University of Alberta

Aerobic Biodegradation of Disulfides Produced from Dibenzothiophene Metabolites

by

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ABSTRACT

Dibenzothiophene (DBT) is a sulfur heterocycle in petroleum. When DBT is degraded through the Kodama pathway, the likely route of DBT biodegradation in petroleum-contaminated environments, three large disulfides are formed. 2-Oxo-2-(2-thiophenyl)ethanoic acid disulfide (disulfide C), 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide (disulfide D), and 2,2'-dithiosalicylic acid (disulfide E), can be detected using high performance liquid chromatography. Soil from near marigold roots contained microorganisms that degraded disulfide C, but the enrichments lost activity upon transfer. Two isolates, strains RM1 and RM6, were capable of degrading disulfide E in combination. Isolate RM1 likely provided vitamin B_{12} to isolate RM6. Benzoic acid was identified as a metabolite in the biodegradation of disulfide E by isolate RM6. Isolate RM6 was identified as a member of the genus *Variovorax* and mineralized disulfide E. Combining *Variovorax* sp. strain RM6 with *Pseudomonas* sp. strain BT1d, which degrades DBT through the Kodama pathway, did not release sulfate from DBT.

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guidance and inspiration
light in all the dark

committee members
push thoughts to grow and blossom
brains are fertile dirt

friendship in the lab
there is no thesis without
lab mates who bring snacks

thank you family
as long as I can go home
I can not be lost

Stephanie Cheng sequenced
made my bacterium reveal
its secret identity

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my husband my heart
sunrise, sunshine, and sunset
I love you always

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DBT	dibenzothiophene
Disulfide C	2-oxo-2-(2-thiophenyl)ethanoic acid disulfide
Disulfide D	2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide
Disulfide E	2,2'-dithiosalicylic acid
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
GC-MS	gas chromatography-mass spectrometry
GSH	glutathione
HFBT	3-hydroxy-2-formylbenzothiophene
HPLC	high performance liquid chromatography
HPLC-MS	HPLC-mass spectrometry
MTBSTFA	*N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide
OD	optical density
PAH	polycyclic aromatic hydrocarbon
PCA	plate count agar
SDS	sodium dodecyl sulfate
SFMM	sulfate-free mineral medium

1. INTRODUCTION

1.1 SULFUR HETEROCYCLES

1.1.1 Sources of sulfur heterocycles and their environmental significance

Sulfur heterocycles are organic compounds with at least one sulfur-containing ring. They exist primarily as saturated rings and thiophenes, and occur naturally as secondary metabolites of some microorganisms and plants (Christensen and Lam 1990). For example, the three-ring thiophene α-terthienyl (Figure 1.1) is abundant in marigold petals, and members of the family Asteracea (Compositae) are frequently associated with the production of one-, two-, and three-ring thiophenes from fatty acids. These metabolites may play a role in defense against predators or pathogens as they are often toxic to other organisms. Marigold root extracts have been used to control a variety of parasitic nematodes in soil, although Topp *et al.* (1998) determined that there is little depression of microbial populations in marigold rhizospheres. Sulfur heterocycles are also found as microbially-produced antibiotics; penicillins have a five membered sulfur-containing ring and cephalosporins, a six membered sulfur-containing ring (Jensen and Demain 1995).

Despite these natural occurrences, sulfur heterocycles found in the environment often originate from human activities. These compounds are found in petroleum, in liquids derived from coal, and in chemicals used in industrial processes. Fossil fuels are a major source of sulfur heterocycles in the environment, with petroleum spills and leakage contaminating terrestrial and aquatic environments. Atlas and Bartha (1998) estimated that more than 3.2 million tonnes of oil enter the oceans annually through production, transportation, refining, and disposal of petroleum.

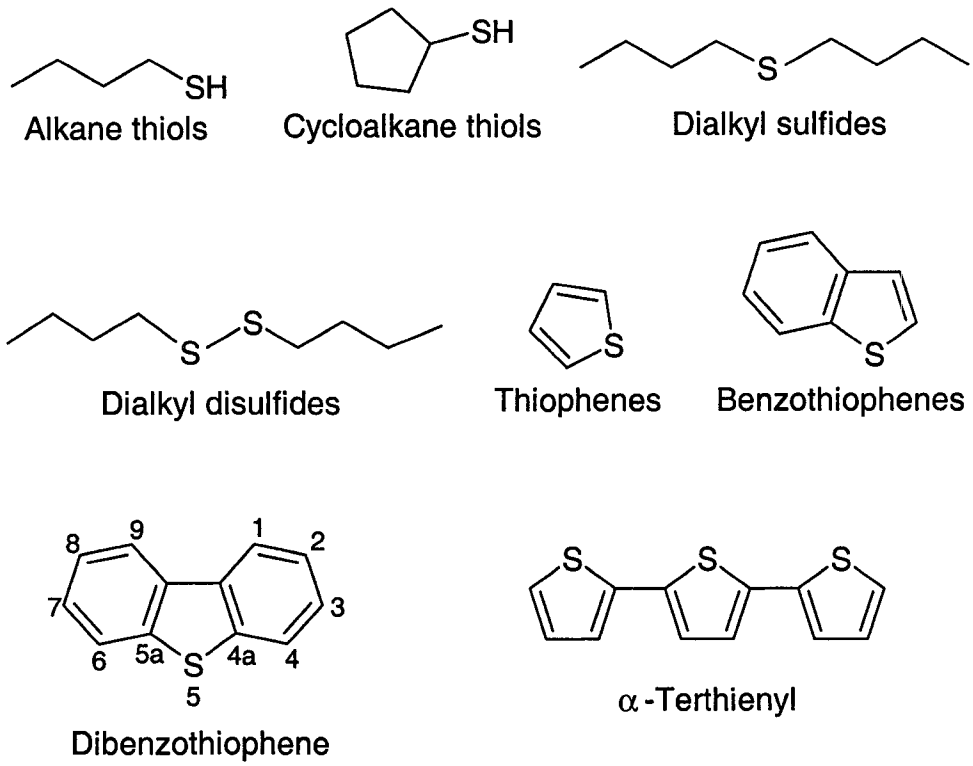


Figure 1.1 Representative structures of various organosulfur compounds (Kropp and Fedorak 1998). The ring numbering convention for DBT is given.

Organically bound sulfur in petroleum is studied for a number of reasons: it causes corrosion and poisons catalysts during the refining process, and it produces sulfur dioxide when sulfur-containing fuels are burned (Sinninghe Damsté and de Leeuw 1990). Combustion of sulfur-containing compounds can contribute to air pollution and acid rain. Oxidation of sulfur released into the atmosphere produces sulfur dioxide, which reacts with oxygen and water to form sulfuric acid (Atlas and Bartha 1998). The elevated levels of sulfur entering the environment with human industrial activity can not be assimilated by ecosystems. In forests, atmospheric inputs of sulfur exceed both natural sulfur

requirements and the ability of the system to biologically accumulate sulfur (Johnson 1984).

Bressler *et al.* (1998) reviewed the biodegradation of sulfur heterocycles, highlighting their environmental significance and the evidence for biological ring cleavage. Dibenzothiophene (DBT) has been studied as the model sulfur heterocycle in petroleum, with many studies identifying DBT metabolites and possible pathways for biodegradation. This information will be reviewed later, with emphasis on the first pathway determined for DBT biodegradation, the Kodama pathway (Kodama *et al.* 1970, 1973). Despite the fact that this pathway is likely to predominate in sulfate-containing environments like the oceans (Kropp and Fedorak 1998), the release of inorganic sulfur from DBT degraded by pure cultures using the Kodama pathway has never been reported.

1.1.2 Nomenclature and bond strengths of sulfur-containing compounds

The structures of some sulfur heterocycles relevant to this discussion are shown in Figure 1.1, with the ring numbering convention indicated for DBT. These compounds include thiophenes, sulfur heterocycles synthesized by plants (Christensen and Lam 1990), and unsubstituted condensed thiophenes such as benzothiophene and DBT found in petroleum and coal derivatives (Jacob 1990).

Bond strength data show that the heteroatomic C-S bonds are weakest in sulfur heterocycles, and therefore they might be susceptible to cleavage during biodegradation (Bressler *et al.* 1998). Also, given that oxygen has the second highest electronegativity of all elements, its addition to the sulfur atom or carbons adjacent to the sulfur atom would further weaken these bonds. Analysis of the pathways for degradation of some sulfur heterocycles shows that the C-S bond is not always cleaved first, and several mechanisms

for thiophene ring cleavage exist (Bressler *et al.* 1998). Consideration of the susceptibility of bonds to cleavage helps to determine the fate of the sulfur atom in cultures degrading sulfur heterocycles. In biological systems enzymes act as catalysts to increase the rate of chemical reactions, but enzymes cannot make possible reactions that are chemically impossible.

1.2 PETROLEUM SULFUR HETEROCYCLES

1.2.1 Biodegradation of petroleum sulfur heterocycles

Sulfur is the third most abundant element in crude oils and bitumens (Speight 1980). Most of the sulfur in crude oils, bitumens, and kerogens is organically bound, related to its initial formation from biomass. Among petroleum sulfur heterocycles, condensed thiophenes are the most common, with benzothiophene, DBT, and their alkylated derivatives being major contributors to petroleum organic sulfur (Speight 1980). Atlas (1981) reviewed the chemistry of petroleum and noted that biodegradation of petroleum increases the sulfur content and polarity of the residual petroleum. This increase in sulfur content can be attributed to the fact that alkyl-condensed thiophenes are particularly resistant to microbial attack in petroleum-contaminated environments (Bence *et al.* 1996, Boehm *et al.* 1981).

Laboratory studies have shown that benzothiophene, DBT, and their alkylated derivatives are biodegradable. Fedorak and Westlake (1983) specifically studied the microbial degradation of organic sulfur compounds in crude oil using laboratory cultures. They found that the order of susceptibility of sulfur heterocycles to biodegradation was: C_2-benzo[b]thiophenes > C_3-benzothiophenes; DBT > C_1-dibenzothiophenes > C_2-dibenzothiophenes. Foght and Westlake (1988) established enrichment cultures growing

on the aromatic fraction of crude oil. While screening for aromatic-degrading pseudomonads, they found one isolate that was capable of mineralizing or oxidizing several polycyclic aromatic hydrocarbons (PAHs), S-, N-, and O-heterocyclic analogues, alkylPAHs, and observed biodegradation of C_2- and C_3-benzothiophenes, DBT, C_1-DBTs, and some C_2-DBTs.

Foght and Westlake (1988) also observed different colonial morphologies for variations in degradative phenotypes. Though there have been many different types of microorganisms isolated with the ability to degrade petroleum components, even within the same species there are variations in the genes and enzymes associated with these transformations. Abed *et al.* (2002) found that microbial communities dominated by cyanobacteria can also be involved in petroleum biodegradation. While studying the microbial community of Wadi Gaza, they found changes in cyanobacterial and bacterial communities accompanied the degradation of aliphatic and aromatic compounds, including DBT.

1.2.2 Toxicity of petroleum sulfur heterocycles

Kropp and Fedorak (1998) reviewed the occurrence, biodegradation, and toxicity of condensed thiophenes found in petroleum. Although several unsubstituted 4-ring polycyclic aromatic sulfur heterocycles have been shown to have mutagenic potential, DBT was inactive in similar studies. Of the sulfur heterocycles tested in rats and mice, DBT is also not particularly carcinogenic. However, DBT was shown to be more toxic to *Daphnia magna* than the structurally similar PAHs phenanthrene and anthracene. Monticello *et al.* (1985) found that the metabolites of DBT biodegradation produced by three *Pseudomonas* strains were more toxic to the bacteria than DBT itself. DBT and its

alkyl derivatives have also been shown to bioaccumulate in tissues of living organisms, especially the C_1-, C_2-, and C_3-substituted DBTs in clams and mussels. Ogata and Fujisawa (1985) found that some organic sulfur compounds, alkyl benzothiophene, DBT, and alkyl DBT, can be transferred from crude oil to oyster and mussel.

The world's oceans are the largest and ultimate receptors of hydrocarbon pollutants, including petroleum sulfur heterocycles. Contamination occurs along transportation routes, with exploration for new petroleum sources, and with spills occurring during routine operations or from oil supertankers. Some historically famous spills include the *Torrey Canyon*, 1967, the *Amoco Cadiz*, 1978 (Atlas *et al.* 1981), and the *Exxon Valdez*, 1989 (Hostettler and Kvenvolden 1994; Bence *et al.* 1996). Not only is floating oil dangerous to birds and marine life, but the dissolved components are equally as hazardous. Aromatic compounds from petroleum can disrupt chemoreception in some marine organisms (Atlas and Bartha 1998). For as long as industrial activity is dependent on the oil industry, the consequences of petroleum extraction, processing, and transportation will require constant attention from researchers, consumers, industries, and governments.

1.3 MECHANISMS OF DBT RING CLEAVAGE

Researchers have studied DBT as the model sulfur compound in petroleum from an engineering perspective, to remove petroleum sulfur that is resistant to chemical treatment, and to better understand the fate of this compound in the environment following contamination. It is estimated that more than 77 tonnes of DBT and methylDBTs were released into the environment during the *Exxon Valdez* spill (Bressler and Fedorak 2001b). DBT biodegradation has recently been reviewed (Bressler *et al.*

1998; Kropp and Fedorak 1998). These authors define three modes of enzymatic attack on DBT that occur under aerobic conditions. Most studies of DBT degradation focus on biodesulfurization through the 4S pathway. This pathway, using microbial enzymes, offers an alternative to chemical and physical treatments for removing the sulfur from DBT. In another mode, DBT carbons 4 and 4a (Figure 1.1) are oxidized by angular dioxygenase attack. Dioxygenase attack can also occur at carbons 1 and 2, analogous to naphthalene degradation. This pathway for DBT degradation is known as the Kodama pathway, the route of DBT biodegradation studied in this thesis and described in section 1.3.3.

1.3.1 Biodesulfurization through the 4S pathway

Many studies of DBT biodegradation focus on biodesulfurization, the selective removal of the sulfur atom that preserves the hydrocarbon quality of the compound (Isbister *et al.* 1988; Krawiec 1990; Kilbane and Jackowski 1992; Kayser *et al.* 1993; Monticello 1994). In this pathway, the sulfur atom is oxidized to the sulfoxide and then the sulfone (Figure 1.2). When the thiophene ring is cleaved, 2'-hydroxybiphenyl-2-sulfinic acid is formed. The sulfur is removed from the ring forming 2-hydroxybiphenyl and sulfite, which rapidly oxidizes to sulfate. This pathway is inhibited by the presence of sulfate (Gray *et al.* 1996), and is not likely to be the major pathway for DBT degradation in sulfate-containing environments such as ocean waters (Kropp and Fedorak 1998).

The most famous bacterium capable of desulfurizing DBT is *Rhodococcus erythropolis* IGTS8, and new *Rhodococcus* strains continue to be identified with the ability to release sulfur from DBT (Kirimura *et al.* 2002; Tanaka *et al.* 2002). The enzymes of this pathway, two monooxygenases and a desulfinase, are encoded by the

genes *dszA*, *dszB*, and *dszC* in a single operon on a circular plasmid in *Rhodococcus* strain IGTS8 (Gray *et al.* 1996). As this pathway occurs during sulfur-limitation, a condition not likely to be present in petroleum-contaminated environments, biodegradation of DBT in the environment would likely proceed through other pathways.

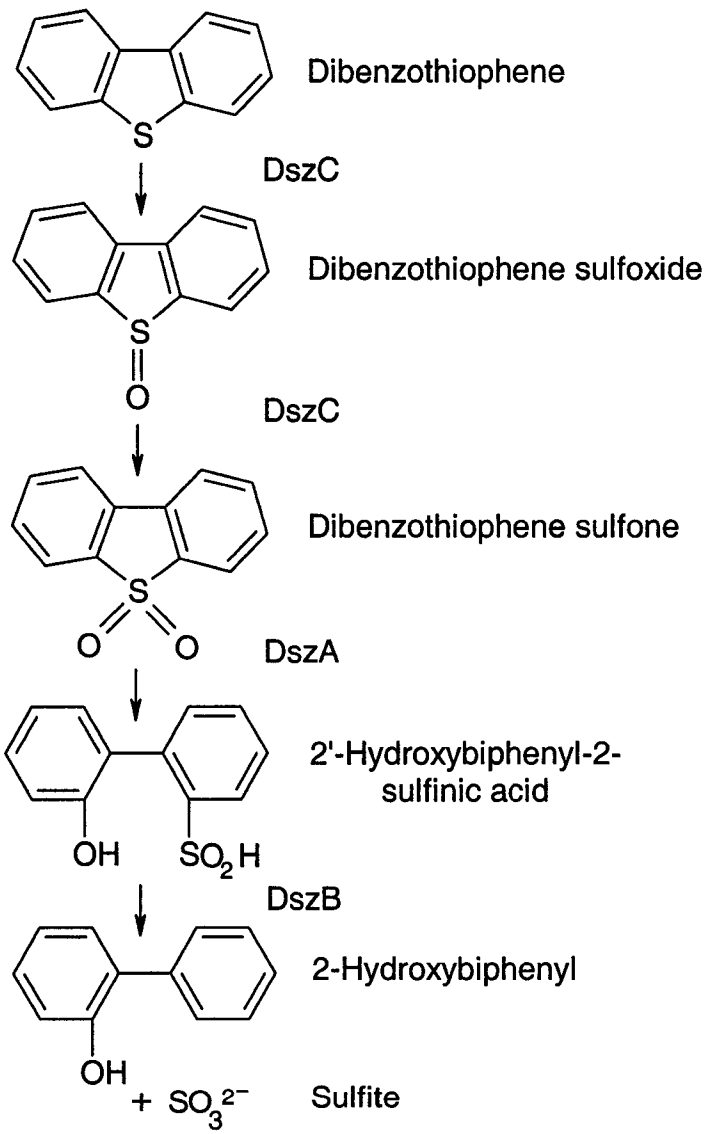


Figure 1.2 The 4S pathway begins with oxidation of the sulfur atom of DBT to form DBT sulfoxide and DBT sulfone. The thiophene ring is cleaved to release 2-hydroxybiphenyl and sulfite. Both DszC and DszA are monooxygenases and DszB is 2-hydroxybiphenyl sulfinate desulfinase.

Previously described as *sox* genes (Denome *et al.* 1994), the *dsz* genes of *Rhodococcus* strain IGTS8 have been cloned and sequenced. A sulfide/sulfoxide monooxygenase encoded by *dszC* catalyzes the stepwise oxidation of DBT to DBT sulfoxide and DBT sulfone. The *dszA* gene product converts DBT sulfone to 2'-hydroxybiphenyl-2-sulfinic acid, which is desulfinated by the enzyme encoded by *dszB*, aromatic sulfinic acid hydrolase (Gray *et al.* 1996). Duarte *et al.* (2001) analyzed bacterial communities in oil-contaminated soil by isolating strains from enrichment cultures and determining their capacity to desulfurize DBT. They found isolates with various partial combinations of the *dsz* genes, and most of their isolates were identified as *R. erythropolis*. The 4S pathway releases sulfite into the medium, which rapidly oxidizes to sulfate.

The kinetics of the abiotic conversion of sulfite to sulfate were studied by Wilkinson *et al.* (1993), Ermakov *et al.* (1997), and Shaikh (1998). Ermakov *et al.* (1997) showed that although the oxidation of sulfite to sulfate (I) is thermodynamically favorable, it does not proceed without catalysts (trace metal contaminants like Fe, Mn, or Cu) or sources of free radicals.

$$SO_3^{2-} + 0.5\ O_2 \longrightarrow SO_4^{2-} \qquad \text{(I)}$$

The uncatalyzed and catalyzed reactions of sulfite oxidation to sulfate proceed through different mechanisms (Wilkinson *et al.* 1993). The uncatalyzed reaction mechanism is dependant on the bisulfite concentration, which exists in equilibrium with sulfite by complex acid-base chemistry (II) (Ermakov *et al.* 1997). The catalyzed reaction mechanism decreases with pH, opposite to the uncatalyzed reaction.

$$HSO_3^- \rightleftharpoons SO_3^{2-} + H^+ \qquad \text{(II)}$$

1.3.2 Angular dioxygenase attack on DBT

DBT biodegradation can also occur through oxidation of the sulfur atom, followed by angular dioxygenation (van Afferden *et al.* 1990, 1993; Bressler and Fedorak 2000). van Afferden *et al.* (1990) studied DBT degradation by *Brevibacterium* sp. DO. They observed the release of sulfite, which was oxidized to sulfate. DBT sulfoxide, DBT sulfone, and benzoate were the major metabolites detected. Their proposed pathway for DBT degradation begins with oxidation of the sulfur atom, and only after the sulfone is formed does angular dioxygenase attack occur at carbons 4 and 4a (Figure 1.3). This pathway results in the complete mineralization of DBT. Because benzoate was released as a metabolite of DBT degradation by *Brevibacterium* sp. DO, van Afferden *et al.* (1993) suggested that the carbon skeleton would be degraded analogous to bacterial degradation of biphenyl. Since the reports by van Afferden *et al.* (1990, 1993) there have been no reports of microorganisms that degrade DBT in the manner shown in Figure 1.3.

1.3.3 The Kodama pathway

Kodama *et al.* (1970, 1973) reported the first pathway for DBT biodegradation. *Pseudomonas abikonensis* or *Pseudomonas jianii* produced 3-hydroxy-2-formylbenzothiophene (HFBT) as the major DBT metabolite (Figure 1.4). There are few reports of bacteria that can grow on DBT using the Kodama pathway in sulfate-containing media (Kropp *et al.* 1997b; Frassinetti *et al.* 1998; Di Gregorio *et al.* 2004). In contrast, there are many studies of bacteria capable of cometabolizing DBT with a variety of other aromatic hydrocarbons (Monticello *et al.* 1985; Foght and Westlake 1988; Saftić *et al.* 1992; Denome *et al.* 1993, Kropp *et al.* 1997a; Kropp *et al.* 1997b).

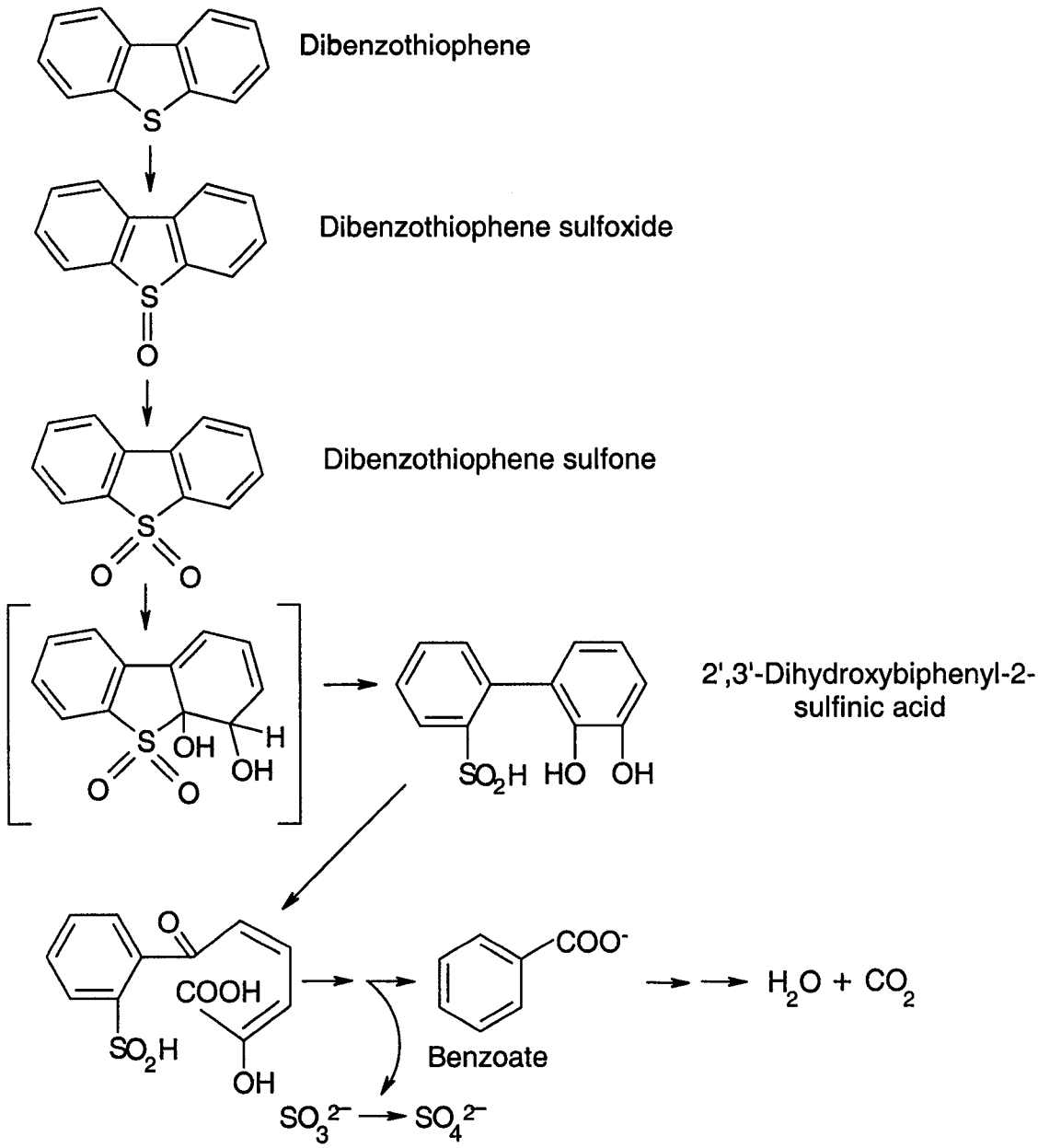


Figure 1.3 DBT biodegradation by *Brevibacterium* sp. DO (after van Afferden *et al.* 1993).

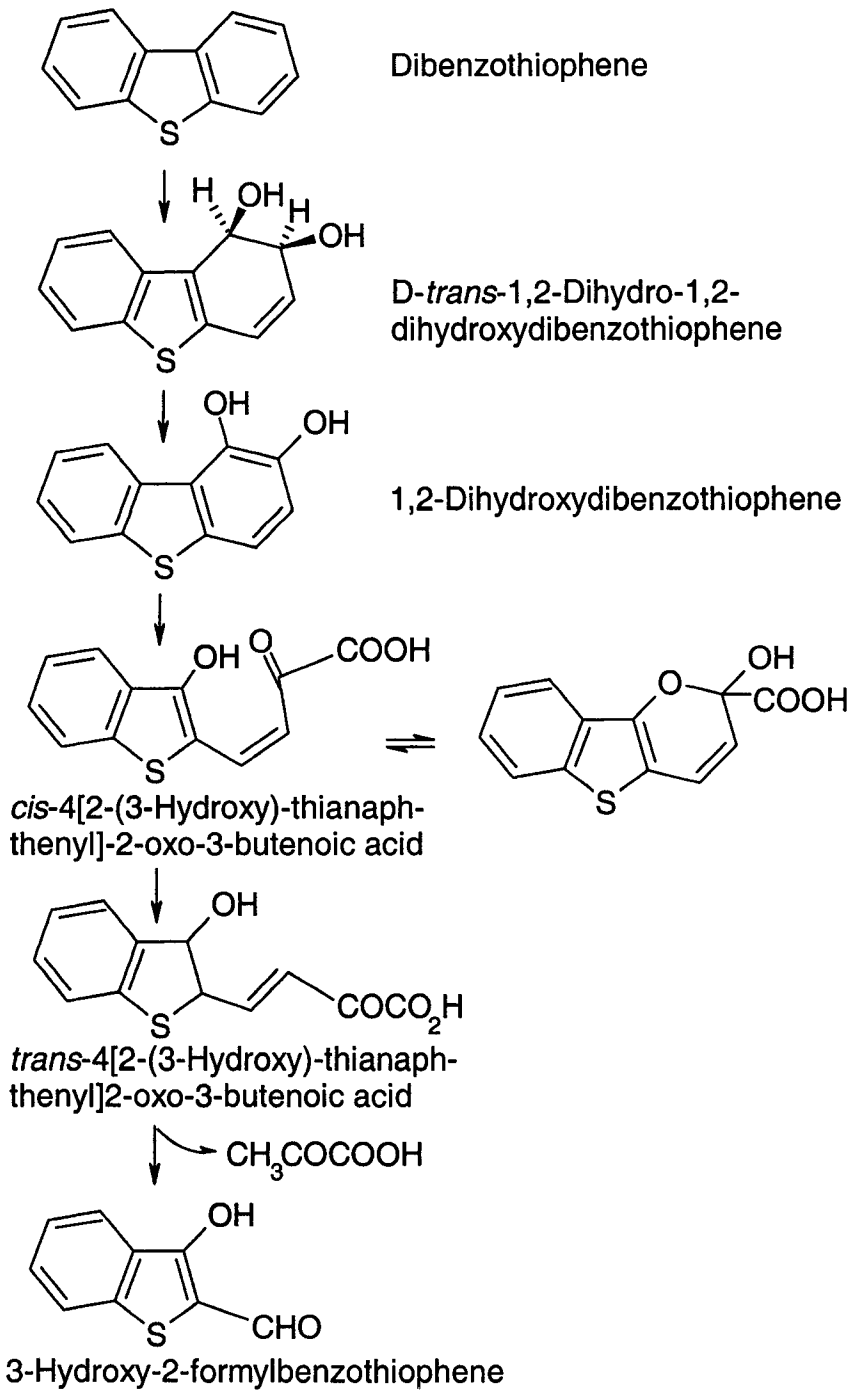


Figure 1.4 The Kodama pathway for DBT biodegradation. The final product of this pathway is 3-hydroxy-2-formylbenzothiophene (HFBT).

Yamada *et al.* (1968) found a yellow metabolite from microbial conversion of petroleum sulfur compounds that was identified as HFBT, the final product of the Kodama pathway, by Kodama *et al.* (1970). Laborde and Gibson (1977) found HFBT in pure cultures of a *Beijernickia* sp. degrading DBT, but HFBT was not further metabolized by this organism. Mormile and Atlas (1988) studied the mineralization of HFBT formed from the biodegradation of DBT using a strain of *Pseudomonas putida.* Their results suggested that HFBT can be further degraded; however, they were unable to show release of sulfur from the thiophene ring under aerobic conditions, and they did not identify any metabolites of HFBT biodegradation. To achieve complete mineralization of DBT, they suggested that a succession of microbial populations would be necessary.

Denome *et al.* (1993) cloned and sequenced the genes for DBT biodegradation. The 9.8-kb DNA fragment from *Pseudomonas* strain C18 consisted of nine open reading frames designated *dox*ABDEFGHIJ. Three open reading frames, *dox*ABD, were identical to the *Pseudomonas putida ndo*ABC genes coding for naphthalene dioxygenase, and DoxG was similar to NahC at both the nucleotide and amino acid levels. From these results, Denome *et al.* (1993) suggested that DBT serves as an alternate substrate for naphthalene-degrading enzymes, allowing the degradation of DBT through the Kodama pathway.

Kodama *et al.* (1970, 1973) observed that DBT was also oxidatively converted into DBT sulfoxide, which is not part of the enzymatic pathway analogous to naphthalene degradation by *Pseudomonas.* Monticello *et al.* (1985) described three *Pseudomonas* isolates that oxidized DBT to water-soluble products via the Kodama pathway, and found that this mode of DBT oxidation was plasmid-associated in at least two of the three

isolates. Di Gregorio *et al.* (2004) used molecular techniques to study *Burkholderia* sp. DBT1, capable of transforming DBT to HFBT, and found two new sets of genes for DBT biodegradation through the Kodama pathway. The DNA sequence of genes involved in the initial steps of DBT biodegradation by isolate DBT1 show only 60% similarity to the highly conserved *nah*-like class of genes.

The Kodama pathway for DBT biodegradation has been known for 30 years, with recent attention focused on accounting for the sulfur in DBT-degrading cultures. Kropp *et al.* (1997b) predicted that the sulfur missing in cultures degrading DBT through the Kodama pathway could exist as highly polar organic compounds. Analysis of the culture supernatants from four *Pseudomonas* isolates that degraded DBT could account for between 31 and 76% of the sulfur from DBT. They detected primarily HFBT in the *Pseudomonas* strain BT1d culture, along with DBT sulfoxide, DBT sulfone, and benzothiophene-2,3-dione. No sulfate was detected from the biodegradation of DBT by these isolates.

Recently, Bressler and Fedorak (2001a) purified HFBT from a DBT-degrading culture of *Pseudomonas* strain BT1d, which grows on DBT as its sole carbon source. They found that, upon storage, HFBT condensed to thioindigo and *cis*-thioindigo, products from DBT biodegradation that had not previously been reported. A mixed culture that had been maintained on Prudhoe Bay crude oil mineralized HFBT to CO_2. Benzothiophene-2,3-dione was detected in an HFBT-degrading mixed culture, the product of 2-mercaptophenylglyoxylate ring closure. Benzothiophene-2,3-dione was previously found in cultures degrading benzothiophene (Bohonos *et al.* 1977; Fedorak and Grbić-Galić 1991). Benzothiophene-2,3-dione has also been detected in DBT-

degrading cultures (Bohonos *et al.* 1977; Kropp *et al.* 1997a) and must be a metabolite of HFBT formed through the Kodama pathway (Bressler and Fedorak 2001a). The Kodama pathway is the focus of this thesis, because the release of the sulfur from DBT has never been observed using cultures that degrade DBT through the Kodama pathway.

1.4 PRODUCTS OF BIOTIC AND ABIOTIC TRANSFORMATIONS OF CONDENSED THIOPHENE METABOLITES

1.4.1 Identification of 2,3-diones from different thiophene compounds

Biodegradation of benzothiophene-2,3-dione has only recently been studied by Bressler and Fedorak (2001b), prompted by the relative frequency with which 2,3-diones are detected in cultures degrading benzothiophenes and DBTs (Table 1.1). Bohonos *et al.* (1977) studied the biodegradation of benzothiophene in freshwater and wastewater samples and found benzothiophene-2,3-dione in culture extracts.

The aerobic biotransformations of benzothiophene and 3-methylbenzothiophene were studied by Fedorak and Grbić-Galić (1991). They found that neither of these sulfur heterocycles could support the growth of a culture previously enriched on 1-methylnaphthalene. With cometabolism, benzothiophene was transformed to benzothiophene-2,3-dione and 3-methylbenzothiophene to 3-methylbenzothiophene sulfoxide and 3-methylbenzothiophene sulfone. This demonstrated that oxidation occurs preferentially at carbons 2 and 3 in benzothiophene, and that when carbon 3 is substituted with a methyl group, the sulfur atom gets oxygenated. Benzothiophene-2,3-dione was found to be chemically unstable, and not detected from benzothiophene transformations in the presence of crude oil. The photooxidation of benzothiophene also yielded

Table 1.1 Identification of 2,3-diones from the biodegradation of different thiophenes.

Reference	Substrate	2,3-Dione	Organisms	Observations
Bohonos *et al.* 1977	Benzothiophene	Benzothiophene -2,3-dione	Freshwater and waste water treatment plant samples	Benzothiophene also transformed to 2,3-dihydoxy-2,3-dihydrobenzothiophene and benzothiophene sulfoxide
Fedorak and Grbić-Galić 1991	Benzothiophene	Benzothiophene -2,3-dione	Mixed enrichment cultures and *Pseudomonas* sp. strain BT1	Cometabolism of benzothiophene yielded 2,3-dione while 3-methyl-benzothiophene produced the sulfoxide and sulfone
Saftić et al. 1992	Methylbenzo-thiophenes	Methylbenzo-thiophene-2,3-diones	*Pseudomonas* sp. strain BT1	The isomers with a methyl group on the benzene ring gave the corresponding 2,3-diones except 7-methylbenzothiophene which gave the 2,3-dione, sulfoxide and sulfone
Eaton and Nitterauer 1994	Benzothiophene	Benzothiophene -2,3-dione	*Pseudomonas putida* RE204	Purified and identified 2,3-dione from 2-mercapto-phenylglyoxalate transformation during extraction of products
Kropp *et al.* 1994a and Kropp *et al.* 1996	Benzothiophene and methyl-benzothiophenes	Benzothiophene -2,3-dione and some methyl-benzothiophene-2,3-diones	*Pseudomonas* strains W1, F and SB(G)	Benzothiophenes with methyl groups on the benzene ring gave the corresponding 2,3-diones
Kropp *et al.* 1997a	Isomers of dimethylDBT	6,7-dimethyl-benzothiophene-2,3-dione and 5-methylbenzothio phene-2,3-dione	*Pseudomonas* strains W1, BT1 and F	The susceptibilities of dimethylDBTs to attack depended on the position of the methyl groups
Bressler and Fedorak 2001b	DBT	Benzothiophene -2,3-dione	*Pseudomonas* strain BT1d	DBT was used as the sole carbon and sulfur source, 2,3-dione detected in acidified culture extracts by GC-MS

Pseudomonas strain BT1 was further investigated by Saftić *et al.* (1992), using six methyl-substituted benzothiophenes as substrates. 2,3-Diones were detected as metabolites from 4-methylbenzothiophene, 5-methylbenzothiophene, and 6-

methylbenzothiophene. Sulfoxides and sulfones were also detected, with metabolites of methylbenzothiophene degradation determined by the position of the methyl substitution. The thiophene ring of benzothiophene is more susceptible to microbial oxidation than the benzene ring.

