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Thermophilic Carbon-Sulfur-Bond-Targeted Biodesulfurization

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Petroleum contains many heterocyclic organosulfur compounds refractory to conventional hydrodesulfurization carried out with chemical catalysts. Among these, dibenzothiophene (DBT) and DBTs bearing alkyl substitutions are representative compounds. Two bacterial strains, which have been identified as *Paenibacillus* strains and which are capable of efficiently cleaving carbon-sulfur (C—S) bonds in DBT at high temperatures, have been isolated for the first time. Upon attacking DBT and its various methylated derivatives at temperatures up to 60°C, both growing and resting cells of these bacteria can release sulfur atoms as sulfate ions and leave the monohydroxylated hydrocarbon moieties intact. Moreover, when either of these paenibacilli was incubated at 50°C with light gas oil previously processed through hydrodesulfurization, the total sulfur content in the oil phase clearly decreased.

Sulfur emission through fossil fuel combustion is a global problem because it is a major cause of acid rain. This concern will become much more grave at least partially owing to the decreasing availability of low-sulfur fuels. Crude oil has a sulfur content of a few percent by weight, and diesel oil treated by hydrodesulfurization processes usually still retains sulfur on the order of a few hundred parts per million. Regulations against sulfur emission must become more strict worldwide in light of the need for environmental protection; in fact, the sulfur level in motor diesel was required to be less than 500 ppm in 1997 in Japan. To decrease the sulfur content in petroleum, the hydrodesulfurization process has been routinely applied in refineries worldwide. This system is carried out with chemical catalysts comprised of metal compounds and under severe conditions such as extremely high temperature and pressure; for example, desulfurization of light gas oil is carried out at temperatures higher than 300°C and at pressures on the order of 10^7 Pa.

The biodesulfurization process using microorganisms and/or enzymes has the advantage of being carried out more safely under mild conditions. Moreover, bioprocessing is in general strictly specific for the substrates. Such high substrate specificities of biological reactions may be most convenient for treatment of petroleum fractions containing a limited number of species of organosulfur compounds. Petroleum fractions contain many types of organosulfur compounds which are resistant to treatment with the chemical catalysts implemented for hydrodesulfurization (4, 19). Dibenzothiophene (DBT) and DBTs bearing alkyl substitutions are accepted as the representatives of such refractory organosulfur compounds (17). For this reason, most trials screening for desulfurizing bacteria have been carried out with DBT as the sole sulfur source to be added to the selection media. Various types of bacteria have been known to degrade DBT via carbon-carbon (C—C) bond-targeted (7, 15) or carbon-sulfur (C—S) bond-targeted (3, 8, 13, 20, 21, 23, 24) reactions. A C—S-bond-targeted reaction is preferable and ideal for desulfurization because it keeps the remaining hydrocarbon molecules fully active as energy sources without any loss of their thermal units. Extensive characterization of genes and enzymes responsible for C—S-bond-targeted DBT degradation has been performed with *Rhodococcus rhodochrous* IGTS8 (6, 16, 25). To date, all of the microorganisms which have been reported to degrade DBT in a C—S-bond-targeted fashion can do it only at mesophilic temperatures, usually near 30°C. On the other hand, distillation and hydrodesulfurization are performed at much higher temperatures in the course of the conventional refining process. If the biodesulfurization reaction, which could be applied at mild but still higher temperatures without cooling the treated petroleum fractions to normal temperature, were integrated into the refining process, it would be far more desirable. Moreover, the rate of chemical reaction is in general increased with increasing the reaction temperature.

Thermophilic degradation of DBT or desulfurization of coal by *Sulfolobus acidocaldarius*, a reduced-sulfur, iron-oxidizing bacterium, has been reported (9–11). However, the thermophilic bacterium should degrade DBT in a C—C-bond-targeted fashion because it utilizes DBT as a sole carbon source. In addition, some *Pseudomonas* strains have been reported to degrade DBT at 55°C in the same fashion, leaving a hydroxylated compound in which a sulfur atom still remains (5).

