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Sulfur-Specific Microbial Desulfurization of Sterically Hindered Analogs of Dibenzothiophene

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Dibenzothiophenes (DBTs) bearing alkyl substitutions adjacent to the sulfur atom, such as 4,6-diethyldibenzothiophene (4,6-DEDBT), are referred to as sterically hindered with regard to access to the sulfur moiety. By using enrichment cultures with 4,6-DEDBT as the sole sulfur source, bacterial isolates which selectively remove sulfur from sterically hindered DBTs were obtained. The isolates were tentatively identified as *Arthrobacter* species. 4,6-DEDBT sulfone was shown to be an intermediate in the 4,6-DEDBT desulfurization pathway, and 2-hydroxy-3,3′-diethylbiphenyl (HDEBP) was identified as the sulfur-free end product.

Biocatalytic processes are noted for their mild operating conditions, high selectivity, and reaction chemistry that cannot be done chemically. Although biodesulfurization has been considered an alternative to hydrodesulfurization for more than 40 years (for reviews, see references 2 and 3), to date no commercially viable bioprocess for organic sulfur removal from fuels has been developed. One of the primary reasons for this lack of success was that the biochemical pathways used for sulfur removal were in fact hydrocarbon degradation pathways. The use of such pathways for sulfur removal results in an unacceptable loss of fuel content, because sulfur removal occurs fortuitously along with the overall degradation of hydrocarbon compounds (4, 10, 11).

Recently, bacteria that remove sulfur from dibenzothiophene (DBT), by first oxidizing the sulfur atom and then cleaving the carbon-sulfur bonds, have been isolated (5, 8, 12, 13, 15, 16). All of these recently isolated strains produce 2-hydroxybiphenyl (HBP) as an end product of DBT desulfurization, with the exception of *Brevibacterium* sp. strain DO, which also degrades the carbon skeleton (15). *Rhodococcus rhodochrous* IGTS8, isolated by Kilbane and Bielaga (8), has perhaps been the most extensively studied and is also the basis for a program, by Energy Biosystems Corp., to develop a commercial microbial desulfurization process (14). A variety of organic sulfur compounds, in addition to DBT, were shown to be substrates for desulfurization by IGTS8; these included thianthrene, phenyl sulfoxide, trithiane, and benzyldisulfide (7). However, none of these recently isolated strains have been shown to desulfurize DBTs bearing alkyl substitutions adjacent to the sulfur atom. These compounds are referred to as sterically hindered with regard to access to the sulfur moiety. We targeted these molecules in an enrichment scheme designed to isolate microorganisms which use sulfur-specific mechanisms for organic sulfur removal. In this paper, we report the isolation of gram-positive bacteria, tentatively identified as *Arthrobacter* species, which selectively remove sulfur from sterically hindered DBTs. These organisms possess a broad substrate range, suggesting their potential use in fuel desulfurization. We describe the biochemical pathway involved in desulfurization and compare the relative desulfurization activity toward DBT and the sterically hindered analog, 4,6-diethyldibenzothiophene (4,6-DEDBT).

