

Comparison of Dibenzothiophene-Degrading Bacteria under Low Oxygen Conditions

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Nine dibenzothiophene (DBT) degrading bacteria were isolated from various crude oil reservoir tanks in Japan. Strains T37 and T38 were identified as *Pseudomonas cepacia* and A76 and 203S as *Pseudomonas stutzeri*. Strains T09, 23S and 32S were suggested to be *Pseudomonas fluorescens*. Strains N-1 and A54 were identified as *Rhizobium* sp. and *Shingomonas* sp., respectively. Their DBT degradation abilities under low oxygen conditions were compared with that of *Xanthobacter polyaromaticivorans* strain 127W, recently isolated in our laboratory. *X. polyaromaticivorans* strain 127W, *Sphingomonas* sp. strain A54, and *Rhizobium* sp. strain N-1 degraded 28.5, 8.8 and 6.3 mg/l of DBT in six days, at DO 3 ppm, respectively. All the seven strains tested in the genus *Pseudomonas* degraded less DBT than *X. polyaromaticivorans* strain 127W, *Sphingomonas* sp. strain A54, and *Rhizobium* sp. strain N-1 under this low oxygen condition.

Key words: dibenzothiophene, degradation, low oxygen condition

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) and heterocyclic aromatic compounds (HAC) are of environmental concern because of their recalcitrant and carcinogenic behavior^(8,9). Although PAH and HAC in surface water or soil particles are susceptible to degradation by various aerobic bacteria, large fractions of PAH and HAC stick to solid particles and settle to the bottom of rivers or lakes where only limited oxygen is available⁽¹³⁾. These fractions remain undegraded for long periods because anaerobic biodegradation proceeds slowly. Even the polluted site soon becomes anoxic due to high oxygen demand for hydrocarbon degradation by bacteria. Thus much interest exists in isolating and studying microorganisms that effectively degrade PAH and HAC under limited oxygen conditions from the viewpoints of developing bioremediation technologies and understanding natural attenuation. Dibenzothiophene (DBT) is a model compound among sulfur-containing HAC in crude oil. Although various bacteria degrade DBT aerobically^(1,2,5,7,10,11,14), little knowledge is available of DBT degradation under low oxygen conditions.

We recently isolated *Xanthobacter polyaromaticivorans*

strain 127W from an anoxic sludge in a crude oil reservoir tank in Fukui⁽⁴⁾. This strain degrades significant amounts of DBT under both aerobic and extremely low oxygen conditions. In this study, we isolated nine additional bacterial strains that degrade DBT aerobically and compared their degradation ability with that of strain 127W under both aerobic and low oxygen conditions. The DBT degradation activity of strain 127W was the most tolerant against oxygen limitation among the strains tested. All strains tested in the genus *Pseudomonas* were less active than *Rhizobium* sp. strain N-1, *Sphingomonas* sp. strain A54, and *X. polyaromaticivorans* strain 127W.

2. Materials and Methods

2.1. Isolation of DBT-degrading strains

Sludge samples were collected from crude oil reservoir tanks in Hokkaido, Aomori, Akita and Okinawa, Japan. An aliquot of each sample was inoculated into a mineral salt medium containing DBT, CSF-DBT, as a sole carbon and sulfur source. CSF-DBT contained in one liter, 4 g of Na₂HPO₄, 4 g of K₂HPO₄, 2 g of NH₄NO₃, 0.1 g of MgCl₂·6H₂O, 0.01 g of CaCl₂·2H₂O, 0.01 g of FeCl₃·6H₂O,

0.05 g of yeast extract, and 100 mg of DBT (add 10 ml of 54 mM DBT in ethanol). The pH was adjusted to 7.0 by HCl. Glucose minimum medium, CSF-G, containing 2% glucose as carbon source, and Luria broth were also used if necessary. Cultivation was at 30°C. After transferring the culture to a new medium three times, bacterial strains were isolated on a CSF-DBT-agar plate containing 15 g/l agar to solidify the medium. When the cells were grown on a CSF-DBT-agar or on a CSF-G-agar plate overlaid with a spray of 1% DBT-diethyl ether solution, each colony was surrounded by a clear halo indicating DBT degradation⁴.

