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Sequence and Molecular Characterization of a DNA Region Encoding the Dibenzothiophene Desulfurization Operon of *Rhodococcus* sp. Strain IGTS8

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Dibenzothiophene (DBT), a model compound for sulfur-containing organic molecules found in fossil fuels, can be desulfurized to 2-hydroxybiphenyl (2-HBP) by *Rhodococcus* sp. strain IGTS8. Complementation of a desulfurization (*dsz*) mutant provided the genes from *Rhodococcus* sp. strain IGTS8 responsible for desulfurization. A 6.7-kb *Taq*I fragment cloned in *Escherichia coli-Rhodococcus* shuttle vector pRR-6 was found to both complement this mutation and confer desulfurization to *Rhodococcus fascians*, which normally is not able to desulfurize DBT. Expression of this fragment in *E. coli* also conferred the ability to desulfurize DBT. A molecular analysis of the cloned fragment revealed a single operon containing three open reading frames involved in the conversion of DBT to 2-HBP. The three genes were designated *dszA*, *dszB*, and *dszC*. Neither the nucleotide sequences nor the deduced amino acid sequences of the enzymes exhibited significant similarity to sequences obtained from the GenBank, EMBL, and Swiss-Prot databases, indicating that these enzymes are novel enzymes. Subclone analyses revealed that the gene product of *dszC* converts DBT directly to DBT-sulfone and that the gene products of *dszA* and *dszB* act in concert to convert DBT-sulfone to 2-HBP.

Acid rain, a major contributor to environmental distress, results in large part from sulfur oxides produced during combustion of fossil fuels. As much as 70% of the sulfur in these fuels may be in the form of heterocyclic organic compounds, such as benzothiophene, dibenzothiophene (DBT), and more complex thiophenes (4). DBT is generally accepted as the model compound for organic sulfur-containing fossil fuel components. These types of fuel components can be removed by physical and chemical methods, but these processes are expensive. Consequently, there has been considerable effort to develop bioprocesses for fossil fuel desulfurization by using DBT as a model compound. There have been numerous reports of microorganisms that metabolize DBT. These organisms include members of the genera *Pseudomonas* (16, 19, 22), *Beijerinckia* (12), *Rhizobium* (14), *Acinetobacter* (14), *Arthrobacter* (10), and *Brevibacterium*. (21). These bacteria, however, suffer from the disadvantage that they metabolize DBT via a ring-destroying oxidative pathway (11) that results in a net carbon loss and reduction in fuel value. In addition, it has been observed that the pathway for naphthalene metabolism (5) closely resembles the ring-destroying DBT-degrading pathway, raising the possibility that further undesired metabolism of structurally related non-sulfur-containing fuel components may occur. Because of the net carbon loss and resulting reduction in fuel value, it is unlikely that these types of biotransformations could lead to an industrial process for fossil fuel desulfurization.

In contrast, workers have recently described two organisms which can desulfurize DBT via a pathway that does not destroy carbon rings. These organisms are *Rhodococcus* sp. strain IGTS8 (9) and *Corynebacterium* sp. strain SY1 (17). Both of these strains convert DBT to 2-hydroxybiphenyl (2-HBP) and sulfite. One proposed pathway for desulfurization by these organisms is the so-called 4S pathway shown in Fig. 1. This pathway involves the sequential metabolism of DBT to DBT-sulfoxide, DBT-sulfone, DBT-sulfonate, and finally 2-HBP and sulfite. A modification of this pathway in which 2′-hydroxybiphenyl-2-sulfinate and 2′-hydroxybiphenyl-2-sulfonate are additional intermediates has recently been described (6).

In this report we describe molecular cloning and characterization of the *Rhodococcus* sp. strain IGTS8 DNA responsible for DBT desulfurization. This DNA includes three genes, *dszA*, *dszB*, and *dszC*, which encode enzymes that are sufficient for conversion of DBT to 2-HBP. On the basis of the results of a subclone analysis of the operon which we identified, we propose a modified, simpler version of the 4S pathway utilized by this organism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All of the strains and plasmids used in this study are described in Table 1. *Rhodococcus* strains were maintained on *Rhodococcus* medium (RM), which contained (per liter) 8.0 g of nutrient broth (Difco), 0.5 g of yeast extract, and 10.0 g of glucose. Recombinant Cm^r *Rhodococcus* strains were selected on RM plates supplemented with 25 μg of chloramphenicol per ml. For expression of the Dsz^+ phenotype, *Rhodococcus* strains were grown in basal salts medium (BSM), which contained (per liter) 2.44 g of KH_2PO_4, 5.57 g of Na_2HPO_4, 2.0 g of NH_4Cl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.001 g of $CaCl_2 \cdot 2H_2O$, 0.001 g of $FeCl_3 \cdot 6H_2O$, 0.004 g of $MnCl_2 \cdot 4H_2O$, 6.4 ml of glycerol, and 5.0 g of glucose. All *Rhodococcus* strains were grown at 30°C. *Escherichia coli* JM109 was grown at 37°C in L broth (Difco). Ap^r transformants were selected on L agar plates supplemented with 100 μg of ampicillin per ml.