Kropp *et al.* (1994a, 1994b) observed the formation of high molecular weight compounds after abiotic condensations in benzo[*b*]thiophene degrading cultures. Three *Pseudomonas* isolates were studied, also capable of degrading methylbenzothiophenes with substitutions on the benzene ring to dimethyl-substituted benzonaphthothiophenes. In a study of three *Pseudomonas* strains for their ability to transform benzothiophene and methylbenzothiophenes, 2,3-diones were observed as metabolites when the methyl group was on the benzene ring, versus sulfoxides when the methyl group was on the thiophene ring. The ring cleavage products of 6- and 7-methylbenzothiophene were detected, and the microbially mediated condensation product was more toxic than the parent compound (Kropp *et al.* 1994a).

Eaton and Nitterauer (1994) studied the biotransformation of benzothiophene by isopropylbenzene-degrading bacteria. 2-Mercaptophenylglyoxylate was identified as a metabolite, though it was not found in a dihydrodiol dehydrogenase-deficient mutant incubated with benzothiophene. Their data indicated that benzothiophene oxidation begins at either ring, with several enzyme-catalyzed reactions, and they were able to predict pathways for benzothiophene transformations by the intermediate compounds detected. Oxidation of the benzene ring by isopropylbenzene-2,3-dioxygenase produced a substrate for the dehydrogenase, followed by the 3-isopropylcatechol-2,3-dioxygenase and the product accumulated, not being a substrate for the next isopropylbenzene

enzyme. These reactions are analogous to naphthalene metabolism, but naphthalene-degraders possess enzymes that can continue to degrade the products that accumulate when isopropylbenzene degraders attack benzothiophene.

Eaton and Nitterauer (1994) also found that oxidation of the thiophene ring produces *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene, whose ring opens spontaneously with aldehyde accumulation in cultures. By abiotic oxidation or other cellular enzymes, 2-mercaptophenylglyoxylate, the open ring form of benzothiophene-2,3-dione, is produced. Thus, the abiotic reactions of DBT metabolites proved to be as important as those mediated by microorganisms in discovering the fate of DBT degraded through the Kodama pathway. Eaton and Nitterauer (1994) suggested that it may be possible to completely metabolize organosulfur compounds by combining enzymes from different pathways. They proposed creating enzyme mixtures by constructing artificial bacterial strains. The use of mixed cultures may be equally as applicable for combining degradative traits. Assembled mixed cultures would more accurately reflect the reality of biodegradation in contaminated environments.

1.4.2 Detection of disulfides produced from DBT metabolites

The investigation of the fate of DBT metabolites led to the discovery of dicarboxylic acid disulfides in culture supernatants (Bressler and Fedorak 2001b). The properties of these compounds and their high molecular weight (despite being products of biodegradation) previously concealed them from researchers studying the fate of DBT degraded through the Kodama pathway. The release of the sulfur, or mineralization of DBT, has not yet been shown for this pathway.

Bressler and Fedorak (2001b) proposed a possible abiotic mechanism for the formation of benzothiophene-2,3-dione from HFBT (Figure 1.5). Through free radical reactions with molecular oxygen, HFBT loses HCHO and H_2O to form the 2,3-dione. At neutral pH, benzothiophene-2,3-dione opens to form 2-mercaptophenylglyoxylate (Figure 1.6).

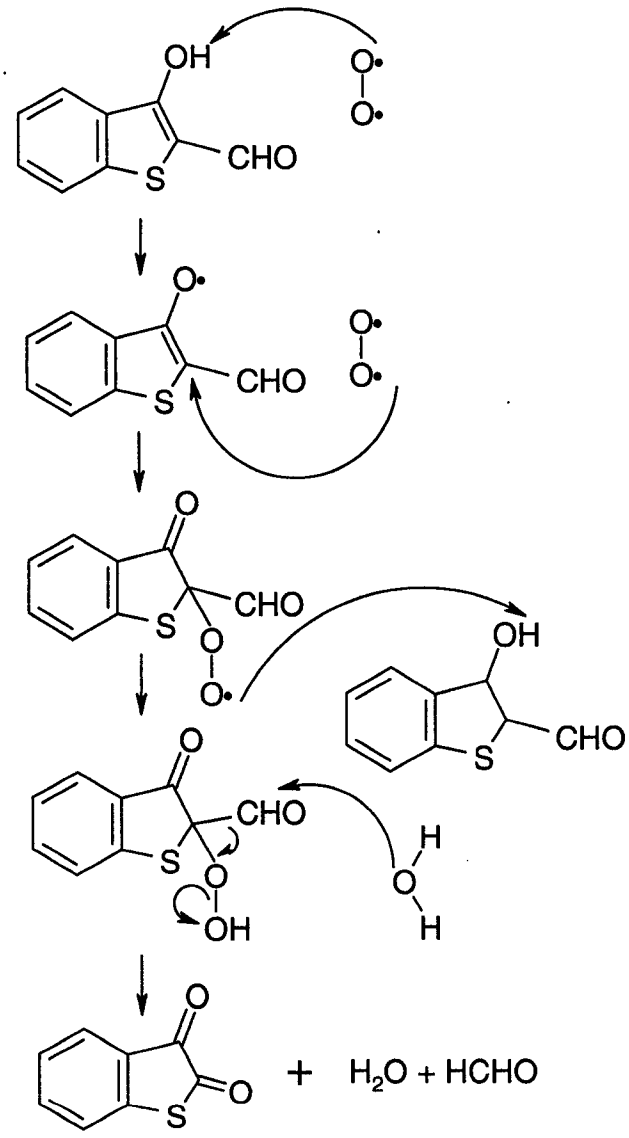


Figure 1.5 A proposed abiotic mechanism for the formation of benzothiophene-2,3-dione from HFBT (Bressler and Fedorak 2001b). In the first steps, HFBT participates in free radical reactions with oxygen and a second molecule of HFBT.

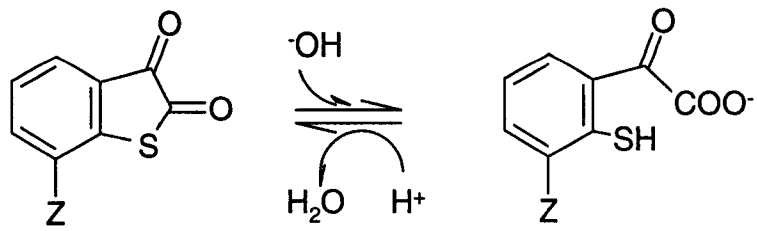


Figure 1.6 Benzothiophene-2,3-dione (Z = H) opens to form 2-mercapto-phenylglyoxylate, dependant upon pH. The same equilibrium exists when Z is a methyl group, and 7-methylbenzothiophene-2,3-dione opens to form 3-methyl-2-mercaptophenylglyoxylate at neutral pH (Bressler and Fedorak 2001b).

In the course of investigating the reactions of benzothiophene-2,3-dione, Bressler and Fedorak (2001b) encountered numerous analytical difficulties. The laboratory procedures generally employed to extract and analyze DBT-degrading cultures (Bressler and Fedorak 2001a, Kropp *et al.* 1997a) were unsuccessful for detecting further metabolites. Bressler and Fedorak (2001b) credit the use of high performance liquid chromatography (HPLC) and HPLC coupled with mass spectrometry (HPLC-MS) for their observations of novel compounds resulting from the reactions of benzothiophene-2,3-dione.

Three disulfides, two novel metabolites of DBT biodegradation, and a third previously observed disulfide (Finkel'stein *et al.* 1997) were detected by Bressler and Fedorak (2001b) in cultures degrading DBT through the Kodama pathway. 2-Mercaptophenylglyoxylate, formed from the 2,3-dione, abiotically condensed to form 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide (Figure 1.7). After abiotic loss of a carbon and oxygen atom, another novel disulfide was formed, 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide. This compound also abiotically lost a carbon and oxygen atom to form 2,2'-dithiosalicylic acid. These three disulfides, referred to as disulfides C, D, and E in Figure 1.8, could account for the missing sulfur in DBT-degrading cultures

predicted by Kropp *et al.* (1997b) because they are highly polar, sulfur-containing organic compounds. The disulfides previously avoided detection because they are highly polar dicarboxylic acids, are poorly soluble in organic solvents, can be adsorbed by commonly used drying agents, and have high molecular masses. During biodegradation, compounds are generally broken down into smaller units, whereas in this case, the metabolites of DBT biodegradation through the Kodama pathway undergo abiotic reactions to yield disulfides with more carbon and oxygen atoms than the parent compound.

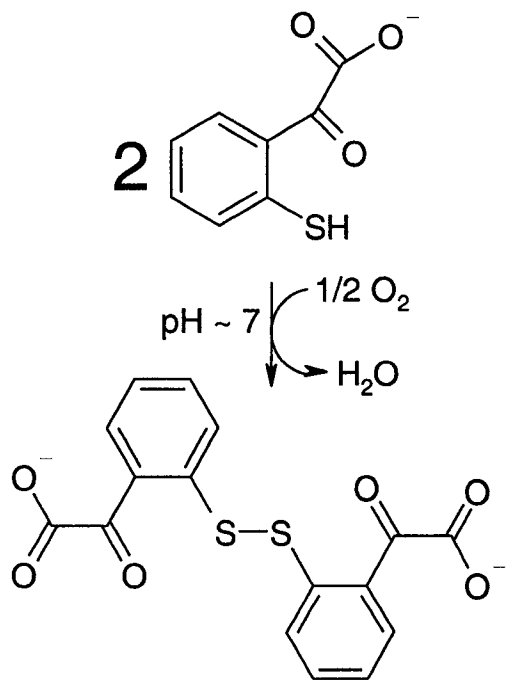
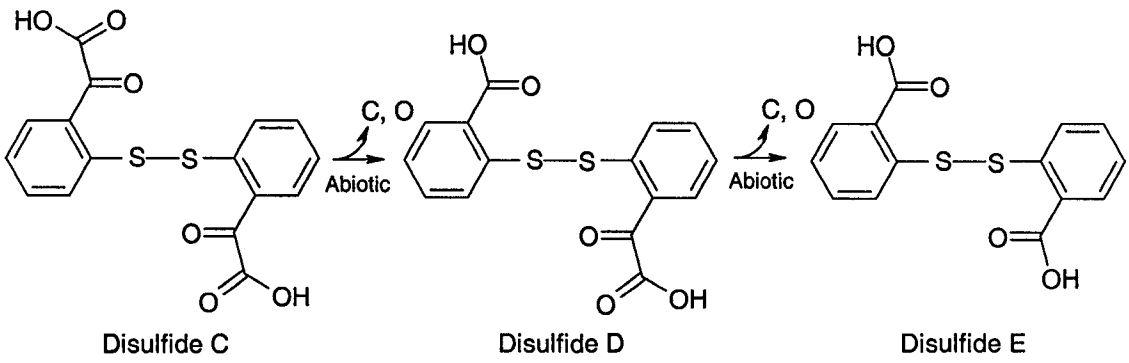


Figure 1.7 Two molecules of 2-mercaptophenylglyoxylate abiotically condense to form a dicarboxylic acid disulfide (Bressler and Fedorak 2001b).



Disulfide C Disulfide D Disulfide E

Figure 1.8 Structures of disulfides C, D, and E. 2-Mercaptophenylglyoxylate abiotically condenses to form 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide, disulfide C. After abiotic losses of carbon and oxygen atoms, 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide, disulfide D, and 2,2'-dithiosalicylic acid, disulfide E, are formed (Bressler and Fedorak 2001b).

2,2'-Dithiosalicylic acid (disulfide E) was also observed by Finkel'stein *et al.* (1997) in DBT-degrading cultures, but they believed it to be formed through oxidation of 2-mercaptobenzoic acid. Bressler and Fedorak (2001b) observed that the abiotic condensation of 2-mercaptophenylglyoxylate to disulfide C occurs quickly, suggesting that although some disulfide E forms from 2-mercaptobenzoic acid dimerization, most of this disulfide originates from abiotic losses of carbon and oxygen atoms from disulfides C and D.

1.4.3 Dicarboxylic acid disulfides lose carbon and oxygen atoms

Disulfides C and D contain α-keto acid moieties similar to phenylglyoxylic acid. There are many reports on the biological reactions of simple α-keto acids, like the familiar tricarboxylic acid cycle intermediate α-ketoglutarate, but few involving molecules that also contain aromatic rings. In the reactions of disulfides C and D to form disulfide E, carbon and oxygen atoms are lost abiotically either as CO or CO_2. The

decarbonylation and decarboxylation of phenylglyoxylic acid have been studied by Siegel and Lanphear (1979a, 1979b), Kuhn and Gorner (1988), Chen *et al.* (1995), and Yonezawa *et al.* (2000). Siegel and Lanphear (1979a) reported that the presence of hydrogen peroxide promoted oxidative decarboxylation, and in its absence, decarbonylation occurred after several days in acidic medium. The oxidative decarboxylation reaction is catalyzed by iron, a trace metal found in the medium in which disulfides were detected by Bressler and Fedorak (2001b). However, the calculated activation energy for decarbonylation is lower than that for decarboxylation (Chen *et al.* 1995). Bressler and Fedorak (2001b), considering the lower activation energy, favored decarbonylation for the disulfide losses of carbon and oxygen atoms.

1.5 REACTIONS AND PROPERTIES OF SOME DISULFIDES

Disulfides are generally less reactive than free thiols (Jocelyn 1972). The rate of thiol-disulfide exchange is pH-dependant, though in most cases exchange is fast and spontaneous near pH 7. Fernandes and Ramos (2004) studied the theoretical mechanism for thiol-disulfide exchange based on previous experimental data and proposed mechanisms. They found that an uncomplicated S_N2 transition state best described the exchange, with the theoretical results in excellent agreement with observed kinetics. Disulfide-disulfide exchange occurs if a catalytic amount of thiol is present, and in most reactions of disulfides, cleavage occurs between the sulfur atoms (Jocelyn 1972).

Disulfides occur in biological molecules like glutathione disulfide and in the disulfide linkages between cysteine residues in protein. Glutathione (GSH) plays a major role in maintaining the redox state of cells, and the enzyme GSH reductase is widely distributed in bacterial, plant, and animal cells (Jocelyn 1972). GSH slowly reduces disulfide groups

in albumin and insulin under physiological conditions. Redox regulation in response to oxidative and nitrosative stresses includes modifications of proteins, including the formation of inter- and intramolecular protein disulfides and mixed disulfides with GSH (Huang and Huang 2002).

Another reaction involving disulfides is sulfitolysis (Cole 1967). In the presence of sulfite, disulfide E would be cleaved to form 2-(S-sulfo)benzoic acid and thiosalicylic acid. Kunert (1989) showed that the fungus *Microsporum gypseum* excretes sulfite into the medium to cleave disulfide bonds in wool, enabling the keratin to be degraded by extracellular proteases.

Though there is no information on the toxicity of disulfides C and D, disulfide E is likely not a highly toxic compound. It has been clinically compared with aspirin as a medication for arthritis patients (Dequeker *et al.* 1980), and is a decomposition product of thiomersal (Tan and Parkin 2000). Thiomersal is an effective antibacterial and antifungal agent widely used as a preservative in liquid pharmaceutical formulations, primarily ophthalmic solutions and opened multidose vial vaccines, where special care should be taken to ensure that it does not complex with other ingredients (Abuqaddom *et al.* 2003). Thiomersal is almost 50% mercury by weight, and is used in some diphtheria, tetnus, hepatitis B, influenza, meningococcal, and rabies vaccines (Goldman *et al.* 2001).

1.6 BIODEGRADATION OF SOME DISULFIDES

There are few reports on the biodegradation of disulfide compounds, despite their prevalence in nature and industrial products. Of environmental concern are the disulfides that occur in polymers like rubber. Spent car tires are a solid waste problem, with biological possibilities for treatment that require breaking the reinforcing disulfide bond

crosslinking (Holst *et al.* 1998). Most studies on rubber degradation focus on protecting exposed rubber from microbial attack, rather than promoting its breakdown. Holst *et al.* (1998) relate desulfurization of rubber to studying oil desulfurization using the 4S pathway for DBT biodegradation. Microorganisms degrade rubber by oxidizing the polymer backbone, defining the search for extracellular enzymes capable of selectively breaking sulfur crosslinking and disulfide bonds.

Dimethyl disulfide is a malodorous compound, produced by the wood-pulp industry, oil refineries, manure, sediments, and sewers, that can be attacked by microorganisms (Visscher and Taylor 1993). A strain of *Thiobacillus* grew chemolithotrophically on dimethyl disulfide as the sole source of carbon and energy (Smith and Kelly 1988). They found methanethiol as a metabolite of dimethyl disulfide under aerobic and anaerobic conditions, proposing that dimethyl disulfide reductase, methanethiol oxidase, formaldehyde dehydrogenase, and formate dehydrogenase would be essential enzymes in the pathway for dimethyl disulfide biodegradation. Diallyl disulfide is an essential oil of garlic that interacts with cholesterol synthesis and reduces chemically induced carcinogenesis. In rats, diallyl disulfide is transformed to allyl mercaptan, allyl methylsulfide, allyl methyl sulfoxide, and allyl methyl sulfone (Germain *et al.* 2002).

Some fungi are also capable of degrading disulfide compounds. A new Basidiomycete that was capable of degrading dimethyl disulfide was isolated by Phae and Shoda (1991) using enrichment techniques with sulfurous gases. They studied the degradation of four gases: hydrogen sulfide; methanethiol; dimethyl sulfide; and dimethyl disulfide. However, the data for dimethyl disulfide degradation do not appear on any of the graphs presented by these authors. Dimethyl disulfide is also the only gas they did not

continue to test in mixed gas experiments, despite reporting enhanced dimethyl disulfide removal by this fungus. Itoh *et al.* (1997) found that both *Coriolus versicolor* and *Tyrmyces palustris* were capable of degrading dibenzyl disulfide. These fungi produced trace amounts of benzyl alcohol and benzaldehyde when given benzyl sulfide or benzyl disulfide.

Disposal of feather waste from the poultry industry also requires disulfide biodegradation, because the mechanical stability of keratin is derived from its disulfide bridges. Riffel *et al.* (2003) isolated a novel feather-degrading bacterium from poultry waste. The organism was identified as a strain of *Chryseobacterium* that could reduce disulfide bonds. Nam *et al.* (2002) identified a thermophilic anaerobe capable of degrading poultry feathers. *Fervidobacterium islandicum* AW-1 produced a thermostable keratinolytic serine protease.

For chicken feathers to disintegrate, extracellular reduction of the disulfide bonds is necessary. Keratin disulfide reduction has been observed with *Streptomyces pactum* (Böckle *et al.* 1995, Böckle and Müller 1997) and *Stenotrophomonas* sp. strain D-1 (Yamamura *et al.* 2002). In these studies, the authors isolated extracellular serine proteases for degrading protein chains, and extracellular disulfide bond-reducing agents. Yamamura *et al.* (2002) isolated a disulfide reductase-like protein capable of increasing keratin degradation by cleaving disulfide bonds. This enzyme could also reduce disulfides in collagen, elastin and oxidized glutathione and cysteine. The disulfide reductase-like protein showed no activity against casein, a substrate lacking disulfide bonds. In the case of keratin, it appears that combinations of enzymes are required for

degradation, where a protease and a disulfide bond-reducing enzyme act cooperatively to release protein sulfur.

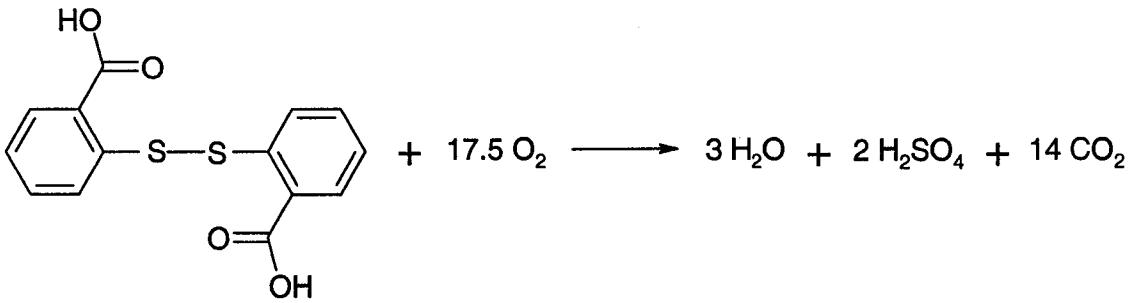
Disulfides also occur in some pesticides. Tetramethylthiuram disulfide is an environmentally persistent pesticide that adversely affects rhizobium-legume symbiosis. Tetramethylthiuram disulfide was biodegradable by a strain of *Pseudomonas aeruginosa* isolated from soil (Shirkot *et al.* 1994). These authors used enrichment techniques with basal salts medium and soil inocula to obtain cultures capable of tetramethylthiuram disulfide degradation. Microorganisms were isolated from these cultures by plating on nutrient agar, and identified using morphological and biochemical tests. Similar enrichment and isolation techniques could be used to isolate other microorganisms capable of degrading disulfides formed from DBT biodegradation.

1.7 BIODEGRADATION OF DISULFIDE E AND 2,3-DIONES

Biodegradation of disulfide E (Figure 1.8) was observed by Bressler (2001) using mixed cultures enriched from garden soil. Four distinct colony types were observed on solid medium, but efforts to purify the isolates and establish pure cultures degrading disulfide E were unsuccessful. This suggested that a combination of bacteria may be required to biodegrade this disulfide. Significant amounts of sulfate, more than 80% of the S in the disulfide, were detected in the mixed cultures degrading disulfide E, indicating mineralization of this compound and thiophene ring cleavage.

Lau (2001) observed the release of sulfate in cultures incubated with 7-methylbenzothiophene-2,3-dione, causing a decrease in pH and death of the cultures. This drop in pH complicates the study of sulfur heterocycle mineralization, because active cultures will cause their own environment to become highly acidic if the medium

is not buffered. Figure 1.9 shows the production of sulfuric acid from disulfide E mineralization.



Disulfide E

Figure 1.9 The equation for disulfide E mineralization.

Lau (2001) reported that 7-methylbenzothiophene-2,3-dione is a metabolite of both 4-methylDBT and 7-methylbenzothiophene degradation. These results suggest that not only would DBT biodegradation result in the formation of disulfides, but also degradation of substituted DBTs, benzothiophene, and substituted benzothiophenes. Analogous to the condensation of 2-mercaptophenylglyoxylate to form disulfide C, other substituted 2,3-diones formed by microbial reactions in petroleum-contaminated environments could similarly open and condense. Biodegradation of DBT and substituted DBTs likely results in the formation of a variety of disulfide compounds.

Though DBT is studied as the model sulfur heterocycle in petroleum, its complete mineralization has not yet been shown via the Kodama pathway. By assembling a mixed culture of a DBT-degrading bacterium, like isolate BT1d, and a population of bacteria capable of mineralizing the disulfides formed abiotically from HFBT, release of the sulfur atom as sulfate may be observed in a DBT-degrading culture.

1.8 PROJECT OBJECTIVES AND EXPERIMENTAL APPROACHES

This project had two major objectives. The first objective was to demonstrate biodegradation of disulfides formed as a result of bacterial degradation of DBT via the Kodama pathway. Figure 1.10 shows an abbreviated pathway for DBT degradation, which indicates the formation of disulfides C, D, and E.

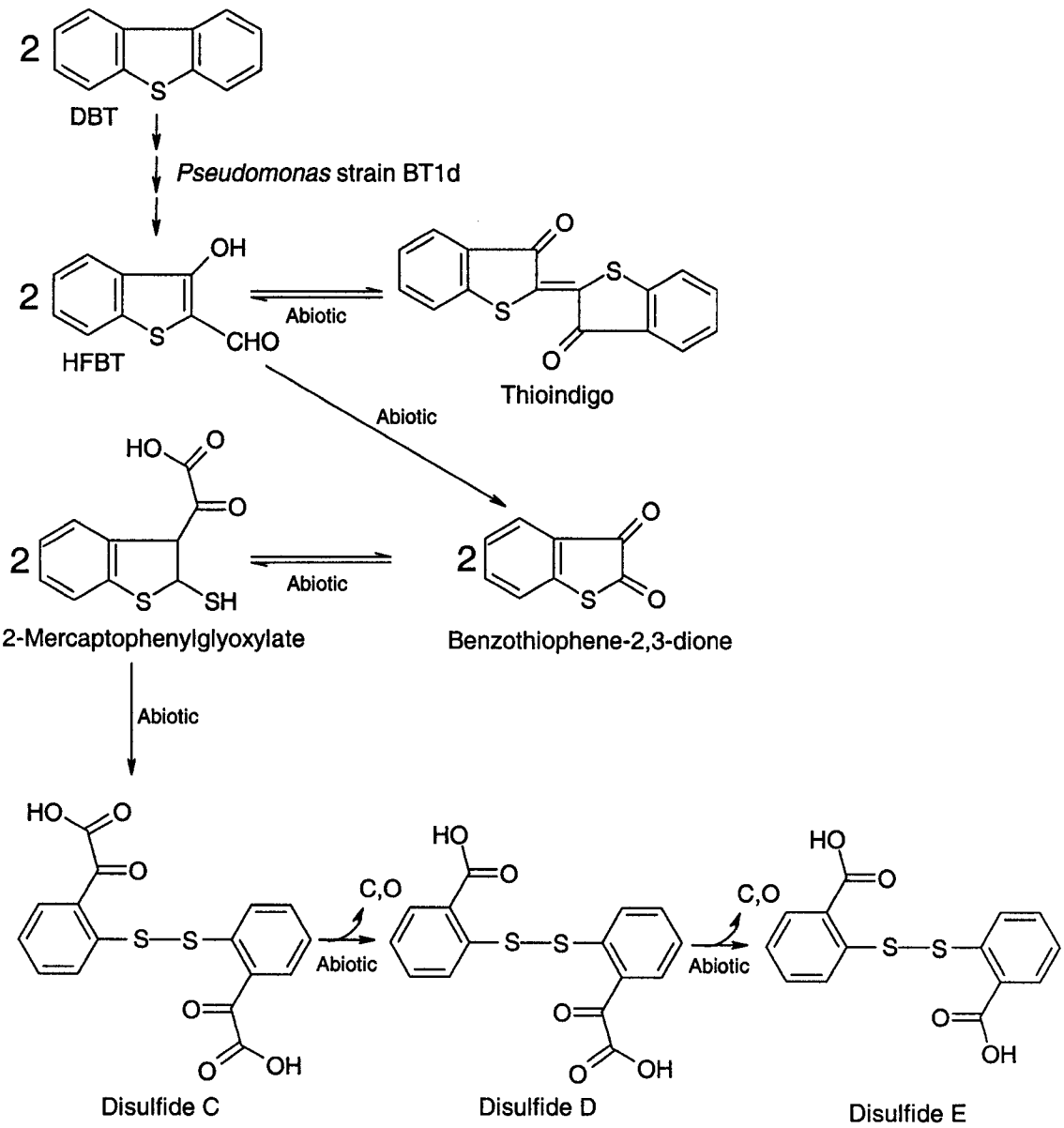


Figure 1.10 A summary of the biotic and abiotic reactions leading to the formation of disulfides C, D, and E (after Bressler and Fedorak 2001b).

Disulfides C and D were provided as sole carbon and sulfur sources in sulfate-free medium, produced abiotically from benzothiophene-2,3-dione. The 2,3-dione was synthesized and when it was incubated in sterile medium the ring opened to form 2-mercaptophenylglyoxylate, abiotically condensing to form disulfide C (Figure 1.10). 2,2'-Dithiosalicylic acid is commercially available, and it was added to sterile medium to search for microorganisms capable of degrading disulfide E. Using the HPLC method described by Bressler *et al.* (2001b), disappearance of disulfides C, D, and E was monitored as they were degraded in cultures. Figure 1.11 shows HPLC chromatograms with retention times and UV scans obtained for disulfides C, D, and E.

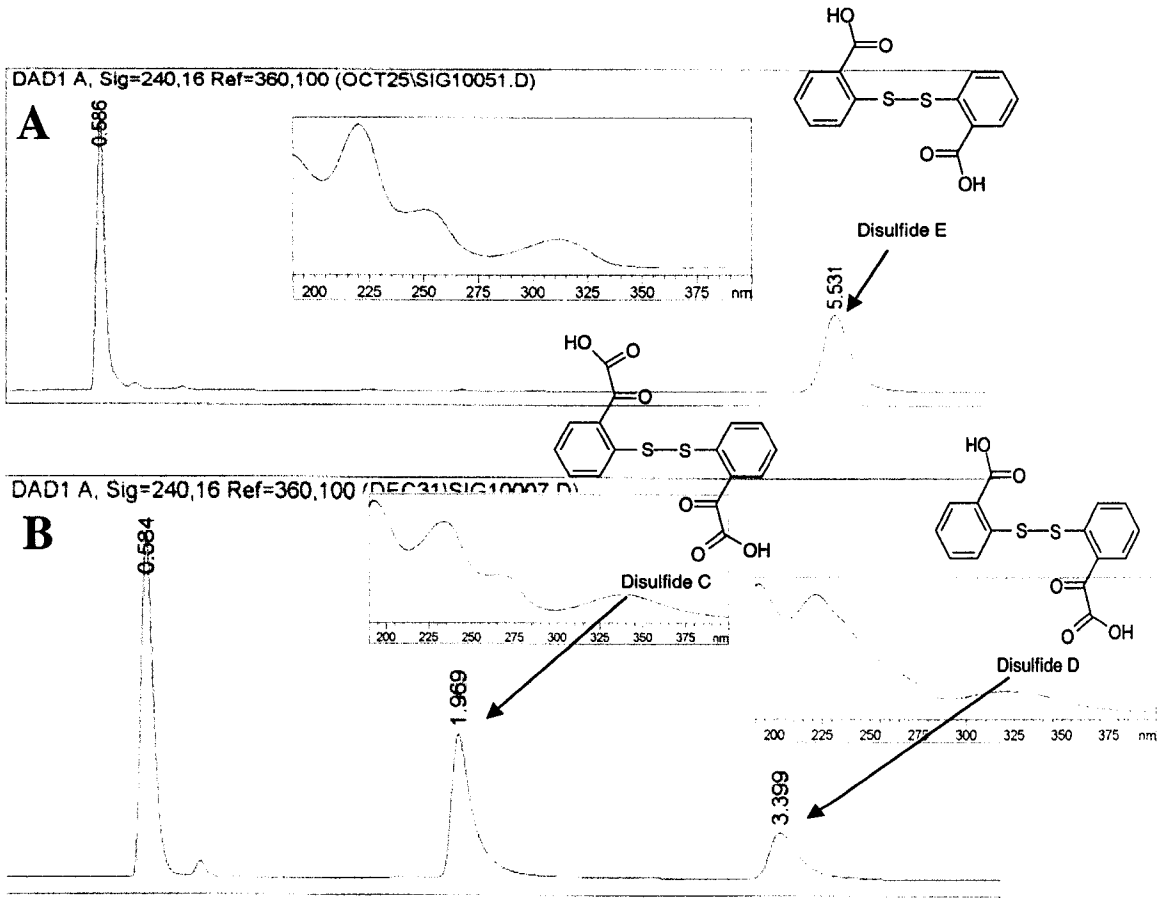


Figure 1.11 HPLC chromatograms at 240 nm and UV scans for disulfides C, D, and E. The retention time of disulfide E was 5.5 min (A), and the retention times of disulfide C and disulfide D were 1.9 min and 3.4 min, respectively (B).

The most likely environment for finding microorganisms capable of degrading disulfides C, D, and E is soils used to cultivate marigolds, plants associated with thiophene compounds. By establishing enrichment cultures using these soils as inocula, microorganisms with the capacity to degrade these disulfides were enriched and isolated.

The second objective of this project was to assemble a mixed culture of *Pseudomonas* strain BT1d with a bacterial isolate that could mineralize disulfide C, D, or E. Strain BT1d cannot release sulfur from DBT (Figure 1.10). To date, there have been no reports of DBT mineralization by bacteria degrading DBT through the Kodama pathway. It was hypothesized that a defined mixed culture could carry out this mineralization.

2. MATERIALS AND METHODS

2.1 CHEMICALS

2,2'-Dithiosalicylic acid (95% purity) was purchased from Lancaster Synthesis (Windham, NH) and DBT was obtained from Fluka (Buch, Switzerland). Thiosalicylic acid, 2-sulfobenzoic acid, mercaptoacetic acid, 4-mercaptobenzoic acid, benzothiophene, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), octyl sulfide, *n*-dodecyl sulfide, benzyl sulfide, phenyl sulfide, thiophenol, phenyl disulfide, benzyl disulfide, and Triton X-100 were purchased from Aldrich (Milwaukee, WI). Pentafluorobenzoic acid and *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) were also obtained from Aldrich.

Plate count agar (PCA) and brain heart infusion medium were purchased from Becton Dickinson and Company (Sparks, MD). Sodium benzoate, sodium acetate, catechol, naphthalene, sodium sulfite, and sodium sulfate were purchased from Fisher Scientific Company (Fair Lawn, NJ) and D-glucose, salicylic acid, metallic zinc, and yeast extract were purchased from BDH Inc. (Toronto, ON). Sodium hydroxide and dibasic potassium phosphate were obtained from Merck (Darmstadt, Germany).

Dichloromethane, methanol, and acetonitrile from Fisher Chemicals were all HPLC grade. The phosphoric acid and acetic acid used in HPLC mobile phases were from Fisher Chemicals. All water used for HPLC was purified using the Milli-Q® system (Millipore, Billerica, MA). Nitrogen for flushing was purchased from Praxair Canada Inc. (Mississauga, ON).

2.2 MEDIA AND GENERAL INCUBATION CONDITIONS

The optimal formulation for sulfate-free mineral medium (SFMM) was investigated by Lau (2001). For the biodegradation of thiophenes, medium that contained $NaNO_3$ as the nitrogen source was best able to support cultures that would cause a decrease in pH as they released sulfur as sulfate from the carbon and sulfur source. The SFMM contained (per L): K_2HPO_4, 0.1 g; NaCl, 0.1 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $NaNO_3$, 2.4 g; and 1 mL of a trace metals solution. The trace metals solution contained (per L): $CaCl_2 \cdot 2H_2O$, 3.7 g; H_3BO_3, 2.5 g; $MnCl_2 \cdot 4H_2O$, 0.87 g; $FeCl_3$, 0.65 g; $ZnCl_2$, 0.44 g; $Na_2MoO_4 \cdot 2H_2O$, 0.29 g; $CoCl_2 \cdot 6H_2O$, 0.01 g; and $CuCl_2 \cdot 2H_2O$, 0.1 mg. A filter sterilized vitamin solution (Collins and Widdel 1986), 1 mL per L of sterilized medium, was also added to supplement the SFMM. The vitamin solution contained (per L): p-aminobenzoic acid, 0.05 mg; thiamine, 0.1 mg; biotin, 0.01 mg; and vitamin B_{12}, 0.05 mg. *Pseudomonas* strain BT1d was grown in B+N8P medium (Kropp *et al.* 1994a) unless otherwise indicated.

The SFMM, 200 mL in 500-mL Erlenmeyer flasks, was sterilized by autoclaving at 121°C and 15 psi. The B+N8P medium, 1 L in 2-L Erlenmeyer flasks, was similarly sterilized. All media were approximately pH 7, measured using a Fisher ACCUMET pH Meter Mode 620 with a narrow probe. Bacteria were grown on PCA plates incubated in the dark at 28°C. To prepare bacteria grown on plates for inoculation into liquid media, approximately 4 mL of sterile phosphate buffer (3 mM, pH 7.3) was used to wash a plate and the cell suspension was aseptically transferred to a sterile 16 mm test tube. The cell suspension was vortexed and 1 mL was added to 200 mL of medium. All flasks of media were capped with foam plugs unless otherwise indicated. Flasks of media were incubated with shaking at 200 rpm in the dark at 28°C unless otherwise indicated. Samples of 1 mL were taken from cultures and stored at -20°C prior to HPLC analysis.

2.2.1 Disulfide C addition to SFMM

Disulfide C is not commercially available and it was synthesized for use as a growth substrate. Two methods were used for the addition of disulfide C to SFMM. One method involved the abiotic reaction of benzothiophene-2,3-dione. Benzothiophene-2,3-dione was synthesized by the method of Hannoun *et al.* (1982), using dichloromethane in place of carbon disulfide. Crystals of benzothiophene-2,3-dione, 200 mg, were dissolved in 10 mL of dichloromethane to prepare a concentrated stock solution. From the stock solution, the appropriate amount of 2,3-dione was added to warm, sterile SFMM and the medium was incubated with shaking at 200 rpm in the dark at 28°C. Formation of disulfide C from the 2,3-dione was confirmed using HPLC. In experiments with disulfide C as the sole carbon and sulfur source in SFMM, 100 mg of $CaCO_3$ was added to each 200 mL portion of SFMM prior to autoclaving to buffer the medium near neutrality.

The second method used the biotransformations of DBT by *Pseudomonas* strain BT1d to produce disulfide C. DBT, 2 g, was added to 1 L of sterile B+N8P medium in a 2-L Erlenmeyer flask. *Pseudomonas* strain BT1d was inoculated into the medium and the culture was incubated with shaking at 200 rpm in the dark at 28°C. *Pseudomonas* strain BT1d degrading DBT through the Kodama pathway was harvested after several months of growth. The cultures were centrifuged in 200-mL portions at 16, 000 g for 10 min in a Sorvall RC-5B refrigerated superspeed centrifuge. The resulting supernatant was extracted three times using dichloromethane and the aqueous fraction was filter sterilized through a 0.45 μm polyvinylidene fluoride membrane (Millipore; Billerica, MA). The presence of disulfides in the aqueous extract was confirmed using HPLC. This extract

was aseptically added to SFMM as a source of disulfide C; 50 mL was added to 200 mL of SFMM.

