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Deep Desulfurization of Diesel Oil and Crude Oils by a Newly Isolated *Rhodococcus erythropolis* Strain

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The soil-isolated strain XP was identified as *Rhodococcus erythropolis*. *R. erythropolis* XP could efficiently desulfurize benzonaphthothiophene, a complicated model sulfur compound that exists in crude oil. The desulfurization product of benzonaphthothiophene was identified as α-hydroxy-β-phenyl-naphthalene. Resting cells could desulfurize diesel oil (total organic sulfur, 259 ppm) after hydrodesulfurization. The sulfur content of diesel oil was reduced by 94.5% by using the resting cell biocatalyst for 24 h at 30°C. Biodesulfurization of crude oils was also investigated. After 72 h of treatment at 30°C, 62.3% of the total sulfur content in Fushun crude oil (initial total sulfur content, 3,210 ppm) and 47.2% of that in Sudanese crude oil (initial total sulfur, 1,237 ppm) were removed. Gas chromatography with pulsed-flame photometric detector analysis was used to evaluate the effect of *R. erythropolis* XP treatment on the sulfur content in Fushun crude oil, and it was shown that most organic sulfur compounds were eliminated after biodesulfurization.

Sulfur oxides released from fossil fuel combustion contribute to acid rain and air pollution (6, 22). The increasingly stringent limitations on SO_x emission are imposing an urgent requirement for refineries to reduce the sulfur content in the fuels they produce. However, a common problem facing refineries around the world is that crude oils are becoming heavier, with higher sulfur contents, which results in higher sulfur levels in both straight-run and secondarily processed diesel oil. Therefore, it is preferable to reduce the sulfur content of crude oil before the oil is refined.

The sulfur-containing compounds in crude oil include sulfides, thiols, thiophenes, substituted benzo- and dibenzothiophenes (BTs and DBTs), benzonaphthothiophene (BNT), and many considerably more complex molecules (21). The condensed thiophenes are the most common forms in which sulfur is present (28). Alkyl DBTs and alkyl BTs are highly recalcitrant to chemical catalysts, especially when alkylated at the 4 and 6 positions (9, 14, 20). Many research efforts have been put into the investigation of biological desulfurization systems using DBTs or alkylated DBTs as model compounds, and the metabolic pathway of desulfurization has been proposed as the so-called "4S" or extended "4SM" pathway (5, 7, 12, 16, 18). Desulfurization of crude oil is also a target for biodesulfurization, but few results have been published (7). Oil refineries usually separate crude oil into several fractions and then desulfurize them separately. Capital savings can be made if most of the sulfur is removed from the crude oil before it is fractionated. Furthermore, the high content of water in crude oil makes biodesulfurization of crude oil more practicable than that of diesel oil and gasoline (30).

In this paper, a newly isolated DBT-desulfurizing mesophilic bacterium, *Rhodococcus erythropolis* XP, is shown to grow in the presence of various DBTs, BNT, and 3-methyl-BT (3-M-BT). The biotransformation of diesel oil and crude oils by resting cells of *R. erythropolis* XP was also evaluated.

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MATERIALS AND METHODS

Bacterial strain and cultivation. Basal salts medium (BSM) supplemented with a 0.5 mM concentration of various organic sulfur compounds as the sole sulfur source was used as a culture medium. BSM contained the following reagents per liter: 4 g glycerol, 2.44 g KH_2PO_4, 14.04 g Na_2HPO_4, 2 g NH_4Cl, 2 ml 10% (vol/vol) $MgCl_2$, 100 μl 1% $FeCl_3$, 100 μl 1% $CaCl_2$, 200 μl vitamin mixture, and 5 ml metal solution (16). A bacterium designated XP was isolated by enrichment culture on BSM supplemented with 0.5 mM DBT. Cultivation was performed at 30°C in BSM supplemented with 0.5 mM DBT, with rotation at 200 rpm, for 42 h. 2-Hydroxybiphenyl (2-HBP) and its derivatives were detected by the Gibbs assay (15) as follows. A 1.0-ml aliquot of the supernatant was transferred to a 10-ml tube containing 200 μl of 1 M $NaHCO_3$ (pH 8.0) and Gibbs reagent (20 μl; 1 mg/ml in ethanol solution). The reaction mixture was agitated at 30°C for 30 min to allow the formation of a blue complex by the Gibbs reagent and aromatic hydroxyl groups such as 2-HBP. The strain was identified by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Strain XP was deposited at the China Center for Type Culture Collection (CCTCC M 205068).

