Elucidation of the metabolic pathway for dibenzothiophene desulphurization by Rhodococcus sp. strain IGTS8 (ATCC 53968)

Christopher Oldfield,1† Olga Pogrebinsky,1 Julie Simmonds,1 Edwin S. Olson2 and Charles F. Kulpa3

Author for correspondence: Christopher Oldfield. Tel: +44 131 455 2217. Fax: +44 131 455 2291. e-mail: c.oldfield@napier.ac.uk

Rhodococcus sp. strain IGTS8 (ATCC 53968) is able to utilize dibenzothiophene (DBT) as a sole source of sulphur. The carbon skeleton of DBT is not metabolized and is conserved as 2-hydroxybiphenyl (HBP), which accumulates in the medium. This phenotype is due to the expression of the plasmid-encoded DBT-desulphurization (dsz) operon, which encodes three proteins, DszA, B and C. In this paper it is shown, using [35S]DBT radiolabelling studies, that sulphur is released in the form of inorganic sulphite. The pathway of DBT desulphurization is described in detail. In summary, DszC catalyses the stepwise S-oxidation of DBT, first to dibenzothiophene 5-oxide (DBTO) and then to dibenzothiophene 5,5-dioxide (DBTO2); DszA catalyses the conversion of DBTO2 to 2-(2'-hydroxyphenyl)benzene sulphinate (HBPSi-); and DszB catalyses the desulphination of HBPSi- to give HBP and sulphite. Studies with cell-free extracts show that DszA and DszC, but not DszB, require NADH for activity. 18O2-labelling studies show that each incorporated oxygen atom is derived directly from molecular oxygen. These results are consistent with the role of DszC as a mono-oxygenase, of DszA as an apparently unique enzyme which catalyses the reductive hydroxylation of DBTO2, leading to cleavage of the thiophene ring, and of DszB as an aromatic sulphinic acid hydrolase.

Keywords: Rhodococcus sp. strain IGTS8, fossil fuel desulphurization, clean technologies, desulphurizing enzymes

INTRODUCTION

Crude oil and its distillates contain significant amounts of low-molecular-mass organosulphur compounds such as alkyl- and cycloalkyl thiols, alkyl- and arylthioethers and aromatic heterocycles based on thiophene. This last group includes thiophene itself, benzothiophene, dibenzothiophene, and their alkylated derivatives. Combustion of these compounds results in the release into the atmosphere of sulphur oxyacids (SOx) and hence to acid rain. Atmospheric SO2 is a major contributing factor to poor air-quality in the city environment and acid rain is a primary cause of global deforestation since it lowers the soil pH to levels intolerable for many trees and plants. As a consequence legislation has been enacted by Scandinavia, the EU, the USA and Japan which requires progressive annual reductions in the sulphur content of petrochemicals (CONCAWE, 1994). Thus there is considerable interest in the development of petrochemical desulphurization technologies. One approach involves the application of micro-organisms selected for their ability to specifically degrade the organosulphur component. A key organism in this context is IGTS8 (ATCC 53968), provisionally identified as a strain of Rhodococcus erythropolis (M. Goodfellow, C. Oldfield, A. S. A. Teboli, M. D. Collins & J. Chun, unpublished results) which is capable of utilizing dibenzothiophene (DBT; see Fig. 4 for structure) as sole
source of sulphur. Previous work has shown that strain IGTS8 degrades DBT to 2-hydroxybiphenyl (HBP). HBP is not further metabolized and IGTS8 is unable to use DBT as a source of carbon (Kayser et al., 1993). A number of alkyl-substituted derivatives of DBT have also been shown to be desulphurized to the corresponding monophenol (Ohshiro et al., 1996a). This ‘desulphurization’ phenotype is conferred by the plasmid-located dsz operon, which encodes three proteins, DszA, B and C (Denome et al., 1993, 1994).

Expression of the dsz operon enzymes is repressed by sulphide, sulphate, methionine and cysteine (Li et al., 1996; Ohshiro et al., 1996b). GC-MS analysis of ethyl acetate extracts of strain IGTS8 culture media containing DBT as sole sulphur source revealed the presence of dibenzothiophene 5-oxide (DBTO), dibenzothiophene 5,5-dioxide (DBTO₂), biphenylene sulthine (BPSi), biphenylene sulftone (BPSo) and 2,2′-dihydroxybiphenyl (DHBP), in addition to HBP (Olson et al., 1993). Based on these observations, several alternative desulphurization pathways were outlined (Gallagher et al., 1993).

This paper reports a detailed characterization of the DBT-desulphurization pathway of strain IGTS8. Experiments with [³⁵S]DBT were carried out to identify the sulphur-containing product of the reaction. In order to identify the precise metabolic route from DBT to HBP, all of the sulphur-containing intermediates identified by Olson et al. (1993) were chemically synthesized and incubated with whole cells of strain IGTS8, with product analysis by GC-MS. The identity of the dsz-operon gene products responsible for each step in the pathway was deduced from experiments in which Escherichia coli subclones producing DszA, DszB or DszC were incubated with the various intermediates. Experiments with cell-free extracts of strain IGTS8 were carried out to deduce which of the dsz operon enzymes required NADH for activity. Finally, [¹⁸O] labelling studies were carried out to ascertain which of the steps in the pathway require molecular oxygen.

**METHODS**

**Materials.** Suppliers were as follows: sulphite oxidase (suspension in 2.3 M (NH₄)₂SO₄), bovine-heart cytochrome c, the buffers HEPES and HEPPS, Gibbst reagent (2,6-dichloroquinone 4-chloromide) and ACS-grade sodium sulphite, Sigma; ACS-grade H₂O₂ (30%), DBT (99+%), DBTO (97%), HBP (sold as 2-phenylphenol, 99+%) and DHBP (99%), Aldrich; t-buty hypochlorite, K₂CO₃, and dichlorodicyanobenzene (DDC), Fluka; n-butyl hypochlorite, K₂CO₃, and dichlorodicyanobenzene (DDC), Fluka; H₂O₂ (30%), DBT, DBTO, DBTO₂, BPSi and BPSo were stored in solid form in a desiccator at room temperature. There was no evidence of decomposition, as judged by GC-MS, over the period of this study.

**Bacteria.** Rhodococcus sp. strain IGTS8 (ATCC 53968) was obtained from J. Kilbane (Institute of Gas Technology, Chicago, IL, USA). Strain CPE648, generated from IGTS8 by UV mutagenesis and lacking the entire dsz operon, was used as a control organism. Strain CPE648 was transformed with plasmid pENOK3 to obtain a strain expressing DszC activity only (Piddington et al., 1995). CPE648 and pENOK3 were obtained from C. Piddington (Panlabs, Inc., Bothell, WA, USA).

