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THIRD QUARTERLY TECHNICAL PROGRESS REPORT

on

**MOLECULAR BIOLOGICAL ENHANCEMENT
OF COAL BIODESULFURIZATION**

Contract No. DE-AC-22-89PC89902

to

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

by

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March 15, 1990

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EXECUTIVE SUMMARY

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

- The Lehigh University strains A4, A15, B24 and C18 were characterized biochemically using Biolog™ microtiter plates containing 95 different potential carbon sources. The results showed that strains A4, A15 and B24 resembled closely the *Pseudomonas putida* ATCC 33015 control strain, but C18 was distinctly different. The strains were also screened for antibiotic resistance and all strains were sensitive to tetracycline at a concentration of 20 µg/ml.
- The Krawiec ultraviolet fluorescence plate assay system was evaluated and adapted for use in this project. The limit of detection of o,o'-biphenol on an agar plate was determined to be between 0.05 to 0.5 µg in a 1 cm spot. A single positive colony could be detected easily in a field of 100 negative colonies. Conversely, a single negative colony could be visualized in a field of 100 positive colonies with this assay.
- The ARCTECH HPLC analytical method was adapted for use in detecting 4-S pathway compounds for the purposes of this project. This HPLC method will be used to further characterize selected recombinants identified by the fluorescence plate assay with respect to 4S pathway activity.
- Excellent transformation of *P. putida* ATCC 33015 plasmid DNA was achieved by electroporation with the broad host range cosmid vector pLAFR3.
- Electrophoretic analysis indicated that plasmids were present in Lehigh strains A15, B24 and C18. No plasmid DNA was detected in the A4 strain.
- The *P. putida* ATCC 33015 pLAFR3 DNA was restricted only by the restriction endonucleases *Pst* I and *Hind* III. Further investigations on determination of which restriction enzymes will cut the DNA of the host strain as well as the Lehigh strains are in progress.

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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.

**RESULTS FOR THE QUARTER -- TASK 2. RECOVERY AND CLONING
OF THE GENES OF THE SULFOXIDE/SULFONE/SULFONATE/SULFATE
PATHWAY FROM NATURAL ISOLATES OF MUTANTS THEREOF**

Strain Characterization

The four strains currently being used on this program are strains A4, A15, B24, and C18 (received from Dr. Steven Krawiec of Lehigh University). All of these strains were reported to show the capacity to degrade dibenzothiophene (DBT) by Krawiec's plate assay. The strain Pseudomonas putida (ATCC 33015) was obtained for use as a control. All of the strains were Gram negative as expected. The strains were tested for tetracycline resistance; all appeared to be sensitive to 20 ug/ml tetracycline.

Biolog™ (Biolog, Inc., 3447 Investment Blvd., Suite 3, Hayward, CA, 94545) plates were used to characterize the metabolic capability of the strains. Biolog™ GN plates are microtiter dishes containing 95 different potential carbon sources (Figure 1). When bacteria are added to the dish, the oxidation of the substrate carbon source leads to a redox change which is indicated by a color change in the well. This test, therefore, identifies the carbon substrates that a strain can utilize. Of course the conditions used to grow the inoculum for the test can affect the outcome so standard conditions must be established. The results of the Biolog™ tests can be used as a metabolic fingerprint of the strains. Major metabolic changes in the strains will be obvious on the plates. Periodically, this test will be repeated to allow us to monitor the strains for metabolic changes over time. This test will be particularly useful during the Strain Stability tests in Task 3 of this project.

It is possible to design plates with alternative carbon sources. It may be useful to develop plates to examine specific degradative capabilities of the desulfurizing strains.