2.2.2 Determining the lowest sulfate concentration at which strain BT1d degrades DBT

The formation of HFBT in B+N8P by *Pseudomonas* strain BT1d degrading DBT was tested with different concentrations of sulfate in the medium to determine the lowest sulfate concentration necessary to support DBT biodegradation. Cultures were prepared with B+N8P medium and 2 mM, 1 mM, 0.5 mM, 0.1 mM, and 0.01 mM sulfate. Samples, 1 mL in sterile Eppendorf tubes, were taken weekly for analysis of HFBT using a spectrophotometer. For UV/VIS measurements, a PU 8740 UV/VIS scanning spectrophotometer with a Magnavox RGB Monitor 80 was used. The UV-visible spectra of the cultures were compared to a sterile control, with attention to the absorption of HFBT at 394 nm (Bressler and Fedorak 2001a).

2.2.3 Disulfide E addition to SFMM

Disulfide E, 200 mg of the commercial preparation, was dissolved in 20 mL of 0.1 M NaOH and the solution was filter sterilized through a 0.22 μm polyvinylidene fluoride membrane (Millipore). The sterile stock solution was stored at 4°C. For most experiments, the SFMM contained 100 mg disulfide E/L.

Disulfide E was also prepared in SFMM through abiotic dimerization of thiosalicylic acid. Thiosalicylic acid was dissolved in methanol and added to SFMM to a final concentration of 100 mg thiosalicylic acid/L. The flasks were incubated with shaking at 200 rpm for several days in the dark at 28°C. Formation of disulfide E was confirmed by HPLC. In experiments with disulfide E as the sole carbon and sulfur source, 10 mg of

$CaCO_3$ was added to each 200 mL portion of SFMM to buffer the medium prior to autoclaving.

2.3 BIODEGRADATION EXPERIMENTS

Enrichment techniques were used to find mixed cultures capable of degrading disulfides C and E. Several attempts were made to enrich cultures that degraded disulfide C, and two soil extracts, yeast extract, brain heart infusion, and vitamins were added to the SFMM. A mixed culture capable of degrading disulfide E, designated culture M, was enriched from marigold soil using enrichment techniques similar to the experiments used to find mixed cultures capable of degrading disulfide C (McInnis 2002). Six different bacteria were isolated from culture M by streaking from the culture onto PCA plates and streaking to purify the different colonies. Testing of the isolates in pure culture and in combinations revealed that although no single isolate was capable of degrading disulfide E in SFMM, a combination of two isolates, designated RM1 and RM6, was capable of degrading disulfide E.

2.3.1 Disulfide C enrichment cultures

Disulfide C enrichment cultures were prepared using SFMM with the disulfide added as the sole carbon and sulfur source. Disulfide C was prepared either from *Pseudomonas* strain BT1d degrading DBT or prepared abiotically in the SFMM from the 2,3-dione. Soils from near marigold roots were used as inocula, with 10 g of each soil added into individual flasks with disulfide C. These enrichment cultures were shaken at 200 rpm in the dark at 28°C. Samples were taken periodically to check the pH of the enrichment cultures and for disulfide analysis by HPLC.

The disulfide C enrichment cultures repeatedly lost activity upon transfer to fresh SFMM with disulfide C as the sole carbon and sulfur source. A variety of supplements was investigated to attempt to preserve the activity of the enrichment cultures. The first soil extract was prepared using soil from a garden of marigolds grown in Sherwood Park, Alberta and a second soil extract was prepared from soil collected near Red Deer, Alberta, designated soil CG. The marigold root extract was prepared using marigolds grown in Edmonton, Alberta, and the roots were first ground using a mortar and pestle. The extraction methods were adapted from Ng *et al.* (2004) and Dügenci *et al.* (2003). The soil or roots were heated to 60°C, stirred overnight, and left to cool at 4°C for several days. The mixture was centrifuged at 16, 000 g for 10 min and the supernatant was filter sterilized using a 0.22 μm Millipore filter. Yeast extract, brain heart infusion, and vitamins were added to the marigold root extract-supplemented cultures. Yeast extract and brain heart infusion were added to SFMM to a final concentration of 1% (w/v) each from filter sterilized, concentrated stock solutions prepared in double distilled H_2O.

2.3.2 Disulfide C and disulfide E in SFMM

The toxicity of disulfide C to the soil enrichments was studied using cultures with various concentrations of disulfide C and a constant amount of disulfide E. Disulfide E was readily degraded by marigold soil enrichments and isolate RM6 when vitamins were added to the SFMM. The influence of the disulfide C concentration on the degradation of disulfide E by isolate RM6 was investigating using a similar approach. Disulfide C and disulfide E were added to SFMM with the vitamin solution using disulfide C formed from abiotic reactions of the 2,3-dione and disulfide E added from the commercial stock solution. Both soil CG, 10 g per flask, and isolate RM6, phosphate buffer cell

suspensions from washed PCA plates, were tested separately with various concentrations of disulfide C: 200 mg/L, 100 mg/L, 50 mg/L, 25 mg/L, and 10 mg/L, and a constant amount of 100 mg disulfide E/L. A sterile control was also established with 50 mg disulfide C/L and 100 mg disulfide E/L. Samples, 1 mL, were taken periodically and stored at -20°C before preparation for analysis by HPLC of the disulfides.

2.3.3 Disulfide E biodegradation experiments

Several experiments were performed to study the biodegradation of disulfide E by isolates RM1 and RM6. The isolates were first inoculated into SFMM with 15 mM sulfate, the concentration of sulfate in B+N8P medium, to confirm that biodegradation of disulfide E formed from DBT could occur in the medium used to grow strain BT1d. Isolate EPWF, obtained from Kathlyn Kirkwood (University of Alberta), was also combined with isolates RM1 and RM6 to determine if isolate EPWF or the bacterial combination could degrade disulfide E. The SFMM was supplemented with vitamins in all experiments using EPWF. When it was observed that isolate RM6 alone could degrade disulfide E if vitamins were added to the SFMM, filter-sterilized supernatants from growing cultures of isolate RM1 were prepared to determine if this microorganism produced a growth factor necessary for isolate RM6 to degrade disulfide E. Experiments with individual vitamins in the SFMM were also performed to find the vitamin required by isolate RM6 to biodegrade disulfide E.

The HPLC chromatograms of the sterile controls and time zero samples with disulfide E all contained a small peak with a retention time of 1.8 min. This contaminant was confirmed by GC-MS to be benzoic acid. Disulfide E was added to the SFMM by the dimerization of thiosalicylic acid, and this preparation was free of benzoic acid. Isolate

RM6 biodegraded disulfide E formed from thiosalicylic acid, and produced benzoic acid as a metabolite. Experiments with pentafluorobenzoic acid were performed to attempt to slow the degradation of disulfide E and detect other intermediates in the biodegradation of disulfide E by isolate RM6. Attempts were made to detect H_2S, which might be formed in cultures of isolate RM6 degrading disulfide E, using a cadmium acetate solution in sealed flasks. Details of each experiment are given below.

2.3.3.1 Biodegradation of disulfide E by isolates RM1 and RM6 in the presence of 15 mM sulfate

The biodegradation of disulfide E by isolates RM1 and RM6 in the presence of sulfate was confirmed using SFMM supplemented with 15 mM sulfate. Isolates RM1 and RM6 were prepared in pure culture in the SFMM, in combination in the SFMM, and compared to mixed culture M transferred to fresh SFMM. A sterile control with 15 mM sulfate was prepared and all flasks of medium received 100 mg disulfide E/L from the commercial stock solution. Samples were taken every 2 d by aseptically transferring 1 mL from each flask to sterile Eppendorf tubes and the samples were stored at -20°C before analysis by HPLC for disulfides.

2.3.3.2 Biodegradation of disulfide E by isolates EPWF, RM1, and RM6

Isolate EPWF was inoculated into SFMM with disulfide E as the sole carbon and sulfur source to attempt to find a pure culture capable of degrading disulfide E. Isolate EPWF was tested in pure culture, in combination with isolate RM1, and in combination with isolate RM6. Growth of isolate EPWF required the addition of a vitamin solution to the SFMM, and all cultures in this experiment were supplemented with the vitamin solution. A sterile control was prepared and all flasks received 100 mg disulfide E/L from the commercial stock solution. The isolate EPWF experiment showed that isolate RM6

was important for the biodegradation of disulfide E, and the experiment was repeated with isolate RM6 in pure culture. One milliliter samples were taken periodically from all flasks and stored at -20°C before analysis by HPLC for disulfides.

2.3.3.3 Biodegradation of disulfide E by isolate RM6 supplemented with the sterile supernatant from growing cultures of isolate RM1

Isolate RM6 biodegraded disulfide E in SFMM supplemented with the vitamin mixture, or with isolate RM1 in the SFMM. To confirm that isolate RM1 produced a growth factor necessary for isolate RM6, the sterile supernatant from growing cultures of isolate RM1 was also used as a supplement for isolate RM6 cultures degrading disulfide E in SFMM. Isolate RM1 was inoculated into SFMM with 2 mM sulfate and 100 mg benzoate/L or 100 mg glucose/L as the sole carbon sources. After 2 d and 4 d, the pure cultures of isolate RM1 growing on benzoate were centrifuged at 16,000 g for 10 min in a Sorvall centrifuge and the supernatant was filter sterilized through a 0.22 μm membrane. After 3 d and 6 d, the supernatants from pure cultures of isolate RM1 growing on glucose were collected as described above. Two milliliters of the supernatant preparations were added to pure cultures of isolate RM6 in SFMM and a sterile control. Disulfide E, 100 mg disulfide E/L from the commercial stock, was added to each flask and samples were taken periodically for disulfide analysis by HPLC.

2.3.3.4 Biodegradation of disulfide E by isolate RM6 supplemented with individual vitamins from the vitamin mixture

Isolate RM6 was inoculated into SFMM with each of the individual vitamins from the vitamin mixture to determine which was required for growth of isolate RM6. The vitamins were prepared individually at the concentration in the vitamin mixture, filter sterilized through a 0.22 μm membrane, and added separately to the SFMM. A sterile

control was prepared with the vitamin mixture, and 100 mg commercial disulfide E/L was added to each flask. Isolate RM6 was also inoculated into SFMM with the vitamin mixture, 2 mM sulfate, and 100 mg disulfide E/L. Samples were taken periodically for analysis by HPLC.

2.3.3.5 Biodegradation of disulfide E formed from thiosalicylic acid dimerization

Benzoic acid was detected as a contaminant, 1% (w/w), in the commercial preparation of disulfide E by HPLC and GC-MS. Samples of the commercial preparation were derivatized using MTBSTFA (St. John *et al.* 1998). Because the contaminant could interfere with the detection of intermediates produced during the biodegradation of disulfide E by isolate RM6, disulfide E was instead formed from the abiotic dimerization of thiosalicylic acid. The SFMM with disulfide E formed from thiosalicylic acid was free of benzoic acid. Pure cultures of isolate RM6 in SFMM were prepared with disulfide E formed from thiosalicylic acid and samples were taken periodically for disulfide and benzoic acid analysis by HPLC. When benzoic acid was detected in the culture by HPLC analysis (2.5 d), the culture was sacrificed and acidified with concentrated HCl to pH < 2. The culture was extracted three times with dichloromethane and the pooled extract was evaporated under reduced pressure to approximately 1 mL and taken to dryness under a nitrogen stream. The extract was derivatized using the MTBSTFA method and analyzed by GC-MS for benzoic acid.

2.3.3.6 Biodegradation of disulfide E in the presence of pentafluorobenzoic acid

Benzoic acid was determined to be an intermediate in the biodegradation of disulfide E by isolate RM6. The addition of pentafluorobenzoic acid, a fluorinated derivative of benzoic acid, to SFMM could slow the biodegradation of disulfide E and allow

intermediates occurring earlier in the pathway to accumulate. Cultures of isolate RM6 degrading disulfide E and benzoic acid in SFMM were prepared with various amounts of pentafluorobenzoic acid present. Flasks with 200 mL of SFMM with 500, 300, 100, or 10 mg pentafluorobenzoic acid/L were prepared using a concentrated stock solution of pentafluorobenzoic acid in dichloromethane. The dichloromethane was allowed to evaporate from the SFMM by incubating the flasks with shaking (200 rpm) in the dark at 28°C for several days. Either disulfide E, 100 mg disulfide E/L, or benzoic acid, 100 mg benzoic acid/L, was added and sterile controls with both pentafluorobenzoic acid and disulfide E or benzoic acid were prepared. In some disulfide E-containing flasks, 2 g acetate/L was added. Isolate RM6 was inoculated into the culture flasks and incubated. One milliliter samples were taken periodically for analysis by HPLC.

2.3.3.7 Biodegradation of disulfide E in sealed flasks to detect H_2S

The search for sulfur-containing metabolites of disulfide E biodegradation led to H_2S, which could be released from disulfide E if the disulfide bond was reductively cleaved (Smith and Kelly 1998). In duplicate 200-mL flasks with center wells, 100 mL of SFMM with vitamins was inoculated with isolate RM6. The center wells were filled with a cadmium acetate solution that contained (per 0.5 L distilled water): cadmium acetate, 15.2 g; and glacial acetic acid, 62.5 mL. The flasks received 100 mg disulfide E/L and were sealed. The center wells were observed over several days for the appearance of a yellow CdS precipitate that would indicate formation of H_2S in the cultures of isolate RM6 degrading disulfide E.

2.3.4 Isolates RM1 and RM6 substrate range experiments

Isolates RM1 and RM6 were capable of biodegrading disulfide E in SFMM, and several experiments were performed with the isolates and pure cultures of isolate RM6 to identify other possible carbon or carbon and sulfur sources. The types of compounds biodegradable by isolate RM6 would give information that may help determine the pathway for disulfide E biodegradation by this isolate. To determine if isolates RM1 and RM6 or pure cultures of these isolates degraded disulfide C, they were inoculated into SFMM with disulfide C as the sole carbon and sulfur source. These isolates were also inoculated into SFMM with DBT as the sole carbon and sulfur to determine if they could degrade DBT. The substrate range of isolates RM1 and RM6 was studied using a variety of carbon and carbon and sulfur sources. The biodegradation of disulfide E and benzoic acid by isolate RM6 in SFMM with vitamins was tested in the presence of 3-chlorobenzoic acid or 3-fluorobenzoic acid to attempt to slow biodegradation of disulfide E to detect intermediates. Other compounds with structures similar to disulfide E and benzoic acid, salicylic acid, catechol, and naphthalene, were also tested as carbon sources for isolate RM6. The biodegradation of other disulfides found in biological systems was studied using pure cultures of isolate RM6 with vitamins in the SFMM. Other disulfides were formed abiotically in SFMM and isolate RM6 degraded a mixed disulfide formed from thiosalicylic acid and 4-mercaptobenzoic acid. Tan and Parkin (2000) detected 2-sulfobenzoic acid from the decomposition of disulfide E under abiotic conditions. Isolate RM6 was inoculated into SFMM with 2-sulfobenzoic acid to determine if this compound could be used as a sole carbon and sulfur source, and the biodegradation of disulfide E by isolate RM6 in the presence of 2-sulfobenzoic acid was studied.

2.3.4.1 Disulfide C or DBT as the sole carbon and sulfur source for isolates RM1 and RM6

Isolates RM1 and RM6 were tested in combination and in pure cultures with and without vitamins with disulfide C as the sole carbon and sulfur source in SFMM. These cultures were observed over a period of 6 weeks for growth in the SFMM. Cultures were also prepared with 50 mg DBT as the sole carbon and sulfur source and inoculated with isolate RM1, isolate RM6, or isolates RM1 and RM6. The flasks were observed for growth and color changes indicative of DBT degradation through the Kodama pathway.

2.3.4.2 Isolates RM1 and RM6 with various carbon or carbon and sulfur sources

Isolates RM1 and RM6 were inoculated into 5 mL of SFMM with different carbon or carbon and sulfur sources. The cultures were incubated on a tube-roller at 28°C. The substrates: glucose; benzoate; acetate; disulfide E; naphthalene; DBT; DTNB; benzyl disulfide; phenyl disulfide; benzyl sulfide; phenyl sulfide; octyl sulfide; *n*-dodecyl sulfide; benzothiophene; or thiophenol, were tested at a concentration of 1 mmol/L in SFMM. For substrates tested as sole carbon sources in SFMM, 2 mM sulfate was added. Water soluble substrates were prepared in concentrated stock solutions and filter sterilized using a 0.22 μm membrane. Water insoluble substrates were prepared in appropriate solvents and added to sterile tubes. The solvents were allowed to evaporate through foam plugs in a fume hood and the SFMM was added. Isolate RM6 was inoculated in a manner similar to the larger culture experiments by washing PCA plates with phosphate buffer and aseptically adding the cell suspension to the sterile tubes. Bacterial growth in the tubes of medium was followed by optical density (OD_{600}) using a spectrophotometer. This experiment was repeated with SFMM supplemented with vitamins.

2.3.4.3 Biodegradation of benzoic acid and disulfide E in the presence of 3-chlorobenzoic acid or 3-fluorobenzoic acid

3-Chlorobenzoic acid or 3-fluorobenzoic acid were added to pure cultures of isolate RM6 in SFMM with benzoic acid or disulfide E present to attempt to slow biodegradation and detect metabolites. Flasks of SFMM were prepared with 100 mg benzoic acid/L and 100 mg/L 3-chlorobenzoic acid or 3-fluorobenzoic acid, and 100 mg disulfide E/L and 100 mg/L 3-chlorobenzoic acid or 3-fluorobenzoic acid. Isolate RM6 was added and vitamins were provided in the medium. A sterile control was prepared with all compounds present. Samples were taken periodically and analyzed by HPLC.

2.3.4.4 Isolate RM6 with salicylic acid, catechol, or naphthalene as sole carbon sources

Substrates similar to disulfide E and benzoic acid, salicylic acid, catechol, and naphthalene, were used to study other types of carbon sources degradable by isolate RM6. Both salicylic acid and catechol were added to SFMM as sole carbon sources. Both substrates were prepared in aqueous stock solutions and filter sterilized using a 0.22 μm membrane. From the concentrated stock solutions the substrates were added separately to 200 mL of SFMM with 2 mM sulfate, for cultures with isolate RM6 and sterile controls, to a concentration of 100 mg salicylic acid/L and 100 mg catechol/L. The catechol stock solution and all flasks containing catechol were wrapped in aluminum foil and kept in the dark as much as possible. Samples were taken periodically from all cultures and sterile controls and analyzed by HPLC. Samples containing catechol were protected from the light during HPLC analysis by covering the windows to the sample tray area with aluminum foil.

Isolate RM6 was also tested for naphthalene degradation using carbon-free plates prepared using SFMM with 2 mM sulfate and Noble Agar (Difco). Both isolate RM6 and *Pseudomonas fluorescens* strain LP6a, a gift from Dr. J. Foght (University of Alberta), were streaked in duplicate onto the carbon-free plates and naphthalene crystals were added to the plate lids as the sole source of carbon. The plates were incubated agar side up in a sealed plastic container at room temperature. The plastic container was kept in a fume hood. The plates were observed for growth over several days of incubation.

2.3.4.5 Isolate RM6 with some disulfides found in biological systems as carbon and sulfur sources

Some disulfides found in biological systems were investigated as sole carbon and sulfur sources in SFMM to determine if isolate RM6 was able to attack a variety of disulfides. Glutathione disulfide, cystine, and cysteine, were added separately to 200 mL of SFMM with vitamins and the cultures were inoculated with isolate RM6. Sterile controls were prepared with glutathione disulfide, cystine, or cysteine and the cultures were monitored for growth using OD_{600}. Samples were also taken periodically for pH analysis.

2.3.4.6 Biodegradation of other disulfides formed abiotically in SFMM

Isolate RM6 was incubated in SFMM with compounds similar to disulfide E to investigate which disulfides were attacked by this bacterium. Thiosalicylic acid was added from a concentrated stock solution in methanol to 200 mL of SFMM with 4-mercaptobenzoic acid prepared similarly so that both compounds were at final concentrations of 100 mg/L in the medium. Abiotically, the compounds dimerized to form disulfide E, 2,4'-dithiosalicylic acid, and 4,4'-dithiosalicylic acid. Isolate RM6 was added to the medium and a disulfide E containing positive control. Samples were taken

periodically for analysis by HPLC. Thiosalicylic acid and mercaptoacetic acid were also used to prepare a mixed disulfide in SFMM. Thiosalicylic acid was prepared in a stock solution using methanol and mercaptoacetic acid was prepared in an aqueous solution and filter sterilized through a 0.22 μm membrane. Both compounds were added to 200 mL of SFMM to final concentration of 100 mg/L and the presence of the mixed disulfide was confirmed using HPLC and GC-MS.

2.3.4.7 Biodegradation of 2-sulfobenzoic acid by isolate RM6

2-Sulfobenzoic acid was detected by Tan and Parkin (2000) as a decomposition product of the abiotic degradation of disulfide E. The biodegradation of disulfide E by isolate RM6 could similarly produce 2-sulfobenzoic acid, which would also be degraded by isolate RM6 before the release of benzoic acid. Isolate RM6 was inoculated into SFMM with vitamins and 2-sulfobenzoic acid as the sole source of carbon and sulfur. 2-Sulfobenzoic acid was prepared in an aqueous stock solution and filter sterilized using a 0.22 μm membrane. Isolate RM6 was also inoculated into SFMM with vitamins and 100 mg 2-sulfobenzoic acid/L, and with both 100 mg 2-sulfobenzoic acid/L and 100 mg disulfide E/L. A sterile control was prepared with both compounds present and 1 mL samples were taken periodically for HPLC analysis.

2.4 IDENTIFICATION AND TAXONOMIC CHARACTERISTICS OF ISOLATES

Isolates RM1 and RM6 were grown on PCA plates and separated from mixed culture M using observations of colony morphology (McInnis 2002). Both isolates were Gram stained and tested with a 3% (w/v) potassium hydroxide solution to confirm the Gram stain results. The oxidase and catalase tests were performed on isolate RM6. Isolate RM6

was observed using a Philips/FEI (Morgagni) transmission electron microscope with CCD camera (TEM-CCD) at the University of Alberta Microscopy Unit in Biological Sciences with the assistance of Randy Mandryk. Isolate RM6 was also tested for nitrate reduction using the method of Eckford and Fedorak (2002) and compared to a *Pseudomonas stutzeri*, obtained from the Department of Biological Sciences bacterial culture collection (University of Alberta), positive control.

2.4.1 Identification using the Biolog system

Isolates RM1 and RM6 were identified using the Biolog system (Biolog Inc., Hayward, CA). The isolates were first grown on Biolog blood agar plates and incubated at 28°C in the dark for 5 d. Colonies from the plates were aseptically transferred to the appropriate Gram positive or Gram negative Biolog growth solution to the specified turbidity. Isolate RM6 was inoculated into Biolog plates with and without addition of the vitamin mixture. The plates were incubated at 28°C in the dark and observed after 24 h and 48 h for comparison to the Biolog database. The Biolog similarity index was calculated by the Biolog software. *Pseudomonas fluorescens* was obtained from the obtained from the Department of Biological Sciences bacterial culture collection (University of Alberta).

2.4.2 16S rDNA sequencing

Genomic DNA extraction, PCR amplification, and sequencing of amplified 16S rDNA were performed by Stephanie Cheng (University of Alberta). A direct, physical lysis method using 10% sodium dodecyl sulfate (SDS) and chloroform was used to extract genomic DNA from isolate RM6 cells. To a 2 mL screw cap tube (Fisher Scientific), 0.5 g of 2.5 mm and 0.1 mm diameter zirconium-silica beads (Biospec

Products, Bartlesville, OK) were added in a biohazard hood. All beads were handled with metal spatulas that had been sterilized in bleach and rinsed twice with sterile Milli-Q water to prevent contamination with DNA. The bead-filled tubes were autoclaved twice on successive days for 20 min. Next, 300 µL each of 100 mM phosphate buffer (pH 8), SDS lysis buffer (10% SDS in 100 mM NaCl, 500 mM Tris, pH 8), and chloroform-isoamyl alcohol (24:1) were added to the tubes. Half a loopful of isolate RM6 cells was picked from a plate using a flamed inoculating loop and suspended in the solution in bead beating tubes. The bacterial samples were homogenized in a 3110 BX Model mini bead beater (Biospec Products) for 40 s at 5000 rpm. Cellular debris was removed by microcentrifugation for 5 min at 13,000 rpm. The DNA-containing supernatant (about 650 µL) was transferred into an autoclaved 1.5 mL microfuge tube and gently mixed with 7 M ammonium acetate to achieve a final concentration of 2.5 M. The coagulated protein was removed by microcentrifugation for 5 min at 13,000 rpm. DNA was precipitated by transferring the supernatant into another autoclaved micofuge tube, adding an equal volume of isopropanol, and incubating the mixture for 30 min at -20°C. The DNA was recovered as a pellet after microcentrifugation for 10 min at 14,000 rpm. The pellet was air-dried and dissolved in 40 µL of autoclaved Milli-Q water. The procedure was performed aseptically at a bench designated for PCR work, using autoclaved or filter sterilized reagents, micropipettes with filtered tips, and while wearing gloves.

The 16S rDNA fragment of isolate RM6 was amplified by PCR using the oligonucleotide primers PB36 (5'-AGRGTTTGATCMTGGCTCAG-3') and PB38 (5'-GKTACCTTGTTACGACTT-3'), corresponding to positions 8-27 and 1492-1509, respectively (Brosius *et al.* 1981). The PCR reaction was carried out in a final volume of

50 μL using 5 μL of genomic DNA, 0.5 μM of each forward and reverse primer, 50 mM Tris HCl (pH 9), 1.5 mM magnesium chloride, 0.4 mM dGTP, dCTP, dATP, and dTTP, 5% dimethyl sulfoxide, and 0.5 U *Taq* polymerase (Roche Diagnostics, Laval, QC). Reactions were performed using a Mastercycler Eppendorf thermocycler (Eppendorf, Hamburg, Germany) for 30 cycles (39 s at 93°C, 60 s at 54°C, 120 s at 73°C) after an initial denaturation of 4 min at 95°C, followed by a final extension at 73°C for 10 min.

The PCR product was cleaned up according to the manufacturer directions from the Roche High Pure PCR clean up kit (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing reactions were performed using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing PCR reactions were carried out to a final volume of 20 μL using 90 ng of purified PCR product, 0.25 μM of each sequencing primer, 1X sequencing dilution buffer (200 mM pH 9 Tris HCl, 5 mM magnesium chloride) and 2 μL of Big Dye terminator sequencing premix.

Seven sequencing reactions were required to obtain nearly full-length 16S rDNA sequences. The seven sequencing oligonucleotide primers are shown in Table 2.1. Reactions were performed using a Mastercycler Eppendorf thermocycler (Eppendorf) for 25 cycles (20 s at 95°C, 15 s at 58°C, 60 s at 68°C). The PCR product was purified from salts via ethanol precipitation according to the manufacturer directions in the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were sent to the Molecular Biology Servicing Unit (University of Alberta) where they were resolved on an ABI 3100 Genetic Analyzer (Applied Biosystems).

Sequence data assembly, analysis, and storage were done using the Canadian Bioinformatics Resource (http://www.cbr.nrc.ca.login.ezproxy.library.ualberta.ca). Raw

sequence data were assembled into contigs with PreGap4 (version 1.1) and Gap4 (version 4.6) in the Staden software package (release 2000.0: J. Bonfield, K. Beal, M. Betts, M. Jordan, and R. Staden, 2000). Contig sequences were compared to known other sequences in GenBank using the FASTA and BLASTn search tools. Phylogenetic trees were created using 22 sequences from the Comamonadacaea family. *Escherichia coli* was used as an out-group. Full-length sequences were downloaded from GenBank and an alignment was generated using ClustalX (Thompson *et al.* 1997). Phylogenetic analysis was done with programs in the PHYLIP software package, version 3.63 (Felsenstein 1993). Specifically, alignments were sampled for bootstrap analysis with Seqboot, distances were calculated with the maximum-likelihood option of Dnadist. Dendrograms were constructed from distance data by neighbor joining with neighbor. Consensus trees were calculated with Consense and branch lengths were superimposed on consensus trees with Fitch. Completed trees were viewed with TreeView (Page 1996). Bootstrap values supporting each node are shown as a percentage of 100 replicates.

Table 2.1 Full complement of primers used to obtain near full-length 16S rDNA sequences. R represents A or G, M denotes A or C, and K represents G or T.

Primer	**Sequence**	**Position (Brosius *et al.* 1981)**
PB36	5'-AGR GTT TGA TCM TGG CTC AG-3'	8-27
16S.1	5'-ACT CCT ACG GGA GGC AGC AG-3'	360-379
16S.2	5'-GTA TTA CCG CGG CTG CTG GCA-3'	539-559
16S.3	5'-GGA TTA GAT ACC CKG GTA GTC C-3'	808-830
16S.4	5'-GGT TAA GTC CCG CAA CGA GC-3'	1125-1144
16S.5	5'-GCT CGT TGC GGG ACT TAA C-3'	1125-1144
PB38	5'-GKT ACC TTG TTA CGA CTT-3'	1492-1509

2.5 WASHED CELL EXPERIMENTS

Benzoic acid was identified as a transient metabolite of disulfide E degradation by isolate RM6 growing in SFMM. Washed cell experiments were prepared using cell

suspensions of isolate RM6 harvested from exponential phase during the biodegradation of disulfide E. The cells were resuspended in phosphate buffer or saline, and experiments were performed under non-growth conditions. When disulfide E was added to washed cell suspensions of isolate RM6, the disulfide was degraded relatively quickly compared to growing cells of isolate RM6. The concentration of benzoic acid observed in the washed cell suspensions of isolate RM6 degrading disulfide E was also much higher than in growing cultures. A variety of washed cell experiments with isolate RM6 were performed to study this isolate and attempt to identify other metabolites in the biodegradation of disulfide E.

2.5.1 Disulfide E in washed cell suspensions of isolate RM6

In initial washed cell experiments, a 1 L pure culture of isolate RM6 in SFMM with vitamins and 100 mg disulfide E/L as the sole carbon and sulfur source was grown to late exponential phase (approximately 4 d). The culture was centrifuged at 16, 000 g for 10 min in 200-mL portions in a Sorvall centrifuge and the cell pellets were washed three times with sterile phosphate buffer. The final cell pellet was resuspended in 20 mL of phosphate buffer with 100 mg disulfide E/L and 20 μL of the vitamin mixture in a 50-mL Erlenmeyer flask. The cell suspension was incubated with shaking at 200 rpm in the dark at 28°C and samples were taken periodically over 24 h for immediate HPLC analysis.

Washed cell suspensions of isolate RM6 were also prepared using 200 mL cultures of isolate RM6 harvested after 4 d of growth in SFMM with vitamins and 100 mg disulfide E/L as the sole carbon and sulfur source. The cultures were similarly centrifuged and washed twice with one of the following: 3 mM phosphate buffer, 0.3 mM phosphate buffer, 3 mM NaCl, or 0.3 mM NaCl. The pellets were resuspended in 20 mL of

phosphate buffer, 0.3 mM phosphate buffer, 3 mM NaCl, 0.3 mM NaCl, or anaerobic phosphate buffer and 20 μL of the vitamin mixture was added. Disulfide E, 100 mg/L, was added to each cell suspension and samples were taken periodically over 48 h for immediate HPLC analysis. The anaerobic phosphate buffer cell suspension was resuspended in a sealed serum bottle flushed with nitrogen to prevent oxygen access to the suspension. Samples that showed disulfide E degradation and benzoic acid formation were pooled and taken to the Mass Spectrometry Laboratory in the Department of Chemistry at the University of Alberta for HPLC-MS analysis.

In a similar washed cell experiment, 200 mL cultures of isolate RM6 growing on disulfide E as the sole source of carbon and sulfur were harvested and washed twice with 0.3 mM NaCl. The pellets were resuspended in sterile 35-mL serum bottles with 20 mL of anaerobic 0.3 mM NaCl prepared by flushing with nitrogen filter sterilized using a 0.22 μm membrane. The serum bottles were sealed with butyl rubber stoppers and various volumes of oxygen (filter sterilized through a 0.22 μm membrane) were added through a sterile syringe. The anaerobic isolate RM6 cell suspensions received 5, 2.5, 1, 0.5, or 0.1 mL of oxygen. All suspensions received 100 mg disulfide E/L and 0.5 mL samples were aseptically taken periodically over 24 h for HPLC analysis. At the end of the experiment, a 1 mL sample was taken from each bottle for sulfate analysis using a turbidimetric method (Kolmert *et al.* 2000). Samples were also taken for disulfide HPLC-MS analysis.

2.5.2 Disulfide E and 2-sulfobenzoic acid in washed cell suspensions of isolate RM6

Isolate RM6 cell suspensions prepared in 3 mM NaCl and 0.3 mM NaCl with vitamins were tested for biodegradation of 2-sulfobenzoic acid. A cell suspension with

100 mg 2-sulfobenzoic acid/L was monitored by HPLC analysis over a period of 50 h. One cell suspension received 100 mg disulfide E/L and after disulfide E degradation was apparent (approximately 6 h), 2-sulfobenzoic acid was added and samples were taken periodically over 48 h. In similar washed cell suspensions, Triton X-100 was added to suspensions with both 100 mg disulfide E/L and 100 mg 2-sulfobenzoic acid/L. Triton X-100 permeabilizes cell membranes (Beller and Spormann 1997), which might help release intermediates into the cell suspension.

2.5.3 Attempts to detect H_2S in washed cell suspensions of isolate RM6

Reductive cleavage of the disulfide bond could release H_2S into washed cell suspensions of isolate RM6 (Smith and Kelly 1998). Washed cell suspensions of isolate RM6 degrading disulfide E were tested for the production of H_2S. To some washed cell suspensions, small pieces of Zn metal were added to the cell suspensions. Preliminary tests of acidified Zn pieces exposed to H_2S produced a yellow color in the cadmium acetate solution when the headspace of the acidified sample was bubbled into the cadmium acetate solution. A similar technique could be employed to detect H_2S in washed cell suspensions of isolate RM6 that degraded disulfide E in the presence of Zn.

2.6 MINERALIZATION EXPERIMENTS

2.6.1 Mineralization of disulfide E by isolate RM6 in SFMM

Isolate RM6 grown on disulfide E as the sole carbon and sulfur source in 200 mL of SFMM with vitamins was used as an inoculum for determining if disulfide E was mineralized by this bacterium. SFMM was prepared with vitamins and 200 mg disulfide E/L, and 15 mL was added to each of 21 sterile 60-mL serum bottles. SFMM was also prepared without disulfide E and 15 mL was added to each of 21 sterile 60-mL serum

bottles to account for carbon dioxide and sulfate carried over from the inoculum. After 6 d of growth in the SFMM, 2 mL of the isolate RM6 pure culture was added to each serum bottle. The bottles were capped with sterile butyl rubber stoppers. At each sample time, over the period of 12 d, three cultures were acidified with concentrated HCl to pH < 2. GC analysis was done on the headspace of each bottle to determine the mg of carbon or the amount of carbon dioxide released from the disulfide by isolate RM6 (Bressler *et al.* 1999). At the end of the experiment, samples were taken for disulfide HPLC analysis and sulfate analysis using the method of Kolmert *et al.* (2000). Standards were prepared containing known amounts of sodium bicarbonate solution, acidified, left to equilibrate overnight, and analyzed by GC for CO_2 (Bressler *et al.* 1999).

2.6.2 Mineralization of disulfide E by cell suspensions of isolate RM6

The washed cell suspensions of isolate RM6 degraded disulfide E and a mineralization experiment was performed to confirm that the cell suspensions also released carbon dioxide and sulfate from disulfide E. A 1-L culture of isolate RM6 in SFMM with vitamins growing on disulfide E as the sole carbon and sulfur source was harvested in late exponential phase and the cells were washed twice with 0.3 mM NaCl. The cells were resuspended in 0.3 mM NaCl in sterile 60 mL serum bottles with vitamins and 100 mg disulfide E/L. The bottles were sealed with butyl rubber stoppers and incubated. The cell suspensions were sampled at various times over a period of 48 h. At each time, three suspensions were acidified and analyzed as outlined in section 2.6.1. Samples for disulfide HPLC analysis were also taken and sulfate was determined using the turbidimetric method. Standards were prepared containing known amounts of sodium

bicarbonate solution, acidified, left to equilibrate overnight, and analyzed by GC as in section 2.6.1.

2.7 OXIDATION OF DISULFIDE E

One potential pathway for disulfide E degradation by isolate RM6 is the oxidation of the disulfide sulfur atoms. To generate compounds that might be intermediates in the biodegradation of disulfide E by isolate RM6, the disulfide was oxidized chemically and by an enzyme preparation. The chemical oxidation of disulfide E followed the method of Willey *et al.* (1981) using hydrogen peroxide in benzene and acetic acid. The chemical oxidation product was analyzed by HPLC and spectral analysis by the Department of Chemistry at the University of Alberta. An enzyme preparation provided by Dr. Michael Pickard at the University of Alberta contained laccase. Using a method similar to that described by Pickard *et al.* (1999), a reaction mixture was prepared that contained 100 mg disulfide E/L and the laccase enzyme. Samples were taken periodically over several days from the enzyme reaction mixture and analyzed immediately by HPLC for disulfide E.

2.8 CELL-FREE EXTRACT EXPERIMENTS

Cell-free extract experiments to attempt to generate metabolites of disulfide E biodegradation by isolate RM6 followed the method used by Allison *et al.* (1995). Isolate RM6 cells were prepared similar to the washed cell experiments in 2 mL of 0.3 mM NaCl and sonicated on ice for eight bursts in 30 s pulses with appropriate cooling periods. After sonication, the cell suspension was centrifuged for 20 min at 4°C in 1 mL portions in 1.5 mL Eppendorf tubes using a microcentrifuge at 14, 000 rpm. In one reaction mixture, 1 mL of supernatant from the cell extract was added to 20 mL of 0.3 mM NaCl with 100

mg disulfide E/L and 8 mM NADH and NADPH. The second 1 mL portion of cell extract was added to 20 mL of 0.3 mM NaCl with 100 mg disulfide E/L without NADH and NADPH. Samples, 200 μL, were taken periodically over 48 h for immediate HPLC analysis. Cell-free extracts were also prepared using sonication coupled with bead beating for 1 min and cell disruption using a french pressure cell with the assistance of Tom Hantos (University of Alberta).

