Technical Progress Report

for

The Biocatalytic Desulfurization Project

Quarterly Report

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Abstract

Research activities in the second quarter have largely been a continuation of efforts previously described in the first quarterly report as well as a degree of redirection of effort as a result of discussions during the first quarterly meeting held in San Diego. Chemical synthesis efforts have been refined and are currently being used to support generation of substrates for evaluation and evolution of enzymes for their oxidation. Analysis of the sulfur species in Petro Star diesel, CED extract and refinement of the speciation data is nearly complete. Molecular biology efforts continue with the cloning, expression and characterization of the DszA and DszC proteins as well as the flavin reductases to support regeneration of the essential FMN cofactors. In addition, we have initiated an evolution effort for the extension and improvement of DszA enzyme activity using Diversa's Gene Site Saturation Mutagenesis (GSSMTM) technology. To support the evolution effort as well as of characterization of enzyme activities on a variety of substrates, a high-throughput mass spectroscopy-based assay has been developed. Two selection/screen strategies for the discovery and evolution of biocatalyst enzyme have been developed and are being evaluated for performance using gene libraries constructed from known biodesulfurization strains and environmental libraries.

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1. Executive Summary

Task 1

Analysis of Petro Star diesel was carried out by separating peroxyacetic acid-oxidized material into discrete fractions using preparative LC. Total sulfur determinations and comparison to compositional mass has enabled the determination of the nature and contribution of individual sulfur-containing species within homologous series (n+1 alkyl substitutions).

Task 8

Similar to the efforts described above, Petro Star-provided CED material has been analyzed and contributions of sulfur species determined. In support of Task 1 and Task 8, as well as additional tasks, synthesis of alkyl-substituted dibenzothiophenes and benzothiophenes for use as analytical standards and model substrates continues.

Tasks 2, 3, 4, 9

To determine the enzymatic characteristics of Dsz proteins available from a variety of organisms and to provide information for subsequent optimization of activities, DszA and DszC proteins, as well as the DszD flavin reductase components, have been cloned, expressed and purified from a number of organisms. In addition, development of a MS-based assay for determination of catalytic rates is in progress to support the quantitative evaluation of the Dsz enzymes and their high-throughput characterization during evolution efforts. Directed evolution of the DszA protein from *Rhodococcus* IGTS8 has been initiated with efforts focused on extension of the substrate range of the enzyme on alkyl-substituted DBTs and BTs. This is carried out by GSSM, a proprietary Diversa technology for the comprehensive mutagenesis of every position within a given protein and evaluation of the effect on enzymatic activity and performance. Discovery efforts also continue with the refinement of a screening/selection strategy for identifying additional *dsz* genes from the environmental gene libraries generated from oil-contaminated sites in Russia, Indonesia and US.

2. Experimental

Chemical assay:

HPLC-MS-MS for the determination BT and DBT sulfone substrates and products was carried out by injecting samples from 96-well plates using a CTC PAL autosampler (Leap Technologies, Carrboro, N.C.) into an LC mobile phase provided by LC-10ADvp pumps (Shimadzu, Kyoto, Japan) at 1.0 mL/min through an Agilent Zorbax C8 column (4.6 x 150 mm) (Agilent Technologies, Palo Alto, CA) to an API 4000 TurboIon spray triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Ion spray and Multiple Reaction Monitoring (MRM) are performed for analytes in the positive ion mode. Initial ionization produces parent ions, fragments of which are observed and quantified. The current method for analysis of DBT sulfone is a seven minute run with a five minute H₂O/acetonitrile gradient from 70% H₂O to 5% H₂O. For methylated DBT sulfones, a five minute isocratic mixture of H₂O / acetonitrile 25-75% is used.

Molecular biology:

Genomic DNAs were prepared using standard methods (1). PCR amplification of sequences to be cloned was carried out using either genomic DNA or purified plasmid as template and *Pfu* polymerase (Stratagene, La Jolla). Optimized amplification conditions were determined by varying incubation temperatures and numbers of cycles. PCR products of the coding regions of DszA and DszC homologues were amplified by PCR and cloned into pASK5 plasmid between *Nco*I and *Bgl*II sites to create a plasmid that expresses native or carboxy-terminal hexahistidine-tagged oxidoreductase. For amino-terminal hexahistidine-tagged proteins, the PCR product was cloned into pASK1 between *Bgl*II and *Hind*III sites.

Construction of hydroxybiphenyl reporter plasmid was carried out as follows. A 3.5kb PCR fragment containing the *hbpRC* genes from *Pseudomonas azelaica* (2) was cloned into the *Eco*RI-*Bam*HI site of reporter plasmid pProbe-OT' (3). Into this plasmid was cloned a 750bp PCR fragment containing a promoterless chloramphenicol acetyltransferase gene into a *Sal*I-*Pst*I site. The final construct is called pHbp-GFP-Cat.

