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Isolation and Characterization of a Dibenzothiophene Degrading Sulfate-Reducing Soil Bacterium

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Sulfate-reducing bacteria have been isolated from soil and their abilities to degrade dibenzothiophene (DBT) were compared with those of type cultures. Among the strains tested a soil isolate M6 showed the highest ability to degrade DBT. Isolate M6 was characterized as a mesophilic obligatory anaerobe. The morphology of the bacterium was vibrioid with the size of 0.4-0.7 μm by 1.0-1.5 μm. Gram reaction was negative and nonsporulating. Desulfoviridin is present. Lactate, pyruvate, ethanol and malate supported growth of the bacterium in the presence of sulfate. Sulfate, sulfite, thiosulfate and sulfur served as electron acceptors for growth. Hydrogenase was present. The mol% of guanine and cytosine of DNA was determined as 56%. The bacterium produced viscous material. From these results, the isolate M6 was identified as *Desulfovibrio desulfuricans*.

Recently attempts are being made to use microorganisms for the removal of organic sulfur from fossil fuels (1, 2, 6, 9-12). Various aerobic bacteria oxidize heterocyclic sulfur compounds to water soluble products (2, 18). On the other hand mixed cultures of anaerobic bacteria reduce the sulfur compounds to hydrogen sulfide (9, 10). Sulfate-reducing bacteria have been identified as the main component of the anaerobic microbial consortia (1).

Sulfate reducing bacteria(SRB), although obligate anaerobes, can be isolated from a wide variety of environments (15). The metabolism of SRB is characterized by the reduction of sulfate, which serves as an electron acceptor, to sulfide. Classification of sulfate-reducing bacteria is based on nutritional and morphological characteristics, and on some chemical criteria, such as the G+C content of the DNA and the presence of specific pigments (16). *Desulfovibrio* and *Desulfotomaculum* are the major genera among 8 genera recognized in SRB (19). Recently sulfate reducers have been found in archaebacteria (17).

Sulfur compounds in the fossil fuel can be classified into thiols, sulfides and thiophenes (12). The relative abundance of dibenzothiophene (DBT) derivatives in fossil fuel has led us to use DBT as the model compound in the microbial desulfurization studies.

This paper describes the selection and the identification of a dibenzothiophene (DBT) degrading sulfate-reducing bacterium.

MATERIALS AND METHODS

Isolation of SRB

Samples were anaerobically inoculated into anaerobic tubes containing Postgate medium E (15) under a stream of oxygen-free-nitrogen gas, and the tube was incubated for 5 days at 30℃. An aliquot from each culture positive for sulfate-reducing activity was transferred to Hungate tube containing molten Postgate medium E with 2% agar (4). Colonies appeared in the tube were purified by repeated transfers onto solid medium E in an anaerobic glove box (Coy Lab., Ann Arbor, MI) with the atmosphere of 95% (v/v) nitrogen plus 5% (v/v) hydrogen.

Other Bacterial Strains Used and Culture Cultivation

Reference strains *Desulfovibrio vulgaris* KCTC 1910 (NCIB 8303), *Desulfovibrio desulfuricans* KCTC 1907 (NCIB 8310), *Desulfotomaculum orientis* KCTC 3198 (NCIB 8382) and *Desulfotomaculum ruminis* KCTC 3183 (NCIB 8452) were obtained from the Korean Collection for Type Cultures (KCTC, Seoul, Korea).

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Postgate media C and E (15) were made anaerobically in anaerobic pressure tubes (Bellco Glass Inc., Vineland, NJ) or in serum vials (Wheaton Scientific, Millville, NJ) and used throughout the study. The growth was initiated by 5% inoculation and incubated at 30℃. Substrate utilization was analyzed using Postgate medium E substituting substrates to be tested for lactate.

