

Identification and Cloning of Genes Involved in Specific Desulfurization of Dibenzothiophene by *Rhodococcus* sp. Strain IGTS8

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The gram-positive bacterium *Rhodococcus* sp. strain IGTS8 is able to remove sulfur from certain aromatic compounds without breaking carbon-carbon bonds. In particular, sulfur is removed from dibenzothiophene (DBT) to give the final product, 2-hydroxybiphenyl. A genomic library of IGTS8 was constructed in the cosmid vector pLAFR5, but no desulfurization phenotype was imparted to *Escherichia coli*. Therefore, IGTS8 was mutagenized, and a new strain (UV1) was selected that had lost the ability to desulfurize DBT. The genomic library was transferred into UV1, and several colonies that had regained the desulfurization phenotype were isolated, though free plasmid could not be isolated. Instead, vector DNA had integrated into either the chromosome or a large resident plasmid. DNA on either side of the inserted vector sequences was cloned and used to probe the original genomic library in *E. coli*. This procedure identified individual cosmid clones that, when electroporated into strain UV1, restored desulfurization. When the origin of replication from a *Rhodococcus* plasmid was inserted, the efficiency with which these clones transformed UV1 increased 20- to 50-fold and they could be retrieved as free plasmids. Restriction mapping and subcloning indicated that the desulfurization genes reside on a 4.0-kb DNA fragment. Finally, the phenotype was transferred to *Rhodococcus fascians* D188-5, a species normally incapable of desulfurizing DBT. The mutant strain, UV1, and *R. fascians* produced 2-hydroxybiphenyl from DBT when they contained appropriate clones, indicating that the genes for the entire pathway have been isolated.

The presence of sulfur in coal and petroleum contributes to corrosion of production and refining equipment, and burning these high-sulfur products emits sulfur oxides to the atmosphere (12). Inorganic sulfur in these fuels can be removed by physical or microbiological treatments (2, 12, 27), but sulfur that is bound covalently to the organic components of coal or petroleum cannot be removed easily. Several attempts have been made to find microorganisms that can enzymatically release organically bound sulfur from these substrates or from dibenzothiophene (DBT), a compound that models the bulk of organic sulfur in these mixtures. Such a reaction could conceivably reduce the sulfur content of high-sulfur fuels without depleting their British thermal unit value.

Two major pathways for the microbial metabolism of DBT have been proposed. Kodama et al. (28, 29) identified intermediates that led them to conclude that DBT is metabolized by a series of oxidations analogous to those by which naphthalene is degraded. In this pathway, dioxygenation of one of the aromatic rings of DBT leads to degradation of the ring without releasing sulfur, to yield the final product, 3-hydroxy-2-formyl-benzothiophene (29). Several bacteria that partially degrade DBT by this route have been isolated (12, 38). The second pathway is one in which the sulfur is oxidized first and then removed from DBT with or without subsequent degradation of its two aromatic rings. Holland et al. (16) identified the first oxidation steps of this pathway in a group of fungi, and Crawford and Gupta (4) showed that

Cunninghamella elegans also oxidizes the sulfur moiety to give DBT-5-oxide (DBT-sulfoxide) and DBT-5-dioxide (DBT-sulfone). In these two cases, no additional degradation products were observed. On the other hand, van Afferden et al. (36) isolated a *Brevibacterium* species that oxidizes the sulfur in DBT but continues to degrade these intermediates to sulfite and benzoate, which is itself degraded.

A very specific version of this second pathway has been described by Kilbane et al. in the gram-positive soil isolate *Rhodococcus* sp. strain IGTS8 (22, 23). In this case, sulfur is extracted from DBT without further degradation of the aromatic carbon structure to yield 2-hydroxybiphenyl (2-HBP) as a final product (20, 22, 23, 32). This reaction is potentially useful for removing the sulfur from compounds in petroleum and/or coal without decreasing their calorific value (2, 12). In fact, Kilbane and Jackowski have shown that incubation of IGTS8 with water-soluble coal-derived material for as little as 24 h removes 30 to 40% of the organic sulfur from that complex substrate while retaining the British thermal unit content (24). Kim et al. (25) have described an anaerobe, *Desulfovibrio desulfuricans* M6, that may also desulfurize DBT by this second type of pathway.

Some of the genes for the DBT degradative pathway have been isolated and characterized (5, 11, 13). However, no genes for the desulfurization pathway have been identified. We now report the isolation from *Rhodococcus* sp. strain IGTS8 of a set of genes that confer a specific desulfurization phenotype to mutants of IGTS8 and to a related organism, *Rhodococcus fascians* D188-5, that is normally unable to desulfurize DBT.

