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Isolation and Characterization of Thermophilic Benzothiophene-Degrading *Mycobacterium* sp.

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Abstract

A bacterial strain, SWU-4, capable of using benzothiophene (BT) as a sole carbon and energy source was isolated from a petroleum-contaminated site in Thailand and identified by 16S rRNA gene sequence analysis to be in the genus of *Mycobacterium*. The strain was Gram-positive, nonspore former, and grew at 50° C. Colonies of the strain on nutrient agar were rod-shaped, smooth with a convex surface, slightly mucoid, and yellow pigmented. The thermophilic *Mycobacterium* sp. strain SWU-4 rapidly degraded 2% (w/v) BT at 50°C. Interestingly, this strain was able to degrade a wide variety of organosulfur compounds including thiophene, bromo(α)thiophene, and 3-methylthiophene in liquid minimum medium at 50°C, which will be beneficial for industrial applications.

Index Entries: Thermophilic *Mycobacterium* sp.; organosulfur-degradation; 16S rDNA.

Introduction

Contaminated soil and water systems have received much attention for several decades. Their hazardous nature and potential impact on human and

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environmental health are beginning to be realized. Over the years, toxic compounds have been discarded, and a normal, common way has been to bury the compounds (oil spill, polyaromatic hydrocarbons [PAHs]/creosote, and metal spill) directly in the soil. The environmental risk of heterocyclic PAH is not as well established as it is for unsubstituted PAHs. Large differences in chemical characteristics and biologic reactivity are likely to exist, e.g., between sulfur heterocyclic PAH (SPAH) and parental PAH. The substitution of a carbon atom by a sulfur atom makes these substances more polar and increases their water solubility. Concentrations of SPAHs found in the environment are one to two orders of magnitude lower than PAH concentration, but their biologic effects can be of similar magnitude *(1)*. Today, when a former industry or polluter is (usually) long gone, the ground is still heavily contaminated and treatment is necessary before the soil can be used again. Biologic treatment is a cheap and effective method compared with other methods, such as incineration, soil washing, and venting *(2)*. The effectiveness of biologic treatment is greatly dependent on the contaminant type, soil properties, microbial activity, and redox conditions. It is necessary to control these parameters carefully to succeed with a biologic treatment *(3,4)*.

Various investigations with pure bacterial cultures and pure organosulfur compounds have demonstrated that benzothiophene (BT) is susceptible to microbial attack *(5)*. Some mesophilic microorganisms that metabolize BT have been isolated. For example, *Pseudomonas aeruginosa* PRG-1 oxidizes BT dissolved in light oil but cannot use this compound as a sole carbon source *(6)*, and a dibenzothiophene-oxidizing isolate, *Pseudomonas alcaligenes* DBT 2, oxidizes BT to water-soluble compound products *(7)*. However, the metabolites of BT oxidation were not identified in either of those studies. In another investigation *(8)*, enrichment cultures from several aquatic environments and from wastewater treatment plant effluents were able to oxidize BT when naphthalene was provided as a sole carbon source.

In this article, we report on the isolation and characterization of the thermophilic BT-degrading *Mycobacterium* sp. from an oil refinery site in Thailand, which utilizes BT as the sole carbon and energy source. In addition, we report on the ability to degrade other organosulfur compounds.

Materials and Methods

Culture Media

Carbon-free minimal medium (CMM) was used as the basal medium and contained (per liter of deionized water) 2 g of Na_2SO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.65 g of K_2HPO_4, 1 g of NH_4Cl, 2 g of $NaNO_3$, and 0.01 g of $FeSO_4$ *(9)*. The final pH of the medium was adjusted to 7.4 with 1 *N* HCl, and the medium was sterilized by autoclaving (121°C for 20 min) prior to the addition of the organosulfur hydrocarbon substrate. The substrate was supplied at 0.1% (w/v) as a solution in acetone:hexane (1:1 [v/v]). Solid medium was prepared by the addition of 2.0% Bacto agar to the respective medium.