Figure 1

A1 water	A2 α -cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D- galactosamine	A8 N-acetyl-D- glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 erythritol	B2 D-fructose	B3 L-fructose	B4 D-galactose	B5 gentiobiose	B6 α -D-glucose	B7 m-inositol	B8 α -lactose	B9 lactulose	B10 meliose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β -methyl glucoside	C3 psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turannose	C10 xyitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 cis-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D- glucosaminic acid	D9 D-glucuronic acid	D10 α -hydroxy butyric acid	D11 β -hydroxy butyric acid	D12 γ -hydroxy butyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α -keto butyric acid	E4 α -keto glutamic acid	E5 α -keto valeric acid	E6 D,L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl- glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl- L-aspartic acid	F12 glycyl- L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenyl alanine	G6 L-proline	G7 L-pyro- glutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D,L-carnitine	G12 γ -amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3- butanediol	H9 glycerol	H10 D,L- α - glycerol phosphate	H11 glucose-1- phosphate	H12 glucose-6- phosphate

Figure 1. GN MicroPlate™

The results of this test are shown in Figures 2 to 6. It is fairly obvious that strains A4, A15, and B24 closely resemble the *P. putida* control. It is very apparent that strain C18 is distinctly different from the other strains. At this time we can not speculate from these results as to what genus or species of bacteria C18 resembles. In the near future we plan to obtain the Biolog™ data base software system which may allow us to determine the genera to which C18 is most closely related.

We have obtained a mixed population of bacteria (ATCC 39327) which is reported to desulfurize coal (US patent 4,659,670). This population will be examined in the desulfurization assays and the results will be reported in subsequent reports.

Analytical Methods Development for 4S Pathway Activity

In order to achieve the primary objectives of Task 2, characterization of the recombinant strains in terms of 4S pathway activity is necessary. In this pathway, dibenzothiophene (DBT) is thought to be converted through a series of single oxidation steps to DBT sulfoxide, DBT sulfone, DBT sulfonate, and finally to o,o'-biphenol, with the release of sulfate. A method to detect organisms having a functional 4S pathway, based on detection of the end-product, o,o'-biphenol, would be particularly useful in the screening of a large number of recombinants. An analytical technique for identification of the 4S intermediates and/or end products could provide for additional, detailed information about the enzymatic steps that are functional in the recombinants, and would be necessary in the determination of the cellular location of the pathway enzymes. During this Quarter, 4S compounds were procured from chemical suppliers, a screening method based on the fluorescence of o,o'-biphenol was developed, and an HPLC system was set up and successfully tested for the detection of 4S intermediates. In addition, characterization of the microbial strains was initiated based on this work.

4S Pathway Compounds

Dibenzothiophene was purchased from Aldrich Chemical Co., Eastman Kodak, and Lancaster Synthesis. Dibenzothiophene sulfoxide was purchased from K&K Laboratories. Dibenzothiophene sulfone was purchased from Aldrich Chemical Co. The compound o,o'-biphenol was purchased from Aldrich Chemical Co., Lancaster

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A4-A

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
water	α -cyclodestin	destin	glycogen	tween 40	tween 80	N acetyl D- galactosamine	N acetyl D- glucosamine	adonitol	L-arabinose	D-arabitol	collibiose
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
i-erythritol	D-fructose	L-fructose	D-galactose	gentiobiose	α D-glucose	m-inositol	α -lactose	lactulose	mallose	D-mannitol	D-mannose
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D-maltose	β -methyl glucoside	palatose	D-raffinose	L-rhamnose	D-sorbitol	sucrose	D-trehalose	tanarose	xylyl	methyl pyruvate	mono-methyl succinate
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
acetic acid	dis-acetic acid	citric acid	formic acid	D-galactonic acid lactone	D-galacturonic acid	D-gluconic acid	D- glucosaminic acid	D-glucuronic acid	α -hydroxy butyric acid	β -hydroxy butyric acid	γ -hydroxy butyric acid
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
p-hydroxy phenylacetic acid	itaconic acid	α -keto butyric acid	α -keto glutamic acid	α -keto valeric acid	D-lactic acid	malonic acid	propionic acid	quinic acid	D-succinic acid	sebacic acid	succinic acid
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
bromo succinic acid	succinamic acid	glucuronamide	alaninamide	D-alanine	L-alanine	L-alanyl- glycine	L-asparagine	L-aspartic acid	L-glutamic acid	glycyl- L-aspartic acid	glycyl- L-glutamic acid
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
L-histidine	hydroxy L-proline	L-leucine	L-ornithine	L-phenyl alanine	L-proline	L-pyro glutamic acid	D-serine	L-serine	L-threonine	D,L-carnitine	γ -amino butyric acid
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
urocanic acid	inosine	uridine	thymidine	phenyl ethylamine	putrescine	2-amino ethanol	2,3- butanediol	glycerol	D,L- α - glycerol phosphate	glucose-1- phosphate	glucose-6- phosphate

