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Selective Desulfurization of Dibenzothiophene by *Rhodococcus erythropolis* D-1

YOSHIKAZU IZUMI,* TAKASHI OHSHIRO, HIROSHI OGINO, YOSHIMITSU HINE, AND MASAYUKI SHIMAO

Department of Biotechnology, Faculty of Engineering, Tottori University, Tottori 680, Japan

Received 7 June 1993/Accepted 20 October 1993

A dibenzothiophene (DBT)-degrading bacterium, *Rhodococcus erythropolis* D-1, which utilized DBT as a sole source of sulfur, was isolated from soil. DBT was metabolized to 2-hydroxybiphenyl (2-HBP) by the strain, and 2-HBP was almost stoichiometrically accumulated as the dead-end metabolite of DBT degradation. DBT degradation by this strain was shown to proceed as DBT → DBT sulfone → 2-HBP. DBT at an initial concentration of 0.125 mM was completely degraded within 2 days of cultivation. DBT at up to 2.2 mM was rapidly degraded by resting cells within only 150 min. It was thought this strain had a higher DBT-desulfurizing ability than other microorganisms reported previously.

Numerous organic sulfur compounds are found in coal and crude oil. When these fossil fuels are combusted, sulfur dioxide is released into the atmosphere, causing acid rain and air pollution. Chemical desulfurization of petroleum is carried out by an expensive hydrogenation process for sulfur compounds that uses inorganic catalysts at a high temperature and a high pressure.

Recently, microbial degradation of organic sulfur compounds has attracted attention for its potential application to the desulfurization of coal and petroleum. Among the organic sulfur compounds present in fossil fuels, dibenzothiophene (DBT) and its derivatives are regarded as representatives. There have been several reports on the isolation of DBT-degrading bacteria by use of DBT as a model compound. A *Brevibacterium* sp. strain mineralized DBT as a sole source of carbon, sulfur, and energy via benzoate to carbon dioxide, sulfite, and water (10). The partial microbial degradation of DBT was demonstrated by other researchers, and metabolites derived from the oxidation of DBT were detected, depending on the strain and culture conditions. Under aerobic conditions, 3-hydroxy-2-formyl benzothiophene was formed by a *Pseudomonas* sp. (5) and 1,2-dihydroxy-1,2-dihydrodibenzothiophene and dibenzothiophene 5-oxide were generated by a *Beijerinckia* sp. (4), and under anaerobic conditions, biphenyl was formed by *Desulfovibrio desulfuricans* M6 (11). The partial DBT degradation product, 3-hydroxy-2-formyl benzothiophene, was degraded completely by a mixed culture (6). Recently, two bacterial strains, *Corynebacterium* sp. strain SY1 (8) and *Rhodococcus rhodochrous* IGTS8 (2), were shown to remove only sulfur from DBT, converting DBT to 2-hydroxybiphenyl (2-HBP). These strains appear promising for their ability to selectively remove organic sulfur without the loss of carbon atoms, which bring about energy. Figure 1 shows the proposed pathways of DBT microbial degradation reported previously.

We have isolated a DBT-degrading bacterium, tentatively identified as *Rhodococcus erythropolis* D-1, which utilizes DBT as a sole source of sulfur. This report describes the desulfurization of DBT by growing and resting cells of strain D-1 and compares the DBT-desulfurizing ability and the DBT metabolic pathway of *R. erythropolis* D-1 with those of *R. rhodochrous* and a *Corynebacterium* sp.

* Corresponding author. Phone: 857-31-5267. Fax: 857-31-0881.

MATERIALS AND METHODS

Microorganisms. Several strains which utilized DBT as a sole source of sulfur were isolated from 150 soil samples from various areas in Japan by the selective enrichment method with medium A (see below) supplemented with DBT at 5.4 mM. Single-colony isolation was repeated on the same medium containing 1.5% agar and finally carried out on a nutrient agar medium. Among the DBT-utilizing strains, strain D-1 was used throughout this work. The strain was maintained on slants of medium A supplemented with DBT at 5.4 mM and containing 1.5% agar.