Chemicals. 3-M-BT was purchased from Lancaster Synthesis, United Kingdom. 4-Methyl-DBT (4-M-DBT) was obtained from ACROS. DBT, 4,6-dimethyl-DBT (4,6-DM-DBT), BNT, and 2-HBP were of the highest quality available and were purchased from Sigma-Aldrich. Fushun crude oil was obtained from Fushun Research Institute of Petroleum and Petrochemicals, SINOPEC, China. Sudanese crude oil was imported from the Republic of the Sudan and obtained from Karamay Petroleum and Petro-Chemicals Corporation, Karamay, China. All other materials were of the highest purity commercially available.

Biotreatment of oils with strain XP. Cells were harvested in the mid-exponential phase of growth and washed twice with 100 mM of potassium phosphate buffer (pH 7.0). The cells were suspended in the same buffer supplemented with 2% glucose as the energy source. Desulfurization of diesel oil and crude oil proceeded as follows. Five milliliters of diesel oil was added to 45 ml of cell suspension at 8 g dry cell weight per liter for 24 h in a 300-ml flask; 3 g crude oil was added to 60 ml of cell suspension at 16 g dry cell weight per liter for 72 h. The oil was separated by centrifugation at 8,000 × *g* for 10 min. Control oils were samples containing oil without bacterial amendment.

Analytical procedures. The molecular structures of the metabolites were analyzed using gas chromatography-mass spectrometry (GC-MS) with an instrument (GCD 1800C; Hewlett-Packard) equipped with a 50-m DB-5MS column (J&W Scientific, Folsom, CA).

Analyses of diesel oil were performed by GC with flame ionization detection

(GC-FID) and GC with atomic emission detection (GC-AED). Hydrocarbon-containing compounds were detected by the FID instrument. The distribution of organic sulfur-containing compounds was determined by the AED instrument (Agilent G2350A; Hewlett-Packard). The total sulfur content of crude oil was determined in triplicate for each sample, using a model 7000 NS ANTEK sulfur analyzer (ANTEK, TX). GC with a pulsed-flame photometric detector (GC-PFPD) (Varian Associates) was performed on 0.2 μl of sample by using a DB-5MS column. The temperature zones for GC were as follows: injector and detector temperature, 280°C; and initial oven temperature, 50°C for 1 min, followed by a 10°C/min temperature ramp to 280°C for a final 10-min hold.

RESULTS

Identification of DBT-desulfurizing bacterium XP. Strain XP was previously identified as a *Rhodococcus* species. Further identification of strain XP was performed by DSMZ.

Strain XP is an aerobic, gram-positive bacterium. The cells were shown to be short branched hyphae, which disintegrated into rod- and coccus-like elements. Colonies were shiny, dissolving, and cream-colored to pink. The mycolic acid pattern was compared with the pattern stored in the *Rhodococcus* mycolic acid database. The pattern of XP showed a high similarity to *R. erythropolis*. The fatty acid pattern revealed unbranched saturated and unsaturated fatty acids plus tuberculostearic acid. Fatty acid composition analysis (MIDI software) identified a high similarity (0.865) to *R. erythropolis*. The riboprint pattern of XP was similar to that of *R. erythropolis* DSM 43066^T. Sequence analysis of the 16S rRNA gene revealed 100% similarity to *R. erythropolis* DSM 43066^T in the diagnostic regions (nucleotides 1 to 500; GenBank accession number DQ074453). Based on conventional markers (morphology), chemotaxonomic markers (fatty acid and mycolic acid patterns), and molecular biological results (riboprint patterns and comparative 16S rRNA gene sequencing), it was concluded that strain XP belongs to the species *R. erythropolis*.

