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Bacterial Transformations of 1,2,3,4-Tetrahydrodibenzothiophene and Dibenzothiophene

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The transformations of 1,2,3,4-tetrahydrodibenzothiophene (THDBT) were investigated with pure cultures of hydrocarbon-degrading bacteria. Metabolites were extracted from cultures with dichloromethane (DCM) and analyzed by gas chromatography (GC) with flame photometric, mass, and Fourier transform infrared detectors. Three 1-methylnaphthalene (1-MN)-utilizing *Pseudomonas* strains oxidized the sulfur atom of THDBT to give the sulfoxide and sulfone. They also degraded the benzene ring to yield 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene. A cell suspension of a cyclohexane-degrading bacterium oxidized the alicyclic ring to give a hydroxy-substituted THDBT and a ketone, and it oxidized the aromatic ring to give a phenol, but no ring cleavage products were detected. GC analyses with an atomic emission detector, using the sulfur-selective mode, were used to quantify the transformation products from THDBT and dibenzothiophene (DBT). The cyclohexane degrader oxidized 19% of the THDBT to three metabolites. The cometabolism of THDBT and DBT by the three 1-MN-grown *Pseudomonas* strains resulted in a much greater depletion of the condensed thiophenes than could be accounted for in the metabolites detected by GC analysis, but there was no evidence of sulfate release from DBT. These 1-MN-grown strains transiently accumulated 3-hydroxy-2-formylbenzothiophene (HFBT) from DBT, but it was subsequently degraded. On the other hand, *Pseudomonas* strain BT1d, which was maintained on DBT as a sole carbon source, accumulated 52% of the sulfur from DBT as HFBT over 7 days, and, in total, 82% of the sulfur from DBT was accounted for by the GC method used. Lyophilization of cultures grown on 1-MN with DBT and methyl esterification of the residues gave improved recoveries of total sulfur over that obtained by DCM extraction and GC analysis. This suggested that the further degradation of HFBT by these cultures leads to the formation of organosulfur compounds that are too polar to be extracted with DCM. We believe that this is the first attempt to quantify the products of DBT degradation by the so-called Kodama pathway.

Organosulfur compounds are a small but significant component of petroleum and coal-derived liquids. Conventional crude oils contain between 0.04 and 5% (wt/wt) sulfur (50), and in general, crude oils of higher density contain a higher percent sulfur. The organosulfur compounds present include thiols, sulfides, and thiophenes, but the sulfur compounds which predominate in the so-called heavy fractions, where sulfur content is the highest, are primarily the condensed thiophenes. Of these, the dibenzothiophenes (DBTs) were recognized to be among the compounds that were most resistant to biodegradation in sediments contaminated with oil from the *Amoco Cadiz* spill (5). The recalcitrance of the DBTs, relative to the other aromatic compounds found in crude oil that are amenable to analysis by gas chromatography (GC), has also been observed in other studies (4, 6, 51) and contributes to the potential of condensed thiophenes to accumulate in the tissues of shellfish in marine environments that become contaminated with crude oil (29, 37). This has led to the suggestion that the DBTs might serve as oil pollution markers (14, 37).

There have been numerous biodegradation studies using DBT as a model condensed thiophene (8, 20, 22, 23, 28, 32, 34). Since the initial studies of Kodama et al. (22, 23) with *Pseudomonas* strains, there have been several reports of bacterial oxidation and cleavage of one of the benzene rings of DBT to yield 3-hydroxy-2-formylbenzothiophene (HFBT) by the so-called Kodama pathway (28, 32, 34). Many of these isolates also oxidize the sulfur atom, giving DBT sulfoxide and sulfone (22, 23, 28, 34), which appears to be a dead-end pathway in these aromatic compound-degrading bacteria.

Other isolates are reported to use DBT as a sulfur source by oxidizing DBT via the sulfoxide and sulfone and then subsequently releasing the sulfur atom as sulfate, leaving 2-hydroxybiphenyl (16, 21, 38). This pathway is of potential use in the development of a microbial process for the biodesulfurization of petroleum because it does not result in extensive degradation of the hydrocarbon. Kinetic data of the metabolism of DBT by *Rhodococcus erythropolis*, which uses this pathway, have been reported (53). van Afferden et al. (52) observed stoichiometric release of sulfate from DBT by a *Brevibacterium* sp. that oxidized DBT via the sulfoxide and sulfone, released sulfite, and then subsequently degraded the desulfurized hydrocarbon. The only metabolite detected in the degradation of the carbon backbone was benzoic acid. Although quantitative data have been reported for isolates which oxidize DBT to the sulfoxide and sulfone, and then further degrade DBT sulfone by one of the two pathways mentioned above, we are not aware of any quantitative studies which establish a mass balance for bacterial degradation of DBT by the Kodama pathway.

As part of a series of investigations into the metabolism of condensed thiophenes (12, 24–27, 42, 43), we now report on studies of the biotransformation of 1,2,3,4-tetrahydrodibenzothiophene (THDBT). This compound is formed during hydroprocessing reactions aimed at the hydrodesulfurization of the DBTs found in petroleum (35, 41) and has been detected as a

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minor constituent of solvent-refined coal liquids (30, 36). THDBT consists of a benzene ring and a cycloparaffin ring fused to opposite sides of a thiophene ring, and as such there are various potential mechanisms that could be utilized for its oxidation by microorganisms.

This report describes the biotransformations of THDBT by three *Pseudomonas* strains while growing on 1-methylnaphthalene (1-MN) and by cell suspensions of a cyclohexane-degrading bacterium. The use of a GC with an atomic emission detector (AED), which gives an equimolar response to sulfur in all organic forms that are amenable to GC analysis (3), allowed quantitative analyses of the metabolites. However, the limited amount of synthesized THDBT did not allow additional investigations. Subsequently, quantitative studies were done with DBT in an attempt to establish a sulfur mass balance for DBT oxidation by four *Pseudomonas* strains which utilize the Kodama pathway of DBT metabolism. These quantitative studies with DBT were motivated by the difficulty experienced in achieving a sulfur mass balance in studies with aromatic hydrocarbon-degrading bacteria and THDBT (present study) or dimethyldibenzothiophenes (26).

MATERIALS AND METHODS

Chemicals. THDBT (≥99% pure by GC) was synthesized by the method of Wilputte and Martin (54). DBT (98%) and 1-MN (97%) were purchased from Fluka (Buchs, Switzerland). Cyclohexane (pesticide grade) was purchased from Fisher Scientific (Fair Lawn, N.J.). DBT sulfone (97%) was purchased from Aldrich (Milwaukee, Wis.). THDBT sulfone was synthesized by refluxing THDBT with H_2O_2 (30%) in acetic acid for 15 min. After recrystallization from ethanol, the THDBT sulfone gave a melting point of 186 to 187°C, which is consistent with the literature melting point of 187 to 188°C (31a), and was >98% pure by GC.

Bacterial cultures and culture methods. Biotransformation experiments were done in 500-ml Erlenmeyer flasks containing 200 ml of mineral medium supplemented with a trace metals solution (25), and the cultures were incubated at 28°C on a rotary shaker at 200 rpm. Following inoculation, each flask received 2 to 4 μl of liquid THDBT (1 μl = 1.0 mg) or 4 mg of DBT dissolved in 100 μl of acetonitrile. For each biotransformation experiment, appropriate uninoculated sterile controls were included to account for any abiotic loss and transformations. Although some evaporation occurred, none of the oxygenated products detected in the culture extracts were found in the sterile controls.

The isolation and characterization of the three 1-MN-degrading *Pseudomonas* strains BT1, W1, and F were described previously (12, 43). The 10-ml inoculum used for biotransformation experiments with each of these isolates was obtained from 1-MN-grown maintenance cultures that were transferred weekly. Following inoculation, the biotransformation cultures received 50 μl of 1-MN as the growth substrate together with the cosubstrate THDBT or DBT. Because isolate BT1 can grow on DBT as its sole carbon and energy source (43), some experiments tested this isolate with DBT, with 1-MN omitted from the medium. The inoculum used for these experiments (10 ml) also came from the 1-MN-grown BT1 maintenance culture. As well, a fourth strain, designated BT1d, was tested in some experiments with DBT. This strain originated from isolate BT1, which was maintained for over 2 years by weekly transfers into mineral medium with 4 mg of DBT as the sole carbon and energy source. Although the colonial morphologies of isolates BT1 and BT1d were identical, their metabolism of DBT was found to be different, as discussed later. In all of the studies with DBT, the maintenance cultures used as inocula were 7 days old and had reached the stationary phase of growth.