By using a screening method excluding C—C-bond-targeting bacteria, we have isolated two thermophilic bacterial strains capable of degrading DBT and several methylated DBTs even in the vicinity of 60°C. Degradation of DBT and its related compounds by these bacteria is optimal at a high temperature (around 55°C) and takes place via specific cleavages of their two C—S bonds. The thermophilic bacteria were also found to further desulfurize light gas oil which had been treated by the hydrodesulfurization process. These data suggest the applicability of thermophilic biodesulfurization to the processing of actual petroleum fractions.

MATERIALS AND METHODS

Bacterial strain. *R. rhodochrous* IGTS8 (ATCC 53968), a well-characterized desulfurizing bacterium (18) cleaving C—S bonds of DBT at mesophilic temperatures, was used as a control bacterium.

Chemicals. DBT, 2,8-dimethyl-DBT, and 2-hydroxybiphenyl (2-HBP) were of the highest quality available and were purchased from Tokyo Kasei Kogyo Co. Ltd. Three kinds of methylated DBTs (4-methyl-DBT, 4,6-dimethyl-DBT, and 3,4,6-trimethyl-DBT) were organically synthesized by and obtained from Nard

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Institute, Ltd. Their structures were confirmed by nuclear magnetic resonance spectroscopy and infrared spectroscopy. These substrates were found to be more than 94% pure by gas chromatography (GC). DBT-sulfoxide and DBT-sulfone were purchased from ICN Biomedicals, Inc., and Aldrich Chemical Co., Inc., respectively. *n*-Tetradecane was purchased from Kanto Chemical Co., Inc. Light gas oil samples processed by hydrodesulfurization and containing 800 ppm of total sulfur were supplied by a Japanese oil company.

Screening and culture conditions. Modified A medium (8) supplemented with 50 μg of yeast extract (Difco) per ml and 5 mg of glucose per ml as a carbon source was used for bacterial growth. Each of the organic sulfur compounds was dissolved in ethanol or *N*,*N*′-dimethylformamide and added to the medium as a sole sulfur source. For solid media, Bacto Agar (Difco) was added to modified A medium to a final concentration of 1.5%. Cell growth was measured by monitoring the optical density at 660 nm (OD_{660}). Calibration of the spectrophotometric response against cell dry weights was performed: one OD_{660} unit corresponds to 0.59 mg of cells (dry weight) per ml. The apparent OD_{660} of the bacterial cultures which were grown in the presence of light gas oil (one-fifth of the volume of the medium) was determined after dilution with acetone-ethanol-H_2O (1:1:1) to obtain homogeneous suspensions.

Microorganisms with the ability to utilize DBT as a sole sulfur source were isolated from soil samples collected from various areas in Japan. A small amount of each soil sample was inoculated into 5 ml of modified A medium and shaken at 50°C. After a few days, aliquots of the cultures were centrifuged and the resulting supernatants were subjected to Gibb's assay (12) to detect 2-HBP produced by microbial degradation of DBT. Single colonies of the bacteria producing 2-HBP efficiently were isolated by repeated streaking on solid agar plates. Finally, two bacterial strains were selected on the basis of their strong ability to degrade DBT at high temperatures. Taxonomical identification of the isolated bacteria was done by the National Collections of Industrial and Marine Bacteria Limited (Aberdeen, Scotland) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

Gibb's assay. Desulfurization activity was monitored by using Gibb's reagent (2,6-dichloroquinone-4-chloroimide; Sigma) as follows. After a resting-cell reaction, 150 μl of the reaction mixture was removed into a well of a microplate and mixed with 30 μl of 1 M $NaHCO_3$ (pH 8.0). Twenty microliters of Gibb's reagent (1 mg/ml in ethanol solution) was then added, and the reaction mixture was agitated at room temperature for 15 to 45 min for full color development. The absorbance of the reaction mixture was determined at 595 nm (model 450 microplate reader; Bio-Rad) and converted to parts per million based on a 2-HBP-generated standard curve.