The recombinant E. coli strains used in this work have already been described (Denome et al., 1994). The host strain was E. coli strain MZ-1 transformed with plasmids pSAD267-1 (expressing DszA), pSAD277-7A (DszB), pSAD269-2A (DszC), or pSAD262-1 (no insert). The plasmids each carry the ampicillin-resistance marker. Expression of the recombinant genes is under the control of the bacteriophage λ promoter and the thermosensitive lacZ′ repressor.

**Culture conditions.** Rhodococcus sp. strain IGTS8 was cultured in standard Rhodococcus medium (SRM), which has the following composition (per litre): NaHPO₄, 4·33 g; KH₂PO₄, 2·65 g; glucose, 20 g; NH₄Cl, 2 g; MgCl₂.6H₂O, 0·64 g; nitritotriacetic acid, 0·1 g; CaCl₂.2H₂O, 33 mg; ZnCl₂, 2·6 mg; FeCl₃.4H₂O, 2·6 mg; EDTA, 1·25 mg; MnCl₂.4H₂O, 1·0 mg; CuCl₂.2H₂O, 0·15 mg; Co(NO₃)₂.6H₂O, 0·125 mg; Na₂B₄O₇.10H₂O, 0·10 mg; (NH₄)₂MoO₄.4H₂O, 0·09 mg. Sodium sulphate was added as a filter-sterilized solution after autoclaving (121 °C, 20 min). The final pH was 7·2 without titration.

Sodium sulphate (1·5 mM) was used as a sulphur source for cultures of IGTS8 expressing no desulphurizing activity. DMSO (1·5 mM, added after autoclaving) was substituted for sulphate to obtain IGTS8 expressing DBT-desulphurizing activity. [Note: DMSO is not a substrate for the DBT-desulphurizing enzyme system. Thus dsz mutants of strain IGTS8, such as CPE648, grow equally well with DMSO. Expression of DBT-desulphurization activity in the presence of DMSO is due to the derepression of the dsz operon in the absence of more readily bioavailable sulphur (sulphide, sulphate, cysteine and methionine (Li et al., 1996).] Cells grown with DMSO were entirely free of DBT metabolites as judged by GC-MS. For certain experiments cells were grown with DBT or one of the putative metabolic intermediates as sole sulphur source, added following autoclaving from a stock solution (40 mM in acetone/tet; except BPSi, 10 mM in ethanol), to a final concentration of 200 μM (final solvent concentration <1%, v/v). Cultures of CPE648 were grown using 1·5 mM sulphate or 1·5 mM DMSO as required.

 Cultures were maintained on SRM agar plates (Bacto Difco agar, 20 g l⁻¹) containing either 200 μM DBTO₂ (strain IGTS8) or 1·5 mM sulphate (strain CPE648), subcultured every week.
Liquid media (250 ml in a 2 l Erlenmeyer flask) were inoculated with a loopful of cells from the plate and incubated in an orbital shaker (250 r.p.m., 30 °C) until the end of the exponential growth phase (approximately 80 h). The specific activity for DBT-desulphurization was maximal at this point (C. Oldfield & J. Simmonds, unpublished data). The cells were recovered by centrifugation (15 000 g for 15 min), washed twice by re-suspension in 10 vols 50 mM HEPPS buffer, pH 8.0, and finally resuspended in the same buffer to a density of approximately OD400 250 (Beckman DU 700 UV-Vis spectrophotometer, 1 cm path-length cell), equivalent to 88 g dry weight l−1. The suspension was kept on ice and used on the day of harvesting.

Standard Gibbs assay for HBP. The stock OD400 250 cell suspension was diluted with 50 mM HEPPS, pH 8.0, to a nominal OD400 of 1.0. This was done by diluting the suspension to give a spectrophotometer reading of OD400 0.1–0.2, and calculating the dilution factor required to obtain an OD400 of 1.0 (OD400 1.0 corresponds to a cell density of 0.35 g (dry wt) l−1). Substrate (25 μM final concentration, introduced from a 40 mM stock in acetone, except for BPSi, 10 mM in ethanol) was added to 40 ml freshly diluted OD400 1.0 cell suspension in a 100 ml stopped Erlenmeyer flask and incubated in an orbital shaker (240 r.p.m., 30 °C) for 60 min. At 10 min intervals 1.5 ml aliquots were removed to Eppendorf tubes and centrifuged (12 000 r.p.m., 5 min) to remove cells. A 1.0 ml aliquot of the supernatant was transferred to a 1 ml disposable spectrophotometer cuvette and stored at 4 °C until the end of the incubation. Gibbs reagent (10 μl, 10 mM in acetone) was then added to each cuvette. A blank solution (HEPPS buffer pH 8.0 plus 10 μl Gibbs reagent) was also prepared. The assays were incubated overnight at 30 °C, for full colour development, and the A550 was measured. The ΔA550 was converted to HBP concentration using a standard curve prepared with authentic HBP in the range 0–30 μM. The linear regression slope of a plot of HBP concentration vs time gave the reaction rate (μM h−1); the specific activity (μmol g−1 h−1) was obtained by dividing the rate by the cell concentration (g dry wt l−1). The substrate concentration used was about twice which could be converted by an OD400 1.0 suspension of cells in 1 h [specific activities were typically of the order of 25–40 μmol (g dry wt)−1 h−1]. Under these conditions the plots of HBP concentration vs time were invariably linear (see Fig. 3).

Strain IGTS8 also converted BPSi to DHBP. This reaction was assayed using the standard Gibbs assay as described above, except that authentic DHBP was used to construct the standard curve.

Spectrophotometric assay for sulphite. Sulphite was estimated on a routine basis using a modification of the sulphite-oxidase-linked assay (oxidation of sulphite to sulphate, linked to the reduction of cytochrome c) described by Beutler (1987). The assay was carried out as described above for HBP except that at the end of the incubation cytochrome c (20 μl of a 5 mM solution in 50 mM HEPPS, pH 8) was added to each cuvette. Assays were incubated at room temperature for 5 min and the A540 was read. Two microlitres of the Sigma sulphite oxidase suspension was then added to each cuvette and the set was incubated at room temperature for a further 5 min. The A550 was read again and the ΔA550 was converted to sulphite concentration using a standard curve prepared with sodium sulphite.