To construct the *cysP* operon (encoding sulfate/thiosulfate permease) deletion in *E. coli*, a composite PCR fragment containing ~1kb on either side of the *cysP* operon was generated by overlap PCR and introduced into the *Eco*RI site of pST98-AS, a vector for the markerless replacement of genes in the *E. coli* chromosome. This construct was then used to generate the chromosomal deletion of the *cysP* operon using the markerless exchange (4) and the mutation confirmed by chromosomal PCR.

Expression and Purification:

E. coli TOP10 cells (Invitrogen, Carlsbad CA) were used as a host to express recombinant oxidoreductases. Freshly transformed colonies were inoculated into LB medium with kanamycin. 1 L cultures were grown until OD₆₀₀~1 and expression was

induced with anhydrotetracycline (10-100 $\mu\text{g/l}$) and carried on overnight at 30°C. Cells were lysed in buffer I (50 mM sodium phosphate; 100mM NaCl supplemented with lysozyme (EpiCentre, Madison, WI) by french-pressure treatment. Cell lysates were clarified by centrifugation at 15,000 x g for 30 min and recombinant protein was bound to pre-equilibrated Ni^{+2} -NTA resins at 4°C for 1 hr. Resins were washed with buffer I containing imidazole (20 to 80 mM) and eluted with 0.5 M imidazole in buffer I. The eluted protein was dialyzed against buffer II (50 mM Tris pH 7.5, 100 mM NaCl) and then against buffer II containing 40% glycerol. Protein was stored at -20°C in buffer II with 40% glycerol and 1mM DTT.

Enzymatic assays:

In vitro DszA assays were carried out using conditions as previously described (5) with slight modifications. Purified proteins (200pmols of DszA and 450 pmols of flavin reductase), as well as crude lysates were incubated with 100 μM DBTO₂, in the presence of 10 μM FMN, 4 mM NADH, 100 mM NaCl, and 25 mM sodium phosphate buffer (pH 7.5). The reaction mixture was shaken at 230 rpm at 30°C. At designated time points the reaction was quenched with equal volume EtOH and then diluted 10X with 1% ammonium hydroxide. Substrate and product concentrations were determined by mass spectrometry.

In vitro flavin reductase assays were carried out as described (5). The reductase activity was measured by the FMN-dependent oxidation of NADH monitored at 340 nm. Reaction was carried on at 30°C in 50 mM phosphate buffer, in the presence of 0.1 mM NADH and 20 μM FMN.

3. Results and Discussion

Chemical synthesis (in support of multiple tasks)

During the second quarter we continued to acquire and generate various benzothiophene (BT) and dibenzothiophene (DBT) precursors, compounds and their alkylated derivatives as well as corresponding sulfones as model substrates and standards for analysis.

Benzothiophenes (BTs).

We have acquired parent benzothiophene and three monomethyl derivatives (2-, 3-, and 5-MeBTs, C₁BTs). From these we have produced three pure sulfones by standard oxidation with peracetic acid (Scheme 1, Figure 1), while the 2-methylbenzothiophene sulfone proved to be unstable.

Additional synthetic work was performed in two areas, 1) synthesis of other monomethyl BTs and 2) further alkylation of the available BT and monomethylBTs. In the first effort, 7-methylBT (C₁BT) was prepared from 2-methylthiophenol in two steps (Scheme 2). Further alkylation was done by two methods: (i) lithiation and alkylation,

and (ii) acylation and Kishner-Wolf reduction (Scheme 3). Lithiation and alkylation is suitable to alkylated BTs only in position 2, and it was used to methylate and ethylate

Scheme 1. Oxidation of benzothiophenes

Scheme 2. Preparation of 7-Methylbenzothiophene

Scheme 3. Further alkylation of benzothiophenes

Scheme 4. Oxidation of dialkylsulfides

Scheme 5. Preparation of DBT sultine

Figure 1. Schema for the synthesis of compounds.

5-MeBT. Acylation is a more universal approach, and it was used to generate precursors for 2-ethylBT (C_2 BT), 3-ethylBT (C_2 BT), 2-methyl-3-ethylBT (C_3 BT), 2,5-dimethyl-3-ethylBT, (C_4 BT) 5-methyl-3,5-diethylBT (C_5 BT), and 2,5-dimethyl-3,6-diethylBT

(C₆BT). Some of these precursors were obtained as mixtures. Reduction of the precursors proceeds with fair to poor yields, and the corresponding procedure will need optimization.

