GENERATION AND CHARACTERIZATION OF *dszB* MUTANTS FROM *Nocardia asteroides* A3H1

THESIS

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GENERATION AND CHARACTERIZATION OF *dszB* MUTANTS

FROM NOCARDIA ASTEROIDES A3H1

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Dibenzothiophene, a model compound for sulfur-containing organic molecules found in fossil fuels, can be desulfurized to 2-hydroxybiphenyl by *Nocardia asteroides* sp. strain A3H1 (A3H1) using the "4S" desulfurization pathway. In the final and rate-limiting step of this pathway, the DszB enzyme catalyzes the hydrolysis of 2-(2'-hydroxyphenyl) benzenesulfinate to form inorganic sulfur and 2-hydroxybiphenyl. The wildtype *dszB* gene from A3H1 was successfully cloned into the pBAD TOPO® and pBAD TOPO®/ThioFusion expression vectors. Cloning efficiency of the wildtype gene into the pBAD TOPO® vector was 86%. The *in vitro* directed evolution of DszB was

initiated using error-prone PCR. An asexual *dszB* mutant library was generated and sixty mutant genes were characterized. The average mutation rate of the *dszB* gene was determined to be 0.75% ± 0.84. Attempts at overexpressing the mutant genes were unsuccessful and no measurable induction of fusion protein expression was observed upon arabinose addition.

CHAPTER I

INTRODUCTION

The industrial extraction of petroleum and the use of fossil fuels as an energy source have led to a significant increase in environmental pollution over the past century. Not only has air quality diminished but soil and groundwater pollution are direct consequences stemming from fossil fuel combustion. Combustion of petroleum derived fuels leads to the atmospheric emission of sulfur oxides which are the major source of acid rain.[1] As much as 70% of the organosulfur in fossil fuels is in the form of polyaromatic sulfur heterocycles (PASHs), such as benzothiophene, dibenzothiophene (DBT), and more complex thiophenes.[1,2] The inorganic sulfur content in fossil fuels can be removed by physical or microbiological treatments but sulfur that is bound covalently to the organic heterocyclic components of petroleum can not be removed easily.[3] Several desulfurization methods have been employed to combat this problem.

Traditionally, hydrodesulfurization (HDS) has been used to remove sulfur from fossil fuels.[4,5] Conventional HDS is a catalytic process that converts organic sulfur to hydrogen sulfide gas by reacting crude oil fractions with an inorganic catalyst and hydrogen at pressures between 150 and 3000 lb/in^2 and temperatures between 290 and 455°C.[3,5] However, this chemistry does not work well on certain sulfur molecules in oil, particularly the PASHs.[1] It is this limitation, in addition to the cost of building and operating HDS units

that has peaked interest in finding a cheaper and more efficient method of desulfurization.

Biocatalytic desulfurization (BDS) could be the answer. BDS has been studied at length by the petroleum industry in recent years as an alternative to hydrodesulfurization. This technology incorporates the use of bacteria that can remove the covalently bound sulfur from the aromatic hydrocarbon skeleton of petroleum without degrading the fuel value. BDS has the potential benefits of lower capital and operating costs while producing substantially less greenhouse gases.[6] Additionally, the Environmental Protection Agency (EPA) proposed a 97% reduction of the sulfur content in diesel to less than 15 ppm by 2006 down from the current specification of 500 ppm.[7] BDS could potentially attain these new EPA mandates for lower sulfur levels in fuels.

Dibenzothiophene (DBT) has been used as a model compound for studies involving BDS[8-11] and two distinct bacterial desulfurization pathways have been elucidated. Many bacteria in the genera *Pseudomonas*[12-14], *Beijerinckia*[15], *Rhizobium*[16], *Acinetobacter*[16], *Arthrobacter*[17], and *Brevibacterium*[18], metabolize DBT through a ring destroying oxidative pathway.[19] This pathway leads to a decrease in fuel quality as both the carbon and sulfur of DBT are incorporated into the cellular biomass. Other bacteria have utilized DBT as a metabolite using a less destructive pathway. The bacterium *Rhodococcus erythropolis* strain IGTS8 (further referred as IGTS8) has been studied extensively in the area of biodesulfurization because it was the first isolated bacterium to use the "4S" pathway for desulfurization of DBT.[11,20] This pathway of desulfurization has also been observed in other bacteria including those in the genera *Corynebacterium*[21], *Gordona*[22], and *Nocardia*.[22] The "4S" pathway involves three enzymes that undergo the sequential metabolism of DBT to DBT-sulfoxide, DBT-sulfone, 2-(2'-hydroxyphenyl) benzenesulfinate, and finally 2-

hydroxybiphenyl and sulfite.[8,12,20, 22-24] The conversion of DBT to 2-hydroxybiphenyl and sulfite is of major interest to the energy industry since this pathway does not destroy the aromatic rings of DBT. Furthermore, this pathway does not destroy the hydrocarbon chain linked to DBT.[1] These two advantages allow the fuel quality to be retained when utilizing the "4S" pathway. It would be advantageous for refineries to integrate this naturally occurring bacterial pathway in their operations, as an alternative to HDS, to diminish acid rain pollution.

A schematic of the desulfurization (dsz) "4S" pathway is shown in Figure 1. The first and second steps in the pathway are catalyzed by the DszC enzyme (DBT-monooxygenase) which sequentially oxidizes DBT to DBT-sulfoxide (DBTO) and DBT-sulfone ($DBTO_2$). The third step in the pathway is catalyzed by the DszA enzyme ($DBTO_2$-monooxygenase) which further oxidizes DBT-sulfone, cleaving the first carbon-sulfur bond and generating 2-(2'-hydroxyphenyl) benzenesulfinate (HPBS). The final step in the pathway, catalyzed by the DszB enzyme, aptly named 2-(2'-hydroxyphenyl) benzenesulfinate desulfinase (HPBS desulfinase), catalyzes the nonoxidative cleavage of the second carbon-sulfur bond to form 2-hydroxybiphenyl (HBP) and sulfite (SO_3^{2-}). This final step in this pathway, catalyzed by DszB, is the slowest and therefore, is considered the rate-limiting step. A fourth enzyme, an NADH:FMN oxidoreductase, regenerates the reduced flavin cofactor, $FMNH_2$, and reduced pyridine, NADH, required in the first three oxidation steps of the pathway.[8] This oxidoreductase is known as the DszD enzyme since it is the fourth enzyme required for the desulfurization pathway *in vivo*.

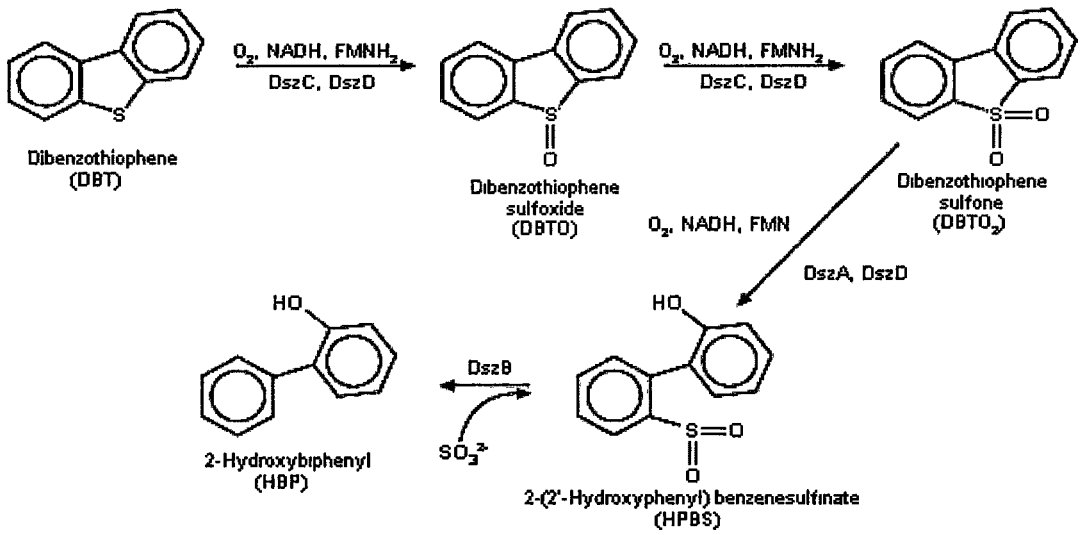

Figure 1. The "4S" pathway for the bacterial desulfurization of DBT and its derivatives. The key enzymes in the pathway are dibenzothiophene monooxygenase (DBT-MO), a tetramer encoded by the *dszC* gene, dibenzothiophene sulfone monooxygenase ($DBTO_2$-MO), a dimer encoded by the *dszA* gene, HPBS desulfinase, a monomer encoded by the *dszB* gene, and an NADH:FMN oxidoreductase encoded by the *dszD* gene.

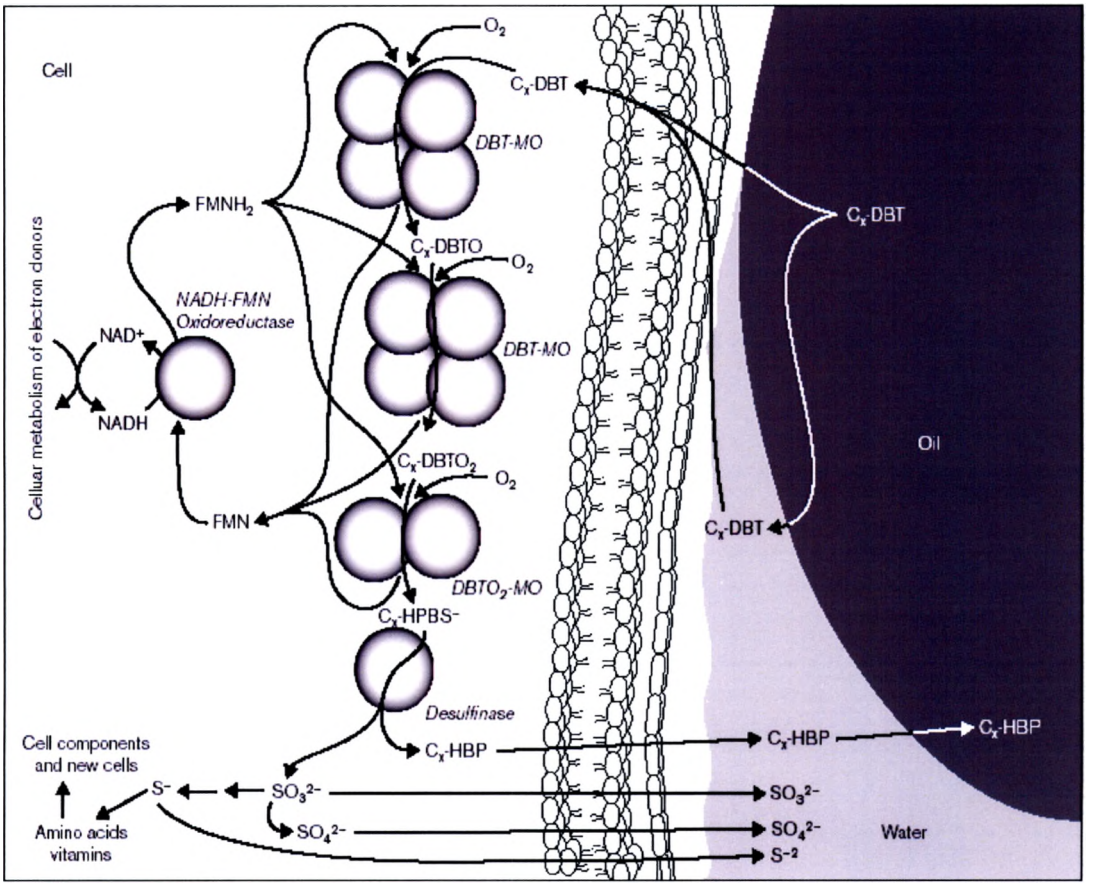

Figure 2. Conceptual diagram of some of the steps in the "4S" desulfurization pathway (see Figure 1). The enzymes involved in this pathway have their quaternary structures represented by spheres. Alkylated derivatives of each compound are represented by the (C_x) prefix.[6]

The molecular characterization of the DNA region that encodes the DBT desulfurization operon of IGTS8 has been reported. A single operon containing three open reading frames (ORFs) is involved in the conversion of DBT to HBP.[19] These three ORFs contain the genes *dszA*, *dszB*, and *dszC*,[19] and reside on a 150 kb endogenous plasmid in IGTS8.[25,26] The order of designation indicates the 5'→ 3' arrangement in the *dszABC* gene cluster.[27] A 385 base pair (bp) fragment located upstream from the *dszA* gene contains both

the promoter and regulatory regions for this operon.[28] Potential ribosome binding sites are present in this 385 bp fragment as well. The stop codon of *dszA* and the initiation codon of *dszB* overlap, indicating that there may be translational coupling of these two genes.[19] Between the *dszB* and *dszC* genes there is a 13 bp gap. Repression of this operon has been observed in the presence of sulfate, methionine and cysteine. The method of repression is believed to be negative feedback with expression only in times of sulfur salvaging. Expression levels of the *dszB* gene are significantly lower than that of the other genes in the *dsz* gene cluster, reiterating the point that its enzyme product, DszB, is the limiting factor for the dsz pathway. The *dszD* gene is not found within the *dszABC* gene cluster and is believed to be under a separate regulatory control mechanism.[29]

Until recently, most research into the "4S" desulfurization pathway has been done on the genera *Rhodococcus*. Currently, many studies are being conducted on other bacterial genera that utilize this dsz pathway.[21,22] Of particular interest is the bacterium *Nocardia asteroides* sp. strain A3H1 (further referred as A3H1) due to its close similarity to IGTS8. The metabolic pathway of DBT degradation to HBP is identical to IGTS8.[22] Another similarity is that these two bacteria share 89.9% homology in the *dszC* gene.[30] DBT-monooxygenase from A3H1 has a higher substrate affinity for otherwise poorly converted, complex alkylated derivatives of dibenzothiophene (C_x-DBTs).[30] Homology has been seen in the other two genes in the *dszABC* gene cluster as well. These characteristics are of particular interest to researchers studying the dsz pathway because many strategies have been developed recently that allow researchers to genetically manipulate bacterial species that have similar phenotypes.