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MATERIALS AND METHODS

Bacterial strains, media, and reagents. *Rhodococcus* sp. strain IGTS8 has been patented and is deposited with the American Type Culture Collection as ATCC 53968 (21); it was provided to us by J. Kilbane. UV1 is a mutant of IGTS8 that is unable to desulfurize DBT (this work). *R. fascians* D188-5 (from J. Desomer) (7) and *R. rhodochrous* ATCC 13808 (type strain from the American Type Culture Collection) do not metabolize DBT. *Escherichia coli* XL1-Blue (from Stratagene) is *recA1 lac thi endA1 gyrA96 hsdR17 supE44 relA1* [F' *proAB lacI^q lacZΔM15 Tn10*]. *E. coli* CS109 (from C. Schnaitman) is W1485 *thi supE* F⁻. *E. coli* S17-1 (from A. Puhler) is a derivative of *E. coli* 294 and is *recA thi pro hsdR res mod⁺* [RP4-2-Tc::Mu-Km::Tn7] (33).

Pseudomonas minimal salts medium (PMS) was prepared by the method of Giurard and Snell (15) and contained 0.2% glycerol, 40 mM phosphate buffer (pH 6.8), 2% Hutner's mineral base, and 0.1% (NH₄)₂SO₄. PMS medium lacking sulfate was prepared with chloride salts in place of sulfate salts. Luria broth (LB) was 1% tryptone (Difco Laboratories), 0.5% yeast extract, and 1% NaCl. All liquid medium incubations were performed with shaking in water baths (New Brunswick Scientific). Ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) were included as selective agents when required. DBT was purchased from Fluka, DBT-sulfoxide was from ICN Pharmaceuticals, and DBT-sulfone was from Aldrich Chemical. Agarose was from Bethesda Research Laboratories.

Plasmid vectors. pLAFR5 was obtained from N. T. Keen (18). pRF29 (from J. Desomer) (8) served as a source of the *Rhodococcus* plasmid origin of replication.

Cosmid library construction. High-molecular-weight DNA was isolated from IGTS8 by the method of Consevage et al. (3), except that cell lysis was accomplished in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing lysozyme (5 mg/ml) and sodium dodecyl sulfate (2%). The DNA was partially digested with *Sau3AI*, and fragments of approximately 20 kb were isolated after centrifugation through a sodium chloride gradient (14). These fragments were ligated into the *Bam*HI site of pLAFR5. In vitro packaging was performed with Gigapack Plus (Stratagene). Packaged cosmids were transduced into *E. coli* S17-1.

DBT spray plate assay. A spray plate assay for the identification of bacteria capable of modifying DBT was originally described by Kiyohara et al. (26) and modified by S. Krawiec (30). We further modified the assay for use with *Rhodococcus* sp. strain IGTS8 as follows. Cells from individual IGTS8 colonies were transferred to LB plates as small (0.5-cm) patches and were incubated at 30°C for 24 to 36 h. Large amounts of cells from these patches were transferred onto PMS-1% agarose plates that had been sprayed previously with a 0.1% DBT solution in ethyl ether to provide the sole source of sulfur. The PMS-DBT plates were incubated at 30°C for 6 to 18 h, and fluorescent products around the patches were detected by viewing under short-wave (254-nm) UV illumination.

Sulfur bioavailability assay. IGTS8 was incubated in PMS medium at 30°C for 24 to 48 h, and the cells were pelleted by centrifugation, followed by two washes with sulfur-free PMS. Washed cells were inoculated into PMS that contained as a sole source of sulfur a 0.2% concentration of one of the following: DBT, DBT-sulfoxide, or DBT-sulfone. The inoculum was adjusted so that the beginning *A*₆₀₀ was 0.02. The culture was incubated at 30°C, and growth was monitored at *A*₆₀₀. For cultures incubated with DBT, the supernatant was

viewed at various intervals under short-wave UV light to check for the production of fluorescent products.

Plasmid isolation and hybridizations. Cosmid DNA (pLAFR5) was isolated from *E. coli* as described by Ish-Horowicz and Burke (17) and from *Rhodococcus* species as described by Singer and Finnerty (34). Large-scale cosmid preparations were carried out by the method of Birnboim and Doly (1). DNA hybridization experiments were performed by the method of Southern (35). DNA was labelled with ³²P-dCTP (Amersham Corp.), using the random primer method of Feinberg and Vogelstein (10).

UV mutagenesis of IGTS8. IGTS8 was incubated overnight in LB at 30°C, and approximately 3,000 CFU were spread onto fresh LB plates. These plates were immediately exposed to short-wave (254-nm) UV light for 5 to 20 s at a distance of 3.5 cm. Plates were incubated at 30°C for 48 h or until colonies developed. Colonies from plates exhibiting >50% cell death were assayed with the spray plate procedure for the ability to metabolize or desulfurize DBT.

Electrotransformation of *Rhodococcus* sp. *Rhodococcus* sp. strain IGTS8 and the UV1 mutant were transformed with plasmid DNA via electroporation (Gene Pulser; Bio-Rad Laboratories). The bacteria were grown in LB for 24 to 48 h at 30°C, diluted to an *A*₆₀₀ of 0.15 with fresh LB, and incubated at 30°C for an additional 4 h. Cells were collected by centrifugation, washed four to five times with 0.3 M sucrose, and finally resuspended to ~5 × 10⁹ cells per ml in 0.5 M sucrose. To an ice-cold 0.2-cm electroporation cuvette (Bio-Rad) was added 40 μl of this bacterial solution. The cells were pulsed at 25 μF and 2.5 kV with the Pulse Controller at 800 Ω and were immediately diluted with 1 ml of LB containing 0.5 M sucrose. The cells were incubated at 30°C for 1 h, plated on LB agar plates plus appropriate antibiotics, and incubated at 30°C until colonies developed. When the plasmid carried the pRF29 *Rhodococcus* plasmid origin of replication, colonies were visible after 48 h. In the absence of the pRF29 origin, colonies appeared after 4 to 5 days.

