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ORIGINAL PAPER

Sulfur from benzothiophene and alkylbenzothiophenes supports growth of *Rhodococcus* sp. strain JVH1

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Abstract *Rhodococcus* sp. strain JVH1 was previously reported to use a number of compounds with aliphatic sulfide bridges as sulfur sources for growth. We have shown that although JVH1 does not use the three-ring thiophenic sulfur compound dibenzothiophene, this strain can use the two-ring compound benzothiophene as its sole sulfur source, resulting in growth of the culture and loss of benzothiophene. Addition of inorganic sulfate to the medium reduced the conversion of benzothiophene, indicating that benzothiophene metabolism is repressed by sulfate and that benzothiophene is therefore used specifically as a sulfur source. JVH1 also used all six isomers of methylbenzothiophene and two dimethylbenzothiophene isomers as sulfur sources for growth. Metabolites identified from benzothiophene and some methylbenzothiophenes were consistent with published pathways for benzothiophene biodesulfurization. Products retaining the sulfur atom were sulfones and sultines, the sultines being formed from phenolic sulfinates under acidic extraction conditions. With 2-methylbenzothiophene, the final desulfurized product was 2-methylbenzofuran, formed by dehydration of 3-(*o*-hydroxyphenyl) propanone under acidic extraction conditions and indicating an oxygenative desulfination reaction. With 3-methylbenzothiophene, the final desulfurized product was 2-isopropenylphenol, indicating a hydrolytic desulfination reaction. JVH1 is the first microorganism reported to use all six isomers of methylbenzothiophene, as well as some dimethylbenzothiophene isomers, as sole sulfur sources. JVH1 therefore possesses broader sulfur extraction abilities than previously reported, including not only sulfidic compounds but also some thiophenic species.

Keywords Aromatic sulfur source · Benzothiophene · Methylbenzothiophene · *Rhodococcus* biodegradation

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Introduction

Benzothiophene and alkyl benzothiophenes are constituents of petroleum and creosote. These sulfur-heterocyclic compounds become environmental contaminants when petroleum or creosote

is released into surface waters, ground waters or soils.

Many biodegradation studies have focused on the biotransformations of benzothiophene (Bohonos et al., 1977; Fedorak and Grbić-Galić 1991; Kropp et al., 1994; Dyreborg et al., 1997; Annweiler et al., 2001), and fewer investigations have addressed the metabolism of alkyl benzothiophenes (Saftić et al., 1992; Kropp et al., 1994). In general, benzothiophene and its alkyl-substituted homologues undergo cometabolism and they are transformed to sulfones (Fedorak and Grbić-Galić 1991; Saftić et al., 1992), sulfoxides (Fedorak and Grbić-Galić 1991; Saftić et al., 1992; Kropp et al., 1994), alcohols (Kropp et al., 1994), and carboxylic acids (Kropp et al., 1994; Annweiler et al., 2001). In most reports, these sulfur heterocycles do not serve as the sole carbon source for microbial growth, however, Watanapokasin et al. (2002) isolated a thermophilic bacterium that grows on benzothiophene as its sole carbon and energy source, and Sandhya et al. (1995) studies a mutated strain of *Nocardioides* that grew on benzothiophene as its sole carbon, sulfur and energy source.

It has been demonstrated that some organosulfur compounds can also serve as a sulfur source for microbial growth (Alves et al., 2005; Marzona et al., 1997; Van Hamme et al., 2004). Thus, in sulfur-deficient environments, the release of sulfur from benzothiophenes could provide this essential growth element to microorganisms.

Two divergent pathways for the microbial extraction of sulfur from benzothiophene have been reported (Fig. 1; Gilbert et al., 1998; Konishi et al., 2000), and two different end products have been identified. In *Gordonia desulfuricans* strain 213E, the sulfinate group is removed with oxygenation of the molecule, giving 2-(2'-hydroxyphenyl)ethan-1-al (Gilbert et al., 1998). This product is recovered as benzofuran due to dehydration under acidic extraction conditions. In *Paenibacillus* sp. strain A11-2, the final product is *o*-hydroxystyrene, produced through desulfination of the molecule, which does not oxygenate the carbon atom (Konishi et al., 2000). Only one microorganism, *Rhodococcus* sp. strain WU-K2R, has been reported to produce both end products

