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Isolation of a unique benzothiophene-desulphurizing bacterium, *Gordona* sp. strain 213E (NCIMB 40816), and characterization of the desulphurization pathway

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***Gordona* sp. strain 213E (NCIMB 40816) grew in pure culture in a mineral salts medium containing fructose as a source of carbon and energy, and benzothiophene (BTH) as the sole source of sulphur. During growth a phenolic compound accumulated, as indicated by the production of a blue colour on addition of Gibb's reagent. Therefore this pathway is analogous to the dibenzothiophene (DBT) desulphurization pathway of *Rhodococcus* sp. strain IGTS8, in which 2-hydroxybiphenyl accumulates during growth with DBT as the sole sulphur source. Ethyl acetate extraction of the culture medium yielded the metabolites benzothiophene *S*-oxide (BTHO), benzothiophene *S,S*-dioxide ($BTHO_2$), benzo[*c*][1,2]oxathiin 6-oxide (BcOTO), 2-(2′-hydroxyphenyl)ethan 1-al (HPEal) and benzofuran (BFU). The deduced pathway for BTH desulphurization is BTH → BTHO → $BTHO_2$ → $HPESi^-$ → HPEal. $HPESi^-$ is (*Z*)-2-(2′-hydroxyphenyl)ethen 1-sulphinate, the stable aqueous-solution form of BcOTO. It was concluded that HPEal was the Gibb's-reagent-reactive phenolic compound which accumulated in the culture medium of strain 213E during growth, and that the presence of BFU was due to partial condensation of HPEal during the ethyl acetate extraction procedure. *Gordona* sp. strain 213E was unable to grow in a mineral salts medium containing fructose as a source of carbon and energy and DBT as the sole sulphur source. BTH-desulphurization-active cells (grown using BTH as sole sulphur source) were unable to desulphurize DBT. Likewise *Rhodococcus* sp. strain IGTS8 was unable to grow using BTH as the sole sulphur source, and DBT-desulphurization-active cells of strain IGTS8 (grown using DBT as sole sulphur source) were unable to desulphurize BTH. This absence of cross-reactivity is discussed in terms of fundamental differences in the chemistry of the DBT- and BTH-desulphurization reactions.**

Keywords: *Gordona* sp., *Rhodococcus* sp., benzothiophene, desulphurizing enzymes, clean fuels

INTRODUCTION

Interest in the application of micro-organisms as a convenient and economical route to the removal of organic sulphur compounds from fossil fuels led to the discovery of *Rhodococcus* sp. strain IGTS8 (Kayser *et al.*, 1993). Strain IGTS8 is able to desulphurize dibenzothiophene (DBT) to 2-hydroxybiphenyl (HBP) and inorganic sulphite (see Fig. 5b). When strain IGTS8 is grown in media containing DBT as the sole source of sulphur, DBT serves as the source of sulphur for biomass, and HBP simply accumulates in the medium

Abbreviations: BcOTO, benzo[*c*][1,2]oxathiin *S*-oxide; BeOTO, benzo[*e*][1,2]oxathiin *S*-oxide; $BcOTO_2$, benzo[*c*][1,2]oxathiin *S,S*-dioxide; BFU, benzofuran; BPSi, dibenz[*c,e*][1,2]oxathiin *S*-oxide; BTH, benzothiophene; BTHO, benzothiophene *S*-oxide; $BTHO_2$, benzothiophene *S,S*-dioxide; DBT, dibenzothiophene; DBTO, dibenzothiophene *S*-oxide; $DBTO_2$, dibenzothiophene *S,S*-dioxide; fruRM, fructose Rhodococcus medium; glyRM, glycerol Rhodococcus medium; HBP, 2-hydroxybiphenyl; HPEol, 2-(2′-hydroxyphenyl)ethen-1-ol; HPEal, 2-(2′-hydroxyphenyl)ethan-1-al; $HPESi^-$, (*Z*)-2-(2′-hydroxyphenyl)ethen 1-sulphinate; $HPESo^-$, (*Z*)-2-(2′-hydroxyphenyl)ethen 1-sulphonate; $HBPSi^-$, 2-hydroxybiphenyl 2′-sulphinate.

(Kayser *et al.*, 1993; Oldfield *et al.*, 1997). Therefore this seems to be a sulphur-scavenging pathway. Other such pathways are known, e.g. the arylsulphatase degradation pathway of *Pseudomonas aeruginosa* (Beil *et al.*, 1995) and the taurine degradation pathway of *Escherichia coli* (van der Ploeg *et al.*, 1998).

DBT is widely regarded as a model compound, representative of the aromatic organosulphur fraction of coal and crude oil. Therefore DBT desulphurization by strain IGTS8 has been the subject of considerable study. The DBT-desulphurization phenotype is conferred by the plasmid-located *dsz* operon, encoding three proteins, DszA, B and C, which are necessary and sufficient for DBT desulphurization in *Rhodococcus* sp. strain IGTS8 and in *Escherichia coli*. The operon has been cloned and sequenced (Denome *et al.*, 1993, 1994) and the metabolic pathway has been elucidated (Olson *et al.*, 1993; Oldfield *et al.*, 1997). The enzymes have been purified and partially characterized (Lei & Tu, 1996; Gray *et al.*, 1996). Genetic analysis of the regulatory region of the *dsz* operon indicates that the primary regulatory circuit is repression by more readily bioavailable sulphur (sulphate, cysteine, methionine) (Li *et al.*, 1996; Ohshiro *et al.*, 1996b), a result which is consistent with the hypothesis that this pathway serves a sulphur-scavenging function.