Field Soil Samples

Oil-contaminated soil samples were collected from the petroleum-contaminated sites in two provinces in Thailand, Chonburi and Rayong, and stored at 4°C until used. Sampling was done randomly in blocks *(10)*. Four random samples were taken from the top layer (10 cm) of each of six plots in the area. The individual samples from each plot were bulked and mixed.

Enrichment and Isolation

BT-degrading bacteria were isolated from the enrichment cultures by the spray-plate technique *(11)* using BT (Wako) as the sole carbon and energy source. Twenty milliliters of CMM with BT (0.1 g/L) in 100-mL Erlenmeyer flasks was inoculated with 0.5 g of soil. The flasks were shaken (200 rpm) at 30°C. After 2 d of inoculation, a 0.2-mL sample was diluted 1:100 (v/v) in fresh medium and incubated under the same conditions. After two transfers, samples were serially diluted, and plated on CMM agar. Immediately, the surfaces of the plated agar were coated lightly with an acetone:hexane (1:1 [v/v]) spray of diluted BT. The plates were then incubated at 50°C for 48 h. BT-degrading bacteria were visualized by a distinct BT clear zone surrounding individual colonies. A strain capable of rapid degradation of BT was selected. These representative colonies were aseptically removed and subcultured in liquid CMM containing BT and incubated at 50°C. Tests for Gram stain, oxidase activity, catalase activity, carbon source utilization, nitrate reduction, and acid fastness (Ziehl-Neelsen method) were carried out by standard procedure *(12)*. Identification was confirmed by the 16S rDNA gene.

Utilization of BT

Growth and utilization of BT were established by demonstrating an increase in bacterial biomass. BT-grown cells from the late exponential growth phase were used as inoculum (10^6–10^7 cells/mL) for tube culture. Three replicate batch cultures were grown in 22-mL test tubes containing 5 mL of CMM supplemented with 2.0% (w/v) BT. Incubation was at 50°C with agitation at 200 rpm for 72 h. Two separate controls were included in the system: substrate control (CMM with BT devoid of bacteria); and a cell growth control, consisting of CMM and a bacterial inoculum without BT supplementation. Cell growth was monitored by measuring turbidity at 660 nm with UV-1200 UV-VIS spectrophotometer (Shimadzu). Calibration of the spectrophotometric response against cell dry weights was performed: one OD_{660} unit corresponds to 0.32 mg of cells (dry wt)/mL.

Utilization of Other Organosulfur Hydrocarbons

Utilization of three other organosulfur hydrocarbons as substrates for growth was tested by inoculating the selected isolate into three replicate 22-mL of test tubes containing 5 mL of CMM with individual compounds

(2% [w/v]) as follows: thiophene (Cica, Kanto, Japan), bromo(α)thiophene (Cica), and 3-methylthiophene (Wako). Incubation was at 50°C with agitation at 200 rpm for 72 h. Cultures were analyzed for an increase in bacterial biomass (OD_{660}).

DNA Extraction

An aliquot (1.5 mL) of an overnight culture was centrifuged for 2 min (12,000*g*), washed with Tris-EDTA (TE) buffer *(13)*, and the pellet was resuspended in 500 µL of TE buffer plus 0.5 g of glass beads (0.1 mm in diameter). After bead beating for 30 s, 30 µL of 10% sodium dodecyl sulfate and 500 µL of Tris-buffered phenol (pH 8.0) were added. The mixture was then centrifuged for 10 min (at 12,000*g*), and the aqueous phase was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1). Finally, the DNA was precipitated with 0.6 vol of isopropanol (5 min, room temperature), centrifuged for 10 min (12,000*g*), and washed with 70% ethanol. The pellet was air-dried and resuspended in 50 µL of TE buffer. Subsequent steps consisted of purification with CsCl to precipitate impurities; precipitation of the DNA with isopropanol; precipitation with potassium acetate (20 µL of an 8M solution per 100 µL), 0.6 vol of isopropanol; and finally, purification over resin spin columns (Wizard DNA cleanup system; Promega, Madison, WI).