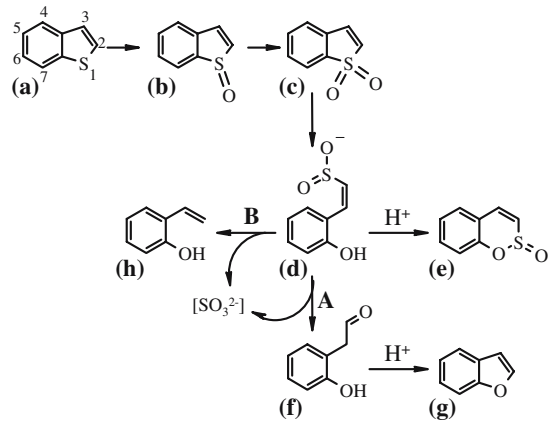

Fig. 1 Divergent pathways of benzothiophene metabolism by (**a**) *G. desulfuricans* strain 213E (Gilbert et al., 1998), and (**b**) *Paenibacillus* sp. strain A11-2 (Konishi et al., 2000): (**a**) benzothiophene; (**b**) benzothiophene *S*-oxide (sulfoxide); (**c**) benzothiophene *S*,*S*-dioxide (sulfone); (**d**) 2-(2'-hydroxyphenyl)ethen 1-sulfinate; (**e**) benzo[*e*][1,2]oxathiin *S*-oxide (sultine); (**f**) 2-(2'-hydroxyphenyl)ethan-1-al; (**g**) benzofuran; (**h**) *o*-hydroxystyrene. Sulfite has not been definitively shown as the final sulfur species. Products (**e**) and (**g**) formed by abiotic reactions under acidic extraction conditions

from the desulfurization of benzothiophene (Kirimura et al., 2002).

Rhodococcus sp. strain JVH1 can use a number of aliphatic sulfides as sulfur sources for growth (Van Hamme et al., 2004). In the current work, we explored whether strain JVH1 could use aromatic sulfides, specifically benzothiophene and alkyl benzothiophenes, as its sulfur source while growing on acetate. This study demonstrates that strain JVH1 can extract sulfur from several benzothiophenes and presents the mechanisms of sulfur removal used by JVH1 for this group of compounds.

Materials and methods

Chemicals

Benzothiophene (99 %), 1,4-dithiane (97 %), 2-methylbenzothiophene (97 %) (2-MBT), and thianthrene (99+ %) were from Aldrich (Oakville, ON, Canada). 3-MBT was from Lancaster (Windham, NH, USA). 5-MBT (98 %) was from Alfa Aesar (Ward Hill, MA, USA). Dichlorom-

ethane and methanol (high-performance liquid chromatography grade) were from Fisher Scientific (Fairlawn, NJ, USA).

The commercial preparation of 1,4-dithiane was further purified by recrystallization from methanol.

Benzothiophene sulfone and 3-MBT sulfone were synthesized as described by Bressler et al. (1999) and 5-fluorobenzothiophene sulfone was synthesized as described by Bressler (2001). The synthesis of the other alkylbenzothiophenes, including the mixture of 4- and 6-MBT, was described by Andersson (1986), and the synthesis of the other alkylbenzothiophene sulfones was described by Kropp et al. (1994).

Growth media and amendments

Plate Count Agar was from BD (Franklin Lakes, NJ), and was prepared using twice-distilled water. Sulfate-free mineral medium with acetate (SFMM) was prepared in acid-washed glassware as previously described (Kirkwood et al., 2005).

Organic sulfur sources were added to sterile SFMM in a minimum volume of methanol. Sulfate was added as an autoclaved solution of Na_2SO_4 in deionized water.

Culture conditions

Rhodococcus sp. strain JVH1 was stored in glycerol stocks at –70°C. To start an experiment, JVH1 was first streaked from frozen stocks onto Plate Count Agar and grown for 3 to 14 days. A single colony was picked from the plate to inoculate 5 ml of SFMM with 1,4-dithiane as the sole sulfur source (0.025 mmol l^{-1}) in roll tubes. After 3 days growth, a portion of the culture was harvested by centrifugation, washed three times and resuspended in an equal volume of phosphate buffer. Portions of the final suspension were used to inoculate triplicate experimental cultures at a concentration of 0.4 % by volume.

