FIFTH QUARTERLY TECHNICAL PROGRESS REPORT

on

**MOLECULAR BIOLOGICAL ENHANCEMENT
OF COAL BIODESULFURIZATION**

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**U. S. DEPARTMENT OF ENERGY
PITTSBURGH ENERGY TECHNOLOGY CENTER**

by

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EXECUTIVE SUMMARY

The following work was accomplished during the Fifth Quarterly Report period:

- A BiologTM plate analysis of a fresh culture of strain C18 obtained from Lehigh University showed that this strain did not correspond to any of the other Lehigh strains or to known strains in the Biolog guide
- A comparison of the antibiotic sensitivity profiles of Lehigh strain C18 obtained from the University of North Dakota with the C18 strain used at Battelle showed that both strains were identical
- Thin layer chromatographic analysis of the culture broth and cells of C18 did not demonstrate the presence of 4S pathway compounds
- A study of dibenzothiphenes (DBT) disappearance in C18 cultures as a function of this indicated that DBT sulfone may have been produced as shown by HPLC analysis. Only very low levels of sulfate were detected by ion chromatography after 9 days of incubation of C18 in Stanier's medium plus glycerol
- The C18-pLAFR-3 library did not electroporate into *Pseudomonas putida* ATCC 33015. Further work is underway to solve this problem.
- Seven *Thiobacillus ferrooxidans* strains have been screened for antibiotic susceptibility in ferrous iron (9K) or tetrathionate media. In general, good susceptibility was detected for ampicillin, carbenicillin (on tetrathionate only) and chloramphenicol. Moderate inhibition was observed for spectinomycin (9K and tetrathionate) and for kanamycin and tetracycline (tetrathionate).
- A 20L culturing system for *T. ferrooxidans* is being assembled and additional cultures are being requested from various sources
- Literature and patent searches to evaluate the status of *Thiobacillus* genetics have been conducted and are being analyzed

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- Future work planned for the next quarter includes preparation of purified DNA for electroporation studies, completion of the time course experiments on metabolites produced from DBT by strain C18, and continuation of work on acquisition, screening and characterization of *T. ferrooxidans* strains for suitability in this program.

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INTRODUCTION

The U.S. Department of Energy (DOE) is investigating the microbial-mediated release of sulfur from coal as viable industrial process for coal desulfurization. The overall DOE goal is to develop an optimized microbiological process that is cost-efficient, operates under mild conditions and is simple to engineer.

It would be desirable to have improved strains that would be capable of removing both inorganic and organic sulfur from coal. This project is directed toward achieving this goal.

OBJECTIVE

The objective of this project is to produce one or more microorganisms capable of removing the organic and inorganic sulfur in coal. The specific technical objectives of the project are to:

- Clone and characterize the genes encoding the enzymes of the "4S" pathway (sulfoxide/sulfone/sulfonate/sulfate) for release of organic sulfur from coal
- Return multiple copies of genes to the original host to enhance the biodesulfurization activity of that organism
- Transfer this pathway into a fast-growing chemolithotrophic bacterium
- Conduct a batch-mode optimization/analysis of scale-up variables.

This report presents the results of research at Battelle during the 5th Quarterly Report period beginning on June 15, 1990.

RESULTS FOR THE QUARTER

Task 2. Recovery and Cloning of the Genes of the Sulfoxide/Sulfone/Sulfonate/Sulfate Pathway from Natural Isolates Mutants Thereof

Strain Characterization

Biolog analysis of C18. A new culture of the soil isolate C18 from Lehigh University was screened for carbon source utilization by Biolog™ plate analysis. This analysis was performed previously for the other strains and what was thought to be C18. The results were read on an ELISA reader and the percent utilization relative to the strongest response minus the blank was calculated for each carbon source.

The results of the analysis of C18 grown on both trypticase soy broth (TSB) and Stanier's medium showed that C18 utilized many of the carbon and energy sources to a moderate extent, but few of them really well. The carbon sources that were utilized the best (>60% of the highest value) in the Stanier's plus glycerol culture are shown in Table 1.

Comparison with the guide from Biolog showed that none of the organisms listed corresponded to C18. Also, C18 does not resemble any of our other Lehigh strains, including the earlier culture, a contaminant, which was mistaken for C18.