Reversed-phase (RP-) HPLC assay for organic metabolites. The assay was carried out as for the Gibbs HBP assay except that 0.75 ml aliquots were removed to Eppendorf tubes at each 10 min time-point and an equal volume of acetonitrile added, with thorough mixing. The cells were removed by centrifugation (12 000 r.p.m. for 5 min). The supernatant was analysed using a Hewlett-Packard series 1050 liquid chromatograph equipped with a diode-array detector and fitted with a Synchropak RP-C18 column (4.6 × 100 mm) and a 10 μl loop. The column was eluted with helium-degassed phosphate buffer (10 mM, pH 6.0)/acetonitrile (1:1, v/v) and washed with acetonitrile between runs. Approximate retention times (min) were as follows: HEPPS buffer, 0.8; DHB, 1.1; DBTO, 1.2; DBTO2, 1.5; HBP, 1.8; BPSi, 1.9; BPSo, 2.1; DBT, 4.6. Any ambiguities in assigning metabolite peaks were resolved by comparing the diode-array spectra with those of standards.

[33]DBT radiolabelling studies. Strain IGTS8 was grown in SRM with either DBF (unlabelled) or Na2SO3 as sole sulphur source. The stock OD400 250 cell suspension was diluted to OD400 1.0 with 50 mM HEPPS, pH 8.0. [33]DBT (supplied as a solution in ethanol) was diluted with unlabelled DBT to give a stock solution (25 mM in ethanol; specific activity 43 mCi mmol−1; 1.59 GBq mmol−1). Sixteen microlitres was added to 40 ml of each cell suspension (final DBT concentration, 10 μM) and incubated in an orbital shaker (250 r.p.m., 30 °C) for 10 min. Aliquots (1 ml) were withdrawn at 2 min intervals and the cells were removed by centrifugation. Supernatants were analysed by ion-exchange chromatography using a Dionex 2000i/SP chromatograph equipped with an anion micromembrane suppresser loaded with 12.5 mM H2SO4. Sample aliquots (50 μl) were eluted from an Ion-Pac AG9-SC analytical column using a mobile phase of 18 mM Na2CO3 and 17 mM NaHCO3 at a flow-rate of 2.0 ml min−1. Fractions of 500 μl were collected and diluted into 10 ml scintillation fluid. Radioactivity in each vial was measured by counting for 10 min in a Beckman LS 5000 scintillation counter, with c.p.m./d.p.m. conversion based on 85% counting efficiency for 35S measured using authenticated standards. Quenching was negligible at the count rates obtained in these experiments. Authentic 34SO2 and 35SO42− eluted at approximately 7.1 and 9.1 min, respectively. Counts were recovered with >95% efficiency.

Isolation of organic metabolites and GC-MS analysis. Strain IGTS8 was grown in SRM with DMSO as sole sulphur source. A 100 ml volume of the OD400 250 cell suspension (approximately 9 g dry wt of cells) was diluted to 400 ml with 50 mM HEPPS buffer, pH 8.0. The required substrate was added to final concentration of 200 μM and the suspension was incubated overnight in an orbital shaker (250 r.p.m., 30 °C). The cells were then removed by centrifugation and the supernatant was titrated to pH 1 with HCl. An equal volume of ethyl acetate was added and the mixture stirred for 4 h. The ethyl acetate phase was recovered and dried by stirring for 1 h with anhydrous MgSO4. The ethyl acetate was removed by rotary evaporation and the solids were redissolved in 3 ml ethyl acetate. Uncharged compounds such as DBT, DBTO, DBTO2, and BPSO partitioned readily into the organic phase and HBP using first condensing to BPSi, a
reaction favoured by low pH (Fig. 1). GC-MS analysis was carried out as described previously (Olson et al., 1993).

**18O₂ labelling studies.** Strain IGTSS was grown with DMSO as sole sulphur source. A 25 ml aliquot of the OD₆₀₀ 250 cell suspension was placed in a 50 ml polypropylene centrifuge tube and capped with a rubber septum carrying a syringe needle. The tube was alternately degassed by vacuum and flushed with helium (3 x 10 min cycles). After the final evacuation the syringe needle was removed. Substrate was added to a final concentration of 200 μM, and ¹⁸O₂ (96 atom%) was introduced to a pressure slightly above 1 atm, using gas-tight microsyringes. The tube was incubated overnight (30°C with shaking) and the reaction products were extracted and analysed by GC-MS as described above.

**Preparation and assay of cell-free extracts.** Strain IGTSS was grown in SRM with DMSO as sole sulphur source. The cells were washed twice with 10 vols 50 mM HEPES, pH 7.5, and resuspended in the same buffer, containing DTT (1 mM) and PMSF (30 μg ml⁻¹), to OD₆₀₀ 250. The cells were broken by a single pass through a French pressure cell at an operating pressure of 2200 p.s.i. (15.2 MPa). MgCl₂ (10 mM), DNase (0.1 mg ml⁻¹) and RNase (0.1 mg ml⁻¹) were added and the mixture was stirred at 4°C for 30 min. Cell debris was removed by centrifugation at 39,800 g for 60 min. The supernatant was diluted to 10 mg protein ml⁻¹ with 50 mM HEPES, pH 7.5. Protein concentrations were estimated using the Bio-Rad protein assay kit. The extract was divided into equal volumes of acetonitrile, vortexed thoroughly and centrifuged at 12,000 r.p.m. for 10 min. The supernatant was diluted with an equal volume of acetonitrile, vortexed thoroughly and analysed by RP-HPLC and IP-HPLC, as described above.

**RESULTS**

Sulphite is the sulphur-containing product of the desulphurization reaction

To identify the sulphur-containing product of the desulphurization reaction, a batch of cells with a specific DBT-desulphurizing activity of 12 μmol g⁻¹ h⁻¹, measured using the standard Gibbs assay, was incubated with [³⁵S]DBT, as described in Methods. Ion-exchange chromatography of the recovered incubation medium revealed the presence of water-soluble radiolabelled material which eluted as a single peak with a retention time of 7.1 min, the same as that of ³⁵SO₃⁻ (Fig. 2a). It colony and incubating overnight in an orbital shaker (250 r.p.m., 30°C). Then 500 ml of SRMS in a 2 l Erlenmeyer flask was inoculated with 10 ml starter culture and incubated under the same conditions to OD₆₀₀ 0.4 (about 4.5 h). The temperature was then raised to 39°C for 2 h to derepress the Dsz proteins. The cells were recovered by centrifugation (15,000 g for 15 min), washed twice with 10 vols SRMS and finally resuspended in SRMS to a nominal OD₆₀₀ of 10. The required substrate was added to a final concentration of 100 μM. After overnight incubation in an orbital shaker (250 r.p.m.; 30°C), 0.75 ml aliquots were transferred to Eppendorf tubes and the cells removed by centrifugation (12,000 r.p.m., 10 min). The supernatant was diluted with an equal volume of acetonitrile, vortexed thoroughly and analysed by RP-HPLC and IP-HPLC, as described above.

