tandem mass spectrometry
LiaH, phage shock protein A homolog

M
M, molar
mA, milliampere
Mbl, MreB-like protein
mg, milligram(s)
mL, milliliter
min, minute(s)
mM, millimolar
mNOS, mammalian nitric oxide synthase
Mn-SOD, manganese-containing superoxide dismutase
Mowse, molecular weight search
MS, mass spectrometry
MS/MS, tandem mass spectrometry
MSB, modified sample buffer
m/z, mass-to-charge

N
NADPH, nicotinamide adenine dinucleotide phosphate
NAG, N-acetylglucosamine
NAM, N-acetylmuramic acid
NDK, nucleoside diphosphate kinase
nm, nanometer(s)
NO, nitric oxide
NOS, nitric oxide synthase

O
OD$_{600}$, optical density measured at 600nm
PBPs, penicillin-binding proteins
pl, isoelectric point
PIC, protease inhibitor cocktail
PspA, phage shock protein A
S
S. aureus, Staphylococcus aureus
SDS, sodium dodecyl sulfate
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec, second(s)
SNP, sodium nitroprusside
T
TE, tris-EDTA buffer
TEMED, N,N,N',N'-tetramethylethlenediamine
TGS Buffer, tris-glycine-SDS buffer
THF, tetrahydrofolate
2-DGE, two-dimensional gel electrophoresis
µg, microgram(s)
μM, micromolar

μl, microliter(s)

V

V-hr, volt hour

v/v, volume percentage

w

W, watts

w/v, weight per volume percentage solution
Dedicated to my Mom, my Dad, & Squeaky
ACKNOWLEDGEMENTS

To Dr. Kim – a.k.a. ‘My Little Ball of Sunshine’ on good days & ‘Dr. Cranky Pants’ on other days – thanks for all your encouragement and patience. Most of all, thanks for the ride.

To Rochelle, Nathaniel, and Jonathan – thanks for allowing me to become a part of the family. I will certainly miss baby-sitting/hanging-out with the boys.

To my Mom & Dad – thanks for all your love, support, and words of wisdom. Wahoo I’m finally done!

To my family – my grandparents, aunts, uncles, cousins, and my brother – thanks for all your support in all its forms. I also want to send a special ‘shout-out’ to my Uncle Pat.

To the YSU PRG – Heather Trenary, Julie Chandler (The Lab Goddesses), Dr. Cooper, and Dr. Walker – thank you for your continued support and giving me a start in the world of proteomics. I will cherish all the memories.

To the many friends I have made along the way at YSU & RIT – thanks for all the memories. All of you have taught me so much and life would have been bleak without you.

To Java Wally’s – thanks for providing the necessary caffeine needed to survive the day.

To my Timmy – because of you I still have my sanity. Thanks for always being a bright spot in my day.
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CHAPTER ONE

Introduction
**Bacillus subtilis**

*Bacillus subtilis*, as depicted in Figure 1.1, is a rod-shaped, endospore-forming bacterium that is commonly found in soil, water sources, and in association with plants.\(^1\)
As a facultative anaerobe capable of anaerobic respiration in the presence of nitrate, and fermentation in the presence of glucose and pyruvate, it is a paradigm for Gram-positive bacteria.\(^1,2\)

The completion of *B. subtilis*’ genome sequence in 1997 lead to the discovery of a Nitric Oxide Synthase-like (NOS-like) protein, which was found within the 4,100 protein-coding genes of *B. subtilis*.\(^1\) This NOS-like protein is analogous to the mammalian NOS (mNOS) protein in sequence, structure, and activity.\(^3,4\) As shown in Figure 1.2, NOS catalyzes the five-electron heme based oxidation of L-arginine, via the stable intermediate N-hydroxy-L-arginine, to produce the transient, radical gas molecule, nitric oxide (NO) and the amino acid citrulline.\(^3,5\) The electron transfer starts with NADPH (nicotinamide adenine dinucleotide phosphate) donating electrons to the reductase domain of mNOS.\(^6\) These electrons proceed via two flavin redox carriers to the oxygenase domain of mNOS, where they interact with the active site heme iron and 6R-tetrahydrobiopterin (BH\(_4\)) to catalyze the oxidation of L-arginine.\(^6\) The oxidation of L-arginine at the active site generates the products citrulline and NO.\(^6\)

In mammals, there are two forms of the NOS enzyme, an inducible NOS (iNOS) and a constitutive NOS (cNOS).\(^3\) When NO is produced by the iNOS, it functions as a cytotoxic agent in the service of immune response.\(^3\) NO produced by the cNOS is known to play a role in intracellular and intercellular signal transduction.\(^3\)
The function of NO in Gram-positive bacteria, such as \textit{B. subtilis}, is not fully known. Kinetic data presented by Struehr \textit{et al.} suggests that bacterial NOS may follow the function of NO formed by the cNOS in mammals.\textsuperscript{4} Table 1.1 represents the kinetic data collected by Stuehr, \textit{et al.}, that compares the turnover number, the binding constant of BH\textsubscript{4}, the binding constant of tetrahydrofolate (THF), and the dissociation constant of L-arginine for \textit{B. subtilis} NOS (bsNOS), the mammalian iNOS, and the mammalian cNOS.\textsuperscript{4} This data shows that the bsNOS closely follows the mammalian cNOS in all categories except for THF binding.\textsuperscript{4} THF replaces BH\textsubscript{4} in bacteria that lack the enzymes to produce BH\textsubscript{4}.\textsuperscript{4} So, while bsNOS and other bacteria can use THF as a replacement for BH\textsubscript{4}, the mNOS enzyme does not have an affinity for THF.\textsuperscript{4}

The mammalian signaling pathway that NO participates in is depicted in Figure 1.3.\textsuperscript{7} Soluble guanylyl cyclase is the only known NO receptor enzyme.\textsuperscript{7} Upon binding, NO activates the synthesis of cyclic guanidine monophosphate (cGMP) from guanidine triphosphate (GTP).\textsuperscript{7} cGMP is a secondary messenger that regulates various cellular effectors, including cGMP-dependent kinases, cGMP-gated ion channels, and cGMP-regulated phosphodiesterases.\textsuperscript{7} These cellular effectors regulate various physiological functions in the cardiovascular and nervous systems.\textsuperscript{7} \textit{B. subtilis} has no homologous gene for soluble guanylyl cyclase, which makes it unlikely that the possible NO signaling pathway in \textit{B. subtilis} is similar to the mammalian signaling pathway.

