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INTERACTIONS OF SELECTED BACTERIAL ISOLATES WITH DBT AND SOLUBILIZED COAL

Diane H. Key, Robert V. Fox, Reggie S. Kase, Michelle S. Willey,
Daphne L. Stoner and Thomas E. Ward

Biotechnology
Idaho National Engineering Laboratory
Idaho Falls, ID 83415

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ABSTRACT

We are studying the interactions of isolated bacteria with dibenzothiophene (DBT), a sulfur-containing model compound, and with a solubilized coal product derived from a high-organic-sulfur lignite. The sensitivity of the tetrazolium assay used to identify and study these strains was improved by substituting tetrazolium violet for triphenyltetrazolium. DBT metabolism by thirteen strains was investigated using qualitative and quantitative GC and GC-MS analyses. Growth medium and incubation time affect the extent of DBT degradation and the production of DBT metabolites. Under specific conditions, seven of the strains produce metabolites which elute close to the position of one or another of the biphenyl standards. However, when these samples are spiked with the standard compounds, the bacterial metabolites do not co-elute with the standards. The modification of solubilized high-organic-sulfur coal by six of these strains was also studied. No selective removal of sulfur relative to carbon was observed.

INTRODUCTION

We have previously identified a number of strains that can metabolize the model compound dibenzothiophene (DBT), using an assay based on the oxidation/reduction indicator dye triphenyltetrazolium chloride (1,5). Approximately 25 DBT degrading strains were identified using this assay. All of the strains are gram-negative rods, and API tests indicated that the majority of the strains are pseudomonads, with several falling in the fluorescent pseudomonad group (5). Our goal is to use these strains, or components derived from them, to remove organic sulfur from coal. Biological removal of sulfur from coal may offer advantages

over chemical and physical methods in terms of capital investment, operating costs and lowered environmental pollution, since the processes take place at ambient temperature and pressure and do not involve continuous addition of hazardous and costly materials.

The objective of the present set of experiments was to investigate the various metabolites produced from DBT by these strains and to correlate DBT metabolism with ability to remove sulfur from a soluble product derived from high-organic-sulfur coal. Soluble coal retains many of the structural features of particulate coal and is more accessible to the desulfurizing activities of microbes, making it a good substrate for studying the desulfurizing capabilities of microorganisms. This report describes our research in these areas.

MATERIALS AND METHODS

STRAINS AND MEDIA

Three basic media were used: HS, SDP and M9. HS contained: Na_2HPO_4, 5.24 g/l; KH_2PO_4, 2.77 g/l; NH_4Cl, 1.0 g/l; $MgSO_4 \cdot 7H_2O$, 0.25 g/l; and yeast extract, 0.25 g/l; pH 7.0 (12). M9 contained: Na_2HPO_4, 6.0 g/l; KH_2PO_4, 3.0 g/l; NH_4Cl, 1.0 g/l; $MgSO_4$, 2.0 mls of a 1M solution; glucose, 10.0 mls of a 20% solution; $CaCl_2$, 0.1 ml of a 1M solution; pH 7.4. SDP has been previously described (11). For solid media, 15 g/l of agar was added. Dibenzothiophene was dissolved at a concentration of 0.5% in acetone or chloroform and sprayed on previously poured plates. The acetone/chloroform was allowed to evaporate, leaving the DBT as a crystalline sheen on the surface of the plate. All chemicals were reagent grade. Water from a Barnstead Nanopure unit was used throughout.

TETRAZOLIUM ASSAY FOR DBT METABOLISM

The assay using triphenyltetrazolium (TTC) to identify DBT degrading bacteria has been previously described (5). To test whether different tetrazolium compounds could increase the sensitivity/discrimination of the assay, blue tetrazolium,

iodonitrotetrazolium, MTT tetrazolium, and tetrazolium violet were tested at a concentration of 0.025 gm/l. Effects of varying the yeast extract concentration on the color response of the assay were also examined.

QUANTITATIVE ASSAY FOR DBT DEGRADATION AND METABOLITE PRODUCTION

DBT was dissolved in chloroform at a concentration of 0.5%, 100 μl was transferred to a 30 ml Corex screw-cap tube, and the chloroform was allowed to evaporate. Five mls of cells, grown overnight in the absence of DBT and resuspended in fresh medium to an $O.D._{595}$ of 0.3, were added. The tubes were shaken on a roller drum (Bellco, 50-60 RPM) at 30°C. After incubation for various times, 100 μl of 0.5% thianthrene in chloroform was added. The mixture was extracted three times with 2 mls of chloroform, the extracts combined, and 1.0 ml of the combined extracts placed in a vial containing 10 μl of 1.0% dimethylnaphthalene.

