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ORIGINAL PAPER

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Thermophilic biodesulfurization of naphthothiophene and 2-ethylnaphthothiophene by a dibenzothiophene-desulfurizing bacterium, *Mycobacterium phlei* WU-F1

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Abstract Naphtho[2,1-*b*]thiophene (NTH) is an asymmetric structural isomer of dibenzothiophene (DBT), and NTH derivatives can be detected in diesel oil following hydrodesulfurization treatment, in addition to DBT derivatives. *Mycobacterium phlei* WU-F1, which possesses high desulfurizing ability toward DBT and its derivatives over a wide temperature range (20–50 °C), could also grow at 50°C in a medium with NTH or 2-ethylNTH, an alkylated derivative, as the sole source of sulfur. At 50 °C, the resting cells of WU-F1 degraded 67% and 83% of 0.81 mM NTH and 2-ethylNTH, respectively, within 8 h. By GC-MS analysis, 2-ethylNTH-desulfurized metabolites were identified as 2-ethylNTH sulfoxide, 1-(2′-hydroxynaphthyl)-1-butene and 1-naphthyl-2-hydroxy-1-butene, and it was concluded that WU-F1 desulfurized 2-ethylNTH through a sulfur-specific degradation pathway with the selective cleavage of carbon–sulfur bonds. Therefore, *M. phlei* WU-F1 can effectively desulfurize asymmetric organosulfur compounds, NTH and 2-ethylNTH, as well as symmetric DBT derivatives under high-temperature conditions, and it may be a useful desulfurizing biocatalyst possessing a broad substrate specificity toward organosulfur compounds.

Introduction

Today, petroleum is treated by hydrodesulfurization (HDS), using chemical catalysts containing metals to remove sulfur compounds, which generate sulfur oxides by combustion, leading to acid rain and air pollution. However, heterocyclic sulfur compounds cannot be completely removed by HDS. Dibenzothiophene (DBT) derivatives are recalcitrant organosulfur compounds and are widely recognized as target compounds for more efficient desulfurization, since they can be detected in diesel oil following HDS treatment. Therefore, the application of a biodesulfurization process using a DBT-desulfurizing microorganism following HDS, mainly for diesel oil, has attracted attention to achieve more efficient desulfurization (Oshiro and Izumi 1999; Monticello 2000).

Some mesophilic DBT-desulfurizing microorganisms have been isolated to date, for example *Rhodococcus* sp. IGTS8 (Gallagher et al. 1993; Olson et al. 1993; Denome et al. 1994; Oldfield et al. 1997), *R. erythropolis* D-1 (Izumi et al. 1994), *R. erythropolis* H-2 (Ohshiro et al. 1995, 1996), *R. erythropolis* KA2-5-1 (Ishii et al. 1998; Yan et al. 2000; Naito et al. 2001) and *Mycobacterium* sp. G3 (Nekodzuka et al. 1997). In addition, three thermophilic DBT-desulfurizing microorganisms have been isolated for high-temperature biodesulfurization of diesel oil following HDS. Konishi et al. (1997) isolated *Paenibacillus* sp. A11-2 desulfurizing DBT at 60 °C and the genes involved in DBT desulfurization were characterized (Ishii et al. 2000). We also isolated two moderately thermophilic DBT-desulfurizing microorganisms, *Bacillus subtilis* WU-S2B (Kirimura et al. 2001) and *M. phlei* WU-F1 (Furuya et al. 2001), which could desulfurize DBT and its derivatives over a wide temperature range (20–50 °C), with highest activity at 50 °C. These bacteria desulfurize DBT through a sulfur-specific degradation pathway with the selective cleavage of carbon–sulfur (C-S) bonds without reducing the energy content (Gallagher et al. 1993; Olson et al. 1993; Oldfield et al. 1997).

