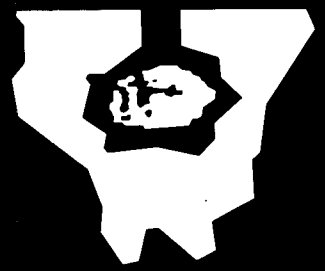

Coal Extraction and Utilization Research Center

Southern Illinois University at Carbondale

SIU

DOE/PC/88861--T9

DE89 009576

MICROBIAL REMOVAL OF ORGANIC SULFUR FROM COAL (BACTERIAL DEGRADATION OF SULFUR-CONTAINING HETEROCYCLIC COMPOUNDS)

B. Klubek

FINAL REPORT
January 1 - December 31, 1988

Southern Illinois University at Carbondale
Carbondale, Illinois 62901

Submitted to:
U.S. Department of Energy

Contract No. DE-FC22-88PC88861

MICROBIAL REMOVAL OF ORGANIC SULFUR FROM COAL (BACTERIAL DEGRADATION OF SULFUR CONTAINING HETEROCYCLIC COMPOUNDS)*

by

Brian Klubek
Department of Plant and Soil Science

Southern Illinois University at Carbondale
Carbondale, Illinois 62901

Final Report
January 1 - December 31, 1988

Submitted to
U.S. Department of Energy
Contract Number DE-FC22-88PC88861

March 1989

* This project is cost-shared between the U.S. Department of Energy and the Illinois Coal Development Board.

DISCLAIMER

ABSTRACT

The presence of substantial levels of sulfur in coal is a major source of air pollution, and considerable efforts are being made to devise a cost-effective way of removing the sulfur. One method is to mutate a laboratory species, *Escherichia coli,* an organism which is genetically well-understood and whose pathways for the metabolism of sulfur-containing amino acids have been extensively investigated. Such thiophene degraders can be genetically analyzed and the genes involved can be cloned in order to amplify their products. Dr. David Clark, Department of Microbiology, is evaluating these mutants of *E. coli.* The second approach is the development of naturally occurring bacteria capable of thiophene desulfurization. Characterization of the degradation of model compounds, enhancement of the desulfurization potential of the isolated strains via mutagenesis, and studies with crushed coal will comprise the approach used in this study. The screening of soil isolates for the potential to desulfurize thiophenic and other sources of organic sulfur will identify the best strains for the microbial removal of organic sulfur from coal. Ultimately, the genes responsible for thiophene degradation by the isolated strains will be transferred to an *E. coli* strain creating a single organism capable of degrading a broad spectrum of thiophene compounds.

INTRODUCTION

Utilization of domestic coal within the U.S. has increased dramatically since 1979. Coal production is expected to double between 1978 and 1990, with Appalachian and Midwest fields as the largest single sources (Dugan and Apel 1978). One problem associated with the utilization of such coal is the emission of SO_2 during its direct combustion. Physical and chemical methods of coal desulfurization are either expensive or result in a loss of fine coal particles (Meyers 1977, Wheelock 1977, Elliot 1978). The sulfur content of appalachian and midwestern coal varies from 3.0 to 5.5% (Dugan and Apel 1978). This sulfur is a mixture of inorganic (mostly pyrites) and organic (thiols, sulfides, disulfides, and thiophene groups) (Wheelock 1977, Chandra et al. 1979).

Microbial desulfurization of coal before combustion should cost less and be more energy efficient than high-temperature chemical processes (Dugan and Apel 1978, Detz and Barvinchak 1979). The removal of sulfur compounds from coal via microbial catalysis can also be accomplished without any loss of fine coal particles. The large deposits of high-sulfur coal east of the Mississippi River and the presence of eastern markets provide economic incentives for the removal of sulfur from coal.

Both *Thiobacillus* and *Sulfolobus* are capable of converting the inorganic sulfur in coal into soluble sulfate which can be washed out. The goal of this project is to complement the microbial removal of inorganic sulfur in coal by transforming organic sulfur into sulfate.

METHODS

Bacterial Strains and Growth Medium

Approximately 40 different strains were isolated that had the ability to grow on a selected thiophenic or organic sulfur compound. These strains were then

characterized for their desulfurization potential (Klubek and Clark 1987). From this initial screening, 21 strains were chosen for further characterization. In addition, two mutant strains of *E. coli* developed in Dr. David Clark's laboratory were included in these characterization studies. For the natural isolates, the cultures were maintained on a mineral salts medium supplemented with benzoate, thiophene carboxylic acid, or cystine (Table 1). Table 2 lists the strains chosen for further screening and characterization studies.

Table 1

Mineral Salts Medium Used for Growth of Isolated Strains*

Medium Composition	Concentration (g/L)
KH_2PO_4	4.0
Na_2HPO_4	4.0
NH_4NO_3	1.2
$MgCl_2$	0.08
$CaCl_2 \cdot 2H_2O$	0.001
$FeCl_3 \cdot 6H_2O$	0.001
Distilled Water	1000 mL

*Adjust pH to 7.0 with concentrated HCl; final concentration of organic S source is 0.05%; for a solidified medium, 10 g Gelrite® (Scott Laboratory) and 1 g $MgCl_2$ is used.