Protein engineers are now looking at taking advantage of genetic recombination between similar species with distinct phenotypes in order to try to directly evolve proteins that have enhanced functionality. Traditionally, site-directed mutagenesis has been the method employed for enhancing the kinetics of enzymes, whether the need was altered specificity for a substrate or enhanced catalytic rate. Site-directed mutagenesis is considered the "rational" approach to protein engineering. This method has proven useful in determining amino acids involved in the active sites of countless enzymes.[31] In order to carry out "rational" protein engineering, structural information based on modeling is utilized to determine a specific amino acid that is involved in the function of choice. The codon is changed to one coding for a different amino acid and the effects of the mutation on protein function are tested. However, changing one specific amino acid to a handful of other specific amino acids is labor intensive and the chance of significantly enhancing the properties of an enzyme is low.[32] Adding to the problem is the fact that many amino acid residues that directly affect the rate of catalysis are found far from the active site catalytic residues.[33]

Directed evolution is seen as an alternative to the traditional methods of protein engineering. Often referred to as the "irrational" approach to protein engineering, many research groups are now using this technology to effectively enhance the kinetics of enzymes. Several methods, including random mutagenesis PCR[34] and DNA shuffling[35], have been utilized to effectively evolve enzymes. Directed evolution involves the generation of a genetic mutant library followed by subsequent screening techniques to determine which recombined genotypes show the enhanced phenotypic function. A rapid and sensitive screen is necessary due to the vast number of mutants that are generated by this process.

The dsz pathway and specifically the DszB enzyme is an optimal target for directed evolution studies. The DszB enzyme catalyzes the rate-limiting step in the dsz pathway and has low expression levels. This makes the DszB enzyme a good candidate for mutagenesis and *in vitro* directed evolution. If the specificity or the catalysis of this enzyme could be enhanced several fold over the wildtype, then desulfurization of DBT could be accomplished efficiently and the use of BDS and biocatalysts would become feasible at the industrial level. In this research, random mutagenesis in the form of error-prone PCR was used to generate a mutant library of the *dszB* gene from A3H1. The mutant genotypes were then cloned into an expression vector and transfected into competent *E. coli* cells. The generated library was characterized by sequencing. Two different activity screens were tested and protein expression levels were measured.

CHAPTER II

MATERIALS

All chemicals and reagents used were molecular biology grade or better and were purchased from Fisher Scientific (Pittsburgh, PA), Sigma-Aldrich Corporation (St. Louis, MO), Mallinckrodt, Inc. (Paris, KY), AMRESCO Inc. (Solon, OH) or EMD Chemicals, Inc. (Gibbstown, NJ). Bacto™ agar, Bacto™ tryptone, and Bacto™ yeast extract were purchased from Difco Laboratories (Sparks, MD). Ampicillin sodium salt was purchased from AMRESCO Inc. (Solon, OH). Macroscale measurements of powdered reagents were carried out using a Mettler College 300 Balance from Mettler-Toledo International, Inc. (Columbus, OH). Microscale measurements of powdered reagents were carried out using an American Scientific LLC (Columbus, OH) S/P® 20 Electronic Microbalance. All solutions and media were pH equilibrated using an Orion 250A Portable pH Meter purchased from Thermo Electron Corporation (Waltham, MA). A Corning, Inc. (Cambridge, MA) Laboratory Stirrer/Hot Plate was used for all solution mixtures. Fifteen and fifty milliliter (mL) screw cap centrifuge tubes were purchased from VWR International (Buffalo Grove, IL). Microcentrifuge tubes, both 0.7 mL and 1.5 mL, along with 0.2 mL PCR tubes were purchased from VWR International (Buffalo Grove, IL) and Ambion, Inc. (Austin, TX). All micropipetting was done using either VWRBrand® Automatic Variable Volume

Micropipets from VWR International (Buffalo Grove, IL) or Oxford® Autoclavable Micro Pipettes from Nichiryo America, Inc. (Flanders, NJ). All pipette tips were purchased from VWR International (Buffalo Grove, IL). Frozen bacterial cultures were kept in 4 mL Cryogenic Vials from Corning, Inc. (Cambridge, MA) and were placed in a -80°C freezer from Harris Manufacturing Company (Asheville, NC).

Microcentrifuge tubes, Oxford® micropipettes, solutions, reagents and media were sterilized in a Hirayama HICLAVE HV-50 autoclave from Amerex Instruments, Inc. (Lafayette, CA). Heat-sensitive aqueous solutions were sterilized using 0.2 μm Surfactant Free Cellulose Acetate (SFCA) syringe filters from Corning, Inc. (Cambridge, MA) and organic solutions were sterilized using Nalgene™ 0.2 μm Nylon syringe filters from Nalge Nunc International (Rochester, NY). An AirGard™ 201 Laminar Airflow Workstation provided a sterile work environment and was purchased from NuAire, Inc. (Plymouth, MN). A VWR International (Buffalo Grove, IL) Heated/Cooled Circulating Water Bath was used for all water bath incubations. Cells were both shaken and incubated in a Gyromax™ Orbital Incubator/Shaker from Amerex Instruments, Inc. (Lafayette, CA). Agitation of reaction mixtures was done using a Vortex Maxi Mix® II vortex mixer from Barnstead International (Dubuque, IA). Macroscale centrifugation was done using a Beckman Coulter, Inc. (Fullerton, CA) J2-21 Refrigerated High Speed Centrifuge. An Adams™ Compact II Centrifuge from Beckton Dickinson and Company (Sparks, MD) was used for medium volume (2-15 mL) centrifugation. A HERMLE Z180 M Microcentrifuge from National Labnet Co. (Woodbridge, NJ) was used for all microscale centrifugation.

Plasmid DNA purification was done with either the Wizard® *Plus* Minipreps DNA Purification System from Promega Corporation (Madison, WI) or the QIAprep® Miniprep

System purchased from QIAGEN Inc. (Valencia, CA). A Promega Corporation (Madison, WI) VacMan® Laboratory Vacuum Manifold was also used in plasmid DNA purification. The restriction enzymes, *PstI* and *NcoI*, were purchased from New England Biolabs, Inc. (Beverly, MA). DNA precipitation was done using a DNA110 SpeedVac® coupled with a GP110 Gel Pump™ both from Savant Instruments, Inc. (Holbrook, NY). Purified plasmid DNA was sent off to Davis Sequencing (Davis, CA) for sequence determination.

Agarose gel electrophoresis was carried out using Agarose I purchased from AMRESCO Inc. (Solon, OH). The power supply for gels was an ACCU P O W E R model 300 power supply from VWR International (Buffalo Grove, IL). Horizontal gels were run in a Mini-Sub® Cell GT apparatus from Bio-Rad Laboratories, Inc. (Hercules, CA). Both 100 base pair (bp) and 500 bp ladders were used as molecular weight standards and were purchased from Invitrogen Corporation (Carlsbad, CA). The 100 bp ladder contained bands from 100 bp to 2072 bp. The 500 bp ladder contained bands from 500 bp to 8500 bp.

The wildtype *dszB* gene from *Nocardia asteroides* sp. strain A3H1 was received in a pBAD TOPO® vector, as a gift from Enchira Biotechnology Corporation (The Woodlands, TX). Custom primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and sequencing primers were obtained from Invitrogen Corporation (Carlsbad, CA). Primers are summarized in Table 1. Melting temperatures for each designed primer were determined using OligoAnalyzer version 3.0 from Integrated DNA Technologies, Inc. (Coralville, IA). Error-Prone PCR reactions were conducted using the GeneMorph™ PCR Mutagenesis Kit purchased from Stratagene (La Jolla, CA). PCR was performed in a GeneAmp® PCR System 2400 from Applied Biosystems (Foster City, CA). *Taq* polymerase was purchased from Invitrogen Corporation (Carlsbad, CA). dNTPs were purchased from

Novagen (Madison, WI). The Wizard® PCR Preps DNA Purification System from Promega Corporation (Madison, WI) was used to purify PCR products. The pBAD TOPO® TA Expression Kit and the pBAD/TOPO® ThioFusion™ Expression Kit were used as cloning and expression systems and were purchased from Invitrogen Corporation (Carlsbad, CA). The cell line used for all transfections was One Shot® TOP10 chemically competent *E. coli* cells from Invitrogen Corporation (Carlsbad, CA).

Table 1. Designed primers and sequencing primers

Primer Designation	Primer Orientation	Melting Temperature (°C)	Nucleotide Sequence
A3H1	Forward	73.8	5'-ATGGCAGGCCGCCTCAGCCCCGG-3'
CSA3R	Reverse	72.1	3'-GGCCTTAAAGACTTGTGGTCGGAGT TGGCAGTGGCTACT-5'
Trx Sequencing	Forward	56.0	5'-TTCCTCGACGCTAACCTG-3'
pBAD Sequencing	Reverse	48.0	3'-GGACTATGTCTAATTTAG-5'

Cell rupture by sonication was performed using a Vibra-Cell™ Ultrasonic Processor from Sonics & Materials, Inc. (Newtown, CT). The cell lysis reagent Y-PER® Yeast Protein Extraction Reagent was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Lysozyme was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

All NuPAGE™ Novex Bis-Tris polyacrylamide gel electrophoresis reagents, gels, buffers, and equipment were purchased from Invitrogen Corporation (Carlsbad, CA). An XCell *Sure-Lock*™ Mini-Cell electrophoresis module was used for vertical polyacrylamide gel electrophoresis. NuPAGE™ 4-12% Bis-Tris gels were used for all SDS-PAGE. NuPAGE™ 4x LDS sample buffer, NuPAGE™ 20x MES SDS Running Buffer, and

NuPAGE™ Antioxidant were used for all SDS-PAGE reactions. Mark 12™ Unstained Standard was used as a protein molecular weight standard and was purchased from Invitrogen Corporation (Carlsbad, CA). The Mark 12 Unstained Standard™ has twelve proteins that are spaced at 200 kDa (myosin), 116.3 kDa (β-galactosidase), 97.4 kDa (phosphorylase B), 66.3 kDa (bovine serum albumin), 55.4 kDa (glutamic anhydrase), 36.5 kDa (lactate dehydrogenase), 31.0 kDa (carbonic anhydrase), 21.5 kDa (trypsin inhibitor), 14.4 kDa (lysozyme), 6.0 kDa (aprotinin), 3.5 kDa (insulin B chain), and 2.5 kDa (insulin A chain). The Perfect Protein™ Markers 15-150 kDa was purchased from Novagen (Madison, WI). The Perfect Protein™ Markers molecular weight standard contained seven precisely sized proteins at 150 kDa, 100 kDa, 75 kDa, 50 kDa, 35 kDa, 25 kDa, and 15 kDa. An Orbital Benchtop Shaker from Lab-Line Instruments, Inc. (Melrose Park, IL) was used to gently shake polyacrylamide gels being stained with SimplyBlue™ Safe Stain purchased from Invitrogen Corporation (Carlsbad, CA).

Western blotting (immunoblotting) of polyacrylamide gels was done using a Blot Module coupled with an XCell *Sure-Lock*™ Mini-Cell electrophoresis module. The blotting was done using Sponge Pads purchased from Invitrogen Corporation (Carlsbad, CA), along with Bio-Dot® SF Filter Paper and Trans-Blot® Transfer Medium (0.2 μm nitrocellulose) both from Bio-Rad Laboratories (Hercules, CA). Anti-Thio™ antibody purchased from Invitrogen Corporation (Carlsbad, CA) was used as the primary antibody probe. A secondary antibody, Anti-Mouse IgG (whole molecule)-Alkaline Phosphatase antibody and a specific alkaline phosphate substrate, Sigma *Fast*™ BCIP/NBT, were both purchased from Sigma-Aldrich Corporation (St. Louis, MO). SeeBlue® Prestained Standard and

MagicMark™ Western Protein Standards from Invitrogen Corporation (Carlsbad, CA) were used as protein markers to monitor electrophoretic transfer and development.

Ultraviolet (UV) and visible quantifications were done using a Beckman Coulter, Inc. (Fullerton, CA) DU-7400 Diode Array Spectrophotometer and a SpectraMax® 190 Microplate Spectrophotometer from Molecular Devices Corporation (Sunnyvale, CA). Fluorescent DNA quantitation was done using a Hoechst 33258 dye in a Hoefer™ DyNA Quant™ 200 Fluorometer from Amersham Biosciences (SF) Corporation (San Francisco, CA). Further fluorescent DNA quantitation was completed utilizing the PicoGreen® reagent from Molecular Probes, Inc. (Eugene, OR). This reagent was used in conjunction with an FLx800 Microplate Fluorescence Reader from Bio-Tek Instruments, Inc. (Winooski, VT). A Luminescence Spectrometer LS50-B from PerkinElmer, Inc. (Shelton, CT) was used for all fluorescence assays determining enzyme kinetics. Costar® UV and visible 96-well microplates were purchased from Corning, Inc. (Cambridge, MA). Microfluor® 2 fluorescence 96-well microplates were purchased from DYNEX Technologies, Inc. (Chantilly, VA).

Kodak Digital Science™ Image Station 440 CF from Eastman Kodak Company Scientific Imaging Systems (Rochester, NY) was used to image polyacrylamide gels and agarose gels. A Bio-Rad Gel Doc 1000 from Bio-Rad Laboratories (Hercules, CA) was also used to image agarose gels. Before imaging, agarose gels were viewed on a FOTO/UV® 300 Ultraviolet Transilluminator from Fotodyne, Inc. (New Berlin, WI).

The microplate analysis software used to quantify DNA on the microplate spectrophotometer was SoftMax® Pro version 2.6 from Molecular Devices Corporation (Sunnyvale, CA). Fluorescence DNA quantitation software was KC4™ Signature version 2.7

from Bio-Tek Instruments, Inc. (Winooski, VT). The software used to analyze gel images was either Kodak 1D™ Image Analysis version 3.6.1 from Eastman Kodak Company Scientific Imaging Systems (Rochester, NY) or Multi-Analyst™ version 1.0.1 from Bio-Rad Laboratories (Hercules, CA). Software utilized for kinetic data analysis included EnzFitter version 2.014.0 from Biosoft (Cambridge,UK) and Microsoft Excel from Microsoft Corporation (Redmond, WA). The FLWinLab™ software version 3.00 from PerkinElmer, Inc. (Shelton, CT) was used to operate the luminescence spectrometer. Plasmid DNA sequence data were viewed and manipulated using MacVector™ version 7.0 sequence analysis software from Oxford Molecular Group, Inc. (Campbell, CA). The ClustalW program within MacVector® was used for multiple sequence alignment of nucleotides and proteins.

CHAPTER III

METHODS

CELL CULTURE TECHNIQUES

Cell Growth

All transfected bacteria were grown using Luria-Bertani media (LB) media containing 100 μg/mL ampicillin. One liter of LB media contains 950 mL distilled, deionized water (ddH_2O), 10 g Bacto™ tryptone, 5 g Bacto™ yeast extract, and 10 g NaCl. The pH was adjusted to 7.0 with 3 M NaOH and the final volume was brought up to 1 L with distilled, deionized water (ddH_2O).[36] When plates were needed, 15 g of Bacto™ agar was added to this recipe before sterilization. Ampicillin was added to cooled LB media after autoclave sterilization. Ampicillin (amp) was made by dissolving 2.5 g of ampicillin sodium salt into 50 mL of ddH_2O and then filtering the resulting stock solution (50 mg/mL) with a 0.2 μm SFCA syringe filter. Isolated bacterial colonies were selected from plates, plucked with sterile inoculating loops and grown in culture tubes containing 5 mL LB broth and 100 μg/mL amp. These inoculated culture tubes were incubated in an orbital shaker set at 200 rpm and 37°C for 24 hours. For batch culture, 250 mL Erlenmeyer flasks were used containing 50 mL LB media with 100 μg/mL amp. Culture flasks were inoculated with 100 μL bacterial culture and were incubated in an orbital shaker (200 rpm) at 37°C for 48 hours.