Studies thus far have only examined the response of \textit{B. subtilis} to exogenous NO at levels sufficient to induce oxidative stress in the cells.\textsuperscript{8} These treatments, correspondingly, showed an up-regulation of genes involved in oxidative stress response.\textsuperscript{8} Signaling levels of NO are usually much lower than the levels of NO used in
previous studies. The objective of this study to elucidate the functional role of NO in *B. subtilis* through analysis of protein expression changes in response to inhibition of bsNOS. Substrate analog inhibitor aminoguanidine will be used at constitutive levels to inhibit the production of NO. This will therefore reveal, by subtraction, the affected proteins in the possible NO mediated pathway of *B. subtilis*. In the end, the proteins affected by NO inhibition will be determined through proteomic analysis and lead to the elucidation of a relevant signaling pathway for NO in *B. subtilis*. The fact that other Gram-positive bacteria, such as *Bacillus anthracis* and *Staphylococcus aureus* contain a homologous NOS-like protein and that structurally the bacterial NOS and the mammalian NOS are different enough for inhibitor discrimination, gives importance to finding the role of NO in *B. subtilis*.³ If the NO pathway proves to be crucial to bacterial viability, these studies could provide an opportunity for the development of antibacterial agents to aid in the fight against the growing number antibiotic-resistant bacteria.

**Proteomics**

Proteomics by two-dimensional gel electrophoresis (2-DGE) will be used to analyze the protein expression changes produced by NO inhibition via the inhibitor aminoguanidine. Two-dimensional gel electrophoresis provides a means for separation and visualization of thousands of proteins simultaneously on one 2-D gel.⁹,¹⁰,¹¹ Essentially, 2-DGE provides a snapshot of the proteins in a cell at a given time relative to the external and internal conditions at that time.⁹,¹¹ In 2-DGE, proteins are first separated horizontally by isoelectric focusing (IEF) and then vertically by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Figure 1.4 depicts a generic proteomic experiment schematic.¹⁰,¹¹
Before the first dimension of 2-DGE can be executed, the protein sample must be converted to an appropriate physiochemical state. The protein sample preparation method is crucial to the quality and protein resolution in the final 2-DGE product. It is essential that the protein sample preparation method preserves the native charge and molecular weight of the constituent proteins. Generally, the proteins of a sample need to be solubilized, disaggregated, denatured, and reduced.

In the first dimension, IEF separates a protein mixture according to isoelectric point (pI), i.e. the pH at which a protein carries no net charge, along a continuous pH gradient. The pI of a protein is based on its amino acid sequence; the charges of acidic and basic amino acid side chains differ based on the pH of their environment. A protein’s net charge is obtained by adding up the charges of the side chains, and the N and C terminal ends of the amino acid.

Prior to the 1980s, capillary gels were used for IEF. Carrier ampholytes and an electric current were applied to the capillary gels generating a pH gradient. After the pH gradient formed, the protein sample was then loaded. Major problems in this method of IEF included uneven buffering capacity, uneven conductivity, unknown chemical environment, batch to batch variation, and cathodic drift, which resulted in extensive loss of proteins at the gel cathodic end upon prolonged runs. These problems made gel to gel reproducibility difficult. In addition to yielding poor reproducibility, capillary gels are flimsy and hard to handle.

Gel to gel reproducibility greatly improved with the introduction of immobilized pH gradients (IPG) in 1982. Buffering compounds which are covalently bound to
porous, polyacrylamide gels generate the pH gradients within IPG strips.\textsuperscript{11,15} This allows the pH gradients to remain stable over extensive periods of times at high voltages, which is necessary for high-resolution separation of proteins.\textsuperscript{11,15} IPGs are also easier to manipulate due to their being cast on plastic backing sheets that are cut into mechanically stable strips.\textsuperscript{11,15} Limiting factors of IPG strips include their inability to manage large or hydrophobic proteins, under-representation of proteins found in the extreme acidic or basic regions, and a low protein load capacity.\textsuperscript{11,13,15} The development of methods of protein pre-fractionation has helped in removing some of the limitations of IPGs.\textsuperscript{10,11} Protein pre-fractionation can reduce the complexity of protein mixtures and/or isolate specific sub-sets off proteins, which allows for higher protein load.\textsuperscript{10,11} Some protein pre-fractionation methods include reversed-phase HPLC, ion exchange chromatography, or affinity chromatography.\textsuperscript{10,11}

In the second dimension, SDS-PAGE separates focused proteins according to molecular weight. Polyacrylamide forms a porous gel through the polymerization of acrylamide monomer into long chains.\textsuperscript{11,13} These long chains are cross-linked by the bifunctional compound N,N’-bisacrylamide when it reacts with free functional groups at the acrylamide chain termini.\textsuperscript{11,13} The polymerization is initiated by the reaction of N,N,N’,N’-tetramethylethylenediamine (TEMED) forming free radicals from ammonia persulfate.\textsuperscript{11,13} Because TEMED must be in free base form for the free radicals to form, the solution of polyacrylamide is buffered at a relatively acidic pH.\textsuperscript{11,13} The pores of the gel act as a sieve by slowing the movement of larger proteins while allowing smaller proteins to move more quickly.\textsuperscript{11,13} Pore size is increased or decreased by reducing or increasing the concentration of acrylamide.\textsuperscript{11,13} The IPGs containing the focused
proteins from the first dimension are equilibrated with SDS-containing buffers prior to the start of the second dimension.\textsuperscript{11,16} SDS is an anionic detergent that denatures proteins and gives a negative charge to the protein in proportion to its length.\textsuperscript{11,13,16} Because the SDS gives the proteins an equal net negative charge the proteins are able to be separated by molecular weight when an electric current is applied.\textsuperscript{11,13,16} Also included in the SDS-containing buffers is a thiol reducing agent, such as dithiothreitol (DTT) or β-mercaptoethanol to reduce disulfide bonds.\textsuperscript{11,13,16}

Once the proteins from a sample have been separated by 2-DGE they are visualized. Common protein detection methods include Coomassie Brilliant Blue (CBB) R-250, silver staining, and fluorescent protein staining.\textsuperscript{11,17} In this study, CBB will be used to stain the gels.

