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THE PHYSIOLOGY AND THE CHARACTERISTICS OF THE UPPER PATHWAY OF ANAEROBIC TOLUENE DEGRADATION IN A NEW BACTERIUM AZOARCUS TOLULYTICUS STRAIN TOL-4

presented by

Joanne C. Chee-Sanford

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THE PHYSIOLOGY AND THE CHARACTERISTICS OF THE UPPER PATHWAY OF ANAEROBIC TOLUENE DEGRADATION IN A NEW BACTERIUM *AZOARCUS TOLULYTICUS* STRAIN TOL-4

By

Joanne C. Chee-Sanford

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ABSTRACT

THE PHYSIOLOGY AND THE CHARACTERISTICS OF THE UPPER PATHWAY OF ANAEROBIC TOLUENE DEGRADATION IN A NEW BACTERIUM *AZOARCUS TOLULYTICUS* STRAIN TOL-4

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A toluene-degrading denitrifier, *Azoarcus tolulyticus* strain Tol-4, was one of eight similar strains isolated from petroleum-contaminated aquifer sediment in Northern Michigan. Strain Tol-4 is a motile, Gram negative rod, capable of aerobic and anaerobic (denitrifying) growth using toluene as a substrate. These strains are related to a newly recognized group of toluene-degrading denitrifiers consisting of the genera *Azoarcus* and *Thauera*. When Tol-4 was grown anaerobically on toluene 68% of the carbon from toluene was mineralized to CO_2 and 30% was incorporated into biomass. The doubling time on toluene was 4.3 h, the V_{max} was 50 $\mu mol \cdot min^{-1} \cdot g\ protein^{-1}$, and the cellular yield was 49.6 $g \cdot mol\ toluene^{-1}$. The stoichiometry of anaerobic toluene degradation was: $C_7H_8+5.43\ NO_3^-+0.44\ NH_3+5.43\ H^+ \text{---> } 4.78\ CO_2+2.73\ N_2+5.79\ H_2O+0.44\ C_5H_7O_2N$. Benzylsuccinate and *E*-phenylitaconate accumulated during anaerobic toluene degradation, accounting for less than 2% of the carbon from toluene. These compounds were also produced when cells were grown on hydrocinnamate and cinnamate, but not on intermediates stemming from hydroxylation reactions. These findings suggested an anaerobic toluene degradation pathway involving an oxidative addition of acetyl-CoA to the methyl group of toluene to first form hydrocinnamoyl-CoA, followed by oxidation to form cinnamoyl-CoA, then benzoyl-CoA. The presumed oxidation of benzylsuccinate to form *E*-phenylitaconate would be analogous to the oxidation of hydrocinnamate to form

cinnamate. Monofluoroacetate addition to cultures grown on toluene resulted in a significant increase in production of benzylsuccinate and *E*-phenylitaconate. These results suggested that the formation of these two compounds came after a second acetyl-CoA addition to cinnamoyl-CoA. Labeled cinnamate, hydrocinnamate, benzylsuccinate, and *E*-phenylitaconate were detected when Tol-4 was grown on ^{14}C-acetate and toluene. There was no evidence for direct methyl group oxidation. Further experiments also indicated that benzylsuccinate and *E*-phenylitaconate could be part of the main pathway of toluene degradation and not dead-end metabolites. Cell-free anaerobic toluene degrading activity was obtained for Tol-4 that was oxygen-sensitive and dependent on using both soluble and particulate fractions. The best activity was obtained when acetyl-CoA was added. Aromatic CoA ligase activities were detected for benzoate, hydrocinnamate, and cinnamate, but not for benzylsuccinate or *E*-phenylitaconate. In addition, a unique CoA transferase for benzoate involving acetyl-CoA was detected.

To my loving and supportive family.

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INTRODUCTION

Overview

Over the last two decades there have been a number of investigations to determine the biodegradability of aromatic compounds under anaerobic conditions (Young 1984; Berry et al. 1987; Evans and Fuchs 1988; Grbic-Galic 1990; Smith 1990; Grbic-Galic 1991; Londry and Fedorak 1992; Elder 1994; Fuchs et al. 1994). Among these compounds are benzoate and substituted benzoates, phenolics, chlorinated aromatics, and alkylated and non-alkylated aromatic compounds. It is now apparent that anaerobic microorganisms play a very important role in the degradation of these compounds in nature, particularly in subsurface environments where oxygen concentrations are low.

Many of these aromatic compounds are ubiquitous in the environment and are derived from both natural and anthropogenic sources. In nature, aromatic compounds are breakdown products of lignin, the second most abundant naturally occurring polymer on earth (Colberg and Young 1982). Fungi are predominantly responsible for lignin degradation, releasing a variety of aromatic products, which can then subsequently serve as possible substrates for microbial degradation in anaerobic environments. Cinnamic acids and hydroxycinnamic acids are commonly derived from lignin degradation (Healy AEM 39:436-444). Benzene, toluene, ethylbenzene, and xylenes, collectively known as BTEX compounds, are constituents of petroleum and are natural hydrocarbon

combustion products. Biogenic accumulation of toluene has also been reported, particularly in anoxic environments, from substrates such as phenylalanine and phenylacetate (Juttner and Henatsch 1986). Many chlorinated benzoates and phenols are also produced biologically (Gribble 1992).

Non-oxygenated aromatic compounds such as BTEXs pose a particular problem in anaerobic degradation. The recalcitrance of these compounds is due in large part to the arrangement of the π electrons, resulting in a large (negative) resonance energy associated with the benzene nucleus. BTEX compounds are considered very chemically stable, however these compounds can be attacked by strong chemical oxidants (Tang and Kochi 1973). Disrupting the high stability of the aromatic ring thus is the most difficult barrier to microbial biodegradation of these compounds. Benzene poses an additional problem because the structure contains no functional group that may facilitate chemical reaction. The presence of ring substituents with electron-withdrawing properties such as oxygen (e.g., in benzoates or phenols) may disrupt the resonance structure of the benzene nucleus and at the same time, can confer greater overall chemical reactivity. Even the presence of an alkyl group affects the resonance structure of the benzene nucleus, thus enhancing the possibility for chemical reaction to occur.

The presence of BTEX compounds in the environment, primarily from petroleum sources, poses a human health hazard. These compounds are among the twenty chemicals most often found on the National Priority List and, consequently, they are regulated by the U.S. EPA. Leaking underground storage tanks and fuel spills have contributed to soil and groundwater contamination world-wide. Because BTEXs are relatively more soluble than other constituents of gasoline, their occurrence in aquifers is not surprising. Toxicity tests have found that BTEX fractions of the total volatile hydrocarbons account for most of the toxicity in gasoline-contaminated groundwater (Carroquino et al. 1992).

Studies involving the toxicity of BTEX compounds, particularly benzene and to a lesser extent toluene, have shown detrimental human health effects ranging in severity from leukemias to minor dermal and central nervous system effects (Dean 1985; Fishbein 1985).

A number of technologies have been examined in the remediation of BTEX-contaminated sites. Many of these studies have focused on optimizing aerobic processes, where oxygen, in the form of O_2 or hydrogen peroxide, is added. Oxygen serves as a terminal electron acceptor for microbial metabolism as well as being a cosubstrate in the catalysis of the compound. The addition of gaseous oxygen is expensive and is usually the rate limiting factor in aerobic transformations due to its low solubility and diffusional constraints in subsurface environments (Zehnder and Stumm 1988; Hutchins et al. 1991). The disadvantages of using hydrogen peroxide include decomposition due to catalysis by transition metals like iron, or by bacterial catalase activity. Peroxide breakdown results in oxygen species that are toxic to microorganisms.

Oxygen-deprived conditions are prevalent in subsurface environments. Nitrate is an attractive alternative terminal electron acceptor in these anaerobic systems due to its high solubility, aqueous mobility, and potentially comparable rates of degradation to those measured under aerobic conditions. Few *in situ* studies have been reported that involve the use of nitrate to promote the bioremediation of fuel contaminants. Despite some success, very little information is available on the factors controlling these activities in the subsurface (Hutchins et al. 1991; Mikesell et al. 1991; Gersberg et al. 1995). These known factors include nutrient and electron acceptor availability, indigenous microbial populations, and the distribution and concentrations of the contaminants at the site. Other potential electron acceptors in anaerobic environments are Fe(III), $SO_4^=$, and CO_2; however, the practicality of

bioremediation under these electron acceptor conditions has not been demonstrated.

The aerobic degradation of BTEX compounds has been widely demonstrated in the past. Numerous review articles have been published to describe the aerobic metabolism of monoaromatic compounds (Gibson and Subramanian 1984; Dagley 1986; Harwood and Gibson 1988; Williams and Sayers 1994). Several aerobic pathways have been elucidated for the degradation of these compounds, and all involve oxygen as a reactant in steps mediated by oxygenases. Oxygen is required for both the initial step of the degradative pathway, and for subsequent aromatic ring cleavage. Under oxygen-free conditions, the activation and subsequent ring cleavage of aromatic compounds must be biochemically and mechanistically different from aerobic processes. It has now been clearly shown that all BTEXs can be degraded under anaerobic conditions. In addition to nitrate, a number of anaerobic enrichment studies have demonstrated degradation of monoaromatic compounds under sulfidogenic (Beller et al. 1991; Haag et al. 1991; Beller et al. 1992; Edwards et al. 1992; Rabus et al. 1993), methanogenic (Wilson et al. 1986; Grbic-Galic and Vogel 1987; Edwards et al. 1994), and iron(III)-reducing conditions (Lovley et al. 1989; Lovley and Lonergan 1990). Pure cultures of toluene degraders have been obtained under iron-reducing (Lovley and Lonergan 1990), sulfate-reducing (Rabus et al. 1993; Beller 1995) and denitrifying conditions (Dolfing et al. 1990; Evans et al. 1991; Schocher et al. 1991; Fries et al. 1994; Rabus and Widdel 1995). These studies demonstrate that a wide variety of microorganisms exist in nature with the ability to degrade non-oxygenated monoaromatic compounds. These studies further suggest the practical potential of anaerobic transformation processes in the bioremediation of BTEX-contaminated sites.

Although studies have shown the biodegradability of these types of compounds under anaerobic conditions, little is known of the enzymatic reactions involved in the anaerobic oxidation of aromatic compounds by microbes. Limited studies with pure cultures have resulted in debate over the intermediates involved in the anaerobic degradation of BTEXs. Of the BTEX compounds, toluene has been the most widely studied under anaerobic conditions, although the other alkylated benzenes have also been shown to be degraded. The aromatic ring reduction mechanismn followed by hydrolytic ring cleavage, and the intermediates involved in anaerobic benzoate degradation have recently become more clearly understood (Dispensa et al. 1992; Hartel 1993; Koch et al. 1993; Elder 1994; Fuchs et al. 1994; Perrotta and Harwood 1994). The anaerobic degradation of compounds that are thought to be metabolized via benzoate as an intermediate would likely involve such reactions once benzoate was formed. Prior to the presumed formation of benzoate in the degradation of alkylbenzenes, the biochemistry of the oxygen-independent initial reactions is still unknown. Several bacterial isolates have been used in attempts to determine the activating steps involved in anaerobic BTEX degradation, and certain preliminary pathways have been suggested. In addition to the biochemistry of anaerobic BTEX degradation, we now known that several physiological groups of bacteria are capable of anaerobic BTEX degradation, including denitrifiers, sulfate reducers, and Fe(III)-reducers.

The work I present here describes the isolation and characterization of a toluene-degrading denitrifier, strain Tol-4, as well as provides evidence for its anaerobic toluene degradation pathway. Strain Tol-4 is one of fifteen related microorganisms isolated at MSU that anaerobically degrade toluene under denitrifying conditions. This group has been established as a new species, *Azoarcus tolulyticus*, and strain Tol-4 has been designated the type strain.

Following my isolation of strain Tol-4, my research addressed the following main questions:

1. **What are the physiological characteristics that typify strain Tol-4 and its relationship to other known anaerobic BTEX degraders?**

2. **What are the intermediates and possible reaction mechanisms involved in the upper pathway of anaerobic toluene degradation by strain Tol-4?**

3. **Can cell-free anaerobic toluene degradation activity be established for strain Tol-4, and if so, what are the characteristics and requirements for this activity?**

Background

General Principles

The metabolism of soluble, low molecular weight aromatic compounds by bacteria in the absence of molecular oxygen presents a number of intriguing aspects. First, there exists very few insights into how bacteria activate seemingly inert compounds without an initial oxygenase reaction. In particular, the intermediates and mechanisms of the first few reactions involved have been difficult to identify. The specificities of these enzymes are completely unknown.

In general, three main steps occur in the anaerobic metabolism of soluble low molecular weight aromatic compounds. These steps are presented juxtaposed to an aerobic metabolic scheme in Figure 1.1. The first step involves activation of the compound in order to facilitate further degradation reactions. Since highly reactive molecular oxygen is absent, such reactions could involve various activating cosubstrates such as water, CO_2, CoA or CoA thioesters, ATP

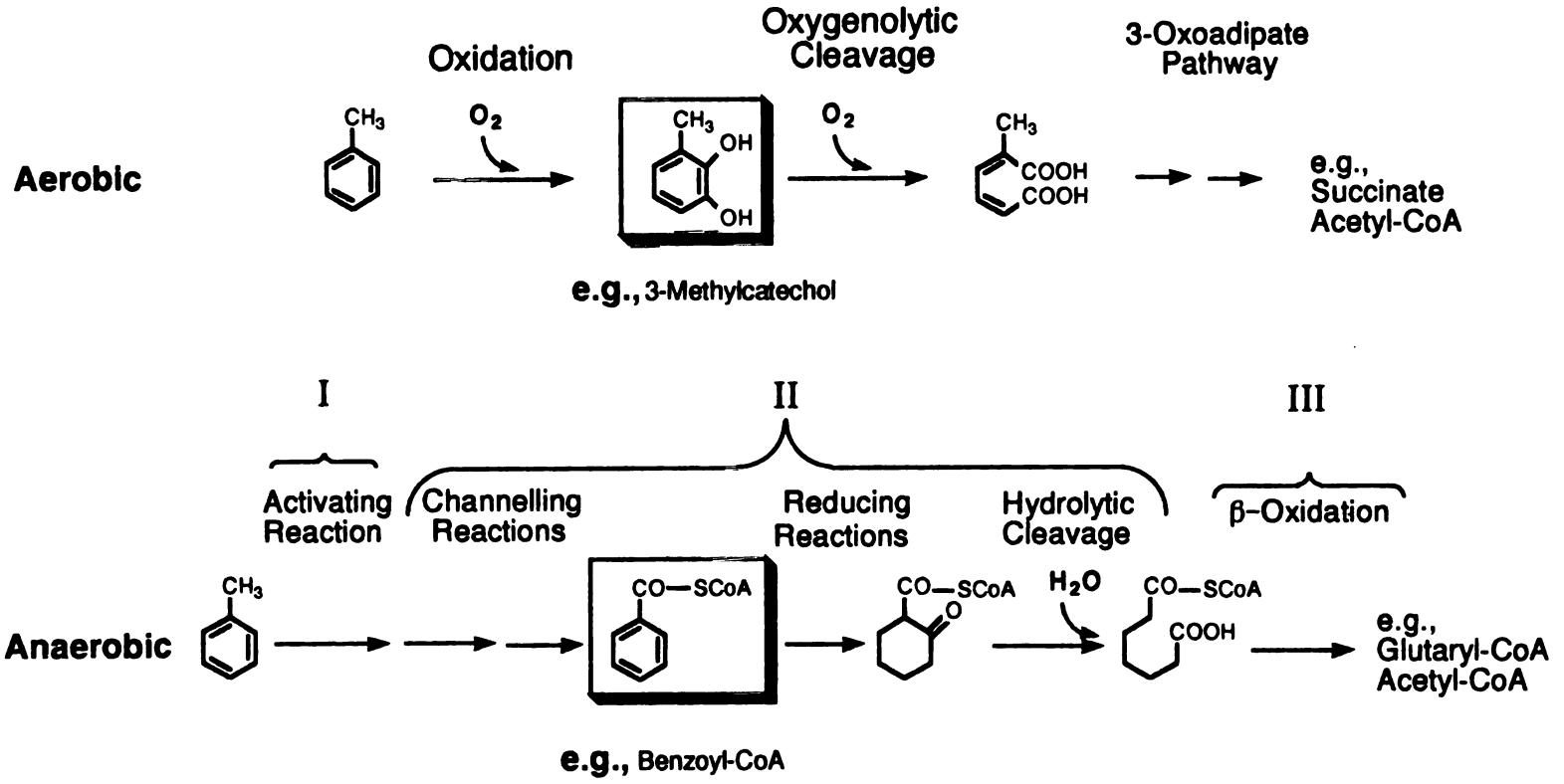


Figure 1.1. Microbial strategies for monoaromatic compound degradation. One pathway for aerobic toluene degradation and the three steps involved in anaerobic toluene degradation are illustrated.

or other high energy substrates, other coenzymes, and possibly other reactions. In the second step, the products of these initial activating steps are then directed towards the formation of a few central intermediates by channelling reactions, where the ring nucleus then undergoes reductive attack and subsequent ring cleavage. These central intermediates are thought to include benzoyl-CoA, resorcinol (dihydroxybenzene), phloroglucinol (trihydroxybenzene) (Figure 1.2.), and possibly others (Fuchs et al. 1994). The third step is the conversion of the alicyclic compounds generated following cleavage into common metabolites such as acetyl-CoA and CO_2. The last step probably involves conventional cellular pathways.

Although common biochemical principles exist for anaerobic aromatic compound degradation, variations in the strategies for aromatic metabolism are expected. Organisms with the facultative ability to degrade a particular aromatic compound may incorporate aspects of the aerobic pathway in an anaerobic pathway. We can also expect that organisms belonging to distinct physiological and phylogenetic groups could have some variations or even quite different mechanisms for degradation of a common substrate. There may also be organisms that have evolved as specialists for one or a few aromatic compounds while others are able to degrade a large variety of aromatics. At present, generalizations about the biochemistry of anaerobic aromatic compound degradation are premature, however, based on the limited studies done so far, many of these reactions promise to be unique.

Step 1: Activation of the aromatic compound

It has now been established that under anaerobic conditions simple oxygenated aromatic compounds like benzoate are biologically transformed via reductive reactions which convert the aromatic ring into an alicyclic ring (Dutton

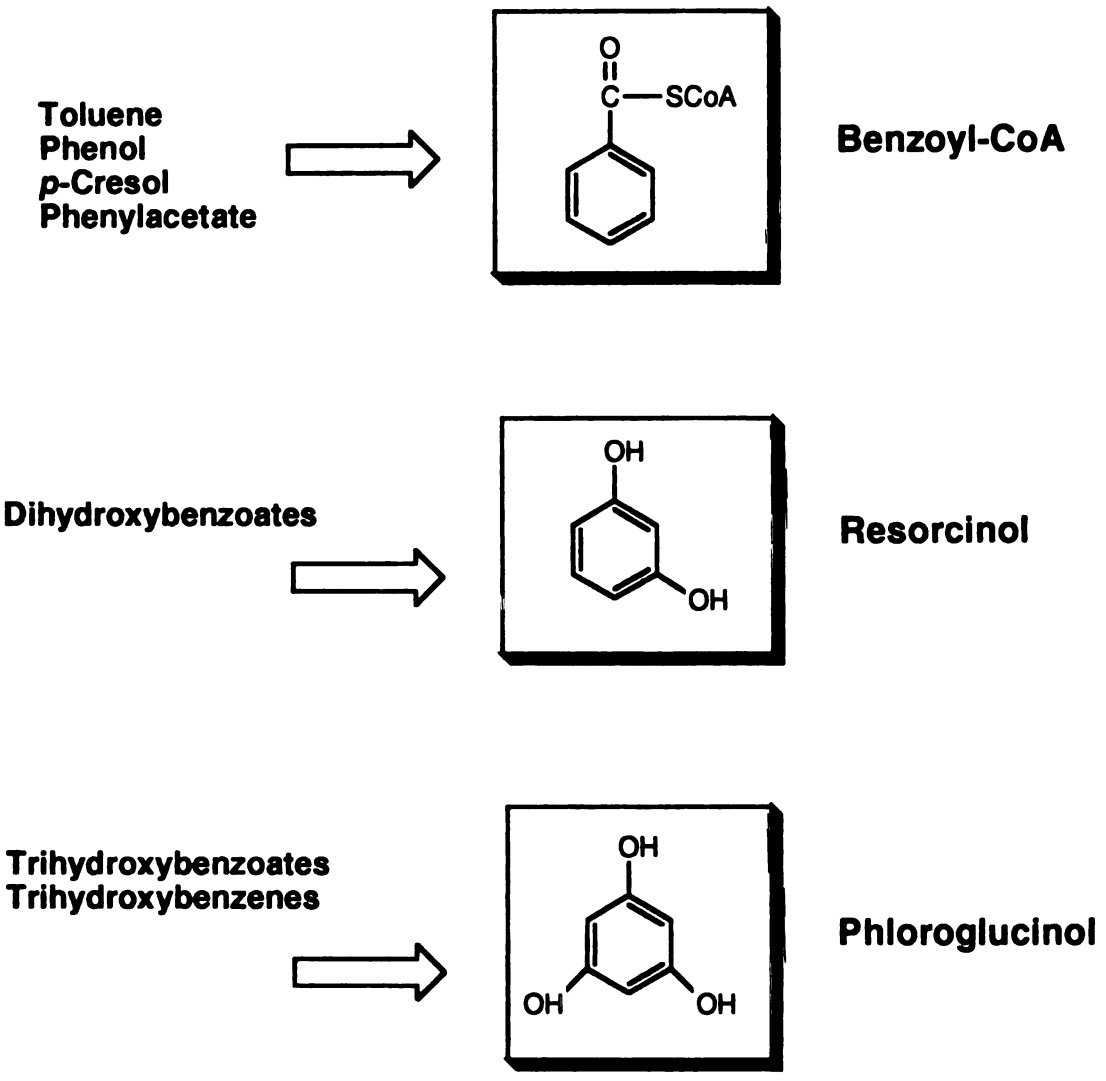


Figure 1.2. Central intermediates (boxed) in anaerobic monoaromatic compound degradation.

and Evans 1969). Hydrolytic ring cleavage occurs at the alicyclic carbon carrying the oxygenated substituent, differing significantly from aerobic ring cleavage reactions catalyzed by oxygenases. Following hydrolytic cleavage, the original aromatic substrate is finally converted to an aliphatic acid and subsequently degraded to common end products like CO_2, CH_4, and biomass.

In the case of non-oxygenated aromatics like toluene, the product of the same initial reductive reactions would be a completely reduced alicyclic ring, which would be even less reactive than the original aromatic ring (Morrison and Boyd). A more advantageous approach for microorganisms would be to first oxidize the aromatic compound. Initial oxidation reactions would produce electrons, which could ultimately be used to generate energy. Although there have been reports of methylcyclohexane and methycyclohexene as products in methanogenic consortia degrading toluene and benzene (Grbic-Galic and Vogel 1987), there is evidence that oxidation reactions may be more prevalent, particularly in the degradation of toluene. Figure 1.3. summarizes the initial steps for anaerobic toluene and benzene degradation based on metabolites detected in methanogenic consortia (Vogel and Grbic-Galic 1986; Grbic-Galic and Vogel 1987). Methylcyclohexene and methylcyclohexane have not been detected in pure cultures, but only in mixed methanogenic consortia where more complex reactions may be occurring. The predominant reactions include ring or methyl group hydroxylation, ring or methyl group carboxylation, ring reduction, and demethylation. Of these reactions, only hydroxylation has been shown usingpure cultures under anaerobic conditions. In addition, there is chemical precedence for reactions such as hydroxylation of the ring or methyl group of toluene, to occur.

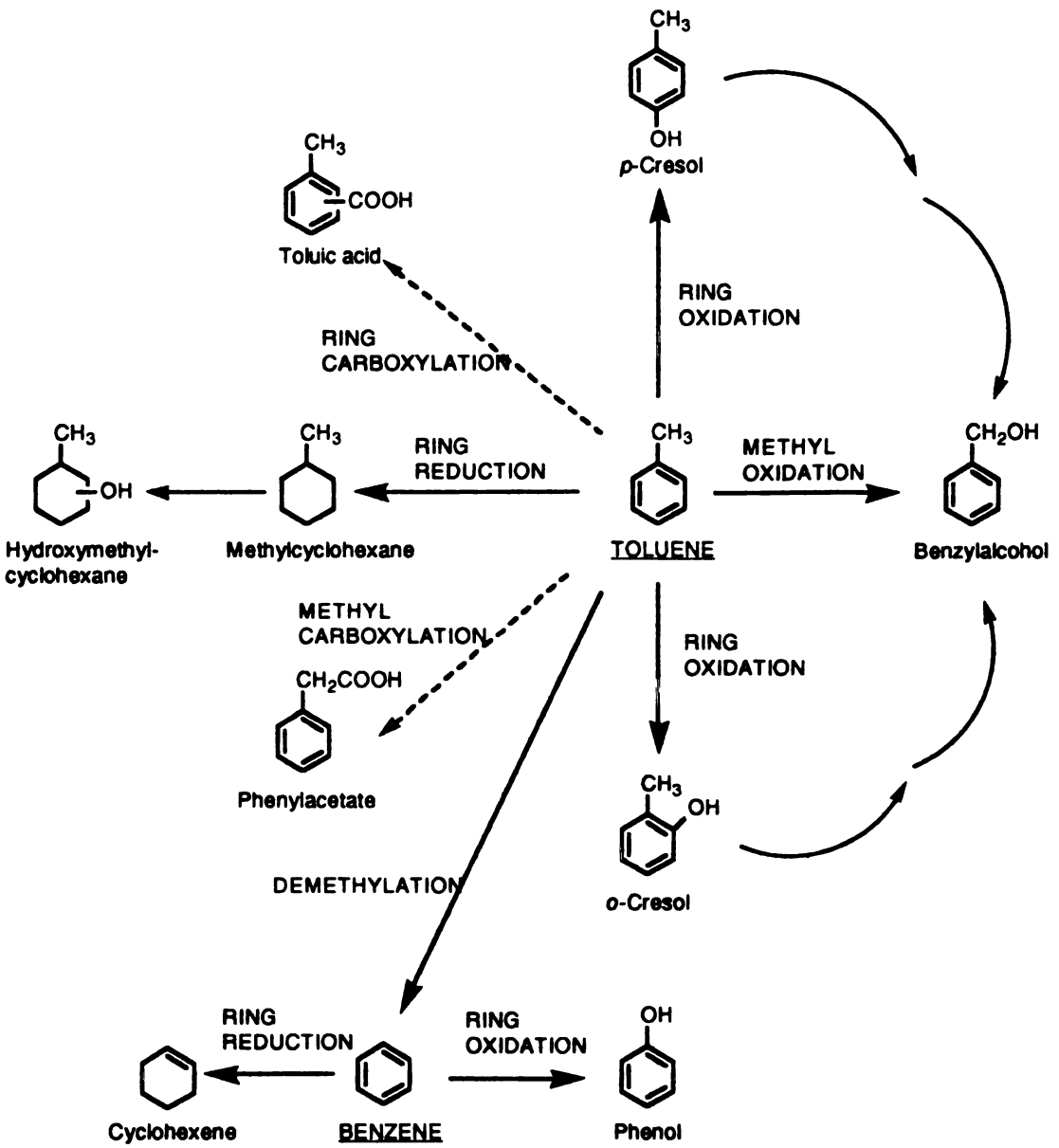


Figure 1.3. Initial steps of toluene and benzene degradation postulated from methanogenic consortia studies. Dark arrows point to compounds actually detected during degradation and dotted arrows point to hypothesized intermediates.

Chemistry of oxidative reactions:

The mechanism of hydroxylation of the aromatic ring has been hypothesized to involve strong metal oxidants in an initial electrophilic addition on the ring to yield an aryl-metal complex, which is then followed by a nucleophilic attack (e.g., by water) and reductive elimination of the metal (Grbic-Galic 1990). Abiotic chemical reaction studies using strong metal oxidants demonstrate the presence of an electrophilic mechanism involving formation of a bond between the metal center and toluene ring nucleus (Fukuzumi and Kochi 1981). The orientation of the substitution is dependent upon the existing substituent group (Morrison and Boyd 1973). The methyl group has electron-donating characteristics which tends to neutralize the positive charge on the ring and the dispersal of the charge stabilizes the carbonium ion. The addition of the metal center to the ring results in the formation of a hybrid carbonium ion intermediate (Figure 1.4., I, II, III). When aryl-metal substitution occurs at positions *para* or *ortho* to the methyl group, the most stable carbonium ions occur when the charge is on the carbon carrying the methyl group (Figure 1.4., Ib and IIc). The higher stability of these ions leads to rapid formation of *p*-cresol and *o*-cresol following nucleophilic attack by a molecule of water. However, when the substitution occurs at the *meta* position, the positive charge occurs on ring carbons other than the carbon carrying the methyl substituent (Figure 1.4., IIIa, b, c). The resulting hybrid is less stable than those formed from *o*- or *p*-substitutions, thus *m*-cresol is less likely to form relative to the formation of *o*-cresol and *p*-cresol. Infact, the order preference of hydroxyl ring substitution on toluene, *p*->*o*->*m*-, is similar to what has been observed biologically under methanogenic conditions (Grbic-Galic 1990; Grbic-Galic 1990).

