GENERATION AND SCREENING OF DszB MUTANTS FOR ENHANCED CATALYTIC ACTIVITY

THESIS

Presented to the Graduate Council of
Southwest Texas State University
in Partial Fulfillment of
the Requirements

For the Degree

Master of Science

By

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August 2003

ACKNOWLEDGEMENTS

I would like to express tremendous thanks to Dr. Linette Watkins, my graduate advisor, for her support. She has been a teacher and mentor.

This manuscript was submitted on June 30, 2003.

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The presence of a metabolic pathway for dibenzothiophene desulfurization has been reported in various bacterial organisms.[1, 2, 3] The role of this pathway is to oxidize and remove sulfur from the organosulfur compounds, such as dibenzothiophene (DBT). The activation of the dibenzothiophene desulfurization (Dsz) pathway is a result of the induction of the plasmid-encoded *dsz* operon (cluster of genes) in absence of sulfate and sulfur-containing amino acids.[1, 4] The Dsz pathway has been the focus of increased study

due to its potential to purify crude oil. The Dsz pathway involves four steps using four enzymes (DszA, DszB, DszC and DszD). In the final and rate-limiting step, DszB catalyzes the hydrolysis of 2-(2-hydroxyphenyl)benzenesulfinate (HPBS) to form inorganic sulfur and 2-hydroxybiphenyl (HBP).[2] As the rate-limiting enzyme in the pathway, DszB does not display the magnitude of enzymatic activity necessary for the efficient mass purification of crude oil. The goal of the research described is to develop a method to increase the activity of the DszB enzyme using directed evolution.

A mutant *dszB* gene library was generated from *R. erythropolis* strain IGTS8. It was ligated into the pBAD TOPO® vector and screened for activity. The screen indicated that there were four mutants with activity significantly above wild-type activity, seven mutants with activity similar to wild-type and 82 mutants with activity significantly below wild-type activity. One mutant with enhanced activity was selected for further characterization.

CHAPTER I

INTRODUCTION

Desulfurization of fossil fuels has been traditionally accomplished through hydrodesulfurization (HDS).[1] However, organosulfur compounds, such as dibenzothiophene (DBT) and its derivatives remain present in HDS treated fuel. The combustion of fossil fuel containing compounds such as DBT results in the production of sulfur oxides, a contributing factor to acid rain.[1] Due to increasingly stringent regulations of sulfur levels in fuels, it is necessary to either develop additional methods for the further removal of sulfur from HDS treated fuel or to completely replace this process. Over the past decade biodesulfurization (BDS) has become a leading candidate for an alternative method of fuel desulfurization.[2] Identification of microorganisms which express a catabolic desulfurization pathway has thus far been limited to the taxonomic group actinomycetes.[2]

Rhodococcus erythropolis strain IGTS8 is a member of the actinomycetes taxonomic group and expresses an extensively studied desulfurization pathway, termed the "4S pathway" or the Dsz pathway.[3,4,5,6] Expression of the Dsz pathway is repressed in the presence of sulfur-containing amino acids or sulfate and is required only for sulfur salvaging.[1] Thus, the pathway ultimately leads to the release of sulfur via cleavage of two C-S bonds in organosulfur compounds such as DBT. DBT is the accepted model compound

of Dsz pathway because of its high concentration in HDS treated fuel. The Dsz pathway has been determined to involve four steps using four enzymes (Figure 1).[3]

In the first two steps, DszC (DBT-monooxygenase), a 45-kDa enzyme, catalyzes the sequential oxidation of DBT first to DBT-sulfoxide and then to DBT-sulfone ($DBTO_2$).[3] Next, DszA (dibenzothiophene 5, 5-dioxide-monooxygenase), a 50-kDa enzyme, oxidizes DBT-sulfone forming 2-(2'-hydroxyphenyl) benzenesulfinate (HPBS).[3] The final and rate-limiting step is the desulfurization of HPBS, and is catalyzed by DszB (2-(2'-hydroxyphenyl)benzene sulfinate desulfinase), a 39-kDa enzyme, yielding the products 2'-hydroxybiphenyl (HBP)and sulfite.[3] The first three enzymatic reactions of the Dsz pathway, catalyzed by DszC and DszA, require flavin reductase to generate the essential reduced flavin ($FMNH_2$) for the oxidation reactions.[3] The flavin reductase is known as DszD and is the fourth enzyme in the Dsz pathway.[7]

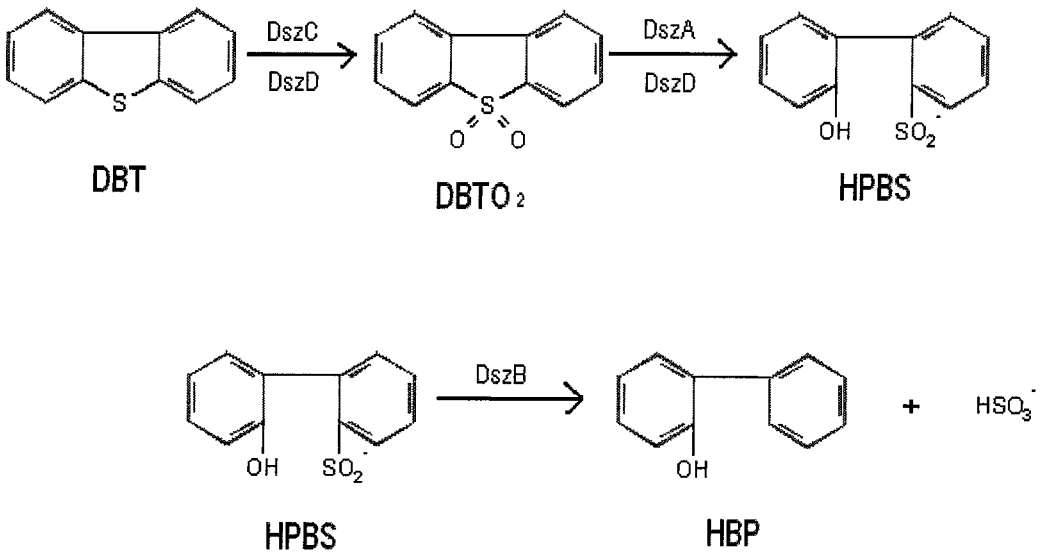

Figure 1: *Rhodococcus erythropolis strain IGTS8* contains a four enzyme, three step desulfurization pathway for DBT.