The cyclohexane degrader strain CB1323, a gift from Celgene, Inc. (Warren, N.J.), was capable of growth on plates of mineral medium containing 1.5% Noble agar (Difco, Detroit, Mich.) incubated with cyclohexane vapors in a sealed 3-liter container. However, better growth was obtained on plates of solidified Luria broth (LB; consisting per liter of distilled water of 15 g of Difco agar, 10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, and 10 g of NaCl, with the pH adjusted to 7.5), which were also incubated in the presence of cyclohexane vapors. The inoculum of strain CB1323 for biotransformation experiments was obtained by aseptically washing the growth of a 3-day-old culture from an LB agar plate with 9 ml of sterile phosphate buffer (pH 7.2, 10 mM) into each flask for the biotransformation experiment. Five replicate cell suspensions were incubated at the same time. THDBT was the only carbon source added to these cell suspensions, and after 2 days of incubation, they were pooled prior to extraction.

Solvent extraction. Cultures, cell suspensions, and sterile controls were acidified with 2 M H_2SO_4 to a pH of <2 and extracted with dichloromethane (DCM; 4 times 20 ml) to recover substrates and products. The DCM extract was dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. For quantitative experiments by GC-AED analysis, known amounts of one or two of the following commercially available organosulfur compounds (Aldrich) were added as internal standards prior to extraction: thianthrene (99%), phenyl sulfoxide (97%), and phenyl sulfide (98%). The standards used were chosen so that they would not coelute during GC analysis with THDBT, DBT, or the products detected in preliminary screenings.

GC analyses. To screen for the presence of sulfur-containing metabolites, the extracts were analyzed by capillary GC with a 30-m DB-5 column in a Hewlett-Packard (HP; Mississauga, Ontario, Canada) model 5890 GC equipped with a flame ionization detector (FID) and a sulfur-selective flame photometric detector (FPD). Details of the operating conditions were given previously (12). The methods routinely used for GC-mass spectrometry (GC-MS) and GC-Fourier transform infrared spectroscopy (GC-FTIR) have also been described previously (42, 43). To obtain high-resolution GC-MS data, analyses were done at the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta (13).

Quantitative GC analyses were done with a HP 5921A AED connected to a HP 5890 GC. The GC was equipped with a split or splitless injection port, and injections were made by an automatic sampler (model HP 7673). The column used was a HP-5MS with dimensions of 0.25 mm (inside diameter) by 30 m (0.25-μm film thickness). The temperature program used held the column at an initial temperature of 90°C for 2 min before increasing it at 4°C/min to 250°C, where it was held for 18 min. Helium (99.996% pure), after further purification with a VD-1200 helium purifier from VICI Valco Instruments (Houston, Tex.), was used as the carrier and plasma gas. Hydrogen and oxygen were used as auxiliary gases as required. The system was controlled by a HP Chem Station 382 with the HP 35920A software package. Gas selection and detector tuning were computer controlled, whereas the plasma gas flow rate was set manually to 60 ml/min for optimal sensitivity and peak shape in the sulfur trace. The data presented are based on the micromoles of sulfur in each compound detected by the GC-AED analysis.

To facilitate GC-MS identification of some of the metabolites, trimethylsilyl (TMS) derivatives of compounds in culture extracts were made by silylating with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) in acetonitrile as described in the manufacturer's instructions (Pierce, Rockford, Ill.; method 5).

Total sulfur analysis. The DCM extracts of cultures of the *Pseudomonas* strains incubated with DBT were diluted to 1.0 ml with DCM in volumetric flasks, and 200-μl portions of these were applied to filter paper (Whatman no. 42). After evaporation of the DCM, the filter paper samples were combusted in sealed Schöninger oxygen flasks and the SO_2 produced was trapped as H_2SO_4 in 10 ml of water to which 2 drops of 30% hydrogen peroxide had been added. After the addition of 40 ml of 2-propanol, the amount of sulfate present was determined by microtitration with $BaClO_4$ (0.01077 M) with thorin as the end point indicator (15).

Assessment of sulfate release from DBT. Cultures of the three 1-MN-degrading *Pseudomonas* strains were maintained for three sequential 10-ml transfers in 500-ml Erlenmeyer flasks containing 200 ml of sulfate-free medium (18). The transfers were done every 5 days after the cultures had grown, and at each transfer, the three isolates received 50 μl of 1-MN and 4 mg of DBT. In addition, isolate BT1d was grown on 4 mg of DBT without 1-MN. At the time of the third transfer, the cultures were transferred into flasks of medium that contained 50 mg of DBT. After 6 days of incubation, the cultures were acidified with 2 ml of 4 M HCl and centrifuged at 16,300 × *g* for 15 min to remove cells and undissolved DBT. The supernatant was then passed through an ENVI Chrom P solid-phase extraction tube (Supelco, Bellefonte, Pa.) that had been preconditioned with 10 ml of methanol and 10 ml of acidified sulfate-free medium. The solid-phase extraction procedure effectively removed the colored DBT oxidation products from the supernatant. The color of these metabolites would have interfered with the turbidimetric method used to assay for sulfate (2). The solid-phase extraction procedure did not retain sulfate when a standard solution of Na_2SO_4 (15 mg/liter) was passed through the extraction tube.

Lyophilization and methyl esterification. Cultures of the *Pseudomonas* strains incubated with DBT were made alkaline (pH >12) by the addition of 0.5 g of NaOH before lyophilization. After lyophilization, the residue was transferred to a 100-ml round-bottom flask with 40 ml of methanol and 4 ml of concentrated H_2SO_4 and refluxed for 2 h. After cooling and the addition of 40 ml of water, the reaction mixture was extracted with DCM as described above.

RESULTS

Transformations of THDBT by *Pseudomonas* strains. GC-FPD analysis of an extract of an acidified culture of isolate F grown in the presence of THDBT for 3 days revealed the presence of three abundant sulfur-containing metabolites. GC-MS analysis (Fig. 1) showed that metabolite A, with the shortest retention time, had a molecular ion at *m/z* 182 and a base peak at *m/z* 154, which could result from the loss of CO or C_2H_4 $(M-28)^+$, both of which would be fragmentations consistent with the structure shown (Fig. 1). Loss of CO $(M-28)^+$ is characteristic of phenols, and loss of C_2H_4 $(M-28)^+$ is characteristic of cyclic alkanes (49). The molecular weight of metab-

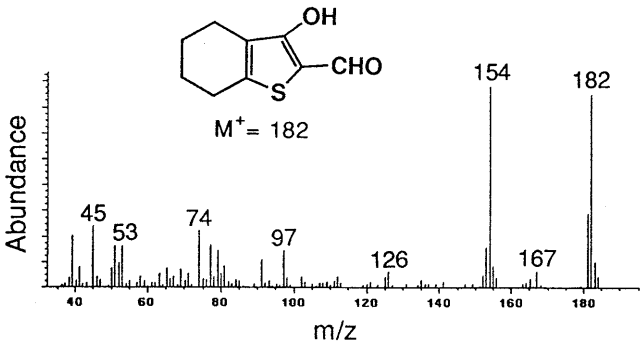

FIG. 1. From GC-MS analysis, the mass spectrum of sulfur-containing metabolite A from a culture of isolate F grown on 1-MN in the presence of THDBT. These results indicate that metabolite A is 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene.

olite A is 4 mass units greater than that of HFBT, which has been previously reported as a product of DBT biotransformation (22, 23, 28, 32, 34), and is consistent with the metabolite being 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene, as shown in Fig. 1. The presence of the hydroxy functional group was verified by preparing the TMS-ether of the metabolite, which gave a weak molecular ion at *m/z* 254 (3%) during GC-MS analysis. The derivative characteristically lost a methyl group $(M\text{-}15)^+$ from the molecular ion (40) to give the base peak at *m/z* 239.

