Dibenzyl Sulfide Metabolism by White Rot Fungi

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Received 15 July 2002/Accepted 6 November 2002

Microbial metabolism of organosulfur compounds is of interest in the petroleum industry for in-field viscosity reduction and desulfurization. Here, dibenzyl sulfide (DBS) metabolism in white rot fungi was studied. *Trametes trogii* UAMH 8156, *Trametes hirsuta* UAMH 8165, *Phanerochaete chrysosporium* ATCC 24725, *Trametes versicolor* IFO 30340 (formerly *Coriolus* sp.), and *Tyromyces palustris* IFO 30339 all oxidized DBS to dibenzyl sulfoxide prior to oxidation to dibenzyl sulfone. The cytochrome P-450 inhibitor 1-aminobenzotriazole eliminated dibenzyl sulfoxide oxidation. Laccase activity (0.15 U/ml) was detected in the *Trametes* cultures, and concentrated culture supernatant and pure laccase catalyzed DBS oxidation to dibenzyl sulfoxide more efficiently in the presence of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) than in its absence. These data suggest that the first oxidation step is catalyzed by extracellular enzymes but that subsequent metabolism is cytochrome P-450 mediated.

Microbial metabolism of organosulfur compounds is of particular interest in the petroleum industry. To date, work has focused mainly on dibenzothiophene (DBT) due to its recalcitrance during hydrodesulfurization. While pure enzymes have been isolated and genetic systems for DBT desulfurization are relatively well known for the bacterium *Rhodococcus erythropolis* IGTS8 (17), relatively little work has been carried out with fungi. Fungi are able to metabolize a wide range of anthropogenic chemicals and hydrocarbons, including polychlorinated biphenyls (2) and polycyclic aromatic hydrocarbons, through the action of cytochrome P-450 and extracellular enzymes (3, 4, 21).

Extracellular enzymes produced by fungi have the potential to be useful biocatalysts due to their broad specificity, ability to attack high-molecular-weight substrates (8), and amenability to immobilization and chemical modification for increased solvent tolerance (20). In terms of metabolism of sulfur compounds, Schreiner et al. (24) found that a ligninase from *Phanerochaete chrysosporium* oxidizes thianthrene to thianthrene monosulfoxide. While Ichinose et al. (12) did not observe such a reaction for DBT and 4-methyl-DBT using laccase, lignin peroxidase, or manganese peroxidase from either *Trametes versicolor* IFO 30340 or *P. chrysosporium* ATCC 34541, they did find that *T. versicolor* can produce the corresponding sulfoxides and sulfones by a cytochrome P-450 mechanism.

Compared to sulfur-containing ring structures such as thiophenes and DBTs, relatively little information is available on the microbial metabolism of compounds with sulfur moieties present within alkyl chains. These structures are important as bridges in the high-molecular-weight asphaltene components of petroleum (16). Therefore, biological attack on sulfides is of

considerable interest for biological heavy-oil viscosity reduction. Bioremediation efforts directed towards Yperite [bis(2-chloroethyl) sulfide; mustard gas] contamination has prompted some work in this area. For example, IGTS8 is able to use 1-chloroethyl sulfide as the sole sulfur source (15). Rhee et al. (22) reported that the DBT-desulfurizing *Gordonia* strain CYKS1 can also use dibenzyl sulfide (DBS) as the sole sulfur source, but the intermediate metabolites were not identified. In a study on Yperite metabolism by fungi, Itoh et al. (14) reported that *T. versicolor* IFO 30340 and *Tyromyces palustris* IFO 30339 metabolize DBS to benzyl alcohol and benzyl mercaptan. Similarly, *Rhodococcus* sp. strain SY1 reportedly converts dibenzyl sulfoxide to benzyl alcohol and toluene (18).

DBS was selected as one possible type of sulfur bridge in the residual (boiling point, >525°C) fractions of heavy crude oils and bitumens. In this study, we examine the general ability of a variety of fungal strains to metabolize DBS, as well as the susceptibility of DBS to oxidation by purified laccase. In addition, the major metabolites are identified and a hypothesis put forth with respect to the involvement of both extracellular enzymes and the cytochrome P-450 system in DBS oxidation.