Enzymes and reagents. Restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and New England Biolabs, Beverly, Mass. T4 ligase and the Klenow fragment of *E. coli* DNA polymerase I were purchased from Bethesda Research Laboratories. HK phosphatase was purchased from Epicentre Technologies, Madison, Wis. All enzymes were used in accordance with the manufacturers' recommendations. The enzyme assay substrates DBT, dibenzothiophene 5-oxide (DBT-sulfoxide), and dibenzothiophene sulfone (DBT-sulfone) and the end product standard 2-HBP were purchased from Aldrich, Milwaukee, Wis.

Mutagenesis of strain IGTS8 and identification of Dsz^- mutants. In order to generate mutants of IGTS8 that could not metabolize DBT, we mutagenized strain IGTS8 with short-wavelength UV light and with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) obtained from Sigma Chemical Co., St. Louis, Mo. In the UV exposure mutagenesis experiments, a model UVG-54 Mineralight lamp (UVP, Inc., San Gabriel, Calif.) was used to kill more than 99% of the cells. In the NTG mutagenesis experiments, cell suspensions were treated with 500 μg of

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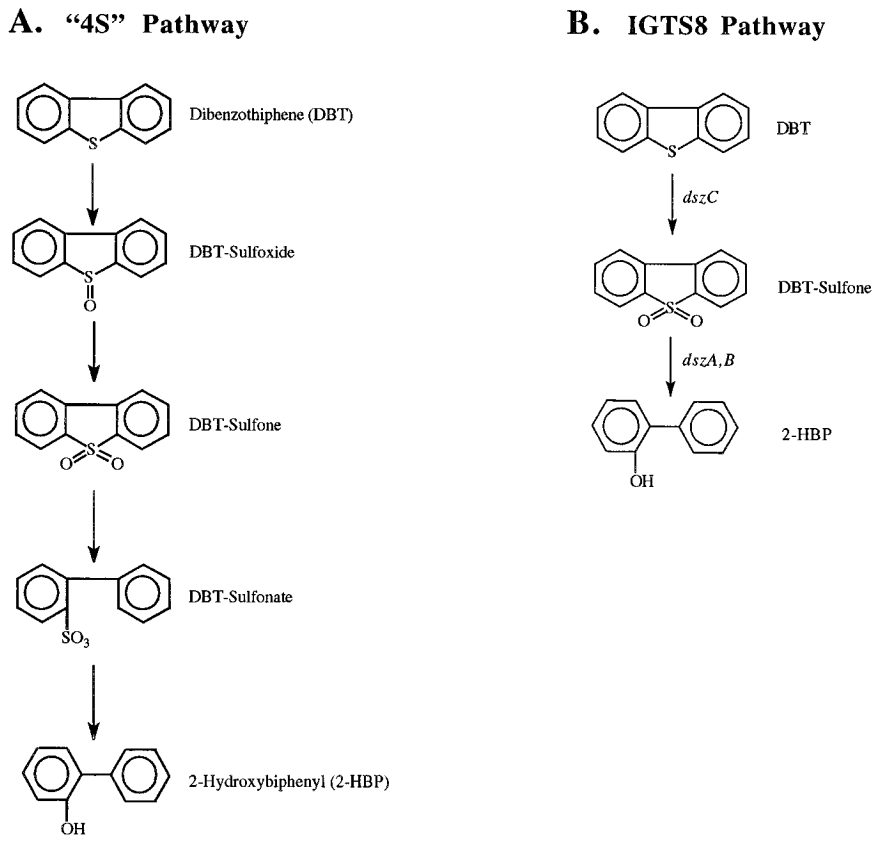

FIG. 1. (A) Hypothetical 4S pathway for desulfurization of DBT. (B) DBT desulfurization pathway defined by *dsz* operon of *Rhodococcus* sp. strain IGTS8.