DBT Degradation Test

Cells of 2 day old 70 ml culture in Postgate medium C were harvested anaerobically and suspended in 65.1 ml of sulfate-free medium C (Postgate medium C without sodium sulfate). The cell suspension was added by 100 mM methyl viologen solution (1.4 m*l*) and 2% DBT solution in dimethylformamide (3.5 m*l*). The headspace of the vial was filled with hydrogen, and the reaction mixture was reduced by 2.5% sodium sulfide solution when needed, before the vial was incubated at 30℃ for 5 days. The decrease of DBT concentration was determined by gas chromatographic method after extraction with butanol.

Cell Morphology

A light microscope, Jenalumer (Jena, Leipzig, GDR) was used for routine culture observation. Transmission electron micrographs were taken using JEM 100CX-2 electron microscope (Jeol Ltd., Tokyo, Japan) after the specimen was fixed by glutaraldehyde and stained by osmium tetraoxide (20). Scanning electron micrographs were taken using SEM S-450 (Hitachi, Tokyo, Japan) after gold coating according to the manufacturer's manual.

Biochemical Test

The presence of desulfoviridin was determined according to Postgate (14) in alkaline medium. The mol percent of guanine plus cytosine (G+C) of deoxyribonucleic acid (DNA) was determined by the thermal melting point method (5) using an automatic recording spectrophotometer (Gilford System, Oberlin, OH). DNA from *Escherichia coli* B was included in each set of analysis as a standard.

Consumption hydrogenase activity was determined spectrophotometrically by monitoring the reduction of methyl viologen at 578 nm (7). Organisms from a 2 day old culture in medium C were centrifuged, washed twice in 0.1 M Tris-HCl buffer (pH 7.6), and suspended in the same buffer to use as the enzyme. The anaerobic reaction mixture (1 ml) contained 2 mM methyl viologen, 2 mM dithiothreitol under 1 atm hydrogen. The reaction was started by adding the cell suspension. Protein content of the cell suspension was measured by Biuret method using bovine serum albumin as the standard (3). One unit of the enzyme activity was defined as the amount of protein which uptake 1 μmol of hydrogen per minute.

Analyses

Sulfide and carbon dioxide concentration were determined gas chromatographically using a Varian Model 3700 GC (Sunnyvale, CA) equipped with a thermal conductivity detector and a teflon column packed with Chromosil 310 (Supelco, Bellefonte, PA). The gas samples for the analyses were taken directly from tubes using pressure lock syringes and injected into the GC. Nitrogen was used as the carrier gas at the flow rate of 25 ml/min. Temperatures of injector, column and detector were 120, 50 and 120℃, respectively. The increase of the gaseous products was used as the indicator of the bacterial growth. The culture turbidity was read using Jasco spectrophotometer model UVIDEC 610 (Tokyo, Japan) after the sulfide precipitates were oxidized by exposing the culture to air. Soluble fermentation products were quantified by gas chromatography (8).

RESULTS AND DISCUSSION

Degradation of DBT

Soil isolates were used in the experiment to measure their abilites of DBT degradation, and the results were compared with those of the type cultures (Table 1). The degree of DBT degradation ranged from 2% upto 42%. Among the tested strains the soil isolate M6 showed the highest ability to degrade DBT. Among the type culture strains *Desulfovibrio* degraded more DBT than strains of *Desulfotomaculum*. Kurita *et. al.* (10) reported that hydrogenase activities of SRB are closely related to their abilities of organic sulfur compounds degradation. *Desulfovibrio* has higher hydrogenase activity than *Desulfotomaculum* for the operation of the energy-conservation mechanism of hydrogen cycling during the reduction of sulfate (13). The reduction of DBT seems to be also related to hydrogenase activity.

