Biodesulfurization of Alkylated Forms of Dibenzothiophene and Benzo thiophene by *Sphingomonas subarctica* T7b

Ida Bagus Wayan Gunam,† Yosuke Yaku,† Makoto Hirano,† Kenta Yamamura,† Fusao Tomita,‡ Teruo Sone,† and Kozo Asano†*

Laboratory of Applied Microbiology, Graduate School of Agriculture, Hokkaido University, Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan; and The University of the Air, Hokkaido Study Center, Kita 17 Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0817, Japan

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*Sphingomonas subarctica* T7b was isolated from soil in Toyotomi, Hokkaido, Japan as an organism capable of desulfurizing aromatic hydrocarbons in light gas oil (LGO) through enrichment culture. *S. subarctica* T7b could grow on mineral salt sulfur-free (MSSF) medium with the *n*-tetradecane oil phase containing dibenzothiophene (DBT), alkyl dibenzothiophenes (alkyl DBTs) or alkyl benzo thiophenes (alkyl BTs) as the sole sulfur source and desulfurize these compounds, but could not utilize the tetradecane as a carbon source. This is the first report of a gram-negative bacterium which can desulfurize 4,6-dibutyl DBT and 4,6-dipentyl DBT. The desulfurized product of DBT produced by this strain was 2-hydroxybiphenyl, as in the case of other DBT-desulfurizing bacteria. *S. subarctica* T7b could desulfurize LGO and the sulfur content was decreased to 41% within 36 h.

[Key words: desulfurization, dibenzothiophene, alkyl dibenzothiophene, alkyl benzo thiophene, light gas oil, *Sphingomonas subarctica*]

The emission of sulfur-oxides (SO₃) to the atmosphere through the combustion of petroleum causes environmental problems such as air pollution and acid rain. Atmospheric SO₃ is a major factor of air pollution in city environments and is also a major cause of acid rain which is a primary cause of global deforestation. To reduce these emissions, the amount of organic sulfur compounds in fossil oils should be reduced during refining processes (1).

Organic sulfur compounds in the middle-distillate fractions, such as diesel and fuel oil, consist of alkylated benzo thiophenes and alkylated dibenzothiophenes. The removal of such compounds is considerably difficult using conventional physicochemical desulfurizing methods or a hydrodesulfurization process (1). In practice, a hydrodesulfurization process is costly because of the use of hydrogen gas at a high temperature and pressure. As an alternative method, microbial desulfurization or the biodesulfurization of petroleum has attracted much attention over the last decade (2–4), and the application of microbial reaction has been proposed for the development of a moderate desulfurization process (5).

DBT-degrading microbes with C-S bond cleavage activity are expected to be useful as biocatalysts for the biodesulfurization of diesel oil (3, 6, 7). Biodesulfurization has been studied using *Rhodococcus* spp. (1, 3, 8–10) and other organisms, such as *Pseudomonas* spp. (11, 12), *Sphingomonas* spp. (13, 14), *Corynebacterium* spp. (7, 15), *Bacillus subtilis* WU-S2B (4), *Gordona* spp. (16, 17), *Mycobacterium* spp. (18, 19), *Pae nirbacterius* sp. A11-2 (20) and *Brevibacterium* sp. (21).

It has been reported that long-chain alkyl DBTs and alkyl BTs are difficult to desulfurize using these desulfurizing microorganisms (5, 22, 23). In the case of desulfurization using *Mycobacterium* sp. G3, it has been shown that it cannot desulfurize 4,6-dibutyl DBT and 4,6-dipentyl DBT in an oil/water two-phase reaction system (5). It was observed that *R. erythropolis* KA2-5-1, a DBT-desulfurizing bacterium, was able to degrade some alkyl BTs such as 3-meth yl BT, 2-ethyl BT and 2,7-diethyl BT. However, this strain was unable to desulfurize DBTs and BTs with longer alkyl chains such as 4,6-dipropyl DBT, 4,6-dibutyl DBT and 4,6-dipentyl DBT, in the oil phase (22).

In this paper, we describe the isolation and characterization of the desulfurizing bacterium *Sphingomonas subarctica* T7b, which has a broader substrate specificity. This is the first report of a gram-negative bacterium that can desulfurize both DBTs and BTs with longer alkyl chains.

**MATERIALS AND METHODS**

**Materials** Alkyl BTs and alkyl DBTs were synthesized and obtained from Nard Institute (Hyogo). DBT was purchased from Tokyo Kasei Kogyo (Tokyo). Dibenzothiophene sulfone was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetradecane was supplied by Wako Pure Chemical (Osaka). 2-Hydroxybiphenyl was purchased from Tokyo Kasei Kogyo. Light gas oil (LGO)
was a gift from the Petroleum Energy Center (PEC), Shizuoka. All other reagents were of analytical grade and used without further purification.

