Gasoline Biodesulfurization
DE-FC07-97ID13570
FINAL REPORT

ENCHIRA
Biotechnology Corporation

RESEARCH & DEVELOPMENT

GASOLINE DESULFURIZATION PROGRAM

DE-FC07-97ID13570

Final Report

Results from 1997-2001

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EXECUTIVE SUMMARY

Nine strains were identified to grow with gasoline as sole sulfur source. Two different genes were cloned from *Gordonia terrae* KGB1 and tested for the ability to support gasoline BDS. The first of these, *fmo*A, was cloned by screening a KGB1 gene library for the ability to convert indole to indigo (a sulfur-regulated capability in KGB1). The *fmo*A gene was overexpressed in a gasoline tolerant strain of *Pseudomonas putida* PpG1 and the recombinant strain was shown to convert thiophene to a dimer of thiophene sulfoxide at rates nearly two orders of magnitude higher than KGB1 could catalyze the reaction. Despite this high activity the recombinant PpG1 was unable to demonstrate any activity against gasoline either in shake flask or in bench-scale gasoline BDS bioreactor.

A second gene (*toe*A) was cloned from KGB1 and shown to support growth of *Rhodococcus erythropolis* JB55 on gasoline. The *toe*A gene was also identified in another gasoline strain *T. wratislaviensis* EMT4, and was identified as a homolog of *dsz*A from *R. erythropolis* IGTS8. Expression of this gene in JB55 supported conversion of DBTO2 (the natural substrate for DszA) to HPBS, but activity against gasoline was low and BDS results were inconsistent. It appeared that activity was directed against C2- and C3-thiophenes. Efforts to increase gene expression by plasmid manipulation, by addition of flavin reductase genes, or by expression in PpG1 were unsuccessful.

The DszC protein (DBT monooxygenase) from IGTS8 has very little activity against the sulfur compounds in gasoline, but a mutant enzyme with a substitution of phenylalanine for valine at position 261 was shown to have an altered substrate range. This alteration resulted in increased activity against gasoline, with activity towards mainly C3- and C4-thiophenes and benzothiophene.

A mutant library of *dsz*B was constructed by RACHITT (W. C. Coco et al., DNA shuffling method for generating highly recombined genes and evolved enzymes. 2001. Nature Biotech. 19:354-359) method of *in vitro* recombination. Methods for analysis were developed and a preliminary analysis of the library was performed.

A preliminary gasoline process design was constructed and process economics were determined based upon assumptions made from experimental results. The projected cost of gasoline BDS was determined to be competitive with current competing technologies.

BACKGROUND

Sulfur in gasoline is not only a source of air pollution, but also plays a significant role in determining the tailpipe emissions of other pollutants, such as nitrogen oxides, carbon monoxide and unburned hydrocarbons. The biological removal of sulfur from gasoline, a process called Biocatalytic Desulfurization (BDS) under development at Enchira Biotechnology Corporation (EBC), is an innovative approach that offers the petroleum industry and the U.S. economy potentially great rewards. The purpose of this project was to develop gasoline BDS technology to the point of readiness for operation in a continuous test unit, which would provide the basis for economically attractive commercial designs.

Most of the sulfur species in gasoline are a result of fluid catalytic cracking (FCC) of gas oils or residuum materials that are relatively high in sulfur. There are three main classes of organic molecules: thiophenes, benzothiophenes, and mercaptans. (Throughout this report we will use the abbreviations T for thiophene, BT for benzothiophene and DBT for dibenzothiophene. The prefix Cx- will be shorthand for unspecified levels of alkylation. A complete list of abbreviations can be found in Appendix 1) Therefore, refineries using FCC to produce gasoline components are concerned with sulfur reduction in gasoline. Sulfur levels can be reduced during refining by hydrodesulfurization (HDS) of the product material or the feed to the FCC unit. However, HDS is an expensive and energy-intensive process, requiring high temperature and pressure. It also requires expensive collateral processes to generate hydrogen and to convert the main process by-product, hydrogen sulfide, from a poisonous, odorous gas into an acceptable by-product form, elemental sulfur. When applied to gasoline produced by FCC, the HDS process saturates some of the olefins that are the primary contributors to octane rating, thereby significantly raising the cost of the product and reducing the quality of the fuel. Using HDS to treat FCC feed does not have the octane debit drawback, but these HDS units are particularly expensive to build and operate.

If BDS can be applied effectively to gasoline, the refiner will be offered a less expensive alternative to HDS that also avoids the drawback of octane degradation. Furthermore, BDS will significantly reduce energy requirements and, consequently, the environmental impact of desulfurizing gasoline (L. Linguist and M. Pacheco. Enzyme-based diesel desulfurization offers energy, CO2 advantages. Oil Gas J. 1999 97:45-48). The majority of U.S. refiners are able to achieve average gasoline sulfur levels of about 300 ppm, but are faced with expensive investments in order to achieve the lower levels (30 ppm) that are being discussed widely by regulatory authorities and the auto industry for introduction by year 2005 (M. Lorenzetti. US refiners, EPA continue maneuvering as low-sulfur fuel deadlines loom. Oil & Gas J. 2001. 19:66-70). Therefore, refiners are looking for new technologies to reduce the cost of environmental compliance and of sulfur removal in particular. Since BDS has the potential advantages of lower capital and operating costs than HDS and no octane debit for the FCC gasoline, refiners have shown interest in this technology (B. L. McFarland et al. Biocatalytic sulfur removal from fuels: applicability for producing low sulfur gasoline. Crit. Rev. Microbiol. 1998 24:99-147).

Conservatively, the U.S. market opportunity for gasoline BDS is about 1.2 million barrels per day. Similar analysis indicates a European market, representing export opportunity for the technology, of about 0.7 million barrels per day. We estimate the size of the Asian market to be at least 0.5 million barrels per day (William Heck, personal communication).

This project was initiated with the premise that BDS for gasoline would require the development of a new biocatalyst that would be selective for the sulfur molecules present in gasoline and not act upon the bulk of the hydrocarbons as well as the design of a specialized processing system. The gasoline application differs from EBC's efforts in middle distillate BDS because of the unique sulfur species, the toxicity of the gasoline to biocatalyst and the potential explosion hazard. Nonetheless, the three critical success factors, rate, extent, and longevity, identified in the middle distillate BDS program (M. A. Pacheco et al. Recent advances in biodesulfurization of diesel fuel. Annual Meeting NPRA: 1999 March 21-23; San Antonio, Texas. Wasington, DC; National Petrochemical and Refiners Association: 1999:AM-99-27) would also apply to gasoline BDS. With an appropriate biocatalyst system in place as a starting point, the next step would be to clone the genes(s) responsible for gasoline BDS and express them in a gasoline-tolerant bacterial strain. This would lead to an evaluation of BDS rate, extent, and longevity, and classical genetic engineering methods as well as novel directed evolution techniques would be brought to bear to improve biocatalytic deficiencies.

The major process challenges to developing gasoline BDS lie primarily in reactor design, and, to a lesser extent, in separations and by-product disposition. The reactor must be designed to maximize mass transfer through contact between gasoline, the aqueous phase, and biocatalyst. Although the low viscosity of gasoline minimizes difficulties for mixing efficiency, mass transport, and fluid flow, its volatility requires special processing equipment for vapor condensation and recovery. It must also minimize the problems associated with toxicity of the gasoline for the biocatalyst and the issues of flammability and product quality degradation resulting from the use of oxygen (essential for the growth of aerobic bacteria and an expected substrate for the desulfurization enzymes). By-product disposition must be accomplished in an environmentally sound and cost-effective manner.

Prior to the launch of the DOE gasoline BDS project, EBC had a modest research effort focused on the BDS of FCC gasoline. This project was distinct from the efforts in middle distillate BDS because of the differences in sulfur compounds, toxicity, and volatility of the two petroleum streams. We decided early on to narrow our scope to an aerobic process, because despite the fact that an anaerobic biocatalyst would offer several process advantages, an analysis of the thermodynamics of these reactions revealed that Ts or BTs would not serve as terminal electron acceptors. Just as HDS requires high temperatures and high hydrogen partial pressures to force the conversion of recalcitrant substrates, it has been shown that anaerobic BDS under ambient conditions is a very inefficient process.

While this project is still in an early stage, we learned that the biocatalyst developed for middle distillate BDS has very little or no selectivity for the sulfur species found in gasoline. We conducted a microbial screening and enrichment program in order to isolate environmentally derived organisms that could use BT as a sole source of sulfur. We isolated several different strains of bacteria producing enzymes that directly oxidize the sulfur atom in BT. (A complete list of these strains is given in Appendix 2. These strains are maintained in the Enchira culture collection and are available for licensing.) Several strains can utilize the sulfur from BT in a growth-dependent manner, indicating that they release the oxidized sulfur in a form (probably sulfate) that can be biologically assimilated. Further enrichment work produced a strain, classified as *Gordonia terrae* and designated as KGB1 that will use either BT or 3-methyl-T as a sole source of sulfur. KGB1 has a number of disadvantages for development as a gasoline BDS biocatalyst, including poor growth characteristics, few available genetic tools, and, perhaps most importantly, it is extremely sensitive to FCC gasoline. These qualities led us to decide that the best approach would be to clone the genes responsible for gasoline BDS from KGB1 for expression in a gasoline tolerant strain.

In addition to the identification of KGB1 as a potential source of BDS genes, we had also developed expertise in sulfur speciation, obtained mainly from our work in middle distillate BDS. Many of these methods (including sulfur speciation techniques and analysis of ^{14}C-labelled sulfur heterocyclic compounds) could be employed to analyze and characterize gasoline. These methods are described in Appendix 6.

Finally, we had developed systems, including sealed bottle assays and pressurized batch stirred reactors (pBSRs), to perform small-scale biotransformations. However, we had begun to recognize the deficiencies of these systems and had begun to envision a process more closely resembling that being developed by EBC for middle distillates. The gasoline would be combined with a suspension of bacterial cells in water in a well-stirred, aerated reactor. It would be essential to maintain intimate contact among all four phases: hydrocarbon, aqueous, cells, and air. Cells would be recycled and provisions would be included for by-product disposition and cell withdrawal and make-up. Multi stage condensers would be used to minimize the loss of gasoline hydrocarbons due to volatilization.

This information base coupled with the ongoing efforts in middle distillate BDS set the stage for the DOE project on gasoline BDS.

INTRODUCTION

The research plan was divided into five main objectives, which were to be completed by the end of three years:

- Develop a gasoline BDS biocatalyst
- Demonstrate lab-scale BDS of gasoline
- Develop a preliminary gasoline BDS process design
- Develop a basic gasoline BDS performance model
- Assess technology economics

These objectives were organized into two main areas: biocatalyst development and process development. A research plan was proposed with a list of milestones set for each area (See Table 1, below). The list of tasks in Table 1 will be used to organize the results and discussion section of this report.

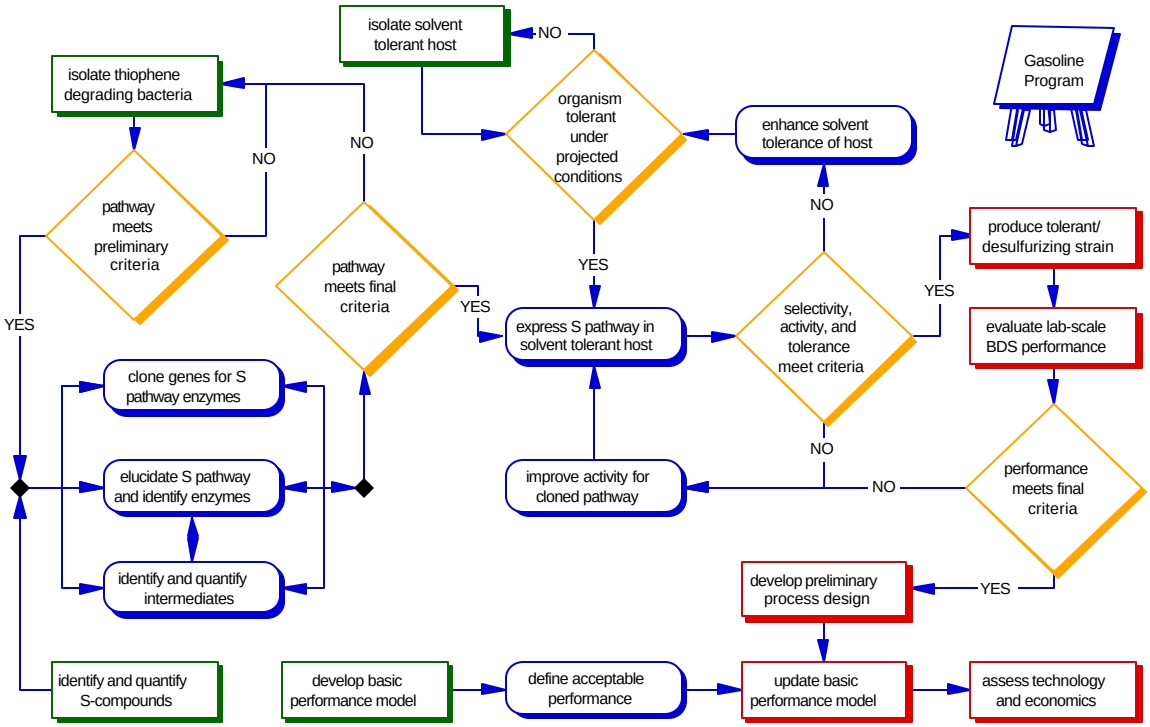
Table 1. Research on Gasoline Desulfurization Process

ID Number	Task / Milestone Description
1	**Biocatalyst Development**
1.1	Isolate Desulfurizing Strains (T, BT)
1.1.1	Identify at least 5 desulfurizing bacteria
1.1.2	Demonstrate in vivo desulfurization (strain#)
1.1.3	Identify S pathway in vivo (±mineralization)(strain#)
1.1.4	Select best desulurizing strain for characterization and development
1.2	Analytical
1.2.1	Identify S compounds (bulk/individually)
1.2.2	Quanitfy S compounds (bulk/individually)
1.2.3	Isolate, identify & quantify intermediates
1.2.4	Develop rapid assay for each reaction intermediate/product
1.2.5	Develop prototype robotic screen
1.3	Enzymology
1.3.1	Review and summarize current knowledge
1.3.2	Elucidate S pathway(s), identify metabolic enzymes
1.3.3	Purify individual pathway enzyme (s)
1.4	Genetics – Characterization
1.4.1	Construct gene banks
1.4.2	Clone genes
1.4.3	Confirm gene/phenotype relationships
1.4.4	Sequence genes
1.4.5	Engineer regulated expression
1.4.6	Complete gene set in selected host
1.5	Genetics – Improvement
1.5.1	Define basis for improvement
1.5.2	Mutagenesis of desulfurization gene cluster
1.5.3	Screen mutant banks
1.5.4	Select “improved” mutant strains
1.5.5	Integrate mutant genes into regulated vectors
1.5.6	Evaluate mutant genes in host strains (s)

Table 1. Research on Gasoline Desulfurization Process	
ID Number	Task / Milestone Description
1.5.7	Complete improved gene set in selected host
1.6	Develop Gasoline Tolerant/Desulfurizing Strain(s)
1.6.1	Build information data base
1.6.2	Identify gasoline tolerant strain(s) at least 5 unique strains
1.6.3	Enhance gasoline tolerance (live/dead ratio>1@24 hr
1.6.4	Develop "model" system for evaluating desulfurization activity
1.6.5	Develop system for evaluating desulfurization activity in gasoline
1.6.6	Enhance stability of desulfurization activity (<10% loss over 24 hr)
1.6.7	Evaluate strategies to enhance tolerance
1.6.8	Specify strain, recommend operating strategy, and operating range
2	**Process development**
2.1	Mathematical modeling
2.2	Reactor testing
2.3	Technology and economic assessment
2.3.1	Construct basis model
2.3.2	Update assessment
2.3.3	Update assessment
2.3.4	Update assessment
2.3.5	Final assessment

It must be noted, however, that this linear list of tasks does not take into consideration the decision-making process required in fundamental research, and the flow chart shown in Figure 1 is a better representation of our approach to carrying out the project. It was understood from the very beginning of this project that any enzymes responsible for growth with sulfur compounds in gasoline might be insufficient (in terms of rate or extent) to be used as the platform for the construction of a biocatalyst, and that it might be necessary to look to novel bacterial isolates for genes better suited for the process. This is, in fact, what happened, as the list of gene candidates was expanded beyond *fmo*A, to include novel genes from thiophene degrading bacteria as well as mutants of *dsz*C isolated from Rhodococcus erythropolis IGTS8. The results and discussion section will deal with each of these candidates separately

Figure 1. Decision tree for organization of gasoline BDS project



RESULTS AND DISCUSSION

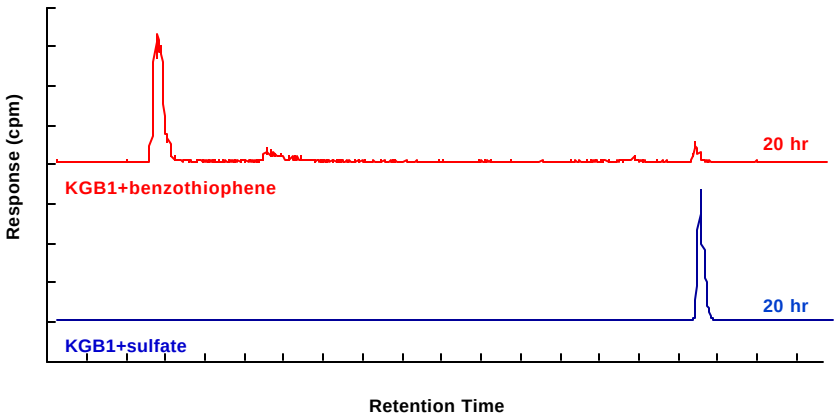
Section 1A. Biocatalyst Development: Fmo

1.1A. Isolate Desulfurizing Strains: Fmo

Growth of bacteria on organosulfur compounds represents a serious technical challenge, because Nocardioform bacteria such as *Rhodococcus* require very little sulfur and can generate colonies even when they cannot assimilate the sulfur compound provided by scavenging more-readily accessible sulfur contaminants present in the agar. Thus it is very easy to obtain false positives when screening for novel sulfur metabolic capabilities. In addition, the hydrocarbons present in gasoline are too toxic to use in most bacterial cultures. Finally, special methods must be used to provide volatile compounds such as Cx-Ts or non-volatile but hydrophobic compounds such as Cx-BTs to aqueous media. Initially, we provided volatile compounds in the vapor phase of plates or liquid cultures and non-volatile compounds dissolved in hexadecane as a second phase for liquid cultures. In this manner, we evaluated many strains for activity on T and BT. Several strains were found to utilize mercaptans and BT as sole sulfur source for growth, but only one strain was found that could utilize BT and T, KGB1. This strain was originally classified as a *Nocardia* sp. by fatty acid analysis, but more recently has been re-classified as a strain of *Gordonia terrae* by 16S-RNA sequence. In addition, to T and BT, KGB1 has been shown to grow with a variety of sulfur compounds including, DMSO, 2-MeT, 3-MeT, 2-EtT, BT sulfone, ethylmercaptan, and propylmercaptan.

Attempts were made to demonstrate gasoline BDS with KGB1, but this strain proved to be especially sensitive to gasoline toxicity. It was possible to demonstrate T and BT biotransformations by KGB1 through the use of ^{14}C-labelled substrates. LC analysis of the aqueous phase with a radiometric detector of a biotransformation mix clearly demonstrated the bioconversion of ^{14}C-labelled T to a very-water soluble product with short retention time (See Figure 2, below). This metabolic capability was shown to be regulated by the growth sulfur source of the cells. Cells grown on BT as sole sulfur source demonstrated the ability to transform T, but cells grown on rich medium did not have that capability. The down-regulation of T metabolic activity on rich medium and the relationship between BT growth and T activity strongly suggested that the KGB1 T pathway was sulfur specific, a critical element in the selection of a source of genes for gasoline BDS. A non-specific enzyme system might attack non-sulfur containing hydrocarbons in gasoline, making it unacceptable as a BDS biocatalyst.

Figure 2. Biotransformation of ^{14}C-labelled thiophene by KGB1



At this point in the project, KGB1 was chosen as the source of genes to build a gasoline biocatalyst. A summary of the characteristics of KGB1 are shown in Figure 3, below.

Figure 3. Characteristics of *Gordonia terrae* strain KGB1

Attributes:

- **grows with several sole S-sources (DMSO, T, 2MT, 3MT, 2ET, BT, BTSO2, n-Et-SH, n-propyl-SH, benzatiazole, & benzodithiol-3-one)**
- **rich media represses desulfurization suggesting S-selectivity**
- **[^{14}C]-T completely transformed to water soluble product(s)**

Limitations:

- **killed quickly when contacting gasoline**
- **growth characteristics**
  - **slow growth**
  - **adheres to glass**
  - **clumps**
- **low desulfurization activity**
- **molecular genetics difficult**

In the second year of the project, we observed that the toxicity of gasoline could be minimized by artificial weathering—removing the most volatile components by flushing the gasoline with nitrogen to reduce the volume by 70% ending with a sulfur concentration of 1000 ppm. Under these conditions all of the sulfur species present in gasoline were recovered although their relative abundances changed. In all experiments, weathered gasoline was diluted with various amounts of hexadecane. Weathered gasoline could be used as a second phase in liquid culture and could also be used to form a stable emulsion with molten agar using Tween-80 as emulsifier. Model compounds dissolved in hexadecane could also be used in emulsion agar plates. Gasoline emulsion plates and sulfur-free hexadecane emulsion plates were used to test a number of soil and sludge isolates from our culture collection. Since some organisms require minute amounts of sulfur, sulfur-free hexadecane emulsion plates were used to distinguish between true utilization of the sulfur source and growth on minimal sulfur contamination. An illustration of these emulsion plates is given in Figure 4, with KGB1 inoculated onto plates with and without weathered gasoline. The use of emulsion plates was a major technical advance in the identification strains with novel sulfur metabolic capabilities.

Figure 4. Growth of gasoline desulfurizing strain on emulsion agar

Weathered gasoline was also used in two-phase shake flask cultures with KGB1, confirming that this strain could indeed utilize gasoline as a sulfur source and allowing for the first time an opportunity to determine the substrate range for this strain on gasoline-related sulfur compounds.

1.2.A. Analytical: Fmo

We developed the analytical capabilities necessary to identify the sufur in model compounds and actual gasoline using GC with sulfur chemiluminescence detection (GC-SCD) and GC with mass spectrometer detection (GC-MS). We developed analytical methods to quantify the bulk sulfur using combustive UV fluorescence. In addition, we identified the target FCC gasoline for use in this project. Two candidate light FCC gasolines contained a range of Cx-Ts and Cx-BTs with total sulfur contents of about 600 ppm.

The two candidate gasolines (FCC Gasoline #1 and #2) were analyzed and found to have slightly different cut points resulting in different amounts of Cx-BTs present (Figure 5). Gasoline #1 contains T, C1-, C2-, and C3-Ts as well as BT, C1-, C2- and some C3-BTs (Table 2). Gasoline #2 contains a similar distribution of organosulfur compounds but only a small amount of BT and no CxBTs. Gasoline #2 was also analyzed by ASTM D-2887/simulated distillation, which showed an even distribution of all components over the temperature range of 20-220^{o}C. Analysis of the different classes of hydrocarbons present revealed that the gasoline contains 20% paraffin, 45% olefins, 10% naphthalenes, and 25% aromatics. Gasoline #2 was selected for further work as it is more representative of the cut produced in most refineries (William Heck, personal communication). Twenty gallons was obtained for use on this project.