Liquid cultures were grown in 5 ml of medium in screw-cap culture tubes fitted with PTFE liners. Solutions of sulfur heterocyclic compounds in methanol were added to the culture medium at a maximum of 20 μl per tube. The same volume of pure methanol was always added to the positive controls with sulfate and to the sulfur-free negative controls to account for any influence of the solvent on the sulfur heterocyclic-containing cultures. Tubes were incubated on a tube roller in the dark at 28 °C. Incubation times for each culture are given in Table 1.

Analytical techniques

Growth in culture tubes was monitored by measuring the optical density at 600 nm (OD_{600}) directly in the tubes with a Spectronic 21 spectrophotometer (Bausch and Lomb, Rochester, NY, USA), using a sterile control tube as a blank, and comparing experimental cultures to sulfur-free negative controls. Because the measurements were made directly in the culture tubes, and not in cuvettes, growth data measured in this way are reported as ''qualitative OD_{600}'', and were only compared within an experiment.

After incubation, all cultures to be extracted were acidified with concentrated HCl to pH < 1, and either extracted immediately or stored at 4°C until extraction. Thianthrene was added as an internal standard and the cultures were extracted with 2 ml of dichloromethane.

Culture extracts were analyzed for organic sulfur compounds by gas chromatography using a Hewlett Packard 5890 series gas chromatograph equipped with an HP-1 capillary column (25 m length, 0.32 mm inner diameter, 0.17 μm film thickness) (Agilent Technologies, Wilmington, DE) and a flame ionization detector (GC-FID).

Metabolites were identified in culture extracts by low-resolution electron impact mass spectrometry using a Hewlett–Packard 5890 series II gas chromatograph with a 5970 series mass selective detector following a DB-5 capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) (J&W Scientific) (GC-MS). Further GC-MS analysis was performed in the Mass Spectrometry Laboratory, Department of Chemistry, University of Alberta.

Results

Use of aromatic sulfur sources

The three-ring aromatic compounds dibenzothiophene, dibenzothiophene sulfone and thianth-

Table 1 Use of monosubstituted and disubstituted benzothiophenes and benzothiophene sulfones by *Rhodococcus* sp. strain JVH1 as sole sulfur sources with acetate as carbon source

Compound[a] (incubation time, days)		Initial concn (mmol L^{-1})	Growth[b]	Substrate loss[d] (%)
Monoalkylbenzothiophenes				
2-MBT	(7)	0.11	+	99 ± 1
3-MBT	(8)	0.09	+	38 ± 6
4-MBT	(11)	0.15	–	33 ± 21
	(10)	0.07	+	72 ± 8
5-MBT	(7)	0.10	+	87 ± 5
4- & 6-MBT	(8)	0.09 total	+	66 ± 3 (4-MBT) 100 (6-MBT)
7-MBT	(11)	0.03	+	24 ± 20
7-EBT	(11)	0.12	–	Not significant
	(10)	0.06	+ (1/3)[c]	64 (1/3)[c]
			– (2/3)[c]	15 (2/3)[c]
Monosubstituted benzothiophene sulfones				
3-MBT sulfone	(8)	0.10	+	100
4-MBT sulfone	(11)	0.08	+	84 ± 14
5-FBT sulfone	(8)	0.10	+	92 ± 13
4- & 6-MBT sulfone	(8)	0.08 total	+	86 ± 4 (4-MBT sulfone) 99 ± 2 (6-MBT sulfone)
Dimethylbenzothiophenes				
2,3-DMBT	(11)	0.14	–	Not significant
	(10)	0.07	–	Not significant
2,7-DMBT	(11)	0.10	–	Not significant
	(10)	0.05	–	35 ± 17
3,5-DMBT	(11)	0.14	–	Not significant
	(10)	0.07	+ (1/3)[c]	87 (1/3)[c]
			– (2/3)[c]	Not significant (2/3)[c]
3,7-DMBT	(11)	0.16	–	Not significant
	(10)	0.08	–	Not significant
4,6-DMBT	(11)	0.09	–	9 ±1
	(7)	0.04	+	89 ± 1

[a] EBT, ethylbenzothiophene; FBT, fluorobenzothiophene

[b] Growth (qualitative OD_{600}) greater than (+) or less than (–) sulfur-free negative control