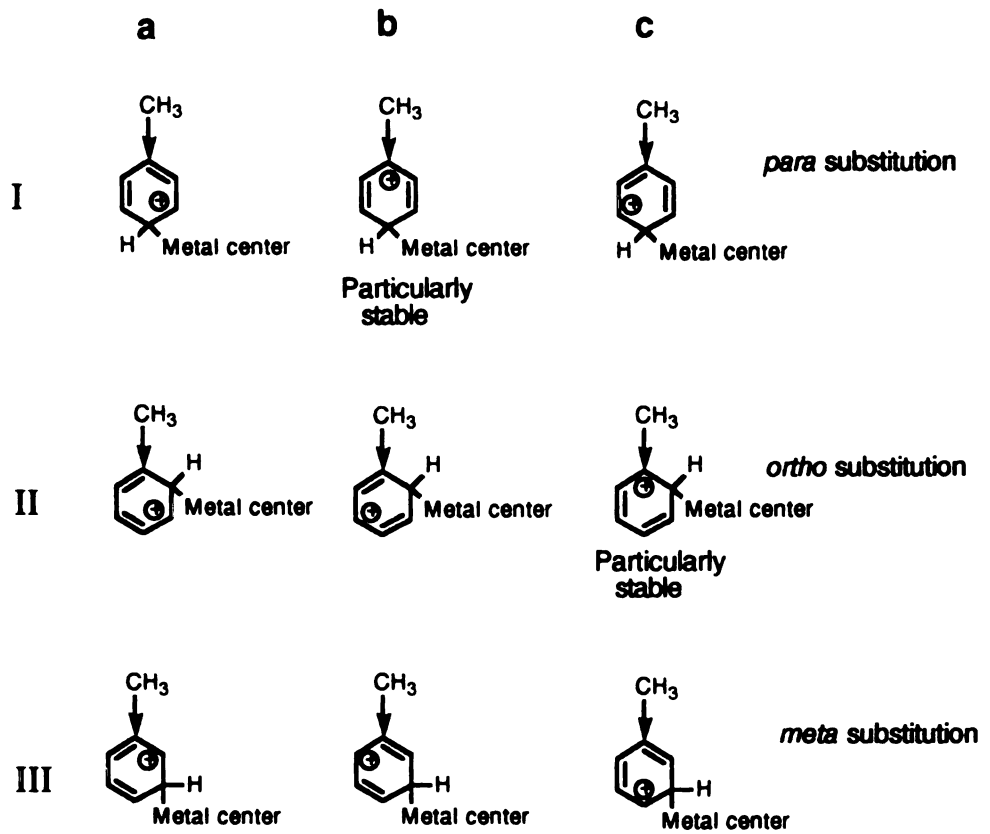


Figure 1.4. Aryl-metal carbonium ion complexes of toluene following electrophilic substitution occurring at the *para, ortho,* and *meta* positions. The actual carbonium ions are hybrids of the three structures, I (a-c) for *para*, II (a-c) for *ortho*, and III (a-c) for *meta*.

Chemical oxidation of an alkyl group substituent on an aromatic compound occurs quite readily and has commonly been used in the synthesis of aromatic carboxylic acids and in the identification of alkylbenzenes. The chemical removal of a benzylic hydrogen can result in the formation of a benzyl radical (shown for toluene in Figure 1.5.). The odd electron is not localized on the side chain but is delocalized and distributed about the ring. Abiotic chemical reactions involving strong chemical oxidants showed the involvement of a charge-transfer mechanism and loss of an electron in the formation of an intermediate cation radical (Figure 1.5.) (Kochi et al. 1973), after which the loss of a proton would readily result in the formation of the benzyl radical. The loss of another electron can result in a structure that may be susceptible to a nucleophilic attack (e.g., by water or acetyl-CoA) on the methylene carbon. Co(III) is a strong oxidant and has been used successfully in chemical studies in the form of Co(III)-acetate or Co(III)-trifluoroacetate (TFA) to oxidize cyclohexane, benzene, and toluene. The general reaction involves the formation of an aryl cation radical and reaction with the acetate moiety to form an ester bond between acetate and either the ring carbon of benzene or cyclohexane, or the methyl carbon of toluene (Tang and Kochi 1973). Co(III) is reduced to Co(II).

Metabolite studies involving the degradation of alkylbenzenes and cresols using pure cultures of anaerobes have demonstrated that either the electrophilic substitution or nucleophilic attack reactions are possible. The reactions that may be important for non-oxygenated monoaromatics are summarized in Figure 1.6.

A. Methylhydroxylation of *p*-cresol

Phenolic compounds with a methyl group *para* to a hydroxyl group can be oxidized via the formation of an alcohol and aldehyde (Hopper 1978). *p*-Cresol dehydrogenase appears to catalyze the hydroxylation of the methyl group

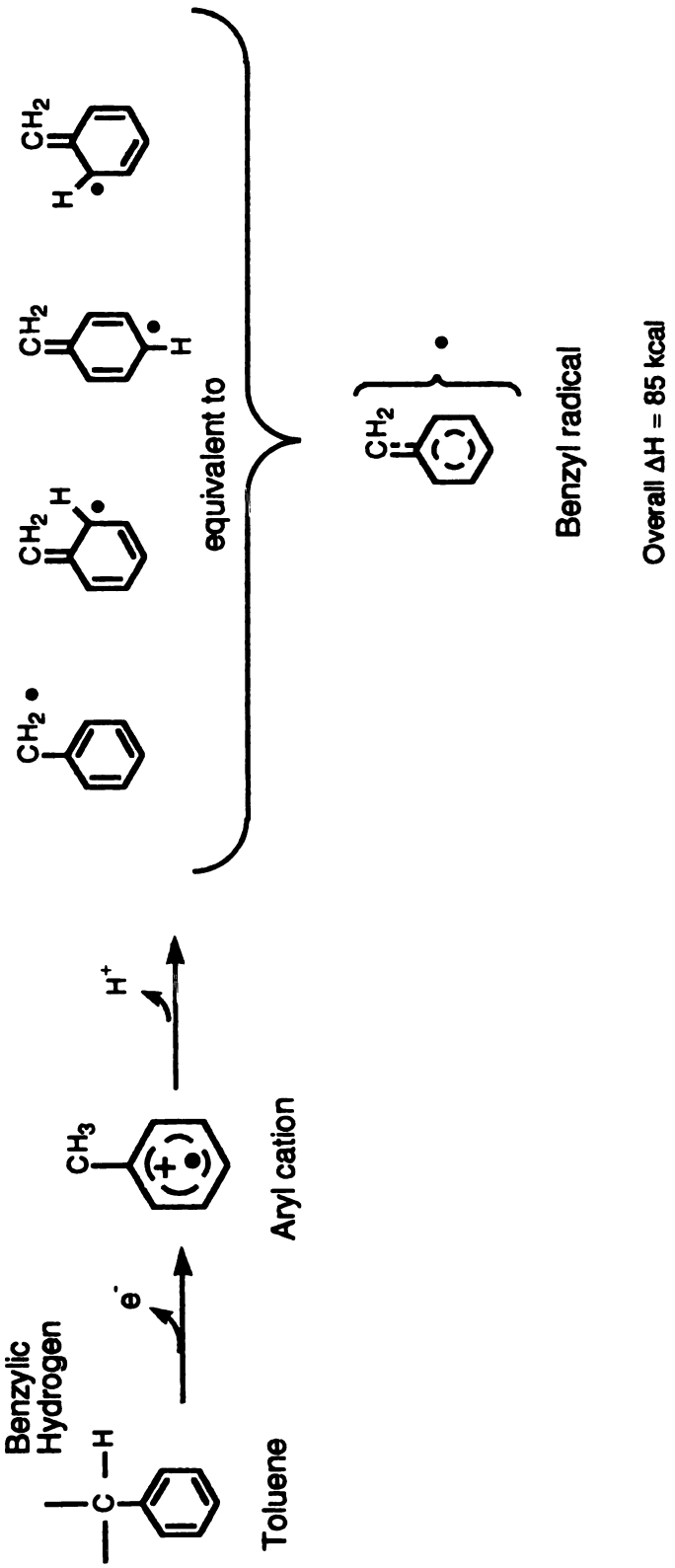
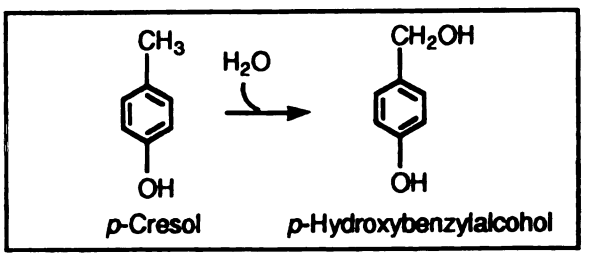
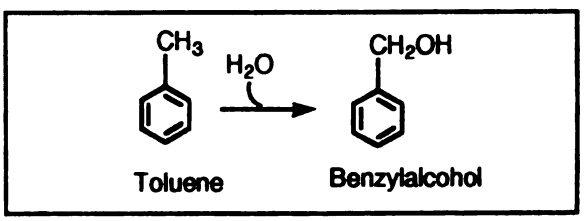


Figure 1.5. Formation of a toluene aryl cation and benzyl radical after loss of a benzylic hydrogen.

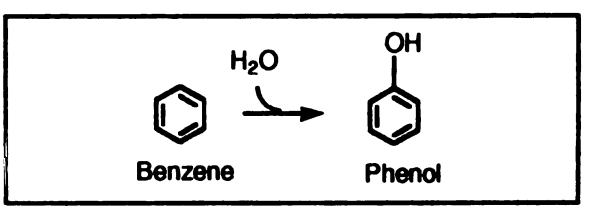
A. Methyl Hydroxylation of *p*-Cresol



B. Methyl Hydroxylation of Toluene



C. Aromatic Ring Hydroxylation



D. Methyl Group Oxidative Addition

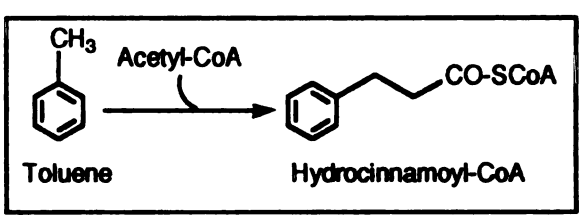


Figure 1.6. Oxidative first steps postulated to be involved in the anaerobic degradation of monoaromatic compounds. Reactions A, B, and D are based on pure culture studies, reaction C is based on methanogenic and denitrifying consortia.

in the absence of oxygen in both aerobic and anaerobic bacteria. A quinone methide intermediate is first formed before water is added as the source of the hydroxyl group. *p*-Cresol methylhydroxylase activity has not been shown in anaerobic toluene-grown pure cultures (Altenschmidt and Fuchs 1991) and *p*-cresol has not been seen as an intermediate of toluene metabolism. These results suggested that toluene is not metabolized via ring hydroxylation at the *para* position. However, ^{18}O-labeled water studies have demonstrated with mixed methanogenic cultures that water is incorporated into toluene to form *p*-cresol (Grbic-Galic and Vogel 1987). Methanogenic consortia, however, involve very different organisms from the pure cultures studied to date, which have primarily involved denitrifiers, sulfidogens, and iron (III)-reducers.

B. Methylhydroxylation of toluene

One of the pathways hypothesized for anaerobic toluene metabolism is a direct methyl group hydroxylation with water as the hydroxyl source. No direct evidence has yet shown the presence of a toluene methylhydroxylase. However, it can be inferred from increased benzylalcohol and benzaldehyde dehydrogenase activity observed in some denitrifying toluene-grown strains that toluene methylhydroxylation could occur to form benzylalcohol, which can be further oxidized to form benzaldehyde and then benzoic acid (Altenschmidt and Fuchs 1992). Benzylalcohol, benzaldehyde, and benzoate have been reported as transient intermediates of toluene degradation in some denitrifying strains (Altenschmidt and Fuchs 1992; Seyfried et al. 1994). The mechanism of a toluene methylhydroxylation is thought to differ from *p*-cresol methylhydroxylation where the *para* hydroxyl group is involved with the formation of the quinone methide intermediate prior to hydroxylation (Hopper et al. 1991). A mechanism of activation by methylhydroxylation might also be expected for

xylenes but there is no evidence yet that any xylenes are metabolized via hydroxylation.

C. Aromatic ring hydroxylation

In mixed methanogenic enrichments, phenol was formed from benzene in which the incorporated oxygen was derived from water (Vogel and Grbic-Galic 1986; Grbic-Galic and Vogel 1987). This anaerobic oxidation was very slow under methanogenic conditions. *p*-Cresol was detected under these conditions when toluene was used as a substrate. While these studies indicate ring hydroxylation can occur, it has not been shown for pure cultures metabolizing non-oxygenated aromatics.

D. Oxidative addition to the methyl group of toluene via CoA adducts

Another hypothesized pathway for toluene and xylenes degradation involves an oxidative addition of acetyl-CoA or succinyl-CoA to the methyl group. This step was indicated by the presence of accumulating products reported as benzylsuccinate and benzylfumarate from toluene metabolism, and 2-methylbenzylsuccinic acid and 2-methylbenzylfumaric acid which accumulated from *o*-xylene metabolism by a denitrifying bacterium (Evans et al. 1992). Such an oxidative addition requires the methyl group to be oxidized to a methylene substituent or formation of a reactive intermediate such as a cation radical structure hypothesized to occur chemically (Figure 1.5.). Evans and coworkers (Evans et al. 1992) suggested the presence of an oxidative addition of acetyl-CoA to the methyl group of toluene to form hydrocinnamic acid (phenylpropionate), which can then be further oxidized via β-oxidation to benzoyl-CoA in the main route of toluene mineralization. The formation of benzylsuccinate and benzylfumarate was hypothesized to result from the analogous oxidative addition of succinyl-CoA to the methyl group of toluene.

Oxidative addition to alkyl substituents of the ring has not been directly demonstrated. Such reactions would likely involve a novel enzyme system.

Step 2. Channeling reactions to central intermediates

After activation, aromatic compounds are thought to be metabolized to a few central intermediates. Reactions to form central intermediates such as benzoyl-CoA, resorcinol, and phloroglucinol, and possibly others, serves two functions. These reactions permit cells to fully metabolize otherwise inert compounds for carbon and energy and to minimize the number of enzymes required to metabolize many similar compounds. Figure 1.7. summarizes several aromatic compounds being channelled towards the formation of benzoyl-CoA. Following the formation of a central intermediate, the aromatic ring is reduced and subsequently cleaved. Many of the proposed channelling reactions are unique in that they represent reactions that have little or no biochemical precedent and, like the initial activation reactions, are likely to represent new classes of enzymes. Several such reactions involving monoaromatic compounds are summarized in Figure 1.8.

E. Lyase reactions and β-oxidation (C_6-C_3 compounds)

These reactions shorten aliphatic side chains of aromatic compounds by cleaving C-C bonds. An example of a well-known lyase reaction is catalyzed by the enzyme tyrosine-phenol lyase (Elsden et al. 1976). Aliphatic chains with three or more carbons are thought to proceed via β-oxidation reactions resulting in the release of acetyl-CoA (Zenk et al. 1980; Elder et al. 1992). Benzoic acids, phenylacetate, or their respective CoA thioesters, are frequently produced as intermediates before reduction of the ring prior to cleavage.

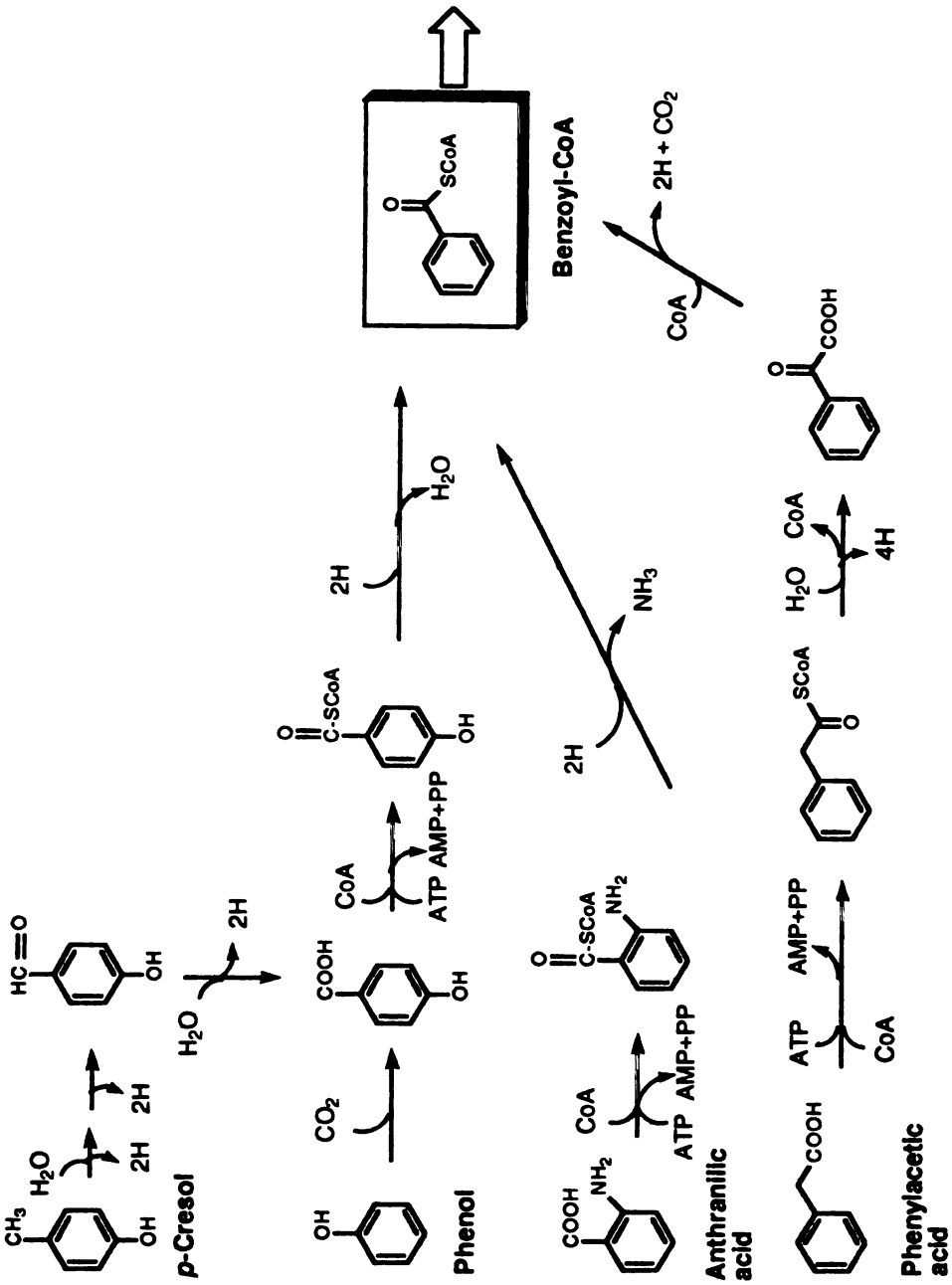
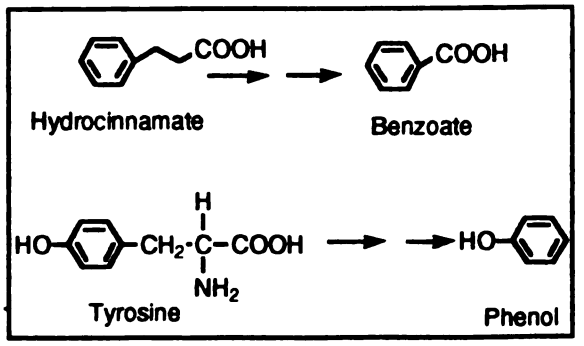
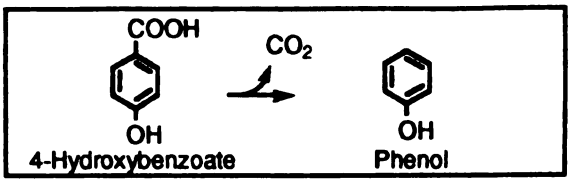


Figure 1.7. Examples of channeling reactions from aromatic substrates leading to the formation of benzoyl-CoA.

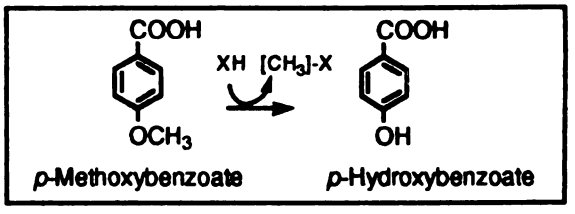
E. β-Oxidations and Lyase Reactions



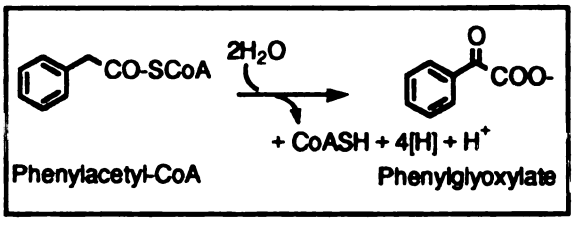
F. Decarboxylations



G. O-Demethylation, aryl ether cleavage



H. α-Oxidation



I. Oxidative Decarboxylation

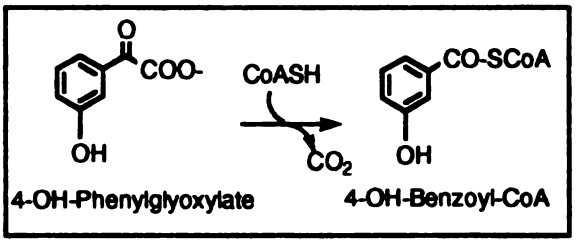
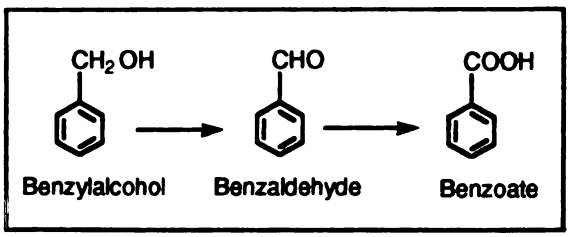
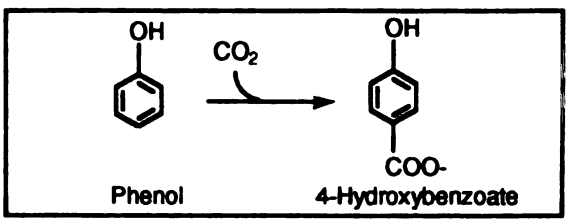


Figure 1.8. Reactions hypothesized to be involved in anaerobic monoaromatic compound degradation that ultimately channel substrates towards a few central intermediates. These reactions are based on pure culture studies.

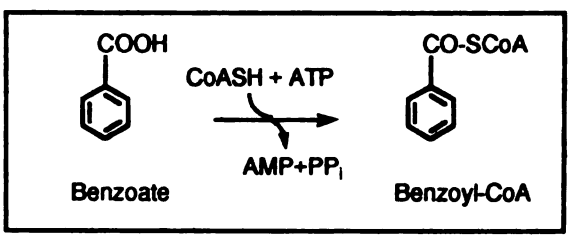
J. Aromatic Alcohol and Aldehyde Dehydrogenases
(may be NAD^+ or $NADP^+$ specific)



K. Carboxylation



L. Coenzyme A Thioester Formation



M. Reductive Dehydroxylation

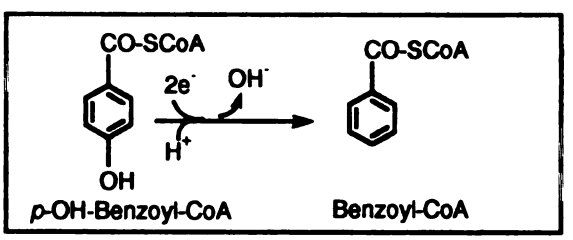


Figure 1.8. (cont.)

F. Decarboxylation reactions (hydroxylated aromatic acids)

These reactions are not commonly part of complete degradation pathways. However, the products of aromatic acid decarboxylations may represent compounds which can be completely metabolized by other members of an anaerobic food web. Phenylacetate can be decarboxylated to toluene, which can proceed as a substrate for complete degradation by another organism. *p*-Hydroxybenzoate can be decarboxylated to form phenol; this decarboxylation appears to be favorable when the hydroxy substituent is *para* to the carboxy group. Enzymes for these types of reactions have not been purified but appear to be specific, inducible, and soluble (Fuchs et al. 1994).

G. O-Demethylation, aryl ether cleavage (aromatic acids with O-methyl ether linkage, phenylmethyl ethers)

O-methyl ether linkages in aromatic acids are common natural compounds and have been found to be metabolized anaerobically by acetogens, which use the reaction to form the methyl group of acetate. Co(I) and vitamin B_{12} are involved in this reaction.

H. α-Oxidation (phenylacetate)

Studies involving the degradation of phenylacetate indicate that the CoA thioester is first formed, which appears to activate the α-methylene carbon, allowing its dehydrogenation and hydroxylation, with water as the source of oxygen (Dangel et al. 1991). The intermediate formed is phenylglyoxylate. This is an interesting reaction since phenylacetate and other aromatic acids with an even number of carbons in the carboxyalkyl side chain represent compounds that cannot undergo classical β-oxidation to form CoA derivatives that would be suseptible to reduction reactions. Phenylacetate cannot be readily reduced while in its CoA thioester form due to the methylene bridge. The CoA ligase for the

thioester formation has been purified. A separate CoA ligase has also been found for 4-hydroxyphenylacetate.

I. Oxidative decarboxylation ((hydroxy)phenylglyoxylates)

This reaction is a likely step for the metabolism of phenylglyoxylates, in which oxidative decarboxylation gives the corresponding benzoyl-CoA (Dangel et al. 1991). 4-Hydroxyphenylglyoxylate has been reported to be oxidatively decarboxylated to form 4-hydroxybenzoyl-CoA in an oxygen-sensitive reaction dependent upon CoA. These reactions are likely to be important in the complete degradation of C_6-C_2 acids.

J. Aromatic alcohol and aldehyde dehydrogenases

Aerobic enzymes catalyzing the conversion of aromatic alcohols to aldehydes and the corresponding benzoic acids have been purified. These enzymes have not been purified from anaerobic bacteria, however, these enzyme activities have been reported in a denitrifying, toluene-degrading strain K172 (Altenschmidt and Fuchs 1991). These reactions have been proposed as steps in anaerobic toluene degradation following an initial methyl group hydroxylation. Other denitrifying toluene degraders can metabolize benzylalcohol and benzaldehyde anaerobically, with benzoic acid formed as an intermediate. The characteristics, specificity, and cosubstrate requirements are not known yet for these enzymes. The aerobic enzymes catalyzing the same dehydrogenase reactions have been purified and are pyridine nucleotide (NAD^+ or $NADP^+$)-dependent.

K. Carboxylation (phenol)

Carboxylation of the ring has been well-studied in phenol metabolism in denitrifying bacteria (Tschech and Fuchs 1987; Tschech and

Fuchs 1989; Dangel et al. 1991). *o*-Cresol and other *ortho*-substituted phenolic compounds, and possibly *m*-cresol, may also undergo carboxylation in the first step of degradation. *Para* carboxylation of phenol has been directly shown but there is less direct evidence for *para* carboxylation of *o*-cresol and catechol. *Ortho* carboxylation has also been shown for hydroquinone. Purification of the enzyme system referred to as phenol carboxylase has been difficult due to its oxygen sensitivity and it is not known yet whether phenylphosphate or another phenol derivative is the physiological intermediate formed prior to the carboxylation reaction.

L. CoA thioester formation (aromatic acids)

The conversion of benzoic acid and substituted benzoates to the corresponding CoA thioesters has been the subject of numerous studies (for reviews see Elder 1994; Villemur 1995). Several of these soluble, relatively specific and inducible ligases have been purified and all seem to be ATP-dependent. The formation of these CoA thioesters allows the cell to trap freely diffusable aromatic acids intracellularly as well as to activate the compound for further metabolism (e.g., the formation of benzoyl-CoA leading to ring reduction). The importance of CoA-mediated reactions is well-known in cellular metabolism and CoA thioester formation is considered key to the anaerobic metabolism of many aromatic compounds. The regulation of benzoate-CoA ligase has been well-studied in *Rhodopseudomonas palustris* (Harwood and Gibson 1986; Kim and Harwood 1991). The enzyme is induced by benzoate, hydroxyl- and methyl-substituted benzoates, and partly reduced alicyclic compounds that are thought to be intermediates in anaerobic benzoate metabolism. It is intriguing to consider CoA thioesters as possible intermediates in the metabolism of other aromatic compounds, particularly with aromatic compounds containing non-oxygenated

substituents like toluene. Enzymes quite distinct from CoA ligases are likely to convert these compounds to the corresponding CoA thioesters, like those proposed in Figure 1.6., D.