CBB R-250 has a protein detection limit as low as five nanograms.\textsuperscript{11,17} It binds nonspecifically to nearly all proteins and even though it is less sensitive than silver stain, it is far more convenient.\textsuperscript{11,17} Staining with CBB R-250 is preferable when relative amounts of protein need to be determined by densitometry since binding of CBB to protein occurs in near stoichiometric fashion.\textsuperscript{11}

Image-acquisition equipment varies from simple light boxes and cameras to sophisticated laser-based fluorescent detectors.\textsuperscript{11,15} For image analysis to be possible, the gel images must be captured electronically.\textsuperscript{11,15} Visual examination of gel images alone does not allow for objective quantification and comparison of the large number of protein spots found on the gel.\textsuperscript{11,15} Using computer software in gel analysis allows for a comprehensive examination of the gel.\textsuperscript{11,15} Image software for 2-D gels quantifies spots, removes background patterns, matches images from related gels, compares intensities
of corresponding spots in related gels, prepares gel data for presentation, and exports gel-image information.\textsuperscript{11,15} Additionally, image software can guide the excision of proteins from gels for further analysis.\textsuperscript{11,15}

In this study, excised proteins will be analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Ohio State University. A generic schematic of a LC-MS/MS experiment is depicted in Figure 1.5. In general, LC-MS/MS allows for the identification of the amino acid sequence of a protein by fragmenting a specific peptide into smaller peptides.\textsuperscript{9} This fragmentation into smaller peptides allows for the amino acid sequence to be deduced, which can be used to identify the protein by searching protein databases.\textsuperscript{9,18,19}

A protein sample must first be digested before it can be analyzed by LC-MS/MS. Typically, proteins are digested with the enzyme trypsin, which breaks the proteins down into peptides of manageable sizes.\textsuperscript{9,18,19} Trypsin breaks down the proteins by cleaving at the C-terminus side of the amino acid residues lysine and arginine unless the residue is followed by a proline.\textsuperscript{9,18,19}

Once the protein spot has been digested it is purified by LC to remove salts and buffers that can interfere with the ionization process.\textsuperscript{18} Specifically, if these salts and buffers are not removed they can form adducts with the sample making mass determination problematic or they can hinder analyte ion formation.\textsuperscript{18}

After purification, the peptides are ionized using electrospray ionization, which generates charged peptides when the liquid protein sample travels across an electric field into the mass spectrometer.\textsuperscript{9,18,19} The charged peptides are desolvated with a dry gas or heat, resulting in gaseous ions.\textsuperscript{9,18,19} These charged peptides are then separated
according to their charge-to-mass (m/z) ratios by the first mass analyzer. This creates a list of the most intense peptide peaks called the parent ions. Each parent ion is individually directed into a collision cell where it is fragmented primarily along the peptide backbone into a daughter ion by collision induced dissociation (CID). CID is the collision of the individual parent ions with a gas, typically Nitrogen or Argon. The daughter ions are then separated according to their m/z ratios by the second mass analyzer. CID creates a ladder of fragment ions that represents the amino acid sequence of the peptide. The nomenclature b-ion and y-ion indicates the type of ions that have been generated from the fragmentation of the parent ion. A b-ion maintains the charge on the N-terminus of the ion after parent ion fragmentation and a y-ion maintains the charge on the C-terminus after fragmentation. The data obtained from the second mass analyzer is then interpreted to identify the protein.

Identification of proteins from MS/MS data is carried out using bioinformatics technology. This technology allows for the MS data to be interpreted by software programs and protein databases. The databases store predicted peptide sequences produced from known protein sequences based on the enzyme used to digest it. Data obtained experimentally can be put into the database and matched to the theoretical data.
Figure 1.1: *Bacillus subtilis* cells.\textsuperscript{21}
Figure 1.2: Schematic of NO Synthesis by NOS.\textsuperscript{5}
Table 1.1: Stuehr, et al. Kinetic Data.
<table>
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<th>Enzyme</th>
<th>Turnover # (x10^{-2} min^{-1})</th>
<th>$K_m$ for BH$_4$ (µM)</th>
<th>$K_m$ for THF (µM)</th>
<th>$K_d$ for Arg (µM)</th>
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<tr>
<td>bsNOS</td>
<td>11±1</td>
<td>0.1±0.02</td>
<td>0.4±0.05</td>
<td>50±5</td>
</tr>
<tr>
<td>iNOS</td>
<td>31±3</td>
<td>10±2</td>
<td>20±5</td>
<td>97±10</td>
</tr>
<tr>
<td>cNOS</td>
<td>10±1</td>
<td>0.03±0.01</td>
<td>&gt;10,000</td>
<td>55±4</td>
</tr>
</tbody>
</table>
Figure 1.3: NO/cGMP signaling cascade in mammals.
Figure 1.4: Generic Proteomic Experiment Schematic.\textsuperscript{10,11}
Cell/Tissue/Fluid

Solubilization of Proteins

Separation of Proteins by 2-DGE

Computer-Assisted Analysis of 2D Gel

Enzymatic Digestion of Proteins of Interest

Amino Acid Sequencing by LC-MS/MS

Database Search with MS/MS Data

Identification of Protein of Interest
The protein sample is digested by trypsin, which breaks the sample down into peptides of manageable size. These peptides are then purified by liquid chromatography and ionized by electrospray ionization. The charged peptides are then separated according to their m/z ratios by the first mass analyzer. This creates a list of the most intense peptide peaks, the parent ions. These parent ions are individually directed into a collision cell where they are primarily fragmented along the peptide backbone, generating daughter ions. The daughter ions are separated according to their m/z ratios by the second mass analyzer. b-ions represent daughter ions that maintained the charge on the N-terminus after peptide fragmentation and y-ions are those that maintained the charge on the C-terminus. The amino acid sequence is determined from the daughter ions and subjected to a database search for identification of the protein.
Protein Digestion

Tryptic Digest

Liquid Chromatography

Electrospray Ionization (ESI)

Selected Parent Ion

1st Mass Analyzer

m/z, amu (parent ions)

Collison Cell

2nd Mass Analyzer

m/z, amu (daughter ions)

Amino Acid Sequence
QYLRMNMNSDL

Protein Identification
MSIIGRFKDIMSANIL
DCAENPEKMVDQYLRMN
NSDLAKVKAETAVE
EQRAKREYHENDMEE

Database Search
CHAPTER TWO

Methods
**Chemicals, Reagents, and Media.**

All chemicals, reagents, and media were obtained from Amresco, Inc., Bio-Rad, Inc., Fischer Scientific, Sigma Chemical Co., or VWR International, Inc.

**Cell Culture.**

*B. subtilis*, strain 168 (American Culture Type Collection), was grown aerobically at 37°C in Luria-Bertani Broth (LB) with vigorous shaking. Conditions of inhibition were achieved by the addition of aminoguanidine hydrochloride (AG) to exponentially growing cells at an OD$_{600}$ of 0.300. The final concentration of AG was 8mM. At an OD$_{600}$ of approximately 1.00 the cells were collected by centrifugation at 3,000rpm for 10min at room temperature. Residual broth was removed by washing cells 3x with TE (10mM Tris, 1mM EDTA, pH 8.0) buffer and centrifuging at 5,000rpm for 5 min at 4°C.