Media and growth conditions. Medium A is a sulfur-free synthetic medium containing 5 g of glucose, 0.5 g of KH_2PO_4, 4 g of K_2HPO_4, 1 g of NH_4Cl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 0.01 g of NaCl, 10 ml of metal solution, and 1 ml of vitamin mixture in 1,000 ml of distilled water (pH 7.5). The metal solution contained 0.5 g of $FeCl_2 \cdot 4H_2O$, 0.5 g of $ZnCl_2$, 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $Na_2MoO_4 \cdot 2H_2O$, 0.05 g of $CuCl_2$, 0.05 g of $Na_2WO_4 \cdot 2H_2O$, and 120 mmol of HCl in 1,000 ml of distilled water. The vitamin mixture was composed of 400 mg of calcium pantothenate, 200 mg of inositol, 400 mg of niacin, 400 mg of pyridoxine hydrochloride, 200 mg of *p*-aminobenzoic acid, and 0.5 mg of cyanocobalamin in 1,000 ml of distilled water (9). Unless otherwise indicated DBT was dissolved in ethanol (27 mM) and added to sterilized medium A. The nutrient agar medium was composed of 10 g of peptone, 7 g of meat extract, 5 g of yeast extract, 3 g of NaCl, and 15 g of agar in 1,000 ml of distilled water. Cultivation was carried out at 30°C in test tubes containing 5 ml of medium with reciprocal shaking (300 strokes per min) or in 2-liter flasks containing 500 ml of medium with reciprocal shaking (100 strokes per min).

Resting-cell reactions. Strain D-1 was grown in medium A containing DBT at 0.136 mM in 2-liter flasks for 2 days. Cells were harvested at 4°C by centrifugation at 10,000 × *g* for 15 min, washed once with 0.85% NaCl, and stored at −20°C until use. The cells were suspended in 0.1 M potassium phosphate buffer (pH 7.0). DBT-ethanol solution was added to 1 ml of the cell suspension in a test tube (18 by 180 mm),

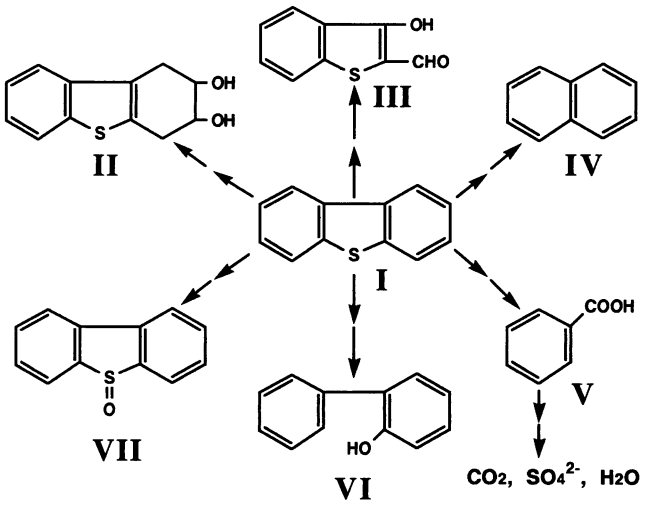

FIG. 1. Proposed pathways of DBT degradation. (I) DBT. (II) 1,2-Dihydroxy-1,2-dihydrodibenzothiophene. (III) 3-Hydroxy-2-formyl benzothiophene. (IV) Biphenyl. (V) Benzoic acid. (VI) 2-HBP. (VII) Dibenzothiophene 5-oxide.