NTG per ml for enough time so that 30 to 50% of the cells were killed. Combination mutagenesis in which we used both NTG and UV light was also performed; in these experiments an overall mortality rate of more than 99.9% was obtained. Colonies that survived the mutagenesis treatments were transferred to RM plates and screened for the Dsz^- phenotype by replica plating them onto BSM agarose plates supplemented with 1.2 ml of a saturated ethanol solution of DBT per liter. After 24 h, production of 2-HBP was visualized under UV illumination as fluorescent zones in the agarose surrounding Dsz^+ colonies.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description[a]	Source or reference[b]
Rhodococcus sp. strains		
IGTS8	Dsz^+	9
CPE-648	Dsz^- mutant derivative of IGTS8	This study
GPE-362	Dsz^- mutant derivative of IGTS8	This study
R. fascians 12974	Type strain, Dsz^-	ATCC
E. coli JM109	*recA1 endA1 lacI*^{q} *lacZ*Δ*M15* Δ(*pro-lacAB*)	ATCC
Plasmids		
pUC19	Ap^r, cloning vehicle	BRL
pRF29	*R. fascians-E. coli* shuttle vector, Cm^r Ap^r	3
pRR-6	*Rhodococcus* sp.-*E. coli* shuttle vector, Cm^r Ap^r	This study
pTOXI-1	6.7-kb *Sau*3A fragment from IGTS8 containing the *dsz* operon in pRR-6	This study
pENOK-1	4.0-kb *Sph*I fragment of pTOXI-1 in pRR-6	This study
pENOK-2	3.6-kb *Sac*I fragment of pTOXI-1 in pRR-6	This study
pENOK-3	1.1-kb *Xho*I deletion of pTOXI-1	This study
pENOK-16	4.0-kb *Bst*BI-*Sna*BI fragment of pTOXI-1 in pRR-6	This study
pENOK-*Nsi*	4-bp deletion of pTOXI-1 at *Nsi*I site	This study
pENOK-19	2.1-kb *Not*I-*Sna*BI deletion of pTOXI-1	This study
pENOK-20	2.8-kb PCR-amplified fragment of pTOXI-1 spanning *dszB* and *dszC*, fused to the Cm^r gene promoter of pRR-6	This study
pDR540	*E. coli* expression vector utilizing *tac* promoter	Pharmacia
pDRDsz	4.0-kb *Dra*I-*Sna*BI fragment of pTOXI-1 in pDR540	This study

[a] Dsz^+, ability to utilize thiophenic compounds, such as DBT, as sole sources of sulfur by the selective cleavage of carbon-sulfur bonds; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

[b] ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratories.

Visualization of 2-HBP production was considerably easier when we used BSM plates solidified with agarose instead of agar. This was a consequence of the relative transparency of the agarose plates, which allowed fluorescence to be observed more readily. Mutants which did not appear to produce 2-HBP in plate prescreening examinations were incubated in BSM supplemented with DBT as the sole source of sulfur, examined by the sulfur bioavailability assay, and analyzed for DBT derivatives by high-performance liquid chromatography (HPLC). Because BSM is a defined minimal medium, a duplicate control culture which contained inorganic sulfur (20 mM Na_2SO_4) was grown in order to distinguish true Dsz$^-$ mutants from auxotrophic mutants. Mutants which grew poorly in both the control and experimental media were assumed to be auxotrophic mutants and were not characterized further.

Sulfur bioavailability assay. In the sulfur bioavailability assay we examined the ability of an organism to liberate organically bound sulfur from a substrate used as the sole source of sulfur for growth (8). In this assay, BSM, which contained no sulfur, was supplemented with DBT as the sole source of sulfur. The ability of a strain to liberate sulfur was assessed by determining its viability in this medium, as monitored by A_{600}.

HPLC identification of DBT and DBT metabolites. DBT metabolites were detected by HPLC. Cells were incubated for 3 to 5 days at 30°C in 250-ml shake flasks containing 25 ml of BSM supplemented with either 100 mM DBT, 100 mM DBT-sulfoxide, or 100 mM DBT-sulfone as the substrate. Reagent alcohol was added to culture broth preparations (1:1, vol/vol) in order to extract DBT and its metabolites. Extracted broth preparations were clarified by centrifugation, and the supernatants were analyzed by using a 4μ phenyl Novapak column (Waters, Milford, Mass.) and an isocratic flow of a 60% acetonitrile–H_2O mobile phase delivered at a flow rate of 1.5 ml/min. DBT and DBT-sulfone contents were monitored at 233 nm. DBT-sulfoxide and 2-HBP contents were monitored at 248 nm. To determine specific activities in sulfur repression studies, cells were grown for 36 to 40 h as described above in medium supplemented with $(NH_4)_2SO_4$, collected by centrifugation, resuspended in 50 mM KH_2PO_4 (pH 7.0), and incubated with DBT for 1 to 2 h before analysis.