Figure 5. Analysis of two candidate FCC gasolines

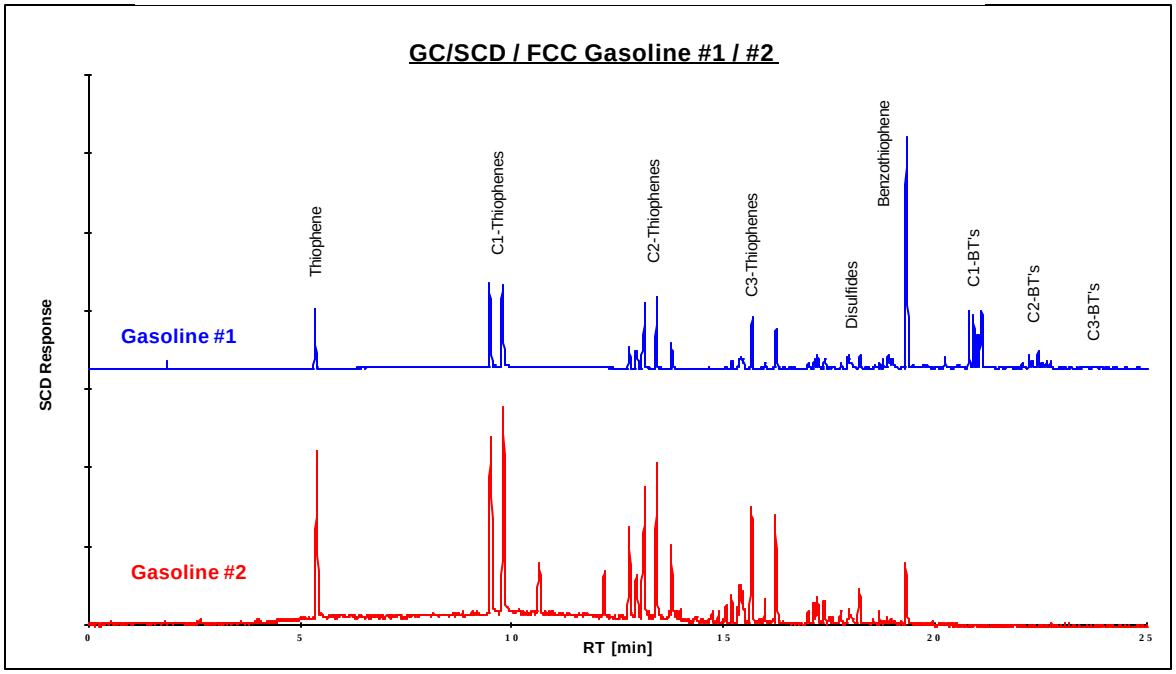


Table 2. Detailed sulfur speciation of two candidate FCC gasolines

Analytes	Gasoline #1 [mg S/g]	% of Total	Gasoline #2 [mg S/g]	% of Total
Tetrahydro thiophene	**0**	0	**19**	3
Thiophene	**27**	5	**53**	8
2-Methyl thiophene	**34**	6	**53**	8
3-Methyl thiophene	**53**	9	**69**	11
2-Ethyl thiophene	**9**	2	**15**	2
3-Ethyl thiophene	**0**	0	**50**	8
2,5-Dimethyl thiophene	**13**	2	**35**	5
2,4 & 2,3-Dimethyl thiophene	**0**	0	**50**	8
3,4-Dimethyl thiophene	**0**	0	**18**	3
Methyl ethyl thiophenes	**0**	0	**35**	5
Trimethyl thiophenes	**0**	0	**70**	11
Thiophenes subtotal	**136**	**23**	**467**	**72**
Benzothiophene	**88**	15	**27**	4
Methyl benzothiophenes	**68**	12	**0**	0
Dimethyl benzothiophenes	**21**	4	**0**	0
Trimethyl benzothiophenes	**3**	1	**0**	0
Benzothiophenes subtotal	**180**	**31**	**27**	**4**
CS2	**5**	1	**2**	0
Methanethiol	**0**	0	**1**	0
Ethanethiol	**0**	0	**13**	2
n-Propylthiol	**0**	0	**6**	1
Isopropylthiol	**0**	0	**3**	0
Thiols subtotal	**0**	**0**	**23**	**4**
Methyl sulfide	**0**	0	**1**	0
Ethyl methyl sulfide	**0**	0	**1**	0
n-Propyl sulfide	**0**	0	**2**	0
Sulfides subtotal	**0**	**0**	**4**	**1**
Ethyl disulfide	**1**	0	**0**	0
Ethyl propyl disulfide	**19**	3	**0**	0
Methyl isopropyl disulfide	**37**	6	**0**	0
Methyl sec-butyl disulfide	**4**	1	**0**	0
Ethyl isopropyl disulfide	**7**	1	**0**	0
Ethyl sec-butyl disulfide	**5**	1	**0**	0
n-Propyl disulfide	**9**	2	**0**	0
Isopropyl disulfide	**14**	2	**0**	0
Disulfides subtotal	**96**	**16**	**0**	**0**
Trismethyl thiomethane	**3**	1	**0**	0
Unidentified volatile sulfur	**170**	29	**130**	20
Total	**590**	**100**	**653**	**100**

Total Sulfur by ANTEK	**535 mg S/g**	**627 mg S/g**
Density @ 15°C	**0.748 g/ml**	**0.748 g/ml**
API Gravity @ 15°C	**57.6**	**57.7**

1.3A. Enzymology: Fmo

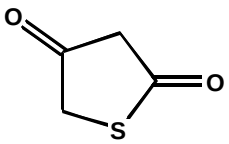
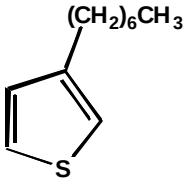
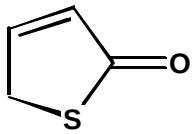
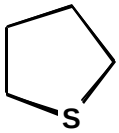
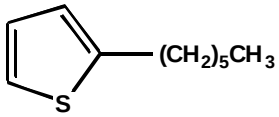
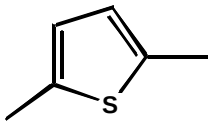
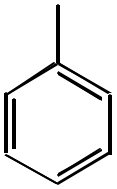
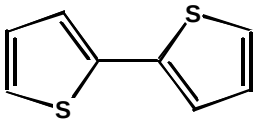
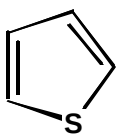
The goal of the biochemistry effort was to survey the nature and complexity of the Fmo enzymology, to provide information that would be necessary to maximize activity and to generate biocatalyst improvement strategies. The sequencing of the gene cloned from KGB1 and identified as a member of the *fmo* (an abbreviation for flavin-containing monooxygenase) gene family provided us with preliminary biochemical information such as the

requirement for NAD(P)H for activity and a broad range of substrates and products. Fmos have been shown to catalyze the oxidation of nucleophilic nitrogen, sulfur, and phosphorus atoms in a wide variety of compounds, evidence that Fmo from KGB1 would be sulfur specific and not attack the hydrocarbons in gasoline in a non-specific fashion.

The protein appears to oxidize both T and indole very slowly even in lysates which contain approximately 20% Fmo protein with reaction rates measured on the order of hours. KGB1 Fmo protein expressed in the Gram negative hosts *E. coli* and *P. putida* is found primarily in the soluble fraction, and thus is different than the eukaryotic Fmo protein associated with the membrane

As a prelude to trying to purify this activity we investigated *in vivo* activity versus a number of different molecules (Table 3). One objective behind these experiments was to ascertain whether the Fmo protein was selectively acting on sulfur containing molecules representative of those found in gasoline or if it also acted on aromatic molecules like toluene. Another objective was to try various compounds like alkylated Ts, which were considered less polar and less volatile and therefore better suited for the development of a routine analytical method. Finally, some molecules like thiotetronic acid were being tested for biotransformation within the framework of a working hypothesis regarding the intermediates of the desulfurization of T assuming the Fmo protein was the first enzyme in the pathway. While T was converted only in the *fmo* positive strain the other molecules were either unchanged or were otherwise removed by both the *fmo* plus and minus strains. This activity is reasonably attributed to the *Pseudomonas* host, which has been shown to possess activity against a number of different aromatic molecules and therefore may not be the ideal host to use in this type of study.

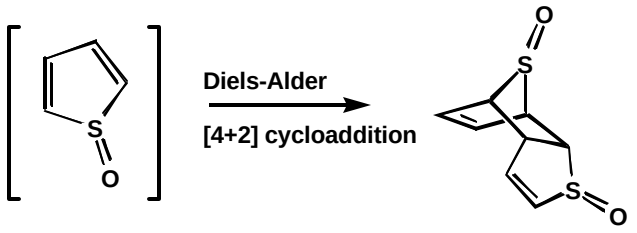
Table 3. ***In vivo*** **activity of** ***P. Putida*** **strain G1 with and without Fmo activity**

		COLUMN RETENTION TIME (min)			
		*fmo*A$^-$ sample		*fmo*A$^+$ sample	
SUBSTRATE	STRUCTURE	T = 0 h	T=14 h	T= 0 h	T=14 h
Thiotetronic Acid		3.2	V_0	3.2	V_0
3-HexylT		U	U	U	U
2(5*H*)-Thiophenone		1.8	7.0	1.8	6.2-10
TetrahydroT		6.1	V_0	6.0	V_0
2-HexylT		U	U	U	U
2,5-Dimethyl-T		U	V_0	14.3	V_0
Toluene		12.9	V_0	12.7	V_0
2,2'-BiT		16.2	V_0	16.1	V_0
[^{14}C]T		7.8	7.8	7.8	V_0

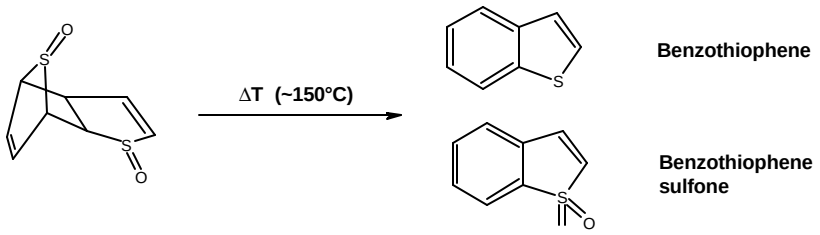
Legend 1. Abbreviations: V0, void volume; U, undetermined.

Acting on the assumption that endogenous metabolic activities in *Pseudomonas* may have been responsible for the apparent turnover of some of the compounds used in this study, we repeated it using *E. coli*. DH10ß expressing the Fmo protein. The results were similar (data not shown), indicating that the activity could be attributed to Fmo.

The *fmo* from rat liver cloned in yeast is reported to catalyze sulfoxidation reactions as well as hydroxylation at nitrogen. There are no examples of hydroxylation at carbon. An example in the literature of the breadth of substrate specificity and reactions catalyzed by the rat liver Fmo protein acted as a guide to the development of working hypotheses regarding the desulfurization pathway for Cx-Ts in gasoline as it exists in KGB1 or at least as a model for the Fmo protein reaction. It is important to note the nonenzymatic reactivity of the sulfoxidation products of T and BT as dieneophiles in intermolecular cycloaddition reactions and as electrophilic intermediates susceptible to intracellular nucleophilic molecules as shown in Scheme 1. This reaction was ultimately confirmed after a great deal of effort due to further rearrangements that took place on the GC column. The T S-oxide dimer is unstable at high temperatures, yielding a mixture of BT and BT sulfones (Scheme 2).

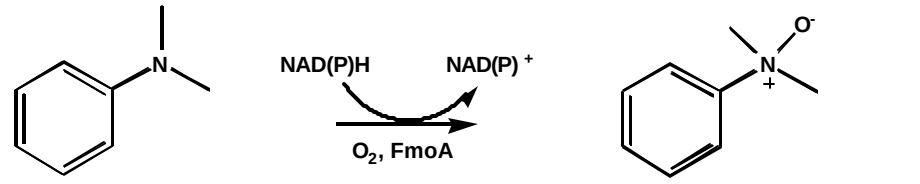


Scheme 1



Scheme 2

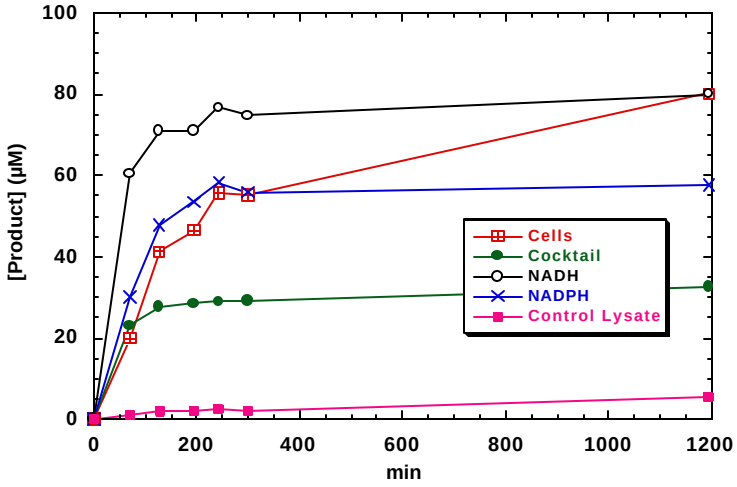
As suggested from its name, Fmo protein from rat liver is reported to have a prosthetic bound FAD and NADPH dependent N,N-dimethylaniline (NNDMA) N-hydroxylase activity as illustrated in Scheme 3.



Scheme 3

Therefore, Fmo protein from KGB1 presumably contains a bound flavin and has a catalytic requirement for NAD(P)H. This latter point was confirmed with cell-free lysates of recombinant *P. putida* carrying the *fmo* gene (Figure 6). In this experiment it can be seen that ^{14}C-labelled thiophene can be oxidized by whole cells or by crude lysates only in the presence of either NADH or NADPH or a combination of the two (cocktail).

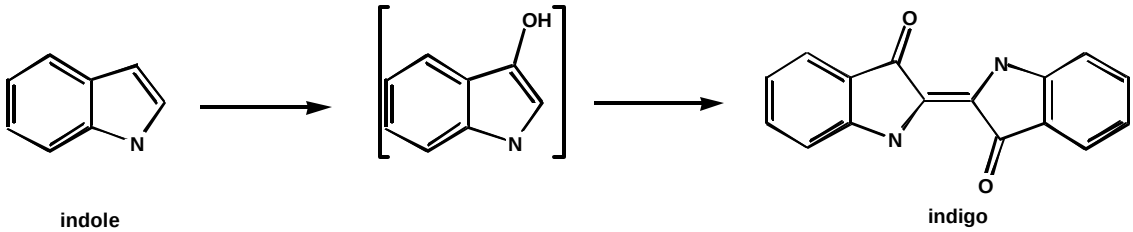
Figure 6. Conversion of ^{14}C-labelled thiophene by whole cells or crude lysates of *P. putida* (pEXY10)



1.4.A. Genetics-Characterization: Fmo

The classical approach to identify the KGB1 genes involved in BT and T metabolism would involve the isolation of mutants that can no longer grow on these compounds. These mutants would then be complemented with plasmids containing fragments of wild-type KGB1 DNA, and the plasmids that would support growth would be expected to contain the gene that was defective in the mutant strain. Unfortunately, this approach did not work with KGB1 because technical difficulties of delivering these sulfur compounds (prior to the discovery of emulsion plates) and poor growth of KGB1 in general make it nearly impossible to establish sulfur limitation.

A second approach was based upon the observation that KGB1, grown on BT or T as sulfur source, can convert indole into indigo, but sulfate-grown cells cannot. The conversion of indole to indigo (Scheme 4) is a common feature of oxygenases (For example DBT monooxygenase, DszC, can catalyze this reaction.), and it seemed likely that indigo formation in KGB1 was catalyzed by an enzyme responsible for BT and T metabolism. This led us to construct a KGB1 gene library and test individual clones for the ability to convert indole to indigo. As a secondary screen, positive clones would be screened for activity against BT or T.



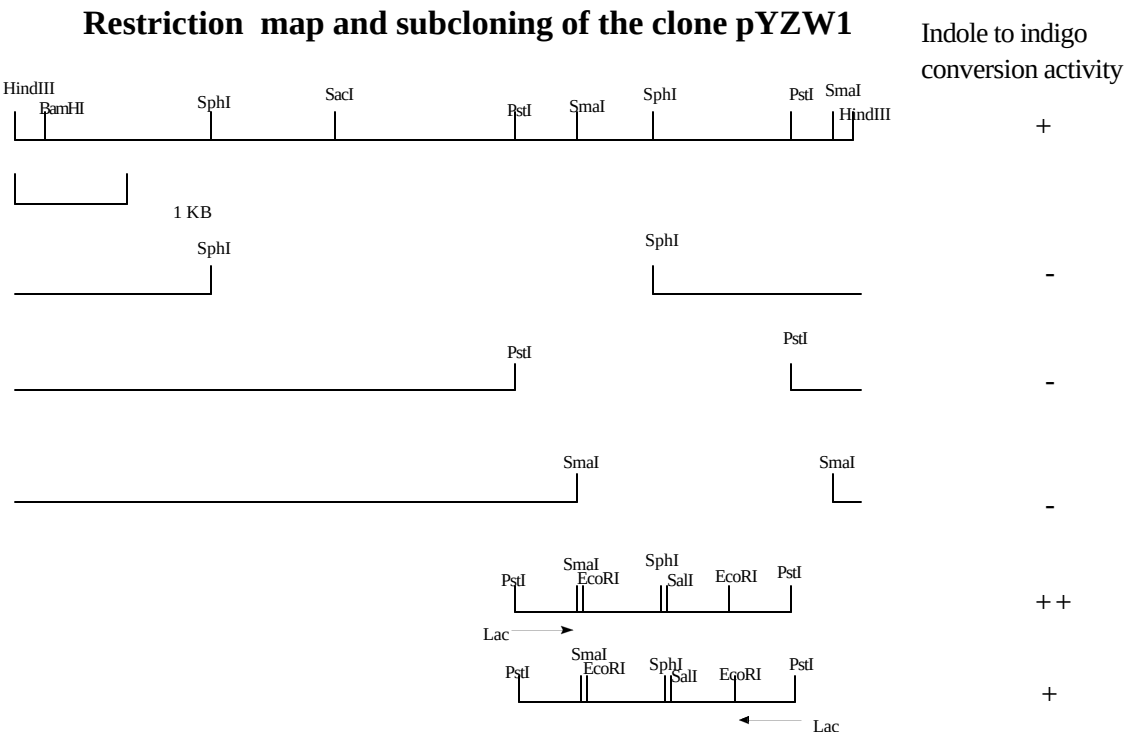
Scheme 4

The genomic DNA of strain KGB1 was isolated and partially digested by *Hin*dIII. The cloning vector used was pEX92, a pUC18-based vector containing the *fre* gene (a flavin oxidoreductase gene that has been shown to work with dszA and dszC from IGTS8). A *fre* background was used here in case the BT or T oxidation system was similar to the Dsz system. The vector was completely digested by *Hin*dIII, mixed with the genomic partial digest, and ligated. The ligation mixture was then electroporated into *E. coli* strain DH10B. The cells were plated on LB + Amp + IPTG and incubated at 37^{o}C overnight and then removed to room temperature.

From 20,000 colonies screened, 2 blue colonies were found, and these two clones had the same DNA insertion, a 7.5 kb *Hin*dIII fragment. This clone was named pYZW1. A simple restriction map is shown in Figure 7. The blue pigment was extracted by chloroform and the spectrum matches that of the standard, indigo. The activity of the clone against BT was tested through detecting the oxidation product of ^{14}C-BT. A very tiny amount of product was detected (retention time close to BTO or BTO2) while the negative control showed no product.

The data from restriction mapping were used to construct several subclones. As shown in Figure 7, none of them retained the ability to turn indole into indigo. Accordingly, the 2.4 kb *Pst*I fragment was cloned in either orientation into vector pNEB193 (a derivative of pUC19, with more cloning sites). One of the orientations had five times more activity than the other indicating the direction of the open reading frame.

Figure 7. Cloning indigo gene(s) from KGB1 into *E. coli*.



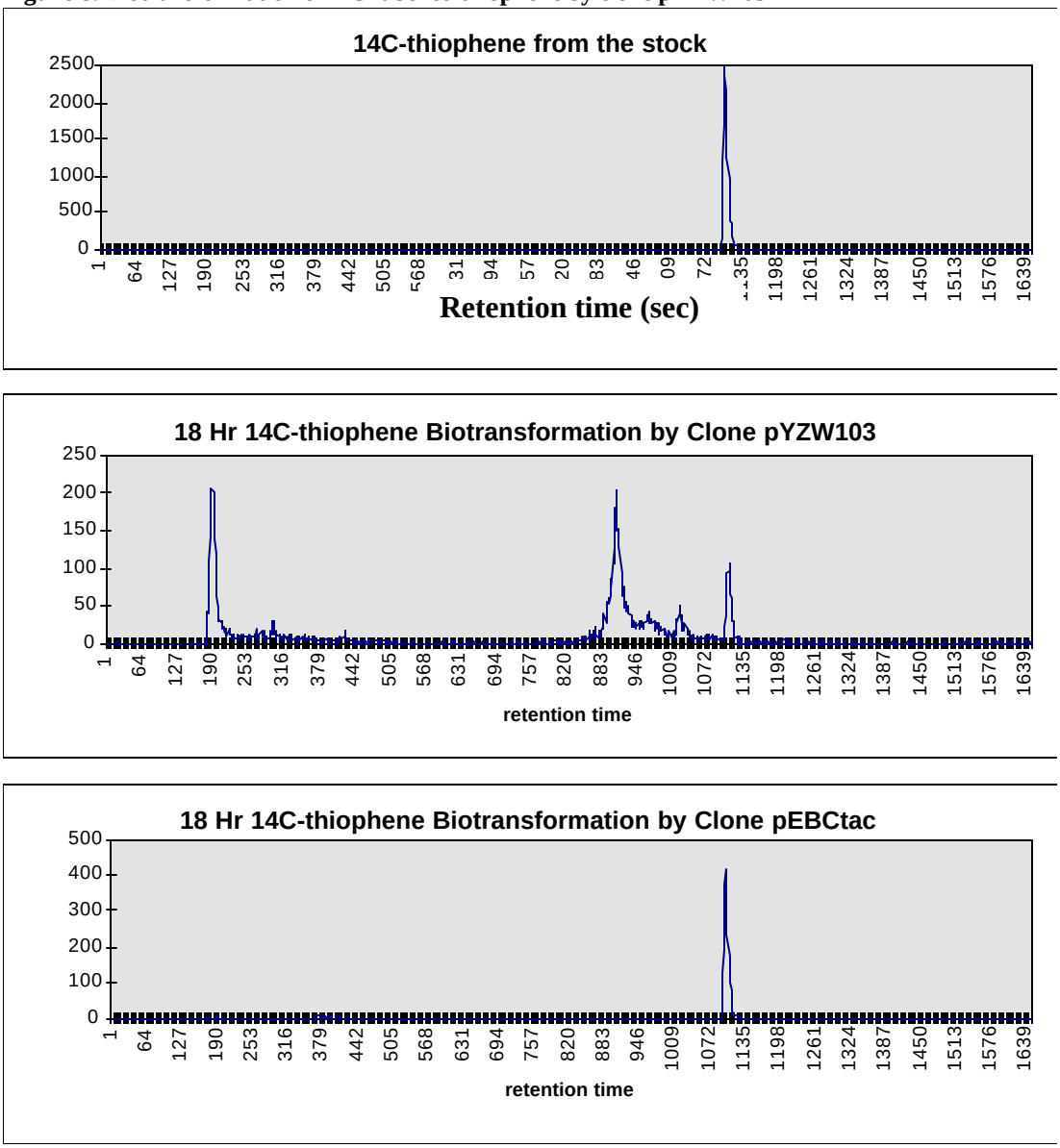
A clone named pYZW102 was constructed at the same time as the clone pYZW101. The insertion orientation of this clone is opposite that of pYZW101. A *Hin*dIII-*Xba*I fragment containing the *Pst*I fragment, responsible for the indigo phenotype, from this clone was cloned into the *E. coli* expression vector pEBCtac. The new construct was named pYZW103and transferred into *E. coli* strain DH10

E. coli cells containing clone pYZW103 were grown in LB medium and induced with 1 mM IPTG. A negative control was done using the cells containing the vector pEBCtac alone. Cells were washed and resuspended in phosphate buffer containing 50% LB. The final cell OD_{600} is 7.0. 100 uM ^{14}C-T was added to 4 ml cells in a sealed bottle. The bottle was shaken at 30^{o}C for 18 hrs. Then 1 ml sample supernatant was used for HPLC analysis using a radiometric detector.

The HPLC results are shown in the Figure 8. Two major products at the retention time of ~190 s and ~900 s were detected from ^{14}C-T biotransformation by clone. The negative control shows no product. Both clone pYZW103 and

wild type KGB1 can transform ^{14}C-T to a product at the retention time of ~190 s. Clone pYZW103 showed very little conversion of ^{14}C-BT biotransformation (data not shown).

Figure 8. Biotransformation of ^{14}C-labelled thiophene by clone pYZW103



The complete sequence of both strands of the 2.4 kb *Pst*I fragment was determined. Two ORFs were found in this region. ORF1 runs from bp131 to bp1495 and codes for a 455 amino acid protein with the calculated molecular weight of 51, 800. ORF2 runs from 1559 to 2347 and codes for a 263 amino acid protein with the calculated molecular weight of 28,891. The sequence for both ORFs show a very high percentage of guanines and cytosines at the third position of each codon, which is evidence that the ORFs are expressed. The region from 1 to 130 is high in adenines and thymines (57%), which could mean that this can serve as promoter region. A possible ribosome binding site GAGGA is found in front of the ATG starting codon for ORF1.

As noted above, the ORF1 encoded protein has homology to dimethylaniline monooxygenases (also named **f**lavin-containing **mo**nooxygenases, Fmo) from mammals. No protein from bacteria or other prokaryotic cells was found to have homology to ORF1 by conventional sequence database searching. As reported in the literature, the identities of all known homologous Fmo forms are between 52 and 57%. There is 29% identity and 52.9% similarity between ORF1 and the closest eukaryotic Fmo protein. Therefore, the ORF1 encoded protein is distinct from the others. Because of the similarity between ORF1 and eukaryotic *fmo* genes we gave this gene the name *fmo*A. The N- and C-terminal parts of the two proteins match each other very well. The principle regions of non-homology between the proteins is in the middle of the pair of proteins. There are two putative pyrophosphate-binding sequences (GxGxxG) in FmoA. In ORF1, one is found in the N-terminus, GAGPSG. These sequences are indicative of FAD (particularly when found near the N-terminus of a protein) or NAD binding sites.

ORF2 encoded protein has homology to many ATP binding proteins involved in substrate transport. The best matched one is BraF, high-affinity branched-chain amino acid transport ATP-binding protein from *Pseudomonas aeruginosa*. The amino acid sequence alignment of the two proteins is shown in the following table. There is 38.7% identity and 64.4% similarity between the two proteins.

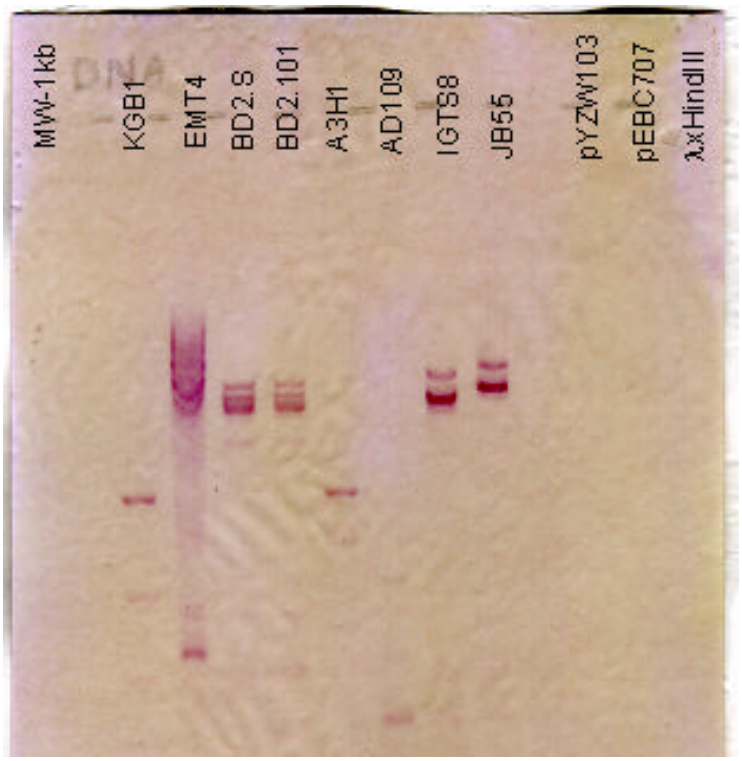
The summary of the ORFs are listed in the following table.