Estimation of degradation of DBT and methylated DBTs by growing cells. DBT or its methylated compounds were dissolved in *n*-tetradecane. *Paenibacillus* sp. strain A11-1 or A11-2 cells were shaken at 50°C in modified A medium containing DBT or one of the methylated DBTs at a final concentration of 3.1 mM until the OD_{660} of the culture reached 1.15. In the course of cultivation, aliquots of the culture were removed. The bacterial cultures were centrifuged to remove cells, and the resulting supernatants were acidified to pH 2.0 by the addition of 6 N HCl followed by extraction with ethyl acetate (0.5 volume of ethyl acetate/volume of supernatant). A portion of the ethyl acetate layer was used for GC analysis. Fluorene was included as an internal standard in all experiments involving extraction with ethyl acetate. GC quantification of DBT, methyl-DBT compounds, and 2-HBP was performed by reference to standard curves plotted with a series of dilutions of these pure DBT-related compounds. Ranges of concentrations effective for quantification of DBT-related compounds were from 10 to 250 μg/ml. The coefficients of variation observed with all of the above-mentioned DBT-related compounds were 1.0 to 4.9%.

DBT, methylated DBT compounds, and their metabolites were also prepared from the *n*-tetradecane layer by a solid-phase extraction technique (22) and analyzed by GC (GC-17A chromatograph; Shimadzu), GC-mass spectrometry (MS) (Magnum apparatus; Finnigan Mat), and atomic emission spectrometry (5890II/5921A apparatus; Hewlett-Packard) (22). The molecular masses of the desulfurization products were deduced from their mass spectra. For detection of 2-HBP, a colorimetric Gibb's assay was also used. Determination of total sulfur in the *n*-tetradecane or light gas oil phase was made according to American Society for Testing and Materials method D5453 by the pyro-UV fluorescence method using a 7000S sulfur analyzer (Antek Instruments, Inc.). The lower limit of detection of total sulfur by this method was about 0.05 ppm to a precision of about ±40 ppb. Determination of sulfur content in light gas oil-cell suspensions was carried out with the supernatant oil phase obtained by centrifugation of the suspensions.

Temperature-dependent DBT degradation by growing cells. Temperature-dependent bacterial growth was examined by use of an automated temperature gradient incubation shaker (Bio-Scanner; Ohtake Co. Ltd.). Five milliliters of modified A medium supplemented with 50 μg of yeast extract per ml and DBT at a final concentration of 0.25 mM was preheated in screw-cap test tubes for 1 h to warm it to the defined incubation temperatures. One hundred microliters of the preculture of strain A11-2 was added to each of the tubes, and the tubes were shaken at defined temperatures for 48 h. Bacterial growth was estimated by measuring the turbidity at 660 nm. The cultures were acidified, and the degradation products were extracted with ethyl acetate. GC analysis of the extracts was carried out to determine the concentrations of DBT and 2-HBP by reference to the standard curves.

Resting-cell reaction. Bacteria were grown in modified A medium containing 3.1 mM DBT. Cells were harvested in the late logarithmic phase at 4°C by centrifugation at 8,000 × *g* for 5 min, washed twice with 0.1 mM potassium phosphate buffer (pH 7.0), and stored at −80°C until use. The cells were suspended in 0.1 mM potassium phosphate buffer (pH 7.0). Nine microliters of ethanol solution of DBT (54 mM) was added to 0.6 ml of the cell suspension in a 10-ml screw-cap test tube to a final concentration of 0.80 mM DBT. The reaction was allowed to proceed at 50°C or the temperatures indicated in Fig. 4 with inverted shaking in a rotating incubator at 50 rpm for 1 h. After the reaction, the bacterial suspensions were centrifuged to remove cells and the resulting supernatants were acidified to pH 2.0 by the addition of 6 N HCl followed by extraction with 0.5 volume of ethyl acetate. A portion of the ethyl acetate layer obtained from each reaction mixture was used for GC analysis.