Plasmid DNA isolation and manipulation. Plasmids were isolated from *E. coli* and *Rhodococcus* strains by alkaline lysis methods (1, 15). Strain JM109 was transformed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as recommended by the manufacturer. *Rhodococcus* strains were also transformed by electroporation as described by Desomer et al. (3). The *Rhodococcus-E. coli* shuttle vector pRR-6 contains the *Rhodococcus* origin of plasmid replication and a chloramphenicol resistance marker (Cm^r) from plasmid pRF29 (3). The origin of replication and Cm^r were removed from pRF29 as a 6.9-kb *Xho*I-*Xba*I (partial) fragment. The ends were made blunt with the Klenow fragment and ligated to *Sal*I-*Xba*I-cut pUC18. A unique *Nar*I site was available for cloning in pRR-6. *Nar*I-cut ends were compatible with 4-base recognition endonuclease *Taq*I. All pENOK subclones were maintained in vector pRR-6. The DNA of pENOK-20 spanning open reading frames (ORFs) 2 and 3 was amplified by the PCR. Primers RAP-1 (5′-GC**GAA**TTCCGCACCGAGTACC-3′; positions 2062 to 2082) and RAP-2 (5′-ATCCA**TA**TGCGCACTACGAATCC-3′; positions 4908 to 4886) were synthesized with an Applied Biosystems model 392 DNA-RNA synthesizer (the nucleotides in boldface type were altered from the nucleotides in the pTOXI-1 template sequence in order to create restriction sites for subcloning). Amplification was carried out with a GeneAmp kit (Perkin Elmer Cetus) by using the *Taq* polymerase and a Perkin Elmer Cetus model 9600 thermocycler. The Cm^r gene promoter of pRF29 was ligated to the amplified product as an *Xba*I-*Eco*RI fragment. For pDRDsz, vector pDR540 was cut with *Bam*HI, and the ends were made blunt with the Klenow fragment. To this vector, a 4.0-kb *Dra*I-*Sna*BI fragment spanning the *dsz* operon from pTOXI-1 was ligated. The *Dra*I site lies 16 bp upstream of the cluster.

Plasmid library construction. IGTS8 genomic DNA was isolated essentially by the sodium dodecyl sulfate lysis method of Hunter (7). Treatment with 15 mg of lysozyme per ml greatly improved DNA yields. The genomic DNA was partially cut with *Taq*I in order to produce fragments that were 0.5 to 23 kb long. Cut DNA was electrophoresed through 0.8% low-melting-temperature agarose, and fragments more than 5 kb long were isolated and purified by standard methods (15). Vector pRR-6 was cut with *Nar*I and dephosphorylated with alkaline phosphatase to prevent religation of the vector. The size-fractionated genomic DNA was ligated to cut and dephosphorylated pRR-6.

DNA sequencing and analysis. The insert of pTOXI-1 was sequenced by primer walking on a double-stranded template, using a Sequenase 2.0 dideoxy sequencing kit (US Biochemical). The initial priming was with the −40 universal primer on subclones of pTOXI-1. A DNA sequence was generated from each strand. All computer sequence analyses were performed by using GeneWorks software (Intelligenetics, Mountain View, Calif.).

Nucleotide sequence accession number. The nucleotide sequence which we determined has been deposited in the Genome Sequence Data Base data library under accession number L37363.

RESULTS

Isolation and characterization of Dsz$^-$ mutants. As indicated in Table 2, wild-type strain IGTS8 can convert DBT, DBT-sulfoxide, and DBT-sulfone to 2-HBP. Following mutagenesis of IGTS8, approximately 2,000 survivors were examined individually to determine whether they had lost the ability to convert DBT to 2-HBP. We identified two mutants, CPE-648 and GPE-362, that could no longer metabolize DBT. Both of these organisms grew slowly with DBT as the sole sulfur source, presumably because there were trace amounts of sulfur on the glassware or in the medium components; however, as shown in Table 2, mutants CPE-648 and GPE-362 were completely unable to metabolize DBT, even after extended incubation (6 to 10 days). Further characterization indicated that the mutants also lacked the ability to convert DBT-sulfoxide and DBT-sulfone to 2-HBP (DBT-sulfonate is not commercially available and therefore was not tested). After we cloned the desulfurization genes (see below), we performed a Southern hybridization analysis (data not shown) and determined that both CPE-648 and GPE-362 are deletion mutants in which all of the desulfurization pathway is missing.

TABLE 2. Conversion of substrate to 2-HBP[a]

Strain	Conversion of:		
	DBT	DBT-sulfoxide	DBT-sulfone
IGTS8	+	+	+
CPE-648	−	−	−
GPE-362	−	−	−
CPE-648/pTOXI-1	+	+	+
GPE-362/pTOXI-1	+	+	+
R. fascians/pTOXI-1	+	+	+
JM109/pTOXI-1	−	−	−
JM109/pDRDsz	+	+	+

[a] Strains were grown in BSM supplemented with 100 mM substrate for 3 to 5 days. +, complete conversion of substrate to 2-HBP, as determined by HPLC; −, no conversion of substrate to 2-HBP.