DNA Amplification

Polymerase chain reactions (PCRs) were performed in a thermal cycler (model PTC 200 DNA Engine; MJ). The assay was performed in a 50-µL volume containing a mixture of 20 ng of genomic DNA; 1× PCR buffer (10 m*M* Tris-HCl, pH 8.0; 50 m*M* KCl; 0.1% gelatin); 200 µ*M* each of dATP, dCTP, dGTP, and dCTP; 1.0 m*M* $MgCl_2$, 10 pmol of primer pair, and 5 U of *Taq* DNA polymerase (Perkin-Elmer). Negative controls (PCR mixture without added genomic DNA) were included in all PCRs. 16S rDNA was amplified from genomic DNA by PCR with forward primer GM5F (5′-CCTACGGGAGGCAGCAG-3′) and reverse primer DS907R (5′-CCCCGTCAATTCCTTTGAGTTT-3′) *(14)*. An initial denaturation step (94°C, 3 min) followed by 30 cycles consisting of 94°C for 30 s, primer annealing at 59°C for 30 s, extension at 68°C for 40 s, and a final extension at 68°C for 7 min was used. The amplification products were purified after agarose gel electrophoresis with the Wizard PCR Prep (Promega) for automated dye-dideoxy terminator sequencing with a 373A DNA sequencing system (Applied Biosystems).

Phylogenetic Analysis

The 16S rDNA sequence of our isolate was aligned against those retrieved from the GenBank using Clustal W *(15)*. The divergence between pairs of sequences was estimated using Kimura's *(16)* two-parameter model. A neighbor-joining tree based on divergence between pairs of

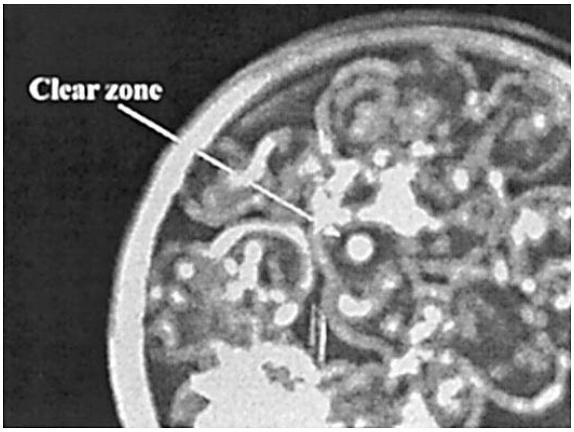

Fig. 1. BT-degrading bacterial colony and clear zone of BT utilization on CMM agar coated with BT.

samples was constructed *(17)* using the Neighbor routine in Phylip 3.56c *(18)*. In addition, aligned sequences were bootstrapped 2000 times using Seqboot. The consensus neighbor-joining and maximum parsimony trees were constructed to illustrate relationships among isolates. All phylogenetic programs described were routined in Phylip *18)*.

Results

Isolation and Characterization of Thermophilic BT-Degrading Bacteria

Five bacterial colonies were obtained from petroleum-contaminated sites in Chonburi and Rayong provinces, Thailand, by standard culture enrichment techniques using BT as the sole source of carbon and energy. When these colonies were grown on BT-coated agar plates, clear zones were visualized indicating the BT-degrading ability (Fig. 1). However, when these strains were tested for their BT-degrading ability in liquid medium, only strain SWU-4 was able to degrade BT rapidly. Morphologically, strain SWU-4 was a Gram-positive, catalase-positive, oxidase-negative, strongly acid fast from glucose fermentatively, nonspore former and grew at 50°C. Colonies of the strain on nutrient agar were rodshaped, smooth with a convex surface, slightly mucoid, yellow pigmented, with a size of 0.7–1.1 μm by 1.3–2.1 μm. The strain did not grow on adonitol, arabinose, cellobiose, dulcitol, galactose, inositol, malonate, raffinose, rhamnose, sorbitol, and starch. Results from bacteriologic and biochemical characterization of the organism, as shown in Table 1, suggest that the strain SWU-4 belongs to the genus *Mycobacterium*. DNA sequence analysis of PCR-amplified 16S rDNA as compared with those of *Mycobacterium phlei* (Fig. 2) confirmed the result that strain SWU-4 did indeed belong to the genus *Mycobacterium*.

