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MICROBIAL EXTRACTION OF SULFUR FROM MODEL COAL ORGANOSULFUR COMPOUNDS

ABSTRACT

Several hundred bacterial cultures isolated from a variety of natural sites were screened for their ability to desulfurize the model coal organosulfur compounds, dibenzothiophene (DBT) and DBT-sulfone. A sulfur-stress assay, in which DBT-sulfone was the only bioavailable source of sulfur, was used to screen and select for organisms that selectively desulfurized the organic-sulfur substrate. Only two new isolates, UMX9 and UMX3, and strain IGTS-8, a *Rhodococcus rhodochrous* provided by the Institute for Gas Technology (Chicago, U.S.A.) as a reference culture, would grow on DBT or DBT-sulfone as a sole source of sulfur. Under sulfur-stress conditions, a desulfurized product identified as 2-hydroxybiphenyl (2-phenylphenol) was detected only for UMX9 and IGTS-8. Biodesulfurization activity for all three organisms occurred only for growing cultures, and was depressed by free sulfate, although more so for UMX3 and IGTS-8 than for UMX9. None of the three cultures exhibited good growth on DBT, DBT-sulfone, or 2-phenylphenol as sole sources of carbon. Taxonomic studies revealed UMX3 to be similar to IGTS-8, whereas UMX9 only exhibited *Rhodococcus*-like features. Comparative tests for carbohydrate utilization revealed that only UMX9 would grow on glucose, and that only IGTS-8 would grow on L-arabinose. Assays of biodesulfurization activity as a function of temperature or pH revealed further differences between UMX9 and UMX3/IGTS-8. Under optimized assay conditions for each organism, UMX9 exhibited up to 30% greater biodesulfurization activity than did IGTS-8 and UMX3, which were similar in activity.

INTRODUCTION

Combustion of sulfur-containing fossil fuels results in the liberation of sulfoxides into the atmosphere where interactions with water produce sulfuric acids. Precipitation laden with the acids forms "acid rain", which poses a multitude of threats to the environment. The continued use of coals in general, and high-sulfur coals in particular, will require technologies designed to eliminate or reduce sulfur emissions. Two approaches can be taken: elimination of sulfur from post-combustion gases or pre-combustion coal desulfurization. The predominant forms of sulfur in coals are inorganic pyrite crystals existing as aggregates cloistered among - but not tightly bound to - the organic coal matrix, and "organic" sulfur bound to carbon atoms. The bulk of pyritic sulfur can be removed by a variety of chemical, physical, and biological technologies, but cost-effective and efficient methods for removing organic sulfur are still in developmental stages.

In recent years, there have been several indications for use of bacteria to remove organic sulfur from model coal organosulfur compounds (1-8) or even of coal itself (1,4,6,7), in a process that we will call **biodesulfurization**. A pathway was proposed (1,9) for selective enzymatic cleavage of carbon-sulfur bonds in thiophenic model compounds, with emphasis on dibenzothiophene (DBT). The proposed pathway (Fig 1) has been called the "4S" pathway in reference to a sequence of DBT sulfur oxidations via DBT-5-oxide, DBT-5-dioxide (-sulfone), 2-(2-hydroxyphenyl) benzenesulfonic acid, and liberation of free sulfate ion, resulting in the desulfurized product, 2,2'-dihydroxybiphenyl (2,2'-biphenol). A variant of the 4S pathway

Fig. 1. Representation of proposed "4S" pathway whereby dibenzothiophene is oxidatively biodesulfurized. Either one (or both?) of 2,2'-biphenol or 2-phenylphenol are possible products.

leading to 2-hydroxybiphenyl (2-phenylphenol) has been reported by Kilbane (1). Another possible pathway of biodesulfurization was reported (5,10) in which ring cleavage accompanies or follows biodesulfurization, with benzene and derivatives occurring as transient intermediates. For effective biodesulfurization of fuel coals, a desired activity would be specific cleavage of carbon-sulfur bonds. The fact of attendant ring cleavage of model compounds might be inconsequential because such compounds probably do not exist in coal. For example, a microorganism that removes sulfur from DBT along with degradation of the parent substrate might attack only carbon-sulfur bonds in coal, if the desulfurization activity is independent of carbon-carbon bond cleavage. A catalyst system that specifically cleaves carbon-sulfur bonds might be used effectively to desulfurize coal without unacceptable loss of organic carbon. Thus, the use of model compounds represents one approach by which potential coal bio- desulfurizers can be obtained and characterized for further testing on coals, and possible genetic-engineering applications. We note that the cultures reported by Kilbane (1,2) to biodesulfurize coals are active desulfurizers of model compounds.