Dibenzothiophenes (DBTs).

The pool of already prepared DBTs includes 4-MeDBT (C₁DBT), 4,6-diMeDBT (C₂DBT), 4-EtDBT (C₂DBT), 4,6-diEtDBT (C₄DBT), 4-PrDBT (C₃DBT), 4,6-diPrDBT (C₄DBT), 2,8-diMeDBT (C₂DBT), 3,7-diMeDBT (C₂DBT), and 2,4,8-triMeDBT (C₃DBT). All these DBTs have been converted into corresponding sulfones. In addition, several precursors for alkylated DBTs and a mixture of monoethylDBTs have been prepared. Precursors include a mixture of monoacylated 4-MeDBT (C₃DBT), mixture of bisacylated 4-MeDBT (C₅DBT), 2-acyl-3,7-diMeDBT (C₄DBT), mixture of bisacylated 3,7-diMeDBT (C₆DBT), a mixture of monoacylated 4,6-diMeDBT (C₄DBT), and mixture of bisacylated 4,6-diMeDBT (C₆DBT).

Other compounds.

We acquired seven commercially available symmetric dialkyl sulfides (butyl through decyl) and prepared a series of corresponding sulfones (Scheme 4). Analysis of GC-MS chromatograms of the sulfones confirms our initial observation that these compounds are not present in CED of PetroStar diesel. Currently, we use the dialkyl sulfones as internal standards for quantitative analysis of CED fractions.

We have also prepared DBT sulfone (anhydrous hydroxybiphenylsulfinate) (Scheme 5) as a standard for analysis of enzymatic reaction products.

Chemical Analysis (Tasks 1, 8 and support of other tasks)

CED Analysis. As described in the previous report, we are analyzing CED of Petrostar diesel by separating it into fractions by preparative HPLC and further analyzing these fractions by GC/MS/SCD. Distribution curves were plotted for sets of compounds with masses differing by 14 m/z units based on relative intensity of MS signals and absolute normalized intensities of SCD signals. These curves showed that members of these series of compounds appeared initially at lower concentrations in early HPLC fractions then in increased amounts in later fractions; they went through a maximum and declined to undetectable levels. Such distribution curves may be expected for elution of homologues series of compounds belonging to a certain class. Recognition of similar patterns allowed us to validate presence of previously assigned homologous series of alkylated DBTs, BTs, thiophenes, monocyclic and bicyclic sulfides in the Petro Star CED. Plotting SCD data from all HPLC fractions produced a two-dimensional chromatogram of the CED sample (Figure 2).

Figure 2. Two-dimensional chromatogram of sulfur species in CED

Assay development.

We have chosen HPLC-MS-MS as a method of choice for determination of concentration of BT and DBT and sulfone substrates because of its accuracy and flexibility (Table 1). These methods are essential for both the kinetic characterization of enzyme activities as well as the development of high-throughput procedures for enzyme evolution. The method that has been developed allows for quantitation of the substrates and products in the range of concentrations between 2 and 100 μ molar.

Sulfones	MRM masses	
	Parent ion	Observed fragment
DBT	217.3	152.1
4-MeDBT	231.2	152.1
4,6-diMeDBT	245.2	165.2
4-EtDBT	245.2	165.1
4-diEtDBT	273.2	181.0
2,4,8-triMeDBT	259.0	165.1
BT	167.1	103.1
3-MeBT	181.1	91.0
5-MeBT	181.1	91.1

Table 1. MS-MS data for analysis of sulfone derivatives of DBT and BT compounds.

Molecular Biology and Biochemistry (Tasks 2, 4 and 9)

Cloning, Expression and Purification of Dsz Proteins

As the focus of our evaluation and optimization of the biodesulfurization proteins is the *in vitro* characterization of enzymatic activities, we continue our efforts to clone express and purify each of the available DszA, -C and -D proteins. During the second quarter we have nearly completed this effort on the seven DszA homologue proteins that are available (Table 2). Three DszD homologues have also been expressed and purified for evaluation of activity and interaction with DszAs in the *in vitro* assay. In addition, three *dszC* genes were subcloned and are currently being expressed and purified.