Table 1
Biochemical and Morphologic Characteristics of
Thermophilic BT-Degrading *Mycobacteri*um Sp. Strain SWU-4

	Characteristics	Results[a]
Bacteriologic tests:	Flagella	–
	Gram reaction	+
	Morphology	Rod-shape smooth with a convex surface; no endospore forming, yellow
Growth tests:	Glucose	+
	Lactose	–
	Maltose	+
	Sucrose	–
	Xylose	+
	Arabinose	+
	Mannitol	+
	Galactose	–
	Citrate	+
Biochemical tests:	Oxidase	–
	Catalase	+
	Nitrate reduction	–
	Acid fast	+

[a]+, good growth or activity; –, poor or no growth or activity.

Utilization of BT

Utilization of BT as the sole source of carbon and energy was demonstrated by the increase in biomass (dry wt) (Fig. 3) when *Mycobacterium* sp. strain SWU-4 was cultivated on CMM containing 2.0% (w/v) BT. Maximum growth was achieved after 60 h at 50°C.

Substrate Specificity

Many organosulfur compounds such as thiophene, bromo(α)thiophene and 3-methylthiophene are detected in fossil fuels after hydrodesulfurization treatment. As shown in Table 2, *Mycobacterium* sp. strain SWU-4 grew in the CMM medium with thiophene, bromo(α)thiophene, or 3-methylthiophene replacing BT as the sole source of carbon and energy at 50°C.

Discussion

Organic sulfur compounds are found in fossil fuel, the combustion of which causes serious environmental problems, such as acid rain. At the refinery, hydrodesulfurization is currently performed to remove sulfur compounds from fossil fuels. This process is done at high temperatures and processed by metal catalysis and is effective for removing inorganic sulfur

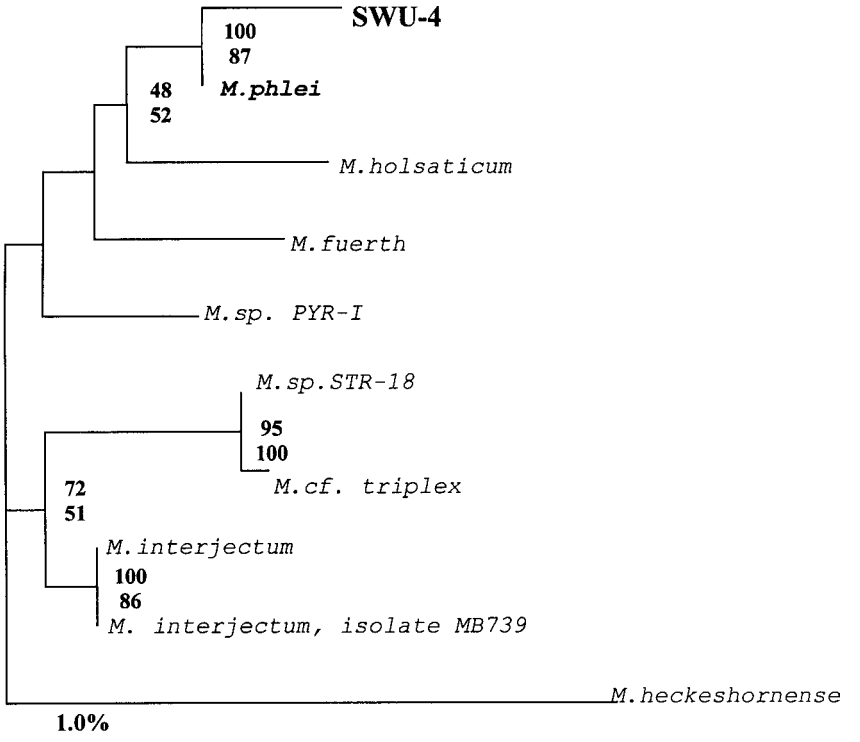

Fig. 2. A tree illustrating relationships between different *Mycobacterium* isolates based on sequence divergence of 16S rDNA. Values at the node (only greater than 50% are shown) represent the percentage of times that the particular node occurred in 2000 trees (neighbor-joining, maximum parsimony) generated by bootstrapping the original sequences.