(a) + = positive

++ = strong positive

Figure 2. Strain A4 Reactions on Biolog™ Plates

A15-a 12-19-89

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
water	α -cyclobutanol	desferal	glycogen	weon 40	weon 80	N-acetyl-D-galactosamine	N-acetyl-D-glucosamine	adonitol	L-arabinose	D-arabitol	colobiose
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
i-arabitol	D-fructose	L-glucose	D-galactose	gentiobiose	α -D-glucose	m-inositol	α -lactose	lactulose	maltose	D-mannitol	D-mannose
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D-maltobiose	β -methyl glucoside	pektose	D-raffinose	L-rhamnose	D-sorbitol	sucrose	D-trehalose	uranose	xylyl	methyl pyruvate	mono-methyl succinate
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
acetic acid	dis-succinic acid	citric acid	formic acid	D-galactonic acid lactone	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid	D-glucuronic acid	α -hydroxy butyric acid	β -hydroxy butyric acid	γ -hydroxy butyric acid
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
p-hydroxy phenylacetic acid	isocaproic acid	α -keto butyric acid	α -keto glutamic acid	α -keto valeric acid	D-l-lactic acid	malonic acid	propionic acid	quinic acid	D-pyrocacetic acid	sebacic acid	succinic acid
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
bromo succinic acid	succinamic acid	glucuronamide	alaninamide	D-alanine	L-alanine	L-alanyl-glycine	L-asparagine	L-aspartic acid	L-glutamic acid	glycyl-L-aspartic acid	glycyl-L-glutamic acid
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
L-histidine	hydroxy L-proline	L-leucine	L-ornithine	L-phenyl alanine	L-proline	L-pyrro-glutamic acid	D-serine	L-serine	L-threonine	D-L-carnitine	γ -amino butyric acid
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
urocanic acid	inosine	uridine	thymidine	phenyl ethylamine	putrescine	2-amino ethanol	2,3-butanediol	glycerol	D,L- α -glycerol phosphate	glucose-1-phosphate	glucose-6-phosphate

Figure 3. Strain A15 Reactions on Biolog™ Plates

B24-A

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A1	water	A2	α-cyclodextrin	A3	desferal	A4	glycogen	A5	wean 40	A6	wean 80	A7	N-acetyl D-glucosamine	A8	N-acetyl D-glucosamine	A9	adonitol	A10	L-arabinose	A11	D-arabitol	A12	cellobiose
B1	erythritol	B2	D-fructose	B3	L-fucose	B4	D-galactose	B5	gentiobiose	B6	α-D-glucose	B7	m-inositol	B8	α-lactose	B9	lactulose	B10	maltose	B11	D-mannitol	B12	D-mannose
C1	D-melibiose	C2	β-methyl glucoside	C3	pelucose	C4	D-raffinose	C5	L-rhamnose	C6	D-sorbitol	C7	sucrose	C8	D-trehalose	C9	turanose	C10	xylyl	C11	methyl pyruvate	C12	mono-methyl succinate
D1	acetic acid	D2	dis-acetic acid	D3	citric acid	D4	formic acid	D5	D-galactonic acid lactone	D6	D-galacturonic acid	D7	D-gluconic acid	D8	D-glucosaminic acid	D9	D-glucuronic acid	D10	α-hydroxy butyric acid	D11	β-hydroxy butyric acid	D12	γ-hydroxy butyric acid
E1	phosphoryl phenylacetic acid	E2	itaconic acid	E3	α-keto butyric acid	E4	α-keto glutaric acid	E5	α-keto valeric acid	E6	D,L-lactic acid	E7	malonic acid	E8	propionic acid	E9	quinic acid	E10	D-succinic acid	E11	sebacic acid	E12	succinic acid
F1	trans-succinic acid	F2	succinamic acid	F3	glucuronamide	F4	alaninamide	F5	D-alanine	F6	L-alanine	F7	L-alanyl-glycine	F8	L-asparagine	F9	L-aspartic acid	F10	L-glutamic acid	F11	glycyl-L-aspartic acid	F12	glycyl-L-glutamic acid
G1	L-histidine	G2	hydroxy L-proline	G3	L-leucine	G4	L-ornithine	G5	L-phenyl alanine	G6	L-proline	G7	L-pyro glutamic acid	G8	D-serine	G9	L-serine	G10	L-threonine	G11	D,L-carnitine	G12	γ-amino butyric acid
H1	uracanic acid	H2	inosine	H3	uridine	H4	thymidine	H5	phenyl ethylamine	H6	putrescine	H7	2-amino ethanol	H8	2,3-butanediol	H9	glycerol	H10	D,L-α-glycerol phosphate	H11	glucose-1-phosphate	H12	glucose-6-phosphate