Comparison of Lehigh strains sent from North Dakota with our strains. Dr. Kevin Young, of the University of North Dakota (UND), sent us samples of the cultures that UND has been using for this project and the Battelle strains were sent to him. A comparison of the antibiotic sensitivity profiles of these strains indicated that they were identical. These strains are being prepared for fluorescence analysis.

**Table 1. UTILIZATION OF CARBON
AND ENERGY SOURCES BY STRAIN C-18
AS DETERMINED BY BIOLOG™ ANALYSIS**

Carbon Source	% Utilization
L-arabinose	79
D-galactose	80
α -D-glucose	72
m-inositol	61
cis-aconitate	95
citric acid	87
D-galacturonic acid lactone	66
D-galacturonic acid	85
D-glucosaminic acid	78
D-glucuronic acid	78
α -ketoglutaric acid	74
D-saccharic acid	100
D,L-lactate	85
L-serine	66
glycerol	77

% Utilization - values reported in Table 1 are expressed in terms of the percent positive reaction in the Biolog™ test relative to the carbon source best utilized (D-saccharic acid).

The spray-plate assay of 4S metabolites using the Lehigh strains. Dibenzo-thiophene (DBT), DBT sulfoxide and DBT sulfone were sprayed onto two-day old cultures of the four Lehigh strains (A4, A15, B24 and C18) on Stanier's plus glycerol plates. Hexane:acetone 3:2 was used as the solvent system to deliver all of the metabolites to the plates. All of the DBT-sprayed cultures showed fluorescence except for C18. The DBT-sulfoxide-sprayed cultures did not show apparent fluorescence. It was difficult to see any fluorescence on the DBT-sulfone-sprayed plates because the fluorescence from the DBT sulfone standard was so extreme. However, Kodama products were only produced on plates sprayed with DBT and not with the other two compounds. Perhaps a liquid follow-up culture would establish whether or not further metabolites could be detected from these compounds by TLC or HPLC. It appears that 4S pathway metabolites are not precursors to Kodama products or they could function as inhibitors of formation of Kodama products.

Thin layer chromatographic (TLC) analysis of 4S standards. The 4th Quarterly Report presented the results of experiments in which the culture broth and cells of C18 were examined for the metabolites by extraction using the method of Kodama et al (1970) and compared to our 4S standard compounds by the TLC analysis method used in our laboratory. Since no compounds were observed which co-chromatographed with the standards (DBT, DBT sulfoxide, DBT sulfone and o,o'-biphenol), it was concluded that either none of these compounds was present, or that the extraction procedure affected the resulting mobility of the standards by altering the charge on the molecules. The latter hypothesis was tested by extracting the 4S standards by the same procedure as used for the culture medium followed by analysis by TLC. These mobilities were then compared with those of the unextracted standards. The results showed that the 4S standards did not change in mobility due to the extraction procedure.

Time point production of DBT metabolites of C18 and A4 in glucose and glycerol medium by TLC analysis. A recent quarterly report from University of North

Dakota indicated that o,o'-biphenol was only produced from cultures containing glucose and not glycerol. Experiments were conducted to confirm this observation. All of our C-18 cultures were grown previously on Stanier's medium plus 0.2% glycerol for subsequent analysis. Consequently, strains A4 and C18 were grown on Stanier's medium plus 0.2% glucose or glycerol with DBT added at 0.005 g/L in the medium. A4 did not accumulate any DBT intermediates which co-migrated with the 4S standards using either glucose or glycerol as a carbon source. C18 grown on glucose in the presence of DBT also did not show any metabolites which corresponded to the 4S metabolites. At the time of this report, TLC analysis shows that C18 cultures grown on glycerol produce a compound which co-migrates with DBT sulfone, even 2.5 h after inoculating the culture into the DBT containing medium. This putative DBT sulfone metabolite does seem to increase over time as well. Further analyses of the samples which appear to accumulate DBT sulfone over time by HPLC/fluorescence are in order to more conclusively identify this intermediate.

Time course point production of DBT metabolites of C18 and A4 in glucose and glycerol media. An experiment was conducted to trace the disappearance of DBT and the appearance of metabolites, including sulfate, in liquid cultures of C18 as a function of time. In this experiment, C18 was grown for three days in Stanier's medium with 0.2% glycerol. The grown cells were then washed twice in 10 mM phosphate buffer (pH 7) with 0.2% glycerol to remove sulfate in the medium prior to the addition of DBT. The washed cells were resuspended in the sulfate-free phosphate buffer with glycerol, and added to tubes containing DBT (100 ppm final concentration). Control cultures lacking DBT were included for comparison. At specified times during the study, duplicate tubes were prepared for analysis.