The DNA sequence of DszABC contains an operon (dsz operon) consisting of three genes (*dszA*, *dszB* and *dszC*).[8] The dsz gene cluster is 3722-bp sequence on a 150-kb plasmid.[8] The *dszA* gene is 1361 bases in length and encodes DszA.[2,4,8] The *dszB* gene (Figure 2) is 1098 bases in length and encodes DszB.[2,4,8] The *dszC* gene is 1251 bases in length and encodes DszC.[2,4,8] The order the genes appear from 5'-3' on the 150 kb plasmid is: *dszA-dszB-dszC*.[2,4,8] The *dszA* stop codon overlaps the start codon for *dszB*, suggesting translational coupling.[1] A thirteen base-pair gap occurs between *dszB* and *dszC*.[1] Immediately upstream from the *dszA* gene is a 385-bp regulatory region (-1 to -385), which contains the transcription start site (-46) and the promoter region (a 77-bp stretch from -44 to -121).[1] A repressor protein is believed to bind to the promoter region in the presence of cysteine, methionine, or sulfate.[1] However, the repressor protein has yet to be isolated. The gene responsible for the expression of DszD is 579-bp in length and is not encoded by the dsz operon nor is it present on the plasmid containing the dsz operon.[9,10]

```
          5' ATGACAAG GCGCGTCGAC CCCGCAAACC CCGGTTCAGA ACTCGATTCC
GCCATCCGCG ACACACTGAC CTACAGCAAC TGCCCGGTAC CCAACGCTCT GCTCACGGCA
TCGGAATCGG GCTTCCTCGA CGCCGCCGGC ATCGAACTCG ACGTCCTCAG CGGCCAGCAG
GGCACGGTTC ATTTCACCTA CGACCAGCCT GCCTACACCC GTTTTGGGGG TGAGATCCCG
CCACTGCTCA GCGAGGGGTT GCGGGCACCT GGGCGCACGC GTCTACTCGG CATCACCCCG
CTCTTGGGGC GCCAGGGCTT CTTTGTCCGC GACGACAGCC CGATCACAGC GGCCGCCGAC
CTTGCCGGAC GTCGAATCGG CGTCTCGGCC TCGGCAATTC GCATCCTGCG CGGCCAGCTG
GGCGACTACC TCGAGTTGGA TCCCTGGCGG CAAACGCTGG TAGCGCTGGG CTCGTGGAGC
GCGCGCGCCT TGTTGCACAC CCTTGAGCAC GGTGAACTGG GTGTGGACGA CGTCGAGCTG
GTGCCGATCA GCAGTCCTGG TGTCGATGTT CCCGCTGAGC AGCTCGAAGA ATCGGCGACC
GTCAAGGGTG CGGACCTCTT TCCCGATGTC GCCCGCGGTC AGGCCGCGGT GTTGGCCAGC
GGAGACGTTG ACGCCCTGTA CAGTTGGCTG CCCTGGGCCG GGGAGTTGCA AGCCACCGGG
CGGGCGGGAG TGGTGGATCT CGGCCTCGAT GAGCGCAATG CCTACGCCAG TGTGTGGACG
GTCAGCAGCG GGCTGGTTCG CCAGCGACCT GGCCTTGTTC AACGACTGGT CGACGCGGCC
GTCGACGCCG GGCTGTGGGC ACGCGATCAT TCCGACGCGG TGACCAGCCT GCACGCCGCG
AACCTGGGCG TATCGACCGG AGCAGTAGGC CAGGGCTTCG GCGCCGACTT CCAGCAGCGT
CTGGTTCCAC GCCTGGATCA CGACGCCCTC GCCCTCCGTT AGCGCACACA GCAATTCCTG
CTCACCAACA ACTTGCTGCA GGAACCCGTC GCCCTCGATC AGTGGGCGGC TCCGGAATTT
CTGAACAACA GCCTCAATCG CCACCGATAG 3'
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Figure 2: DNA sequence of the gene from *Rhodococcus erythropolis strain IGTS8*, which codes for HPBS desulfinase (DszB).

Given the ability of the Dsz pathway to remove sulfur from DBT and other organosulfur compounds, the incorporation of this biocatalytic tool in commercial fossil fuel purification may result in a more efficient and cost effective means of sulfur removal than HDS. However, the regulation of expression of the pathway and the kinetics of each step in the pathway are limited and must be maximized before incorporation into a mass commercial process. The replacement of the promoter region has been shown to eliminate pathway repression.[3] Despite the ability to bypass the repression of the pathway, the expression of the genes is not equal.[1] The *dszB* gene is expressed at lower levels than those of the *dszA* and *dszC* genes.[1] Not only is the *dszB* gene expressed at lower levels but its product (DszB) has the lowest catalytic activity of the enzymes that precede it.[3] The expression of *dszB* has been enhanced in expression vectors with and without *dszA* and *dszC* and shows promise of utilizing this effect in the expression of the entire operon. If biodesulfurization is to be used as an alternative form of desulfurization not only does the *dszB* expression level need to be enhanced but also the catalytic activity of DszB. The ability to increase the catalytic activity of the DszB enzyme would prove to be invaluable.

Two approaches toward increasing the catalytic activity of an enzyme are directed protein evolution or rational design.[11] In recent years, rational protein (re-)design and directed evolution have been at the forefront of protein engineering (or reengineering). The rational protein design involves iterative computer design and site-directed mutagenesis.[12] However, this approach is rendered useless by the absence of detailed structural and mechanistic information.[12] A quite different approach to enzyme redesign is directed evolution, which does not require this information.[12] Directed evolution (often called the irrational approach to protein design) mimics the evolutionary processes of mutation and

selection (or screening) *in vitro*.[13] The growing appreciation that many enzyme functions are not confined to amino acids near the active site (but are influenced by residues far from the active sites) is causing protein engineering groups to favor directed evolution. The reengineering of enzymes to enhance performance using random mutagenesis techniques, such as error-prone PCR[14] and DNA shuffling[15,16,17] is proving to be useful. Directed evolution has been applied not only to acquire a desired function of an enzyme but to investigate structure/function relationships as well.[18] Directed evolution experiments generate mutant libraries and these mutant libraries accumulate effectual mutations over generations of randomization and selection. Furthermore, the use of directed evolution over rational protein design is advantageous for those who have a limited amount of information of the catalytic mechanism, structure and specificity of the target enzyme.[12]

Given the limited information on the three dimensional structure and mechanism of the DszB enzyme, any chance of enhancing its catalytic activity and obtaining a better understanding of the mechanism exists in the use of directed evolution. Various techniques of generating DNA libraries using random mutagenesis ('irrational') have been described.[16,18,19,20] Here we describe the generation of a mutant *dszB* DNA library using an error-prone PCR method, developed by Stratagene®.[21] The wild-type *dszB* gene from *R. erythropolis* strain ISTS8 was used as a template for the error-prone PCR reaction. The resulting *dszB* mutant library was then cloned into an expression vector, transfected into *E. coli* cells and subjected to a fluorimeteric screening assay. The mutants which appeared to have enhanced enzymatic activity were then isolated. An enhanced isolate was characterized, over expressed and run on an affinity column.

CHAPTER II

MATERIALS

Difco Bactoagar, Bacto-Tryptone, sodium chloride and Bacto-Yeast extract were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Ampicillin sodium salt was purchased from Amresco® (Solon, Ohio). The cell lysis reagent Y-PER-S was purchased from Pierce Biotechnology, Inc. (Rockford, IL). All other chemicals and reagents used were research grade or better and were purchased from Aldrich® Chemical Company (Milwaukee, Wisconsin), Fisher Scientific (Pittsburgh, Pennsylvania), VWR (Buffalo Grove, IL) or Sigma® Chemical Company (St. Louis, Missouri).