![Fig. 1. The pH-dependent interconversion between HBPSi and BPSi. The HBPSi → BPSi equilibrium is shifted right on decreasing the pH. At pH 1, BPSi is the sole species. The interconversion was followed by UV spectrophotometry (Hanson & Kemp, 1981).](image-url)

![Fig. 2. Sulphite production from [³⁵S]DBT by Rhodococcus sp. strain IGTSS. (a) Strain IGTSS was grown in SRM with unlabelled DBT as sole sulphur source. Cells were washed twice in 10 vols HEPES buffer, pH 8.0, and resuspended to a density of OD₆₀₀ 1.0 in the same buffer. [³⁵S]DBT (specific activity 43 mCi mmol⁻¹) was added to a final concentration of 10 μM and the preparation was incubated in an orbital shaker (250 r.p.m., 30°C) for 10 min. Aliquots (1 ml) were removed at 2 min intervals and the cells removed by centrifugation. The supernatants were analysed by ion-exchange chromatography as described in Methods. The peak at 7.1 min corresponds to ³⁵SO₃⁻ (calibration using Na₂³⁵SO₃). Times of incubation of strain IGTSS with [³⁵S]DBT: ●, 0 min; ○, 2 min; □, 4 min; ▲, 6 min; △, 8 min. (b) Plot of medium ³⁵SO₃⁻ concentration vs incubation time. The slope corresponds to a specific activity of 14 μmol (g dry wt)⁻¹ h⁻¹.](image-url)
Dibenzothiophene desulphurization pathway

Fig. 3. Time-course of the conversion of (a) DBT, (b) DBTO and (c) DBTO₂ to HBP, and of (d) PSO to DHBP, by whole cells of *Rhodococcus* sp. strain IGTS8. Cells were grown to the end of the exponential phase in SRM with DBTO as sole sulphur source, washed twice with 10 vols 50 mM HEPPS buffer, pH 8.0, and finally resuspended in the same buffer. The suspension was portioned into 40 ml aliquots and the required substrate was added to a final concentration of 25 μM. At each time-point, aliquots were withdrawn for analysis by RP-HPLC, Gibbs assay and sulphite oxidase assay, as described in Methods. ○, DBT; △, DBTO; □, DBTO₂; ▽, HBP; ▼, PSO; ●, DBHP. Molar mass-balance, calculated by summing the molar substrate and product(s) concentrations at each time point. In these experiments only substrate and desulphurized product (HBP or DHBP) could be detected, except in the incubation with DBT, where DBTO was also observed. The lower limit for detection was approximately 0.5 μM.

was therefore concluded that sulphur was released from DBT as sulphite.

The plot of sulphite produced vs time was linear (Fig. 2b) with a slope of 5 μM h⁻¹, corresponding to a specific desulphurization activity of 14 μmol (g dry wt)⁻¹ h⁻¹. This compared well with the specific activity calculated from the assay of HBP production.

No radiolabelled sulphite was produced in incubations of sulphate-grown IGTS8 with [³⁵S]DBT under otherwise identical conditions, and these cells likewise produced no detectable HBP.

**Whole cells of *Rhodococcus* sp. strain IGTS8 desulphurize DBT, DBTO and DBTO₂ to HBP and sulphite with equal efficiency**

Strain IGTS8, but not the *dsz* mutant CPE648, grew in SRM containing DBT, DBTO or DBTO₂ (200 μM) as the sole source of sulphur. HBP accumulated in the medium during growth, reaching a final concentration of 200 μM, as determined by RP-HPLC, by the end of the exponential growth phase (C. Oldfield & J. Simmonds, unpublished data).

The kinetics of DBT, DBTO and DBTO₂ desulphurization by strain IGTS8 was determined (Fig. 3, Table 1). Each of these compounds was desulphurized to HBP and sulphite with approximately the same specific activity as measured in terms of HBP or sulphite accumulation in the medium (Table 1). With DBT, reproducible transient accumulation of DBTO was observed (Fig. 3a). With DBTO and DBTO₂, no peak other than HBP was apparent in any of the HPLC traces. The desulphurization of DBTO by strain IGTS8 in these experiments contradicts an earlier report (Denome *et al.*, 1994), but was reproducible in this study.

The rate of disappearance of substrate from the medium was faster than the rate of HBP accumulation (Table 1). However after overnight incubation the HBP concentration reached the maximum value (25 μM) expected assuming a 1:1 substrate:HBP reaction stoichiometry (data not shown). This phenomenon was also observed in growing cultures, where the substrate was entirely consumed in the early stages of growth but the HBP concentration did not reach the theoretical maximum until the end of the exponential growth phase (C. Oldfield & J. Simmonds, unpublished data). It was therefore concluded that the substrate became transiently adsorbed to the cell envelope prior to conversion to HBP.

**DszC oxidizes DBT to DBTO₂**

When *E. coli* strain MZ-1(pSAD269-2A), expressing *dszC*, was incubated overnight in SRMS with DBT or DBTO (100 μM), the substrate concentration was
Table 1. Specific desulphurization activity of DBT and its metabolites by whole cells of *Rhodococcus* sp. strain IGTS8

The assay methods are indicated in parentheses. See legend to Fig. 3 for details of incubations. Specific activities were calculated as described in Methods, using the slopes of the plots shown in Fig. 3. Specific activities calculated in terms of product formation (HBP or DHBP and sulphite) for different batches of cells grown under the same conditions were reproducible to ±5%. Specific activities estimated on the basis of substrate disappearance were always fractionally larger than those estimated by product formation, but the variance was higher; this was attributed to a variable degree of binding of the substrate to the bacterial cell wall (see text).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity [μmol (g dry wt)⁻¹ h⁻¹]</th>
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<tbody>
<tr>
<td></td>
<td>Substrate consumption (HPLC)</td>
</tr>
<tr>
<td>DBT</td>
<td>57</td>
</tr>
<tr>
<td>DBTO</td>
<td>219</td>
</tr>
<tr>
<td>DBTO₂</td>
<td>54</td>
</tr>
<tr>
<td>BPSo*</td>
<td>48</td>
</tr>
</tbody>
</table>

* The products of BPSo desulphurization are DHBP and sulphite.