For the preparation of samples, 1 ml of culture was withdrawn from each tube, centrifuged, and the supernatants stored frozen for sulfate analysis. The pellets were returned to the culture to avoid any loss of particulate DBT. Acetonitrile was then added at 60% final concentration to the remaining cultures, and the samples were vortexed and

then stored in a refrigerator for HPLC analysis. The HPLC method used in analysis of samples was described in the 3rd (March, 1990) Quarterly Report.

Sulfate was monitored by suppression ion chromatography, which has sensitivity in the ppb-ppm range. The system consisted of a 2120i Dionex Ion Chromatograph equipped with a conductivity detector, an AS4 anion exchange column (Dionex), and fiber suppressor. The separations were conducted using an isocratic eluent buffer (3 mM NaHCO_3 + 2.4 mM Na_2CO_3 at 2 ml/min). Preliminary tests showed that phosphate buffers (pH 7, 10-20 mM) would not interfere with the sulfate analysis (see Figure 1).

Production of colored products was not evident after 24 hours of incubation of C18 with DBT in the phosphate-glycerol medium. It had been consistently observed that colored material (presumably Kodama intermediates) appeared during the degradation of DBT by the Lehigh strains in more complete media. Thus, it appeared likely that some cofactors (i.e., metals) may be required in the medium for DBT degradation. A sulfate-free trace metals solution for denitrifying organisms (Table 2) was then added to all culture tubes (at 24 hours). The production of colored material (the culture became brownish-red) was evident in the tubes containing DBT after overnight incubation, indicating that the DBT was probably being metabolized and that certain metals are likely involved in a Kodama-like DBT degradation pathway. It has not been determined if this

Figure 1. NON-INTERFERENCE BETWEEN PHOSPHATE AND SULFATE IN ION CHROMATOGRAPHY

**Table 2. SULFATE-FREE TRACE METALS SOLUTIONS
FOR DBT DEGRADATION EXPERIMENT**

Solution A		Solution B	
NH ₄ Cl	53.45 g	MnCl ₂ · 4H ₂ O	0.5 g
CaCl ₂ · 2H ₂ O	1.5 g	H ₃ BO ₃	0.05 g
MgCl ₂	0.4 g	ZnCl ₂	0.05 g
FeCl ₂ · 4H ₂ O	0.4 g	CuCl ₂ · 2H ₂ O	0.03 g
H ₂ O ^(a)	1 liter	NaMoO ₄ · 2H ₂ O	0.01 g
		CoCl ₂ · 6H ₂ O	0.5 g
		Na ₂ S ₂ O ₃	0.05 g
		NiCl ₂ · 6H ₂ O	0.05 g
		H ₂ O ^(a)	1 liter

(a) deionized water

is the case for 4S metabolism. The colored compounds disappeared after extended incubation (5 days).

Very low levels of sulfate (0.4 ppm or less) were seen in all of the samples over the 9 day incubation period (Figure 2). It was unclear if there was a significant difference between sample sulfate levels in cultures with or without DBT. Only a small amount of DBT (approximately 5 ppm) was degraded in this experiment, which could account for the low sulfate levels observed. If all of the DBT (100 ppm) were degraded with the stoichiometric release of sulfate, 51 ppm sulfate would have been expected.

HPLC analysis of the samples suggested that DBT sulfone was produced by C18 during DBT degradation. As shown in Figure 3, a sample (217 h incubation) which showed a putative DBT sulfone peak was spiked with a DBT sulfone standard; this resulted in a co-eluting peak. Fluorescence detection was employed to further analyze the samples. The detector (Kratos Spectroflow 980 Programmable Fluorescence Detector) was set at 254 nm excitation, with a 418 nm cutoff emission filter. One fluorescing compound that eluted near or at the point of DBT sulfoxide was observed in both DBT spiked and control samples at 217 hours (Figure 4). The putative sulfone peak seen by UV detection in DBT amended samples at 217 hours was also observed by fluorescence detection. An additional fluorescing compound was observed in DBT spiked samples which eluted between DBT sulfoxide and DBT sulfone. This compound may be of importance relative to the plate screening assay since it may be responsible along with other compounds for the characteristic fluorescence. For comparative purposes, Figure 5 presents an overlay of the 4S compound standards (UV detection) and a 217 hour DBT+ culture sample chromatogram generated using fluorescence detection. The standards chromatogram is slightly offset to the left (by a few seconds) since the sample passed through the UV detector before the fluorescence detector.