Frozen Permanents

Isolated colonies were selected from LB + amp agar plates, inoculated in 5 mL LB liquid media containing 100 μg/mL amp, and incubated in an orbital shaker (200 rpm) for 24 hours at 37°C. Four milliliters (mL) of each cell culture was added to a 15 mL screw cap centrifuge tube. One-hundred forty microliters (μL) of sterile filtered dimethyl sulfoxide (DMSO) was added to 4 mL of cell culture, vortexed, and placed on ice for 15 min. An additional 140 μL of DMSO was added after incubation and the solution was vortexed again. The bacterial cultures were then aliquoted in 4 mL cryovials and stored at -80°C.

MOLECULAR BIOLOGY TECHNIQUES

Agarose Gel Electrophoresis

Stock 50x Tris-Acetate-EDTA (TAE) buffer was prepared using 242 g Tris-base, 57.1 mL glacial acetic acid, and 100 mL 0.5 M EDTA. The pH was adjusted to 8.0 and final volume was brought to 1 L with ddH_2O.[36] The stock 50x TAE buffer was diluted to 1x using ddH_2O. A 40 mL, 0.8% agarose gel was prepared using 0.32 g agarose I and 40 mL 1x TAE buffer in a 125 mL Erlenmeyer flask. The gel solution was heated in a microwave three times on high setting for 30 seconds each time, swirling after each heating. The gel was then poured into a leveled gel molding rig and allowed to cool. After congealing, the gel was placed into a horizontal gel electrophoresis apparatus, immersed in 1x TAE buffer, and loaded with prepared samples. Loading samples were prepared by mixing sample DNA with 6x dye (40% sucrose, 0.25% bromophenyl blue, 0.25% xylene cyanol FF, and ddH_2O to 10 mL).[36] A 100 base pair (bp) ladder, stock concentration 1 μg/μL, was used as a molecular

weight standard for 100 to 2,200 base pairs. A 500 bp ladder, stock concentration 1 μg/μL, was used as a molecular weight standard for 500 to 8500 base pairs. Both ladders were diluted to a working concentration of 0.1μg/μL by adding 3 μL ladder DNA (1 μg/μL), 5 μL 6x dye, and 22 μL sterile TE buffer to a sterilized microcentrifuge tube. The agarose gel was electrophoresed at 80 volts for 60-70 minutes. After electrophoresis, the gel was immersed in a 10 μg/mL ethidium bromide (EtBr) solution (30 μL of 10 mM EtBr in 300 mL of ddH_2O) for 20 minutes to incur staining. After staining, the gel was placed in a 300 mL distilled water (diH_2O) solution for 20 minutes to destain. The gel was then viewed on a transilluminator prior to being imaged.

Polymerase Chain Reaction

All PCR reactions were run according to the following conditions. The following reagents were added, in order, to a PCR tube: 5 μL of 10x PCR buffer, 5 μL of 25 mM $MgCl_2$, 100-200 ng of each primer (A3H1 and CSA3R), 1 μL of 10 mM dNTP mix, 70 ng DNA template, 3% (v/v) of DMSO, and sterile ddH_2O to 49 μL. The "hot start" method was employed for all PCR experiments. The "hot start" method is initiated by heating the PCR samples to 94°C and pausing the PCR program. During the pause, 1 μL of *Taq* polymerase (5 U/μL) was added to each sample bringing the final volume in each tube to 50 μL. The tubes were immediately placed back into the thermocycler and the PCR program was continued. The first denaturation step heated the samples at 94°C for 5 minutes. The run was continued for 35 cycles under the following conditions: 94°C for 15 seconds, 52°C for 30 seconds, and 72°C for 3 minutes. Upon completion of the cycles, the reaction was held at 72°C for 7 minutes to ensure full length extension of PCR products. The reaction products

were purified using the protocol outlined for the Wizard® PCR Preps Purification System[37]. Purified PCR products were qualitatively analyzed by agarose gel electrophoresis. Remaining PCR reaction products were stored at 4°C.

$MnCl_2$ Error-Prone PCR

High mutation frequency parameters outlined in the GeneMorph® instruction booklet[38] were followed for the $MnCl_2$ error-prone PCR. No Mutazyme® reaction buffer or Mutazyme DNA Polymerase® was used for the $MnCl_2$ error-prone PCR reactions. The following reagents were added, in order, to a PCR tube: 5 μL of 10x PCR buffer, 5 μL of 5 mM $MnCl_2$, 0.25 μL of each primer, A3H1 and CSA3R (250 ng/μL each), 1 μL of 40 mM dNTP mix, 473 pg DNA template, 3% (v/v) DMSO, and sterile ddH_2O to 49 μL. The "hot start" method of enzyme addition was employed. The samples were brought up to 96°C and paused. During the pause, 1 μL of *Taq* polymerase (5 U/μL) was added to each PCR tube bringing the final reaction volume to 50 μL. The PCR tubes were immediately placed back in the thermocycler and the pause was disengaged allowing the PCR tubes to run through the thermocycling conditions. The initial denaturation step was set at 96°C for 30 seconds. The run was then continued for 30 cycles under these conditions: 96°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The reaction was then held at 72°C for 10 minutes to finish extension of PCR products. The reaction products were purified with the protocol from the Wizard® PCR Preps Purification System[37] and analyzed by agarose gel electrophoresis. Remaining PCR reaction products were stored at 4°C.

TOPO® Cloning and Transfection

PCR products were ligated into the pBAD TOPO® vector (Figure 3) and the pBAD/Thio TOPO® vector (Figure 4) using the TOPO® cloning reaction protocol.[39,40] These systems take advantage of the *araBAD* operon. The P_{BAD} promoter is both positively and negatively regulated by the product of the *araC* gene.[41,42,44] AraC is a transcriptional regulator that forms a complex with L-arabinose. In the absence of L-arabinose the AraC dimer contacts the O_2 and I_1 half sites of the *araBAD* operon, forming a 210 bp DNA loop.[39,40] A schematic of the transcriptional regulation of the P_{BAD} promoter is shown in Figure 5. Additionally, these vector cloning systems utilize topoisomerase I which mediates directional cloning of double strand DNA. Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5-CCCTT in one strand.[45] The energy from the phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase.[46] A diagram of the phosphodiester cleavage is shown in Figure 6. For the TOPO® cloning reaction, 4 μL of PCR product, 1 μL Salt Solution (1.2 M NaCl, and 0.06 M $MgCl_2$) and 1 μL TOPO® vector were mixed and incubated at room temperature for 5 minutes. The reaction mixture was then placed on ice until the transfection reaction was performed. The ligated vector was transfected into One Shot® TOP10 chemically competent *E. coli* by gently mixing 2 μL of the TOPO® cloning reaction product with one vial of cells and incubating the mixture on ice from 5 to 30 minutes. The cells were then heat shocked for 30 seconds in a 42°C water bath. The tubes were transferred back to

ice and 250 μL of room temperature SOC media was added. SOC media was prepared using 950 mL ddH_2O, 20 g Bacto™ tryptone, 5 g Bacto™ yeast extract, 5 g NaCl, and 10 mL of 250 mM KCl. The media was adjusted to pH 7.0 and brought to 1 L with ddH_2O. The media was autoclaved and after sterilization, 2 mL of sterile 1 M glucose was added.[36] After addition of SOC media, the tubes were then shaken horizontally at 200 rpm and 37°C for one hour. The now transfected cells were plated on LB plates containing 100 μg/mL amp. Two different volumes of the transfection reaction were plated, 50 μL and 150 μL, and incubated overnight at 37°C. From the resulting plate, isolated colonies were selected and grown overnight in culture tubes containing 5 mL LB broth and 100 μg/mL amp. Plasmid DNA was purified from these bacterial cultures and qualitatively analyzed by agarose gel electrophoresis. Further analysis of each plasmid was done using PCR reactions and restriction digests.

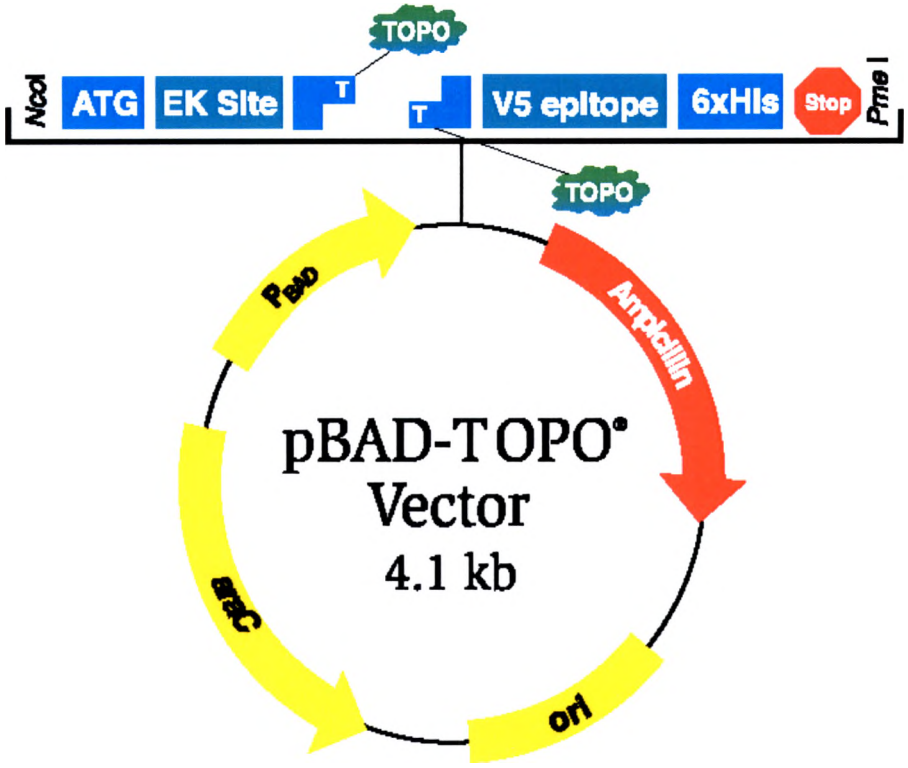

Figure 3. Map of pBAD TOPO® cloning vector. The *araBAD* promoter (P_{BAD}) provides tight, dose-dependent regulation of heterologous gene expression.[43] The *araC* gene encodes the regulatory protein for tight regulation of the P_{BAD} promoter.[42,44] This vector also includes an ampicillin resistant gene (β-lactamase), an Enterokinase (EK) recognition site, a C-terminal V5 epitope tag and a C-terminal polyhistidine region.[39]

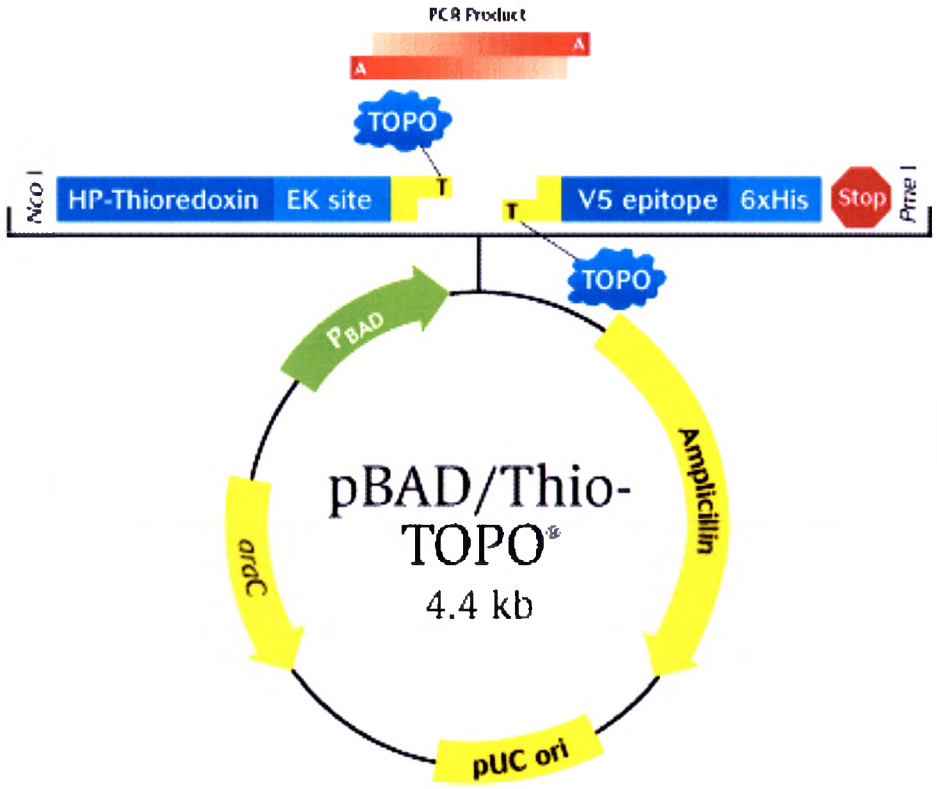

Figure 4. Map of pBAD/Thio TOPO® cloning vector. The *araBAD* promoter (P_{BAD}) provides tight, dose-dependent regulation of heterologous gene expression.[43] The *araC* gene encodes the regulatory protein for tight regulation of the P_{BAD} promoter.[42,44] A pUC origin lies between bases 854-871. The vector also includes an ampicillin resistant gene (β-lactamase), an Enterokinase (EK) recognition site, a C-terminal V5 epitope tag and a C-terminal polyhistidine region. The Histidine Patch (HP)-Thioredoxin peptide on the N-terminus of the fusion protein allows for one step affinity purification of the protein.[40]