**Homogenization.**

After collection, cells were spun dry and suspended in modified sample buffer (MSB; 7M Urea, 4% [w/v] CHAPS, 1% [w/v] dithiothreitol [DTT]) containing 1% (w/v) protease inhibitor cocktail (PIC). The cells were lysed by ultrasonication at 16W for a total of 90sec in three 30sec cycles with cooling in an ice bath between each cycle. Lysed cells were centrifuged for 60min at 13,200rpm and 4°C. The supernatant was removed and placed in a new tube for protein quantification.

**Bradford Assay.**

Protein quantification was done using a modified Bradford assay$^{22}$. Each reaction tube contained 80µl water, 10µl 0.1M HCl, 4mL Bradford reagent$^{23}$, and the required protein sample. The absorbance of each sample was recorded at 595nm. A standard curve was constructed using bovine albumin serum (BSA) at concentrations ranging
from 10µg to 40µg. The protein concentration of the homogenate was calculated using the standard curve and the absorbance of the homogenate.

**Two-Dimensional Gel Electrophoresis.**

ReadyStrip IPG strips (Bio-Rad) of 17cm in size and of the pl range 4 – 7 were passively rehydrated for electrophoresis in the first dimension. A total volume of 300µl of rehydration buffer was used to rehydrate the strip. This volume was made up of rehydration buffer (RB; 8M urea, 1% CHAPS, 15mM DTT, 0.2% BioLytes (BioRad), 0.001% bromophenol blue) and 250µg of protein homogenate. The strips were covered in mineral oil and left to rehydrate for 12 – 18hr with gentle agitation. After rehydration, the IPG strips were placed into a focusing tray containing electrode wicks (wetted with Millipore water) and overlaid with mineral oil. Isoelectric focusing was performed using a Protean IEF Cell (BioRad) at 20°C for 60,000V-hr. Once focusing was complete, the IPG strips were equilibrated with equilibration buffer I (6M urea, 2% sodium dodecyl sulfate [SDS], 0.375M Tris-HCl pH 8.8, 20% [v/v] glycerol, 130mM DTT) and equilibration buffer II (6M urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% [v/v] glycerol, 135mM iodoacetamide) for 10min each using gentle agitation.

The equilibrated strips were dipped in 1x Tris-Glycine-SDS (TGS) buffer (25mM Tris base, 192mM glycine, 0.1% SDS) before loading on 12% polyacrylamide gels for separation via vertical SDS-PAGE. Once the strips were loaded, overlay agarose (0.5% low melt agarose in TGS buffer with 0.001% bromophenol blue) was used to keep the strips in place. The gels were then loaded into a Protean II xi cell (BioRad). A 10-24mA
constant current was applied to the apparatus until the dye front was approximately 1 cm from the bottom of the gels.

**Staining.**

Following electrophoresis, the gels were stained with Coomassie stain (5% Coomassie Brilliant Blue G-250, 45% methanol, 10% acetic acid) for at least 3 hr with gentle agitation. Destaining followed with a mixture of methanol and acetic acid (40% methanol, 10% acetic acid) to wash away the background color leaving only protein spots. Once destained, the gels were imaged using a flatbed scanner (hp ScanJet 5370C). Stained gels were stored in 5% (v/v) acetic acid.

**Image Analysis.**

Digital images from three independent trials were analyzed using PDQuest 2-D Analysis Software (version 8.0.1; Bio-Rad) to create a matchset for each experimental condition. The matchsets were created using the program’s automated detection and matching wizard. This wizard filtered the images, determined the spot detection limits, and detected and matched the spots. The matchsets resulted in a master gel for each experimental condition. A master gel is a composite gel of the triplicate set. Comparison of the master gels identified differentiated proteins.

**Spot Excision.**

Protein spots of interest were excised for sequencing by mass spectrometry (MS). As before, 2-DGE was run on protein homogenates. Once the proteins were separated, the gels were placed in 500 mL fixing solution (50% ethanol, 40% water, 10% acetic acid; v/v) for 1 hr at room temperature. The fixing solution was removed and 500 mL of wash solution (50% methanol, 40% water, 10% acetic acid) was added. The
gels were left in wash solution overnight with gentle agitation at room temperature. Upon removal of the wash solution, the gels were stained for at least 3hr with Coomassie stain. Destaining was done with a mixture of methanol and acetic acid (50% methanol, 40% water, 10% acetic acid) at room temperature. The stained gels were stored in 5% (v/v) acetic acid.

Excision of the protein spots was done using sterile 2500 µl pipette tips. The gel pieces were then placed into sterile 1.5mL microcentrifuge tubes and covered in 5% (v/v) acetic acid. Excised spots were submitted to the Ohio State University Mass Spectrometry and Proteomics Facility (Columbus, OH; http://www.ccic.ohio-state.edu/MS/proteomics.html) for sequencing by MS.

**Mass Spectrometry.**

Collaborators at the Ohio State University MS and Proteomics Facility followed standard protocols to analyze the gel plugs. Submitted samples were prepped for MS sequencing via the following procedure. The excised gel plugs were digested with sequencing grade trypsin from Promega (Madison, WI) or sequencing grade chymotrypsin from Roche (Indianapolis, IN) using the Montage In-Gel Digestion Kit from Millipore (Bedford, MA) following manufactures recommended protocol. In short, the excised plugs were trimmed as close as possible to minimize background polyacrylamide material. The plugs were then washed in 50% methanol/5% acetic acid for 1hr. A second wash was done before the gel plugs were dehydrated in acetonitrile. The gel plugs were rehydrated and incubated with DTT solution (5mg/mL in 100 mM ammonium bicarbonate) for 30min prior to the addition of 15mg/mL iodoacetamide in 100mM ammonium bicarbonate solution. Iodoacetamide was incubated with the gel
plugs in the dark for 30min before being removed. The gel plugs were washed again
with cycles of acetonitrile and ammonium bicarbonate (100mM) in 5min increments. A
speed vac was used to dry the gel plugs. After drying, the protease is driven into the gel
plugs by rehydrating them in 50µL of sequencing grade modified trypsin or
chymotrypsin at 20µg/mL in 50mM ammonium bicarbonate for 10min. After this time
period, 20µL of 50mM ammonium bicarbonate was added to the gel plugs and the
mixture was incubated at room temperature overnight. To extract the peptides from the
polyacrylamide, the gel plugs are washed with 50% acetonitrile and 5% formic acid
several times. The washes are pooled together and concentrated to 25µL in a speed
vac.