GC-FTIR analysis of the underivatized metabolite A gave the FTIR spectrum shown in Fig. 2a. The aromatic C—H stretching absorption in the FTIR spectrum of THDBT at 3,069 cm^{-1} (Fig. 2b) is missing from the FTIR spectrum of metabolite A (Fig. 2a), whereas the saturated C—H stretching absorptions observed at 2,863 and 2,945 cm^{-1} in the parent compound (Fig. 2b) are still present in the FTIR spectrum of the metabolite (Fig. 2a). This suggests that the aromatic ring of THDBT has been degraded while the saturated ring has been left unaltered to give metabolite A. However, the saturated C—H stretching absorption at 2,863 cm^{-1} in the metabolite also overlaps with the aldehydic C—H stretching absorption observed in Fig. 2a as a doublet at 2,844 cm^{-1} and approximately 2,770 cm^{-1} (49). The other strong absorption of metabolite A (Fig. 2a) at 1,638 cm^{-1} is due to the carbonyl group and is similar to the strong absorption previously reported for HFBT at 1,640 cm^{-1} (43). Thus, this evidence further suggests that metabolite A from THDBT is 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene.

The second and third sulfur-containing metabolites detected by GC-FPD analysis of the isolate F culture extract nearly coeluted with one another but were sufficiently resolved for GC-MS and GC-FTIR analyses. The mass spectrum of the more abundant of these two metabolites (metabolite B) showed a molecular ion at *m/z* 204 (Fig. 3a), which is 16 mass units greater than that of THDBT, suggesting the incorporation of a single atom of oxygen into the substrate. However, no TMS-ether of this metabolite was formed when the extract was treated with BSA, ruling out the possibility that it is a hydroxy-substituted isomer of THDBT. Thus, the metabolite was thought to be THDBT sulfoxide. This conclusion was verified by GC-FTIR analysis, which showed that metabolite B absorbed strongly at 1,081 cm^{-1}, an absorption characteristic of sulfoxides (49).

The later of these two metabolites to elute (metabolite C) gave the mass spectrum shown in Fig. 3b. This spectrum and the GC retention time of the metabolite were the same as those of an authentic standard of THDBT sulfone. GC-FTIR analysis of metabolite C showed that it gave strong absorptions at 1,331 and 1,167 cm^{-1}, which are characteristic of sulfones (49). Thus, metabolite C is THDBT sulfone.

Isolate W1 produced the same three abundant metabolites from THDBT as the isolate F culture did, and the extracts of both of these isolates showed similar amounts of metabolites and an abundant peak in the chromatograms corresponding to unoxidized THDBT. However, GC-FPD analyses of the extracts of 3-day-old cultures of isolate BT1 incubated with THDBT showed only trace amounts of residual THDBT and of a number of novel sulfur-containing metabolites that were not abundant enough to identify. 3-Hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene (metabolite A), which was also present in trace amounts, was detected in 3-day-old BT1 culture extracts. In extracts of BT1 cultures that had been incubated for 1 or 2 days, 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene was present in greater abundance, but only trace amounts of the novel sulfur-containing metabolites were

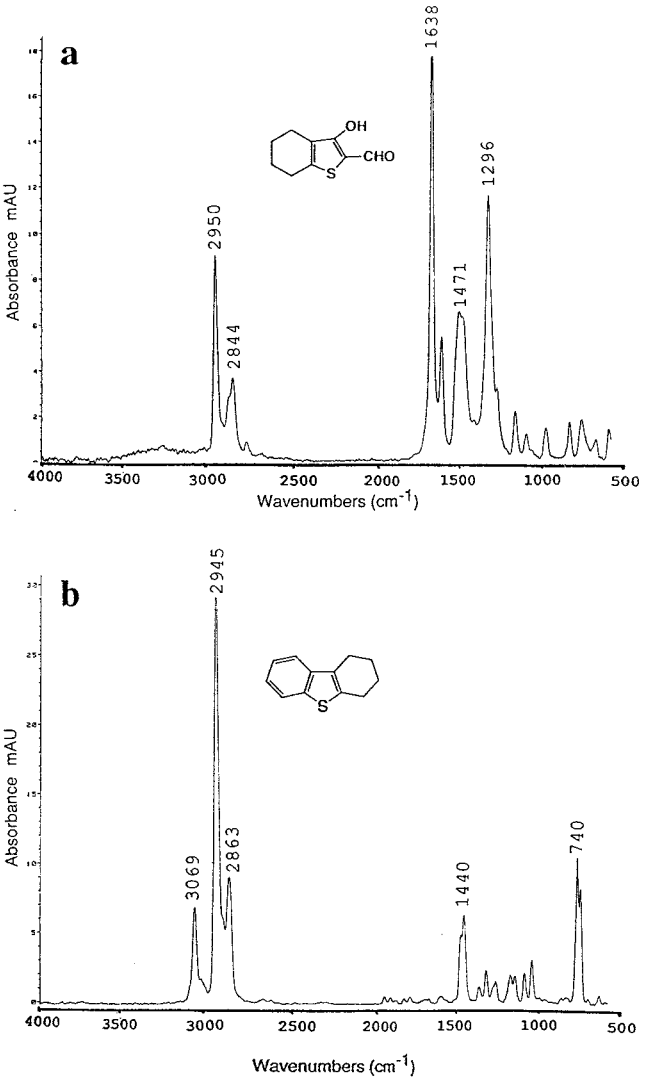

FIG. 2. From GC-FTIR analyses, the FTIR spectra of sulfur-containing metabolite A from a culture of isolate F grown on 1-MN in the presence of THDBT (a) and of THDBT (b). These results indicate that metabolite A is 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene. mAu, milliabsorbance units.

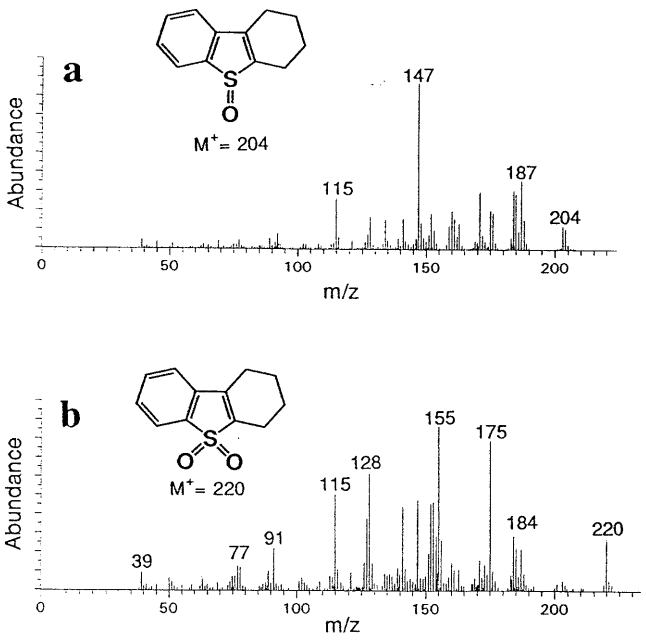

FIG. 3. From GC-MS analyses, the mass spectra of sulfur-containing metabolite B (a) and metabolite C (b) from a culture of isolate F grown on 1-MN in the presence of THDBT. These results indicate that metabolites B and C are the sulfoxide and sulfone of THDBT, respectively.