Biodesulfurization of heterocyclic sulfur compounds. When strain XP was cultured in BSM containing various compounds as the sole sulfur source, it grew well with DBT, 4-M-DBT, 4,6-DM-DBT, BNT, and 3-M-BT. Each sulfur compound was added as an *N*,*N*′-dimethylformamide (DMF) solution to a final concentration of 0.5 mM. All of the liquid cultures produced a blue result in the Gibbs assay (15), indicating the presence of aromatic hydroxyl groups (data not shown).

The end product of BNT metabolism was determined to be α-hydroxy-β-phenyl-naphthalene (Fig. 1). Moreover, 2-hydroxybiphenyl (2-HBP) was detected as a metabolite of DBT, 2-hydoxy-3′-methyl-biphenyl and 2-hydroxy-3-methyl-biphenyl were detected as metabolites of 4-M-DBT, and 2-hydroxy-3,3′-dimethyl-biphenyl was confirmed as a metabolite of 4,6-DM-DBT. 3-M-BT sulfone and 2-isopropenylphenol were the two main metabolites of 3-M-BT (data not shown). Therefore, *R. erythropolis* XP converted DBT and its derivatives via a traditional "4S" pathway, similar to other biodesulfurizing organisms (11, 23).

Deep desulfurization of hydrodesulfurized diesel oil. The feasibility of diesel oil desulfurization was investigated using hydrodesulfurization (HDS)-treated diesel oil (total organic sulfur, 259 ppm) obtained from the Institute of Fushun Petroleum and Petro-chemicals. A GC-AED chromatogram of the diesel oil samples treated by resting cells revealed extensive depletion of sulfur compounds across the entire boiling range of the oil (Fig. 2). Sulfur compounds with longer retention

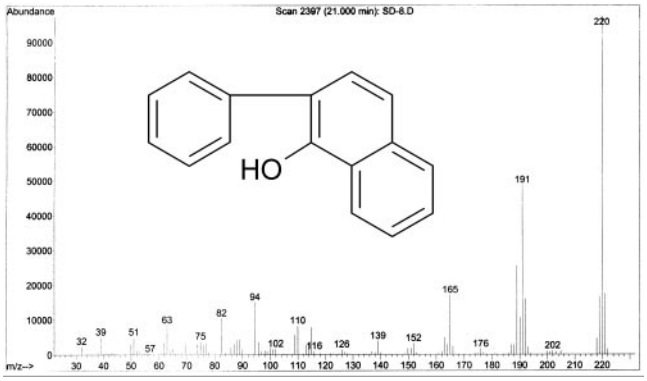

FIG. 1. MS spectrum of benzonaphthothiophene metabolite. The metabolite was identified as α-hydroxy-β-phenyl-naphthalene (molecular weight, 220). The spectrum corresponds to the peak detected at a retention time of 21.00 min.

times than DBT were also desulfurized. A GC-FID chromatogram of the diesel oil samples from the reaction systems showed little change in the resolvable peaks (data not shown), demonstrating that the alkane value of the diesel oil was not lost during this bioprocess. The total organic sulfur content of the diesel oil after treatment by resting cells of *R. erythropolis* XP was 14 ppm, corresponding to a reduction of 94.5%.

Biodesulfurization of crude oils by *R. erythropolis* XP. *R. erythropolis* XP was also capable of removing sulfur compounds from Fushun crude oil and Sudanese crude oil. After treatment by resting cells of XP, about 62.3% of the total sulfur in Fushun crude oil and 47.2% in Sudanese crude oil were removed. The total sulfur content of Fushun crude oil was decreased from 3,210 ppm to 1,210 ppm and that of Sudanese crude oil was decreased from 1,237 ppm to 653 ppm. GC-PFPD was also used to evaluate the sulfur removal from Fushun crude oil. As shown in Fig. 3, almost all sulfur compounds in the detectable level were removed.