Table 2. Metabolism of DBT desulphurization pathway intermediates by whole cells of recombinant *E. coli* strain MZ-1 expressing proteins of the *dsz* operon of *Rhodococcus* sp. strain IGTS8

Recombinant strains of *E. coli* strain MZ-1, expressing individual proteins of the *dsz* operon of *Rhodococcus* sp. strain IGTS8, were grown and assayed as described in Methods. Figures in parentheses are micromolar concentrations estimated using RP-HPLC, except for HBP⁺⁺ (++, present, (−), absent, as determined by IP-HPLC). Products are shown in bold type. It was concluded that the concentration values for each strain/substrate combination did not sum to 100 μM due to binding of substrates and/or products to the cells (see text). ND, Not determined.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Metabolites accumulated with substrate (100 μM) shown:</th>
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<tbody>
<tr>
<td></td>
<td>DBT</td>
</tr>
<tr>
<td>MZ-1(pSAD267-1) (DszA)</td>
<td>DBT (65)</td>
</tr>
<tr>
<td></td>
<td>DBTO (0)</td>
</tr>
<tr>
<td></td>
<td>DBTO₂ (0)</td>
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<td>HBP (0)</td>
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<tr>
<td>MZ-1(pSAD277-7A) (DszB)</td>
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<td>HBPSi⁻⁻</td>
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<td></td>
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<td>MZ-1(pSAD269-2A) (DszC)</td>
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<td>HBP (0)</td>
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<tr>
<td>MZ-1(pSAD267-1) + MZ-1(pSAD277-7A) (DszA + DszB)*</td>
<td>ND</td>
</tr>
</tbody>
</table>

* In this experiment *E. coli* strain MZ-1(pSAD267-1), expressing *dszA*, and strain MZ-1(pSAD277-7A), expressing *dszB*, were grown and derepressed separately. Equal volumes of the washed suspensions were then mixed for the incubation.
greatly reduced and DBTO₂ was detectable in the supernatants (Table 2). In incubations with DBT, a small amount of DBTO was also detected. No other compounds were detected by RP- or IP-HPLC. No DBTO₃ was detected if dszC was not first de-repressed by incubation at 39 °C (data not shown).

When strain MZ-1(pSAD267-1), expressing dszA, or strain MZ-1(pSAD277-7a), expressing dszB, was incubated overnight in SRMS with DBT or DBTO, some disappearance of substrate was noted (Table 2), but no products were detected. Disappearance of substrate was once again attributed to binding of the substrate to the cell and for this reason assignments of enzymes on the reaction pathway were made exclusively on the appearance of product.

Based on these data, it was concluded that expression of the dszC gene-product alone was sufficient to confer on E. coli the ability to oxidize DBT or DBTO to DBTO₂. Since DBTO, as well as DBT, is a substrate for this enzyme, and given that DBTO accumulates transiently in incubations of both strain MZ-1, expressing dszC, and strain IGTSS, with DBT (Fig. 3a), it was concluded that DszC catalyses the sequential oxidation DBT → DBTO → DBTO₂. The results of the ¹⁸O₂ labelling studies, described below, were consistent with this interpretation.

**DszA converts DBTO₂ to HBPSi⁻ and DszB converts HBPSi⁻ to HBP**

GC-MS analysis of ethyl acetate extracts of supernatants from incubations of strain IGTSS (whole cells) with DBT, DBTO and DBTO₂ revealed the presence of BPSo at concentrations well below the HPLC detection limit (approx. 1 μM), confirming the results of the earlier study (Olson et al., 1993).

BPSo is unstable in aqueous media at neutral pH, and would be present solely as its hydrolysed form, HBPSi⁻ (Fig. 1). It was reasonable to conclude that HBPSi⁻ is the penultimate intermediate in the desulphurization pathway since HBPSi⁻ → HBP would only require the cleavage of a single C–S bond.

When E. coli strain MZ-1(pSAD267-1), expressing dszA, was incubated overnight in SRMS with DBTO₂, DBTO₂ disappeared completely from the medium, as judged by RP-HPLC of the supernatant, and HBPSi⁻ was detected using IP-HPLC (Table 2). The presence of BPSo in ethyl acetate extracts of the acidified supernatant, as revealed by GC-MS, was consistent with the presence of HBPSi⁻ in the untreated supernatant (data not shown).

When DBTO₂ was incubated with cell suspensions of strain MZ-1 expressing dszB or dszC, there was some disappearance of DBTO₃, once again attributed to binding of the substrate to the cells (Table 2), but HBPSi⁻ was never detected in the supernatant and BPSo was never detected in ethyl acetate extracts. It was therefore concluded that expression of dszA was necessary and sufficient to confer on strain MZ-1 the ability to convert DBTO₂ to HBPSi⁻.

No HBP was detectable in the HBPSi⁻-containing supernatant from the incubation of DBTO₃ with strain MZ-1(pSAD267-1) expressing dszA. However, when the supernatant was incubated overnight at 30 °C with strain MZ-1(pSAD277-7A), expressing dszB, HBP was produced. This result was only obtained if the cells had first been incubated at 39 °C to derepress dszB (data not shown). Similarly, incubation of DBTO₂ with a mixture of strain MZ-1(pSAD267-1), expressing dszA, and strain MZ-1(pSAD277-7A), expressing dszB, grown and de-repressed separately, resulted in considerable HBP production (Table 2).

To confirm that HBPSi⁻ was an intermediate in the pathway, the ability of Rhodococcus sp. strain IGTSS to use this compound as a sulphur source was investigated. Strain IGTSS grew in SRM with HBPSi⁻ as sole sulphur source. At the end of the exponential growth phase, HBP was the sole detectable product in the medium, as determined by RP-HPLC, and no HBPSi⁻ was detectable by IP-HPLC (data not shown).

Strain IGTSS grown in SRM with DMSO as sole sulphur source was unable to convert HBPSi⁻ (Table 3). However, considerable HBPSi⁻-desulphurizing activity was observed in cell-free extracts (Table 3). It was therefore concluded that DszB is an intracellular enzyme, and that HBPSi⁻ was not converted under standard assay conditions because the cell envelope is relatively impermeable to the charged HBPSi⁻ molecule. Strain IGTSS grew in SRM with HBPSi⁻ as sole sulphur source and it was concluded that passage of HBPSi⁻ across the cell envelope was not rate-limiting for growth.

It was concluded that DBTO₃ was desulphurized in two steps, DBTO₂ → HBPSi⁻ → HBP, catalysed by DszA and DszB, respectively, and that the complete pathway is DBT → DBTO → DBTO₂ → HBPSi⁻ → HBP + sulphite (Fig. 4).