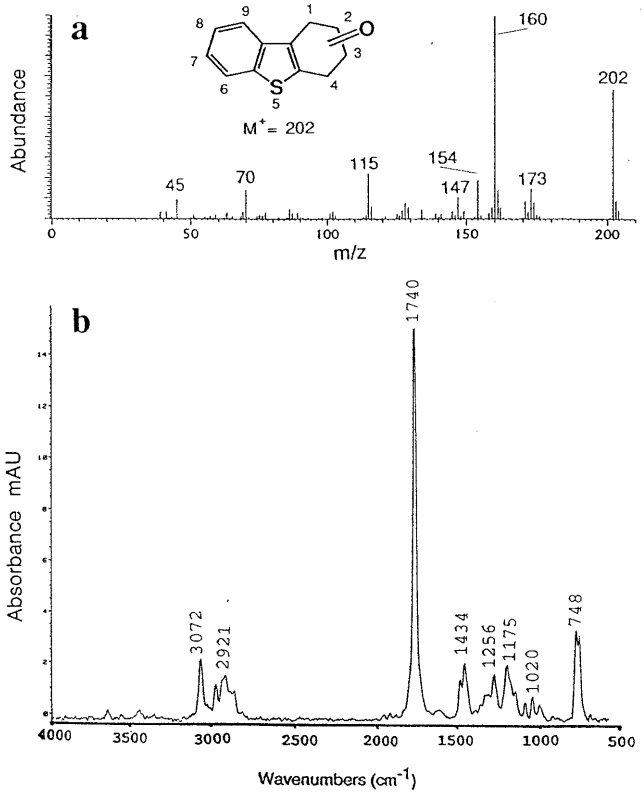

FIG. 4. Mass spectrum (a) and FTIR spectrum (b) of sulfur-containing metabolite D from the analyses of the extract of the cell suspension of strain CB1323 incubated with THDBT. The results indicate that one of the methylene carbon atoms of THDBT has been oxidized to a carbonyl group, yielding a cyclic ketone. mAU, milliabsorbance units.

ever observed, even with the shorter incubation times, and so they could not be identified.

The sulfur-selective GC-AED analyses of extracts of cultures that had been incubated for 3 days and extracted after the addition of internal standards was used to quantify the amounts of THDBT and sulfur-containing metabolites present (Table 1). The recovery of THDBT from the sterile control was 92% of the 16.5 μmol of THDBT added (Table 1). After 3 days of incubation, ≤13% (2.1 μmol) of the THDBT was recovered as residual substrate from each of the active cultures (Table 1). The percentage of the sulfur added as THDBT that was recovered as 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene and THDBT sulfoxide and sulfone, which were quantified together because they were not adequately resolved during GC-AED analyses, is shown in Table 1 for each of the isolates. The sulfoxide and sulfone of THDBT were not detected in extracts of isolate BT1. The difference between the total recovery of THDBT and sulfur-containing metabolites in the culture extracts and the corresponding sterile control ranged from 58.2 to 91.1% (Table 1). Clearly, other metabolites were produced but not detected by these analytical methods.

TABLE 1. Quantification of THDBT and sulfur-containing metabolites as determined by GC-AED analyses of DCM extracts of acidified cultures of three *Pseudomonas* strains incubated for 3 days with 1-MN and 16.5 μmol of THDBT

Strain	% Of sulfur from THDBT recovered as:			Total recovery (%)	Recovery in sterile control (%)	Difference[b]
	THDBT	THHFBT[a]	THDBT sulfoxide and sulfone			
W1	13	4.8	16	33.8	92	58.2
F	7.4	3.3	21	31.7	92	60.3
BT1	0.7	0.2	ND[c]	0.9	92	91.1

[a] THHFBT, 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene.
[b] Difference between total recovery in culture extracts and that in the corresponding sterile control.
[c] ND, not detected.

Transformations of THDBT by strain CB1323. Cell suspensions of strain CB1323 that had been pregrown in the presence of cyclohexane vapors were shown to oxidize THDBT, producing three sulfur-containing metabolites (referred to as D, E, and F below) over a 2-day incubation.

GC-MS analysis gave the mass spectrum shown in Fig. 4a for metabolite D. The strong molecular ion at *m/z* 202 is consistent with the chemical formula of $C_{12}H_{10}OS$ that was determined by high-resolution GC-MS analysis. The base peak at *m/z* 160 $(M-42)^+$ results from loss of CH_2CO. GC-FTIR analysis gave the spectrum shown in Fig. 4b for metabolite D. The presence of C—H stretching between 2,850 and 2,979 cm^{-1} and at 3,072 cm^{-1} indicates that both saturated and aromatic C—H bonds were present in the molecule although the relative intensity of the saturate C—H stretching was decreased relative to that of unoxidized THDBT (Fig. 2b). The strong absorption at 1,740 cm^{-1} is due to the presence of the oxygen atom in a carbonyl group (Fig. 4b). Because the chemical formula for metabolite D of $C_{12}H_{10}OS$ is one oxygen atom greater and two hydrogen atoms less than that of THDBT, it is likely that one of the methylene carbon atoms in the saturated ring was oxidized to a ketone. This is indicated by the structure shown for metabolite D in Fig. 4, although it is not known which of the methylene groups was oxidized to give the cyclic ketone. However, the loss of CH_2CO to give the fragment at *m/z* 160 $(M-42)^+$ in

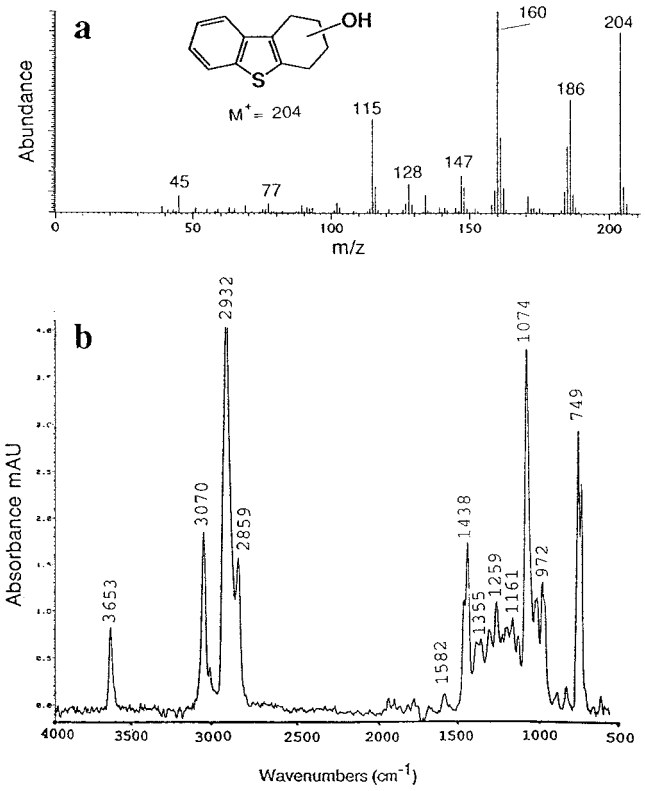

FIG. 5. Mass spectrum (a) and FTIR spectrum (b) of sulfur-containing metabolite E from the analyses of the extract of the cell suspension of strain CB1323 incubated with THDBT. The results indicate that the metabolite is an isomer of hydroxy-substituted THDBT, bearing the hydroxy group on the saturated ring. mAU, milliabsorbance units.

the mass spectrum of metabolite D (Fig. 4a) has also been observed in the mass spectrum of 3,4-dihydro-2(1*H*)-naphthalenone but not in the mass spectrum of 3,4-dihydro-1(2*H*)-naphthalenone (19). This may indicate that the carbonyl group of metabolite D is not located at a position alpha to the thiophene ring of THDBT (i.e., position 1 or 4) but rather is located at a position beta to the thiophene ring (i.e., position 2 or 3). This assignment is further supported by the carbonyl absorption of metabolite D at 1,740 cm^{-1}, which is consistent with the carbonyl absorption of saturated ketones (1,725 to 1,745 cm^{-1} in the vapor phase) but not consistent with the carbonyl absorption of conjugated ketones (1,690 to 1,720 cm^{-1} in the vapor phase) (31).

Metabolite E gave the mass spectrum shown in Fig. 5a with a strong molecular ion at *m/z* 204, consistent with the chemical formula $C_{12}H_{12}OS$ determined by high-resolution GC-MS analysis. This product resulted from the incorporation of a single atom of oxygen into THDBT ($C_{12}H_{12}S$), and GC-FTIR analysis proved that it was present as a hydroxyl group since the metabolite absorbs strongly at 3,653 cm^{-1} (Fig. 5b). The FTIR spectrum (Fig. 5b) also suggests that the hydroxyl group is located on the saturated ring because of the relatively strong intensity of the aromatic C—H stretching at 3,070 cm^{-1}. Other evidence for this assignment comes from the fragmentation pattern of metabolite E observed in Fig. 5a. The fragment at *m/z* 186 results from the loss of H_2O $(M-18)^+$, and this fragmentation is also observed in the mass spectrum of cyclohexanol (49) and 1,2,3,4-tetrahydro-1-naphthalenol (19). The base peak at *m/z* 160 $(M-44)^+$ results from loss of CH_2CHOH. Thus, metabolite E appears to be a hydroxy-substituted THDBT which bears the hydroxy group on the saturated ring, although the exact position is not known. However, metabolite E likely bears the hydroxy substituent at the same position beta to the thiophene ring (position 2 or 3), as was suggested above for the further-oxidized metabolite D.