2.9 SULFITOLYSIS EXPERIMENTS

Another potential pathway for disulfide E biodegradation by isolate RM6 could involve sulfite attack at the disulfide bond. Experiments were performed to generate and study the sulfitolysis product, which would be degraded by isolate RM6 if it was an intermediate in disulfide E biodegradation. Sulfitolysis was done by abiotically incubating 250 mg disulfide E/L in 100 mL of water with 38 mM sulfite in 158 mL serum bottles capped with butyl rubber stoppers. The reaction mixture was incubated with shaking at 200 rpm in the dark at 28°C. Samples were taken for HPLC analysis of disulfide E over several days. Samples showing new peaks by HPLC analysis were taken to the Mass Spectrometry Laboratory in the Department of Chemistry for HPLC-MS analysis. The sulfitolysis product was formed similarly in 0.3 mM NaCl.

Washed cells of isolate RM6 in 20 mL of 0.3 mM NaCl with the disulfide E sulfitolysis product were monitored for 48 h by immediate HPLC analysis. Washed cell suspensions with 100 mg disulfide E/L and the sulfitolysis product and 100 mg disulfide E/L were also monitored by HPLC.

A mineralization experiment with growing cells of isolate RM6 and the sulfitolysis product was attempted. SFMM with 100 mg disulfide E/L was incubated with 0.15 g of

Na_2SO_3 to form the sulfitolysis product and a mineralization experiment was set up as previously described (section 2.6.1). A culture of isolate RM6 degrading disulfide E in SFMM was used as the inoculum, and a positive control of isolate RM6 mineralizing disulfide E was included. Triplicate cultures were analyzed by GC for carbon dioxide after 0 d and 28 d of incubation.

2.10 COMBINING ISOLATES BT1d AND RM6

The second objective of this project was to combine isolates BT1d and RM6 to determine if this mixed culture could mineralize DBT. The isolates were inoculated into SFMM with vitamins in pure culture and in combination, with 50 mg DBT as the sole carbon and sulfur source. A sterile control was prepared with 50 mg DBT in the SFMM. Samples, 2 mL, were taken weekly for several months and stored at -20°C. The samples were acidified to pH 2 and extracted three times using dichloromethane before sulfate analysis using the turbidimetric method (Kolmert *et al.* 2000). After 4 months of incubation, samples were taken monthly for sulfate analysis. HPLC analysis was used to confirm the presence of the disulfides in the mixed culture.

2.11 ANALYTICAL METHODS

Disulfide E, benzoic acid, salicylic acid, catechol, the mixed disulfides, and 4,4'-dithiosalicylic acid were analyzed using an Agilent (Wilmington, DE) 1100 series HPLC with an autosampler, thermostated column compartment, and a UV-visible diode array detector connected to an Agilent Chemstation operating with software for LC 3D systems. A low-volume column inlet filter (2 μm pore size) protected the analytical LiChrosopher 100 RP-18 column (5 μm particle size, 125 mm x 4 mm, Agilent). The mobile phase used by Bressler and Fedorak (2001b) to separate the disulfides formed

from DBT biodegradation was modified to contain acetonitrile:water (35:65) with phosphoric acid (5 mL/L) and KH_2PO_4 buffer (1 M, 5 mL/L). The mobile phase was run at 1.5 mL/min and the effluent was monitored at 240 nm. Using the same equipment, 2-sulfobenzoic acid was detected using the method of Gonçalves and Fedorak (1996) with an acetonitrile:water (10:90) mobile phase with acetic acid (5 mL/L) and KH_2PO_4 buffer (1 M, 1 mL/L).

Carbon dioxide was analyzed by injecting 0.5 mL of headspace gas into a Hewlett Packard 5890 Series II gas chromatograph with a thermal conductivity detector and a 4 m x 0.3 cm O.D. stainless steel column packed with Poropak R (mesh size 80/100, Waters Associate Inc., Milford, MA) (Bressler *et al.* 1999). Helium, 23 mL/min, was the carrier gas and the oven, injector, and detector were at 35, 37, and 80°C, respectively. Acetate was analyzed by the GC method of Fedorak *et al.* (2002).

For low resolution GC-MS, a Hewlett Packard 5890 series II GC with a series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA) was used. The GC temperature program was 90°C for 1 min, 5°C/min to 280°C for 21 min. Samples were derivatized using MTBSTFA prior to GC-MS analysis. Analysis by HPLC-MS was performed by the Department of Chemistry Mass Spectrometry Laboratory using an Agilent Technologies 1100 mass selective detector with the assistance of Don Morgan. The mobile phase was a gradient of water:methanol (90:10) to 100% methanol with 1% HAc over 10 min at a flow rate of 0.25 mL/min. The effluent was monitored at 240 nm and 10 μL was injected. Infrared spectrometry was performed by the Department of Chemistry Spectral Services and Microanalytical Laboratory with the assistance of Wayne Moffat.

3. RESULTS AND DISCUSSION

Based on the work of Bressler and Fedorak (2001b), a significant portion of DBT sulfur would end up as large disulfides in sulfate-containing environments like the oceans. In their review of the biodegradation of condensed thiophenes found in petroleum, Kropp and Fedorak (1998) listed the two main reasons we study these compounds: to discover microbial processes that remove sulfur from petroleum, and to understand how organosulfur compounds are biodegraded so that their fates can be predicted in contaminated environments. With this latter goal in mind, this thesis describes the biodegradation of disulfides formed from DBT biodegradation.

3.1 BIODEGRADATION OF DISULFIDE C

Disulfide C, 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide, is the first disulfide formed from the dimerization of 2-mercaptophenylglyoxylate in cultures of *Pseudomonas* strain BT1d degrading DBT (Bressler and Fedorak 2001b). It is a symmetric disulfide with two phenylglyoxyl moieties (-$C_6H_4COCOOH$). The α–keto acid can undergo abiotic reactions (decarboxylation or decarbonylation) to lose a carbon and oxygen to yield disulfide D, 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide, which similarly loses a carbon and oxygen to form disulfide E, 2,2'-dithiosalicylic acid. This reaction is pH dependant, and happens slowly at pH 7.

In laboratory cultures at neutral pH, bacteria would be exposed to disulfide C, formed from benzothiophene-2,3-dione or extracts of *Pseudomonas* strain BT1d degrading DBT, for months before significant amounts of disulfide E would be formed. In the environment, disulfide C is likely the first disulfide encountered by organisms exposed to

the products of DBT biodegradation through the Kodama pathway. The conversion of disulfides C and D to disulfide E, which undergoes no further abiotic changes, is the rate limiting step if disulfides C and D cannot be biodegraded by indigenous microbes.

3.1.1 Disulfide C enrichment cultures

To determine if microorganisms from soil were capable of using disulfide C as a sole carbon and sulfur source in SFMM, enrichment cultures were established with soils from marigold gardens as inocula. Figure 3.1 shows the loss of disulfide C in SFMM with six different soils used as inocula. The soil enrichments were capable of degrading disulfide C in 21 d. The loss of disulfide C in the sterile control was accompanied by an increase in the concentrations of disulfides D and E. The abiotic conversion of disulfide C to disulfide D is pH dependant (Bressler 2001), and the initial enrichment culture medium was approximately pH 7.5. Although some of the loss of disulfide C observed in the soil cultures can be attributed to abiotic reactions, after 21 d in the SFMM, disulfides C, D, and E were below detectable concentrations in the enrichment cultures soil CG, soil CC, soil SY, and soil S. The medium for subsequent enrichments was approximately pH 7.

Three of the most active enrichment cultures from those summarized in Figure 3.1 were transferred to fresh SFMM with disulfide C. These cultures (transfer 1) were monitored for loss of disulfide C. Only the enrichment culture with soil CG continued to degrade disulfide C (Figure 3.2). Disulfide C was below detectable concentration in 14 d, significantly more quickly than in the initial enrichment with soil CG (Figure 3.1). This culture was similarly transferred and monitored for disulfide C concentration (Figure 3.3). Disulfide C degradation continued for the first 8 d, and at 60 mg disulfide C/L, no further loss of the disulfide was observed after an additional 20 d of incubation.

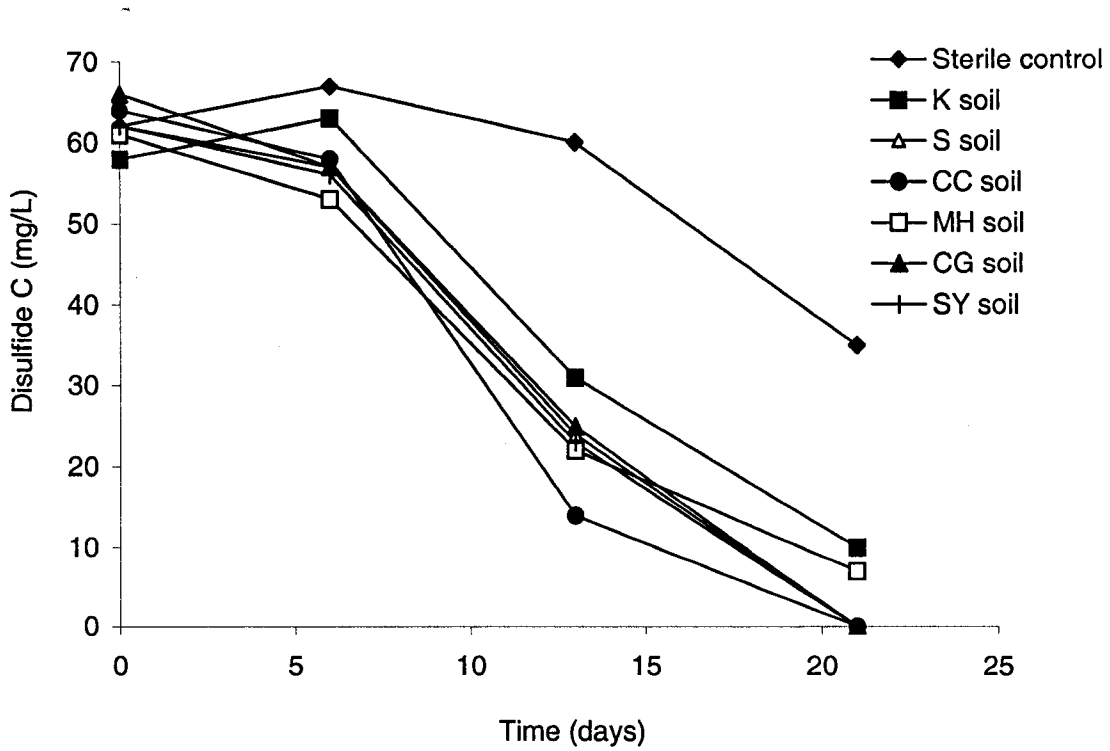


Figure 3.1 Degradation of disulfide C by enrichment cultures with different soils in SFMM and disulfide C from benzothiophene-2,3-dione.

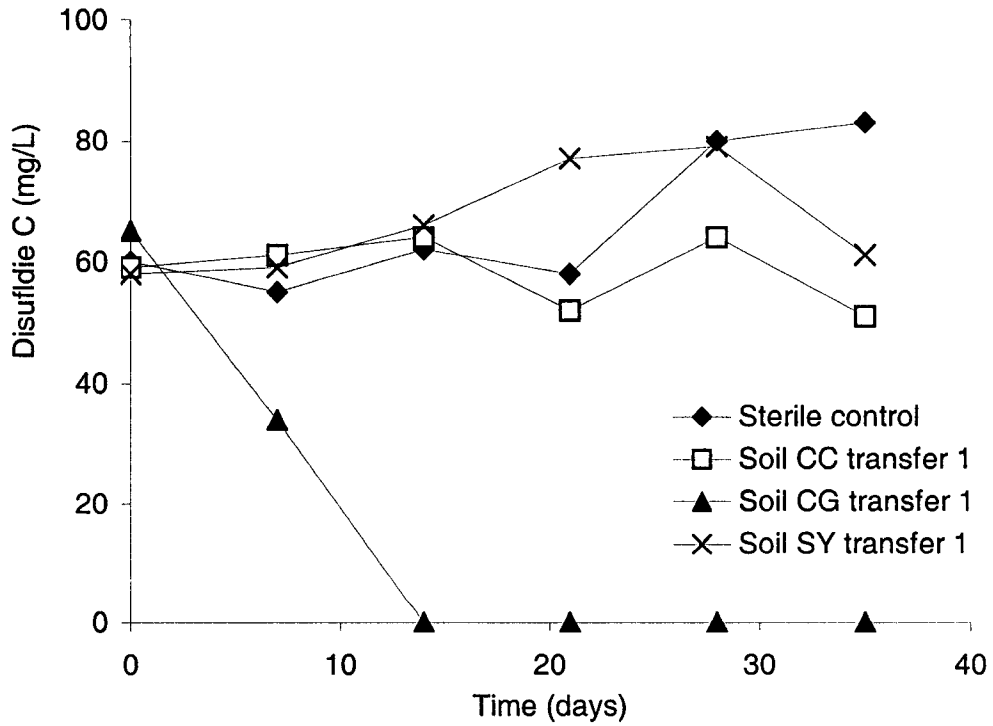


Figure 3.2 Disulfide C concentrations in the first transfers of three soil enrichments in SFMM with disulfide C from benzothiophene-2,3-dione.

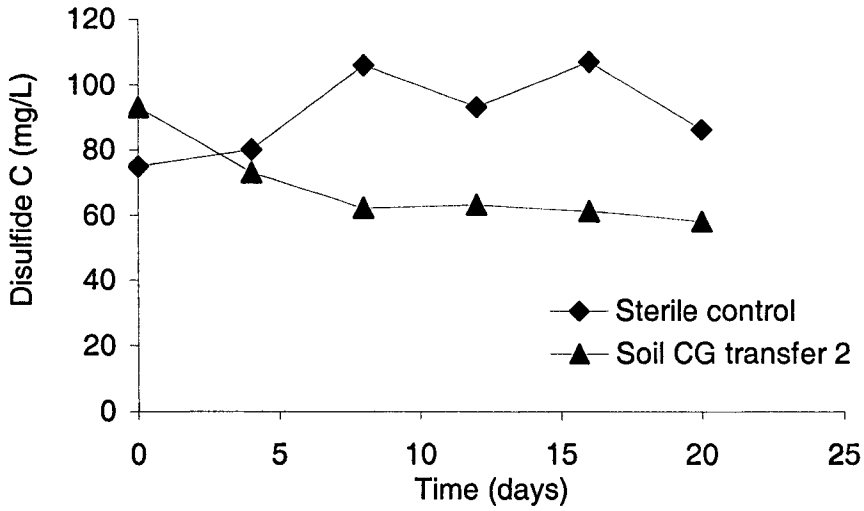


Figure 3.3 Disulfide C concentrations in the second transfer of soil CG to fresh SFMM with disulfide C from benzothiophene-2,3-dione.

The loss of disulfide C degradation following two transfers of the soil CG culture into fresh medium suggested that the process may have diluted out a component of the soil essential for activity. A soil extract was prepared using a different marigold soil source, due to the limited availability of soil CG. Various amounts of the soil extract were added to transfers of soil CG transfer 1 culture (the active transfer from Figure 3.2) to determine if a second transfer enrichment culture would retain activity. No loss of disulfide C was observed in any of the transfers compared to the sterile control over 50 d (data not shown). Despite the lack of success using the soil extract, the observation that subsequent transfers lost activity suggested that the cultures were losing something over time that was essential to their ability to degrade disulfide C. Other supplements were tested for their ability to preserve disulfide C degradation following transfers of soil CG enrichments.

3.1.1.1 Enrichments with the vitamin mixture, yeast extract, and brain heart infusion

Growth factors are often added as supplements to media to promote the growth of different microorganisms. Watson *et al.* (2001) investigated the growth requirements of *Sinorhizobium meliloti*, which is often supplemented with yeast extract and plant or animal extracts, and identified two vitamins in the yeast extract that were absolutely required in synthetic media for the growth of the *S. meliloti* strains tested. Similar to the enrichments established previously, the SFMM was prepared but supplemented with a vitamin mixture, yeast extract, and brain heart infusion, and inoculated with soil CG.

The first two enrichments of soil CG degraded disulfide C (Figure 3.4). Similar to the experiments without the supplements, the first transfer was able to degrade disulfide C over the course of 30 d, but the second transfer could not maintain this activity and 60 d after transfer, 50 mg disulfide C/L remained in the culture. However, the concentration of disulfide C did decrease between days 85 and 120, when the experiment was terminated. The disulfide concentration remained quite constant in the sterile control over the duration of the experiment.

The culture soil CG transfer 2 (Figure 3.4) was not followed past 120 d because it appeared that conversion of disulfide C to disulfides D and E was primarily responsible for the loss of disulfide C observed in the culture. Both disulfides D and E were observed in the HPLC chromatograms for samples taken from the second transfer of the soil CG culture. The biodegradation of disulfide D or disulfide E by the soil culture could increase the abiotic conversion of disulfide C to the smaller disulfides.

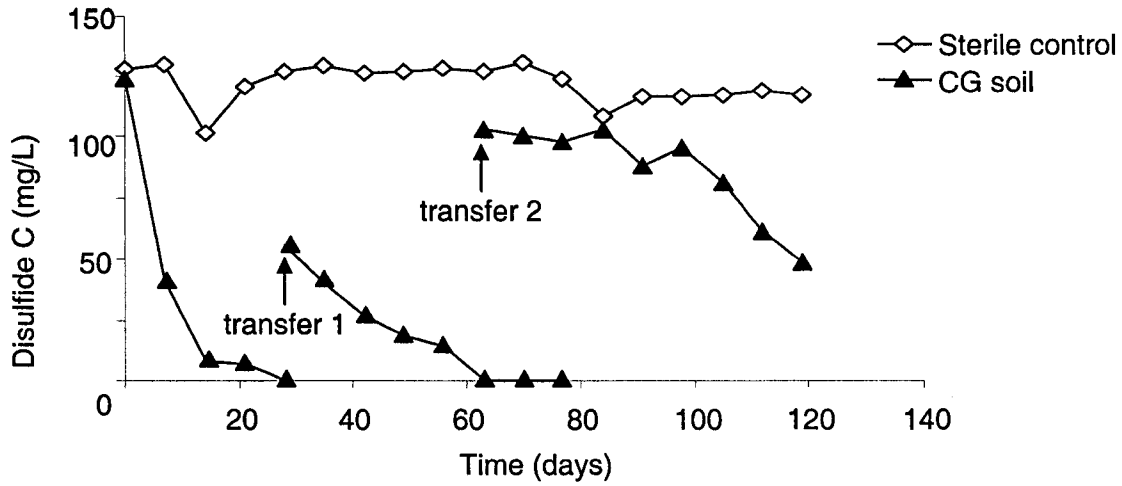


Figure 3.4 Disulfide C concentrations in an enrichment culture with soil CG and the vitamin mixture, yeast extract, and brain heart infusion provided in the SFMM. The soil CG enrichment culture was transferred after 30 d (transfer 1), and again after 62 d of incubation (transfer 2).

A different approach was taken to see if the biodegradation of disulfide C could be sustained. This involved resupplementing an active culture after disulfide C was depleted. The enrichment culture soil CG transfer 1 (Figure 3.4) was re-fed disulfide C from a concentrated stock of benzothiophene-2,3-dione in SFMM. This culture was able to continue to degrade disulfide C (Figure 3.5). After 45 and 75 d, disulfide C was added from the stock solution and degraded by the maintenance culture. This culture was clearly capable of degrading disulfide C formed from benzothiophene-2,3-dione providing that the substrate was added to the existing culture. This supported the idea that a component of the soil not provided in the minimal medium was integral to maintaining the activity of the culture. After 120 d of incubation, disulfides from extracts of *Pseudomonas* strain BT1d degrading DBT were added to the maintenance culture (Figure 3.5). Disulfide degradation was observed, but it ceased after 150 d.

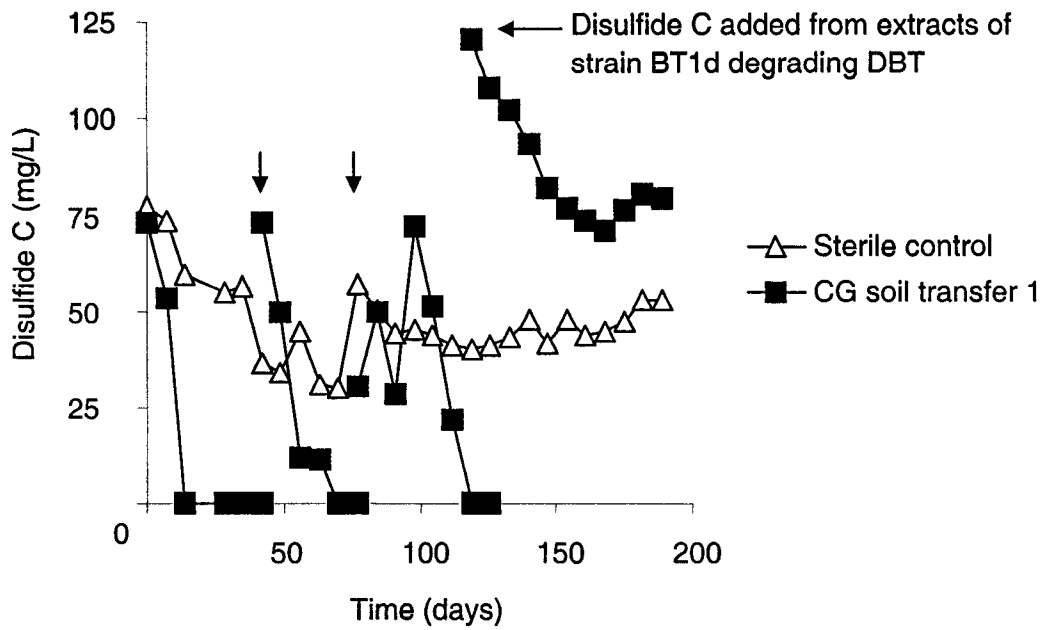


Figure 3.5 Maintenance of soil CG transfer 1 with disulfides added to the SFMM from benzothiophene-2,3-dione three times, at 0 d, 48 d, and 75 d. The CG maintenance culture was given disulfides from *Pseudomonas* strain BT1d degrading DBT extracts after 120 d.

The maintenance culture lost the ability to degrade disulfide C after incubating for several months. The pH of the culture was checked to ensure that the enrichment had not dropped in pH as sulfate was released from the biodegradation of the disulfide, and was confirmed to be neutral. The addition of the *Pseudomonas* strain BT1d extract may have played a role in the loss of activity, as the degradation of DBT through the Kodama pathway by *Pseudomonas* spp. also produces DBT sulfoxide (Kodama *et al.* 1973) and DBT sulfone (Mormile and Atlas 1989), compounds to which the culture had not been previously exposed. The autooxidation of thiols to disulfides, as would occur in the medium during the formation of disulfide C, generates active oxygen species (O_2^- and H_2O_2) that may have been toxic to the culture (Munday 1985). Also, over time the unidentified growth factor that limits the ability of these cultures to be transferred to fresh SFMM may have been depleted. Lastly, as the maintenance culture continued to degrade

disulfide C, the bacteria were generating metabolic waste products that accumulated and may have limited the growth of the disulfide C degrading maintenance culture.

3.1.1.2 Marigold root extract addition to disulfide C degrading cultures

Using methods similar to those described to generate root extracts from ginseng and ginger (Ng *et al.* 2004), a healthy marigold plant was obtained and the roots ground to make a marigold root extract. This root extract was used as a supplement to new soil CG enrichment cultures degrading disulfide C (Figure 3.6). At the time the marigold root extract was being prepared, another batch of soil CG was obtained from Red Deer, Alberta and used to make a soil extract that was also used as a supplement. This new soil CG was used as the inoculum and a killed soil control was also included to ensure that sorption to the soil or some abiotic mechanism was not responsible for the loss of disulfide C observed in cultures compared to the sterile control.

The new soil CG enrichment culture degraded disulfide C in 45 d, and the soil CG culture with the soil and root extracts degraded disulfide C in 38 d (Figure 3.6). The killed soil control in Figure 3.6 showed that although some of the disulfide associated with the soil, it is clearly biotic processes that caused the loss of disulfide C to below detectable concentrations. By *t* test comparison (Kleinbaum and Kupper 1978), the slope for the loss of disulfide C in the killed soil control is significantly different ($p<0.0002$) from the slopes for the linear portions of disulfide C loss in both soil CG cultures. Interestingly, the slopes of the cultures degrading disulfide C in the absence or presence of the soil and root extracts are also significantly different. The addition of the marigold soil and root extracts enhances the biodegradation of disulfide C by microorganisms in soil CG.

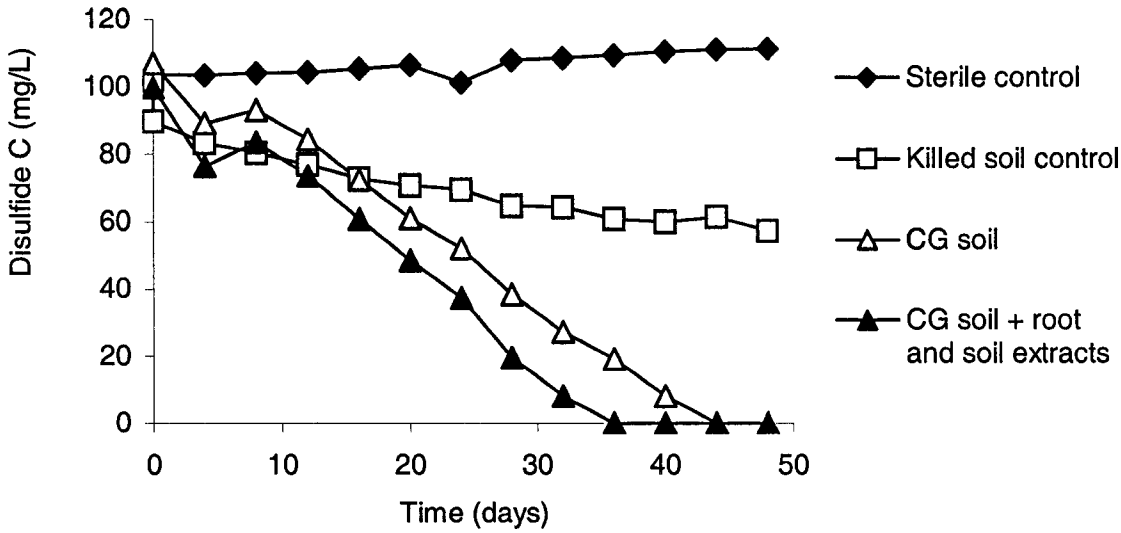


Figure 3.6 Disulfide C concentrations in a single experiment with soil CG enrichment cultures in SFMM with disulfides from benzothiophene-2,3-dione. One enrichment culture was provided with soil CG and the root and soil extracts. The killed soil was autoclaved three times before addition to the SFMM with disulfide C.

Both of the disulfide C-degrading cultures (Figure 3.6) were transferred to fresh SFMM with disulfide C as the sole carbon and sulfur source, and the culture that had been supplemented was again provided with the soil and root extracts. Both of the transferred cultures were able to degrade disulfide C and were transferred again after 50 d (Figure 3.7). When the cultures were transferred a second time, both lost their ability to degrade disulfide C. Disulfide C was added to the transfer 1 cultures from the concentrated stock solution, but the cultures did not continue to degrade disulfide C (Figure 3.7). The disulfide C enrichment cultures with soil CG degraded disulfide C consistently when inoculated with soil, but the activity could not be preserved when the cultures were transferred.

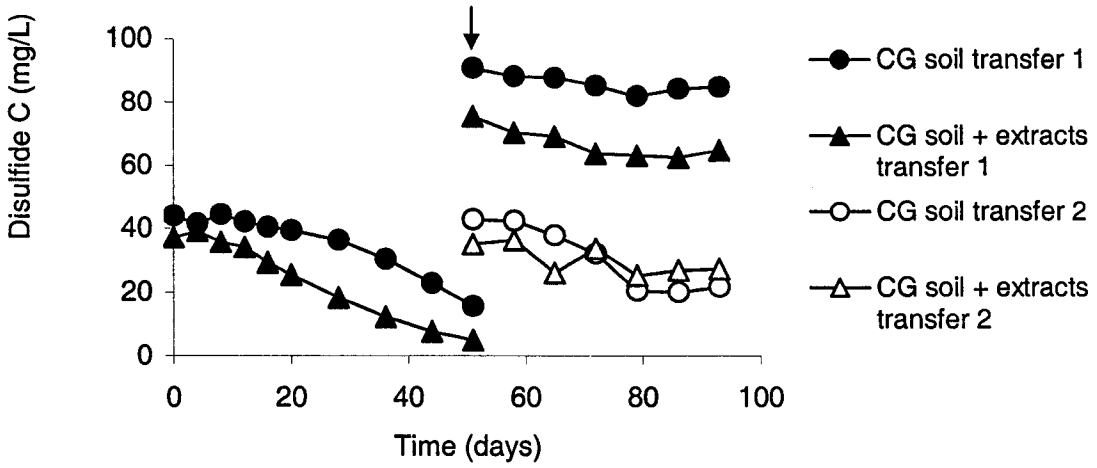


Figure 3.7 Disulfide C concentrations in soil CG enrichment culture transfers. Enrichment cultures in SFMM were provided with soil and marigold root extracts. On day 50, more disulfide C was added to the transfer 1 cultures (indicated by the arrow), which were also transferred to fresh SFMM with disulfide C (transfer 2).

Bohonos *et al.* (1977) were also unable to maintain active mixed cultures attacking benzothiophene using enrichment techniques. The failure of the vitamin solution, yeast extract, brain heart infusion, soil and marigold root extracts to support the transferred cultures does not rule out the possibility that the soil contained a growth factor necessary for the microorganisms to degrade disulfide C. Another possibility is a trace metal not provided in the defined trace metal solution added to the SFMM. The bacterium, or bacteria, capable of degrading disulfide C may also be dependant upon another microorganism in the culture that grew very poorly and was diluted upon transfer to fresh SFMM. The toxicity of disulfide C has not been determined because the disulfide is not commercially available, but either disulfide C or its degradation products could be toxic to the mixed cultures. The investigation of growth factors and the role of communities in the biodegradation of compounds of interest will be discussed in later sections.

3.1.2 Biodegradation of disulfide C in the presence of disulfide E

Disulfide C biodegradation proved difficult to study, because it is not commercially available, unlike disulfide E, and the cultures could not be coaxed to survive transfer to fresh medium. Thus, the microorganisms in soil CG capable of degrading disulfide C could not be isolated. The possibility exists that despite the significant loss of disulfide C in initial enrichments of soil CG, the microorganisms were actually degrading disulfide D or E, enhancing the conversion of disulfide C to disulfides D and E. This possibility seems more likely in the transferred enrichments, because the initial soil CG cultures did not accumulate other disulfides, whereas the subsequent transfers often showed more disulfide D as disulfide C was degraded (data not shown). In these transfers, disulfide C degradation occurred slowly, perhaps limited by the conversion of disulfide C to disulfides D and E. The loss of disulfide C in soil CG enrichments must be related to the activity of microorganisms, because disulfide C is significantly more degraded in soil cultures than in the killed soil control (Figure 3.6).

Soil CG was inoculated into SFMM with various concentrations of disulfide C and 100 mg disulfide E/L in each flask (Figure 3.8). The commercial availability of disulfide E, its occurrence in pharmaceutical products that could be released into the environment, and its smaller size suggest that disulfide E might be easier for microorganisms to degrade than disulfide C. This experiment tested the toxicity of disulfide C to soil CG enrichments. If increasing concentrations of disulfide C limited the ability of soil CG cultures to degrade the constant amount of disulfide E, disulfide C may be toxic to the soil microorganisms. No information regarding the toxicity of disulfides C and D is currently available in the literature. Disulfide E is likely not particularly toxic as it occurs

as a decomposition product of thiomersal (Tan and Parkin 2000) and its magnesium salt was tested as a rheumatoid arthritic medication (Dequeker *et al.* 1980).

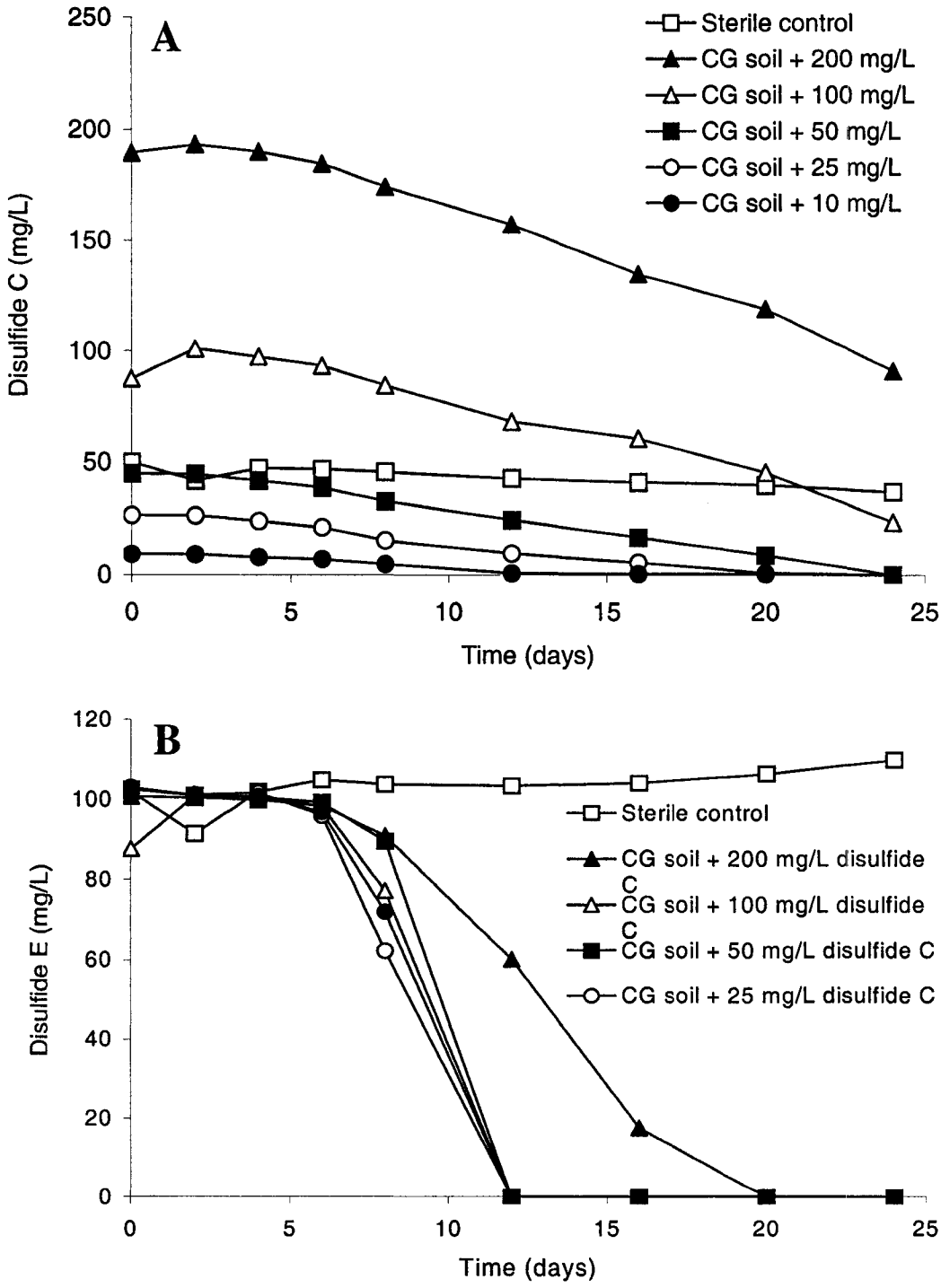


Figure 3.8 Degradation of various concentrations of disulfide C by soil CG (A) and a fixed concentration of disulfide E in the presence of various concentrations of disulfide C (B) in SFMM.

The soil CG enrichment cultures degraded 50, 25, and 10 mg disulfide C/L in 24 d (Figure 3.8A). The soil CG enrichment cultures partially degraded 200 and 100 mg disulfide C/L over 25 d (Figure 3.8A). The soil CG cultures were capable of degrading 100 mg disulfide E/L in 12 d in the presence of 100, 50, 25, and 10 mg disulfide C/L (Figure 3.8B). The culture with 200 mg disulfide C/L was able to degrade disulfide E in 20 d. Disulfide E was easier for microorganisms in soil CG to degrade than disulfide C, which inhibited the soil culture at the highest concentration. At the concentrations used in soil CG enrichments, disulfide C did not appear to be toxic to the soil cultures. Although this experiment did not determine if soil CG cultures degrade disulfide E exclusively, it did demonstrate that disulfide E is easier for these soil microorganisms to degrade than disulfide C, and that disulfide E is likely quickly degraded in the environment.