Cloning of the *dsz*$^+$ gene cluster. CPE-648 was transformed with a plasmid library of IGTS8 genomic DNA constructed in shuttle vector pRR-6. Complemented mutants were selected by determining growth in liquid minimal medium containing DBT as the sole sulfur source; this sulfur bioavailability selection procedure allowed only cells that could desulfurize DBT to grow significantly. (The sulfur bioavailability assay was performed in liquid medium because agar contains enough sulfur to allow growth of the mutant strains.) In addition to viable growth in liquid medium containing DBT as the sole sulfur source, transformants also produced 2-HBP, whereas control cultures transformed with plasmid alone did not. Single-colony isolates were obtained by spreading aliquots of the liquid cultures on RM plates supplemented with chloramphenicol, and plasmid DNAs were prepared from three independent clones. A preliminary restriction mapping analysis revealed that the three clones contained overlapping sequences. Only the smallest clone, pTOXI-1, was mapped in detail, and its restriction map is shown in Fig. 2.

pTOXI-1 was used to retransform CPE-648 and to transform *Rhodococcus fascians*, a related organism that does not desulfurize DBT. All of the CPE-648 and *R. fascians* transformants obtained with pTOXI-1 could convert DBT to 2-HBP, indicating that the genetic information required for DBT desulfurization resides on this plasmid.

DNA sequence analysis of the pTOXI-1 insert. Since we found that pTOXI-1 contained enough genetic information to specify DBT desulfurization, the insert was sequenced; 5.5 kb of this DNA sequence is shown in Fig. 3. An analysis of the DNA sequence data revealed that there were three ORFs which were transcribed in the same orientation. The genes encoded by the three ORFs were designated *dszA*, *dszB*, and