Table 2

List of Strains Used in Characterization Studies

Strain	Organic Sulfur Source for Growth
B_{wt}, 89-N, 89-T, 89-S, 89-R, 89-B	Dibenzothiophene (DBT)
DBTS-2, 4, 18, 19B, 22B, 26	Dibenzothiophene Sulfone (DBTS)
BSA-54, 56	Benzene Sulfonic Acid (BSA)
CYI 61, 64	Cystine (CYI)
LGA, 84-B2, 85-A2, NAR30, NAR41	Thiophene Acetic Acid (TCA)

Determination of Desulfurization Potential

For each group of strains that were selected for desulfurization activity, four replicates of each strain were grown in a broth culture with the desired organic sulfur (model) compound in a randomized complete block design. A non-inoculated flask was used as a control. All of the flasks were incubated at room temperature for 15 days under constant aeration, as previously described (Klubek and Clark 1986). The flow of air through each flask was 52 mL min^{-1}. Following incubation, 10 mL of the broth culture was centrifuged and the collected supernatant was tested for sulfate colorimetrically with $BaCl_2$. For the determination of H_2S, the zinc acetate solution was treated with 2% starch and titrated with 0.025 M I_2 in KI.

A 10 mL subsample was also taken at the beginning and end of each experiment to determine the rate of growth of each group of strains. Isolates growing on DBT and DBTS were plated on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Growth colonies were counted after 7 days of incubation. The growth rates of those isolates specific for BSA, CYI, or TCA were determined by absorbance on a Klett-Summerson colorimeter.

The determination of the final concentration of the model compound was attempted by acidifying the broth culture to a pH of 2.0 followed by extraction with HPLC grade ethyl ether or benzene. The extract was then evaporated to dryness with an air stream. The residue was then dissolved in a 50% mixture of HPLC grade acetylnitrile and water. The final concentration was 10 mL. The concentration of the model compound was then determined by a Waters Associates UV-diode array 990•HPLC. A general purpose column (μBONDAPAK C_{18}, Waters Associates) was used for these separations, with a flow rate of 2 mL/min. The injection volume

(depending on the type of concentration of the model compound) varied from 5 to 25 µL.

Characterization of Selected Strains for Model Compound Specificity

Following the second screening of our selected strains, 11 strains were chosen to assess their ability to grow on and desulfurize 19 model compounds. The same mineral salts medium described in Table 1 was used. The final concentration of each model compound was 0.05%. The presence or absence of growth was evaluated after 7 days of incubation. A rapid assay method was developed to detect the formation of sulfate-sulfur. A 6 mm hole (well) was made in the solidified medium (test plate) adjacent to the growth of a test organism. To 10 mL of deionized (HPLC grade) water, 1 mL of an acid seed solution (6 N HCl containing 20 ppm S as K_2SO_4) and 0.5 g $BaCl_2 \cdot 2\ H_2O$ crystals were added. This test solution was then added to each well on a test plate and incubated for 3 hr. A positive test for sulfate-sulfur gave a diffuse white precipitate around the well. Positive and negative control plates were also used for comparison.

Determination of Plasmid DNA

To determine if the desulfurization activity of our natural isolates was plasmid associated, the 11 strains were grown and plasmid DNA was isolated and run on electrophoretic gels (Maniatis et al. 1982). Those isolates possessing plasmids were then treated with a variety of curing agents (novobiocin, acriflavine, acridine orange, quinacrine, and mitomycin C) on gradient plates. Those strains showing gradient growth were rescued on yeast extract-peptone-succinate agar (1 g/L each plus 2 g KCl, pH 6.8). The rescued strains were then tested for growth on the appropriate model compound.

Screening *Thiobacillus sp.*, *T. novelus*, and *T. perometabolis* for Desulfurization Activity on Model Compounds

Cultures of *Thiobacillus* sp. (ATCC-27793), *T. novellus* (ATCC-8093) and *T. perometabolis* (ATCC-23370) were grown on media described by Starkey (1934), McCarthy and Charles (1975), and London and Rittenberg (1967), respectively. These cultures were then used to inoculate plates employing the mineral salts medium described in Table 1 amended with a variety of inorganic and organic sulfur compounds.

Coal Toxicity Experiments

Strains B_{wt}, DBTS-4 and 18, BS-54 and 56, CYI-64 (all natural isolates), and *E. coli* strains DC625 (parent strain), NAR30 and NAR40 were used to inoculate a 50 mL succinate (0.5%) mineral salts medium amended with 0.05, 0.5, or 5 g of coal. The control was the succinate-mineral salts medium minus the addition of coal. Coal, either Illinois No. 5 or No. 6, was ground to $\leq$ 10μm particle size and added to the succinate-minerals salts solution before autoclaving. Following inoculation with each test strain, subsamples were taken and plated on a 1% glucose -0.3% yeast extract -0.3% peptone growth medium at the start of the experiment and up to 7 days of incubation at 30° C. Each treatment was replicated 3 times.

Statistical Analysis

Where appropriate, the data was statistically analyzed by an analysis of variance (ANOVA), and mean separation was achieved by employing either a Duncan's Multiple Range Test (Duncan 1955) or the Least Significant Difference (LSD) Test (Little and Hills 1978). Acceptable error, unless noted elsewhere, was 5%.

RESULTS

Determination of Desulfurization Potential

Table 3 summarizes the growth rate characteristics of the isolated strains assessed for the desulfurization of dibenzothiophene (DBT), dibenzothiophene sulfone (DBTS), and benzene sulfonic acid (BSA). The DBT and DBTS strains showed very slow generation times of 1.09 to 3.96 days when grown with their respective model compounds and aerated with a flow rate of 52 mL min^{-1}. However, the BSA strains were determined (in a separate study) to have more rapid generations times of 1.01 (BSA 54) and 1.41 hr (BSA 56).

Table 3

Growth Rate Characteristics of Selected Strains that Degrade Dibenzothiophene, Dibenzothiophene Sulfone, or Benzene Sulfonic Acid.