R. fascians D188-5 was transformed by electroporation in a similar manner, but due to its slower growth rate, it was incubated in LB overnight until it reached an *A*₆₀₀ of ~2.0. The cells were washed and resuspended in distilled water instead of sucrose. The Pulse Controller was set at 400 Ω, and the recovery period after electroporation was in LB for 4 h before plating onto selective media. Successful transformation of *R. fascians* D188-5 with *E. coli* plasmids required that the DNA be methylated in vitro beforehand, using the CpG methylase *SssI* (New England BioLabs).

Gas chromatography (GC) and mass spectroscopy (MS). Cells were incubated overnight in LB medium at 30°C, and 100 μl was used to inoculate 50 ml of PMS minimal medium. The culture was incubated at 30°C for 4 days and washed twice with sulfur-free PMS, and the pelleted cells were inoculated into 50 ml of PMS that contained 0.1% DBT as the sole source of sulfur. These cells were incubated at 30°C for 24 h, and the entire culture was stored frozen at -20°C. For assays involving *R. fascians* D188-5, incubation times were increased two- to threefold.

Sample preparation and chemical analyses were performed as described before (32). Briefly, each sample supernatant (~50 ml) was thawed, and residual insoluble material was removed by centrifugation. The cleared supernatant was acidified with HCl to pH 1.0 and then extracted three times with 50 ml of ethyl acetate. Insoluble material from the centrifugation step was also extracted with ethyl acetate. The ethyl acetate extracts were combined, dried over anhy-

drous calcium chloride, and filtered, and ethyl acetate was removed by rotary evaporation. A known amount of internal standard (octadecane in chloroform solution) was added to the sample, which was then analyzed by GC-flame ionization detection and GC-Fourier transform infrared-MS. In some samples, the acidic components in the ethyl acetate extract or in the postextraction aqueous layer were methylated by treatment with an ether solution of diazomethane.

The analyses were performed on a serially interfaced GC-Fourier transform infrared-MS system as described previously (9, 31). This system consisted of the Finnegan ion trap (ITD 800) operated with the automatic gain control on and the Nicolet 20SXB Fourier transform infrared spectrometer. GC was conducted with a DB5 column (30 m by 0.32 mm; 1.0- μ m phase thickness) with a 2.0-ml/min helium carrier flow rate measured at 330°C. On-column injections were utilized for sample introduction because the sulfoxides and sulfones are thermally unstable and they decompose in split or splitless injectors (37). The oven temperature program was as follows: 40°C injection, followed by increases in temperature at rates of 20°C/min to 80°C, 5°C/min to 200°C, and 10°C/min to 330°C and hold for 5 min. GC-flame ionization detection analyses were performed with an HP 5880A gas chromatograph with a similar column and program for flow rate and oven temperature.

RESULTS AND DISCUSSION

Isolation of a desulfurization-negative mutant of *R. rhodochrous* IGTS8. When cloning from a foreign bacterial genus into *E. coli*, not all genes are expressed, nor are all protein products active. To assure that cloned desulfurization genes would be expressed in the host cell, we isolated a mutant of *Rhodococcus* sp. strain IGTS8 that could no longer desulfurize DBT. Using this mutant as a cloning recipient ensured that the cellular environment was appropriate for gene expression and protein function and allowed us to screen for cloned desulfurization genes by complementation.

Rhodococcus sp. strain IGTS8 was mutagenized by exposure to UV light, and 1,000 survivors were screened for the ability to produce a UV-fluorescent product in the DBT spray plate assay. Three potential desulfurization-negative mutants were identified and then reevaluated in the sulfur bioavailability assay. Two mutants (designated UV1 and UV23) could not use DBT or DBT-sulfone as a sole source of sulfur. When grown in the presence of DBT, mutant UV1 could not metabolize DBT to 2-HBP or to any other potential intermediate, as measured by GC-MS analysis. Therefore, strain UV1 was used as the host for complementation studies to identify clones that carried desulfurization genes.

Cosmid cloning of desulfurization genes. DNA from *Rhodococcus* sp. strain IGTS8 was used to construct a library in the cosmid vector pLAFR5. This library was transduced into *E. coli* S17-1, and plasmids were isolated from approximately 25,000 colonies. These cosmids were electroporated into *Rhodococcus* sp. strain UV1, a desulfurization-negative mutant of IGTS8, with an efficiency of ~300 transformants per μ g of DNA. Various numbers of UV1 transformants were pooled and incubated for 18 h at 30°C, after which the cells were washed twice and resuspended in sulfate-free PMS. Approximately 7×10^8 pooled cells were inoculated into 100 ml of PMS with DBT as the sole source of sulfur. A predicted product of the DBT desulfurization reaction is 2-HBP, which is fluorescent when exposed to UV light. Therefore, batch cultures were grown at 30°C, and the supernatants were observed for fluorescence. Approxi-

mately 3,300 UV1 transformants were screened in four separate batches. In one batch (representing ~600 transformants), a UV-fluorescent product appeared in the supernatant after 5 days of incubation. Individual colonies were isolated, and 12 of these continued to produce a fluorescent product when exposed to DBT.