Table 4. Summary of the ORF1 and ORF2 cloned from KGB1

ORFs	nucleotide position	Amino Acid length	homologous proteins	identity	similarity
ORF1	131-1495	455	dimethylaniline monooxygenase	29.2%	52.9%
ORF2	1559-2347	263	High-affinity branched-chain amino acid transport ATP-Binding protein	38.7%	64.4%

Although *fmo* genes had never before been found in prokaryotic sources, we carried out Southern analysis on a number of microorganisms able to grow on gasoline components as a sole sulfur source. In this technique with the *fmoA* gene from KGB1 was labeled and used as a probe to find homologous genes in other organisms. Southern blots were made and probed (Figure 9). These blots were hybridized and washed at high stringency. They demonstrate that the *fmo*A gene is not only present in KGB1, but very similar genes appear to be present in most of the other strains tested, including strains not capable of growing on gasoline as sulfur source (*Nocardia* A3H1 and *Sphingomonas* AD109). The *Rhodococcus* strains IGTS8 and JB55 were tested for their ability to convert [^{14}C]-labeled 2-methyl T to a less volatile compound and neither of these strains had as much activity as KGB1. Many more strains of EBC's collection have now been analyzed for the presence of *fmoA* like genes and most do hybridize to the *fmoA* gene of KGB1 when tested under high stringency.

Figure 9. Southern blot of genomic DNA from various gasoline strains using KGB1 *fmo*A gene as probe



The 2.4 kb fragment from pYZW101 was cloned into a *Pseudomonas* expression vector and given the name pYZW113 (See Figure 10). This vector makes use of the *nah*H promoter which is controlled by the presence of salicylate. This plasmid was cloned into the solvent tolerant P. putida strain PpG1, and transformants were identified by the ability to convert indole to indigo. Expression of the *fmo*A gene in PpG1 grown in the presence of salicylate was shown to be quite high as determined both from analysis of total soluble protein on SDX-PAGE gels (Figure 11) and by conversion of ^{14}C-labelled T (Figure 12), but as with E. coli carrying pYZW103, this strain had very little activity towards BT.

Figure 10. Construction of plasmid pYZW113

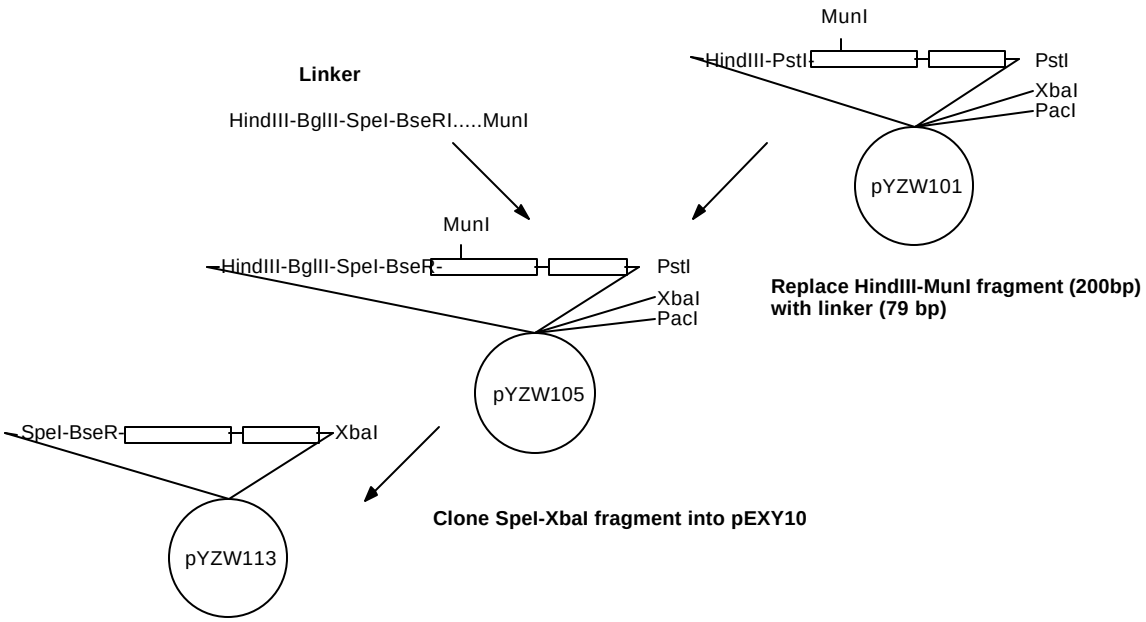
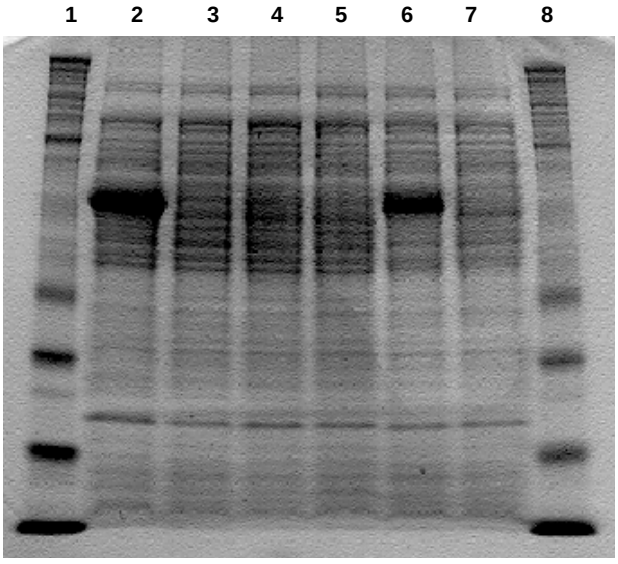


Figure 11. Overexpression of fmoA gene in P. putida



Lane

1 MW marker

2 PpG1 (pYZW113) induced by salicylate

3 PpG1 (pYZW113) uninduced

4 PpG1 (pEXY10) induced by salicylate

5 PpG1 (pEXY10) uninduced

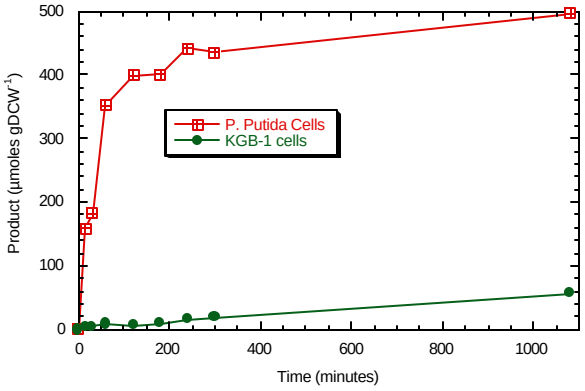
6 PpKT2440 (pYZW113) induced by salicylate

7 PpKT2440 (pYZW113) uninduced

8 MW marker

Figure 12. Conversion of 14C-labelled thiophene by recombinant *P. putida* and *R. erythropolis* expressing *fmo*A gene from KGB1

Strain	µmol/gDCW/min
KGB1	0.007
P. putida G1	0.002
P. putida G1/ pYZW113	0.28
JB55	0.0001
JB55::fmoA	0.04



With the *fmo*A gene overexpressed in a gasoline tolerant (*P. putida* PpG1) host it was finally possible to determine the rate and extent of gas BDS using this enzyme system. The technique for maintaining continuous cultures of the wild-type strain of PpG1 in the presence of a gasoline phase had already been developed (See Section 1.6, below), and now it was applied with PpG1/pYZW113 (fmo+). Chemostats containing PpG1 (*fmo*A-) and PpG1/pYZW113 (*fmo*A+) were run for more than 4 months. Straight FCC gasoline was fed to the chemostats over this period in an attempt to maintain a concentration of 5% v/v, although this varied due to flow interruptions. Optical density readings from the reactors while gasoline was present generally varied between 5 and 10 (A_{600}). The cultures were able to maintain these levels during constant exposure to gasoline.

The actual percentage of gasoline within the chemostats was determined experimentally. This method involved extracting a sample of chemostat medium with pentane and obtaining a straight gasoline sample by a freeze/thaw/centrifugation method. Both sample were run on the GC/SCD and the total FID peak areas were compared to derive a ratio. With this method, differences in the FID profile (and thus the total peak area) due to differences in the amount of weathering were minimized. For periods when measurements were taken, the gasoline in the chemostats varied from 2 to 7%, with the majority of the readings falling between 3 and 5%. The degree of weathering was dependent on how well the condenser system was operating. The amount of weathering of the gasoline within the reactor ranged widely, from 0.8 to 0.1, as determined by the ratio of FID peak areas. This range of weathering values translates to approximately 70-30% of the gasoline weight remaining. When the condenser system was operating optimally, a significant amount of T, the most volatile sulfur component in FCC gas, was retained.

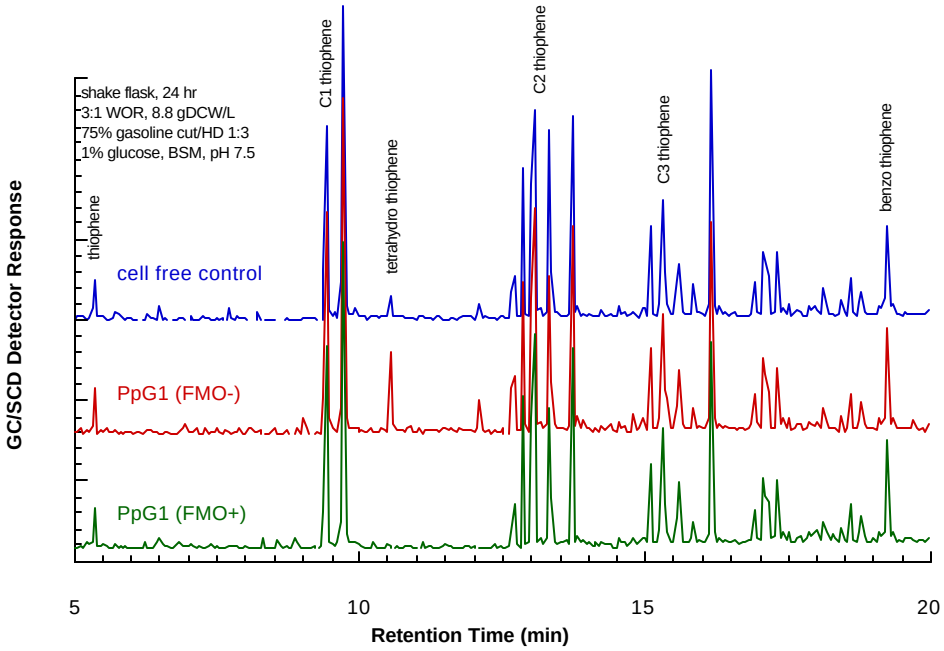
Indole tests with isolated colonies showed that the *fmo*A+ strain was maintained in the chemostat when the inducer (salicylate) was present, but the percentage of fmo+ bacteria dropped to a steady state level of approximately 40%. After salicylate was added, there was no evidence that the *fmo*A+ chemostat was removing sulfur from the organic phase. Interpretation of the results of a steady-state experiment was complicated by problems with the condenser system, but GC-SCD chromatograms with similar weathering ratios taken from uninduced and induced chemostats did not show the removal of any specific sulfur peaks, including T. In addition, samples removed from the induced chemostat and tested by radioactive 2-methyl-T assays did not show activity, even though approximately 40% of the cells in the reactor contained the *fmo*A gene.

Following up on these results, we evaluated the effect of gasoline on the rate of model compound turnover by PpG1 expressing the *fmoA* gene. In whole cell assay the presence of 5% percent (vol./vol.) gasoline dramatically slowed the rate of 2-methyl T turnover. The cells were viable under these conditions consuming glucose, and the cells could be recovered and re-plated. These results are consistent with gasoline specifically inhibiting the ability of

these cells to catalyze the oxidation of 2-methyl T. It may be that the presence of gasoline interferes with 2-methyl T uptake that some component(s) of gasoline are competitive inhibitor(s) of the *fmoA* gene product versus 2-methyl T

One last effort was made to demonstrate gasoline BDS with the *fmo*A gene product in PpG1, using weathered gasoline. Cells of PpG1 with and without the plasmid pYZW113 were grown and treated with salicylate to induce expression of *fmo*A on plasmid pYZW113. The cell cultures were mixed with weathered gasoline at a 3:1 water:oil ratio (WOR) and incubated with shaking for 24 hours. The oil phase was recovered and analyzed by GC-SCD. The chromatograms of oil recovered from fmo+, fmo- and cell free controls (shown in Figure 13) demonstrate that there was virtually no gasoline BDS activity in the fmo+ cells. The failure of these recombinant cells to remove a significant amount of sulfur from the weathered gasoline was difficult to explain although it is likely due to the presence of so many different compounds in gasoline relative to the model system used to detect Fmo enzyme activity. It is possible that there are substances in gasoline that act as inhibitors or inactivators of the Fmo enzyme. At this point, the work on *fmo*A was discontinued in favor of developing new enzyme systems from gasoline strains.

Figure 13. GC-SCD chromatograms of weathered gasoline treated by recombinant *P. putida* carrying *fmo*A gene from KGB1



1.5.A. Genetics-Improvement: Fmo

Although we have demonstrated that the *fmo*A gene product from KGB1 was capable of converting T and Me-T to a water soluble product, there was no evidence that the enzyme could transform these or other Cx-Ts when present in a gasoline matrix. This lack of activity provided no basis for improvement. Rather, the decision was made to develop other enzyme systems that would be more likely to provide a better starting point for gasoline BDS biocatalyst development.

Section 1B. Biocatalyst Development: New Strains

1.1.B. Isolate Desulfurizing Strains: New Strains

Through the use of weathered gasoline used in emulsion plates and in two-phase shake flask liquid cultures, we have identified nine strains (Table 5) in our culture collection that are capable of growth with gasoline as sulfur source. These strains, which include KGB1, were chosen for further evaluation to supply new genes to be developed for gasoline BDS. One strain (XJ5) was capable of growth at 50° C, and the remaining strains were all mesophiles. Three of the strains (9.27.3, 9.20.5, and 9.20.6) were isolated from soil used for growing marigolds. Marigolds are known to excrete thiophenic compounds from the roots to act as insect repellants, and it was thought that this soil would be pre-enriched for thiophene-degrading strains.

Table 5. Gasoline isolates with characterization based on the 16S-RNA sequences.

Isolate	Source	Best match	% Identity
KGB1	BT enrichment culture	*Gordonia terrae*	100
EMT1	Methyl-T enrichment culture	*Gordonia rubropertinctus*	98.3
EMT2	Methyl-T enrichment culture	*Rhodococcus coprophilus*	95.4
EMT4	Methyl-T enrichment culture	*Tsukemurella wratislaviensis*	100
670-1	Biodesulfurized middle distillate enrichment culture	*Tsukemurella wratislaviensis*	99.8
XJ5	DBT enrichment culture	*Nocardia farcinica*	100
9.20.5	Methyl-T enrichment culture	*Gordonia terrae*	100
9.20.6	Methyl-T enrichment culture	*Rhodococcus erythropolis*	99.1
9.27.3	Methyl-T enrichment culture	*Rhodococcus erythropolis*	99.1

Weathered gasoline was recovered from shake flask cultures of the nine strains listed in Table 5 and analyzed for total sulfur removed during growth. The results from this screen are summarized in Table 6. Cell-free controls were run with media both containing and lacking yeast extract. Strains XJ5, 670-1, and 9.20.6 clearly stood out as the best at gasoline BDS under these condition. GC-SCD analysis confirmed the total sulfur values (Figures 14 and 15).

Table 6. Total sulfur removed from weathered gasoline during growth

	Cell Free Control	Growth – Yeast Extract	Cell Free Control	Growth + Yeast Extract
EMT2	140 ppm	141 ppm	ND	ND
EMT4	140 ppm	132 ppm	101 ppm	89 ppm
XJ5	86 ppm	46 ppm	80 ppm	25 ppm
KGB1	140 ppm	118 ppm	101 ppm	61 ppm
670-1	ND	ND	90 ppm	50 ppm
9.27.3	ND	ND	90 ppm	76 ppm
9.20.5	ND	ND	90 ppm	70 ppm
9.20.6	ND	ND	90 ppm	62 ppm

Figure 14. GC-SCD chromatograms of weathered gasoline after treatment with thermophilic isolates

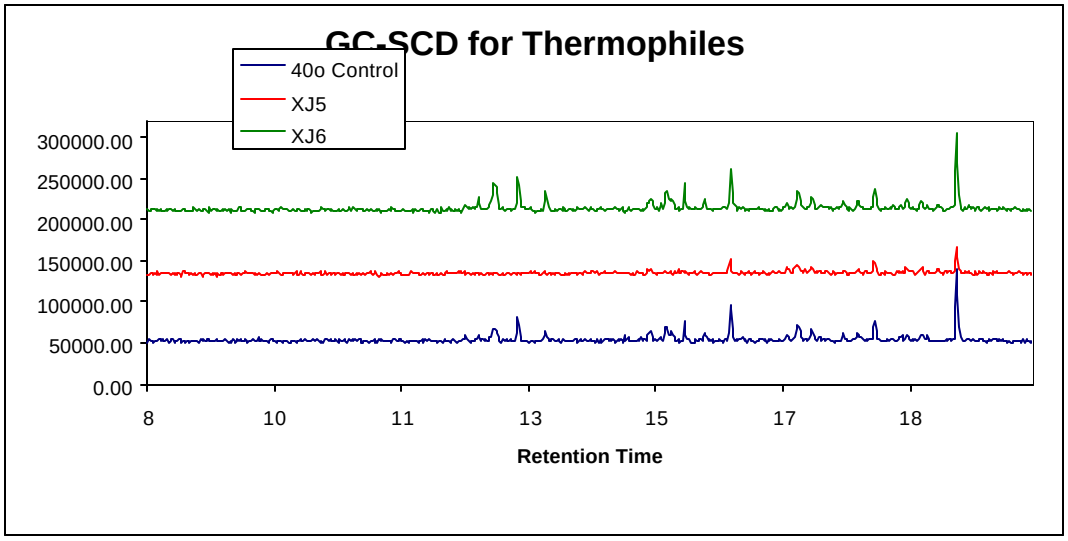
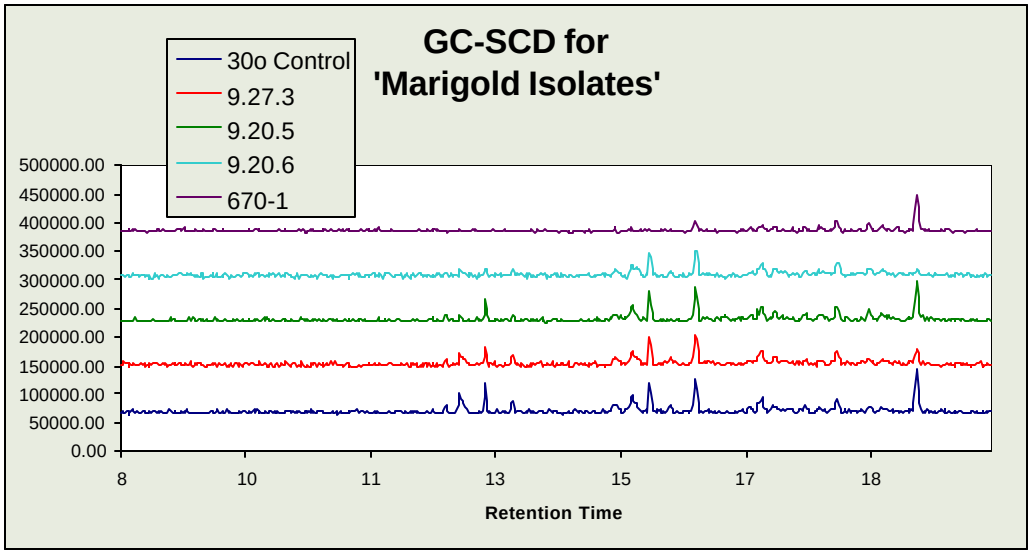
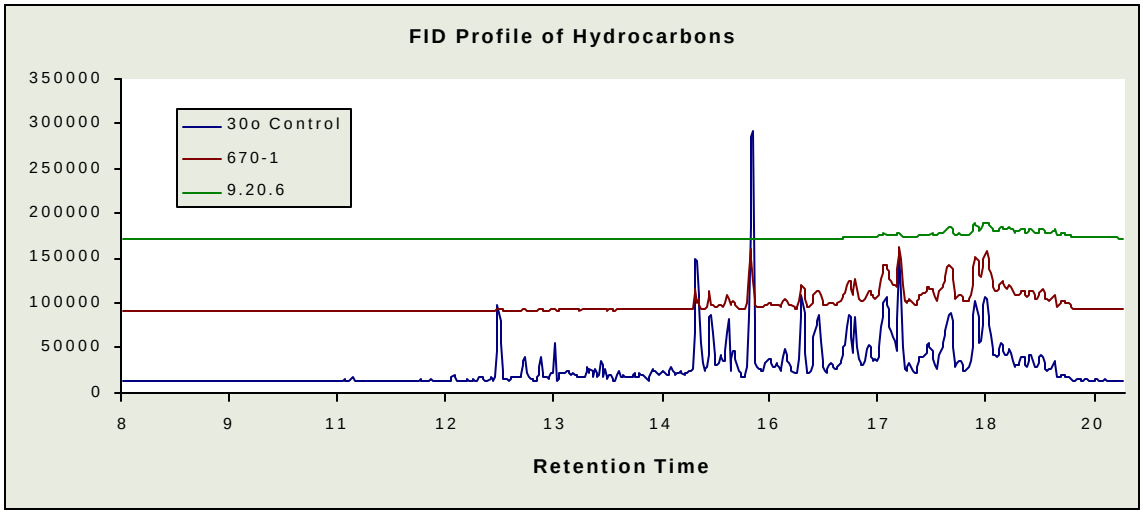


Figure 15. GC-SCD chromatograms of weathered gasoline after treatment with mesophilic strains



Further evaluation of the oil recovered from some of these cultures by GC-FID indicated that not only were the sulfur compounds being removed but that the significant changes in the hydrocarbon content was occurring as well, as in shown in Figure 16. Experimental results for strain XJ-5 were very similar (data not shown).

Figure 16 Hydrocarbon Profile from Desulfurization Assays



As was noted earlier, optimal desulfurization performance of these strains is achieved when the cultures are grown in the presence of weathered gasoline as the sole source of sulfur. One potential problem with this approach is that unknown catabolic pathways in these strains could also be induced, resulting in degradation of the hydrocarbon matrix. This could explain the results presented in Figure 16. It is also possible that the enzymatic mechanism for sulfur removal from the gasoline involves attack of the carbon atoms of the organosulfur molecules. It would then be possible for this pathway to act upon molecules in the gasoline that do not contain sulfur. It would be very difficult, if not impossible, to discriminate between these two mechanisms in the wild-type strains.

Given the difficulties encountered with the chemostat cultures, and the inability to determine the cause of the hydrocarbon removal by the wild-type strains, emphasis was placed on isolation of the genes responsible for the sulfur removal by these isolates. Identification, cloning, and overexpression of the T pathways in a naïve host would likely eliminate these difficulties. These topics will be discussed below.

1.2.B. Analytical: New Strains

No new analytical methods were developed for this approach.

1.3.b. Enzymology: New Strains

The enzymology of gasoline BDS observed in these strains was not evaluated.

1.4.B. Genetics-Characterization: New Strains

One strategy for identifying the enzyme(s) responsible for T desulfurization is to randomly mutate the cell's DNA, or "knock out" T pathway gene(s), and screen for mutants that can no longer utilize T as a sulfur source. The

pathway enzyme(s) in these mutants no longer function properly and have to be complemented with functional enzymes, translated from a gene library, before the mutant's T oxidation is restored.

Different approaches were taken to mutagenize the organisms. Initially, we attempted to carry out transposon mutagenesis. Transposon mutants are useful because DNA sequence reactions starting at the known transposon sequence can immediately identify the gene that has been inactivated. We worked with the "TNP Transposome™ Kit" from Epicentre, which has been proven to work with Mycobacterium, closely related to the gasoline isolates, but this approach was discontinued because of the low number of transformants. Thousands of mutants would be necessary to expect a reasonable chance of knocking out a specific T-oxidizing enzyme. The efficiency of the transposon transformation in the gasoline isolates was about a hundred times too low to make it work.

With the failure of transposon mutagenesis, we turned to more classical mutagenic methods. N-methyl-N-nitro-N'-nitrosoguanidine (NTG) mutagenesis failed, but UV irradiation proved to be an effective method. A killing efficiency was achieved of over 90%, and stocks were frozen at -80^{o}C. Each of the mutant libraries was replica plated on rich medium and minimal medium plus sulfate or minimal medium plus 2-methyl T. Colonies that appeared on the rich medium and the sulfate medium, but not on the T medium, were presumed to have mutations in the T pathway and were selected for further evaluation.

With knock out mutants in hand, the second step in localizing the T pathway gene(s) would be to add back an intact gene from a gene-library, which complements the disabled protein and restores function. Genomic DNA was isolated and library preparations were constructed from several T-utilizing strains, including the three strains selected for further development, XJ-5, 9.20.6, and 670-1. These libraries were prepared in the hygromycin shuttle vector p16R2. Several transformations were done to generate sufficient libraries to complement the UV knockout mutants.

UV mutants of strains 670-1, 9.20.6, and XJ-5 were transformed with genomic DNA libraries from its parent strain and plated on minimal medium using 2-methyl T or gasoline as the sole source of sulfur. Colonies appearing under these growth conditions were expected to have genomic DNA inserts that complemented the mutation in the T pathway. Colonies from this initial growth on plates were selected and re-plated under the same growth conditions to verify the complementation. Plasmid DNA from 8 of the 670-1 complemented mutants was isolated and again transformed into the knockout mutants for final verification that the target gene(s) was identified. Five of the 8 complemented mutants grew successfully on 2-methyl T, and the plasmid DNA from these strains was amplified using PCR to generate sufficient quantities of DNA to facilitate DNA sequencing. Amplification of three of these products was successful.