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MATERIALS AND METHODS

Media. Mineral salts sulfur-free medium (MSSF) was used for isolation and growth of microorganisms which use a sulfur-specific mechanism to obtain sulfur from organic sulfur compounds. MSSF was supplemented with a nonhydrocarbon carbon source to discourage the enrichment of microorganisms which obtain sulfur by mineralization of the organic sulfur compounds found in petroleum. MSSF contained the following components per liter: 0.4 g of KH_2PO_4, 1.6 g of K_2HPO_4, 1.5 g of NH_4Cl, 0.17 g of $MgCl_2 \cdot 6H_2O$, 0.09 g of $CaCl_2 \cdot 2H_2O$, 1 ml of vitamin solution, and 5 ml of mineral solution. The vitamin solution contained (per 100 ml of deionized water) 10 mg of thiamine, 5 mg of *p*-aminobenzoic acid, 5 mg of vitamin B_{12}, and 1 mg of biotin. The mineral solution contained (per liter of deionized water) 1.5 g of nitrilotriacetic acid (dissolved in 500 ml of H_2O and adjusted to pH 6.5 with 10 M KOH), 5.1 g of $MgCl_2 \cdot 6H_2O$, 0.66 g of $MnCl_2 \cdot 2H_2O$, 1.0 g of NaCl, 1.0 g of $FeCl_3 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 6H_2O$, 0.01 g of $CuCl_2 \cdot 6H_2O$, 0.08 g of $ZnCl_2$, 0.05 g of $AlCl_3$, 0.01 g of H_3BO_3, and 0.04 g of $Na_2MoO_4 \cdot 2H_2O$. Sodium acetate (J. T. Baker) and glucose (Sigma) were added as carbon sources as indicated. Organic sulfur compounds were prepared as 54 mM stocks in ethanol and added as indicated after cooling the medium to 54°C. MSSF plates were prepared from MSSF containing 0.8% SeaKem Gold agarose (FMC Corp.), a low-free-sulfate agarose.

Isolation of desulfurization bacteria. Microorganisms with the ability to selectively remove sulfur from the sterically hindered organic sulfur compound 4,6-DEDBT were isolated from marine sediments by enrichment culture. Marine sediment (10 g) was mixed with 100 ml of MSSF on a rotary shaker and allowed to settle for 5 min. A 1-ml volume of the supernatant was removed and centrifuged at 14,000 × *g* for 5 min, and the pellet was resuspended in 1 ml of MSSF for use as the inoculum. Enrichment cultures were established by adding a 250-μl volume of inoculum to 50 ml of MSSF containing sodium acetate and glucose (5.0 g/liter each) as carbon sources, 4,6-DEDBT (130 mg/liter) as the sole sulfur source, and 0.005% yeast extract as an additional vitamin supplement. Enrichment cultures were incubated on a rotary shaker at 300 rpm at 25°C. Cultures were serially transferred by inoculating 50 μl of a previously grown culture into 200 ml of fresh medium. Microorganisms capable of using 4,6-DEDBT as the sole sulfur source were isolated from transfer enrichment cultures as single colonies on solidified MSSF containing 4,6-DEDBT (130 mg/liter) as the sole sulfur source.

Growth measurements and taxonomy. Growth was measured gravimetrically by dry cell weight and turbidimetrically with a Klett Summerson meter with a green filter or by visual comparison with sterile controls. General taxonomic characteristics were determined by standard methods as described by Benson (1). Fatty acid analysis was performed by Microbial ID, Inc., Newark, Del.

Growth on organic sulfur compounds. Cultures were grown until mid-log phase in liquid MSSF containing 10 g of sodium acetate per liter and 1 mM sulfate as the sulfur source. The cells were then washed with 6 mM potassium phosphate buffer (pH 7.0) to remove residual sulfate and immediately used as inocula for growth experiments at a 1:50 dilution. Growth on sulfur compounds was evaluated in MSSF containing 10 g of sodium acetate per liter and 0.54 mM organic sulfur compound. Cultures were incubated at 25°C with shaking at 250 rpm.

Time course experiments were performed as described above with 500-ml cultures. Duplicate 50-ml volumes were taken on days 0, 4, 8, and 12 for analysis. When DBT and 4,6-DEDBT were supplied individually, a concentration of approximately 0.6 mM was used; when they were supplied as a mixture, a concentration of approximately 0.26 mM each compound was used.