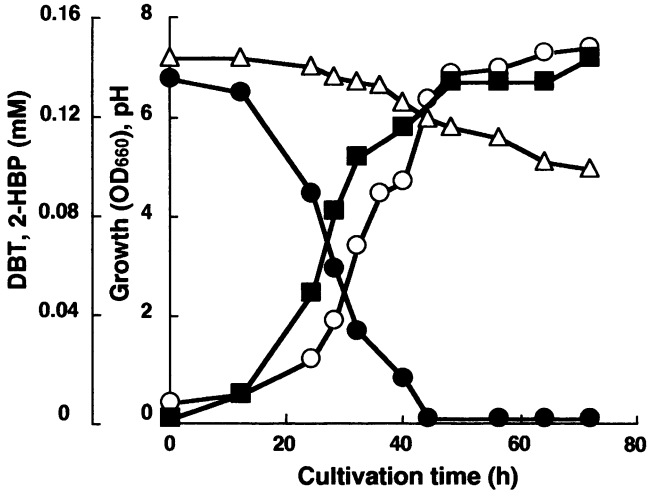

FIG. 2. DBT utilization by *R. erythropolis* D-1. The strain was cultivated in medium A supplemented with 0.125 mM DBT as a sole source of sulfur. Symbols: ○, growth; △, pH; ●, DBT; ■, 2-HBP. OD_{660}, optical density at 660 nm.

and the resting-cell reaction was allowed to proceed at 30°C with reciprocal shaking at 300 strokes per min.

Analytical methods. Growth was measured turbidimetrically at 660 nm. Determination of DBT and its metabolites was carried out by gas chromatography. The culture broth or the reaction mixture with resting cells in a test tube was acidified to pH 2.0 with 1 N HCl and extracted with an 0.8 volume of ethyl acetate. A portion of the ethyl acetate layer was removed and centrifuged, and 5 μl of the supernatant was injected into a gas chromatograph (GC-14A; Shimadzu, Kyoto, Japan) with a flame ionization detector. The gas chromatograph was equipped with a glass column (3.2 mm by 1 m) packed with Silicone OV-17, 2% Chromosorb WAW DMCS 80/100 (GL Sciences, Tokyo, Japan). The flow rate of the nitrogen carrier gas was 15 ml/min. The column temperature was programmed from 120 to 250°C at 5°C/min. The injector and detector temperatures were maintained at 250 and 335°C, respectively. DBT and its metabolites were also determined by high-performance liquid chromatography (HPLC) with an 880-PU pump and an 875-UV variant-wavelength UV monitor (both from JASCO, Tokyo, Japan) and a Lichrospher RP-18 column (particle size, 5 μm) (4 by 250 mm; E. Merck AG, Darmstadt, Germany). Detection was based upon the A_{280}. The mobile phase was 20 mM potassium dihydrogen phosphate, the pH of which was adjusted to 2.5 with phosphoric acid-methanol (1:4 [vol/vol]). The flow rate was 1 ml/min. Sulfate was determined with $BaCl_2$ by the method of Dodgson (1).

Chemicals. DBT was obtained from Wako Chemicals, Osaka, Japan. 2-HBP was purchased from Nacalai Tesque, Kyoto, Japan. DBT sulfone was obtained from Aldrich, Milwaukee, Wis. All other chemicals were of analytical grade, commercially available, and used without further purification.

RESULTS

Taxonomic characteristics of strain D-1. Among the isolated strains, one strain, designated D-1, showed the most potent DBT-utilizing ability. The taxonomic characteristics of the strain were investigated at the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, United Kingdom). Strain D-1 was a gram-positive, immotile bacterium which formed coccoid and short rod cells. The strain was catalase positive and oxidase negative and did not produce acid from glucose fermentatively. The strain grew at 37°C but not at 41°C. Colonies of the strain on nutrient agar were round, regular, entire, smooth, slightly mucoid, pale orange-pink, opaque, and low convex. The diamino acid of the cell wall of the strain was *meso*-diaminopimelic acid. Cells of the strain contained mycolic acids and 10-methyl octadecanoic acid (tuberculostearic acid). The strain utilized inositol, mannitol, sorbitol, sodium adipate, sodium citrate, glycerol, trehalose, *p*-hydroxybenzoic acid, D-mannose, acetamide, sodium lactate, and sodium glutamate but not maltose, rhamnose, *m*-hydroxybenzoic acid, sodium benzoate, L-tyrosine, or D-galactose as a sole source of carbon. Therefore, although its utilization of maltose and mannose was atypical, we tentatively identified the strain as *R. erythropolis*.