Attempts to recover cosmid DNA from these isolates failed, so Southern hybridizations were performed to determine whether the cosmids had become integrated into the chromosome of strain UV1. Total DNA was isolated from seven transformants and digested with *Eco*RI. After agarose electrophoresis and blotting, the fragments were hybridized with ³²P-labelled probes derived from pLAFR5. In all transformants tested, pLAFR5 probes hybridized to a DNA fragment of ~20 kb in size. Vector-derived probes did not hybridize to the control IGTS8 genome (data not shown). Therefore, the desulfurization-positive cosmid clones had apparently integrated into the chromosome of strain UV1. Desomer et al. have reported previously that nonreplicative vectors can be inserted into the chromosome of *R. fascians* by illegitimate recombination (6). Our observations with *Rhodococcus* sp. strains IGTS8 and UV1 support such an insertion mechanism for the cosmid vector pLAFR5. Thus, even if specific cloning vectors are not available for a species of *Rhodococcus*, this integration phenomenon allows the use of classical gram-negative bacterial vectors, though the frequency of integration is very low.

Since the plasmids had integrated into the chromosome, the genomic DNA connected to either side of the plasmid cloning site must represent *Rhodococcus* sp. strain IGTS8 sequences that were able to complement the desulfurization-negative mutation in strain UV1. (This would be true regardless of whether the mode of integration was by homologous or illegitimate recombination.) We recovered sequences that flanked the inserted plasmid from three desulfurization-positive transformants by digesting genomic preparations with *Eco*RI or *Bam*HI. These enzymes cut pLAFR5 once in the polylinker region so that an intact sequence of pLAFR5 could be recovered, linked to a neighboring chromosomal fragment from IGTS8. The digested DNA was ligated to itself (at a concentration of ~20 ng/ μ l) and was transformed into *E. coli* S17-1. Sixteen tetracycline-resistant colonies were obtained, seven from the *Bam*HI digestion and nine from the *Eco*RI digestion. Restriction enzyme analysis revealed that all *Eco*RI-rescued clones contained a 2.1-kb fragment of IGTS8 DNA. The *Bam*HI-rescued clones contained a 1.65-kb fragment from IGTS8.

The 2.1-kb IGTS8 DNA from the *Eco*RI rescue experiment was used as a template to make a labelled DNA probe, which was hybridized to colony lifts of the original cosmid library in *E. coli*. Of 5,000 colonies screened, 17 hybridized with the IGTS8 probe. Cosmid DNA was isolated from each clone and transformed into strain UV1. Three of the 17 DNA preparations complemented the desulfurization-negative phenotype.

A restriction map for this region was constructed, using *Eco*RI and *Hind*III (Fig. 1). Probes from the 2.1-kb IGTS8 DNA hybridized to the 4.5-kb *Eco*RI fragment. All cosmid clones that conferred the desulfurization-positive phenotype contained the entire 4.5-kb *Eco*RI fragment and portions of the 4.5-kb *Eco*RI-*Hind*III and 18-kb *Eco*RI fragments. These results indicated that the desulfurization genes lay within a 15-kb region defined by the overlapping areas of cosmids GE1-C, GE1-H, and GE1-K (Fig. 1).

Subcloning the desulfurization genes. The 4.5-kb *Eco*RI and the 4.5-kb *Eco*RI-*Hind*III fragments were subcloned into