The concentrated fraction of aromatic compounds (CA) was produced by the fractionation of commercial light gas oil, according to the method of Schiller and Mathiason (24). Light gas oil (sulfur concentration = 280 ppm) was applied onto a hexane-treated aluminum oxide 90 active neutral column (Merck, Darmstadt, Germany). After the removal of aliphatic hydrocarbons with hexane, ethanol was applied onto the column to elute the aromatic hydrocarbons. The ethanolic eluate was then concentrated by heating on a mantle heater at 80°C. The final concentrated fraction of aromatic hydrocarbons contained sulfur at approximately 144,000 ppm (determined at the Petroleum Energy Center).

**Biochemical analysis**

**Species identification** The isolate was identified by microscopic observation, measurement of various biochemical parameters using the API 20E bacterial identification system (BioMerieux, Hazelwood, MO, USA), and by determining the DNA sequence of a 1400-bp fragment of the 16S rRNA gene. For the sequencing, the 16S RNA gene was amplified from genomic DNA isolated using ISOLPLANT (Nippon Gene), with a pair of primers 27f (5′-AGAGTTTATCTGCTGCTCAG-3′) and 1492r (5′-GGCTACCTTGTTATCTGACT-3′). The sequences determined were subjected to a similarity search (BLAST) on the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/). The DNA sequence determined in this study has been deposited into the DDBJ/EMBL/GentBank database under the accession no. AB237654.

**Bio-desulfurization assay** Seed culture was carried out in a test tube containing 5 ml of MSSF-CA medium with reciprocal shaking at 273 strokes per min at 27°C for 4 d. Six milliliters of MSSF-TD medium containing various sulfur compounds was inoculated with 0.1 ml of the seed culture and cultured under the same conditions as in the seed culture. After the incubation, the organic layer of n-tetradecane or LGO and the water layer were separated by centrifugation at 20,000 × g for 10 min at 4°C. An uninoculated medium, which was treated in the same manner was used as a control.

**LGO desulfurization assay in jar fermentor** As a seed culture, S. subarctica T7b was grown in test tubes containing MSSF-CA until the OD600 reached 2.5, using the same conditions as in the bio-desulfurization assay described above. Forty-five milliliters of the seed culture was inoculated into the mixture of 750 ml of MSSF medium and 250 ml of commercial light gas oil, in a 2.0-1 jar fermentor (Tokyo Rika Kikai, Tokyo). The cultivation was performed under the conditions of 27°C, 400 rpm, 0.9/min aeration, with pH controlled at 7.0.

**DBTs and BTs quantification** The measurements of the amounts of DBT, alkyl DBTs and alkyl BTs were performed using a gas chromatograph equipped with a flame photometric detector (GC-FPD). The amounts of DBT, and alkyl DBTs in growth culture were analyzed using a gas chromatograph (GC-17A; Shimadzu, Kyoto) equipped with a DB-17 fused-silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). The flow rate of the nitrogen carrier gas was 15 ml/min, the column temperature was set at 250°C, and the injector and detector temperatures were maintained at 260°C. For the analyses of the desulfurization tests of LGO, a GC-14A (Shimadzu) apparatus equipped with a Zebron ZB-1 column (60 m × 0.25 mm, 0.25 µm; Phenomenex, Torrance, CA, USA) was used under the following conditions: the initial temperature of the column oven was 220°C, which was heated from 220°C to 280°C at a rate of 3°C/min. The injection and detector temperatures were maintained at 300°C. The content of each alkyl DBT in the LGO was calculated by comparing the GC areas with those of authentic alkyl DBTs. The decrease in total sulfur content was estimated from the decrease in the sum of each sulfur peak area determined by GC-FPD, as described by Belnámez-Moldonado and Yang (25).

**Detection of phenolic compounds** The metabolite hydroxybiphenyl was identified by gas chromatography-mass spectrometry (GCMS-QP5000; Shimadzu). Hydroxybiphenyl qualification was performed with Gibbs reagent (2,6-dichloroquinone-4-chloroimide; Sigma, St. Louis, MO, USA) as follows. After centrifugation of bacterial cultures, 1.5 ml of the supernatant was mixed with 0.3 ml of 1 M NaHCO3 (pH 8.0). Gibbs reagent (0.2 ml of 1 mg/ml ethanolic solution) was then added, and the reaction mixture was agitated at room temperature for 15–45 min. The absorbance of the reaction mixture was measured at 595 nm as described by Konishi et al. (20). Alkyl hydroxybiphenyl produced in culture supernatants with various sulfur compounds was detected by the same method.