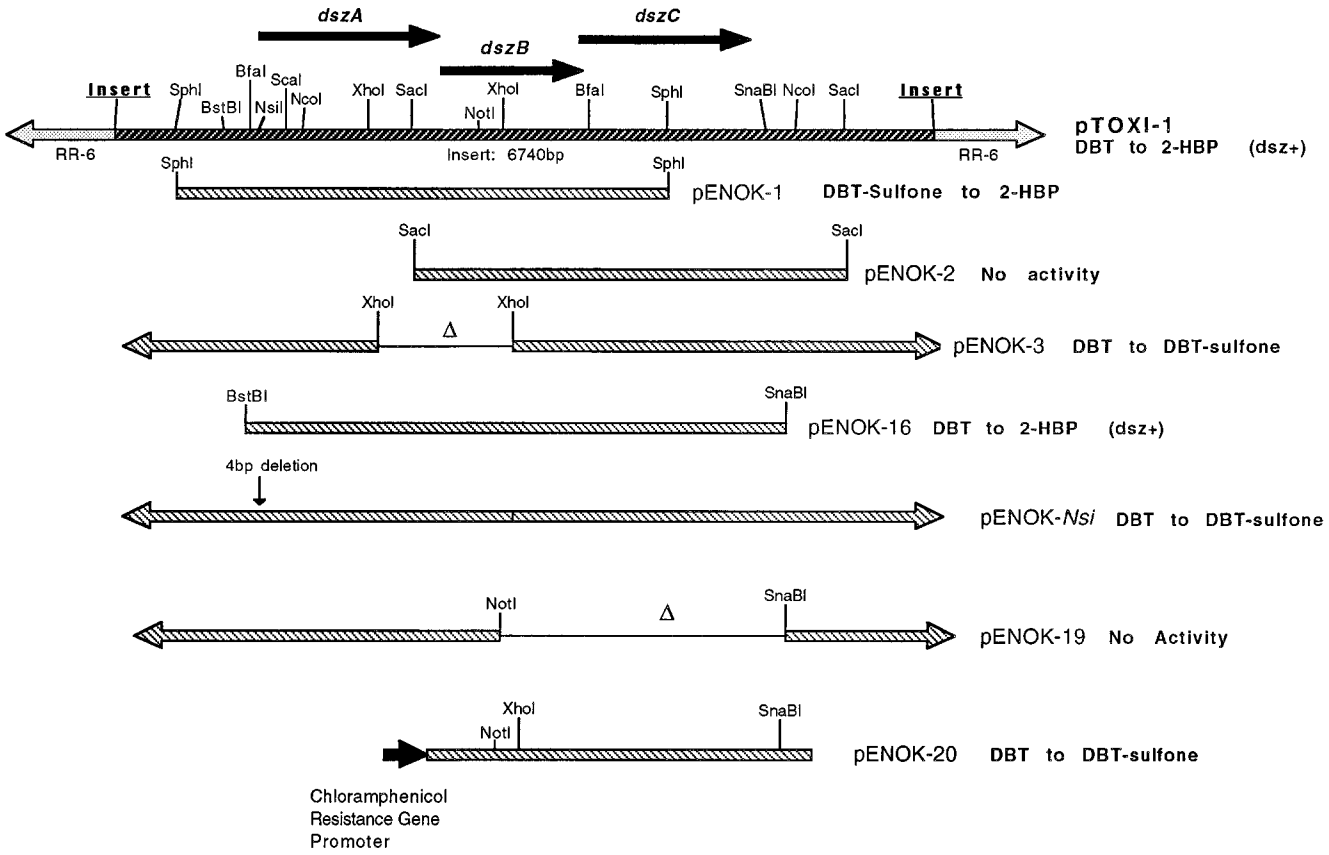

FIG. 2. Restriction map of cloned 6.7-kb insert of pTOXI-1 and subclones. All subclones were maintained in shuttle vector pRR-6 for introduction into *Rhodococcus* sp.

dszC; the predicted molecular masses of the proteins encoded by these three genes were 49.5, 38.9, and 45.1 kDa, respectively. The termination codon of *dszA* and the initiation codon of *dszB* overlapped, indicating that there may be translational coupling of these two genes. Between *dszB* and *dszC* there was a 13-bp gap. Potential ribosome binding sites were also present upstream of each putative ATG initiation codon (Fig. 3). The spacing and orientation of the three genes suggested that they were expressed as an operon, a suggestion which was also supported by the results of subclone analyses and promoter replacement analyses (see below). The genes and their predicted protein products were used to search the GenBank 83, EMBL 39 and Swiss-Prot 28 databases for homologous sequences. Using the FastP and FastN methods of Pearson and Lipman (13, 18) with default alignment score cutoff values of 100, we detected no significant levels of homology. A more stringent search (K_{TUP} = 1 for protein; K_{TUP} = 3 for DNA) involving potentially related sequences, such as oxygenase, dioxygenase, and sulfur metabolism-related sequences, also failed to detect significant levels of homology, thus indicating that the *dszA*, *dszB*, and *dszC* genes encode novel enzymes.

Expression in *E. coli* and maxicell analysis of *dsz* operon-encoded proteins. Previous experiments had shown that the cluster is not expressed in *E. coli* from the native *Rhodococcus* promoter. Therefore, we constructed vector pDRDsz, in which the *E. coli tac* promoter replaced the native promoter. We found that transformants of *E. coli* JM109 harboring pDRDsz grown in Luria-Bertani medium at 30°C produced 2-HBP from DBT when they were induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The specific activity was comparable to that obtained with IGTS8. Thus, the desulfurization-specific enzymes were stable and active in *E. coli*, and any required cofactors were also present. Also, replacement of the native promoter eliminated sulfur repression of the cluster, as shown by successful expression in a rich medium. Activity was much lower when transformants were grown at 37°C.

We also utilized expression in *E. coli* to demonstrate that the three ORFs encode proteins by performing a maxicell analysis (20). Proteins encoded by genes on plasmid DNA could be specifically radiolabeled in UV-irradiated *E. coli* cells. A maxicell analysis of JM109 harboring vector pDRDsz revealed that three novel protein bands were obtained compared with vector-only controls. The positions of these bands (49.5, 33.0, and 45 kDa) correlated well with the predicted molecular masses for the deduced amino acid sequences (49.5, 38.9, and 45.1 kDa for DszA, DszB, and DszC, respectively). Taken as a whole, the *E. coli* expression data indicated that the three predicted ORFs of pTOXI-1 encode three proteins which constitute the desulfurization pathway.

Functional analysis of the cluster. In order to determine the functions of the three genes, we subcloned various fragments of the full-length cluster, transformed them into deletion mutants, and determined the resulting phenotypes by performing a HPLC analysis in which DBT and DBT-sulfone were used as the substrates. Figure 2 shows a map of the subclones used in this analysis.

The minimal sequences required for desulfurization were

FIG. 3. DNA sequence and translation from pTOXI-1. The regions enclosed in boxes are potential Shine-Dalgarno ribosome binding sites.

FIG. 3—*Continued.*

defined by pENOK-16. This construction contains a 4-kb *Bst*BI-*Sna*BI fragment which begins 234 bp upstream of *dszA* and ends 80 bp downstream of *dszC*. Transformants of CPE-648 with pENOK-16 converted DBT to 2-HBP, thus clearly demonstrating that the three genes are sufficient for conversion of DBT to 2-HBP in strain IGTS8.

In order to assign specific functions to individual genes, *dszA* and *dszB* were first disabled while *dszC* was left intact. This was

accomplished in pENOK-3 by deleting a 1.1-kb *Xho*I fragment that spanned *dszA* and *dszB*. On the basis of DNA sequence data we predicted that this deletion would create an in-frame fusion between *dszA* and *dszB* such that a fusion protein terminating at the end of *dszB* would result. Thus, as a consequence of the in-frame deletion, polar effects on the expression of *dszC* were not anticipated. Transformants harboring pENOK-3 (*dszA dszB dszC*$^+$) converted DBT to DBT-sulfone. No production of 2-HBP was detected when either DBT or DBT-sulfone was used as the substrate. Thus, *dszC* apparently encodes a sulfur dioxygenase that catalyzes the conversion of DBT to DBT-sulfone.