**DszA also catalyses the conversion of BPSo to DHBP + sulphite**

BPSo was implicated as an intermediate in DBT metabolism following its recovery in very small amounts from culture media of strain IGTSS grown on DBT (Olson et al., 1993). When whole cells of strain IGTSS grown on DBT, DBTO₂ or DMSO were incubated under standard assay conditions with authentic BPSo, DHBP was the sole product detectable in the supernatant as determined by RP-HPLC (Fig. 3d). The identity of the 1.1 min RP-HPLC peak as DHBP was confirmed by GC-MS (data not shown). The specific BPSo-desulphurizing activity of whole cells of strain IGTSS, measured in terms of DHBP production, was similar to that for HBP production from DBT, DBTO and DBTO₂ (Table 1). The sulphur-containing product of BPSo desulphurization was sulphite, as determined by sulphite oxidase assay, and the specific activity measured in terms of sulphite production was similar to that measured for DHBP production (Table 1).

Studies with recombinant E. coli showed that BPSo desulphurization was catalysed exclusively by DszA.
Table 3. Activity of DBT-desulphurization pathway enzymes in whole cells and cell-free extracts of \textit{Rhodococcus} sp. strain IGTS8

Cells were grown to the end of the exponential phase in SRM with DMSO as sulphur source. For whole-cell assays, cells were washed twice with 10 vols 50 mM HEPPS buffer, pH 8.0, and assayed under standard assay conditions, with HBP (or DHBP) estimation by RP-HPLC. For cell-free assays cells were washed twice with 10 vols 50 mM HEPES buffer, pH 7.5, and resuspended in the same buffer to OD₆₀₀ 250. The cell extract (10 mg protein ml⁻¹ in 50 mM HEPES, pH 7.5) was prepared and assayed as described in Methods, with product analysis by RP-HPLC. ND, Not determined.

| Reaction catalysed | dsz operon proteins involved | Specific activity
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Whole cells [µmol (g dry wt)⁻¹ h⁻¹]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minus NADH</td>
</tr>
<tr>
<td>DBT → HBP</td>
<td>DszA, B and C</td>
<td>19.8</td>
</tr>
<tr>
<td>DBTO → HBP</td>
<td>DszA, B and C</td>
<td>23.0</td>
</tr>
<tr>
<td>DBTO₂ → HBP</td>
<td>DszA and B</td>
<td>24.5</td>
</tr>
<tr>
<td>BPSo → DHBP</td>
<td>DszA only</td>
<td>31.0</td>
</tr>
<tr>
<td>HBPSI⁻ → HBP</td>
<td>DszB only</td>
<td>0*</td>
</tr>
<tr>
<td>DBT → DBTO₂</td>
<td>DszC only†</td>
<td>ND</td>
</tr>
</tbody>
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* The inability of whole cells to metabolize HBPSI⁻ was taken to indicate that the cell envelope of strain IGTS8 was relatively impermeable to this substrate (see text).
† Recombinant \textit{Rhodococcus} sp. strain CPE648 (dsz) transformed with plasmid pENOK3, expressing dszC only, was used for this experiment.

Fig. 4. Metabolic pathway for the desulphurization of DBT to HBP and sulphite by \textit{Rhodococcus} sp. strain IGTS8. Actual reaction stoichiometries with respect to oxygen and NADH consumption were not measured and values given are those required for a balanced equation (see Discussion).
Dibenzothiophene desulphurization pathway

c(DBTOf80)

Dsz
C

I

DBT02f*O](Dsz A

@0

mh-

220

IM

=2ia

Fig. 5. 

18O labelling of BPSi recovered from the incubation of DBTO with whole cells of Rhodococcus sp. strain IGTS8 in the presence of 18O2. Strain IGTS8 was grown to the end of the exponential growth phase in SRM with DMSO as a sulphur source. Cells were washed and resuspended in 50 mM HEPPS buffer, pH 8.0, to OD600 250. DBTO (200 μM) was added and the suspension was incubated overnight under 18O2, as described in Methods. GC-MS analysis of BPSi recovered by ethyl acetate extraction revealed singly and doubly labelled BPSi in 1:1 ratio, with the phenolic oxygen labelled in both cases (see text). Therefore the phenolic oxygen and one of the sulphinate oxygens of the parent HBPSi- were labelled and hence derived from molecular oxygen.

BPSO was not a substrate for DszB or DszC (Table 2). Thus DszA catalysed two reactions, DBTO2 → HBPSi- and BPSO → DHBP.

DszA and DszC catalyse NADH-dependent reactions

Ohshiro et al. (1994) reported that the conversion of DBT to HBP by cell-free extracts of R. erythropolis strain D1 was strongly stimulated by NADH and it was concluded that at least one of the enzymes of the pathway must be NADH-dependent. To identify the NADH-dependent step(s), the individual enzymes were assayed in cell-free extracts of strain IGTS8, in the presence and absence of NADH. The results of these experiments are given in Table 3. The DszB-catalysed reaction, HBPSi- → HBP, was NADH-independent and the DszA-catalysed reaction, BPSO → DHBP, was NADH-dependent. The conversion of DBTO2 to HBP, which requires both DszA and DszB (Fig. 4), was NADH-dependent and it was concluded that the DszA-catalysed step, DBTO2 → HBPSi-, was NADH-dependent since the following step, HBPSi- → HBP, catalysed by DszB, was NADH-independent. Conversion of DBT and DBTO to HBP was NADH-dependent, but this did not prove that the DszC-catalysed conversion of DBT to DBTO2 required NADH, due to the dependence of the following, DszA-catalysed, step (DBTO2 → HBPSi-). Therefore, the production of DBTO2 from DBT was NADH-dependent in cell-free extracts of Rhodococcus sp. strain CPE362(pENOK3), which produced DszC only (Table 3). Based on these results, it was concluded that DszC and DszA, but not DszB catalyse NADH-dependent reactions (Fig. 4).

No significant stimulation of activity in cell-free extracts was observed on addition of NADPH, as found by Ohshiro et al. (1994) (data not shown).

The phenolic oxygen of HBP is derived from molecular oxygen

Strain IGTS8 was incubated with unlabelled DBT, DBTO or DBTO2 in an 18O2 atmosphere and the recovered metabolites were analysed by GC-MS to ascertain their oxygen labelling patterns. The HBP recovered from all three incubations was exclusively 18O-labelled (molecular ion peak at m/e = 172). No unlabelled HBP (molecular ion peak at m/e = 170) could be detected and it was concluded that the phenolic oxygen was derived exclusively from molecular oxygen. The possibility of rearrangement involving transfer of a sulphone oxygen to carbon during C-S bond cleavage was excluded on the basis that no unlabelled HBP was produced in incubations with DBTO or DBTO2.