Growth on carbon sources. The carbon source utilization profile of bacterial isolates was determined by using 0.1% (by weight) of the test compound in liquid MSSF supplemented with $MgSO_4 \cdot 7H_2O$ (0.2 g/liter) in place of $MgCl_2$. Growth

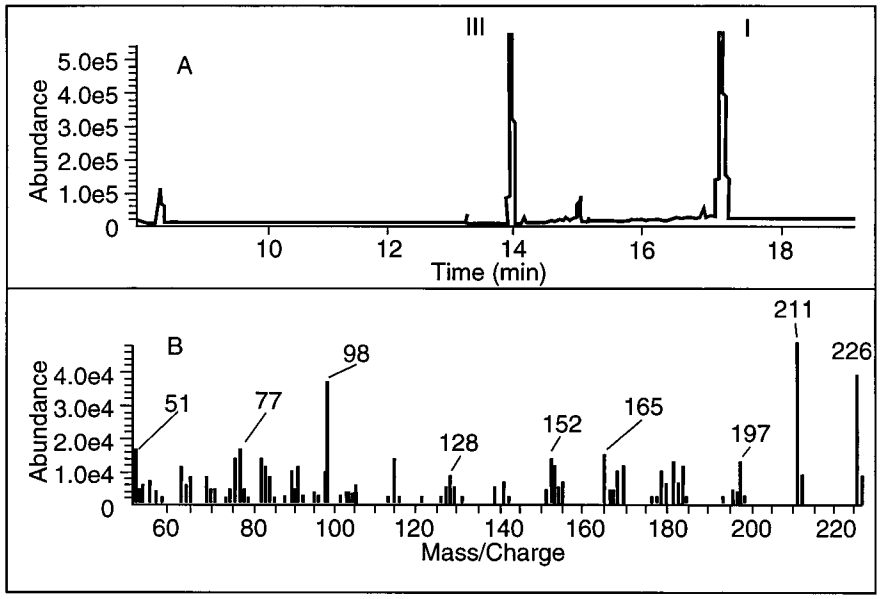

FIG. 1. GC-MS analysis of an extract from the primary enrichment culture. 4,6-DEDBT was the sole sulfur source in these cultures. (A) Total ion chromatogram of the culture extract showing 4,6-DEDBT (I) and HDEBP (III). (B) Mass spectrum of HDEBP (molecular weight, 226).

was determined by measuring the change in turbidity (Klett value) after 5 days of incubation.

Analysis of organic sulfur compounds and metabolites. Organic sulfur compounds and their metabolic products were determined by the analysis of methylene chloride extracts of culture samples. A 50-ml volume of a bacterial culture was acidified with 5 N HCl to pH 1.0, and an extraction efficiency standard was then added as follows: for cultures with DBT as the sulfur source, 0.65 mg of 4,6-DEDBT was added; for cultures with 4,6-DEDBT as the sulfur source, 0.5 mg of DBT was added; for cultures with both DBT and 4,6-DEDBT as sulfur sources, 0.85 mg of 4-ethyldibenzothiophene (4-EDBT) was added. The samples were subsequently extracted with three 25-ml volumes of methylene chloride. For time course experiments, a 1.0-ml volume of a fluorene internal standard (8.7 mg/ml in toluene) was added to the methylene chloride extract to normalize for actual injection volumes. The volume of the sample extract was reduced to approximately 1 ml by evaporation of the solvent methylene chloride with a stream of N_2.

Gas chromatography-mass spectrometry (GC-MS) analysis was used for initial identification of products. GC-MS analysis was performed on an HP 5890A gas chromatograph with a 5970 mass selective detector in electron impact mode and a model 9000 300 data station. The GC column used was a Supelco SE-54 column (60 m by 0.25 mm [inner diameter] by 0.25 μm [film thickness]). The temperature program was 150°C for 1 min followed by a 10°C/min ramp rate to a final temperature of 300°C, with a final hold for 15 min. The mass scan range was 50.0 to 300.0 amu.