Naphtho[2,1-*b*]thiophene (NTH) is an asymmetric structural isomer of DBT (Kropp et al. 1997) and NTH derivatives can be detected in diesel oil following HDS treatment, in addition to DBT derivatives, although the NTH derivatives are minor components, in comparison with DBT derivatives (unpublished data). Therefore, NTH derivatives may also be target compounds for more efficient desulfurization. However, there are no data related to NTH desulfurization by the bacteria which can desulfurize DBT and/or benzothiophene (Gilbert et al.

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1998; Kobayashi et al. 2000; Konishi et al. 2000; Matsui et al. 2000). To date, there is only one report of NTH biodegradation (Kropp et al. 1997), reporting that *Pseudomonas* sp. W1 could degrade NTH. However, this bacterium utilized NTH as the carbon source, reducing the energy content, and the sulfur atom was not removed from NTH during the degradation.

In this paper, we describe the thermophilic desulfurization of NTH and 2-ethylNTH by *M. phlei* WU-F1, which possesses high desulfurizing ability toward DBT and its derivatives over a wide temperature range (20–50 °C). We examined the desulfurizing ability of growing and resting cells of WU-F1 and found that WU-F1 could effectively desulfurize NTH and 2-ethylNTH through a sulfur-specific degradation pathway with the selective cleavage of C-S bonds at high temperature (50 °C).

Materials and methods

Cultivation and resting-cell reaction

M. phlei WU-F1 was isolated for its ability to grow in a medium with DBT as the sole source of sulfur at 50 °C (Furuya et al. 2001). Cultivation of WU-F1 was done using AF medium (Furuya et al. 2001) supplemented with 0.27 mM (corresponding to 50 ppm NTH) of a heterocyclic sulfur compound, such as NTH, 2-ethylNTH, DBT or 4,6-dimethylDBT, as the sole source of sulfur and 1% (v/v) *n*-tridecane for each suspension of heterocyclic sulfur compounds. WU-F1 was cultivated at 50 °C in test tubes (18×180 mm) containing 5 ml of the medium supplemented with one of the heterocyclic sulfur compounds and *n*-tridecane, with reciprocal shaking at 240 strokes/min. For the resting-cell reaction, WU-F1 was cultivated at 45 °C for 45 h in 500-ml Erlenmeyer-flasks containing 200 ml of AF medium with 0.27 mM DBT or 0.1 g/l Na_2SO_4 as the sole source of sulfur. The cells were harvested by centrifugation at 10,000 *g* for 10 min at 4 °C, washed twice with 0.1 M potassium phosphate buffer (pH 7.6) and suspended in the same buffer. The optical density at 660 nm (OD_{660}) of the cell suspension was adjusted to 50. Nine microliters of *n*-tridecane solution containing one of the heterocyclic sulfur compounds was added to an L-shaped test tube containing 0.6 ml of the cell suspension, to give a final concentration of 0.81 mM heterocyclic sulfur compound. The resting-cell reaction was performed at 50 °C with reciprocal shaking at 180 strokes/min.

Analytical methods

NTH, 2-ethylNTH, DBT and 4,6-dimethylDBT were determined using high-performance liquid chromatography (HPLC, type LC-10 A; Shimadzu, Kyoto, Japan) equipped with a Puresil C18 column (Waters, Milford, Mass.), as described by Kirimura et al. (2001). The molecular structures of metabolites after 2-ethylNTH desulfurization were analyzed using gas chromatography-mass spectrometry (GC-MS, type 5890II; Hewlett Packard, Mississauga, Ontario, Canada) equipped with a 30-m type HP-5 column (Hewlett Packard) as described by Kirimura et al. (2001).

Chemicals

DBT was purchased from Tokyo Kasei (Tokyo, Japan). NTH (naphtho[2,1-*b*]thiophene), 2-ethylNTH and 4,6-dimethylDBT were kindly supplied by the laboratory of the Japan Cooperation Center, Petroleum (Shizuoka, Japan). All other reagents were of analytical grade and commercially available.