In the next construction, *dszC* was disabled while *dszA* and *dszB* were left intact. This was accomplished in pENOK-1 by subcloning a 4.0-kb *Sph*I fragment that spanned *dszA* and *dszB* but truncated *dszC*. pENOK-1 (*dszA*$^+$ *dszB*$^+$ *dszC*) transformants converted DBT-sulfone to 2-HBP, but failed to metabolize DBT. This finding is consistent with the results obtained with pENOK-3 and directly demonstrates that *dszA* and *dszB* are sufficient to convert DBT-sulfone to 2-HBP.

From the results of these experiments it was clear that *dszC* is responsible for conversion of DBT to DBT-sulfone and that *dszA* and *dszB* are responsible for the conversion of DBT-sulfone to 2-HBP. In order to determine the specific functions of *dszA* and *dszB*, we next disrupted one of the genes by using conditions under which the other gene should be expressed. First, a small frameshift deletion was introduced into *dszA* to create pENOK-*Nsi*. This mutation was created by introducing a 4-bp deletion at the unique *Nsi*I site located 23 bp downstream from the predicted start of *dszA*. Transformants harboring this plasmid converted DBT to DBT-sulfone, but not to 2-HBP. Because the *dszC*-encoded oxygenase was clearly expressed in this clone, it seemed likely that *dszB* would also be expressed. Thus, the *dszB* gene product alone was apparently not capable of further metabolism of DBT-sulfone. However, because of the possibility of translational coupling between *dszA* and *dszB*, we were concerned that *dszB* might be poorly expressed as a consequence of the *dszA* frameshift mutation. Therefore, pENOK-20 was created in a second attempt to examine the function of *dszB* and *dszC* independent of the function of *dszA*. pENOK-20 contained a PCR-amplified fragment containing *dszB* and *dszC* fused to the chloramphenicol resistance promoter from pRF29. All transformants obtained with pENOK-20 (*dszA dszB*$^+$ *dszC*$^+$) converted DBT to DBT-sulfone, demonstrating that *dszC* was expressed in this construction. However, none of the transformants was capable of further metabolism of DBT-sulfone. This is consistent with the results obtained with pENOK-*Nsi* (see above), and together these data strongly suggest that the *dszB* gene product cannot utilize DBT-sulfone as a substrate.

In order to determine whether the *dszA* gene product could metabolize DBT-sulfone, we constructed pENOK-19 by deleting a *Not*I-*Sna*B1 fragment that spanned *dszB* and *dszC*. In this subclone only *dszA* should have been active. Transformants harboring pENOK-19 (*dszA*$^+$ *dszB dszC*) were not able to metabolize either DBT or DBT-sulfone, indicating that the *dszA*-encoded gene product was also not able to use DBT-sulfone. Thus, no further metabolism of DBT-sulfone was observed when either *dszB* (pENOK-*Nsi* and pENOK-20) or *dszA* (pENOK-19) was expressed separately, whereas expression of both *dszA* and *dszB* simultaneously (pENOK-1) resulted in conversion of DBT-sulfone to 2-HBP. These data indicate that the *dszA* and *dszB* gene products act in concert to convert DBT-sulfone to 2-HBP.

We also examined the activity of pENOK-2, which contained a 3.6-kb *Sac*I fragment. This fragment contained *dszB* and *dszC*, but contained truncated *dszA*. An analysis of pENOK-2 transformants revealed that no metabolism of either DBT or DBT-sulfone occurred. This finding is consistent with the hypothesis that the three genes are expressed as an operon as this construction lacked the promoter region.

Sulfur repression of desulfurization. Previous experiments performed with strain IGTS8 had demonstrated that no desulfurization activity could be detected in a rich medium such as RM. We studied whether high levels of inorganic sulfur in the medium resulted in either gene repression or inhibition of the desulfurization enzymes. When strain IGTS8 was grown in BSM supplemented with 40 mg of sulfate per liter, we observed an 80% reduction in the specific activity of 2-HBP production from DBT. When we used constructions in which alternative promoters, such as the promoter from the chloramphenicol resistance gene, replaced the native promoter, we observed no repression of activity in the presence of >2 g of sulfate per liter. Thus, regulation by sulfur acts by repression of the native *dsz* promoter.