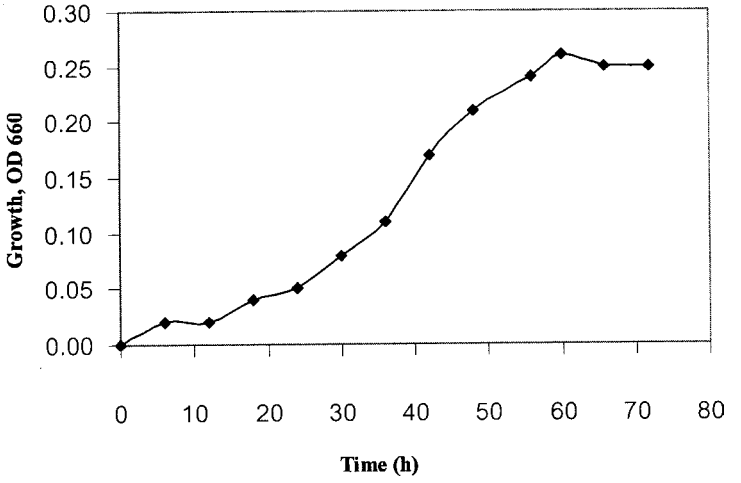

Fig. 3. Growth curve of *Mycobacterium* sp. strain SWU-4 cultured in CMM broth containing BT as sole carbon and energy sources.

Table 2
Bacterial Growth on Various Single Pure Organosulfur Compounds as Sole Source of Carbon and Energy[a]

Substrate	OD_{660} [b]
No substrate (control)	—
Thiophene	0.32 ± 0.04
Bromo(α)thiophene	0.27 ± 0.02
3-Methylthiophene	0.55 ± 0.07

[a]*Mycobacterium* sp. SWU-4 was incubated in CMM medium (5 mL) containing 20 g/L (w/v) of organosulfur compounds in 22-mL test tubes at 50°C for 72 h.

[b]Three measurements were obtained. The values are means ± SD.

and simple organic sulfur compounds. As legislative limits on sulfur emission have become tighter, the need to remove polycyclic sulfur compounds from fuel has become more pressing. The sulfur-specific pathway has been extensively studied by using two *Rhodococcus* strains: *R. erythropolis* IGTS8 *(19)* and *R. erythropolis* D-1 *(20)*.

In the present study, samples were collected from petroleum-contaminated sites located in Chonburi and Rayong provinces, Thailand, a point source for petrogenic chemicals entering Thai Bay. We speculated that hot climate, high inorganic and organic nutrient levels, and a long history of exposure to high concentrations of petroleum hydrocarbons would favor these sites as possible sources of bacteria able to degrade organosulfur compounds. Herein, we have reported on the isolation and characterization of a thermophilic *Mycobacterium* sp. that readily degraded BT and other organosulfur compounds. It was identified as a *Mycobacterium* sp. on the basis of its cellular and colony morphology, Gram-positive and strong acid-fast staining, mycolic acids, diagnostic biochemical reactions, and 16S rDNA analysis. *Mycobacterium* sp. is common in the environment *(21)* and has been reported to degrade PAHs *(22)*. Since hydrodesulfurization treatment is supplied at high temperature, thermophilic organosulfur compound-degrading microorganisms are more advantageous than mesophilic ones for this application. In addition, the *Mycobacterium* sp. strain SWU-4 can be used for other applications including wastewater treatment.

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