3.1.3 Summary of biodegradation studies with disulfide C

This work clearly demonstrates that disulfide C was depleted from soil enrichment cultures (Figures 3.1 and 3.5). Although disulfide C does not appear to be toxic to the enrichment cultures, the cultures repeatedly lost activity upon transfer to fresh medium. The addition of various supplements, vitamins, yeast extract, brain heart infusion, soil extracts, and a marigold root extract, did not help soil enrichment cultures survive transfers. No other studies have attempted to demonstrate biodegradation of disulfide C. The biotransformation of L-phenylalanine into benzaldehyde by *Trametes suaveolens* involves intermediates similar to disulfide C (Figure 3.9). Phenylglyoxylic acid, with an α–keto acid, was converted into mandelic acid, benzaldehyde, benzyl alcohol, and p-hydroxybenzaldehyde by *T. suaveolens* CBS 334.85 (Lomascolo *et al.* 2001). The fungus was grown in basal medium and aromatic metabolites were detected using HPLC. The

occurrence of the α–keto acid in intermediates of amino acid transformations suggests that microorganisms with the ability to degrade these functional groups also exist in the environment. Further experiments are necessary to study the biodegradation of disulfide C by soil enrichment cultures. Enrichment cultures inoculated with petroleum-contaminated soils or with a more extensive array of growth factors may help establish mixed cultures capable of biodegrading disulfide C.

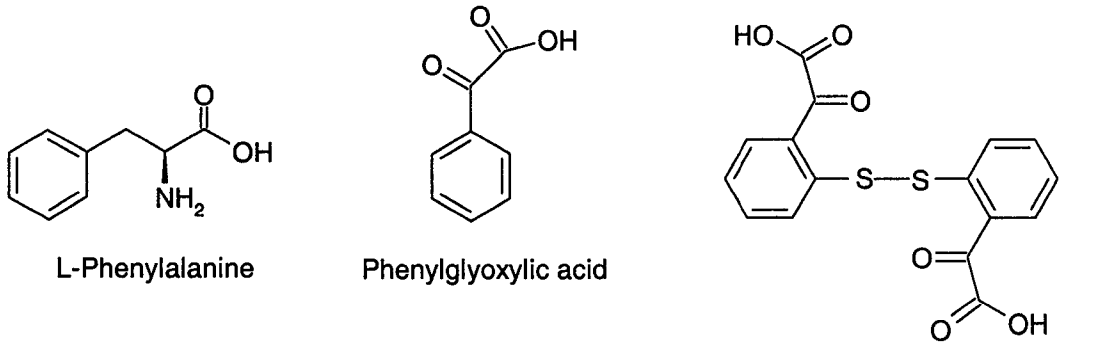


Figure 3.9 Structures of L-phenylalanine, phenylglyoxylic acid, and disulfide C.

3.2 BIODEGRADATION OF DISULFIDE E

Disulfide E is commercially available and easier to study as a carbon and sulfur source in SFMM than disulfides C and D. The biodegradability of disulfide E was demonstrated by Bressler (2001), who found that mixed cultures enriched from garden soil were capable of mineralizing disulfide E. No bacterium isolated from the mixed culture was able to degrade disulfide E in pure culture, and Bressler (2001) suggested that a combination of bacteria might be required to biodegrade disulfide E.

McInnis (2002) used enrichment techniques to attempt to find a mixed culture capable of biodegrading disulfide E. Soils from marigold gardens were used as inocula in SFMM with disulfide E as the sole carbon and sulfur source. Microorganisms in several

marigold soils were capable of degrading disulfide E. The most active mixed culture, designated culture M, was transferred several times to dilute out the soil and some of the bacteria not involved in disulfide biodegradation. Culture M continuously degraded disulfide E upon transfer to fresh SFMM and mineralized disulfide E, releasing 93% of the sulfur as sulfate and 68% of the carbon as carbon dioxide (McInnis 2002).

McInnis (2002) streaked mixed culture M onto PCA plates and six different colonial types were observed. These isolates were picked and purified, and designated isolates RM1 through RM6. No isolate was capable of degrading disulfide E in pure culture. Combinations of the isolates were tested in SFMM with disulfide E as the sole carbon and sulfur source and monitored by HPLC. Together, isolates RM1 and RM6 biodegraded disulfide E (McInnis 2002). These observations were confirmed at the beginning of this project and isolates RM1 and RM6 were selected for further study.

3.2.1 Biodegradation experiments using isolates RM1 and RM6

Isolates RM1 and RM6 degraded disulfide E in combination only, and were investigated for their ability to use other compounds as carbon and sulfur sources. Once isolates had been found that could reliably degrade disulfide E, the goal was to understand their growth requirements to combine them with *Pseudomonas* strain BT1d cultures degrading DBT. This mixed culture might be able to mineralize DBT degraded through the Kodama pathway.

3.2.1.1 Substrate range of isolates RM1 and RM6

Isolates RM1 and RM6 were cultured on solid medium and tested with various carbon and carbon and sulfur sources in SFMM. Isolate RM1 was capable of using glucose, benzoate, and acetate as carbon sources, whereas isolate RM6 grew very poorly

on these compounds (Table 3.1). Isolate RM6 grew as flocs in liquid culture, making the determination of growth difficult by observation and impossible by OD measurements.

Table 3.1 Substrates used as carbon or carbon and sulfur sources in SFMM by isolates RM1, RM6, or a combination of isolates RM1 and RM6. Growth was determined using turbidity and scored "+" if growth was observed, "±" if weak growth was observed, and "-" if no growth was observed.

Substrate	Isolate RM1	Isolate RM6	Isolate RM1 and Isolate RM6
Glucose	+	±	+
Benzoate	+	±	+
Acetate	+	±	+
Disulfide E	-	-	+
Naphthalene	-	-	-
Benzothiophene	-	-	-
DBT	-	-	-
DTNB	-	-	-
Thiophenol	-	-	-
Benzyl sulfide	-	-	-
Phenyl sulfide	-	-	-
Octyl sulfide	-	-	-
n-Dodecyl sulfide	-	-	-
Benzyl disulfide	-	-	-
Phenyl disulfide	-	-	-

3.2.1.2 DBT and disulfide C in SFMM with isolates RM1 and RM6

Isolates RM1 and RM6 were incubated in SFMM to determine if they could use DBT or disulfide C as carbon and sulfur sources. The isolates were inoculated as pure cultures or in combination, with either DBT or disulfide C, and observed for growth and color changes. In the medium with DBT as the sole carbon and sulfur source, no growth or color changes were observed in the pure cultures or the mixed culture after several weeks of incubation. Isolates RM1 and RM6 also could not use disulfide C, alone or in combination, as a sole carbon and sulfur source in SFMM. The ability to degrade disulfide E did not enable the mixed culture to attack DBT, suggesting that the enzymes used by isolates RM1 and RM6 for disulfide degradation were different from those used by *Pseudomonas* strain BT1d to degrade DBT. A mixed culture would be necessary for DBT mineralization by bacteria using the Kodama pathway.

3.2.2 Identification of isolates RM1 and RM6

Isolates RM1 and RM6 had very distinct colony morphologies when grown on PCA. Isolate RM1 grew as a large, irregular, orange colonies and isolate RM6 grew as small, circular, concave, yellow colonies. Isolate RM1 was Gram positive, rod-shaped, and catalase positive. Isolate RM6 was Gram negative, rod-shaped, catalase positive, and weakly oxidase positive. Identification of the isolates was first attempted using the Biolog system, which compares a metabolic fingerprint of the unknown microorganism responding to various carbon sources to a database of known bacteria. Different Biolog plates were required for Gram positive or Gram negative organisms.

3.2.2.1 Biolog results

Biolog identified isolate RM1 as a strain of *Rhodococcus coprophilus* (Table 3.2). After the first Biolog test, isolate RM6 could not be identified. As culture work with isolates RM1 and RM6 continued, it became apparent that isolate RM6 had an absolute requirement for vitamin B_{12} (section 3.2.4.2). The Biolog procedure was repeated for isolate RM6 with vitamins in the inoculum, and isolate RM6 was identified as a member of the genus *Variovorax* (Table 3.2).

Table 3.2 Biolog results for isolates RM1 and RM6. The Biolog similarity index is calculated by the Biolog software and determines the level of identification. After 24 h of incubation in the Biolog plates, the similarity index must be at least 0.50 to be an acceptable identification. *Pseudomonas fluorescens*, from the Biological Sciences bacterial culture collection (University of Alberta), was also tested as a positive control.

Isolate	Biolog identification	Similarity index
RM1	*Rhodococcus coprophilus*	0.50
RM6	*Roseomonas* genomospecies 4	0.45
RM6 + vitamins	*Variovorax paradoxus*	0.75
Pseudomonas fluorescens	*Pseudomonas fluorescens*	0.69

Another interesting isolate from our laboratory was identified as a strain of *Variovorax paradoxus*. *Variovorax paradoxus* strain WP1 is capable of using sulfolane, a component of the Shell Sulfinol® process for sweetening natural gas, as a sole carbon and sulfur source (Greene *et al.* 2000). Interestingly, although *Variovorax* isolates RM6 and WP1 share similar biochemical traits, *Variovorax* sp. strain RM6 has a much smaller range of usable carbon sources (Table 3.3).

Table 3.3 Comparison of isolate RM6 to *V. paradoxus* strain WP1 based on colonial morphology and biochemical traits.

Comparison	Isolate RM6[a]	Isolate WP1
Colony morphology on PCA	Yellow, circular, concave, small, dry colonies	Yellow, circular, convex, large, gooey colonies
Catalase	Positive	Positive
Oxidase	Weak positive	Weak positive
Uses (as carbon source):		
Succinate	-	+
Acetate	+	+
Arabinose	+	+
Citrate	-	±
Gluconate	-	+
Glucose	+	+
Glycerol	-	+
Inositol	-	-
Lactose	-	-
Malate	-	+
Maltose	-	-
Mannitol	+	+
Mannose	-	+
Phenylacetate	Not tested	-
Rhamnose	-	-
Sorbitol	-	-
Sucrose	-	-

[a] Carbon source results obtained from Biolog analysis.

Members of the genus *Variovorax* are often found in mixed cultures biodegrading various compounds. In enrichment cultures from Arctic soil growing on pyrene and phenanthrene, Eriksson *et al.* (2002) found *Polaromonas*, *Sphingomonas*, *Alcaligenes*, *Caulobacter*, and *Variovorax*. Also using Arctic soil enrichments, Eriksson *et al.* (2003) identified *Acidovorax*, *Bordetella*, *Pseudomonas*, *Sphingomonas*, and *Variovorax* in

mixed cultures degrading PAHs. *Variovorax paradoxus* VAI-C, which degrades acyl-homoserine lactones (bacterial quorum sensing signals), was found to be involved in a coenrichment with a member of the genus *Arthrobacter*, suggesting that consortia may play a role in mineralizing the molecules of quorum sensing (Flagan *et al.* 2003).

3.2.2.2 16S rDNA sequencing

The 16S rDNA fragment of isolate RM6 was amplified from extracted genomic DNA and sequenced. The sequence was compared to other 16S rDNA sequences in the database GenBank. Isolate RM6 was shown to be a member of the family Comamonadacaeae, within the β-Proteobacteria division. The nearest database neighbors were environmental isolates from the genus *Variovorax* (Table 3.4). The isolate RM6 16S rDNA sequence was 99% similar to two *Variovorax* isolates, WDL1 and 55. *Variovorax* sp. strain WDL1 was isolated directly from a mixed culture capable of degrading the herbicide linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) (Dejonghe *et al.* 2003). *Variovorax* sp. strain 55 was isolated from a soil microbial community (Macur *et al.* unpublished). Phylogenetic analysis (Figure 3.10) showed that isolate RM6 grouped with members of the genus *Variovorax*. Currently, there is only one species in the genus *Variovorax*, *V. paradoxus*, formerly *Alcaligenes paradoxus* (Willems *et al.* 1991). Figure 3.11 is a TEM image of *Variovorax* sp. strain RM6.

Table 3.4 Nearest neighbors of the 16S rDNA sequence from isolate RM6.

Nearest neighbor	**Percent similarity (%)**	**GenBank Accession number**
Variovorax sp. WDL1	99%	AF538929
Variovorax sp. 55	99%	AY238498
Uncultured *Variovorax* sp. Cl-15-TB2-I	98%	AY599727
Variovorax sp. TUT1027	98%	AB098595
Variovorax sp. KS2D-23	98%	AB196432
Uncultured *Variovorax* sp. 4f48	98%	AY177770

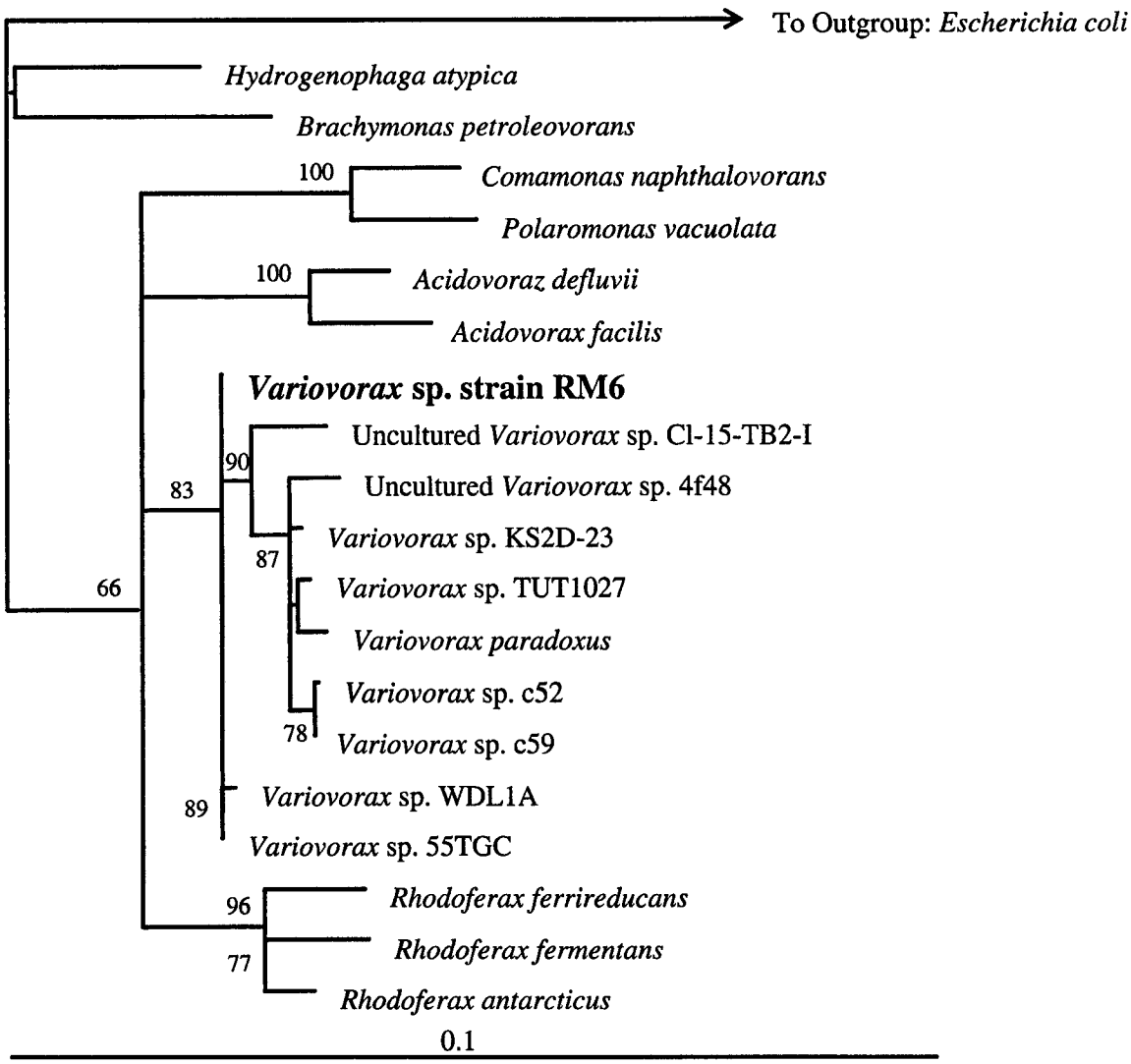


Figure 3.10 Neighbor-joining phylogenetic tree constructed with 19 Comamonadaceae isolates from GenBank based on their 16S rDNA sequences. *E. coli* was used as the outgroup. Bootstrap values greater than 50% are indicated at the nodes (100 replications). The bar stands for 0.1 substitutions per nucleotide position.

Figure 3.11 TEM image of isolate RM6 cells grown on disulfide E.

3.2.2.3 Nitrate reduction test

Lin *et al.* (2002) isolated a microorganism that produced *N*-acyl-D-amidohydrolase, an enzyme involved in the hydrolysis of *N*-acyl-D-amino acids, and identified isolate Iso1 as a strain of *V. paradoxus* using the Biolog system and 16S rDNA gene sequence analysis. Their isolate was positive for nitrate reduction. The genus *Variovorax* is divided into biovar I and biovar II strains, where the biovar II strains show nitrate reduction activity (Holding 1986). Scholten and Stams (2000) investigated a nitrate-reducing, rod-shaped, weakly motile Gram negative bacterium whose closest relative was *V. paradoxus*. The nitrate-reducing bacterium, strain ANRB-Zg, was isolated from freshwater sediment and grew optimally on acetate at 20-25°C. In the presence of acetate and nitrate, strain ANRB-Zg oxidized thiosulfate to sulfate. Isolate RM6 was tested under nitrate-reducing conditions, and compared to a *Pseudomonas stutzeri* positive control.

Variovorax sp. strain RM6 grew in the medium but did not reduce nitrate. *Variovorax* sp. strain RM6 must be a *Variovorax* biovar I strain.

3.2.3 Degradation of disulfide E by isolates RM1 and RM6

Interestingly, isolates RM1 and RM6 could only degrade disulfide E in combination in SFMM. If the isolates were added to a culture of *Pseudomonas* strain BT1d that was degrading DBT, they would have to degrade disulfide E in the presence of sulfate because the medium for culturing isolate BT1d contained 15 mM sulfate. Culture experiments with isolate BT1d showed that the amount of sulfate could be reduced to 0.01 mM in the medium and the degradation of DBT would produce HFBT through the Kodama pathway. Isolates RM1 and RM6 were tested for disulfide E degradation in the presence of sulfate, and with isolate EPWF, another bacterium isolated in our laboratory capable of degrading sulfur compounds.

3.2.3.1 Disulfide E degradation by isolates RM1 and RM6 in the presence of sulfate

Isolates RM1 and RM6 were capable of degrading disulfide E in the presence of sulfate (Figure 3.12). In the absence of sulfate, the mixture of isolates degraded disulfide E in 10 d. In the presence of 15 mM sulfate, the mixture of isolates RM1 and RM6 biodegraded disulfide E in 18 d. In pure culture, the isolates were unable to degrade disulfide E in the SFMM.

3.2.3.2 Disulfide E degradation by isolates RM1, RM6, and EPWF

Experiments with isolate EPWF showed that this bacterium did not degrade disulfide E in SFMM. Isolate EPWF was isolated from enrichment cultures using a medium with vitamins, and the vitamin mixture was added to the SFMM for all experiments with isolate EPWF. In combination, isolates EPWF and RM6 degraded disulfide E in SFMM

with the vitamin mixture. Isolates EPWF and RM1 did not degrade disulfide E in SFMM with the vitamin mixture. The experiment with isolate EPWF was repeated, and a pure culture of *Variovorax* sp. strain RM6 in SFMM with vitamins was included.

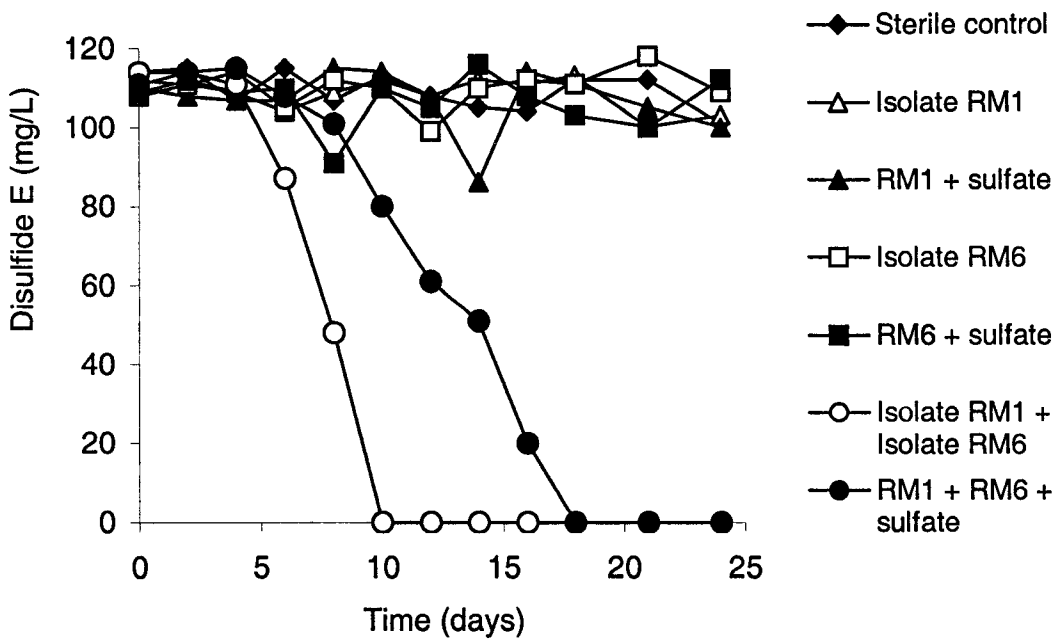


Figure 3.12 Concentrations of disulfide E in cultures of isolates RM1 and RM6 in SFMM with and without 15 mM sulfate. The two isolates were incubated individually and as a mixed culture.

Isolate EPWF did not degrade disulfide E in SFMM or use the disulfide as a sole sulfur source with acetate as a carbon source (Figure 3.13). Three cultures with vitamins in the SFMM degraded disulfide E: a mixed culture of isolates EPWF and RM6, a mixed culture of isolates RM1 and RM6, and a pure culture of *Variovorax* sp. strain RM6 (Figure 3.13). *Variovorax* sp. strain RM6 was capable of biodegrading disulfide E if the vitamin mixture was provided in the SFMM with or without 2 mM sulfate (data not shown). Degradation of DBT by *Brevibacterium* sp. DO also required the addition of a vitamin solution to the mineral medium (van Afferden *et al.* 1990). By adding single

vitamins to the culture medium, van Afferden *et al.* (1990) determined that *Brevibacterium* sp. DO required thiamine as a growth factor.

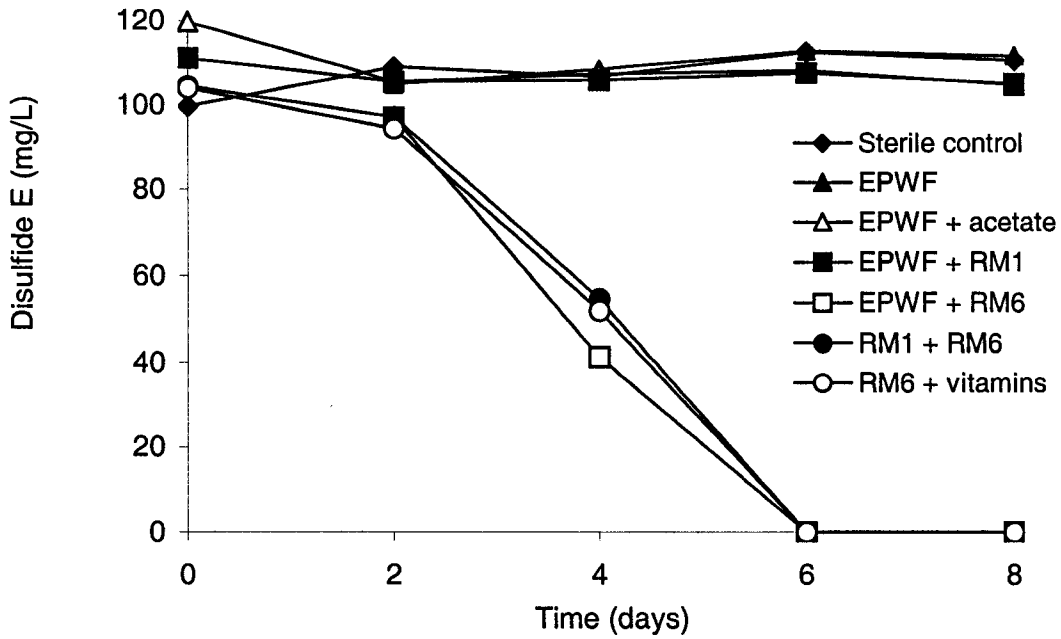


Figure 3.13 Disulfide E concentrations in cultures of isolate EPWF and isolates RM1 and RM6 in SFMM with disulfide E. Acetate was added to one culture of isolate EPWF. Isolate RM6 was incubated in pure culture in SFMM with vitamins.

3.2.4 Isolate RM6 vitamin requirement studies

Variovorax sp. strain RM6 was solely capable of degrading disulfide E if vitamins were provided in the SFMM (Figure 3.13). The importance of isolate RM1 in the mixed culture without vitamins implied that isolate RM1 must provide some vitamin to *Variovorax* sp. strain RM6. To determine if isolate RM1 released a growth factor into the medium, isolate RM1 was grown in SFMM with sulfate, and glucose or benzoate were provided as carbon sources. The RM1 culture supernatants were harvested for use as supplements in cultures of *Variovorax* sp. strain RM6. The particular vitamin required by *Variovorax* sp. strain RM6 was also investigated.

3.2.4.1 Isolate RM1 supernatant as a supplement to SFMM for isolate RM6

The filter sterilized supernatants from isolate RM1 grown on either glucose or benzoate were added to cultures of *Variovorax* sp. strain RM6 in SFMM with disulfide E as the sole carbon and sulfur source. The supernatants from cultures of isolate RM1 enabled isolate RM6 to degrade disulfide E similar to the addition of the vitamin mixture (Figure 3.14). Presumably, isolate RM1 produced some vitamin that is required by *Variovorax* sp. strain RM6.

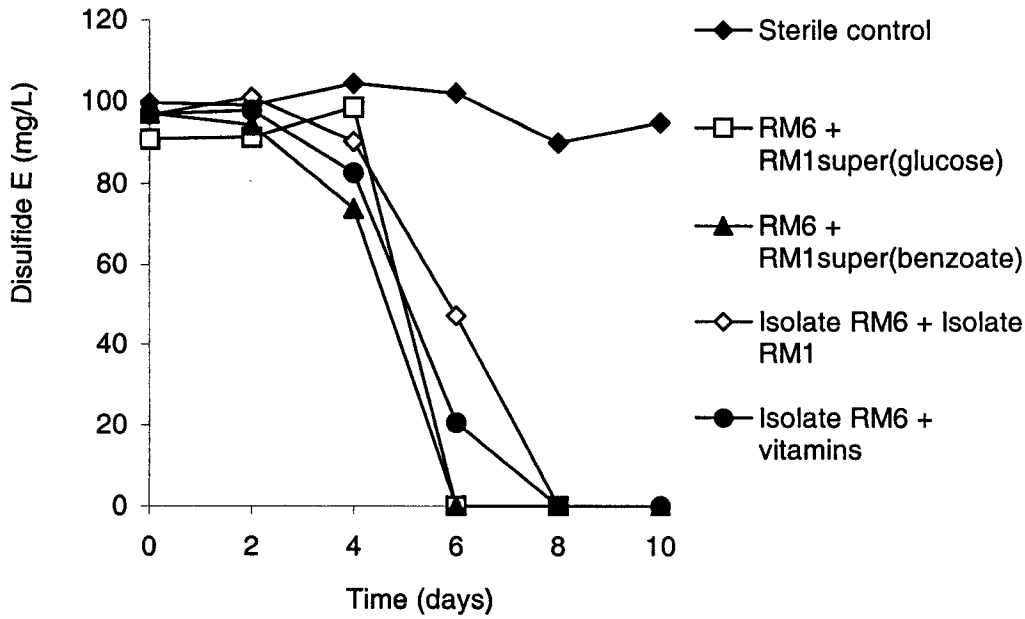


Figure 3.14 Disulfide E concentrations in cultures of isolate RM6 containing one of the following: the sterile supernatant from isolate RM1 grown on glucose, RM1super(glucose), the sterile supernatant from isolate RM1 grown on benzoate, RM1super(benzoate), viable RM1, or the vitamin mixture.

3.2.4.2 Individual vitamins tested as supplements to SFMM for isolate RM6

The vitamin mixture that was used to supplement the SFMM contained four different vitamins: thiamine, p-aminobenzoate, biotin, and vitamin B_{12}. These vitamins were prepared in filter sterilized solutions and added individually to the SFMM. *Variovorax* sp. strain RM6 only degraded disulfide E when vitamin B_{12} was provided in the SFMM

(Figure 3.15) or when cocultured with isolate RM1. Thus, isolate RM1 likely provided vitamin B_{12} to *Variovorax* sp. strain RM6.

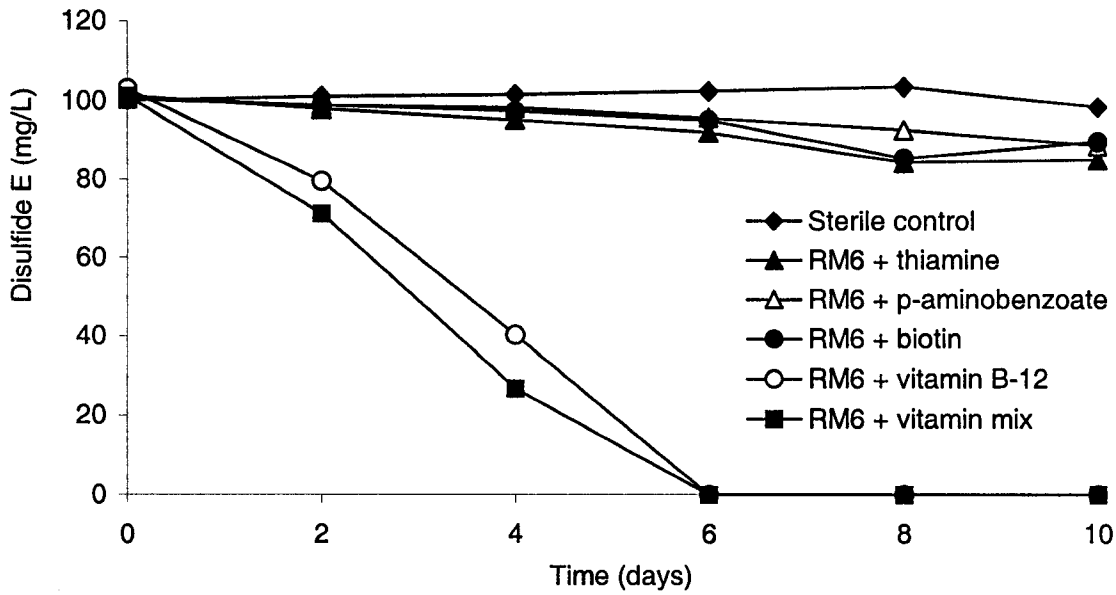


Figure 3.15 Disulfide E concentrations in pure cultures of isolate RM6 supplemented with each of the vitamins individually in SFMM.

The requirement for vitamin B_{12} by microorganisms is not unusual. Watson *et al.* (2001) tested 27 strains of *S. meliloti* that were cultured in rich media with yeast extract. Every strain required either cobalt or methionine supplementation. Vitamin B_{12} is the only vitamin that contains an essential mineral, cobalt, and is required for the biosynthesis of choline and methionine (Gottschalk 1986). Several dehydratases, deaminases, and amino mutases are B_{12}-enzymes that perform rearrangements of substituent groups between two adjacent positions (Gottschalk 1986). Vitamin B_{12} itself can be synthesized through two different pathways, one aerobic and one anaerobic (Scott 2001).

Revillas *et al.* (2000) studied the production of B-group vitamins by two *Azotobacter* strains, because this genus is often associated with the production of water-soluble

vitamins. They found that several B-group vitamins were produced by *Azotobacter* using phenolic monomers as carbon sources at concentrations commonly found in soil. Similarly, Sierra *et al.* (1999) found that two *Rhizobium* strains also produced B-vitamins. Interestingly, despite that *R. meliloti* strains do not generally require the vitamin pantothenic acid as a growth factor, one of the strains tested by Sierra *et al.* (1999) produced large amounts of this vitamin and released it into the culture medium. It is likely that the vitamin is released for use by other organisms in the soil, including the associating legume.

The isolation of *Variovorax* sp. strain RM6 from a mixed culture that also contained a bacterium capable of producing vitamin B_{12} reflects the nature of the community environment. Likely, isolate RM1 is capable of using a metabolite of disulfide E biodegradation by *Variovorax* sp. strain RM6 as a carbon source in the SFMM. The discovery of the vitamin requirement of *Variovorax* sp. strain RM6 allowed for further studies of disulfide E degradation using a pure culture, with the goal of identifying intermediates and determining the pathway for disulfide E biodegradation.

3.2.5 Pure cultures of isolate RM6 degrading disulfide E

Pure cultures of *Variovorax* sp. strain RM6 degrading disulfide E in SFMM with the vitamin mixture were followed by HPLC. *Variovorax* sp. strain RM6 was tested for its ability to degrade other substrates with and without vitamins, and experiments were done to determine if metabolites could be detected during the biodegradation of disulfide E.

3.2.5.1 Degradation of disulfide E, benzoate, and acetate in the presence of vitamins

Variovorax sp. strain RM6 was inoculated into SFMM with disulfide E as a sole carbon and sulfur source, and benzoate and acetate were used as sole carbon sources in

SFMM with and without vitamins. In all of the cultures, *Variovorax* sp. strain RM6 only used the substrate if vitamins were present in the medium (Figure 3.16). Likely, the vitamins are not required specifically for disulfide E biodegradation, but vitamin B_{12} is an absolute requirement for the growth of isolate RM6. All subsequent experiments with *Variovorax* sp. strain RM6 contained vitamins in the SFMM.

3.2.5.2 Attempts to slow biodegradation of disulfide E to detect metabolites

Preliminary experiments with pentafluorobenzoic acid were done to determine if the presence of pentafluorobenzoic acid would slow disulfide E degradation by *Variovorax* sp. strain RM6 allowing for the accumulation of metabolites that could be detected by HPLC. At a concentration of 500 mg pentafluorobenzoic acid/L in the SFMM, no disulfide E degradation occurred (data not shown). When either 200 or 300 mg pentafluorobenzoic acid/L was added to the SFMM, disulfide E degradation by *Variovorax* sp. strain RM6 was not inhibited. However, no metabolites were detected by HPLC in any of the cultures that contained pentafluorobenzoic acid.

The experiment with 300 mg pentafluorobenzoic acid/L was repeated, but the pentafluorobenzoic acid was added after 2 d, rather than at time zero. If pentafluorobenzoic acid interfered with disulfide biodegradation, it would slow disulfide degradation as *Variovorax* sp. strain RM6 entered exponential phase (having synthesized the enzymes for disulfide degradation during the 2 d lag). *Variovorax* sp. strain RM6 was also inoculated into SFMM with disulfide E and acetate, to determine if this isolate would use disulfide E as a sulfur source only, perhaps accumulating the products of disulfide degradation after the sulfur was removed. A culture with disulfide E, acetate, and pentafluorobenzoic acid was also included in the experiment, attempting to slow

disulfide degradation coupled with removal of the disulfide sulfur, which might allow a metabolite to accumulate to high enough levels for detection by HPLC.

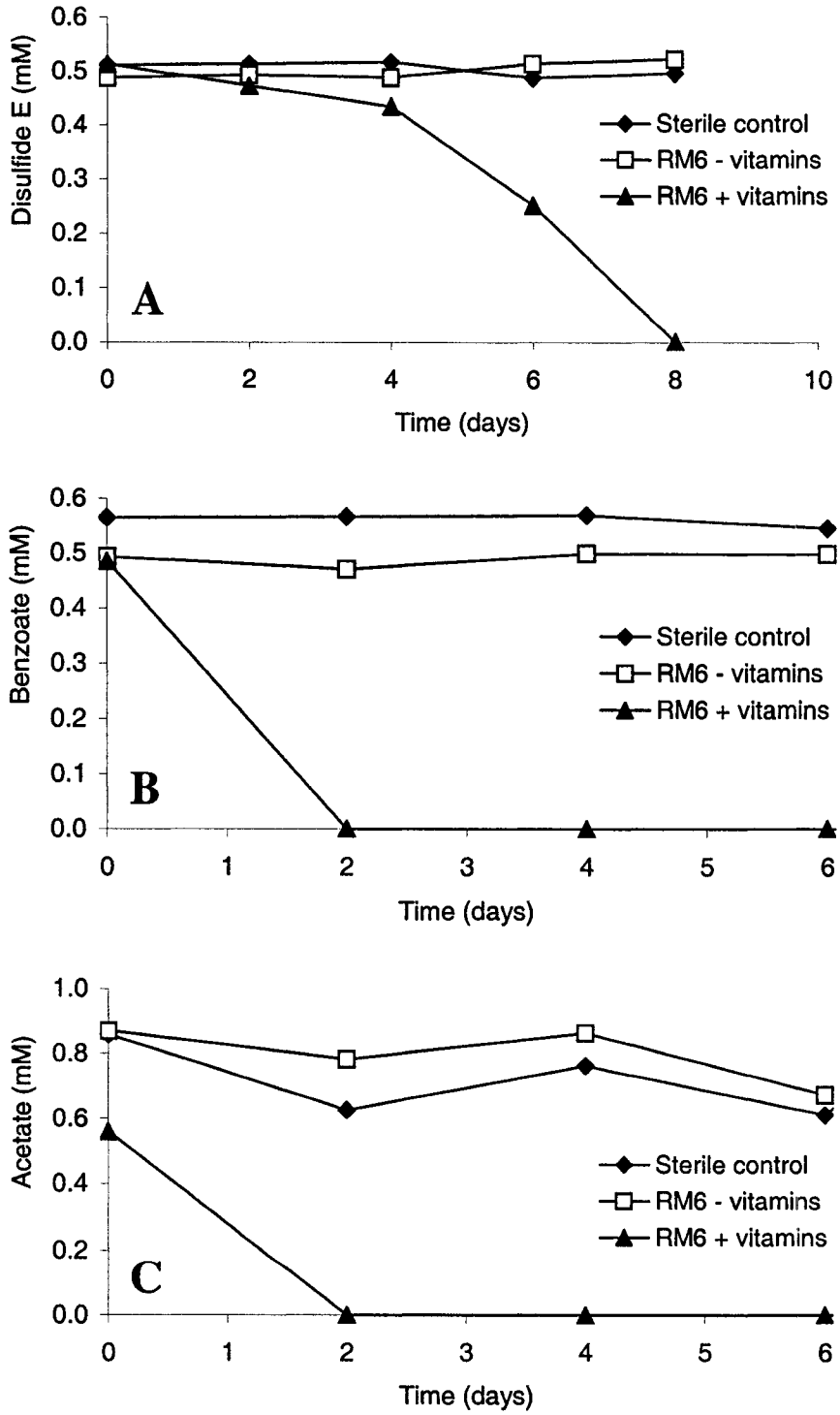


Figure 3.16 Substrate depletion in cultures of isolate RM6 incubated with disulfide E (A), benzoate (B), or acetate (C) with and without vitamins.