Figure 4. Strain B24 Reactions on Biolog™ Plates

C18-A 12-19-89

A1	water	A2	α -cyclodextrin	A3	dextrin	A4	glycogen	A5	Tween 40	A6	Tween 80	A7	N-acetyl-D-glucosamine	A8	N-acetyl-D-glucosamine	A9	adonitol	A10	L-arabinose	A11	D-arabitol	A12	cellobiose
B1	erythritol	B2	D-fucose	B3	L-fucose	B4	D-galactose	B5	gentiobiose	B6	α -D-glucose	B7	m-inositol	B8	α -lactose	B9	lactulose	B10	mallose	B11	D-mannitol	B12	D-mannose
C1	D-melibiose	C2	β -methyl glucoside	C3	palcose	C4	D-raffinose	C5	L-rhamnose	C6	D-sorbitol	C7	sucrose	C8	D-trehalose	C9	turannose	C10	xyliol	C11	methyl pyruvate	C12	mono-methyl succinate
D1	acetic acid	D2	dis-acetic acid	D3	citric acid	D4	lactic acid	D5	D-galactonic acid lactone	D6	D-galacturonic acid	D7	D-gluconic acid	D8	D-glucosaminic acid	D9	D-glucuronic acid	D10	α -hydroxy butyric acid	D11	β -hydroxy butyric acid	D12	γ -hydroxy butyric acid
E1	p-hydroxy phenylacetic acid	E2	itaconic acid	E3	α -keto butyric acid	E4	α -keto glutaric acid	E5	α -keto valeric acid	E6	D,L-lactic acid	E7	malonic acid	E8	propionic acid	E9	quinic acid	E10	D-succinic acid	E11	sebacic acid	E12	succinic acid
F1	bromo succinic acid	F2	succinamic acid	F3	glucuronamide	F4	alaninamide	F5	D-alanine	F6	L-alanine	F7	L-alanyl-glycine	F8	L-asparagine	F9	L-aspartic acid	F10	L-glutamic acid	F11	glycyl-L-aspartic acid	F12	glycyl-L-glutamic acid
G1	L-histidine	G2	hydroxy L-proline	G3	L-leucine	G4	L-ornithine	G5	L-phenyl alanine	G6	L-proline	G7	L-pyroglutamic acid	G8	D-serine	G9	L-serine	G10	L-threonine	G11	D,L-carnitine	G12	γ -amino butyric acid
H1	urocanic acid	H2	inosine	H3	uridine	H4	thymidine	H5	phenyl ethylamine	H6	putrescine	H7	2-amino ethanol	H8	2,3-butanediol	H9	glycerol	H10	D,L- α -glycerol phosphate	H11	glucose-1-phosphate	H12	glucose-6-phosphate