Routine quantitation of products was performed by GC in a Hewlett-Packard 5880A gas chromatograph with a split injection (He carrier, 250:1), and flame ionization detector. Data were integrated with a level 4 microprocessor. The column used was an HP PONA (50 m by 0.2 mm [inner diameter] by 0.5 μm [film thickness]). The temperature program was the same as described for the GC-MS analysis. All compounds were identified by authentic standards, except for 4,6-DEDBT sulfoxide and 2-hydroxy-3,3′-diethylbiphenyl (HDEBP), which were confirmed by GC-MS analysis on the basis of the fragmentation patterns expected for these compounds. GC analysis with a sulfur-selective sulfur chemiluminescence detector (GC-SCD) further confirmed the identity of HDEBP by demonstrating that it lacked sulfur. Injections of 5.0 μl were made in duplicate, and the areas for each product peak were averaged. Response factors for DBT, 4,6-DEDBT, and HBP, relative to fluorene, were calculated by preparing a series of standards in methylene chloride and comparing the weight/area ratios. As the response factors for DBT and 4,6-DEDBT were virtually identical and an authentic standard for HDEBP was not available, the response factor for HDEBP was assumed to be equivalent to that of HBP.

GC-SCD was performed with an HP5890 gas chromatograph and a Sievers model 350B sulfur detector. The column used in this analysis was a J&W Scientific DB-1 column (30 m by 0.32 mm [inner diameter] by 0.25 μm [film thickness]). The internal standard was thiophene. The temperature program was 30 to 220°C at a ramp rate of 10°C/min with a final hold for 20 min.

Sulfur compounds. 4,6-DEDBT, 4-EDBT, and 4,6-dimethyldibenzothiophene (4,6-DMDBT) were synthesized as described by Katrizky and Perumal (6). 4,6-DEDBT sulfone was synthesized as follows. A 2.00-g portion of 4,6-DEDBT was added to a 50-ml round-bottom flask equipped with a hot plate/magnetic stirrer, water bath, and thermometer. Then 11 g of glacial acetic acid (Fisher) was added, and stirring was started. To this suspension, 4 ml of 30% hydrogen peroxide was added slowly. The water bath was heated to 70°C. After 10 min, the crystals were dissolved into a heterogeneous mixture. The mixture was stirred for another 1 h until white solids precipitated. The flask was allowed to cool, and the solids were filtered. The crystals and flask were then rinsed with a mixture of 11 g of cold acetic acid and 50 ml of water. The synthesis product was confirmed as 4,6-DEDBT sulfone by GC-MS. All other organic sulfur compounds and hydrocarbons were purchased from Aldrich.

RESULTS

Isolation and taxonomy of strains. Initial evidence of sulfur-specific desulfurization activity was obtained by GC-MS analysis of a sample taken from a primary enrichment culture grown on 4,6-DEDBT as the sole sulfur source and both glucose and acetate (50 g/liter each) as carbon sources. One primary metabolic product, with a molecular weight of 226, was detected and identified as HDEBP on the basis of the expected fragmentation pattern ($M^+ = m/z$ 226, with fragment ions at m/z 211 due the loss of CH_3 and 197 due to the loss of CH_3 and CH_2) (Fig. 1). Analysis of the sample by GC with a flame ionization detector and GC-SCD confirmed that the product did not contain sulfur.

Transfer cultures were plated onto solidified MSSF containing 4,6-DEDBT as the sulfur source. Individual colonies, obtained from these plates, were then tested in liquid MSSF for the ability to grow on 4,6-DEDBT as the sole sulfur source. Microbial isolates able to utilize 4,6-DEDBT as the sole sulfur source in liquid culture were obtained from cream colonies with two distinct morphologies, the first being round with regular margins, smooth, and highly mucoid, and the second being round with irregular margins and rough (nonmucoid). Isolates from both colony types were aerobic, nonsporeforming, nonmotile, gram-positive bacteria. The cell morphology of the isolates went through a cycle, consisting of an irregular rod form, which interconverted with a coccoid form. The irregular rods were largely in club- and V-shaped forms; branching was not observed. The isolates were catalase positive and oxidase