[c] If triplicates differed, number in each category is indicated

[d] Relative to sterile controls; avg ± SD, $n = 3$, unless otherwise indicated

rene did not serve as sulfur sources for growth of JVH1, and growth was equivalent to sulfur-free negative controls (Fig. 2). Growth of cultures with the two-ring aromatic compound benzothiophene (Fig. 2), accompanied by over 90 % loss of the compound, was an unexpected result, contradicting the results of Van Hamme et al. (2004). Benzothiophene sulfone also supported growth of JVH1 as the sole sulfur source (Fig. 2). Additional tests showed that JVH1 grew readily with benzothiophene at concentrations up to 0.25 mmol l^{-1}, although limited growth was observed at 0.5 mmol l^{-1}, probably reflecting its toxicity to bacteria as reported by others (Bohonos et al., 1977; Fedorak and Grbić-Galić 1991; Seymour et al., 1997).

Rhodococcus sp. strain JVH1 was incubated with each of the six MBT isomers as the sole sulfur sources for growth, as well as five dimethylbenzothiophene (DMBT) isomers and substituted benzothiophene sulfones. JVH1 was able to grow with all of the monosubstituted benzothiophenes and benzothiophene sulfones tested, at concentrations of approximately 0.1 mmol l^{-1} (Table 1). Concentration-dependent inhibition of growth was observed with some alkylbenzothiophenes. At 0.15 mmol 4-MBT l^{-1}, growth was suppressed relative to the sulfur-free

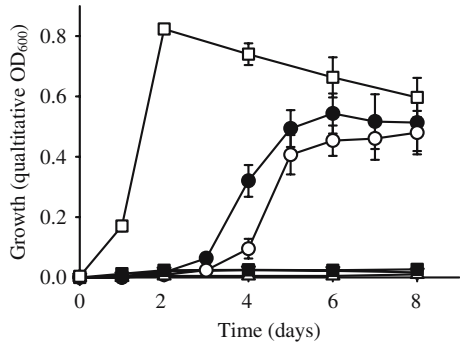

Fig. 2 Growth of *Rhodococcus* sp. strain JVH1 with thiophenic sulfur compounds or sulfate as sole sulfur sources and acetate as carbon source (average ± standard deviation, $n = 3$). Closed and open circles, benzothiophene and benzothiophene sulfone, 0.1 mmol l^{-1}; closed and open triangles, dibenzothiophene and dibenzothiophene sulfone, 0.01 mmol l^{-1}; closed and open squares, sulfur-free negative controls and positive controls with sulfate (1 mmol l^{-1})

negative controls, but the cultures did grow with 0.07 mmol 4-MBT l^{-1}. Similarly, JVH1 did not grow with 0.12 mmol 7-ethylbenzothiophene l^{-1}, and only one of the triplicate cultures grew with 0.06 mmol 7-ethylbenzothiophene l^{-1} (Table 1).

Because JVH1 was able to use all of the MBT isomers, it was hypothesized that JVH1 would also be able to use DMBT isomers as sulfur sources. However, the DMBT isomers tested were apparently more inhibitory than the MBT isomers and no growth was observed at the higher concentrations tested (Table 1). Only 4,6-DMBT supported growth in triplicate cultures at 0.04 mmol l^{-1}, whereas 3,5-DMBT supported growth at 0.07 mmol l^{-1} in only one of the triplicate cultures. The results in Table 1 also suggest that steric hindrance may influence the enzymatic extraction of sulfur from DMBTs having methyl groups on carbons 2 and/or 7 (Fig. 1), because these isomers did not support growth.

Metabolites from benzothiophenes

Culture extracts of *Rhodococcus* sp. strain JVH1 grown with benzothiophene and MBTs were analyzed by GC-FID and GC-MS to identify any accumulated metabolites. The proposed metabolites (Table 2) are consistent with the published pathways for the desulfurization of benzothiophene and MBTs (Gilbert et al., 1998; Konishi et al., 2000), and include sulfones and sultines. Two additional end products were tentatively identified. 2-Methylbenzofuran was identified in the 2-MBT extract by comparison of the GC-FID retention time to an authentic standard. In the 3-MBT cultures, the final product may be 2-isopropenylphenol. An authentic standard was not available, but the mass spectrum indicated a molecular weight of 134 and a fragmentation pattern (Table 2) consistent with reported spectra for 2-isopropenylphenol (Kobayashi et al., 2000) and the isomer 4-isopropenylphenol (Hunter et al., 2004).