**RESULTS**

**Isolation of desulfurizing bacterium** Strain T7b was isolated from an oil-contaminated soil of Toyotomi, Hokkaido, Japan by enrichment culture in MSSF medium supplemented with CA as the sole sulfur source. This strain was rod shaped with the dimensions of 0.8–1.2 µm by 0.8–1.0 µm, gram-negative, non-spore-forming, catalase positive, aerobic, motile and did not produce acid from glucose by fermentation. It could grow at 27°C as yellow colonies on MSSF-CA agar medium. The culture was positive for the utilization of citrate, urea, l-arabinose, D-xylose, galactose, glucose, fructose, cellobiose, maltose, trehalose, and gentiobiose (Table 1). The sequence of the 16S rRNA of strain T7b showed 99.9% homology with S. subarctica KF17 (accession no. X94102) (26). From these results, this strain was identified as S. subarctica and named S. subarctica T7b.

**Bio-desulfurization activity of S. subarctica T7b** The time course of the growth and DBT desulfurization by S. subarctica T7b in MSSF-TD medium with DBT as the sole sulfur source are shown in Fig. 1. After 120 h of cultivation, the cellular OD540 reached 2.2, and the concentrations of residual DBT and 2-hydroxybiphenyl in the culture medium were 14.9 ppm (93.7% desulfurization) and 169.7 ppm, respectively. It was confirmed that 2-hydroxybiphenyl accumulated as a metabolite of DBT desulfurization. According to the GC-MS analysis, the molecular
mass of 2-hydroxybiphenyl was 170. The disappearance of substrate from the medium was faster than the accumulation of 2-hydroxybiphenyl.

No growth occurred in the absence of a sulfur source in the organic layer or without the addition of glucose to the MSSF medium (data not shown). These results indicate that this strain utilized dibenzothiophene as a sulfur source but not as a carbon source. In addition, the strain could not utilize the solvent n-tetradecane.

The effects of various sulfur compounds on the activity of DBT desulfurization were examined. S. subarctica T7b was grown in MSSF-TD medium supplemented with DBT in the presence of other organic and inorganic sulfur compounds such as Na$_2$SO$_4$, MgSO$_4$, l-methionine, l-cysteine (100 ppm) and glucose (10 g/l). After 4 d of cultivation, the residual amounts of DBT in the MSSF-TD medium were measured. All sulfur compounds repressed DBT degradation (Table 2).

The medium containing MgSO$_4$ showed the highest growth rate of S. subarctica T7b and the final optical density (OD$_{660}$) was 4.3. On the other hand, the medium containing methionine showed a lower growth rate of S. subarctica T7b but a higher degree of DBT desulfurization than the other media.

**TABLE 2. DBT desulfurization in presence of various sulfur compounds**

<table>
<thead>
<tr>
<th>Sulfur source$^a$</th>
<th>OD$_{660}$</th>
<th>DBT desulfurization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBT + Na$_2$SO$_4$</td>
<td>4.07</td>
<td>4.2</td>
</tr>
<tr>
<td>DBT + MgSO$_4$</td>
<td>4.34</td>
<td>13.8</td>
</tr>
<tr>
<td>DBT + methionine</td>
<td>3.54</td>
<td>15.5</td>
</tr>
<tr>
<td>DBT + cysteine</td>
<td>4.13</td>
<td>7.0</td>
</tr>
<tr>
<td>DBT</td>
<td>1.96</td>
<td>91.3</td>
</tr>
</tbody>
</table>

$^a$ S. subarctica T7b was cultured in 6 ml of MSSF-TD medium at 27°C with shaking at 273 rpm for 4 d. Na$_2$SO$_4$, MgSO$_4$, methionine, and cysteine (100 ppm) were added to the medium containing 250 ppm DBT.

**TABLE 3. Desulfurization (%) of DBT, alkyl-DBT and alkyl-BT in MSSF-TD medium**

<table>
<thead>
<tr>
<th>Sulfur compound</th>
<th>Individual$^b$</th>
<th>Mixture$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 h</td>
<td>72 h</td>
</tr>
<tr>
<td>DBT</td>
<td>91</td>
<td>36</td>
</tr>
<tr>
<td>7-Ethyl BT</td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>5,7-Dimethyl BT</td>
<td>57</td>
<td>28</td>
</tr>
<tr>
<td>7-Propyl BT</td>
<td>41</td>
<td>31</td>
</tr>
<tr>
<td>4,6-Dimethyl DBT</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>4,6-Diethyl DBT</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>4,6-Dipropyl DBT</td>
<td>33</td>
<td>38</td>
</tr>
</tbody>
</table>

$^b$ All sulfur compounds were mixed in the same test tube and the total concentration of sulfur was 250 ppm.