A number of other DBT-desulphurizing rhodococci have been reported. These include *R. erythropolis* strains D-1 (Izumi *et al.*, 1994), H-2 (Ohshiro *et al.*, 1996a) and N-36 (Wang & Krawiek, 1996; Wang *et al.*, 1996); *Rhodococcus* sp. strain SY1 (Omori *et al.*, 1995), first reported as *Corynebacterium* sp. strain SY1 (Omori *et al.*, 1992); strains UM3 and UM9, provisionally identified as rhodococci (Purdy *et al.*, 1993); *Rhodococcus* sp. strains B1, If, Ig and Ih (Denis-Larose *et al.*, 1997); and strain ECRD-1, which was initially classified as a strain of *Arthrobacter* (Lee *et al.*, 1995) and is now reclassified as *R. erythropolis* strain ATCC 55310 (Denis-Larose *et al.*, 1997).

Other DBT-desulphurizing isolates have been classified as *Agrobacterium* strain MC501 (Constanti *et al.*, 1996), *Mycobacterium* strain G3 (Nekodzuka *et al.*, 1997) and *Paenibacillus* strain A11-2 (Konishi *et al.*, 1997). All of these isolates are reported to desulphurize DBT to HBP, but are otherwise poorly characterized.

The rhodococcal DBT-desulphurization enzymes have a fairly relaxed specificity for members of the DBT family and will desulphurize alkyl- or aryl-substituted DBT derivatives to the corresponding monophenol (Lee *et al.*, 1995; Ohshiro *et al.*, 1996a). However, these isolates seem to be incapable of desulphurizing benzothiophene (BTH). Thus strains IGTS8, ECRD-1 and N1-36 are unable to grow in mineral salts media containing BTH as sole source of sulphur (Kayser *et al.*, 1993; Lee *et al.*, 1995; Wang & Krawiec, 1996). This result has important implications for the development of a microbial process for the desulphurization of crude oil and its distillates, since the aromatic organic sulphur fraction is comprised of both dibenzothiophenes and benzothiophenes. Therefore a screening programme was carried out with the objective of isolating micro-organisms capable of desulphurizing BTH.

This paper describes the isolation and characterization of *Gordona* sp. strain 213E (NCIMB 40816), which is capable of desulphurizing BTH and is therefore complementary in its role in fuel desulphurization to that of DBT-desulphurizing isolates. The basic growth properties of strain 213E on BTH are described; characterization of BTH metabolites, obtained by ethyl acetate extraction of culture media, is presented and a pathway for BTH desulphurization is proposed. Experiments were carried out to confirm that strain IGTS8 was incapable of desulphurizing BTH (Kayser *et al.*, 1993) and it was also found that strain 213E was incapable of desulphurizing DBT. On this basis it is postulated that strains IGTS8 and 213E respectively express DBT- and BTH-specific desulphurizing enzyme systems.

METHODS

Materials. The buffers HEPES and HEPPS and Gibb's reagent (2,6-dichloroquinone 4-chloroimide) were obtained from Sigma. DBT (99+%), $DBTO_2$ (97%), HBP (sold as 2-phenylphenol; 99+%), BTH (sold as thianaphthene; 99%) and BFU (sold as 2,3-benzofuran, 99%) were obtained from Aldrich. Bacto and Noble agar were obtained from Difco. Glucose, fructose and glycerol were obtained from Merck.

Bacteria. *Rhodococcus* sp. strain IGTS8 (ATCC 53968) was obtained from J. Kilbane (Institute of Gas Technology, Chicago, IL, USA). *Gordona* sp. strain 213E (NCIMB 40816) was isolated in this work (see below).

Culture medium. Rhodococcus medium (RM) has the following composition (per litre): Na_2HPO_4, 4·33 g; KH_2PO_4, 2·65 g; NH_4Cl, 2 g; $MgCl_2.6H_2O$, 0·64 g; nitrilotriacetic acid, 0·1 g; $CaCl_2.2H_2O$, 33 mg; $ZnCl_2$, 2·6 mg; $FeCl_2.4H_2O$, 2·6 mg; EDTA, 1·25 mg; $MnCl_2.4H_2O$, 1·0 mg; $CuCl_2.2H_2O$, 0·15 mg; $Co(NO_3)_2.6H_2O$, 0·125 mg; $Na_2B_4O_7.10H_2O$, 0·10 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0·09 mg; final pH 7·2 without titration. After autoclaving (121 °C, 20 min), filter-sterilized aqueous glycerol or fructose was added to a final concentration of 10 g l^{-1}, to give, respectively, glycerol Rhodococcus medium (glyRM) or fructose Rhodococcus medium (fruRM). The sulphur source was added to a final concentration of 200 μM. DBT or BTH was added from stock solutions in acetone (40 mM); sodium sulphate was added from a filter-sterilized stock solution in water (40 mM).

Strain IGTS8 was maintained at 30 °C on fruRM agar plates (Difco Noble agar, 20 g l^{-1}) containing either DBT or sulphate, and strain 213E was maintained on fruRM agar containing either BTH or sulphate. Both strains were subcultured each week.