DBS oxidation by *Trametes trogii* and *Trametes hirsuta* at a wide range of substrate concentrations. *T. trogii* and *T. hirsuta* (University of Alberta Microfungus Collection and Herbarium, Devonian Botanical Gardens, University of Alberta, Edmonton, Alberta, Canada) were capable of oxidizing DBS at initial substrate concentrations up to 4.6 mM, the aqueous solubility limit (Fig. 1). Increasing the DBS concentration from 0.4 to 4.6 mM decreased the oxidation extent from 90 to 30% in each case. These experiments were carried out with glucose-malt-yeast extract medium (GMV) that contained, per liter of distilled water, 10 g of glucose, 3.5 g of malt extract (Difco, Detroit, Mich.), 2.5 g of yeast extract (Difco), and 0.5 g of MgSO₄ · 7H₂O. In all cases, inocula were prepared by homogenizing 1 cm² of mycelium from a potato-dextrose agar plate (Difco) in an Omnimixer (Sorvall, Norwalk, Conn.) for 5 to

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FIG. 1. Effect of DBS concentration on the extent of removal by *T. hirsuta* UAMH 8165 and *T. trogii* UAMH 8156.

10 s and growing it for 3 days at 28°C in 500-ml Erlenmeyer flasks containing 200 ml of GMY. Erlenmeyer flasks (125 ml) containing 25 ml of GMY were inoculated with 4% (vol/vol) homogenized culture and grown on an orbital shaker set to 150 rpm for 3 days prior to the addition of 100 μ l of the appropriate DBS, dibenzyl sulfoxide, or dibenzyl sulfone stock solution prepared in methanol.

All experiments were performed in triplicate, and error bars on graphs indicate standard deviations. For high-performance liquid chromatography (HPLC) analysis, phenylanthracene was added as an extraction standard and the contents of each flask were extracted by the addition of 25 ml of acetone and homogenization for 60 s. The homogenate was centrifuged (10,000 $\times g$ for 10 min), and the supernatant was analyzed by reverse-phase HPLC. A model 201TP reverse-phase C_{18} column (Vydac Inc., Hesperia, Calif.) was used with a Waters (Milford, Mass.) model 600 controller. Elution was achieved by running a gradient at 1 ml/min, starting with a 30:70 solution of acetonitrile-water, for 5 min and increasing the gradient to 95:5 by 40 min. Substrates and products were detected with a Waters model 996 photodiode array detector. DBS, dibenzyl sulfoxide, and dibenzyl sulfone all displayed absorbance maxima at 227 nm, so quantification was carried out at this wavelength. Acetone, acetonitrile, and dichloromethane (HPLC grade) were obtained from Fisher Chemicals (Fair Lawn, N.J.). Dibenzyl sulfoxide was from Fluka Chemika (Buch, Switzerland), and DBS and dibenzyl sulfone were from Aldrich Chemical Company, Inc. (Milwaukee, Wis.).

Good DBS recovery (87%) was obtained from killed and cell-free sterile controls. Two major metabolites were detected in each culture, and retention times and UV scans were used to tentatively identify these two metabolites as dibenzyl sulfoxide and dibenzyl sulfone (not shown). Other trace metabolites, eluting around benzoic acid, were present in different combinations in the various cultures. As metabolite identification was desired, an initial DBS concentration of 0.8 mM, with

which approximately 80% oxidation was achieved, was chosen for further study.

Major metabolite identification. In order to verify the identities of the major metabolites as dibenzyl sulfoxide and dibenzyl sulfone, both *T. trogii* and *T. hirsuta* cultures were incubated with 0.8 mM DBS for 48 h. Cultures (100 ml of GMY in 500-ml Erlenmeyer flasks) were extracted with dichloromethane (100 ml). Extracts were dehydrated over Na_2SO_4 and concentrated before being analyzed on a Hewlett-Packard model 5890 series II gas chromatograph with a 5970 series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific, Folsom, Calif.). The gas chromatograph temperature program used for analysis was 90°C for 2 min, followed by an increase of 15°C/min to 270°C. Retention times and mass spectra of commercially available chemical standards (data not shown) were used to positively identify the oxidation products dibenzyl sulfoxide and dibenzyl sulfone as the major metabolites. No other low-molecular-weight metabolites detected by HPLC have been identified at this time, though a compound with the same elution time and UV scan as those of benzoic acid has been observed in trace amounts.

Oxidation time course studies. With knowledge of the major oxidation products, time course studies for *T. trogii* and *T. hirsuta* were carried out and the results were compared to those for the well-characterized *P. chrysosporium* strain ATCC 24725 (American Type Culture Collection, Manassas, Va.) and two strains previously reported to metabolize DBS (14): *T. versicolor* IFO 30340 and *Tyromyces palustris* IFO 30339 (Institute for Fermentation, Osaka, Japan). Except with *Tyromyces palustris*, which did not grow well under the given conditions, DBS removal began within the first 4 to 8 h and was accompanied by the accumulation and subsequent removal of dibenzyl sulfoxide (Fig. 2). Typically, once the dibenzyl sulfoxide concentration approached 0.2 mM and began to decline, dibenzyl sulfone started to accumulate. The DBS oxidation rate was higher for *T. trogii* and *T. versicolor* than for *T. hirsuta* and *P. chrysosporium* and without a significant lag period, although, after the 7-day incubation, dibenzyl sulfoxide accumulated to a concentration of 0.3 mM in the culture medium. This result contrasted with the slower DBS oxidation by *T. hirsuta* and *P. chrysosporium*. However, these cultures did not accumulate dibenzyl sulfoxide beyond 3 days. Prior to dibenzyl sulfone formation, the initial rate of DBS oxidation matched the rate of dibenzyl sulfoxide formation, typically on the order of 2.5 μ mol/h. In each case, the total mass balance decreased over time as the dibenzyl sulfoxide was metabolized. Trace levels of low-molecular-weight products were detected but did not accumulate.