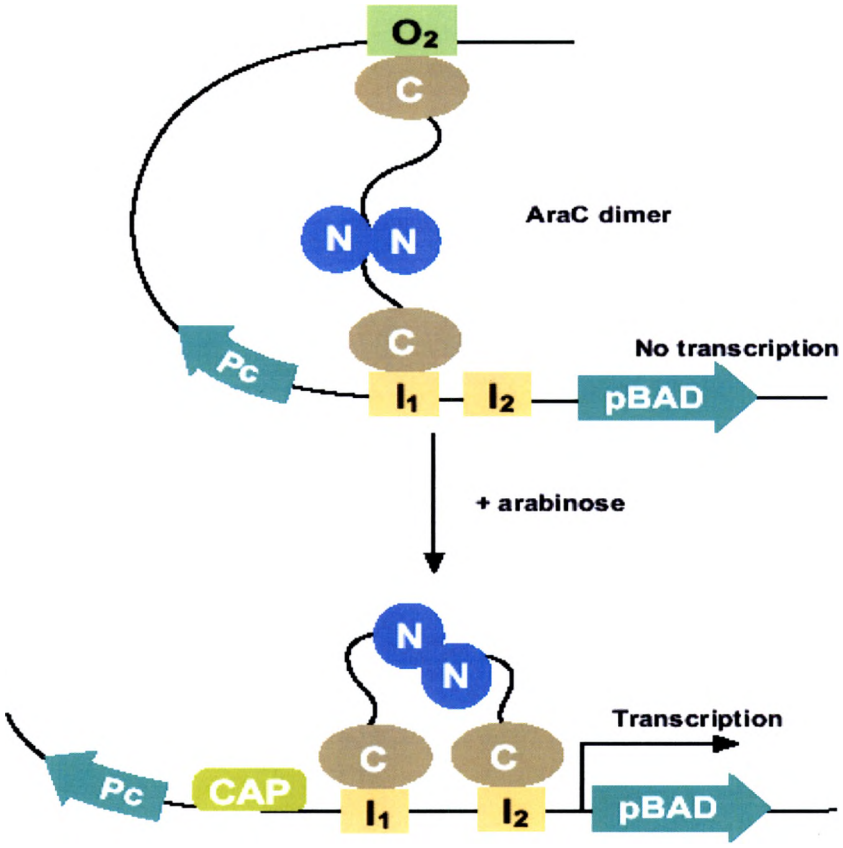

Figure 5. Schematic showing the regulation of the P_{BAD} promoter. For maximal transcriptional activation arabinose binds to AraC and causes the protein to release the O_2 site. This releases the DNA loop and allows transcription to begin. Additionally, the cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC at I_1 and I_2 allowing transcription to begin.[39,40]

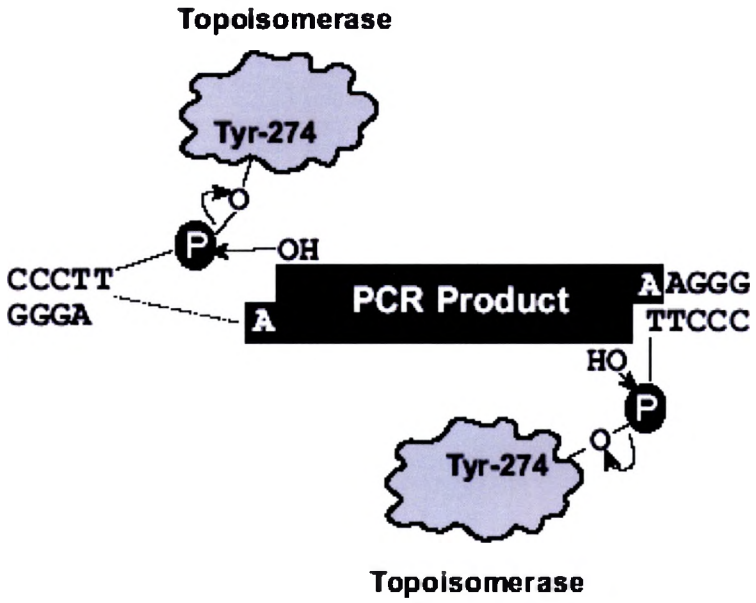

Figure 6. Topoisomerase chemical cloning reaction. Diagram of the phosphodiester bond cleavage by the 5' hydroxyl of the PCR product upon ligation into the vector.[39,40]

Plasmid DNA Purification

All plasmids were purified from 5 mL overnight bacterial culture using the Wizard® *Plus* Minipreps DNA Purification System[47] or the QIAprep® Miniprep System[48] and subsequently analyzed by agarose gel electrophoresis. Both the Wizard® and the QIAprep® systems utilize alkaline lysis of the bacterial cells. The lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step followed by the purification of double stranded plasmid DNA using minicolumns. The minicolumns were fabricated with either a nitrocellulose or silica-gel membrane. The purified plasmid DNA was qualitatively analyzed by agarose gel electrophoresis.

Plasmid Designation

The wildtype *dszB* gene, from *N. asteroides* strain A3H1, ligated into the pBAD TOPO® vector, was kindly provided by Enchira Biotechnology Corporation. This plasmid was designated pA3H1. Additionally, the wildtype *dszB* gene was cloned into the pBAD/Thio TOPO® vector and the product was designated pAThio. Newly generated plasmids were named pNOGO, pALIB(X), and pATHLIB(X). A description of all plasmids used throughout this study is provided in Table 2.

Table 2. Plasmids generated and used in this study

Plasmid	Description	Source
pBAD/Thio	Positive expression control. Control vector expressing a 16 kDa HP-thioredoxin fusion protein	Invitrogen
pNIC	Negative control plasmid. Circularized pBAD TOPO® without gene insert.	This study
pNOGO	Negative control plasmid. 500 bp control PCR product inserted into the pBAD TOPO® vector	This study
pA3H1	Positive control plasmid. Wild-type (*N. asteroides* A3H1) *dszB* gene inserted in the pBAD TOPO® vector	Enchira
pALIB(X)[a]	Experimental plasmid. Mutated A3H1 *dszB* gene inserted into the pBAD TOPO® vector	This study
pAThio	Positive control plasmid. Wild-type (*N. asteroides* A3H1) *dszB* gene inserted into the pBAD/Thio TOPO® vector	This study
pATHLIB(X)[a]	Experimental plasmid. Mutated A3H1 *dszB* gene inserted into the pBAD/Thio TOPO® vector	This study

[a] The X indicates the number assigned to the gene isolated from the library

Restriction Digests

All restriction digests were performed under the following conditions. Each reaction contained 2 μL *PstI* and/or 2 μL *NcoI* restriction endonuclease, 6 μL 5x KGB buffer (100 mM potassium glutamate, 1.0 M Tris-acetic acid (pH 7.6), 1.0 M Mg^{+2} acetic acid, 10 mg/mL BSA and 14.3 M β-mercaptoethanol), 6 μL plasmid DNA, and sterile ddH_2O to a final volume of 30 μL. The reactions were incubated in a 37°C water bath for 3 hours. Upon completion of the incubation, the digested DNA was ethanol (EtOH) precipitated. The DNA precipitation involved the addition of 75 μL (2.5x digest reaction volume) of 100% EtOH chilled at -20°C. The resulting mixture was vortexed and centrifuged at 13,000 rpm at room temperature for 10 minutes. The supernatant was then pipetted off and the tube was placed in a SpeedVac® for 10 minutes to evaporate remaining EtOH. Seven microliters of sterile Tris-EDTA (TE) buffer (0.12 g Tris, 0.03 g EDTA and ddH_2O to 100 mL, pH 8.0)[36] was added to the precipitated DNA along with 2 μL of 6x dye. The resulting mixture was then loaded and analyzed by agarose gel electrophoresis.

Pilot Expression

The protocol for the pilot expression was obtained from the pBAD/TOPO® ThioFusion™ Expression Kit.[40] A single transfected *E. coli* colony containing either the pBAD/Thio expression control plasmid or the wildtype pAThio plasmid from the cloning and transfection reactions were inoculated in 2 mL of LB broth containing 100 μg/mL amp and incubated overnight at 37°C with shaking (200 rpm) to an $OD_{600} \sim 2$. The next day, five labeled tubes containing 10 mL LB and 100 μg/mL amp were each inoculated with 100 μL of the overnight culture. The cultures were grown for three hours until they reached mid-log

phase (OD_{600} ~ 0.5). One milliliter of cells from each tube was removed, pelleted by centrifugation and the supernatant was aspirated. These cell pellets were labeled as the zero time point and stored at -20°C. A 10-fold serial dilution of 20% arabinose in sterile ddH_2O was used for induction. Arabinose was added to the five 9 mL cultures for both sets of samples. The final arabinose concentrations in tubes 1 through 5 were 0.00002%, 0.0002%, 0.002%, 0.02% and 0.2 %, respectively. The cultures were incubated for three hours at 37°C after addition of arabinose. At four hours a 1 mL aliquot was taken from each tube, pelleted by centrifugation and the supernatant was removed by aspiration. The samples were allowed to incubate for an additional six hours in a 37°C shaking incubator. Another 1 mL aliquot was removed from each tube at ten hours after induction. The aliquot was pelleted by centrifugation and the supernatant was removed by aspiration. All the cell pellets were kept in the -20°C freezer until SDS-PAGE analysis.

PROTEIN CHEMISTRY TECHNIQUES

Pilot Expression Cell Lysis

The cell pellets from the pilot expression experiment were thawed and resuspended in 500 μL of Lysis Buffer (50 mM potassium phosphate pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100 and 10 mM imidazole). The samples were placed on ice and sonicated for 10 seconds at 100 amps. The remaining solution was centrifuged at 13,000 rpm at room temperature for 1 minute to pellet the cell debris. The supernatant was transferred to a new microcentrifuge tube and was prepared for analysis by SDS-PAGE.

Y-PER® Cell Lysis

Yeast Protein Extraction Reagent, Y-PER®, uses a mild detergent formula to lyse bacterial cells. Overnight bacterial cell culture was pelleted by centrifugation at 3200 rpm for 5 minutes at 4°C. The cell pellet was immediately resuspended with the Y-PER® Plus reagent. For *E. coli* cells 2.5-5.0 mL of the Y-PER® Plus reagent was used per 1 g of pelleted cells. A few crystals of protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF), were added to the sample during lysis to inhibit protein degradation. The mixture was agitated for 20 minutes at room temperature. The cellular debris was collected by centrifugation at 3200 rpm for 5 minutes. Ten microliters of the supernatant was used for SDS-PAGE analysis. The remaining supernatant was stored at -20°C.

Pro-Bond™ Cell Lysis

The Preparation of Bacterial Cell Lysate-Native Conditions was used as outlined in the ProBond™ Purification System Protocol.[49] The stock 5x Native Purification Buffer contains 250 mM $NaHPO_4$ and 2.5 M NaCl. This solution was diluted to a working concentration of 1x before use. Batch cultures (50 mL) induced with 0.2% arabinose for 24 hours were harvested by centrifugation at 3200 rpm with a benchtop centrifuge at 4°C. The supernatant was decanted and the cell pellet was resuspended in 8 mL of Native Binding Buffer, pH 8.0. Native binding buffer contains 30 mL 1x Native Purification Buffer and 100 μL of 3 M imidazole (pH 6.0). To the resuspended cells, 8 mg of lysozyme was added and the solution was incubated on ice for 30 minutes. A sonicator was used to sonicate the chilled solution using six 10-second bursts at 100 amps with a 10-second cooling period between each burst. The resulting slurry was centrifuged at 3200 rpm at 4°C for 15 minutes

to pellet the cell debris. The supernatant was transferred to a 15 mL screw cap centrifuge tube. Ten microliters of the supernatantwas removed and prepared for SDS-PAGE analysis. The remaining supernatant was stored at -20°C.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The pilot expression supernatant from the sonicated samples was mixed with an equal amount of 2x lithium dodecylsulfate (LDS) sample buffer diluted from the 4x stock (1.09 M glycerol, 141 mM Tris-Base, 106 mM Tris-HCl, 73 mM LDS, 0.51 mM EDTA, 0.22 mM Serva Blue G-250, and 0.175 mM phenol red) and heated for 5 minutes in a 70°C water bath. Batch culture cell lysate samples were prepared using reducing conditions which included 62 μL of supernatant, 25 μL of 4x LDS sample buffer, 3 μL sterile ddH_2O and 10 μL of 0.5 M dithiothreitol (DTT) to a final volume of 100 μL. The samples were denatured by heating at 70°C for 5 minutes. All prepared samples and either the Perfect Protein™ Markers or Mark 12 Unstained Standard™ were loaded onto a pre-cast NuPAGE® 4-12% Bis-Tris polyacrylamide gel. Ten microliters of each prepared sample or 5 μL of the molecular weight standard was loaded into the wells of the polyacrylamide gel. The gel was run at 200 volts for 35 minutes in 1x NuPAGE® MES SDS running buffer diluted from the 20x stock (500 mM 2-(*N*-morpholino)ethane sulfonic acid, 50 mM Tris-base, 3.5 mM SDS, 1 mM EDTA, pH 7.3). Under reducing conditions, polyacrylamide gels were electrophoresed with 200 mL of running buffer containing 500 μL of NuPAGE® Antioxidant loaded into the inner chamber. The outer chamber was filled with 600 mL of 1x NuPAGE® MES SDS running buffer. After electrophoresis, the gel was removed from the cassette and washed for 5 minutes with diH_2O. The gel was then stained for 1 hour using the SimplyBlue™ Safe Stain

while being agitated on a benchtop orbital shaker. After staining, the gel was destained in a diH_2O solution overnight while being rotated by an orbital shaker. The gel was then imaged and submitted for band analysis.

Western Immunoblot

The immunoblotting protocol from the Anti-Thio™ Antibody manual[50] was used for the Western Blot. Protein samples from expression controls and arabinose induced experimental samples were prepared and loaded onto a pre-cast NuPAGE® 4-12% Bis-Tris polyacrylamide gel. A SeeBlue® Prestained Standard was used to monitor electrophoretic transfer of proteins to the nitrocellulose membrane and MagicMark™ Western Protein Standards was used to monitor membrane development. The gel was electrophoresed for 35 minutes at 200 volts. The proteins were then transferred to a 0.2 μm nitrocellulose membrane electrophoretically. The nitrocellulose membrane, along with the blotting pads were soaked in transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3) before electrophoresis. The sandwich containing the gel, blotting pads, and filter paper was electrophoresed at 20 volts for 60 minutes. The membrane was then incubated, for one hour at room temperature with shaking, in blocking buffer (3 % (w/v) Non-fat dry milk in transfer buffer) followed by one hour incubation with shaking in a 1:5000 Anti-Thio™ antibody solution. The enzyme-linked secondary antibody was diluted 1:2000 with transfer buffer and the membrane was immersed in this solution for one hour at room temperature with shaking. The protein bands on the membrane were visibly detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

ANALYTICAL TECHNIQUES

DNA Quantitation

DNA quantitation of purified plasmid DNA was determined by ultraviolet (UV) absorbance at 260 nm in a 1 mL quartz cuvette on a Beckman DU 7400 Diode Array Spectrophotometer and in a 96-well microplate using a SpectraMax® 190 Microplate Spectrophotometer. Absorbance values were used to calculate DNA concentration according to the following equation: (A_{260}) x (50 μg/mL) x dilution factor. Fluorescent quantitation of plasmid DNA concentration was determined by using the Hoechst 33258 assay on the Hoefer™ DyNA Quant™ Fluorometer with fixed wavelengths at λ_{ex} = 365 nm (excitation wavelength) and λ_{em} = 460 nm (emission wavelength). Plasmid DNA was diluted in a glass cuvette 1:1000 with the low range (A) assay solution (0.1 μg/μL Hoechst H 33258 dye in 1x TNE). Tris-Na^{+2}-EDTA (TNE) 10x stock solution contained 100 mM Tris, 10 mM EDTA $Na^{+2} \cdot 2H_2O$, and 2M NaCl in a final volume of 1 L. The 10x TNE buffer was adjusted to pH 7.4 with concentrated HCl. The Hoechst dye stock solution (1 mg/mL) was made by dissolving 10 mg of Hoechst H 33258 into 10 mL of diH_2O. Further fluorescence plasmid DNA quantitation was done using the PicoGreen® reagent. In 0.7 μL microcentrifuge tubes, the PicoGreen® DNA standard was 2-fold serially diluted with sterile 1x TE. Ten dilutions of this standard DNA were added to a 96-well microplate. These dilutions were used to generate the standard curve. One microliter of each purified plasmid DNA sample was added to 99 μL of sterile TE in a microcentrifuge tube and this 100 μL mixture was transferred to a designated well on a 96-well microplate. In a dark room, 0.5 μL of PicoGreen® and 99.5μL of 1x TE was added to each standard and sample well. The resulting

mixtures were incubated at room temperature in a dark room for 5 minutes. The microplate was then covered and transported to the FLx800 Microplate Fluorescence Reader for DNA quantification using the following wavelengths, λ_{ex} = 485 nm and λ_{em} = 528 nm.