DISCUSSION

Biodesulfurization of DBT and its derivatives by DBT-desulfurizing bacteria has been reported in many publications. Almost all of the bacteria reported could degrade DBT to 2-HBP or its derivatives through a sulfur-specific pathway. Li et al. reported that *Mycobacterium* sp. strain X7B, a facultative thermophilic bacterium, could further convert 2-HBP to 2-methoxybiphenyl, and the initial steps of this metabolism were similar to those in other bacteria (16). Using [^{35}S]DBT radiolabeling, Oldfield et al. confirmed that sulfur was released from DBT in the form of inorganic sulfite (25), which was then oxidized to sulfate (22). The fate of the sulfur atom in DBT was also investigated in this study. Resting cells of strain XP were employed to treat 0.5 mM DBT, and then the liquid culture was centrifuged. White precipitation was detected in the centrifuged supernatant upon the addition of $BaCl_2$ in HNO_3 solution (18), suggesting that sulfate had also been formed.

From the standpoint of practical biodesulfurization, special attention should be paid to what is left in the oil after chemical desulfurization. Among these refractory organic sulfur compounds are DBT derivatives, such as alkyl-substituted DBTs

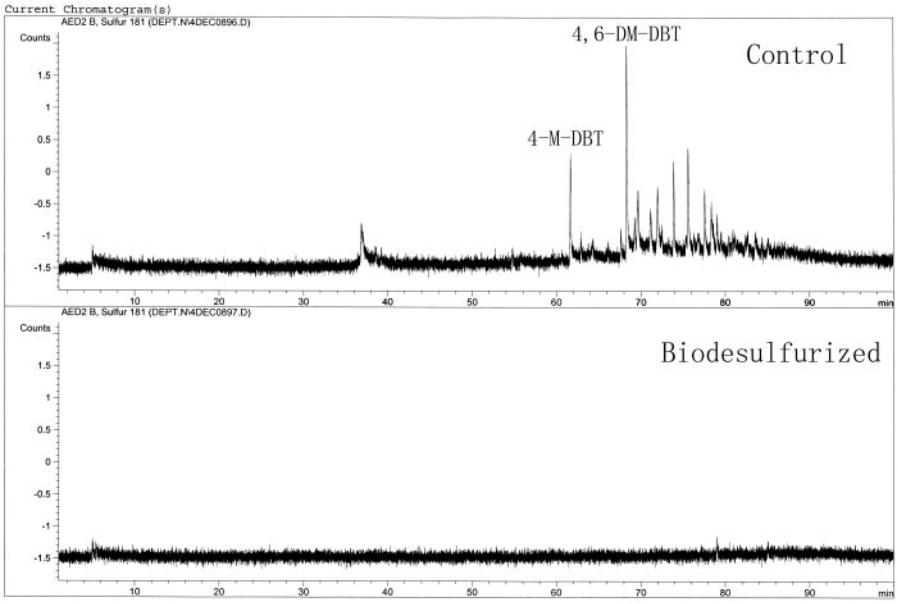

FIG. 2. GC-AED chromatograms of control and *R. erythropolis* XP-desulfurized diesel oils. The 4-M-DBT and 4,6-DM-DBT peaks are indicated in the figure. The oil-phase-to-aqueous-phase ratio was 1:9 (vol/vol). A resting cell suspension (8 g dry cells liter^{-1}) served as the aqueous phase. The control sample was an oil sample without bacterial amendment and operated under the same conditions as the desulfurized sample.

and BTs remaining in HDS-treated diesel oil, which are harder to remove than DBT by conventional HDS treatment (4, 13, 24). Strain XP could efficiently desulfurize DBT and alkyl-substituted DBTs in diesel oil, resulting in a low residue of sulfur content (14 ppm), with a 94.5% reduction of total sulfur. Comparable sulfur reductions have been reported previously for the desulfurization of diesel oils by a *Gordona* sp. using the sulfur-specific pathway at 30°C: the total sulfur content of a middle-distillate unit feed (MDUF) decreased from 0.15% (wt/wt) to 0.06% and that of a light gas oil decreased from