Metabolite F also appears to be a hydroxy-substituted THDBT. It has the same molecular weight of 204 (Fig. 6a) and the same chemical formula of $C_{12}H_{12}OS$. As well, GC-FTIR analysis shows a strong O—H stretching absorption at 3,653 cm^{-1} (Fig. 6b). However, the data suggest that the hydroxy group is located on the benzene ring of THDBT, not on the saturated ring as proposed for metabolite E. The base peak at *m/z* 176 in the mass spectrum of metabolite F (Fig. 6a) results from the loss of CO $(M-28)^+$. This fragmentation is commonly observed in the mass spectra of phenols (49) and has been observed in the mass spectra of 5,6,7,8-tetrahydro-1-naphthalenol and 5,6,7,8-tetrahydro-2-naphthalenol (19). Furthermore, the aromatic C—H stretching at 3,034 cm^{-1} in the FTIR spectrum of metabolite F (Fig. 6b) is weaker and broader than it is in metabolite E (Fig. 5b). Thus, metabolite F appears to be a hydroxy-substituted THDBT which bears the hydroxy group on the benzene ring, although the exact position is unknown.

When the extract of the cell suspension of strain CB1323 was treated with BSA, the two hydroxy-substituted products (metabolites E and F) both reacted to give the respective TMS-ethers with molecular ions at *m/z* 276 observed in the mass spectra from GC-MS analysis of the derivatized extract. The earlier derivative to elute was presumed to be that of metab-

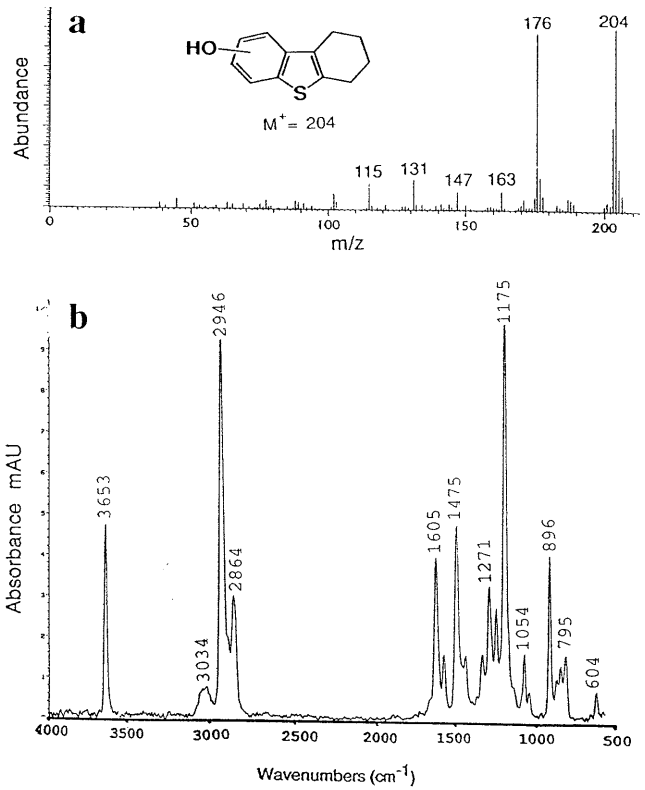

FIG. 6. Mass spectrum (a) and FTIR spectrum (b) of sulfur-containing metabolite F from the analyses of the extract of the cell suspension of strain CB1323 incubated with THDBT. The results indicate that the metabolite is an isomer of hydroxy-substituted THDBT, bearing the hydroxy group on the benzene ring. mAU, milliabsorbance units.

olite E since the abundant ions at *m/z* 186 (68%) and *m/z* 160 (100%) were also observed in the mass spectrum of the underivatized metabolite E (Fig. 5a). The mass spectrum of the TMS-ether of metabolite F gave the parent peak at *m/z* 276 (100%), with fragments at *m/z* 261 $(M-15)^+$ (17%), *m/z* 248 $(M-28)^+$ (40%), and *m/z* 233 $(M-43)^+$ (10%).

GC-AED analysis was used to quantify the amounts of metabolites D, E, and F produced by five replicate cell suspensions of strain CB1323 that each contained 16.5 μmol of THDBT. These were incubated for 2 days and pooled prior to extraction. Results showed that 44.6 μmol (54%) of the initial 82.5 μmol of THDBT added remained in the extract and that 8 μmol (9.6%), 3.2 μmol (3.9%), and 4.6 μmol (5.6%) of the added THDBT were present as metabolites D, E, and F, respectively. The recovery of THDBT from a single sterile control that received 16.5 μmol of THDBT was 73% (12 μmol). Assuming that the evaporative loss from the individual cell suspensions was the same as that from the sterile control (27%), the analytical method accounted for 82.7 μmol (100%) of sulfur added as THDBT.

Transformations of DBT by *Pseudomonas* strains. The metabolites from DBT oxidation that were detected and identified by GC analyses of extracts of acidified cultures of *Pseudomonas* strains W1, F, BT1, and BT1d were the same as those identified in previous studies, so their identification is not described here. The metabolites detected were HFBT (22, 23, 28, 32, 34, 43), benzothiophene-2,3-dione (7), and DBT sulfoxide and sulfone (22, 23, 28, 34). Strains W1, F, and BT1 have also been reported to oxidize methyldibenzothiophenes to give methyl-substituted HFBTs and benzothiophene-2,3-diones (43). In addition, some of the methyldibenzothiophenes were oxidized to the corresponding sulfones (43).

GC-AED analyses of extracts of cultures of each of the *Pseudomonas* strains incubated with DBT for 2 to 9 days gave quantitative data on the kinetics of DBT oxidation to the observed metabolites. Figure 7a shows the oxidation of DBT by a culture of isolate W1 growing on 1-MN in the presence of DBT. This isolate and isolate F, which gave results similar to isolate W1 so they are not shown, cannot use DBT as a growth substrate but cometabolize it while growing on 1-MN. The recoveries of DBT and the various metabolites after 7 days of incubation are summarized in Table 2 for both isolates. Neither isolate W1 nor isolate F completely degraded the DBT, with 9.6% (2.0 μmol) and 12% (2.5 μmol) remaining after 7 days, respectively. In addition, while HFBT concentrations were appreciable after 2 days of incubation (Fig. 7a), this accumulation was transient, resulting in only 0.6% (0.13 μmol) and 0.7% (0.15 μmol) of the sulfur from DBT persisting as this product in cultures of isolates W1 and F, respectively, after 7 days. Thus, the metabolite HFBT is further degraded by these isolates. Only small amounts of the benzothiophene-2,3-dione were ever detected over the 7-day incubation (Fig. 7a; Table 2). The major products of these isolates were the sulfoxide and sulfone of DBT, which together accounted for 17% (3.6 μmol) and 24% (5.0 μmol) of the sulfur from DBT in cultures of isolate W1 and F, respectively (Table 2). The sulfoxide and sulfone were quantified together because they coeluted from the GC column.