RESULTS AND DISCUSSION

Screening of bacteria desulfurizing at high temperatures. The purpose of our screening was to find bacteria which could cleave heterocyclic organosulfur compounds refractory to the hydrodesulfurization process in a C—S-bond-targeted fashion at high temperatures. For this purpose, we used a semisynthetic medium containing DBT as a sole source of sulfur to grow and select desulfurizing bacteria. Enrichment culturing by shaking soil samples with the selection medium at 50°C for 2 or 3 days was performed four times. At the end of the enrichment culturing, several samples gave fully turbid cultures. Most of the microbial cultures were positive in Gibb's assay, which had been employed for detection of phenolic compounds, like 2-HBP (12). GC analysis with solvent extracts from these cultures showed an extensive decrease in the amount of DBT and the generation of a large peak at 3.74 min (data not shown). The corresponding reaction product was identified as 2-HBP by comparing the chromatographic patterns between it and the standard and its fluorescence under UV light and by MS analysis. In the MS analysis, the product which peaked at 3.74 min in the GC gave a signal at *m*/*z* 170 corresponding to the calculated molecular mass of 2-HBP. On the basis of the production of 2-HBP and DBT-sulfoxide by incubation of the bacterial cultures with DBT, it can be concluded that DBT degradation takes place through the cleavages of C—S bonds in the organosulfur compound.

Colony isolation of desulfurizing bacteria was carried out on agar plates containing the selection medium. In extensive morphological and physiological tests, the two bacteria showed properties similar to ones belonging to the genus *Bacillus*. As a result of sequence analysis of the 16S rRNA genes of these bacteria, it was confirmed that both of them were paenibacilli. The genus *Paenibacillus* has been established by reclassification of the bacteria phylogenetically removed from *Bacillus* groups (1, 2). The partial sequences of the 16S rRNA genes of the two desulfurizing bacteria showed a maximum of 91 to 92% homology with those of other paenibacilli. In addition, there is no member having the same microbiological properties as those of our bacterial strains among the known paenibacilli. Therefore, these desulfurizing paenibacilli should belong to new species of *Paenibacillus. Bacillus* groups contain a few species of thermophilic bacteria. Among them, *Bacillus stearothermophilus* and *Bacillus caldolyticus* are the representatives. However, no sufficient homology in the sequences of the 16S rRNA genes between them and the desulfurizing paenibacilli was found.

Thermophilic biodesulfurization of DBT. Figure 1 shows the time course of desulfurization of DBT at 50°C by *Paenibacillus* sp. strain A11-2. Production of 2-HBP appeared to proceed in parallel with the increase in biomass. However, the relationship between the decrease in DBT recovered and the increase in 2-HBP produced does not seem stoichiometric. This may be caused by a time lag between the DBT uptake by the bacterial