2.2. DBT degradation under low oxygen conditions

Exponentially growing cultures in Luria broth were harvested, were washed once with sterile water, and were suspended in a small volume of water. CSF-DBT (10 ml) in a 20 ml glass vials (No. 5, Maruemu, Tokyo) was inoculated with the cell suspension at a final OD₆₆₀ of 0.1, and 50 mg/l (0.27 mM) DBT was added as a substrate. A low oxygen condition was prepared as follows: vials containing CSF-DBT and the cells were left in an anaerobic chamber (EAN-101, Tabai Espec, Osaka) for a defined time after degassing twice by N₂ gas and once by an anaerobic gas mixture (CO₂/H₂/N₂=5 : 5 : 90 with DO<0.02 ppm). Dissolved oxygen, (DO) in the medium decreased as the length of time kept in the anaerobic chamber increased. After about 2 hr, DO decreased to 3 ppm. The vials were tightly sealed with butyl rubber septa with aluminum crimps and were gently shaken at 30°C for two to six days. An aerobic degradation experiment was done in vials sealed in the atmosphere.

2.3. Gas chromatography (GC) analysis

The reaction mixture was acidified to pH 2.0 with 6 M HCl and DBT was extracted with one volume of ethyl acetate containing 0.12 mM of fluorene as an internal standard. To eliminate trace contamination of air and absorption of hydrocarbons into the butyl rubber stopper, we used neither a needle to take samples from the vial nor transferred samples to another vial. Instead, the whole reaction culture was directly subjected to extraction in the original vial at each degradation time. Part of the ethyl acetate layer was analyzed by using a GC system GC-14A equipped with a 30-m non-polar capillary column CBP-1 (Shimadzu, Kyoto) and a flame ionization (FID) detector (GC/FID) or JEOL JMS-DX303 mass spectrometer (JEOL, Tokyo, GC/MS). The temperature and carrier gas flow conditions were as described elsewhere⁴.

3. Results and Discussion

3.1. Isolation of DBT-degrading bacteria

We expected to isolate bacteria that actively degrade DBT under low oxygen conditions. Bottom sludge samples in the crude oil tank were used to isolate such bacteria, because they are insulated from air. Remaining oxygen should

have been consumed by aerobic bacteria. Sludge samples from tanks, T09, T37, T38 (Hokkaido), No. 32 (Aomori), TK-23 (Akita), #1 (Niigata), TA-54/TA-76/TK-203 (Okina-wa) contained bacteria that degraded DBT. Strains that most effectively degraded DBT were chosen from each tank and were named after the name of the tank: T09, T37, T38, 32S, 23S, N-1, A54, A76, and 203S.

3.2. Identification of bacterial strains

All strains were revealed Gram-negative bacteria. All strains, except A54 and N-1, showed similar results in the following API tests: oxidase, catalase, and nitrate reductase were positive, and indole production and the Voges-Proskauer test were negative. Based on these characteristics, strains T09, T37, T38, 32S, 23S, A76, and 203S were suggested to belong to the genus *Pseudomonas* (15). Further physiological characteristics were found by using an API system (Biomérieux, Cedex, France). Strains T09, 23S, and 32S were suggested to be *P. fluorescens* with identification scores 92.9%, 94.9%, and 95.6%, respectively. Strains T37 and T38 were identified as *P. cepacia* with identification at 99.8% and 99.9%, respectively, and strains A76 and 203S were *P. stutzeri* both with identification at 99.9%. *P. fluorescens*, *P. cepacia*, and *P. stutzeri* are well studied bacterial groups in hydrocarbon degradation pathways. Strain A54 was identified as *Sphingomonas paucimobilis* with identification at 98.4%. However, strain N-1 could not be identified by using the API system because all scores were low. The 16S rRNA gene was amplified by PCR and the nucleotide sequence was determined and analyzed by using BLAST search program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequence of the 16S rRNA gene from strain N-1 (DDBJ/EMBL/GenBank; AB182639) had the highest similarity with *Rhizobium galegae* at identification 97.8% and *R. leguminosarum* at identification 95.6%, indicating that the strain belongs to *Rhizobium*.

3.3. DBT degradation under aerobic and low oxygen conditions

Isolated strains were tested for DBT degradation under both aerobic and low oxygen (DO=3.0 ppm) conditions (Fig. 1). *X. polyaromaticivorans* strain 127W and *P. stutzeri* strain T102, that were previously isolated strains⁴, were used as comparative strains. Fig. 1a shows that strains A76, A54, and 127W degraded 50 mg/l DBT completely in three days under aerobic conditions. When the DO was reduced to 3.0 ppm, strains A76, 203S, T102, T37, 23S, and T09 lost almost all their degradation abilities. But strains N-1, A54, and 127W degraded 6.3, 8.8, and 28.5 mg/l of DBT, respectively. Strain 32S also showed a degradation ability, but at a lower level than strains N-1, A54, and 127W.