The three PCR products from the 670-1 complementation mutants were submitted for DNA sequencing. Sequences were obtained and a BLAST search of public databases was conducted to identify homologous gene sequences. Results showed a gene with 60 percent similarity with *nysC* of *Streptomyces noursei* for the first sequence, 42 percent similarity with feruloyl-CoA synthetase of *Amycolatopsis* sp. for the second sequence, and 62 percent similarity with CDA peptide synthetase of *Streptomyces coelicolor* for the third sequence. Unfortunately, all three sequences were different, an unexpected result if the actual T target gene would have been identified. In addition, none of the homologous sequences identified from the BLAST search of the public databases identified gene products that "make sense" relative to T conversion.

Several more attempts indicated that the UV-mutants had a tendency to revert to wild-type phenotype when attempts were made to select clones that could grow on 2-methyl T. This resulted in the generation of many false-positive clones, and ultimately prevented us from identifying any plasmid carrying gene(s) necessary for growth on 2-methyl T.

A parallel approach to the knock-out/complementation strategy described in the previous section is to clone the T genes directly into a solvent tolerant host. The procedure involves making a genomic library from the strains harboring the target genes, and transforming the DNA into a naïve solvent-tolerant host. This library would then be screened for T oxidizing activity, and the DNA from the positive strains isolated and sequenced. This approach has the advantage of avoiding secondary effects that are often encountered with a knock-out/complementation strategy, but has the disadvantage that cloning and expression in a foreign host can sometimes be difficult. This latter point proved to be the case. Genomic DNA from each of the three strains was isolated and prepared for cloning. Most of the work on this task was with the shuttle vector pEBC1150. Tests were completed to confirm that this vector could

be transformed into *E. coli* and *P. putida*, and to confirm that the vector would replicate in these two species. However, efforts to ligate DNA fragments from the three target strains into this vector were unsuccessful, and numerous control experiments did not allow us to identify the source of the problem. Two additional shuttle vectors were then tried. The first (p16R2) has a hygromycin antibiotic marker, which was found to be useless in PpG1 since the strain itself was found to be naturally resistant to hygromycin. The second (pLS88; ATCC) had several useful antibiotic markers, but could not replicate in PpG1.

Rhodococcus erythropolis JB55 (a derivative of IGTS8, with the *dszABC* operon deleted) was chosen to be the host for gene libraries prepared from KGB1 and EMT4. JB55 is unable to grow with either thiophene or gasoline as a sulfur source and so would depend upon expression of appropriate gene(s) from gasoline isolate libraries to grow on gasoline. With this method several clones have been isolated from different organisms. By sequence analysis, some of the clones appeared to be over expressed sulfate transporters, which allows the organism to scavenge for minute amounts of sulfate, present as very low levels of contaminations in chemicals. These clones were not studied any further, since they appear to be independent for gasoline sulfur and therefore not interesting for this study.

Other clones however contained open reading frames similar to the *dszA* from diesel desulfurizing organisms. The gene *dszA* codes for DBT sulfone monooxygenase, responsible for the carbon sulfur bond cleavage. The genes found were approximately 50 % similar to *dszA* from IGTS8 and were named *toeA*. An alignment of *toeA* of KGB1 and EMT4 is given in Figure 17. A database search revealed 20 similar proteins, of which most have a hypothetical function. In Figure 18 a phylogenetic tree represents all those enzymes and their evolutionary relation to one another. The two *toeA* genes from KGB1 and EMT4 cluster together and appear to be most closely related to the six enzymes with proven desulfurizing activity.

The complementing DNA consisted of roughly 5 kb, while the *toeA* gene is less than 1.5 kb (see Figure 19) This made it necessary to truncate the 5 kb to just the *toeA* gene, to justify its responsibility. This was done in combination with and without the flavin reductase gene *dszD*. If the situation is analogous to diesel desulfurization, over expression (the host does contain one copy already on the chromosome) of this gene could dramatically increase the activity of the *toeA* product.

Figure 17. Sequence homology between toeA genes isolated from KGB1 and EMT4

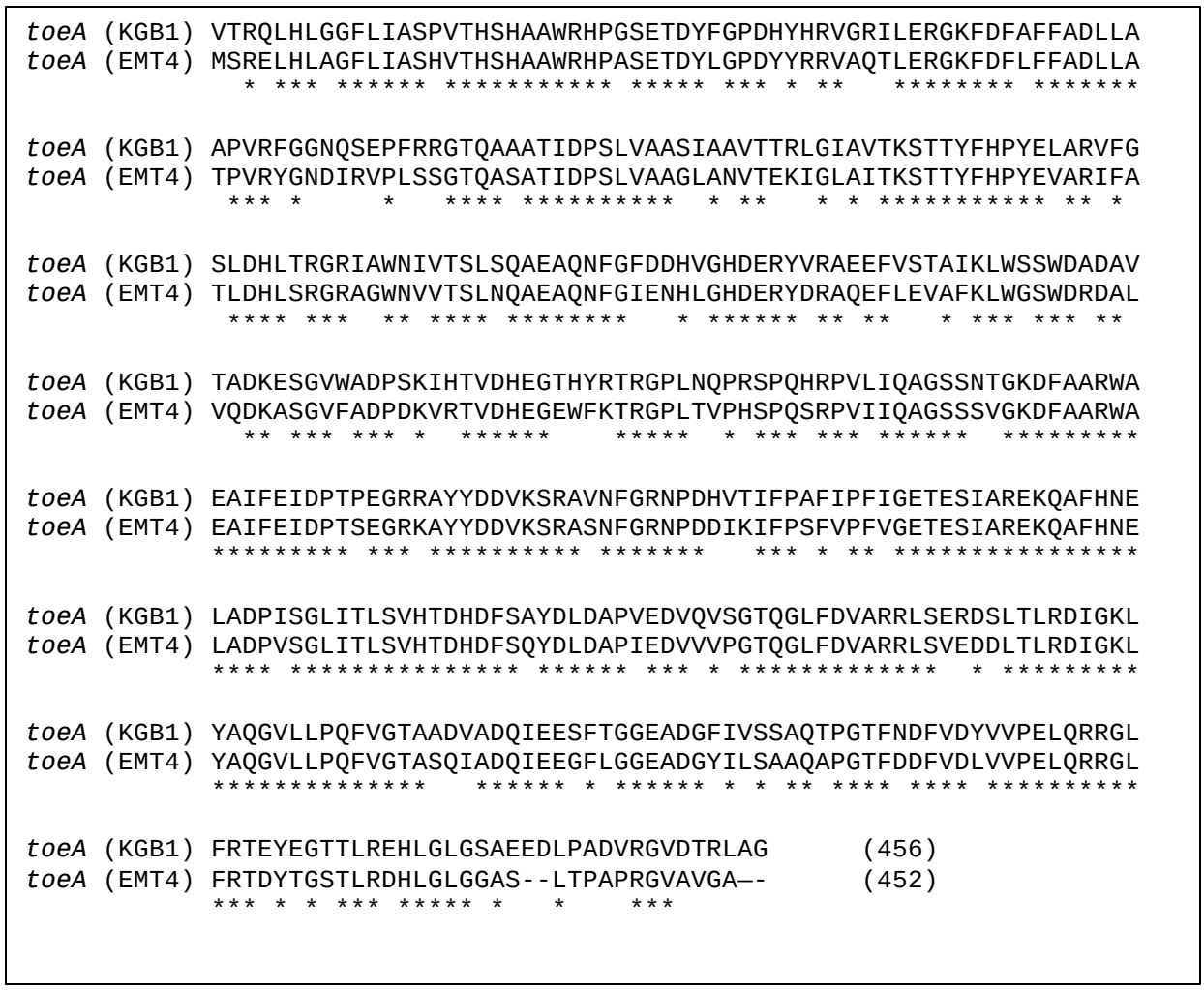
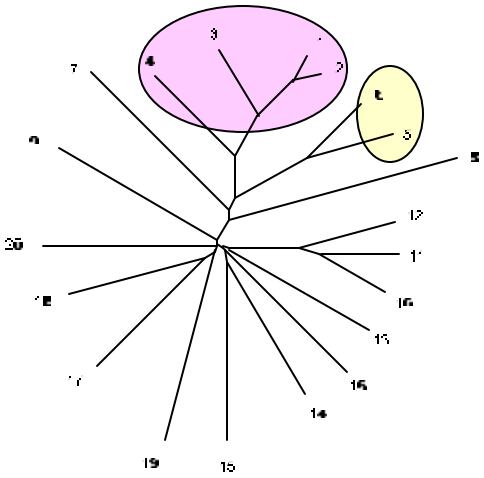
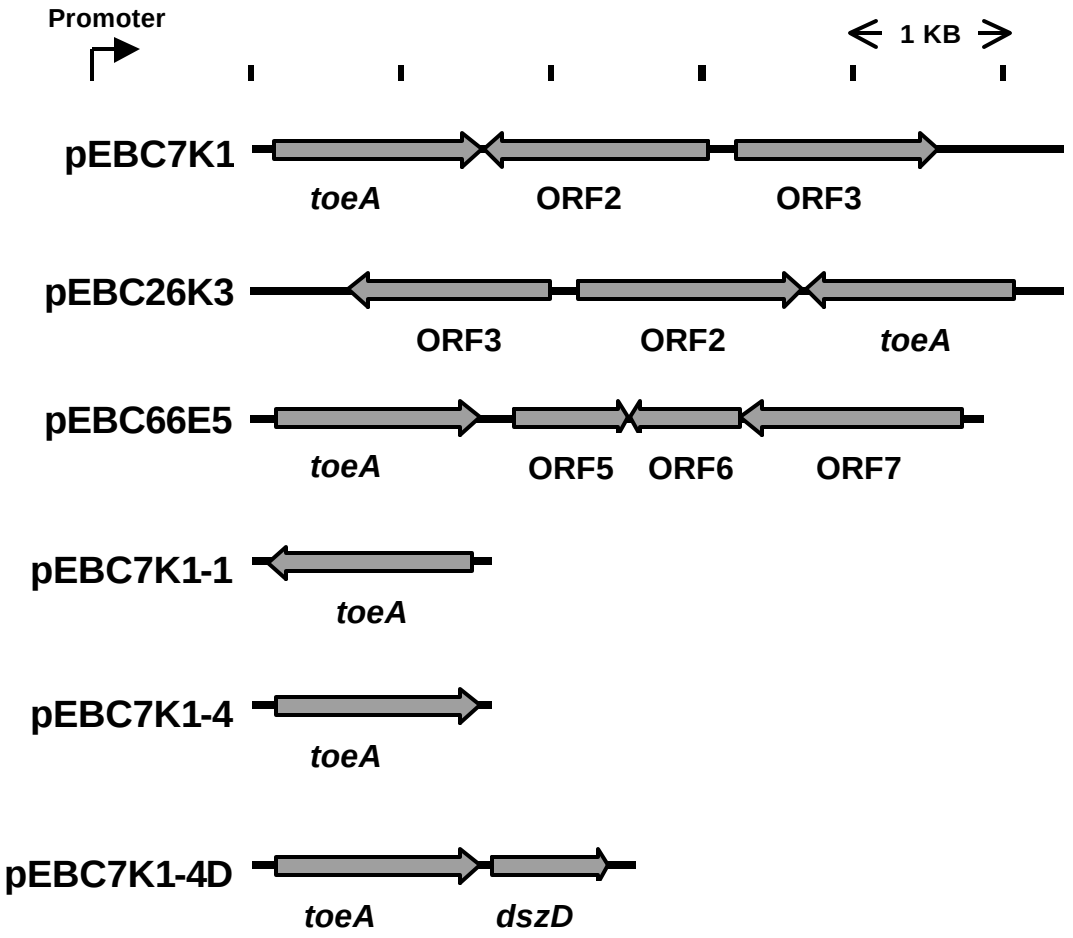


Figure 18. Phylogenetic tree of sequences most similar to *toeA*



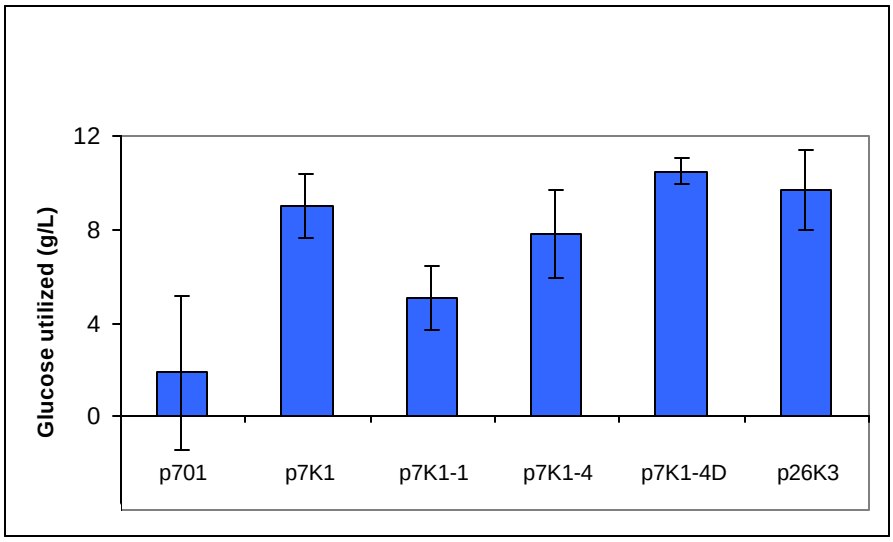
1	DSZ-A	*Rhodococcus erythropolis* IGTS8
2	DSZ-A	*Nocardia* A3H1
3	DSZ-A	*Sphyngomonas* AD109
4	TDS-A	*Paenibacillus* sp.
5	TOE-A	*Gordonia terrae* KGB1
6	TOE-A	*Tsukamurella wratislaviensis* EMT4
7	DSZ-A	*Mesorhizobium loti*
8	PA2326	*Pseudomonas aeruginosa*
9	NTA-MO-A(c)	*Mesorhizobium loti*
10	NRD	*Bradyrhizobium japonicum*
11	NTA-MO-A(b)	*Mesorhizobium loti*
12		*Sphingomonas aromaticivorans*
13	NTA-MO-A	*Chelatobacter heintzii*
14	NTA-MO-A(d)	*Mesorhizobium loti*
15	NTA-MO-A(e)	*Mesorhizobium loti*
16	NTA-MO-A(a)	*Mesorhizobium loti*
17	FUO	EDTA-degrading bacterium BNC1
18		*Bacillus subtilis*
19	YtnJ	*Bacillus subtilis*
20		*Streptomyces pristinaespiralis*

Figure 19. Inserts with open reading frames of plasmids complementing *R. erythropolis* JB55 when grown on gasoline as sole sulfur source.



Growth results on gasoline emulsion plates indicate that the *toeA* gene is responsible for complementing JB55. All of the plasmids carrying *toe*A allowed JB55 to grow two-phase liquid culture with weathered gasoline as sole sulfur source. Quantitation of growth by standard means (optical density, dry cell weight) was not possible because of the presence of an oil phase, but it was possible to measure growth by analyzing glucose remaining in culture. Figure 20 shows the glucose utilized in a shake flask experiments run with three replicates of each culture. Clearly, the plasmids containing *toe* A (p7K1, p7K1-1, p7K1-4, p7K1-4D, and p26K3, described in Figure 19) support higher and more consis tent growth than the empty vector control (p701).

Figure 20. Growth of JB55 constructs on weathered gasoline



The weathered gasoline was recovered from a number of growth assays for total sulfur analysis and for sulfur speciation by GC-SCD. The data from these experiments was difficult to interpret due to lack of sensitivity, low activity, and variations in volatilization, but after evaluating a number of replicas, we believe that JB55 carrying the *toeA* gene from KGB1 can remove certain sulfur (mainly C2- and C3-Ts) containing compounds from gasoline. A typical set of chromatograms is given in Figure 21. Attempts to raise activity by adding the dszD gene to the plasmid (pEBC7K1-4D) failed, suggesting either that FMNH2 is not required or that the background level of dszD in JB55 is sufficient to support this low level of activity.

The correlation between the *toeA* genes and the *dszA* genes is interesting with respect to the pathway identified for the desulfurization of diesel. In that pathway, the *dszA* gene product is part of a multiple enzyme pathway, while results to date indicate *toeA* is solely responsible for the oxidation of Cx-Ts.

The similarity between *toeA* and *dszA* is also indicated by the activity of ToeA with $DBTO_2$, the substrate for DszA in DBT desulfurization. In Table 7, the activity of KGB1 ToeA in different constructs is listed as production of hydroxybenzene sulfinate (HPBS). The cells were grown with DMSO as sulfur source because this has been shown to be the best sulfur source for expression of genes under the control of the ΔG promoter (included in all plasmids derived from p701). *R. erythropolis* BKO53, which has high DszA but no DszB activity, and thus can convert DBTO2 to HPBS, was used as the control. As seen in Table 7, the conversion of DBTO2 by ToeA is much lower than that seen with DszA. The promoter of *toeA* by itself appears to be not active under these conditions and the gene is under control of the ΔG promoter on the vector. The addition of *dszD* to *toeA* increases the DBTO2 activity only slightly and not significant with respect to the detection limit.

Figure 21. GC-SCD chromatograms of oil phase of growth assay experiments.

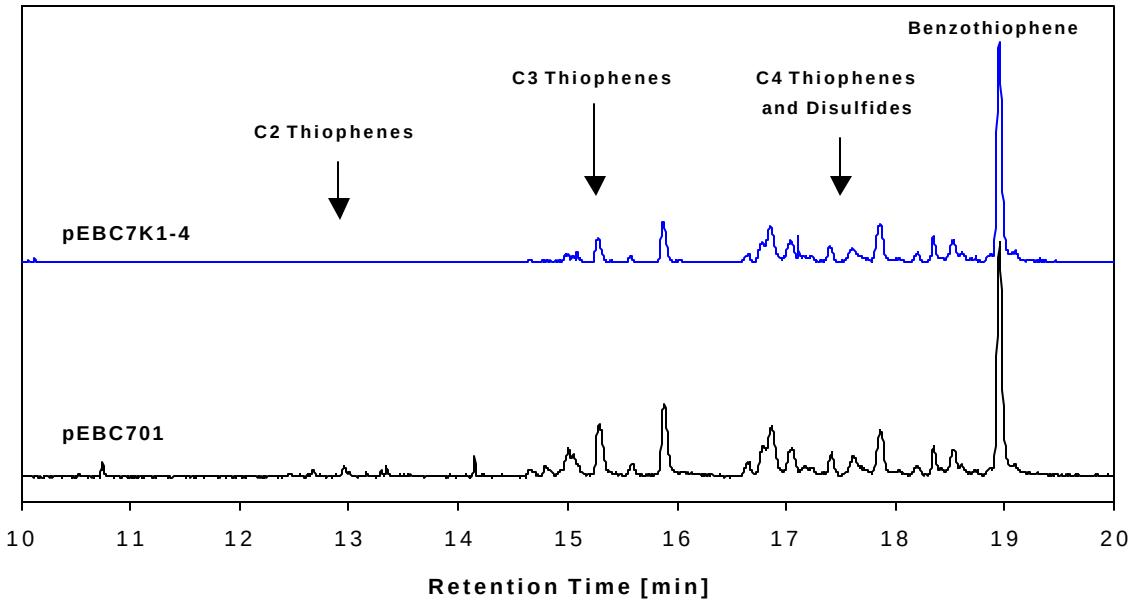


Table 7. HPBS production from $DBTO_2$

Strain	HPBS produced from 300 µM $DBTO_2$
BKO53 (*dszA*)	223 µM
JB55	0
JB55 x pEBC701	0
JB55 x pEBC26K3	0
JB55 x pEBC7K1	45
JB55 x pEBC7K1-1	0
JB55 x pEBC7K1-4	29
JB55 x pEBC7K1-4D	45

The strain which is intended to be used as biocatalyst in the gasoline desulfurization is PpG1, selected previously for its tolerance towards a gasoline matrix. Different constructs were made using combinations of *toe*A with or without either *dsz*D from IGTS8 or *fre* from *E. coli* (both encoding NAD(P)H-FMN oxidoreductases) and making use of different promoters, proven to be active in P. putida. Growth experiments with these constructs did not give any indication of activity towards the sulfur compounds. None of the peaks in the GC-SCD traces decreased after 4 days incubation. In addition, none of these constructs was able to transform DBTO2. There are many reasons why this did not happen, most likely the expression problem of the genes in this host. This is not uncommon for heterologous expression and will require some additional molecular engineering.

1.5.B. Genetics-Improvement: New Strains

The uncharacterized gasoline BDS genes from strains XJ5, 670-1, and 9.20.6 cannot be further exploited without a successful cloning strategy. Strain 670-1 is especially interesting because it appears to have a very wide substrate range, attenuating all but the BT peak. All of these strains show a worrisome capacity for transformation of nonsulfur-containing hydrocarbons in gasoline, and if that capacity is part of the uncharacterized BDS system, then this approach would likely be dropped, although it may be possible to use directed evolution techniques to limit the substrate specificity to organosulfur molecules.

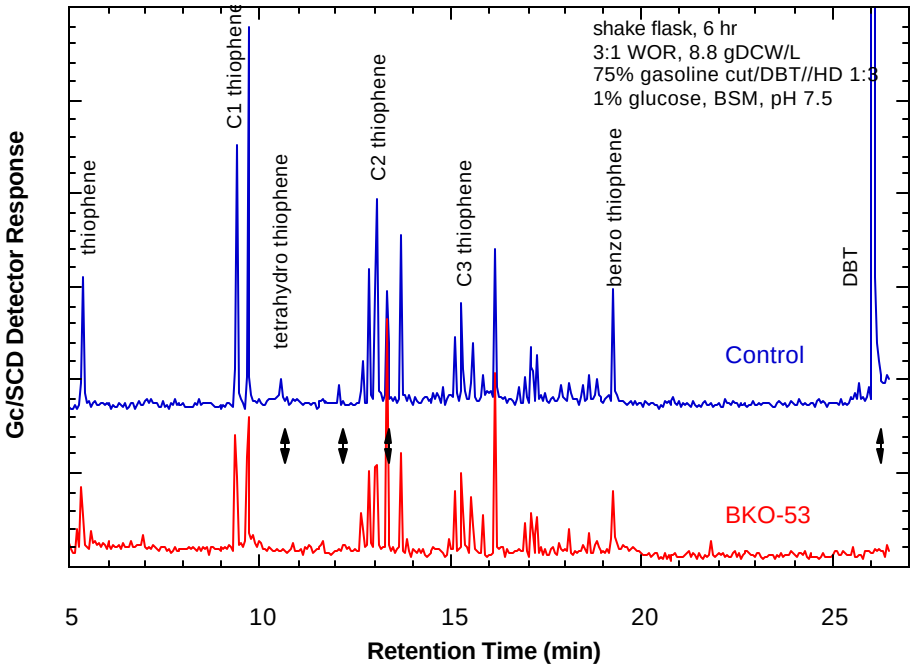
The *toe*A genes from KGB1 and EMT4 are only at the very early stages of development. The rates and extents for gasoline BDS with *toe*A from KGB1 expressed in JB55 are clearly insufficient, and we have not yet demonstrated gene expression in PpG1. It is not clear at this stage how well the genes are expressed, but it appears that the use of the G promoter did very little to improve the rate of DBTO2 transformation or gasoline BDS, which suggests that the original KGB1 promoter drives gene expression even in JB55. If this is the case, then it is likely that the *toe*A expression level is quite low, and significant improvement can be obtained by manipulating this strain.

Section 1C. Biocatalyst Development: Dsz

1.1.C. Isolate Desulfurizing Strains: Dsz

Through the use of weathered gasoline, we have learned that *dsz*-based biocatalysts are capable of removing some sulfur compounds from gasoline and that *dsz*+ strains of *Rhodococcus* can grow with weathered gasoline as sulfur source whereas *dsz*- strains cannot. Figure 22, below shows the results of a resting cell assay with BKO53, a high Dsz activity derivative of IGTS8, using weathered gasoline spiked with DBT. We can see that the DBT peak is completely removed and that there are small changes in minor peaks such as tetrahydrothiophene as well as some attenuation of the BT peak. In previous work we found that the Dsz system was able to desulfurize highly alkylated BTs, but was not efficient at transforming lightly alkylated BTs. BT itself was poorly transformed, and alkylated Ts were not transformed at all. It was clear that the wild type Dsz system was insufficient for gasoline desulfurization, but our success with directed evolution of DszC suggested a new strategy for gasoline BDS employing a selection for mutated *dsz* genes with altered substrate specificity capable of removing a more significant level of sulfur from gasoline.

Figure 22. GC-SCD chromatogram of weathered gasoline after treatment with Dsz+ *R. erythropolis* strain BKO53



1.2.C. Analytical: Dsz

No new analytical methods were developed for this approach.

1.3.C. Enzymology: Dsz

The Dsz system is well characterized with regards to its enzymology (B. R. Folosm et al. Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by *Rhodococcus erythropolis* I-19. Appl. Environ. Microbiol. 1999. 65:4967-4972. K. A. Gray et al., Molecular mechanisms of biocatalytic desulfurization of fossil fuels. Nature Biotechnology 1996 14:1705-1708).

1.4.C. Genetics-Characterization: Dsz

The Dsz system is well characterized with regards to its genetics. (T. Oshiro and Y. Izumi. Microbial desulfurization of organic sulfur compounds in petroleum. Biosci. Biotechnol. Biochem. 1999. 63:1-9. M. Li et al. Genetic analysis of the *dsz* promoter and associated regulatory regions. J. Bacteriol. 1996. 178:6400-6406).

1.5.C.i. Genetics-Improvement of dszC

The Dsz system of IGTS8 and other strains is effective for removing DBT and its alkylated derivatives, i.e. the major sulfur components in diesel. The Dsz system contains two monooxygenase enzymes. DszC (DBT monooxygenase) catalyzes the addition of two oxygen atoms to the sulfur atom in DBT to generate the sulfone. DszA (DBT sulfone monooxygenase) catalyzes the breaking of a carbon-sulfur bond to generate a sulfinic acid.