Figure 5. Strain C18 Reactions on Biolog™ Plates

33015-A

12-19-89

A1	water	A2	α -cyclohexanone	A3	glycerol	A4	glycerol	A5	weon 80	A6	N-acetyl D-glucosamine	A7	N-acetyl D-glucosamine	A8	adenitol	A9	L-arabinose	A10	D-arabitol	A12	cellobiose
B1	D-erythritol	B2	D-fructose	B3	D-fructose	B4	D-galactose	B5	D-glucose	B6	D-glucose	B7	D-glucose	B8	D-lactose	B9	D-mannitol	B10	D-mannitol	B12	D-mannose
C1	D-maltose	C2	β -methyl glucoside	C3	glucose	C4	D-rhamnose	C5	D-sorbitol	C6	D-sorbitol	C7	sucrose	C8	D-xelabose	C9	xyliol	C10	methyl pyruvate	C12	mono methyl succinate
D1	acetic acid	D2	dis-succinic acid	D3	citric acid	D4	formic acid	D5	D-galacturonic acid	D6	D-galacturonic acid	D7	D-glucuronic acid	D8	D-glucuronic acid	D9	D-glucuronic acid	D10	β -hydroxy butyric acid	D12	γ -hydroxy butyric acid
E1	p-hydroxy phenylacetic acid	E2	itaconic acid	E3	α -keto butyric acid	E4	α -keto glutaric acid	E5	α -keto valeric acid	E6	D,L-lactic acid	E7	malonic acid	E8	propionic acid	E9	quinic acid	E10	D-succinic acid	E12	succinic acid
F1	bromo succinic acid	F2	succinic acid	F3	glutaronamide	F4	alaninamide	F5	D-alanine	F6	L-alanine	F7	L-alanyl-glycine	F8	L-asparagine	F9	L-aspartic acid	F10	L-glutamic acid	F12	glycyl-L-glutamic acid
G1	L-histidine	G2	hydroxy L-proline	G3	L-leucine	G4	L-ornithine	G5	L-phenyl alanine	G6	L-proline	G7	L-pyro glutamic acid	G8	D-serine	G9	L-serine	G10	L-threonine	G12	γ -amino butyric acid
H1	urocanic acid	H2	histidine	H3	uridine	H4	thymidine	H5	phenyl ethylamine	H6	putrescine	H7	2-amino ethanol	H8	2,3-butanediol	H9	glycerol	H10	D,L- α -glycerol phosphate	H12	glucose-6-phosphate

Figure 6. *Pseudomonas putida* ATCC 33015 reactions on Biolog TM Plates

Synthesis, and Sigma Chemical Co. It should be noted that o,o' biphenol is listed by suppliers as 2,2' dihydroxybiphenyl (Lancaster Synthesis), 2,2' biphenol (Aldrich), and o,o'-biphenol (2,2' dihydroxybiphenol) (Sigma). We will use the nomenclature o,o'- biphenol in this and subsequent reports. Dibenzothiophene sulfonate was not commercially available. Each chemical, except for DBT sulfoxide, yielded a single peak on HPLC chromatograms (see subsequent section), indicating that the compounds were of high purity. DBT sulfoxide, however, was grossly contaminated with DBT sulfone.

There are no known suppliers of highly pure DBT sulfoxide. A method for purification of this compound was obtained from Carroll Collier, Senior Chemist of ARCTECH, Inc., Alexandria, VA. This chromatographic method is based on the selective elution of DBT sulfone followed by elution of the sulfoxide. Using ARCTECH's procedure as a guideline, Battelle will attempt to purify gram quantities of DBT sulfoxide when this compound is needed for pathway analysis of recombinant organisms.

Development of a Recombinant Screening Method

A screening assay was developed by Dr. Steven Krawiec at Lehigh University for the presumptive detection o,o'-biphenol on solid cultures. Based on this assay, a method was developed for the screening of recombinants for DBT degradation to o,o'-biphenol. Following the screening procedure, a more definitive HPLC assay (see subsequent section) will be used to further characterize selected recombinants with respect to 4S pathway activity.

Basis for Assay. In the assay developed by Dr. Krawiec, organisms to be tested for 4S activity are plated out on agar medium. Grown colonies are then sprayed with a DBT/ether solution. After a short incubation period (approximately 1-2 hours), the plate is viewed under a hand held mineral (short wave UV) light. With some cultures, a material which fluoresces bright purple can be visualized around the perimeter of colonies (and sometimes in the colonies). Control plates (no DBT) are included to verify that the colonies do not themselves fluoresce; preliminary work at Battelle confirmed that none of the organisms under study fluoresce under short wave UV light. Under excitation of short wave UV light, DBT and DBT sulfoxide do not fluoresce, DBT sulfone fluoresces dark purple, and o,o'-biphenol fluoresces a vivid

purple. Thus, the fluorescing material indicates the possible presence of the product, o,o'-biphenol. The limit of detection of o,o'-biphenol on an agar plate was determined to be between 0.05-0.5 ug in a 1 cm diameter spot. The assay is known to be sensitive to the growth medium employed, the concentration of DBT applied, the duration of contact with DBT, the size of colony, and efficiency of DBT conversion by the organism.