Isolation of *Gordona* sp. strain 213E. *Gordona* sp. strain 213E was isolated by enrichment culture in glyRM + BTH using as an inoculum a soil sample taken from the vicinity of an oil shale spoil heap located at a disused mine located at West Calder (West Lothian, Scotland). One hundred millilitres of glyRM + BTH in a 250 ml Erlenmeyer flask was inoculated with 20 g soil and incubated in an orbital shaker (120 r.p.m., 30 °C) for 14 d. This culture was enriched for BTH-degrading organisms by fivefold serial transfer of 1 ml into 50 ml glyRM + BTH, in a 100 ml Erlenmeyer flask, with 14 d incubation at 30 °C. A 100 μl aliquot from the fifth transfer

was serially diluted onto glyRM+BTH plates (15 g Noble Agar l^{-1}) and these were incubated at 30 °C until average colony sizes of 5 mm were obtained. Representative single colonies were used to inoculate 20 ml aliquots of glyRM+BTH in 50 ml Erlenmeyer flasks. The flasks were incubated (120 r.p.m., 30 °C) until $OD_{600} > 9{\cdot}0$ (about 5 d). Only flasks inoculated with colonies designated 213E and 213F yielded this level of growth. Serial dilution and plating out of these cultures onto glyRM+BTH agar plates gave, after 5 d incubation, predominantly one organism in each case, as judged by colony morphology and Gram staining. Selected colonies from each plate, grown in glyRM+BTH, yielded only one organism by the same criteria. The two isolates were designated as strains 213E and 213F and deposited as NCIMB 40816 and 40817, respectively. In terms of BTH-desulphurizing activity, strain 213F was identical to strain 213E and will not be discussed further.

Growth studies. Fifty millilitres of medium, containing the appropriate sulphur source, in a 100 ml Erlenmeyer flask was inoculated with cells from a single colony of strain 213E (from a fruRM+BTH agar plate) or of strain IGTS8 (from a fruRM+DBT agar plate). The flasks were incubated in an orbital shaker (120 r.p.m., 30 °C). At intervals 3 ml of culture was withdrawn for measurement of biomass (OD_{600}) and estimation of accumulated phenolic compounds by Gibb's assay, as follows. A 1·5 ml sample of culture was put into an Eppendorf tube, centrifuged (12000 r.p.m., 5 min) to pellet cells and the supernatant frozen (−20 °C) until the end of the experiment. Then 1·0 ml was transferred to a 1 ml disposable spectrophotometer cuvette (1 cm pathlength) and 10 µl Gibbs reagent (10 mM in acetone) was added. A blank solution (fruRM+10 µl Gibbs reagent) was also prepared. After overnight incubation at 30 °C the A_{610} was measured, with dilution if necessary to keep A_{610} below 1·0. A_{610} was converted to [HBP] using a standard curve, prepared under the same conditions, with authentic HBP in the range 0–30 µM. HPEal is not commercially available and it was not possible to construct a standard curve. However it was noted that fruRM+BTH recovered following growth of strain 213E well into stationary phase (150 h) always gave $A_{610} = 0{\cdot}75$, approx., on addition of Gibb's reagent followed by overnight incubation at 30 °C. Therefore, with the assumption that [HPEal] = 200 µM (i.e. complete conversion of BTH to HPEal), $\varepsilon_{610} = 3800\ M^{-1}\ cm^{-1}$ for the Gibb's-HPEal adduct. Therefore [HPEal] was estimated from the expression $[HPEal]/\mu M = 10^6\ A_{610}/3800$ (where A_{610} was measured in a 1 cm pathlength cell).

Isolation of organic metabolites and GC–MS analysis. Cultures of strain IGTS8 or strain 213E were grown in fruRM to the end of the exponential phase (60 h). Cells were removed by centrifugation at 3000 r.p.m. (1600 *g*) for 30 min. The supernatant was titrated to pH 1 with 50 % HCl and extracted three times with an equal volume of ethyl acetate. The extracts were pooled, dried by stirring for 1 h with anhydrous $MgSO_4$ (100 g l^{-1}), and the ethyl acetate removed by rotary evaporation. The solids were redissolved in 3·0 ml ethyl acetate. GC–MS analysis of metabolites was carried out using a Hewlett Packard 5890 series II gas chromatograph coupled to a 5972 series mass-selective detector. The gas chromatograph was fitted with a Hewlett Packard capillary column HP-5MS, length 30 m. The carrier gas was helium. Run conditions were as follows: start temperature 40 °C held for 2 min, followed by 10 °C min^{-1} ramp rate to a final temperature of 240 °C. Where possible, structural assignments were confirmed using the on-line G1035A Wiley PBM library (John Wiley & Sons, New York).

Studies on the metabolism of DBT and BTH by pre-grown cells. DBT-desulphurization-active cells of strain IGTS8 were grown in fruRM+DBT, and BTH-desulphurization-active cells of strain 213E were grown in fruRM+BTH, to the end of the exponential phase (60 h). The cells were recovered by centrifugation at 4200 r.p.m. (1700 *g*) for 15 min, washed twice with and finally resuspended in, 50 mM HEPPS buffer, pH 8, to OD_{600} 10. Each suspension was divided into two aliquots, and to each of these either BTH or DBT was added, from 40 mM stocks in acetone, to a final concentration of 200 µM. After overnight incubation at 30 °C on an orbital shaker (120 r.p.m.) the cells were removed by centrifugation and the supernatants were extracted with ethyl acetate for GC–MS analysis.

RESULTS

Gordona sp. strain 213E grows in a mineral salts medium containing fructose as a source of carbon and energy and BTH as the sole source of sulphur

When grown on fruRM+BTH Noble Agar plates for 3 d, colonies of strain 213E were pink, mucoid, with entire margins and umbonate elevation, and stained as slender Gram-positive rods of irregular length. Strain 213E was obligately aerobic, non-motile, partially acid-fast, catalase-positive and oxidase-positive. Glucose,