Figure 2. RELEASE OF SULFATE DURING THE INCUBATION OF C18 WITH DBT

Figure 3. EVIDENCE FOR THE PRESENCE OF DBT SULFONE IN DBT-SPIKED C18 CULTURES

Figure 4. HPLC CHROMATOGRAMS OF C18 SUPERNATANTS WITH AND WITHOUT DBT ADDITION, GENERATED USING FLUORESCENCE DETECTION

Figure 5. OVERLAY OF 4S COMPOUND STANDARDS (UV DETECTION) AND AND C18 SUPERNATANTS (FLUORESCENCE DETECTION) AFTER INCUBATION WITH DBT

Two unidentified peaks were observed in ion chromatographs of C18 culture supernatants which eluted after sulfate, at 7.8 and 10.6 minutes; the peak at 10.6 minutes (compound No. 2) appeared only in cultures with DBT at 5-9 days, when the colored compounds disappeared (Figure 6). Compound No. 2 is likely a low molecular weight, water soluble anionic product of DBT degradation. Methods such as Inductively Coupled Plasma Emission Spectrometry (ICP) would be useful to determine if this compound contains sulfur.

Another C18 time-course experiment is in progress, in which the growth medium contained a reduced level (75 μ M, or 10 ppm) of sulfate and a normal level of trace metals. The level of sulfur is expected to result in near or complete exhaustion of exogenous sulfur during the late phase of growth. DBT was added to the cultures after several days of growth. These conditions may result in the higher conversion of DBT. Interestingly, no formation of colored products was observed during incubation of this culture with DBT. The analytical results of this experiment are pending.

C18 library manipulations. The C18-pLAFR3 library was prepared for electroporation into *Pseudomonas putida* ATCC 33015. After several attempts to electroporate the library into *Pseudomonas*, it was noticed that clones were not obtained from the library. We are now planning to isolate CsCl gradient density centrifugation-purified DNA of optimal quality for electroporation to attempt to solve this problem. Electroporation will be needed to move cloned DNA (such as the UND DNA segment/vector system) into *Pseudomonas* subsequently in the modified Task 3 of this program.

The C18-pLAFR3 library was screened for tryptophan conversion to indole. No blue colonies were observed in the DH5 α MCR library when the plates were sprayed with tryptophan on Luria broth medium. This experiment was performed because the UND group reported that their indole-forming clones were also fluorescent when subcloned into *Pseudomonas*.

**Figure 6. ION CHROMATOGRAM OF A C18 SUPERNATANT
AFTER INCUBATION WITH DBT. PEAK NO. 2
AT 10.6 MINUTES APPEARED ONLY IN CULTURES WITH DBT.**

Task 4. Expression in Chemolithotrophs

Culturing of *Thiobacillus ferrooxidans*

The Ohio State University project team is presently culturing several strains of *Thiobacillus ferrooxidans* and purifying them from single colonies from both iron and tetrathionate media. Also, *T. ferrooxidans* colonies are being selected with copper sulfate (approximately 1 g/L in the 9K medium). The usual co-contaminants of *T. ferrooxidans* will not tolerate high concentrations of copper sulfate, although *T. ferrooxidans* is quite resistant to copper toxicity.

Antibiotic Sensitivity Screening of *Thiobacillus ferrooxidans* Strains

The seven strains of *T. ferrooxidans* (shown in Tables 3 and 4) were screened for susceptibility to several antibiotics. This screen consisted of a disk test; antibiotics of a known concentration were added in a constant volume to all the disks and the disks placed in the center of a freshly inoculated pour plate. The growth medium used was ferrous iron (9K) or tetrathionate. Antibiotic susceptibility was demonstrated by a zone of clearing around the disk after the plate cultures were incubated at 30°C for a week.