Reagents were sterilized in a HICLAVE HV-50 autoclave from Amerex Instruments Inc. (Lafayette, California). Sterile conditions were provided for bench work by a Laminar Flow Hood model NU-201-430 from NUAIRE (Plymouth, Minnesota). Cells were both shaken and incubated in a Gyromax Orbital Incubator Shaker from Amerex Instruments Inc. (Lafayette, California). Enzyme and other reagents were concentrated and filtered using Centricon YM-10 and YM-30 centrifugal filters from Millipore™ Corporation (Bedford, Massachusetts).

The *dszB* gene was received in a pBAD TOPO® vector, as a gift from Enchira Biotechnology Corporation (The Woodlands, Texas). Primers were purchased from Integrated DNA Technologies® Inc. (Coralville, Indiana) and Invitrogen™ Life

Technologies (Carlsbad, California) (Table 1). Error-Prone PCR reactions were conducted using the Genemorph™ Mutagenesis kit purchased from Stratagene (La Jolla, California). PCR was performed in a Gene Amp PCR system model 2400 thermal cycler from Applied Biosystems (Foster City, California). Dimethyl sulfoxide (DMSO) was purchased from Sigma® Chemical Company (St. Louis, Missouri). Wizard® *Plus* Minipreps DNA Purification System and Wizard® PCR Preps DNA Purification System were both purchased from Promega (Madison, Wisconsin). The QIAprep Miniprep System was purchased from QIAGEN Inc. (Valencia, California).

Table 1: Primer sequences and descriptions.

Name	Sequence	Direction	Melting Temperature	Description
LMW6R	5'-CTATCGGTGGCGATTGAGGCTGTTG TTCAGAAATTCCGGAGCCGCCCACTG ATCGAGGGCG-3'	Reverse	79 2°C	Native gene primer adding bases missing in pEBS16
LMW6F	5'-ATGACAAGCCGCGTCGACCCCGC3'	Forward	69 6°C	Native gene Primer
Trx Forward Sequence Primer	5'-TTCCTCGACGCTAACCTG-3'	Forward	56°C	Primer complimentary to vector 5' of inserted gene
pBAD Reverse Sequence Primer	5'-GATTTAATCTGTATCAGG-3'	Reverse	48°C	Primer complimentary to vector 3' of inserted gene

Agarose gel electrophoresis was performed using agarose purchased from Invitrogen™ Life Technologies (Carlsbad, California). Horizontal were run in a multi-sub

cell GT apparatus from Bio-Rad Laboratories (Hercules, California). Both 500 base pair markers and 100 base pair markers were used as molecular weight standard and were purchased from New England Biolabs® Inc. (Beverly, Massachusetts).

The restriction enzymes *Pst*I and *Nco*I were purchased from New England Biolabs® Inc. (Beverly, Massachusetts). pBAD TOPO® TA Expression Kit, pBAD/TOPO® ThioFusion™ Expression Kit, *Taq* Polymerase, ProBond™ purification, Enterokinase (EKMax™), Anti-Thio® (antibody), EK-Away™ (for Enterokinase removal) resin and *E. coli* strains One Shot® Top-10 and BL-21 were purchased from Invitrogen™ Life Technologies (Carlsbad, California).

All NuPage® gel electrophoresis reagents, gels, and equipment were purchased from Invitrogen™ Life Technologies (Carlsbad, California). A Novex XCELL-11 electrophoresis module was used for polyacrylamide gel electrophoresis. Perfect Protein™ Markers 15-150 kDa were purchased from Novagen (Madison, Wisconsin). The Perfect Protein™ Markers molecular weight standard contained seven precisely sized proteins at 150,000 kDa, 100,000 kDa, 75,000 kDa, 50,000 kDa, 35,000 kDa, 25,000 kDa and 15,000 kDa.

A Beckman DU-7400 UV spectrophotometer was used for all ultraviolet-visible determinations. A Perkin Elmer Luminescence spectrophotometer LS50-B was used for all fluorimetric assays. A Beckman J2-21 centrifuge was used for all macroscale centrifugations. A HERMLE Z180 M centrifuge from National Labnet Co. (Woodbridge, NJ) was used for all microscale centrifugations. A Kodak Digital Science™ Image Station 440 CF from Eastman Kodak Company (New Haven, Connecticut) was used to image polyacrylamide gels and agarose gels. A Bio-Rad Gel

Doc 1000 from Bio-Rad Laboratories (Hercules, California) was also used to image agarose gels.

The software used to analyze gel images was either Kodak 1D version 3.5.3 from Eastman Kodak Company (New Haven, Connecticut) or Multi Analyst version 1.0.1 by Bio-Rad Laboratories (Hercules, California). The FLWinLab program version 3.00 from Perkin Elmer was used to operate the spectrofluorimeter. Microsoft Excel® was used to organize and graph data.

CHAPTER III

METHODS

Cell Culture Experimental

Cell Growth

All cells were plated onto Luria-Bertani Media (LB) plates containing 100 μg/mL ampicillin (amp) and incubated for overnight at 37°C. LB (1 liter) contains: 950 mL H_2O, 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, and 10 g NaCl, and the pH was adjusted to 7.0 with 3 M NaOH.[22] Colonies were selected and grown in culture tubes containing 5 mL LB broth and 60 μg/mL ampicillin and incubated in a shaker (200 rpm) for 24 hours at 37°C.

Frozen Permanents

Colonies were isolated from LB agar plates containing 60 μg/mL amp, inoculated in 5 mL LB liquid media containing 60 μg/mL amp, and incubated in a shaker (200 rpm) for 24 hours at 37°C. One-hundred forty microliters of DMSO were added to 4 mL LB/amp/cell culture and placed on ice for 15 min, at which time an additional 140 μL DMSO were added. The samples were then placed in cryotubes and stored at -80°C.

Molecular Biology Methods

DNA Concentrations

Concentrations of isolated plasmid and primer DNAs were determined by UV absorbance at 260 nm in 1 mL quartz cuvettes on a Beckman DU 7400 UV-Vis spectrophotometer. Samples were diluted 1:100 with ddH_2O prior to the absorbance measurement. Absorbance values were used to calculate DNA concentration using the following equation: (A_{260}) x (50 μg/mL) x dilution factor (100).

Agarose Gel Electrophoresis

A 50 mL, 0.8% agarose gel was prepared using 0.4 g agarose and 50 mL 1x TAE buffer. The stock 50x TAE buffer was prepared using: 242 g Tris-base, 57.1 mL glacial Acetic Acid, 100 mL 0.5 M EDTA (pH 8.0), and diluted with water to 1x.[22] The gel solution was heated in a microwave on high setting for 30 seconds three times, swirling after each heating. The gel was then poured into a mold and allowed to set. The mold was placed into a horizontal gel electrophoresis apparatus, surrounded by 1xTAE buffer, and loaded with samples prepared in 6x dye buffer (40% sucrose, 0.25% bromophenyl blue, 0.25% xylene cyanol FF, and water to 10 mL).[22] TE buffer contains: 0.12 g Tris, 0.03 g EDTA, water to 100 mL, pH 8.0.[22] A 100 base pair marker (3 μL marker, 5 μL 6x dye, and 21 μL TE buffer) was used as a molecular weight standard for 100 to 2,200 base pairs. The gel was electrophoresed at 70 volts for 30 to 90 minutes. After electrophoresis the gel was stained with an ethidium bromide solution (25 μL of 10 mM ethidium bromide in 250 mL of H_2O).

Plasmid Isolation, Purification and Naming

All plasmids, except those sent for sequencing, were isolated using a Wizard® *Plus* Minipreps DNA Purification System[23] and analyzed by agarose gel electrophoresis. Plasmids were purified for sequencing using the QIAprep Miniprep System.[24] Newly generated plasmids were named pNOGO, pMUTA(X) and pMTHIO(X). The plasmids pEBS16 and pBTHIO were previously generated. A description of all plasmids is provided in Table 2.

Table 2: Plasmid names and descriptions.