Morphology of Sulfate-Reducing Soil Isolate M6

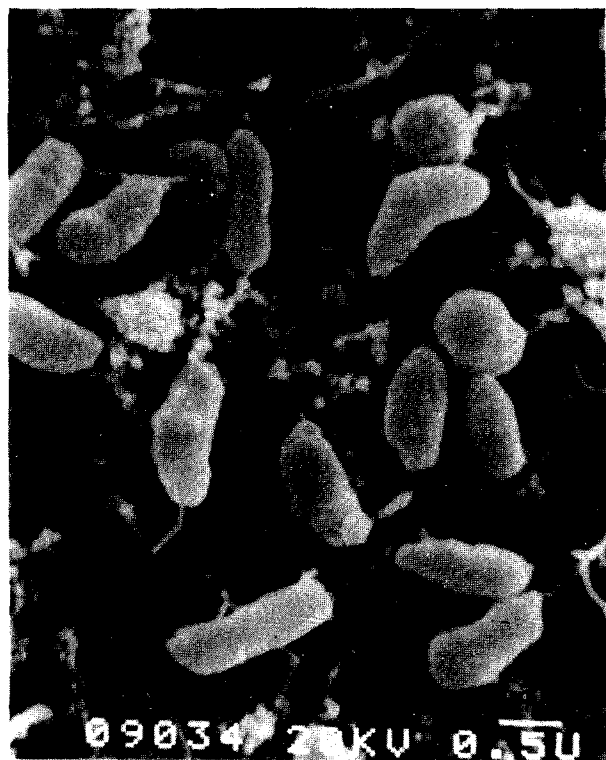
The isolate was strictly anaerobic, Gram-negative, motile, nonsporulating, and mesophilic dissimilatory SRB.

Table 1. Degradation of DBT by sulfate-reducing bacteria

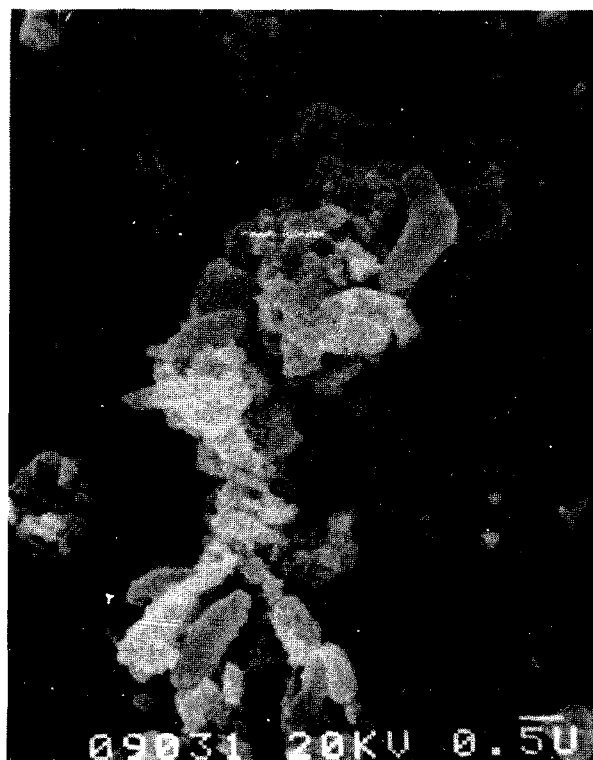
Strain	Degradation of DBT (%)
Isolate M6	42
Isolate M8	6
Isolate S1	17
Isolate SA	21
Isolate 5	12
Desufovibrio vulgaris KCTC 1901	15
Desufovibrio desulfuricans KCTC 1907	19
Desulfotomaculum ruminis KCTC 3183	2
Desulfotomaculum orientis KCTC 3198	6

Cells in a young culture gave a uniform suspension, but when the culture became older than a week, the cells clumped indicating that the bacterium produces extracellular polymer.