Our earlier reports (11,12) established that numerous microorganisms are capable of non-desulfurizing, ring-cleavage attack on DBT ("Kodama"-type pathway [13]), whereas others removed DBT-sulfone from solution without production of detectable desulfurized products, thus without confirmed biodesulfurization.

Our goal was to obtain a variety of naturally occurring bacterial cultures capable of desulfurizing model coal organosulfur compounds, evidenced by ability to utilize such compounds as sole source of sulfur. We report here the results of our tests on a variety of microbial cultures for specific biodesulfurization activity and the confirmed biodesulfurization activity of two new microbial isolates, UMX3 and UMX9.

EXPERIMENTAL

Organisms

We derived new cultures (usually mixed) from source samples collected from fresh or weathered, crude or refined oils (mixed with soils or aquatic habitats), activated sludge, weathered coals, and about 150 isolates collected by Dr. Al Mikell (University of Alabama, University of Mississippi) from a variety of superfund sites. We also obtained from culture collections other bacteria already reported to exhibit biodesulfurization activity. Professor Steven Krawiec, Lehigh University, U.S.A., provided cultures A4, B24, C18, C1, C2, D20, E1, F14, and F23, whereas Dr. John J. Kilbane, Institute of Gas Technology, Chicago, U.S.A. provided culture IGTS-8. Taxonomic studies were by standard methods for microbial identification and by the computerized MIDI Microbial Identification System referenced to software libraries of fatty-acid profiles of known strains.

Screening for Biodesulfurization Activity

For standard screening, we used a sulfur-stress assay in which the only available sulfur was organic (adapted from the method of Kilbane [1]). The sulfur-stress medium consisted of sulfur-free mineral salts and vitamins plus DBT or DBT-sulfone as sole sulfur source. The defined, sulfur-free medium was the "21C" medium (14) in which all sulfate salts were replaced with chloride salts; pH was adjusted to 7.0. Filter-sterilized glucose (0.5% wt/vol) and glycerol (0.5% vol/vol) were added to the autoclaved and cooled medium. When used, crystals of DBT (Aldrich, U.S.A.) were added as 1 mg/ml medium (equivalent to 0.05% if completely soluble). Deionized water (reverse osmosis water passed through Nanopure II (Barnstead, U.S.A.) cartridges to yield 16 megohm) was used for all preparations. We washed assay glassware with HCl to eliminate residual sulfur, which did not exceed 1.0 ppm in the sulfur-stress medium. (Sulfate analyses were made using a Dionex Model 2000i ion chromatograph) Saturated aqueous solutions containing 0.16 mM DBT-sulfone were prepared by adding an excess of DBT-sulfone (Aldrich, U.S.A.) crystals to deionized water and allowing same to equilibrate at 30° C overnight. The DBT-sulfone-saturated solution was used to avoid interferences in direct measurements of O.D. and absorbance caused by excess, undissolved crystals of organic-sulfur substrates (DBT-sulfone has higher water-solubility than does DBT).

A standard formulation of the 21C medium would contain up to 2.5 mM sulfate. We considered the sulfur-stress medium at beginning of culture to represent a sulfur-sufficient growth environment; that is, there was potentially sufficient organic sulfur available initially to support good growth of any organism capable

of efficient biodesulfurization. Thus, the assay environment would be one of sulfur-stress because the usually available sulfate would be absent, thus stress-selective for biodesulfurizers.