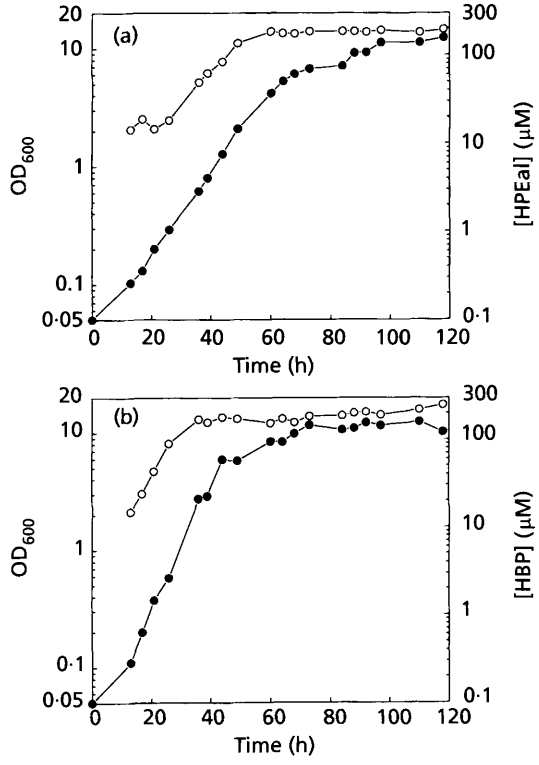

Fig. 1. Growth curves for desulphurizing bacterial strains. (a) *Gordona* sp. strain 213E grown in fruRM+BTH; (b) *Rhodococcus* sp. strain IGTS8 grown in fruRM+DBT. ●, Cell density measured as OD_{600} (1 cm pathlength cuvette); ○, concentration of accumulated HPEal or HBP, as determined by Gibb's assay.

mannitol, pyruvate, citrate and glycerol could be substituted for fructose in this medium. 16S rRNA analysis indicates that strain 213E belongs to the genus *Gordona* (S. Kim, C. Oldfield & M. Goodfellow, unpublished).

Strain 213E grew in fruRM + BTH at 30 °C with a mean doubling time of 9 h and a phenolic compound accumulated in the medium, as indicated by the production of a blue colour on addition of Gibb's reagent. The phenolic compound was identified as HPEal by GC–MS analysis (see below). Complete desulphurization correlates roughly with the end of exponential growth in this sulphur-limited medium (Fig. 1a). This behaviour can be compared (Fig. 1b) with that of strain IGTS8, which grew in fruRM + DBT with a doubling time of 7 h and HBP (identity confirmed by GC–MS analysis; data not shown) accumulated as expected (Kayser *et al.*, 1993; Oldfield *et al.*, 1997).

Strains 213E and IGTS8 grew in fruRM + sulphate with doubling times of 3 h and 4 h, respectively. Therefore growth with an organosulphur source was rather slower than with a more readily bioavailable inorganic source of sulphur. No blue colour was produced following addition of Gibb's reagent to culture medium supernatant. When strain 213E was grown in fruRM containing 200 μM of each of sulphate and BTH, no blue colour was produced on addition of Gibbs reagent to the medium, indicating that BTH desulphurization did not occur (data not shown). Therefore it was concluded that BTH was not metabolized by strain 213E in the presence of sulphate.

Characterization of metabolites extracted from culture supernatants of strain 213E grown in fruRM + BTH enables prediction of a metabolic pathway for BTH desulphurization

When strain 213E was grown in fruRM + BTH to mid- or end-exponential phase, GC analysis of ethyl acetate extracts of the culture medium revealed a total of seven peaks (a)–(g) which were absent from extracts of cultures grown on fruRM + sulphate (Fig. 2). The structure of each compound was deduced by MS (Fig. 3a–g) as follows:

Peaks (a) and (b). Benzofuran (BFU) and benzothiophene (BTH), respectively, assigned on the basis of GC retention times (Fig. 2) and mass spectra (Fig. 3a, b) identical to those of commercially available standards.

Peak (c). 2-(2′-Hydroxyphenyl)ethan-1-al (HPEal); molecular ion peak at $m/e = 136$; high-abundance fragment-ion at $m/e = 107$ corresponds to loss of CHO (aldehyde moiety) from the molecular ion. Fragment-ions at $m/e = 91$, 90 and 89 correspond to loss of phenolic oxygen plus 0, 1 or 2H, respectively from the $m/e = 107$ fragment-ion, and the fragment-ion at $m/e = 77$ is $C_6H_5^+$ (Fig. 3c). Actually HPEal could be expected to exist as a mixture of HPEal and *cis*- and *trans*-2-(2′-hydroxyphenyl)ethen-1-ol (*cis*- and *trans*-HPEol), due to keto–enol tautomerization. For this particular interconversion no value for the equilibrium constant, $K_{eq} = ([cis\text{-enol}] + [trans\text{-enol}])/[\text{keto}]$ was

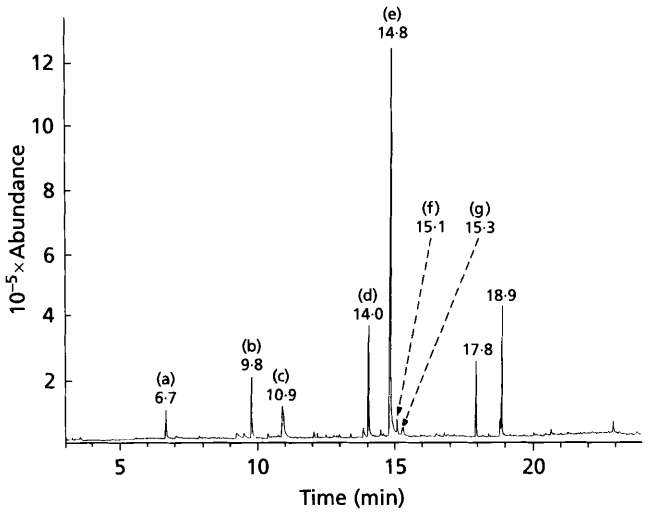

Fig. 2. Gas chromatogram of an ethyl acetate extract of the culture medium of strain 213E containing BTH (200 μM) as sole sulphur source. The culture was grown in 50 ml fruRM + BTH in a 100 ml Erlenmyer flask, incubated in an orbital shaker (120 r.p.m.; 30 °C) to the end of the exponential phase (approx. 60 h) and extracted with ethyl acetate as described in Methods.

found in the literature. However for the closely related couple, 2-phenylethen-1-ol ↔ 2-phenylethan-1-al, $K_{eq} = 1{\cdot}3 \times 10^{-3}$ in aqueous solution at neutral pH and 20 °C (Chiang *et al.*, 1989). Therefore, this compound can be regarded for practical purposes as HPEal.