Carbon Source. A study was conducted to determine a suitable carbon source for use in the screening assay. Glucose, glycerol, and succinate (each at 0.2% were tested, based on discussions with the contracts at Lehigh University and the University of North Dakota. Stanier's mineral salts and Noble agar was used in preparation of the plates. These media were streaked with the 4S positive A4, A15, B24, and C18 strains, and the 4S negative *P. putida* ATCC 33015 strain. After growth (C-18 had negligible growth), the plates were sprayed with a 0.3% (w/v) DBT/ether solution (approximately 0.5 second). After incubation with DBT, fluorescence was detected with strains A4, A15, and B24, but only when the organisms were grown on the glycerol plates. Additional colored compounds appeared on several plates after prolonged incubation of the cultures with DBT (see subsequent section, Initial Pathway Characterization).

Colony Size and DBT Concentration. An experiment was conducted to determine the effect of colony size and DBT concentration on fluorescence. Strain A4 was grown up in Luria Broth, diluted in phosphate buffered saline (PBS), and plated onto Stanier's agar plates containing glycerol. At day three of incubation at room temperature, the colonies (approximately 10 per plate) were approximately 2 mm in diameter, and were 3-4 mm in diameter at day 6. Plates at these incubation times were sprayed with a 0.15% (w/v) DBT/ether solution for 1, 5, or 10 seconds, using 1 second bursts with 5 seconds in between bursts to allow for evaporation of the ether. The spray rate was approximately 750 ul/sec, or 1.1 mg DBT/sec. After 1 hour, the plates were observed under UV light. Fluorescence was most apparent with the larger colonies, perhaps due to a higher concentration of o,o'-biphenol. The lowest concentration of DBT tested gave the best results; no fluorescence was observed on

plates sprayed for a total of 10 seconds. The higher concentration of DBT may have obscured the fluorescence or possibly could have been at an inhibitory concentration.

Grid Size for Plate Assay. A study was conducted to determine the number of colonies per plate suitable for the detection of single fluorescence positive or negative colonies. In the previous experiment, DBT sprayed colonies which were 3-4 mm in diameter fluoresced within the colonies and in a 6 mm diameter zone (total) around the colonies. Based on this data, approximately 200 colonies could theoretically be distinguished on a 100 mm diameter plate. Grids were constructed at colony densities of 25, 50 or 100 per plate, and consisted of single 4S positive (A4) or single 4S negative (*P. putida* ATCC 33015) strains, in a field of organisms with the opposite trait. The plates were incubated at room temperature until the colonies reached 3-4 mm in diameter, and then sprayed with DBT. Under short wave UV light, a single positive colony was easily visible in a field of 100 negative colonies, and a single negative colony was also easily seen in a field of 100 positives. It may be possible to construct grids of 200 colonies, but colonies grow to a smaller size when crowded. The plate assay at its present stage of development can be effectively used to screen thousands of recombinants.

Alternate Medium for C-18. An attempt was made to substitute another medium for the glycerol/Staniens plates for testing of C-18, since this organism grows poorly on the glycerol medium. Although C-18 grew on Luria Broth-Agar (LB), fluorescence was not detected when conducting the spray test. An attempt was also made to improve growth of C-18 on the glycerol medium by employing the stab technique, which produces a decreasing oxygen gradient down the stab. This technique proved ineffective. Growth of C-18 in liquid Stanier-glycerol medium was also poor.

Screening of Liquid Cultures. A feasibility study was done to determine if ELISA plates could be used for fluorescence screening of small (0.25ml) liquid cultures. Standards of o,o'-biphenol were easily seen in the plate wells when spiked into water and observed under UV light, but when spiked into cultures, background fluorescence

was intense. Therefore, use of ELISA plates is not appropriate for fluorescence screening.

