

MICROBIAL DESULFURIZATION OF DIBENZOTHIOPHENE AND ITS DERIVATIVES

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INTRODUCTION

The serious environmental problem of acid rain is at least partly caused by the combustion of sulfur compounds present in the fossil fuels, releasing sulfur dioxide into the atmosphere. Though inorganic sulfur can be reduced by physical or chemical means, none of them can be applied to removing organic sulfur from petroleum. Therefore, microbial processes that can do so have recently received much focus. Dibenzothiophene (DBT) and its derivatives have been widely used as model organic sulfur compounds in petroleum (1). Three pathways of DBT degradation have been reported. The first is the ring-destructive pathway, in which the sulfur of DBT remains (2-5), the second is the completely destructive pathway, in which DBT is mineralized to carbon dioxide, sulfite and water (6), and the third is the sulfur-specific pathway, in which only sulfur is removed from DBT (7-9) as illustrated in Fig. 1. Strains having the second or third pathways should be applied to the microbial desulfurization process. In other studies, we also isolated a DBT-degrading bacterium, *Rhodococcus erythropolis* D-1, which has the sulfur-specific pathway (10) and observed an enzyme system catalyzing this conversion (11). Many research groups have since studied the desulfurization of DBT by the sulfur-specific pathway (12-14). The genes involved in DBT degradation have been identified (15-17). However, there has so far been little report on the DBT desulfurization in the presence of hydrocarbon. Since petroleum should ideally be desulfurized, we isolated a strain with the capacity to desulfurize DBT in the presence of hydrocarbon. Here we describe the desulfurization of DBT by growing whole cells of *Rhodococcus erythropolis* H-2 in the presence of n-tetradecane (TD) and other hydrocarbons. We also describe the desulfurization of substituted DBTs which actually exist in petroleum by *R. erythropolis* H-2 in the presence of TD.

MATERIALS AND METHODS

Medium A-1 was the same as medium A described elsewhere (10) except that glucose was omitted. Cells were cultivated at 30°C in test tubes containing 5 ml of medium or in 2-liter flasks containing 500 ml of medium with reciprocal shaking (300 rpm for test tubes and 100 rpm for flasks).

To isolate bacteria which could desulfurize DBT in petroleum, several soil samples from various areas in Japan were transferred to test tubes containing medium A-1 supplemented with 5.4 mM DBT as a sole source of sulfur and 0.5% TD. Single colony isolation was repeated on the same medium containing agar. Among the DBT-utilizing strains in the presence of TD, we selected strain H-2.

Strain H-2 was cultivated in medium A-1 with 0.5% glucose and 0.27 mM DBT in 2-liter flasks for 2 days. Cells were harvested at 4°C by centrifugation at 10,000 x g for 15 min, washed once with 0.85% NaCl and resuspended in the same solution. The suspension was lyophilized and kept at -20°C until use. The reaction mixture contained, in 1 ml, TD, DBT which was dissolved in TD, 0.1 M potassium phosphate buffer (pH 7.0) and lyophilized cells. The reaction proceeded in test tubes at 30°C with reciprocal shaking (300 rpm).

DBT and 2-HBP were determined by gas chromatography or high performance liquid chromatography as described (10). TD was measured by gas chromatography under the same conditions. When the strain was cultivated in the medium with hydrocarbon, the cells floated on the surface of the medium. Therefore, growth could not be measured turbidimetrically. We centrifuged the culture broth at 15,000 x g for 45 min and the cell pellet was resuspended in 0.85% NaCl containing 5% polyoxyethylene lauryl alcohol ether (Brij 35). Cell growth was determined by measuring the optical density of this suspension. OD660 was proven to be proportional to the number of viable cells.

RESULTS AND DISCUSSION

Characterization of a DBT-utilizing bacterium in the presence of hydrocarbon

Among the isolates, a strain designated H-2 utilized DBT most rapidly in the presence of TD. The taxonomic properties were examined at the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, Scotland, United Kingdom). As a result, the strain was identified as *Rhodococcus erythropolis*. There are some differences between our previous strain D-1 (10) and the present strain H-2 grown on carbon source such maltose, L-tyrosine and D-mannose: in strain H-2, these were possibly positive. Since this strain assimilated TD as a carbon source in addition to DBT as a sulfur source, several hydrocarbons were investigated to determine whether or not they could support the growth of *R. erythropolis* H-2. As shown in Table 1, this strain grew on n-alkanes with carbon chains longer than C8 with and without glucose, whereas it did not grow on n-hexane, styrene, p-xylene, cyclooctane and toluene even in the presence of glucose.