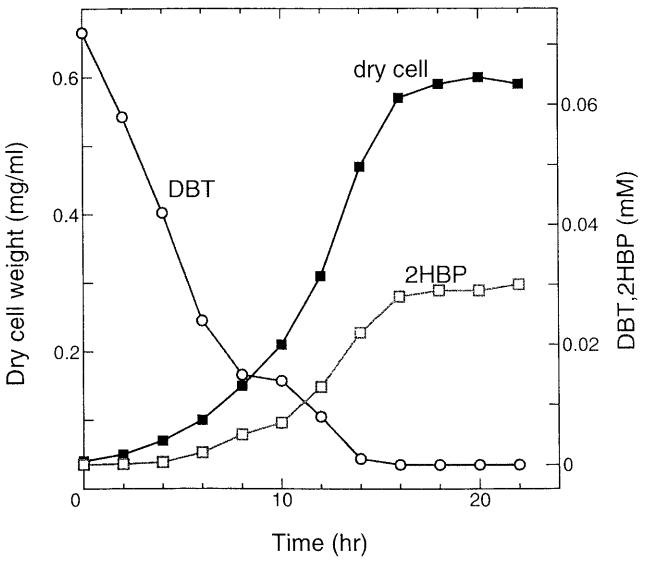

FIG. 1. Time course of DBT degradation by growing cells of *Paenibacillus* sp. strain A11-2 at a high temperature. *Paenibacillus* sp. strain A11-2 was grown at 50°C, and ethyl acetate extracts were analyzed by GC as described in Materials and Methods. Quantities of starting material (DBT) and desulfurized end product (2-HBP) are shown with cell dry weight. The data for intermediates are not presented. Data are the mean results of the analysis of duplicate samples. The average standard deviation for all data points was 5% or less.

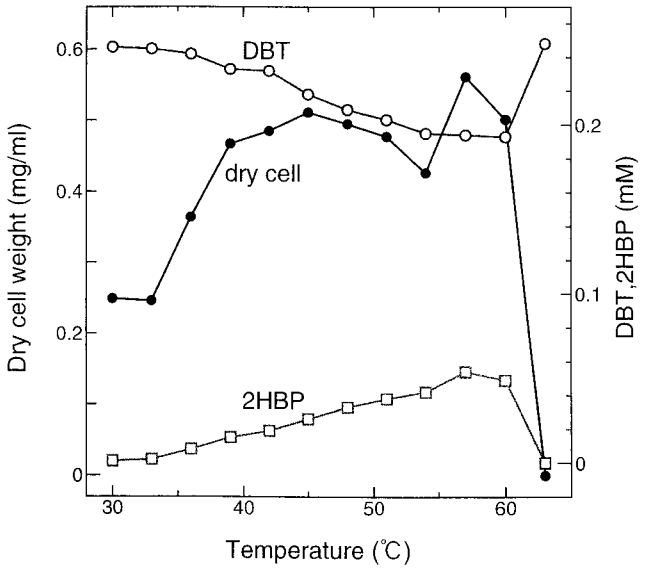

FIG. 2. Temperature dependence of DBT degradation by growing cells of *Paenibacillus* sp. strain A11-2. Changes in both the amounts of 2-HBP produced in the culture and DBT recovered from the culture are indicated, with cell dry weight as a function of incubation temperature. The results are for the final concentration of 2-HBP, the product, at the incubation temperature indicated. Production of 2-HBP was directly related to the biomass of culture produced. Duplicates agreed to less than 5% of the mean. One OD_{660} unit of the culture of *Paenibacillus* sp. strain A11-2 corresponds to 0.59 mg (dry weight) of cells per ml.

cells and the release of 2-HBP produced as well as adsorption of DBT onto them. We found significant levels of adsorption of DBT to the bacterial cells upon incubation in control experiments.

Temperature-dependent desulfurization by the paenibacilli isolated was examined by culturing them at 30 to 63°C (Fig. 2). Both *Paenibacillus* sp. strains A11-1 and A11-2 grew significantly in the medium containing DBT as the sole sulfur source at a wide range of temperatures, from 30 to 60°C. Their growth was clearly temperature dependent and seemed to reach a maximum at temperatures near 58°C. We observed that the rate of growth of the paenibacilli was lower at 54°C than at lower and higher temperatures; the reasons have never been clear, though. On the other hand, virtually no growth of these paenibacilli was observed at 63°C. Following a similar pattern, the amounts of 2-HBP produced by *Paenibacillus* sp. strain A11-2 cells were increased with increasing culturing temperatures (Fig. 2). The maximal specific desulfurizing activity (the amount of 2-HBP generated per milligram [dry weight] of cells) was observed at 54°C with this *Paenibacillus* strain. Practically no desulfurizing activity with these paenibacilli was detected at temperatures over 63°C due to inhibition of bacterial growth. It should be noted that the apparent specific desulfurizing activity of *Paenibacillus* sp. strain A11-2 was still high at 60°C, and significant amounts of DBT still remained in the culture. This suggests that desulfurizing enzyme activities of the *Paenibacillus* strains might be able to be shown at much higher temperatures. The increase in temperatures from 33 to 42°C caused a rapid increase in biomass but affected the desulfurization reaction insignificantly. This may be ascribed in part to weak desulfurizing activities of the bacterial enzymes at such low temperatures. In the examinations of bacterial growth and desulfurization, we performed control experiments with bacterial cells incubated in the presence of ethanol or *n*-tetradecane but without DBT in duplicate flasks. In all these cases, no practical increase in biomass was observed in the absence of DBT at any temperature examined.