In previous work performed at Enchira, we determined that Cx-BTs and CxTs were transformed by the IGTS8 Dsz system poorly or not at all. The inability of these compounds to be transformed indicated that the DszC enzyme was unable to attack them. When sulfones of several Cx-BTs and Cx-Ts were synthesized, they were readily transformed by the Dsz system. This suggested that DszA was able to transform the sulfones and that the bottleneck in biodesulfurization of light compounds was the DszC enzyme. If a DszC enzyme could be mutated to transform BTs and Ts, it might improve the extent of BDS of straight-run diesels as well as provide a suitable starting point for gasoline BDS.

We designed a chemostat selection experiment to generate DszC mutants that could transform lightly alkylated BTs and/or alkylated Ts. This consisted of continuously growing an IGTS8 derivative strain in a sulfur-limited flow through bioreactor in the presence of 5-MBT and trimethylthiophene. If a mutation arose which allowed the transformation of one of these compounds, that mutant would be able to grow to large numbers and become the dominant population in the reactor. Using this strategy, a mutant strain, *R. erythropolis* C4-224, was isolated that had developed the ability to transform 5-MBT. There was no evidence that mutants capable of transforming an alkylated T ever arose in the chemostat.

C4-224 was genetically characterized. DNA sequence analysis of the *dsz* genes indicated a single mutation in the gene encoding the DszC enzyme, *dszC*, and two mutations in the gene encoding the DszA enzyme, *dszA*. Because it had previously been determined that DszC was the bottleneck in Cx-BT biodesulfurization, it was hypothesized that the *dszC* mutation was the cause of the new 5-MBT-transformation phenotype. The mutation in *dszC* was a substitution of a thymine for a guanine at codon 261 that caused an amino acid change in the DszC enzyme. The amino acid valine was replaced with phenylalanine at codon 261, i.e. Val261Phe mutation. If this mutation was indeed the cause of the new phenotype, then additional mutations at that locus might produce even bigger shifts in the directed evolution of DszC toward gasoline biodesulfurization. However, we first needed to demonstrate that the Val261Phe mutation was indeed responsible for the 5-MBT gain-of-function.

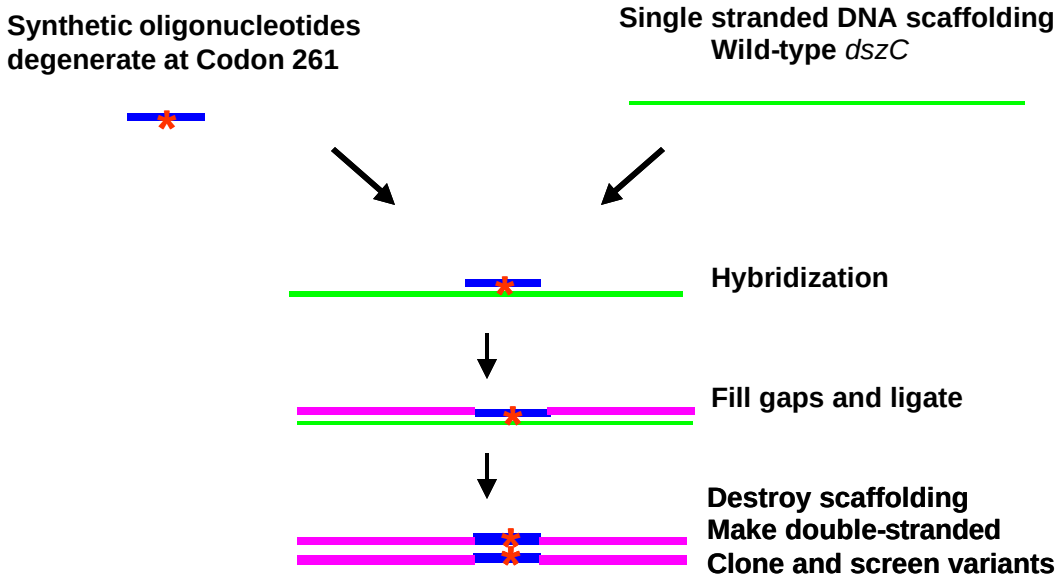
To verify that the 5-MBT phenotype is conferred by the *dszC* mutation, the mutant *dszC* gene (Val261Phe) was isolated from C4-224 by the polymerase chain reaction (PCR) and then inserted into a vector containing a wild-type *dszA, B, and D* genes; i.e., a genetic background containing none of the other mutations observed in the chemostat

mutant strain. The new plasmid construct was termed pJA103. Analysis of pJA103 confirmed that the Val261Phe mutation caused the 5-MBT-utilization phenotype. When pJA103 was transformed into a Dsz$^-$ strain, *R. erythropolis* JB55, it conferred upon it the ability to transform and grow on 5-MBT. Whole cell assays were performed with *R. erythropolis* JB55(pJA103) and confirmed that the strain transformed 5-MBT. Cells were incubated with 5-MBT in dodecane (150 ppm-S) at a 6:1 water:oil ratio. After 24 hours, the dodecane was collected and analyzed for total sulfur by combustive UV fluorescence. *R. erythropolis* JB55(pJA103) removed 65 ppm-S 5-MBT from the oil in this assay. This transformation was slow, but reproducible. In addition, JB55(pJA103) was more efficient than IGTS8 at transforming BT. However, it was not able to desulfurize Cx-Ts. JB55(pJA103) did not lose its ability to transform DBT. It transformed DBT at 1 mole $gDCW^{-1}$ min^{-1}, a specific activity comparable to the equivalent wild-type Dsz system.

The Val261Phe DszC mutant was now theoretically better able to desulfurize at least BT in gasoline (See Figure 22). This mutation demonstrated the importance of codon 261 to expanding the substrate range of DszC into lighter compounds. To attempt to develop a biocatalyst for gasoline biodesulfurization, we decided to perform saturation mutagenesis at codon 261 to generate additional mutations at this locus. Our goal was to generate a set of mutants with all possible amino acid substitutions at codon 261. Since a phenylalanine substitution at this position produced a shift in substrate specificity to include 5-MBT, it was thought that other amino acid substitutions at this position might create a shift in substrate specificity to include Cx-Ts.

A library was constructed containing clones randomized with respect to the sequence at codon 261. The library was generated by a modified RACHITT procedure. This procedure is illustrated in Figure 23. The *dszC* gene was made to be single stranded by exonuclease digestion and served as a template. Synthetic oligonucleotides (donor fragments) were annealed to the template across codon 261. The oligonucleotides were designed to be random, or degenerate, at codon 261, but to be DszC-homologous with regard to amino acid coding at all other positions. The annealed complexes were extended and repaired to generate full-length upper strands, and then the template strands were destroyed. The remaining top strands, containing the randomization at codon 261, were amplified by PCR, inserted into a vector containing *dszABD*, and then transformed into *R. erythropolis* JB55. In this way, a randomized codon 261 library was generated, containing approximately 16,000 individual clones. Theoretically, this library was large enough to contain with certainty at least one clone with each of the possible codon substitutions.

Figure 23. Modified RACHITT procedure for saturation mutagenesis of *dszC*.



Several attempts were made to screen the mutant library for candidate clones that could desulfurize Cx-Ts or weathered gasoline. The library was plated onto agar emulsion plates with Cx-Ts entrained in the oil phase. A mixture of 2-methyl-T, 3-methyl-T, and 2,5-di-methyl-T was used with each compound at 1,000 ppm-S in the oil phase of the emulsion plate. It was expected that those mutants that could transform Cx-T would develop into colonies and manifest themselves on the plates. In addition to the Cx-T plate selection, the mutant library was also spread onto agar emulsion plates containing trimethyl-T, 5-MBT, and weathered gasoline, respectively. Approximately 16,000 clones were plated onto each of these selection plates. In addition, plates with no sulfur added were inoculated as a negative control, and plates with DBT were inoculated as a positive control. No colonies arose on the Cx-T or trimethyl-T selection plates within one week. Many thousands of colonies arose on the positive control DBT plates. Colonies arose on the Cx-T or trimethyl-T selection plates after one week, but similar colonies also arose on the no sulfur negative control plates. These types of colonies typically arise on sulfur-limited plates after one week and are considered to be of a scavenger phenotype. These colonies were not analyzed. Many colonies arose on the weathered gasoline plate. However, a similar number of colonies arose when an IGTS8 control was spread on the weathered gasoline plates. This indicated that there was a sulfur component in the weathered gasoline that supported growth of cells with the wild-type Dsz system, and this selection was not appropriate for our purpose.

Interestingly, no colonies arose on the 5-MBT emulsion plates within one week. In a library of 16,000 mutants, it was expected that several hundred clones would contain the Val261Phe mutation previously demonstrated to confer the ability to grow on 5-MBT. The original chemostat isolate that was able to utilize 5-MBT, however, grew very slowly and took more than one week to manifest itself on an agar plate. Therefore, it is likely that the mutants for which we were selecting grew too slowly to form colonies within a reasonable period of time and before they would be masked by the appearance of colonies of the scavenger phenotype. Also, in the case of Cx-Ts, there was no direct evidence that a mutation in DszC allowing formation of a Cx-T sulfone would lead to growth. It may be for these compounds that the products of biodesulfurization are accumulated and not utilized as a sulfur source for growth.

In addition to the plates described above, over 1,900 clones from the mutant library were plated into individual agar-containing wells of 96-well plates. These plates were incubated in the presence of Cx-T vapors in an enclosed chamber. The Cx-T vapors were generated from a mixture of 2-methyT, 3-methyT, and 2-ethylT. This strategy was attempted because it was thought that perhaps Cx-Ts were volatilizing too rapidly when entrained in emulsion agar plates. Again, no Cx-T degraders were observed to grow within one week. After one week, false positives began to grow.

As described above, selections to isolate mutants capable of transforming Cx-Ts were not successful. As a result, it was decided to begin DNA sequencing across codon 261 of random isolates from the mutant library. The goal of this exercise was to identify and collect one mutant with each of all possible amino acid substitutions at codon 261. This mutant set could then be tested in whole cell assays at the shake flask level to determine the potential for Cx-T transformation. Whole cell assays do not require growth and can detect low level of activity toward a substrate that is inefficiently transformed.

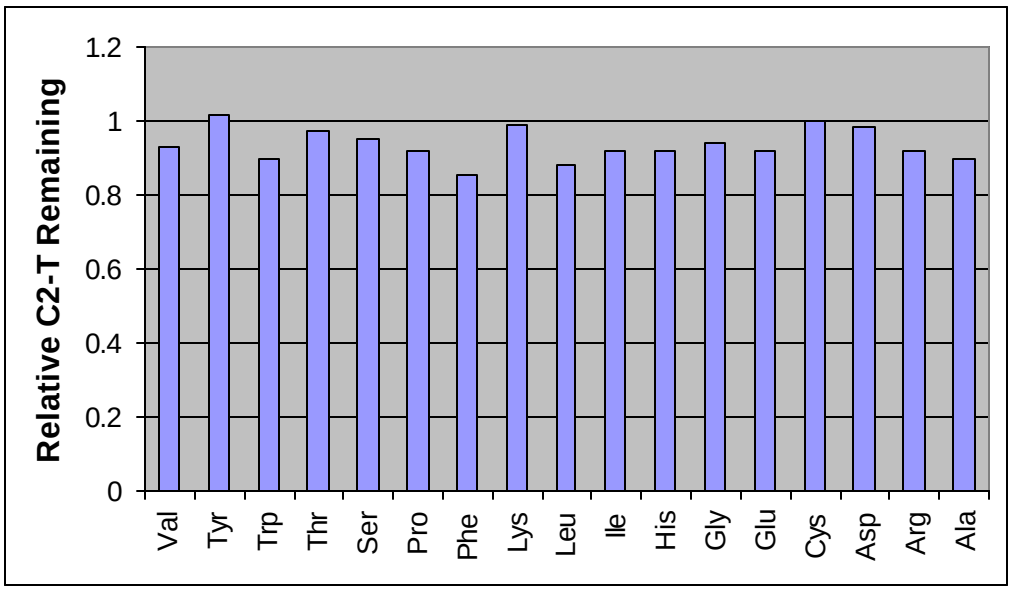
Sequencing of 321 clones revealed that the library was quite randomized at codon 261. Mutants representing 53 codons were identified and collected as summarized in Figure 24. At least one codon was collected for each of the twenty amino acids.

Figure 24. Summary of codon 261 mutants identified by DNA sequencing and collected.

	T	C	A	G	
	Phe	Ser	Tyr	Cys	U
T	Phe	Ser	Tyr	Cys	A
	Leu	Ser	Stop	Stop	C
	Leu	Ser	Stop	Trp	G
	Leu	Pro	His	Arg	U
C	Leu	Pro	His	Arg	A
	Leu	Pro	Gln	Arg	C
	Leu	Pro	Gln	Arg	G
	Ile	Thr	Asn	Ser	U
A	Ile	Thr	Asn	Ser	A
	Ile	Thr	Lys	Arg	C
	Met	Thr	Lys	Arg	G
	Val	Ala	Asp	Gly	U
G	Val	Ala	Asp	Gly	A
	Val	Ala	Glu	Gly	C
	Val	Ala	Glu	Gly	G

Seventeen of the codon 261 amino acid substitution mutants were initially screened for activity toward the sulfur compounds in weathered gasoline. Whole cell weathered gasoline assays were performed. The weathered gasoline was spiked with DBT and 5-MBT as control compounds. After 24 hours, the weathered gasoline phase was collected and analyzed by GC-SCD. There was virtually no removal of any of the sulfur molecules in weathered gasoline by any of the mutants. A sample of the results, those for the C2-T class of gasoline sulfur components, is shown in Figure 25.

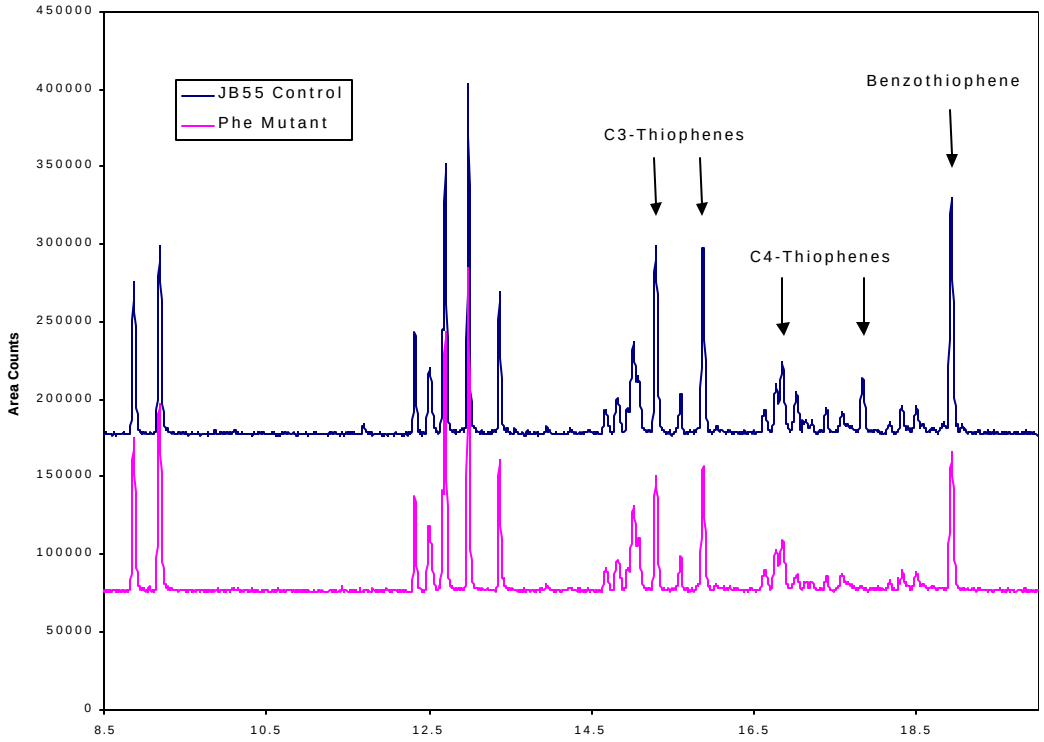
Figure 25. C2-T Removal from Weathered Gasoline by codon 261 mutants.



DBT was removed by many of the clones, indicating that the weathered gasoline was not toxic and that the Dsz system was active. However, some of the mutants did not transform DBT. It was assumed that the loss of activity in these samples was due to the nature of the codon 261 mutation in these clones. This was later confirmed by model compound assays with DBT as described below. There was no significant removal of spiked 5-MBT by any of the mutants, including the Val261Phe mutant despite the fact that this mutant had been shown to transform 5-MBT in model systems. It may be that 5-MBT has poor affinity for the mutant DszC enzyme and that the concentration of 5-MBT spiked into the gasoline was too low for efficient transformation. Alternatively, 5-MBT transformation may have been competitively inhibited by one or more of the scores of organosulfur compounds present in the weathered gasoline.

A complete set of 19 mutants, each with a different amino acid substitution at codon 261, was retested in whole cell assays for transformation of weathered gasoline. As a control, a strain with the wild-type sequence (valine) at codon 261 was also tested. No significant losses in total sulfur were observed for any of the mutants. However, GC-SCD chromatograms showed the attenuation of C3-T, C4T,and BT peaks in the assay for the Val261Phe mutant (see Figure 26). These results were consistent through several repetitions of the experiment.

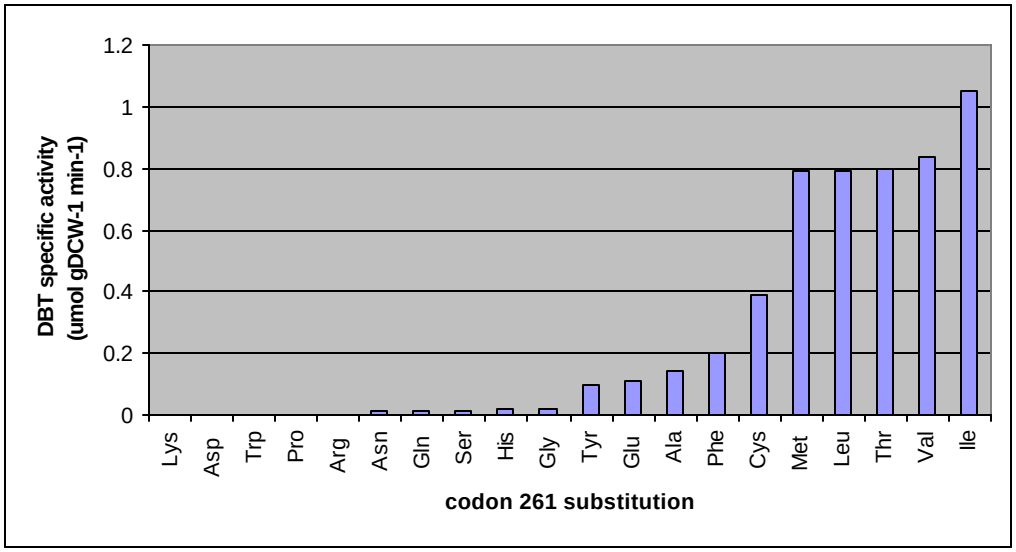
Figure 26. GC-SCD chromatogram of gasoline before and after BDS with Val261Phe mutant



The same set of 19 mutants was tested in whole cell assays for the ability to transform 5-MBT, BT, and DBT. The Val261Phe mutant was the only mutant that was able to transform 5-MBT. It was also the only mutant able to transform BT more efficiently than the wild-type control. Interestingly, the specific activity on DBT (Figure 27) of many of the mutants was reduced and in some cases, eliminated. This confirms the importance of codon 261 for substrate binding and specificity. Note that in this assay the phenylalanine mutant had less than 25% of the wild-type activity on DBT though it had gained the ability to transform 5-MBT. It is possible that the ability to broaden the DszC substrate specificity to include lighter compounds may need to be at the expense of DBT activity. This

would not be problematic for development of a gasoline desulfurization biocatalyst since DBT is not a component of gasoline. Although the isoleucine mutant appeared to have a higher DBT activity than the wild-type valine strain, this observation was not confirmed upon retesting.

Figure 27. Summary of DBT specific activity of mutants with the specified amino acid substitution at codon 261 of DszC.



Additional broadening of the substrate range of DszC is required to allow transformation of Cx-Ts. In addition, 5-MBT transformation by Val261Phe mutant is slow and requires significant improvement in rate. Because all of the possible mutations at codon 261 have been exhausted, additional improvements must be gained by mutating additional *dszC* loci and then selecting for the desired phenotype using methods similar to those described in this report. A structural model of DszC is not yet available, so a rational approach to additional mutagenesis is not possible at this time. Random mutagenesis and/or *in vitro* recombination are tools available for generating additional mutants. *In vitro* recombination of the mutant *dszC* gene generated in this work with other alleles may generate chimera with combinations of beneficial traits, e.g. better rates on 5-MBT and/or increased substrate specificity to include Cx-Ts.

The work described in this section has been submitted for publication. The manuscript is scheduled to be published in Applied and Environmental Microbiology, February, 2002.

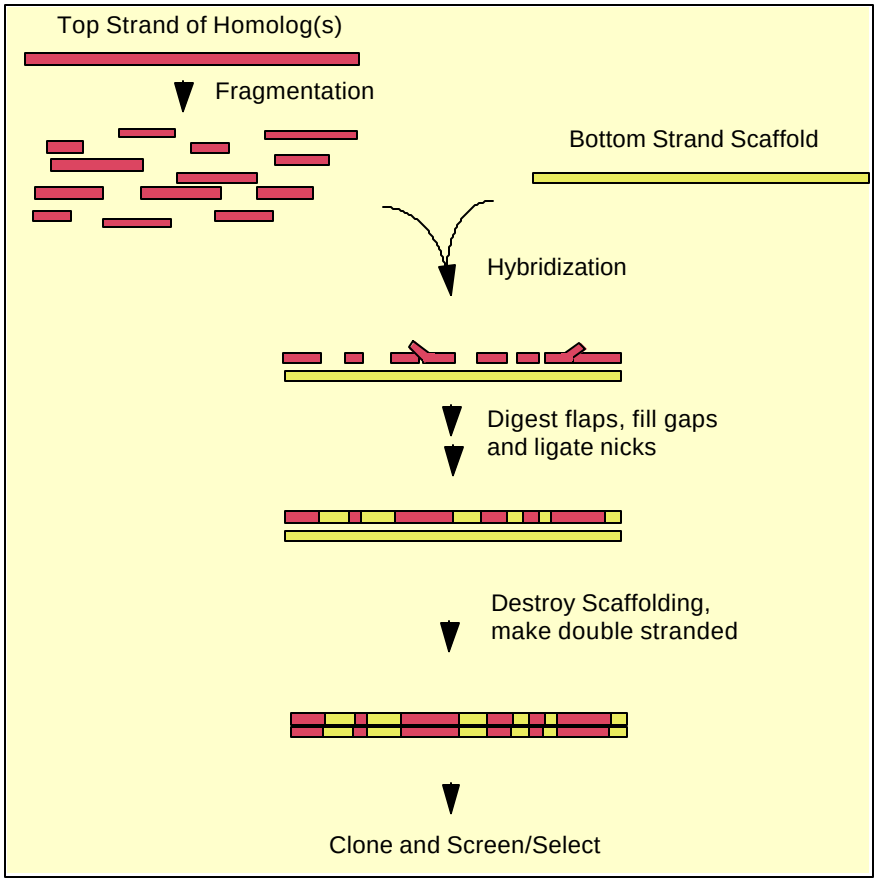
1.5.C.ii. Genetics-Improvement of dszB

The IGTS8 gene *dszB* encodes the enzyme that catalyzes the conversion of 2-(2-hydroxyphenyl)-benzenesulfinate (HPBS) to 2-hydroxybiphenyl (HBP) and inorganic sulfur. This desulfination is the final and rate-limiting step (more than an order of magnitude slower than that those of DszC and DszA) in the Dsz pathway that results in the removal of sulfur from DBT, and it would be expected to be the rate limiting step in a gasoline BDS process using a mutated *dsz*C gene as described above. This section documents the results of our efforts to apply the RACHITT technology to *dszB* to improve the biocatalytic properties of the desulfinase enzyme with an ultimate goal of constructing a commercial biocatalyst.

In order to use the RACHITT technology (Figure 28), at least two homologous sequences are required. At the beginning of this project, three wild-type *dsz*B homologs were available: IGTS8 (*Rhodococcus*), A3H1 (*Nocardia*), and AD109 (*Sphingomonas*) along with two mutants derived from IGTS8 (Q65H and A176V). These two single

substitution mutants were obtained using random mutagenesis techniques and they were shown to have higher activity than the wild-type. More genetic diversity was desired, however, and so the first phase of the project was the isolation of additional *dszB* homologs from a variety of different environmental soil samples. This would allow us to have a larger panel of homologs to use in our pursuit of improved *dszB* using RACHITT.

Figure 28. Schematic of RACHITT procedure



Eight variant *dsz*ABC clusters were isolated by PCR from different environmental samples. These clusters were cloned into pUC-based vectors. Each of the eight clones was partially sequenced in order to determine the level of homology to the known dszB genes. These results are summarized in Table 8 below:

Table 8. Relationship among *dsz*B clones obtained by PCR from environmental samples

		HOMOLOGY(%)		
CLONE	NUCLEOTIDES SEQUENCED	A3H1	IGTS8	AD109
SS13-1	410	96	89	68
SS13-3	170	99	92	62
SS13-8	470	100	-	-
SS13-6	315	98	91	69
JSP-3	510	96	91	71
JSP-1	780	97	91	72
JSP-2	400	96	87	64
JSP-8	620	96	92	68

The data in Table 8 demonstrate that each *dszB* sequence is highly homologous to A3H1. In fact, sample SS13-8 and A3H1 are identical to each other at the 5'- end (470 nucleotides), but further sequencing of the rest of the gene is required to determine whether SS13-8 is actually A3H1. JSP-1 and JSP-3 are identical to one another at the 5'- 510 nucleotides, but are distinct sequences from A3H1. Like SS13-8, further sequencing would be necessary to determine if JSP-1 and JSP-3 are actually one in the same. The other six sequences are definitely unique based on the partial sequencing. Based on the homology data, it was clear that there were many candidates that could be used in RACHITT method of *in vitro* recombination with the *dsz*B gene from IGTS, 8A3H1, or AD109.