M. Reductive dehydroxylation (hydroxybenzoates)

Hydroxyl functions *para* to a carboxyl group on an aromatic ring can be reductively dehydroxylated but require CoA thioester formation in order to proceed (Taylor et al. 1970; Grbic-Galic 1991). This reaction is important for the complete metabolism of phenol, 4-hydroxybenzoate, *p*-cresol and 4-hydroxyphenylacetate and involves the intermediate 4-hydroxybenzoyl-CoA to be further dehydroxylated to form benzoyl-CoA. The enzyme involved in the dehydroxylation of 4-hydroxybenzoyl-CoA has been purified and involves an iron-sulfur protein and a reduced electron donor.

Other types of biochemical reactions used to channel aromatic substrates towards the formation of central intermediate include reductive deamination, reductive dehalogenation, transhydroxylation, and nitro group reduction. Removal of sulpho or sulphonic acid ring substituents and anaerobic oxidation of polyaromatic hydrocarbons are currently unknown.

Step 3. Anaerobic ring cleavage

The last step of anaerobic aromatic metabolism involves reactions leading to the formation of central metabolic intermediates such as acetyl-CoA (Figure 1.9.). These reactions are thought to proceed via a series of β-oxidation reactions to release three molecules of acetyl-CoA and CO_2. The degradation pathway of benzoyl-CoA has been studied extensively in *R. palustris* and in a few denitrifiers (for review, see Elder 1994; Fuchs et al. 1994).

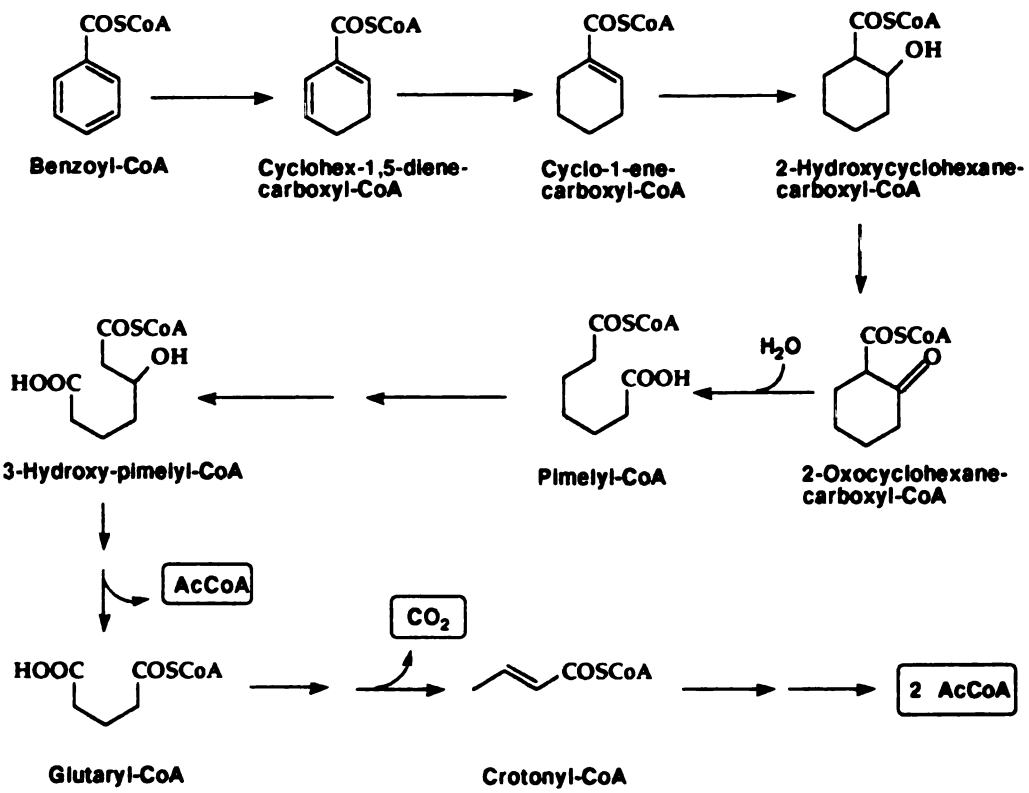


Figure 1.9. Anaerobic benzoate degradation pathway showing the reactions following the formation of benzoyl-CoA, including the hydrolytic cleavage of the ring and the subsequent products formed. This pathway is derived from studies with *R. palustris* and strain K172.

Anaerobic alkylbenzene-degrading bacteria

Studies have shown the ability of bacterial cultures with the ability to degrade alkylbenzenes under anaerobic conditions. This catabolic ability has been found in methanogenic consortia and in individual bacterial isolates including sulfidogens, iron reducers, photosynthetic bacteria, and most frequently, denitrifiers. The theoretical calculations for the energy resulting from metabolism of one mole of toluene is quite comparable for aerobes, denitrifiers, and iron (III) reducers (Table 1.1.). Individual isolates have been useful in studying the pathway of anaerobic toluene degradation, however, all the studies so far have demonstrated that the pathway is neither simple nor straightforward. Conventional methods of simultaneous adaptation and isotope trapping have given indirect evidence of toluene degradation intermediates. Enzyme activity assays give further evidence of the mechanisms that may be involved. Direct detection of metabolites have given the most definite proof of the intermediates that could be involved in anaerobic toluene mineralization.

Fermentation and methanogenesis:

Fermentation occurs under anaerobic conditions by facultative and obligate anaerobic bacteria. Organic compounds are used as electron donors and electron acceptors. Most natural aromatic compounds containing oxygen and/or nitrogen can be fermented under anaerobic conditions. Although methanogens support their own growth by reducing simple compounds such as CO_2, acetate, or other C_1 compounds to methane, methanogenesis occurs during the degradation of more complex aromatic compounds. Hydrogen (H_2) is commonly the electron donor. In anaerobic environments where other electron acceptors are present in low concentrations, fermenters and acetogens degrade more complex compounds to methane precursors. Obligately anaerobic

Table 1.1. Stoichiometry and energetics of toluene degradation[a]

	Free energy (kJ/reaction)
Toluene oxidation half-reaction:	
$C_7H_8 + 21\ H_2O$ -------> $7\ HCO_3^- + 18\ H_2 + 7\ H^+$	+479.5
Overall reactions:	
O_2 respiration:	
$C_7H_8 + 9\ O_2 + 3\ H_2O$ -------> $7\ HCO_3^- + 7\ H^+$	-3,791.0
Denitrification:	
$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+$ -------> $7\ HCO_3^- + 3.6N_2 + 0.6\ H_2O$	-3,554.3
Iron (III) reduction:	
$C_7H_8 + 36\ Fe^{3+} + 21\ H_2O$ -------> $7\ HCO_3^- + 43\ H^+\ 36\ Fe^{2+}$	-3,629.5
Sulfate reduction:	
$C_7H_8 + 4.5\ SO_4^{2-} + 7\ H_2O$ -------> $4.5\ HS^- + 7\ HCO_3^- + 2.5\ H^+$	-204.1
CO_2 reduction:	
$C_7H_8 + 7.5\ H_2O$ -------> $2.5\ HCO_3^- + 4.5\ CH_4 + 2.5\ H^+$	-130.7

[a]Equations are simplified and do not account for conversion of carbon from toluene into microbial biomass or other metabolites. The values for the Gibbs free energies under standard conditions and pH 7 were obtained from Thauer et al. (1977).

methanogens, in turn, continually remove fermentation products, resulting in an overall thermodynamically favorable process; CO_2 and CH_4 are the terminal products in this whole process. Growth is generally slow and degradation of aromatic compounds is carried out by undefined consortia of bacteria.

Many of the earlier studies with aromatic compound degradation involved methanogenic consortia (Vogel and Grbic-Galic 1986; Wilson et al. 1986; Grbic-Galic and Vogel 1987). The observation that benzoate could be degraded in the absence of molecular oxygen under methanogenic conditions was made more than sixty years ago (Tarvin and Buswell 1934). Since then, a number of aromatic compounds other than aromatic acids have been shown to be metabolized by methanogenic mixed cultures including benzene, toluene, and xylenes (Vogel and Grbic-Galic 1986). Studies involving ^{18}O-labeled water showed ^{18}O incorporation into *p*-cresol and phenol in cultures fed toluene and benzene, respectively (Vogel and Grbic-Galic 1986). Further work with the same methanogenic consortium showed the transient presence of benzylalcohol, benzaldehyde, benzoate, *o*-cresol, *p*-cresol, and methylcyclohexane in cultures where toluene was added. These intermediates indicated a complexity of reactions that may occur in mixed cultures, but more notably, the initial reactions involved in the first oxidative attack on toluene under anaerobic conditions could involve hydroxylation of the methyl group or of the ring, or possibly even ring reduction. The same study also confirmed the earlier observation that ring hydroxylation could be involved in benzene metabolism since phenol was again detected. More recent studies have demonstrated toluene and *o*-xylene degradation under methanogenic conditions (Edwards and Grbic-Galic 1994) and that toluene degradation may be proceeding via methyl group hydroxylation to form benzylalcohol, benzaldehyde, and benzoate (Edwards et al. 1994).

Sulfate reduction:

In nature where sufficient levels of sulfate are available, (e.g., marine environments), some strictly anaerobic sulfidogenic bacteria are known to couple the reduction of sulfate to the oxidation of a wide variety of compounds including aromatics (Widdel 1988). Organic compounds can be completely mineralized to CO_2 during sulfate respiration. Benzene, toluene, and xylenes were found to be completely mineralized to CO_2 in sulfate-reducing enrichments (Beller et al. 1991; Haag et al. 1991; Edwards et al. 1992). Pure cultures of sulfate-reducers have been isolated; strain Tol2 (Rabus et al. 1993) and PRTOL1 (Beller 1995). Simultaneous adaptation studies done with Tol2 involving a number of hypothetical intermediates of toluene degradation did not support ring or methyl-group hydroxylation of toluene as an activating reaction. Rabus and coworkers suggest the possibility of an oxidative condensation of the toluene methyl group with acetyl-CoA, however, no experimental evidence was presented to support this (Rabus et al. 1993). Studies involving the sulfate-reducing enrichments from which strain PRTOL1 was obtained demonstrated the presence of two dead-end metabolites, identified as benzylsuccinate and benzylfumarate, when fed toluene (Beller et al. 1992). Subsequent studies using a pure culture of PRTOL1 demonstrated the same products accumulating when grown on toluene. Additionally, 2-methylbenzylsuccinic acid was observed as the primary product of *o*-xylene degradation concomitant with toluene breakdown. The presence of these accumulating compounds appears to support a metabolic pathway involving an initial attack on the toluene methyl group by a CoA adduct. As with strain Tol2, PRTOL1 was unable to use benzylalcohol as a growth substrate.

Iron (III) reduction:

Iron-reducing bacteria obtain energy for growth by oxidizing organic compounds and reducing Fe(III) to Fe(II). A number of organic compounds are included in the substrate range of Fe(III)-reducers, including aromatic compounds (Lovley and Lonergan 1990; Lovley 1991). Strain GS15 is one of the first isolates found to be capable of anaerobically degrading aromatic hydrocarbons. This strain is able to mineralize toluene to CO_2 using Fe(III) as the electron acceptor. No intermediates were detected during toluene metabolism, however the ability to metabolize a number of hypothetical toluene degradation intermediates such as *p*-cresol and benzylalcohol suggests the possibility that hydroxylation reactions could be associated with the initial activation step.

Anoxygenic photosynthesis:

Photoassimilation of organic compounds by the purple non-sulfur bacteria under anaerobic light conditions provides energy and carbon for anabolic reactions. Organic substrates assimilated by these photosynthetic bacteria vary between species; among these are a large number of aromatic acids. *Rhodopseudomonas palustris* has been extensively studied for its benzoate metabolism (Dutton and Evans 1969; Harwood and Gibson 1986; Elder et al. 1992; Elder et al. 1992). Studies have also shown that *R. palustris* has the ability to degrade a number of other aromatic acids and it was postulated that the degradation pathways of aromatic acids which support growth lead to the formation of the central intermediate, benzoyl-CoA (Harwood and Gibson 1986). Toluene was reported to be degraded by strains of *R. palustris* and methyl group hydroxylation has been proposed as the possible degradation pathway (C. S. Harwood, personal communication). No direct evidence demonstrates this

pathway, however, and more recent studies have not been reported for toluene degradation involving photosynthetic bacteria.

Denitrification:

Many bacteria capable of dissimilatory nitrate reduction are facultative anaerobes. This form of respiration involves the reduction of nitrate to nitrous oxide or dinitrogen gas. A wide variety of organic compounds serve as carbon and electron donors for denitrifiers and pure cultures from this physiological group have been the most extensively studied for anaerobic aromatic compound degradation, including phenolics and BTEX compounds. The oxidation of any given organic compound coupled to nitrate reduction yields energetic benefits that are nearly comparable to that of oxygen respiration. The first reliable report of alkylbenzene degradation coupled to denitrification was made more than a decade ago (Kuhn et al. 1985). Benzene degradation (Major et al. 1988) and naphthalene and acenaphthalene degradation (Mihelcic and Luthy 1988) have also been reported to occur under denitrifying conditions but pure cultures of denitrifying bacteria with these abilities have not yet been obtained. Several known denitrifying strains are capable of degrading toluene under either strictly denitrifying conditions or under both denitrifying and aerobic conditions.

Strains T and K172 are two toluene-degrading denitrifying strains that have been characterized extensively (Tschech and Fuchs 1987; Dolfing et al. 1990). Strain T is somewhat more versatile in its BTEX degradation range than K172; the former reportedly is capable of degradaing *m*-xylene and *p*-xylene under denitrifying conditions. Strain T further differs from K172 in the ability of the former to degrade toluene under both aerobic and anaerobic conditions. Numerous studies with these two strains have shown data in support of direct oxidation of the methyl group of toluene in which benzylalcohol, benzaldehyde,

and benzoate have been detected as metabolites (Altenschmidt and Fuchs 1992; Seyfried et al. 1994) (Figure 1.10.A.), and in the case of strain T, methyl oxidation of *m*-xylene to form 3-methylbenzaldehyde and 3-methylbenzoate (Seyfried et al. 1994). 3-Methylbenzylalcohol was not reported as an intermediate. Altenschmidt and Fuchs reported the conversion of ^{14}C-toluene to ^{14}C-benzylalcohol and ^{14}C-benzaldehyde in studies with K172; the maximum concentration measured of these products accounted for approximately 10% of the toluene that was degraded (Altenschmidt and Fuchs 1992). ^{14}C-benzoate was not reported in this study even though benzaldehyde was transient. Seyfried and coworkers used dense cell suspensions of strains T and K172 to show a maximum concentration of benzaldehyde and benzoate at 15 μM and 5 μM, respectively, after 1 mM toluene was degraded (Seyfried et al. 1994). Benzylalcohol was not detected in this study. These data have been difficult to interpret as being unequivocal proof that strains K172 and T metabolized toluene primarily via direct methyl oxidation because the low amounts of the products detected could also result from minor reactions of toluene. Alternatively, as proponents of this pathway would suggest, the low levels of products observed could represent some steady state levels of these compounds during normal toluene metabolism.

One notable feature of K172 among the many anaerobic toluene degraders is the strain's preference for benzylalcohol as a growth substrate. Studies with most strains found benzylalcohol inhibitory to toluene degradation while K172 preferentially consumed benzylalcohol before toluene when both substates were present. Altenschmidt and Fuchs suggested that this diauxic growth indicated efficient regulation of the initiating step of toluene degradation (Altenschmidt and Fuchs 1992). Strain T, however, does not grow on benzylalcohol and it was suggested that benzylalcohol was not normally a free

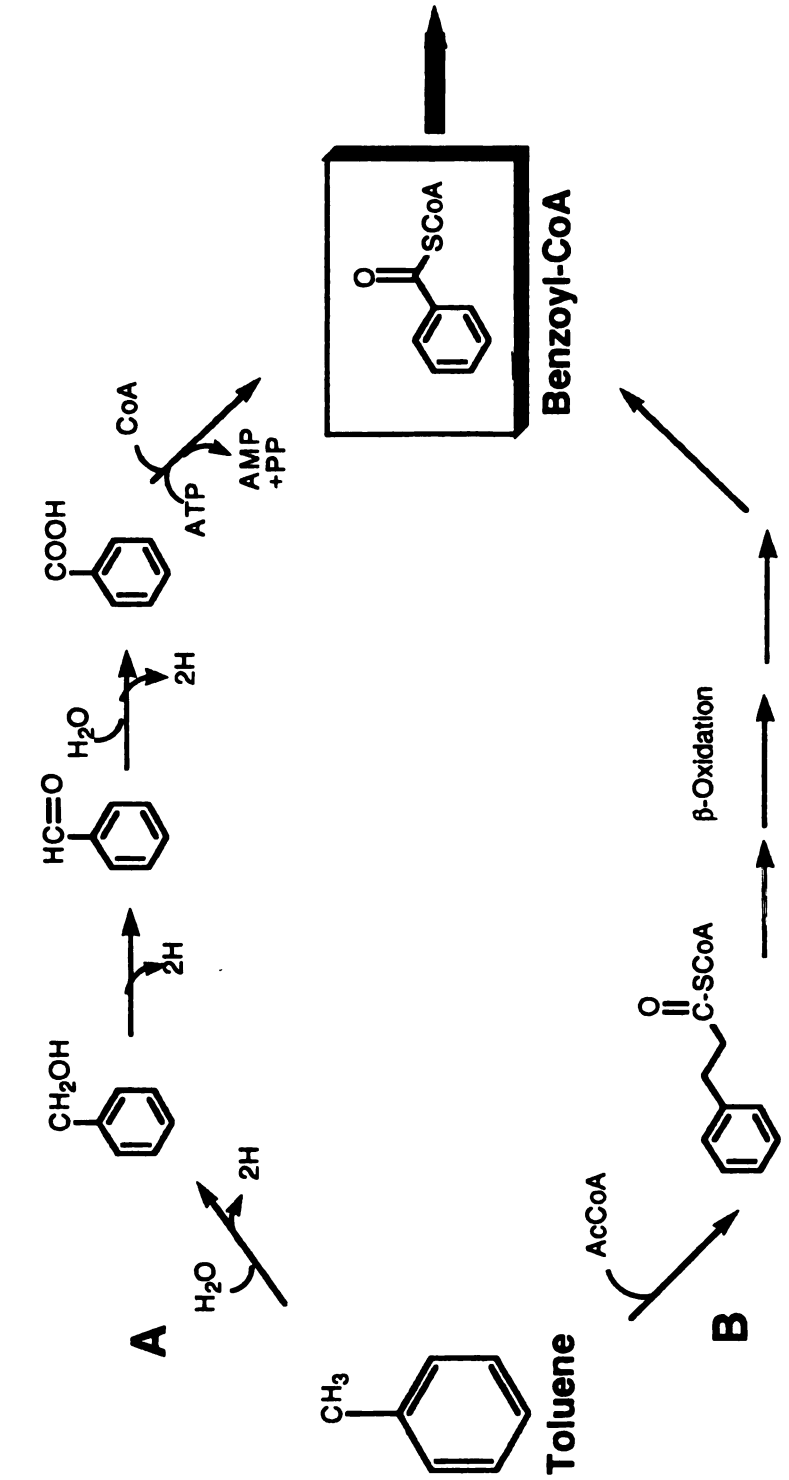


Figure 1.10. Proposed routes for anaerobic toluene degradation leading to benzoyl-CoA based on pure cultures of isolates (A) K172 and T (Altenschmidt and Fuchs 1992, Seyfried et al. 1994), and (B) T1 (Evans et al. 1992).

intermediate in toluene degradation (Seyfried et al. 1994). It was further suggested that while the initial step for toluene degradation was the same for strains K172 and T, differences in substrate specificities and benzylalcohol growth might indicate that different initial enzymes are involved. Seyfried and coworkers also reported benzylsuccinate and benzylfumarate accumulating from toluene metabolism (0.5%) in both strains K172 and T, but suggested these to be from nonspecific side reactions likely to be catalyzed by a separate enzyme than the one initiating toluene degradation. Experimental evidence is lacking for this. Further support of direct methyl oxidation comes from biochemical studies using cell extracts of K172. Benzylalcohol dehydrogenase and benzaldehyde dehydrogenase activities were present in toluene-grown cells (Altenschmidt and Fuchs 1991; Dangel et al. 1991).

Several new strains of denitrifying alkylbenzene degraders were isolated recently that showed similar characteristics to K172 in regards to anaerobic toluene metabolism (Rabus and Widdel 1995). These isolates were not able to degrade alkylbenzenes aerobically and most could metabolize benzylalcohol. Strain ToN1 was specific for toluene degradation, EbN1 was able to degrade toluene and ethylbenzene; mXyN1 was able to degrade toluene and *m*-xylene; PbN1 was able to degrade ethylbenzene and propylbenzene but not toluene. Substrate utilization and simultaneous adaptation studies suggested that these strains might also degrade alkylbenzenes via a direct methyl group oxidation to the corresponding alcohols. Rabus and Widdel further proposed a unique step involving the oxidation of the alcohols to the corresponding ketones, which could then be further metabolized via carboxylation to β-ketoacids (Rabus and Widdel 1995). An analogous carboxylation step has been shown in the anaerobic degradation of ketones by denitrifiers (Platen & Schink, 1989). The β-ketoacids would then be activated by CoA-esters and then β-oxidized to benzoyl-CoA

before ring cleavage occurs. The inhibition of *m*-xylene and toluene degradation by benzylalcohol in mXyN1, however, allowed these authors to speculate that if hydroxylation is involved in the initial step, then a different mechanism which does not involve a free alcohol intermediate must be postulated. This may be the case for those strains which also show inhibition of toluene by benzylalcohol.

Besides direct methyl group oxidation, one other pathway had been suggested for toluene degradation based on pure culture studies. The proposed pathway for strain T1 involves oxidative condensation via acetyl-CoA to form hydrocinnamoyl-CoA (phenylpropionyl-CoA) (Evans et al. 1992) (Figure 1.10.B.). In addition, T1 can also transform *o*-xylene in the presence of toluene (Evans et al. 1991). Evans and coworkers were the first to detect benzylsuccinate and benzylfumarate as accumulating products from toluene degradation in T1; up to 17% of the carbon from toluene was converted to these products. The identity of these compounds led the authors to propose a possible mineralization pathway involving an acetyl-CoA attack on the toluene methyl group, analogous to a proposed attack by succinyl-CoA which was hypothesized to result in the observed accumulation of benzylsuccinate and benzylfumarate as dead-end metabolites. *o*-Xylene was co-metabolized in the presence of toluene to form 2-methyl-benzylsuccinate and 2-methyl-benzylfumarate as accumulating products. T1 does not metabolize *o*-xylene as a growth substrate. Following the formation of hydrocinnamoyl-CoA, Evans and coworkers suggest that reactions analogous to β-oxidation might occur to form benzoyl-CoA. Benzylalcohol and benzaldehyde were not observed as intermediates in any of the studies done with T1, but benzoate was detected (Frazer et al. 1993). Fluoroacetate, added as an inhibitor of the TCA cycle, resulted in inhibition of toluene degradation and inhibition of benzylsuccinate and benzylfumarate production. The latter result supported the hypothesis that succinyl-CoA was the key reactant in the formation

of the accumulating products. The formation of benzylsuccinate and benzylfumarate was also specific to toluene metabolism and not hydrocinnamate or benzaldehyde metabolism in strain T1. The degradation of toluene was induced by the presence of toluene but not by hydrocinnamate or pyruvate. Such a pathway involving CoA adducts as intermediates in the toluene pathway may explain the difficulty in detecting soluble metabolites of toluene degradation in studies with the anaerobic toluene degraders thus far. The observation of benzoate accumulation in studies with T1 supports either pathway, i.e., direct methyl group oxidation or acetyl-CoA attack. In the latter pathway, it is likely that benzoyl-CoA is the direct intermediate formed and any free acid intermediates detected may be due to non-specific thioesterase activities present in the cells. Although a pathway involving oxidative addition to the methyl group of toluene seems feasible and consistent with the evidence from studies with T1, no direct evidence for the pathway exists, i.e., the formation of hydrocinnamoyl-CoA and cinnamoyl-CoA.

My goals in this study were to characterize the new denitrifying bacterium that I isolated, strain Tol-4, and to provide new insight into the pathway of anaerobic toluene metabolism in this strain. The following chapters will describe Tol-4 in detail; its physiological characteristics and some unique features in its anaerobic toluene degradation metabolism.

List of References

Altenschmidt U and Fuchs G (1991) Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. 156: 152-158.

Altenschmidt U and Fuchs G (1992) Anaerobic toluene oxidation to benzylalcohol and benzaldehyde in a denitrifying *Pseudomonas* strain. J. Bact. 174: 4860-4862.

Beller HR (1995) Anaerobic metabolism of toluene and other aromatic compounds by sulfate-reducing soil bacteria. Ph.D. Thesis, Stanford University.

Beller HR, Edwards EA, Grbic-Galic D, Hutchins SR and Reinhard M (1991) Microbial degradation of alkylbenzenes under sulfate-reducing and methanogenic conditions. In: EPA Project Summary, Vol EPA/600/S2-91/027 U.S. Environmental Protection Agency, Washington D.C.

Beller HR, Grbic-Galic D and Reinhard M (1992) Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. Appl. Environ. Microbiol. 58: 786-793.

Beller HR, Reinhard M and Grbic-Galic D (1992) Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 58: 3192-3195.

Berry DF, Francis AJ and Bollag J-M (1987) Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol. Rev. 51: 43-59.

Carroquino MJ, Gersberg RM, Dawsey WJ and Bradley MD (1992) Toxicity reduction associated with bioremediation of gasoline-contaminated groundwaters. Bull. Environ. Contam. Toxicol. 49: 224-231.

Colberg PJ and Young LY (1982) Biodegradation of lignin-derived molecules under anaerobic conditions. Can. J. Microbiol. 28: 886-889.

Dagley S (1986) Biochemistry of aromatic hydrocarbon degradation in Pseudomonas. In: J. R. Sokatch and L. N. Ornstron (Ed), The Bacteria, Academic Press, New York.

Dangel W, Brackman R, Lack A, Mohamed M, Koch J, Oswald B, Seyfried B, Tschech A and Fuchs G (1991) Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch. Microbiol. 155: 256-62.

Dean BJ (1985) Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mut. Res. 154: 153-181.

Dispensa M, Thomas CT, Kim M-K, Perrotta JA, Gibson J and Harwood CS (1992) Anaerobic growth of *Rhodopseudomonas palustris* on 4-hydroxybenzoate is dependent on aadR, a member of the cyclic AMP receptor protein family of transcriptional regulators. J. Bacteriol. 174: 5803-5813.

Dolfing J, Zeyer J, Binder-Eicher P and Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154: 336-341.

Dutton PL and Evans WC (1969) The metabolism of the aromatic compounds by *Rhodopseudomonas palustris*. Biochemistry Journal 113: 525-536.

Edwards EA, Edwards AM and Grbic-Galic D (1994) A method for detection of aromatic metabolites at very low concentrations: application to detection of metabolites of anaerobic toluene degradation. Appl. Environ. Microbiol. 60: 323-327.

Edwards EA and Grbic-Galic D (1994) Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60: 313-322.

Edwards EA, Wills LE, Reinhard M and Grbic-Galic D (1992) Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate reducing conditions. Appl. Environ. Microbiol. 58: 794-800.

Elder DJE and Kelly DJ (1994) The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions: Unifying trends and new perspectives. FEMS Microbiol. Rev. 13: 441-468.

Elder DJE, Morgan P and Kelly DJ (1992) Anaerobic degradation of *trans*-cinnamate and ω-phenylalkanecarboxylic acids by the photosynthetic bacterium *Rhodopseudomonas palustris*: evidence for a β-oxidation mechanism. Arch. Microbiol. 157: 148-154.

Elder DJE, Morgan P and Kelly DJ (1992) Evidence for two differentially regulated phenylpropenoyl-Coenzyme A synthetase activities in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. 98: 255-260.

Elsden SR, Hilton MG and Waller JM (1976) The end products of the metabolism of aromatic amino acids by clostridia. Arch. Microbiol. 107: 283-288.

Evans PJ, Ling W, Goldschmidt B, Ritter ER and Young LY (1992) Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58: 496-501.

Evans PJ, Mang DT, Kim KS and Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57: 1139-1145.

Evans WC and Fuchs G (1988) Anaerobic degradation of aromatic compounds. Annu. Rev. Microbiol. 42: 289-317.

Fishbein L (1985) An overview of environmental and toxicological aspects of aromatic hydrocarbons, II. Toluene. Sci. Total Environ. 42: 267-288.