Model Compound/ Strain	Log_{10} Count/mL		Absorbance, Klett Units		Generation Time (days)
	Initial	Final	Initial	Final	
DBT					
B_{wt}	2.18	5.25			1.47*
89-N	0.92	5.08			1.09
89-T	3.35	7.00			1.24
89-S	4.22	5.90			2.69
89-R	4.18	5.32			3.96
89-B	2.99	5.47			1.82
DBTS					
2	6.74	8.80			2.19
4	6.53	8.45			2.35
18	6.69	9.11			1.87
19B	6.71	8.18			3.07
22B	6.38	8.91			1.78
26	7.48	8.92			3.14
BSA					
54			13.5	35.0	1.01†
56			8.3	23.7	1.41

*da.
†hr.

Table 4 summarizes the sulfur balance sheet for DBT desulfurization. The strains that showed the greatest percent degradation of DBT also showed the lowest percent desulfurization. Of the six strains tested, B_{wt} and 89-N showed the lowest percent degradation of DBT (4.4 and 35.1%, respectively) but the highest percentage for desulfurization activity (1.5 and 1.1%, respectively).

Table 4

Sulfur Balance Sheet for the Desulfurization Potential of Selected Strains that Degrade Dibenzothiophene Sulfone

Strain	DBT Concentration (µg/mL)		% Degradation	$SO_4^=$-S Concentration (µg/mL)		Net Change $SO_4^=$-S Production (µg/mL)	% Desulfurization*
	Initial	Final		Initial	Final		
B_{wt}	500	458 ab†	4.4	1.9	3.1	1.2 a†	1.5
89-N	500	323 bc	35.1	2.8	3.9	1.1 b	1.1
89-T	500	48 d	91.0	1.8	2.5	0.7 b	0.3
89-S	500	207 cd	59.3	2.3	2.8	0.5 b	0.9
89-R	500	126 d	75.9	2.8	3.1	0.3 b	0.3
89-B	500	86 d	82.9	1.3	0.9	-0.4 c	0
Non-inoculated Control	500	498 a	0	2.1	1.0	-1.1 d	0

*Based on 87µg S/mL as DBT.

†Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan's Multiple Range Test.

Table 5 summarizes the sulfur balance sheet for DBTS desulfurization. All six strains were equivalent in their ability to degrade and desulfurize DBTS. For this set of strains, the percent desulfurization varied from 21.8 to 30.8%.

Table 6 summarizes the sulfur balance sheet for BSA desulfurization. Both strains were equally effective in the desulfurization of BSA, varying 57.8 to 71.1%. However, the percent degradation of BSA could not be determined since the

Table 5

Sulfur Balance Sheet for the Desulfurization Potential of Selected Strains that Degrade Dibenzothiophene Sulfone

Strain	DBTS Concentration (µg/mL)		% Degradation	$SO_4^=$-S Concentration (µg/mL)		Net Change $SO_4^=$-S Production (µg/mL)	% Desulfurization*
	Initial	Final		Initial	Final		
19 B	500	23.0 b†	95.4	5.1	28.0	22.9 a†	30.8
2	500	25.4 b	94.9	4.5	26.0	21.5 a	29.1
22 B	500	17.7 b	96.5	6.3	26.0	19.7 a	26.6
26	500	14.7 b	97.1	2.3	21.9	19.6 a	26.5
4	500	18.2 b	96.4	4.8	23.5	18.7 a	25.2
18	500	11.6 b	97.7	6.0	22.1	16.1 a	21.8
Non-inoculated Control	500	480 a	4.0	2.3	4.8	2.5 b	3.4

*Based on 74µg S/mL as DBT.

† Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan's Multiple Range Test.

Table 6

Sulfur Balance Sheet for the Desulfurization Potential of Selected Strains that Degrade Benzene Sulfonic Acid

Strain	DBTS Concentration (µg/mL)		% Degradation	$SO_4^=$-S Concentration (µg/mL)		Net Change $SO_4^=$-S Production (µg/mL)	% Desulfurization*
	Initial	Final		Initial	Final		
56	500	0	N.D.†	9.0	80.8	71.8 a‡	71.1
54	500	0	N.D.	8.9	67.6	58.7 a	57.8
Non-inoculated Control	500	0	N.D.	3.6	6.0	2.3 b	2.4

*Based on 101µg S/mL as BSA.

† Not determined.

‡ Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan's Multiple Range Test.

chromatogram of this compound was missing in both inoculated treatments and the non-inoculated control.

Table 7 summarizes the desulfurization potential of selected strains that degrade cystine. Strain CYI-64 showed a significantly higher desulfurization potential than CYI-61 (62.5 versus 36.9%). The percent degradation of cystine (like BSA) could not be determined since the colorimetric procedure for this amino acid failed to detect the presence of either cystine or cysteine in both inoculated treatments and the non-inoculated control.

Table 7

Desulfurization Potential of Selected Strains that Degrade Cystine

Strain	Net Change in $SO_4^=$ Production (µg/mL)	% Desulfurization*
CYI-64	83.1 a†	62.5
CYI-61	49.1 b	36.9
Non-inoculated Control	1.7 c	1.4

*Based on 133µg S/mL as cystine.

† Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test.

Table 8 summarizes the desulfurization potential of thiopheneacetic acid (TAA) by soil isolates and mutant strains of *E. coli*. None of these strains showed the potential to desulfurize TAA. HPLC chromatography showed the presence of TAA with only a minor shoulder to the chromatographic peak.