To determine the 18O2 labelling pattern of HBPSi- it was necessary to take into account the fact that this compound was recovered as BPSi, with loss of a sulphinate oxygen (Fig. 1). In a control experiment unlabelled BPSi was hydrolysed in H218O, and then recondensed by addition of HCl to pH 1. The BPSi recovered by ethyl acetate extraction revealed singly and doubly labelled BPSi in 1:1 ratio, with the phenolic oxygen labelled in both cases (see text). Therefore the phenolic oxygen and one of the sulphinate oxygens of the parent HBPSi- were labelled and hence derived from molecular oxygen.
Fig. 6. Suggested pathway for the desulphurization of DBT to DHBP. The committing step would be the oxidation of HBPSi⁻ to HBPSO⁻. At neutral pH and physiologically relevant temperatures HBPSO⁻ would spontaneously, and presumably rapidly, condense to give BPSo, a substrate for DszA (Table 1).

A pathway for the desulphurization of DBT to DHBP

DHBP was detected as a product of the action of DszA on BPSo (Table 1). A suggested pathway to DHBP is shown in Fig. 6. The committing step would be the oxidation of HBPSi⁻ to HBPSO⁻. At neutral pH and physiologically relevant temperatures HBPSO⁻ would spontaneously, and presumably rapidly, condense to give BPSo, a substrate for DszA (Table 1).

Fig. 7. ¹⁸O labelling profile of BPSo. It was hypothesized that BPSo could be derived from HBPSi⁻ by direct mono-oxygenation of the sulphinate moiety, yielding HBPSo⁻, which would spontaneously condense, with loss of a water molecule, to give BPSo. If this were the case, BPSo obtained from the incubation of strain IGTS8 with DBTO under ¹⁸O₂ would be triply and doubly labelled in the ratio 1:2. In fact the recovered material was singly, doubly and triply labelled in the ratio 8:86:14, and it was concluded that BPSo was not formed as a result of mono-oxygenase action on HBPSi⁻.

Finally, DBTO recovered from the incubation of unlabelled DBTO was distributed between singly labelled (m/e = 218) and doubly labelled (molecular ion peak at m/e = 220) species, in 1:1 ratio (Fig. 5). Fragment ions were present at m/e = 187 and 188, again corresponding to the loss of HC¹⁸O or C¹⁸O from singly labelled BPSo (m/e = 218), and at m/e = 189 and 190, corresponding to loss of same from the doubly labelled (m/e = 220) species. Therefore the phenolic oxygen of HBPSi⁻ was derived from molecular oxygen. This meant that only one of the sulphinate oxygens could be labelled and the presence of singly and doubly labelled BPSo in 1:1 ratio was consistent with the loss with equal probability of either the labelled or the unlabelled sulphinate oxygen from HBPSi⁻ on condensation (Fig. 5). Since the starting material was unlabelled DBTO it was therefore concluded that both the second (sulphone) and third (phenolic) oxygens were derived from ¹⁸O₂.

It was further postulated that oxidation of HBPSi⁻ to HBPSO⁻ could be due to the action of a cellular mono-oxygenase. Sufficient BPSo for mass-spectroscopic analysis was recovered from the incubation of strain IGTS8 with unlabelled DBTO under ¹⁸O₂. This is the same incubation which yielded doubly labelled HBPSi⁻ (Fig. 5) and if HBPSO⁻ originated by mono-oxygenation of HBPSi⁻ the recovered BPSo would be expected to be triply and doubly labelled in 1:2 ratio (Fig. 7). However, the material was singly, doubly and triply labelled (molecular ion peaks at m/e = 234, 236 and 238, respectively) in the ratio 8:86:14, far from the expected value. In fact, ratios consistent with the simple mono-oxygenation of HBPSi⁻ were never obtained in any incubation which yielded enough BPSo for mass-spe-
trosopic analysis and it was concluded that HBPSO is not derived from HBPSi by this route. However, this result does not necessarily invalidate the pathway given in Fig. 6, since other mechanisms of HBPSi oxidation cannot be ruled out (see Discussion).

**Molecular oxygen is incorporated in the DszA-catalysed conversion of BPSO to DHBP**

When strain IGTS8 was incubated with unlabelled BPSO in an \(^{18}\)O atmosphere the DHBP produced was exclusively singly labelled (molecular ion peak at \(m/e 188\)). Therefore the original phenolic oxygen was retained and the new one was derived by direct incorporation of molecular oxygen.

**DISCUSSION**

The \(dSZ\) operon encodes three proteins, DszA, B and C, which are necessary and sufficient to confer the DBT desulphurization phenotype on *Rhodococcus* sp. strain IGTS8. The products of the reaction are HBP and sulphite. The reaction requires molecular oxygen and NADH. Although the functional stoichiometry with respect to these reactants has not yet been measured, the overall reaction is reasonably written (for neutral pH):

\[
\text{DBT} + 3 \text{O}_2 + 4 \text{NADH} + 2 \text{H}^+ \rightarrow \text{HBP} + 4\text{NAD}^+ + 3 \text{H}_2\text{O} + 4 \text{SO}_3^-.
\]

The four-step pathway is shown in Fig. 4.

The oxygen-labelling studies presented here are entirely consistent with the recent study by Lei & Tu (1996) on purified DszC. The data show unequivocally that DszC is a mono-oxygenase which is able to catalyse the sequential sulphoxidation reaction, DBT \(\rightarrow\) DBTO \(\rightarrow\) DBTO\(_2\) (Fig. 4). The balanced overall reaction is (for neutral pH):

\[
\text{DBT} + 2 \text{O}_2 + 2 \text{NADH} + 2 \text{H}^+ \rightarrow \text{DBTO}_2 + 2 \text{NAD}^+ + 2 \text{H}_2\text{O}.
\]

Recent work by our group (Gray et al., 1996; Xi et al., 1997) and by Lei & Tu (1996), has shown that purified DszC requires reduced FMNH\(_2\) as a cosubstrate (the purified enzyme contains no bound FMN cofactor). An NADH-dependent FMN oxidoreductase has been identified in strain IGTS8. This 25 kDa protein, which is not encoded by the \(dSZ\) operon, presumably serves as a source of FMNH\(_2\) for DszC *in vivo* (Gray et al., 1996). Since DszC is active in *E. coli* strain MZ-1(pSAD269-2A), which contains \(dSZ\) only (this work; Denome et al., 1994), it seems that a native *E. coli* FMN oxidoreductase is available to supply FMNH\(_2\) to DszC in the recombinant strain.

The requirement for FMNH\(_2\) as co-substrate implies the involvement of flavin 4a-hydroperoxide as the oxygen donor to the substrate and the same general reaction mechanism as for other flavin-dependent mono-oxygenases (Ballou, 1984). However, it is worth noting that DszC is exceptional in that FMNH\(_2\) is required as a co-substrate, whereas the classical flavin-dependent mono-oxygenases utilize a bound FAD cofactor. DszC is also exceptional in that it can oxidize DBT to the sulphone; more usually the oxidation of organic sulphides, by both flavin-monooxygenases and haem-dependent enzymes such as horseradish peroxidase or cytochrome P450, proceeds only as far as the sulphoxide, with further oxidation to the sulphone occurring much more slowly, or not at all (Holland, 1988; Dordick et al., 1991). The substrate specificity of DszC has not been fully investigated, but the purified enzyme is also capable of catalysing the oxidation of benzy1 sulphide to the sulphone (Lei & Tu, 1996), thus implying a fairly relaxed substrate specificity.