C—S-bond-specific cleavages of methylated DBTs. Petroleum contains alkylated DBT derivatives which are much more refractory to hydrodesulfurization than DBT itself. To determine the capability of the bacteria to degrade refractory DBT derivatives through cleavage of C—S bonds and removal of sulfur, monomethyl-, dimethyl-, and trimethyl-DBT were chemically synthesized starting with DBT (for monomethyl-DBT and dimethyl-DBT) or dimethyl-DBT (for trimethyl-DBT). Two kinds of dimethyl-DBT (2,8-dimethyl-DBT and 4,6-dimethyl-DBT) which differed from each other in the positions of the two methyl groups attached to the DBT moiety were used as the substrates. To demonstrate the capability of the bacteria to degrade the methylated DBT molecules in the presence of hydrocarbons which are comprised in actual petroleum, they were dissolved in *n*-tetradecane and added to the growth medium. When either of the DBT-degrading paenibacilli was shaken in medium containing any of the methylated DBTs as the sole sulfur source at 50°C, it grew well in all cases. Specific degradation of the methylated DBT molecules by the paenibacilli was further investigated through a series of analyses of the solvent extracts prepared from the oil phases of the bacterial cultures. Quantitative analysis of total sulfur content demonstrated the extensive decreases (26.0% for 4-methyl-DBT, 25.5% for 4,6-dimethyl-DBT, 23.5% for 2,8-dimethyl-DBT, and 29.5% for 3,4,6-trimethyl-DBT) in the amounts of sulfur in the oil phase.

GC-atomic emission spectrometry of the degradation products showed that all of the methylated DBTs examined had lost their sulfur atoms (data not shown). GC and GC-MS also provided data suggesting that the metabolites of these methylated DBTs were desulfurized. Figure 3 shows the gas chromatogram and the mass spectrum of the metabolite of 4,6-