Table 8

Desulfurization Potential of Selected Strains that Degrade Thiopheneacetic Acid

Strain	Net Change in $SO_4^=$ Production (µg/mL)	% Desulfurization*
DC625	1.02 a†	0.82
85-AZ	0.42 b	0.34
NAR30	0.33 b	0.26
LGA	0.15 bc	0.12
84-B2	-0.17 cd	0
NAR41	-0.38 d	0
Non-inoculated Control	0.25 b	0.66

*Based on 125µg S/mL as TCA.

† Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test.

Characterization of Selected Strains for Model Compound Specificity

Table 9 summarizes the specificity of selected soil isolate strains for 19 model compounds. Poor to good growth was determined for strain B_{wt} when grown in the presence of 17 out of 18 model compounds. However, the presence of sulfate was detected only when this strain was grown in the presence of cystine or benzene sulfonic acid. With the exception of 2-formyl-3-hydroxybenzothiophene, strains DBTS-2, 19B, 22B, and 26 showed poor to good growth on all of the tested model compounds. Like isolate B_{wt}, all of these strains showed a positive test for sulfate when grown in the presence of cystine and benzene sulfonic acid. Strains BSA-54 and 56, like the DBTS strains, grew in the presence of all 18 model compounds. Strain BSA-56, but not BSA-54, grew in the presence of 2-formyl-3-hydroxybenzothiophene. In addition, this strain also showed a positive test for sulfate. Both

Table 9

Characterization of Isolated Strains for Model Composed Specificity*

Model Compound		Strain						
		B_{wt}	DBTS-2	DBTS-19B	DBTS-22B	DBTS-26	BSA-54	BSA-56
1-Napthol-3,6-Disulfonic Acid	G	+/-	+/-	+	+/-	+/-	+	+
	S	-	-	-	-	-	-	-
3-Nitrobenzene Sulfonic Acid	G	+/-	+	+	+	+	+/-	+/-
	S	-	-	-	-	-	-	-
2-Sulfobenzoic Acid Hydrate	G	+	+	+	+	+	+	+
	S	-	-	-	-	-	-	-
Thiophene Acetic Acid	G	+/-	+	+	+	+	+/-	+/-
	S	-	-	-	-	-	-	-
1,2-Napthoquinone-4-Sulfonic Acid	G	+	+/-	+	+	+/-	+	+
	S	-	-	-	-	-	-	-
Tetramethylene Sulfone	G	+/-	+	+	+	+	+	+
	S	-	-	-	-	-	-	-
5-Sulfo-Salicylic Acid	G	+	+	+/-	+	+	+	+
	S	-	-		-	-	-	-
2-Aminobenzene Sulfonamide	G	+	+	+	+	+	+	+/-
	S	-	-	-	-	-	-	-
1-Napthratene Sulfonic Acid	G	+	+	+	+	+	+	+
	S	-	-	-	-	-	-	-

*G = Growth after 7 days incubation; S = sulfate formation.

†ND = Not determined.

Table 9

Characterization of Isolated Strains for Model Composed Specificity*

Model Compound		Strain						
		B_{wt}	DBTS-2	DBTS-19B	DBTS-22B	DBTS-26	BSA-54	BSA-56
2-Napthratene Sulfonic Acid	G	+	+/-	+	+	+/-	+/-	+/-
	S	-	-	-	-	-	-	-
Cysteic Acid	G	+/-	+/-	+	+	+/-	+	+
	S	-	-	-	-	-	-	-
Cystine	G	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+
Benzene Sulfonic Acid	G	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+
Thiophene Carboxylic Acid	G	+/-	+/-	+	+	+	+/-	+/-
	S	-	-	-	-	-	-	-
p-Toluene Sulfuric Acid	G	+	+	+	+	+	+	+
	S	-	-	-	-	-	-	-
Sulfonic Acid	G	ND†	+/-	+	+	+/-	+/-	+/-
	S	ND	-	-	-	-	-	-
Thiourea	G	+	+	+	+	+	+	+
	S	-	-	-	-	-	-	-
Dimethyl-sulfoxide	G	+	+/-	+	+	+	+/-	+
	S	-	-	-	-	-	-	-
2-Formyl-3-hydroxy-benzothiophene	G	-	+	-	-	-	-	+
	S	-	-	-	-	-	-	+

*G = Growth after 7 days incubation; S = sulfate formation.

†ND = Not determined.

strains showed a positive sulfate test when grown in the presence of cystine and benzene sulfonic acid.

Determination of Plasmid DNA

Table 10 summarizes the analysis of plasmid DNA from selected soil isolates.

Table 10

Analysis of Plasmid DNA from Soil Isolates

Strain	Number of Plasmids	Size of Plasmid(s) (kb)
B_{wt}	1	10-12
89-B	1	10
89-Q	1	10
89-R	1	10
89-T	1	10
DBTS-2	2	10, 92
DBTS-19B	2	10, 92
DBTS-22B	1	10
DBTS-26	1	10
BSA-54	Unknown	ND*
BSA-56	Unknown	ND

*ND = Not determined.

With the exception of strains DBTS-2, BSA-54, and BSA-56, all of the selected strains showed the presence of at least one plasmid in the 10 to 12 kb range. Strains DBTS-2 and 19B appear to have two plasmids, one megaplasmid (92 kb) and a smaller plasmid (10 kb) similar to the plasmid detected in the other soil isolates. Strains BSA-54 and 56 may have a very large megaplasmid (approximately 100 kb) and requires restriction endonuclease digestion for the confirmation of this plasmid.