Frazer AC, Ling W and Young LY (1993) Substrate induction and metabolite accumulation during anaerobic toluene utilization by the denitrifying strain T1. Appl. Environ. Microbiol. 59: 3157-3160.

Fries MR, Zhou J-Z, Chee-Sanford JC and Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60: 2802-2810.

Fuchs G, Mohamed MES, Altenschmidt U, Koch J, Lack A, Brackmann R, Lochmeyer C and Oswald B (1994) Biochemistry of anaerobic biodegradation of aromatic compounds. In: C. Ratledge (Ed), Biochemistry of Microbial Degradation, Kluwer Academic Publishers, The Netherlands.

Fukuzumi S and Kochi JK (1981) Electrophilic aromatic substitution: charge-transfer excited states and the nature of the activated complex. J. Am. Chem. Soc. 103: 7240-7252.

Gersberg RM, Korth KG, Rice LE, Randall JD, Bogardt AH, Dawsey WJ and Hemmingsen BB (1995) Chemical and microbial evaluation of *in-situ* bioremediation of hydrocarbons in anoxic groundwater enriched with nutrients and nitrate. World J. Microbiol. & Biotech. 11: 549-558.

Gibson DT and Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: D. T. Gibson (Ed), Microbial Degradation of Organic Compounds, Marcel Dekker, Inc., New York.

Grbic-Galic D (1990) Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface, and freshwater sediments. In: J. M. Bollag and G. Stotzky (Ed) Soil Biochemistry: Vol 6 (pp 117-189), Marcel Dekker, Inc., New York and Basel

Grbic-Galic D (1990) Methanogenic transformation of aromatic hydrocarbons and phenols in groundwater aquifers. Geomicrobiol. J. 8: 167-200.

Grbic-Galic D (1991) Anaerobic microbial degradation of aromatic hydrocarbons. In: E. C. Donaldson (Ed), Microbial Enhancement of Oil Recovery- Recent Advances, Proceedings of the 1990 International Conference on Microbial Enhancement of Oil Recovery, (pp 145-161), Elsevier Science Publishers B.V., New York.

Grbic-Galic D and Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. 53: 254-260.

Gribble GW (1992) Naturally occurring organohalogen compounds - a survey. J. Nat. Prod. 55: 1353-1395.

Haag F, Reinhard M and McCarty PL (1991) Degradation of toluene and *p*-xylene in anaerobic microcosms: evidence for sulfate as a terminal electron acceptor. Environ. Toxicol. Chem. 10: 1379-1389.

Hartel U, Eckel E, Koch J, Fuchs G, Linder D, and Buckel W (1993) Purification of glutaryl-CoA dehydrogenase from *Pseudomonas* sp., and enzymes involved in the anaerobic degradation of benzoate. Arch. Microbiol. 159: 174-181.

Harwood CS and Gibson J (1986) Uptake of benzoate by *Rhodopseudomonas palustris* grown anaerobically in light. J. Bacteriol. 165: 504-509.

Harwood CS and Gibson J (1988) Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. 54: 712-717.

Healy JBJ, Young LY and Reinhard M (1980) Methanogenic decomposition of ferulic acid, a model lignin derivative. Appl. Environ. Microbiol. 39: 436-444.

Hopper DJ (1978) Incorporation of [^{18}O] water in the formation of *p*-hydroxybenzyl alcohol by the *p*-cresol methylhydroxylase from *Pseudomonas putida*. Biochem. J. 175: 345-347.

Hopper DJ, Bossert ID and Rhodes-Roberts ME (1991) *p*-Cresol methylhydroxylase froma denitrifying bacterium involved in anaerobic degradation of *p*-cresol. J. Bacteriol. 173: 1298-1301.

Hutchins SR, Downs WC, Wilson JT, Smith GB, Kovacs DA, Fine DD, Douglass RH and Hendrix DJ (1991) Effect of nitrate addition on biorestoration of fuel-contaminated aquifer: Field demonstration. Groundwater 29: 571-580.

Juttner F and Henatsch JJ (1986) Anoxic hypolimnion is a significant source of biogenic toluene. Nature 323: 797-798.

Kim M-K and Harwood CS (1991) Regulation of benzoate-CoA ligase in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. 83: 199-204.

Koch J, Eisenreich W, Bacher A and Fuchs G (1993) Products of enzymatic reduction of benzoyl-CoA, a key reaction in anaerobic aromatic metabolism. European Journal of Biochemistry 211: 649-661.

Kochi JK, Tang RT and Bernath TB (1973) Mechanisms of aromatic substitution. Role of cation-radicals in the oxidative substitution of arenes by cobalt (III). J. Am. Chem. Soc. 95: 7114-7123.

Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJB and Schwarzenbach RP (1985) Microbial transformations of substituted

benzenes during infiltration of river water to groundwater: laboratory column studies. Environ. Sci. Technol. 19: 961-968.

Londry KL and Fedorak PM (1992) Benzoic acid intermediates in the anaerobic biodegradation of phenols. Can. J. Microbiol. 38: 1-11.

Lovley DR (1991) Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev. 55: 259-287.

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Philips EJP and Siegel DJ (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339: 297-300.

Lovley DR and Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56: 1858-1864.

Major DW, Mayfield CI and Barker JF (1988) Biotransformation of benzene by denitrification in aquifer sand. Ground Water 26: 8-14.

Mihelcic JR and Luthy RG (1988) Microbial degradation of acenaphthalene and napthalene under denitrification conditions in soil-water systems. Appl. Environ. Microbiol. 54: 1188-1198.

Mikesell MD, Olsen RH and Kukor JJ (1991) Stratification of anoxic BTEX-degrading bacteria at three petroleum-contaminated sites. In: R. E. Hinchee and R. F. Olfenbuttel (Ed), *In Situ* Bioreclamation, (pp 351-362), Butterworth-Heinemann, Stoneham, MA.

Morrison RT and Boyd RN (1973) Organic Chemistry 3rd Edition, Allyn and Bacon, Inc., Boston.

Perrotta JA and Harwood CS (1994) Anaerobic metabolism of cyclohex-1-ene-1-carboxylate, a proposed intermediate of benzoate degradation, by *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. 60: 1775-1782.

Platen H and Schink B (1989) Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J. Gen. Bacteriol. 135:883-891.

Rabus R, Nordhaus R, Ludwig W and Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59: 1444-1451.

Rabus R and Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163: 96-103.

Schocher RJ, Seyfried B, Vazquez F and Zeyer J (1991) Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. 157: 7-12.

Seyfried B, Glod G, Schocher R, Tschech A and Zeyer J (1994) Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60: 4047-4052.

Smith MR (1990) The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation 1: 191-206.

Tang R and Kochi JK (1973) Cobalt (III) trifluoroacetate: an electron transfer oxidant. J. Inorg. Nucl. Chem. 35: 3845-3856.

Tarvin D and Buswell AM (1934) The methane fermentation of organic acids and carbohydrates. J. Amer. Chem. Soc. 56: 1751-1755.

Taylor BF, Campbell WL and Chinoy I (1970) Anaerobic degradation of the benzene nucleus by a facultatively anaerobic microorganism. J. Bacteriol. 102: 430-437.

Thauer RK, Jungermann K and Decker K (1977) Energy conservation in chemotrophic anaerobes. Bacteriol. Rev. 41:100-180.

Tschech A and Fuchs G (1987) Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch. Microbiol. 148: 213-217.

Tschech A and Fuchs G (1989) Anaerobic degradation of phenol via carboxylation to 4-hydroxybenzoate: *in vitro* study of isotope exchange between $^{14}CO_2$ and 4-hydroxybenzoate. Arch. Microbiol. 152: 594-599.

Villemur R (1995) Coenzyme A ligases involved in anaerobic biodegradation of aromatic compounds. Can. J. Microbiol. 41: 855-861.

Vogel TM and Grbic-Galic D (1986) Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. 52: 200-202.

Widdel F (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In: A. J. B. Zehnder (Ed), Biology of Anaerobic Microorganisms, (pp 469-586), John Wiley and Sons, Inc., London.

Williams PA and Sayers RR (1994) The evolution of pathways for aromatic hydrocarbon oxidation in Pseudomonas. Biodegradation 5: 195-217.

Wilson BH, Smith GB and Rees JF (1986) Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. 20: 997-1002.

Young LY (1984) Anaerobic degradation of aromatic compounds. In: D. T. Gibson (Ed), Microbial Degradation of Organic Compounds, (pp 487-523), Marcel Dekker, Inc., New York.

Zehnder AJB and Stumm W (1988) Geochemistry and biogeochemistry of anaerobic habitats. In: A. J. B. Zehnder (Ed), Biology of Anaerobic Microorganisms, (pp 1-38), Wiley and Sons, New York.

Zenk MH, Ulbrich B, Busse J and Stockigt J (1980) Procedure for the enzymatic synthesis and isolation of cinnamoyl-CoA thiolesters using a bacterial system. Anal. Biochem.101: 182-187.

Chapter 2

THE PHYSIOLOGICAL CHARACTERISTICS OF *AZOARCUS TOLULYTICUS* STRAIN TOL-4

Introduction

Numerous studies have shown that aromatic hydrocarbons are metabolized under denitrifying, methanogenic, Fe(III)- and sulfate-reducing conditions (for reviews see Young 1984; Grbic-Galic 1990; Elder and Kelly 1994; Fuchs et al. 1994). Until pure cultures were obtained, little was known about the diversity of bacteria that have the ability to degrade aromatic hydrocarbons in the absence of oxygen. Table 2-1 summarizes all the pure cultures reported to date, along with which BTEX substrates and electron acceptors are used. Given the recent rate of progress, it will not be surprising if more isolates are obtained with additional unique catabolic capabilities, particularly the ability to anaerobically degrade benzene and polynuclear aromatic compounds.

One of the first isolates obtained in pure culture was strain GS15, now known as *Geobacter metalloreducens*, a member of the delta subclass of Proteobacteria. This isolate metabolizes toluene, phenol, and *p*-cresol using Fe(III) oxide as the electron acceptor (Lovley and Lonergan 1990). It was further shown that this strain could also use toluene as an electron donor while reducing Mn(IV) to Mn(II) and also while reducing nitrate to ammonium. Since the discovery of GS15, numerous other bacteria from physiologically distinct groups have been isolated with the ability to degrade various alkylbenzenes as well as phenolics and aromatic acids. Many of the studies investigating the

Table 2.1. Description of bacterial isolates and their ability to degrade BTEX compounds

Isolate	Identification	Electron acceptor(s)[a]	Source	Benzene		Toluene		Ethylbenzene		o-Xylene		m-Xylene		p-Xylene		Ref(s)
				Aer	Ana	Aer	Ana	Aer	Ana	Aer	Ana	Aer	Ana	Aer	Ana	
Tol-4	*Azoarcus tolulyticus*	O_2, NO_3^-, NO_2^-, N_2O	Gasoline contaminated sandy aquifer sediment, 24-25 m depth, N. MI	-	-	+	+	+	-	-	-	-	-	-	-	1,2
BL-2, BL-3, BL-4, BL-11	nd	NO_3^-	Gasoline contaminated sandy aquifer sediment, 24-25 m depth, N. MI	nd	nd	nd	+	nd	nd	nd	nd	nd	nd	nd	nd	Chap.3
2a-1	nd	NO_3^-	Gasoline-contaminated sandy aquifer sediment, 8.5-10 m depth, N. MI	nd	nd	nd	+	nd	nd	nd	nd	nd	nd	nd	nd	Chap.3
3a-1, 7a-1,	nd	NO_3^-	Gasoline-contaminated sandy aquifer sediment, 2.4-4m depth, N. MI	nd	nd	nd	+	nd	nd	nd	nd	nd	nd	nd	nd	Chap. 3
Td-1	*Azoarcus tolulyticus*	O_2, NO_3^-	Hydrocarbon-contaminated soil, WA	-	-	+	+	+	-	-	-	-	-	-	-	2
Td-2	*Azoarcus tolulyticus*	O_2, NO_3^-	Hydrocarbon-contaminated soil, WA	-	-	+/-	+	-	-	-	-	-	-	-	-	2
Td-15	*Azoarcus tolulyticus*	O_2, NO_3^-	Home compost soil, MI	-	-	+	+	+	-	-	-	-	+	-	-	2
Td-17	*Azoarcus tolulyticus*	O_2, NO_3^-	Industrial waste soil, Sao Paulo, Brazil	+	-	+	+	+	-	-	-	-	-	-	-	2
Td-19	*Azoarcus tolulyticus*	O_2, NO_3^-	Industrial waste soil, Sao Paulo, Brazil	-	-	+/-	+	-	-	-	-	-	-	-	-	2
Td-21	*Azoarcus tolulyticus*	O_2, NO_3^-	Muck soil, MI	+	-	+	+	+	-	-	-	-	-	-	-	2
T1	(*Thauera aromatica*)	O_2, NO_3^-	Gasoline-contaminated soil, CA	nd	-	+	+	nd	-	nd	+[b]	nd	-	nd	-	3,4
K172	*Thauera aromatica*	NO_3^-, N_2O	Municipal sewage sludge	-	-	-	+	-	-	-	-	-	-	-	-	5

Table 2.1. (cont.).

Isolate	Identification	Electron acceptor(s)[a]	Source	Benzene Aer	Benzene Ana	Toluene Aer	Toluene Ana	Ethylbenzene Aer	Ethylbenzene Ana	o-Xylene Aer	o-Xylene Ana	m-Xylene Aer	m-Xylene Ana	p-Xylene Aer	p-Xylene Ana	Ref(s)
S100	nd	NO_3^-, N_2O	Contaminated sediment	nd	nd	-	+	nd	nd	nd	nd	nd	nd	nd	nd	5,6
S2	nd	NO_3^-, N_2O	Contaminated sediment	nd	nd	-	+	nd	nd	nd	nd	nd	nd	nd	nd	5,6
T	nd	O_2, NO_3^-, N_2O	River sediment	nd	-	+	+	nd	-	nd	-	nd	+	nd	+[c]	6,7
EbN1	*Azoarcus/ Thauera*	NO_3^-	Ditch and river mud homogenate, Germany	nd	nd	-	+	-	+	-	-	-	-	-	-	8
PbN1	*Azoarcus/ Thauera*	NO_3^-	Ditch and river mud homogenate, Germany	nd	nd	-	-	-	+	-	-	-	-	-	-	8
ToN1	*Azoarcus/ Thauera*	NO_3^-	Ditch and river mud homogenate, Germany	nd	nd	-	+	-	-	-	-	-	-	-	-	8
mXylN1	*Azoarcus/ Thauera*	NO_3^-	Ditch and river mud homogenate, Germany	nd	nd	-	+	-	-	-	-	-	+	-	-	8
Tol2	*Desulfobacula toluolica*	$SO_4^=$	Marine sediment	nd	nd	-	+	-	-	-	-	-	-	-	-	9
PRTOL1	nd	$SO_4^=$, $SO_3^=$, S_2O_3	Fuel-contaminated soil, Patuxent River, MD	-	-	-	+	-	-	-	+[b]	-	-	-	+[b]	10
GS15	*Geobacter metallo-reducens*	Fe(III), Mn(IV), NO_3^-	Hydrocarbon-contaminated groundwater	nd	nd	-	+	nd	nd	nd	nd	nd	nd	nd	nd	11,12

[a]Electron acceptors reported to be used for BTEX degradation.
[b]Cometabolized in the presence of toluene.
[c]*p*-Xylene was degraded only in dense cell cultures.
+/- = weak growth
nd = not determined
[1]Chee-Sanford et al. 1992, [2]Fries et al. 1994, [3]Evans et al. 1991, [4]Evans et al. 1991, [5]Schocher et al. 1991, [6]Bonting et al. 1995, [7]Dolfing et al. 1990, [8]Rabus and Widdel 1995, [9]Rabus and Widdel, 1993, [10]Beller1995, [11]Lovley et al. 1989, [12]Lovley 1990.

pathway of anaerobic aromatic metabolism have focused on denitrifying strains.[(DeSoete 1983; Lovley et al. 1989; Dolfing et al. 1990; Evans et al. 1991; Evans et al. 1991; Schocher et al. 1991; Bonting et al. 1995)].

The known denitrifying strains so far appear to comprise a branch in the beta subclass of the Proteobacteria, with close relationships to two recently described genera, *Azoarcus* and *Thauera.* Within this group, however, there are distinct physiological differences such as substrate utilization capabilities and other growth characteristics suggesting several lines of descent leading to the different species. Members of the genus *Azoarcus* are characterized by their ability to fix nitrogen (Reinhold-Hurek et al. 1993) while the known species of *Thauera*, *Thauera selenatis*, was reported not to fix nitrogen (Macy et al. 1993). Members of both genera are denitrifiers. The majority of the aromatic-degrading denitrifiers can anaerobically degrade toluene, indicating that this metabolic trait is relatively conserved. Strain KB740, recently described as *Azoarcus evansii*, is not included in Table 2.1. because it is reported to anaerobically degrade a variety of aromatic acids but not alkylbenzenes, including toluene (Anders et al. 1995). 16S rDNA analysis showed that the nucleotide sequence similarity between KB740 and *Azoarcus tolulyticus* strain Td3 is 99.4% (J. Urbance, personal communication). The inability of strain KB740 to degrade toluene may not be so unusual considering that within the known genus *Azoarcus, Azoarcus* sp. strains VB32^T and S5b2 were reported not to degrade toluene (Zhou et al. 1995) but are known to metabolize a variety of aromatic acids (Reinhold-Hurek et al. 1993). However, the phylogenetic placement of strains VB32^T and S5b2 shows them to more distant from the cluster comprising KB740 and the Td isolates (Figure 2.1.).

Another notable distinction among these anaerobic toluene degraders is whether they can metabolize toluene aerobically. Strain K172 cannot grow

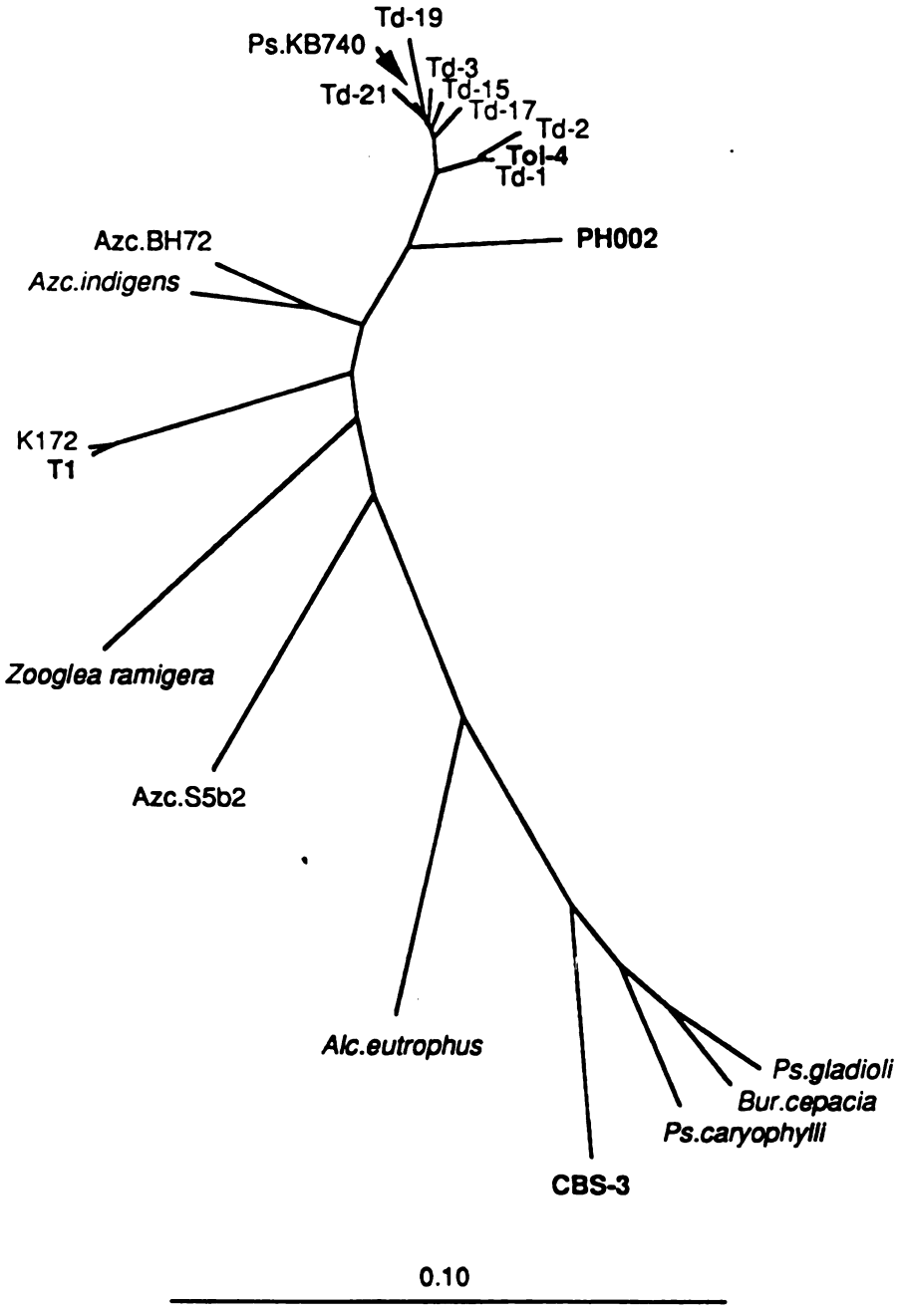


Figure 2.1. Phylogenetic tree based on 16S rRNA gene sequences showing several aromatic compound-degrading denitrifiers including Tol-4, Td strains, KB740, K172, and T1. The tree was generated using the distance matrix method of DeSoete (1983) courtesy of J. Urbance, Michigan State University, 1996.

aerobically on toluene while T1 can. These two strains are 99.5% similar in their16S rRNA gene sequences (J. Urbance, personal communication). Strain K172 has been reported as a new species of the genus *Thauera*, *Thauera aromatica* (Anders et al. 1995), and based on 16S rRNA analysis, it is very likely that strain T1 also belongs to this group. Both strains K172 and T1 have been extensively studied and two distinctly different pathways are postulated for their anaerobic toluene degradation (Chapter 1). Until the pathway(s) become more clearly defined, we cannot rule out the possibility of similar pathways in these two organisms. It is also conceivable that even if the intermediates of toluene degradation are found to be the same for these isolates, the enzymes and mechanisms may be still be different.

New alkylbenzene-degrading denitrifiers were recently discovered by Rabus and Widdel and reported to be members of the *Azoarcus* and *Thauera* branch (Rabus and Widdel 1995). EbN1 and PbN1 are both able to degrade ethylbenzene under denitrifying conditions. These isolates are the first to be reported that can anaerobically degrade alkybenzenes with alkyl chains of greater than one carbon. PbN1 is also capable of degrading propylbenzene, but cannot metabolize toluene, while strain EbN1 can. The new strains mXylN1 and ToN1 also degrade toluene, with the former also being able to metabolize *m*-xylene. ToN1 was found to cluster by 16S rDNA sequence analysis with KB740 and several Td isolates while mXyN1 clustered with strain K172.

The Td isolates, strain Tol-4, and the other unidentified isolates obtained from Michigan aquifer sediments (Table 2.1.) can all degrade toluene under both aerobic and anaerobic conditions (Fries et al. 1994). The Td isolates and Tol-4 form a tight cluster by phylogenetic and phenotypic analyses and it is likely that all the unidentified isolates from Northern Michigan also belong in this cluster. Repetitive extragenic palindromic (REP)-PCR patterns of the unidentified

isolates are identical, or nearly so, to the REP-PCR pattern of Tol-4 (personal communication, E. Alexander). These isolates, while forming colonies that are morphologically distinct from one another, represent very closely related strains, i.e., same genospecies. Similarly, it has also been suggested that strains S100, S2, and K172 (Table 2.1.) represent a second genospecies (Anders et al. 1995).

The large group of denitrifiers that have been described so far form a relatively cohesive phylogenetic group with distinct physiological differences among its members. The strains can be placed in three general metabolic categories: 1) isolates capable of anaerobic toluene degradation and possibly other alkylbenzenes and aromatics, 2) isolates incapable of anaerobic toluene degradation but able to metabolize other alkylbenzenes and aromatics, and 3) isolates incapable of metabolizing any of the alkylbenzenes but able to metabolize other aromatic compounds.

In addition to GS15, other anaerobic toluene degraders have been found in the delta subclass of the Proteobacteria. *Desulfotobacula toluolica* strain Tol2 (Rabus et al. 1993) and PRTOL1 (Beller 1995) have been recently identified as strains capable of degrading toluene under sulfate-reducing conditions. These two strains are distinct from one another. PRTOL1 may represent an entirely new genus and has a more versatile alkylbenzene degradation capability than strain Tol2.

This chapter describes the enrichment, isolation, and general physiological characteristics of *Azoarcus tolulyticus* strain Tol-4. Some of these data can be found in Chee-Sanford et al. (Chee-Sanford et al. 1992), Fries et al. (Fries et al. 1994), and Zhou et al. (Zhou et al. 1995) and are summarized together in this chapter for easier reference. Other characteristics of Tol-4 more specific to toluene and other aromatic compound degradation are presented in Chapter 3 of this thesis.

Material and Methods

Enrichment and isolation. Sediment (20 g) was added to 160 ml serum bottles sealed with Teflon-lined butyl rubber stoppers containing aerobically-prepared phosphate buffered basal salts (BS) solution (Owens and Keddie 1969) amended with 20 mM KNO_3 under a headspace of O_2-free argon. The pH of the medium was adjusted to 7.0. Toluene (99.8%, Sigma) was added neat to achieve a final concentration of 1000 µM (92 ppm). After the initial enrichment, BS was prepared anaerobically under an O_2-free argon headspace and amended to a final concentration of 5 mM nitrate and final toluene concentration of 540 µM (50 ppm) for subsequent transfers and growth of pure cultures isolated from the enrichments. Incubation of all cultures was at 25°C or 30°C. All anaerobic liquid cultures were grown in serum bottles sealed with Teflon-lined stoppers and incubated in an inverted position. Unless otherwise stated, all experiments were done using the above anaerobic protocol.

To isolate denitrifying toluene degraders, the primary enrichment cultures were incubated in sealed serum bottles. Secondary and tertiary transfers of 20% (v/v) inoculum were made into fresh anaerobically-prepared BS plus NO_3^- and toluene medium for the enrichments showing depletion of toluene. The active tertiary enrichments were serially diluted and plated onto BS plus NO_3^- agar (2% (w/v) Difco Noble agar). Plates were incubated in sealed anaerobic jars that were flushed with O_2-free nitrogen. Toluene vapors were introduced by adding 1.0 ml toluene to sterile cotton in one petri dish per stack of ten culture plates. To obtain pure cultures, individual colonies were restreaked at least three times from single, well-isolated colonies on half-strength TSA (Difco) plus 20 mM NO_3^-. Cultures were grown both aerobically and anaerobically. For anaerobic incubation, jars containing plates were incubated in a Coy anaerobic

chamber (Coy Manufacturing Co., Ann Arbor, MI) at room temperature. Cells from individual colonies were inoculated into 20 ml anaerobic BS plus 5 mM NO_3^- and 0.5 mM toluene and checked for toluene depletion. All transfers were made in the anaerobic chamber and the tubes were inverted to minimize any vapor loss during incubation. Anaerobic growth on toluene by isolate Tol-4 was verified by growth in BS plus NO_3^- medium with 0.1 mM amorphous FeS, added as a reductant and scavenger of oxygen (Brock and O'Dea 1977). Cultures of Tol-4 were maintained on either anaerobic BS plus NO_3^- medium containing toluene or on aerobic M-R2A agar (Fries et al. 1994).

Characterization of strain Tol-4. Cell dimensions and morphology were determined by phase-contrast microscopy using cells of Tol-4 grown anaerobically on toluene. Scanning electron microscopy (SEM) was used to photograph cells (SEM facility, Michigan State University). Inocula for all physiological tests were prepared from cells grown aerobically on M-R2A medium at 30°C for 24 h. Gram staining and tests for catalase and cytochrome *c* oxidase activities were performed using standard methods (Smibert and Krieg 1981). Denitrification was tested by growing cells in M-R2A-NO_3^- broth medium modified by excluding glucose and starch, and monitoring for depletion of NO_3^- and production of gases. M-R2A broth prepared either aerobically, or anaerobically, and supplemented with 10 mM NO_3^- were used to determine growth curves at 30°C. Optical density was measured at time intervals at 600 nm using a spectrophotometer. Nitrogen-fixing capability was determined after growth in nitrogen-free medium and evaluating nitrogenase activity by the acetylene reduction assay (Fries et al. 1994). The temperature range was determined by growth on M-R2A at temperatures from 4 to 45°C. Salt tolerance and pH range were tested by growth using M-R2A amended either by addition of

0 to 10% NaCl or by pH-adjustment from pH 3 to 10. All assays were done in duplicate.