Gene Sequencing

Plasmids were purified using both the Wizard® *Plus* Minipreps DNA Purification System and the QIAprep® Miniprep System. Plasmid DNA quantitation was determined using the PicoGreen® fluorescent reagent. Plasmids were concentrated to 150 ng/μL by EtOH precipitation and resuspended in 8 μL sterile ddH_2O. Eight microliters of the sample, 20 μL of 0.1 μg/μL Trx forward sequencing primer and 20 μL of 0.1 μg/μL of the pBAD reverse sequencing primer were sent to Davis Sequencing. Additionally, the custom reverse PCR primer, CSA3R, was sent in a 20 μL volume at a concentration of 0.12 μg/μL. The sequence data were analyzed with the MacVector™ program. Sequence alignments and mutation characterization was determined for each mutagenized *dszB* gene.

Gibb's Assay

Modification of the previously performed Gibb's assay[51] was used to screen for the formation of 2-hydroxybiphenyl (HBP). Gibb's reagent (10 mg/mL *N* 2,6-Trichloro-*p*-benzoquinoneimine in EtOH)[51] binds to phenol rings and produces blue color at visible wavelength, λ = 610 nm. The standard curve for the product, HBP, was established by setting up a gradient of increasing HBP concentrations from 0 μM to 50 μM in a 96-well microplate. The final concentrations of HBP used in each well were 0.1, 1, 10, 20, 40 and 50 μM with 0, 2.2, 22, 44, 88 and 110 μL of 0.1 mM HBP added to each well, respectively. The

buffer used for this assay was 1 M $NaHCO_3$. Five microliters of the Gibb's reagent (10 mg/mL) was added to each well being assayed. The order of addition of reagents was $NaHCO_3$ buffer, Gibb's reagent, and HBP to bring the final volume in each well to 220 μL. The plate was allowed to incubate for 20 minutes at room temperature and then was read on a microplate spectrophotometer at λ = 610 nm. Each assay was done in triplicate and the resulting data was plotted in Microsoft Excel to give the standard curve of HBP.

Standard Fluorescence Assay

A fluorescence assay was done to measure DszB activity. DszB converts 2-(2'-hydroxyphenyl) benzene sulfinate (HPBS) to 2-hydroxybiphenyl (HBP). HBP formation can be directly monitored by fluorescence. HPBS was synthesized by mixing 43.2 mg of biphenosultine with 10 mL of 44 mM NaOH overnight. The pH of the soluble product was adjusted to between 8 and 9 with HCl. The resulting 20 mM HPBS solution was diluted to a working concentration of 100 μM for the standard assay. The fluorimeter was set to the following parameters: λ_{ex}= 288 nm, λ_{em} = 414 nm, excitation slit width 5.0 mm, and emission slit width 5.0. Each run was done in triplicate in a Microfluor® 96-well microplate. The assay contained 140 μL of 10x Buffer A (250 mM NaH_2PO_4 and 1 M NaCl, pH 7.4), 10 μL of 100 μM HPBS, 20 μL enzyme and diH_2O to 200 μL. Negative controls were missing enzyme or substrate. The microplate was incubated on a plate warmer at 33°C for 3 minutes before being placed in the fluorimeter. Product (HBP) formation was monitored over time by increase in fluorescence. The fluorimeter data was then plotted in Microsoft Excel to determine the rate of HBP formation.

CHAPTER IV

RESULTS & DISCUSSION

Purified pA3H1 Plasmids

The wildtype *dszB* gene from A3H1 was ligated into the pBAD TOPO® vector to generate pA3H1. The pA3H1 plasmid was a gift from Enchira Biotechnology Corporation (The Woodlands, TX). Transfection of pA3H1 into TOP10 *E. coli* cells and purification of pA3H1 is outlined in the Methods section. The purified plasmid ran as a single band as seen on the agarose gel shown in Figure 7.

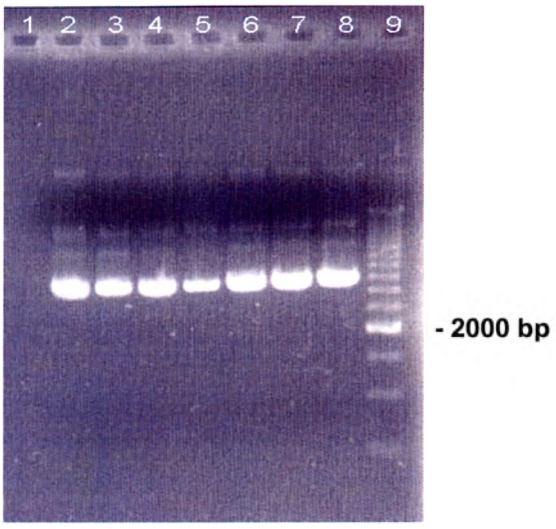

Figure 7. Ethidium bromide stained agarose gel of purified pA3H1 plasmid DNA. Lanes 2-8: pA3H1 plasmid DNA samples; Lane 9: 500 bp ladder.

Isolation of the dszB Gene from pA3H1

The wildtype *dszB* gene was amplified from the pA3H1 plasmid by PCR as described in Methods. The primers, A3H1 and CSA3R (Table 1), were used for gene amplification. The size of the PCR product was qualitatively determined using agarose gel electrophoresis (Figure 8). A band at approximately 1098 bp can be observed in Lane 7. This corresponds to the molecular size of the *dszB* gene.

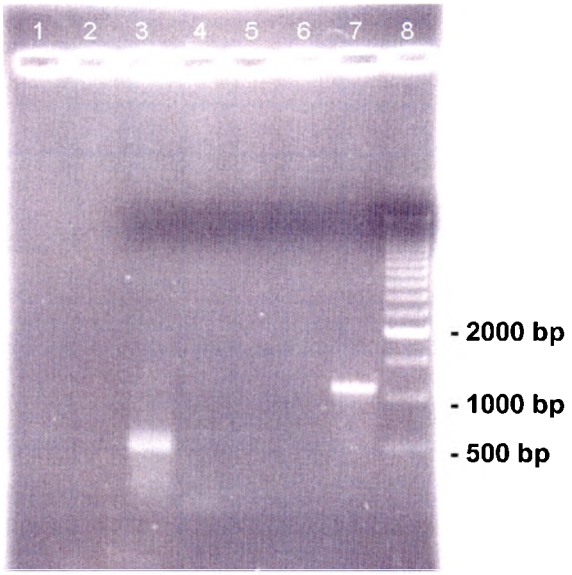

Figure 8. Ethidium bromide stained agarose gel of PCR products using pA3H1 as the template. Lane 3: Positive PCR control (500 bp); Lane 4: Negative PCR control (no template); Lane 7: Product of amplified *dszB* gene from pA3H1; Lane 8: 500 bp ladder.

Error-Prone PCR Experiments

Error-prone PCR experiments were carried out as described in Methods. $MnCl_2$ was used in the reaction instead of $MgCl_2$ to decrease the fidelity of the polymerase reaction. The

wildtype pA3H1 plasmid was used as the template and A3H1 and CSA3R were used as the primers. The product of the PCR experiment was run on agarose gel to determine the product size (Figure 9). Bands are present for the duplicated experimental samples at approximately 1098 bp corresponding to the length of the wildtype *dszB* gene.

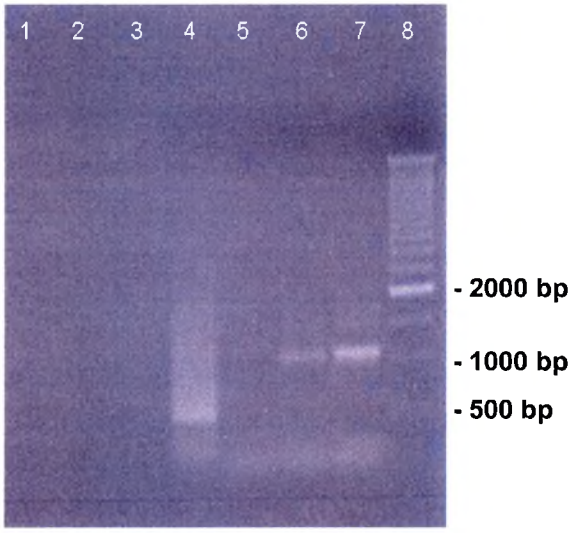

Figure 9. Ethidium bromide stained agarose gel of $MnCl_2$ error-prone PCR. Lane 4: Positive PCR control (500 bp); Lane 5: Negative PCR control (no template); Lane 6-7: A3H1 *dszB*fragments produced by PCR with $MnCl_2$; Lane 8: 500 bp ladder.

Screening of the dszB Mutant Library

The mutagenized fragments were cloned into the pBAD TOPO® vector and transfected into TOP10 competent *E. coli* as outlined in Methods. Two different methods of screening the library were tested, a colorimetric microplate assay and a fluorimetric assay. The colorimetric microplate assay was based on the Gibb's assay.[51] The Gibb's reagent forms a blue color in the presence of a phenol. A standard curve of the HBP product was produced using a modified Gibb's assay as outlined in the Methods. The data from three

trials were averaged and the standard curve was calculated to be $y = 0.0029x + 0.1349$ with an average R^2 value of 0.9902. Once the standard curve of HBP in buffer was determined, a product formation screen using LB media and bacterial cells was tested. This microplate assay could not accurately distinguish between the phenolic substrate, HPBS, and the phenolic product, HBP, in LB media. Due to the assay's low sensitivity, the microplate assay was discontinued.

The fluorescence screen used the standard assay to measure relative activity of mutant DszB enzymes. Bacterial cells were lysed using the Y-PER® protocol as outlined in Methods. The resulting lysate was used to determine relative activity of each mutant relative to the wildtype. The lysate was added to the standard assay mixture in a 96-well microplate and fluorescence was monitored over time. The relative activities were normalized to wildtype and plotted. The normalized relative activities showed an increase in seven out of eighty mutants screened by this microplate assay (data not shown). However, upon further characterization it was determined that the mutants with increased activity did not have the *dszB* gene inserted into their respective plasmid DNAs. This conclusion prompted termination of the activity screen. The regeneration and characterization of a new *dszB* mutant library was undertaken.

Characterization of dszB PCR Products Cloned into pBAD TOPO®

PCR products from the amplification of *dszB* under native and error-prone conditions were cloned into the pBAD TOPO® vector and transfected into TOP10 competent *E. coli* cells as outlined in Methods. Isolated colonies were arbitrarily numbered and screened for gene insertion. The agarose gels in Figures 9-16 show the resulting purified plasmid DNA

from each of the numbered colonies. The plasmids resulting from cloning reactions with the native *dszB* gene and pBAD TOPO® are labeled pA3H1 I through XXII and are listed in Table 3. These plasmids were used to calculate cloning efficiency and generate positive control plasmids. Eleven of the twenty-two purified plasmids listed in Table 3 are shown in Figures 10 and 11. The plasmids resulting from cloning reactions with the mutant *dszB* gene and pBAD TOPO® are labeled pALIB1-60. Lanes containing plasmid with a gene insert have a single band between 3000 and 3500 bp. Lanes containing plasmid without an insert have a single band 2500 and 3000 bp.

Table 3. Purified plasmid DNA from duplicate purification reactions

Plasmid Designation	Cloning Reaction	Contains Insert
pA3H1 I	1	Yes
pA3H1 II	1	Yes
pA3H1 III	1	Yes
pA3H1 IV	1	Yes
pA3H1 V	2	No
pA3H1 VI	3	Yes
pA3H1 VII	3	Yes
pA3H1 VIII	4	No
pA3H1 IX	4	No
pA3H1 X	5	Yes
pA3H1 XI	5	Yes
pA3H1 XII	1	Yes
pA3H1 XIII	1	Yes
pA3H1 XIV	1	Yes
pA3H1 XV	5	Yes
pA3H1 XVI	5	Yes
pA3H1 XVII	5	Yes
pA3H1 XVIII	5	Yes
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pA3H1 XX	5	Yes
pA3H1 XXI	5	Yes
pA3H1 XXII	5	Yes

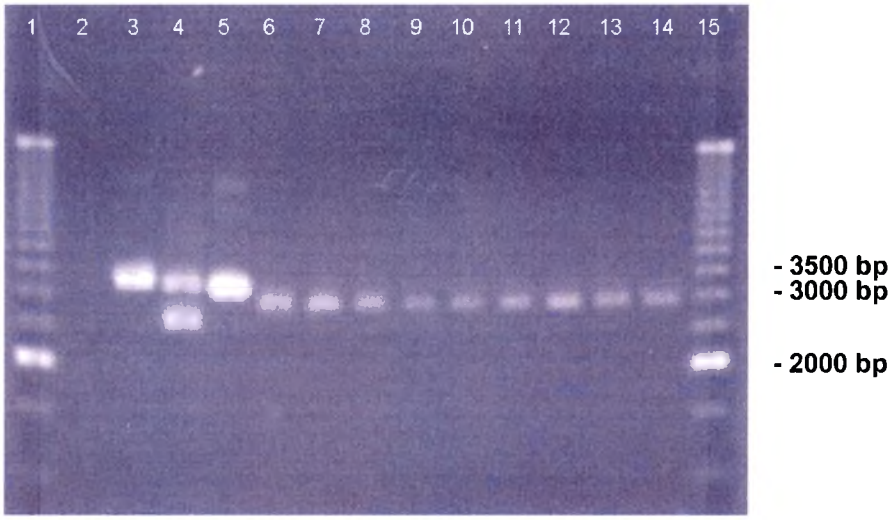

Figure 10. Ethidium bromide stained agarose gel of pA3H1 I-VII and pALIB1-3. Lane 1: 500 bp ladder; Lane 2: pA3H1 (I); Lane 3: pA3H1 (II); Lane 4: pA3H1 (III); Lane 5: pA3H1 (IV); Lane 6: pA3H1 (V); Lane 7: pALIB3; Lane 8: pALIB3; Lane 9: pALIB2; Lane 10: pALIB2; Lane 11: pALIB1; Lane 12: pALIB1; Lane 13: pA3H1 (VI); Lane 14: pA3H1 (VII); Lane 15: 500 bp ladder.