Development of HPLC Analytical Technique. The fluorescence screening method described above will be used to identify recombinants appearing to have a complete 4S pathway, leading to the production of o,o'-biphenol. Further analysis of the fluorescing material is necessary to confirm the presence of o,o'-biphenol. Moreover, another technique is necessary to determine which step(s) of the 4S pathway may be nonfunctional in recombinants. An HPLC technique which was developed at ARCTECH is well suited for the separation and identification of 4S pathway intermediates, and was chosen for detailed analysis of the recombinants generated in this program.

The HPLC setup was based on the use of a reverse-phase, isocratic solvent system. The mobile phase, which was blanketed with helium, consisted of 0.01M phosphoric acid (46%), tetrahydrofuran (18%), acetonitrile (18%), and dioxane (18%). The column selected was a DuPont Zorbax ODS 4.6 mm x 25 cm C-18 analytical column, fitted with a Zorbax ODS 4mm x 1.25 cm guard column. Components of the HPLC system included a 10 ul injection loop, Beckman Model 110A pump, Altex Controller set at 1.2 ml/min, LCD/Milton Roy spectroMonitor D Variable Wavelength Detector set at 242 nm, and a Hewlett Packard HP3396A Integrator.

Excellent sensitivity and low baseline noise was achieved using a small (10 ul) injection loop. Baseline resolution of the 4S pathway intermediates was achieved. A typical chromatogram is given in Figure 7. All of the 4S compounds were identified by comparison of the retention times of the mixture with retention times of individual standards. Each standard, except for DBT sulfoxide, yielded single peaks on HPLC chromatograms, indicating that the compounds were of high purity. DBT sulfoxide, however, was grossly contaminated with DBT sulfone (Figure 8).

The HPLC setup is fully developed and ready to use for detailed analysis of 4S compounds produced by liquid cultures after exposure to DBT. Sample preparation will consist of addition of acetonitrile to the culture to a final concentration of 60%, followed by vortexing of the mixture and centrifugation to remove particulates. The supernatant containing unreacted DBT and any intermediates will then be injected onto the HPLC column.

Figure 7. HPLC Chromatogram of 4S Pathway Compounds

Figure 8. impurity of DBT Sulfoxide, as Indicated by Two HPLC Peaks

Initial Pathway Characterization

Results from studies conducted in the development of the plate assay indicated that the end product of DBT degradation was dependent on the carbon source used to grow the organisms. The appearance of fluorescing material was dependent on the carbon source glycerol. In addition, distinctive colors appeared on the plates after prolonged (several hours) incubation with DBT. As shown in Figure 9, a dark red color appeared with cultures grown on glycerol, an orange color was observed with glucose grown cultures, and a bright yellow color was seen with cultures grown on succinate. It is not known whether the orange pigment was made up of a combination of the red and yellow compounds. In one study on the bioconversion of DBT, Hou and Laskin (1976) isolated and purified an orange pigment, and identified it as 4[2-(3-hydroxy)-thianaphthanyl]-2-hydroxy-3-butanoic acid. These investigators also partially purified two related red compounds. All of these materials appeared to contain an intact sulfur moiety; it is unclear how such compounds would fit into a sequential pathway containing o,o'-biphenol as an intermediate. In light of this, it is probable that the orange material is different from the material isolated by Hou and Laskin, or that side reactions occur during the biodegradation of DBT by the organisms under this study. Interestingly, all of the 4S positive strains which grew on plates showed similar results. The 4S negative culture did not fluoresce and did not produce any colored products.

Cloning and Identification

Electroporation

Pseudomonas putida ATCC 33015 was studied for electroporation transformation of plasmid DNA using the BRL Cell-Porator. *P. putida* ATCC 33015 was transformed with the broad host range cosmid vector pLAFR3 at an efficiency of 1.62×10^8 cfu/ μ g plasmid with a field strength of 9.99 kV/cm using the protocol of Smith and Iglewski (1989). The results with the *P. putida* strain are very encouraging, since the pLAFR3 vector is the vector that will be used for cosmid library construction. This

Figure 9. Chromophoric Materials Produced by Strains
During Incubation with DBT

transformation efficiency is also 10^4 higher than the highest transformation efficiency previously published (Wirth et al., 1989).