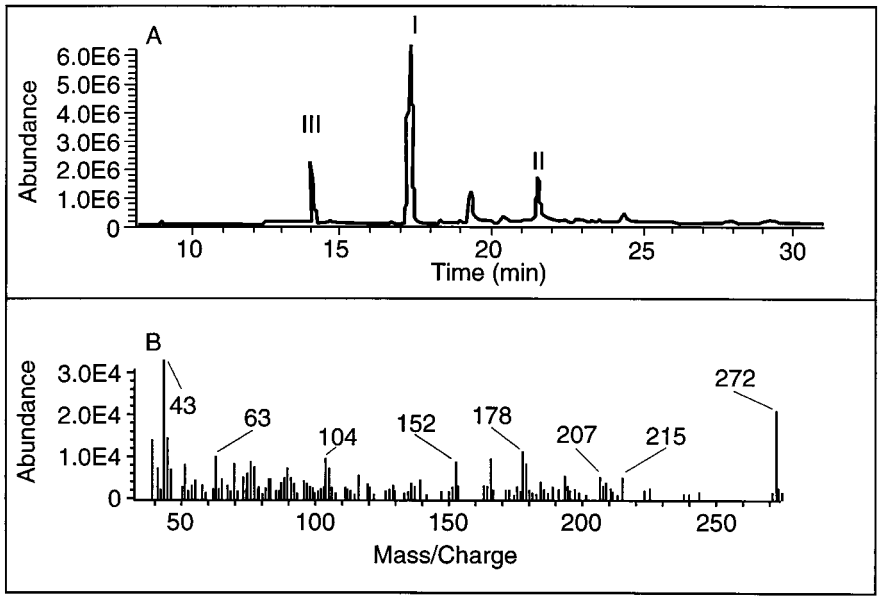

FIG. 2. GC-MS analysis of an extract from an ECRD-1 culture grown on 4,6-DEDBT as the sole sulfur source. (A) Ion chromatogram of the culture extract showing 4,6-DEDBT (I), 4,6-DEDBT sulfone (II), and HDEBP (III). (B) Mass spectrum of 4,6-DEDBT sulfone (molecular weight, 272).

negative. Unlike *Rhodococcus* species, these isolates were resistant to lysozyme. No vitamin additions were required for growth of either isolate, and growth occurred at 4°C. The fatty acid profile of the strains was most similar to that of *Arthrobacter paraffineus* ATCC 15590; however, the presence of tuberculostearic acid (10Me18:0) in this organism and the desulfurization isolates leads to some doubt whether they are true *Arthrobacter* species. The desulfurization isolates have been tentatively identified as *Arthrobacter* species. The nonmucoid isolate was designated ECRD-1 and was used for most of the subsequent characterizations.

Metabolism of 4,6-DEDBT and DBT by isolates. Both ECRD-1 and the mucoid strain were capable of utilizing 4,6-DEDBT or DBT as the sole sulfur source for growth. Analysis of metabolites by GC-MS showed that for both strains, the main products of 4,6-DEDBT and DBT metabolism were HDEBP and HBP, respectively. ECRD-1 was also shown to produce low levels of 4,6-DEDBT sulfone from 4,6-DEDBT (Fig. 2). The mucoid strain also produced low levels of 4,6-DEDBT sulfone from 4,6-DEDBT as well as traces of 4,6-DEDBT sulfoxide. For both strains, the HDEBP and HBP accumulated in the medium and their levels did not decline significantly throughout stationary phase, indicating that they were dead-end products. Additional peaks, which have not been identified, have been observed in GC chromatograms of samples taken from cultures grown on DBT or 4,6-DEDBT as the sulfur source. These peaks represent a small fraction of the total mass and may not represent metabolic products of 4,6-DEDBT metabolism. Nonetheless, a complete mass balance was not obtained. Tests to determine if thermal decomposition of HBP and HDEBP in the GC injection port was responsible for the incomplete mass balance revealed that this was not the case. HBP is the active ingredient in the disinfectant Lysol and, perhaps not surprisingly, was found to be toxic to strain ECRD-1 at a concentration of 50 mg/liter when applied to freshly inoculated cultures. Attempts to grow strain ECRD-1 on 10 mg of HBP per liter as the sole carbon source failed, while control cultures with acetate as the carbon source grew in the presence of an identical amount of HBP. To examine whether the sulfone was an intermediate in the desulfurization pathway, ECRD-1 was grown on DBT sulfone and 4,6-DEDBT sulfone as the sole source of sulfur. The products of growth on DBT sulfone and 4,6-DEDBT sulfone were HBP and HDEBP, respectively, indicating that they were in fact desulfurization pathway intermediates. From the observed products formed by the metabolism of 4,6-DEDBT and DBT, a proposed desulfurization pathway for ECRD-1 was established (Fig. 3).