Table 11 summarizes the attempt to cure the detected plasmids of the isolated strains with intercalating dyes and antiobiotics. With the exception of mitomycin C, strain B_{wt} failed to grow in the presence of the curing agents. Strains DBTS-2 and BSA-56 failed to grow on plates amended with novobiocin, while strains DBTS-19B

Table 11

Plasmid Curing with Intercalating Dyes and Antiobiotics

Strain	Curing Agent*				
	AC	AO	Q	N	MC
B_{wt}	-	-	-	-	+ +
89-Q	-	-	-	ND†	+ +
DBTS-2	-	+ +	+ +	-	+ +
DBTS-19B	+ +	±	+ +	G	+ +
DBTS-22B	+ +	±	+ +	G	+ +
DBTS-26	G	G	+ +	G	+ +
BSA-56	+ +	+ +	±	-	+ +

* 10µg/mL of curing agent was added to yeast extract-peptone-succinate agar. AC = Acriflavin; AO = Acridine Orange; Q = Quinicrine; N = Novobiocin; MC = Mitomycin C.
- No growth
± Poor growth
+ Good growth
+ + Confluent growth
G Gradient growth
† ND = Not determined.

and 22B showed gradient growth on novobiocin plates. Strain DBTS-26 showed gradient growth on plates amended with acridine orange, acriflavine, and novobiocin. Isolates showing gradient growth were transferred to yeast extract-peptone-succinate (YEPS) agar to rescue growing cells. Growth from these plates was then tested on a mineral salts medium amended with benzoic acid (0.25%) or dibenzothiophene sulfone (0.10%). All of the rescued isolates showed growth on both types of plates, with hydrolysis of dibenzothiophene sulfone.

Screening of Thiobacillus Strains for S Oxidation Activity

Table 12 summarizes the growth of *Thiobacillus* strains on several sources of inorganic sulfur. *Thiobacillus sp.* grew on all four sources of sulfur and was positive for sulfate formation. With the exception of sodium lauryl sulfate, *T. novellus* grew on the remaining inorganic sources of sulfur testing positive for sulfate formation.

T. perometabolis grew on all four inorganic sources of sulfur. Sulfate production could not be determined for this strain due to the presence of the anion in the growth medium. For all three strains, the formation of sulfate from thiosulfate was determined from both cultures.

Table 12

Growth of *Thiobacillus* Strains and Formation of Sulfate on Inorganic S Compounds*

Strain	Sodium Hydrosulfite		Sulfonic Acid		Sodium Lauryl Sulfate		$Na_2S_2O_3$	
	G	S	G	S	G	S	G	S
Thiobacillus sp. (ATCC-27793)	+	+	+	+	+	+	+	+
T. novellus (ATCC-8093)	+	+	+	+	-	-	+	+
T. perometabolis (ATCC-23370)	+	ND†	+	ND	+	ND	+	+

*G = Growth after 7 da incubation; S = Sulfate formation.
†ND = Not determined.

Table 13 summarizes the growth and formation of sulfate by the *Thiobacillus* strains when grown on 14 sources of organic sulfur. *Thiobacillus sp.* grew in the presence of all 14 sources of organic sulfur; however, sulfate production was positive only for cysteic acid and cystine. *T. novellus* did not grow in the presence of dibenzothiophene or dibenzothiophene sulfone, but did indicate the formation of sulfate when in the presence of 1, 2-napthoquinone-4-sulfonic acid. *T. perometabolis* grew in the presence of all 14 model compounds; however, sulfate production could not be determined due to the presence of anion in the growth medium.

Table 13

Growth of *Thiobacillus* Strains and Formation of Sulfate on Organic S Compounds*

	Strain					
	Thiobacillus sp. (ATCC-27793)		*T. novellus* (ATCC-8093)		*T. perometabolis* (ATCC-23370)	
	G	S	G	S	G	S
Thiophene Acetic Acid	+	+/−	+	−	−	−
1,2-Napthoquinone-4-Sulfonic Acid	+	+/−	+	+	+	ND†
Cysteic Acid	+	+	+	+/−	+/−	ND
Cystine	+	+	+	+/−	+	ND
Thiophene Carboxylic Acid	+	+/−	+	−	+	ND
2-Naphratene Suflonic Acid	+	+/−	+	−	+	ND
p-Toluene Sufuric Acid	+	−	+	+/−	+	ND
1-Naphratene Sulfanic Acid	+	−	+	+/−	−	−
Dimethyl Sulfoxide	+/−	+/−	+	−	+	ND
Dibenzothiophene	+	−	−	−	+	ND
Dibenzothiophene sulfone	+	+/−	−	−	+	ND
2-Formyl-3hydroxy-benzothiophene	+	+/−	+	−	+	ND
Sulfanilic Acid	+	+/−	+	+/−	+/−	ND
Thiourea	+	+/−	+	−	+	ND

*G = Growth after 7 days incubation; S = Sulfate formation.
†ND = Not Determined

Coal Toxicity Experiments

The results of coal toxicity studies (Illinois No. 5 and No. 6 coals) on selected soil isolates and mutant strains of *E. coli* are summarized in the Appendix. Depending on the type of coal and the strain tested, significant differences in cell counts were obtained between the control and the coal slurry treatments. In general, the cell counts for the 0.1 and 1% slurry treatments were equal to or greater than the counts obtained from the control. However, for all treatments, an increase in cell

counts was obtained following 6 to 7 days of incubation, indicating the lack of any toxic effect on the test strains by either coal.