Effect of sulfate on utilization of benzothiophene and benzothiophene sulfone

The effect of sulfate concentration on the degradation of benzothiophene and benzothiophene sulfone by JVH1 was tested to determine whether metabolism of these thiophenic compounds was sulfur-specific. Degradation of both compounds was significantly repressed at sulfate concentrations of 0.05 mM or higher (Fig. 3). The extent of degradation decreased from 100 % with no sulfate to 11 % with 5 mM sulfate for benzothiophene, and from 93 % to 15 % for benzothiophene sulfone.

Discussion

The ability of *Rhodococcus* sp. strain JVH1 to extract sulfur from benzothiophenes contradicts the results of Van Hamme et al. (2004). JVH1 was shown to be sensitive to the concentration of benzothiophene and of alkylbenzothiophenes. The published negative result (Van Hamme et al., 2004) was therefore likely due to the inhibitory effect of benzothiophene at higher concentrations. As previously reported, dibenzothiophene did not serve as a sulfur source for JVH1 (Van Hamme et al., 2004). Although many bacteria do use both benzothiophene and dibenzothiophene as sulfur sources (Alves et al.,

Table 2 Metabolites formed during growth of *Rhodococcus* sp. strain JVH1 in medium with acetate as carbon source plus benzothiophene or methylbenzothiophenes as sulfur source

Substrate	Proposed products Major ions, *m/z* (abundance, %)	Evidence
Benzothiophene	Benzothiophene sulfone 166 (31, M^+), 137 (100), 118 (18), 109 (54)	Comparison of GC-FID retention time to authentic standard Comparison of mass spectrum to published spectrum (Gilbert et al. 1998)
	Sultine 166 (3, M^+), 118 (100), 90 (51), 63 (52)	Comparison of mass spectrum to published spectrum (Gilbert et al. 1998)
2-Methyl benzothiophene	2-Methyl benzothiophene sulfone 180 (33, M^+), 137 (100), 115 (40), 109 (47)	Comparison of mass spectrum to authentic standard
	Sultine 180 (5, M^+), 132 (100), 103 (13), 77 (19)	Comparison of mass spectrum to published spectrum for sultine formed from benzothiophene (Gilbert et al. 1998)
	2-Methylbenzofuran (MS data not available)	Comparison of GC-FID retention time to authentic standard
3-Methyl benzothiophene	2-Isopropenylphenol 134 (35, M^+), 119 (23), 115 (8), 105 (11), 91 (100)	Mol wt from mass spectrum Comparison of MS fragmentation to published data for 2-isopropenylphenol (Kobayashi et al. 2000) and 4-isopropenylphenol (Hunter et al. 2004)
5-Methyl benzothiophene	5-Methyl benzothiophene sulfone 180 (41, M^+), 151 (100), 131 (30), 123 (26), 115 (20)	Comparison of mass spectrum to published spectrum (Kropp et al. 1994)

2005; Chang et al., 1998; Kayser et al., 2002; Marzona et al., 1997; Tanaka et al., 2002), it is not uncommon for bacteria capable of using benzothiophene to be unable to extract sulfur from dibenzothiophene [for example, *G. desulfuricans* strain 213E (Gilbert et al., 1998) and others (Kirimura et al., 2002; Matsui et al., 2000, 2001; Tanaka et al., 2001)], and for bacteria that can extract sulfur from dibenzothiophene to be unable to use benzothiophene [for example, *Rhodococcus erythropolis* strain IGTS8 (Arensdorf et al., 2002) and others (Izumi et al., 1994; Kobayashi et al., 2000)]. These reports are primarily based on growth assays, and a genetic basis for the observed differences in substrate ranges has not been clearly established. Arensdorf et al. (2002) showed that a mutation in *dszC*, the gene for dibenzothiophene monooxygenase in *R. erythropolis* strain IGTS8, was required for use of 5-MBT, which was not a substrate for the wild-type enzyme. In other microorganisms, the apparent inability to use benzothiophene may in fact be due to an inhibitory effect of the compound, as observed for *Rhodococcus* sp. strain JVH1.