Once the gel plugs were digested, MS sequencing was performed with Capillary-
liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) on a
Thermo Finniagan LTQ mass spectrometer equipped with a nanospray source operated
in positive ion mode. The LC system was an UltiMate™ Plus system from LC-Packings
A Dionex Co (Sunnyvale, CA) with a Famos autosampler and Switchos column
switcher. Solvent A was water with 50mM acetic acid and solvent B was acetonitrile. For
the first injection, 5µL of each sample was injected onto the trapping column (LC-
Packings A Dionex Co, Sunnyvale, CA), and then washed with 50mM acetic acid. The
injector port was switched to inject and the peptides were eluted off the trap onto the
column. Chromatographic separations were done using a 5 cm and 75µm ID ProteoPep
II C18 column (New Objective, Inc. Woburn, MA) packed directly in the nanospray tip. A
gradient of 2% - 80% B was used to elute the peptides directly off the column onto the
LTQ system. Elution was done over a 50 min period, with a flow rate of 300nL/min. The total run time was 60mins.

The MS/MS was acquired according to standard conditions established by the lab. In short, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200°C was used. The scan sequence of the mass spectrometer was based on the TopTen™ method. This method uses a full scan analysis, recorded between 350 – 2000Da and a MS/MS scan to generate product ion spectra. From the product ion spectra, amino acid sequence is determined in consecutive instrument scans of the ten most abundant peaks in the spectrum. The CID fragmentation energy is set to 35%. Dynamic exclusion is enabled with the following parameters: a repeat count of 30sec, exclusion duration of 350sec, and a low mass width of 0.5Da and a high mass width of 1.50Da.

MS sequence information from the MS/MS data was processed by converting the raw dta files into a merged file (.mgf) using MGF creator (merge.pl, a Perl script). The first scan number, last scan number, number, number of intermediate scans, number of grouped scans, and minimum number of ions were set to blank, blank, 1, 0, and 8, respectively. Resulting mgf files were searched using Mascot Daemon by Matrix Science version 2.2.1 (Boston, MA) and the database searched against the full SwissProt database version 54.1(283454 sequences; 104030551 residues). Because the data was acquired on an ion trap mass analyzer, the mass accuracy of the precursor ions was set to 2.0Da. The fragment mass accuracy was set to 0.5Da. Considered modifications (variable) were methionine oxidation and carbamidomethy cysteine. Two missed cleavages for the enzyme were allowed. Peptides with a score of
less than 20 were filtered and protein identification required bold red peptides. Protein identifications were checked manually. Any proteins with a Mascot score of 50 or higher, with a minimum of two unique peptides from one protein having a $-b$ or $-y$ ion sequence tag of five residues or better, were accepted.
CHAPTER THREE

Results
The dose curve used to determine the effect of aminoguanidine concentration on \textit{B. subtilis} growth is shown in Figure 3.1. Concentrations of aminoguanidine used were 0mM, 1mM, 2mM, 4mM, 8mM, 10mM, and 20mM. The highest concentration of aminoguanidine that produced a curve similar to growth of \textit{B. subtilis} in the absence of aminoguanidine was selected for experimental use. Cellular growth in 1mM, 2mM, 4mM, 8mM, and 10mM aminoguanidine all closely followed normal growth. The 20mM aminoguanidine growth curve exhibited an increased doubling time (i.e. slowing of growth) around 300min relative to growth in the absence of aminoguanidine. To be conservative, a concentration of 8mM aminoguanidine was selected for experimental use. Hereafter, cultures grown in the absence of aminoguanidine will be referred to as the untreated cultures and those cultures grown in the presence of 8mM aminoguanidine will be referred to as the treated cultures.

Initial 2-DE experiments studied the protein profiles of untreated \textit{B. subtilis} cultures and treated \textit{B. subtilis} cultures over the pI range of 3 – 10 on 7cm, 10% polyacrylamide gels (Figure 3.2). Homogenization of the \textit{B. subtilis} cells typically gave protein concentrations ranging from 4.0µg/µl to 6.0µg/µl, which yielded enough protein to load 100µg of protein on each 7cm IPG strip. It was observed that a majority of the expressed proteins fell in the mid- pI ranges. This observation is consistent with previous work on the \textit{B. subtilis} proteome. Studies by Eymann \textit{et al.} found that two-thirds of the proteins expressed in growing \textit{B. subtilis} cells lie in the pI range of 4 – 7.\textsuperscript{24} It was also observed that most of the proteins were situated in the mass range of 73kDa to 15kDa on the 10% polyacrylamide gel, which covers the mass range of 99kDa to 15kDa. Therefore, efforts were focused on more effectively resolving and identifying
differentiated proteins in the pI range of 4–7 on 17cm, 12% polyacrylamide gels, which resolves proteins in the mass range of 75kDa to 10kDa. These separation parameters will enhance protein separation horizontally and vertically.

Representative 2DGE results for untreated *B. subtilis* cultures and treated *B. subtilis* cultures over the pI 4–7 on 17cm, 12% polyacrylamide gels are depicted in Figure 3.3. A protein load of 250µg was loaded on each IPG strip. These gels were run in triplicate and subjected to analysis using the PDQuest software system. Matchset analysis revealed 121 detectable spots from both untreated and treated cultures. Direct comparison of the matchset master gels, seen in Figure 4, revealed 3 protein spots of interest on the master gel representing the untreated cultures. These 3 protein spots were completely absent in the treated gel, indicating that they may have been down-regulated by the inhibition of NO via aminoguanidine.

The 3 spots unique to the untreated cultures along with a spot common to both the untreated and treated cultures were excised for MS sequencing (Figure 3.4). Proteins were identified by Nano-LC/MS/MS in combination with Mascot, a protein database search engine, and are represented in Table 3.1.

The Mascot search engine finds the most suitable peptide match for each spectra by matching peaks in each fragmentation pattern to the calculated ions from the *in silico* digest, a computational prediction of peptide fragmentation patterns. Mowse (molecular weight search) scoring algorithm is used to produce a probability based score called a Mascot score. The Mascot score indicates the probability that the match was a random error and is equal to -10*Log(P), where P is the absolute probability. Thus, the higher the Mascot score, the lower the probability that the match
is a random event. Each peptide listed in Table 3.1 possessed a Mascot score greater than 50, indicating identity or extensive homology (p<0.05).