DBT utilization by strain D-1. Figure 2 shows the time course of DBT utilization by the strain in medium A supplemented with DBT at 0.125 mM as a sole source of sulfur. The strain showed maximum growth (about 7 units of optical density at 660 nm) at 42 h of cultivation, concurrent with the depletion of DBT. A metabolite detected in ethyl acetate extracts of the cultures was identified as 2-HBP by cochromatography with authentic 2-HBP, by gas chromatography, and by HPLC. 2-HBP accumulation was almost equimolar to DBT depletion in the culture and did not decrease during stationary-phase cultivation up to 75 h (Fig. 2). This fact indicated that 2-HBP is a dead-end metabolite of DBT degradation by this strain. In addition to 2-HBP, a metabolite detected in trace amounts by gas chromatography and HPLC was identified as DBT sulfone. There was a transient appearance of DBT sulfone in cultures grown on DBT between the early exponential and late log phases of growth. The pH decreased during the cultivation.

Growth specificity of alternate sulfur sources. The strain was cultivated in medium A with 25 mg of various sulfur compounds per liter as a sole source of sulfur for 7 days (Table 1). The strain grew well on DBT, DBT sulfone,

TABLE 1. Sulfur compound specificity of *R. erythropolis*[a]

Sulfur compound	Result after the following cultivation time:			
	3 days		7 days	
	Growth (OD_{660})	pH	Growth (OD_{660})	pH
None	0.1	7.2	0.1	7.1
DBT	6.4	4.9	6.6	4.5
DBT sulfone	3.0	6.5	5.0	6.0
Thiophene	0.3	7.1	0.6	6.9
2-Methylthiophene	0.1	7.2	0.2	7.1
3-Methylthiophene	0.1	7.2	0.1	7.2
2-Ethylthiophene	0.2	7.2	0.3	7.1
Benzothiophene	0.1	7.1	0.2	7.1
Thiophene 2-carboxylic acid	0.1	7.2	0.1	7.2
Thiophene 2-acetic acid	0.1	7.2	0.1	7.2
Thianthrene	0.2	6.9	0.5	6.7
Thioxanthen-9-one	1.1	6.6	4.1	6.4
Benzenesulfonic acid	0.1	7.2	0.1	7.2
Benzenesulfinic acid	0.1	7.2	0.1	7.2
p-Toluenesulfonic acid	0.1	7.2	0.1	7.2
Dimethyl sulfate	0.1	7.2	0.2	7.2
Dimethyl sulfone	5.8	5.2	6.8	4.1
Dimethyl sulfoxide	0.1	7.2	0.1	7.2
Dimethyl sulfide	0.2	7.2	0.1	7.2
Methanesulfonic acid	5.3	4.2	6.1	4.1
2-Mercaptoethanol	4.1	5.4	4.8	4.7
Ammonium sulfate	4.3	6.1	5.1	5.8
Sulfur	4.8	6.1	5.8	5.8

[a] Each sulfur compound was added to medium A at an initial concentration of 25 mg/liter. Volatile compounds, thiophene, 2-methylthiophene, 3-methylthiophene, 2-ethylthiophene, and benzothiophene, were fed to the cultures at a concentration of 25 mg/liter again after 3 days of cultivation. OD_{660}, optical density at 660 nm.

thioxanthen-9-one, dimethyl sulfone, methanesulfonic acid, 2-mercaptoethanol, elemental sulfur, and ammonium sulfate, all yielding a decrease in the pH of the medium. However, the growth of the strain on the thiophene derivatives tested was negligible.

DBT degradation by resting cells. The desulfurization of DBT was investigated with various amounts of resting cells (Fig. 3). The conversion of DBT to 2-HBP appeared the most efficient in reaction mixtures containing 21 mg of cells. It was almost stoichiometric in the resting-cell reaction mixture (data not shown). However, in reaction mixtures with a much higher cell density, DBT degradation was observed to be significantly retarded. This fact may indicate that oxygen is a limiting factor for DBT degradation by the cells. The availability of a water-insoluble substrate (DBT) to the resting cell population may have caused the scattering of datum points in Fig. 3. Sulfate was detected in reaction mixtures with resting cells, but the amount of sulfate detected was small and not stoichiometric to the amount of DBT converted to 2-HBP (data not shown). DBT degradation in reaction mixtures containing various amounts of DBT was examined. DBT at up to 2.2 mM (approximately 400 mg/liter) was completely degraded within 150 min (data not shown). Almost the same degradation rate was observed in reaction mixtures containing up to about 3 mM DBT (data not shown), indicating no substrate inhibition by DBT.