GAS CHROMATOGRAPHY

The samples were run on a Hewlett-Packard Model 5890A gas chromatograph equipped with a Model 7673A autosampler, a J & W Scientific DB5 column (30 meters) and an FID detector. Carrier gas was helium with a column flow rate of 11 mls/min. The temperature program involved a double ramp starting at 170°C for 4 minutes, followed by an increase to 205°C at 5°C per minute, and then by an increase to 212°C at 2°C per minute, with a 1.25 minute hold at 212°C. Finally, there was an increase to 270°C at 30°C per minute and a 10 minute hold at 270°C. The injector temperature was 260°C, and the detector was set at 290°C. Under these conditions biphenyl, dimethylnaphthalene, 2-hydroxybiphenyl, 2,2'-dihydroxybiphenyl, DBT and thianthrene eluted at 2.42, 2.64, 3.65, 5.65, 7.50 and 11.36 minutes respectively. Various control tubes contained all components except cells or DBT. The areas under the DBT peaks were corrected for variations in injection volume, as reflected in the areas of the dimethylnaphthalene peaks, and for variations in extraction efficiency, as reflected in the areas of the thianthrene peaks. The percent DBT remaining was calculated relative to uninoculated controls. The amount of metabolites or presumed metabolites produced was determined from standard curves produced from samples containing known amounts of the metabolites.

EXPERIMENTS ON SOLUBILIZED COAL

Mequinenza lignite was provided by Dr. R. Menendez, Instituto Nacional de Carbon y sus Derivados, Oviedo, Spain. Soluble coal was prepared by stirring 20 g of coal (<200 mesh) in 10 l of 200 mM NaOH for one month at room temperature. After treatment, the particulate coal was allowed to settle. The liquid was decanted, centrifuged to remove solids, and filtered through a 0.5μm nitrocellulose filter (Millipore Corp.). The soluble coal was precipitated by adjusting the pH to 2.5 with concentrated phosphoric acid and collected by centrifugation at 25,000XG for 20 minutes. The precipitate was washed with dilute phosphoric acid and lyophilized. The dry powder was redissolved in growth medium at a concentration of 2.5 mg/ml (0.25%). Bacterial cultures grown overnight at 30°C were harvested by centrifugation and resuspended in 100 ml of the growth medium/soluble coal solution to give an $O.D._{595}$ of 0.1. Cultures were incubated with shaking (150 rpm) at 30°C and sampled at 0, 2, 7, and 14 days. To prepare samples for analysis, aliquots were centrifuged, the supernatant filtered through a 0.2μm filter to remove cells, and the soluble coal recovered by acid (H_3PO_4) precipitation and centrifugation. Precipitated material was washed with water and lyophilized. The recovered treated coal was analyzed for elemental composition using a Model EA 1108 Elemental Analyzer (Carlo Erba).

RESULTS AND DISCUSSION

1. IMPROVEMENT OF TETRAZOLIUM ASSAY FOR DBT DEGRADING ACTIVITY

Previously, a technique based on the oxidation/reduction indicator dye triphenyltetrazolium chloride (TTC) was adapted to identify environmental isolates capable of metabolizing DBT (1,5). In this assay, DBT metabolizing colonies are pinkish in the presence of DBT and white in the absence of DBT, presumably due to extra metabolic energy produced by degradation of the DBT. The color difference in this assay is subtle. If it is to be used in experiments involving mutagenesis and cloning by complementation, where one must be able to distinguish a small number of cells with one phenotype from a majority of cells with the opposite phenotype, the sensitivity/discrimination of the assay must be improved. To that end, four tetrazolium compounds were substituted for

TTC as described in Materials and Methods. The results showed that the clearest color difference between growth in the presence and absence of DBT was obtained with tetrazolium violet. The optimal yeast extract concentration varied from strain to strain but was either 0.25 or 0.5 gm/L.