Protein spots 1 and 5 (Table 3.1; Figure 3.4) were excised as landmark proteins because they are common to both untreated and treated cultures, well resolved, and well defined. Excising landmark proteins ensures that the gels were matched properly during analysis. Protein spot 1 was excised from the treated gel and protein spot 5 was excised from the untreated gel. Both spots were identified as phosphocarrier protein HPr from *B. subtilis*, indicating that the gels were matched properly during analysis. The sequence coverage for protein spots 1 and 5 was 62% and the Mascot scores were 395 and 414, respectively.

Protein spots 2, 3, and 4 (Table 3.1; Figure 3.4) were expressed only in the gels representing the untreated *B. subtilis* cultures. MS results identify protein spot 2 as a nucleoside diphosphate kinase (NDK), protein spot 3 as an MreB-like protein, and protein spot 4 as a heat shock protein homolog. With Mascot scores of 409 for protein spot 2, 721 for protein spot 3, and 760 for protein spot 4, these protein identifications show a very high fidelity. The percent amino acid sequence coverage for each of these protein spots was 51% for protein spot 2, 41% for protein spot 3, and 62% for protein spot 4.
Figure 3.1: *B. subtilis* Dose-Response Curve with Aminoguanidine. Comparison of normal *B. subtilis* growth with growth of *B. subtilis* in 1mM, 2mM, 4mM, 8mM, 10mM, & 20mM aminoguanidine.
B. subtilis Dose-Response Curve with Aminoguanidime

OD
0 60 120 180 210 240 270 300 330 360 390
Time (min)

0mM AG
1mM AG
2mM AG
4mM AG
8mM AG
10mM AG
20mM AG
Figure 3.2: Initial *B. subtilis* 2D-Gels.
*B. subtilis* proteins were separated horizontally on 7cm IPG strips, pI 3 – 10, and vertically on 10% polyacrylamide gels: (a) Untreated Gel (b) Treated Gel
Figure 3.3: Representative *B. subtilis* 2D-Gels. *B. subtilis* proteins separated horizontally on 17cm IPG strips, pI 4 – 7, and vertically on 12% polyacrylamide gels: (a) Untreated Gel (b) Treated Gel
Figure 3.4: *B. subtilis* Matchset Master Gels. 
(a) Untreated Master Gel (b) Treated Master Gel
**Table 3.1:** Proteins Identified by Nano-LC-MS/MS in Combination with Mascot.
<table>
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<tr>
<th>Spot</th>
<th>Protein Description</th>
<th>NCBI Accession (Version)</th>
<th>Theo. pI/Mr (kDa)</th>
<th>Expt. pI/Mr (kDa)</th>
<th>Species</th>
<th>Mascot Score</th>
<th>NP/PD</th>
<th>MS/MS Peptide Sequence</th>
<th>SC (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>Phosphocarrier protein HPr</td>
<td>P08877 (GI:131533)</td>
<td>4.86/9.2</td>
<td>5.1/23.0</td>
<td>Bacillus subtilis</td>
<td>395</td>
<td>37/1851</td>
<td>MAQKTFKVTA DSGIHARPAT VLVTASKYD ADVNLEYNGK TVNKLKIMGV MSLGIAKGAE ITISASGADEN DALNALEET MKSEGLGE</td>
<td>62%</td>
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<td>Nuceloside Diphosphate Kinase (NDK)</td>
<td>P31103 (GI:18266849)</td>
<td>5.67/16.8</td>
<td>6.2/26.0</td>
<td>Bacillus subtilis</td>
<td>409</td>
<td>25/2476</td>
<td>MEKTIFMVKP DGVQRQLIGD ILSRFERKGL QLAGAKLMRV TEQMAEKHYA EHOQPFFFGVE LVEFITSGPV FAMVWEGENV IETRQLIGK TNPKEALPGT IRGDYGMFVG KNIHGDSL ESAEREINIF FNKEELVSYQ QLMAGWY</td>
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<tr>
<td>Spot</td>
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<td>Theo. pl/Mr (kDa)</td>
<td>Expt. pl/Mr (kDa)</td>
<td>Species</td>
<td>Mascot Score</td>
<td>NP/PD</td>
<td>MS/MS Peptide Sequence</td>
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<td>P39751 (GI:729993)</td>
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<td>6.3/54.0</td>
<td>Bacillus subtilis</td>
<td>721</td>
<td>21/2452</td>
<td>MFARDIGIDL GTANVLHIHK GKGIVLNEPS VVALLDKNNSGK VLAVGEEARR MVGRTPGNIV AIRPLKDGVI ADFEVTEAMGL KHFINKLNVK GLFSKPRMLI CCPNITTSVE QKAKEAEXES1 SGGKHVYLEE EPKVAAIAGAG MEIFQPSGNNM VVDICGGTTDT IAVISMGDIV TSSSIKMAKD KFDMEILNYI KREYKLLIGE RTAEDIKIKV ATVFPDARHE EISIRGRDMV SGLPRITTIVN SKEVEEALRE SVAVIVQAARK QVLERTPPEL SADIIDRGVI ITGGGALLNG LDOLLAEELK VPVLVAENPM DCVAIGTGVMD NMMSDKLKPRK VRKLS</td>
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Table 3.1 (cont.)

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<th>Species</th>
<th>Mascot Score</th>
<th>NP/PD</th>
<th>MS/MS Peptide Sequence</th>
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<td>4</td>
<td>Phage shock protein A homolog (LiaH)</td>
<td>P54617 (GI:3123254)</td>
<td>5.87/25.2</td>
<td>6.4/46.0</td>
<td><em>Bacillus subtilis</em></td>
<td>760</td>
<td>40/2120</td>
<td>MSIIGRFKDI MSANINALLD KAENPEKMVD QYLGRNMNSDL AKVKAETAAV MAEEQRAKRE YHECOADMKEK MESYAMKALQ AGNESDARKF LERKTSLESK LSELOQAAQIQI AATNAAOMRK MHDKLVSDIG ELEARKNIK AKKAVAKTQE RMNKLGASVS STSQSMSAFG RMEDKVNKAL DQANAMAEILN SAPQDDMALD SAKYDYGSS QVDDELAAKL AKMMLDK</td>
<td>62%</td>
</tr>
<tr>
<td>Spot</td>
<td>Protein Description</td>
<td>NCBI Accession (Version)</td>
<td>Theo. pI/Mr (kDa)</td>
<td>Expt. pI/Mr (kDa)</td>
<td>Species</td>
<td>Mascot Score</td>
<td>NP/PD</td>
<td>MS/MS Peptide Sequence</td>
<td>SC (%)</td>
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</tr>
<tr>
<td>5</td>
<td>Phosphocarrier protein HPr</td>
<td>P0877 (GI:131533)</td>
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<td>5.1/23.0</td>
<td><em>Bacillus subtilis</em></td>
<td>414</td>
<td>34/2241</td>
<td>MAQKTFKVTA DSGIHARPAT VLVQTASKYD ADVNLEYNGK TVNLKSMGV MSLGIAKGAE ITIASSGADE NDALNALEET MKSEGLGE</td>
<td>62%</td>
</tr>
</tbody>
</table>