DISCUSSION

A new DBT-degrading bacterium, tentatively identified as *R. erythropolis* D-1, utilizes DBT as a sole source of sulfur.

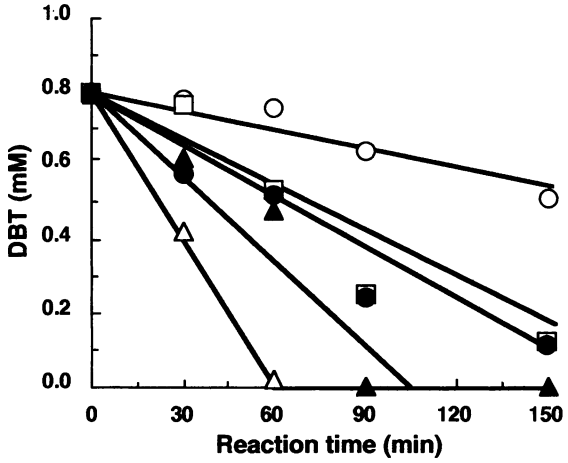

FIG. 3. Effect of cell density on DBT degradation by resting cells of *R. erythropolis* D-1. The reaction mixture contained DBT at an initial concentration of 0.8 mM. Symbols (indicating cell density, in mg [dry weight] per ml): ○, 4.2; ●, 11; △, 21; ▲, 42; □, 84.

The strain showed efficient DBT-desulfurizing ability. DBT at an initial concentration of 0.125 mM was completely degraded within 2 days by growing cells, whereas 2.2 mM DBT was converted to 2-HBP within 150 min by resting cells. Such an efficient conversion of DBT has not been reported for other DBT-oxidizing microorganisms. For example, it took 48 h for 5.4 mM DBT to be reduced to 10% by resting cells of *R. rhodochrous* IGTS8 (2), but reaction conditions were not identical.

The metabolism of DBT by aerobic microorganisms can be generally classified into three types. In type 1 metabolism, the carbon skeleton of DBT is partially oxidized, with the C-S bond remaining intact (3). In type 2 metabolism, DBT serves as the sole source of carbon, sulfur, and energy, with mineralization proceeding through benzoate (10). In type 3 metabolism, DBT serves as the sole source of sulfur. DBT is desulfurized by the selective cleavage of the C-S bond, resulting in the accumulation of 2-HBP (2, 7, 8). This study shows that DBT degradation by strain D-1 belongs to type 3 metabolism. DBT was stoichiometrically converted to 2-HBP by this strain, indicating that 2-HBP represents a dead-end metabolite of DBT metabolism. Sulfate production during the degradation of DBT by resting cells was not stoichiometric to the amount of DBT converted to 2-HBP, probably because sulfate was incorporated by the cells. This strain did not grow on DBT as a sole source of carbon (data not shown). In addition to 2-HBP, a trace amount of DBT sulfone was detected in cultures grown on DBT. This strain utilized DBT sulfone as a sole source of sulfur. In the resting-cell reaction, added 1 mM DBT sulfone was converted to 0.3 mM 2-HBP after 120 min, and no DBT was detected as a by-product (data not shown). Therefore, DBT degradation by this strain was assumed to proceed as DBT → DBT sulfone → 2-HBP. The metabolic pathway is similar to that proposed for DBT degradation by *Corynebacterium* sp. strain SY1 (8) and *R. rhodochrous* IGTS8 (2, 7). With this type of DBT degradation, there is no loss of the carbon atoms of DBT, and only the DBT sulfur atom is eliminated. This type of DBT degradation may be of potential advantage for the microbial desulfurization of fossil fuels and can be extended to other DBT derivatives, such as 4,6-dialkyl-DBT, which are actual forms of DBT present in such fuels.

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