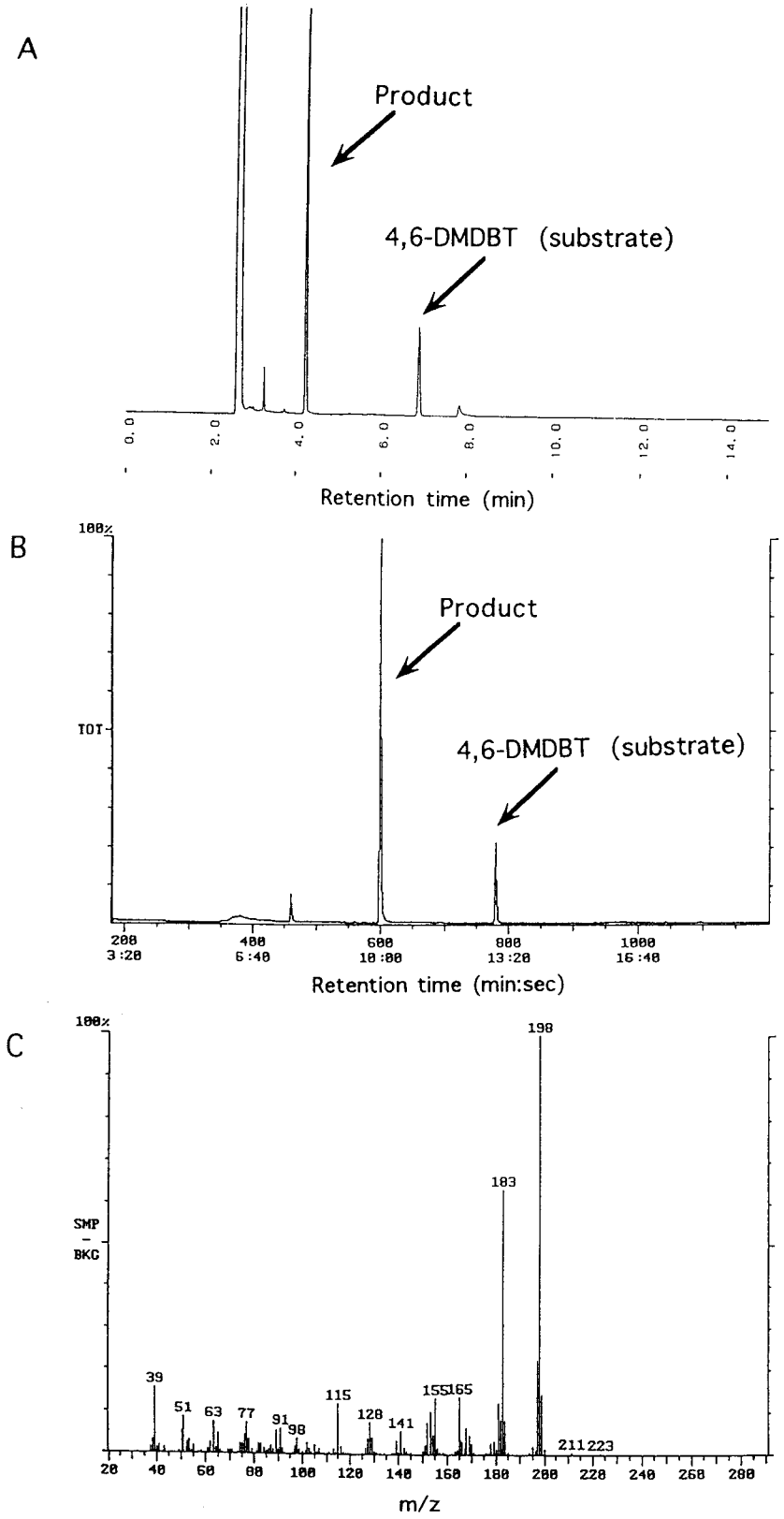

FIG. 3. Analysis of the bacterial metabolites of 4,6-dimethyl-DBT. (A) Gas chromatogram of the degradation products of 4,6-dimethyl-DBT. (B) GC-MS chromatogram of the degradation products of 4,6-dimethyl-DBT (DMDBT). (C) Mass spectrum of the main peak in panel B.

dimethyl-DBT which was produced by *Paenibacillus* sp. strain A11-2 at 50°C. Mass ions at *m/z* 198 corresponding to the molecular mass of monohydroxyl dimethyl biphenyl were detected. With the metabolites from 4-methyl-DBT, 2,8-dimethyl-DBT, and 3,4,6-trimethyl-DBT, main ion peaks were detected at *m/z* 184, 198, and 212, respectively. These mass ions correspond to the molecular masses of monohydroxyl methyl biphenyl, monohydroxyl dimethyl biphenyl, and monohydroxyl trimethyl biphenyl, respectively. When the bacteria were shaken in medium lacking any sulfur source at 50°C as a control, neither bacterial growth nor a decrease in sulfur content was found in any experiment. In conclusion, the results presented suggest that the thermophile can degrade various types of methylated DBT compounds as well as DBT itself in a C—S-bond-targeted fashion.

Light gas oil is known to contain small amounts of sulfur and limited species of heterocyclic organosulfur compounds composed mainly of alkylated DBT derivatives (19). When *Paenibacillus* sp. strains A11-1 and A11-2 were cultured in the presence of light gas oil containing 800 ppm of total sulfur at a high temperature, both strains grew to apparent OD_{660}s of 1.0. In contrast to this, no increase in the OD_{660} of the culturing medium was observed when light gas oil was omitted from it. In conformity with the stimulated bacterial growth, the content of sulfur in the oil phase was significantly decreased (7 to 11%), indicating that both *Paenibacillus* strains can desulfurize at high temperatures from the processed light gas oil. The bacterial cells may utilize sulfur released from these compounds for their growth.