*a*Spot: spot number corresponds to labeled spots in Figure 4; Protein Description: description of matched protein; NCBI Accession (Version): accession number and submission version of matched protein from NCBI database; Theo. pI/Mr (kDa): theoretical isoelectric point and molecular mass based on amino acid sequences of the identified protein; Expt. pI/Mr (kDa): experimental isoelectric point and molecular mass estimated from the 2DGE gels; Species: the bacterial species of the matched protein; Mascot Score: score obtained from the Mascot search for each match; NP: the number of matched peptides; PD: the number of peptides detected; MS/MS Peptide Sequence: peptide sequence of identified protein with amino acids identified by Nano-LC-MS/MS in bold; SC: percent amino acid sequence coverage for the identified protein.
CHAPTER FOUR

Discussion
Studies of the role of NO in bacteria have indicated that it may serve as a signaling molecule as it does in mammals.\textsuperscript{4} Soluble guanylyl cyclase is the NO receptor in mammalian cell. There is no homologous gene to this receptor in \textit{B. subtilis}. This indicates that, if NO does serve a signaling function in \textit{B. subtilis}, the effects of NO activity will likely not resemble those seen in mammalian systems. Therefore, the goal of this study was to elucidate proteins involved in a possible NO signaling pathway in the bacterium \textit{B. subtilis} using proteomic analysis.

A dose-response curve comparing the growth of untreated \textit{B. subtilis} cells to the growth of \textit{B. subtilis} cells in various concentrations of aminoguanidine determined that an 8mM aminoguanidine concentration was the maximum amount of aminoguanidine that could be added to growing cells without significantly changing the growth pattern. This ensured that the protein expression changes would reflect the action of NO as a signaling agent rather than protein expression changes reflecting the effects of aminoguanidine toxicity.

The identification of 3 protein spots unique to the untreated cultures provides preliminary data that supports the possibility of NO having a role in bacterial signal transduction. These three protein spots were down-regulated in the treated cultures via NO inhibition by aminoguanidine and were identified as nucleoside diphosphate kinase (NDK), MreB-like (Mbl) protein, and phage shock protein A homolog (LiaH).

NDK has a theoretical pl/Mr of 5.67/16.8kDa and with a mascot score of 409 the probability of the identification being a random match is approximately $P = 10^{-41}$. This protein catalyzes the transfer of $\gamma$-phosphoryl groups from a nucleoside triphosphate to a nucleoside diphosphate.\textsuperscript{27} The role of NDK in cellular metabolism is crucial in
maintaining pools of nucleoside triphosphates for the synthesis of DNA and RNA. \textsuperscript{27} Under anaerobic conditions in a related bacterial species, \textit{Bacillus halodenitrificans}, NDK was found to be involved in a protein complex, referred to as the green protein complex (GPC).\textsuperscript{27} This complex consists of NDK and a manganese-containing superoxide dismutase (Mn-SOD), both in an inactive state.\textsuperscript{27} Upon exposure to air, the complex dissociates and the two proteins become active.\textsuperscript{27} Under anaerobic conditions, bacterial growth rate is much lower than growth in the presence of oxygen, which dictates a low demand for NDK.\textsuperscript{27} Due to the absence of NDK under conditions of NO inhibition, we believe that NDK may be complexed in the absence of NO and that NO may play a role in dissociating this complex. This is further supported by crystallographic studies of the GPC that indicate the presence of a heme center capable of binding NO.\textsuperscript{27} Overall, these results show that NO may have a valuable role in the regulation of NDK.

The Mbl protein has a theoretical pl/Mr of 5.77/36.0kDa and produced a Mascot score of 721, which translates into a probability of approximately $P = 10^{-72}$ that the match is random. This protein is a second determinant of the MreB protein found in \textit{B. subtilis}. Studies involving \textit{mre} genes in \textit{B. subtilis} have provided data indicating a function in cell wall biosynthesis. Gram-positive bacteria such as \textit{B. subtilis}, contain a large amount of peptidoglycan, which consists of linear chains of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) sugar residues linked by $\beta$-(1,4)-glycosidic bonds and oligopeptides that form the cross-links between the linear chains giving a mesh-like structure outside the plasma membrane in bacteria (Figure 4.1a).\textsuperscript{28} The bonds between the oligopeptide cross-links are formed by penicillin-binding
proteins (PBPs). In Stewart’s proposed model for the role of the B. subtilis Mre proteins in cell wall biosynthesis (Figure 4.1b), the MreB protein and Mbl protein form helical coils in the cell that contact the cell membrane at the MreCD complexes. MreCD complexes form contacts with PBPs, which couples the Mbl and MreB proteins to the cell elongation PBPs.

The MreB protein is believed to interact with PBP 2B, the enzyme responsible for peptidoglycan synthesis at the division septum. This interaction involves regulating the switch to septal peptidoglycan synthesis mediated by PBP 2B at the midcell and polar potential division sites. Mechanistically, MreB would down-regulate PBP 2B septum-specific peptidoglycan synthesis, therefore allowing other PBPs to mediate lateral wall extension. When it is time for the cell to divide, MreB would then up-regulate PBP 2B septum specific peptidoglycan synthesis and down-regulate the other PBPs involved in mediating lateral wall extension. Loss of the MreB results in failure to repress PBP 2B activity. Failure to repress PBP 2B activity leads to loss of lateral wall synthesis, which consequently leads to a weakening of the lateral cell wall, loss of osmostability and swelling of the cell followed by lysis.