DISCUSSION

Determination of Desulfurization Potential

The determined generation times reported in this study were based upon samples taken at the beginning and end of each experimental trial. This was done to avoid contamination of each replicated flask. Since only two samplings were used, these reported growth rates are estimates of the true generation time of these soil isolates. Nevertheless, for the DBT and DBTS degrading bacteria, a 1 to 4 log increase in the plate counts were determined over the 15-day incubation period. Under the described experimental conditions, the reported generation times are an accurate assessment of the growth of these isolated strains. For strains BS-54 and 56, the reported generation times were determined in a separate study. Several flasks were sacrificed during the 15-day incubation period to avoid contamination of the replicated flasks. Hence, these generation times represent the true growth rates of these isolates.

The DBT degradation study indicates that the best strains for desulfurization are those strains with the lowest percentage of DBT decomposition (B_{wt} and 89-N). HPLC-diode array analysis of the samples indicated (depending on strain) 2 to 5 additional degradation products from DBT. One of these additional degradation products was 2-formyl-3-hydroxy-benzothiophene. Since very little sulfate was released from DBT, these data suggest that most of the sulfur remains in the organic form in a variety of degradation products. These results further suggest that strain B_{wt} is the best candidate for future mutation studies since this strain may specifically attack the sulfur component of the DBT molecule.

Studies with dibenzothiophene sulfone (DBTS) indicated a high rate of decomposition but a desulfurization rate of 20 to 30%. HPLC-diode array analysis indicated the presence of 2 to 3 additional degradation products. It can be assumed that the remaining sulfur is in the organic form associated with these intermediate products. The amount of available oxygen may be a limiting factor in the desulfurization of DBTS. Future studies will assess the effect of shaking rate (stationary, 50, 100, 150, and 250 rpm) on the growth and desulfurization potential of the DBTS strains. Currently, all six strains are good candidates for future mutation studies to enhance desulfurization activity.

Degradation studies with benzene sulfonic acid (BSA) indicated an unknown degradation product in the inoculated treatments and the non-inoculated control. The failure to detect BSA in any of the treatments suggests that BSA had undergone a physical and/or chemical change due to autoclaving. Nevertheless, a 60 to 70% desulfurization rate was determined regardless of the form of organic sulfur. These results suggest that these strains may used as co-inoculants for coal desulfurization studies based on their ability to utilize simple forms of organic sulfur.

Degradation studies with cystine (CYI) showed decay rates of 35 to 60%; however, the presence of cystine or cysteine could not be detected in either of the inoculated treatments or the non-inoculated control. This again suggests that CYI may have undergone physical and/or chemical change due to autoclaving, but both strains were able to desulfurize this compound. However, since this compound is relatively easy to degrade, no further characterization of these strains was determined.

Degradation studies with thiophene acetic acid (TAA) showed no desulfurization activity by any of the strains tested. The previous positive tests for H_2S by the soil isolates may have been due to the presence of volatile aliphatic sulfides, or to the fact that the desulfurization activity of these strains was plasmid associated

but was lost after several transfers for maintenance of stock cultures. Strains NAR30 and NAR41 (mutants of *E. coli*) possess no desulfurization activity despite reports by D. Clark to have isolated the *thd a, c,* and *d* genes. HPLC-diode array analysis showed the presence of the TAA peak modified by a slight shoulder, suggesting that the compound may undergo the initial stages of desulfurization. D. Clark has confirmed, by NMR studies, that his mutants of *E. coli* do not desulfurize either TCA (thiophene carboxylic acid) or TAA but simply modify the electron resonance of the sulfur atom.

Characterization of Selected Strains for Model Compound Specificity

All of the tested strains grew in the presence of most or all of the 19 model compounds, but formed sulfate only with CYI or BSA. These data suggest that selection of strains for enhanced activity be limited to B_{wt} and the DBTS strains since all of these strains have demonstrated the ability to desulfurize CYI and BSA. The data further suggest that these soil isolates are tolerant to a wide variety of aromatic compounds, showing no inhibited growth. In addition, these strains have been observed to grow (although poorly) on a mineral salts medium without the addition of an added carbon source. This result indicates that these bacteria are scavengers and may be capable of growth in the presence of aromatic compounds that are toxic to most other bacteria.

Determination of Plasmid DNA

Most of the soil isolates were determined to have at least a single plasmid in the 10 to 12 kb range. However, confirmation that these plasmids are associated with desulfurization activity has yet to be demonstrated. The inability of 10 μg/mL of acridine orange, acriflavin, quinicrine, and novobiocin to inactivate the desulfurization activity of the DBTS strains suggests that either a different curing method or agent be used, or that the genes responsible for desulfurization are on the genome.

In very recent studies, acridine orange and acriflavin at 100 µg/mL inhibited the growth of DBTS-22 and 26, but not B6-56 or DBTS-2. Similarly, quinicrine at 100 µg/mL did not inhibit the growth of the latter two strains, but it did yield gradient growth for the former two strains. Acridine orange, acriflavin, and quinicrine concentration as high as 1,000 µg/mL failed to yield gradient growth for strains BSA-56 and DBTS-2. These results again indicate the ability of these strains to grow in the presence of inhibitory or toxic substances. Those strains that have shown gradient have yet to show a loss of desulfurization activity. Currently, we are passing these strains on a glucose-yeast extract-peptone medium with the intent to lose the plasmid by deleting the model compound.