Growth of strain ECRD-1 on hydrocarbons and heterocyclic compounds. The ability of ECRD-1 to degrade hydrocarbons and heterocyclic compounds was evaluated with a range of compounds as the sole source of carbon for growth. ECRD-1 was unable to utilize toluene, naphthalene, biphenyl, fluorene,

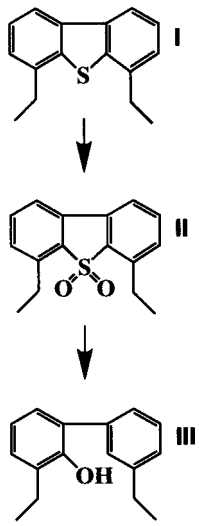

FIG. 3. Proposed pathway for the desulfurization of 4,6-DEDBT by ECRD-1, showing 4,6-DEDBT (I); the intermediate, 4,6-DEDBT sulfone (II); and the final product, HDEBP (III).

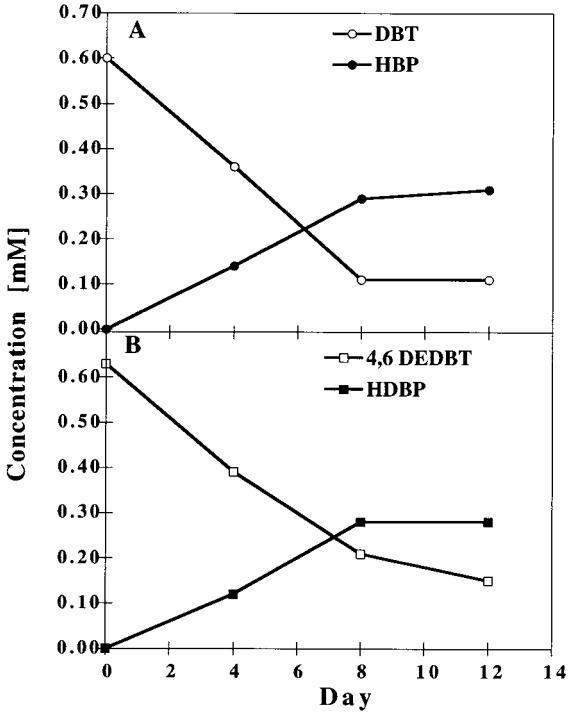

FIG. 4. Time course of desulfurization by ECRD-1. (A) Metabolism of DBT as the sole sulfur source. (B) Metabolism of 4,6-DEDBT as the sole sulfur source. Quantities of starting materials and desulfurized end products are shown. Unidentified products were not quantified. Data are the mean of results of the analysis of duplicate samples. The average relative standard deviation for all data points was 6% or less.

tetralin, phenanthrene, salicylic acid, or hexane. DBT, pyridine, indole, and quinoline also did not support growth of ECRD-1. In contrast, benzoic acid and the *n*-alkanes dodecane and hexadecane served as carbon sources for growth of ECRD-1.