The addition of pentafluorobenzoic acid after 2 d did slow disulfide degradation by *Variovorax* sp. strain RM6 (Figure 3.17). Disulfide E was degraded in 4 d without pentafluorobenzoic acid present, and in the presence of pentafluorobenzoic acid the disulfide was degraded in 6 d. Disulfide E was not degraded by *Variovorax* sp. strain RM6 in the presence of acetate. No new peaks were observed by HPLC in the cultures containing disulfide E and acetate with or without pentafluorobenzoic acid.

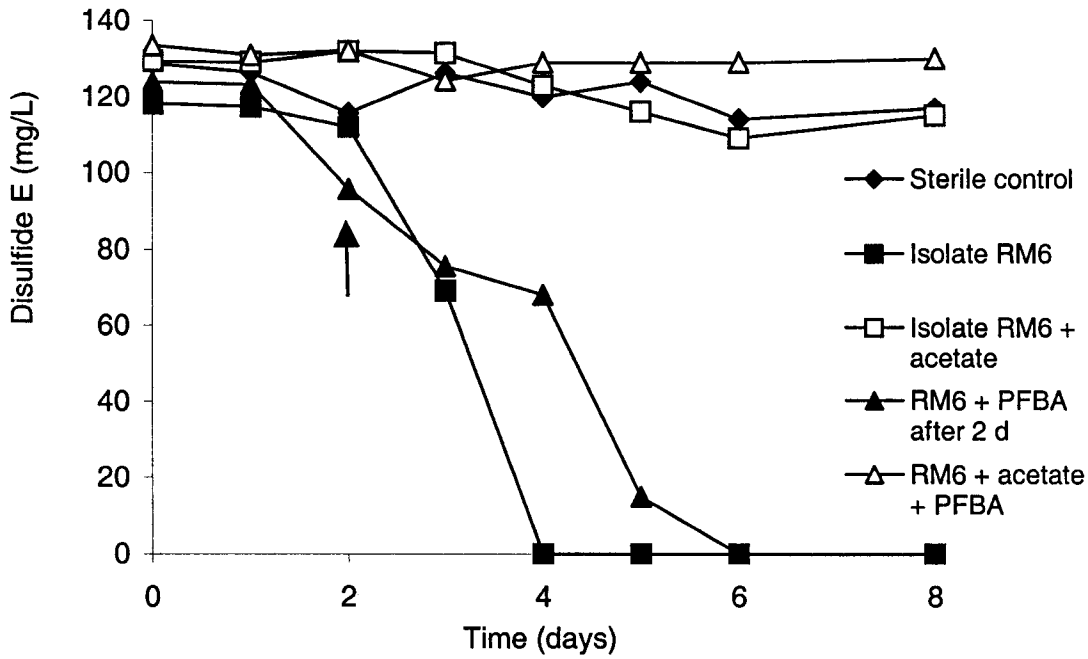


Figure 3.17 Isolate RM6 incubated in SFMM plus vitamins with disulfide E and pentafluorobenzoic acid (PFBA). In one flask, pentafluorobenzoic acid was added after 2 d (indicated by the arrow). Acetate was added to a flask of isolate RM6 and one flask was prepared with isolate RM6, disulfide E, acetate, and pentafluorobenzoic acid.

Other compounds were investigated for their ability to slow biodegradation of disulfide E by *Variovorax* sp. strain RM6. Earlier experiments demonstrated that isolate RM6 could use benzoate as a sole carbon source in SFMM with sulfate and vitamins. Both 3-chlorobenzoic acid and 3-fluorobenzoic acid were added to SFMM with benzoate and disulfide E. *Variovorax* sp. strain RM6 could not degrade either of these substituted

benzoic acids, but could degrade benzoate in the presence of 3-chlorobenzoic acid or 3-fluorobenzoic acid. Interestingly, *Variovorax* sp. strain RM6 could not degrade disulfide E in the presence of 3-chlorobenzoic acid or 3-fluorobenzoic acid. Because no new peaks were observed in the HPLC chromatograms, other experimental approaches were used in attempts to detect metabolites of disulfide E biodegradation.

3.2.5.3 Degradation of disulfide E formed from thiosalicylic acid

HPLC chromatograms from pure cultures of *Variovorax* sp. strain RM6 with disulfide E in SFMM showed a small peak at 1.8 min in time zero samples. The retention time of disulfide E using this HPLC method was approximately 6 min. The small peak was gone by the next day, suggesting that it was a contaminant of the commercial disulfide E preparation and degradable by the isolate. The retention time and UV scan of the contaminant was similar to benzoic acid. GC-MS analysis of benzoic acid standards and preparations of disulfide E extracted from SFMM and derivatized with MTBSTFA confirmed that the contaminant was benzoic acid.

In order to avoid the presence of this contaminant, so that metabolites at a similar retention time could be observed, disulfide E was prepared in the SFMM from thiosalicylic acid dimerization. Time zero samples of SFMM with disulfide E prepared from thiosalicylic acid were free of benzoic acid as determined by HPLC. *Variovorax* sp. strain RM6 was added to triplicate flasks of SFMM with this disulfide E preparation. After 1 d of incubation, a peak was observed at 1.8 min in the HPLC chromatograms for all flasks. The 1.8 min peak was scanned and showed a UV scan consistent with benzoic acid. The formation of benzoic acid in the triplicate cultures of *Variovorax* sp. strain RM6 degrading disulfide E was transient (Figure 3.18). Further experiments were done to

confirm that benzoic acid was a metabolite in the biodegradation of disulfide E by *Variovorax* sp. strain RM6.

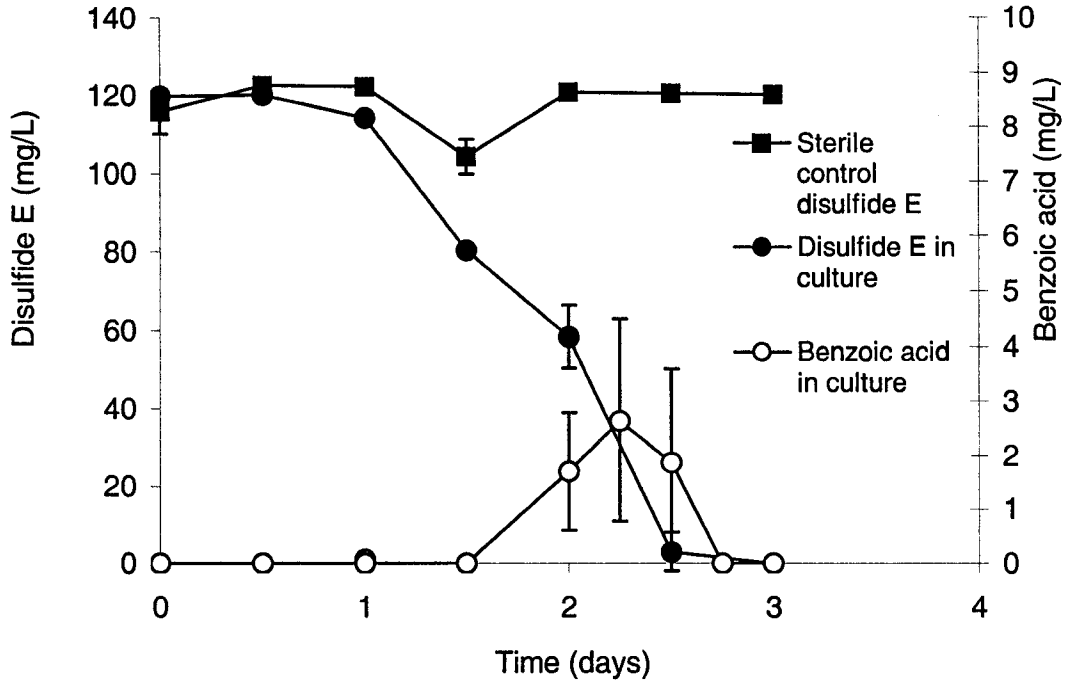


Figure 3.18 Benzoic acid, tentatively identified using retention time and UV scans, formed in triplicate cultures of isolate RM6 degrading disulfide E formed from thiosalicylic acid dimerization.

3.2.5.4 GC-MS and HPLC-MS confirm that benzoic acid is a metabolite of disulfide E degradation by isolate RM6

The retention time and UV scan of the transient metabolite were both consistent with a benzoic acid standard. The experiment with triplicate cultures of *Variovorax* sp. strain RM6 degrading disulfide E formed from thiosalicylic acid dimerization was repeated, and when the 1.8 min peak was observed, the cultures were extracted to obtain the metabolite. The culture extracts and a standard of benzoic acid were derivatized with MTBSTFA and GC-MS analysis was performed. The metabolite and standard mass spectra confirmed that benzoic acid was produced in cultures of *Variovorax* sp. strain RM6 degrading disulfide E (Figure 3.19). Coinjection of the derivatized culture sample and the standard

produced one peak by GC-MS, consistent with benzoic acid. Samples of *Variovorax* sp. strain RM6 degrading disulfide E were also analyzed by HPLC-MS, which confirmed the presence of disulfide E and benzoic acid. Thus, benzoic acid is an intermediate in the biodegradation of disulfide E by *Variovorax* sp. strain RM6.

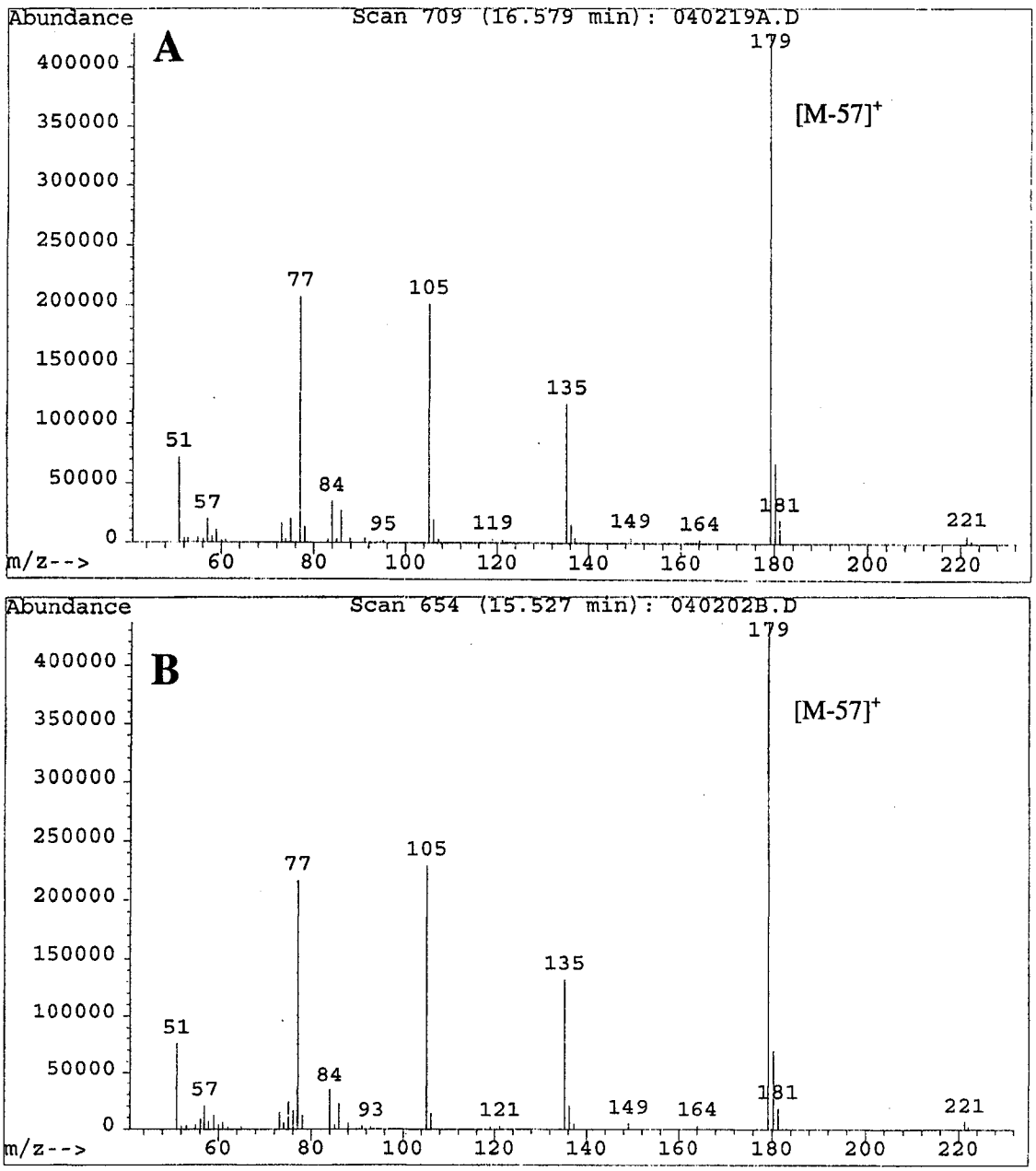


Figure 3.19 MTBSTFA-derivatized benzoic acid standard (A) and metabolite from cultures of isolate RM6 degrading disulfide E in SFMM (B) analyzed by GC-MS.

3.2.6 Possible pathways for the biodegradation of disulfide E by isolate RM6

When benzoic acid was confirmed to be formed during the degradation of disulfide E, a literature search was performed to find disulfide degradation pathways that might produce benzoic acid from disulfide E. Although the mechanism of disulfide biodegradation is not well known, there are some studies suggesting how a particular disulfide might be degraded by different microorganisms or processes. Three hypothetical pathways for disulfide E biodegradation, that would produce benzoic acid as an intermediate, were investigated and experiments were designed to attempt to discover how *Variovorax* sp. strain RM6 degraded disulfide E. The literature review revealed that the removal of the sulfur, and whether it occurs before or after oxidation, is the key step in understanding the biodegradation of disulfides. The three potential mechanisms discussed here for the biodegradation of disulfide E are the reduction of the disulfide bond, sulfitolysis of disulfide E, and oxidation of the disulfide sulfur atoms.

3.2.6.1 Reduction of the disulfide bond

Smith and Kelly (1998) proposed a pathway for the biodegradation of dimethyl disulfide by *Thiobacillus thioparus* strain E6 that began with the reductive cleavage of the disulfide into two molecules of methanethiol. Isolate E6 produced methanethiol from dimethyl disulfide under anaerobic conditions, and the rate of methanethiol production was significantly faster in the presence of additional NADH. Aerobic suspensions of isolate E6 degraded the metabolite methanethiol four times faster than anaerobic suspensions.

These observations led Smith and Kelly (1998) to predict that the first step in the pathway for dimethyl disulfide degradation was oxygen-independent, but that subsequent

steps required oxygen. They proposed that an NADH-stimulated reductase formed methanethiol from DMDS, and that a methanethiol oxidase reacting with oxygen would release the sulfur as H_2S. The H_2S would be quickly oxidized to sulfate in aerobic cultures. However, Smith and Kelly (1998) did not detect H_2S in either aerobic or anaerobic incubations. If a similar pathway occurred for the biodegradation of disulfide E by *Variovorax* sp. strain RM6, the disulfide would be cleaved to form thiosalicylic acid after reduction of the disulfide bond (Figure 3.20). Removal of H_2S from thiosalicylic acid would form benzoic acid, which could be further biodegraded. Thiosalicylic acid can be detected using the HPLC method used to analyze for disulfide E, but the thiosalicylic acid was quickly abiotically oxidized to form the disulfide under aerobic conditions.

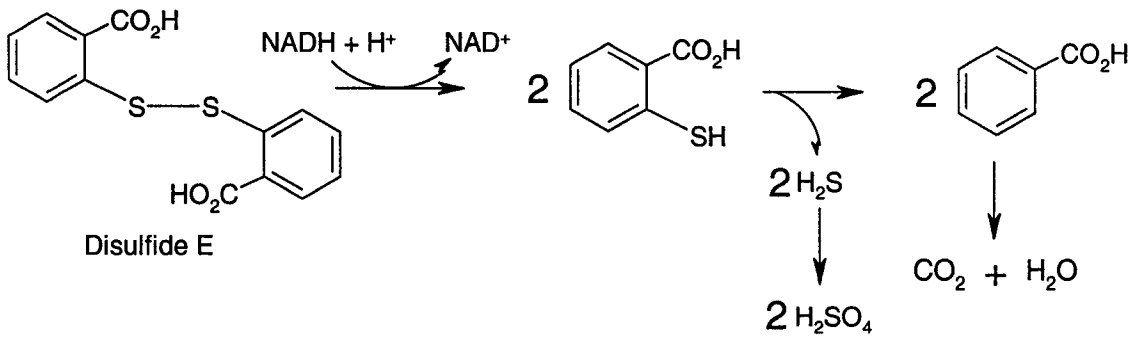


Figure 3.20 Hypothetical pathway for the biodegradation of disulfide E by disulfide bond reduction and production of H_2S (after Smith and Kelly 1998).

Visscher and Taylor (1993) also studied the biodegradation of dimethyl disulfide by a marine bacterium. Although they observed the formation of sulfate from dimethyl sulfide, diethyl sulfide, and dibenzyl sulfide, they did not report the percent of sulfate recovered from the biodegradation of dimethyl disulfide, diethyl disulfide, or dibenzyl disulfide by their isolate. Interestingly, these authors supplemented their marine isolate with additional vitamin B_{12}.

Riffel *et al.* (2003) isolated a keratinolytic bacterium capable of reducing the disulfide bonds that give keratin its high mechanical stability. Over time, thiols were formed by *Chryseobacterium* strain kr6 degrading keratin. *Fervidobacterium islandicum* AW-1 is a keratinase-producing thermophilic anaerobe (Nam *et al.* 2002). Isolate AW-1 likely contains an outer membrane-bound enzyme responsible for keratinolytic activity, although the exact mechanism of keratinolysis is unknown. A disulfide reductase-like protein was isolated from a *Stenotrophomonas* sp. capable of degrading keratin (Yamamura *et al.* 2002). The disulfide reductase increased keratin degradation by cleaving disulfide bonds, and could also reduce disulfides in collagen, elastin, and oxidized glutathione and cysteine.

Dibenzyl disulfide was degraded by the fungi *Coriolus versicolor* and *Tyromyces palustris*, producing trace amounts of benzyl alcohol and benzaldehyde (Itoh *et al.* 1997). Although benzoic acid was not detected, Itoh *et al.* (1997) proposed a metabolic pathway for the biodegradation of dibenzyl disulfide through reduction of the disulfide bond to form benzyl sulfide, formation of benzoic acid, and subsequent mineralization. Although they implied that fungi should be able to cleave a C-S bond, the authors did not suggest a mechanism for the removal of the sulfur from benzyl sulfide. If reductive cleavage of the disulfide bond is used by *Variovorax* sp. strain RM6, thiosalicylic acid or H_2S should be detected in cultures of the isolate degrading disulfide E.

3.2.6.2 Sulfitolysis of disulfide E

Some fungi excrete sulfite into the growth medium, and presumably into the environment, to cleave disulfide bonds. *Microsporum gypseum* used sulfitolysis to attack disulfide bonds in wool, enabling the keratin to be degraded by extracellular proteases

(Kunert 1989). In the presence of sulfite, disulfide E would be cleaved to form 2-(S-sulfo)benzoic acid and thiosalicylic acid (Figure 3.21).

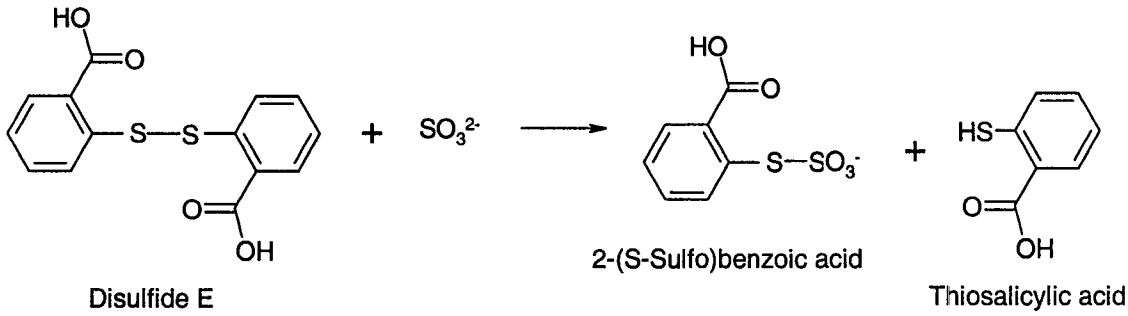


Figure 3.21 Sulfitolysis of disulfide E.

The sulfitolysis pathway also produces thiosalicylic acid, which would likely re-dimerize to form disulfide E in the highly aerobic cultures. Thiosalicylic could also be attacked by enzymes of *Variovorax* sp. strain RM6, similar to the reductive cleavage pathway (Figure 3.20). The key to determing if the sulfitolysis pathway occurs during the biodegradation of disulfide E is to detect the sulfitolysis product 2-(S-sulfo)benzoic acid. This compound can be detected by the HPLC method used to determine the concentration of disulfide E and benzoic acid. *Variovorax* sp. strain RM6 should also be able to degrade 2-(S-sulfo)benzoic acid if this compound is an intermediate in the biodegradation of disulfide E.

3.2.6.3 Oxidation of the disulfide sulfur atoms

Biodegradation of organosulfur compounds often involves oxidations, of both the carbon and sulfur atoms (Kropp and Fedorak 1998). Oxidation of sulfur atoms yields sulfoxides and sulfones, functional groups which can be used to help identify a metabolite. *In vivo* metabolism of diallyl disulfide by rats produced primarily allyl methyl sulfoxide and allyl methyl sulfone (Germain *et al.* 2002). In the aerobic and anaerobic

metabolism of cysteine, the disulfide cystine is formed. Other biological forms of cysteine include cystine sulfinic acid, which can be metabolized to release sulfate, thiocysteine, thiocystine, and cystathionase (Wlodek *et al.* 2001). Interestingly, vitamin B_6 and folic acid are required for the biodegradation of sulfur amino acids cysteine and homocysteine in the liver. Many modifications of amino acids in stress responses involve oxidations mediated by GSH and GSH disulfide (Huang and Huang 2002).

Oxidation of symmetric disulfides using hydrogen peroxide and methyltrioxorhenium as a catalyst generated the thiosulfinate, thiosulfonate, and over longer periods of time, the sulfonic acid (Wang and Espenson 2000). Similar results were obtained when GSH disulfide was electrochemically oxidized (Terashima *et al.* 2003). Tan and Parkin (2000) studied the decomposition of thiomersal under abiotic conditions in the presence of the ethylmercuric ion. Thiomersal formed thiosalicylic acid, which dimerized to disulfide E. They detected 2-sulfinobenzoic acid and 2-sulfobenzoic acid formed from the abiotic degradation of disulfide E.

A postulated pathway for the biodegradation of disulfide E through a similar mode, where the disulfide sulfur atoms are oxidized and the molecule is cleaved to form 2-sulfobenzoic acid, is shown in Figure 3.22. Tan and Parkin (2000) detected benzoic acid as a minor decomposition product of the abiotic degradation of disulfide E. Figure 3.22 shows the release of the sulfur from disulfide E as sulfite, which is oxidized to sulfate, forming benzoic acid. During the biodegradation of DBT through the 4S pathway, studied extensively in *Rhodococcus erythropolis* IGTS8, an aromatic sulfinic acid hydrolase desulfinates 2'-hydroxybiphenyl-2-sulfinic acid (Gray *et al.* 1996). By a similar mechanism, *Variovorax* sp. strain RM6 might produce a desulfinating enzyme

capable of releasing sulfite from an intermediate in disulfide E biodegradation. The sulfite would be quickly oxidized to sulfate in the highly aerobic cultures.

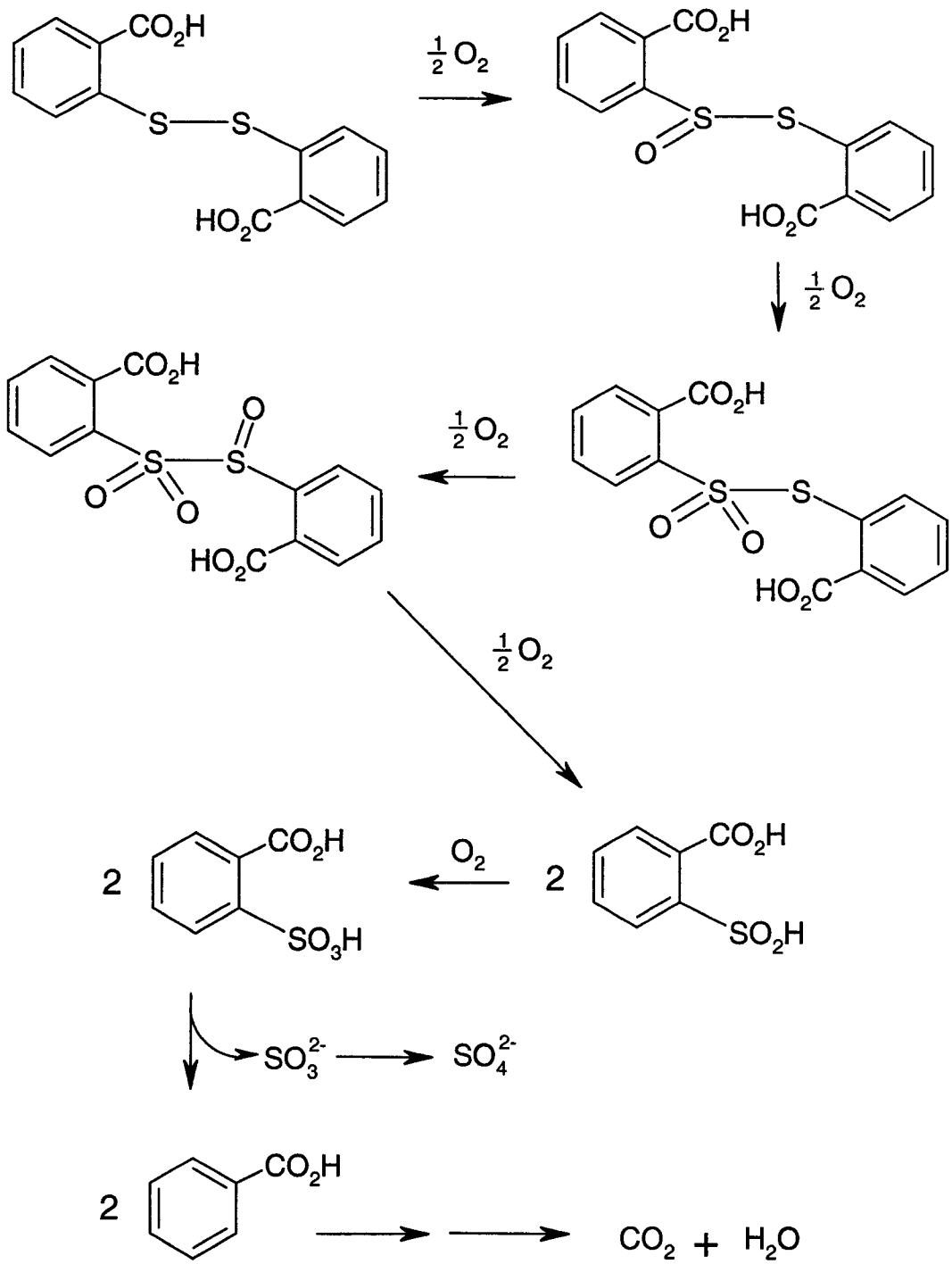


Figure 3.22 A possible pathway for the oxidation of disulfide E to form 2-sulfobenzoic acid, benzoic acid, and sulfate.

To determine if the oxidation pathway is used by *Variovorax* sp. strain RM6, intermediate disulfides with additional oxygen atoms or 2-sulfobenzoic acid might be detected. If *Variovorax* sp. strain RM6 degrades 2-sulfobenzoic acid by the proposed oxidation pathway, benzoic acid would be detected in cultures degrading 2-sulfobenzoic acid. Gonçalves and Fedorak (1996) studied the biodegradation of 2-sulfobenzoic acid by *Pseudomonas* sp. strain SB(W). Using their HPLC method to detect 2-sulfobenzoic acid, *Variovorax* sp. strain RM6 was inoculated into SFMM with 2-sulfobenzoic acid as a sole carbon and sulfur source or with disulfide E and 2-sulfobenzoic acid, and the cultures were monitored by HPLC. *Variovorax* sp. strain RM6 degraded 2-sulfobenzoic acid in 20 d in the absence of disulfide E (Figure 3.23). In the presence of disulfide E, the isolate degraded 2-sulfobenzoic acid in 4 d (Figure 3.23). The disulfide was also degraded in 4 d. The presence of disulfide E in the medium enhanced the biodegradation of 2-sulfobenzoic acid. This implied that in the biodegradation of disulfide E, enzymes may be produced that are also used to attack 2-sulfobenzoic acid. Although this experiment did not determine if 2-sulfobenzoic acid is an intermediate in the biodegradation of disulfide E, it did confirm that *Variovorax* sp. strain RM6 can degrade 2-sulfobenzoic acid.

The degradation of 2-sulfobenzoic acid in the presence of disulfide E was repeated in triplicate. One set of flasks contained 2-sulfobenzoic acid and disulfide E, and a second set of flasks contained disulfide E only. Because disulfide E degradation generally lagged for 2 d, 2-sulfobenzoic acid was added to the disulfide E flasks after 2 d to determine if 2-sulfobenzoic acid degradation would occur at a faster rate if the enzymes for disulfide E degradation were active. Disulfide E was degraded by *Variovorax* sp. strain RM6 in 3 d with 2-sulfobenzoic acid present at the time of inoculation, and in approximately 3.5 d

with 2-sulfobenzoic acid added after 2 d (Figure 3.24A). The degradation of disulfide E did not appear to be affected by the presence of 2-sulfobenzoic acid. When 2-sulfobenzoic acid was initially present in the SFMM with disulfide E, *Variovorax* sp. strain RM6 degraded it in 3 d (Figure 3.24B). When 2-sulfobenzoic acid was added after 2 d, it was degraded by *Variovorax* sp. strain RM6 in approximately 3.5 d with little or no lag period (Figure 3.24B). This experiment did not determine if 2-sulfobenzoic acid is a metabolite in the biodegradation of disulfide E by *Variovorax* sp. strain RM6, although it did confirm the result of the previous experiment, because 2-sulfobenzoic acid degradation occurred quickly in the presence of disulfide E. It is possible that a product of disulfide E degradation is responsible for enhancing the rate of 2-sulfobenzoic acid biodegradation, if their pathways share a common intermediate like benzoic acid or salicylic acid.

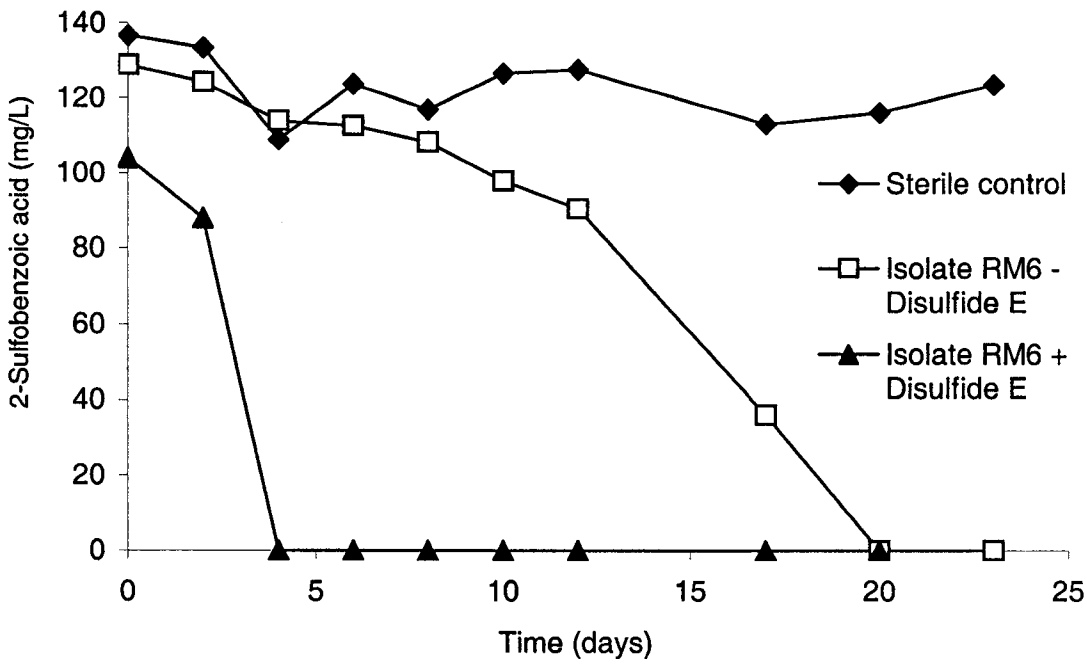


Figure 3.23 Isolate RM6 incubated in SFMM with 2-sulfobenzoic acid, with or without disulfide E.

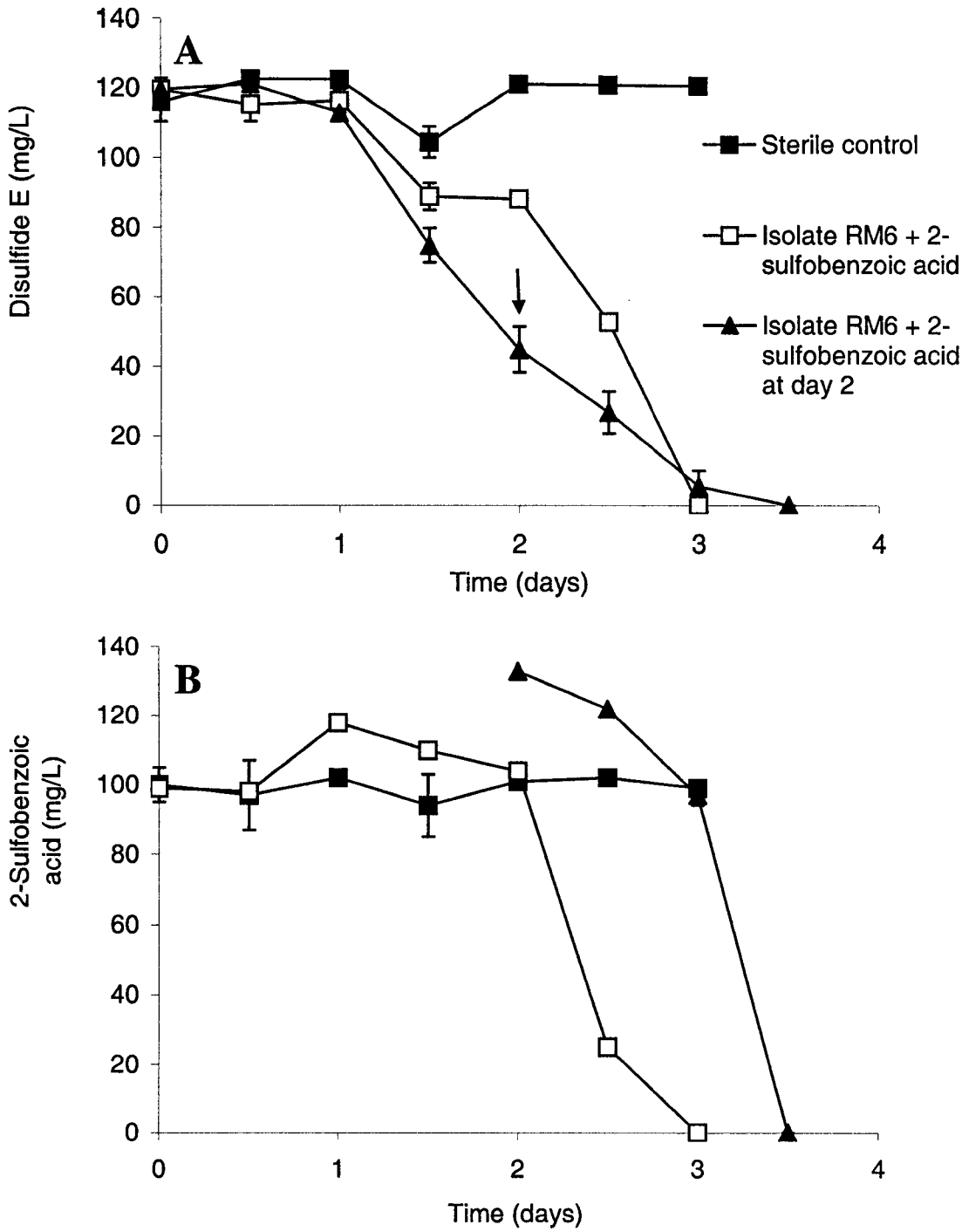


Figure 3.24 Isolate RM6 incubated in SFMM with 2-sulfobenzoic acid and disulfide E, and with isolate RM6 degrading disulfide E and 2-sulfobenzoic acid added after 2 d as indicated by the arrow in panel A. Disulfide E concentration is shown in panel A, and 2-sulfobenzoic acid concentration is shown in panel B.