Growth of *R. erythropolis* H-2 in the medium containing DBT and TD

The strain was cultivated in medium A-1 with TD as a sole source of carbon and DBT as a sole source of sulfur. The strain showed maximal growth (OD660=ca. 3.0) after 2 days of cultivation. DBT completely disappeared before this point. The metabolite 2-HBP was formed from DBT and it was almost equimolar to the amount of DBT degraded. The level of TD decreased slightly, and the pH decreased concomitantly with the increase of cell growth.

DBT degradation by whole cell reactions

To prepare whole cells for DBT degradation, *R. erythropolis* H-2 was cultivated in medium containing either 0.5% glucose or 0.5% TD as a carbon source. Cells were lyophilized after harvesting and used for each reaction by resting cells. When the whole cell reactions proceeded with 50% TD for 4 h, the DBT degradation rates by cells pregrown in glucose and TD were 60 and 33%, respectively. Therefore, the following studies of whole cells reactions were performed using cells grown in glucose. DBT degradation was investigated using various amounts of lyophilized cells. The reaction proceeded most efficiently when the cells were added to the reaction mixture at a concentration of 80 mg/ml. However, DBT degradation was suppressed in the reaction mixture at elevated concentrations of the lyophilized cells. The limitation of oxygen may lower DBT degradation as found in *R. erythropolis* D-1 (10). DBT degradation in reaction mixtures containing various amounts of TD or DBT were examined. The reaction proceeded more efficiently with, than without TD. Even with as much as 70% TD, the degradation was enhanced compared with the situation without TD. The optimal concentration of TD was about 40%. TD at a concentration higher than 80% suppressed the degradation. In a reaction mixture supplemented with 40% TD and 80 mg/ml of the lyophilized cells, DBT up to 3 mM was completely degraded within 4 h. Figure 2 shows the time course of DBT degradation and 2-HBP accumulation. The amount of 2-HBP formed was almost stoichiometric to that of DBT degraded. It seemed that the level of TD was slightly decreased.

Degradation of DBT and its derivatives by whole cell reactions

R. erythropolis H-2 was cultured in medium AG with DBT or its derivatives (Fig. 3) as the sole source of sulfur at 50 mg/l. The strain grew more or less on the four aromatic sulfur compounds tested: Growth (OD660) on DBT, 2,8-dimethyldibenzothiophene (2,8-dimethylDBT), 4,6-dimethyldibenzothiophene (4,6-dimethylDBT) and 3,4-benzodibenzothiophene (3,4-benzoDBT) in 4-day culture: 5.7,4.7, and 1.7, respectively. Though 3,4-benzoDBT was not a good sulfur source for this strain, the two dimethylDBTs as well as DBT also supported the growth of this strain.

The reaction using lyophilized cells cultured with DBT, proceeded with DBT derivatives at 1 mM in the presence of TD. New peaks appeared on all the elution HPLC profiles with concomitantly decreasing substrate peaks. When DBT, 3,4-benzoDBT, 2,8-dimethylDBT, and 4,6-dimethylDBT were the substrates, the retention times of the new peaks were 3.5, 5.2, 4.6, and 5.2 min, respectively. The new peak in the DBT reaction profile corresponded to 2-HBP. The products in the reaction mixture using 2,8-dimethylDBT and 4,6-dimethylDBT as substrates were analyzed by gas chromatograph-mass spectrometry. Mass ions at m/z 198 corresponding to the molecular mass of monohydroxy dimethylbiphenyls were detected. With 3,4-benzoDBT, the mass ion of the product at m/z 220 was also obtained. These results indicated that the microbial desulfurization of these DBT derivatives and of DBT proceeded in a similar manner and gave the corresponding hydroxylated biphenyls as products. And it was interesting to know whether the hydroxy group of the 3,4-benzoDBT product was attached to the benzene, or the naphthalene ring. To identify their exact structures, the products from 3,4-benzoDBT, 2,8-dimethyl DBT and 4,6-dimethylDBT were purified from the reaction mixtures and analyzed by NMR.

In the case of 3,4-benzoDBT, signals were observed at δ 5.54 (s, 1 H), 7.04-7.09 (m, 1 H), 7.13 (d, 2 H, $J=7.5$), 7.24-7.25 (m, 1 H), 7.26-7.27 (m, 2 H), 7.29-7.30 (m, 1 H), 7.33 (d, 1 H, $J=8.2$), 7.35-7.36 (m, 1 H), 7.65 (d, 1 H, $J=8.0$), and 8.51 (d, 1 H, $J=8.2$). Since the signals at 7.07, 7.13 and 7.26 ppm were specific for one substituted benzene and those at 7.24 and 7.33 ppm were specific for 1,2,3,4-substituted benzene, this spectrum suggested that the hydroxy group is attached to the naphthalene ring. Therefore, we proposed that the structure of the product from 3,4-benzoDBT is a-hydroxy-b-phenylnaphthalene (Fig. 4).