2. USE OF GAS CHROMATOGRAPHY TO INVESTIGATE DBT DEGRADATION AND METABOLITE PRODUCTION

Thirteen of the originally isolated candidate strains were analyzed for the extent of DBT degradation and type of metabolite produced (partial data-Table 1). It can be seen that different media have a significant effect on the extent of DBT degradation and the production of various metabolites. In general, the poorer media, using glycerol or succinate, result in increased degradation of the DBT. We have not been able to discern a pattern for medium effects on the production of metabolites. Seven isolates, DK 1, 2', 5', 6', 14', 19 and 22, produced significant metabolite peaks that eluted close to the position of one or another of the biphenyl standards on GC analysis. Unfortunately, none of these metabolites co-eluted with those standards when the samples were spiked with them. Recent GC-MS analyses of a few of these spiked samples show that these bacterial metabolites are not related to biphenyl (Dr. Malvina Farcasiu, Pers. Comm.).

A significant amount of work has been done on bacterial metabolism of DBT (2-10). However, most of the interactions have not been the type that would be useful for coal desulfurization, since the attack on DBT involves side ring cleavage and not release of sulfur from the molecule. The most reliable report of a bacterial strain with this latter capability is that of Dr. John Kilbane, who has reported isolating a strain of Rhodococcus with the ability to desulfurize DBT, using an alternative isolation technique of selection in sulfur limited medium supplemented with DBT (6).

3. INVESTIGATION OF BACTERIAL INTERACTIONS WITH SOLUBILIZED COAL

Six of the DBT degrading isolates, DK 1, 2', 5', 6', 19 and 22, were examined for their ability to modify the organosulfur component of a water soluble coal

product derived from Mequinenza lignite. Compared to particulate coal, soluble coal increases the accessibility of the organosulfur moieties to the bacteria and simplifies the experimental procedure, yet still presents bacteria with the complexity and variety of sulfur forms present in coal. Mequinenza lignite solubilized by alkaline treatment, contains approximately 9% organic sulfur. The elemental analysis of the solubilized material was very similar to the starting material, except for the ash component. Sulfur form analysis indicated that this soluble coal product is essentially free of sulfate (0.17%) and pyritic sulfur (<0.01%).

Elemental analysis of the soluble coal product after incubation with the six isolates showed that the carbon/sulfur ratios do not change significantly after bacterial treatment, and thus that these strains did not modify the organosulfur component of soluble coal (Table 2). These results are consistent with previous results which indicated that such bacteria are unable to modify a soluble coal product derived from Illinois No. 6 coal (13).

There could be a number of explanations for the fact that no effect on the organosulfur component of solubilized coal was observed. The culture conditions utilized may not have been correct. For instance, medium composition had a significant effect on the interaction of these bacteria with DBT (Table 1) and may also affect their ability to modify soluble coal products. The thiophenic sulfur content of this solubilized Mequinenza lignite product is not known. The abundance of thiophenic sulfur in the soluble coal product may affect the microorganisms ability to remove significant amounts of organosulfur. In addition, the appropriateness of DBT as a model compound for isolation of microorganisms capable of removing organic sulfur from coal has not been rigorously examined. Model compounds are appropriate for isolation of candidate microorganisms from environmental samples and for genetic and enzymatic studies because the substrate is defined, homogeneous and can be analyzed in detail. However, after identification of candidate microbes using model compounds, their ability to desulfurize coal should be clearly established before significant effort is expended on genetic and biochemical studies.

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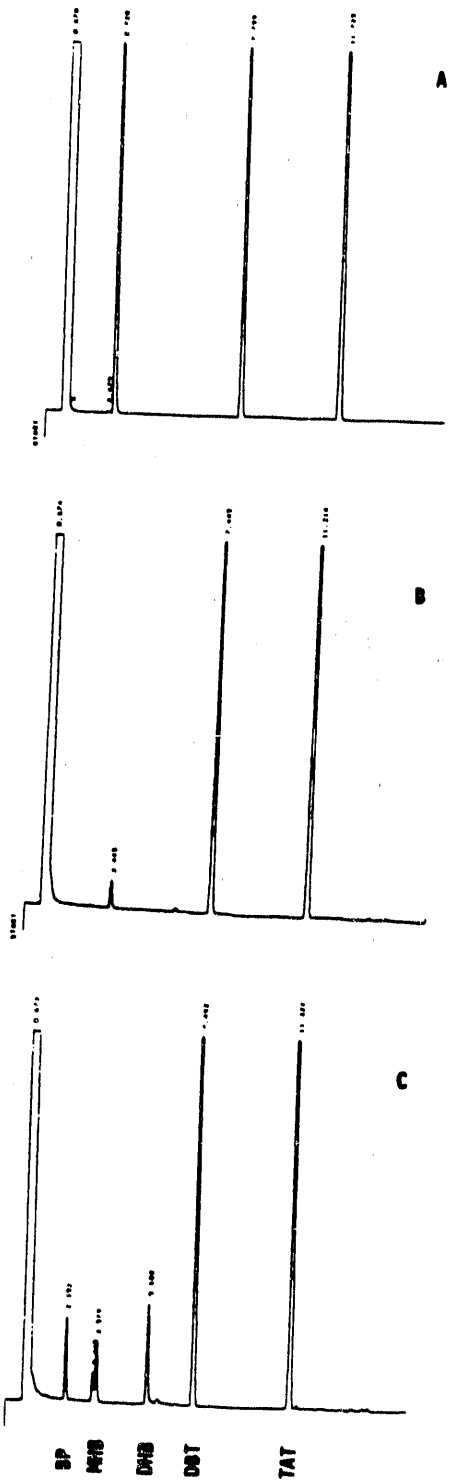