To search for disulfide intermediates that had been oxidized to form the sulfoxides and sulfones in Figure 3.21, a chemical oxidation of disulfide E was performed (Willey *et al.* 1981). The oxidized disulfides should be detected using the HPLC method for disulfide E, but as these compounds are not commercially available, the chemical oxidation would help determine approximate retention times and types of intermediates possible. The chemical oxidation product was analyzed by HPLC and four new peaks were observed in the chromatogram. The oxidized sample was taken for infrared spectroscopy analysis to detect sulfoxide and sulfone functional groups. Unfortunately, although carboxyl groups were observed in the oxidized sample, no sulfoxide or sulfone functional groups were present and the products of disulfide E oxidation could not be identified. No peaks at the retention times detected in the chemically oxidized disulfide E sample were observed in cultures of *Variovorax* sp. strain RM6 degrading disulfide E.

Disulfide E was also biologically oxidized using an enzyme preparation. A pure preparation of laccase, an extracellular enzyme produced by fungi, catalyzed the oxidation of dibenzyl sulfide to dibenzyl sulfoxide (Van Hamme *et al.* 2003). A similar laccase preparation was obtained from M.A. Pickard. In the reaction mixture with laccase and disulfide E, the disulfide was removed and changes were observed in the peaks eluting from the HPLC with retention times of less than 1 min. The biological oxidation of disulfide E appeared to produce polar intermediates that eluted quickly but were not well resolved. No similarities were observed between the results for the chemical and biological oxidations of disulfide E. Benzoic acid was not formed in the reaction mixture with laccase attacking disulfide E. Other experiments were needed to search for

metabolites in the degradation of disulfide E to confirm which pathway was used by *Variovorax* sp. strain RM6.

3.2.7 Washed cell experiments to attempt to determine the pathway for disulfide E degradation

Kroon and Ginkel (2001) established a mixed culture from domestic waste water sludge that was capable of degrading dodecyldimethylamine. From their mixed culture they isolated two bacteria, *Burkholderia cepacia* sp. strain ADM12D and *Stenotrophomonas maltophila* sp. strain DMA1, that in combination were capable of mineralizing dodecyldimethylamine. They used washed cell experiments to follow the degradation of dodecyldimethylamine and formation of intermediates. Briefly, the isolates were grown on dodecyldimethylamine, or an intermediate, and harvested by centrifugation. The cells were washed with phosphate buffer three times and resuspended in the same buffer with the compound of interest. They observed degradation of dodecyldimethylamine by isolate ADM12D over a period of 8 h. Isolate ADM12D formed dimethylamine, which was mineralized by isolate DMA1.

Using a similar washed cell procedure, *Variovorax* sp. strain RM6 cell suspensions were prepared with disulfide E as the sole carbon and sulfur source. Washed cell experiments were also attempted to detect H_2S and to determine if the sulfitolysis product or 2-sulfobenzoic acid were degraded by cell suspensions of *Variovorax* sp. strain RM6. The goal of the washed cell experiments was to help elucidate the pathway for disulfide E biodegradation by *Variovorax* sp. strain RM6.

3.2.7.1 Preliminary washed cell experiments detected benzoic acid

In the first attempts to perform washed cell experiments, a 1 L culture of *Variovorax* sp. strain RM6 grown on disulfide E was harvested, washed, and resuspended in 100 mL of phosphate buffer with disulfide E. The washed cells degraded disulfide E in less than 10 h, and a second addition of disulfide E was supplied to the suspension after 20 h (Figure 3.25). Following this addition, benzoic acid was formed in the cell suspension.

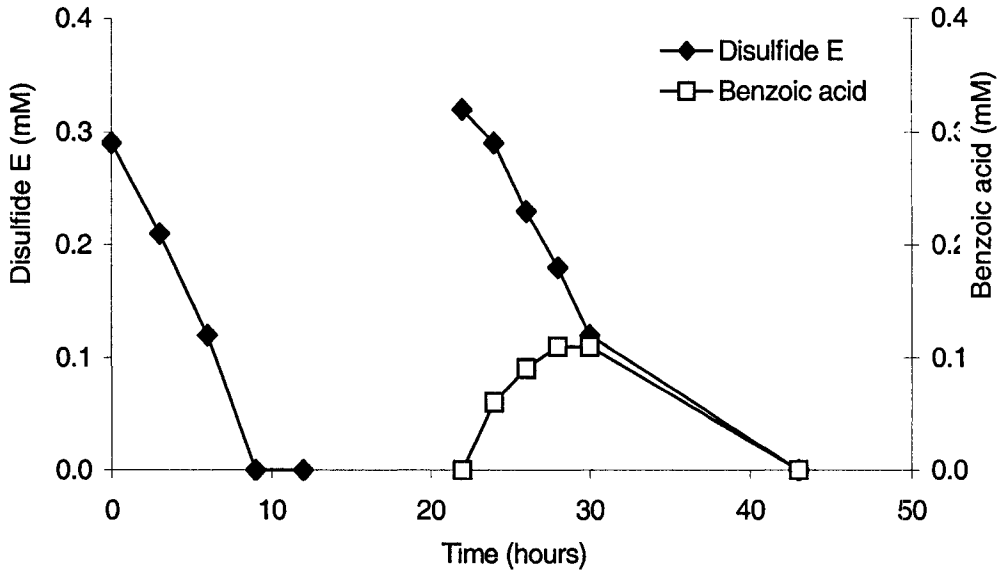


Figure 3.25 Disulfide E and benzoic acid concentrations in a washed cell suspension of isolate RM6. Disulfide E added at 0 h and 22 h.

After the initial washed cell experiments detected benzoic acid, experiments were done to determine if saline solutions could be used instead of phosphate buffer because the HPLC-MS method could not be used for samples that contained phosphate. However, washed cell suspensions of *Variovorax* sp. strain RM6 in physiological saline (150 mM) did not degrade disulfide E. Both 3 mM NaCl and 0.3 mM NaCl were used to make washed cell suspensions. The 0.3 mM NaCl washed cell suspensions degraded disulfide E slowly, and often accumulated benzoic acid rather than degrading the metabolite along

with the disulfide. The washed cell procedure using 0.3 mM NaCl was used to search for metabolites unless otherwise indicated.

3.2.7.2 Attempts to detect H_2S

Cripps (1973) detected H_2S by thin layer chromatography in aerobic cultures degrading thiophene-2-carboxylate. Cells growing and in a resting suspension metabolizing thiophene-2-carboxylate produced the characteristic odor of H_2S in that study. Despite the rapid degradation of disulfide E in washed cell suspensions of isolate RM6, no H_2S odor was detected. Initial experiments to detect H_2S were done using growing cultures of *Variovorax* sp. strain RM6 in SFMM with disulfide E as the sole carbon and sulfur source, followed by washed cell experiments to attempt to detect H_2S.

Cultures of *Variovorax* sp. strain RM6 with disulfide E were placed into the bottom of flasks with center wells. The wells were filled with a cadmium acetate solution that detected H_2S by the formation of a yellow precipitate. The flasks were sealed and monitored by observation for several days. No yellow color was observed. After 7 d, the cell suspensions had dropped to pH 3.5 and acetate was detected in the medium by GC analysis. Although some disulfide E degradation occurred in the cultures with cadmium acetate in the center wells (data not shown), likely some of the acetic acid in the cadmium acetate solution splashed into the medium and drastically dropped the pH of the cultures. No H_2S was detected.

A washed cell experiment was attempted using zinc metal. Initial tests showed that if pieces of zinc were exposed to a sulfide solution, the metal reacted with the sulfide and upon acidification, the presence of H_2S could be confirmed using the cadmium acetate solution. Pieces of zinc consistent in size and appearance were added to cell suspensions

of *Variovorax* sp. strain RM6 with disulfide E. If H_2S was produced, sulfide in the suspensions would react with the metal. The metal could be acidified and the headspace gas collected and bubbled into the cadmium acetate solution. Unfortunately, no degradation of disulfide E occurred in the presence of the zinc pieces. The presence of zinc inhibited the ability of the washed cells to degrade disulfide E. Choudhury and Srivastava (2001) reviewed zinc resistance in bacteria, noting that although zinc toxicity is generally low compared to Hg, Cd, Cu, Ni, Co, and Pb, it can inhibit electron transport in bacteria.

Experiments to detect H_2S, including olfactory detection, were not successful. The possibility that *Variovorax* sp. strain RM6 used reductive cleavage of the disulfide bond has not been ruled out by the lack of evidence for H_2S production. In this pathway (Figure 3.20), thiosalicylic acid would also be formed as an intermediate, but this compound was not detected by HPLC. The reactions may occur so quickly that detection of the intermediates prior to benzoic acid is impossible using the techniques applied here.

3.2.7.3 Washed cell experiments with disulfide E and the sulfitolysis product

Another potential pathway for disulfide E biodegradation by *Variovorax* sp. strain RM6 is the sulfitolysis of disulfide E (Figure 3.22). A solution of disulfide E and sulfite was incubated in a sealed serum bottle and samples were periodically removed for HPLC analysis. With loss of disulfide E, a new peak at 0.8 min was formed in the HPLC chromatogram. A peak at 2.6 min, consistent with the retention time and UV scan of thiosalicylic acid, was also formed. Analysis by HPLC-MS in the Department of Chemistry at the University of Alberta showed a peak at 9 min in the HPLC

chromatogram with a mass spectrum consistent with the sulfitolysis product 2-(S-sulfo)benzoic acid.

Washed cell suspensions of *Variovorax* sp. strain RM6 were incubated with 2-(S-sulfo)benzoic acid, disulfide E, or both 2-(S-sulfo)benzoic acid and disulfide E. Because the sulfitolysis product, 2-(S-sulfo)benzoic acid, is not commercially available, a standard curve could not be prepared and the peak area counts were reported in Figure 3.26 for all compounds. In one washed cell suspension, disulfide E was degraded by the isolate and benzoic acid was formed (Figure 3.26A). In a second suspension, 2-(S-sulfo)benzoic acid was added as a carbon and sulfur source, but no degradation was observed over 24 h (Figure 3.26B). The small amount of benzoic acid observed at time zero in Figure 3.26B is present because the commercial preparation of disulfide E, which contains some benzoic acid, was used to form the sulfitolysis product.

In a washed cell suspension with both disulfide E and 2-(S-sulfo)benzoic acid, only disulfide E was degraded over the 24 h (Figure 3.26C). Interestingly, the rate of disulfide E degradation was slower in the presence of the sulfitolysis product than in its absence (Figure 3.26A), and no benzoic acid was produced. The amount of the sulfitolysis product present in the washed cell suspension appeared to increase over the 24 h. However, it is unclear if this increase is due to disulfide E reacting with residual sulfite to produce more 2-(S-sulfo)benzoic acid, if the sulfitolysis product is produced as a metabolite, or if the increase is insignificant and would not be observed in a repeat of this experiment with triplicate washed cell suspensions. Although *Variovorax* sp. strain RM6 did not degrade 2-(S-sulfo)benzoic acid, the presence of the sulfitolysis product in washed cell suspensions did influence the biodegradation of disulfide E.

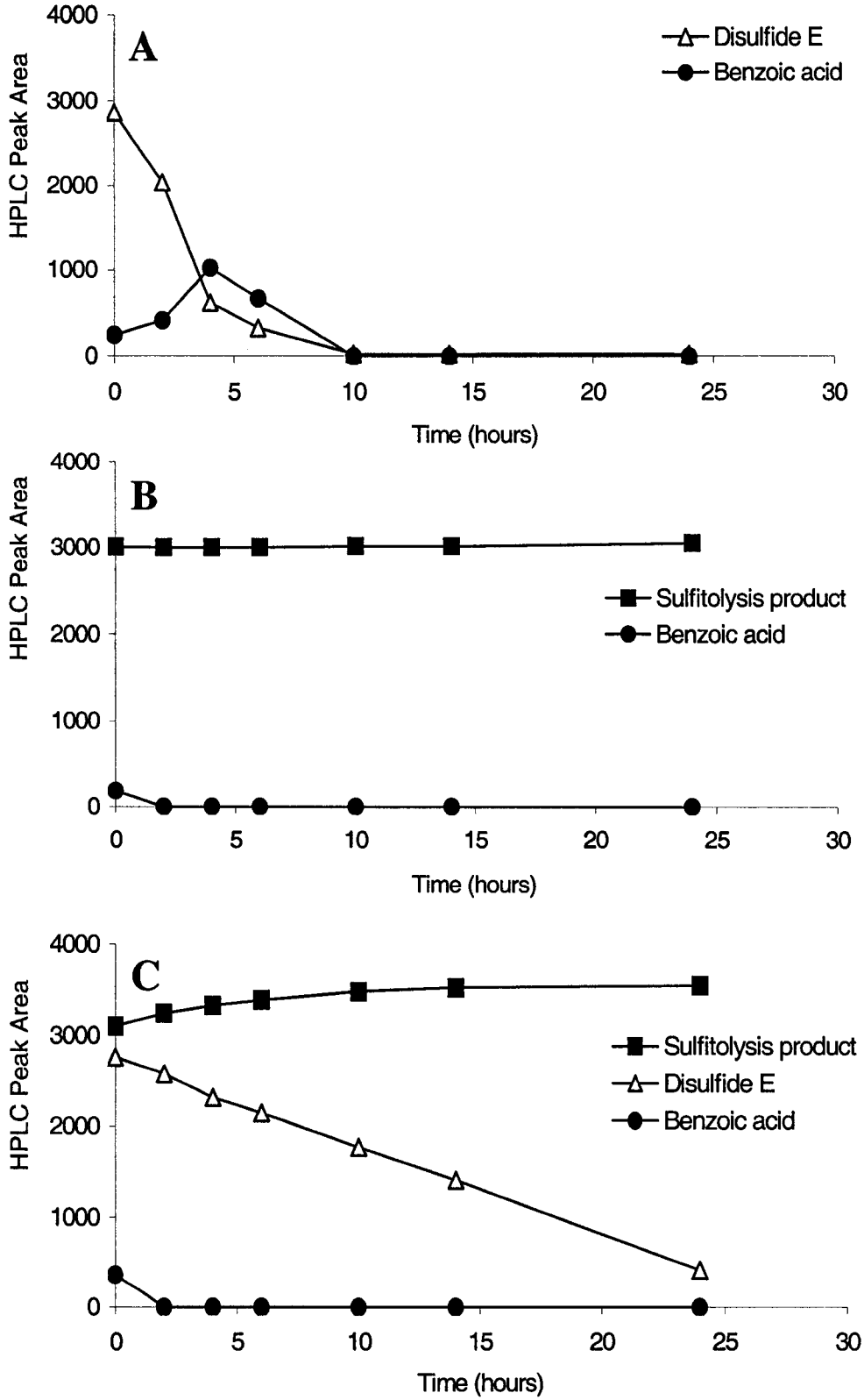


Figure 3.26 A single experiment with washed cells of isolate RM6 in 0.3 mM NaCl with disulfide E (A), the sulfitolysis product (B), or both disulfide E and the sulfitolysis product (C).

Although the washed cell sulfitolysis experiment was not repeated, it appeared that *Variovorax* sp. strain RM6 could not degrade 2-(S-sulfo)benzoic acid. This does not, however, eliminate the sulfitolysis pathway as a candidate for disulfide E degradation by *Variovorax* sp. strain RM6. 2-(S-Sulfo)benzoic acid could be formed inside the cell following sulfitolysis but may not be taken up from the external medium by *Variovorax* sp. strain RM6 for use as a carbon and sulfur source. If sulfitolysis occurs inside the cell, the product may be acted upon by intracellular enzymes. Extracellular enzymes would be washed away during the preparation of the washed cell suspensions, so disulfide E degradation by *Variovorax* sp. strain RM6 is likely an intracellular process once the disulfide, or a modified form of the disulfide, is taken up by the cells.

3.2.7.4 Washed cell experiments with disulfide E and 2-sulfobenzoic acid

Three washed cell experiments were performed with disulfide E and 2-sulfobenzoic acid to determine if 2-sulfobenzoic acid is an intermediate in the biodegradation of disulfide E by *Variovorax* sp. strain RM6. The first washed cell experiment with 2-sulfobenzoic acid examined the ability of *Variovorax* sp. strain RM6 washed cells grown on disulfide E to degrade 2-sulfobenzoic acid added to a washed cell suspension degrading disulfide E for 4 h. This washed cell experiment was carried out in phosphate buffer, in which disulfide E degradation occurs faster than in washed cell suspensions using 0.3 mM NaCl. The addition of 2-sulfobenzoic acid slowed the degradation of disulfide E, which was degraded in 24 h (Figure 3.27). The degradation of 2-sulfobenzoic acid lagged for 8 h, but was complete in 24 h. Benzoic acid was produced in the washed cell suspension (Figure 3.27). The lag observed when a disulfide E-degrading washed cell suspension was fed 2-sulfobenzoic acid suggested that the enzymes in the disulfide E

degrading pathway are not used to degrade 2-sulfobenzoic acid. Different enzymes specific for the biodegradation of 2-sulfobenzoic would have been synthesized during the 8 h lag. 2-Sulfobenzoic acid did not appear to be a metabolite of disulfide E biodegradation by *Variovorax* sp. strain RM6.

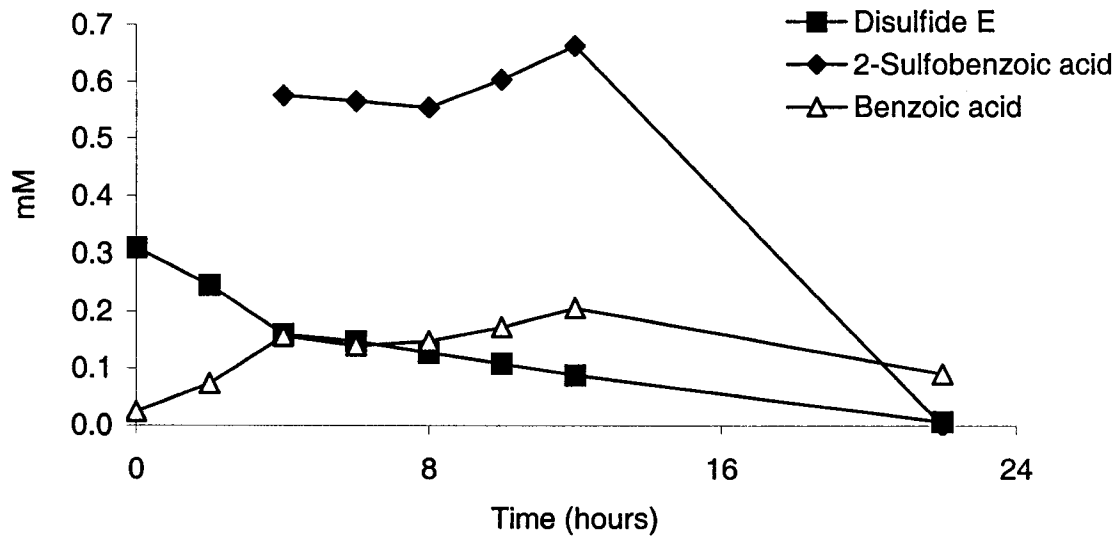


Figure 3.27 A single experiment with washed cells of isolate RM6 degrading disulfide E and producing benzoic acid, supplemented with 2-sulfobenzoic acid after 4 h.

A second washed cell experiment was attempted with 2-sulfobenzoic acid and *Variovorax* sp. strain RM6 grown on disulfide E. This experiment also included a positive control with disulfide E as the sole carbon and sulfur source in the SFMM, and a washed cell suspension with both 2-sulfobenzoic acid and disulfide E. The disulfide in the positive control was degraded in 70 h (Figure 3.28A). Over this time period, only half of the disulfide E was degraded in the cell suspension with both compounds, and the 2-sulfobenzoic acid was also only partially removed in this suspension (Figure 3.28B). 2-Sulfobenzoic acid was not significantly degraded by the washed cells of *Variovorax* sp. strain RM6 grown on disulfide E (Figure 3.28B). Although washed cells grown on disulfide E did not degrade 2-sulfobenzoic acid, suggesting that it is not a metabolite in

the pathway for disulfide E biodegradation, 2-sulfobenzoic acid was only degraded in the presence of disulfide E. The degradation of disulfide E stimulated the degradation of 2-sulfobenzoic acid. Benzoic acid was formed in all cell suspensions of *Variovorax* sp. strain RM6 degrading disulfide E (data not shown).

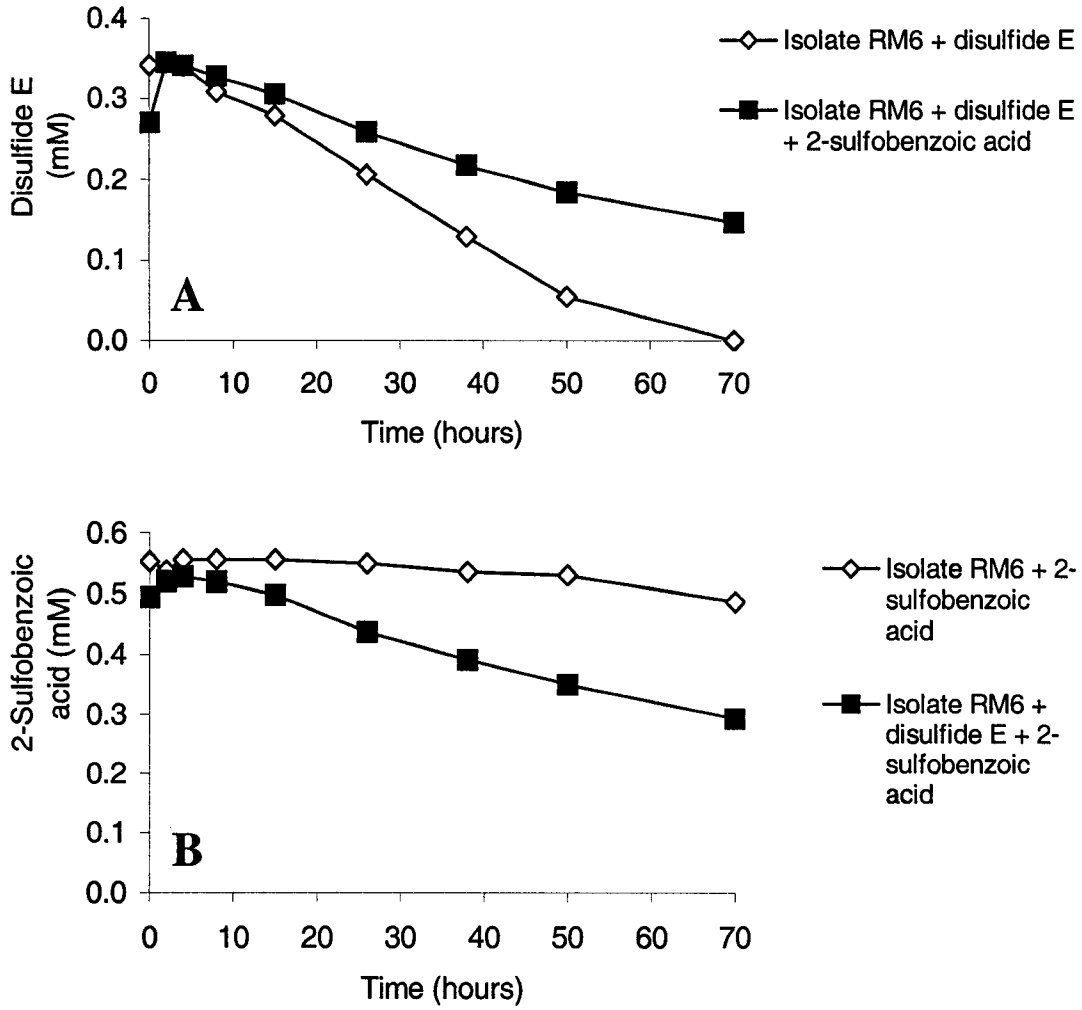


Figure 3.28 A single experiment with washed cells of isolate RM6 with disulfide E (A), 2-sulfobenzoic acid (B), or disulfide E and 2-sulfobenzoic acid.

In the third washed cell experiment, Triton X-100 was added to cell suspensions with 2-sulfobenzoic acid and disulfide E to permeabilize the membrane (Beller and Spormann 1997). *Variovorax* sp. strain RM6 washed cell suspensions were provided with disulfide

E, 2-sulfobenzoic acid, or disulfide E and 2-sulfobenzoic acid. Disulfide E was degraded slowly when Triton X-100 was present, and 2-sulfobenzoic acid was not degraded. Benzoic acid was formed in all suspensions of *Variovorax* sp. strain RM6 washed cells degrading disulfide E. No new peaks were observed in the HPLC chromatograms for cell suspensions degrading disulfide E in the presence of Triton X-100.

3.2.7.5 Washed cell experiments with various amounts of oxygen

In a preliminary experiment with an anaerobic washed cell suspension of *Variovorax* sp. strain RM6, no degradation of disulfide E was observed. This experiment suggested that the first step in disulfide E degradation might be oxygen-dependant. A washed cell experiment with reduced partial pressure amounts of oxygen was attempted, hoping that the limited amount of oxygen might allow the disulfide to be partially oxidized without further mineralization. Then, the oxidized metabolite might accumulate and could be detected by HPLC.

The washed cell suspension with 0 mL of oxygen added after flushing with N_2 likely contained some atmospheric oxygen because disulfide E was partially consumed (Figure 3.29A). No reducing agent was added to the washed cell suspensions of *Variovorax* sp. strain RM6. Disulfide E degradation in the washed cell suspensions with additional oxygen was related to the amount of oxygen supplied (Figure 3.29A). Benzoic acid was formed in the washed cell suspensions (Figure 3.29B). No new peaks were observed in the HPLC chromatograms for the washed cell suspensions with various amounts of oxygen.

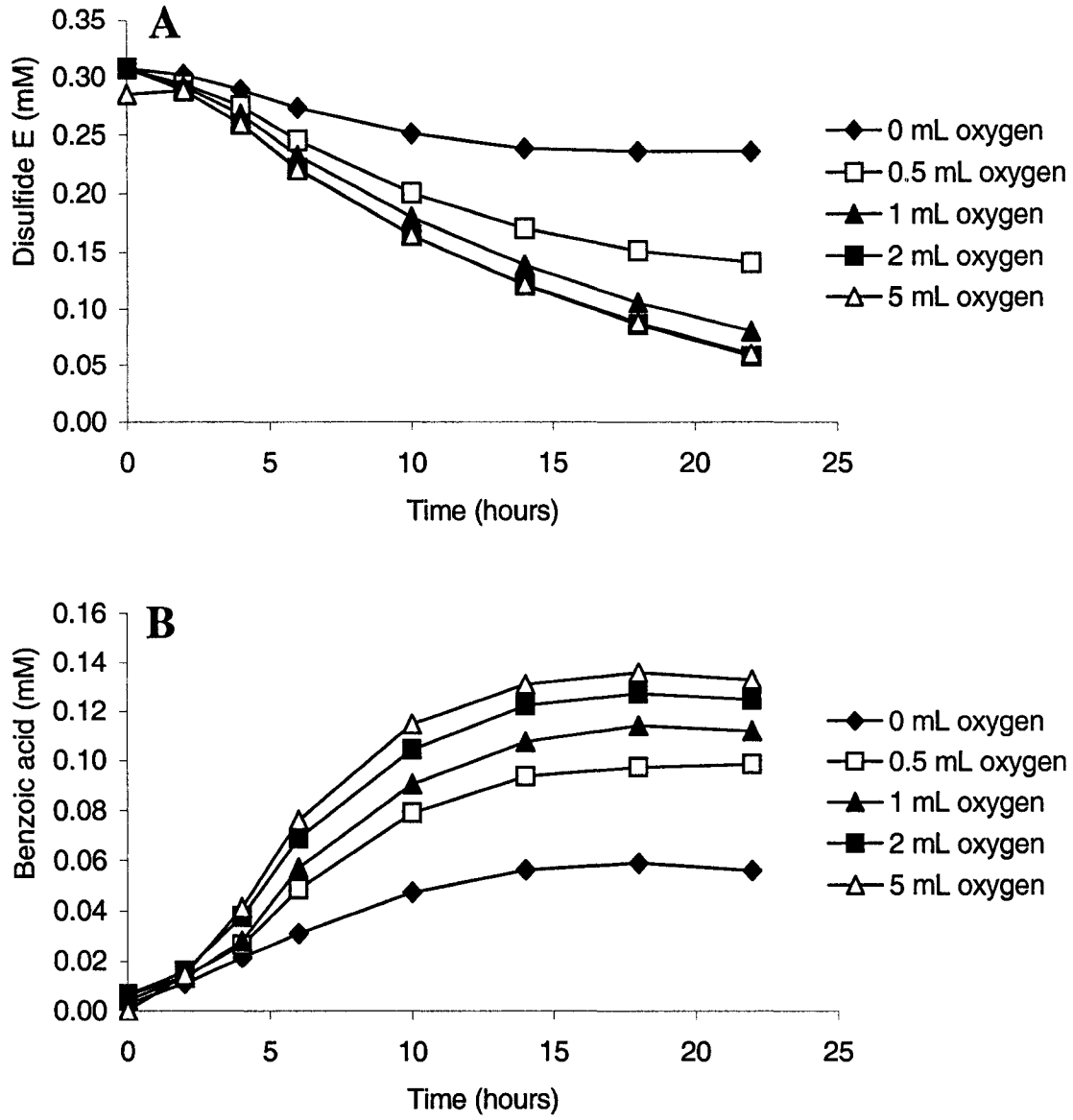


Figure 3.29 A single experiment with isolate RM6 washed cells flushed with N_2 and incubated with various amounts of oxygen, degrading disulfide E (A) and producing benzoic acid (B).

3.2.8 Degradation of other substrates by *Variovorax* sp. strain RM6

Studying the degradation of other substrates by *Variovorax* sp. strain RM6 helped to not only characterize the bacterium, but to also establish which types of enzymatic reactions could be possible. *Variovorax* sp. strain RM6 contains a limited suite of

enzymes, the functions of which may overlap, giving clues about the attack on disulfide E by this bacterium and the pathway used to degrade the disulfide.

In the reductive pathway the disulfide bond is reduced and a thiol, thiosalicylic acid, is formed (Figure 3.20). 2-Mercaptobenzothiazole, a condensed thiophene with a nitrogen atom in the 5-membered ring and a thiol group, formed two metabolites when attacked by *Rhodococcus rhodochrous* (Haroune *et al.* 2004). The first metabolite was a *cis*-dihydrodiol derivative of 2-mercaptobenzothiazole that would result from the activity of a hydroxylating dioxygenase. The second metabolite resulted from a hydroxylation of the aromatic ring at position 6, likely from the action of a monooxygenase. *R. rhodochrous* is a Gram positive organism and very different from *Variovorax* sp. strain RM6. However, another strain of *V. paradoxus* degraded homovanillate using a dioxygenase and aromatic ring hydroxylation (Allison *et al.* 1995), so *Variovorax* sp. strain RM6 may possess similar hydroxylating enzymes.

Catechol and salicylic acid have hydroxyl groups, and may be degraded by *Variovorax* sp. strain RM6 if similar compounds are formed during the biodegradation of disulfide E. The attack on other disulfides by *Variovorax* sp. strain RM6 may also help to determine which pathway is used by this bacterium to degrade disulfide E.

3.2.8.1 Naphthalene, catechol, and salicylic acid as sole carbon sources in SFMM with sulfate

Naphthalene was provided as a sole carbon source in SFMM with sulfate, and *Variovorax* sp. strain RM6 did not grown in the medium. Turbidity is a poor indicator for the growth of *Variovorax* sp. strain RM6, because this organism often grows in flocs. Minimal medium plates were prepared with sulfate and naphthalene crystals were added to the dishes as the sole carbon source. *Variovorax* sp. strain RM6 was streaked onto

plates, and *P. fluorescens* was used as a positive control. *Variovorax* sp. strain RM6 did not grow on plates with naphthalene as the sole carbon source, and *P. fluorescens* grew as predicted. Therefore, *Variovorax* sp. strain RM6 could not grow on naphthalene vapors.

Variovorax sp. strain RM6 was added to SFMM with catechol and 2 mM sulfate, and to SFMM with catechol and disulfide E. Catechol was detected using the same HPLC method as disulfide E. *Variovorax* sp. strain RM6 did not degrade catechol compared to the sterile control, in the presence or absence of disulfide E. Catechol is extremely light sensitive, and melanized over time when exposed to minimal light. Using procedures to minimize light exposure, the catechol remained in the test cultures and sterile control long enough to determine that *Variovorax* sp. strain RM6 did not degrade this compound.

Variovorax sp. strain RM6 was inoculated into SFMM with salicylic acid and 2 mM sulfate, with salicylic acid, 2 mM sulfate, and benzoic acid, and with salicylic acid and disulfide E. The salicylic acid was removed in all cultures after 2 d of incubation. *Variovorax* sp. strain RM6 was capable of using salicylic acid as the sole carbon source with 2 mM sulfate, with benzoic acid in the medium, and with disulfide E as the sole sulfur source. Benzoic acid and disulfide E were degraded in 3 d. Hong *et al.* (2004) found that their isolate, *Pseudomonas veronii* PH-03, could use both catechol and salicylic acid as sole carbon sources. Benzoic acid was not detected in the HPLC chromatograms of *Variovorax* sp. strain RM6 degrading salicylic acid. Salicylic acid was degraded by the bacterium, but may not be an intermediate in disulfide E biodegradation.

3.2.8.2 Other disulfides as sole carbon and sulfur sources in SFMM

Glutathione disulfide, cysteine, and cystine were added to SFMM with *Variovorax* sp. strain RM6 as sole carbon and sulfur sources. *Variovorax* sp. strain RM6 did not grow in

any cultures with these disulfides. Other disulfides that were not commercially available were also investigated. Using thiosalicylic acid and 4-mercaptobenzoic acid incubating in SFMM, three disulfides were formed abiotically in the medium: disulfide E, 2,4'-dithiosalicylic acid, and 4,4'-dithiosalicylic acid (Figure 3.30).

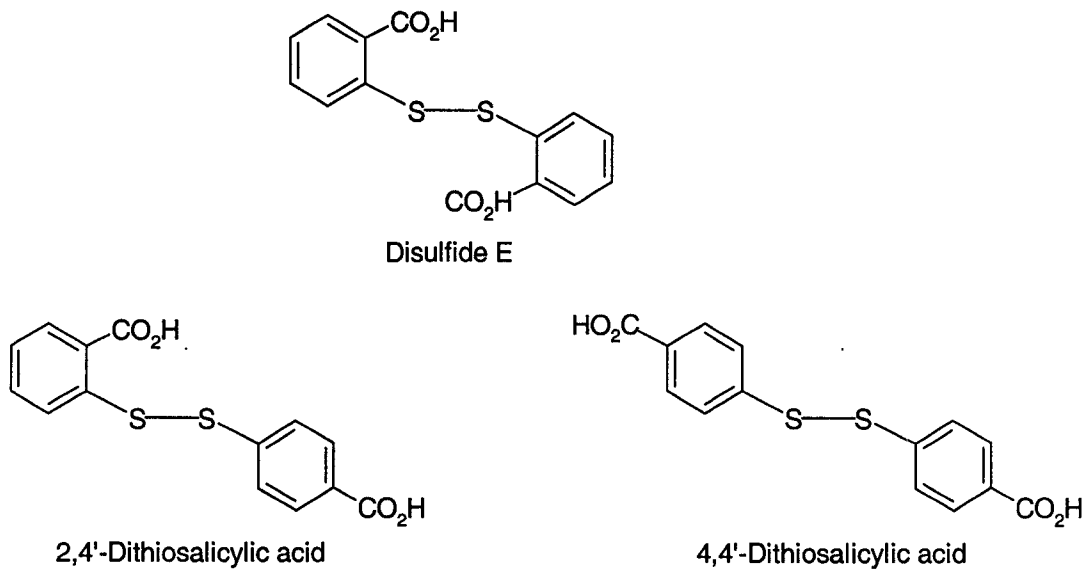


Figure 3.30 Chemical structures of disulfide E, 2,4'-dithiosalicylic acid, and 4,4'-dithiosalicylic acid.

All of the disulfides in Figure 3.30 were detected by the HPLC method. 4,4'-Dithiosalicylic acid was solely formed in SFMM from 4-mercaptobenzoic acid dimerization and used as a comparison to the compound formed in the mixture of thiosalicylic acid and 4-mercaptobenzoic acid. Presumably, the third peak formed in the mixture was 2,4'-dithiosalicylic acid. *Variovorax* sp. strain RM6 degraded disulfide E only, and did not attack 2,4'-dithiosalicylic acid or 4,4'-dithiosalicylic acid. This was surprising, because the two disulfides are structural isomers of disulfide E. If the reductive pathway occurs, and the disulfide bond is first reduced to form the thiol, disulfide E and 2,4'-dithiosalicylic acid would both form thiosalicylic acid as an

intermediate. Both of these compounds would show loss by HPLC if the disulfide bond was reduced by *Variovorax* sp. strain RM6 and thiosalicylic acid was further degraded.