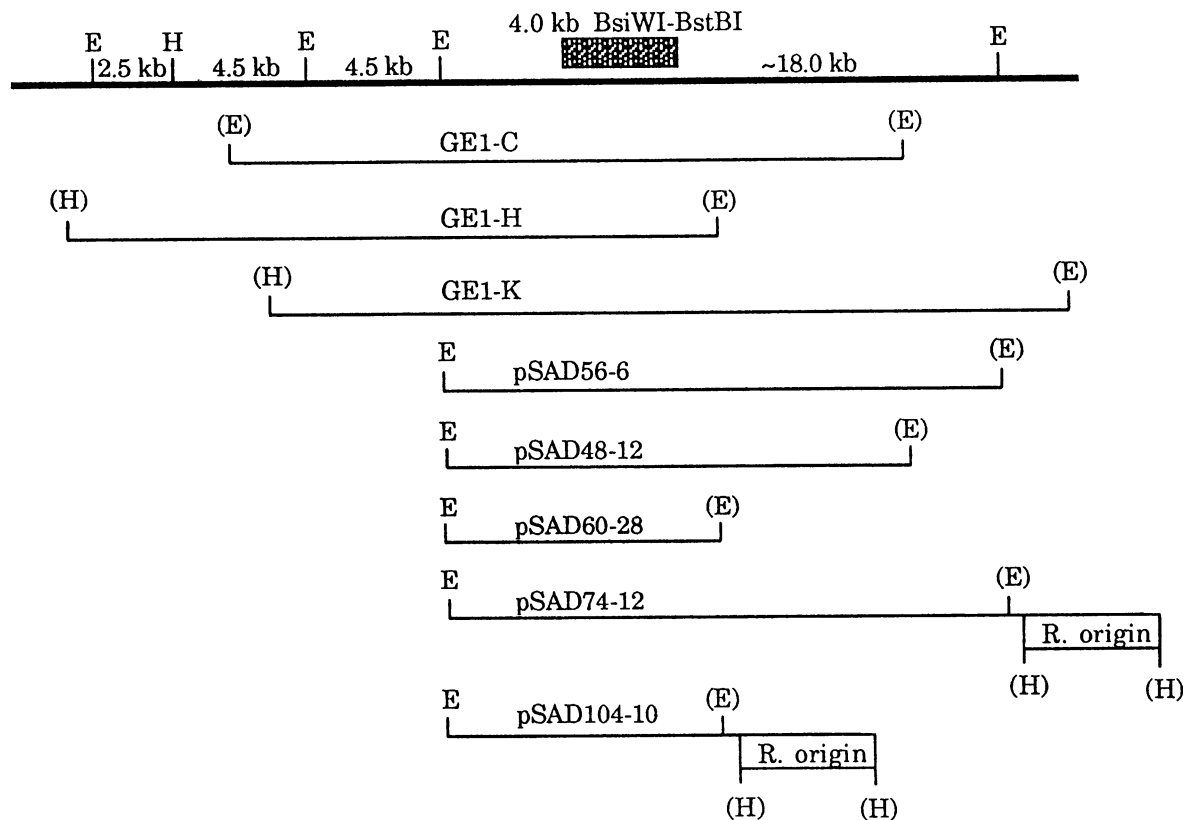


FIG. 1. Restriction map of desulfurization clones derived from *Rhodococcus* sp. strain IGTS8. The upper line represents part of a composite restriction map assembled from 17 cosmid clones. Numbers on that line are the lengths between adjacent restriction enzyme sites. The IGTS8 DNA from three of these cosmids (GE1-C, GE1-H, and GE1-K) is denoted by lines underneath the composite map. When transformed into the desulfurization-negative mutant *Rhodococcus* sp. strain UV1, each of these cosmids restored the desulfurization phenotype. Five subclones generated from these three cosmids are also shown schematically. The shaded box above the composite map denotes the location of the 4.0-kb *BsiWI-BstBI* DNA fragment that is the smallest fragment which complements the desulfurization-negative phenotype of strain UV1. Parentheses indicate the location of restriction sites that are derived from vector sequences: E, *EcoRI*; H, *HindIII*; R. origin, *Rhodococcus* origin of replication from plasmid pRF29.

pLAFR5, but neither fragment complemented the desulfurization mutation of strain UV1. The 9.0-kb *EcoRI* fragment from GE1-H, the 15.0-kb *EcoRI-HindIII* fragment from GE1-C, and the 18-kb *EcoRI* fragment from GE1-K were subcloned into pLAFR5 to yield plasmids pSAD60-28, pSAD48-12, and pSAD56-6, respectively. When transformed into UV1, all three produced UV-fluorescent products from DBT in the spray plate assay, consistent with the localization of the desulfurization phenotype as determined by restriction mapping. Construction of additional subclones from this region narrowed the location of the relevant genes to a 4.0-kb *BsiWI-BstBI* fragment (shaded section in Fig. 1).

Nature of the mutation in strain UV1. Genomic blots of *EcoRI*-digested IGTS8 and UV1 DNA were hybridized with probes produced from the 2.1-kb *EcoRI*-rescued fragment of IGTS8. No hybridization to UV1 DNA was detected, indicating that the UV1 mutation is a large deletion and not a simple point mutation. Southern blotting of DNA separated by pulsed-field gel electrophoresis indicates that the desulfurization genes of IGTS8 reside on a plasmid and that this plasmid is absent from strain UV1 (5a).

A *Rhodococcus* plasmid origin of replication increases transformation of UV1. Electroporation of UV1 with pSAD48-12 typically resulted in a low transformation efficiency (~550

per μg of DNA), and only about 50% of the transformants exhibited the desulfurization-positive phenotype (presumably because DNA had been lost or rearranged during recombination with the chromosome). To improve the transformation efficiency, we cloned a 4.5-kb *HindIII* fragment from pRF29 into the *HindIII* site of pSAD48-12, resulting in pSAD74-12. This 4.5-kb fragment contains a *Rhodococcus* plasmid origin of replication, which allowed pSAD74-12 to replicate as a plasmid in strain UV1. This clone transformed UV1 with an efficiency of greater than 10^4 transformants per μg of DNA. Nearly 100% of these transformants exhibited the desulfurization phenotype. Even so, very often such small amounts of plasmid were recovered from DNA preparations of these transformants that no plasmid was visible on agarose gels. There was, however, enough plasmid in these preparations to transform *E. coli* S17-1, from which large amounts of the plasmid could be prepared. These results emphasize the need for improved techniques of plasmid isolation from rhodococci.