Isolate BT1 is capable of growth with either 1-MN or DBT as the sole carbon and energy source, so the transformation of DBT by this strain was tested both with and without 1-MN in the medium. The results of these tests after 7 days are summarized in Table 2, and Fig. 7b shows the oxidation of DBT as the sole carbon source over 9 days. A notable difference between isolates W1 and F and isolate BT1 was that DBT degradation proceeded to a greater extent in the cultures of isolate

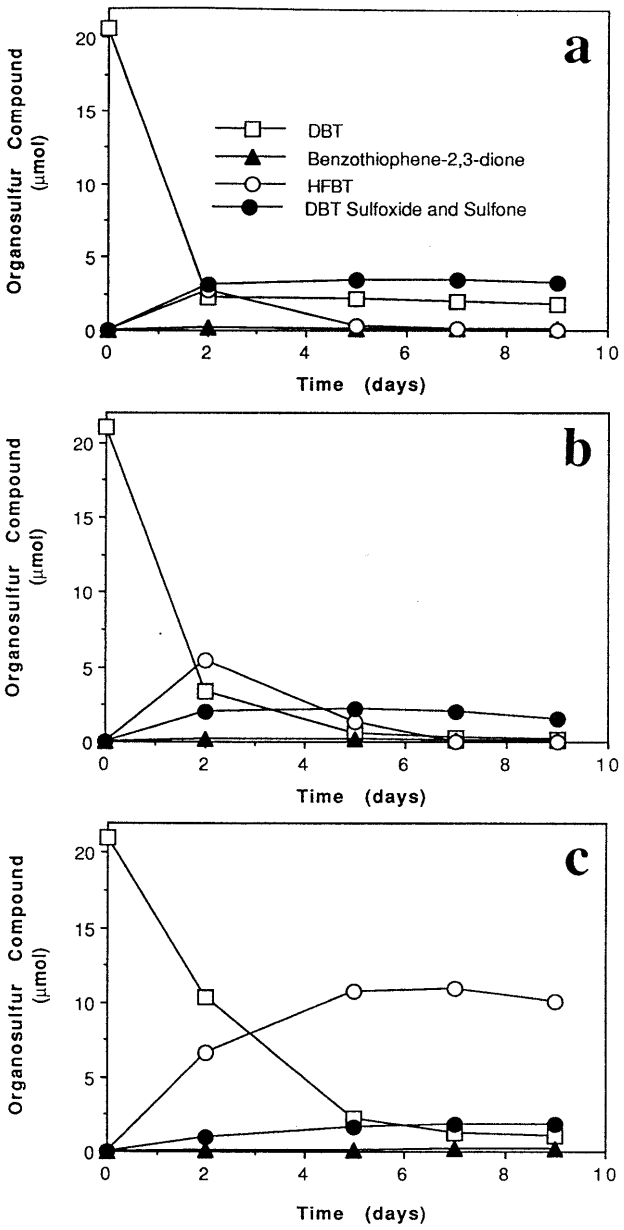

FIG. 7. Transformation of DBT to metabolites detected by GC-AED analysis of a culture of isolate W1 grown on 1-MN in the presence of DBT (a), a culture of isolate BT1 grown on DBT as the sole source of carbon and energy (b), and a culture of isolate BT1d grown on DBT as the sole source of carbon and energy (c).

BT1, regardless of whether 1-MN was included in the medium. For example, only 0.8% (0.18 μmol) of the DBT remained when 1-MN was included (Table 2, line 3), and 1.3% (0.29 μmol) remained when 1-MN was omitted (Table 2, line 4). As was observed with isolates W1 and F, the accumulation of HFBT in BT1 cultures was transient (Fig. 7b), with further degradation yielding only trace amounts of this product after 7 days of incubation regardless of whether 1-MN was included in the culture (Table 2, lines 3 and 4). In addition, only trace amounts of benzothiophene-2,3-dione were ever detected (Fig. 7b; Table 2).

When isolate BT1, pregrown on 1-MN, was incubated with DBT as the sole carbon source, oxidation of the sulfur atom

TABLE 2. Quantification of DBT and sulfur-containing metabolites as determined by GC-AED analyses of DCM extracts of acidified cultures of four *Pseudomonas* strains incubated for 7 days

Strain	Growth substrate	% Of sulfur from DBT recovered as:				Total recovery (%)	Recovery in sterile control (%)	Difference[a]
		DBT	HFBT	DBT sulfoxide and sulfone	Benzothiophene-2,3-dione			
W1[b]	1-MN	9.6	0.6	17	0.5	27.7	88	60.3
F[b]	1-MN	12	0.7	24	1.3	38.0	88	50.0
BT1[c]	1-MN + DBT	0.8	0.2	1.6	0.2	2.8	88	85.2
	DBT	1.3	0.2	9.4	0.3	11.2	86	74.8
BT1d[b]	DBT	5.9	52	8.9	0.8	67.6	86	18.4

[a] Difference between total recovery in culture extracts and that of the corresponding sterile control. The difference is the percentage of sulfur from DBT that escaped detection by the methods used.
[b] Twenty-one micromoles of DBT initially in the culture.
[c] Twenty-two micromoles of DBT initially in the culture.

gave the sulfoxide and sulfone as the major products (9.4%; 2.1 μmol) (Table 2, line 4), as was observed with isolates W1 and F. However, when 1-MN and DBT were in the medium, only 1.6% (0.35 μmol) of the sulfur originally present as DBT accumulated as the sulfoxide and sulfone (Table 2, line 3). Thus, with 1-MN in the BT1 cultures, either less DBT was sulfoxygenated or further degradation of the sulfoxygenated metabolites was stimulated, so that less of these products accumulated over the 7-day incubation.

The ability of strains W1, F, and BT1 to further transform DBT sulfone (17 μmol) was tested. Isolates W1 and F were unable to transform this sulfone over a 7-day incubation period when grown with 1-MN and the sulfone in the medium. The same was true for isolate BT1 incubated with DBT sulfone as the sole carbon and energy source. However, when isolate BT1 was grown on 1-MN in the presence of DBT sulfone, 55% (9.4 μmol) of the sulfone was depleted from the medium in 7 days. However, metabolites from oxidation of DBT sulfone were not detected by the extraction and analytical methods used. The ability of isolate BT1 to further degrade DBT sulfone, when provided with 1-MN as growth substrate, contributes to the large portion of organosulfur from DBT (85.2%) (Table 2, line 3) that was not detected by the DCM extraction and GC analytical methods used. The increase in the amount of sulfoxide and sulfone detected in cultures of isolate BT1 without 1-MN over that detected in cultures which included 1-MN (7.8%; 1.7 μmol) accounted for the smaller portion of organosulfur from DBT that was undetected in cultures where 1-MN was excluded (74.8%) (Table 2, line 4).

Large fractions of organosulfur from DBT were not detected by DCM extraction and GC analysis of cultures of isolates W1, F, and BT1 (between 50.0 and 85.2%) (Table 2). With 1-MN-grown cultures of isolate BT1, this was partly due to the further degradation of DBT sulfone. Evidence that a large fraction of this undetected sulfur from DBT resulted from the further degradation of HFBT by isolates W1, F, and BT1 was obtained with strain BT1d. The DCM extraction and GC analysis methods used accounted for 81.6% of the sulfur from DBT provided as the sole carbon source to cultures of strain BT1d after 7 days of incubation and after evaporative losses also observed in the sterile control were accounted for (Table 2). A large portion of this (52%; 11 μmol) was present in culture extracts as HFBT which accumulated in cultures (Fig. 7c). While some further degradation of HFBT did occur to yield the trace amount of benzothiophene-2,3-dione that was detected (Fig. 7c; Table 2), clearly much greater amounts of HFBT accumulated with this strain. The direct result is that for strain BT1d, only 18.4% of the sulfur from DBT is unaccounted for by the methods used. Thus, for strains W1, F, and BT1, which accumulated HFBT only transiently, the further degradation of HFBT is likely a major contributing factor to the large portions of organosulfur from DBT that were not detected by the methods used (50.0 to 85.2%).

The sulfur from DBT that escaped detection by GC-AED analysis of culture extracts could exist as metabolites that are extractable with DCM under the conditions used but are too polar to be analyzed by the GC method used. This was tested in a separate experiment comparing the recovery of DBT and its metabolites as determined by GC-AED analysis with the recovery determined by total sulfur analysis of DCM extracts of acidified cultures. Table 3 shows that the two methods of analysis gave recoveries that were within 12% of each other for the five cultures used in this comparison. In addition, neither method gave consistently higher recoveries. Thus, there was not a large amount of DCM-extractable organosulfur from DBT that was not amenable to detection by the GC-AED method.