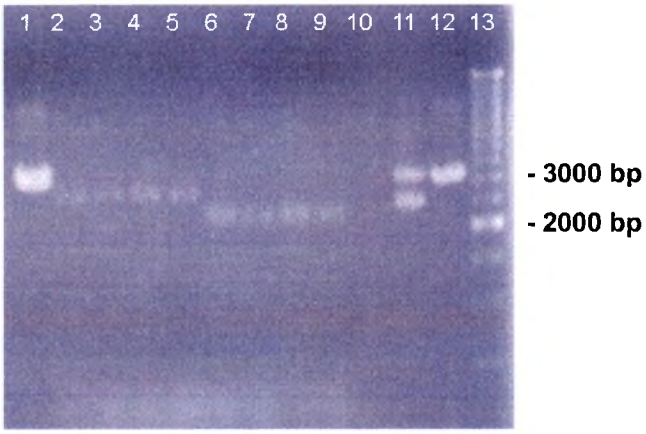

Figure 11. Ethidium bromide stained agarose gel of pA3H1 IV, V, and VIII-XI and pALIB4-7. Lane 1: pA3H1 (II); Lane 2: pALIB7; Lane 3: pALIB6; Lane 4: pALIB5; Lane 5: pALIB4; Lane 6: pA3H1 (VIII); Lane 7: pA3H1 (IX); Lane 8: pNIC; Lane 9: pNOGO; Lane 10: pNOGO; Lane 11: pA3H1 (X); Lane 12: pA3H1 (XI); Lane 13: 500 bp ladder.

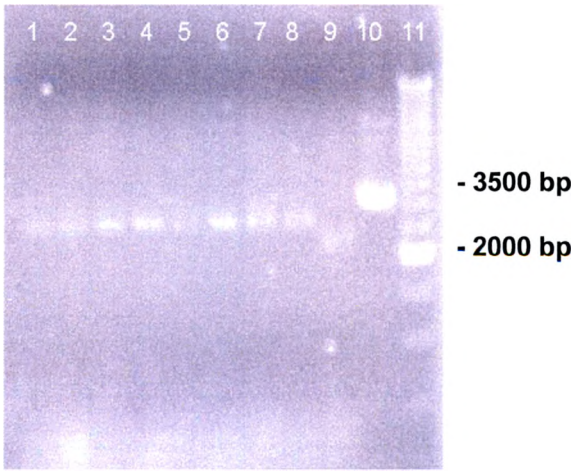

Figure 12. Ethidium bromide stained agarose gel of pALIB8-15. Lane 1: pALIB15; Lane 2: pALIB14; Lane 3: pALIB13; Lane 4: pALIB12; Lane 5: pALIB11; Lane 6: pALIB10; Lane 7: pALIB9; Lane 8: pALIB8; Lane 9: pNIC; Lane 10: pA3H1 (II); Lane 11: 500 bp ladder.

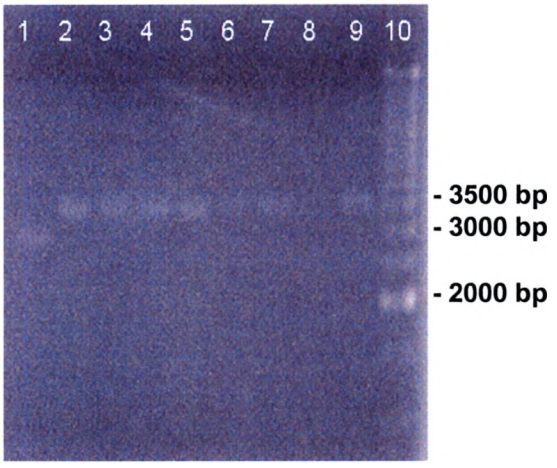

Figure 13. Ethidium bromide stained agarose gel of pALIB16-22. Lane 1: pNIC; Lane 2: pALIB22; Lane 3: pALIB21; Lane 4: pALIB20; Lane 5: pALIB19; Lane 6: pALIB18; Lane 7: pALIB17; Lane 8: pALIB16; Lane 9: pA3H1 (II); Lane 10: 500 bp ladder.

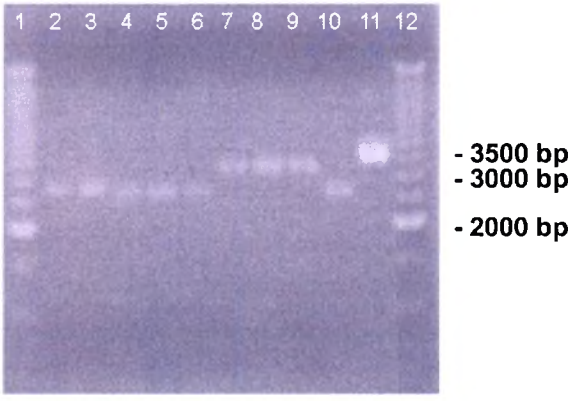

Figure 14. Ethidium bromide stained agarose gel of pALIB23-30. Lane 1: 500 bp ladder; Lane 2: pALIB30; Lane 3: pALIB29; Lane 4: pALIB28; Lane 5: pALIB27; Lane 6: pALIB26; Lane 7: pALIB25; Lane 8: pALIB24; Lane 9: pALIB23; Lane 10: pNIC; Lane 11: pA3H1 (II); Lane 12: 500 bp ladder.

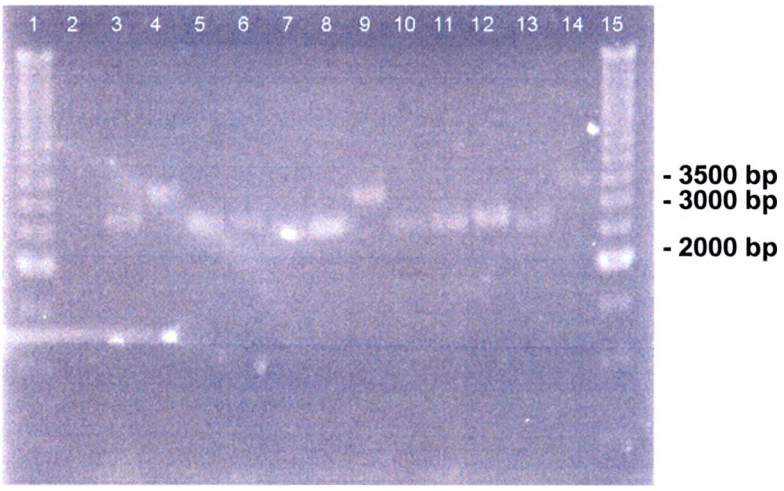

Figure 15. Ethidium bromide stained agarose gel of pALIB31-41. Lane 1: 500 bp ladder; Lane 2: pALIB41; Lane 3: pALIB40; Lane 4: pALIB 39; Lane 5: pALIB38; Lane 6: pALIB37; Lane 7: pALIB36; Lane 8: pALIB35; Lane 9: pALIB34; Lane 10: pALIB33; Lane 11: pALIB32; Lane 12: pALIB31; Lane 13: pNIC; Lane 14: pA3H1 (II); Lane 15: 500 bp control.

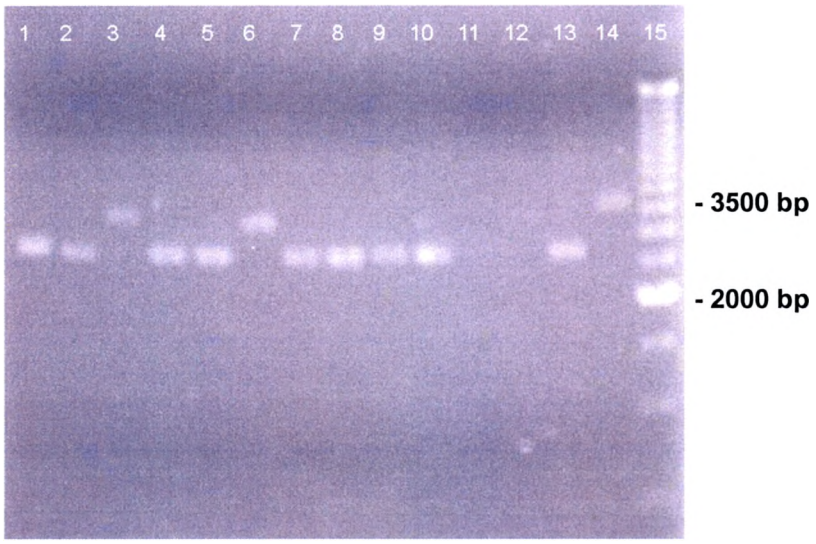

Figure 16. Ethidium bromide stained agarose gel of pALIB42-50. Lane 1: pNIC; Lane 2: pALIB50; Lane 3: pALIB49; Lane 4: pALIB48; Lane 5: pALIB47; Lane 6: pALIB46; Lane 7: pALIB45; Lane 8: pALIB44; Lane 9: pALIB43; Lane 10: pALIB42; Lane 11: pALIB41; Lane 12: pALIB16; Lane 13: pNIC; Lane 14: pA3H1 (II); Lane 15: 500 bp ladder.

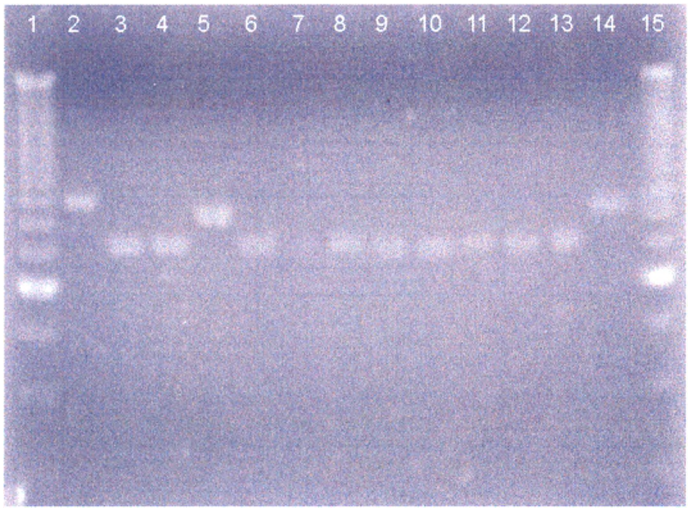

Figure 17. Ethidium bromide stained agarose gel of pALIB51-60. Lane 1: 500 bp ladder; Lane 2: pA3H1 (II); Lane 3: pALIB60; Lane 4: pALIB59; Lane 5: pALIB58; Lane 6: pALIB57; Lane 7: pALIB56; Lane 8: pALIB55; Lane 9: pALIB54; Lane 10: pALIB53; Lane 11: pALIB52; Lane 12: pALIB51; Lane 13: pALIB41; Lane 14: pALIB16; Lane 15: 500 bp ladder.

Overall twenty-two colonies from six cloning reactions were tested for insertion of the wildtype *dszB* gene. Plasmids were purified from these twenty-two colonies and nineteen were found to have an inserted gene. This represents a cloning efficiency of 86%. From the eleven plasmids that were used to establish a positive control, pA3H1 II was used as positive control.

Overall sixty colonies from one cloning reaction were tested for insertion of the mutant *dszB* gene. Plasmids were purified from those sixty colonies and ten were found to have an inserted gene. This represents a cloning efficiency of 17%. A gel showing all ten plasmids containing a mutagenized *dszB* insert is shown in Figure 18.

The pBAD expression system utilizes vector bound Topoisomerase I to aid in cloning efficiency. Topoisomerase I has a nontemplate-dependent terminal transferase that adds a single deoxyadenosine to the 3' end of PCR products.[39,40] Topoisomerase I is released from the vector upon insertion of the PCR product due to attack by the 5' hydroxyl of the PCR product which breaks the covalent bond between the vector and enzyme.[46] The TOPO® cloning system is reported to have a cloning efficiency of close to 95%[40], but efficiency that high has never been observed with the *dszB* gene. The cloning efficiency of the *dszB* gene from IGTS8 with both pBAD TOPO® and pBAD/Thio TOPO® was determined to be closer to 50%.

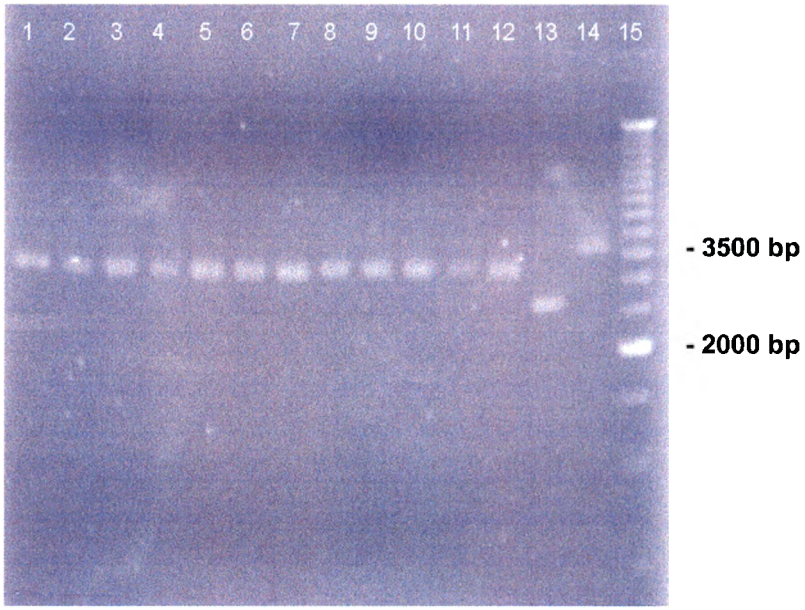

Figure 18. Ethidium bromide stained agarose gel of plasmids containing inserted mutant *dszB* genes. Lane 1: pALIB58; Lane 2: pALIB39; Lane 3: pALIB34; Lane 4: pALIB25; Lane 5: pALIB24; Lane 6: pALIB23; Lane 7: pALIB22; Lane 8: pALIB21; Lane 9: pALIB20; Lane 10: pALIB19; Lane 11: pALIB18; Lane 12: pALIB16; Lane 13: pNIC; Lane 14: pA3H1 (II); Lane 15: 500 bp ladder.