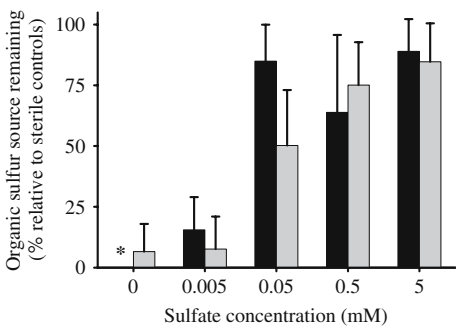

Fig. 3 Effect of added sulfate on degradation of benzothiophene (black bars) or benzothiophene sulfone (grey bars) by *Rhodococcus* sp. strain JVH1 after 5 days incubation (average + standard deviation, $n = 3$). Each organic sulfur compound provided 0.05 mmol S l^{-1} to the culture. (* No benzothiophene was detected in cultures incubated with benzothiophene and no added sulfate)

The differing results obtained with JVH1 illustrate the limitations of growth assays for assessing

substrate ranges. The present work shows that a single test is not sufficient to determine that a compound is not used; instead, multiple concentrations should be tested if necessary, and replicates must be included for each condition tested. For example, lowering the concentrations of 7-ethylbenzothiophene and of 3,5-DMBT (Table 1) yielded growth of JVH1, but only in one of three replicate cultures for each compound. The advantage of growth assays when testing sulfur sources, however, is that a positive result indicates release of sulfur from the compound, even in the absence of detectable metabolites. Although resting-cell or cell-free extract experiments showing loss of a compound may appear to indicate broader substrate ranges than growth tests, the observed loss of substrate may only be due to sulfur oxidation and not sulfur extraction.

Rhodococcus sp. strain JVH1 is the first microorganism tested with and reported to grow on all six isomers of MBT, as well as some DMBT isomers, as sole sulfur sources for growth. For example, *R. erythropolis* strain KA2-5-1 could use 3-MBT, 2-ethylbenzothiophene, and 2,7-diethylbenzothiophene, but not benzothiophene, 2-, 5-, or 7-MBT, 7-ethylbenzothiophene, or 5,7-DMBT (Kobayashi et al., 2000); whereas *Gordonia rubropertinctus* strain T08 could use benzothiophene, 2- and 5-MBT, and 2-ethylbenzothiophene, but not 3- or 7-MBT, 7-ethylbenzothiophene, or 2,7- or 5,7-diethylbenzothiophene (Matsui et al., 2001), although the effect of concentration was not tested in these studies. 4- and 6-MBTs can be oxidized cometabolically (Kropp et al., 1994; Saftić et al., 1992), but have not previously been tested as sulfur sources for bacterial growth.

Although the position of the methyl group in MBT seemed to affect the toxicity of the isomers, it did not affect the ultimate ability of *Rhodococcus* sp. strain JVH1 to use these substrates. The inability of JVH1 to use some DMBT isomers is therefore more likely due to their inhibitory effect than to an enzymatic limitation. The negative effect of the compounds on culture growth is also consistent with inhibition by the alkylbenzothiophenes rather than recalcitrance. Alkylation is known to increase the acute toxicity of small aromatic hydrocarbons such as benzene, naphthalene and phenanthrene (Neff, 1979). For benzothiophenes, methylation decreases aqueous solubility and increases toxicity, whereas oxidation to sulfones or diones increases aqueous solubility and decreases toxicity for both benzothiophene and MBTs (Seymour et al., 1997). Oxidation of benzothiophenes to their sulfones may therefore be an essential detoxification step for JVH1. Lower aqueous concentrations due to reduced aqueous solubility, and lower reaction rates due to mass transfer limitations and possibly steric hindrance for the more hydrophobic MBTs and DMBTs, would limit the efficiency of detoxification, and could therefore be responsible for the inability of JVH1 cultures to grow on some of these isomers. Although lower concentrations of the DMBT isomers were not tested due to limitations in measurement, they might be expected to support growth, as long as enough sulfur was still available to the culture.

The effect of sulfate concentration on the degradation of organic sulfur compounds can be used as an indicator of sulfur-specific activity (Kirkwood et al., 2005), because the presence of sulfate typically results in repression of the ability to use alternate sulfur sources (Serbolisca et al., 1999; van der Ploeg et al., 2001). The desulfurization of aliphatic sulfides by *Rhodococcus* sp. strain JVH1 is repressed by sulfate (Kirkwood, 2006). Sulfate also exerted a regulatory effect on the use of the aromatic sulfur compounds benzothiophene and benzothiophene sulfone by JVH1 (Fig. 3), indicating a sulfur-specific mode of attack.