Because some sulfur from DBT escaped detection by the methods used, the isolates were tested to determine if sulfate was being released from DBT. The cultures were incubated for three serial transfers in sulfate-free medium to dilute the sulfate in the maintenance medium to negligible background levels, and the amount of DBT added at the time of the third transfer was increased to 50 mg so that if sulfate was released,

TABLE 3. Comparison of the recoveries of organosulfur from DBT as determined by GC-AED analyses and by total sulfur analyses of DCM extracts of acidified cultures of four *Pseudomonas* strains incubated with 22 μmol of DBT for 7 days

Strain	Growth substrate	% Recovery by:	
		GC-AED analysis	Total sulfur analysis
W1	1-MN	22	31
F	1-MN	50	45
BT1	1-MN + DBT	4	14
	DBT	10	22
BT1d	DBT	64	58

TABLE 4. Comparison of the recoveries of organosulfur from DBT by DCM extraction of acidified cultures and by lyophilization[a]

Strain	Growth substrate	Organosulfur recovered (μmol) by:		Additional organosulfur found with lyophilization[c] (μmol)
		DCM extraction	Lyophilization[b]	
W1	1-MN	8.7	21.7 (5.6)	7.4
F	1-MN	12.5	18.2 (3.5)	2.2
BT1	1-MN + DBT	4.6	17.0 (3.9)	8.5
	DBT	6.8	19.9 (NA)	13.1
BT1d	DBT	16.8	19.6 (NA)	2.8

[a] The four *Pseudomonas* strains were incubated with 22 μmol of DBT for 7 days. Recovery was determined by total sulfur analysis of extracts obtained by the two methods.

[b] Values in parentheses represent the micromoles of organosulfur that were also observed by total sulfur analysis of controls that did not receive DBT. An appropriate control was not available (NA) for the cultures of isolates BT1 and BT1d with DBT as the growth substrate because omission of DBT yielded no growth of the cultures.

[c] Additional sulfur found = (amount found by lyophilization) − (amount found in control + amount found in DCM extract).

it would be more likely to be detected above the background levels. The detection limit of the turbidimetric assay used was 1 mg of sulfate per liter, which would have detected sulfate release from ≥0.8% of the 50 mg of DBT. However, no sulfate was detected with any of the isolates incubated with 1-MN in the presence of DBT or with isolate BT1d incubated with DBT alone.

Thus, it appeared that the sulfur from DBT that could not be detected was present in organic forms in aqueous cultures but was too polar to be extracted with DCM. To test this hypothesis, duplicate cultures of all three isolates grown on 1-MN in the presence of DBT, and of isolates BT1 and BT1d grown with DBT as the sole carbon and energy source, were established by use of the usual culture methods. After 7 days of incubation, one of each of the cultures was acidified and extracted with DCM, the other was lyophilized, and the residue was methyl esterified prior to extraction. Total sulfur analysis of the extracts was done to compare the recoveries of sulfur from DBT by the two methods (Table 4). A series of controls, which consisted of each of the isolates grown on 1-MN without DBT, were also taken through the lyophilization and methyl esterification procedure to account for any organosulfur products recovered by this method that result from microbial incorporation of sulfate provided in the medium into organic compounds.

As shown by the data in Table 4, lyophilization and methyl esterification gave increased recovery of organosulfur over that obtained by DCM extraction of acidified cultures. The increases ranged from 2.2 to 13.1 μmol of sulfur or from 10 to 60% of the initial 22 μmol of DBT added. Thus, there was a considerable portion of organosulfur that was not recovered by DCM extraction of acidified cultures but is recovered by lyophilization and methyl esterification.

DISCUSSION

THDBT is a minor constituent of some fossil fuel derivatives (30, 36), and while it is not itself a significant environmental contaminant or priority pollutant, its chemical structure incorporates a variety of molecular features offering an interesting study of bacterial transformations. The oxidation of THDBT by cell suspensions of the cyclohexane degrader CB1323 that had been pregrown in the presence of cyclohexane vapors yielded products analogous to those reported for the initial steps in the metabolism of cyclohexane (39). Just as monooxygenation of cyclohexane and subsequent dehydrogenation yield cyclohexanol and cyclohexanone as intermediates in the bacterial metabolism of cyclohexane, strain CB1323 also oxidized a methylene group in the alicyclic ring of THDBT to give a hydroxy-substituted THDBT (metabolite E), and subsequent dehydrogenation yielded the corresponding ketone (metabolite D). It is not known which of the methylene groups in the saturated ring was oxidized to give these products.

In the metabolism of cyclohexane, these reactions are followed by a biological Baeyer-Villiger monooxygenation to give a lactone, which is subsequently hydrolyzed to give adipic acid (39). However, the analogous ring cleavage reactions were not evident in the oxidation of THDBT by strain CB1323. The quantitative data obtained by GC-AED analysis showed that 100% of the sulfur in the substrate THDBT was accounted for, based on the assumption that the evaporative loss in the viable cultures was the same as that in the sterile control (27%).

Other compounds that contain both an aromatic and an alicyclic ring have been used in biodegradation studies which have focused primarily on bacteria that are able to degrade aromatic compounds. For example, the carbazole-degrading bacterium *Pseudomonas* sp. strain LD2 cometabolized 1,2,3,4-tetrahydrocarbazole when carbazole was provided as a growth substrate but could not utilize this compound as the sole carbon and energy source (17). Although over 60% loss of the 1,2,3,4-tetrahydrocarbazole was observed, no metabolites from its oxidation were detected by GC analysis.

Schreiber and Winkler (44) studied *Pseudomonas stutzeri* AS39, which was capable of growth on tetralin (1,2,3,4-tetrahydronaphthalene) and naphthalene but not on cyclohexane, cyclohexanol, or cyclohexanone. They reported the oxidation of tetralin to 1-tetralone [3,4-dihydro-1(2*H*)-naphthalenone] and 1-tetralol (1,2,3,4-tetrahydro-1-naphthalenol), which are analogous to metabolites D (Fig. 4) and E (Fig. 5) found from THDBT.

Sikkema and de Bont (46) described the ability of eight bacterial isolates to utilize tetralin as the sole carbon and energy source. All but one of the strains were able to grow on one or more aromatic hydrocarbons, but none could utilize cyclohexane as the sole carbon and energy source. Reported metabolites included products of oxidation of the alicyclic ring, i.e., 1,2,3,4-tetrahydro-1-naphthalenol and 3,4-dihydro-1(2*H*)-naphthalenone, and products of oxidation of the aromatic ring, i.e., 5,6,7,8-tetrahydro-1-naphthalenol and 5,6,7,8-tetrahydro-2-naphthalenol (47, 48). Strain CB1323, used in our study, also oxidized both the aromatic and alicyclic rings of THDBT, yielding metabolites D, E, and F.

The cometabolism of THDBT by the 1-MN-degrading *Pseudomonas* strains resulted in oxidation, cleavage, and degradation of the benzene ring of THDBT to form 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene (Fig. 1). The assignment of these functional groups to positions 3 and 2 is based on analogy to the published Kodama pathway of DBT metabolism (22, 23), which proceeds via dioxygenase attack at positions 1 and 2 of DBT to give 1,2-dihydroxy-1,2-dihydrodibenzothiophene, followed by dehydrogenation to give 1,2-dihydroxydibenzothiophene. This intermediate then undergoes *meta* cleavage to open the benzene ring of DBT, and degradation of the opened ring yields a molecule each of pyruvate and HFBT (22, 23, 28, 32, 34). In the current study, the data suggest that *Pseudomonas* strains W1, F, and BT1 utilize an analogous pathway to oxidize the benzene ring of THDBT. This is not sur-

prising since these isolates metabolize DBT via HFBT by use of the Kodama pathway, with further transformation presumably yielding benzothiophene-2,3-dione. These isolates degrade the unsubstituted ring of methyldibenzothiophenes (43) and 3,4-dimethyldibenzothiophene (26) to give methyl and dimethyl-substituted isomers of HFBT and benzothiophene-2,3-dione. The DBT-oxidizing cultures of Bohonos et al. (7) also transformed DBT to HFBT and benzothiophene-2,3-dione. However, 4,5,6,7-tetrahydrobenzothiophene-2,3-dione was not observed in these studies with THDBT.