Peak (d). Benzo[*e*][1,2]oxathiin *S*-oxide (BcOTO); molecular ion peak at $m/e = 166$ and high-abundance fragment-ion at $m/e = 118$ corresponding to loss of S=O (Fig. 3d). This is the common mode of sultine fragmentation under electron impact (Bowie *et al.*, 1966; Fields & Meyerson, 1966). (See Fig. 4.) BcOTO is the condensed 'sultine' form of the alkenyl sulphinate, (*Z*)-2-(2′-hydroxyphenyl)ethen 1-sulphinate ($HPESi^-$). The situation here is analogous to the case in DBT desulphurization, where $HBPSi^-$ is actually isolated as the sultine, dibenz[*c*,*e*][1,2]oxathiin *S*-oxide (BPSi; Oldfield *et al.*, 1997). Sultines are stable in aqueous solution only at pH <2 and are formed under the conditions of the ethyl acetate extraction procedure. At physiologically relevant (neutral) pH, these compounds exist exclusively as the sulphinate (Hanson & Kemp, 1981).

Peak (e). Benzothiophene *S*-oxide (BTHO); weak molecular ion peak at $m/e = 150$; the high-abundance fragment-ion at $m/e = 134$ is BTH^+ derived by loss of the sulphinyl oxygen (Fig. 3e). The low abundance of molecular ion, due to the lability of the S=O double bond, is typical of aromatic sulphoxides.

Peak (f). Benzothiophene *S*,*S*-dioxide ($BTHO_2$); molecular ion peak at $m/e = 166$; fragment-ion peaks at $m/e = 150$ and $m/e = 134$, consistent with the loss of O and 2O, respectively, from the molecular ion peak (Fig. 3f). The high-abundance $m/e = 118$ and $m/e = 137$