Plasmid name	Plasmid description
PEBS16	Positive control. Truncated wild-type (*R. erythropolis* strain IGTS8) *dszB* gene inserted in pBAD TOPO® vector
PNOGO	Negative control. Control PCR product inserted into pBAD TOPO® vector
pMUTA(X)*	Experimental. Mutated *dszB* gene inserted in pBAD TOPO® vector
pMTHIO(X)*	Experimental. Mutated *dszB* gene inserted in pBAD/TOPO® ThioFusion™ vector
PBTHIO	Positive control. Wild-type (*R. erythropolis* strain IGTS8) *dszB* gene inserted in pBAD/TOPO® ThioFusion™ vector

*X= arbitrarily numbered mutants generated

PCR

All PCR reactions were optimized to the following conditions. The following reagents were mixed on ice: ~50 ng template, 1.5 µL of DMSO, 1 µL of 10 mM dNTP

mix, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 5 μL of 10x PCR buffer (supplied with Taq), 5 μL of 25 mM $MgCl_2$ and water to 50 μL. These reagents were heated to 94°C in a PCR machine and incubated for 5 minutes, a "hot start". The run was paused and 1 μL of Taq polymerase was added. The run was then continued for 35 cycles: 94°C for 15 seconds, 52°C for 30 seconds, and 72°C for 3 minutes. The reaction was then held at 72°C for 7 minutes to finish extension on any incomplete DNA chains. The reaction products were analyzed by agarose gel electrophoresis and stored at -20°C. All PCR products were purified using Wizard® *Plus* PCR Purification System.[23]

Restriction Digests

All restriction digests were performed under similar conditions. The total volume of each reaction was 20 μL and contained: 1 μL restriction enzyme, 4 μL 5x KGB buffer (100 mM potassium glutamate, 1.0 M Tris-Acetic acid (pH 7.6), 1.0 M Mg Acetic acid, 10 mg/mL BSA and 14.3 M βME) 4 μL DNA, and 11 μL sterile water. The reactions were incubated in a water bath for 3 hours at 37°C. The reaction products were analyzed by agarose gel electrophoresis.

Error-Prone PCR

All error-prone PCR reactions were optimized to the following conditions. The following reagents were mixed on ice: 1 μL pEBS16 (10 pg/μL-100 pg/μL), 1.5 μL of DMSO, 1 μL of 40 mM dNTP mix, 0.5 μL (250 ng/μL) of each primer, and 5 μL of 10x Mutazyme reaction buffer (supplied with GeneMorph™) and water to 50 μL. These

reagents were heated to 94°C in a PCR machine and incubated for 5 minutes, a "hot start". The run was paused and 1 μL of Mutazyme DNA polymerase (2.5 U/μL) was added. The run was then continued for 35 cycles: 94°C for 15 seconds, 52°C for 30 seconds, and 72°C for 3 minutes. The reaction was then held at 72°C for 7 minutes to finish extension on any incomplete DNA chains. The reaction products were analyzed by agarose gel electrophoresis and stored at -20°C.

pBAD TOPO® Cloning

The PCR products were ligated into the pBAD TOPO® vector[25] (Figure 3) using the Topo cloning reaction[25] (Figure 4). Four microliters of PCR product, 1 μL Topo 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 transformation reaction was performed. The ligated vector and insert were transformed into One Shot TOP10 *E. coli* by gently mixing 2 μL of the Topo cloning reaction product with one vial of cells and incubating on ice for 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 added. SOC media was prepared using: 950 mL H_2O, 20 g bacto-tryptone, 5 g yeast extract, 0.5 g NaCl, and 10 mL 250 mM KCl. The media was adjusted to pH 7.0 and brought to 1 L with water. The media was sterilized and 2 mL of sterile 1 M Glucose was added.[22] The tubes were then shaken (200 rpm) horizontally at 37°C for one hour and the cells were plated on a LB plate containing 100 μg/mL ampicillin. Colonies were selected and grown in culture tubes containing 5 mL LB broth and 100 μg/mL ampicillin. DNA was isolated using a

Wizard DNA prep kit and analyzed by agarose gel electrophoresis, PCR, and restriction digests.

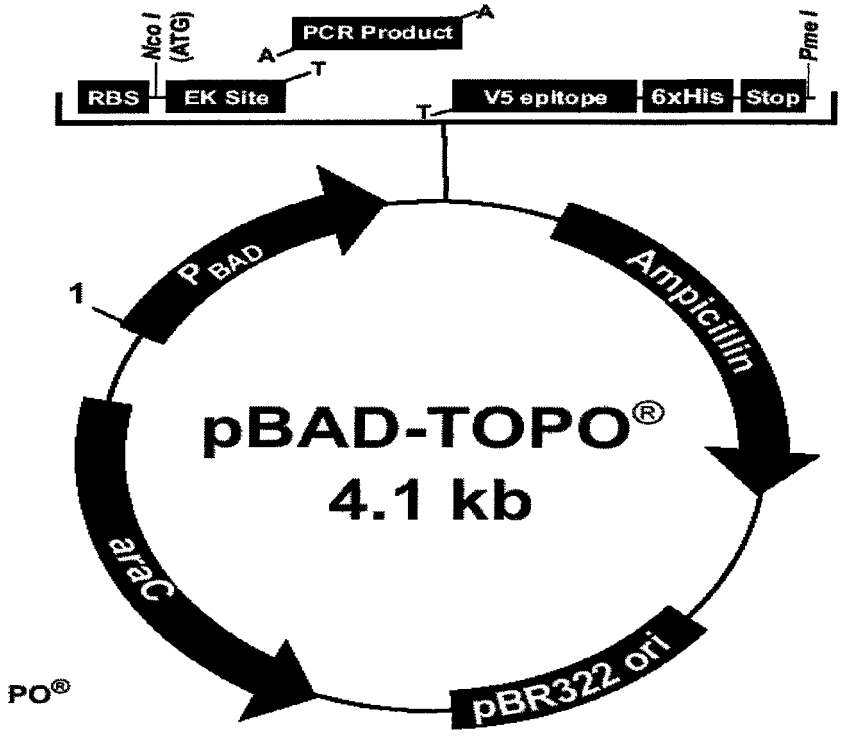

Figure 3: Map of pBAD TOPO® cloning vector. The vector includes an ampicillin resistant gene, 6xHis tag and arabinose inducible promoter.

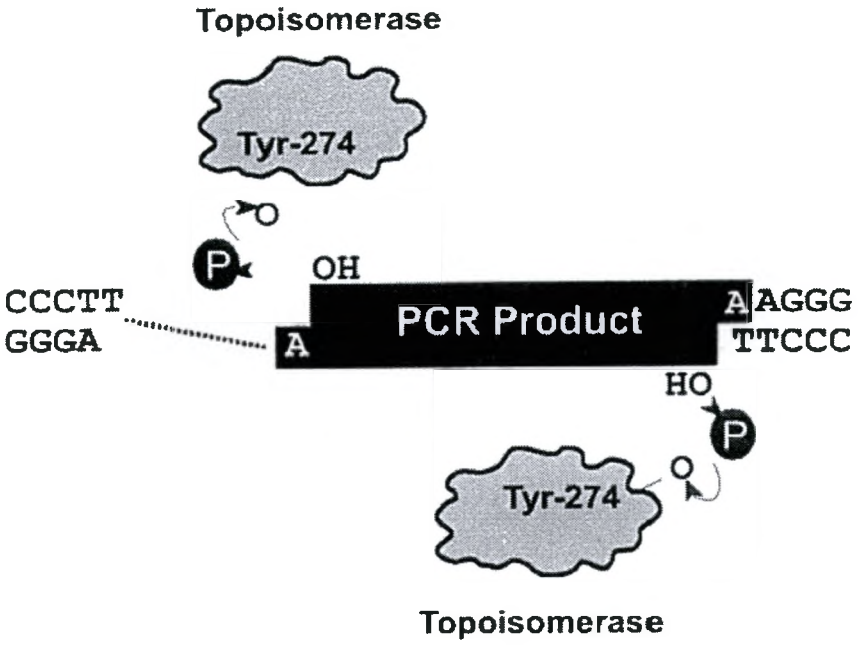

Figure 4: Schematic of the Topoisomerase chemical cloning reaction.

pBAD/TOPO® ThioFusion™ Cloning

The cloning reaction, using the vector pBAD/TOPO® ThioFusion™ (Figure 5), was conducted using the identical conditions as the pBAD TOPO® reaction.[26]

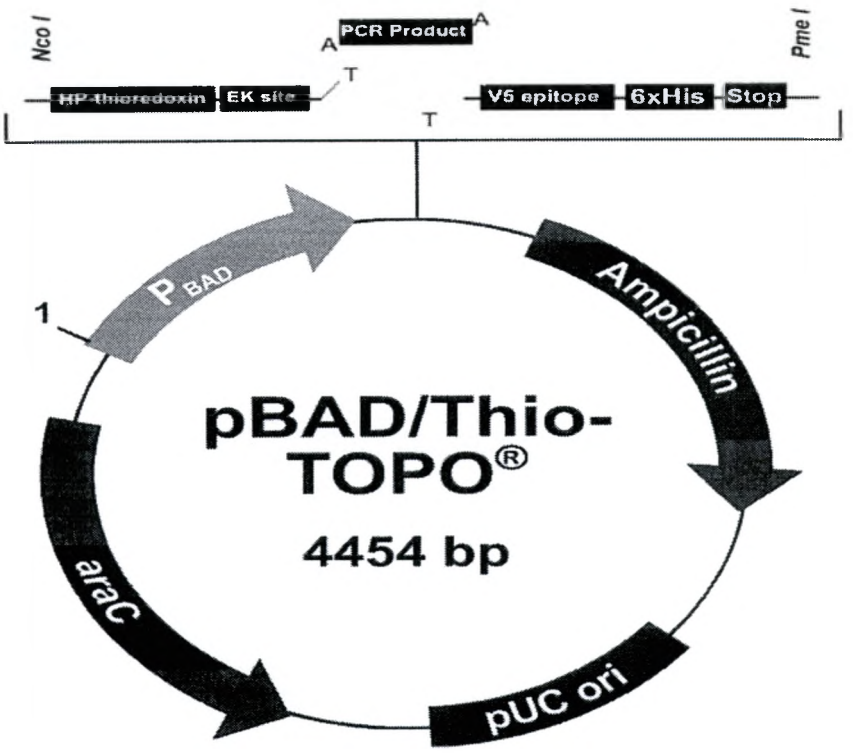

Figure 5: Map of pBAD/TOPO® ThioFusion™ cloning vector. The vector includes an ampicillin resistant gene, 6xHis tag and arabinose expression induction gene. The inclusion of the Histidine Patch-Thioredoxin peptide on the N-terminus of the fusion protein allows for one step affinity purification of the protein.[26]

Generation of Negative Control Plasmid (pNOGO)

The control gene insert supplied with pBAD/TOPO® ThioFusion™ Cloning Kit was amplified under the following conditions: 1 μL control gene insert (10 μg/μL), 10 x PCR buffer (supplied with kit), 0.5 μL 50 mM dNTPs, 0.5 μL of both forward and reverse control primers (150 ng/μL) and H_2O to a final volume of 49 μL. These reagents were heated to 94°C in a PCR machine and incubated for 5 minutes, a "hot start". The

run was paused and 1 μL of *Taq* Polymerase (1 U/μL) was added. The run was then continued for 35 cycles: 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 3 minutes. The reaction was then held at 72°C for 30 minutes to finish extension of any incomplete DNA chains. The reaction products were analyzed by agarose gel electrophoresis and stored at -20°C.

Sequencing of Mutants

Plasmids were purified using QIAprep Miniprep System.[24] Concentrations were determined using a fluorimeteric assay. Plasmids were concentrated to 250-500 ng/μL by ethanol precipitation. Eight microliters of the sample and 1 μL of Trx forward sequencing primer and pBAD reverse sequencing primer (supplied with pBAD/TOPO® ThioFusion™ Cloning kit), were sent to Davis Sequencing (Davis, CA) for sequencing.

Protein Chemistry Methods

Protein Concentrations

Protein concentrations were measured by the method developed by Kalb and Bernlohr using the following equation: $\mu g/mL \text{ protein} = 183(A_{230}) - 75.8\ (A_{260})$.[27] Absorbances at 230 and 260 nM were measured in 1 mL quartz cuvettes on a Beckman DU 7400 UV-Vis spectrophotometer. Samples were diluted until absorbances were less than 1.000 AU.

Sodium Dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were prepared using ~10 μg of protein, 4x lithium dodecylsulfate (LDS) sample buffer (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 2% β-mercaptoethanol. The samples were then denatured by heating at 90°C for 10 minutes. The samples and Perfect Protein™ Markers molecular weight standard were loaded onto a pre-cast NuPAGE 4-12% bis-Tris polyacrylamide gel. The gel was run at 200 volts (constant voltage) for 35 minutes in MES-SDS running buffer (500 mM 2-(*N*-morpholino)ethane sulfonic acid, 50 mM Tris-base, 3.5 mm SDS, 1 mM EDTA, pH 7.3). The gel was removed from the cartridge and fixed with 50% methanol, 7% acetic acid solution for 10 minutes, stained overnight using Colloidal Blue stain, and destained with water.

Fluorimetric Enzyme Assay