The initial stoichiometric conversion of DBS to dibenzyl sulfoxide in each culture, followed by the formation of dibenzyl sulfone, indicates that the primary metabolic pathway is selective sulfur oxidation. The presence of low-molecular-weight products in some cultures indicate that the C—S bond was probably cleaved subsequent to sulfur oxidation. This pathway is reminiscent of the initial stages of the 4S pathway described for DBT-desulfurizing bacteria (17).

However, it does contrast with the data presented by Itoh et al. (14), who proposed that the *T. versicolor* and *Tyromyces palustris* strains used here metabolize DBS by direct cleavage of the C—S bond to form trace amounts of benzyl alcohol and

FIG. 2. DBS metabolism by *T. troglodytes* UAMH 8156 (A), *T. hirsuta* UAMH 8165 (B), *P. chrysosporium* ATCC 24725 (C), *T. versicolor* IFO 30340 (D), and *Tyromyces palustris* IFO 30339 (E). ■, DBS control; □, DBS; ○, dibenzyl sulfoxide; ●, dibenzyl sulfone.

benzyl mercaptan. Similarly, Faison et al. (7) reported that the coal-solubilizing fungus *Paecilomyces* sp. strain TLi formed a variety of products—including stilbene [1,1'-(1,2-ethenediyl) dibenzene], benzyl alcohol, toluene, and benzyl mercaptan—via an unidentified free-radical mechanism. The formation of these products is questionable under oxidizing conditions, and the formation of dibenzyl sulfoxide and dibenzyl sulfone shown here is more likely. Indeed, sulfur-specific oxidations were observed in the study by Faison et al. (7), where DBT was shown to be metabolized to 2,2'-dihydroxybiphenyl via a sulfoxide and sulfone while ethyl phenyl sulfide and diphenyl sulfide were converted to sulfones. In addition, later work with *T. versicolor* and *Tyromyces palustris* with DBT and 4-methyl-DBT showed that only *T. versicolor* could metabolize these substrates and that the primary metabolic products were sulfoxides and sulfones (12). Finally, a variety of *para* substitution aryl-alkyl sulfides have been shown to be oxidized to primarily sulfoxides in the presence of hydrogen peroxide and a lignin peroxidase isolated from *P. chrysosporium* ATCC 24725 (1).

Since the initial conversion of DBS to dibenzyl sulfoxide was found to be stoichiometric, it appears that the sulfur, rather than the aromatic rings, is preferentially oxidized in this system. This possibility contrasts with the ring oxidation and cleavage products formed from diphenyl ethers by *T. versicolor* SBUG 1050 (10). However, in order to conclusively verify that the C—S bond is the first site of bond cleavage, then benzyl sulfinic acid should be isolated from the culture medium. Given the relative strengths of C—C, C—S, and oxidized C—S bonds (5, 9), it is likely that cleavage occurs at this site, although no confirmatory metabolite was identified.

Extracellular enzymes and cytochrome P-450 inhibition. In an effort to determine the major enzymatic system involved in DBS metabolism, extracellular enzyme levels were measured (25, 27, 28). Neither manganese peroxidase nor lignin peroxidase activity was detected, although laccase activity (0.15 U/ml) was detected in the three *Trametes* cultures.

Next, the cytochrome P-450 inhibitor 1-aminobenzotriazole (19), which did not affect cell yields on GMY (data not shown), was added to *T. troglodytes* cultures exposed to DBS, dibenzyl sulfoxide, or dibenzyl sulfone (Fig. 3). DBS was oxidized to the sulfoxide, and in contrast to what occurred with inhibitor-free controls, no dibenzyl sulfone was formed. No oxidation was observed in killed controls. Similar results were obtained for *T. hirsuta* (data not shown). When dibenzyl sulfoxide was added without DBS, dibenzyl sulfone was formed only in the absence of 1-aminobenzotriazole. When added alone, dibenzyl sulfone did not induce activity in the culture and was not metabolized in any way (Fig. 3).