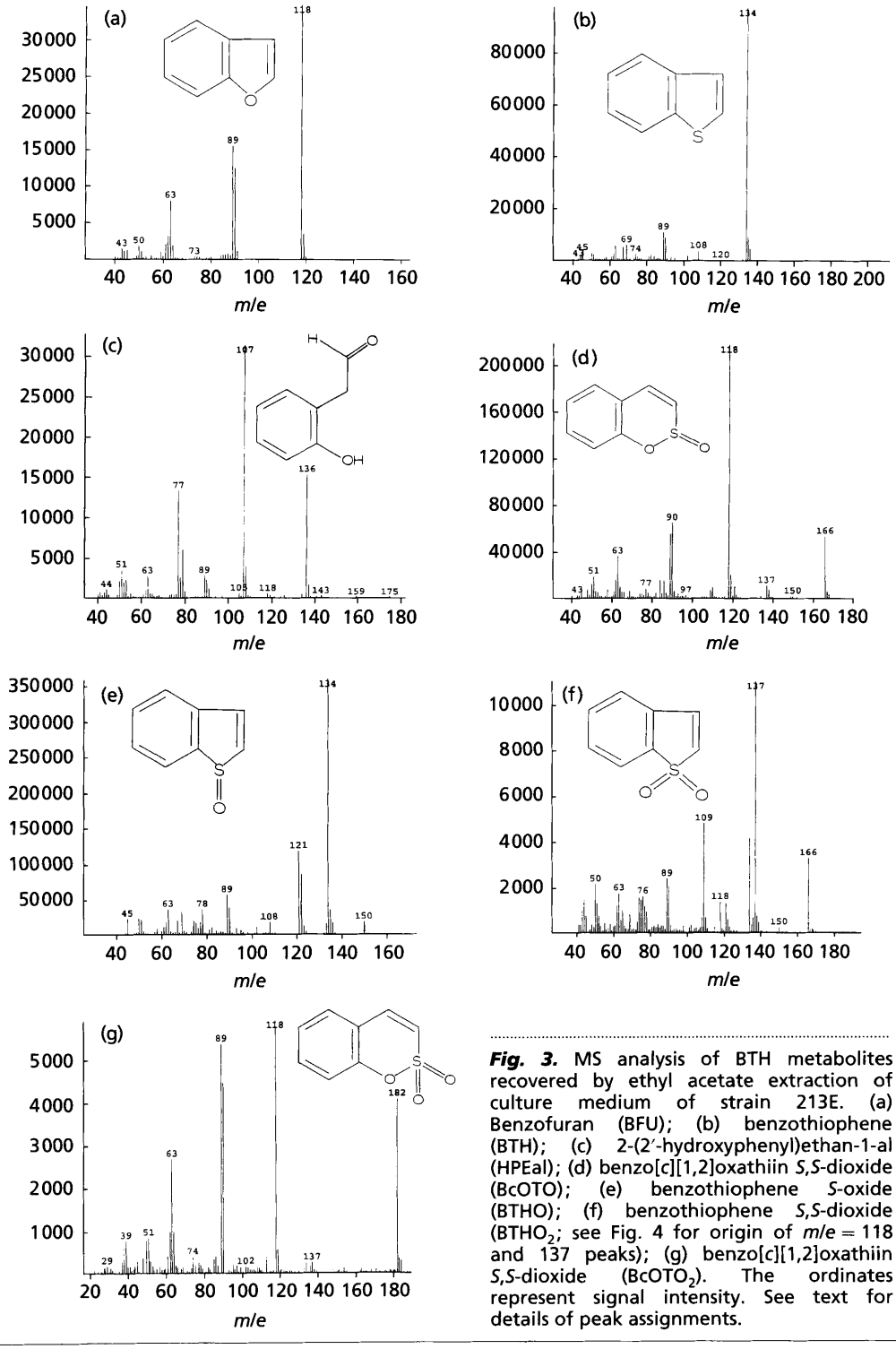

Fig. 3. MS analysis of BTH metabolites recovered by ethyl acetate extraction of culture medium of strain 213E. (a) Benzofuran (BFU); (b) benzothiophene (BTH); (c) 2-(2′-hydroxyphenyl)ethan-1-al (HPEal); (d) benzo[c][1,2]oxathiin *S,S*-dioxide (BcOTO); (e) benzothiophene *S*-oxide (BTHO); (f) benzothiophene *S,S*-dioxide ($BTHO_2$; see Fig. 4 for origin of $m/e = 118$ and 137 peaks); (g) benzo[c][1,2]oxathiin *S,S*-dioxide ($BcOTO_2$). The ordinates represent signal intensity. See text for details of peak assignments.

peaks are also consistent with the identity of this species as $BTHO_2$, given that this molecule may convert to either of two isomeric sultine species, under electron-impact, prior to fragmentation (Bowie *et al.*, 1966; Fields & Meyerson, 1966; Olson *et al.*, 1993). As shown in Fig. 4, the $m/e = 118$ peak arises from isomerization of $BTHO_2$ to benzo[*c*][1,2]oxathiin *S*-oxide (BcOTO) followed by loss of S = O (cf. Fig. 3d), and isomerization of $BTHO_2$ to benzo[*e*][1,2]oxathiin *S*-oxide (BeOTO) followed by loss of CHO is responsible for the $m/e = 137$ peak.

Peak (g). Benzo[*c*][1,2]oxathiin *S,S*-dioxide ($BcOTO_2$); molecular ion peak at $m/e = 182$ and abundant fragment ion peak at $m/e = 118$ corresponding to loss of SO_2 (Fig. 3g). This is a common mode of sultone fragmentation under electron impact.

Other peaks in Fig. 2 correspond to compounds which

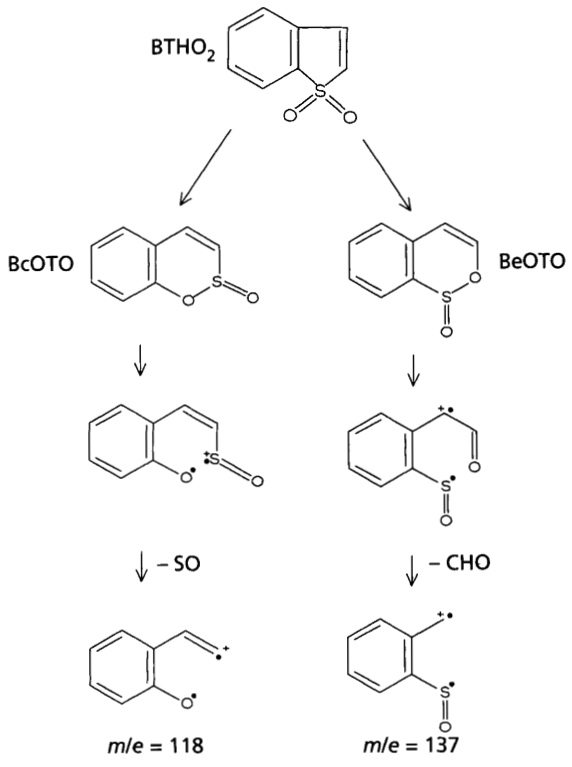

Fig. 4. Fragmentation behaviour of $BTHO_2$ under electron-impact. The mass spectrum in Fig. 3(f) was consistent with the identity of the molecule as $BTHO_2$ on the basis that this molecule can isomerize either to BcOTO or to BeOTO, prior to fragmentation, so that the resulting mass spectrum contains fragment-ions characteristic of both of these molecules, in addition to those characteristic of $BTHO_2$ itself. The left-hand route indicates the fragmentation pattern of BcOTO, characterized by the strong *m/e* = 118 fragment-ion due to loss of S=O and the right-hand route indicates the fragmentation pattern of BeOTO, characterized by the strong *m/e* = 137 fragment-ion due to loss of CHO.

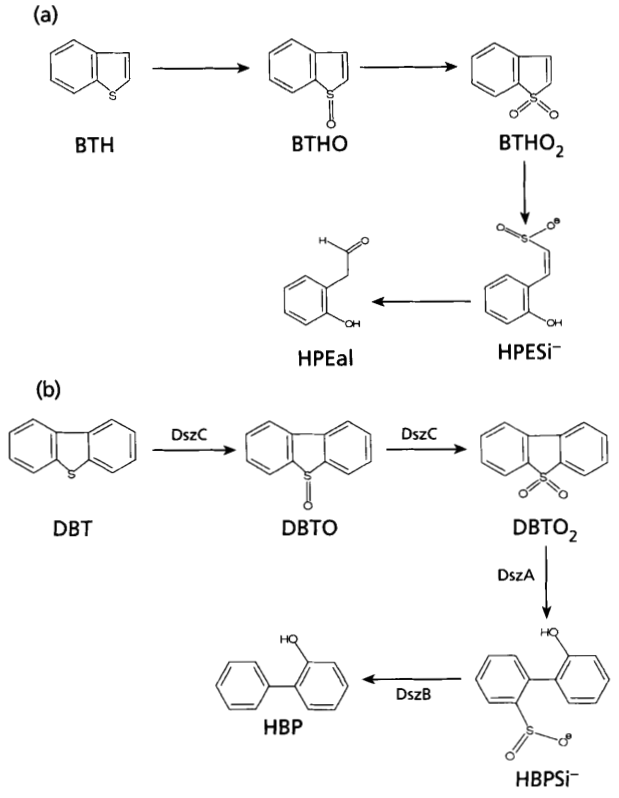

Fig. 5. Comparison of the proposed BTH desulphurization pathway with the DBT desulphurization pathway. (a) Proposed BTH desulphurization pathway for *Gordona* sp. strain 213E. (b) DBT desulphurization pathway of *Rhodococcus* sp. strain IGTS8 (Oldfield *et al.*, 1997).