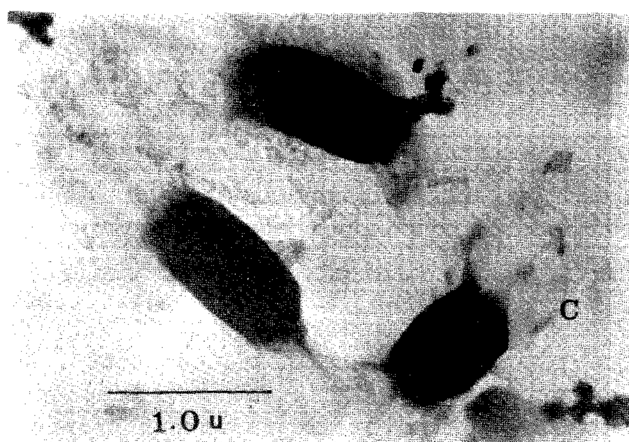
Young (2 day old) and old (a week old) culture were used to take electron micrographs (Fig. 1). Scanning electron microscopic observation showed single cells in young culture (Fig. 1A) whilst clumped cells were predominant in the old culture (Fig. 1B). The cells had vibrioid shape with the size of 0.4-0.5 μm by 1.0-1.5 μm. The cells of a week old culture were place on a grid and dried to observe without staining using a transmission



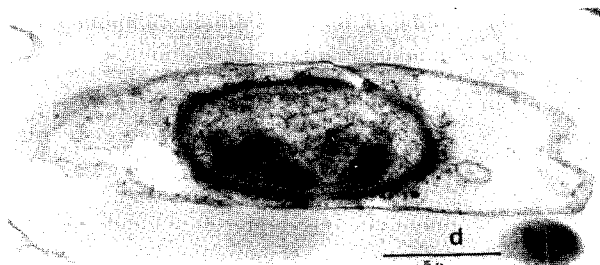
(A)



(B)



(C)



(D)

Fig. 1. Electron micrographs of the sulfate-reducing isolate M6.
Scanning electron micrographs of 3 days old (A) and 7 days old cultures (B) were taken after the sample was coated by gold. Cells taken from the 7 day old culture was observed using a transmission electron microscope with (D) and without (C) thin-sectioning.

Table 2. Characteristics of sulfate reducing isolate M6.

Characteristcs	Isolate M6	*Desulfovibrio desulfuricans*	*Desulfovibrio vulgaris*
Cell			
morphology	vibrioid	vibrioid	vibrioid
diametre (μm)	0.4-0.7	0.5-1.0	0.5-1.0
length (μm)	1.0-1.5	3.0-5.0	3.0-5.0
Growth on			
pyruvate+sulfate	+	+	+
lactate+sulfate	+	+	+
malate+sulfate	+	+	+
acetate+sulfate	−	−	−
ethanol+sulfate	+	+	−
glucose+sulfate	−	+, −	−
pyruvate	+	+	−
malate	−	−	−
Desulfoviridin	+	+	+
Optimum temperature (℃)	30-35	34-37	34-37
Mol % G+C, DNA	56	55	61
Hibitane resistance (mg/ml)	10	10-50	2.5

+: growth or present, −: no growth

electron microscope (Fig. 1C). Extracellular material was observed around the bacterial cells. Similar culture was used in the electron microscopic observation after fixation, thin-sectioning, and staining (Fig. 1D). The old cells were found to be encapsulated by the extracellular material.

Biochemical Properties

DNA was prepared from cells grown on Postgate medium C for 3 days before the melting point was determined. The mol% of guanine and cytosine was calculated as 56% from the DNA melting point. The reduction of methyl viologen was started immediately after its addition to the culture. The specific uptake hydrogenase activity of the whole cell suspension was 21.0 unit/mg cell protein. The cell suspension in alkaline solution gave red fluorescence indicating the presence of desulfoviridin which functions as sulfite reductase in species of *Desulfovibrio*.

Growth Characteristics

The isolate M6 was tested for its growth on carbon sources and its growth inhibition by hibitane, and compared with the type cultures (19). The results are summarized in Table 2.

The isolate utilized lactate, pyruvate, malate and ethanol as the electron donor with sulfate as the electron acceptor, but not glucose and acetate. In the absence of sulfate, growth was observed on pyruvate but not on malate. The isolate used sulfate, sulfite, thiosulfate and elementary sulfur as the electron acceptor. The isolate could grow in postgate medium C containing upto 10 mg/l of hibitane. The optimum temperature for the growth was 30-35℃.