Other heterotrophic media used for testing growth were nutrient agar (Difco), LB agar, PTYG agar (Difco), SM agar (Reinhold-Hurek et al. 1993), and BS agar supplemented with 1 mM each of acetate, succinate, citrate, and oxalacetate. Selenate respiration was determined using anaerobic BS medium supplemented with 10 mM SeO_4. To determine the ability of cells to grow on H_2 autotrophically, 100 ml BS medium was added to sealed serum bottles along with 50 ml of H_2 and 50 ml of air, and CO_2 was supplied by adding 10 mM bicarbonate. API/NFT tests incubated at 30°C were used to test cells for substrate utilization and enzyme activities according the the manufacturer's directions (BioMeriuex). Results were recorded after 48 h. Additional substrate testing was done in aerobically prepared BS medium (10 ml) supplemented with a final carbon concentration of 1 mM of the following individual compounds: glucose, maltose, mannose, malate, ethanol, acetate, succinate, lactate, pyruvate and benzoate. Optical density at 600 nm was used to assess growth. All assays were done in duplicate.

Results and Discussion

Enrichment and isolation. Strain Tol-4 was isolated along with seven other strains that had the ability to degrade toluene under denitrifying conditions. These isolates are listed as the first eight strains in Table 2.1. The success of the isolation can be attributed to the strategy of using low nitrate (5 mM) and toluene (50 or 100 ppm) concentrations. Numerous other studies to enrich for denitrifiers or various denitrifying hydrocarbon degraders have commonly reported nitrate concentrations of up to 20 mM and carbon sources added at 500-1000 ppm.

The gasoline-contaminated sandy aquifer sediments, which were the origin of these eight strains, were low in nutrients and populations were not likely to have been exposed to high concentrations reflected in traditional enrichment strategies. This strategy of using low concentrations of electron donor and acceptor was employed in the isolation of the Td isolates by Fries *et al* (Fries et al. 1994) in this lab with similar success.

After repeated passages of active toluene-degrading cultures on toluene medium, eight different colony types emerged when streaked onto half-strength TSA plus nitrate (Table 2.2). These colonies demonstrated the ability to degrade toluene when inoculated back into BS plus NO_3^- and toluene medium. While the original mixed enrichments were able to degrade a toluene addition of 100 ppm, this concentration of toluene appeared to be toxic to the pure cultures under both aerobic and anaerobic conditions. The toluene concentration was lowered to 50 ppm and this procedure was successful and used routinely for growth. Increased biomass was obtained by respiking toluene and nitrate as needed. No additional vitamins or cofactors were required by Tol-4 for growth on toluene. There appeared to be a requirement for metals; the absence of the trace metals mixture from the medium slowed but did not completely inhibit toluene metabolism. The specific metals required for toluene degradation were not determined.

From the eight isolates obtained, strain Tol-4 was selected for further studies because of its more rapid rate of toluene utilization under denitrifying conditions in comparison to the other seven strains.

Phenotypic characteristics of strain Tol-4. Strain Tol-4 is a motile rod, fairly uniform in size when grown on toluene (1.2 μm x 0.2 μm) (Figure 2.2.). Cells are slightly longer and may form chains when grown on M-R2A or other

Table 2.2. Colony descriptions of the toluene-degrading strains isolated in this study

Strain	Colony description[a]
Tol-4	Beige, round and opaque, can be raised or flat, 1-3 mm in diameter
BL-2	Beige, mucoidal, 3-4 mm in diameter
BL-3	White, ruffled edges, 5 mm in diameter
BL-4	Beige, round opaque, 1-2 mm in diameter
BL-11	Yellow, shiny, round, 2-3 mm in diameter
2a-1	Beige, round, can be raised or flat with darker centers, 2-3 mm in diameter
3a-1	Beige, raised, opaque, darker centers, 4-5 mm in diameter
7a-1	White, round, darker centers, 2-3 mm in diameter

[a]Descriptions are based on cells grown aerobically on half-strength TSA plus NO_3^- at 30°C for 48 h.

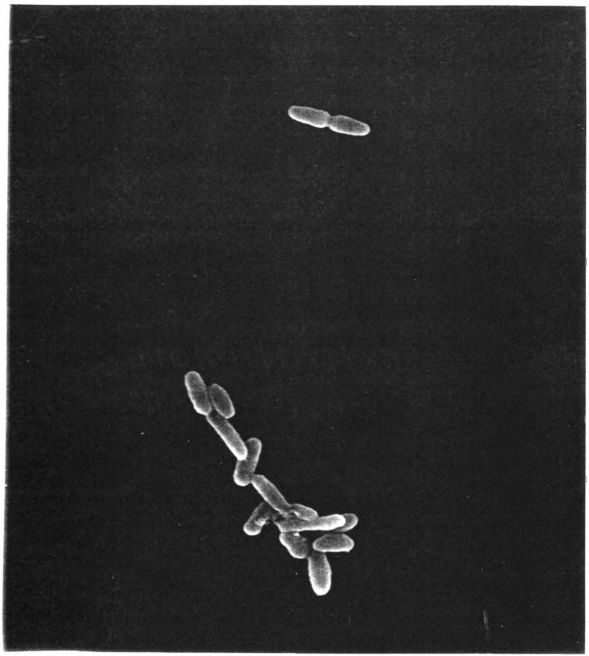


Figure 2.2. Scanning electron micrograph of *Azoarcus tolulyticus* strain Tol-4 after anaerobic growth on toluene for 24 h at 30°C. Photo was taken courtesy of SEM facility, Michigan State University.

heterotrophic media. A summary of the phenotypic characteristics of Tol-4 is in Table 2.3. The colony morphology of strain Tol-4 when grown aerobically on M-R2A was typically yellow and translucent, with darker centers and ruffled edges. Small, uniform colonies can be observed after 24 h at 30°C and after 48 h, fully-grown colonies ranged in size from 2-3 mm in diameter. Cells are gram negative, catalase and oxidase positive. Strain Tol-4 is capable of aerobic respiration and denitrification. Selenate was not used as an electron acceptor, a unique feature reported for *Thauera selenatis* (Macy et al. 1993). Growth under aerobic and anaerobic conditions was similar in MR2A broth (Figure 2.3.). The acetylene reduction assay demonstrated that Tol-4 is able to fix dinitrogen gas, a key taxonomic feature of the genus *Azoarcus*.

Substrate utilization for growth (Table 2.3.). One notable nutritional characteristic of Tol-4 and the Td isolates is the rather fastidious nature of the cells when growing on various standard heterotrophic laboratory media, especially solid media. M-R2A yields uniform colonies under both aerobic and anaerobic conditions, where the Tol-4 colony appearance is distinct from the other seven isolates obtained from Northern Michigan and the Td isolates, and is usually diagnostic. Growth also occurs readily on nutrient agar, but colonies are generally smaller and nondescript. Tol-4 is also able to grow on SM agar, a medium reported to be used for culturing other previously described species of rhizosphere-associated *Azoarcus* (Reinhold-Hurek et al. 1993), however growth of Tol-4 (and the Td isolates) is slow and colonies are small. TSA was reported as a good growth medium for previously described *Azoarcus* strains but Tol-4 growth is slow and colonies are non-uniform in appearance. PTYG, LB agar, and minimal salts agar containing glucose or a mixture of TCA cycle organic acids and acetate all yielded poor or no growth. Tol-4 can grow on benzoate

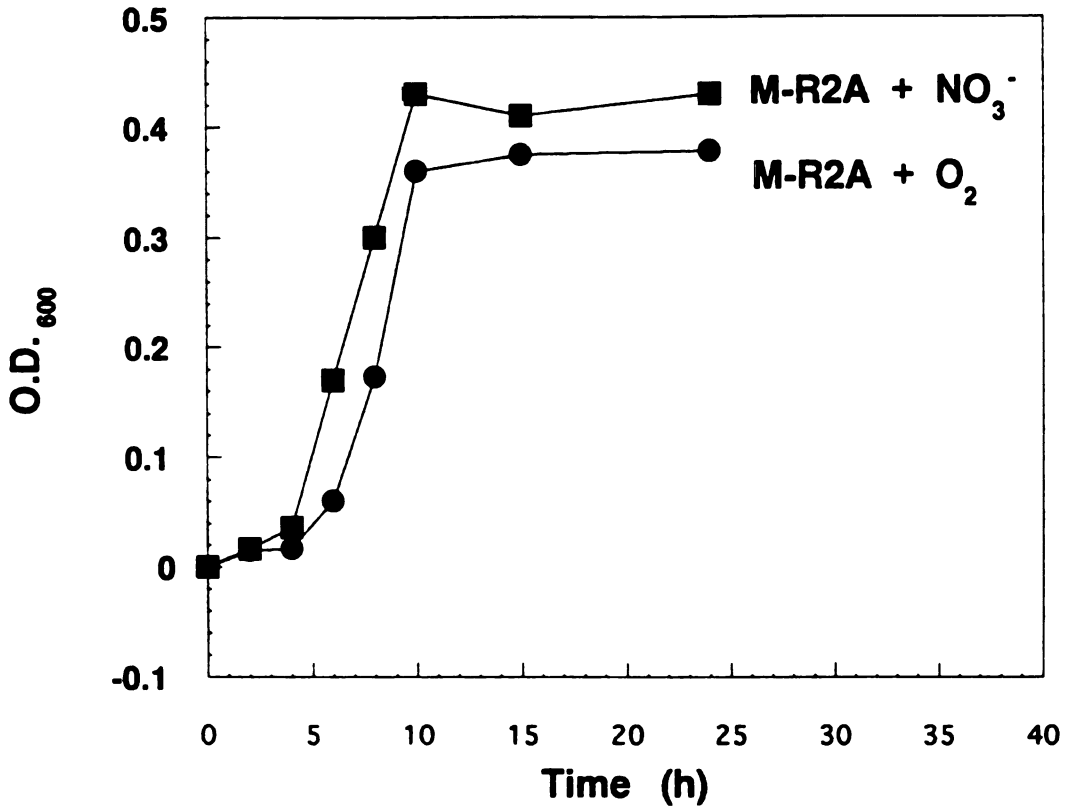


Figure 2.3. Growth curves of strain Tol-4 grown aerobically and under denitrifying conditions on M-R2A at 30°C.

Table 2.3. Characteristics of *Azoarcus tolulyticus* strain Tol-4 (Chee-Sanford et al. 1992, Fries et al. 1994, and Zhou et al. 1995)

Characteristic	Result
Cell description	Uniform rods (1.2 μm x 0.2 μm) when grown on toluene, slightly longer and tends to form chains when grown on M-R2A
Colony description	Yellow, translucent, rough edged with darker centers, approx. 2-3 mm diameter when grown aerobically at 30°C on M-R2A
Gram stain	-
Catalase	+
Oxidase	+
Motility	+
Nitrogen fixation	+
Oxygen respiration	+
N-oxide respiration:	
NO_3^- ---->N_2	+
NO_2^- ---->N_2	+
N_2O ---->N_2	+
Selenate respiration	-
H_2 autotrophy ($H_2/CO_2/O_2$)	-
Heterotrophic media (30°C):	
M-R2A	+
Tryptic Soy Agar (half-strength)	+w
Nutrient Agar	+
SM (Reinhold-Hurek et al. 1993)	+
Peptone/Tryptone/Yeast Extract/Glucose Agar	+w
BS/Acetate/Succinate/Citrate/Oxalacetate Agar	-
LB Agar	-
pH range for growth	pH 6.0-9.0
Temperature range for growth	15°C-37°C
NaCl range	0-1%

Table 2.3. (cont.).

Characteristic	Result
Substrate[a]:	
Glucose	+
Maltose	+
Mannose	-
Malate	+
Ethanol	+
Acetate	+
Succinate	+
Lactate	+
Pyruvate	+w
Benzoate	+
API/NFT[a,b]:	
Glucose	+
L-Arabinose	-
D-Mannose	-
D-Mannitol	-
N-Acetyl-D-glucosamine	-
Maltose	+
D-Gluconate	-
Caproate	-
Adipate	+
Malate	+
Citrate	-
Phenylacetate	-
Tryptophanase	-
Arginine dihydrolase	-
Urease	-
Esculin hydrolysis	+
Gelatinase	-
β-Galactosidase	-

[a]Substrates were tested aerobically at 30°C.
[b]48 h results were recorded.
w = weak growth

readily as a carbon source under both aerobic and denitrifying conditions, however in the same medium with agar added, no growth occurs. Cells can, however, grow on both liquid and solid medium containing toluene. The pH range for growth in M-R2Awas pH 6.0-9.0, temperature range 15°-37°C, and cells could grow in the presence of 0-1% NaCl, but not at 2% or higher NaCl concentrations.

Of the various carbohydrates tested, Tol-4 could utilize many sugars, organic acids and alcohols. The use of sugars by Tol-4 as carbon sources differs from reports for previously described *Azoarcus* strains (Reinhold-Hurek et al. 1993). In addition, Zhou et al. (Zhou et al. 1995) reported that Tol-4 and the Td isolates were unable to oxidize any of the substrates in the standard BIOLOG assay, including ones which supported growth in either the API/NFT assay or tube assay. Media conditions appear to be critical in assessing growth for Tol-4 and the other members of *Azoarcus tolulyticus*. It does not appear that Tol-4 requires additional growth supplements such as vitamins and other cofactors since the BS medium used in the tube assay lacked these additions and several substrates tested produced growth. Tol-4 also was unable to grow on CO_2 and H_2 under aerobic conditions, while *Thauera selenatis* was reported to grow autotrophically (Macy et al. 1993). Data from the API/NFT assay also indicate that Tol-4 was positive for esculin hydrolysis, but negative for tryptophanase, arginine dihydrolase, urease, gelatinase, and β-galactosidase activities.

In general, the major characteristics of strain Tol-4 show it to be a member of the genus *Azoarcus*. Tol-4 and members of the proposed group *Azoarcus tolulyticus* are toluene-degrading denitrifiers with varying substrate utilization patterns. Tol-4 also differs markedly from the other related toluene-degrading denitrifiers such as *Thauera aromatica* strain K172, which does not degrade toluene aerobically and was reported not to fix nitrogen gas (Anders et al. 1995).

Even among the growing group of more closely related toluene-degrading denitrifiers, one known strain, *Azoarcus aromatica* strain KB740, does not degrade toluene. The close phylogenetic relationship of all the toluene-degrading denitrifiers known to date suggest that a similar pathway for anaerobic toluene degradation in these isolates is possible. However, because significant differences do exist even among the members of this group of organisms and with the sulfate- and iron-reducing isolates, it is also reasonable to suggest that there may be more than one pathway involved, or perhaps that there are shared aspects of pathways. Strain Tol-4 is a good model to use to study the pathway due to its rapid growth on toluene anaerobically and its use as a representative of at least fifteen of the known toluene-degrading denitrifiers.

Acknowledgments

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List of References

Anders H-J, Kaetzke A, Kampfer P, Ludwig W and Fuchs G (1995) Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. Inter. J. System. Bacteriol. 45: 327-333.

Beller HR (1995) Anaerobic metabolism of toluene and other aromatic compounds by sulfate-reducing soil bacteria. Ph.D. Thesis, Stanford University.

Bonting CFC, Schneider S, Schmidtberg G and Fuchs G (1995) Anaerobic degradation of *m*-cresol via methyl oxidation to 3-hydroxybenzoate by a denitrifying bacterium. Arch. Microbiol. 164: 63-69.
Brock TD and O'Dea K (1977) Amorphous ferrous sulfide as a reducing agent for culture of anaerobes. Appl. Environ. Microbiol. 33: 254-256.

Chee-Sanford JC, Fries M and Tiedje JM (1992) Anaerobic degradation of toluene under denitrifying conditions in bacterial isolate Tol-4. In: Abstracts of the 92nd Annual Meeting of the American Society for Microbiology 1992, (p. 374), American Society for Microbiology, Washington D.C.

DeSoete G (1983) On construction of "optimal" phylogenetic trees. Zeitschnift fur Naturforshung, Section C. Biosciences (Tubingen) 38: 156-158.

Dolfing J, Zeyer J, Binder-Eicher P and Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154: 336-341.

Elder DJE and Kelly DJ (1994) The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions: Unifying trends and new perspectives. FEMS Microbiol. Rev. 13: 441-468.

Evans PJ, Mang DT, Kim KS and Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57: 1139-1145.

Evans PJ, Mang DT and Young LY (1991) Degradation of toluene and *m*-xylene and transformation of *o*-xylene by denitrifying enrichment cultures. Appl. Environ. Microbiol. 57: 450-454.

Fries MR, Zhou J-Z, Chee-Sanford JC and Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60: 2802-2810.

Fuchs G, Mohamed MES, Altenschmidt U, Koch J, Lack A, Brackmann R, Lochmeyer C and Oswald B (1994) Biochemistry of anaerobic biodegradation of aromatic compounds. In: C. Ratledge (Ed), Biochemistry of Microbial Degradation, Kluwer Academic Publishers, The Netherlands.

Grbic-Galic D (1990) Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface, and freshwater sediments. In: J. M. Bollag and G. Stotzky (Ed), Soil Biochemistry: Vol 6, (pp 117-189), Marcel Dekker, Inc., New York and Basel.

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Philips EJP and Siegel DJ (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339: 297-300.

Lovley DR and Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56: 1858-1864.

Macy JM, Rech S, Auling G, Dorsch M, Stackebrandt E and Sly LI (1993) *Thauera selenatis* gen. nov., sp. nov., a member of the beta subclass of *Proteobacteria* with a novel type of anaerobic respiration. Inter. J. System. Bacteriol. 43: 135-143.

Owens JD and Keddie RM (1969) The nitrogen nutrition of soil and herbage coryneform bacteria. J. Appl. Bact. 32: 338-347.

Rabus R, Nordhaus R, Ludwig W and Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59: 1444-1451.

Rabus R and Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163: 96-103.

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K and De Ley J (1993) *Azoarcus* gen. nov., nitrogen-fixing Proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Inter. J. System. Bacteriol. 43: 574-584.

Schocher RJ, Seyfried B, Vazquez F and Zeyer J (1991) Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. 157: 7-12.

Smibert RM and Krieg NR (1981) General characterization. In: P. Gerhardt, R. G. E. Murray, R. N. Costilowet al (Ed), Manual of Methods for General Bacteriology, (pp 409-443), American Society for Microbiology, Washington, D.C.

Young LY (1984) Anaerobic degradation of aromatic compounds. In: D. T. Gibson (Ed), Microbial Degradation of Organic Compounds, (pp 487-523), Marcel Dekker, Inc., New York.

Zhou J-Z, Fries MR, Chee-Sanford JC and Tiedje JM (1995) Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene: description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. 45:500-506.

Chapter 3

EVIDENCE FOR ACETYL COENZYME A AND CINNAMOYL COENZYME A IN THE ANAEROBIC TOLUENE MINERALIZATION PATHWAY IN *AZOARCUS TOLULYTICUS* TOL-4

The work described in this chapter was done by J. C. Chee-Sanford, with the exception of contributions by J. W. Frost to the pathway mechanism illustrated in Figure 6., M. R. Fries in the establishment of early growth conditions for Tol-4, and J. -Z. Zhou in the 16S rDNA analysis to establish the identity of the species.

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Evidence for Acetyl Coenzyme A and Cinnamoyl Coenzyme A in the Anaerobic Toluene Mineralization Pathway in *Azoarcus tolulyticus* Tol-4

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A toluene-degrading denitrifier, *Azoarcus tolulyticus* Tol-4, was one of eight similar strains isolated from three petroleum-contaminated aquifer sediments. When the strain was grown anaerobically on toluene, 68% of the carbon from toluene was found as CO_2 and 30% was found as biomass. Strain Tol-4 had a doubling time of 4.3 h, a V_{max} of 50 μmol · min^{-1} · g of $protein^{-1}$, and a cellular yield of 49.6 g · mol of $toluene^{-1}$. Benzoate appeared to be an intermediate, since F-benzoates accumulated from F-toluenes and [^{14}C]benzoate was produced from [^{14}C]toluene in the presence of excess benzoate. Two metabolites, *E*-phenylitaconic acid (1 to 2%) and benzylsuccinic acid (<1%), accumulated from anaerobic toluene metabolism. These same products were also produced when cells were grown on hydrocinnamic acid and *trans*-cinnamic acid but were not produced from benzylalcohol, benzaldehyde, benzoate, *p*-cresol, or their hydroxylated analogs. The evidence supports an anaerobic toluene degradation pathway involving an initial acetyl coenzyme A (acetyl-CoA) attack in strain Tol-4, as proposed by Evans and coworkers (P. J. Evans, W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young, Appl. Environ. Microbiol. 58:496–501, 1992) for another toluene-degrading denitrifier, strain T1. Our findings support a modification of the proposed pathway in which cinnamoyl-CoA follows the oxidation of hydrocinnamoyl-CoA, analogous to the presumed oxidation of benzylsuccinic acid to form *E*-phenylitaconic acid. Cinnamic acid was detected in Tol-4 cultures growing in the presence of toluene and [^{14}C]acetate. We further propose a second acetyl-CoA addition to cinnamoyl-CoA as the source of benzylsuccinic acid and *E*-phenylitaconic acid. This pathway is supported by the finding that monofluoroacetate added to toluene-growing cultures resulted in a significant increase in production of benzylsuccinic acid and *E*-phenylitaconic acid and by the finding that [^{14}C]benzylsuccinic acid was detected after incubation of cells with toluene, [^{14}C]acetate, and cinnamic acid. Evidence for anaerobic toluene metabolism by methyl group oxidation was not found, since benzylsuccinic acid and *E*-phenylitaconic acid were not detected after incubation with benzylalcohol and benzaldehyde, nor were benzylalcohol and benzaldehyde detected even in ^{14}C trapping experiments.

Benzene, toluene, ethylbenzene, and xylenes, collectively known as BTEX compounds, are primary contaminants of concern in aquifer water and sediments where petroleum leakages and spills have occurred. The toxicities of these compounds range in severity from causing leukemias to causing minor dermal and central nervous system effects (10). Biological schemes for cleanup of these contaminants have been designed to optimize rates of degradation by providing adequate oxygen to the habitat. Oxygen serves as an electron acceptor and a cosubstrate in the metabolism of these compounds but is usually the limiting factor in aerobic treatment because of its low solubility in water and diffusional constraints in subsurface environments (27, 34, 41). Nitrate is an attractive alternative bacterial electron acceptor because of its high solubility in water, mobility in soil, and potential for rates of degradation comparable to those under aerobic conditions.

In recent years, a number of denitrifying toluene degraders have been isolated (1, 6, 11, 17, 20, 35); however, only a few have been extensively characterized. Furthermore, defining the anaerobic toluene degradation pathway and its biochemical features has proven to be a challenge, resulting in only partial characterization of the pathways in the better-studied strains (2, 7, 17, 19, 36). Most of the denitrifying toluene degraders can also catabolize toluene under aerobic conditions (17, 20, 35). Anaerobic degradation of toluene has also been observed under Fe(III)-reducing (30), methanogenic (13, 23, 24, 40), and sulfidogenic (4, 13) conditions, demonstrating a wide range of electron acceptors that might support the anaerobic biodegradation of BTEX compounds.

The pathways and mechanisms for anaerobic metabolism of aromatic compounds, including toluene, are of considerable interest, since this metabolism must be accomplished without the involvement of oxygenases. Two pathways have been suggested for anaerobic toluene metabolism on the basis of studies with pure cultures. One pathway involves methyl group hydroxylation (2, 36), and the other involves a coenzyme A (CoA) esterification reaction (16), as the postulated first step. Evidence obtained from a mixed methanogenic consortium also indicated toluene degradation via methyl hydroxylation (12). Other pathways that could conceivably occur include hydroxylation of the ring nucleus (39), ring reduction (22), demethylation followed by ring reduction or hydroxylation (18, 24), or carboxylation, similar to phenol metabolism (37, 38). No evidence for any of these last four mechanisms has been observed in pure culture studies. Of the group of BTEX compounds, toluene has been the focus of most studies, and elucidation of its pathway may lead to further understanding of the degradation of other nonoxygenated monoaromatic compounds.

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This paper describes anaerobic toluene metabolism by a new bacterium, strain Tol-4, which was isolated from petroleum-contaminated aquifer sediment obtained from northern Michigan. Besides Tol-4, seven other aquifer strains were isolated in this study. A phylogenetic analysis based on the 16S rRNA sequence and certain physiological characteristics identified strain Tol-4 as a member of a genus of free-living nitrogen fixers, *Azoarcus* (42). It has recently been shown in this laboratory that this strain is one of eight other toluene-degrading denitrifiers (20), isolated from a variety of different sources, that form a new species, *Azoarcus tolulyticus* (42). Strain Tol-4 is able to degrade toluene under anaerobic (denitrifying) conditions and has been proposed as the type strain for this group. In this paper, we detail its physiological characteristics specific to toluene metabolism, including the complete stoichiometry for toluene degradation under denitrifying conditions. We also provide evidence for important modifications in the acetyl-CoA pathway of anaerobic toluene metabolism, namely, the involvement of cinnamoyl-CoA and a second acetyl-CoA addition to form a minor accumulating product, identified as *E*-phenylitaconic acid (32).

MATERIALS AND METHODS

Aquifer sediment sampling and characterization. BTEX-contaminated aquifer sediments collected from three sites (Bear Lake, Wexford, and Kalkaska) in northern Michigan were used for enrichment of denitrifying toluene degraders. All sites contained petroleum from oil well production and processing operations. The sediments were collected from the saturated zone in intact cores (1.5 m by 5 cm) drilled by Hunter/Keck, Inc., Cadillac, Mich., using a hollow stem auger (inner diameter, 4.25 in. [ca. 10.8 cm]). The Kalkaska sediment was sand and gravel, while the other two were primarily sand. Cores were kept sealed at 4°C until use. Sediment (10 g) was dried at 105°C until a constant weight to determine dry weight. BTEX concentrations were measured by gas chromatography with 20 g of sediment (33). Nitrate concentrations were analyzed after extracting 10 g of sediment with 100 ml of 2 M KCl. Slurries were shaken at room temperature for 1 h and filtered through no. 42 Whatman filters, and the supernatant was analyzed for NO_3^- by high-pressure liquid chromatography (HPLC) as described below.

Enrichment and isolation. The procedure used for enrichment and isolation of toluene-degrading denitrifying strains in this study was that described by Fries et al. (20), with some modifications. The toluene concentration in the initial enrichments was 1,000 μM (92 ppm), and the isolates were grown and maintained at 540 μM (50 ppm). Anaerobic growth on toluene by isolate Tol-4 was verified by growth in basal salts (BS) medium plus NO_3^-, with 0.1 mM amorphous FeS added as a reductant and scavenger of oxygen (5). Cultures of Tol-4 were maintained either on anaerobic BS-NO_3^- medium containing toluene or on aerobic M-R2A agar (20).

Degradation of toluene and other substrates. Strain Tol-4 was grown anaerobically on 0.54 mM toluene in BS-NO_3^- medium for 48 h, centrifuged at 10,000 × *g* for 15 min, and washed twice with sterile BS. Approximately 10^9 cells per ml were added to 20 ml of anaerobic BS-NO_3^- medium plus 0.54 mM toluene in Balch tubes under an O_2-free N_2 atmosphere. Uninoculated controls and triplicate cultures were used. The optical density at 600 nm was measured with a spectrophotometer (Turner, Amsco Instrument Co.), and the concentrations of toluene, nitrate, and nitrite were measured. Aerobic growth on toluene was determined by using aerobically prepared BS-toluene medium (prepared in serum bottles containing 20 ml of air headspace). The rate of anaerobic toluene consumption was measured with dense stationary-phase cell cultures. Protein was measured colorimetrically after alkaline hydrolysis of cells by the Folin reaction (25). To determine the cell yield, 300-ml cultures of Tol-4 were anaerobically grown under toluene-limiting conditions in BS plus 5 mM nitrate in sealed 500-ml flasks until toluene was completely consumed. Dry weights were determined by filtering cells and then drying them at 105°C for 2 h before weighing.

Cells grown in [U-^{14}C]toluene (specific activity, 10.2 mCi/mmol; >98%; Sigma) diluted in cold toluene in 150 ml of BS-NO_3^- medium were used to determine a carbon balance. [^{14}C]toluene was added neat (approximately 0.001 μCi/ml) 24 h prior to inoculation. Subsamples of 1 to 5 ml were removed and treated according to one of the following protocols: (i) whole sample, pH 12; (ii) unfiltered sample, N_2 purged; (iii) unfiltered sample, pH 12, N_2 purged; (iv) unfiltered sample, pH 2, N_2 purged; (v) filtered sample; (vi) filtered sample, N_2 purged; (vii) filtered sample, pH 12, N_2 purged; and (viii) filtered sample, pH 2, N_2 purged. Adjustments in pH were made by addition of either 10 N NaOH (pH 12) or 10 N HCl (pH 2), and filtered samples were collected after filtration through 0.45-μm-pore-size filters. Samples which were purged were done so under a steady stream of N_2 gas for 10 min. The radioactivity in each subsample was measured by scintillation counting (model 1500 Tri-Carb Liquid Scintillation Analyzer; Packard Instrument Co.). Radioactivity associated with cells was measured by using cells trapped on 0.45-μm-pore-size filters and included both purged and unpurged filters. Separate cultures of cells were grown anaerobically on toluene under nitrate-limiting conditions to determine nitrogen and electron balances. Toluene, nitrate, nitrite, nitrous oxide, and dinitrogen gas were measured. All experiments were done in triplicate.