The sulfur-stress assay consisted of sets of three different preparations of 10 ml nutrient medium in culture tubes. SET 1 contained sulfur-stress medium plus organic sulfur substrate plus cells; SET 2 contained medium plus cells but no source of sulfur; SET 3 contained medium plus organic sulfur substrate plus sulfate (0.16 mM). Growth was measured as increase in optical density (O.D.) at 660 nm. Growth in SET 1 tubes compared to little to no growth in SET 2 vs. abundant growth in SET 3 represented presumptive positive biodesulfurization activity with minimal inhibition by the organic-sulfur substrate.

We isolated new cultures from source samples by plating dilutions of same onto Tryptic Soy agar (TSA; Difco, U.S.A.) and incubating at 30° C. Suspensions of source-sample consortia or pure cultures then were transferred to TS broth (12 g/l) and incubated for 48-72 h, after which they were washed twice in several volumes of 0.01 N phosphate buffer and resuspended in sulfur-stress medium to yield an initial O.D. of < 0.02. Cultures that exhibited sustained and healthy growth on DBT-sulfone as sole sulfur source after at least three transfers we considered to be biodesulfurizers. For tests on biodesulfurization of different organic-sulfur substrates, 0.5% wt/vol ethanol, which supported good growth of each culture and increased solubility of the organic sulfur substrates, replaced glycerol and glucose.

Characterization of Biodesulfurization Activity

We used time-course assays to characterize and compare biodesulfurization activity among active cultures. At each 20 h increment up to 140 h of incubation under sulfur-stress conditions, samples were withdrawn for analysis from five replicates each of each set of tubes; data were calculated as means of the five replicates for each time point.

For monitoring and quantifying DBT, DBT-sulfone, and 2-phenylphenol, we used gas chromatography and UV spectroscopy. For GC analysis, we acidified suspensions with HCl then extracted twice with one-half volume dichloromethane, after which the aqueous phase was removed with Whatman IPS phase-separation paper. The pooled extracts were transferred to 50 ml wide-mouth jars, allowed to evaporate, then redissolved in 1 ml of dichloromethane. Thianthrene was added as an internal standard. Our extraction efficiencies were about 80% for DBT-sulfone, 83% for 2-phenylphenol, and 93% for DBT. Confirmation of chemical identities (for GC peaks having retention times coinciding with standards) was by GC/mass spectrometry incorporating a commercial software library of chemical spectra as well as a custom library of our standards.

For some assays, we used UV scans in the range of 200-400 nm to monitor and quantify either DBT-sulfone or 2-phenylphenol. This method allowed a direct measurement without problems associated with extractions and sample handling. The instrument was a Milton Roy Model 1201 coupled with computerized SpecScan software for analysis of absorbance data. Samples (1.5 ml) were drawn from sulfur-stress culture tubes and centrifuged for 10 min at 10,000 x g to yield an optically clear supernatant for direct absorbance readings and quantification from standard curves.

We prepared resting-cell suspensions by growing each organism to stationary phase, centrifuging at 15,000 x g for 20 min, washing with 0.02 N phosphate buffer, centrifuging again, then resuspending in sulfur-free medium and incubated for 24 h to expend carryover reserves. Ten milliliters of resting-cell suspension (0.45-.55 O.D.) was layered onto crystalline DBT-sulfone in culture dishes. The DBT-sulfone plates were prepared by spraying 1 ml of a 2.5 mg/ml ethyl ether solution per plate; evaporation resulted in a finely dispersed coating of DBT-sulfone. After incubation, the cultures were extracted and analyzed as described for gas chromatography.

RESULTS AND DISCUSSION

Of several hundred cultures tested, only two new isolates (designated UMX3 and UMX9) exhibited sustained growth on DBT or DBT-sulfone as sole sulfur source. Most other cultures tested would not grow upon initial inoculation of the sulfur-stress medium; a few would grow weakly upon the first inoculation, but did not grow after the second or third transfer. We attribute the initial but unsustained growth of some

cultures to utilization of carryover sulfur coupled perhaps with low sulfur requirements or efficient uptake mechanisms.