Screening of Thiobacillus Strains for Sulfur Oxidation Activity

All three species of *Thiobacillus* showed growth and sulfate formation in the presence of inorganic sources of sulfur. However, growth was evident (depending on the species) in the presence of most or all of the model compounds tested. Sulfate formation was again species dependent and only occurred with cystine, cysteic acid, or 1, 2-napthoquinone-4-sulfonic acid. Nevertheless, these species of *Thiobacillus*, like our soil isolates, demonstrated the ability to grow in the presence of aromatic compounds that may otherwise be inhibitory or toxic to other bacteria. These strains of bacteria are additional candidates for mutagenesis to enhance desulfurization activity from organic sulfur compounds.

Coal Toxicity Experiments

The coal toxicity experiments showed that growth occurred in all three coal slurries despite the significant differences in the cell counts between the control and slurry treatments. Since growth was always observed over the 6- to 7-day incubation period, the differences in the counts can only be attributed to the adsorbance of the bacterial inoculum to the coal particles. Given the observed

tolerance of the soil isolates to aromatic compounds, it was not surprising to observe the growth of these bacteria in coal slurries of up to 10%. Future coal desulfurization studies can now employ coal slurries of at least 10%.

SUMMARY

Twenty-one strains were evaluated for their desulfurization potential on their respective model compounds. From these studies, 11 strains were chosen for further study evaluating their ability to grow and desulfurize a broad spectrum of aromatic sulfur compounds, and to detect the presence of plasmid DNA. Based on these results, 6 strains, (B_{wt}, DBTS-2, 19B, 22B, 26, and BSA-56) were chosen for mutagenesis experiments to enhance desulfurization activity. However, these mutation experiments are dependent upon the verification that desulfurization activity is plasmid associated. Three species of *Thiobacillus* have also been evaluated for their ability to desulfurize organic sulfur compounds and appear to be additional candidates for microbial coal desulfurization. *E. coli* mutants NAR30 and NAR41 failed to desulfurize thiophene acetic acid, suggesting that these strains may be able to initiate only the first step in sulfur oxidation.

REFERENCES

Chandra, D., R. Prodosh, A. K. Mishra, J. N. Chakrabarti, and B. Sengupta. 1979. Microbial removal of organic sulfur from coal. Fuel 58:549-560.

Detz, C. M., and G. Barvinchak. 1979. Microbial desulfurization of coal. Mining Congress Journal 65:75-86.

Dugan, P. R., and W. A. Apel. 1978. Microbial desulfurization of coal. In Metallurgical applications of bacterial leaching and related microbiological phenomena, eds. L. E. Murr, A. E. Torma, and J. A. Brierly, pp. 230-250. New York: Academic Press.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11:1-42.

Elliot, R. C., ed. 1978. Coal desulfurization prior to combustion. Park Ridge, New Jersey: Noyes Data Corporation.

Klubek, B., and D. Clark. 1986 Microbial removal of organic sulfur from coal. Coal Technology Laboratory Final Report. Carbondale: Southern Illinois University at Carbondale, Coal Research Center.

Klubek, B., and D. Clark. 1987. Microbial removal of organic sulfur from coal. Coal Technology Laboratory Final Report. Carbondale: Southern Illinois University at Carbondale, Coal Research Center.

Little, T. M., and F. J. Hills. 1978. Agricultural experimentation design and analysis. New York: John Wiley and Sons, Inc.

London, J., and S. C. Rittenberg. 1967. *Thiobacillus perometabolis* nov. sp., a non-autotrophic thiobacillus. Archiv fur Mikrobiologie 59:218-225.

Maniatis, T., E. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

McCarthy, J. T., and A. M. Charles. 1975. Properties and regulations of ribose diphosphate carboxylase from *Thiobacillus novellus*. Archiv fur Mikrobiologie 105:51-59.

Meyers, R. A. 1977. Coal desulfurization. New York: Marcel Dekker.

Starkey, R. L. 1934. Cultivation of organisms concerned in the oxidation of thiosulfate. Journal of Bacteriology 28:365-386.

Wheelock, T. D., ed. 1977. Coal desulfurization. Chemical and physical methods. In ACS Symposium Series 64. Washington, D.C.: American Chemical Society.

APPENDIX

Table A1

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain B_{wt}*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD‡
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	8.40a	8.31ab	8.23b	8.09c	0.11
2	9.49a	9.37c	9.43b	9.32d	0.04
3	9.42a	9.44a	9.42a	8.96b	0.16
6	9.39a	9.23a	4.93b	8.94ab	4.02§
Illinois No. 6 Coal					
0	ND†	ND	ND	ND	ND
1	ND	7.69a	7.83a	7.54b	0.15
2	8.09d	8.91a	8.84b	8.50c	0.07
3	8.71c	9.20a	9.16ab	9.10b	0.08
6	8.64c	9.31a	9.16b	9.08b	0.11

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†ND = No Data.

‡Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

§Significant at $P \leq 0.10$

Table A2

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain DBTS-4*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD‡
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	8.59a	8.57a	8.42b	8.06c	0.05
1	9.60a	9.62a	9.50b	9.27c	0.08
2	9.62a	9.62a	9.56b	9.45c	0.05
5	9.66ab	9.63b	9.49c	9.69a	0.06
Illinois No. 6 Coal					
0	8.26	9.38	8.40	8.35	NS§
1	ND†	9.16b	9.66a	9.23b	0.10
2	9.25	9.16	9.20	9.38	NS
3	9.41b	9.42b	9.86a	7.52c	0.02
7	9.41b	9.34b	9.51a	7.95c	0.07

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†ND = No Data.