Range of sulfur compounds used by strain ECRD-1. A variety of organic sulfur compounds were tested as sole sulfur sources for growth of ECRD-1. All compounds tested were also tested for toxicity by adding sulfate in addition to the test compound. ECRD-1 grew in all cases when sulfate was present, indicating that none of the compounds tested were toxic at the concentrations used. In addition to 4,6-DEDBT, DBT, DBT sulfone, 4-DEDBT, 4,6-DMDBT, phenothiazine, and dimethyl sulfoxide supported good growth. Benzothiophene, 2-phenylbenzothiophene, and 2-(1-naphthyl)benzothiophene also supported growth, but less efficiently than the dibenzothiophenes did. Thiophene and the substituted derivatives 2-phenylthiophene and 2-(1-naphthyl)thiophene did not support growth. Benzylphenyl sulfide and phenyl sulfide were also tested; however, only benzylphenyl sulfide supported growth. Interestingly, cultures grown on benzylphenyl sulfide required a 3-week period of adjustment before growth ensued. Subsequent transfers from this culture resulted in growth on benzylphenyl sulfide at a rate comparable to that for growth on DBT. Isolates from transfer cultures grown on this compound retained the ability to rapidly metabolize benzylphenyl sulfide, even if passaged on DBT, in marked contrast to the parent ECRD-1 strain.

Competitive growth on DBT and 4,6-DEDBT. The relative desulfurization activities of ECRD-1 toward DBT and 4,6-DEDBT were compared to determine if a preference existed for either compound. ECRD-1 grown on DBT or 4,6-DEDBT individually demonstrated essentially equivalent rates of desulfurization for each compound (Fig. 4). However, when ECRD-1 was grown on a mixture of the two compounds at equal concentrations, a clear preference for DBT over 4,6-DEDBT was observed (Fig. 5A). 4,6-DEDBT was attacked only after the majority of the DBT had been desulfurized, at which time the rate of 4,6-DEDBT desulfurization proceeded at a rate equivalent to that observed when it was provided alone. To investigate whether the preference for DBT over 4,6-DEDBT was due to a greater hydrophobicity of 4,6-DEDBT, ECRD-1 was grown on an equal mixture of the two compounds in the presence of 0.1% (by weight) of the nonionic surfactant Tween 80. The profile of DBT and 4,6-DEDBT desulfurization in the presence of Tween 80 was essentially equivalent to that observed without Tween 80 (Fig. 5B), the slightly greater reduction in 4,6-DEDBT being largely attributable to a lower initial concentration of DBT.

Relationship of desulfurization to biomass production. Desulfurization activity of both ECRD-1 and the mucoid strain was inhibited by 1 mM SO_4^{2-} (data not shown). This suggests that the removal of sulfur is tied to the sulfur requirements of the cell, which are typically on the order of 1% of cell dry weight for bacteria. Table 1 shows the amount of sulfur removed per gram of dry cell weight by ECRD-1 after growth to stationary phase (12 days of growth) on the indicated sulfur compounds. Sulfur removal per gram of dry cell weight was between 6 and 8 mg (0.6 and 0.8% of cell dry weight) in all cases examined, consistent with the sulfur requirements of the bacterial cell.