cannot be BTH metabolites. By reference to library spectra, the peaks at 17·8 and 18·9 min were assigned respectively as a benzene 1,2-dicarboxylate diester and as hexadecanoic acid. These peaks also appear in ethyl acetate extracts of cells grown on fruRM + sulphate (data not shown).

Based on the identities of these metabolites, it was possible to propose a metabolic pathway for BTH desulphurization by strain 213E (Fig. 5a). The proposed sequence is: BTH → BTHO → $BTHO_2$ (activation of sulphur to facilitate thiophene ring-opening); $BTHO_2$ → $HPESi^-$ (thiophene ring-opening), and finally $HPESi^-$ → HPEal (desulphination). As already indicated, $HPESi^-$ is the stable form of BcOTO in aqueous solution at neutral pH. It was therefore concluded that HPEal is the final organic product of BTH desulphurization and that this is the Gibb's-reagent-reactive phenolic compound which accumulates in culture media during growth of strain 213E on BTH (Fig. 1a). The presence of BFU was attributed to partial dehydration of HPEal during the ethyl acetate extraction procedure (Joule *et al.*, 1995).

$BcOTO_2$ was not included in the pathway shown in Fig. 5(a). It was concluded that this was a dead-end metabolite formed by oxidation of BcOTO: a side-reaction which is irrelevant for the main desulphurization pathway.

Strain 213E does not desulphurize DBT and strain IGTS8 does not desulphurize BTH

Strain 213E did not grow in fruRM + DBT and it was concluded that this isolate was unable to desulphurize DBT. Similarly, strain IGTS8 did not grow in fruRM + BTH and it was concluded that the organism was unable to desulphurize BTH (this latter result confirms an earlier observation; Kayser *et al.*, 1993). When DBT-desulphurization-competent cells of strain IGTS8 were incubated overnight in 50 mM HEPPS buffer, pH 8, containing BTH, ethyl acetate extraction yielded none of the expected BTH metabolites, BTHO, $BTHO_2$, BcOTO or HPEal. Similarly, when BTH-desulphurization-competent cells of strain 213E were incubated overnight in 50 mM HEPPS buffer, pH 8, containing DBT, ethyl acetate extraction yielded none

of the expected DBT metabolites, DBTO, $DBTO_2$, BPSi (the condensed sultine form of $HBPSi^-$) or HBP. This kind of experiment was carried out several times and the result was reproducible. Therefore it was concluded that strains IGTS8 and 213E are respectively unable to metabolize BTH and DBT.

DISCUSSION

Gordona sp. strain 213E is a novel BTH-desulphurizing bacterium

This is the first report of a bacterium which is able to desulphurize BTH. *Gordona* sp. strain 213E grew in a mineral salts medium with fructose as a source of carbon and energy and with BTH as the sole sulphur source. During growth a phenolic compound, identified as HPEal, accumulated and it was concluded that this was the unused carbon skeleton of BTH.

No phenolic compounds were produced by cultures of strain 213E grown in fruRM + sulphate, or in fruRM containing 200 μM each of BTH and sulphate, as determined by Gibb's assay. It was concluded from the latter result that expression of the BTH-desulphurizing enzymes was repressed by sulphate, a source of more readily bioavailable sulphur, and therefore that this is a sulphur-scavenging pathway. There is a parallel here with *Rhodococcus* sp. strain IGTS8, which is able to scavenge sulphur by desulphurizing DBT using a pathway which does not degrade the carbon skeleton (Fig. 5b), and which is also repressed by sulphate (Li *et al.*, 1996).

The existence of separate BTH- and DBT-specific desulphurization pathways is consistent with predicted differences in the chemistry of the two substrates

Using metabolites (a)–(f) (Figs 2 and 3) a pathway for BTH desulphurization was constructed (Fig. 5a). This pathway can be compared with that for DBT desulphurization by *Rhodococcus* sp. strain IGTS8 (Fig. 5b). Thus the first step, BTH → BTHO → $BTHO_2$, mirrors the sequence DBT → DBTO → $DBTO_2$ and it was concluded that strain 213E must produce an *S*-oxygenase equivalent to DszC.

The following step, $BTHO_2$ → $HPESi^-$, the thiophene ring-opening reaction triggered by hydroxylation of the aryl C–S carbon, likewise mirrors the step $DBTO_2$ → $HBPSi^-$. The presence of BcOTO (the condensed form of $HPESi^-$ present in ethyl acetate extracts) was taken as direct evidence for ring-opening by cleavage of the aryl C–S bond, rather than of the alkenyl C–S bond. In principle, ring opening could also proceed by way of cleavage of the alkenyl C–S bond. However, if this were the case, the isomeric BeOTO (the sultine of 2-[(*Z*)-2′-hydroxyethen]benzene 1-sulphinate) should be expected instead of, or in addition to, BcOTO. BcOTO and BeOTO are distinguishable by their mass spectra (Fig. 4). BeOTO was never found, despite detailed examination of numerous gas chromatograms, and therefore it was concluded that opening of the thiophene ring of $BTHO_2$ occurs exclusively by cleavage of the aryl C–S bond. On this basis it was also concluded that strain 213E must produce an enzyme which in this respect is identical to DszA (Fig. 5b).