Aromatic substrates for testing utilization by Tol-4 under anaerobic conditions were added at a final concentration of 0.5 mM to 40 ml of cells pregrown on toluene (72 h). Substrate concentrations were monitored periodically for up to 2 weeks. The degradation of substrates was compared with that of controls, which consisted of uninoculated BS-NO_3^- medium along with the corresponding compound. Toluene; ethylbenzene; benzene (>99.9%; Aldrich); *o*-, *m*-, and *p*-xylenes (98 to >99.9%; Aldrich); *o*-, *m*-, and *p*-cresols (99.9%; Aldrich) were added neat. Phenol; catechol; resorcinol; 4-methylcatechol; benzylalcohol (99%; Sigma); benzaldehyde; benzoate; *o*-, *m*-, and *p*-hydroxybenzylalcohols; *o*-, *m*-, and *p*-hydroxybenzaldehydes; and *o*-, *m*-, and *p*-hydroxybenzoates (Sigma) were added from 20 mM stock solutions to obtain a 0.5 mM final concentration. Hydrocinnamic acid, *trans*-cinnamic acid (Sigma), benzoate, and benzyl succinate were prepared in dilute NaOH as 10 mM stocks and added to the same final concentration. *E*-Phenylitaconic acid was synthesized and characterized as described previously (32).

Analog and substrate inhibition studies. Cultures of Tol-4 (150 ml) were pregrown anaerobically on 80 μmol of toluene and respiked with 80 μmol of toluene more plus 15 μmol of either *o*-fluorotoluene, *m*-fluorotoluene, or *p*-fluorotoluene (Sigma). Similar cultures but with the addition of benzylalcohol, benzaldehyde, or benzoate instead of the fluorinated toluenes were prepared. Nitrate was added as needed. Additional toluene and other substrates were added when depletion occurred. Subsamples were removed at regular intervals to analyze by gas and liquid chromatography for substrate disappearance and metabolites. Retention times of peaks were compared with those of authentic compounds.

Tol-4 cells were pregrown anaerobically on toluene as described above before the addition of 10, 100, or 1,000 μM monofluoroacetate (MFA). Subsamples of 1 ml were removed periodically for analysis by HPLC. Each MFA concentration was incubated as triplicate cultures. Control cultures included ones inoculated with cells with toluene added alone and a sterile control containing toluene and 1,000 μM MFA.

Isotope trapping studies using ^{14}C-labeled compounds. Dense anaerobic toluene-grown cultures (150 ml) were spiked with [*methyl*-^{14}C]toluene (500 μM, 3.3 nCi/ml) and 100 μM benzaldehyde or benzoate. Samples were removed at intervals for scintillation counting and also acidified (to pH 2 with HCl) and filtered (0.45-μm pore size) for HPLC analysis. Samples were analyzed immediately to minimize loss due to chemical oxidation. Cultures (100 ml) were also prepared by using cold toluene and [^{14}C]carboxy-acetate (10 μM, 10 nCi/ml) both with and without 100 μM *trans*-cinnamic acid. Samples were removed at intervals from these cultures for scintillation counting and for organic extraction and concentration before HPLC analysis.

Chemical analyses. Toluene and fluorinated toluenes were analyzed by gas chromatography with a flame ionization detector (Varian model 3700). Headspace samples (50 μl) were injected onto a DB-624 column (30 m by 0.543 mm; J & W Scientific), using a helium carrier (1 kg/cm^2). Toluene analysis was made with 90°C column, 200°C injector, and 200°C detector temperatures. Fluorinated toluenes were analyzed similarly but with a 50°C column temperature. Quantitation was based on comparisons with aqueous standards.

To prepare samples for metabolite analysis, culture fluids were centrifuged at 10,000 × *g* for 20 min. Cells were discarded, and the supernatant was acidified to pH 1 with H_3PO_4. The samples were solvent extracted three times with 25 ml of diethyl ether. Extracts were pooled and dried with $NaSO_4$, and the solvent was evaporated under a stream of carbon-filtered argon. The residue was dissolved in methanol-water (1:1) and filtered through 0.45-μm-pore-size filters.

Other aromatic substrates and soluble metabolites were separated and analyzed by HPLC with a UV detector (Hewlett-Packard series 1050) and a LiChrosorb RP-18 (10-μm) Hibar RT column (250 by 4 mm) (EM Separations, Gibbstown, N.J.). Culture fluids were directly filtered through 0.45-μm-pore-size filters into vials with Teflon-lined caps. Samples of 40 to 100 μl were analyzed. The solvent system was 0.1% H_3PO_4–methanol (60:40) at a flow rate of 1.5 ml/min. Wavelengths used were 218, 230, and 270 nm. Authentic standards were prepared in aqueous solutions. Retention times (in minutes) are as follows: toluene, 30.0; benzoate, 6.5; benzylalcohol, 4.3; benzaldehyde, 7.2; hydrocinnamic acid, 9.4; *trans*-cinnamic acid, 12.7; *E*-phenylitaconic acid, 9.5; and DL-benzylsuccinic acid, 7.5. ^{14}C-labeled compounds were detected by using a radiotracer detector (1N/US Systems, Inc.) along with the HPLC system for organic compounds described above.

Nitrate and nitrite concentrations were measured at 210 nm by HPLC with a Partisil 10 SAX column (Whatman) and a mobile phase of 50 mM phosphate (pH 3.0). Samples were filtered (0.45-μm pore size) and diluted 100-fold. Nitrous oxide was measured in a gas chromatograph equipped with a ^{63}Ni electron capture detector (Perkin-Elmer 910 gas chromatograph; 95% argon–5% methane carrier; flow rate, 15 ml · min^{-1}). Headspace samples of 0.5 ml were injected onto a stainless steel Porapak Q column (1.8 m by 0.32 cm), and 55°C column,

TABLE 1. Aquifer sediments used for enrichments and denitrifying toluene-degrading isolates obtained from each sediment

Source[a]	Depth (m)	Description	Initial NO_3^- concn (ppm [dry wt])	Isolate(s)
Bear Lake	24–25	Fine- to medium-grain sand; 5% moisture	0.47	Tol-4, BL-2, BL-3, BL-4, BL-11
Wexford	8.5–10	Fine- to medium-grain sand with small gravel; 10% moisture	0.23	2a-1
Kalkaska	2.4–4	Sand and gravel, silt; 11.5% moisture	0.34	3a-1, 7a-1

[a] BTEX compounds were detected in all samples but were present at less than 10 ppb (sediment basis) each.

60°C injector, and 300°C detector temperatures were used. N_2 gas was measured with a gas chromatograph (Carle Gas Chromatograph, Porapak Q column, argon carrier) equipped with a thermal conductivity detector.

RESULTS

Enrichment and isolation of strains. Benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes were detected in all three aquifer sediments, but the concentrations were very low (<10 ppb). Nitrate was also detected in the three sediments (Table 1). Eight strains of denitrifying toluene degraders were isolated from the three sediments (Table 1). The eight strains were differentiated by colony and cell morphologies on M-R2A. All strains were confirmed to grow anaerobically on toluene by observing turbidity increases and toluene consumption in BS-NO_3^- medium plus toluene under a headspace of oxygen-free argon. Strain Tol-4 was selected for further characterization since it had the highest rate of anaerobic toluene degradation among the eight strains. In addition, strain Tol-4 grew aerobically on toluene.

To verify the ability of strain Tol-4 to grow and degrade toluene under strict anaerobiosis with NO_3^- as the electron acceptor, amorphous FeS was added as a reductant to anaerobically prepared BS-NO_3^- medium plus toluene. The presence of the reductant and incubation of the tubes in an anaerobic chamber eliminated any possibility of oxygen in the cultures. Separate cultures grown in the absence of reductant showed that anaerobic consumption of toluene was directly dependent on *N*-oxides, since toluene removal ceased completely when NO_3^-, NO_2^-, and N_2O were depleted and resumed only when more NO_3^- was added (Fig. 1). Dinitrogen gas was the final N-containing product of denitrification. Strain Tol-4 was also able to use NO_2^- and N_2O as sole electron acceptors for its anaerobic growth on toluene, but growth was poor compared with growth with NO_3^- as the electron acceptor (data not shown).

Characteristics of growth on toluene. Additional characteristics of anaerobic growth on toluene, including a stoichiometric balance, were determined for Tol-4. Concentrations of toluene above 0.54 mM (50 ppm) were inhibitory. The maximum cell density under anaerobic conditions as measured by optical density (600 nm) was reached at approximately 45 h, and toluene reached a nondetectable concentration by 40 h (Fig. 2). Strain Tol-4 reduced nitrate to nitrite first; a nearly stoichiometric accumulation of nitrite often occurred under toluene-limiting conditions. The doubling time was 4.3 h.

Strain Tol-4 degraded 50 μmol of toluene per min per g of protein when grown anaerobically on toluene. On the basis of the actual cell yield (49.6 g per mol of toluene) obtained from anaerobic growth on toluene, and assuming half reactions for cell formation and substrate oxidation of $C_5H_7O_2N$ (standard cell formula [26]) + $8H_2O \rightarrow 5CO_2 + NH_3 + 20H^+ + 20e^-$, $C_7H_8 + 14H_2O \rightarrow 7CO_2 + 36H^+ + 36e^-$, and $2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$, 24.4% of the electrons from toluene would have been used for biomass synthesis, or the

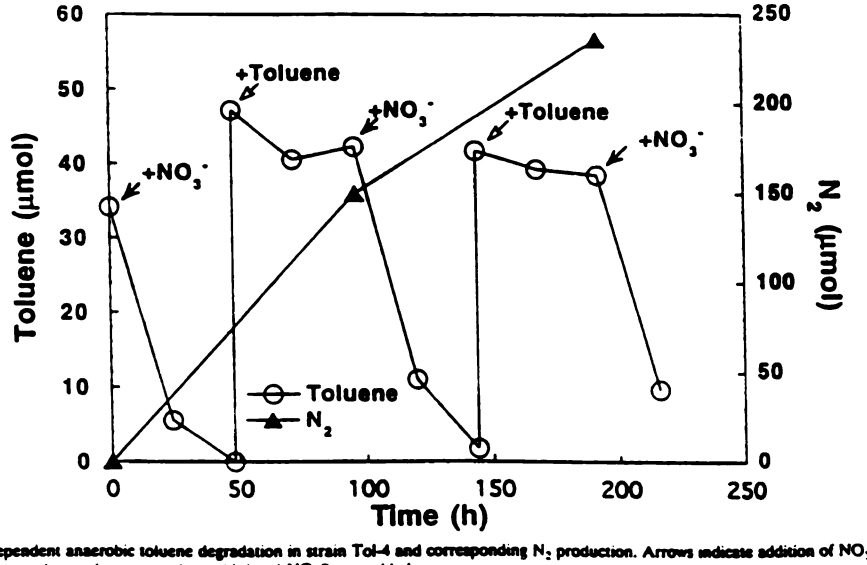

FIG. 1. Nitrate-dependent anaerobic toluene degradation in strain Tol-4 and corresponding N_2 production. Arrows indicate addition of NO_3^- or toluene. NO_3^-, NO_2^-, and N_2O were not detected at times when additional NO_3^- was added.

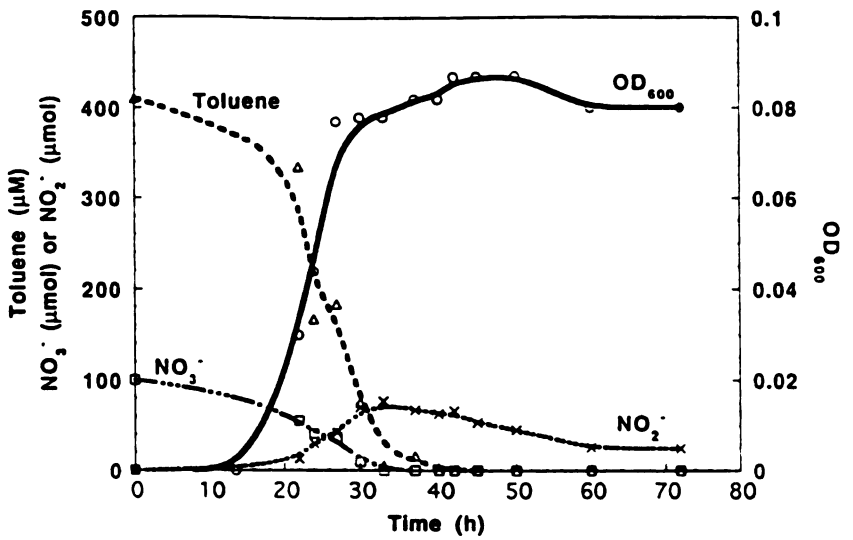

FIG. 2. Pattern of growth of strain Tol-4, toluene consumption, and depletion of nitrate. The doubling time was calculated to be 4.3 h. OD_{600}, optical density at 600 nm.

fraction of electrons used for biomass synthesis (f_s) equals 0.244 (8, 31). The following stoichiometry is obtained: C_7H_8 + 5.43 NO_3^- + 0.44NH_3 + 5.43H^+ → 4.78CO_2 + 2.73N_2 + 5.79H_2O + 0.44$C_5H_7O_2N$.

The stoichiometry was supported by the nitrogen and electron balances obtained when cells were grown anaerobically on toluene under NO_3^--limiting conditions (Table 2). The total N recovered as denitrification products was 96%. The N unaccounted for was probably a small amount of N_2. On the basis of the total amount of electrons from toluene and the electrons accounted for in electron acceptors, the fraction of the electrons transferred to electron acceptors (f_e) is 0.791. The sum of the two experimentally obtained values, f_e (0.791) and f_s (0.244), is 1.035, which matched closely the theoretical sum f_e + f_s = 1.000.

Cells grown anaerobically on [^{14}C]toluene demonstrated that carbon from toluene was mineralized to CO_2 and incorporated into biomass (Table 3). The remaining 2.3% of label that was unaccounted for may be present as nonvolatile, soluble metabolites but in amounts too small to quantify. The percent carbon mineralized was 68%, which matched precisely the value predicted from the stoichiometry given above. The percent carbon incorporated into cells was 29%, which also correlated well with the 31% predicted from the same stoichiometric balance. This stoichiometry also confirms that oxygen is not involved in the reaction.

TABLE 2. Nitrogen and electron balances for strain Tol-4 grown anaerobically on toluene under nitrate-limiting conditions

Substrate	Product	Amt used or produced (μmol)	Amt of electrons transferred (μmol)
Toluene		42	1,512
NO_3^-		554	
	NO_2^-	470	940
	N_2O	32	256
	N_2	ND[a]	
Total N and electrons recovered		534	1,196

[a] ND, not detected.

Other aromatic substrate use. Several other aromatic compounds were used by strain Tol-4 as growth substrates under the same conditions used for anaerobic toluene degradation (Table 4). Benzene; ethylbenzene; *o*-, *m*-, and *p*-xylenes; *o*- and *m*-cresols; phenol; resorcinol; and *o*-hydroxybenzoate were not used by inocula pregrown on toluene. Catechol and *p*-cresol were consumed after a lag period of 24 h. Hydrocinnamic acid; *trans*-cinnamic acid; benzylalcohol; benzaldehyde; benzoate; *o*-hydroxybenzylalcohol and -benzaldehyde; *m*-hydroxybenzylalcohol, -benzaldehyde, and -benzoate; and *p*-hydroxybenzylalcohol, -benzaldehyde, and -benzoate were all degraded immediately. Some of these substrates were metabolized more slowly, as they were not completely removed in 2 weeks. Benzylsuccinic acid was not metabolized at the 500 μM concentration tested.

Metabolite production and substrate inhibition of toluene degradation. When cells were grown on toluene alone, no transient metabolites were detected. However, a small amount of an accumulating metabolite consistently appeared in culture fluids when cells were grown anaerobically on toluene. This compound was identified as *E*-phenylitaconic acid (32) and was not detected during aerobic growth on toluene. Culture fluids

TABLE 3. Carbon-14 balance for strain Tol-4 grown anaerobically under toluene-limiting conditions

Location of ^{14}C label	Value	
	dpm (10^3)	%
Initial toluene	360 ± 2.2	100
Remaining toluene	2.55 ± 0.032	0.7
CO_2	243 ± 1.20	67.6
Cells	106 ± 1.45	29.4
Total label recovered	352	97.7

TABLE 4. Substrate use, as measured by HPLC, under anaerobic denitrifying conditions with a toluene-grown inoculum of strain Tol-4

Substrate[a]	% Loss (time)[b]
Toluene	>99 (72 h)
p-Cresol[c]	>99 (2 wk)
Catechol[c]	37 (2 wk)
Benzylalcohol	>99 (72 h)
Benzaldehyde	>99 (72 h)
Benzoate	>99 (72 h)
o-OH-benzylalcohol	91 (1 wk)
o-OH-benzaldehyde	>99 (2 wk)
m-OH-benzylalcohol	76 (2 wk)
m-OH-benzaldehyde	>99 (1 wk)
m-OH-benzoate	98 (2 wk)
p-OH-benzylalcohol	>99 (2 wk)
p-OH-benzaldehyde	99 (2 wk)
p-OH-benzoate	34 (2 wk)
Hydrocinnamic acid	>99 (5 days)
trans-Cinnamic acid	>99 (5 days)

[a] Substrates were added at a final concentration of 0.5 mM. Benzene, ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene, *o*-cresol, *m*-cresol, phenol, resorcinol, and *o*-OH-benzoate were not degraded.

[b] Percent losses are maximum values at the time indicated and after subtraction for abiotic losses as determined with the uninoculated controls.

[c] Depletion began after a 24-h lag.

TABLE 5. Metabolites detected during anaerobic growth on toluene or with other substrates in the presence or absence of toluene

Substrate(s)[a]	Metabolites detected[b]	Time of detection (h)
Toluene	Benzylsuccinic acid	48
	E-Phenylitaconic acid	48
Benzoate	None	
Hydrocinnamic acid	Benzylsuccinic acid	96
	E-Phenylitaconic acid	96
trans-Cinnamic acid	Benzylsuccinate	96
	E-Phenylitaconic acid	96
DL-Benzylsuccinate	None	
Benzylalcohol	None	
Toluene + benzylalcohol	None	
Benzaldehyde	None	
Toluene + benzaldehyde	Benzoate	3 (transient)
	E-Phenylitaconic acid	48
o-Cresol	None	
Toluene + *o*-cresol	*E*-Phenylitaconic acid	48
	o-Hydroxybenzoate	48
m-Cresol	None	
Toluene + *m*-cresol	None	
p-Cresol	None	
Toluene + *p*-cresol	*E*-Phenylitaconic acid	48
	p-Hydroxybenzoate	48
Toluene + *o*-F-toluene	*E*-Phenylitaconic acid	24
	o-F-Benzoate	24
	o-F-Phenylitaconic acid[c]	24
Toluene + *m*-F-toluene	*E*-Phenylitaconic acid	24
	m-F-Benzoate	24
Toluene + *p*-F-toluene	*E*-Phenylitaconic acid	24
	p-F-Benzoate	24

[a] All compounds were tested at 500 μM, with the exception of the fluorinated toluenes, which were tested at 100 μM. The inoculum was grown anaerobically on toluene before testing.

[b] Metabolites were identified by finding HPLC retention times the same as those of authentic standards. Benzylsuccinate was found in concentrations ranging from 2.4 to 3.0 μM, and *E*-phenylitaconic was found in concentrations ranging from 3.8 to 6.0 μM at the times indicated. Benzoate compounds were not quantified.

[c] Identity based on similarities to *E*-phenylitaconic acid in accumulation and retention time.

were analyzed for metabolites after growth on a variety of possible intermediates and substrate analogs, some in combination with toluene (Table 5). When cells metabolized benzylalcohol, benzaldehyde, and benzoate in the absence of toluene, no detectable metabolites, including *E*-phenylitaconic acid, were observed. Either benzylalcohol or benzaldehyde added to cultures growing on toluene altered the rate of toluene degradation (Fig. 3). The addition of benzaldehyde in the presence of toluene resulted in the transient appearance of a small amount of benzoate (<20 μM), and toluene metabolism was slowed. The presence of benzylalcohol completely inhibited toluene metabolism, and the metabolism of benzylalcohol was also stopped. In isotope trapping experiments, a small amount of [^{14}C]benzoate (7 μM) appeared transiently after 30 min when cold benzoate was added with [^{14}C]toluene (Table 6). [^{14}C]benzoate was not detected in cultures containing labeled toluene and cold benzaldehyde. ^{14}C-labeled benzylalcohol and benzaldehyde were not detected at any time in these cultures.

Cultures grown on either hydrocinnamic acid or *trans*-cinnamic acid produced *E*-phenylitaconic acid and benzylsuccinic acid (Table 5). In isotope trapping experiments using toluene and [^{14}C]acetate, a small transient peak tentatively identified by HPLC as cinnamic acid (0.1 μM) appeared after 10 min (Table 6). Cultures which contained toluene, cinnamic acid, and [^{14}C]acetate produced [^{14}C]benzylsuccinic acid after 30 min.

o- and *p*-cresols were metabolized in the presence of toluene, with some *o*- and *p*-hydroxybenzoate, respectively, detected. The latter compound accumulated (Table 5). *o*-Hydroxybenzoate was not metabolized when added alone to cultures, but this compound was only transient when cells were grown on *o*-cresol in the presence of toluene. *E*-Phenylitaconic acid was detected only when toluene was metabolized. *m*-Cresol inhibited toluene degradation and was not metabolized in the presence of toluene. Growth on hydrocinnamic acid and *trans*-cinnamic acid resulted in production of *E*-phenylitaconic acid plus a second metabolite that we identified as benzylsuccinate on the basis of it having the HPLC retention time and UV absorption characteristics of the authentic standard.

Fluorinated toluenes were used as structural analogs to toluene. In the presence of toluene and *o*-, *m*-, or *p*-fluorotoluene, *o*-, *m*-, and *p*-fluorobenzoate, respectively, accumulated (Table 5). Only in cultures containing *o*-fluorotoluene did a product accumulate along with the usual (nonfluorinated) *E*-phenylitaconic acid. This product was presumed to be an analogous fluorinated *E*-phenylitaconic acid because its retention time and UV absorption ratios at wavelengths of 218, 230, and 275 nm were similar to those of *E*-phenylitaconic acid. The amount of product was too small to confirm this structure by other methods.

To test the effect of tricarboxylic acid cycle intermediates on anaerobic toluene metabolism, MFA was added to cultures growing on toluene. MFA readily condenses with oxaloacetate to form fluorocitrate, a specific and potent inhibitor of aconitase, which is near the entry point of the tricarboxylic acid cycle (3). In cultures containing 10, 100, and 1,000 μM concentrations of MFA, toluene metabolism was slowed (Fig. 4a). The larger amounts of benzylsuccinic acid and *E*-phenylitaconic acid that accumulated correlated with higher MFA concentrations (Fig. 4b and c).

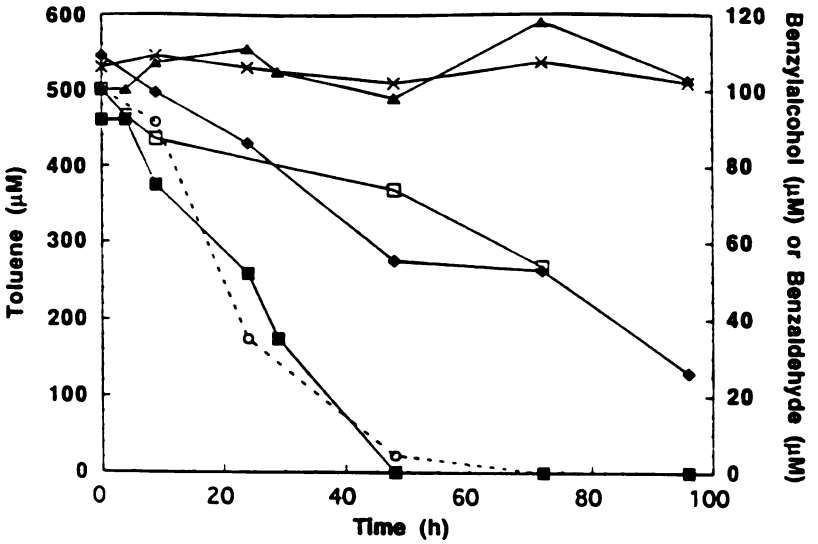

FIG. 3. Benzylalcohol consumption in the presence of toluene (▲) and its effect on toluene degradation (×), benzaldehyde consumption in the presence of toluene (■) and its effect on toluene degradation (◆) under anaerobic conditions, benzylalcohol degradation in the absence of toluene (□), and toluene degradation in a control culture containing toluene alone (○).

DISCUSSION

Denitrifiers capable of anaerobic toluene metabolism may be common in BTEX-exposed aquifers, since they were isolated from all three sites studied. All sources used in our enrichments were primarily sand taken from 2- to 25-m depths and were low in organic matter content and microbial density, yet all yielded denitrifying toluene degraders. Five of eight isolates, including Tol-4, were enriched from the deepest sediment core drilled. We have isolated other denitrifying toluene degraders, but they have been isolated from a variety of surface environments which were more carbon rich and supported a larger microbial population (20). Finding these organisms in a variety of surface and subsurface environments suggests that these organisms and this catabolic process must be widespread.

The absence of oxygen involvement in anaerobic toluene degradation was confirmed by growth of Tol-4 in FeS-reduced BS-NO_3^- medium and routine incubation of cultures in an anaerobic chamber. Our experiments have consistently shown that toluene degradation proceeded only when *N*-oxides were present as electron acceptors.

Toluene was completely mineralized to CO_2 and converted into biomass by strain Tol-4. The carbon, electron, and nitrogen balance predicts well the actual amount of carbon mineralized and cells produced from toluene degradation under denitrifying conditions. In the ^{14}C-labeling study, 98% of the label could be recovered as CO_2 and cell material. By difference, the 2% of label that was unaccounted for was thought to include the water-soluble metabolite *E*-phenylitaconic acid. Direct analysis showed that strain Tol-4 converted between 1 and 2% of the toluene carbon to *E*-phenylitaconic acid (32). Benzylsuccinic acid accumulated in even smaller amounts than *E*-phenylitaconic acid; it was detected in growth medium after several feedings of toluene and concentration of solvent extracts of large volumes of culture fluid. In contrast, strain T1, another toluene-degrading denitrifier, was reported to convert 17% of the carbon from toluene to benzylsuccinic acid and benzylfumaric acid (16, 19). The difference between our strains and strain T1 in the quantities of these dioic acids produced may be due to differences in growth conditions or factors affecting the flux of intermediates leading to the formation of the accumulating products.

TABLE 6. Metabolites produced by cells from ^{14}C-isotope trapping

Substrates[a]	Metabolite	Amt (μM)
[^{14}C]toluene + benzaldehyde	None	
[^{14}C]toluene + benzoate	[^{14}C]benzoate	7
Toluene + [^{14}C]acetate	Cinnamic acid	0.1
Toluene + [^{14}C]acetate + cinnamic acid	[^{14}C]benzylsuccinic acid	ND[b]

[a] The concentrations of substrates used were as follows: toluene, 500 μM; benzaldehyde, 100 μM; benzoate, 100 μM; acetate, 10 μM; and cinnamic acid, 100 μM.
[b] ND, not determined.