Plasmid Screening of Lehigh University Strains

The four strains A4, A15, B24 and C18 were examined for the presence of plasmids using the method of Martin et al. (1981). All strains grew well in "A" medium. Preliminary results using conventional electrophoresis revealed that plasmids were present in A15, B24 and C18. No plasmid DNA was detected in the A4 strain. Follow-up experiments are planned using a pulsed field gel or CHEF gel analysis to more accurately determine the size and number of these plasmids.

Antibiotic Screening of Lehigh Strains

The four strains were screened for antibiotic resistance to ampicillin^(Amp), chloramphenicol^(Cm), kanamycin^(Kan) and tetracycline^(Tet). The results were as follows (r = resistant, s = sensitive):

A4 (Cm^r, Kan^s, Amp^r, Tet^s)
A15 (Cm^r, Kan^s, Amp^s, Tet^s)
B24 (Cm^r, Kan^s, Amp^s, Tet^s)
C18 (Cm^s, Kan^r, Amp^r, Tet^s)

Concentrations of antibiotics tested were as follows: Cm, 5-20 µg/mL; Kan, 50-200 µg/mL; Amp, 100 µg/mL; Tet, 20 µg/mL. Antibiotic resistance profiles reveal that A15 and B24 are very similar, in that respect. This observed antibiotic resistance could be due to markers carried on endogenous plasmids, which is an avenue which we will pursue in subsequent experimental work. All strains were sensitive to tetracycline at 20 µg/mL.

Strain Acquisition

The cosmid cloning host, DH5 α MCR, was acquired. DH5 α MCR yields cosmid clones at five times the efficiency of other *E. coli* strains such as ED8767 (unpublished results), so acquisition of this strain was most desirable. Also, a potential *Thiobacillus* host was identified, *Thiobacillus cuprinus*, a new sulfur-oxidizing strain recently isolated and characterized by K.O Stetter (Huber and Stetter, 1990). This organism oxidizes inorganic sulfur substrates, but not ferrous ion. *T. cuprinus* is a facultative organotrophic acidophile with metal leaching characteristics similar to *T. ferrooxidans* and a faster growth rate. We have requested this strain from Dr. Stetter.

Plasmid Preparation

The alkaline extraction plasmid preparation method of Birnboim and Doly (1979) was used to identify pLAFR3 transformants of *P. putida* ATCC 33015. Since this method gave acceptable preparations of plasmid DNA from this strain, this method was also used on the Lehigh strains. At the present time, the electrophoresis parameters on the CHEF apparatus are being optimized. Accordingly, no conclusions can be made on the application of this procedure to the Lehigh strains until we complete a CHEF electrophoresis characterization of these samples is completed.

After the pLAFR3 plasmid from *P. putida* ATCC 33015 was prepared, we subjected this plasmid to digestion with the restriction enzymes *Eco* R1, *Bam* H1, *Hind* III, *Pst* I, *Sal* I, and *Bgl* II. Only *Pst* I and *Hind* III were able to restrict the *P. putida* ATCC 33015 pLAFR3 DNA. This bias in the capability of certain restriction endonucleases probably reflects the DNA modification system of ATCC 33015; methylation of certain adenine and cytosine residues inhibit restriction of many of the enzymes listed above. Since we are planning to make libraries in this *P. putida* strain, we need to determine which restriction enzymes will cut the DNA of not only the host, but also the DNA of the Lehigh strains before we can prepare our cosmid vector for cloning. We are undertaking these studies at the present time.

FUTURE WORK

Additional work planned include:

- The construction of HPLC standard curves for the 4S compounds
- Analysis of fluorescing material in DBT spray plates to confirm the presence of o,o'-biphenol
- Analysis of liquid cultures of each strain for 4S compounds after short-term (1 hour) incubation with DBT
- Initiation of construction of growth curves for the Lehigh strains to relate Absorbance (600 nm) to viable cell counts for use in the calculations of mutation frequencies.

Cloning and Identification

Future experiments planned include:

- Genomic cosmid library construction from the Lehigh strains
- Electroporation of such cosmid libraries into *P. putida* ATCC 33015
- Screening for DBT desulfurization clones from the cosmid library transformants
- Characterization of the plasmids from the Lehigh strains and determination of antibiotic resistance and/or DBT desulfurization genes on such plasmids
- Plasmid curing of the Lehigh strains and the determination of these effects on their phenotype.

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