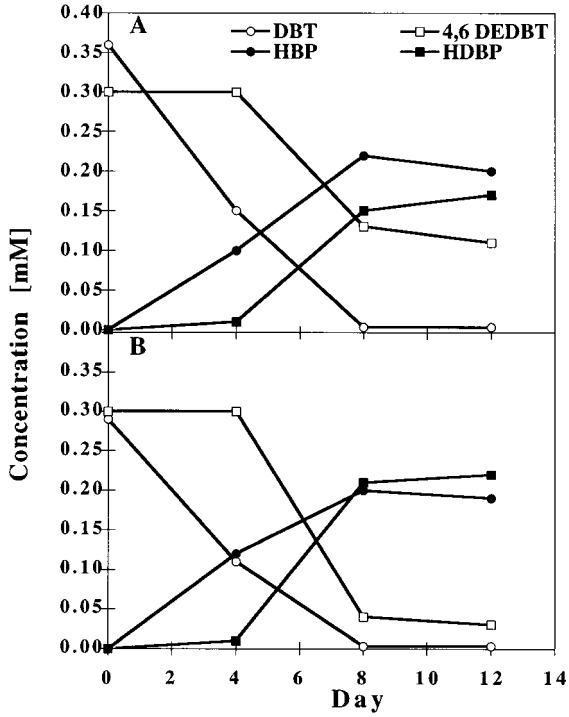

FIG. 5. Time course of desulfurization by ECRD-1. (A) Metabolism of a mixture of DBT and 4,6-DEDBT as sole sulfur sources. (B) Metabolism of a mixture of DBT and 4,6-DEDBT as sole sulfur sources, in the presence of 0.1% (by weight) Tween 80. Quantities of starting materials and desulfurized end products are shown. Unidentified products were not quantified. Data are the mean of results of the analysis of duplicate samples. The average relative standard deviation for all data points was 4% or less.

TABLE 1. Amount of sulfur removed per gram of dry cell weight for ECRD-1[a]

Sulfur source	Amt (mg/g of dry cell wt) of S removed for[b]:	
	DBT	4,6-DEDBT
DBT	6.4	
4,6-DEDBT		7.9
DBT + 4,6-DEDBT	4.7	2.5
DBT + 4,6-DEDBT + 0.1% Tween 80	3.4	3.1

[a] Cultures were grown in MSSF with the indicated sulfur compounds supplied at an individual or combined concentration of 0.54 mM.

[b] Sulfur removal was based on the depletion of the organic sulfur compounds in culture extracts and was calculated individually for each organic sulfur compound. Data are the mean of results of the analysis of duplicate samples. All duplicate measurements had a relative standard deviation of 6% or less.

DISCUSSION

ECRD-1, tentatively identified as an *Arthrobacter* species, performs a sulfur-selective desulfurization of organic sulfur compounds, including DBT and sterically hindered analogs, via a pathway similar to that of other recently isolated gram-positive organisms (5, 8, 12, 13, 15, 16). This pathway does not appear to result in hydrocarbon degradation and therefore should not significantly reduce the fuel value of the desulfurized compound. Although the possibility of HBP degradation by strain ECRD-1 cannot be ruled out yet, it appears unlikely because of the accumulation of HBP during DBT metabolism, its toxicity, and its apparent inability to serve as a carbon source. The incomplete mass balance we observed when analyzing the metabolism of DBT and 4,6-DEDBT may be a result of an inefficient extraction of the desulfurized end products or the inability to detect unknown intermediates by the analytical systems employed.

Although ECRD-1 readily attacks sterically hindered DBTs, a clear preference is shown for unsubstituted DBT. A more complete analysis of the selectivity of this and other systems is required to predict the effect of desulfurization on a mixture of substrates.

Examination of the substrate range of ECRD-1 shows that benzylphenyl sulfide and those compounds containing a DBT or benzothiophene nucleus were attacked whereas 2-phenylthiophene, 2-(1-naphthyl)thiophene, and phenyl sulfide were not. Interestingly, similar patterns of substrate specificity have been reported for other sulfur-selective desulfurizing bacteria, including *Rhodococcus rhodochrous* IGTS8 (7), *Rhodococcus erythropolis* D-1 (5), and *Corynebacterium* sp. strain SY1 (13). The observed substrate range may be a result of how readily the sulfur atom is oxidized. A similar argument was used by Knecht (9) to explain the substrate profile of an *Arthrobacter* isolate which used DBT as the sole source of carbon, energy, and sulfur. Knecht postulated that the isolate used the sulfur atom as the initial site of oxidation in the metabolism of DBT and that the relative susceptibility of the sulfur moiety toward oxidation determined the substrate profile of the organism. This organism was unable to metabolize DBT in the absence of a coisolated *Pseudomonas* species, suggesting that the *Arthrobacter* species may have provided sulfur from DBT while the *Pseudomonas* species provided carbon in the form of DBT degradation products. If this was the case, the *Arthrobacter* species obtained by Knecht may have been the first oxidative sulfur-selective desulfurization organism isolated.

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