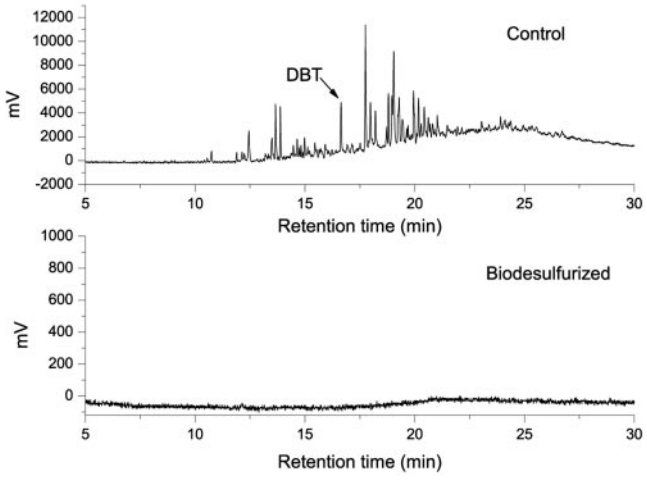

FIG. 3. GC-PFPD chromatograms of the sulfur content in Fushun crude oil. The biodesulfurized sample was treated with resting cells of XP, and the control sample was an oil sample without bacterial amendment. The sample and the control were treated under the same conditions. The DBT peak is indicated in the figure. The oil-phase-to-aqueous-phase ratio was 1:20 (wt/vol). A resting cell suspension (16 g dry cells liter^{-1}) served as the aqueous phase.

0.3% (wt/wt) to 0.15%, and the specific desulfurization rates for MDUF and light gas oil were 0.17 and 0.15 mg sulfur g dry cell weight^{-1} h^{-1} (26), respectively. When diesel oil was treated with *Nocardia* sp. strain CYKS2, the sulfur content decreased from 0.3 to 0.24% (wt/wt) in 48 h (1). *R. erythropolis* I-19, a genetically engineered bacterium, reduced the sulfur in oxidized MDUF from 1,850 to 615 ppm, a 67% reduction, and the specific rate of MDUF biotransformation was 0.15 mg sulfur g dry cell weight^{-1} h^{-1} (4). As much as 52% of the sulfur content was removed from a 12-fold-diluted crude straight-run light gas oil fraction by growing cells of *Mycobacterium phlei* WU-0103, and the sulfur concentration was reduced from 1,000 to 475 ppm (8). It should be noted that *R. erythropolis* XP could reduce 94.5% of the sulfur content in hydrodesulfurized diesel oil, in which most of the easily removed sulfur compounds had been eliminated, with only refractory sulfur compounds such as 4-M-DBT and 4,6-DM-DBT (Fig. 2) remaining. These compounds are generally refractory to HDS, with reactivities >10 times lower than that of DBT (13). A comparative specific biodesulfurization rate (0.12 mg sulfur g dry cell weight^{-1} h^{-1}) was also achieved in this study.

Comparative experiments were carried out to provide information relevant to the biodesulfurizing trait of strain XP. Three biodesulfurizing bacteria (*Rhodococcus erythropolis* XP, *Rhodococcus* sp. strain SDUZAWQ [19], and *Mycobacterium goodii* X7B [17]) isolated by our group were compared. From a GC-AED analysis of the control sample as well as the desulfurized diesel oil samples, the relative biodesulfurization rates for XP, SDUZAWQ, and X7B were 100%, 64%, and 87%, respectively. Furthermore, the remaining highly alkylated derivatives of DBT in diesel oil samples were also compared (Table 1). The degradation rates of monoalkylated DBTs were similar, and the more alkyl carbons were present on the DBT ring, the more refractory the compound was to