Isolates UMX3 and UMX9, both gram-positive (or variable) coccoid rods, continued growth after repeated transfers on sulfur-stress medium, as did IGTS-8. None of the three organisms grew on sulfur-free medium (SET 2 tubes), and each, by estimate of turbidity, grew as well on organic sulfur only (SET 1 tubes) as on organic sulfur plus added sulfate (SET 3 tubes). Cultures of UMX3 and UMX9 were isolated from a consortium of six different bacteria, which together - but not in individual cultures - would grow on DBT or DBT-sulfone as sole source of sulfur. We presume that the (predominant) non-desulfurizing consortium members were growing at the expense of sulfur liberated by UMX3 and UMX9, neither of which existed in conspicuous numbers, and were isolated only after repeated subculturing of dilutions onto sulfur-stress media. We considered UMX3, UMX9, and IGTS-8 to exhibit true biodesulfurization and therefore focused further work on these three organisms.

As a function of time of incubation, disappearance of DBT-sulfone occurred as cell density increased for all three cultures. Figures 2-4 contain representative time-course assays of growth, disappearance of DBT-sulfone, and appearance of 2-phenylphenol for each of the three organisms assayed under sulfur-stress conditions. Both UMX9 and IGTS-8 produced 2-phenylphenol (2-hydroxybiphenyl) from DBT or DBT-sulfone. Identity of 2-phenylphenol was confirmed by GC/MS. All three organisms generated other, DBT-sulfone-dependent, unidentifiable products, but none produced colored, "Kodama"-type products from either DBT or DBT-sulfone. No 2-phenylphenol was detected for UMX3, although disappearance of DBT-sulfone tracked that of IGTS-8. Only growing cultures exhibited biodesulfurization; washed, resting cells did not remove DBT or DBT-sulfone from solution after over 140 h of incubation, and no products were detected. Representative amounts of DBT-sulfone removed (mM), 2-phenylphenol produced (mM), and cell density (O.D.) reached by each organism after 140 h incubation were respectively, UMX9: (0.12), (0.06), (0.4); IGTS-8: (0.10), (0.05), (0.27); UMX3: (0.12), (0.0), (0.27). For UMX9, disappearance of DBT-sulfone and appearance of 2-phenylphenol diminished after 70-80 h of incubation, compared to steadily increasing rates for IGTS-8. We note also that the amounts of 2-phenylphenol generated by UMX9 were about one-half that of DBT-sulfone removed from solution, whereas for IGTS-8, closer to equimolar amounts of substrate removed and product produced were detected. In the absence of cells, no changes in 2-phenylphenol or DBT-sulfone occurred.

After about 100 h of incubation, sulfur became limiting for all three organisms (but more so for UMX3 and IGTS-8 than for UMX9). In separate tests of sulfur-dose responses, growth was greater beyond 100 h in the presence of 2.0 mM vs. 0.16 mM sulfate. At the beginning of incubation sulfur was in excess, as available from DBT-sulfone, thus the data of Figs. 2-4 provide information on biodesulfurization activity under both sulfur-sufficient and sulfur-deficient conditions. When sulfur became limiting, rate of desulfurization of DBT-sulfone apparently increased for both UMX3 and IGTS-8, but almost ceased for UMX9. We are not now able to explain this apparent anomaly.