From these results, it appears that cytochrome P-450 is responsible for the oxidation of dibenzyl sulfoxide to dibenzyl sulfone. This inhibitor has been shown to disrupt DBT sulfoxidation by *Cunninghamella elegans* ATCC 36112 (23), polycyclic aromatic hydrocarbon metabolism by *Pleurotus ostreatus* (3, 4), diphenyl ether metabolism by *T. versicolor* SBUG 1050 (10), and 2,4,6-trinitrotoluene metabolism by *Bjerkandera adusta* DSM 3375 (6). In the last case, 1-aminobenzotriazole did not affect the mineralization of [14 C]glucose, which is in agreement with the observation made here that growth proceeded well on GMY in the presence of the inhibitor. When dibenzyl sulfoxide was added to *T. troglodytes* or *T. hirsuta* cultures, no activity was

FIG. 3. Effect of the cytochrome P-450 inhibitor 1-aminobenzotriazole (0.5 mM) on DBS metabolism by *T. troglia*. DBS, dibenzyl sulfoxide, or dibenzyl sulfone was added as the initial substrate, and metabolites were quantified after a 3-day incubation.

induced, probably due to the oxidized state of the substrate. Ichinose et al. (11, 12) observed a similar phenomenon with *T. versicolor* IFO 30340, which oxidized DBT and DBT-sulfoxide but not DBT-sulfone. Recently, Ichinose et al. (13) identified cytochrome P-450 genes in *T. versicolor* IFO 30340 and observed up-regulation in the presence of DBT and 4-methyl-DBT-5-oxide.

Since the initial oxidation step is not exclusively dependent on cytochrome P-450 and no abiotic oxidation was involved, it appears that another enzyme or enzyme system is involved. To verify that extracellular enzymes were capable of oxidizing DBS to dibenzyl sulfoxide, a concentrated culture supernatant from *T. troglia* (for the preparation, see reference 21) with 5 U of laccase activity per ml was used (Fig. 4A). Stoichiometric DBS conversion to dibenzyl sulfoxide was observed without dibenzyl sulfone formation. The reaction rate was accelerated with the free-radical mediator ABTS [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate)], while ABTS alone did not effect a reaction. Neither dibenzyl sulfoxide nor dibenzyl sulfone was oxidized by the extract when it was added as the starting substrate, even in the presence of ABTS.

Pure laccase from *Coriolopsis gallica* (for the preparation, see reference 21) also catalyzed DBS (50 μ M) oxidation to dibenzyl sulfoxide without dibenzyl sulfone formation (Fig. 4B). In the absence of ABTS, the dibenzyl sulfoxide concentration reached 2 to 4 μ M with minimal DBS removal. With 1.0 mM ABTS, the stoichiometric conversion of DBS to dibenzyl sulfoxide occurred within 24 h. Once again, neither dibenzyl sulfoxide nor dibenzyl sulfone was oxidized when it was added as starting substrate, even in the presence of ABTS. Similar results were found for *Pleurotus ostreatus* laccase (data not shown).

Based on the concentrated culture supernatant and pure laccase activity, coupled with the fact that laccase activity was detected in the *Trametes* cultures, it may be that this extracellular enzyme is responsible for the first oxidation step in these strains. This is not to say that cytochrome P-450 cannot oxidize

FIG. 4. (A) DBS oxidation by concentrated *T. troglia* culture supernatant in the presence (filled symbols) and absence (open symbols) of 1.0 mM ABTS. $\blacktriangle$, DBS; $\blacksquare$, dibenzyl sulfoxide. (B) DBS oxidation by *Coriolopsis gallica* UAMH 8260 laccase in the presence (filled symbols) and absence (open symbols) of 1.0 mM ABTS. $\blacktriangle$, DBS; $\blacksquare$, dibenzyl sulfoxide.

DBS. Vazquez-Duhalt et al. (26) have shown that equine heart cytochrome *c* oxidizes DBS to dibenzyl sulfoxide. The marked increase in DBS oxidation in the presence of the free-radical mediator ABTS is typical for laccase-mediated reactions (21).

In summary, it is proposed that the C—S bond in the alkyl bridge of DBS is the target of oxidation by the fungal cultures tested and that metabolism proceeds from DBS to dibenzyl sulfoxide and then dibenzyl sulfone prior to C—S bond cleavage. More work will be required to verify this, but it appears that ring oxidation and opening occurs only after this cleavage. The metabolic conversion of dibenzyl sulfoxide to dibenzyl sulfone appears to be mediated by cytochrome P-450, while extracellular enzymes, or a combination of extracellular enzymes and cytochrome P-450, may mediate the initial oxidation of DBS.

Partial funding for NCUT has been provided by the Canadian Program for Energy Research and Development (PERD), the Alberta Research Council, and the Alberta Energy Research Institute. Additional support for this study came from the University of Alberta, AEC Oil and Gas, Imperial Oil Resources, and PanCanadian Resources.

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