PCR of Candidate Insertion Plasmids

A PCR experiment was done on the plasmids containing an insertion to verify the presence of a *dszB* gene. Each pALIB(X) plasmid shown in Figure 18 was used as a template for its respective PCR reaction. The PCR conditions are stated in Methods and the primers, A3H1 and CSA3R, were used for these PCR reactions. The PCR products were qualitatively measured using agarose gel electrophoresis and are shown in Figures 19 and 20. PCR products for all experimental samples can be seen at approximately 1098 bp which corresponds to the full length gene.

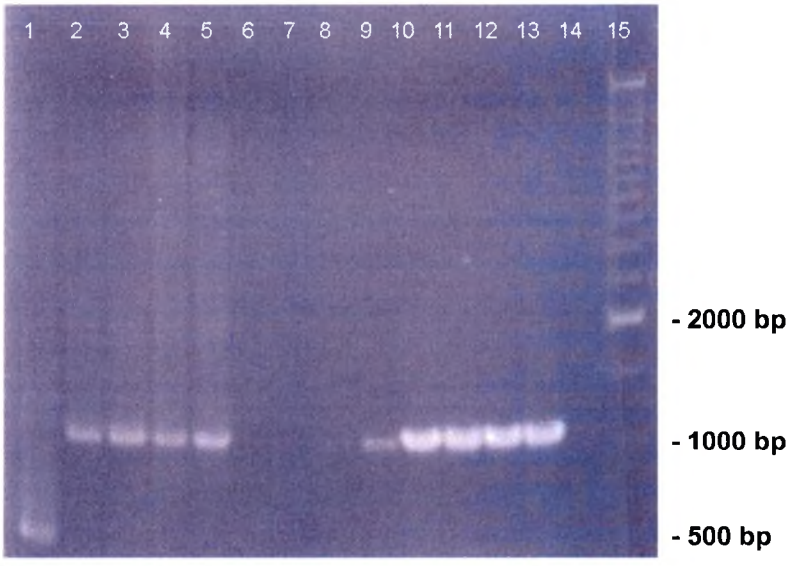

Figure 19. Ethidium bromide stained agarose gel of PCR products from pA3H1 (II) and pALIB(X) samples. Lane 1: Positive PCR control (500 bp); Lanes 2-13: Mutant *dszB* gene from pALIB58, pALIB39, pALIB34, pALIB25, pALIB24, pALIB23, pALIB22, pALIB21, pALIB20, pALIB19, pALIB18, pALIB16, respectively; Lane 14: Wildtype *dszB* gene from pA3H1 (II); Lane 15: 500 bp ladder.

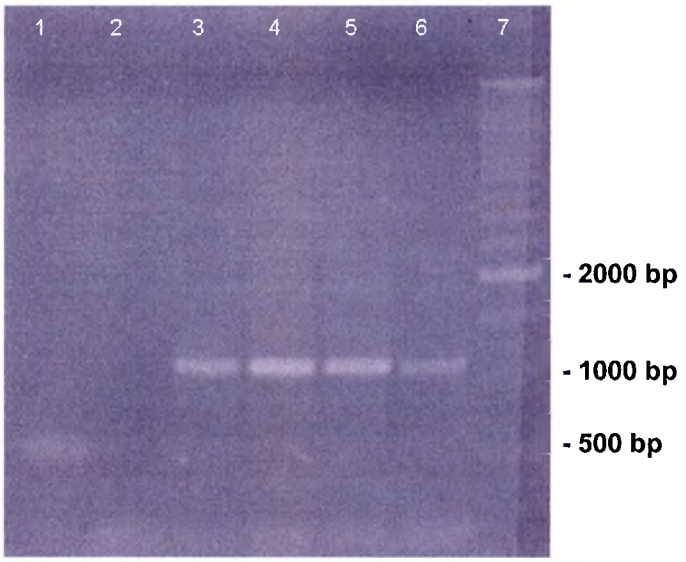

Figure 20. Ethidium bromide stained agarose gel of PCR products from pA3H1 (II) and pALIB22-24. Lane 1: Positive PCR control (500 bp); Lane 2: Negative PCR control (no template); Lane 3: Mutant *dszB* gene from pALIB24; Lane 4: Mutant *dszB* gene from pALIB23; Lane 5: Mutant *dszB* gene from pALIB22; Lane 6: Wildtype *dszB* gene from pA3H1 (II); Lane 7: 500 bp ladder.

Generation of pAThio and pATHLIB(X) Samples

Each purified *dszB* gene, mutants and wildtype, was cloned into the pBAD/Thio TOPO® expression vector and transfected into TOP10 competent *E. coli* as outlined in Methods. The resulting plasmids were designated pATHLIB(X) and the wildtype was designated pAThio. Two identical samples (α and β) for each generated plasmid were purified and checked by agarose gel electrophoresis. Figures 21 and 22 show the resulting purified plasmid DNA. All plasmids can be observed at approximately 3500 bp.

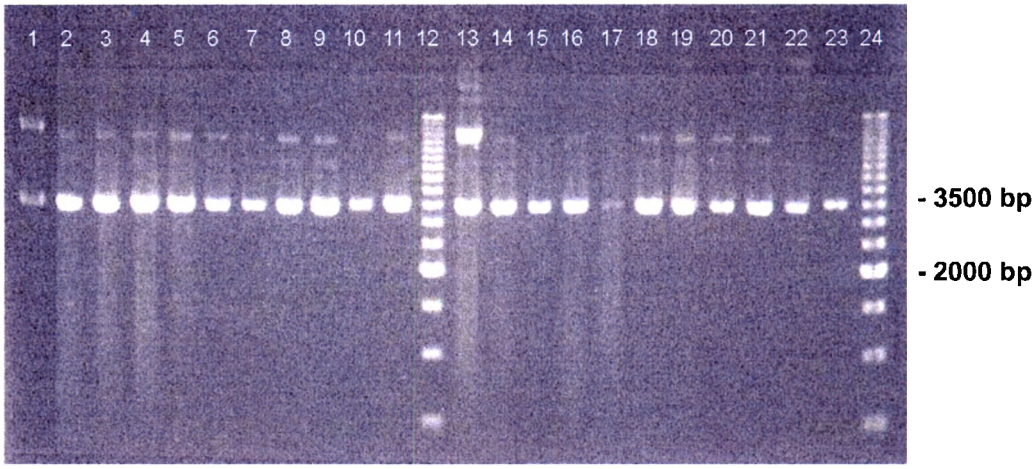

Figure 21. Ethidium bromide stained agarose gel of all pAThio and pATHLIB(X) samples. Lane 1: pATHLIB58 (β); Lane 2: pATHLIB39 (β); Lane 3: pATHLIB34 (β); Lane 4: pATHLIB25 (β); Lane 5: pATHLIB24 (β); Lane 6: pATHLIB23 (β); Lane 7: pATHLIB22 (β); Lane 8; pATHLIB21 (β); Lane 9: pATHLIB20 (β); Lane 10: pATHLIB18 (β); Lane 11: pAThio (β); Lane 12: 500 bp ladder; Lane 13: pATHLIB58 (α); Lane 14: pATHLIB39 (α); Lane 15: pATHLIB34 (α); Lane 16: pATHLIB25 (α); Lane 17: pATHLIB24 (α); Lane 18: pATHLIB23 (α); Lane 19: pATHLIB22 (α); Lane 20: pATHLIB21 (α); Lane 21: pATHLIB20 (α); Lane 22: pATHLIB18 (α); Lane 23: pAThio (α); Lane 24: 500 bp ladder.

Restriction Digest of pAThio and pATHLIB(X) Samples

To check the orientation of the wildtype and each mutant *dszB* gene within each plasmid, restriction digests were done. The restriction endonucleases utilized were *NcoI* and *PstI*. Single digests were conducted using only *NcoI*. The restriction sites within the gene (*PstI*) and the vector (*NcoI*) are positioned such that a correctly oriented gene should yield

DNA fragments of 1373 bp and 4179 bp after a double digestion. Incorrectly oriented genes will yield DNA fragments of 415 bp and 5137 bp. The following gels show the wildtype pAThio digestion (Figure 22), and pATHLIB(X) sample digestions (Figures 23 and 24).

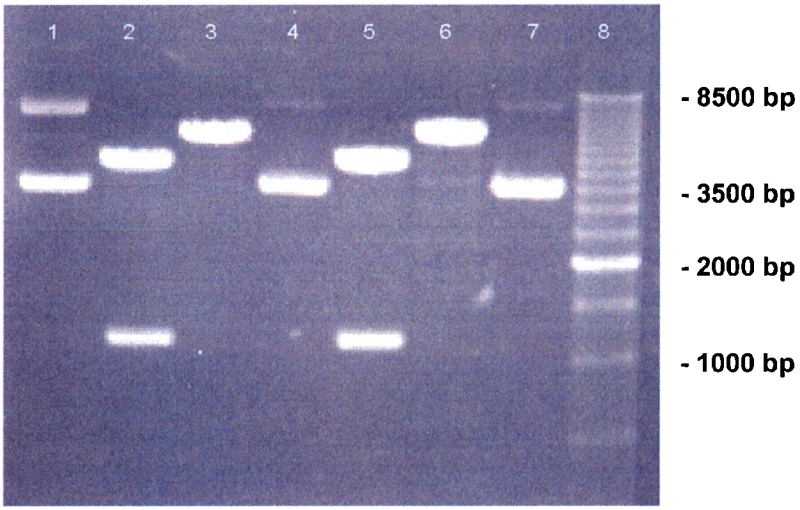

Figure 22. Ethidium bromide stained agarose gel of restriction digests on pAThio α and β. Lane 1: No enzyme control; Lane 2: pAThio (β) double digest; Lane 3: pAThio (β) *NcoI* digest; Lane 4: uncut pAThio (β); Lane 5: pAThio (α) double digest; Lane 6: pAThio (α) *NcoI* digest; Lane 7: uncut pAThio (α); Lane 8: 500 bp ladder.

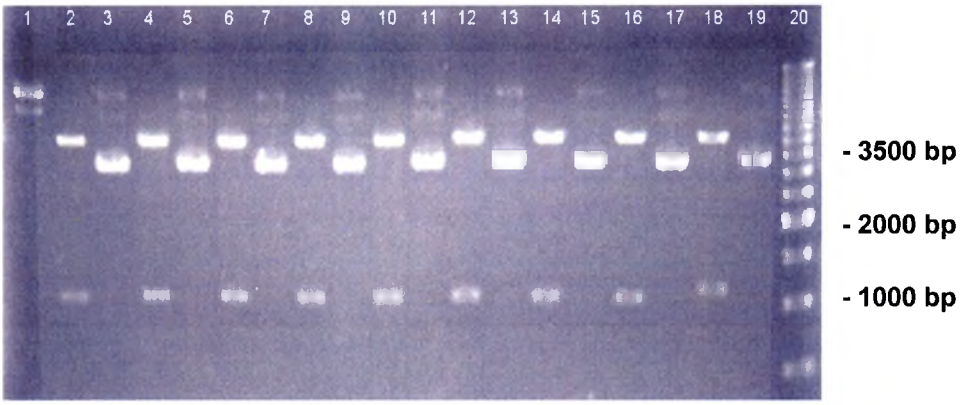

Figure 23. Ethidium bromide stained agarose gel of restriction digests on pATHLIB18 and 20-23. Lane 1: No enzyme control; Lane 2: pATHLIB23 (α) double digest; Lane 3: uncut pATHLIB23 (α); Lane 4: pATHLIB22 (β) double digest; Lane 5: uncut pATHLIB22 (β); Lane 6: pATHLIB22 (α) double digest; Lane 7: uncut pATHLIB22 (α); Lane 8: pATHLIB21 (β) double digest; Lane 9: uncut pATHLIB21 (β); Lane 10: pATHLIB21 (α) double digest; Lane 11: uncut pATHLIB21 (α); Lane 12: pATHLIB20 (β) double digest; Lane 13: uncut pATHLIB20 (β); Lane 14: pATHLIB20 (α) double digest; Lane 15: uncut pATHLIB20 (α); Lane 16: pATHLIB18 (α) double digest; Lane 17: uncut pATHLIB18 (α); Lane 18: pAThio (β) double digest; Lane 19: uncut pAThio (β); Lane 20: 500 bp ladder.

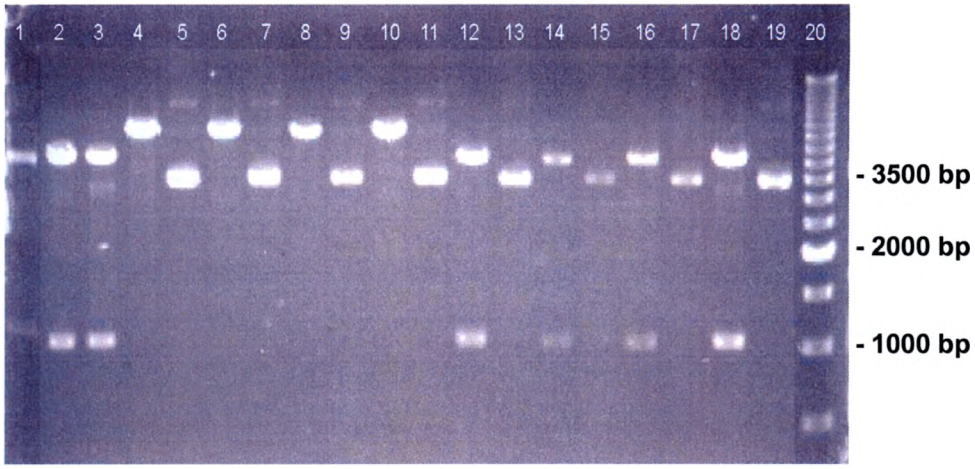

Figure 24. Ethidium bromide stained agarose gel of restriction digests on pATHLIB23, 24, 25, 34, 39 and 58. Lane 1: pAThio (β) double digest; Lane 2: pATHLI58 (β) double digest; Lane 3: pATHLIB58 (α) double digest; Lane 4: pATHLIB39 (β) double digest; Lane 5: uncut pATHLIB 39 (β); Lane 6: pATHLIB39 (α) double digest; Lane 7: uncut pATHLIB39 (α); Lane 8: pATHLIB34 (β) double digest; Lane 9: uncut pATHLIB34 (β); Lane 10: pATHLIB34 (α) double digest; Lane 11: uncut pATHLIB34 (α); Lane 12: pATHLIB25 (β) double digest; Lane 13: uncut pATHLIB25 (β); Lane 14: pATHLIB25 (α) double digest; Lane 15: uncut pATHLIB 25 (α); Lane 16: pATHLIB24 (α) double digest; Lane 17: uncut pATHLIB 24 (α); Lane 18: pATHLIB23 (β) double digest; Lane 19: uncut pATHLIB23 (β); Lane 20: 500 bp ladder.