Resting-cell reaction. Biotransformations of organic compounds with growing cultures has the advantage that the growth of bacterial cells can be a reliable indicator of their utilization of the chemicals as their essential nutritional components. However, such biotransformations by growing cells are usually conducted in complex growth media which can interfere with analysis of products owing to the presence of a complicated mixture of other metabolites produced by the biocatalysts and the medium components. To avoid these problems, we used resting cells of paenibacilli for quantitative analysis of their specific desulfurizing activities.

The resting cells of the paenibacilli also were able to desulfurize DBT to produce 2-HBP at a broad range of temperatures (37 to 60°C) (Fig. 4), similar to what occurred with growing cells (Fig. 2). Maximal desulfurization was observed at a temperature near 55°C. In these experiments, the suspensions of the resting cells were preheated at the actual reaction temperature prior to the addition of the substrate DBT. Therefore, one can exclude the possibility that DBT degradation might have transiently proceeded during heating to the reaction temperatures. The resting-cell reaction without an addition of DBT to the reaction mixture did not produce 2-HBP at all. This confirms that desulfurization did take place during the reaction and that no detectable carryover of 2-HBP previously produced by the cells grown in the presence of DBT was in the reaction mixture.

In addition, we examined the temperature dependence of desulfurization by the control bacterial strain *R. rhodochrous* IGTS8, which is known to degrade DBT efficiently through a C—S-bond-targeted reaction at mesophilic temperatures (12, 14). As shown in Fig. 4, desulfurization with *R. rhodochrous* IGTS8 reached a maximum at 30°C and no significant production of 2-HBP occurred at 50°C or higher. The relative desulfurizing activity was appreciably higher than that exhibited by the *Paenibacillus* strains. These results strongly suggest that bacteria growing efficiently and degrading DBT in a C—S-bond-targeted fashion at mesophilic temperatures, and even their resting cells, cannot perform significant desulfurization at temperatures over 50°C.

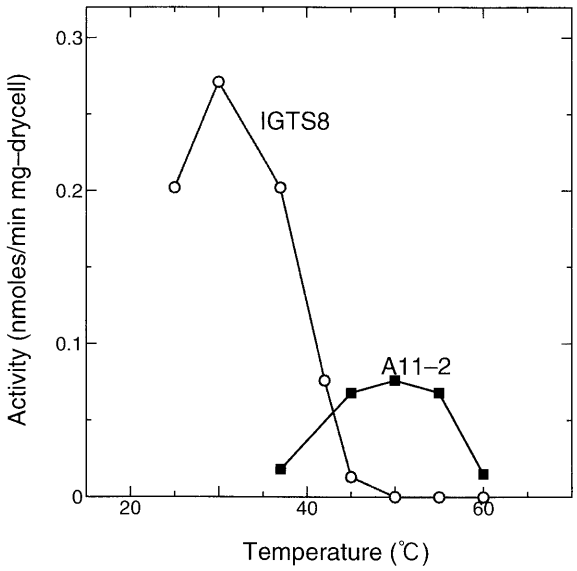

FIG. 4. Temperature dependent resting-cell reaction with cultures of *Paenibacillus* sp. strain A11-2 and *R. rhodochrous* ATCC 53968 (IGTS8). Specific activities of the resting cells of IGTS8 and A11-2 at the temperatures indicated are shown. Duplicates agreed to less than 5% of the mean.

The results presented in this paper represent the first isolation of microbes desulfurizing thermophilically in a C—S-bond-targeted fashion and demonstrate their applicability to an actual petroleum fraction. One promising application of the thermophilic biodesulfurization would be deeper desulfurization of petroleum fractions, like the light gas oil examined in this research, which contained highly refractory organosulfur.

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