All of the *dsz*B variants were cloned into the vector pBAD-TOPO for expression in *E. coli*. In these experiments, cells harboring *dsz*B genes closely related to that of A3H1 had very low activity and were able to convert HPBS to HBP only after very long incubation times. Even at the long time points, the extent of conversion was very low (5%). A number of things could account for this observation, such as low expression, improper folding, toxicity due to the overexpressed protein, or solubility. Growth studies indicated that high levels of desulfinase were toxic to *E. coli*. The A3H1-like clones grew well, but the IGTS8-like clones grew poorly unless lower levels of the inducer arabinose were used. Low *in vivo* activity precluded the use of this approach for the screening of these variants.

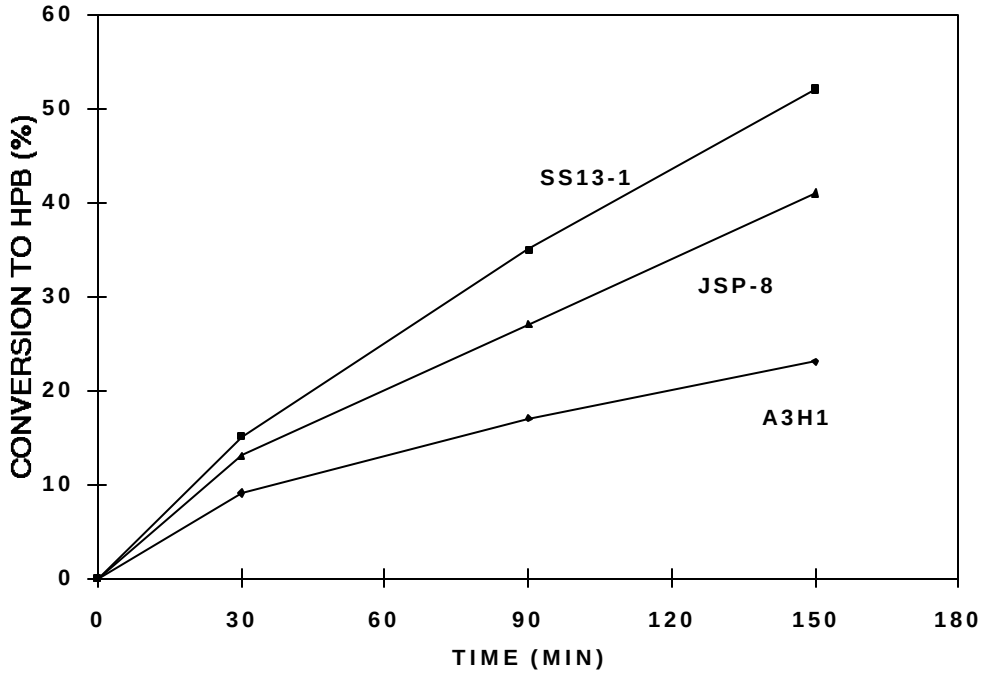
Crude lysates were prepared from the above-mentioned *dszB* clones using lysozyme and slow thawing on ice. Determination of total protein in the lysates indicated that better yields were obtained from bacteria harboring A3H1 or the A3H1-like *dszB* variants. In one experiment, reactions between the various lysates and HPBS were set up and incubated at 37 ^{o}C. Reactions contained 366 µM HPBS (~93 nmol) and ~4 nmol protein in a total volume of 250 µl, and were stopped after 1h by the precipitation of protein using an equal volume of acetonitrile. Following centrifugation the supernatants were analyzed for recovery of HPBS and the formation of HBP. In general, HBP formation was inefficient. Lysates containing *dszB* from IGTS8 or IGTS8 mutants showed no activity. However, lysates containing A3H1 *dszB* did show activity, as did five of the eight A3H1-like variants, though the conversion of HPBS was low.

Table 9. Desulfinase activity in crude lysates

Lysate	% conversion of HPBS to HBP
A3H1	8
SS13-1	18
SS13-3	9
JSP-1	6
JSP-3	10
JSP-8	19

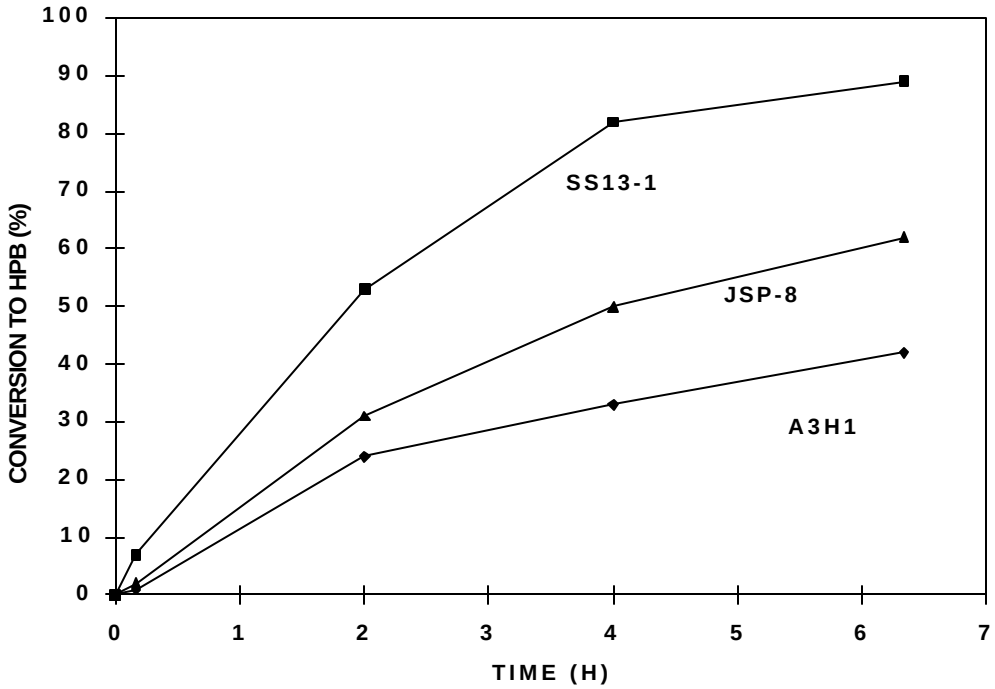
Additional bioconversion experiments using the crude lysates were carried out with the two best variants (SS13-1 and JSP-8) with extracts from cells overexpressing A3H1 and IGTS8 as controls. In time course experiments, 4 nmol of protein was incubated with 50 nmol HPBS in a volume of 250 µl (final [HPBS] was 200 µM) and reactions were stopped at 0, 30, 90 and 150 min by extraction of the protein with an equal volume of acetonitrile. As before, the IGTS8 lysates didn't convert any HPBS to HBP even after prolonged incubation. In contrast, time-dependent conversion of HPBS to HBP was observed for A3H1 and the variants. These data are shown in Figure 29 (expressed as % conversion). As before, the variants were more active than A3H1 or IGTS8. It's possible that the variants are more stable than A3H1 as they continue to produce HBP even at 150 min.

Figure 29. Conversion of HPBS by *E. coli* lysates containing DszB enzymes



Additional time course studies were carried out, and the ability of the extracts to convert HPBS to HBP was measured out to 21 hours. Results are shown in Figure 30. The reactions were set up and stopped as before (~4 nmol protein + 50 nmol HPBS (200 µM)).

Figure 30. Conversion of HPBS by *E. coli* lysates containing DszB enzymes



The data suggest that the SS13-1 and JSP-8 extracts have improved desulfinase activity (initial rates) and may be more stable as well. Extracts containing SS13-1 converted nearly all of the HPBS by 4 h. It should be pointed out that only 3 nmol of JSP-8 extracts were used in these reactions, so the performance of this variant may be understated. Not shown in the plot are the values for the 21 h time point. At 21 h, no HPBS could be detected in the reactions containing SS13-1 or JSP-8, suggesting the reaction went to completion. However, ~50% of the HPBS still remained in the reactions with A3H1. The extracts used in these experiments were analyzed by 10% PAGE and Coomassie Blue staining, and the banding intensity indicated that the same amounts of DszB was present in each of the 3 extract preparations.

Attempts were made to solve the problem of overexpressing the IGTS8 gene in *E. coli* by switching to a Dsz- strain of *Rhodococcus erythropolis* JB55. In this strain, the IGTS8 *dsz*B gene leads to higher desulfinase activity than the A3H1 *dsz*B gene, suggesting the IGTS8 DszB is more active than the A3H1 DszB. SDS-protein gels indicated that the protein levels were much higher in the IGTS8 clone than in the A3H1 clone, and so the activity was a function of gene expression levels. Future work in screening will require an expression system in which all dszB genes are expressed equally. Under those circumstances, improved activity will be due to improved enzymes not improved expression.

RACHITT was carried out using *dsz*B genes from IGTS8 and A3H1. Both genes were manipulated to generate transient template and donor fragments.

The final PCR indicated products present for each template (IGTS8 and A3H1), and no product was formed in the "no donor" reactions. Reactions were gel-purified and cloned into the pBAD-TOPO vector for restriction digests and sequencing to determine the degree of *in vitro* recombination. Following transformation of each library into DH10B *E. coli*, it was determined that the reaction 1 library contained about 15,000 members and the reaction 4 library roughly 40,000.

Restriction analysis of 36 clones picked at random from the RACHITT libraries indicated successful *in vitro* recombination. Roughly half of the clones examined showed an altered digestion pattern using only one or two restriction enzymes (6-base cutters). Ten clones were sequenced, five from the IGTS8 transient template reactions and five from the A3H1 transient template reactions. All 5 of the IGTS8 transient template reactions showed altered

restriction patterns, while only four of the five A3H1 transient template reactions were confirmed by restriction analysis. The fifth gave a restriction pattern similar to that of A3H1 and served as a non-biased or non-screened clone. The sequencing results clearly indicate that each of the ten clones is chimeric and contain multiple crossovers (mossaicisms). The 5'- 360 nucleotides, representing about one-third of the gene, are shown below for each sequence. Sequences 1-5 represent the reactions where IGTS8 was the transient template and 6-10 represent reactions where A3H1 was the transient template. Nucleotides depicted in red indicate IGTS8 sequence and nucleotides in blue represent A3H1 sequence. Note that the crossover points are only estimates of the precise locations where recombination occurred. Also note that each sequence contained at least one point mutation not originating from recombination. This is an expected event due to the multiple PCR steps involved with RACHITT.

1)
ATGGCAGGCCGCCTCAGCCCCGGAAACCCCGGTTCAGAACTCGATTCCGCCATCCGCGACACACTGACCTACAGCAACTGCCCGAT
ACCCAACGCGCTGCTCACGGCGTGGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAACTCGACATCCTCAGCGGCAAGCAGGGAA
CGGTTCATTTCACCTACGACCAGCCTGCCTACACCCGTTTTGGGGGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCT
GGGCGCACGCGTCTACTCGGCATCACCCCGATCCTGGGGCGTCAGGGCTTCTTCGTCGGCGATCGCAGCCCGATCACAGTGGCCGC
CGACCTTGCCGGACGT

2)
ATGACAAGCCGCGTCGACCCCGCAAACCCCGGTTCAGAACTTGATACCGGCATCCTCGACACACTGACCTACAGCAACTGCCCGAT
ACCTAACGCGCTGCTCACGGCGTGGGAATCAGGTTTCCTCGACGCCGCCGGCATCGAACTCGACGTCCTCAGCGGCAAGCAGGGAA
CGGTCCACTTCACCTACGACCAGCCTGCCTACACCCGTTTTGGGGGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCC
GGACGCACGCGTCTACTCGGCATCACCCCGATCCTGGGGCGTCAGGGCTTCTTTGTCCGCGACGACAGCCCGATCACAGTGGCCGC
CGACCTTGCCGGACGC

3)
ATGACAAGCCGCGTCGACCCCGGAAACCCCGGTTCAGAACTTGATACCGGCATCCTCGACACACTGACCTAC
AGCAACTGCCCGATACCCAACGCTCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAA
CTCGACGTCCTCAGCGGCAAGCAGGGAACGGTCCACTTCACCTACGGCCAGCCCGCCTACACCCGTTTTGGG
GGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCCGGACGCACGCGTCTACTCGGCATCACCCCG
ATCCTGGGGCGTCAGGGCTTCTTCGTCGGCGACGACAGCCCGATCACAGTGGCCGCCGACCTTGCCGGACGC

4)
ATGGCAGGCCGCCTCAGCCCCGGAAACCCCGGTTCAGAACTTGATACCGCCATCCGTGACACACTGACCTACAGCAACTGCCCGAT
ACCCAACGCGCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAACTCGACATCCTCAGCGGCAAGCAGGGAA
CGGTCCACTTCACCTACGACCAGCCCGCCTACACCCGTTTTGGGGGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCT
GGGCGCACGCGTCTACTCGGCATCACCCCGCTCTTGGGGCGCCAGGGCTTCTTCGTCGGCGATCGCAGCCCGATCACAGTGGCCCG
CGACCTTGCCGGACGC

5)
ATGACAAGTCGCGTCGACCCCGCAAACCCCGGTTCAGAACTTGATACCGGCATCCTCGACACACTGACCTAC
AGCAACTGCCCGATGCCCAACGCGCTGCTCACGGCGTGGGAATCAGGTTTCCTCGACGCCGCCGGCATCGAA
CTCGACATCCTCAGCGGCAAGCAGGGAACGGTCCACTTCACCTACGACCAGCCTGCCTACACCCGCTATGGC
GGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCTGGGCGCGCGCGTCTACTCGGCATCACCCCG
ATCCTGGGGCGTCAGGGCTTCTTCGTCGGCGATCGCAGCCCGATCACAGTGGCCGCCGACCTTGCCGGACGT

6)
ATGACAAGCCGCGTCGACCCCGCAAACCCCGGTTCAGAACTTGATACCGGCATCCTCGACACACTGACCTAC
AGCAACTGCCCGGTACCCAACGCTCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAA
CTCGACATCCTCAGCGGCAAGCAGGGAACGGTCCACTTCACCTACGACCAGCCCGCCTACACCCGCTATGGC
GGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCCGGACGCACGCGTCTACTCGGCATCACCCCG
ATCCTGGGGCGTCAGGGCTTCTTCGTCGGCGATCGCAGCCCGATCACAGTGGCCGCCGACCTTGCCGGACGC

7)
ATGACAAGCCGCGTCGACCCCGGAAACCCCGGTTCAGAACTCGATTCCGCCATCCGCGACACACTGACCTAC
AGCAACTGCCCGGTACCCAACGCTCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAA
CTCGACGTCCTCAGCGGCCAGCAGGGCACGGTTCATTTCACCTACGACCAGCCCGCCTACACCCGCTATGGC
GGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCCGGACGCACGCGTCTACTCGGCATCACCCCG
CTCTTGGGGCGCCAGGGCTTCTTTGTCCGCGACGACAGCCCGATCACAGCGGCCGCCGACCTTGCCGGACGC

8)

ATGACAAGCCGCCTCAGCCCCGGAAACCCCGGTTCAGAACTTGATACCGGCATCCTCGACACACTGACCTACAGCAACTGCCCGGT
ACCCAACGCTCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAACTCGACGTCCTCAGCGGCAAGCAGGGAA
CGGTCCACTTCACCTACGACCAGCCCGCCTACACCCGCTATGGGGGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCC
GGACGCACGCGTCTACTCGGCATCACCCCGATCCTGGGGCGTCAGGGCTTCTTTGTCGGCGATCGCAGCCCGATCACAGTGGCCGC
CGACCTTGCCGGACGC

9)
ATGACAAGCCGCCTCAGCCCCGGAAACCCCGGTTCAGAACTTGATACCGGCATCCTCGACACACTGACCTACAGCAACTGCCCGGT
ACCCAACGCTCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAACTCGACGTCCTCAGCGGCCAGCAGGGCA
CGGTTCATTTCACCTACGACCAGCCTGCCTACACCCGTTTTGGGGGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCT
GGGCGCACGCCCGGTGGTGGATCTCGGCCT
(#9 sequence is a little shorter due to ambiguity past 300 nucleotides)

10)
ATGACAAGCCGCGTCGACCCCGCAAACCCCGGTTCAGAACTCGATTCCGCCATCCGCGACACACTGACCTAC
AGCAACTGCCCGGTACCCAACGCTCTGCTCACGGCATCGGAATCGGGCTTCCTCGACGCCGCCGGCATCGAA
CTCGACATCCTCAGCGGCAAGCAGGGAACGGTCCACTTCACCTACGACCAGCCCGCCTACACCCGCTATGGC
GGTGAGATCCCGCCACTGCTCAGCGAGGGGTTGCGGGCACCCGGACGCACGCGTCTACTCGGCATCACCCCG
CTCTTGGGGCGCCAGGGCTTCTTTGTCCGCGACGACAGCCCGATCACAGTGGCCGCCGACCTTGCCGGACGC

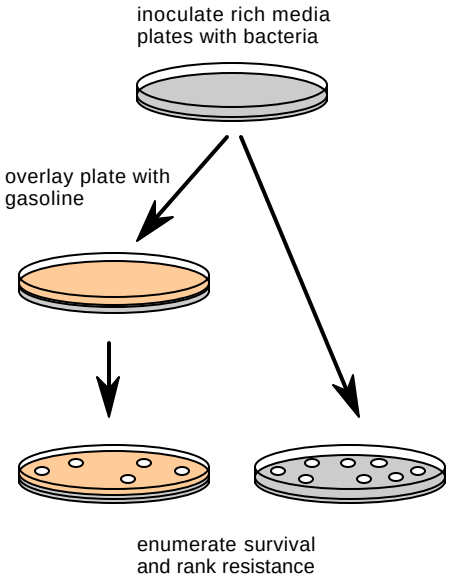
In addition to the frequent crossovers in the chimeric sequences, these data suggest little to no parental contamination. More sequencing could be carried out on additional clones to provide further evidence of this. Regarding the extent of chimeragenesis, it can be estimated that between 10 and 20 crossovers occurred on each template during these reactions.

Attempts to screen dszB variants for improved activity proved to be problematic in both *E. coli* and *R. erythropolis* due to variable levels of gene expression. This problem can be solved using different vectors or promoters and will need to be solved for any high throughput assay. The dszB genes from IGTS8 and A3H1 were successfully shuffled by the RACHITT method and generated libraries containing in excess of 50,000 variants. These libraries appear to be highly chimeric based on sequence data from unselected clones. Additional libraries can be constructed using more of the genetic diversity now available. It is to be expected that desulfinase variants with significant improvements in biocatalytic properties can be obtained by screening these libraries.

1.6 Develop Gasoline Tolerant/Desulfurizing Strain

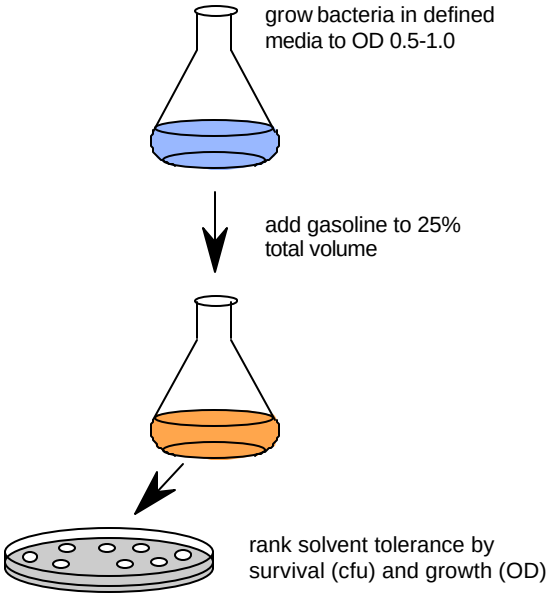
Fifty soil isolates and 17 Pseudomonas strains were screened for gasoline tolerance. Of these, only 11 strains, all *Pseudomonas* spp., survived when grown on rich media plates with a gasoline overlay (Figure 31). Most of these strains were highly sensitive to gasoline toxicity when liquid cultures were challenged with gasoline. However, one strain, *Pseudomonas putida* PpG1, outperformed the others under this set of conditions, with only one log of killing (compared to 2 or more logs with other strains) after 20 hours. Despite the initial killing upon addition of gasoline, PpG1 and others demonstrated an ability to recover and return to exponential growth after 20 hours (Figure 32). Based upon these screens, we chose PpG1 as the best candidate for further development as gasoline BDS host. All of the data collected supported the observations reported in the literature that solvent tolerance is only possible when bacteria are actively growing. This conclusion fits well with our process model calling for continuous growth in the gasoline BDS reactor (Section 2).

Figure 31. Solvent tolerance plate screen



- **Numerous environmental isolates and in-house strains screened**
- **50% grew within 5 days**
- **25% grew within 24 hrs**
- **14% had >10% survival**

Figure 32. Solvent tolerance culture screen

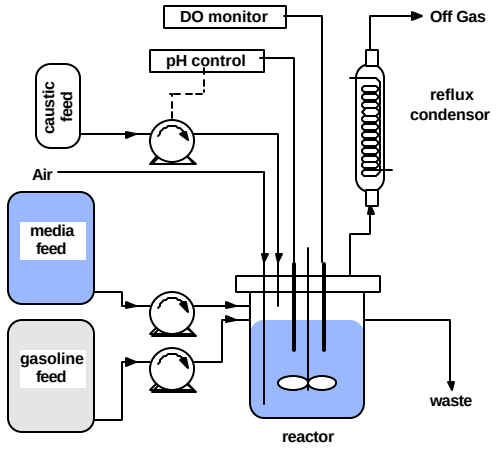


Strain Tested	Rank
P. putida PpG1	1
P. aureofaciens	2
P. aeruginosa PG2O1	3
P. aeruginosa PAO1	4
P. putida Idaho	5
P. putida MT-2	6
P. putida KT2440	7
P. putida PPS1093	8
P. fluorescens NCIB11764	failed
P. chlororaphis ATCC9446	failed
P. denitrificans ATCC1294	failed
P. mendocina ATCC25411	failed

Continuous culture experiments were performed in a prototype bench scale reactor (Figure 33) to evaluate the use of growing cells to overcome the toxic effects of gasoline. A continuous culture of PpG1 was started with a 24 hour residence time using standard growth medium with glucose as the carbon source (Figure 34). The gasoline content in the chemostat was then increased stepwise until a free phase of gasoline was present. Though the composition and distribution of organic compounds in the gasoline shifted due to volatilization of the lower boiling component, it was estimated that the gasoline content reached approximately 5% at the conclusion of this experiment. At each increase in gasoline content, up to the point where free gasoline was observed, the biomass content of the chemostat decreased, which is consistent with published reports of the need for energy to sustain viability in the presence of toxic solvents. In these reports, toxicity increases as the concentration of solvent increases up to the point of saturation. From that point on, solvent sensitivity does not increase with increases in the solvent:water ratio.

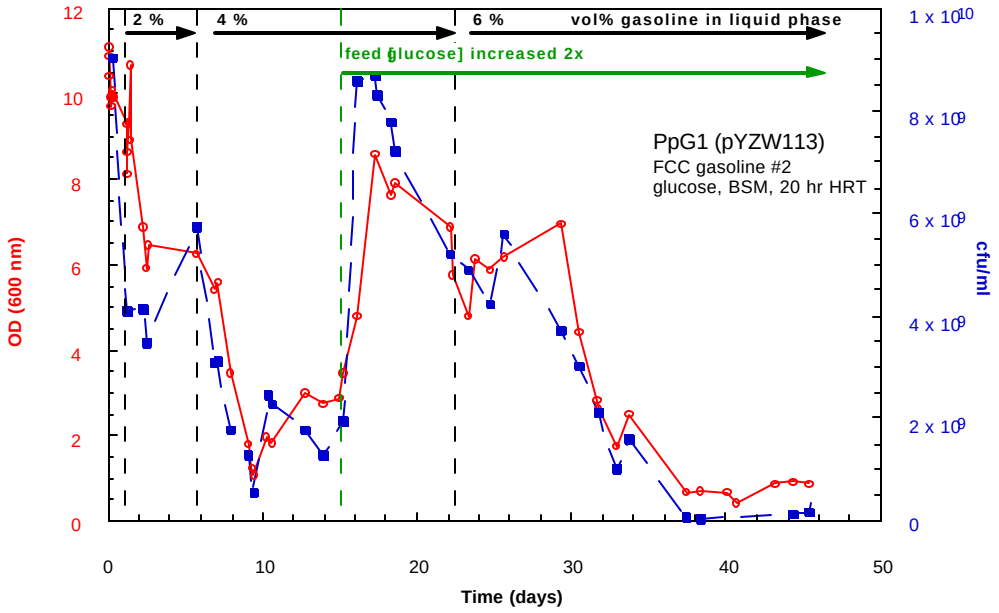
The experiment described above used only a single stage condenser at +5°C, and a significant amount of hydrocarbon vapors were lost to volatilization. Subsequent continuous culture experiments (described in Section 1.4.A) made use of two stage condensers set at +5°C and -70°C. These experiments suffered from difficulties in maintaining the ultra-cold chiller -70°C and from problems with water vapors escaping the first stage to form ice plugs in the second stage. These problems led to a great deal of variability in the hydrocarbon content of the gasoline phase. Ultimately, these problems were solved through the use of minimal airflow and condenser temperature programs that maximized the gasoline hydrocarbon recovery with no ice plugs. Under these conditions, a stable culture of PpG1 was maintained for more than 20 days with hydraulic retention times of 12-48 hours and gasoline making up 1-10% of the liquid volume in the reactor.

Figure 33. Lab-scale bioreactor.