The standard assay was used for all activity measurements of HPBS desulfinase. Water, 10x buffer A (250 mM NaH_2PO_4, 1 M NaCl, pH 7.4), and 1 mM HPBS were mixed in a 96 well plate to a final volume of 200 μL and a final concentration of 10 μM HPBS. The reaction was initiated with the addition of cell lysate. Product formation was monitored over time using a Perkin Elmer LS50B Luminescence spectrophotometer by measuring the increase in fluorescence at 414 nm (λ_{exc} = 288 nm). The slope of a plot of the fluorescence intensity versus time was used to determine the rate of product formation. The relative enzyme activity in the cell lysates was determined by dividing the slopes (calculated by Excel®) by the A_{280} values of the lysate.

Cell Lysis

Cells were harvested from a 50 mL culture by centrifugation (e.g., 5000 x g for 5 minutes) and resuspended in native binding buffer (250 mM $NaPO_4$ and 2.5 NaCl). Eight milligrams of lysozyme were added to the suspended cells and incubated on ice for 30 minutes. Chromosomal DNA was sheared by sonication using six 10 second bursts at high intensity with 10 second cooling periods between each burst. The lysate was centrifuged at 3,000 x g for 15 minutes to pellet the cellular debris. The supernatant was transferred to a fresh tube. Sixty-two microliters of the lysate were used for SDS analysis and the remaining lysate was stored at -20°C.

Pilot Expression Reaction

A single recombinant *E. coli* colony strains One Shot® Top-10 was inoculated in 2 mL of LB containing 100 μg/mL ampicillin and incubated overnight at 37°C with shaking to stationary phase (OD_{600} = 2). Next, 0.1 mL of the overnight culture was added to five tubes containing 10 mL LB and 100 μg/mL ampicillin. The cultures were grown for five hours to mid-log phase (OD_{600} = 0.6). One milliliter of cells from each tube was removed, pelleted by centrifugation and supernatant was aspirated. The cell pellets were labeled zero point and stored at -20°C. Arabinose was added to the five 9 mL cultures. The final arabinose concentrations in tubes 1-5 were 0.00002%, 0.0002%, 0.002%, 0.02% and 0.2 %, respectively. The five cultures were then incubated for four hours at 37°C. At four hours 1 ml samples were taken from each sample and analyzed by SDS.

ProBond Affinity Purification

E. coli cells containing pBAD/Topo® ThioFusion™ cloned plasmids were grown for one day in 50 mL cultures of LB media containing 100 μg/mL ampicillin. Cells were harvested by centrifugation at 10,000 rpm in a Beckman J2-21 centrifuge for one hour, washed twice with minimal salts, and pelleted by centrifugation again. The cell pellet was stored at -70°C and/or resuspended in 8 mL of native buffer (50 mM sodium phosphate, 0.5 M NaCl pH 8.0), and lysed. The lysate was centrifuged at 3,000 rpm for 15 minutes. The supernatant was loaded onto a 10 mL ProBond nickel chelating column.[28] The column was gently shaken at 4°C for one hour to allow for protein binding. The column was then washed with 8 mL of native wash buffer (0.02 M Imidazole, 50 mM sodium phosphate, 0.5 M NaCl pH 8.0). Native wash buffer was replaced with native elution buffer (0.25 M Imidazole, 50 mM sodium phosphate, 0.5 M NaCl pH 8.0). The column was washed with 8 mL of native elution buffer. Fractions were collected during the wash by aspirating the supernatant on the column and every 1 mL was collected during the elution. Fractions collected during elution were assayed for activity using the standard assay and analyzed by SDS-PAGE.

CHAPTER IV

RESULTS

Isolation of *dszB* gene from pEBS16

The *dszB* gene was isolated from the pEBS16 plasmid by PCR amplification. The primers LMW6F and LMW6R were used as primers. The PCR product was verified on an agarose gel (Figure 6). A band approximately 1100 base pairs can be observed in Lane 3.

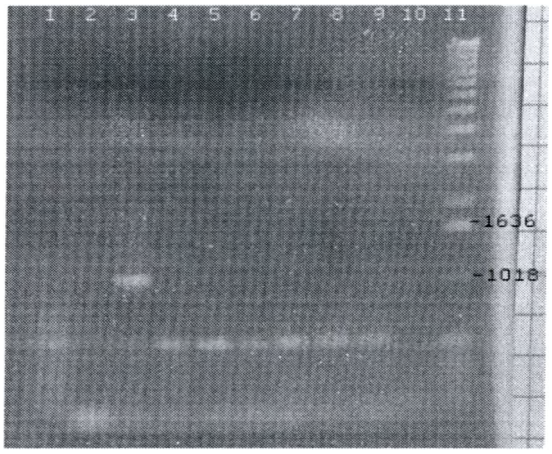

Figure 6: Agarose gel of PCR products using pEBS16 as a template and stained with ethidium bromide. Lane 1: Positive control; Lane 2: Negative control; Lane 3: Product of amplified *dszB* gene from pEBS16; Lanes 4-10: Primer dimers formed in the unsuccessful PCR amplification of *dszB* gene; Lane 11: 1 kb marker (The highest intensity band at approximately the center of the gel is 1636 bp).

Generation of Mutant DNA Library

The Mutant DNA Library was generated according to the protocol described in the Methods section. The pEBS16 plasmid was used as the template for the error-prone PCR reaction, LMW6F and LMW6R were used as primers and Mutazyme DNA polymerase was added. The PCR reaction resulted in a product approximately 1100 bp in length, seen in both Figure 7 (Lane 3) and Figure 8 (Lane 2).

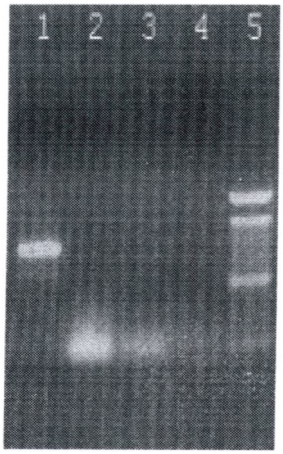

Figure 7: Agarose gel of error-prone PCR products using *dszB* as the template and stained with ethidium bromide. Lane 1: Product of error-prone amplification of *dszB* gene; Lane 2: Positive control; Lane 3: Positive control; Lane 4: Negative control (no template); Lane 5: 100 base pair marker (The lowest molecular weight, high intensity band is 600 bp).

Generation of pNOGO

The plasmid pNOGO was generated as described in the Methods section. A control insert (Invitrogen™ Life Technologies) was amplified and analyzed by agarose gel electrophoresis (Figure 8). A band of approximately 600 base pairs can be observed in Lane 2. The PCR product was cloned into the pBAD TOPO® vector and transfected into *E. coli* strain One Shot® Top-10 cells. Colonies obtained were grown in overnight tubes and the pNOGO plasmid was isolated.

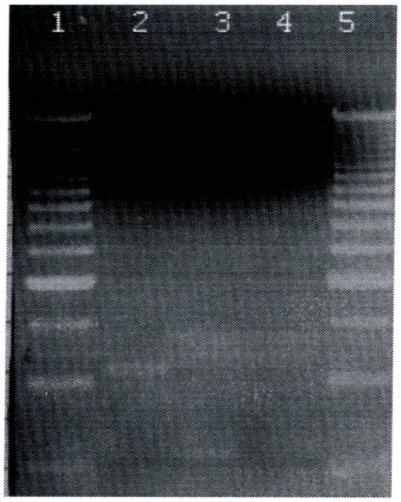

Figure 8: Agarose gel of error-prone PCR products using *dszB* as the template and non-error-prone PCR products using the control insert as the template. The Gel was stained with ethidium bromide. Lane 1: A 500 base pair marker; Lane 2: Product of amplified *dszB* gene; Lane 3: Control insert product; Lane 4: Negative control; Lane 5: 500 base pair marker (The broadest and highest intensity band is 2000 bp).

Generation of pMUTA

The pMUTA plasmids were generated as described in the methods section. The PCR products generated by error0prone synthesis were cloned into the pBAD TOPO® vector and transfected into *E. coli* strain One Shot® Top-10 cells. Colonies obtained were grown in overnight tubes and the pMUTA plasmids isolated and verified on an agarose gel.

Screening of pMUTA(1-103)

Relative activity was determined as described in the Methods section. One hundred and three individual colonies containing the pMUTA plasmid were isolated, lysed and screened for HBP formation. Four mutants displayed enhanced activity, seven mutants at about the same as pEBS16 activity and 82 significantly below pEBS16 activity (Figure 9). The plasmids of the four mutants with enhanced activity (Lane 2: pMUTA38, Lane 3-4: pMUTA91, Lane 5-6: pMUTA92 and Lane 7-8: pMUTA93) were purified and verified on an agarose gel (Figure 10). A band corresponding to approximately 2500 base pairs can be observed in Lanes 2, 5, 6, 7 and 8. In Lanes 3-4 two bands approximately 2500 base pairs and 3500 base pairs can be observed.

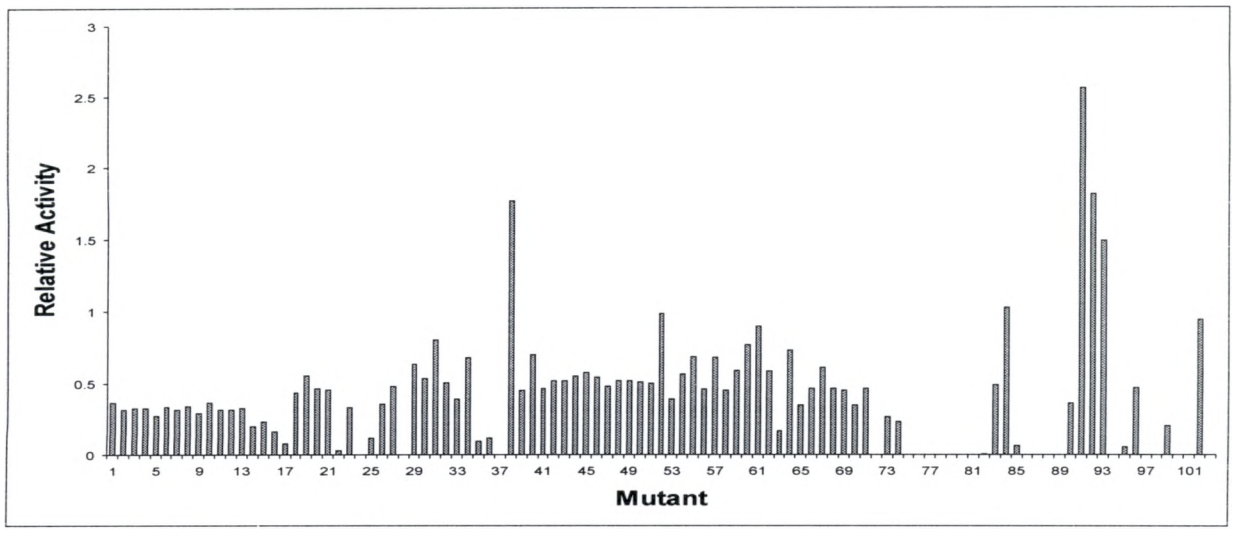