3.4. Classification of isolates based on the DBT-degrading activity

Our experimental results clearly show that all strains in genus *Pseudomonas* require more oxygen than *Rhizobium*,

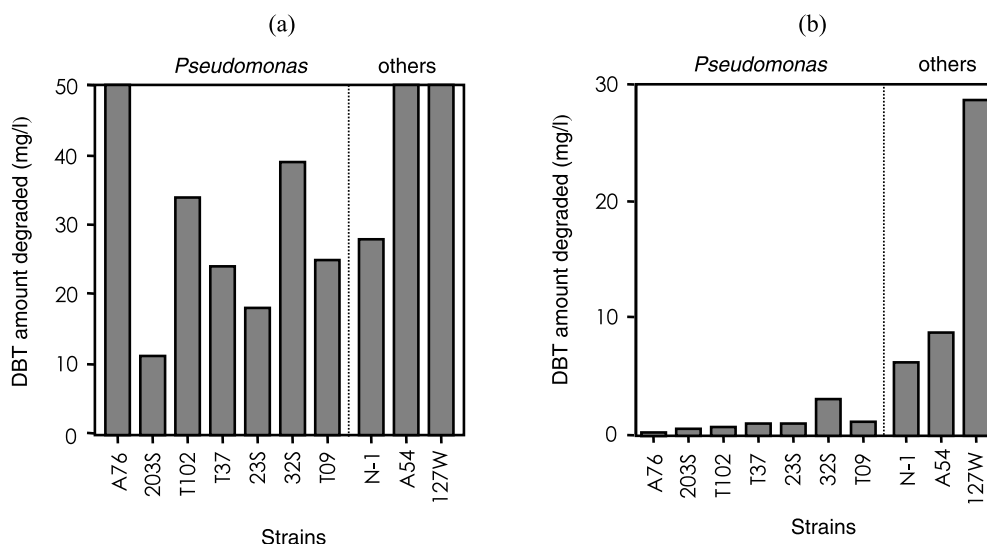


Fig. 1. Comparison of DBT degradability of strains under aerobic and a low oxygen conditions. Each value is an average of three independent experiments.

(a) Degradation of DBT after 3 days under aerobic conditions (DO was continuously over 7 ppm). (b) Degradation of DBT after 6 days under a low oxygen condition (DO=3 ppm).