Description of the Isolate M6

The sulfate-reducing isolate M6 is a Gram-negative vibrio, and does not produce endospores. The bacterium, uses sulfate, sulfite, thiosulfate and elementary sulfur as the electron acceptors, and oxidizes lactate incompletely to acetate. Widell and Pfennig (19) defined the group of bacteria with the above characteristics *Desulfovibrio*.

Desulfovibrio M6 possesses desulfoviridin and tolerates 10 mg/l of hibitane. The mol% G+C of DNA in the bacterium is 56%. Among the *Desulfovibrio* species

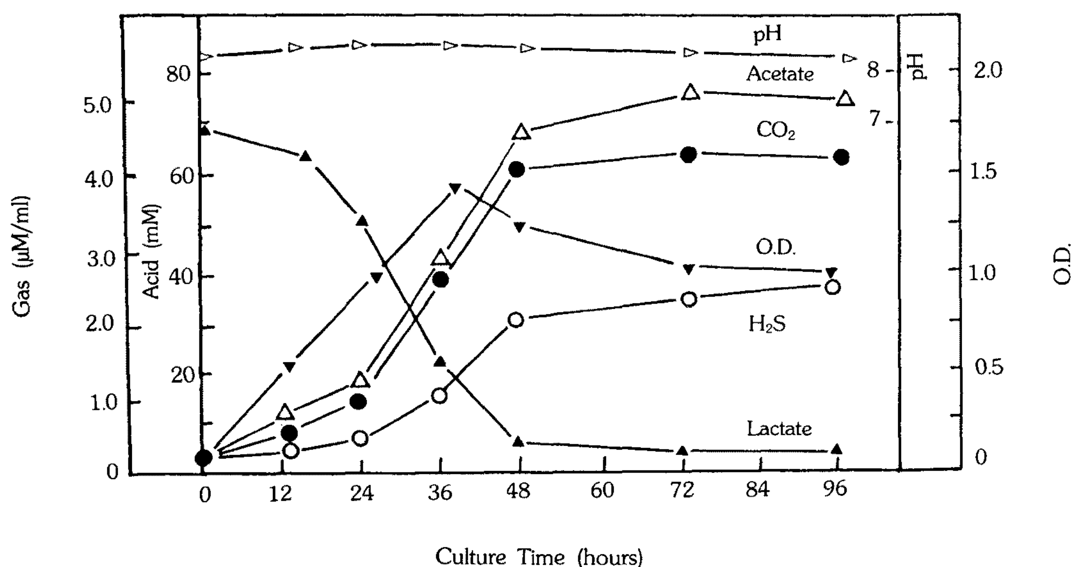


Fig. 2. Growth *Desulfovibrio desulfuricans* M6 on Postgate medium C.

which possess desulfoviridin, *Desulfovibrio desulfuricans* is most similar to *Desulfovibrio* M6 in terms of hibitane tolerance, substrate utilization. Mol % G+C of *D. desulfuricans* is recorded as 55% whilst that of *Desulfovibrio* M6 is 56%. Other differences include the cell size and the isolate's ability to produce extracellular polymer at the stationary phase. *D. gigas* and *D. vulgaris* are known to produce mannose based extracellular polysaccharide in the stationary phase. (15), but they are known to tolerate 2.5 mg/l of hibitane. From these comparisons the dibenzothiophene degrading soil isolate M6 is identified as *Desulfovibrio desulfuricans.* The strain has been deposited to the Korean Collection for Type Cultures (KCTC 2490).

Growth of *D. desulfuricans* M6 and Degradation of DBT

D. desulfuricans M6 was grown in the Postagate medium C, and the changes in substrate, products and culture turbidity were monitored (Fig. 2). Lactate is incompletely oxidized to acetate and carbon dioxide. pH remained nearly constant at neutral during the growth. The culture turbidity reached maximum in 36 hours before decreased. The decrease in the culture turbidity was due to cell clumping.

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