Figure 9: Relative activity of screened pMUTA(1-103). The activity of the screened mutants were measured and compared against pEBS16 (positive control) yielding a relative activity.

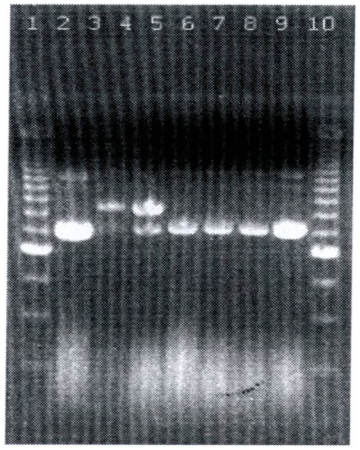

Figure 10: Agarose gel of purified pMUTA38, pMUTA91, pMUTA92 and pMUTA93 and stained with ethidium bromide. Lane 1: A 500 base pair marker; Lane 2: pMUTA38; Lanes 3-4: pMUTA93; Lanes 5-6: pMUTA91; Lanes 7-9: pMUTA92; Lane 10: 500 base pair marker (The broadest and highest intensity band is 2000 bp).

Generation of pMTHIO38, pMTHIO91, pMTHIO92, pMTHIO93

The plasmids pMTHIO38, pMTHIO91, pMTHIO92, pMTHIO93 were generated as described in Methods. The *dszB* gene mutants numbered 38, 91, 92 and 93 were cloned into the pBAD/THIO TOPO® vector and transfected into *E. coli* strain One Shot® Top-10 cells. Colonies obtained were grown in overnight tubes and pMTHIO38, pMTHIO91, pMTHIO92, pMTHIO93 plasmid were isolated and verified on an agarose gel (Figure 11 and 12). In Figure 11, a band at approximately 2500 base pairs can be observed in lanes 4-15. In Figure 12, a band corresponding to approximately 2500 base pairs can be observed in lanes 4, 6 and 8. Also in Figure 12, a band at approximately 3000 base pairs can be observed in lanes 5 and 7.

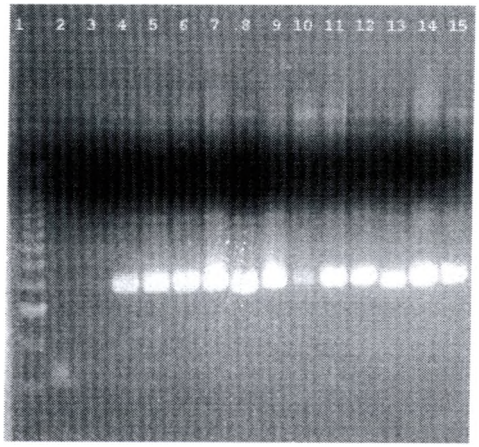

Figure 11: Agarose gel of purified pMTHIO38, pMTHIO91, pMTHIO92 and pMTHIO93 (A) and stained with ethidium bromide. Lane 1: A 500 base pair marker (The broadest and highest intensity band is 2000 bp); Lane 2: No sample; Lane 3: No sample; Lanes 4-7: pMTHIO38; Lanes 8-12: pMTHIO91; Lanes 13-15: pMTHIO92.

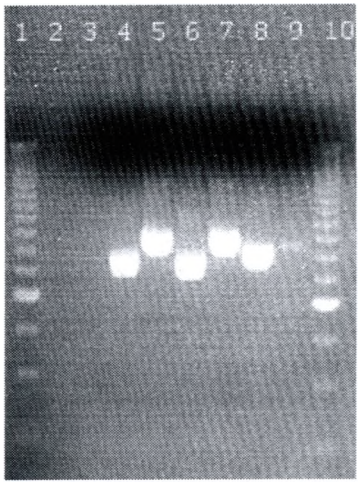

Figure 12: Agarose gel of purified pMTHIO38, pMTHIO91, pMTHIO92 and pTHIO93 (B) and stained with ethidium bromide. Lane 1: A 500 base pair marker (The broadest and highest intensity band is 2000 bp); Lane 2: Negative control; Lane 3: No sample; Lanes 4: pMTHIO92; Lanes 5: pMTHIO93; Lane 6: pMTHIO92; Lanes 7: pMTHIO93; Lanes 8: pMTHIO92; Lane 9: pBTHIO (positive control); Lane 10: A 500 base pair marker.

Gene Presence Verification Using PCR

A PCR reaction was run to verify the presence of the *dszB* gene in the pBAD/TOPO® ThioFusion™ vector and pBAD TOPO®. The PCR reaction was run under the same conditions as discussed in Methods. pMTHIO38, pMUTA38, pMTHIO91, pMUTA91, pMTHIO92, pMUTA92, pMUTA93 or pMTHIO93 was used as a template. The primers LMW6F and LMW6R were used in the reaction. The products were analyzed on an agarose gel (Figure 13). The PCR products from the amplification of pMUTA93 (Lane 10) and pMTHIO93 (Lane 13) are the only products observed corresponding to approximately 1100 base pairs.

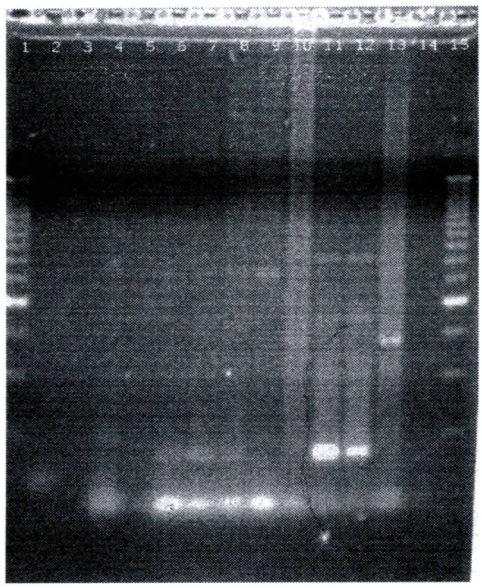

Figure 13: Agarose gel of purified pMTHIO38, pMTHIO91, pMTHIO92 and pTHIO93 and stained with ethidium bromide. Lane 1: A 500 base pair marker (The broadest and highest intensity band is 2000 bp); Lane 2: Negative control; Lane 3: Positive control; Lanes 4: pBAD/TOPO® ThioFusion™ -no insert; Lanes 5: No sample; Lane 6: pMUTA38; Lanes 7: pMTHIO38; Lanes 8: pMUTA91; Lane 9: pMTHIO92; Lane 10: pMUTA93; Lane 11: pMTHIO91; Lane 12: pMUTA92; Lane 13: pMTHIO93; A 500 base pair marker.

Restriction Digest

The mutant pMTHIO93 was digested using the restriction enzymes *Nco*I and *Pst*I. The products of the restriction digest were visualized on an agarose gel (Figure 14). The double digest reaction (in lane four of the gel) displayed bands at approximately 1400 bp and 4000 bp.

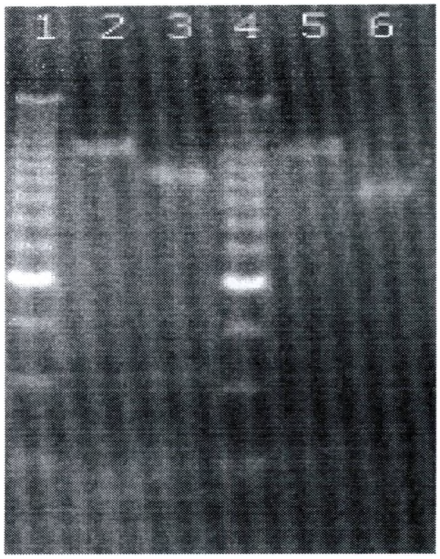

Figure 14: Restriction digest of pMTHIO93 stained with ethidium bromide. Lane 1: A 500 base pair marker (The broadest and highest intensity band is 2000 bp); Lane 2: *Nco*I digested pMTHIO93; Lane 3: *Nco*I and *Pst*I digested pMTHIO93; Lane 4: A 500 base pair marker; Lane 5: *Pst*I digested pMTHIO93; Lanes 6: Uncut pMTHIO93.

Gene Sequencing

The plasmid pMTHIO93 was isolated and sequenced as described in Methods. Plasmids were isolated from *E. coli* cells, concentrated and shipped to Davis Sequencing for analysis. Data obtained from Davis Sequencing indicated that there are two mutations in the *dszB* gene in pMTHIO93, a T→C mutation at position 479 and G→A mutation at position 343.

Pilot Expression Study

A pilot expression study was conducted as described in the methods section. Five *E. coli* colonies transformed with the pMTHIO93 plasmid were subjected to varying arabinose concentrations for a period of four hours. The cells were lysed and the lysate was analyzed by SDS-PAGE. All samples analyzed by SDS-PAGE both before and after addition of varying arabinose concentrations were quantitatively the same (Figure 15 and Figure 16).

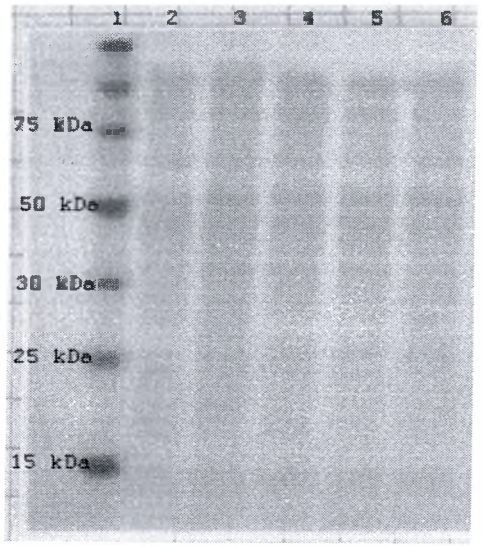

Figure 15: SDS-PAGE gel pilot expression study stained (A) with Coomasie Blue. Lane 1: Protein marker; Lane 2: Zero time point 0.00002% arabinose; Lane 3: Zero time point 0.0002% arabinose; Lane 4: Zero time point 0.002% arabinose; Lane 5: Zero time point 0.02% Lane 6: Zero time point 0.2 % arabinose.

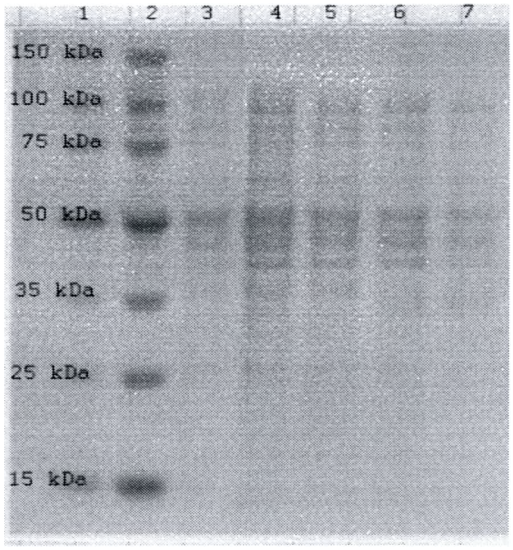

Figure 16: SDS-PAGE gel pilot expression study (B) stained with Coomasie Blue. Lane 2: Protein marker; Lane 3: Four hour time point 0.00002% arabinose; Lane 3: Four time hour point 0.0002% arabinose; Lane 4: Four hour time point 0.002% arabinose; Lane 5: Four hour time point 0.02% Lane 6: Four hour time point 0.2% arabinose.

Purification

A lysate from *E. coli* colony strains One Shot® Top-10 containing the pMTHIO93 plasmid was prepared. The transformed cells (with the pMTHIO93 plasmid) were incubated, harvested, lysed and purified on a ProBond affinity column. The lysate was loaded on the column, the column was washed and bound protein was eluted with native elution buffer. Samples were collected through the course of the purification and analyzed by SDS-PAGE (Figure 17 and 18).

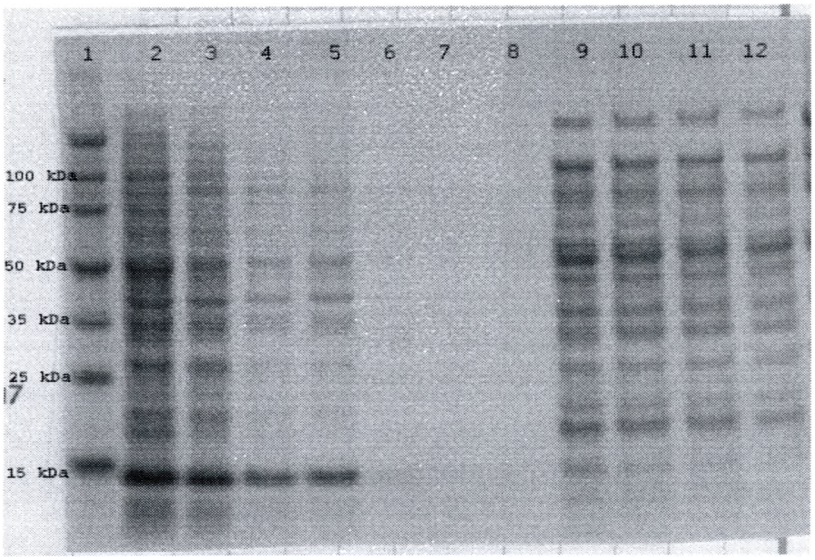

Figure 17: SDS-PAGE gel of protein purification (A) stained with Coomasie Blue. Lane 1: Protein marker; Lane 2: Lysate; Lane 3: Load; Lane 4: Wash 1; Lane 5: Wash 2; Lane 6: Wash 3; Lane 7: Wash 4; Lane 8: Wash 5; Lane 9: Elution fraction 1; Lane 10: Elution fraction 2; Lane 11: Elution fraction 3; Lane 12: Elution fraction 4.

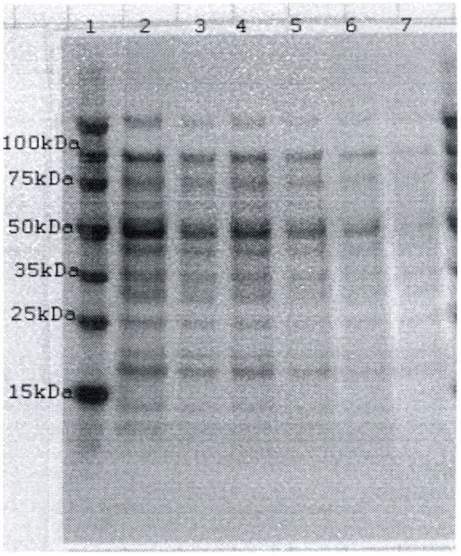

Figure 18: SDS-PAGE gel of protein purification (B) stained with Coomasie Blue. Lane 1: Protein marker; Lane 2: Elution fraction 5; Lane 3: Elution fraction 6; Lane 4: Elution fraction 7; Lane 5: Elution fraction 8; Lane 6: Elution fraction 9; Lane 7: Elution fraction 10.

CHAPTER V

DISCUSSION

Directed evolution mimics the evolutionary processes of mutation, recombination and selection[13] and does not require detailed structural and mechanistic information about the enzyme being studied.[12] In addition, it takes into account the growing appreciation that many enzyme functions are not confined to amino acids near the active site, but are influenced by residues far from the active sites. Directed evolution uses random mutagenesis techniques, such as error-prone PCR[14] and DNA shuffling[15,16,17] to generate mutant libraries. After several generations of randomization and selection these mutant libraries accumulate effectual mutations.