The desulfurization phenotype is not expressed in *E. coli*. *E. coli* S17-1 was transformed with pSAD48-12, and desulfurization activity was measured with the spray plate assay. No positive colonies were identified. It was possible that the *E. coli* polymerase could not recognize the IGTS8 promoter(s)

TABLE 1. Metabolites produced from DBT by bacteria transformed with subclones derived from *Rhodococcus* sp. strain IGTS8

Metabolite ^a	Amt ^b in given <i>Rhodococcus</i> species (plasmid)		
	<i>R. rhodochrous</i> IGTS8	UV1(pSAD104-10) ^c	<i>R. fascians</i> D188-5(pSAD74-12)
DBTO	+	0	0
DBTO ₂	0	0	0
Sulfonic acid	+	++	++
Sulfenic acid	0 or tr	0	+
2-HBP	+++++	+++++	+++++

^a Products were identified by GC-MS, as outlined in Materials and Methods. DBT-O, DBT-5-oxide; DBT-O₂, DBT-5,5-dioxide; sulfonic acid, 2'-HBP-2-sulfonic acid (detected as the sultone: dibenz[*c,e*][1,2]oxathiin 6-oxide); sulfenic acid, 2'-HBP-2-sulfenic acid (detected as the sultone: dibenz[*c,e*][1,2]oxathiin 6,6-dioxide).

^b The presence of metabolites is reported in relative amounts from very large (+++++) to very small (+) or trace amounts. The actual values measured per experimental culture were as follows: +++++, 6 to 7 μg; ++, 1 μg; +, 0.1 μg; tr, 0.05 μg.

^c 9.0-kb *Eco*RI DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.

^d 15.0-kb *Eco*RI-*Hind*III DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.

in pSAD48-12, so we placed the IGTS8 DNA under control of the *E. coli lac* promoter. The 15-kb *Eco*RI-*Hind*III IGTS8 fragment from pSAD48-12 was subcloned into the pBlue-script vectors SK⁻ and KS⁻ so that the IGTS8 fragment was cloned in both orientations with respect to the *lac* promoter. Neither clone expressed the desulfurization phenotype in *E. coli* XL1-Blue. We do not yet know whether this stems from poor transcription or translation of the cloned genes or whether the overproduced proteins are inactive in *E. coli*.

The desulfurization genes are expressed in *R. fascians*. Since the cloned genes were either not expressed or produced inactive proteins in *E. coli*, we attempted to express the genes in other *Rhodococcus* species. *R. fascians* D188-5 exhibited no desulfurization in the DBT spray plate assay or in the sulfur bioavailability assay. At first, we were unable to transform *R. fascians* with the desulfurization-positive plasmid pSAD74-12. Other *Rhodococcus* species are known to have endogenous restriction systems that cleave DNA at *Sal*I-like restriction sites. Since pSAD74-12 contained multiple *Sal*I recognition sequences, we used a CpG methylase, *Sss*I, to methylate pSAD74-12 in vitro. With methylated pSAD74-12 DNA we obtained transformants of *R. fascians* D188-5 with an efficiency of about 7×10^3 transformants per μg of DNA. These transformants displayed the desulfurization phenotype in the spray plate assay, and GC analysis of liquid medium supernatant revealed the formation of 2-HBP from DBT. Thus, the cloned genes encoded enzymes of the desulfurization pathway.

We were unable to transform pSAD74-12 into a second species, *R. rhodochrous* ATCC 13808, despite the use of an unmethylated or a CpG-methylated plasmid. It is possible that the electroporation conditions for ATCC 13808 were not optimal, though a wide range of conditions was tested. It seems more likely that ATCC 13808 has a restriction system that is not inhibited by CpG methylation.

2-HBP is the major desulfurization product. The predominant metabolite produced from DBT by *Rhodococcus* sp. IGTS8 is 2-HBP, with small amounts of 2-HBP-sulfenic acid and 2-HBP-sulfonic acid (identified as the sultone and sultone, respectively, by GC-MS analysis) (32). These products were also produced by IGTS8 in this work (Table 1). Neither *R. fascians* D188-5 nor the IGTS8-derived mutant, UV1, produced these products from DBT. However, when *R. fascians* D188-5 was transformed with plasmid pSAD74-12 and when the UV1 strain was transformed with plasmid pSAD104-10, these bacteria produced products from DBT that were identical to those identified for *Rhodococcus* sp.

strain IGTS8 (Table 1). In particular, 2-HBP was produced in large quantities, indicating that specific desulfurization of DBT was mediated by products of genes cloned from IGTS8.