TABLE 1. Comparative data for XP, SDUZAWQ, and X7B treatments of diesel oil[a]

Sulfur compounds[c]	Amt of sulfur compound in diesel oil (ng/μl) with indicated treatment[b]			
	Control	XP	SDUZAWQ	X7B
C1DBTs	28.7	1.0	2.7	1.3
C2DBTs	94.8	3.6	36.7	5.7
C3DBTs	100.7	4.4	57.3	22.9
C4 + C5DBTs	54.3	34.9	54.7	42.6

[a] All experiments were carried out according to the details in Materials and Methods, except that the reaction temperature for X7B was 45°C.
[b] Detected by gas chromatography with an atomic emission detector.
[c] C1DBTs, methyl-DBTs; C2DBTs, dimethyl-DBTs; C3DBTs, trimethyl-DBTs.

biodegradation. Strain XP has an excellent ability to remove highly alkylated DBTs from diesel oil and could reduce 95.6% of trimethyl-DBTs. In contrast, only 43.1% and 77.3% reductions were achieved by strains SDUZAWQ and X7B, respectively, under the same conditions (Table 1). More heavily substituted DBT derivatives containing four or five alkyl carbons on the DBT ring showed as much as 35.7% reduction by strain XP, while no degradation activity was apparent with strain SDUZAWQ. *Rhodococcus erythropolis* XP was superior to the mesophilic *Rhodococcus* sp. strain SDUZAWQ. The gene clusters responsible for biodesulfurization in the two *Rhodococcus* strains were also investigated. The gene cluster sequence of XP (GenBank accession number AY278323) showed high similarity (>99%) with that of SDUZAWQ (GenBank accession number AY789136). Therefore, extensive desulfurization may not rely on gene differences. The more complicated sulfur compound BNT was also used as a model to demonstrate desulfurization ability. The specific degradation rate by XP was 5.84 μM BNT g dry cell weight^{-1} h^{-1}, while only 5.43 μM BNT g dry cell weight^{-1} h^{-1} was degraded by SDUZAWQ. The ability of strain XP to efficiently remove highly alkylated sulfur compounds may contribute to its deep biodesulfurization of diesel oil (Table 1).

Biodesulfurization of crude oil is also a target for sulfur removal. Setti et al. reported using an *n*-alkane-degrading *Pseudomonas* sp. strain to degrade the sulfur compounds found in a deasphaltenated heavy oil (27). Fedorak et al. used mixed microbial populations to degrade sulfur heterocycles in Prudhoe Bay crude oil (2, 3). The wild-type strain *Rhodococcus* sp. strain IGTS8 was also investigated for biodesulfurization of crude oil. While analyses of the crude oil before and after biodesulfurization by IGTS8 did not reveal a decrease in total sulfur, it was indicated that IGTS8 may be capable of biodesulfurization of refined products such as gasoline and diesel oil, but not crude oil (10). In this study, a pure bacterium capable of degrading DBT via a sulfur-specific pathway was adopted to selectively desulfurize crude oils. Significant decreases in the sulfur contents of crude oils (62% and 47%) were obtained with *R. erythropolis* XP. This may be due to the excellent ability of strain XP to transform more complicated sulfur compounds.

Biotreatments of petroleum, especially biodesulfurization of derived fractions of petroleum such as diesel oil, light gas oil, and gasoline, all face the same problem, that is, the ratio of water to oil is high, which will significantly increase the reaction volume and make practical operation less advisable (1, 8, 16, 17). In addition, techniques such as water flooding are used to increase oil recovery in the oil fields. Up to 87% of Chinese oil fields are flooded (29), and therefore a high content of water exists in the recovered crude oil. In old Chinese oil fields, there is >85% water in the recovered crude oil (30). It would be more practical to incubate microorganisms in this water-oil system before the water is removed. *R. erythropolis* XP metabolized a broad range of organic sulfur compounds in crude oils, suggesting its potential application to the desulfurization of fossil fuels.

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