### Desulfurization activity of S. subarctica T7b on various sulfur compounds

S. subarctica T7b was isolated from the enrichment culture of the CA fraction and was thus expected to have a wide substrate specificity for sulfur sources. Table 3 shows the result of the desulfurization of individual compounds or mixtures of DBT, 7-ethyl BT, 5,7-dimethyl BT, 7-propyl BT, 4,6-dimethyl DBT, 4,6-diethyl DBT and 4,6-dipropyl DBT in n-tetradecane by growing cells of S. subarctica T7b. Although the desulfurization activity was different among the compounds, strain T7b could desulfurize all the compounds tested individually. Desulfurization at 72 h of incubation was the highest (57%) for 5,7-dimethyl BT and the lowest (7%) for 4,6-diethyl DBT. Desulfurization activity on the mixed compounds was similar to that of the individual compounds, but the desulfurization rate of DBT was low in the mixed compounds possibly because of the coexistence of long-chain alkyl DBTs and BTs which have a negative effect on the desulfurization. After 96 h of cultivation, the desulfurization rates of DBT,
Next, we tested the desulfurization activity of *S. subarctica* T7b on the derivatives of BT and DBTs with longer alkyl chains. *R. erythropolis* KA2-5-1, which is known to be a DBT-desulfurizing microorganism, was used for comparison of desulfurization properties (Table 4). *S. subarctica* T7b could desulfurize most of the sulfur compounds used in this experiment, whereas *R. erythropolis* KA2-5-1 could not utilize or desulfurize some alkyl DBTs as the sole sulfur source, particularly alkyl DBTs with longer alkyl chains such as 4,6-dimethyl DBT, 4,6-dipropyl DBT, and also alkyl BTs with longer alkyl chains such as 2-dodecyl BT and 7-dodecyl BT. On the other hand, for alkyl DBTs with shorter alkyl chains such as 4,6-dimethyl DBT, 4,6-dipropyl DBT, and 4,6-dimethyl DBT, *R. erythropolis* KA2-5-1 showed a higher desulfurization activity than *S. subarctica* T7b. Gibbs assay colorimetrically detected the phenolic compounds that were produced from DBTs, BTs and their derivatives by *S. subarctica* T7b.

### Desulfurization of light gas oil (LGO) by batch culture

The feasibility of LGO desulfurization was investigated using a commercial hydrodesulfurized LGO (contains 280 ppm total sulfur, determined at the Petroleum Energy Center), in a jar fermentor. The desulfurization of sulfur compounds, including 4,6-dimethyl DBT that is known as the dominant sulfur compound contained in LGO, was investigated (Fig. 2). The amount of sulfur in the LGO was reduced down to 41% within 36 h. The amount of each sulfur compound decreased sharply until 36 h of cultivation, but from 36 h to 72 h of cultivation, a slight decrease was detected. Up to 66% of 4,6-dimethyl DBT was desulfurized within 36 h. Benzoisophene derivatives such as 7-ethyl BT and 7-propyl BT were desulfurized almost completely at this time.

### DISCUSSION

In this study, we isolated *S. subarctica* T7b, which can utilize DBT, alkyl DBTs and alkyl BTs as the sole source of sulfur. Many bacterial species can degrade DBTs or BTs as a sole source of sulfur, whereas only a few bacteria can degrade both alkyl DBTs and alkyl BTs normally with not very long chains (not more than C3), such as *Paenibacillus* sp. A11-2 (3), *R. erythropolis* KA2-5-1 (9), *Mycobacterium phlei* GTIS10 (18), *Mycobacterium goodie* X7B (19), and *Rhodococcus* sp. KT462 (27). *S. subarctica* T7b could desulfurize alkyl DBTs and alkyl BTs with longer alkyl chains, such as 4,6-dipropyl DBT, 4,6-dibutyl DBT, 4,6-dipentyl DBT, 4-hexyl DBT, 2-dodecyl BT, 4-dodecyl BT and 5-docetyl BT. This is the first report on a bacterium which can desulfurize longer alkyl chain DBTs and BTs. The mechanism of this broader substrate specificity is unknown. To isolate *S. subarctica* T7b, the aromatic fraction of LGO...
(CA) was an important substance used as a sole sulfur source in the enrichment culture medium. This CA contained various sulfur compounds including alkyl DBTs and BTs (data not shown). Thus, this is a possible reason why the microorganism could utilize a wide variety of alkyl DBTs, alkyl BTs and their derivatives.

During the cultivation of this bacterium with DBT as a sole source of sulfur, a significant degradation of DBT and the accumulation of a phenolic compound identified as 2-hydroxybiphenyl were observed. In addition, *S. subarctica* T7b could utilize dibenzothiophene sulfone instead of DBT as the sole source of sulfur and accumulated 2-hydroxybiphenyl (data not shown). Moreover, Gibbs reagent assay showed that phenolic compounds were also produced from phenyl (data not shown). Moreover, Gibbs reagent assay as the sole source of sulfur and accumulated 2-hydroxybiphenyl (data not shown). Moreover, Gibbs reagent assay showed that phenolic compounds were also produced from phenyl (data not shown).

**REFERENCES**