The NMR signals in the case of 2,8-dimethylDBT and 4,6-dimethylDBT were assigned to 2-hydroxy-5,5'-dimethylbiphenyl and 2-hydroxy-3,3'-dimethylbiphenyl, respectively.

The amounts of products formed by whole cells were tentatively calculated assuming that the peak areas per mole of each product on the HPLC were the same as that of 2-HBP. Each substrate was thus converted to the corresponding product. The initial rates of degradation and desulfurization of 2,8-dimethylDBT, 4,6-dimethylDBT and 3,4-benzoDBT were about 120, 60, 20% that of DBT. The 2,8- and 4,6-dimethylDBTs were completely degraded within 6 h. *Arthrobacter* sp. readily attacked the sterically hindered 4,6-diethylDBT (18). Generally, there seems to be no steric hindrance of such alkyl groups against these enzyme systems. The chemical desulfurization rate for alkyl-substituted DBTs is much slower than that for DBT and it has been thought that the desulfurization of alkyl-substituted DBTs would also be less easy than that of DBT. Thus, these results indicate the feasibility of the practical microbial desulfurization of petroleum.

Although 3,4-benzoDBT was degraded slowly, the amount of the substrate was reduced to 0.1 mM after 12 h (Fig. 5). As described above, NMR analysis indicated that the hydroxy group of the identified product was attached to the naphthalene ring. These results suggest that the enzyme system involved in the microbial DBT desulfurization could distinguish between two carbon-sulfur bonds of 3,4-benzoDBT. The steric hindrance caused by the naphthalene ring might lead to this specificity.

Thus, the present work demonstrated that a new strain, identified as *R. erythropolis* H-2, utilized DBT as a sole source of sulfur and converted it to 2-HBP stoichiometrically even in the presence of hydrocarbon. This strain grew well in n-alkanes with relatively long carbon chains but not in hydrocarbons with higher toxicity to the organism such as toluene. The limiting log P value for the growth of our isolated strain was about 4.9 (log P value of n-octanol). From other experimental data (19), *R. erythropolis* H-2 revealed high tolerance against solvents compared with other Gram-positive bacteria, but it had less tolerance than Gram-negative bacteria such as *Pseudomonas* strains. Also in the whole cell reactions, DBT degradation proceeded in the presence of hydrocarbon and was enhanced by adding TD. TD may facilitate contact between DBT and cells since DBT is water immiscible.

The present strain was also found to have an ability to efficiently function in the presence of hydrocarbon and desulfurize DBT and DBT derivatives to form 2-HBP and the corresponding hydroxylated biphenyls, respectively. Therefore, the strain should be useful for the practical microbial desulfurization of petroleum.

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TABLE 1. Growth in the presence of hydrocarbons. The strain was cultivated in medium A-1 with 0.5% hydrocarbons with or without 0.5% glucose in test tubes for 4 days.

Hydrocarbon	(log <i>P</i> value)	+Glucose		-Glucose	
		Growth (OD660)	pH	Growth (OD660)	pH
None		4.8	4.6	n.t. ^{a)}	n.t. ^{a)}
<i>n</i> -Hexadecane	(7.0<)	4.9	4.2	5.2	5.1
<i>n</i> -Tetradecane	(7.0<)	5.6	3.8	4.9	3.8
<i>n</i> -Dodecane	(7.0)	2.9	4.2	3.0	4.8
<i>n</i> -Decane	(6.0)	1.7	5.7	2.5	6.4
<i>n</i> -Nonane	(5.5)	0.8	5.7	0.8	5.8
<i>n</i> -Octane	(4.9)	0.3	6.5	0.3	6.6
Cyclooctane	(4.5)	0	6.9	0	6.9
<i>n</i> -Hexane	(3.9)	0	7.0	0	6.9
<i>p</i> -Xylene	(3.1)	0	7.0	0	7.0
Styrene	(2.9)	0	6.9	0 </td <td>6.9</td>	6.9
Toluene	(2.8)	0	6.9	0	7.0

a) not tested

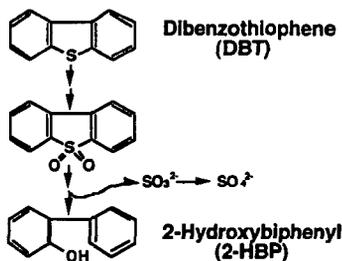


FIGURE 1. Proposed sulfur-specific pathway of dibenzothiophene (DBT)