‡Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

§NS = Non-Significant

Table A3

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain DBTS-18*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD‡
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	7.80	7.88	7.67	8.08	NS§
1	8.75	8.79	8.61	8.87	NS
2	9.11b	ND†	9.23a	8.76c	0.12
3	9.14	9.17	9.16	7.30	NS
6	9.07a	9.03ab	8.80c	8.69b	0.37
Illinois No. 6 Coal					
0	7.80	7.89	7.85	7.81	NS
1	9.05a	8.77b	8.94ab	8.74b	0.25#
2	10.2a	10.2a	10.1b	10.1b	0.09
6	9.64a	9.32b	9.21b	9.20b	0.20

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†ND = No Data.

‡Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

§NS = Non-Significant

#Significant at $P \leq 0.10$

Table A4

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain BS-54*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD†
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	7.80b	7.88a	7.71c	7.29d	0.07
2	8.21b	8.12b	8.32a	8.17b	0.09
3	8.15b	8.04b	8.36a	8.09b	0.14
7	8.14b	8.03b	8.36a	8.09a	0.09
Illinois No. 6 Coal					
0	8.25	8.23	8.53	8.37	NS‡
1	8.68	9.42	8.80	8.81	NS
2	8.95	8.99	8.87	9.02	NS
3	9.05	9.06	9.12	9.21	NS
6	9.45	9.31	9.23	9.20	NS

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

‡NS = Non-Significant

Table A5

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain BS-56*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD†
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	7.28a	7.25a	7.16a	6.70b	0.15
1	8.99	9.14	9.02	8.85	NS‡
2	9.40	9.35	9.29	9.34	NS
3	9.51a	9.44b	9.39c	9.28d	0.04
6	9.51a	9.52a	9.57a	9.28b	0.98
Illinois No. 6 Coal					
0	8.53	8.65	8.54	8.49	NS
1	8.97c	9.08bc	9.42a	9.09b	0.12
2	9.26b	9.61a	9.06c	9.23b	0.81
3	9.54a	9.67a	9.16b	9.02b	0.21
6	9.15a	9.32a	9.09a	8.82b	0.26

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

‡NS = Non-Significant

Table A6

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain CYI-64*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD†
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	8.33	8.36	8.39	8.30	NS‡
2	9.59a	9.49b	9.31d	9.39c	0.06
3	9.58a	9.37b	9.33b	9.40b	0.08
6	9.44a	9.26b	8.90d	9.03c	0.06
Illinois No. 6 Coal					
0	10.4	10.3	10.4	10.3	NS
1	9.97b	10.0ab	10.1a	9.95b	0.13
2	10.1b	10.3a	9.52c	10.2b	0.13
3	10.4a	10.5a	10.4a	10.2b	0.12
7	9.77b	9.48c	10.23a	9.88b	0.11

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

‡NS = Non-Significant.

Table A7

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain DC625*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD†
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	8.34ab	8.48a	8.34ab	8.20b	0.18
1	ND§	8.58a	8.66a	8.36b	0.16
2	8.81b	8.50c	9.08a	8.72b	0.12
3	8.75b	8.50c	8.99a	8.65bc	0.18
7	8.59a	8.23b	8.49a	8.30b	0.18
Illinois No. 6 Coal					
0	5.69	5.33	8.30	5.49	NS‡
1	8.22a	8.05b	8.10b	8.05b	0.07
2	8.53a	8.30c	8.47b	8.50ab	0.05
3	8.75ab	8.49c	8.63bc	8.85a	0.15
6	8.71a	8.11b	8.52a	8.75a	0.28

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

‡NS = Non-Significant.

§ND = No Data.

Table A8

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain NAR30*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD†
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	8.32ab	8.27a	8.40ab	8.19b	0.16‡
1	8.69a	8.44b	8.62a	8.08c	0.13
2	9.04a	8.49c	8.91b	8.24d	0.12
3	9.09a	8.45c	8.98b	8.10d	0.09
7	8.50a	8.25b	8.61a	8.04c	0.17
Illinois No. 6 Coal					
0	8.41	7.74	7.93	8.00	NS§
1	8.65b	8.67ab	8.57b	8.79a	0.13
2	8.75	8.89	8.94	8.86	NS
3	8.90a	8.38b	8.41b	8.72a	0.22
7	8.36	8.40	8.61	8.39	NS

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

‡Significant at $P \leq 0.10$.

§NS = Non Significant.

Table A9

Coal Toxicity of Illinois No. 5 and No. 6 Coals on Strain NAR41*

Incubation Time (days)	Count, Log_{10} mL^{-1}				
	Control	%Coal			Cell Count LSD†
		0.1	1.0	10.0	
Illinois No. 5 Coal					
0	8.44a	8.16b	8.36a	8.34ab	0.19
1	8.39ab	8.19b	8.45a	8.49a	0.24‡
2	8.40b	8.50b	8.47b	8.73a	0.15
3	8.35bc	8.62a	8.24c	8.52ab	0.24
7	7.80ab	7.49b	8.13a	8.09a	0.52‡
Illinois No. 6 Coal					
0	7.56	7.58	7.47	7.78	NS§
1	8.45a	8.56a	8.54a	8.34b	0.19
2	8.66	8.75	8.63	8.57	NS
3	8.67	8.72	8.74	8.60	NS
6	8.06d	8.77a	8.63b	8.19c	0.08

*Counts were made on glucose-yeast extract-peptone agar (1%, 0.3%, and 0.3%, respectively). Plates were incubated at room temperature for 3 to 5 days.

†Means in the same row followed by the same letter (left to right) are not significantly different ($P \leq 0.05$) according to the Least Significant Difference (LSD) test.

‡Significant at $P \leq 0.10$.

§NS = Non-Significant.