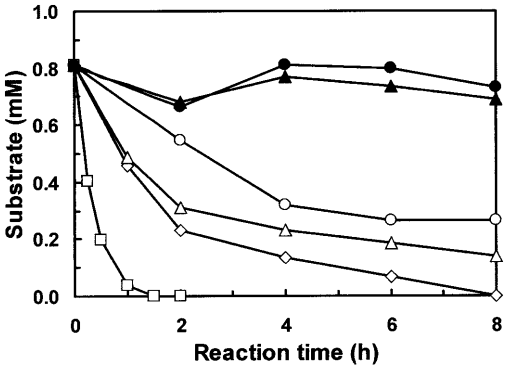

Fig. 1 Degradation of heterocyclic sulfur compounds at 50 °C by resting cells of *Mycobacterium phlei* WU-F1. The resting cells were prepared through cultivation in AF medium (Furuya et al. 2001) with dibenzothiophene (DBT; ○, △, □, ◇) or Na_2SO_4 (●, ▲) as the sole source of sulfur. The reaction mixture contained 0.6 ml of cell suspension (OD_{660} = 50), 0.81 mM of a heterocyclic sulfur compound and 9 µl of *n*-tridecane. ○, ● Naphtho[2,1-*b*]thiophene (NTH). △, ▲ 2-ethylNTH. □ DBT. ◇ 4,6-DimethylDBT

Results

Desulfurization of NTH and 2-ethylNTH

M. phlei WU-F1 grew at 50 °C in AF medium with NTH or 2-ethylNTH as the sole source of sulfur. Growth (OD_{660}) of WU-F1 on NTH for 5 days was 1.0 and that on 2-ethylNTH for 2 days was 3.0. The growing cells of WU-F1 degraded 39% of 0.27 mM NTH within 5 days. Moreover, the growing cells degraded 64% of 0.27 mM 2-ethylNTH within 2 days. These results showed that WU-F1 exhibited a faster growth and higher degradation rate for 2-ethylNTH than for NTH. WU-F1 also exhibited a growth and degradation rate for 2-ethylNTH comparable to those for DBT and 4,6-dimethylDBT (data not shown).

The time-course of the degradation of heterocyclic sulfur compounds at 50 °C by the resting cells of WU-F1 was examined. As shown in Fig. 1, the resting cells of WU-F1 grown with DBT degraded 67% and 83% of 0.81 mM NTH and 2-ethylNTH, respectively, within 8 h. These results indicated that the desulfurizing enzymes produced during cultivation with DBT also catalyzed the desulfurization of both NTH and 2-ethylNTH. In contrast, the resting cells of WU-F1 grown with Na_2SO_4 instead of DBT as the sole source of sulfur degraded neither NTH nor 2-ethylNTH, indicating that the enzymes necessary for the desulfurization of NTH and 2-ethylNTH were not produced during cultivation with Na_2SO_4. The degradation rate for 2-ethylNTH by the resting cells of WU-F1 was higher than that for NTH, although the degradation rate for 2-ethylNTH was lower than those for DBT and 4,6-dimethylDBT.