The final step in the proposed pathway is the desulphination of $HPESi^-$ to HPEal. In the strain IGTS8 DBT-desulphurization pathway, desulphination occurs by DszB-catalysed hydrolysis: $HBPSi^- + H_2O$ → HBP + sulphite (Fig. 5b). Although aryl sulphinates, such as $HBPSi^-$, can be desulphinated in this way the alkenylsulphinate, $HPESi^-$, cannot and therefore strain 213E is not expected to produce an enzyme equivalent to DszB. It is proposed instead that $HPESi^-$ desulphination occurs by oxygenase-catalysed hydroxylation of the C–S carbon, which would permit the expulsion of sulphite. The immediate product of this reaction would be an enol (either *cis*- or *trans*-HPEol, depending on the precise enzymic mechanism), which would tautomerize to HPEal on release from the active site. As already indicated the keto–enol equilibrium is expected to lie strongly in favour of HPEal. The sulphur-containing product of BTH-desulphurization has not yet been identified and these studies await the availability of [^{35}S]BTH.

Differences in chemistry of DBT and BTH desulphurization cannot be the only explanation for the lack of cross-specificity between the two pathways

As already discussed, the DBT- and BTH-desulphurization mechanisms differ crucially in the nature of the sulphinic acid generated following thiophene ring-opening. On this basis alone it is reasonable to expect that DBT and BTH desulphurization would require separate pathways. However, the earlier steps, viz. *S*-oxygenation of the substrate to give the dioxide, followed by opening of the thiophene ring by hydroxylation of an aromatic C–S carbon, are common (Fig. 5) to both pathways and on this basis it was reasonable to expect desulphurization of BTH by strain IGTS8 to proceed as far as $HPESi^-$. Nevertheless, when pre-grown, DBT-desulphurization-competent, strain IGTS8 was challenged with BTH there was no production of BTHO, $BTHO_2$ or $HPESi^-$ and it was concluded that BTH was not a substrate for the entry-point *S*-oxygenase (DszC). On the same basis, whilst there was no reason to expect the strain 213E $HPESi^-$-desulphinating enzyme to desulphurize $HBPSi^-$, it was reasonable to hypothesize that strain 213E would metabolize DBT at least as far as $HBPSi^-$. When BTH-desulphurization-competent strain 213E was challenged with DBT, however, there was no production of DBTO, $DBTO_2$ or $HBPSi^-$ and it was concluded that DBT was not a substrate for the strain 213E equivalent of DszC.

Therefore it was concluded that the mutual exclusivity of the two pathways arises not only from the need for different chemistry at the final desulphination step, but

also from a correspondingly narrow substrate specificity of the entry-point *S*-oxygenase.

Conclusions

Gordona sp. strain 213E possesses a novel BTH-desulphurizing capability. From the point of view of development of fossil fuel desulphurization technology, this activity is complementary to that of DBT-desulphurizing isolates such as *Rhodococcus* sp. strain IGTS8. Fossil fuels contain both benzothiophenes and dibenzothiophenes and it is therefore necessary to target both families of compounds in order to achieve maximal desulphurization. The existence of separate DBT- and BTH-specific pathways is consistent with the need for different kinds of chemistry to carry out the final, desulphinating step; however, this cannot be the whole story, since BTH was not sulphoxidized by strain IGTS8 and DBT was not sulphoxidized by strain 213E, despite the fact that there is common chemistry at this initial stage (compare Fig. 5a and b). Therefore the enzyme responsible, DszC, and its equivalent in strain 213E, seem to possess specificities which are complementary to those of the corresponding desulphinating enzymes. Confirmation of this observation and its understanding in terms of the substrate specificity of the purified enzymes is highly desirable and is being pursued in this laboratory.

Both the DBT- and BTH-specific pathways undoubtedly perform a sulphur-scavenging role for the cell. The extent to which these organisms rely on these pathways in their natural environment is not yet known and it would be interesting to determine the *in situ* expression levels of these enzymes. Sulphur-scavenging pathways have been characterized in other organisms, including *Pseudomonas aeruginosa* (arylsulphatase degradation pathway; Beil *et al.*, 1995) and *Escherichia coli* (taurine degradation pathway; van der Ploeg *et al.*, 1998). However, the DBT and BTH desulphurization pathways are unusual in that the carbon skeleton generated in the desulphurization is not mineralized. DBT desulphurization is an energetically expensive process, estimated to require 4 mol NADH per mol DBT desulphurized (Oldfield *et al.*, 1997) and this is probably also the case for BTH desulphurization. Therefore it seems unusual that the carbon skeleton is not mineralized in order to recoup the energy invested. This peculiarity is compounded by the fact that mono- and binuclear aromatic catabolic pathways are known in the rhodococci (Asturias *et al.*, 1994; Kosono *et al.*, 1997; Masai *et al.*, 1997). Mineralization of HBP and HPEal would certainly result in the release of sufficient energy to compensate for that consumed in the desulphurization reaction; nevertheless, there are no reports of strains of *Rhodococcus* sp. or *Gordona* sp. which are able to both desulphurize DBT (or BTH) and subsequently mineralize the carbon skeleton.

Thus the existence of the desulphurization phenotype raises some interesting questions relevant to the ecology of such organisms, and in particular of the *Rhodococcus–Gordona–Tsukamurella* family. For these reasons, as well as for their potential role in fossil fuel desulphurization, these strains are deserving of further study.

Addendum

Since this article was accepted the isolation of a DBT-desulphurizing strain of *Gordona* sp. has been reported (Rhee *et al.*, 1998).

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