The accumulation of benzylsuccinic acid and benzylfumaric acid from toluene by strain T1 was the key evidence which led Evans and coworkers to propose an anaerobic toluene pathway involving an initial acetyl-CoA attack on toluene to form hydrocinnamoyl-CoA (also known as phenylpropionyl-CoA) as the first intermediate (16). They further proposed that an analogous reaction between toluene and succinyl-CoA would be followed by hydrolysis of the CoA ester to form benzylsuccinic acid, which could then be further oxidized to the dead-end metabolite benzylfumaric acid. The identification of *E*-phenylitaconic acid as the accumulating metabolite produced by strain Tol-4 during anaerobic toluene metabolism (32) and the position of the double bond in this metabolite relative to the position of this double bond in benzylfumaric acid lead to several possible modifications to the previously proposed pathway for toluene mineralization. The production of benzylsuccinic acid along with *E*-phenylitaconic acid by strain Tol-4 reasonably suggests an oxidation reaction whereby benzylsuccinic acid (Fig. 5, structure III) is directly oxidized to form

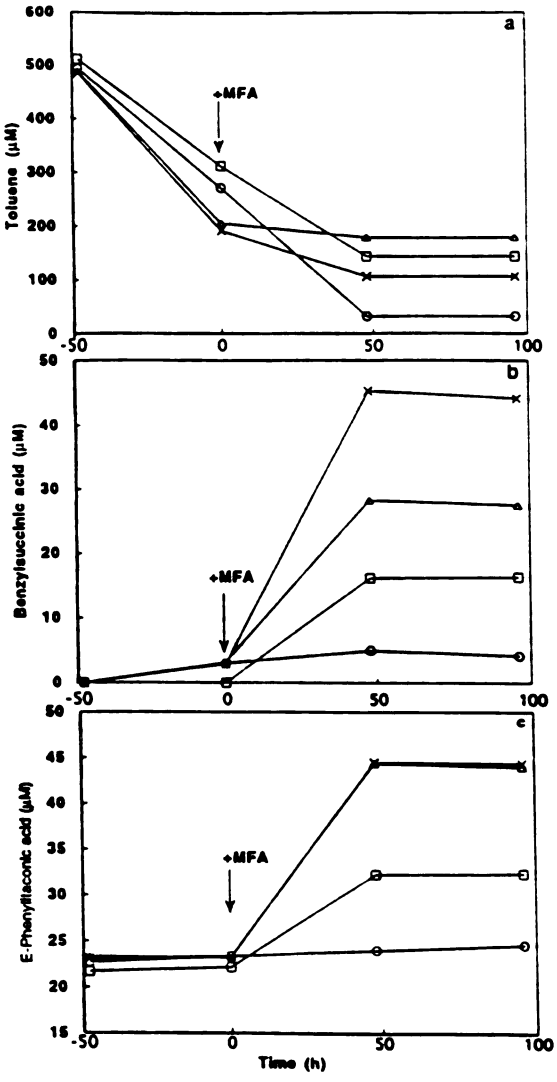

FIG. 4. Effects of 10 μM (□), 100 μM (△), and 1,000 μM (×) MFA on anaerobic toluene-growing cultures. O, toluene only. (a) toluene consumption; (b) benzylsuccinate production; (c) *E*-phenylitaconic acid production.

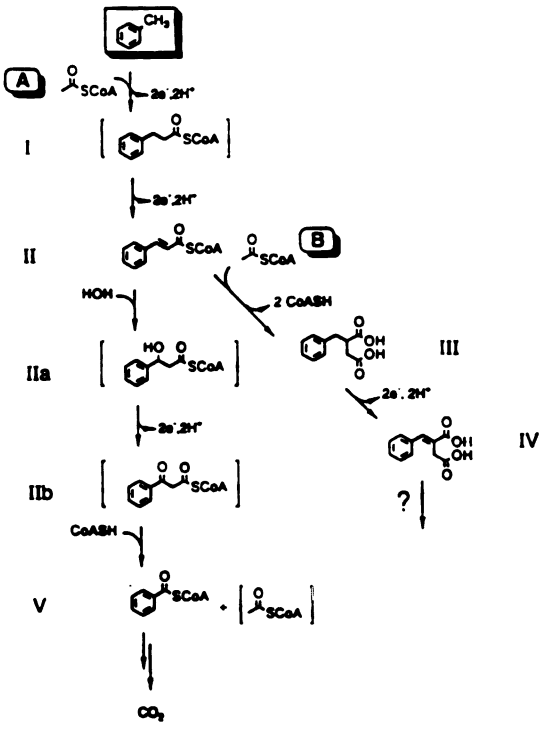

FIG. 5. Proposed mineralization pathway (A) and formation of *E*-phenylitaconic acid (B) from the anaerobic degradation of toluene by strain Tol-4. I, hydrocinnamoyl-CoA; II, cinnamoyl-CoA; IIa, β-hydroxycinnamoyl-CoA; IIb, β-ketocinnamoyl-CoA; III, benzylsuccinic acid; IV, *E*-phenylitaconic acid; V, benzoyl-CoA. Brackets indicate hypothetical intermediates. Cinnamic acid and benzoic acid, rather than the corresponding CoA thioesters, were detected.

E-phenylitaconic acid (Fig. 5, structure IV). Strain Tol-4 did not appear to metabolize benzylsuccinic acid; however, this may be due to the absence of transport systems to take up the compound or to the lack of a ligase to form the corresponding CoA ester.

The oxidation step occurring between benzylsuccinic acid and *E*-phenylitaconic acid could provide a lead on how the two-carbon analog hydrocinnamic acid is metabolized. The same dehydrogenase mechanism, and even perhaps the same enzyme, could oxidize hydrocinnamoyl-CoA (Fig. 5, structure I) to cinnamoyl-CoA (Fig. 5, structure II). To evaluate our pathway hypothesis, we fed *trans*-cinnamic acid as well as hydrocinnamic acid to Tol-4. The cells grew on both substrates and produced both benzylsuccinic acid and *E*-phenylitaconic acid from both substrates, consistent with the hypothesis. The larger amount of benzylsuccinic acid detected, compared with that formed from toluene, might be due to a larger pool of both proposed reactants, acetyl-CoA and cinnamic acid (Fig. 5, path B), when hydrocinnamic acid and cinnamic acid were used than would be the case when toluene is the substrate (Fig. 5, path A). Further evidence to suggest the involvement of cinnamic acid (or its CoA ester) was its detection in cultures incubated with toluene and [^{14}C]acetate. Also consistent with the hypothesis of a branch pathway forming *E*-phenylitaconic acid was the detection of [^{14}C]benzylsuccinic acid when cells were incubated with toluene, [^{14}C]acetate, and *trans*-cinnamic acid.

Cultures grown in the presence of [^{14}C]toluene and benzoate resulted in the transient appearance of [^{14}C]benzoate. Labeled benzoate was not detected when benzaldehyde was added with [^{14}C]toluene in parallel studies. The evidence for benzoate as an intermediate was further supported by the production of F-benzoates from F-toluenes and hydroxylated benzoates from cresol metabolism. The appearance of benzoate may still be consistent with our proposed pathway, since it is not yet clear whether benzoate is formed directly as an intermediate in the pathway prior to CoA esterification or whether it is formed in equilibrium with its CoA ester during toluene metabolism. A small amount of benzoic acid was re-

ported to be excreted transiently during *trans*-cinnamic acid metabolism by a photosynthetic bacterium, *Rhodopseudomonas palustris* (15). This excretion and subsequent uptake were suggested to be linked to cell regulation and the photometabolism of aromatic acids in these cells, which involve aromatic CoA ligase activities (14). Similar CoA ligase reactions may also occur in Tol-4 as part of the mechanism for its aromatic acid degradation. The small amounts of benzoate and cinnamic acid detected in Tol-4 studies may suggest a similar regulatory mechanism.

Altenschmidt and Fuchs proposed that strain K172 degraded toluene anaerobically via methyl group oxidation to form benzylalcohol followed by oxidation to benzaldehyde (2). Seyfried and coworkers supported this scheme by detecting benzaldehyde and benzoate and, in addition, reported the appearance of benzylsuccinic acid and benzylfumaric acid from anaerobic toluene metabolism by strain K172 and another bacterium, strain T (36). The amount of benzylsuccinic acid and benzylfumaric acid produced by strains K172 and T was reported to be 0.5%. Our studies showed that benzylalcohol and benzaldehyde were used as substrates by Tol-4; however, no detectable metabolites, including *E*-phenylitaconic acid and benzylsuccinic acid, were ever seen. In fact, benzylalcohol was not metabolized in the presence of toluene and, furthermore, inhibited toluene metabolism in Tol-4. In addition, incubating [^{14}C]toluene with benzaldehyde did not produce any labeled intermediates, including benzylalcohol and benzaldehyde. These results and the absence of benzylsuccinic acid and *E*-phenylitaconic acid as products of benzylalcohol or benzaldehyde degradation do not lend support to a methyl hydroxylation pathway for toluene metabolism in strain Tol-4. However, we cannot completely rule out reactions involving methyl group oxidation because of the difficulty of making negative conclusions based on the use of exogenous substrates, especially when toxicity might have been involved, such as appeared to be the case with benzylalcohol.

Succinyl-CoA was proposed to be the cosubstrate with toluene in the formation of benzylsuccinic acid in strain T1 (16). The addition of MFA to strain T1 resulted in the inhibition of both toluene utilization and formation of benzylsuccinic acid and benzylfumaric acid (19). These results were consistent with a hypothesis of an MFA-induced decline in succinyl-CoA availability. We found that MFA, when added to Tol-4 cultures, resulted in the inhibition of toluene degradation but a stimulation in benzylsuccinic acid and *E*-phenylitaconic acid production. The increased production of benzylsuccinic acid and *E*-phenylitaconic acid may be due to the increased availability of acetyl-CoA to act as a substrate in both pathways A and B, since its use in the tricarboxylic acid cycle was blocked (Fig. 5). This is in contrast to what might be expected if succinyl-CoA was key to the formation of the accumulating metabolites. A reaction between monofluoroacetyl-CoA and either toluene or cinnamic acid could proceed through steps leading to the formation of both fluorinated benzylsuccinyl-CoA and *E*-phenylitaconyl-CoA, both of which may be blocked from further reactions. Our analysis would be unable to distinguish between the combined fluorinated and nonfluorinated analogs of benzylsuccinic acid and *E*-phenylitaconic acid if the fluorinated analogs were indeed produced. We also did not observe an accumulation of benzoate in the presence of MFA as reported for strain T1.

Our results also argue against other hypothesized anaerobic toluene degradation pathways. The mechanism involving hydroxylation of the ring nucleus to form cresols is unlikely, since simultaneous adaptation and trapping studies using cresols and toluene-induced cells either did not show use of the compound, showed a lag phase before onset of degradation, or failed to produce transient or accumulating products. These results suggested that ring hydroxylation reactions with toluene were doubtful as first-step reactions. Carboxylation of the ring, similar to the *para*-carboxylation mechanism in phenol degradation, is also not likely, since Tol-4 does not catabolize phenol nor was toluene degradation stimulated when CO_2 was provided in a bicarbonate-carbonate-buffered system (data not shown). Since benzene was not detected as a transient metabolite and was not utilized for growth, it is also unlikely that demethylation of toluene occurred.

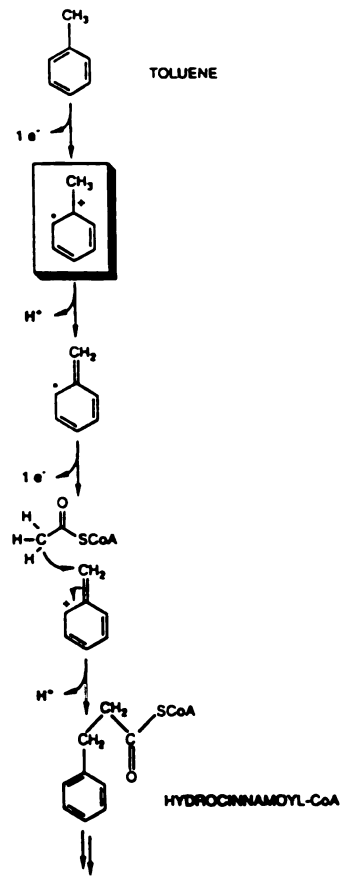

FIG. 6. Proposed mechanism of aryl cation (boxed structure) formation represented by the first step of the reaction between toluene and acetyl-CoA to form hydrocinnamoyl-CoA.

The results obtained from our studies suggest that the pathway illustrated in Fig. 5 is a reasonable one. In addition, this pathway gains further support on the basis of its chemical feasibility. One chemical mechanism suggested for toluene oxidation under anaerobic conditions involves the generation of an aryl cation radical (22, 28). This single electron transfer reaction is then followed by reaction with a nucleophile, as in the first step of the proposed pathway involving acetyl-CoA (Fig. 6). Cation radicals could also, in theory, be generated during the oxidation of hydrocinnamoyl-CoA to form cinnamoyl-CoA, as well as in the analogous oxidation of benzylsuccinyl-CoA to form *E*-phenylitaconyl-CoA. Rather than

speculate on the exact nature of these enzyme-mediated reactions, we arbitrarily assign such oxidations as involving the release of two electrons and two protons from a metabolite. Further oxidation of cinnamoyl-CoA (Fig. 5, structure II) may follow a mechanism analogous to fatty acid β-oxidation, which was suggested by Evans and coworkers to be involved in the further mineralization of hydrocinnamoyl-CoA (phenylpropionyl-CoA) (16). 1,4-Addition of water to cinnamoyl-CoA would form β-hydroxycinnamoyl-CoA (Fig. 5, structure IIa). Oxidation of the alcohol and reaction of the resulting β-ketocinnamoyl-CoA (Fig. 5, structure IIb) with CoASH would generate benzoyl-CoA and acetyl-CoA. Studies performed with *R. palustris* suggested an analogous β-oxidation mechanism in its metabolism of hydrocinnamic acid and *trans*-cinnamic acid (15). The intermediates formed in anaerobic toluene degradation prior to ring reduction steps may also be central in the metabolism of other aromatic compounds. Such channelling strategies may be important in anaerobic metabolism of aromatic compounds, notably in pathways leading to benzoyl-CoA (1, 9, 21, 29).

Our proposed pathway (Fig. 5) places the branch point leading to the formation of the dioic acid products at cinnamoyl-CoA (structure II). An alternative branch point at hydrocinnamoyl-CoA could also be chemically feasible; however, this was ruled out, in large part because of resulting products that would be isomeric to but chemically distinct from benzylsuccinic acid and *E*-phenylitaconic acid. Additionally, since significant percentages of toluene are apparently converted to dioic acid by-products by a variety of anaerobic toluene degraders, attributing the formation of these compounds to dead-end metabolism may be premature. Benzylsuccinic acid and *E*-phenylitaconic acid, in the form of their CoA esters, may actually occupy key positions along the main pathway for anaerobic toluene mineralization. Such a pathway would be essentially similar to the one we propose for toluene mineralization except that the CoA adduct generated along with benzoyl-CoA from further oxidation of *E*-phenylitaconic acid (or its CoA ester) would be succinyl-CoA rather than acetyl-CoA.

The challenge presented to microorganisms with compounds like toluene is in the ability of the organisms to mediate reactions that begin with destabilizing the highly conjugated, stable aromatic structure in order to facilitate further catabolism and a potential gain in energy. Coenzymes are widely known to provide the requisite chemical reactivities in many enzyme-mediated reactions. A reaction between acetyl-CoA and toluene in the first step of mineralization would be one way to activate the structure for further oxidation while possibly precluding the cell from having to use a high-energy phosphate bond (e.g., that in ATP hydrolysis). The chemical mechanism in Fig. 6 shows the release of two electrons in the steps leading to the formation of hydrocinnamoyl-CoA, which may potentially be used in energy-gaining reactions by the cell. The release of one molecule of acetyl-CoA in the oxidation step leading to benzoyl-CoA formation (Fig. 5) would also allow the cells to recycle this substrate for further toluene catabolism. The nature of the first enzyme involved in an addition of acetyl-CoA is intriguing but yet unknown, and there is not yet direct evidence that hydrocinnamoyl-CoA is produced from this reaction. Also intriguing is the possibility that benzylsuccinic acid and *E*-phenylitaconic acid, seemingly common products among the anaerobic toluene degraders, play major roles as intermediates in toluene mineralization.

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REFERENCES

1. **Altenschmidt, U., and G. Fuchs.** 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. 156: 152–158.
2. **Altenschmidt, U., and G. Fuchs.** 1992. Anaerobic toluene oxidation to benzylalcohol and benzaldehyde in a denitrifying *Pseudomonas* strain. J. Bacteriol. 174:4860–4862.
3. **Becker, W. M.** 1983. Aerobic production of ATP: the TCA cycle, p. 323–358. *In* G. Zubay (ed.), Biochemistry. Addison-Wesley Publishing Co., Inc., Reading, Mass.
4. **Beller, H. R., D. Grbic-Galic, and M. Reinhard.** 1992. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. Appl. Environ. Microbiol. 58:786–793.
5. **Breck, T. D., and K. O'Dea.** 1977. Amorphous ferrous sulfide as a reducing agent for culture of anaerobes. Appl. Environ. Microbiol. 33:254–256.
6. **Chee-Sanford, J. C., M. Fries, and J. M. Tiedje.** 1992. Anaerobic degradation of toluene under denitrifying conditions in bacterial isolate Tol-4, p. 374. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
7. **Chee-Sanford, J. C., and J. M. Tiedje.** 1994. Detection and identification of metabolites produced by the denitrifying isolate *Azoarcus* sp. (Tol-4), p. 447. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
8. **Criddle, C. S., L. M. Alvarez, and P. L. McCarty.** 1991. Microbial processes in porous media, p. 641–691. *In* J. Bear and M. Y. Corapcioglu (ed.), Transport processes in porous media. Kluwer Academic Publishers, Dordrecht, The Netherlands.
9. **Dangel, W., R. Brackman, A. Lack, M. Mohamed, J. Koch, B. Oswald, B. Seyfried, A. Tschech, and G. Fuchs.** 1991. Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch. Microbiol. 155:256–262.
10. **Dean, B. J.** 1985. Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mutat. Res. 154:153–181.
11. **Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach.** 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154:336–341.
12. **Edwards, E. A., A. M. Edwards, and D. Grbic-Galic.** 1994. A method for detection of aromatic metabolites at very low concentrations: application to detection of metabolites of anaerobic toluene degradation. Appl. Environ. Microbiol. 60:323–327.
13. **Edwards, E. A., and D. Grbic-Galic.** 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60: 313–322.
14. **Elder, D. J. E., and D. J. Kelly.** 1994. The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions: unifying trends and new perspectives. FEMS Microbiol. Rev. 13:441–468.
15. **Elder, D. J. E., P. Morgan, and D. J. Kelly.** 1992. Anaerobic degradation of *trans*-cinnamate and ω-phenylalkanecarboxylic acids by the photosynthetic bacterium *Rhodopseudomonas palustris*: evidence for a β-oxidation mechanism. Arch. Microbiol. 157:148–154.
16. **Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young.** 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58:496–501.
17. **Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young.** 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57:1139–1145.
18. **Evans, W. C., and G. Fuchs.** 1988. Anaerobic degradation of aromatic compounds. Annu. Rev. Microbiol. 42:289–317.
19. **Fraser, A. C., W. Ling, and L. Y. Young.** 1993. Substrate induction and metabolite accumulation during anaerobic toluene utilization by the denitrifying strain T1. Appl. Environ. Microbiol. 59:3157–3160.
20. **Fries, M. R., J.-Z. Zhou, J. C. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802–2810.
21. **Fuchs, G., M. E. S. Mohamed, U. Altenschmidt, J. Koch, A. Lack, R. Brackmann, C. Lochmeyer, and B. Oswald.** 1994. Biochemistry of anaerobic biodegradation of aromatic compounds, p. 513–553. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. **Grbic-Galic, D.** 1990. Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface, and freshwater sedi-

ments, p. 117–189. *In* J. M. Bollag and G. Stotzky (ed.), Soil biochemistry, vol. 6. Marcel Dekker, Inc., New York.

23. **Grbic-Galic, D.** 1990. Methanogenic transformation of aromatic hydrocarbons and phenols in groundwater aquifers. Geomicrobiol. J. 8:167–200.
24. **Grbic-Galic, D., and T. M. Vogel.** 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. 53:254–260.
25. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
26. **Hoover, R., and N. Porges.** 1952. Assimilation of dairy wastes by activated sludge. Sew. Ind. Waste 24:306–312.
27. **Hutchins, S. R., W. C. Downs, J. T. Wilson, G. B. Smith, D. A. Kovacs, D. D. Fine, R. H. Douglass, and D. J. Hendrix.** 1991. Effect of nitrate addition on biorestoration of fuel-contaminated aquifer: field demonstration. Groundwater 29:571–580.
28. **Kochi, J. K., R. T. Tang, and T. B. Bernath.** 1973. Mechanisms of aromatic substitution. Role of cation-radicals in the oxidative substitution of arenes by cobalt(III). J. Am. Chem. Soc. 95:7114–7123.
29. **Lochmeyer, C., J. Koch, and G. Fuchs.** 1992. Anaerobic degradation of 2-aminobenzoic acid (anthranilic acid) via benzoyl-coenzyme A (CoA) and cyclohex-1-enecarboxyl-CoA in a denitrifying bacterium. J. Bacteriol. 174: 3621–3628.
30. **Lovley, D. R., and D. J. Lonergan.** 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56:1858–1864.
31. **McCarty, P. L.** 1975. Stoichiometry of biological reactions. Prog. Water Technol. 7:157–172.
32. **Migaud, M. E., J. C. Chee-Sanford, J. M. Tiedje, and J. W. Frost.** 1996. Benzylfumaric, benzylmaleic, and *Z*- and *E*-phenylitaconic acids: synthesis, characterization, and correlation with a metabolite generated by *Azoarcus tolulyticus* Tol-4 during anaerobic toluene degradation. Appl. Environ. Microbiol. 62:974–978.
33. **Ree, V. D., M. J. Lacy, and J. D. Stuart.** 1989. Manual headspace method to analyze for the volatile aromatics of gasoline in groundwater and soil samples. Anal. Chem. 61:2584–2585.
34. **Schink, B.** 1988. Principles and limits of anaerobic degradation: environmental and technological aspects, p. 771–846. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.
35. **Schocher, R. J., B. Seyfried, F. Vazquez, and J. Zeyer.** 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. 157:7–12.
36. **Seyfried, B., G. Glod, R. Schocher, A. Tschech, and J. Zeyer.** 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60:4047–4052.
37. **Tschech, A., and G. Fuchs.** 1987. Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch. Microbiol. 148: 213–217.
38. **Tschech, A., and G. Fuchs.** 1989. Anaerobic degradation of phenol via carboxylation to 4-hydroxybenzoate: in vitro study of isotope exchange between $^{14}CO_2$ and 4-hydroxybenzoate. Arch. Microbiol. 152:594–599.
39. **Vogel, T. M., and D. Grbic-Galic.** 1986. Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. Appl. Environ. Microbiol. 52:200–202.
40. **Wilson, B. H., G. B. Smith, and J. F. Rees.** 1986. Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. 20:997–1002.
41. **Zehnder, A. J. B. and W. Stumm.** 1988. Geochemistry and biogeochemistry of anaerobic habitats, p. 1–38. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.
42. **Zhou, J.-Z., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene: description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. 45:500–506.

CHAPTER 3 APPENDICES

The following appendices resulted from my work involving the characterization of the natural metabolites formed during anaerobic toluene degradation by strain Tol-4. These compounds were identified as benzylsuccinate and *E*-phenylitaconate. *E*-phenylitaconate was distinguished from three other isomers, *Z*-phenylitaconate, benzylfumarate, and benzylmaleate, described in Migaud *et al.* 1995 (Appendix A). This work resulted in the unequivocal identification of this metabolite and helped lead to the development of the anaerobic toluene degradation pathway proposed in this chapter.

APPENDIX A

BENZYLFUMARIC, BENZYLMALEIC, AND *Z*- AND *E*-PHENYLITACONIC ACIDS: SYNTHESIS, CHARACTERIZATION, AND CORRELATION WITH A METABOLITE GENERATED BY *AZOARCUS TOLULYTICUS* TOL-4 DURING ANAEROBIC TOLUENE DEGRADATION.

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Benzylfumaric, Benzylmaleic, and *Z*- and *E*-Phenylitaconic Acids: Synthesis, Characterization, and Correlation with a Metabolite Generated by *Azoarcus tolulyticus* Tol-4 during Anaerobic Toluene Degradation

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E*-Phenylitaconic acid has been isolated as a metabolite generated by *Azoarcus tolulyticus* Tol-4 along with benzylsuccinic acid during anaerobic degradation of toluene. Strain Tol-4 converted 1 to 2% of toluene carbon to *E*-phenylitaconate and benzylsuccinate (10:1). The identification of *E*-phenylitaconic acid was based on ^{1}H nuclear magnetic resonance (NMR) characterization of degradation products derived from ^{13}C-labeled toluene followed by comparison of spectroscopic and chromatographic data for the isolated, unlabeled metabolite with those for chemically synthesized benzylfumaric acid, benzylmaleic acid, *E*-phenylitaconic acid, and *Z*-phenylitaconic acid. Spectroscopic comparisons included ^{1}H NMR, ^{13}C NMR, and nuclear overhauser effect correlations. High-pressure liquid chromatography (HPLC) retention times and HPLC coinjections with synthetic dioic acids provided another reliable line of evidence for structure assignment. The formation of *E*-phenylitaconic acid differs from previous reports of benzylfumaric acid generation along with benzylsuccinic acid during anaerobic microbial degradation of toluene. This has important implications relevant to elaboration of the metabolic route for anaerobic toluene degradation by strain Tol-4 and related organisms. Similar amounts of *E*-phenylitaconic acid were also produced by seven other strains of *A. tolulyticus*.

Benzylsuccinic acid and benzylfumaric acid (compound 1a [Fig. 1]) have been reported to accumulate during anaerobic degradation of toluene under denitrifying conditions by strain T1 (8), *Pseudomonas* sp. strain T (18), and *Thauera aromatica* K172 (1, 18) as well as under sulfate-reducing conditions by strain PRTOL (3, 4). A newly characterized microbe, *Azoarcus tolulyticus* Tol-4 (5), has likewise been discovered to accumulate two metabolites during anaerobic degradation of toluene under denitrifying conditions. One of these metabolites was identified as benzylsuccinic acid (5). However, identification of the second metabolite proved to be more elusive. There was little doubt that the unknown metabolite was either a phenylmethylbutenedioic acid (compounds 1a and 2a [Fig. 1]) or a phenylmethylenebutanedioic acid (compounds 3a and 4a [Fig. 1]). However, beyond the carbon backbone of the second metabolite, the location and substitution pattern of the double bond were open to question. Benzylfumaric acid [*E*-(phenylmethyl)butenedioic acid] (compound 1a), benzylmaleic acid [*Z*-(phenylmethyl)butenedioic acid] (compound 2a), *E*-phenylitaconic acid [*E*-(phenylmethylene)butanedioic acid] (compound 3a), and *Z*-phenylitaconic acid [*Z*-(phenylmethylene)butanedioic acid] (compound 4a) all had to be considered as candidate structures for the second metabolite (Fig. 1).

The previous identification (8, 18) of benzylfumaric acid as a product formed during anaerobic microbial degradation of toluene relied primarily on characterization by using electron impact mass spectrometry (EIMS). However, the use of this spectroscopic technique to distinguish between the structures of the individual phenylmethylbutenedioic acids (compounds 1a and 2a) and phenylmethylenebutanedioic acids (compounds 3a and 4a) was potentially problematic. Double-bond migration and interconversion of *E* and *Z* isomers are phenomena that are documented to occur during MS analysis of olefins (7). Double-bond migration and isomerization would not be a problem with nuclear magnetic resonance (NMR) analysis. However, literature ^{1}H and ^{13}C NMR spectral information was available for only one of the dioic acids (12). The derivatization techniques used in previous analyses of metabolites formed during anaerobic toluene degradation were also a cause for concern. Acid-catalyzed migration and isomerization of the double bonds during derivatization of the dioic acids to the corresponding diesters would greatly complicate the assignment of structures to the degradation metabolites.

Our efforts to identify the unknown metabolite relied heavily on synthesized, authentic samples of benzylfumaric acid (compound 1a), benzylmaleic acid (compound 2a), *E*-phenylitaconic acid (compound 3a), *Z*-phenylitaconic acid (compound 4a), and the corresponding dimethyl diesters (compounds 1b to 4b) (Fig. 1). Published synthetic routes to individual dioic acids and diesters span approximately 30 years of chemical literature (2, 6, 11, 13, 15). Some protocols required substantial optimization to afford the desired products. One instance of misassigned ^{1}H NMR data in the literature (6) was discovered subsequent to synthesis and characterization of all of the dioic acids and diesters. Dioic acids 1a, 3a, and 4a were synthesized as the corresponding dimethyl diesters 1b, 3b, and 4b, and this was followed by hydrolysis under basic conditions to afford the free dioic acids. Because of problematic double-bond migration during base hydrolysis of its dimethyl diester 2b, benzylmaleic acid (compound 2a) was synthesized by deprotection of the corresponding trimethylsilyl diester.

Derivatization of the synthesized dioic acids as dimethyl diesters and subsequent MS analyses were carefully examined.

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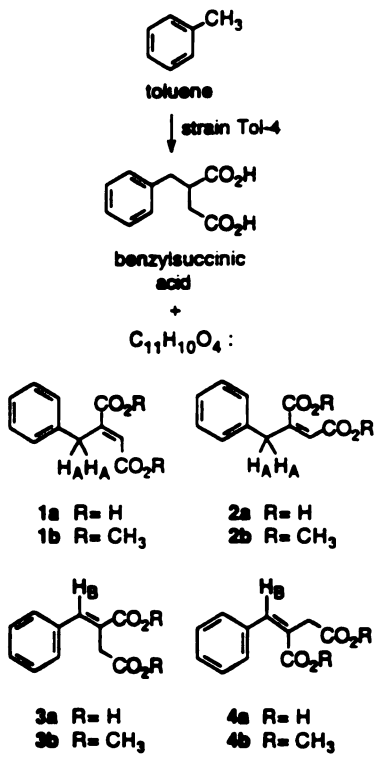

FIG. 1. Possible structures for the second metabolite formed during anaerobic degradation of toluene by *A. tolulyticus* Tol-4.