The interaction of the Mbl protein with the PBPs via the MreCD protein complex, is important to the regulation of lateral wall extension, as well as the spatial configuration of the wall. In the absence of the MreCD protein complex, the coupling of Mbl to the PBPs is lost, which results in a loss of the helical lateral wall extension peptidoglycan synthesis and leaves only septation as a mechanism for cell wall incorporation. Overall, loss of MreCD leads to the same consequences seen in the loss of the MreB protein.
The down-regulation of the Mbl protein via inhibition of NO suggest that NO has a role in the activation of the Mbl protein. Given the above information, the loss of the Mbl protein would suggest an interruption in cell wall biosynthesis, which could lead to an overall loss in cell viability. The fact that the dose-response curve of *B. subtilis* cells grown is 8mM aminoguanidine (i.e. those with down-regulated Mlb) did not show a decrease in growth when compared with the untreated *B. subtilis* growth curve indicates that the loss of Mbl did not contribute to an overall loss of cell viability. Even though the down-regulation of Mbl may cut down on the constituents in the glycan layer, other MreB determinants present in *B. subtilis* may enable maintenance of the cell wall integrity.

LiaH, with a theoretical pI/Mr of 5.87/25.2Kda, appears to be down-regulated in the absence of NO. With a mascot score of 760, the probability that this protein is a random match is \( P = 10^{-76} \). This protein is a homolog of the phage shock protein A (PspA) found in *E. coli*. LiaH is part of the lia locus depicted in Figure 4.2, which consists of the six genes *liaIH-liaGFSR*.\(^{30,31}\) The genes *liaGFSR* are kept at a low level of expression through a weak constitutive promoter upstream of *liaG*.\(^{30,31}\) By comparison, initiation of the expression of the *liaIH* operon by *liaI* promoter is entirely LiaR-dependent.\(^{30,31}\) This LiaR-dependent expression is known as LiaRS-TCS, which stands for lipid II-interacting antibiotics response regulator and sensor – two component system with the two components being the *liaI* and *liaH* genes.\(^{30,31}\) The LiaRS-TCS is part of the regulatory network that responds to cell envelope stress in *Bacillus subtilis*.\(^{30,31}\)
The cell envelope consists of the plasma membrane and the cell wall, and functions to protect the cell against threats from the environment. Specifically, the cell envelope gives the cell its shape, counteracts the high inner osmotic pressure, and provides a sensory interface and molecular sieve between the bacterial cell and its surroundings, acting as a go between for both information flow and controlled transport of solutes. These functions of the cell envelope are crucial to the survival of bacterial cells, which makes it an appealing target for antibiotics.

The cell envelope stress response system in *Bacillus subtilis* is just starting to be actively researched. Thus far, studies have not fully exposed the physiological role of LiaH. It is speculated that LiaH may play a two roles in the cell envelope stress response system. One speculated role is that LiaH acts as a weak negative modulator of the LiaRS-TCS in conjunction with LiaF. LiaF has been identified as a strong inhibitor of the LiaRS-TCS in *B. subtilis*. This speculation is supported by similarities that LiaH shares with its *E. coli* homolog, PspA, which is known to inhibit cell envelope stress response by inhibiting the transcriptional activator of PspF, the expected equivalent of LiaF in *B. subtilis*.

The second speculated role of LiaH is that it maintains the cell envelope integrity when under stress. Again this speculation is based on the similarities that LiaH shares with its *E. coli* homolog, PspA and the fact that it is overexpressed under conditions of stress. In the *E. coli* cell envelope stress response system, PspA maintains cell envelope integrity by anchoring itself to the cell membrane. PspA attachment to the cell membrane in *E. coli* is mediated by protein-protein interaction with two transmembrane proteins. Co-transcriptional expression of liaI, known to code
for a putative membrane protein, with liaH suggests that LiaI may serve as the membrane anchor for LiaH.\(^\text{30}\)

Down-regulation of LiaH in the absence of NO suggests that NO may play a role in the activation of LiaH. The exact connection between NO and LiaH cannot be determined from these results, but further evidence supporting LiaH having a role as a weak negative modulator of the LiaRS-TCS is offered. This evidence exists in the fact that LiaH was found to be expressed in the untreated gel as a faint (i.e. not strongly expressed) protein spot.\(^\text{30}\) The proteins in the untreated cultures were taken from \textit{B. subtilis} cells grown in an unstressed environment. Had the environment caused stress to the cells, the LiaH protein spot would have been stained more intensely.
**Figure 4.1:** (a) Simple schematic of a Gram-positive bacterial cell wall. NAG (N-acetylglucosamine) & NAM (N-acetylmuramic acid) make up the peptidoglycan linear chains and oligopeptides form the cross-links between the linear chains.\(^{32}\) (b) Diagram of Stewart’s proposed model for the role of *B. subtilis* Mre proteins in cell wall biosynthesis. Shows how the Mlb & MreB helical coils come into contact with the MreCD complexes at the cell membrane (cm) allowing for interaction with PBPs.\(^{29}\)
PBPs

(a)

NAG
NAM
Oligopeptide Cross-Links

Peptidoglycan Layer

Plasma Membrane

Bacterial Cell Wall

Bacterial Plasma Membrane

(b)

PG

PBPs

MreCD Complex

MreB

Mbl
Figure 4.2: The lia locus of *B. subtilis*. Under conditions of cell envelope stress LiaF releases LiaS causing LiaR to bind upstream at the *lia* promotor (P_{lia}). P_{lia} induction results in transcription of *liaH*. The induction of *liaH* causes stressed induced expression of *liaGFSR*, therefore triggering the system to respond to cell envelope stress. (b) lia Gene Expression. The dashed arrow represents the genes expressed in the *lia* locus under normal growth conditions (-stress). These conditions produce the expression of the genes *liaGFSR* by the weak constitutive promoter P_{liaG} which is located up-stream of *liaG*. Inactivation of the cell envelope stress response system is maintained by P_{liaG}-dependent expression of *liaGFSR* and the inhibitory activity of LiaF. The solid arrows represent the genes expressed in the *lia* locus under conditions of cell envelope stress (+stress). Representation of *liaI* and *liaH* gene expression by a larger solid arrow indicates their over expression during times of cell stress.
Cell Envelop Stress

(a)

(b)

+l stress

- stress

liaT liaH liaG liaF liaS liaR

P_{lia} P_{liaG}

LiaS LiaR

+l stress

- stress
CHAPTER FIVE

Conclusions
Preliminary studies of the inhibition of NO via aminoguanidine in *B. subtilis* have revealed the down-regulation of three proteins. There have been many studies done on the function of NO in mammalian cells, but little is known about its function in Gram-positive bacteria. The down-regulation of these three proteins presents evidence that NO functions as a signaling molecule in *B. subtilis*. Functionally, these down-regulated proteins are involved in important cellular processes but the loss of their function does not appear to impact the viability of the cell, as measured by growth curves. Further proteomic studies will provide a better understanding of the role NO is playing and lead to the determination of a relevant signaling pathway for NO in *B. subtilis*.  


REFERENCES