IGTS8 cannot use DBT-sulfoxide as a sulfur source. *Rhodococcus* sp. strain IGTS8 was incubated in minimal medium with one of the following as the sole source of sulfur: DBT, DBT-sulfoxide, or DBT-sulfone. IGTS8 was incapable of utilizing the sulfur supplied by DBT-sulfoxide but grew well in the presence of DBT or DBT-sulfone. DBT-sulfoxide was not toxic to cells when grown in a rich medium (LB) (data not shown). Therefore, either IGTS8 cannot transport or

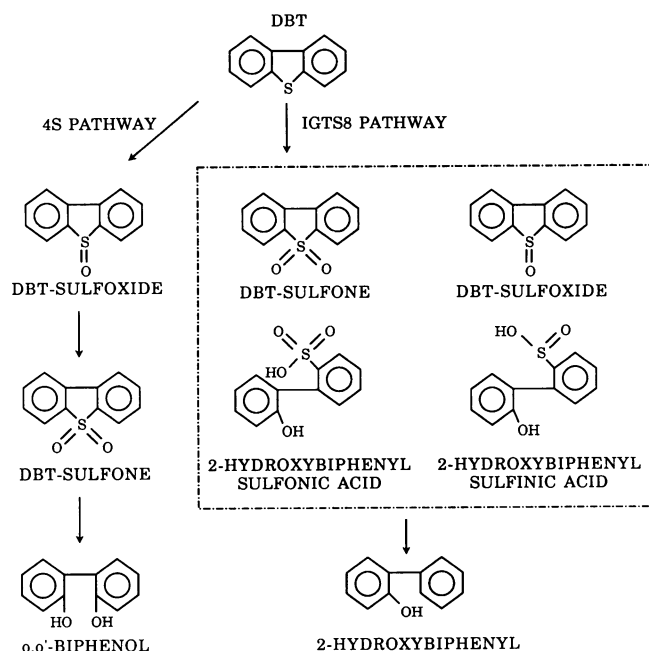


FIG. 2. Structures of chemical intermediates and possible pathways for the desulfurization of DBT. The 4S pathway (left column) is a theoretical pathway for DBT desulfurization (19). There are no experimental data for the existence of the 4S pathway. The boxed structures represent possible intermediates in a variation of the 4S pathway that is consistent with the evidence of this report and that of Olson et al. (32). The exact relationship among the four boxed compounds in the desulfurization pathway is not known.

metabolize extracellular DBT-sulfoxide or else DBT-sulfoxide is not an intermediate in the desulfurization pathway.

Potential pathway for desulfurization of DBT. This report is the first to demonstrate that the "desulfurization only" reaction is the product of a specific set of gene products and thus establishes that the pathway is distinct from the genes and reactions of the previously characterized "degradative" metabolism of DBT. A theoretical pathway that accomplishes this specific desulfurization is referred to as the 4S pathway (Fig. 2) (19). *Rhodococcus* sp. strain IGTS8 apparently produces the sulfinic and sulfonic metabolites from DBT and does not accumulate sulfoxide and sulfone (32). Our results confirm the presence of the sulfinic and sulfonic in the parent organism and extend the observation to organisms that have received certain genes (Table 1). Thus, this isolate of *Rhodococcus* sp. apparently uses a variation of the 4S pathway for this desulfurization reaction (Fig. 2). Identification of the cloned gene products and assays of their individual activities should clarify the relationships between the intermediates of this new pathway.

Since *Rhodococcus* sp. strain IGTS8 can desulfurize the organic sulfur contaminants in soluble coal-derived products (24), further manipulation of the genes that encode this pathway may increase the extent and efficiency of the reaction to a point at which it could become an economical method to desulfurize coal-derived liquids or petroleum.