DISCUSSION

We cloned the genes responsible for DBT desulfurization in *Rhodococcus* sp. strain IGTS8 by complementing *dsz* deletion mutants. DNA sequence and molecular subclone analyses revealed that the desulfurization pathway consists of three genes, *dszA*, *dszB*, and *dszC*. The organization of these genes initially suggested that they are expressed as an operon. The three genes are transcribed in the same direction; the termination codon for *dszA* and the initiation codon for *dszB* overlap; and there is only a 13-bp gap between *dszB* and *dszC*. However, such an organization does not constitute proof of an operon, as it is also not uncommon for promoters to overlap with adjacent structural genes in bacteria. Expression studies were also complicated by the fact that *dszA* and *dszB* had to be coexpressed in order to observe the activity of these genes. However, there is evidence which supports the hypothesis that the cluster is expressed as an operon. First, disabling or removing the promoter region prevented expression of all measurable enzymatic activities. Second, replacing the promoter region with alternative promoters relieved the sulfur repression normally observed at each step of desulfurization. And third, replacing the native promoter region with the *E. coli tac* promoter allowed expression of DBT desulfurization in *E. coli.*

Rhodococcus sp. strain IGTS8 desulfurizes DBT by a modification of the 4S pathway. The first step in the pathway is catalyzed by a sulfur dioxygenase encoded by *dszC*. In this single enzyme reaction DBT is converted directly to DBT-sulfone, without the production of DBT-sulfoxide as proposed for the 4S pathway. Thus, DBT-sulfoxide is not a diffusible intermediate in the IGTS8 desulfurization pathway. Feeding studies performed with subclones and DBT-sulfoxide were not feasible because commercial DBT-sulfoxide preparations are contaminated with significant amounts of DBT and DBT-sulfone. Also, when DBT-sulfoxide is incubated with nondesulfurizing organisms (CPE-648, GPE-362, *R. fascians*, *E. coli* JM109), is rapidly oxidized to DBT-sulfone.

The second step in the desulfurization pathway is cleavage of the carbon-sulfur bonds in DBT-sulfone to yield 2-HBP. We found that the gene products of *dszA* and *dszB* acting in concert are responsible for this activity. It is interesting that the termination codon of *dszA* and the initiation codon of *dszB* overlap, suggesting that translational coupling may occur. Translational coupling is believed to aid coordinated expression of related genes. This suggests that there may be a requirement for stoichiometric amounts of the gene products

and/or physical interaction between the gene products. Consistent with the latter possibility, our results showed that both gene products must be expressed simultaneously in order for DBT-sulfone to be further metabolized.

In the 4S pathway DBT-sulfonate is also a proposed intermediate. In a more recent report, Gallagher et al. suggested that 2′-hydroxybiphenyl-2-sulfinate and 2′-hydroxybiphenyl-2-sulfonate are also intermediates in the IGTS8 desulfurization pathway (6). Our results do not support the hypothesis that these compounds are diffusible intermediates. Prolonged incubation (6 to 10 days) of DBT-sulfone with cultures that express only *dszA* or *dszB* does not result in further metabolism of DBT-sulfone (i.e., the mass balance is maintained as DBT-sulfone), whereas cultures that express both genes rapidly convert DBT-sulfone to 2-HBP. However, our results do not rule out the possibility that there are transient enzyme-bound intermediates which may occasionally be liberated.

It is also interesting that none of the desulfurization genes or the deduced encoded proteins exhibited significant levels of sequence similarity with any of the sequences obtained from the GenBank, EMBL, and Swiss-Prot databases. Thus, enzymes which we studied are unique in that they currently have no apparent counterparts in the databases. This is most likely due to the paucity of information concerning sulfur dissimilatory pathways.

In a recent report, Denome et al. (2) have also described the cloning of the *Rhodococcus* sp. strain IGTS8 desulfurization cluster by similar methods. Although no sequence data are provided, the restriction maps of Denome et al. indicate that the same cluster was cloned. There are, however, several differences between our data and the data of these authors. Denome et al. reported that IGTS8 cannot utilize DBT-sulfoxide as a sulfur source, whereas in our experiments we observed that it could. The reasons for this discrepancy are not clear. Denome et al. reported that the *dsz* cluster is not expressed in *E. coli* from either the native or *lac* promoters. We also found that the native promoter is not recognized in *E. coli*, but, in contrast, found that the cluster was expressed efficiently from the *tac* promoter. This discrepancy can be explained by the fact that in the study of Denome et al., the cluster appeared to have been fused to the *lac* promoter approximately 4 kb upstream of the coding sequences, whereas in our constructions the *tac* promoter was fused 16 bp upstream of the cluster.

The next challenge is to use the cloned genes to engineer strains that can more efficiently remove organic sulfur from DBT and, by extension, from fossil fuels. Preliminary work has shown that increased specific activity for desulfurization of DBT can be obtained by increasing the copy number and that sulfur repression can be alleviated by promoter replacement.

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