When we added 0.166 mM MgSO_4 to SET 1 tubes (also containing 0.166 mM DBT-sulfone), UMX3 and IGTS-8 removed only about 0.018 mM DBT-sulfone during the first 20 h, after which activity ceased for the duration of the 140 h incubation (Figs. 5-7). Under the same conditions, UMX9 removed about 0.076 mM DBT-sulfone during 140 h, with an initial (20 h) rate equal to that in the absence of sulfate (Figs. 2-4), followed by a slower but constant rate of removal. We were not able to detect 2-phenylphenol for any of the cultures. Because the inocula were taken from actively desulfurizing cultures, it is possible that the initial rates of DBT-sulfone removal were due to residual catalyst carried over in the inoculum. We interpret these data to mean that free sulfate blocks desulfurization of DBT-sulfone by UMX3 and IGTS-8, and at least suppresses activity for UMX9. Moreover, the data also indicate that neither UMX3 nor IGTS-8 attack DBT-sulfone other than by specific carbon-sulfur bond cleavage under sulfur-stress conditions. However, the continued removal of DBT-sulfone without production of 2-phenylphenol by UMX9 indicated other mechanisms of activity, which might include nondesulfurizing ring degradation. If such other mechanisms of DBT-sulfone removal also occur during desulfurization under sulfur-stress conditions, then amounts of 2-phenylphenol produced would be less than DBT-sulfone removed, just as we observed (Fig. 2-4) for UMX9. Indeed, the total DBT-sulfone removed under 140 h, sulfur-stress conditions (about 0.12 mM) approximated the combined amounts (about 0.13 mM) of 2-phenylphenol produced (about 0.06 mM) under sulfur-stress plus DBT-sulfone removed (about 0.07 mM) with sulfate present (Figs. 5-7). We therefore suggest that for UMX9, at least two mechanisms of attack on DBT-sulfone occur concurrently under sulfur-stress conditions;

one being a sulfur-specific attack on carbon-sulfur bonds, another being by unknown mechanisms whereby the substrate is removed from solution. Only growing cultures removed DBT-sulfone from solution; resting-cell suspensions did not alter DBT-sulfone concentrations, did not grow, and did not produce detectable products from DBT-sulfone.

Growth for all three cultures in the presence of both sulfate and DBT-sulfone (Fig. 5-7) was about the same as that when only DBT-sulfone provided sulfur (Figs. 2-4). It is apparent that the efficiency of bioextraction of sulfur from DBT-sulfone was sufficient to overcome sulfur-limited growth. It also is apparent that DBT-sulfone - at 0.166 mM - was not inhibitory to growth of any of the organisms.

In companion experiments, either DBT-sulfone (0.16 mM, without added sulfate) or 2-phenylphenol (0.16 mM, plus 0.16 mM sulfate) was supplied as sole source of organic carbon to each of the three organisms. Only negligible amounts (< 0.02 mM) of 2-phenylphenol disappeared (within the first 20-40 h of 140 h incubation) from solution in all three cultures. Kilbane (16) noted that 2-phenylphenol became associated with cells, resulting in misleading concentration measurements. It is possible that our observed slight reductions in 2-phenylphenol were due to non-metabolic cell-substrate interactions. Between 0.04 and 0.05 mM DBT-sulfone was removed in all cultures. Growth also was negligible for all three cultures on 2-phenylphenol or DBT-sulfone: 140 h O.D. = 0.10-0.12, about equal to that without added organic carbon, in which case residual organic carbon apparently allowed for some growth. The removal of up to 0.05 mM DBT-sulfone by the low-density cultures indicates some activity on the substrate, which apparently did not support growth.

We were unable to detect changes in free sulfate concentrations either during or after 140 h incubation (stationary phase) of each organism under sulfur-stress conditions with DBT-sulfone as sulfur source. van Afferden et al. (5) reported on the concomitant production of sulfite and sulfate and biodesulfurization of DBT by *Brevibacterium* sp. DO, which apparently also degraded and used the DBT as carbon, energy, and sulfur source. We presume that for UMX3, UMX9, and IGTS-8, as sulfur was liberated, it became incorporated into cellular biomass. We consider the biodesulfurization activity to be highly responsive to metabolic sulfur requirements. Under sulfur-stress conditions, expression of biodesulfurization reactions would provide sulfur (presumably as sulfate) to growing cultures. The absence of excess sulfur (measured as sulfate) after stationary phase (and the absence of biodesulfurization by resting cells, even without free sulfate) indicates a catalytic system operative only during cellular growth. Kilbane (16), however, reported on active biodesulfurization by non-growing (pregrown) cell suspensions of IGTS85, a derivative of IGTS-8; IGTS85 also actively desulfurized DBT in the presence of high levels of sulfate.

Under sulfur-stress conditions, each of the organisms would grow on DBT-5-oxide, methylene blue, 3-carboxythiophene, benzothiophene, thianaphthene, and trithiane as sole sulfur sources. None of the organisms would utilize sulfur from either 3-aminophenylsulfone or diphenyl sulfide.