The pathway used by these *Pseudomonas* strains to oxidize DBTs to analogs of HFBT is biochemically similar to the pathway reported for naphthalene catabolism (11). Other research has shown that a single genetic pathway controls the metabolism of DBT, naphthalene, and phenanthrene in a soil *Pseudomonas* sp. (9). The oxidative activity of naphthalene dioxygenase is not restricted to aromatic ring dioxygenation with the subsequent dehydrogenation and ring cleavage reactions but has also been reported to catalyze monooxygenations of benzylic methyl or methylene moities and sulfoxygenations of the sulfur atom of organic sulfur compounds (1, 45). The monooxygenation of the C-1 and C-4 methylene groups of THDBT, which are not benzylic but are in a position alpha to the aromatic thiophene ring, by the dioxygenases of the three *Pseudomonas* strains was not observed to occur as might be expected from previous reports of monooxygenation of benzylic methylene groups by naphthalene dioxygenase cloned from NAH7 (45) and from studies of tetralin metabolism (see above). However, the oxidation of the sulfur atom of THDBT to give the sulfoxide and sulfone was observed with 1-MN-grown cultures of isolates W1 and F, and these were the most abundant products detected. The sulfoxide and sulfone of THDBT were not detected with isolate BT1. However, all three isolates were shown to oxidize DBT to the sulfoxide and sulfone as has been reported as an alternate dead-end route for other bacteria that utilize the Kodama pathway to metabolize DBT (22, 23, 28, 34). This oxidation of DBT to the sulfoxide and sulfone was a dead-end pathway for isolates W1 and F; however, isolate BT1 was capable of further degradation of DBT sulfone if 1-MN was present as a growth substrate. It is possible that the sulfoxide and sulfone were not detected in cultures of isolate BT1 grown on 1-MN in the presence of THDBT because the sulfone of THDBT was also further metabolized.

Quantitative GC-AED analyses of extracts of the three *Pseudomonas* strains incubated for 3 days with THDBT and 1-MN showed that only 9 to 42% of the sulfur in THDBT was detected by the analytical methods used. GC-FPD analyses of extracts from cultures incubated for shorter times suggested that 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene was more abundant prior to the day 3 GC-AED analysis. However, the nonlinear response of the FPD does not allow quantitation, and GC-AED analysis was not done for these earlier sample times. But it appeared that 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene accumulated transiently and was subsequently degraded by isolate BT1. Unfortunately, the amount of the synthesized THDBT available was insufficient to do further quantitative studies with this substrate.

Because a sulfur mass balance was not achieved in these studies with aromatic hydrocarbon-degrading bacteria and THDBT, and in other studies with dimethyldibenzothiophenes (26), we chose to pursue additional quantitative studies with commercially available DBT. In general, the amounts of DBT remaining and the amounts of the HFBT, sulfoxide, and sulfone that were produced from DBT were comparable to the amounts of THDBT and the analogous metabolites that were observed for each isolate incubated with 1-MN as the growth substrate. The amounts of tetrahydroHFBT that were observed to form with isolates W1 and F was slightly larger than the amounts of HFBT. Although the analog of benzothiophene-2,3-dione was not observed to form from THDBT, this metabolite from DBT was observed only in very small amounts (0.2 to 1.3% of the DBT added to the cultures), so it does not significantly influence the sulfur mass balance. In addition, the fact that the sulfoxide and sulfone of THDBT were not observed in 1-MN-grown cultures of isolate BT1 did not significantly influence the sulfur mass balance, because in 1-MN-grown cultures of isolate BT1 incubated with DBT, only 1.6% of the DBT was found in the form of sulfoxide and sulfone. Therefore, similar results were obtained with DBT as were observed with THDBT. That is, the sulfur present as the observed metabolites was much less than the amount of substrate organosulfur that was depleted in cultures that were grown on 1-MN (for an example, see Table 2).

As far as we are aware, quantitative studies that establish a sulfur mass balance for bacterial degradation of DBT via the Kodama pathway have not been reported. Kodama et al. (22, 23) identified metabolites from pure cultures of *Pseudomonas jianii*, and the yields of three of the purified metabolites were reported. By our calculations, the metabolites accounted for 31% of the DBT added to the culture. However, the amount of DBT remaining at the end of the incubation period was not reported, so whether a mass balance existed is not known.

Maintaining isolate BT1 on DBT as the sole carbon source for over 2 years yielded a strain, designated BT1d, that had altered DBT-degrading activity, leading to a difference in the abundance of HFBT remaining in batch cultures. The highest recovery of organosulfur was in the culture of isolate BT1d grown with 21 μmol of DBT as the sole carbon and energy source (Table 2). After 7 days of incubation, HFBT was the most abundant product, accounting for 52% of the sulfur from DBT. Although some of the HFBT was degraded further, presumably yielding a small amount of benzothiophene-2,3-dione (0.8%), HFBT accumulated in the culture without extensive further degradation taking place (Fig. 7c). This accumulation of HFBT resulted in a high total recovery of sulfur from DBT by the DCM extraction and GC methods used in this study (81.6% after accounting for evaporative loss). The low total recoveries of sulfur from DBT (14.8 to 50.0% after evaporative losses are taken into account) found with cultures that accumulated HFBT transiently suggest that further degradation of HFBT leads to the formation of metabolites that are too polar to be extracted with DCM and to be analyzed by the GC methods used. However, the further degradation of HFBT did not lead to a corresponding increase in the amount of benzothiophene-2,3-dione detected (Table 2). There is only one report on HFBT biodegradation (33) which demonstrated CO_2 release from this compound, but no sulfate release was detected.

Eaton and Nitterauer (10) showed that benzothiophene was microbially oxidized via dioxygenase attack at positions 2 and 3 and that subsequent reactions, including ring cleavage, led to the formation of 2-mercaptophenylglyoxalate, which cyclized to give benzothiophene-2,3-dione by acid-catalyzed dehydration. Our cultures were routinely acidified prior to extraction, and so the benzothiophene-2,3-dione detected in these extracts was likely present in the culture at neutral pH as 2-mercaptophenylglyoxalate. Thus, detection of this dione indicates that cleavage of the thiophene ring likely occurred. The small amounts of benzothiophene-2,3-dione that were detected, even in culture conditions where further degradation of HFBT did occur, suggest that 2-mercaptophenylglyoxalate may also be further

degraded to compounds that are not detected by the methods used in this study.

Our experimental results show that the amounts of metabolites detected from DBT degradation by DCM extraction and GC-AED did not account for the amounts of the substrate depleted. They also suggest that HFBT, 2-mercaptophenylglyoxalate (detected as benzothiophene-2,3-dione), and DBT sulfone were likely biodegraded under certain conditions. Thus, additional experiments were undertaken to determine the fate of the sulfur in DBT. Total sulfur analyses of DCM extracts indicated that there was not a large portion of sulfur from DBT in the extract that was not detectable by GC-AED analysis. In addition, there was no evidence of sulfate release from DBT by any of the *Pseudomonas* strains. Thus, it appears that the missing sulfur from DBT is present as organic metabolites that are too polar to recover by liquid-liquid extraction with DCM. This hypothesis was supported by the fact that lyophilization and methyl esterification gave increased recovery of total sulfur over that obtained by simple DCM extraction of acidified cultures (Table 4). Some of the missing organic sulfur likely results from further degradation of HFBT, 2-mercaptophenylglyoxalate, and DBT sulfone, and this possibility is the subject of ongoing investigations in our laboratory.

The amounts of DBT sulfoxide and sulfone that were produced in cultures of strain BT1 incubated with and without 1-MN were quite different (Table 2), suggesting that cosubstrates may have a significant effect on the metabolism of organic contaminants. Of course, in petroleum-contaminated environments, DBTs are present with numerous saturated and aromatic hydrocarbons. The complex mixture of compounds present in petroleum will likely influence the fate of the sulfur present as condensed thiophenes. Biodegradation studies that use pure cultures and pure compounds are useful for the determination of metabolic pathways for the destruction of organic contaminants and are a step towards the identification of metabolites whose possible formation in contaminated environments may influence the ability of bioremediation to reduce the toxicity of a contaminated site. However, this work demonstrates that cosubstrates may significantly influence the degradation and environmental fate of contaminants, such as condensed thiophenes, typically found in a complex aromatic matrix. While the same metabolites were detected regardless of whether 1-MN was included in the pure culture studies described in this report, the amounts of metabolites present and the effect on the overall sulfur mass balance were significant. This emphasizes the importance of taking a quantitative approach and pursuing a mass balance in studies of the biodegradation of organic contaminants. There are relatively few studies of the biodegradation of condensed thiophenes that have emphasized this approach.

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