All the plasmids with an inserted mutant *dszB* gene were checked for their orientation within the vector. A linear gene has a 50% chance of being inserted in the correct orientation because poly-T tails are present at both ends of the cloning site in the vector. The restriction

digests show that all ten purified plasmids contain the *dszB* gene. From these ten plasmids that had an insert, eight were correctly oriented within the pBAD/Thio TOPO® vector. This is an orientation efficiency of 80% for the mutants. These plasmids were sequenced to determine the mutations incurred from the error-prone PCR.

DNA Sequencing

The wildtype *dszB* gene is a 1098 nucleotide sequence, which codes for 366 amino acids that make up the DszB enzyme. The wildtype *dszB* gene was ligated into the pBAD/Thio TOPO® and transfected into TOP10 cells and this plasmid was designated pAThio. Plasmid DNA was purified from overnight bacterial culture as outlined in Methods. Purified plasmid DNA for each mutant was fluorimetrically quantitated using PicoGreen® as outlined in Methods. A 150 ng/μL concentration of each plasmid was prepared before shipment to Davis Sequencing. The sequencing primers, Trx forward primer and the pBAD reverse primer (Table 1), which bind to regions flanking the inserted gene sequence were sent with the plasmid DNA samples. Only reverse sequences were returned from Davis Sequencing as the Trx forward sequencing primer did not yield results with any samples. The sequences were manipulated using the MacVector™ program and mutation characterization and homology between the sequences were compared. Another sequencing reaction was carried out with pAThio and the pATHLIB(X) plasmids. The CSA3R reverse primer (Table 1), which binds to the 3' end of the *dszB* gene, was used as a sequencing primer to sequence the 5' end of the gene. Using this primer, more of the gene sequence was obtained than the previous sequencing experiment and together an almost complete nucleotide sequence of each mutant gene was obtained.

Mutation rates were calculated for the mutant sequences and are shown in Table 4. The error rates ranged from 0.19%-2.38% with the average being 0.75% ± 0.84. The pATHLIB34 plasmid had the highest mutation rate with 2.38%. Three mutants have a mutation rate of ~0.2%. The mutation rate for the IGTS8 *dszB* mutants from previous work was 0.24%, which is one-third the average mutation rate in this study. Conditions set forth in the GeneMorph™ protocol for high mutation frequency (>7 mutations/kb) was used for the error-prone PCR. A lower concentration of template in the reaction accounts for the high mutation frequency observed with the A3H1 *dszB* gene. Furthermore, $MnCl_2$ severely reduces the fidelity of *Taq* polymerase to incorporate the correct deoxynuclueotides into the growing DNA strand.

A summary of the mutations for each sequence is shown in Table 4. Of the 51 base changes observed, 39% were silent mutations. Amino acid changes from the remaining mutations were not entirely random. For instance, one base change resulting in a L261R mutation occurred in nine of the ten samples. Perhaps the original gene sequence is incorrect. Other common amino acid changes between some of the sequences include L53F, Q79L, G209D, and Q210R. Since there is no obvious selection process at work in this system, it most likely suggests that the common mutations between unique sequences occurred in an early cycle of the error-prone PCR.

Table 4. Summary of mutation data

Mutant	Nucleotide Changes	Sequence Size	Mutation Rate (%)	Substitutions		
				Common	Other	Silent
pATHLIB23	2	1035	0.19	L261R		1
pATHLIB24	2	1032	0.19	L216R L52F		
pATHLIB25	2	1007	0.2	L261R Q65L		
pATHLIB22	2	901	0.22	L261R	G102A	
pATHLIB58	3	1098	0.27	L261R Q65L		
pATHLIB21	2	341	0.59	L261R		1
pATHLIB20	5	1066	0.47	L261R L52F	S80C	2
pATHLIB18	7	897	0.78	L261R	R90G	5
pATHLIB39	22	1044	2.19	L261R G209D Q210R	G17S I51T P66S T69A S80C P86H G134S Q175R F223L V241N	9
pATHLIB34	24	1007	2.38	L261R G209D Q210R	S80C E192Q F223L V241M D285G G287S T290A	12
			Mean = 0.75% ± 0.84			

Pilot Expression Experiment

The pilot expression study was conducted as outlined in Methods. The plasmid pAThio α was used as the experimental sample. TOP10 cells without vector were used as a negative control and pBAD/Thio was used as the positive expression control vector. The positive control vector should yield a 16 kDa HP-thioredoxin fusion protein. The DszB-HP-thioredoxin fusion protein should yield a 53 kDa protein. Samples were taken before induction with arabinose and at 4 and 10 hours after induction. All samples were prepared and run using SDS-PAGE as outlined in Methods. The gel showing the proteins present 10 hours after induction is shown in Figure 25.

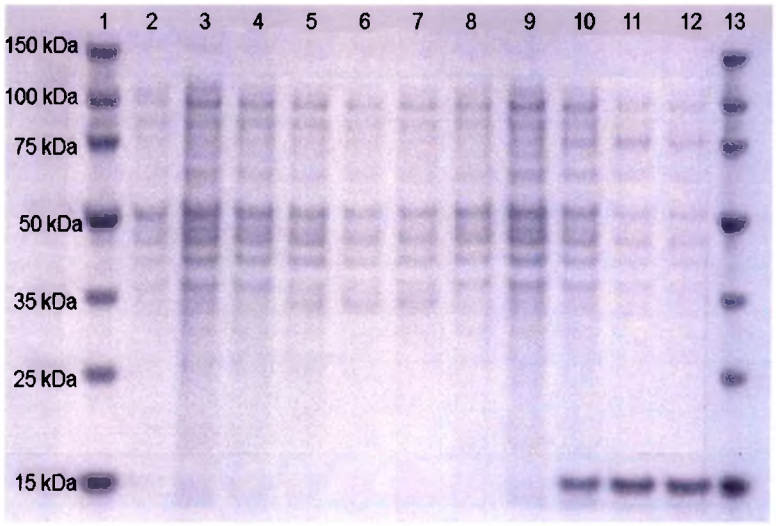

Figure 25. SimplyBlue™ stained polyacrylamide gel at 10 hours after induction with arabinose. Lane 1: Perfect Protein™ Marker; Lane 2: Negative control; Lane 3: pAThio α (0.00002% arabinose); Lane 4: pAThio α (0.0002% arabinose); Lane 5: pAThio α (0.002% arabinose); Lane 6: pAThio α (0.02% arabinose); Lane 7: pAThio α (0.2% arabinose); Lane 8: pBAD/Thio positive control (0.00002% arabinose); Lane 9: pBAD/Thio positive control (0.0002% arabinose); Lane 10: pBAD/Thio positive control (0.002% arabinose); Lane 11: pBAD/Thio positive control (0.02% arabinose); Lane 12: pBAD/Thio positive control (0.2% arabinose); Lane 13: Perfect Protein™ Marker.

The only evidence of arabinose induction at 10 hours can be observed in the positive control experiments (Lanes 8-12). The polyacrylamide gel of the 0 hour induction control indicated very little protein was expressed prior to arabinose addition (data not shown). The polyacrylamide gel of the 3 hour induction experiment is similar to the 10 hour induction experiment (data not shown). The bands in the 10 hour induction experiment observed at approximately 16 kDa correspond to the expression of a HP-thioredoxin fusion protein.

Higher concentrations of arabinose resulted in higher expression levels. Relative intensities of the bands were measured and compared. There is not significant induction of any other proteins and it can not be determined from this gel if a 53 kDa DszB-HP-thioredoxin fusion protein is present. Alternate methods of cell lysis were tested in order to increase sensitivity of the experiment.

Protein Extraction

Three different cell lysis protocols were used to extract protein from overnight bacterial culture. Chemical cell lysis (YPER), enzymatic cell lysis (lysozyme), and physical cell lysis (sonication) were used to produce cell lysates. The detailed protocols for each procedure are outlined in the Methods section. Batch cell culture of the wildtype pAThio α and β induced with 0.2%, 0.02%, and 0.002% arabinose were lysed using the lysozyme procedure. All samples were run on SDS-PAGE. Proteins were detected by colloidal stain (Figure 26) and Western blotting (Figure 27).

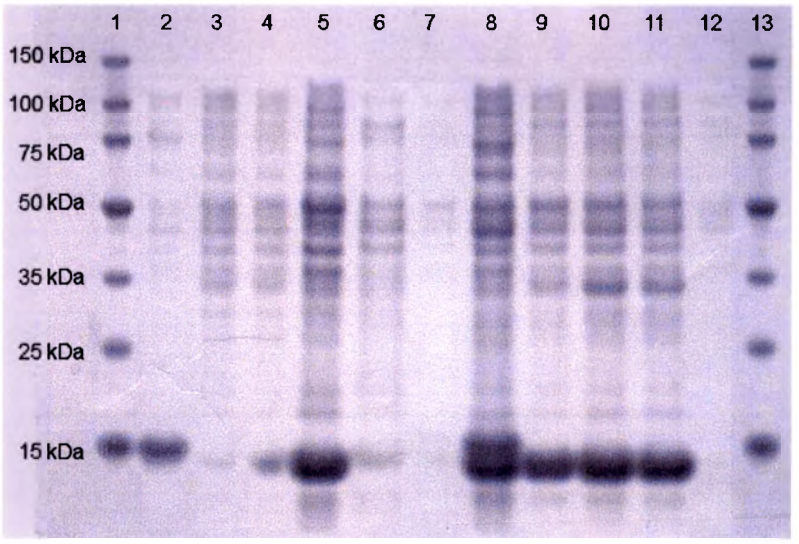

Figure 26. SimpleBlue™ stained polyacrylamide gel of cell lysates from different methods and pAThio α and β induced batch culture cell lysates. Lane 1: Perfect Protein™ Marker; Lane 2: Positive control (0.2% arabinose, 10 hr induction); Lane 3: pAThio α (0.2% arabinose, 10 hr induction); Lane 4: pAThio β (0.2% arabinose, 10 hr induction); Lane 5: pAThio α (lysozyme); Lane 6: pAThio α (YPER); Lane 7: pAThio α (sonication); Lane 8: Positive control (0.2% arabinose, batch culture); Lane 9: pAThio α (0.002% arabinose, batch culture); Lane 10: pAThio α (0.02% arabinose, batch culture); Lane 11: pAThio α (0.2% arabinose, batch culture); Lane 12: Negative control; Lane 13: Perfect Protein™ Marker.

The dark bands present in Lanes 5 and 8-12 at approximately 15 kDa correspond to the lysozyme used to lyse open the cells in these samples. The positive expression control samples (Lanes 2 and 8) show the HP-thioredoxin fusion protein at approximately 16 kDa. The relative intensity of all other bands present are identical and it can not be determined if the DszB-HP-thioredoxin fusion protein is present in the experimental samples. A Western

blot was done to determine if the DszB-HP-thioredoxin fusion protein was present in these samples.

Western Blot Analysis

A Western blot was used to determine if the cells expressed the HP-thioredoxin fusion protein. The Western blot was run in parallel to the SDS-PAGE shown in Figure 26. The Western protocol used antibodies that were specific for HP-thioredoxin tagged proteins. This method was used to see if the DszB-HP-thioredoxin fusion protein was being expressed. The Western protocol is outlined in the Methods section. Distinct bands are present at approximately 16 kDa in the expression control samples (Lanes 2 and 8) correspond to the HP-thioredoxin fusion protein. Bands appearing at approximately 15 kDa correspond to the lysozyme used to lyse the cells. The DszB-HP-thioredoxin fusion protein should appear at 53 kDa.

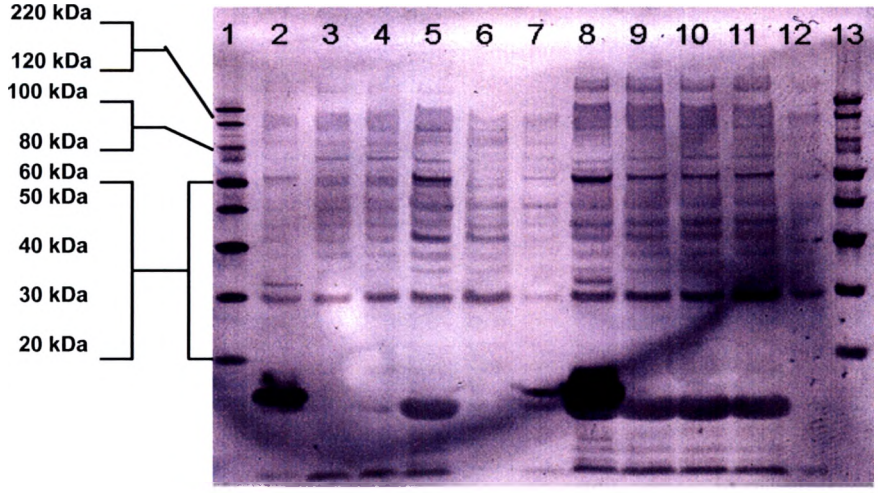

Figure 27. Western blot analysis of duplicate gel loaded as Figure 25. Lane 1: MagicMark™ Western Protein Standard; Lane 2: Positive control (0.2% arabinose, 10hr induction); Lane 3: pAThio α (0.2% arabinose, 10 hr induction); Lane 4: pAThio β (0.2% arabinose, 10 hr induction); Lane 5: pAThio α (lysozyme); Lane 6: pAThio α (YPER); Lane 7: pAThio α (sonication); Lane 8: Positive control (0.2% arabinose, batch culture); Lane 9: pAThio α (0.002% arabinose, batch culture); Lane 10: pAThio α (0.02% arabinose, batch culture); Lane 11: pAThio α (0.2% arabinose, batch culture); Lane 12: Negative control; Lane 13: MagicMark™ Western Protein Standard.

In this Western blot, the order of the lanes is identical to the SDS-PAGE shown in Figure 26. This Western blot shows many bands that were tagged with secondary antibody and subsequently stained after the formation of color once the substrate was introduced. This is due to non-specific binding of the primary and/or secondary antibody to the proteins present. Several approaches could be used to decrease non-specific binding. The incubation times in the antibody solutions could be decreased. Additionally, the substrate incubation

time could be decreased to the time corresponding to the first appearance of colored bands on the nitrocellulose membrane. Although all of these modifications will lower background and increase sensitivity, there is not an observable explanation for the lack of the desired product in this experiment.

Conclusions

The wildtype *dszB* gene from *Nocardia asteroids* A3H1 was successfully cloned into pBAD TOPO® and pBAD/Thio TOPO®. Cloning efficiency of the wildtype gene into the pBAD TOPO® vector was 86%. Error-prone PCR was used to generate a library of *dszB* mutants. Ten mutant genes in pBAD/Thio TOPO® were characterized by restriction analysis and sequencing. Eight of the ten mutants were oriented in the correct direction, 5'→ 3' relative to the P_{BAD} promoter. Mutation frequency ranged from 0.19% to 2.38% with an average mutation frequency of 0.75% +/- 0.84. Mutations were evenly distributed throughout the gene with some mutations occurring in more than one sequence. Attempts at expression of the gene under the P_{BAD} promoter were unsuccessful.

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