The ability of *Rhodococcus* sp. strain JVH1 to grow with each of the possible MBT isomers is consistent with the initial reaction being at the sulfur atom, because alkylation would interfere with attack on the ring carbon atoms. Metabolites identified from benzothiophene and some MBTs (Table 2) were consistent with published pathways for the extraction of sulfur from benzothiophene (Gilbert et al., 1998; Konishi et al., 2000), confirming that JVH1 catalyzes specific sulfur extraction from two-ring aromatic sulfur compounds. The metabolites were also consistent with the reported pathway for desulfurization of *bis*-(3-pentafluorophenylpropyl)-sulfide (PFPS) by JVH1 (Van Hamme et al., 2004). Products retaining the sulfur atom were sulfones [analogous to *bis*-(3-pentafluorophenylpropyl) sulfone]

and sultines. Sultines are formed from the phenolic sulfinate under acidic extraction conditions (Gilbert et al., 1998). The identification of sultines from benzothiophene and 2-methylbenzothiophene is the first direct evidence that a sulfinate is formed from the first carbon–sulfur bond cleavage reaction by JVH1, as was previously proposed (Van Hamme et al., 2004). An alcohol group was also introduced through carbon–sulfur bond cleavage, analogous to the formation of 3-pentafluorophenylpropan-1-ol from PFPS.

Two different desulfurized products were detected in cultures of *Rhodococcus* sp. strain JVH1 grown with MBTs. Acidic extracts of cultures grown on 2-MBT contained 2-methylbenzofuran. This product would be formed by dehydration of 3-(*o*-hydroxyphenyl) propanone under acidic extraction conditions (Joule et al., 1995). A phenolic ketone would result if both carbon–sulfur bond cleavage reactions led to oxygenation of the molecule, as occurs in the removal of the sulfur atom from benzothiophene by *G. desulfuricans* strain 213E (Fig. 1, pathway A; Gilbert et al., 1998). Extracts of cultures grown on 3-MBT contained 2-isopropenylphenol. In this case, only the first carbon–sulfur bond cleavage oxygenates the molecule, and the desulfination reaction would be a hydrolysis, as in the sulfur extraction from benzothiophene by *Paenibacillus* sp. strain A11-2 (Fig. 1, pathway B; Konishi et al., 2000). JVH1 likely possesses both reported pathways for sulfur removal from benzothiophenes. Only one other bacterium, *Rhodococcus* sp. strain WU-K2R, has been reported to possess both pathways (Kirimura et al., 2002). Both *o*-hydroxystyrene and benzofuran were detected in extracts of WU-K2R cultures grown with benzothiophene, and both 2'-hydroxynaphthylethene and naphthofuran were detected in extracts of WU-K2R grown with naphtho[2,1-*b*]thiophene (Kirimura et al., 2002).

The selectivity of the oxidative and hydrolytic desulfination pathways for the various sulfur sources for JVH1 was not determined, therefore, we cannot rule out both pathways operating simultaneously for a given compound. JVH1 may also exhibit both pathways during the extraction of sulfur from aliphatic sulfides, although no evidence for a reductive pathway has yet been observed. The metabolites formed were analogous to the metabolites in the proposed pathway for PFPS desulfurization, suggesting that the enzymes used for thiophenic and sulfidic compounds are at least functionally related. Identification of the desulfurization genes and testing of the purified enzymes is required to show whether more than one desulfurization system is present in this strain, and to establish the enzymatic basis for the two desulfination reactions observed.

Conclusion

This study has clearly shown that strain JVH1 can use several alkylbenzothiophenes as its source of sulfur in medium that is devoid of sulfate. Thus, in sulfate-limited environments that are contaminated with crude oil or creosote, bacteria may be able to extract sulfur, an essential nutrient, from benzothiophenes to support their growth. However, sulfate concentrations as low as 0.05 mM (5 mg l^{-1}) inhibit the removal of sulfur from benzothiophene. In addition, this investigation has demonstrated that elevated concentrations of methyl- and dimethylbenzothiophenes can inhibit the growth of *Rhodococcus* sp. strain JVH1.

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