In general, good antibiotic susceptibility was detected for ampicillin, carbenicillin (on tetrathionate only) and chloramphenicol. Moderate growth inhibition was obtained for spectinomycin (9K and tetrathionate) and for kanamycin and tetracycline on the tetrathionate medium. Antibiotics which did not seem to be appropriate for these strains were gentamicin and neomycin on both media. Strain TFI-35 does not grow on solid media very well; the growth detected on tetrathionate may be due to a contaminant.

Table 3. GROWTH INHIBITION OF *THIOBACILLUS FERROOXIDANS* STRAINS BY ANTIBIOTICS USING THE DISK TEST IN 9K SOLID MEDIUM

Antibiotic	TFI Strain						
	29	35	70	85	90	91	92
	No. = Diameter of Inhibition Zone in mm						
Ampicillin (amp)	73	ng	nd	nd	nd	60	nd
carbenicillin (carb)	10	ng	13	33	ng	12	22
gentimycin (gent)	tr	ng	11	8	-	9	11
tetracycline (tet)	8	ng	7	ng	8	10	20
chloramphenicol (cmp)	47	ng	20	40	ng	47	ng
neomycin (neo)	-	ng	-	-	-	-	-
spectinomycin (spec)	41	ng	23	34	32	29	44
kanamycin (kan)	12	ng	ng	10	24	8	20
ethanol	-	ng	-	-	-	-	-

ng = no growth detected

nd = no data reported

- = no growth inhibition zone

Antibiotic applied to each disk was amp =, 360 µg; carb=, 360 µg; gent=, 300 µg; tet=, 210 µg (ethanol); cmp=, 510 µg (ethanol); neo=, 300 µg; spec=, 750 µg and kan=, 360 µg. Antibiotics were suspended in water unless otherwise noted.

Table 4. GROWTH INHIBITION OF *T. FERROOXIDANS* STRAINS BY ANTIBIOTICS USING THE DISK TEST IN TETRATHIONATE SOLID MEDIUM

	TFI Strain						
	29	35	70	85	90	91	92
Antibiotic	No. = Diameter of Inhibition Zone in mm						
ampicillin	nd	60	58	ng	nd	65	ng
carbenicillin	-	28	nd	30	35	40	30
gentamicin	ng	20	16	24	22	20	26
tetracycline	25	32	26	26	ng	ng	30
chloramphenicol	40	ng	50	50	52	ng	0
neomycin	-	-	-	-	-	-	-
spectinomycin	19	nd	25	25	22	26	30
kanamycin	nd	30	20	28	30	20	23

ng = no growth

nd = no data

- = no inhibition zone

Antibiotics applied to each disk was the same as reported in Table 3.

The rescreening of cultures giving responses, of either substantial inhibition or resistance, will be repeated.

Acquisition of 20 L Culturing Apparatus for *T. ferrooxidans*

The materials for growing moderate scale (20 L) cultures of *T. ferrooxidans* were ordered. This system will consist of a polycarbonate carboy with a ported closure to allow the introduction of sterile air and venting of spent air from the vessel. A membrane filter system will be used to separate the cells.

Thiobacillus Strain Acquisition

We are requesting strains which contain plasmids or genes of interest from various sources including the *T. ferrooxidans* rusticyanin mutant from Martek and the shuttle plasmids pTIF1 and pTFCAT, and the pTFCd8 and pTFCd10 clones from the *T. ferrooxidans* genomic library at Lawrence Livermore National Laboratory.

Literature and Patent Searches

Literature and patent searches were conducted to evaluate the status of *T. ferrooxidans* genetics and also to scan the literature for information about sulfonate and sulfone microbial degradation. Useful results were obtained from all the searches and this information will be analyzed for information on possible *T. ferrooxidans* vectors.

FUTURE WORK

The following work is planned during the next (6th) quarter:

- (1) Completion of Task 2 work which supports the revised workplan, including preparation of purified DNA for electroporation into the C18-pLAFR3 library and the time-course experiment on DBT degradation by C18
- (2) Initiate work on Task 3 -- Expression in *Pseudomonas*
- (3) Continue work on Task 4, including:
 - (a) Plasmid screening of the *T. ferrooxidans* cultures.
 - (b) Metal ion resistance and DBT metabolite screening of the *T. ferrooxidans* cultures on solid media
 - (c) *Thiobacillus* strain acquisition
 - (d) Setup of 20 L *T. ferrooxidans* culturing vessel.

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