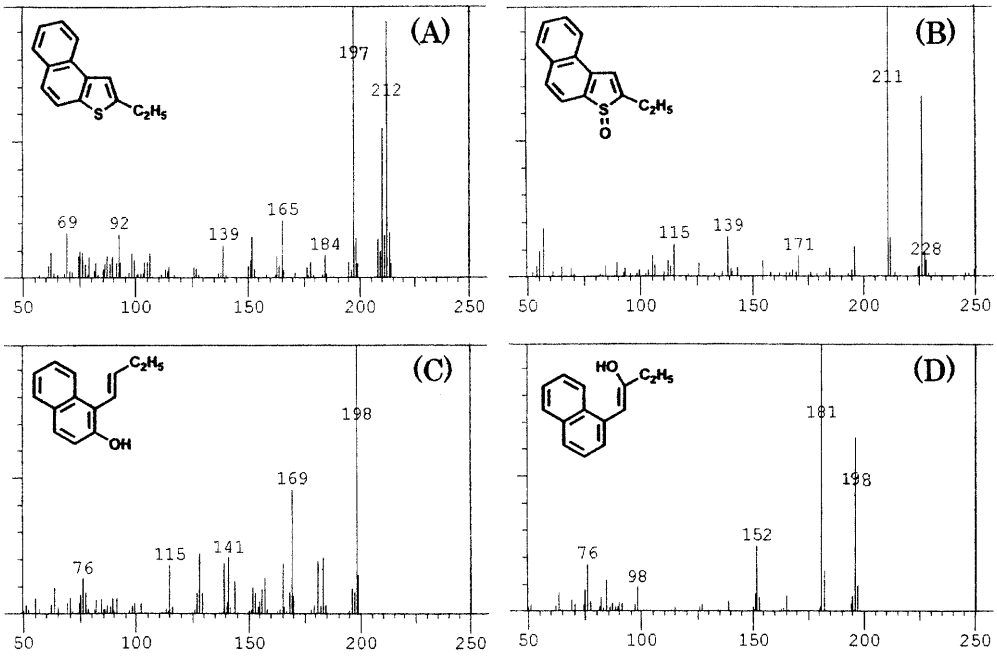

Fig. 2A–D GC/MS analysis of 2-ethylNTH-desulfurized metabolites produced by *M. phlei* WU-F1. **A** 2-EthylNTH, **B** 2-ethylNTH sulfoxide, **C** 1-(2′-hydroxynaphthyl)-1-butene, **D** 1-naphthyl-2-hydroxy-1-butene

2-EthylNTH-desulfurizing pathway

M. phlei WU-F1 was cultivated to the end of the exponential growth phase in AF medium with 2-ethylNTH as the sole source of sulfur. The culture broth was extracted with ethylacetate and the resulting extract containing metabolites after 2-ethylNTH desulfurization was analyzed by GC-MS. As shown in Fig. 2, three compounds (metabolites) were detected in addition to 2-ethylNTH (M^+, *m*/*z*=212; Fig. 2A). Since standard compounds were commercially unavailable, the molecular structures of the metabolites were deduced from these mass spectra. One metabolite is assigned to 2-ethylNTH sulfoxide (M^+, *m*/*z*=228; Fig. 2B). The high-abundance fragment ion at *m*/*z*=211 corresponds to the loss of the oxygen atom from the molecular ion. The other metabolites (Fig. 2C, D) are considered to be compounds including no sulfur atom in their structures. One is assigned to 1-(2′-hydroxynaphthyl)-1-butene (M^+, *m*/*z*=198; Fig. 2C). The fragment ion at *m*/*z*=169 corresponds to the loss of the ethyl group from the molecular ion; and the fragment ion at *m*/*z*=141 corresponds to the further loss of the vinyl group. The other is assigned to 1-naphthyl-2-hydroxy-1-butene (M^+, *m*/*z*=198; Fig. 2D). The high-abundance fragment ion at *m*/*z*=181 might be due to the loss of the hydroxy group from the molecular ion; and the fragment ion at *m*/*z*=152 corresponds to the further loss of the ethyl group.

Based on the deduced structures of metabolites in Fig. 2 and the DBT-desulfurizing pathway previously reported (Gallagher et al. 1993; Furuya et al. 2001), the 2-ethylNTH-desulfurizing pathway by WU-F1 is proposed as shown in Fig. 3. It is concluded that WU-F1 desulfurizes 2-ethylNTH through a sulfur-specific degradation pathway, with selective cleavage of the C-S bonds, similar to the DBT-desulfurizing pathway.