- **single stage CSTR**
- **carbon limited growth to minimize solvent toxicity**
- **vary / control operation**
  - **pH**
  - **temperature**
  - **DO**
  - **WOR**
  - **cell load**
  - **residence time**
- **minimize volatile loss of gasoline range compounds**

Figure 34. Continuous culture of recombinant PpG1 in gasoline bioreactor



Section 2 Process Development

2.1. Mathematical Modeling

Detailed biochemical analysis of a biocatalyst is necessary to develop and test mathematical models. This has not been completed with any of the enzyme systems evaluated for gasoline BDS

2.2. Reactor Testing

A bench scale reactor has been developed (Figure 34) and tested with the gasoline tolerant strain, PpG1, with and without the *fmo*A gene. With the two stage condenser and temperature program in operation, this reactor has been shown to be adequate to maintain a gasoline phase of up to 10% (v/v). Continuous cultures of PpG1 were maintained in this reactor on three separate occasions with stable cultures running for more than four months.

2.3. Technology and economic assessment

A description of a 20,000 BPSD gasoline biocatalytic desulfurization (BDS) unit is provided below, including a process flow diagram at the end of the section.

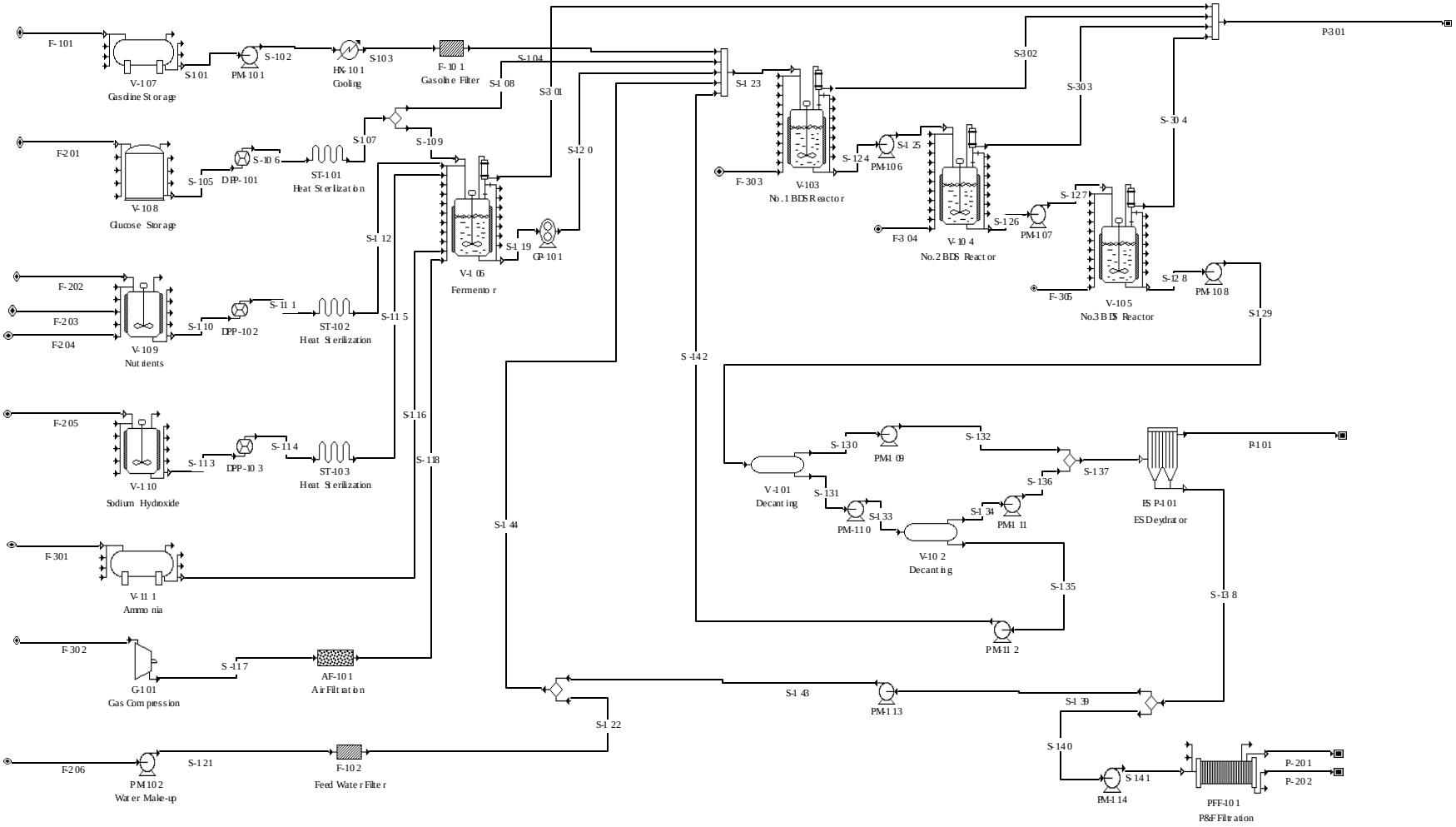
Overview

The conceptual BDS process is envisioned to employ a recombinant strain of *Pseudomonas putida* to convert a portion of the sulfur, contained in a liquid petroleum fuel into a water-soluble compound, thus removing sulfur from the fuel. The use of a recombinant strain requires a number of safeguards designed to prevent release of live biocatalyst from the reactor. These safeguards include design-based biocatalyst containment, chemical and heat kill processes to sterilize spent biocatalyst, offgas filters to remove biocatalyst aerosols. All of these safeguards are based on standard bioprocess methodologies and have been tested in the 5 barrel per day diesel BDS pilot plant operated previously by Enchira. For this generic gasoline BDS unit, the petroleum fuel is gasoline with a total sulfur content of 350 ppmw. Through processing in the BDS unit, the sulfur content of the fuel is reduced to 35 ppmw. Biocatalyst assumptions used in process modeling are listed in Table 10.

Table 10. Biological assumptions used in gasoline BDS process modeling	
Biocatalyst	Recombinant strain of gasoline tolerant *Pseudomonas putida* PpG1
Reaction	Monooxygenation of sulfur-containing hydrocarbons (e.g. conversion of
Maximum reaction rate (Vmax)	15 umole sulfur per minute per gram dry cell weight
Biocatalyst residence time	50 hours
Biocatalyst half saturation point (Km)	100 ppmw sulfur in gasoline
Biocatalyst concentration in reactor	20 gram dry cell weight per liter total liquid volume
Volumetric productivity	150 umoles sulfur per liter per minute (averaged over all three reactors)
Glucose used for biocatalyst	0.45 gram dry cell weight per gram glucose
Glucose used for BDS	10 moles of sulfur per gram glucose

The BDS unit is operated as a continuous flow system at low pressures and moderate temperatures in an aerobic environment. Water is used both as a medium in which to sustain the microorganisms as well as the carrier for the microorganisms within the unit. A process flow diagram is shown in Figure 35.

Figure 35. Process flow diagram for gasoline BDS bioreactor



The BDS reactors are operated at a 50:50 oil-to-water volumetric ratio, a biocatalyst concentration of 20 g/L (dry cell weight, combined phase basis) and a biocatalyst residence time of 50 hours. The majority of the water and biocatalyst supplied to the reaction section is provided by internal recycle. A portion of the biocatalyst inventory is, however, continuously purged from the system with an equivalent amount of fresh biocatalyst grown to maintain biocatalyst activity. The biocatalyst makeup is continuously grown on-site within the system

The main processing sections of the BDS unit include a Feed Section, Reaction Section, and a Separation Section. The Feed Section consists of a series of storage tanks and feed pumps designed to provide all materials to the system. The feeds to the reactors include the raw gasoline feed, oxygen, and the various substrates and nutrients necessary to sustain the activity of the biocatalyst. Also included in the feed system are unit operations to sterilize feed components as well as a fermentation system to grow fresh biocatalyst for makeup to the Reaction Section. The Reaction System consists of three identical, staged flow, continuously-stirred reactors in which the biodesulfurization reactions occur. The streams from the feed section are combined with an internal recycle stream to feed the first reactor, and the emulsified water/oil/biocatalyst effluent from the third reactor is pumped to the Separation Section. The Separation Section consists of two stages of gravity separation, the first to recover the desulfurized gasoline product and the second to recover the bulk of the water and biocatalyst (plus some entrained oil) for recycle to the reactors. The concentrated oil streams are combined for a treatment in an electrostatic precipitator to produce a product gasoline stream free from water and biocatalyst. A portion of the aqueous product is passed through a filter press to concentrate waste biocatalyst for disposal and water to wastewater treatment.

The biocatalyst-containing sections are operated under aseptic conditions. Aseptic operation requires that all inputs to the system be treated to remove or destroy any foreign organisms. Foreign organisms, if introduced into the system, could inhibit the performance of the biodesulfurization organisms, possibly to the point of complete failure. Sterilization of the inputs to the system is accomplished either through removal of contaminants in cartridge filters or by deactivating (killing) the organisms through heating in continuous heat sterilizers. Where sterilization is by means of filtration, a pre-filter is provided to remove relatively large suspended matter that could plug and thus shorten the life of the more expensive bio-filters. Where heat sterilization is employed, the sterilizers would be provided as a complete packaged system consisting of a heater, holding loop and cooler; heating would be provided by a separate circulating loop of steam-heated water.

Feed Section

Feeds to the BDS Unit include the raw gasoline feed, compressed air, oxygen, process water, and various additives and chemicals.

Gasoline: The gasoline charge to the BDS Unit is expected to come from an FCC unit. For proper operation of the downstream bioreactors, the gasoline charge rate must be relatively uniform, its temperature regulated to the operating temperature of the downstream bioreactors, and it must be sterilized.

Gasoline feed to the unit is received into the Gasoline Feed Drum (V-107). V-107 serves as a surge vessel to dampen fluctuations in the gasoline feed flows, thus ensuring a more uniform feed rate to the downstream bioreactors. The drum provides approximately 30 minutes of residence time at the design gasoline feed rate when operating at a maximum level of 80%.

From the Gasoline Feed Drum, the gasoline feed is transferred to the No. 1 BDS Reactor by the gasoline charge pump (PM-101). Prior to entering the reactor, the temperature of the gasoline is reduced to 35°C in the heat exchanger (HX-101), and sterilized in the gasoline filters (F-101). Cooling within the heat exchanger is provided by cooling water from the refinery cooling water system.

Oxygen: Pure oxygen is supplied to each of the bioreactors from a liquid oxygen supply tank. Liquid oxygen rather than air was chosen as the source of oxygen for the reactors in an effort to minimize gas flow through the system, thus minimizing the stripping of hydrocarbons from the reactors. The higher cost of the pure oxygen is justified by avoiding the high cost to condense hydrocarbons in the bioreactor offgas. The oxygen flow rate to the reactors is controlled to balance with the metabolic demand to maintain the oxygen content in the reactors at or below 2 percent

(below the explosive limit for gasoline). At this concentration, the chemical oxidation of olefins will be minimized (William Heck, personal communication)

Compressed Air: Operation of the Fermentor also requires oxygen. Since there is no gasoline present in the fermentor, it is more economical to supply oxygen in the form of air using an Air Compressor (G-101). The compressed air is cooled to the fermentor operating temperature and then sterilized using air filtration (AF-101). Cooling within G-101 is provided by cooling water from the refinery cooling water system.

Process Water: The majority of the water feed to the bioreactors consists of recycled water. However, a small amount of process water is purged to remove the water-soluble organosulfur product from the BDS system. Make-up for this purge is provided in nutrient solutions to the fermentor, but also possible through the Water Make-up pump (PM-102). Water from PM-102 is sterilized by filtration in the Feed Water Filter (F-102).

Additives and Chemicals: Substrates and nutrients are required to produce and sustain the biocatalyst, and chemicals are required for pH control. Glucose serves as the carbon and energy source, and a minimal nutrient solution is supplied to provide nitrogen, phosphorus and micronutrients. Sodium hydroxide is used for pH control.

Glucose is stored in two Glucose Storage tanks (V-108) and fed to the reactor by the glucose feed pump (DPP-101). V-108 provides 48 hours storage of the 50% glucose solution at the design usage rate. The glucose is sterilized prior to entering the system through heat sterilization (ST-101).

The premixed nutrient cocktail is stored in the Nutrient Storage tank (V-109) and fed to the Fermentor by the nutrient feed pump (DPP-102). The tank is designed to provide 48 hours storage of nutrient solution at the design usage rate. The nutrient feed stream is sterilized using heat sterilization (ST-102) prior to entering the fermentor. Ammonia is added as an additional nitrogen source and for pH control and is stored in tank V-111.

Sodium hydroxide is stored in the storage tank (V-110) and fed to the system by pump (DPP-103). The feed stream is sterilized using heat sterilization (ST-103) prior to entering the system.

Fermentor Section

Fifty percent of the biocatalyst make-up to the system is grown in, and provided from, the Fermentor (V-106). The Fermentor operates in a continuous mode with the rate of cell growth adjusted to meet one-half of the make-up requirements of the BDS system.

Water, air, glucose and nutrients are added to the Fermentor at controlled rates to provide the necessary environment for cell growth, and sodium hydroxide is fed to control the pH. All inputs to the fermentor are sterilized. Stoichiometric quantities of glucose and nutrients are supplied to the fermentor in quantities sufficient to produce 50 percent of the biocatalyst make-up. Excess glucose and nutrients are transferred to the BDS reactors where they will be consumed to produce the remaining 50 percent of the biocatalyst make-up. The Fermentor is operated with a hydraulic residence time of 5 hours.

The Fermentor is operated as a continuously-stirred vessel by means of mechanical agitation. The mixer is sized to provide an applied power level of 4-hp/1000 gal of contained fluid. Cooling water is circulated through the vessel jacket to remove the heat generated from the oxidation of the glucose and to control the Fermentor temperature at 30°C. Biocatalyst make-up to the BDS Reactors, in the form of an aqueous slurry of 30 g/L (dry cell weight), is transferred at a controlled rate by pump GP-101.

Reaction Section

Desulfurization of gasoline occurs in three identical BDS Reactors (V-103, V-104, V-105) with a total hydraulic residence time of approximately 36 minutes at the design flows. The reactors are operated in series flow (optimum configuration based upon reaction kinetics), with raw gasoline, biocatalyst make-up and recycle fed to BDS Reactor No.1. The recycle is predominantly water with as much as 3 wt% entrained gasoline. Fresh make-up water can also be supplied to control the desired volumetric oil-water ratio of 50:50. The glucose and nutrient feed rates are controlled to maintain an operating biocatalyst concentration of 20 g/L (dry cell weight, total volume basis) in the

reactors, assuming a biocatalyst purge rate of two percent per hour. The oil/water/biocatalyst emulsion is transferred from the first to the second reactor by pump PM-106, from the second to the third reactor by pump PM-107, and from the third reactor to the Separation Section by pump PM-108.

The BDS reaction kinetics are assumed to follow Michaelis-Menton kinetics with a maximum conversion rate (V_{max}) of 15 µmole/min of sulfur per gram dry cell weight of active biocatalyst, and a saturation constant (K_M) of 100 ppmw sulfur. The reaction mechanism is assumed to include a single oxidation step to convert the organo-sulfur molecules to a water-soluble form (e.g. the conversion of thiophene to thiophene sulfoxide), requiring a molar ratio of substrates of 10:1:11 (sulfur:glucose:oxygen), allowing for biological inefficiencies. The metabolic requirements of the biocatalyst are estimated at 15 µmole/min of glucose and 100 µmole/min of oxygen per gram dry cell weight of biocatalyst (based on Enchira unpublished observations). Based upon stoichiometric oxygen requirements for sulfur conversion, biocatalyst growth and metabolism, oxygen flow rates will be controlled to keep the oxygen concentration in the reactors well below the explosive limit for gasoline. The biocatalyst activity is assumed to decay exponentially with a half-life of 100 hours, and the effective activity of the biocatalyst is estimated to be 50 percent of maximum due to operation at low oxygen tension (< 2% v/v). The reactors will be operated under growth conditions such that 50 percent of the biocatalyst make-up is grown in the bioreactors.

Each of the bioreactors is equipped with a mixer to provide the necessary agitation of the reactor contents. The mixers are sized to provide an applied power of 4 hp/1000 gal of contained fluid. Chilled water is circulated through the reactor vessel jackets and internal coils to remove the heat of reaction and to control the reactor temperature at the desired 30°C. A small amount of nitrogen is used as a sweep gas in the reactor headspace to remove carbon dioxide from the reactors.

Hydrocarbon emissions from the bioreactors were estimated by the process design software used for the process simulation/design. Under the design operating conditions, the offgas from the reactors is predicted to contain a negligible amount of hydrocarbons (0.07% of feed) that can be flared.

Separation Section

The Separation Section serves three functions: 1) to recover the desulfurized gasoline product, 2) to control the purge of water, and 3) to control the purge of biocatalyst. The Separation Section consists of the 1st and 2nd Stage Decanters, an Electrostatic Dehydrator, and a Filter Press.

1st Stage Decanter: The pumped effluent from BDS Reactor No.3 is discharged to the 1st Stage Decanter (V-101). Phase separation within V-101 occurs exclusively by gravity due to the density difference between gasoline and water. The separator is constructed with an internal overflow weir and effluent section for recovery of the separated light liquid (gasoline) phase. The separator is sized to provide a hydraulic residence time of 5 minutes at an operating level of 80 percent.

The recovered gasoline product from V-101 will contain approximately 2 percent water and biocatalyst that must be removed prior to transfer to storage. The V-101 overflow pump (PM-109) will transfer the gasoline from the effluent compartment to the downstream Electrostatic Dehydrator.

The underflow from V-101, representing 60 percent of the total flow and containing approximately 5 percent oil, is transferred to the 2nd Stage Decanter by transfer pump PM-110.

2nd Stage Decanter: The pumped underflow from the 1st Stage Decanter is discharged to the 2nd Stage Decanter (V-102). Phase separation within V-102 occurs exclusively by gravity due to the density difference between gasoline and water. The separator is constructed with an internal overflow weir and effluent section for recovery of the separated light liquid (gasoline) phase. The separator is sized to provide a hydraulic residence time of 15 minutes at an operating level of 80 percent.

The recovered gasoline product from V-102 will represent approximately 6 percent of the total flow to V-102 and contain approximately 50 percent water and biocatalyst. The V-102 overflow pump (PM-111) will transfer the gasoline from the effluent compartment to the downstream Electrostatic Dehydrator. The operation of V-102 is

designed to ensure that the feed to the Electrostatic Dehydrator contains the recommended water content (5%) for optimal performance.

The underflow from V-102, representing 94 percent of the total flow and containing approximately 3 percent oil, is recycled to BDS Reactor No.1 by pump PM-112.

Electrostatic Dehydrator: The majority of the water and biocatalyst retained in the separated gasoline product is removed in the Electrostatic Dehydrator (ESP-101). Recovery is accomplished by imposing an electrical voltage to the fluid that serves to coalesce the entrained water droplets. The hydrocarbon effluent from ESP-101 is forwarded to salt dryers for removal of the last traces of moisture. Approximately 20 percent of the recovered water and biocatalyst is transferred to the Filter Press by pump PM-114, and 80 percent is recycled to BDS Reactor No.1 by pump PM-113.

Filter Press: A plate and frame Filter Press (PFF-101) will be used to recover waste biocatalyst and hydrocarbon for disposal. Two filters are provided for cyclical operation and are sized to filter for 2 hours before emptying the dried filter cake. The cake will contain approximately 82 percent moisture and small amounts of gasoline. The dried cake (11.6 ton/d) can be used as boiler fuel within the refinery. The water effluent (480 GPH) will contain approximately 50 g/L of organo-sulfur product. At this time it is assumed that the sulfur product would be treated in existing wastewater treatment facilities (WWTP), however, recovery of this product may be required prior to discharging water to WWTP.

Table 11. Design Basis

Parameter	Unit	Comments
Hydrocarbon Feed		
Rate	20,000 BPSD	
Specific Gravity	0.8	
Sulfur Content	350 ppmw	
Temperature	20 – 50 deg C	At battery limit
Pressure	10 – 30 psig	At battery limit
Hydrocarbon Product		
Sulfur Content	35 ppmw	
BDS Reaction Kinetics		
Reaction Model	Michaelis-Menton	
Reaction Parameters		
V_{max}	15 µmole S/min/gDCW	Maximum specific utilization rate
K_M	100 ppmw Sulfur	Oil phase concentration
Biocatalyst Half-life	100 hours	Exponential decay
Biocatalyst Metabolism	100 µmole O_2/min/gDCW	
Heat of Reaction	85 kcal/mole Sulfur	
Metabolic Heat of Reaction	110 kcal/mole O_2	
BDS Reactors		
Type	Continuous-stirred	
Number	3	
Arrangement	Series	Series flow on oil & water, cross-flow on oxygen

		oxygen
Oil-to-water Ratio	1:1	Volumetric basis
Hydraulic Residence Time	36 minutes	Based upon total reactor volume
Biocatalyst Concentration	20 gDCW/L	Total volume basis
Biocatalyst Residence Time	50 hours	
Oxygen Supply Rate		
No.1 Reactor	250 kg/hr	
No.2 Reactor	240 kg/hr	
No.3 Reactor	230 kg/hr	
Operating Conditions		
Temperature	30 deg C	
Pressure	5 psig	
pH	7	
Applied Mixer Horsepower	4 hp/1000 gal	
Fermentor		
Type	Continuous-stirred	
Number	1	
Biocatalyst Concentration	30 gDCW/L	
Residence Time	5 hours	
Biocatalyst Yield	0.45 gDCW/g glucose	
Heat of Reaction	110 kcal/mole O_2	
Operating Conditions		
Temperature	30 deg C	
Pressure	5 psig	
pH	7	

Air Supply Rate	220 scfm	
Applied Mixer Horsepower	4 hp/1000 gal	
1st Stage Decanter		
Feed Rate	1,450 gpm	
Overflow Rate	590 gpm	
Hydraulic Residence Time	5 min	
Overflow Composition		
Gasoline	98.0 wt%	
Water	1.9 wt%	Includes nutrients and sulfur product
Biocatalyst	< 0.1 wt%	Dry cell weight basis
Underflow Composition		
Oil	5.1 wt%	
Water	91.4 wt%	Includes nutrients and sulfur product
Biocatalyst	3.5 wt%	Dry cell weight basis
2nd Stage Decanter		
Feed Rate	860 gpm	
Overflow Rate	52 gpm	
Hydraulic Residence Time	15 min	
Overflow Composition		
Gasoline	51.8 wt%	
Water	46.4 wt%	Includes nutrients and sulfur product
Biocatalyst	1.8 wt%	Dry cell weight basis
Underflow Composition		
Oil	2.7 wt%	

Water	93.7 wt%	Includes nutrients and sulfur product
Biocatalyst	3.6 wt%	Dry cell weight basis
Electrostatic Dehydrator		
Product Gasoline		
Sulfur content	35 ppmw	
Gasoline	99.98 wt%	
Contaminants	0.02 wt%	Water, biocatalyst, sulfur product, nutrients
Filter Press		
Biocatalyst Waste		
Discharge Rate	11.6 ton/day	
Percent Solids	18 wt%	
Water Effluent		
Discharge rate	11,500 GPD	
Sulfur Product	48 g/L	

COST ESTIMATES

The total installed cost (TIC) for a generic 20,000 barrels per day gasoline BDS Unit is estimated to be approximately $18.3 million (including offsites) on a U.S. Gulf Coast basis. The TIC estimate includes a 15 percent contingency and is considered to be of ±25 percent accuracy.

The component breakdowns for the TIC estimates are provided in Table 12. A summary of the base equipment costs is provided in Table 13. A summary of the projected operating costs is presented in Table 14.