Gene/Enzyme	Subcloned	Purified
DszD	+	n.a.
DszD-N	+	+
DszD-C	+	+
Fre	+	n.a.
Fre-N	+	+
Fre-C	+	+
HpaC	+	n.a.
HpaC-N	+	+
HpaC-C	+	+

Gene/Enzyme	Subcloned	Purified
IGTS8 DszA	+	n.a.
IGTS8 DszA-N	+	+
IGTS8 DszA-C	+	+
<i>Sphingomonas</i> DszA	+	n.a.
<i>Sphingomonas</i> DszA-N	+	+
<i>Sphingomonas</i> DszA-C	+	+
<i>Nocardia</i> A3HI DszA	+	n.a.
<i>Nocardia</i> A3HI DszA-N	+	+
<i>Nocardia</i> A3HI DszA-C	In progress	
<i>Azotobacter</i> "DszA"	+	n.a.
<i>Azotobacter</i> "DszA" -N	+	+
<i>Azotobacter</i> "DszA" -C	+	+
EMT4 ToeA	+	n.a.
EMT4 ToeA-N	+	+
EMT4 ToeA-C	+	+
670-1 ToeA	+	n.a.
670-1 ToeA-N	+	+
670-1 ToeA-C	+	+
KGB1 ToeA	+	n.a.
KGB1 ToeA-N	+	+
KGB1 ToeA-C	+	+
IGTS8 DszC	In progress	
IGTS8 DszC-N	In progress	
IGTS8 DszC-C	In progress	
<i>Sphingomonas</i> DszC	+	In progress
<i>Sphingomonas</i> DszC-N	+	In progress
<i>Sphingomonas</i> DszC-C	+	In progress
<i>Nocardia</i> A3HI DszC	+	In progress
<i>Nocardia</i> A3HI DszC-N	+	In progress
<i>Nocardia</i> A3HI DszC-C	+	In progress

Table 2. Current progress in cloning and expressing biodesulfurization enzymes.
n.a. not attempted

Characterization of DszA activities

To obtain the specific activity of different DszA enzymes with DBT and BT substrates, a determination of the relative solubility of the substrates and the optimum composition of cosolvents for assays is being carried out. As mentioned above, we have recently developed a mass spectrometry-based assay for the evaluation of enzyme activity. The MS assay will therefore replace the 4-aminoantipyrene total phenol assay that was previously used for initial experiments, but is unsuitable for analysis of a number of the substituted DBTs as well as BTs.

To assess the optimum combination of flavin reductase (HpaC, DszD or Fre, each with either N-terminal or C-terminal HisTag for purification) and DszA proteins, we have begun the evaluation of each of the purified enzymes using DBT sulfone as a model compound. The combinations that produce the highest consistent rates of activities will then be used to evaluate the DszAs on the DBT and BT alkylated derivatives. A preliminary experiment assaying the *Rhodococcus* IGTS8 DszA together with five possible flavin reductases is shown in Table 3.

Flavin Reductase	$\mu\text{M DBTO}_2$ conversion/min/200pmole	$\mu\text{M DBTO}_2$ conversion/min/1nmole
HpaC-N	6.5 +/- 1.3	29.3 +/- 1.9
HpaC-C	7.6 +/- 1.1	38.0 +/- 5.7
Fre-N	6.1 +/- 1.4	30.7 +/- 7.3
Fre-C	3.6 +/- 0.9	17.9 +/- 4.4
DszD-N	5.3 +/- 1.8	26.4 +/- 8.8

Table 3. *Assay of Rhodococcus IGTS8 DszA using different flavin reductases*

GSSM of DszA from *Rhodococcus* IGTS8.

The directed evolution of the *Rhodococcus* IGTS8 *dszA* gene has been initiated. Oligonucleotides for the Gene Site Saturation Mutagenesis have been purchased and are being used to construct all possible mutations at every site in the protein. An ordered library of mutations is in the process of being generated and quality control is being carried out. Once the library of mutations is built and target substrates are identified, a high throughput MS assay will be carried out to identify mutations that extend substrate specificity by determining the ratio of activity on the new substrates to that of DBT sulfone. Initial characterizations will seek to identify increased activities on alkyl substituted DBT and BTs.

Construction of DszA-flavin reductase fusions.

One of the challenges for improved biocatalyst is the coordination of expression and activity of flavin reductase component with DszA and DszC. One strategy for

addressing this challenge the construction a translational fusion of the DszA/C monooxygenases and the flavin reductase. Examples of naturally occurring fusion proteins are the sterol α -demethylase/ferredoxin fusion protein from *Methylococcus capsulatus* (6) and the P450_{BM-3} a *Bacillus megaterium* flavocytochrome, in which the fatty acid hydroxylase component is fused to a diflavin NADPH reductase and has the highest catalytic activity of any known cytochrome P450 (7).

Here we have decided to investigate the possibility of creating a novel fusion protein between the IGTS8 DszA and one of two flavin reductases, DszD and Fre. The two proteins in each fusion are designed to be joined by a flexible alanine-rich linker region and both orientations in the order of components will also generated (i.e. DszA-DszD and DszD-DszA; DszA-Fre and DszD-Fre). As of this report, one of the four combinations has been generated and is currently being characterized for activities of each of the component parts.