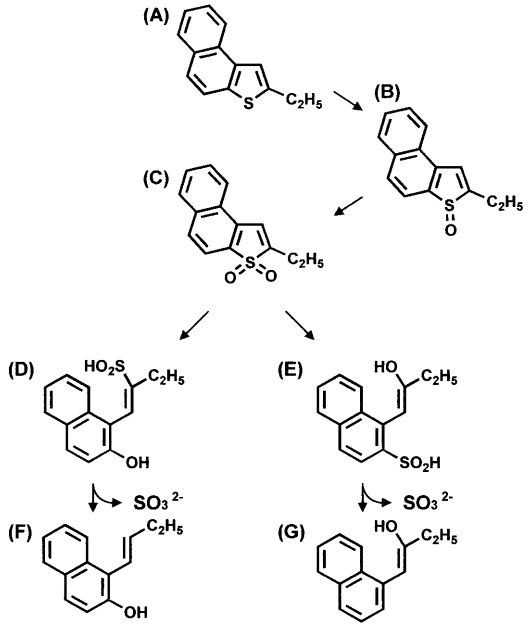

Fig. 3 Proposed pathway of 2-ethylNTH desulfurization by *M. phlei* WU-F1. Compounds *C*, *D* and *E* were not identified and are indicated as postulated metabolites

Discussion

This is the first report describing the biodesulfurization of NTH and 2-ethylNTH through a sulfur-specific degradation pathway. We succeeded in the thermophilic desulfurization of NTH and 2-ethylNTH, using a moderately thermophilic bacterium, *M. phlei* WU-F1, which possessed high desulfurizing ability toward DBT and its derivatives over a wide temperature range (20–50°C; Furuya et al. 2001). From the viewpoint of a practical

process, biodesulfurization at around 50 °C of HDS-treated diesel oil containing various types of DBT and NTH derivatives is advantageous, since cooling treatment of the oil to ambient temperature would be unnecessary.

It is interesting to note that WU-F1 exhibited a faster growth and higher degradation rate for 2-ethylNTH than for NTH (Fig. 1). In comparison with NTH, the ethyl group at the 2-position in the molecule of 2-ethylNTH might increase the affinity of substrate recognition by the desulfurizing enzymes or substrate uptake by WU-F1 cells, leading to the faster growth and higher degradation rate for 2-ethylNTH than for NTH. This property is desirable for biodesulfurization, since HDS-treated diesel oil seems likely to contain various types of alkylated NTH derivatives, including 2-ethylNTH.

We confirmed that WU-F1 produced 2-hydroxybiphenyl only as a sulfur-removed metabolite from DBT, a symmetric organosulfur compound. Moreover, WU-F1 also produced only one sulfur-removed metabolite from an asymmetric 3,4-benzoDBT (Furuya et al. 2001), as reported for *R. erythropolis* H-2 (Ohshiro et al. 1996). In contrast, it is interesting to note that WU-F1 produced two sulfur-removed metabolites (Fig. 3F, G) from 2-ethylNTH, an asymmetric organosulfur compound, probably due to the distinct recognition of two types of C-S bonds in the postulated metabolite, 2-ethylNTH sulfone (Fig. 3C), by the desulfurizing enzyme(s). However, no pronounced metabolites after NTH desulfurization were detected by GC-MS analysis (data not shown). Although we do not have a clear explanation for this, it might be due to the lability of NTH-desulfurized metabolites. However, since WU-F1 utilized NTH as the sole source of sulfur but not as the sole source of carbon (data not shown), it is presumed that NTH was also desulfurized through the sulfur-specific degradation pathway. At present, we are investigating whether or not the "NTH-desulfurizing enzymes" are identical to the "DBT-desulfurizing enzymes" in their properties.

In conclusion, we confirmed that *M. phlei* WU-F1 could effectively desulfurize asymmetric organosulfur compounds, NTH and 2-ethylNTH, as well as symmetric DBT derivatives, through a sulfur-specific degradation pathway with the selective cleavage of C-S bonds under high-temperature conditions. Therefore, *M. phlei* WU-F1 may be a useful desulfurizing biocatalyst possessing a broad substrate specificity toward organosulfur compounds.

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