Table 12. Estimated TIC Summary

Exchangers	$175,000
Reactors	$1,609,000
Separations Equipment	$657,000
Drums & Tanks	$342,000
Pumps	$390,000
Other Equipment	$249,000
Subtotal Equipment	**$3,422,000**
Installation	$1,889,000
Piping	$851,000
Instrumentation	$1,021,000
Insulation	$102,000
Electrical	$340,000
Civil	$408,000
Total Plant Direct Cost	**$8,033,000**
Engineering	$2,004,000
Construction	$2,805,000
TOTAL PLANT COST	**$12,842,000**
License Fee	$500,000
Contingency	$1,926,000
DIRECT FIXED CAPITAL	**$15,268,000**

Table 13. Equipment Costs

EXCHANGERS		Number	Spares	Size	Cost
HX-101	Diesel Cooling Unit	1	0	30 sq m	$51,000
ST-101	Glucose Sterilizer	1	0		$66,000
ST-102	Nutrient Sterilizer	1	0		$38,000
ST-103	NaOH Sterilizer	1	0		$20,000
REACTORS					
V-103	No.1 BDS Reactor	1	0	76,000 L	$453,000
V-104	No.2 BDS Reactor	1	0	76,000 L	$453,000
V-105	No.3 BDS Reactor	1	0	76,000 L	$453,000
V-106	Fermentor	1	0	6,000 L	$250,000
SEPARATIONS EQUIPMENT					
V-101	1st Stage Decanter	1	0	25,000 L	$128,000
V-102	2nd Stage Decanter	1	0	46,000 L	$177,000
ESP-101	Electrostatic Dehydrator	1	0		$320,000
PFF-101	Plate & Frame Filter	2	0	9 sq m	$32,000
DRUMS & TANKS					
V-107	Diesel Feed Drum	1	0	40,000 L	$64,000
V-108	Glucose Storage	2	0	40,000 L	$156,000
V-109	Nutrient Storage	1	0	35,000 L	$69,000
V-110	NaOH Storage	1	0	11,000 L	$35,000
V-111	Ammonia Storage	1	0	1,000 L	$18,000
PUMPS					
PM-101	Diesel Feed	1	1	7 kW	$14,000
PM-102	Water Make-up	1	1	0.1 kW	$6,000
PM-106	Reactor No.1 Effluent	1	1	18 kW	$46,000
PM-107	Reactor No.2 Effluent	1	1	18 kW	$46,000
PM-108	Reactor No.3 Effluent	1	1	18 kW	$46,000
PM-109	1st Stage Decanter Overflow	1	1	7 kW	$26,000
PM-110	1st Stage Decanter Underflow	1	1	11 kW	$34,000
PM-111	2nd Stage Decanter Overflow	1	1	0.6 kW	$14,000
PM-112	2nd Stage Decanter Underflow	1	1	10 kW	$32,000
PM-113	ESP Discharge	1	1	0.2 kW	$18,000
PM-114	Filter Press Feed	1	1	0.1 kW	$14,000
DPP-101	Glucose Feed	1	1	0.1 kW	$4,000
DPP-102	Nutrient Feed	1	1	0.1 kW	$4,000
DPP-103	Sodium Hydroxide Feed	1	1	0.1 kW	$4,000
GP-101	Fermenter Effluent	1	1	0.1 kW	$18,000
G-101	Fermentor Compressor	1	1	20 kW	$64,000
OTHER EQUIPMENT					
AF-101	Fermentor Air Filter	1	0		$4,000
F-101	Diesel Filter	1	0		$50,000
F-102	Water Filter	1	0		$3,000
	Unlisted Equipment				$192,000

Table 14. Operating Cost Estimate

	Costs	Notes
Direct Fixed Capital (DFC)	$15,268,000	
Offsites	$3,053,600	20% of DFC
Total Capital	$18,321,600	
Variable costs		
Electricity	$270,511	$0.10 per kWh
Steam	$4,453	$10 per 1000 kg
Chilled Water	$1,072,620	$0.20 per 1000 kg
Cooling Water	$31,644	$0.03 per 1000 kg
Glucose	$4,322,894	$0.66 per kg
Oxygen	$570,240	$0.10 per kg
Nutrients	$284,134	
Direct Fixed Costs		
Labor	$332,800	
Supervision	$249,680	
Maintenance	$305,360	2% of DFC
Direct Overhead	$262,116	45% of Labor and Supervision
Allocated Fixed Costs		
General Plant Overhead	$577,096	65% of Labor, Supervision, Maintenance
Ins., Env. & Taxes	$229,020	1.5% of DFC
Depreciation	$1,832,160	10% of Total Capital
Royalties	$1,320,000	$0.20 per barrel
Total Annual Cost	$11,664,728	
$/bbl	$1.77	
c/gal	4.2	
Operating days	330	
Capacity (bbl/d)	20,000	

COST SAVING OPPORTUNITIES

Potential cost saving opportunities regarding the design of the BDS unit are summarized below. These items were identified based upon the high percentage of the overall operating cost as the most lucrative opportunities for process improvement. It should be noted that the cost basis for the process was based on biocatalyst assumptions of Vmax = 15 umoles sulfur per minute per gram dry cell weight biocatalyst; Km = 100 ppmw sulfur; $t_{½}$ = 100 hours; and final sulfur concentration = 35 ppmw. All of these assumptions are based on the identification and development of a suitable enzyme system, but to date a suitable system has not yet been discovered. None of the approaches described in this report have led to measurable rates or significant reductions in sulfur levels in gasoline. Thus, there is no point in discussing the potential cost savings that could be attained by exceeding the assumptions used for any of these biocatalytic parameters. Rather, we will focus on two improvements that could be made to the biocatalyst host, *P. putida* PpG1, that would be independent of the enzyme system used for biodesulfurization.

Operating Temperature: The expense for cooling the reactors using chilled water represents almost 10 percent of the total operating cost for the process. If the biocatalyst could be engineered to operate efficiently at 35°C rather than the design temperature of 30°C, then cooling water could be used to remove heat from the reactors rather than chilled water. At current costs, this would amount to a savings of approximately $0.14 per barrel, or 8 percent of the total operating cost.

Glucose Consumption: The requirement for glucose in the system for growth and energy accounts for over 37 percent of the total operating cost for the process. Of that cost, approximately 25 percent is required to generate biocatalyst and 75 percent is for energy requirements (desulfurization and metabolism). One opportunity to reduce this cost would be to use ethanol as an energy source rather than glucose. On an equivalent cellular energy basis, glucose provides 100 mole of reducing equivalent per dollar and ethanol provides 300 mole of reducing equivalent per dollar. If the *Pseudomonas* biocatalyst could be genetically engineered to use ethanol, the cost savings would be $0.33 per barrel, or 18 percent of the total operating cost.

Competitive Desulfurization Technologies

Five key processing technologies are currently being marketed for the desulfurization of FCC gasoline. The licensors include: UOP (ISAL process), ExxonMobil (Octagain), CD Tech (catalytic distillation), IFP (Prime-G), Phillips Petroleum (S-Zorb).

All but the S-Zorb process are variants to what would be considered "traditional" hydrotreating technology, and involve the selective reduction of organo-sulfur compounds using hydrogen and inorganic catalyst at high temperature and pressure. To minimize saturation of olefins, and thus minimize octane loss, these processes first fractionate the feed into a relatively sulfur-free light stream that contains most of the olefins, and a sulfur-rich heavy stream that is hydrotreated. There is some octane loss associated with these processes, although UOP and ExxonMobil include an isomerization catalyst to restore octane value.

Phillips' S-Zorb process involves the selective absorption of sulfur on a solid absorbent. The absorbent is then regenerated with air (oxygen) and the SO_2-bearing gas effluent is fed to an existing claus unit. There is some product loss and some octane loss using this process, but hydrogen consumption is lower than for the other technologies.

The capital and operating costs for the CD Tech, IFP, and Phillips processes are very competitive. Assuming 10 percent straight line depreciation on capital, $3 per 1000 cu ft hydrogen cost, and $6 per 1000 cu ft natural gas, the cost of desulfurization is estimated in the range of $2 per barrel (assuming a 20,000 BPSD unit). Because of the saturation of olefins and subsequent isomerization, the cost for the UOP and ExxonMobil processes is slightly higher.

All of these processes have either been commercially operated or have full-scale demonstration units currently running.

FINAL ASSESSMENT

The estimated cost for gasoline biodesulfurization is under $2 per barrel and would thus be competitive with the competing chemical technologies if we could achieve the activity and specificity called for in the model. The major disadvantage for gasoline BDS is that the competing technologies have proceeded beyond the research stage and are ready for development, whereas further research is necessary to provide even a prototype gasoline BDS biocatalyst. It is unlikely that we have yet identified an appropriate enzyme system to use for the construction of a prototype biocatalyst. The activity of FmoA, ToeA and DszC are all insufficient to demonstrate significant sulfur reduction in gasoline. Rates would need to be improved by at least two orders of magnitude to meet the cost objective. DszC has the advantage that more is known about gene expression and biochemistry, but we know that the Val261Phe *dsz*C mutant gene on pJA103 is already highly expressed. Similarly, the *fmo*A gene was highly expressed in *P. putida* PpG1, but insignificant desulfurization was observed with this biocatalyst. Thus, any improvements in gasoline BDS rates using these enzyme systems would require major enzymatic modifications. The *toe*A gene, on the other hand, may yet be an acceptable starting point for a prototype biocatalyst, because it may be expressed at low levels in all strains so far constructed. If this is the case, two orders of magnitude improvement in rate could conceivably be obtained simply by manipulating the gene expression levels. Further improvements could also be obtained through the use of directed evolution. Improvements in rate would likely result in improvements in extent as well. If further improvements in the extent of gasoline desulfurization were necessary, again, a program in directed evolution could be instituted.

CONCLUSIONS

The research plan was divided into five main objectives. Following is a list of the objectives and a compilation of major activities and results associated with each:

- Develop a gasoline BDS biocatalyst

A1. Nine strains were identified as being capable of growth with gasoline as sulfur source.
A2. A gene (*fmo*A) was isolated from one of these strains (*Tsukeamurella wratislaviensis* KGB1) based on the conversion of indole to indigo.
A3. The *fmo*A gene was overexpressed in *Escherichia coli and Pseudomonas putida* and shown to support thiophene oxidation, though not growth on gasoline.
A4. The product of thiophene oxidation was shown to be thiophene sulfoxide, which spontaneously dimerizes.
A5. Even at high expression levels, the *fmo*A gene did not support gasoline BDS.

B1. Three strains (*Nocardia farcinica* XJ5, *T. wratislaviensis* 670-1, and *Rhodococcus erythropolis* 9.20.6) demonstrated good sulfur removal during growth on weathered gasoline.
B2. These strains also demonstrated degradation of the hydrocarbon components of weathered gasoline.
B3. Attempts to clone the genes responsible for growth on gasoline by complementation of thiophene knock out mutants failed.
B4. Attempts to clone the genes responsible for growth on gasoline by cloning directly into *P. putida* failed.

C1. A gene (*toe*A) was cloned from *T. wratislaviensis* strains KGB1 and EMT4 into *R. erythropolis* JB55and demonstrated to be responsible for growth on gasoline as sulfur source.
C2. The *toe*A gene was shown to be about 50% homologous to dszA from R. erythropolis IGTS8.
C3. Constructs containing the *toe*A gene were capable of conversion of DBTO2 to HPBS
C4. Constructs containing the *toe*A gene appeared to be capable of removal of C2- and C3-thiophenes during growth on weathered gasoline, but this activity was difficult to demonstrate in a consistent manner.
C5. Attempts to improve activity by including a gene for flavin reductase (*dsz*D or *fre*) failed.
C6. Attempts to achieve expression of toeA in *P. putida* failed.

D1. A mutant *dsz*C gene was isolated that supports growth of *R. erythropolis* on 5-methyl benzothiophene.
D2. This mutant was shown to be due to a change from valine to phenylalanine at position 261 of the DszC enzyme.
D3. This mutant was shown to have enhanced gasoline BDS capability relative to the wild type enzyme, mainly removing C3- and C4-thiophenes and benzothiophene.
D4. The rate of gasoline BDS with *R. erythropolis* carrying this gene was too low to measure.
D5. Mutants with all possible amino acids at position 261 were generated, but none of these were better at gasoline BDS than the phenylalanine mutant.

E1. A library of dszB mutants generated by RACHITT gene shuffling was constructed and preliminary evaluation was conducted.

F1. Sixty-seven bacterial strains were screened for gasoline resistance in plate and shake flask assays.
F2. The best of these, *P. putida*, PpG1, was grown in continuous culture for up to four months in the presence of gasoline.
F3. Growth of PpG1 in the presence of gasoline matched published reports of growth of solvent tolerant strains.
F4. The conditions for growth in the presence of gasoline with minimal losses of volatile hydrocarbons was established for use as a bench scale gasoline BDS reactor.

- Demonstrate lab-scale BDS of gasoline

A1. The reactor and operating conditions for demonstration of gasoline BDS have been worked out.
A2. A recombinant strain of PpG1 carrying the *fmo*A gene was used in a continuous process with gasoline, but no desulfurization was observed.

- Develop a preliminary gasoline BDS process design

A1. A preliminary gasoline BDS process design has been proposed based upon our experience of growth of PpG1 in gasoline bioreactor.
A2. The process employs an up-front fermentor for partial biocatalyst make-up, three stirred tank reactors in series, and separations equipment.
A3. Cell recycle will be used to maximize biocatalyst lifetime and growth in place will be used for partial biocatalyst make-up.
A4. The process will use glucose to support growth of biocatalyst as well as supply reducing equivalents for gasoline BDS.
A5. Oxygen will be supplied as pure O_2 to minimize gasoline volatilization; the dissolved oxygen content in the reactor will be maintained below the explosive window for gasoline.

- Develop a basic gasoline BDS performance model

A1. A gasoline BDS performance model has been constructed based upon a 20,000 BPSD unit with sulfur removal from 350 ppmw to 35 ppmw.
A2. The operating expenses are based upon the use of PpG1 as the biocatalyst host and a single monooxygenase step as the reaction.
A3. The biocatalyst is assumed to have a maximum conversion rate of 15 umoles S per minute per gram dry weight; activity will be lower under process conditions because of operation at low sulfur and oxygen concentrations.
A4. The model assumes a 50:50 oil-to-water ratio, biocatalyst concentration of 20 g/L (dry cell weight in combined liquid phase), and biocatalyst residence time of 50 hours.
A5. Estimation of capital and operating costs lead to a prediction of $1.77 per barrel of gasoline.
A6. Cost saving opportunities were identified.

- Assess technology economics

A1. The proposed gasoline BDS process was compared two five existing chemical technologies.
A3. A process model was developed to show how BDS could be cost competitive with existing technologies.
A3. Development of a biocatalyst with sufficient activity remains a major hurdle to compete with existing technologies.

APPENDIX 1 LIST OF ABBREVIATIONS USED IN THIS REPORT

Abbreviation	Definition
16S-RNA	Species of ribonucleic acid polymer found in all ribosomes
Amp	Ampicillin
ASTM	American Society for Testing and Materials
BDS	Biocatalytic desulfurization; biodesulfurization
BT	Benzothiophene
Cx-	Unspecified degree of alkylation
DBT	Dibenzothiophene
DBTO	Dibenzothiophene sulfoxide
DBTO2	Dibenzothiophene sulfone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOE	Department of Energy
EBC	Enchira Biotechnology Corporation (formerly known as Energy BioSystems Corp.
FAD	Flavin adenine dinucleotide
FCC	Fluid catalytic cracking
HDS	Hydrodesulfurization
*Hind*III	A restriction endonuclease used to cut DNA at a specific sequence
HPBS	Hydroxyphenylbenzene sulfinate
LB	Luria-Bertani broth; a common rich medium used to grow bacteria
N,NDMA	N, N-dimethylaniline
NAD(P)H	Reduced adenine dinucleotide or reduced adenine dinucleotide phosphate
NADH	Reduced adenine dinucleotide
NADPH	Reduced adenine dinucleotide phosphate
NTG	N-methyl-N-nitro-N'-nitrosoguanidine
pBSR	Pressurized batch stirred reactor
ppm	Parts per million
RACHITT	RAndom CHImeragenesis on Transient Template
S	Sulfur
T	Thiophene
WOR	Water-oil ratio

APPENDIX 2. LIST OF BACTERIAL STRAINS DESCRIBED IN THIS REPORT

Bacterial Strain Designation	Organism	Description
670-1	*Tsukemurella wratislaviensis*	Soil isolate capable of growth on biodesulfurized middle distillate
9.20.5	*Gordonia terrae*	Soil isolate capable of growth on 2- and 3-MeT vapors
9.20.6	*Rhodococcus erythropolis*	Soil isolate capable of growth on 2- and 3-MeT vapors
9.27.3	*Rhodococcus erythropolis*	Soil isolate capable of growth on 2- and 3-MeT vapors
A3H1	*Nocardia asteroids*	Soil isolate with *dsz*ABC genes, approsimately 90% identical to *dsz*ABC of IGTS8; capable of diesel BDS
AD109	*Sphingomonas sp.*	Soil isolate with *dsz*ABC genes, approsimately 70% identical to *dsz*ABC of IGTS8; capable of diesel BDS
BD2	*Rhodococcus erythropolis*	Dsz- isolate of *R. erythropolis*
BKO53	*Rhodococcus erythropolis*	Derivative of IGTS8 which has been engineered to knock out *dsz*B and to overexpress *dsz*ACD
C4-224	*Rhodococcus erythropolis*	Mutant of *R. erythropolis* JB55 (pEBC388) isolated from model compound chemostat capable of growth on 5-MeBT
DH10ß	*Escherichia coli*	Used as cloning host
EMT1	*Gordonia rubropertinctus*	Soil isolate capable of growth on 2- and 3-MeT vapors
EMT2	*Rhodococcus coprophilus*	Soil isolate capable of growth on 2- and 3-MeT vapors
EMT4	*Nocardia asteroids*	Soil isolate capable of growth on 2- and 3-MeT vapors
IGTS8	*Rhodococcus erythropolis*	Original diesel BDS strain; carries *dsz*ABCD genes
JB55	*Rhodococcus erythropolis*	Derivative of IGTS8; *dsz*ABC genes deleted
KGB1	*Gordonia terrae*	Bacterial soil isolate capable of growth on T and BT
PpG1	*Pseudomonas putida*	Solvent resistant strain; used as cloning host for gasoline BDS genes
XJ5	*Nocardia farcinica*	Thermophilic soil isolate capable of growth on DBT

APPENDIX 3. LIST OF GENES DESCRIBED IN THIS REPORT

Gene Designation	Origin	Description
*dsz*A	IGTS8	Encodes DBTO2 monooxygenase; part of diesel BDS system
*dsz*B	IGTS8	Encodes HPBS desulfinase; part of diesel BDS system
dszB-JSP-1	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-JSP-2	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-JSP-3	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-JSP-8	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-SS13-1	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-SS13-3	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-SS13-6	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
dszB-SS13-8	PCR from DNA obtained from environmental sample	Sequenced but uncharacterized biochemically
*dsz*C	IGTS8	Encodes DBT monooxygenase; part of diesel BDS system
*dsz*D	IGTS8	Encodes NADH:flavin oxidoreductase; part of diesel BDS system
*fmo*A	KGB1	Encodes flavin containing monooxygenase; gene product oxidizes T
fre	*E. coli*	Encodes NAD(P)H:flavin oxidoreductase; can be used in conjunction with DszA and DszC
*toe*A	KGB1	Encodes monooxygenase that supports growth on gasoline as sulfur source

APPENDIX 4. LIST OF VECTORS DESCRIBED IN THIS REPORT

Vector Designation	Origin	Description
p16R2		Mycobacterium-E. coli shuttle vector
pBAD-TOPO		*E. coli* vector
pEBC701		Shuttle vector between *E. coli* and *Rhodococcus*; contains ΔG promoter
pEBCtac		Expression vector for *fmo*A from KGB1 in E. coli
pEX92	pUC18	Cloning vector used to generate KGB1 library in E. coli; also contains *fre* gene from E. coli which encodes NADH:FMN oxidoreductase
pEXY10		E. coli-Pseudomonas shuttle vector used of final cloning of fmoA for expression in PpG1
pNEB193	pUC19,	Used for subcloning *fmo*A from KGB1

APPENDIX 5. LIST OF PLASMIDS DESCRIBED IN THIS REPORT

Plasmid Designation	Origin	Description
pEBC1100	Constructed at EBC	*dszABCD*, kanamycin resistance
pEBC26K3	pEBC701	Clone from KGB1 gene library complementing JB55 on gasoline
pEBC388	Constructed at EBC	*dszABC*, chloramphenicol resistance
pEBC66E5	pEBC701	Clone from EMT4 gene library complementing JB55 on gasoline
pEBC7K1	pEBC701	Clone from KGB1 gene library complementing JB55 on gasoline
pEBC7K1-1	pEBC701	Sub clone of pEBC7K1, with just toeA, in opposite orientation as ΔG promoter
pEBC7K1-4	pEBC701	Sub clone of pEBC7K1, with just toeA, behind ΔG promoter
pEBC7K1-4D	pEBC701	pEBC7K1-4 with dszD gene behind toeA
pJA101	pEBC388	pEBC388 derivative isolated from chemostat mutant *R. erythropolis* C4-104; contains *dszA1* allele
pJA102	pEBC388	Isolated from chemostat mutant *R. erythropolis* C4-224; contains *dszA2* and *dszC1* alleles
pJA103	pEBC1100	pEBC1100 derivative with wild-type *dszC* allele replaced by *dszC1* allele from pJA102
pYZ101	pNEB193	Subclone *fmo*A gene from pYZ1 on 2.4 kb *Pst*I fragment
pYZ102	pNEB193	Same as pYZ101 with 2.4 kb *Pst*I fragment in opposite orientation
pYZ103	pEBCtac	2.4 kb *Pst*I fragment from pYZ101 transferred to this plasmid for expression of *fmo*A gene in E. coli
pYZW1	pEX92	Clone from KGB1 library with 7.5 kb *Hind*III fragment; contains *fmo*A gene; capable of converting indole to indigo and oxidizing T
pYZW113	pEXY10	2.4 kb *Pst*I fragment from pYZ101 transferred to this plasmid for expression of *fmo*A gene in PpG1

APPENDIX 6. LIST OF GENE LIBRARIES DESCRIBED IN THIS REPORT

Library Designation	**Origin**	**Description**
KGB1 library	KGB1	Genomic library of KGB1 cloned into pEX92
XJ-5 UV mutants	XJ-5	Library of random UV-induced mutants
9.20.6 UV mutants	9.20.6	Library of random UV-induced mutants
670-1 UV mutants	670-1	Library of random UV-induced mutants
XJ-5 genomic library	XJ-5	Genomic library of XJ-5 cloned into p16R2
9.20.6 genomic library	9.20.6	Genomic library of 9.20.6 cloned into p16R2
670-1 genomic library	670-1	Genomic library of 670-1 cloned into p16R2
Codon 261 library	JB55 (pEBC1100)	R. erythropolis with plasmid containing wild-type *dsz*ABD and all possible codons at position 261 of *dsz*C
*dsz*B library 1	Chimeric *dsz*B genes in pBAD-TOPO cloned into *E. coli* DH10B	RACHITT library with IGTS8 *dsz*B as transient template and A3H1 *dsz*B as fragments
*dsz*B library 2	Chimeric *dsz*B genes in pBAD-TOPO cloned into *E. coli* DH10B	RACHITT library with A3H1 *dsz*B as transient template and IGTS8 *dsz*B as fragments

APPENDIX 6. ANALYTICAL METHODS

1. HPLC analysis of radio-labeled T and BT bioconversion products

Radio-labeled thiophene and benzothiophene bioconverstion products were measured by HPLC using a Hewlett-Packard 1050 HPLC system. Detection of labeled compounds was achieved using a Packard Radiomatic FLO-ONE A-500 detector placed in series with the standard uv detector. Ultima Flo AP scintillation fluid at a flow rate of 1.5ml/min was used for the radiomatic detector while the uv detector was set at 207nm. Typically 10ul samples were analyzed.

Separation was achieved using a Hypersil, 2.1x100mm column (5 micron) maintained at 40 °C. The mobile phase consisted of two components, component A (50 mM KH_2PO_4 at pH 3.5) and component B (acetonitrile). An elution gradient starting with 10% B in A, increasing to 70% B in A at a rate of 3%/min, with a final hold of 2 minutes was used. The flow rate was maintained at 0.5ml/minute.

2. GC-SCD

Sulfur compounds in gasoline were analyzed with a Hewlett Packard 5890 Series II plus gas chromatograph (GC) equipped with a Sievers Sulfur Chemiluminescence 350B Detector (SCD). Typically 1ul samples were analyzed.

Separation was achieved using a 30 meter RTX-1column with an I.D. of 0.25mm and a film thickness of 0.50µm. The column oven was held at an initial temperature of 35 °C for four minutes, then ramped to 50 °C at 3 °C/min and then to 300 °C at 10 °C/min, with a hold time at 300 °C of five minutes. Helium with a linear velocity of 25cm/second was used as the carrier gas and the injector and detector was held at 250 °C and 320 °C respectively.

3. GC-MS

Mass spectroscopy (MS) of gasoline samples were performed with Hewlett Packard 5890 Series II plus gas chromatograph (GC) equipped with a Hewlett Packard 5972 mass selective detector (MSD). Typically 1ul samples were analyzed.

Separation was achieved using a 30 meter RTX-1column with an I.D. of 0.25mm and a film thickness of 0.50µm. The column oven was held at an initial temperature of 35 °C for four minutes, then ramped to 50 °C at 3 °C/min and then to 300 °C at 10 °C/min, with a hold time at 300 °C of five minutes. Helium with a linear velocity of 25cm/second was used as the carrier gas and the injector and detector was held at 250 °C and 320 °C respectively.

4. Total sulfur by UV fluorescence (ANTEK)

Total sulfur contents of gasoline samples were determined using an Antek 9000 series Total Sulfur Analyzer. Typically 5ul samples were analyzed in triplicate. These samples are combusted in a high temperature furnace and the sulfur oxides detected by uv-fluorescence.

The combustion furnace was set at 1050°C with the inlet argon, inlet oxygen and pyrolysis oxygen flow rates set at 140ml/min, 25ml/min and 450ml/min respectively. Analysis time per replicate was 3 minutes.

5. DNA Sequence

DNA sequences were obtained using an ABI 377-96 DNA sequencer. Long Ranger 5.0% Singel electrophoresis gels from BMA and the DYEnamic ET terminator system from Amersham were used. The dye terminator reaction was performed using a 3 step PCR cycle consisting of: step 1 = 95°C for 20 seconds, step 2 = 50°C for 15 seconds and, step 3 = 60°C for 60 seconds. This was repeated for 30 cycles. AutoSeq G-50 spin columns from Amersham were used for clean-up of the dye terminator reaction products. DNA sequences were analyzed by ABI PRISM software.

6. Percentage of gasoline by GC-FID

Gasoline was analyzed with a Hewlett Packard 5890 Series II plus gas chromatograph (GC) equipped with a flame ionization detector (FID). Typically 1ul samples were analyzed.

Separation was achieved using a 30 meter RTX-1column with an I.D. of 0.25mm and a film thickness of 0.50µm. The column oven was held at an initial temperature of 35 °C for four minutes, then ramped to 50 °C at 3 °C/min and then to 300 °C at 10 °C/min, with a hold time at 300 °C of five minutes. Helium with a linear velocity of 25cm/second was used as the carrier gas and the injector and detector was held at 250 °C and 320 °C respectively.

7. Glucose

Glucose concentrations were determined by HPLC using Hewlett-Packard 1050 HPLC system equipped with a Hewlett-Packard 1047A refractive index detector with multi channel Interface 35900 Analog Digital Converter. Typically 5ul samples were analyzed.

Separation was achieved using BIORAD Aminex HPX-87H ion exclusion 300mm x 7.8mm column maintained at 40 °C. The mobile phase consisted of 5.25uM H_2SO_4. The flow rate was maintained at 0.6ml/minute.

8. $DBTO_2$ to HPBS and HPBS to HPB

Dibenzothiophene bioconverstion products were measured by HPLC using a Hewlett-Packard 1050 HPLC system. Detection was achieved using HP1050 uv detector set at 235nm. Typically 3ul samples were analyzed.

Separation was achieved using a Zorbax, Narrow-Bore SB-Phenyl, 2.1x150mm (5 micron) column maintained at 40 °C. The mobile phase consisted 400 ml of a 1 M H3PO4 at a pH of 2.28, 2400 ml of nano pure H2O, 2200 ml acetonitrile and 7.586 g of tetraheptylammoniumbromide (THA). Isocratic elution at a flow rate of 0.5ml/minute with a total run time of 11 minutes was used.