There are several successful examples of *in vitro* enzyme evolution, including generating enzymes with increased thermostability, enzymes with activity in non-native conditions and enzymes with non-native function. One example of using directed evolution to acquire increased theromstability was done by the Harayama group and began with the isolation of uncharacterized genes from soil samples.[29,30] Using PCR amplified genes from the soil samples, the Harayama group proceeded with the directed evolution of catechol 2,3-dioxygenase(C23O).[30] Degenerate primers were designed based on homologous regions of the C23O genes and used them to amplify gene fragments. These gene fragments were then subcloned into a plasmid containing 5' and 3' fragments of a 'host' C23O, expressed in

E. coli, and screened for improved thermostability relative to the host C23O. Two hybrid genes encoding dioxygenases with greater stability than the plasmid-borne C23O were isolated.[30]

Directed evolution has also been used to create an enzyme capable of functioning at near wild-type activity in high concentrations of organic solvents.[31] The Arnold group used sequential random mutagenesis and screening of the protease subtilisin E to generate an enzyme that functions almost as well in 60% dimethylformamide (DMF) as the wild-type enzyme does in aqueous solution.[31] The results of the experiment yielded nearly a 500-fold increase in total enzyme activity (relative to wild-type enzyme activity in 60% DMF) and a 176-fold increase in specific activity.[31] In 1994, the Arnold group also evolved an efficient *p*-nitrobenzyl (pNB) esterase from an enzyme isolated from *Bacillus subtilis* whose natural function was unknown.[31] The product resulted in an enzyme capable of deprotecting the pNB ester intermediate in the synthesis of loracarbef in DMF and the enzyme activity was later increased 20-fold.[31]

The *dszB* gene has been cloned and extensively characterized,[2,4] but there not as much information known about the DszB enzyme. Our lab has purified DszB to near homogeneity from *R. erythropolis* strain IGTS8 cells and completed the initial characterization of the enzyme.[32] Temperature and pH optima have been determined and kinetic parameters with the substrate 2-(2'-hydroxyphenyl)benzene sulfinate have been measured.[32] Chemical modification studies have established that there is an essential cysteine and tyrosine residue at the active site. Inhibition and analog studies have determined the structural and electronic requirements for the binding to the active site. All of this information has been used to

propose an acid-base mechanism for DszB based on the mechanism of carbon-nitrogen lyases, such as tyrosine phenol lyase. However, despite the physical characterization of the enzyme completed and a proposed mechanism, very little detailed structural information about DszB is known. Thus, in order to enhance the catalytic activity of DszB, directed evolution is the most effective tool available.[11]

A method of directed evolution of the *dszB* gene was developed with the objective of enhancing the catalytic rate of DszB. The *dszB* gene was isolated from *R. erythropolis* strain IGTS8. The plasmid pEBS16 was constructed by Energy Biosystems Inc. by inserting the *dszB* gene from *R. erythropolis* strain IGTS8 into the pBAD TOPO® vector (Invitrogen™). When the pEBS16 plasmid was sequenced, it was determined that 25 bases were missing from the 3' end. In order to ensure that a library of full length mutant genes could be generated from the pEBS16 template, a PCR control reaction was run. Primers were designed to incorporate the missing bases and a PCR reaction was conducted using the newly generated primers and the pEBS16 plasmid as the template. The product of the reaction yielded a product, correlating to the expected size of the full length *dszB* gene (Figure 6).

The efficiency of the PCR reactions of the *dszB* gene were typically very low. PCR reactions amplifying the *dszB* gene were only successful in the presence of 3% dimethyl sulfoxide (DMSO) and using the "hot start" method. In addition, the primers used have very high T_m and the success rate is dependent upon the precise thermal conditions of the PCR reaction. In order to obtain an acceptable amplification of the *dszB* gene, one must consider the high percent of G-C content. The high G-C content requires a high denaturing temperature in order to have an efficient separation of dsDNA. Before optimization the efficiency of the amplification of *dszB* was low with a high yield of undesired primer dimers

(Figure 6). However, with the adjustment of the denaturing temperature and time and the annealing temperature and time, the efficiency of the *dszB* amplification approached 100%.

After PCR conditions were optimized, the error-prone PCR reaction was conducted. Conditions were optimized to yield a mutation frequency of 3-7 mutations/kb. The product of the error-prone PCR reaction correlates to the full-length *dszB* gene, suggesting that the error-prone amplification was successful in amplifying the gene.

In order to screen the mutant library, a negative control plasmid was prepared. The plasmid was generated by amplifying and cloning the control insert into pBAD TOPO® vector. The product of the PCR reaction observed corresponded to the expected size of the control insert (Figure 8).

The product of the error-prone PCR reaction was cloned into pBAD TOPO® and transfected into *E. coli*. The resulting colonies of the cloning and transfection procedure were then isolated, lysed and screened for relative activity. The screening assay measuring the rate of the product (HBP) formation was normalized to the relative protein concentrations of each cell lysate being screened. A total of 103 colonies were screened. The relative activity was determined by setting the positive control to one, averaging the triplicate trial values and calculating the standard deviation of the samples from positive control. The screen resulted in four mutants with activity significantly above positive control, seven mutants at about the same as pEBS16 activity and 82 significantly below positive control.

The mutant *dszB* genes with a relative activity significantly higher than positive control gene were isolated, re-cultured and frozen. Plasmids of the mutants were then isolated to verify the presence of the plasmid. Three of the isolated ran at 2500 bp

(Figure 10), while one plasmid (pMUTA93) had bands at both 2500 bp and 3000 bp, intially suggesting the presence of supercoiled DNA and nicked DNA.

The mutant *dszB* genes in pBAD TOPO® (pMUTA) were then cloned into the pBAD/TOPO® ThioFusion™ vector. The pBAD/TOPO® ThioFusion™ vector encodes a thioredoxin protein segment upstream from the gene insert, generating a fusion protein. Within the folded thioredoxin protein is a His-patch containing four histidine residues. The presence of the His patch allows the one-step IMAC purification to be performed. After purification the thioredoxin protein can be removed from the DszB enzyme by protease digestion with enterokinase. Furthermore, fusion to the thioredoxin protein has been shown to assist in the solubility of expressed proteins.