Discovery of novel biodesulfurization genes

As previously reported, efforts for the discovery of additional biodesulfurization genes has shifted from using a sequence-based approach to an expression-based approach. To this end, we have been exploring two parallel approaches to develop a bacterial host for the discovery of novel enzymes.

In the first approach, we have constructed an *E. coli* mutant strain that is unable to transport extracellular sulfate or thiosulfate. This mutant lacks the sulfate/thiosulfate ATP dependent ABC transport system and is severely inhibited in its ability to use sulfate as a source of sulfur. Figure 4 shows a growth curve of the mutant compared to wild-type on minimal medium with sulfate.

Figure 4. Growth of *Escherichia coli* wild-type (■) and sulfate transport mutant (□) in M9 minimal medium containing 2 mM sulfate with glucose as carbon source.

As this mutant is unable to efficiently incorporate sulfate ions that often contaminate reagents such as mineral salts or the agar/agarose components of microbiological media, it will be tested as a host for the direct selection of biodesulfurization genes from environmental libraries. Recombinant clones expressing DszB-like activity will provide intracellular sulfite to allow for growth dependent upon hydrolysis of hydroxybiphenylsulfinate (product of the oxidation of DBT sulfone by DszA). As all known *dszB* genes are directly linked to both *dszA* and *dszC*, such a strategy should also yield these genes if the DNA insert is of adequate size as isolated from the environmental library. This system may also be used for the selection of evolved variants of Dsz genes that have higher rates of activity or expanded substrate specificity provided they result in increased levels of sulfite production.

An alternative approach that may also be used for both initial discovery as well as later evolution efforts is the use of a bioreporter strain that detects products of the biodesulfurization pathway with high sensitivity. *Pseudomonas azelaica* is a soil organism that is capable of utilizing hydroxybiphenyl as sole source of carbon and energy. To induce the expression of the degradative genes only in the presence of substrate, this bacteria utilizes the regulatory protein HbpR to directly sense HBP and induce the HbpCA operon (2). By incorporating both the *hbpR* gene and the regulated promoter into a transcriptional reporter vector, a very sensitive molecular reporter for HBP can be generated.

With this in mind, we have constructed a reporter that is able to generate a fluorescent signal (using GFP) upon exposure to nanomolar concentrations of HBP. By incorporating this reporter into a host strain and subsequently introducing environmental libraries, recombinant clones expressing DszB activity will hydrolyze HBPS to HBP and can be readily detected by their fluorescence. We are currently incorporating a second reporter (chloramphenicol acetyltransferase) into the construct in order to allow for direct selection of clones by HBP-dependent induction of antibiotic (chloramphenicol) resistance.

4. Conclusions

Effort in the chemical analysis of Petro Star diesel and CED extract are nearing completion. A number of innovative approaches to analyzing and rationalizing the sulfur content of these materials have been carried out. In particular, the extensive preparative fractionation of the material before undertaking MS and the interpretation of the data by considering the presence of homologous series of alkyl substitutions has allowed for a detailed compositional analysis of sulfur species. Tasks 1 and 8 are nearing completion and will culminate in the preparation of a detailed report of the methodologies and findings within the next month.

We are also making significant progress in the cloning and expression of all available biodesulfurization genes and are moving now into the determination of their enzymatic properties in regard to rates and specificities. A significant amount of work has

and is being done on developing *in vitro* analytical techniques to allow for the direct comparisons of enzymes and their potential value in a developed biocatalyst strain. This has proven to be more difficult than originally anticipated, primarily due to the requirement for maintaining solubility of the various substrates to be tested and the need for purifying and stabilizing enzyme activities throughout the course of their characterization. In addition, the development of analytical methods to support that high throughput requirements of a directed evolution effort are also very intensive and require a high degree of validation in terms of robustness and reproducibility before implementation.

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6. List of Acronyms and Abbreviations –

BDS	Biodesulfurization
CED	Conversion Extraction Desulfurization
DBT	Dibenzothiophene
DBTO ₂	Dibenzothiophene sulfone
DNA	Deoxyribonucleic acid
FMNH ₂	Reduced flavin mononucleotide
GC	Gas chromatography
HBP	Hydroxybiphenyl

HBPS	Hydroxybiphenylsulfinate
HPLC	High performance (pressure) liquid chromatography
MS	Mass spectrometry
NADH	Reduced nicotinamide dinucleotide
PCR	Polymerase chain reaction
RP	Reverse phase
SCD	Sulfur chemiluminescence detector