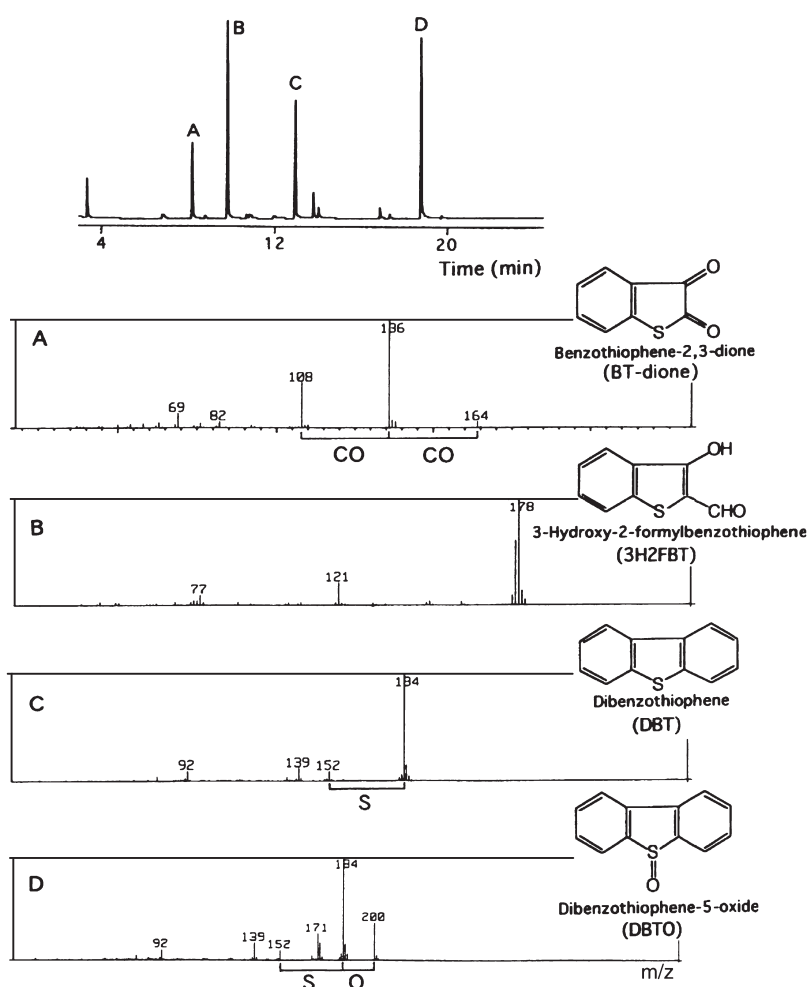


Fig. 2. GC/FID and GC/MS analyses of the degradation products from DBT.

DBT and its degradation products were analyzed after 3 days cultivation of strains 32S and A54. Because the chromatograms are almost identical for these two strains, only the data of strain 32S is shown in the figure. Cultivation was aerobic and extraction of DBT and its related compounds was done with ethyl acetate without fluorene in this case. Column temperature increased from 100°C to 200°C at a gradient of 5°C/min and from 200°C to 290°C at 10°C/min gradient.

Sphingomonas, and *Xanthobacter*. Toluene degradation under limited oxygen condition (DO=2 ppm) by *P. picketti* strain PKO1, *P. fluorescens* strain CFS215, and *Pseudomonas* sp. strain W31 increased by adding 10 mM nitrate to the medium, but degradation by *P. cepacia* and *P. putida* was not affected by nitrate⁶. This event was attributed to higher oxygen affinity of the enzyme responsible, catechol 2,3-dioxygenase, from strains PKO1, CF215, and W31, and an increased gene expression level in response to denitrification under oxygen limitation. In this study, because the CSF-DBT medium contained 25 mM NH₄NO₃, the low degradation ability by *Pseudomonas* strains should not have been due to nitrate starvation. When *Pseudomonas* strains are used, considerable aeration is required for the effective degradation of aromatic hydrocarbons. That is, *Rhizobium*, *Sphingomonas* and especially *X. polyaromaticivorans* could be more cost effective than *Pseudomonas* for DBT degradation under low oxygen conditions. This knowledge may be informative for constructing an effective bioremediation system to clear up accidental spillage of hydrocarbons.

3.5. DBT-degradation pathway of the strains

To understand the DBT-degradation pathway of the strains, the structure of the degradation products was analyzed by using GC/FID and GC/MS (Fig. 2, Table 1). Molecular ion peaks for benzothioephene-2,3-dione (BT-dione, A), 3-hydroxy-2-formyl benzothioephene (3H2FBT, B), DBT (C), and dibenzothioephene-5-oxide (DBTO, D) were clearly observed at *m/z*=164, 178, 184, and 200, respectively. The differences in the *m/z* of the peaks, such as the elimination of the CO group at 28 and at 32 for S, are also used to identify the compounds¹². The most abundant and common product was 3H2FBT, suggesting that all strains degraded DBT by the popular Kodama pathway⁹. Strains 32S and A54 produced DBTO as well as 3H2FBT. These strains should have sulfoxidase-like activity, as suggested for other *Pseudomonas* isolates¹². Several bacterial strains in *Rhodo-*

coccus, *Mycobacterium*, *Phaebacillus* desulfurize DBT by cleaving the C-S bond³. DBTO and dibenzothioephene 5-dioxide are common intermediates in hydroxyl biphenyl and sulfate formation. However, the desulfurization activity is less probable for strains 32S and A54, because neither reduction in amount of DBTO nor production of dibenzothioephene 5-dioxide and hydroxyl biphenyls was observed even after incubation for a long time.

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Table 1. Degradation products of DBT.
Cultivation was at 30°C for 3 days.
Amounts were estimated by the peak area in GC/FID.

	3H2FBT	BT-dione	DBTO
T102	++	+/-	-
T09	++	+/-	-
T23	+	+/-	-
32S	++	+/-	++
A76	+	+/-	-
A54	+	+/-	+
N-1	++	-	-
127W	+++	-	-

T102, T09, T23, 32S, A76, A54, N-1, 127W are the names of the strains.

3H2FBT, 3-hydroxy-2-formylbenzothioephene; BT-dione, benzothioephene-2,3-dione; DBTO, dibenzothioephene-5-oxide.

-, not detectable; +/-, <0.002 mM; +, 0.002~0.05 mM;

++, 0.05~0.1 mM; +++, 0.1 mM<.