Thiosalicylic acid was also combined with mercaptoacetic acid to form another mixture of disulfides (Figure 3.31). The "mixed disulfide" was detected using the same HPLC method used to determine the concentration of disulfide E and benzoic acid. Both disulfide E and the "mixed disulfide" were formed in SFMM and *Variovorax* sp. strain RM6 was inoculated from a plate wash. *Variovorax* sp. strain RM6 degraded the "mixed disulfide" first, in 10 d, and then quickly degraded disulfide E over the next 2 d (Figure 3.32). No new metabolite peaks were observed in the HPLC chromatograms during the degradation of the "mixed disulfide." Benzoic acid was not detected in the culture. As a transient metabolite of disulfide E biodegradation, benzoic acid may have been formed in the culture between sampling points and quickly degraded. Attempts were made to separate the "mixed disulfide" from disulfide E in SFMM, to determine if benzoic acid is produced as a metabolite during the biodegrading of the "mixed disulfide" by *Variovorax* sp. strain RM6, but they were not successful.

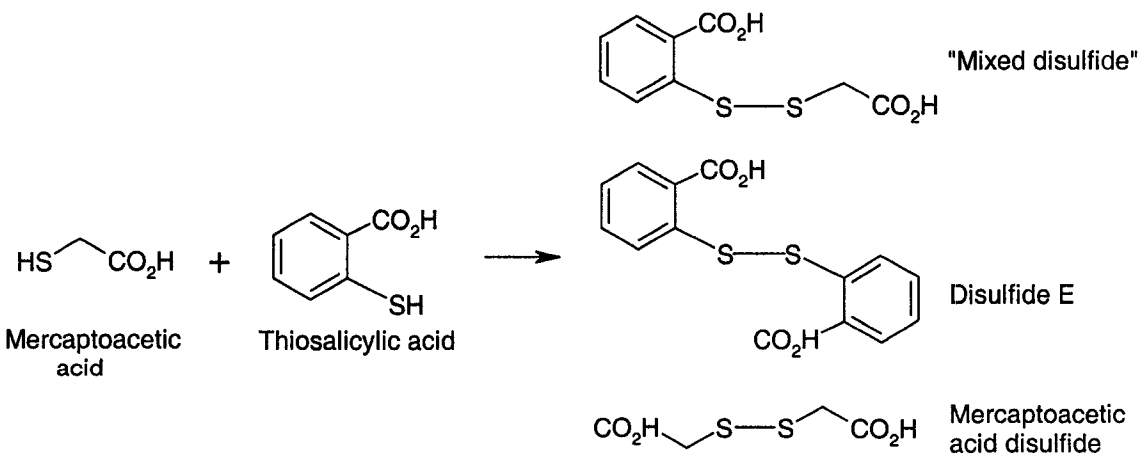


Figure 3.31 The chemical structures of disulfide E, the mercaptoacetic acid and thiosalicylic acid "mixed disulfide", and mercaptoacetic acid disulfide.

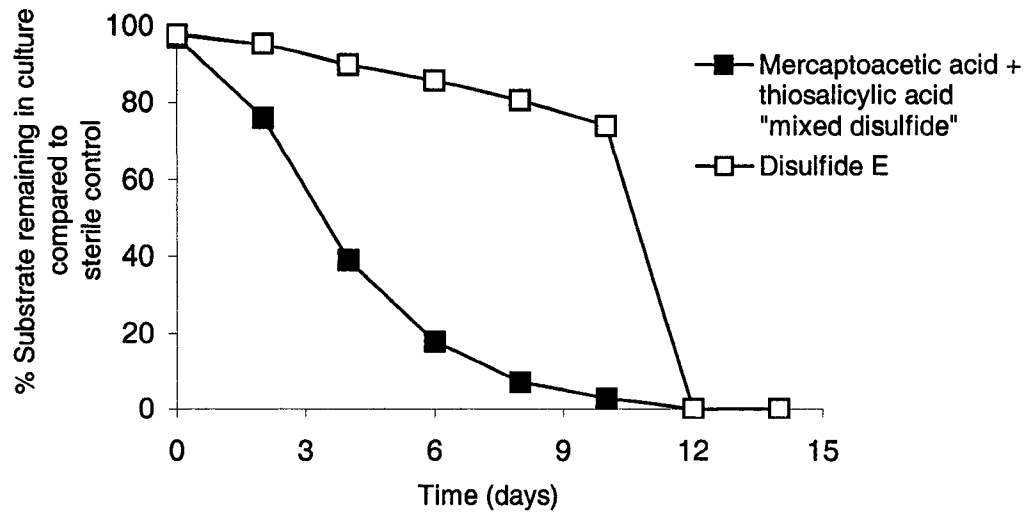


Figure 3.32 Substrate consumption in a culture of isolate RM6 incubated with both the mercaptoacetic and thiosalicylic acid "mixed disulfide" and disulfide E.

Disulfide C was also provided as a sole carbon and sulfur source for *Variovorax* sp. strain RM6 in SFMM. *Variovorax* sp. strain RM6 did not grow in the SFMM with vitamins and disulfide C. *Variovorax* sp. strain RM6 was inoculated into SFMM with acetate and disulfide C as the sole sulfur source only. Isolate RM6 could not use disulfide C as a sole sulfur source or as a carbon and sulfur source. This was also unexpected, because disulfides C and E are very similar.

The degradation of disulfide E by *Variovorax* sp. strain RM6 was studied in the presence of disulfide C. The bacterium was inoculated into SFMM with 100 mg disulfide E/L and various amounts of disulfide C. Disulfide E was degraded in less than 10 d in all of the cultures, seemingly independent of the concentration of disulfide C (Figure 3.33A). Disulfide C did not appear to be toxic to *Variovorax* sp. strain RM6 at the concentrations used in this experiment, or at the concentrations used in the disulfide C enrichment cultures (section 3.1.1). *Variovorax* sp. strain RM6 did not degrade disulfide C in any of the cultures after 25 d (Figure 3.33B). The degradation of disulfide E by *Variovorax* sp. strain RM6 must be very specific. The microorganisms indigenous to environments

exposed to petroleum would likely be exposed to disulfide C first, and clearly disulfide C is biodegradable by microorganisms found in soils from marigold gardens (Figure 3.1).

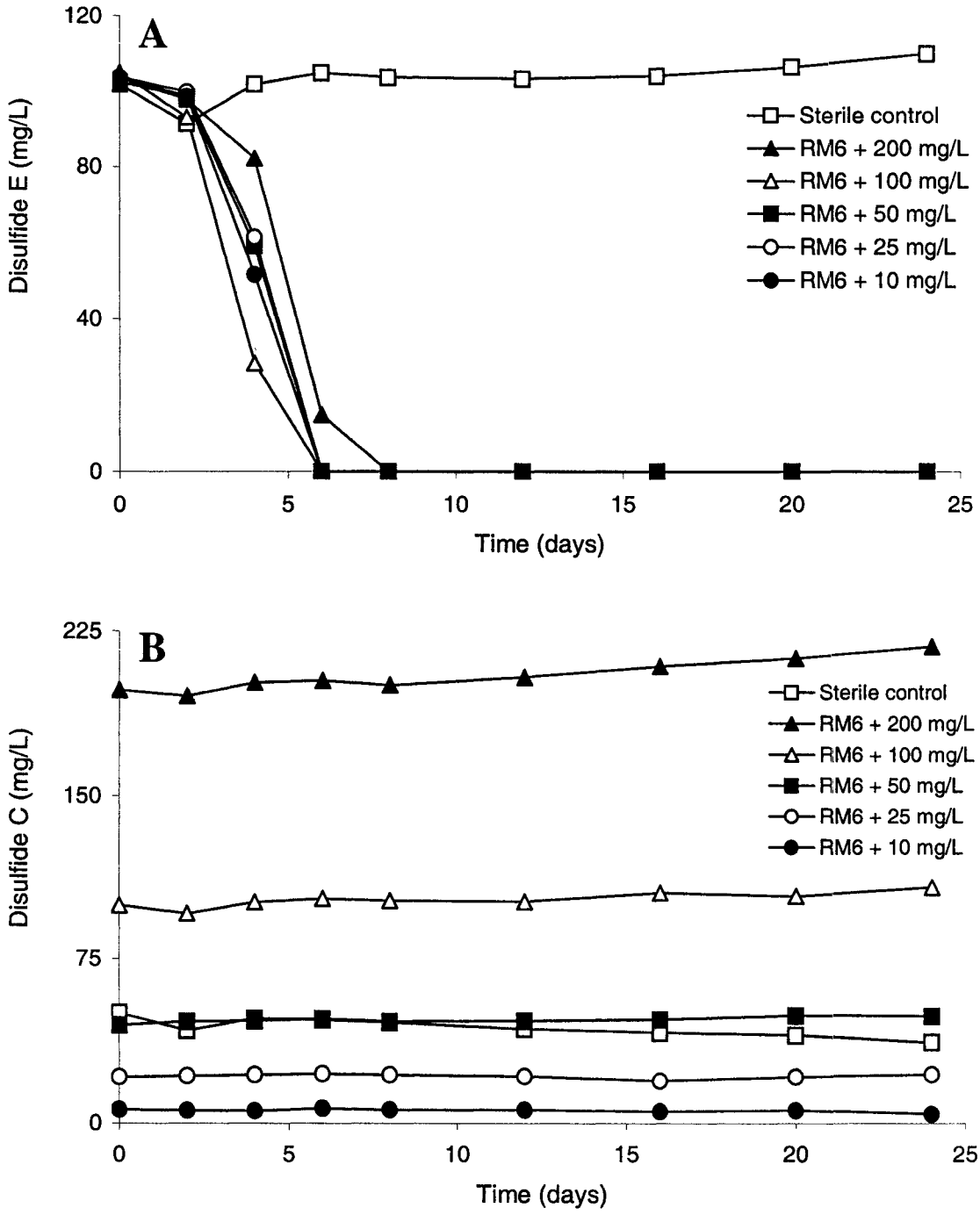


Figure 3.33 Concentrations of disulfide E (A) and disulfide C (B) in cultures of isolate RM6 with various amounts of disulfide C.

Disulfide C slowly converts to disulfide E at neutral pH (Bressler 2001). In the environment, if disulfide C is relatively recalcitrant, the biodegradation of disulfide E by a community of microorganisms would accomplish the final steps in the mineralization of DBT. Not only is a group of microorganisms necessary to carry out different steps in the mineralization of DBT, but other partners in the community may provide essential growth factors to key organisms. The mineralization of DBT would also rely upon the environmental conditions and abiotic reactions. The biodegradation of disulfide E by bacteria might enhance the conversion of disulfide C to disulfides D and E, implying that the rate limiting step for *in situ* DBT mineralization may be abiotic. The pathway for disulfide E biodegradation by *Variovorax* sp. strain RM6 required further investigation.

3.2.9 Cell-free extract experiments

The use of cell-free extracts has enabled many researchers to study the biodegradation of a variety of compounds. Cell-free extracts are prepared by harvesting growing cells, breaking them open, and centrifuging the sample to obtain a supernatant free of cell debris and whole cells but with the enzyme activity of the original culture. The enzyme extracts are used in assays to degrade substrates and detect metabolites.

Chang *et al.* (2001) used cell-free extracts from *Clostridium bifermentans* DPH-1 to study the degradation of tetrachloroethylene, where the enzyme extracts catalyzed dechlorination. Rhee and Fuchs (1999) prepared cell-free extracts of *Thauera aromatica*, a denitrifying bacterium, to study the anaerobic metabolism of phenylalanine. To help determine the pathway for nitrobenzene degradation by *Comamonas* sp. strain JS765, Nishino and Spain (1995) also used cell-free extracts. Enzyme extracts of strain JS765 catalyzed the oxygen-dependant degradation of catechol. The enzyme extracts also

showed catechol-2,3-dioxygenase activity, which allowed the authors to predict *meta* ring cleavage of catechol during nitrobenzene biodegradation. In their studies of homovanillate biodegradation by *V. paradoxus* VP-5, Allison *et al.* (1995) prepared cell-free extracts. The procedure used to liberate enzymes from isolate VP-5 was attempted for *Variovorax* sp. strain RM6, although dithiothreitol, a compound which disrupts disulfide bonds, was not added.

In a preliminary experiment, *Variovorax* sp. strain RM6 cells were sonicated, the suspensions were centrifuged, NADH was added to the supernatant, and the reaction mixture was monitored for disulfide E degradation. Disulfide E degradation was not observed. Microscopic observation found that the pellet contained whole cells, and that the sonication treatment had not broken open the cells. The sonication was repeated for *Variovorax* sp. strain RM6 cells grown on disulfide E and followed by bead beating. The suspension was centrifuged and both the pellet and supernatant, with and without NADH, were monitored for disulfide E degradation. Only the resuspended pellet with NADH degraded disulfide E (Figure 3.34A). Benzoic acid, present at time zero from the commercial preparation of the disulfide, was also observed in the pellet suspension with NADH, and disappeared in several of the test suspensions (Figure 3.34B). Microscopic observation revealed both intact cells and cell debris. The possibility that the disulfide-degrading activity was membrane-associated was considered, but the presence of whole cells in the active pellet preparation suggested that the lysis method was unsuccessful. Sonication was not a very efficient method for breaking *Variovorax* sp. strain RM6 cells, and the cell-free extract experiment was repeated using another cell lysis method.

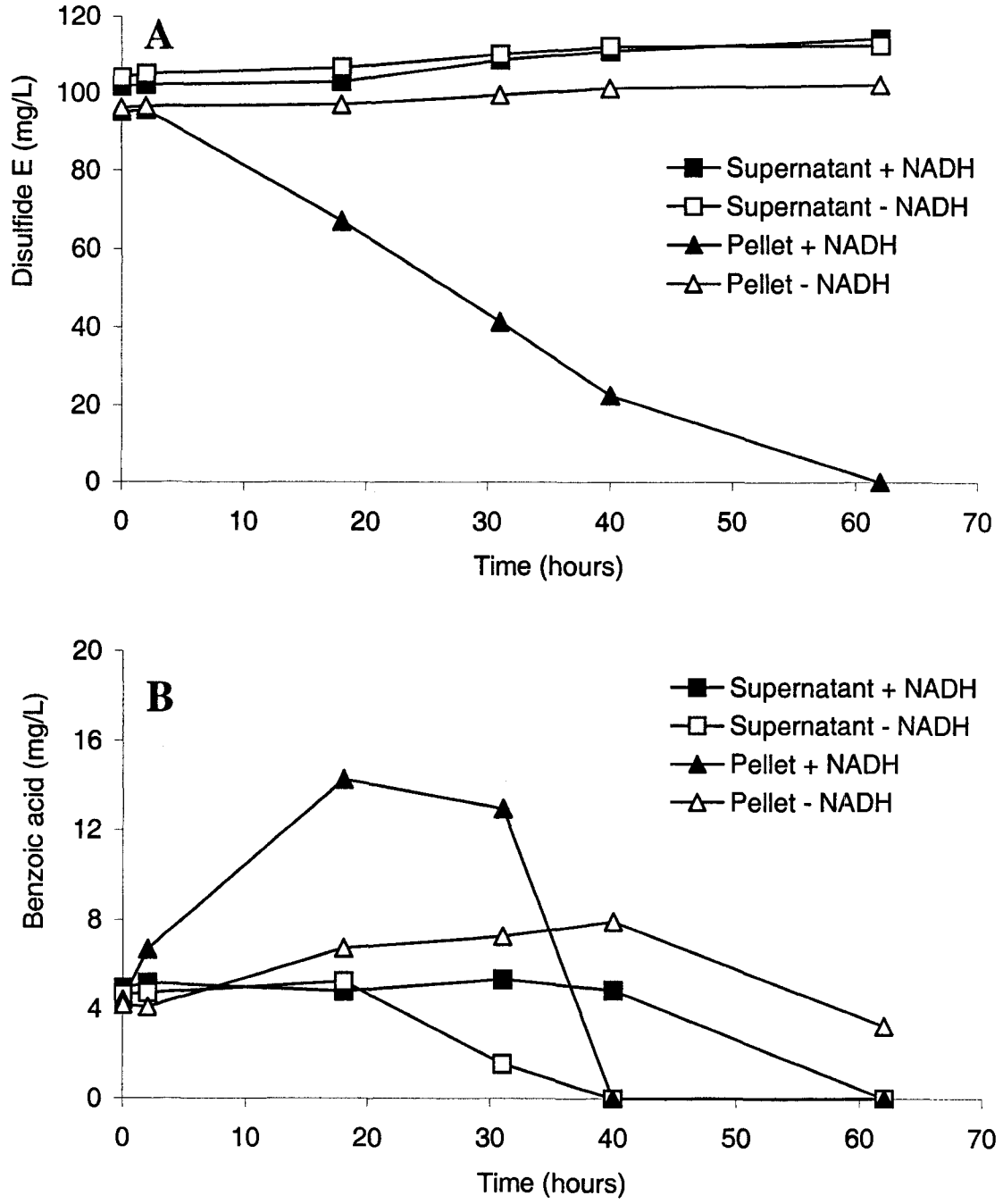


Figure 3.34 Cell-free extract supernatants and pellets with disulfide E (A). Benzoic acid was formed in the reaction mixture degrading disulfide E (B).

Noh *et al.* (2002) used cell-free extracts prepared using a french pressure cell and did not add dithiotheitol to prevent enzyme degradation. The cell-free extracts of *Rhodopseudomonas palustris*, prepared using the french pressure cell, transformed phenol to 4-hydroxyphenylacetate. Washed cells of isolate RM6 were run through a french pressure cell, centrifuged, and the supernatant and pellet tested for disulfide E biodegradation. No disulfide degradation was observed. The attempts to use cell-free extract procedures to study disulfide E degradation by *Variovorax* sp. strain RM6 were not successful.

Further studies to detect intermediates in disulfide E degradation by *Variovorax* sp. strain RM6 might rely on molecular techniques. New research on the biodegradation of DBT through the Kodama pathway involved the use of transposon mutagenensis. Di Gregorio *et al.* (2004) studied mutants of *Burkholderia* sp. DBT1 and identified two new sets of genes for DBT transformation. Foght and Westlake (1996) generated mutants of *P. fluorescens* strain LP6a, an isolate capable of using several PAHs as sole carbon and energy sources, to study the transformation of PAHs by the degradative enzymes. Transposon-induced mutations of *Pseudomonas putida* BS394(pBS216) were used to study naphthalene and salicylic acid biodegradation at low temperatures (Grishchenkov *et al.* 2004). Jang *et al.* (2004) reported the first use of molecular techniques to study dye-decolorization by bacteria. They generated mutants of *Citrobacter* sp. using transposon insertion and found seven mutants defective in malachite green decolorization. Generating random mutants of *Variovorax* sp. strain RM6 could produce strains deficient in stages of disulfide E biodegradation, producing intermediates that might be detected by HPLC, HPLC-MS, or GC-MS.

3.2.10 Mineralization experiments

Although the pathway for disulfide E biodegradation could not be resolved, benzoic acid was determined to be an intermediate and three potential pathways were proposed. For DBT mineralization to be possible by a mixed culture, disulfide E must not only be attacked by the bacterium, but mineralized to carbon dioxide and sulfate. The pathway used to get to these final inorganic compounds, although of considerable interest and mystery, is secondary to determining if the disulfide is actually mineralized by *Variovorax* sp. strain RM6.

3.2.10.1 Mineralization of disulfide E by isolate RM6 in SFMM

Mineralization was first attempted in SFMM with disulfide E as the sole added carbon and sulfur source. Similar to the carbon and sulfur balance experiment performed by Bressler *et al.* (1999), a culture of *Variovorax* sp. strain RM6 degrading disulfide E was used as the inoculum for cultures in serum bottles prepared with SFMM and disulfide E. Serum bottle cultures without disulfide E were also prepared to account for carbon dioxide and sulfate carried over from the inoculum.

Disulfide E was mineralized by *Variovorax* sp. strain RM6 in SFMM releasing, on average over the last 4 d of incubation, 59% of the carbon as carbon dioxide and 88% of the sulfur as sulfate (Figure 3.35). Aerobic cultures generally release approximately 50% of the substrate carbon as carbon dioxide, assimilating the rest of the carbon for biomass production (Maier *et al.* 2000). Bressler *et al.* (1999) found that *Pseudonocardia* sp. strain DB1 biodegraded benzothiophene sulfone and 3-methylbenzothiophene sulfone, releasing 57 and 62% of the carbon as carbon dioxide, respectively. Bressler and Fedorak (2001a) studied an enrichment culture for the ability to mineralize HFBT and found that

43% of the carbon was released as carbon dioxide. *Variovorax* sp. strain RM6 mineralized disulfide E, releasing a similar amount (59%) of carbon dioxide compared to the biodegradation of other organosulfur compounds. The high amount of sulfur released as sulfate (88%) suggests that the disulfide was completely mineralized, rather than accumulating a sulfur-containing intermediate that was not detected by HPLC. A mineralization experiment with the sulfitolysis product showed that *Variovorax* sp. strain RM6 does not appear to release carbon from 2-(S-sulfo)benzoic acid compared to its mineralization of disulfide E (data not shown).

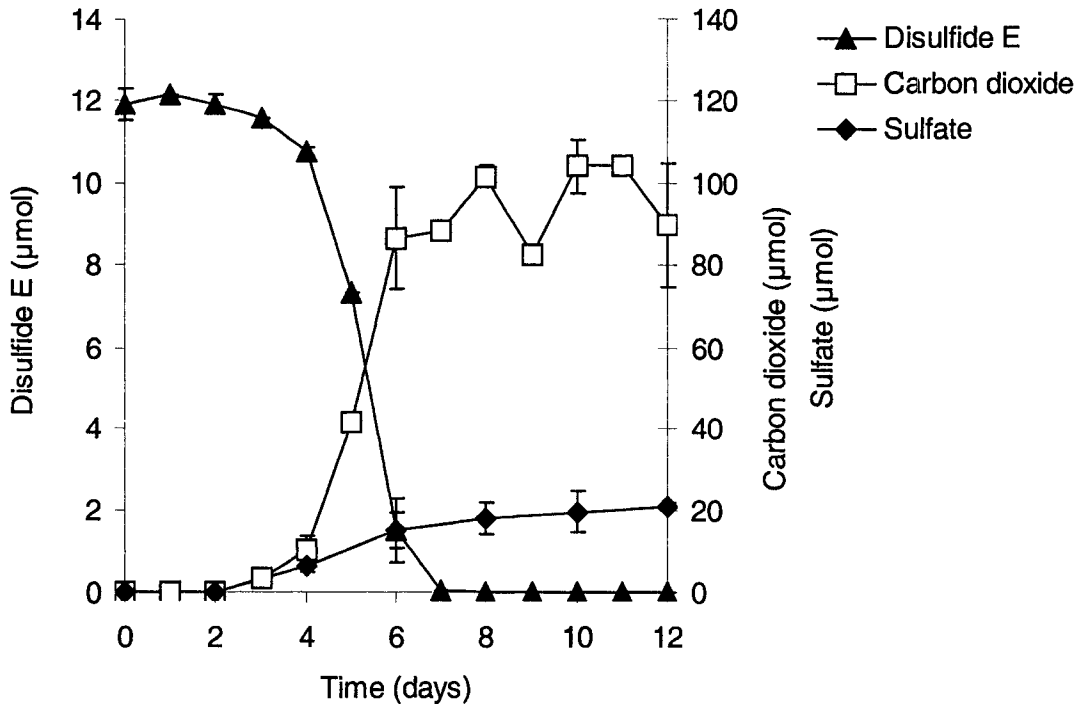


Figure 3.35 Mineralization of disulfide E by isolate RM6 in SFMM. The data points represent means of triplicate cultures and the error bars are one standard deviation from the mean. Some error bars are smaller than the symbols.

3.2.10.2 Mineralization of disulfide E by washed cells of isolate RM6

The washed cell suspensions of *Variovorax* sp. strain RM6 in 0.3 mM NaCl also mineralized disulfide E, releasing 83% of the carbon as carbon dioxide and 74% of the sulfur as sulfate (Figure 3.36). The increased carbon dioxide was predicted from the

washed cells compared to the growing cells, because in a phosphate-free suspension the cells are not growing or assimilating carbon. However, less sulfate was observed from the washed cells mineralizing disulfide E than in the growing culture. The high percent of carbon dioxide produced by the washed cells of *Variovorax* sp. strain RM6 degrading disulfide E suggests that mineralization did occur. A repeat of this experiment would be necessary to determine if the lower yield of sulfate is reproducible.

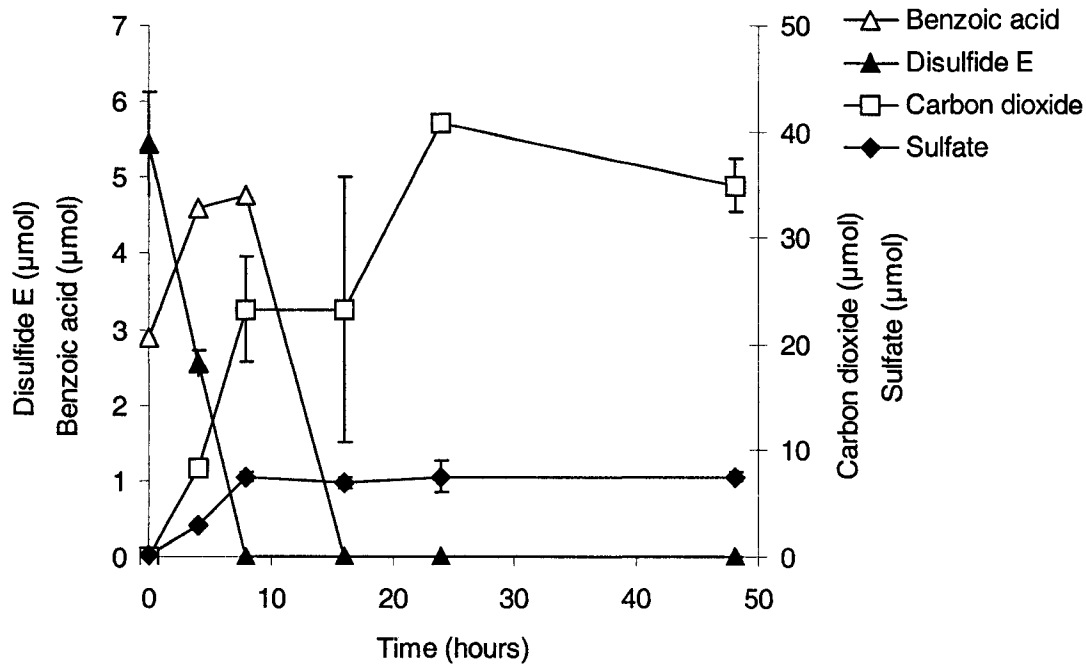


Figure 3.36 Mineralization of disulfide E by isolate RM6 washed cells in 0.3 mM NaCl. The data points represent means of triplicate cultures and the error bars are one standard deviation from the mean. Some error bars are smaller than the symbols.

3.3 COMBINING ISOLATES BT1d AND RM6 IN SFMM WITH VITAMINS

The carbon and sulfur balance experiments with both the *Variovorax* sp. strain RM6 culture and the washed cell suspension gave convincing evidence that this bacterium mineralized disulfide E. A goal of this project was to isolate and identify a microorganism capable of degrading disulfides formed from DBT biodegradation

through the Kodama pathway. *Variovorax* sp. strain RM6 mineralized disulfide E, the last disulfide formed during DBT biodegradation by *Pseudomonas* strain BT1d. Another goal of this project was to combine the disulfide-degrading isolate with *Pseudomonas* sp. strain BT1d to determine if DBT could be mineralized by this mixed culture.

Variovorax sp. strain RM6 was added to SFMM with isolate BT1d and DBT was added as the sole carbon and sulfur source. If all of the DBT sulfur was released as sulfate, 1.4 mM sulfate would be observed in the mixed culture. No significant amount of sulfate was observed in the mixed culture compared to the pure culture of isolate BT1d over 220 d (Figure 3.37). The apparent increase in sulfate observed over time is likely due to the increased absorbance of colored products formed when isolate BT1d degraded DBT.

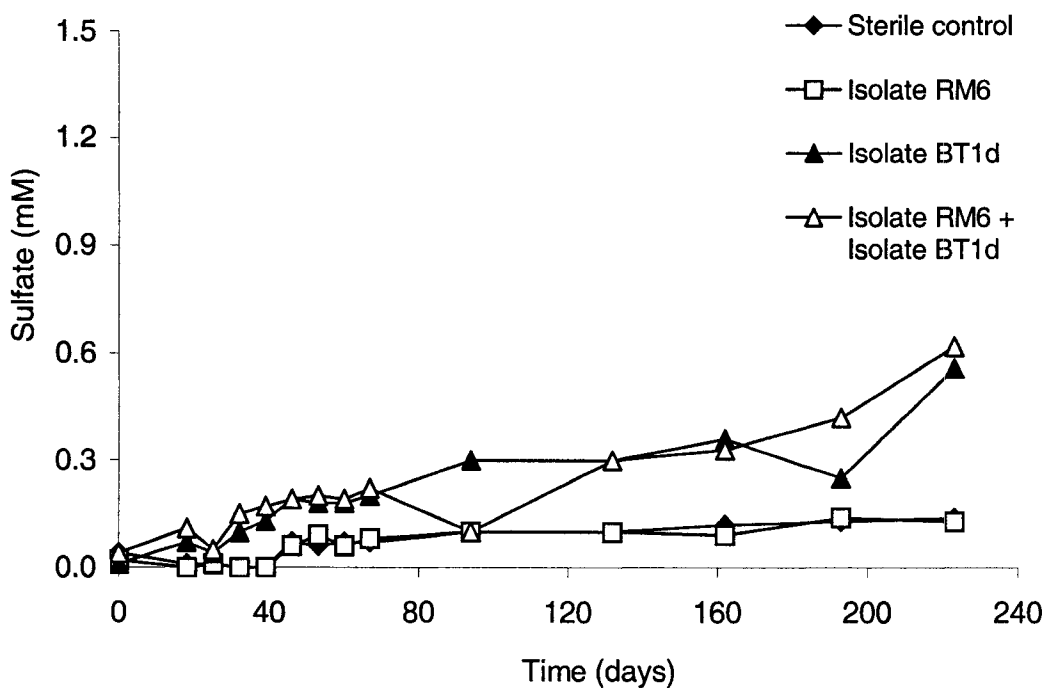


Figure 3.37 Isolates RM6 and BT1d in SFMM with DBT as the sole C and S source. Sulfate analysis was done using the turbidimetric method with extraction to remove some of the colored products formed when isolate BT1d degraded DBT.

Mineralization of dodecyldimethylamine was accomplished using a two-membered bacterial culture. One member of the culture degraded the alkyl chain to form dimethylamine, which was degraded by the second isolate (Kroon and van Ginkel 2001). Similarly, *Pseudomonas* sp. strain BT1d degrading DBT should produce disulfides in the mixed culture, but *Variovorax* sp. strain RM6 did not appear to degrade disulfide E to sulfate in the preliminary test. The cultures were occasionally streaked onto PCA plates, and after 180 d both isolates BT1d and RM6 were still observed on plates from the mixed culture. Although *Variovorax* sp. strain RM6 grew on plates when the mixed culture was streaked, the bacterium may have recovered on the rich medium and after 220 d in the mixed culture it was no longer metabolically active, but simply persisting. HPLC analysis confirmed that disulfides C, D, and E were present in the mixed culture supernatant after 220 d.

Variovorax sp. strain RM6 has not been inoculated into mixed culture with *Pseudomonas* sp. strain BT1d before, and future experiments are required to investigate their relationship. *Pseudomonas* sp. strain BT1d could produce compounds that inhibit *Variovorax* sp. strain RM6. Also, although vitamins were added to the medium, it is unknown if *Pseudomonas* sp. strain BT1d consumes or produces vitamin B_{12}. Because isolate BT1d does not require vitamin supplementation, it likely synthesizes vitamin B_{12}. However, if the vitamin was provided in the medium, *Pseudomonas* sp. strain BT1d may have used the vitamin B_{12} provided and limited the supply available to *Variovorax* sp. strain RM6. Cultures of *Pseudomonas* sp. strain BT1d degrading DBT should be inoculated with *Variovorax* sp. strain RM6 and additional disulfide E should be added. Samples could be taken from this mixed culture for sulfate and HPLC analysis to confirm

that *Variovorax* sp. strain RM6 mineralizes disulfide E under the mixed culture conditions.

During the mineralization of molinate, a thiocarbamate herbicide, a disulfide is produced that cannot be attacked by the bacterium that began the degradation of molinate (Barreiros *et al.* 2003). Other isolates in the mixed culture degraded diethyl disulfide, which is produced by the molinate degrader and is toxic at high concentrations. Disulfide C, which is poorly degraded by bacteria compared to disulfide E, may play a similar role in the environment, limiting the mineralization of DBT. Mineralization of DBT in petroleum-contaminated environments could be dependant upon the formation of disulfide E.

Disulfide E is relatively easy for soil enrichments to degrade. However, in this study a community effort was required, where one isolate produced vitamin B_{12} for the disulfide degrader. Benzoic acid was found to be an intermediate in disulfide E degradation by *Variovorax* sp. strain RM6, but the full pathway, and specifically the cleavage of the disulfide bond, remains unsolved. Disulfide E was mineralized by *Variovorax* sp. strain RM6, but further experiments are required to show DBT mineralization by a mixed culture of isolates BT1d and RM6, or some other isolate that can degrade the disulfides formed from DBT.

4. CONCLUSIONS

DBT is studied as the model sulfur heterocycle in petroleum. The biodegradation of DBT through the Kodama pathway by *Pseudomonas* sp. strain BT1d produces three large disulfides: disulfide C, 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide; disulfide D, 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide; and disulfide E, 2,2'-dithiosalicylic acid. These disulfides are likely produced in petroleum-contaminated environments where sulfate is present and the Kodama pathway likely dominates. Disulfides C, D, and E can be detected using a convenient HPLC method. The goals of this project were to isolate and identify bacteria capable of degrading disulfides formed from DBT biodegradation, and to combine the disulfide degraders with isolate BT1d to obtain a mixed culture capable of mineralizing DBT. There have been no reports of DBT mineralization by bacteria degrading DBT through the Kodama pathway.

4.1 BIODEGRADATION OF DISULFIDE C

Disulfide C, the first disulfide formed from the biodegradation of DBT by *Pseudomonas* strain BT1d, was added to medium as a carbon and sulfur source. Microorganisms in soils from marigold gardens were capable of degrading disulfide C when first inoculated into the medium, but they lost this ability upon transfer to fresh medium. The ability of soil cultures to degrade disulfide C could not be maintained using a marigold soil or root extract, or by the addition of yeast extract, brain heart infusion, or a vitamin mixture. Although disulfide C did not appear to be toxic to the soil cultures at concentrations up to 100 mg disulfide C/L, the enrichment cultures degrading disulfide C could not be grown on this carbon and sulfur source indefinitely. A defined bacterial

culture capable of degrading disulfide C could not be obtained. Further investigations of the biodegradation of disulfide C are required.

4.2 BIODEGRADATION OF DISULFIDE E

Disulfide E is also formed in *Pseudomonas* strain BT1d cultures degrading DBT. Disulfide E, unlike disulfide C, is commercially available and some literature exists regarding its chemical properties. Soil enrichment cultures degrading disulfide E could be transferred to fresh medium without losing activity. Two isolates from a soil enrichment culture that mineralized disulfide E were capable of degrading disulfide E in combination only. These bacteria, designated isolates RM1 and RM6, did not degrade DBT or disulfide C.

Isolate RM1 was identified as a member of the genus *Rhodococcus*, and isolate RM6 was identified as a member of the genus *Variovorax* using the Biolog system and 16S rDNA sequence analysis. Isolate RM1 likely produced vitamin B_{12} for *Variovorax* sp. strain RM6, which was capable of degrading disulfide E in pure culture if this vitamin was provided in the medium. Further disulfide degradation studies were done with pure cultures of *Variovorax* sp. strain RM6 degrading disulfide E.

Benzoic acid was detected as an intermediate in the biodegradation of disulfide E by *Variovorax* sp. strain RM6. Three potential pathways were investigated that could produce benzoic acid during the degradation of disulfide E: reduction of the disulfide bond; sulfitolysis of the disulfide; and oxidation of the disulfide sulfur atoms. No other intermediates were detected using washed cell experiments or attempts with cell-free extracts of *Variovorax* sp. strain RM6. Whether the disulfide bond is cleaved before or

after the sulfur atoms are oxidized remains unclear. The biodegradation of disulfides is generally poorly understood.

Isolate RM6 was capable of mineralizing disulfide E, releasing 59% of the carbon as carbon dioxide and 88% of the sulfur as sulfate. Although *Variovorax* sp. strain RM6 was able to mineralize disulfide E, there was no additional sulfate observed in a mixed culture with isolates RM6 and BT1d degrading DBT.

4.3 FULFILLMENT OF OBJECTIVES

The first objective of this project, to isolate and identify bacteria capable of degrading disulfides formed from DBT biodegradation, was fulfilled by the isolation of *Variovorax* sp. strain RM6. This bacterium mineralized disulfide E. *Variovorax* sp. strain RM6 required vitamin B_{12} and produced benzoic acid during the biodegradation of disulfide E. This is the first evidence of a bacterium that degrades a disulfide produced from the biodegradation of DBT through the Kodama pathway. Further experiments are necessary to determine the pathway used by *Variovorax* sp. strain RM6 to degrade disulfide E.

Variovorax sp. strain RM6 was combined in an initial experiment with *Pseudomonas* sp. strain BT1d to test the second objective of this project, the release of inorganic S from DBT. The mixed culture did not release sulfate from DBT. Further experiments are required to successfully establish a mixed culture with isolates BT1d and RM6 that will mineralize DBT.

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