The colonies resulting from cloning the *dszB* genes in pBAD/TOPO THIO were isolated and the plasmids purified. In Figure 11 and 12, the plasmid sizes may be observed. Only the pMTHIO93 plasmid correlated to the expected size of *dszB* inserted into pBAD/TOPO® ThioFusion™ vector. With this information and the information ascertained from Figure 10 it is possible that the *dszB* gene was never present in other mutants. To verify this hypothesis, a second PCR reaction was conducted on all isolated plasmids. The only PCR products that corresponded to the *dszB* gene size of 1098 bp were isolated from mutant 93 (Figure 13). It was concluded that the other plasmids might not have contained the *dszB* gene. Given this conclusion, only mutant 93 was characterized further.

Cloning of genes into the pBAD/TOPO® ThioFusion™ vector is not directional and therefore there are two possible orientations of the inserted gene. The orientation of pMTHIO93 was determined using the restriction enzymes cuts. A single digest and double digest of the mutant 93 plasmid was conducted. The results of the digest may be observed in

Figure 14. Since the *Pst*I site is internal and 70 bases downstream from the 3' end of the *dszB* gene and the *Nco*I site is in the vector 369 bases upstream the 5' end of the *dszB* gene, the results of the double digest indicate that the gene was inserted in the correct orientation.

Sequencing of the gene was only successful using the reverse sequencing primer. Over a thousand bases were sequenced including 130 bases of the vector. Sequencing results indicate two mutations are present. The error-prone PCR reaction was run under conditions designed to yield 0.3%-0.7% mutation rate. The percent of the mutation observed in the *dszB* was 0.24%. At this mutation rate there is a 60% chance a third mutation has occurred in the un-sequenced portion *dszB* gene between bases 1-253. The mutations that were identified are silent mutations (TTG (Leu) to CTG (Leu) and GTG (Val) to GTA (Val)). However, it is possible that with sequencing of the 5' end of the gene an additional mutation will be found that results in an amino acid change. In the future, a lower concentration of template DNA should be used in the mutagenesis reaction in order to increase the mutation frequency.

The pBAD/TOPO® ThioFusion™ expression system is a vector designed for expression in *E. coli* and is driven by araBAD promoter (pBAD).[33] The *araC* gene product encoded on the pBAD/Thio-TOPO plasmid positively regulates this promoter.[33] Recombinant proteins are expressed as fusions to His-Patch thioredoxin for solubility, high-level expression and simple purification. The *araBAD* promoter used is both positively and negatively regulated by the product of the *araC* gene.[33] *araC* is a transcriptional regulator that forms a complex with L-arabinose. In the absence of arabinose the AraC dimer contacts the O_2 and I_1 sites of the *araBAD* operon, forming a 210 bp DNA loop. For maximum transcriptional activation two events are required.[33] Optimal arabinose concentrations must be present to bind the AraC and cause the protein to release the O_2 site and bind the I_2 site

which is adjacent to the I_1 site. This releases the loop and allows transcription to begin.[33] Also, the cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC to I_1 and I_2.[33] In order to determine the optimal concentrations of arabinose required for expression of the fusion protein to be expressed a pilot expression experiment was conducted. The pilot expression study indicated that there is no effect of arabinose concentration on the number or intensity of protein bands. The expected size of a thioredoxin-DszB fusion protein is approximately 53 kDa. The results obtained from this study are not conclusive, however a band at approximately 53 kDa is present at all concentrations of arabinose.

The ProBond™ Purification System is used to purify both 6xHis-tagged and His-patch thioredoxin recombinant proteins expressed in bacteria, insect and mammalian cells.[34] The system is designed around a high affinity and selectivity of ProBond™ resin (Ni^{2+} charged) for recombinant fusion proteins that have been tagged with a His-patch thioredoxin.[34] The ProBond™ purification was used here to isolate the mutant DszB protein fused to a His-patch thioredoxin protein. The results of the purification showed no significant purification of any single protein (Figure 17 and Figure 18).

The data collected from the preceding experiments are not conclusive and cannot definitively answer whether or not a mutant was generated with enhanced activity. The screening was not efficient and fell well short of being high-through put. Not only was the approach in screening inefficient in analyzing a mass quantity of mutant genes, the instrumentation used to screen for product formation was flawed. The fluorimeter produced a high error due to high quantity of scatter. The scatter was determined to be 50% in a standard deviation. With this the potential of false positives is present without question. The

method or approach in screening for such beneficial mutations must be evaluated. A type of visual screen of mutant colonies on agarose gel must be introduced to eliminate colonies with a non-functional gene product prior to the flourimetric screen. An assay must also be developed to complement the fluorimeteric screen to verify the data obtained from the fluorimeter. Not only will adjustments need to be made on the screening aspect of such a study but the cloning efficiency must be maximized. The expected cloning efficiency in this experiment was 95% without considering directionality. When considering directionality the cloning efficiency drops to under 50%. We observed approximately a 25% cloning efficiency. Several approaches may be taken to increase the cloning efficiency, including the increase of gene insert to vector ratio and the incubation of the cloning reaction time on ice.

Expression experiments and purification experiments were inconclusive. One way of definitively determining the expression of our protein fused with thioredoxin is by immunochemistry coupled with western blotting. Several approaches may be taken to optimize purification, such as recharging the resin with tighter binding ions (i.e. Zn or Co), removing the stop codons on the 3' end of the *dszB* gene and/or developing a multiple step purification procedure.

The *dszB* gene contains high G-C content, which results in a uniquely difficult gene to manipulate. Despite all the difficulties, many protocols were tested and several were optimized including: restriction digestion, error-prone PCR, PCR of the gene, cloning and transfection. Using these techniques it will be possible to routinely generate mutant libraries, screen and characterize their products.

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VITA

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