Thirty-four of the forty-eight tested carbohydrates would not support growth of any of the three organisms. Twelve substrates were utilized by all three organisms. Differences in utilization occurred for two substrates: only UMX9 would grow on D-glucose, whereas only IGTS-8 would grow on L-arabinose. We consider carbohydrate utilization pathways to be independent of biodesulfurization reactions, thus the data indicate fundamental differences among the three organisms. Although IGTS-8 and UMX3 appear identical morphologically, the differences in utilization of L-arabinose and production of 2-phenylphenol from DBT-sulfone must be taken into account. Kilbane (15) identified strain IGTS-8 as *Rhodococcus rhodochrous*. Our MIDI analyses yielded the best similarity index of only 0.221 for UMX3 with *R. rhodochrous* (an index of >0.5 would indicate a good match). There was no numerical match with any library listing for UMX9, although its pink colonies and general morphology indicate a *Rhodococcus*-like organism.

Both UMX3 and IGTS-8 gave significantly (0.05% level, ANOVA) greater desulfurization of DBT-sulfone at (initial) pH 6.0 vs. pH 7 or 8 (similar profiles), whereas UMX9 gave best activity at pH 7.0. Figure 3 contains profiles of growth and biodesulfurization activity as functions of temperature. Under optimum conditions for each organism, UMX9 consistently exhibited CA. 30% greater biodesulfurization than did either UMX3 or IGTS-8.

From our experiences with the many natural samples screened, we must conclude that the variety of biodesulfurizing bacteria in nature is limited. Such organisms would have a competitive advantage in environments where inorganic sulfur is growth limiting, but in which organic sulfur is available. However

sulfur probably rarely is growth limiting in nature, therefore one would not expect to encounter many habitats favoring biodesulfurization. Our initial aim was to establish a culture collection of biodesulfurizing bacteria, with focus on the proposed 4S mechanisms of DBT/DBT-sulfone desulfurization resulting in the product, 2,2'-dihydroxybiphenyl. We were unsuccessful in obtaining a variety of biodesulfurizing cultures. Indeed, both of our active isolates appear to be species of *Rhodococcus*, just as is IGTS-8, and the only biodesulfurized product that we identified was 2-hydroxybiphenyl, the same as produced by IGTS-8. However, we have been encouraged by the recent report by Krawiec (16) who described new biodesulfurizing isolates that also produce 2-hydroxybiphenyl from DBT.

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Fig. 2. Time-course assay of UMX3 growing on DBT-sulfone as a sole source of sulfur. Reaction conditions: 30° C, pH 7.0, sulfur-stress medium plus glucose and glycerol, static cultures in tubes. DBT-sulfone was monitored by UV absorbance; 2-phenylphenol by gas chromatography. Data points are means of five replicates; the error bar for O.D. of UMX3 at 140 h represent maximum standard error (e.g., no S.E. exceeded ± 0.01 units for any parameter measured).

Fig. 3. Time-course assay of UMX9 growing on DBT-sulfone as sole source of sulfur. All parameters as for Fig. 2.

Fig. 4. Time-course assay of IGTS-8 growing on DBT-sulfone as sole source of sulfur. All parameters as for Fig. 2.

Fig. 5. Time-course measurement of biodesulfurization of DBT-sulfone by UMX3 under non-sulfur-stress conditions. (0.16 mM sulfate added to sulfur-stress culture medium). Data points are means of five replicates; no S.E. exceeded ± 0.0079 (140 h O.D.). Reaction conditions as for Fig. 2.

Fig. 6. Time-course measurement of biodesulfurization of DBT-sulfone by UMX9 under non-sulfur-stress conditions. All parameters as for Fig. 2 and 5.

Fig. 7. Time-course measurement of biodesulfurization of DBT-sulfone by IGTS-3 under non-sulfur-stress conditions. All parameters as for Fig. 2 and 5.

Fig. 8. Biodesulfurization of DBT-sulfone as a function of temperature. Reaction conditions as for Fig. 2 for 100 h cultures. Data are for means of four replicates.

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