Figure 1. Gas Chromatography Analysis. Panel A: Uninoculated Control; Panel B: Strain DK6′ incubated with DBT in M9 Glycerol medium; Panel C: Same as Panel B except spiked with three biphenyl standards. BP-Biphenyl, MHB--2-hydroxybiphenyl, DHB--2,2′-dihydroxybiphenyl, TAT--thianthrene.

Table 1

DBT METABOLISM BY SELECTED STRAINS

STRAIN	SDP GLUCOSE	SDP GLYCEROL	SDP SUCCINATE
DK2'	DHB - 1 ppm 5' oxide - 1 ppm 61% DBT degraded A only - 24 hrs.	MHB - 1 ppm DHB - 3 ppm 5' oxide - 2 ppm 75% DBT degraded A & B - 24 hrs.	no metabolites seen 13% DBT degraded
	Biphenyl - 3 ppm DHB < 1 ppm 5' oxide < 1 ppm 17% DBT degraded B only - 24 hrs	DHB - 1 ppm 5' oxide - 1 ppm 42% DBT degraded A & B - 24 hrs.	
	MHB - 2 ppm 5' oxide < 1 ppm 40% DBT degraded A & B - 24 hrs.		
DK19	biphenyl - 3 ppm DHB - 1 ppm 38% DBT degraded A & B 24 hrs.	biphenyl - 4 ppm < 10% DBT degraded A only - 4 hrs.	no metabolites seen > 90% DBT degraded
		biphenyl - 1 ppm MHB < 1 ppm 31% DBT degraded A & B - 24 hrs.	
DK22	biphenyl - 1 ppm MHB < 1 ppm DHB - 1 ppm 5' oxide - 1 ppm 49% DBT degraded A & B - 24 hrs.	biphenyl - 1 ppm MHB < 1 ppm DHB - 2 ppm 5' oxide - 1 ppm > 99% DBT degraded A & B - 24 hrs.	5' oxide - 3 ppm 97% DBT degraded A & B - 24 hrs.
	MHB - 1 ppm DHB - 3 ppm 5' oxide - 2 ppm 32% DBT degraded A & B - 24 hrs.	DHB < 1 ppm 16% DBT degraded A & B - 4 hrs.	
		DHB - 1 ppm 5' oxide - 1 ppm 42% DBT degraded A & B - 24 hrs.	

MHB, DHB, 5' oxide--Metabolite peak observed eluting close to the position of the standard 2-hydroxybiphenyl, 2,2'-dihydroxybiphenyl or dibenzothiophene-5-oxide; A & B--Metabolite appeared in both of the duplicate cultures.

Table 2

C/S RATIOS OF BIOLOGICALLY TREATED SOLUBLE COAL

STRAIN/MEDIUM	DAY 0	DAY 2	DAY 7	DAY 14
CON M9	6.39	6.64	6.34	6.25
DK1 M9	6.45	6.25	6.43	6.40
DK5′ M9	6.18	6.68	5.66	6.35
DK6′ M9	6.25	6.25	6.37	6.45
CON SDP	6.38	7.90	7.40	6.61
DK2′ SDP	6.88	6.28	6.54	6.68
DK19 SDP	6.77	7.58	6.67	6.58
DK22 SDP	6.41	6.36	6.28	6.49

CON--Uninoculated control

END

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