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REFERENCES

- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1413-1423.
- Bos, P., and J. G. Kuenen. 1990. Microbial treatment of coal, p. 343-377. In H. L. Ehrlich, and C. L. Brierley (ed.), *Microbial mineral recovery*. McGraw-Hill, New York.
- Conseville, M. W., R. D. Porter, and A. T. Phillips. 1985. Cloning and expression in *Escherichia coli* of histidine utilization genes from *Pseudomonas putida*. *J. Bacteriol.* 162:138-146.
- Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. *Curr. Microbiol.* 21:229-231.
- Denome, S. A., and K. D. Young. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas*: complete DNA sequence of an upper naphthalene catabolic pathway. Submitted for publication.
- Denome, S. A., and K. D. Young. Unpublished data.
- Desomer, J., M. Crespi, and M. Van Montagu. 1991. Illegitimate integration of non-replicative vectors in the genome of *Rhodococcus fascians* upon electrotransformation as an insertional mutagenesis system. *Mol. Microbiol.* 5:2115-2124.
- Desomer, J., P. Dhaese, and M. Van Montagu. 1988. Conjugative transfer of cadmium resistance plasmids in *Rhodococcus fascians* strains. *J. Bacteriol.* 170:2401-2405.
- Desomer, J., P. Dhaese, and M. Van Montagu. 1990. Transformation of *Rhodococcus fascians* by high-voltage electroporation and development of *R. fascians* cloning vectors. *Appl. Environ. Microbiol.* 56:2818-2825.
- Diehl, J. W., S. B. Kleinjan, and E. S. Olson. 1990. A gas chromatographic/Fourier infrared spectroscopy/mass spectrometry/atomic emission detection/flame ionization detection system. *Spectrosc. Int. J.* 8:43-72.
- Feinberg, A. P., and B. Vogelstein. 1984. Addendum: a technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266-267.
- Finnerty, W. R., and M. Robinson. 1986. Microbial desulfurization of fossil fuels: a review. *Biotechnol. Bioeng. Symp.* 16:205-221.
- Foght, J. M., P. M. Fedorak, M. R. Gray, and D. W. S. Westlake. 1990. Microbial desulfurization of petroleum, p. 379-407. In H. L. Ehrlich and C. L. Brierley (ed.), *Microbial mineral recovery*. McGraw-Hill, New York.
- Foght, J. M., and D. W. S. Westlake. 1990. Expression of dibenzothiophene-degradative genes in two *Pseudomonas* species. *Can. J. Microbiol.* 36:718-724.
- Frischauf, A.-M. 1987. Digestion of DNA: size fractionation. *Methods Enzymol.* 152:183-189.
- Giurard, B. M., and E. E. Snell. 1981. Biochemical factors in growth, p. 79-111. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
- Holland, H. L., S. H. Khan, D. Richards, and E. Riemland. 1986. Biotransformation of polycyclic aromatic compounds by fungi. *Xenobiotica* 16:733-741.
- Ish-Horowitz, D., and J. F. Burke. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* 9:2989-2998.
- Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 70:191-197.
- Kilbane, J. J. 1989. Desulfurization of coal: the microbial solution. *Trends Biotechnol.* 7:97-101.
- Kilbane, J. J. 1990. Sulfur-specific microbial metabolism of organic compounds. *Resources Conserv. Recycling* 3:69-79.
- Kilbane, J. J. April 1992. Mutant microorganisms useful for cleavage of organic C-S bonds. U.S. patent 5,104,801.
- Kilbane, J. J., and B. A. Bielage. 1990. Genetic study of biodesulfurization, p. 2/15-2/32. In S. Yunker and K. Rhee (ed.), *Proceedings: 1990 First International Symposium on the Biological Processing of Coal*. Electric Power Research Institute, Inc., Palo Alto, Calif.
- Kilbane, J. J., A. Maka, C. Akin, and V. J. Srivastava. 1989. Microbial coal desulfurization, solubilization, and gasification research at IGT, p. 2/87-2/105. In S. Yunker (ed.), *Proceedings: 1989 Symposium on Biological Processing of Coal and Coal-Derived Substances*. Electric Power Research Institute, Inc., Palo Alto, Calif.
- Kilbane, J. J., II, and K. Jackowski. 1992. Biodesulfurization of water-soluble coal-derived material by *Rhodococcus rhodochrous* IGTS8. *Biotechnol. Bioeng.* 40:1107-1114.
- Kim, H. Y., T. S. Kim, and B. H. Kim. 1990. Degradation of organic sulfur compounds and the reduction of dibenzothiophene to biphenyl and hydrogen sulfide by *Desulfovibrio desulfuricans* M6. *Biotechnol. Lett.* 12:761-764.
- Kiyohara, H., K. Nagao, and K. Yana. 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl. Environ. Microbiol.* 43:454-457.
- Klein, J., M. Beyer, M. van Afferden, W. Hodek, F. Pfeifer, H. Seewald, E. Wolff-Fischer, and H. Jüntgen. 1988. Coal in biotechnology, p. 497-567. In H.-J. Rehm and G. Reed (ed.), *Biotechnology*, vol. 6b. VCH Verlagsgesellschaft, Weinheim, Germany.
- Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. Part III. Isolation and identification of products from dibenzothiophene. *Agric. Biol. Chem.* 34:1320-1324.
- Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. 1973. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. *Agric. Biol. Chem.* 37:45-50.
- Krawiec, S. 1990. Bacterial desulfurization of thiophenes: screening techniques and some speculations regarding the biochemical and genetic bases. *Dev. Ind. Microbiol.* 31:103-114.
- Olson, E. S., and J. W. Diehl. 1987. Serially interfaced gas chromatography/Fourier transform infrared/ion trap mass spectrometer system. *Anal. Chem.* 59:443-448.
- Olson, E. S., D. C. Stanley, and J. R. Gallagher. 1993. Characterization of intermediates in the microbial desulfurization of

- dibenzothiophene. *Energy Fuels*, 7:159–164.
33. **Simon, R., M. O'Connell, M. Labes, and A. Pühler.** 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol.* 118:640–659.
 34. **Singer, M. E., and W. R. Finnerty.** 1988. Construction of an *Escherichia coli*-*Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* spp. *J. Bacteriol.* 170:638–645.
 35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.
 36. **van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper.** 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. *Arch. Microbiol.* 153:324–328.
 37. **Vignier, V., F. Berthou, and D. Picart.** 1983. Influence of injection systems on the gas chromatographic analysis of oxygenated dibenzothiophenes. *J. High Resolution Chromatogr. Chromatogr. Commun.* 6:661–665.
 38. **Young, K. D.** 1993. Genetic manipulation of coal-biotransforming microorganisms, p. 171–211. *In* D. L. Crawford (ed.), *Microbial transformations of low rank coals*. CRC Press, Inc., Boca Raton, Fla.