DszA catalyses two reactions, DBTO\(_2\) \(\rightarrow\) HBPSi\(^-\) and BPSO \(\rightarrow\) DHBP + sulphite. Both require molecular oxygen and are NADH-dependent. The balanced overall reactions at neutral pH are:

\[
\text{DBTO}_2 + 4 \text{NADH} + 2 \text{H}^+ + \text{O}_2 \rightarrow 2 \text{NAD}^+ + \text{H}_2\text{O} + \text{HBPSi}^-\quad (3)
\]

and

\[
\text{BPSO} + 2 \text{NADH} + \text{O}_2 \rightarrow 2 \text{NAD}^+ + \text{DHBP} + \text{SO}_4^{2-}\quad (4)
\]

There is no net oxidation in the conversion of DBTO\(_2\) to HBPSi\(^-\) and a reasonable enzymic mechanism for this reaction would be a base-catalysed hydrolysis (attack of the C-S carbon by hydroxide followed by expulsion of sulphinate as the better leaving group). Indeed HBPSi\(^-\) is one product of the reaction of DBTO\(_2\) with KOH/crown ether (Squieres et al., 1981). However, since DszA required NADH for activity in cell-free extracts, and since the phenolic oxygen introduced via the DszA-catalysed reaction was always derived from molecular oxygen, it was concluded that DszA must use a mono-oxygenation, rather than a hydrolytic, mechanism. Gray et al. (1996) have shown that this enzyme, like DszC, utilizes FMNH\(_2\), apparently derived from the 25 kDa NADH-dependent FMN oxidoreductase, as a co-substrate. A reasonable mechanism for DszA is shown in Fig. 8. FMNH\(_2\) would bind to DszA and then react with molecular oxygen to give the 4a-hydroperoxide. This molecule would attack a C-S carbon of the substrate (DBTO\(_2\) in Fig. 8), leading to its cleavage by expulsion of sulphinate. The resulting peroxide intermediate would be decomposed by attack of hydride, provided by a molecule of NADH, to yield the products HBPSi\(^-\) and 4a-hydroxy FMNH, which would spontaneously lose a water molecule to regenerate FMN and leave the active site.

The same mechanism applied to BPSO (eqn 4) would yield directly the O-sulphite ester of DHBP, which would hydrolyse to DHBP and sulphite with a half-life of seconds (Oae, 1991). The mechanism applied to BPSO is consistent with the formation of sulphite (Table 1) and with \(^{18}\)O\(_2\)-labelling experiments which showed that the phenolic oxygen incorporated at this step was derived from molecular oxygen.
The overall reaction stoichiometries given in eqns 3 and 4 are consistent with this mechanism, given that one molecule of NADH would be consumed in the initial reduction of FMN.

**DszB catalysed the conversion of HBPSi⁻ to HBP + sulphite (Table 2). The reaction did not require NADH (Table 3). On this basis DszB is classified as an aromatic sulphinic acid hydrolase catalysing the reaction**

\[
\text{HBPSi}^- + \text{H}_2\text{O} \rightarrow \text{HBP} + \text{SO}_3^- + 2 \text{H}^+ 
\]

(5)

A reasonable mechanism invokes the nucleophilic attack of a base-activated water molecule on the sulphinate sulphur (Fig. 9).

It was concluded that BPSo was not an intermediate on the DBT → HBP pathway (Fig. 4), since the desulphurized product is DHBP, not HBP, and no other fate for BPSo could be ascertained. Therefore a second pathway was proposed (Fig. 6) to explain the appearance of BPSo and the other desulphurized product, DHBP, in culture media of strain IGT8 grown on DBT (Gallagher et al., 1993). This pathway was reasonable because the final step, BPSo → DHBP + sulphite, has been shown to be catalysed by DszA, and HBPSi⁻, once formed, is expected to spontaneously, and probably very rapidly, condense to BPSo. Although the ¹⁸O labelling data do not support conversion of HBPSi⁻ to HBPSo⁻ by mono-oxygenase action, the pathway (Fig. 6) is not invalidated since it is possible that HBPSi⁻ is oxidized by another mechanism. It is known, for example, that aromatic sulphinic acids may undergo a complex disproportionation reaction which leads to a mixture of products, including the corresponding sulphonic acid (Kice & Bowers, 1962). Further studies are required in order to ascertain the relevance of such reactions and hence clarify this second pathway.

Whatever the nature of the alternative pathway, desulphurization of DBT to DHBP was quantitatively insignificant compared with desulphurization to HBP under standard assay conditions. GC-MS analysis of ethyl acetate extracts of many different samples showed that the HBP:DHBP ratio was variable, but always >100:1. Furthermore, there was no evidence for growth-dependent metabolic switching between HBP- and DHBP-yielding pathways, as suggested by Gallagher et al. (1993): DHBP formation from DBT was quantitatively insignificant in both exponentially growing and stationary-phase cultures (data not shown). Since the specific activity of whole-cells and cell-free extracts of strain IGT8 for BPSo → DHBP was similar to that for DBT → HBP (Tables 1 and 3), the rate-limiting step in this second pathway must precede BPSo formation.

This work has permitted several ambiguities apparent in earlier studies to be resolved. Firstly, it has been shown that DBTO is an intermediate in the pathway in vivo (a point of minor controversy; Denome et al., 1994). Secondly, DBTO₂ is an intermediate on the main pathway to HBP; Gallagher et al. (1993) placed DBTO₂ on a pathway to DHBP. Thirdly, again referring to the pathway of Gallagher et al. (1993), BPSo, not HBPSo⁻, is the immediate precursor of DHBP.

The pathway for DBT desulphurization by strain IGT8 may be compared with that for *Corynebacterium* sp. strain SY1 (Omori et al., 1992). The sequence DBT →
DBTO → DBTO₂ → X → HB₃P + sulphite was deduced, and it was suggested that X was biphenyl 2-sulphonate. On the basis of the work presented here, X is HB₃PSί⁻. It is almost certainly the case that the DBT-desulphurization pathways of Rhodococcus sp. strain IGTS8, R. erythropolis strain D-1 and Corynebacterium sp. strain SY1 are identical, and that the dsz operon is common to all three of these isolates.

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