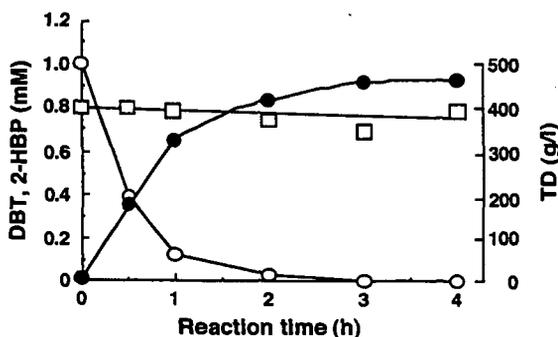


Figure 2. Time course of DBT degradation and 2-hydroxybiphenyl (2-HBP) accumulation in the whole cell reaction. \circ DBT, \bullet 2-HBP, \square TD

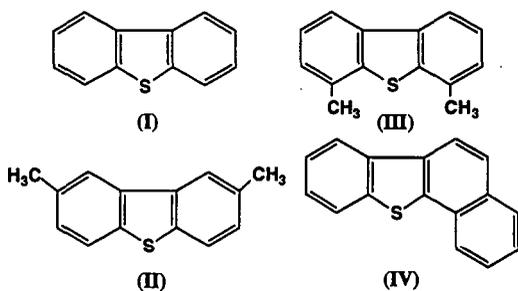


FIGURE 3. Structure of DBT and its derivatives. (I) DBT; (II) 2,8-dimethylDBT; (III) 4,6-dimethylDBT; (IV) 3,4-benzoDBT.

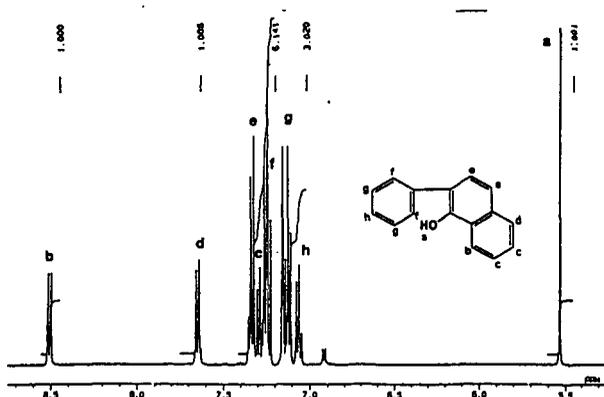


FIGURE 4. ¹H-NMR spectrum of the product from 3,4-benzoDBT.

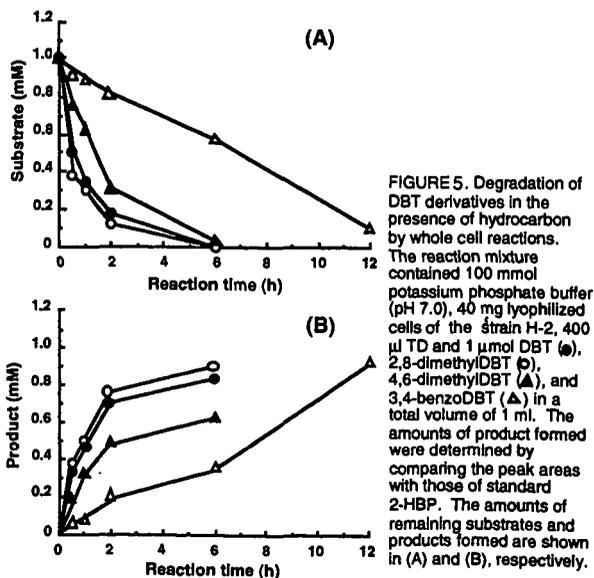


FIGURE 5. Degradation of DBT derivatives in the presence of hydrocarbon by whole cell reactions. The reaction mixture contained 100 mmol potassium phosphate buffer (pH 7.0), 40 mg lyophilized cells of the strain H-2, 400 μ l TD and 1 μ mol DBT (●), 2,8-dimethylDBT (◐), 4,6-dimethylDBT (◑), and 3,4-benzoDBT (Δ) in a total volume of 1 ml. The amounts of product were determined by comparing the peak areas with those of standard 2-HBP. The amounts of remaining substrates and products formed are shown in (A) and (B), respectively.