Ultimately, synthesized phenylmethylenebutanedioic acids and diesters were distinguished from phenylmethylbutenedioic acids and diesters by high-pressure liquid chromatography (HPLC) separation and use of ^{1}H NMR and nuclear overhauser effect (NOE) correlations. These techniques also differentiated between *E* and *Z* isomers within each class of dioic acid and diester. The combined use of ^{1}H NMR, ^{13}C NMR, and HPLC comparisons with synthetic samples ultimately led to the assignment of *E*-phenylitaconic acid (compound 3a) as the structure of the metabolite formed along with benzylsuccinic acid during anaerobic degradation of toluene by strain Tol-4. The identification of *E*-phenylitaconic acid has prompted the proposal of a modified toluene mineralization pathway for strain Tol-4 (5).

MATERIALS AND METHODS

Culture preparation of *A. tolulyticus* Tol-4. Cultures (100 ml) of strain Tol-4 were grown anaerobically in basal salts medium (17) plus 5 mM NO_3^- with 500 μM toluene (98%; Sigma) under a headspace of argon. Cultures were incubated in sealed serum bottles at 30°C. For metabolite analysis, toluene and NO_3^- were added as needed until a total of 500 μmol of toluene was consumed. [*methyl*-^{13}C]toluene (99%; Cambridge Isotope Laboratories) was substituted in order to assess ^{13}C-labeled metabolites by the procedure described above. Separate cultures grown in aerobically prepared basal salts-toluene medium in the absence of NO_3^- were also used for metabolite analysis. Cultures were extracted with ethyl acetate and concentrated for analysis as specified below.

To test the ability of strain Tol-4 to metabolize the dioic acids, cells were grown on 500 μM toluene as described above for 24 h at 30°C. After gas chromatography confirmed toluene depletion, toluene and dioic acids were added to final concentrations of 200 μM toluene and 50 μM *E*-phenylitaconic acid, *Z*-phenylitaconic acid, benzylfumaric acid, or benzylmaleic acid. Samples (1 ml) were removed for HPLC analysis at time zero and then daily for 1 week. After 1 week, cultures were extracted with diethyl ether as specified below. Control cultures consisted of anaerobic toluene-grown cells, one culture to which additional toluene was added, and a second culture to which dioic acids were added. The dioic acids were added from stock solutions prepared in anaerobic basal salts medium and stored at 4°C until use. Authentic undiluted compounds were stored at −20°C.

Metabolite extraction. Cultures were centrifuged at 10,000 × *g* for 20 min at 4°C, and cells were discarded. The supernatant was acidified by addition of 2.5 ml of 10 M H_3PO_4 followed by extraction three times with either ethyl acetate or diethyl ether. The organic fraction was dried with Na_2SO_4 and the solvent was removed under a stream of argon. The residue was dissolved in 1 ml of CH_3OH-H_2O (1:1) and filtered (0.45-μm pore size). Chromatographic analysis and purification of metabolites for spectroscopic characterization followed isocratic elution of a LiChrosorb RP-18 column (10-μm pore size; 4-mm inner diameter [i.d.]; 25-cm length) with H_2O (0.1% in H_3PO_4)-CH_3OH (60:40) with a Hewlett-Packard 1050 HPLC. The metabolite fraction was collected and extracted with hexane as described above. After concentration, the residue was dissolved in ethyl acetate (3 ml) and stored at 4°C until analyzed.

General chemistry. See reference 14 for general experimental information dealing with synthetic manipulations. Photochemical reactions were run in a Rayonet apparatus with an RMR 300-nm light source. Gas chromatography (GC) data were collected on a Hewlett-Packard 5890 apparatus with a DB-1 column (0.25-mm i.d.; 30-m length). HPLC purifications of synthetic dioic acids and diesters were performed on a Rainin instrument with a Microsorb reverse-phase C_{18} semipreparative column (5-μm pore size, 21.4-mm i.d.; 25-cm length). Measurements of retention times and conjections of synthetic dioic acids and diesters employed a Microsorb reverse-phase C_{18} analytical column (5-μm pore size; 4.6-mm i.d.; 25-cm length). ^{1}H NMR spectra were recorded on either a 300- or 500-MHz spectrometer. Chemical shifts for ^{1}H NMR are reported in parts per million relative to internal tetramethylsilane (δ = 0.0 ppm) when $CDCl_3$ was the solvent and relative to $HD_2C(O)CD_3$ (δ = 2.04 ppm) when acetone-d_6 was the solvent. ^{13}C NMR spectra were recorded at 75 or 125 MHz. Chemical shifts for ^{13}C NMR spectra are reported in parts per million relative to $CDCl_3$ (δ = 77.0 ppm) or $CD_3C(O)CD_3$ (δ = 29.8 ppm) in acetone-d_6. Rotating frame overhauser effect spectra were recorded in the phase-sensitive mode at 500 MHz and a controlled temperature (±0.1°C). A mixing time of 0.08 s was used, while the pulse delay was maintained at 2 s. A spectral window of about 4,000 Hz was used in both the f_1 and f_2 dimensions, and increments of 16 scans were collected. The synthetic procedures and identifying characteristics for each of the synthesized standards follow.

Dimethyl *E*-(phenylmethyl)butenedioate (compound 1b) and dimethyl *Z*-(phenylmethyl)butenedioate (compound 2b). A 2 N solution of benzyl magnesium chloride (12.0 mmol) in dry tetrahydrofuran was added dropwise over 10 min to a suspension of CuI (2.28 g, 12.0 mmol) in tetrahydrofuran (25 ml) kept at −40°C under Ar (15). The heterogeneous solution was stirred for 1 h at −40°C. Dimethyl acetylenedicarboxylate (1.42 g, 10.0 mmol) in tetrahydrofuran (5 ml) was subsequently added to the reaction mixture, which immediately turned dark red. After the mixture was stirred for 2 h at −40°C, the reaction was quenched by addition of a saturated NH_4Cl solution (40 ml), the mixture was slowly warmed to room temperature, and the organic soluble products were extracted three times with ether (40 ml). The combined organic layers were washed with brine, dried, and concentrated to an oil which was purified first by radial chromatography (hexane) and then by reverse-phase HPLC. Dimethyl benzylfumarate (compound 1b) (0.73 g, 31%) and dimethyl benzylmaleate (compound 2b) (0.94 g, 40%) were obtained as oils. The characteristics of dimethyl benzylfumarate were as follows: ^{1}H NMR ($CDCl_3$) δ 7.30 to 7.05 (m, 5 H), 6.80 (s, 1 H), 4.13 (s, 2 H), 3.73 (s, 3 H), 3.66 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 166.9, 166.0, 145.9, 138.0, 128.8, 128.3, 126.7, 126.3, 52.5, 51.8, 32.9; MS *m/z* (relative intensity) EI 91 (12), 115 (92), 174 (35), 202 (100), 234 (14, M^+); electron impact high-resolution MS [HRMS (EI)] calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0890; combustion analysis calculated (Anal. Calcd) for $C_{13}H_{14}O_4$, C 66.66 and H 6.02. Found C 66.62 and H 6.03. The characteristics of dimethyl benzylmaleate were as follows: ^{1}H NMR ($CDCl_3$) δ 7.15 to 7.35 (m, 5 H), 5.66 (s, 1 H), 3.77 (s, 3 H), 3.70 (s, 3 H), 3.66 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 168.7, 165.4, 149.0, 135.5, 129.3, 128.7, 127.2, 121.1, 52.3, 51.8, 40.0; MS *m/z* (relative intensity) EI 91 (20), 115 (100), 174 (35), 202 (98), 234 (5, M^+); HRMS (EI) calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0893; Anal. Calcd for $C_{13}H_{14}O_4$, C 66.66 and H 6.02. Found C 66.39 and H 6.03.

Dimethyl *E*-(phenylmethylene)butanedioate (compound 3b). Na metal (0.51 g, 22.0 mmol) was slowly added to CH_3OH (50 ml) maintained at 0°C under Ar (11). After complete disappearance of Na, dimethyl succinate (1.61 g, 11.0 mmol), immediately followed by benzaldehyde (1.06 g, 10 mmol), was added. The solution was warmed to room temperature, stirred for 1 h, and then heated at reflux for 2 h. After the reaction mixture was cooled to 0°C, a saturated NH_4Cl solution (25 ml) was added; this was immediately followed by acidification to pH 2 with the dropwise addition of HCl (1 N). The aqueous layer was then extracted three times with ether (50 ml), and the combined organic fractions were washed with brine, dried, and concentrated to an oil which was purified by radial chromatography (hexane). Pure dimethyl *E*-phenylitaconate (compound 3b) was obtained as a yellow oil (1.52 g, 65%): ^{1}H NMR ($CDCl_3$) δ 7.85 (s, 1 H), 7.40 to 7.45 (m, 5 H), 3.77 (s, 3 H), 3.66 (s, 3 H), 3.52 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ

171.4, 167.6, 141.9, 134.8, 125.9, 128.8, 128.5, 125.8, 52.1, 52.0, 33.3; MS *m/z* (relative intensity) EI 91 (25), 115 (95), 174 (67), 202 (82), 234 (100, M^+); HRMS (EI) calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$: C 66.66 and H 6.02, found C 66.94 and H 6.09.

Dimethyl *Z*-(phenylmethylene)butanedioate (compound 4b). Dimethyl *E*-phenylitaconate (0.34 g, 1.45 mmol) was added to a solution of $CHCl_3$ (10 ml) and acetone (5 ml). After dissolved O_2 was removed by bubbling Ar through the solution for 20 min, the solution was irradiated (2) at 300 nm for 12 h at room temperature. Dimethyl *Z*-phenylitaconate was isolated as the single product of the reaction along with some unreacted material. Purification by radial chromatography (hexane) led to the isolation of dimethyl *Z*-phenylitaconate (compound 4b) (0.20 g, 60%): ^1H NMR ($CDCl_3$) δ 7.45 to 7.35 (m, 5 H), 6.88 (s, 1 H), 3.71 (s, 3 H), 3.63 (s, 3 H), 3.47 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 171.1, 168.1, 139.5, 135.4, 128.5, 128.2, 128.1, 128.0, 126.3, 52.0, 51.6, 40.6; MS *m/z* (relative intensity) EI 91 (23), 115 (100), 174 (72), 202 (83), 234 (97, M^+); HRMS (EI) calculated for $C_{13}H_{14}O_4$ (M^+) 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$: C 66.66 and H 6.02. Found C 66.49 and H 6.08.

Bis(trimethylsilyl)-2-butynedioate. Acetylene dicarboxylic acid (5.70 g, 50.0 mmol) was dissolved (10, 16) under Ar in 33 ml of a tetrahydrofuran-hexane solution (1:2, vol/vol) and slowly added to a solution of hexamethyldisilazane (5.40 g, 33.5 mmol) dissolved in hexane (25 ml). The mixture was stirred for 15 min and then filtered. Pure bistrimethylsilyl acetylenedicarboxylate was obtained as a yellow oil after removal of the solvents (8.97 g, 97%): ^1H NMR ($CDCl_3$) δ 0.24 (s); ^{13}C NMR ($CDCl_3$) δ 150.6, 74.8, −0.5.

***E*-(Phenylmethyl)butenedioic acid (compound 1a).** Dimethyl benzylfumarate (compound 1b) (0.23 g, 1.00 mmol) was dissolved in 10 ml of a tetrahydrofuran-aqueous NaOH (10 mM) solution (4:1, vol/vol). The deprotection was quenched after 12 h of stirring at room temperature by acidification with 5 ml of aqueous HCl (1 N). After extraction of the organic soluble dioic acid three times with ether (10 ml), the combined ether fractions were washed with brine and dried. Pure benzylfumaric acid (compound 1a) was obtained as an oil (0.20 g, 97%) after removal of the solvents: ^1H NMR (CD_3COCD_3) δ 7.31 (d, J = 10 Hz, 2 H), 7.24 (dd, J = 10, 10 Hz, 2 H), 7.14 (dd, J = 10, 10 Hz, 1 H), 6.85 (s, 1 H), 4.17 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 167.9, 167.1, 146.7, 139.4, 129.6, 129.0, 127.8, 127.0, 33.2; MS *m/z* (relative intensity) fast atom bombardment (FAB) 205 (100, M − H^+), 161 (40), 117 (4), 91 (0.5); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 (M − H^+), found 205.04959.

***Z*-(Phenylmethyl)butenedioic acid (compound 2a).** A solution of 2 N benzyl magnesium chloride (11.5 mmol) in dry tetrahydrofuran was added dropwise over 10 min to a suspension of CuI (2.20 g, 11.5 mmol) in tetrahydrofuran (40 ml) maintained at −40°C under Ar (15). The heterogeneous solution was stirred for 1 h at −40°C. Bistrimethylsilyl acetylenedicarboxylate (2.48 g, 9.61 mmol) in tetrahydrofuran (10 ml) was subsequently added to the reaction mixture, which immediately turned dark red. After the mixture was stirred for 2 h at −40°C, the reaction was quenched by addition of a saturated NH_4Cl solution (40 ml) immediately followed by acidification to pH 2 with dropwise addition of HCl (1 N). The heterogeneous solution was then slowly warmed to room temperature, and the organic soluble products were extracted three times with ether (40 ml). Benzylmaleic acid (compound 2a) was the only dioic acid observable in the crude extract after the combined organic layers were washed with brine, dried, and concentrated. Pure benzylmaleic acid was obtained by reverse-phase HPLC purification as an oil (0.79 g, 40%): ^1H NMR (CD_3COCD_3) δ 7.22 to 7.35 (m, 5 H), 5.84 (s, 1 H), 3.71 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 169.5, 166.9, 149.6, 137.5, 130.1, 129.4, 127.6, 122.7, 40.8; MS *m/z* (relative intensity) FAB 205 (100, M − H^+), 161 (25), 117 (2.5), 91 (2); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 (M − H^+), found 205.05080.

***E*-(Phenylmethylene)butanedioic acid (compound 3a).** Dimethyl *E*-phenylitaconate (compound 3b) (0.23 g, 1.00 mmol) was dissolved in 10 ml of a tetrahydrofuran-aqueous NaOH (10 mM) solution (4:1, vol/vol). After 15 min of stirring at room temperature, the deprotection was quenched by acidification to pH 2 with 1 N aqueous HCl. The organic soluble dioic acid was immediately extracted three times with ether, and the organic layer was then washed with brine and dried. After removal of the solvents, *E*-phenylitaconic acid (compound 3a) was crystallized from $CHCl_3$ as a white solid (0.19 g, 93%): ^1H NMR (CD_3COCD_3) δ 7.92 (s, 1 H), 7.35 to 7.55 (m, 5 H), 3.56 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 172.4, 168.8, 141.8, 136.1, 129.9, 129.7, 129.5, 127.8, 33.8; MS *m/z* (relative intensity) FAB 205 (100, M − H^+), 161 (43), 117 (6), 91 (5); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 (M − H^+), found 205.05037.

***Z*-(Phenylmethylene)butanedioic acid (compound 4a).** Dimethyl *Z*-phenylitaconate (compound 4b) (0.23 g, 1.00 mmol) was dissolved in 10 ml of a tetrahydrofuran-aqueous NaOH (10 mM) solution (4:1, vol/vol). The deprotection was quenched after 12 h of stirring at room temperature by acidification with 5 ml of aqueous HCl (1 N). After extraction of the organic soluble dioic acid three times with ether (10 ml), the combined ether fractions were washed with brine and dried. Pure *Z*-phenylitaconic acid (compound 4a) was obtained as an oil (0.18 g, 88%) after removal of the solvents: ^1H NMR (CD_3COCD_3) δ 7.4 (d, J = 10 Hz, 2 H), 7.20 to 7.34 (m, 3 H), 6.89 (s, 1 H), 3.44 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 173.0, 169.6, 138.5, 136.7, 129.6, 128.6, 41.5; MS *m/z* (relative intensity) FAB 205 (35, M − H^+), 161 (17), 117 (9), 91 (1); HRMS (FAB) calculated for $C_{11}H_9O_4$ 205.05008 (M − H^+), found 205.05091. Another method (13) to synthesize *Z*-phenylitaconic acid (compound 4a) from *E*-phenylitaconic acid (compound 3a) was used to confirm the structural assignment of compound 4a.

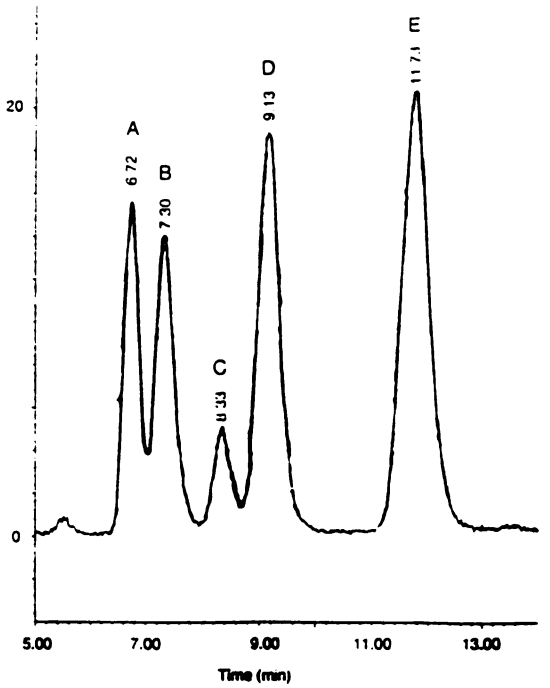

FIG. 2. HPLC analysis and retention times (minutes) of a mixture containing chemically synthesized *Z*-phenylitaconic acid (A), benzylmaleic acid (B), benzylfumaric acid (C), benzylsuccinic acid (D), and *E*-phenylitaconic acid (E). Samples were analyzed on a reverse-phase C_{18} analytical column with UV detection at 218 nm and an isocratic eluting solvent composed of 60% phosphate buffer (0.1% H_3PO_4 in water) and 40% methanol.

E-Phenylitaconic acid (0.21 g, 1 mmol) was dissolved in 10 ml of an acetone-H_2O solution (1:1, vol/vol) containing 1.1 equivalents of Na_2HCO_3. Dissolved O_2 was removed by bubbling Ar through the solution for 20 min, after which the solution was irradiated for 12 h. The reaction mixture was extracted three times with ether (15 ml), and the combined ether fractions were washed with brine, dried, and concentrated. *Z*-Phenylitaconic acid (0.12 g, 60%) was separated from *E*-phenylitaconic acid (0.08 g, 40%) by reverse-phase HPLC.

RESULTS AND DISCUSSION

^{13}C labeling experiments. Anaerobic degradation of [*methyl*-^{13}C]toluene (Fig. 2) by strain Tol-4 provided the first clues relevant to the identity of the second metabolite. The ^{13}C-labeled unknown metabolite was isolated by HPLC with a C_{18} reverse-phase column and then analyzed by ^1H NMR. If the metabolite was benzylfumaric acid (compound 1a), a methylene carbon would be ^{13}C labeled. The ^1H NMR resonance of the protons (H_A; Fig. 1) attached to the ^{13}C-labeled methylene carbon would then be split into a large doublet relative to the same resonance in a metabolite derived from unlabeled toluene. Rather surprisingly, the ^1H NMR resonance displaying the expected splitting caused by ^{13}C labeling occurred at a frequency well downfield of what could be assigned to a methylene proton. This was not consistent with the degradation product being either benzylfumaric acid (compound 1a) or benzylmaleic acid (compound 2a). Such a chemical shift was, however, consistent with ^{13}C labeling of a vinyl carbon (H_B; Fig. 1) of *E*-phenylitaconic acid (compound 3a) or *Z*-phenylitaconic acid (compound 4a). Additional evidence was needed to confirm that the unknown metabolite was a phenylmethyl-

enebutanedioic acid (compound 3a or 4a) and to differentiate between an *E* or *Z* olefin substitution pattern. This necessitated the synthesis and detailed spectroscopic characterization of each phenylmethylbutenedioic acid (compounds 1a and 2a) and phenylmethylenebutanedioic acid (compounds 3a and 4a).

Derivatization and EIMS Analysis. Our original strategy for identifying the second metabolite formed during anaerobic degradation of toluene by *A. tolulyticus* Tol-4 was based on the previously reported derivatization (8) of the metabolites to form dimethyl diesters. These esterified components were then to be analyzed by GC according to retention times and coinjection with synthesized samples. GC interfaced with EIMS was to provide further avenues for analysis with interpretation and correlation of fragmentation patterns. Synthetic dimethyl benzylfumarate (compound 1b), dimethyl benzylmaleate (compound 2b), dimethyl *E*-phenylitaconate (compound 3b), and dimethyl *Z*-phenylitaconate (compound 4b) were separated by GC with baseline resolution.

However, there remained the potential problem of deceptive GC analyses. Double-bond isomerizations were so problematic during attempted hydrolysis of dimethyl benzylmaleate (compound 2b) that an independent route to benzylmaleic acid (compound 2a) had to be developed. Similar isomerizations might occur during derivatization of the dioic acids under acidic conditions. This possibility prompted examination of the previously employed derivatization methods (8), which included treatment with methanol (MeOH)-H_2SO_4-H_2O (2:1:1, vol/vol), MeOH-H_2SO_4 (1:1, vol/vol), and MeOH in the presence of BCl_3.

Treatment of benzylfumaric acid (compound 1a), benzylmaleic acid (compound 2a), and *E*-phenylitaconic acid (compound 3a) at 50°C for 20 min with MeOH-H_2SO_4-H_2O (2:1:1, vol/vol) led to the exclusive formation of dimethyl diesters 1b, 2b, and 3b with no apparent double-bond migration or isomerization. However, treatment of *Z*-phenylitaconic acid (compound 4a) under these same reaction conditions resulted in only partial derivatization, with no dimethyl *Z*-phenylitaconate (compound 4b) observable by GC. Similar results were observed when dioic acids 1a, 2a, 3a, and 4a were treated at 50°C for 20 min with MeOH-H_2SO_4 (1:1, vol/vol). Overall, reaction of the dioic acids 1a, 2a, 3a, and 4a with BCl_3 at 50°C for 20 min proved to be the most useful derivatization protocol. Each of the four dioic acids was dimethylated without any detectable double-bond migration or isomerization.

The next concern was the EIMS analysis of derivatized metabolites. A key argument in previous work (8) was the presence of a tropylium ion ($C_7H_7^+$) at *m/z* 91, which was interpreted as being indicative of the benzyl substituent in dimethyl benzylfumarate (compound 1b) and dimethyl benzylmaleate (compound 2b). The tropylium ion's presence seemed inconsistent with the absence of a benzyl substituent in dimethyl *E*- and *Z*-phenylitaconate (compounds 3b and 4b). The synthesis of dimethyl diesters 1b, 2b, 3b, and 4b provided an opportunity to study MS fragmentation patterns in detail. A fragment at *m/z* 91 consistent with a tropylium ion was observed in the MS for dimethyl benzylfumarate (compound 1b) and dimethyl benzylmaleate (compound 2b). However, a similarly intense fragment at *m/z* 91 was also observed for dimethyl *E*-phenylitaconate (compound 3b) and *Z*-phenylitaconate (compound 4b). In fact, the EIMS fragmentation patterns of compounds 1b, 2b, 3b, and 4b were essentially identical. Ionization evidently was introducing enough energy into these systems for double-bond migration and isomerization to occur.

Identification of the second metabolite. Fortunately, dioic acids 1a, 2a, 3a, and 4a were separable with nearly baseline resolution by HPLC with a C_{18} reverse-phase column (Fig. 2).

TABLE 1. ^1H NMR chemical shift values

Proton	Chemical shift (ppm) in compound:			
	1a	2a	3a	4a
H_B (vinyl)	6.85	5.84	7.92	6.89
H_A (methylene)	4.17	3.71	3.56	3.44

The HPLC retention times and coinjection with synthesized dioic acids along with high-field NMR analysis provided the independent lines of evidence needed for the identification of toluene degradation products. By circumventing the previously employed metabolite derivatizations, HPLC and NMR provided the appealing option of direct analysis of the solution matrix attendant with anaerobic microbial degradation of toluene.

The ^1H NMR chemical shifts (Table 1) of the vinyl and methylene proton resonances were generally the most useful for identification of dioic acid and diester structures. Critical supporting evidence for the ^1H NMR and ^{13}C NMR assignments followed from use of rotating frame overhauser effect spectroscopy. The observed NOEs are summarized in Table 2. Measured NOEs attendant with irradiation of vinyl protons (H_B) were the most diagnostic.

With synthesized samples of the dioic acids on hand, multiple lines of spectroscopic evidence for the assigned dioic acid structures, and baseline HPLC resolution of the dioic acids, attention turned to the unknown metabolite formed by *A. tolulyticus* Tol-4. The isolated quantities of the metabolite were adequate for ^{13}C NMR, ^1H NMR, and assignment of NOE correlations. Measured retention times in addition to coinjection with synthetic samples of compounds 1a, 2a, 3a, and 4a by using an HPLC fitted with a C_{18} reverse-phase column provided the necessary confirming data. On the basis of this information, the second metabolite formed along with benzylsuccinic acid during anaerobic degradation of toluene by *A. tolulyticus* Tol-4 is *E*-phenylitaconic acid (compound 3a). These two metabolites were not produced during aerobic toluene degradation.

Previous reports of formation of benzylfumaric acid (compound 1a) (8, 18) need to be reconciled with the results of this

TABLE 2. NOE intensities

Compound and proton	NOE intensity[a] of proton:		
	H_A (methylene)	H_B (vinyl)	H_C (aromatic)
1a			
H_A		w	m
H_B	w		a
H_C	a	a	
2a			
H_A		s	m
H_B	s		a
H_C	a	a	
3a			
H_A		a	m
H_B	a		m
H_C	m	m	
4a			
H_A		m	a
H_B	s		m
H_C	a	m	

[a] s, strong; m, medium; w, weak; a, absent.

study. The sample derivatization techniques used in those earlier studies are not likely to be problematic. In our hands, incomplete dimethyl diester formation was observed when some of the previously employed derivatization conditions were used, although double-bond migration and isomerization were not observed. Reliance on MS analysis for structure identification is, however, best avoided. The identical MS spectra obtained for synthesized samples of compounds 1b, 2b, 3b, and 4b clearly indicate that MS characterization is of limited utility in identifying the structures of these dioic acids. Resolution of the microbial products of anaerobic toluene metabolism in other organisms should now be straightforward, since all products can be resolved by HPLC analysis. Examination of isolated dioic acids by ^{1}H NMR and use of NOE experiments can take advantage of the chemical shift values and NOE correlations reported in this account.

Dioic acid metabolism and biosynthesis. No degradation of benzylfumaric acid, benzylmaleic acid, *E*-phenylitaconic acid, or *Z*-phenylitaconic acid could be detected even after a 1-week incubation of these dioic acids with strain Tol-4. All four dioic acids were stable in culture fluids and during acidification and extraction on the basis of comparison with the HPLC retention times of chemically synthesized dioic acids. A unique feature of benzylmaleic acid was its complete inhibition of toluene metabolism when added to the culture fluid of strain Tol-4. Toluene metabolism was not discernibly affected when strain Tol-4 was cultured in the presence of the other three dioic acids.

Strain Tol-4 converted 1 to 2% of the toluene carbon to *E*-phenylitaconic acid and benzylsuccinic acid in a 10:1 ratio. This differs markedly from the case for strain T1, which was reported to convert up to 17% of the toluene carbon to benzylsuccinic acid and benzylfumaric acid (8). A 0.5% conversion of toluene into these same dioic acids has been observed for strains K172 and T (18). Seven other strains of *A. tolulyticus* with the ability to degrade toluene anaerobically (9, 19) also synthesized similar amounts of a product identified by its HPLC retention time as *E*-phenylitaconic acid during toluene metabolism (data not shown).

Although strain Tol-4 was unable to metabolize benzylsuccinic acid added to its culture medium, benzylsuccinic acid could still be an intermediate during *E*-phenylitaconic acid biosynthesis. *E*-Phenylitaconic acid is always generated by strain Tol-4 in a sizable excess relative to benzylsuccinic acid, which is consistent with oxidation of an intermediate pool of benzylsuccinic acid to *E*-phenylitaconic acid. The inability of strain Tol-4 to metabolize extracellular benzylsuccinic acid may be due to cellular uptake limitations. In addition, the coenzyme A (CoA) derivatives of the dioic acids postulated for the toluene pathway would likely be derived from the conjugation of toluene with acetyl-CoA (5) or even succinyl-CoA. Strain Tol-4 may lack the ligase activity necessary for conversion of free dioic acids into the CoA derivatives required for metabolism.

The formation of *E*-phenylitaconic acid (compound 3a) may be an important clue for defining the pathway of anaerobic toluene metabolism. The outline of a modified microbial route based on this finding is provided in the accompanying paper (5). While strain Tol-4 produces *E*-phenylitaconic acid, it is not clear whether the strains studied in other laboratories produce this product or benzylfumaric acid. Resolving this question could help define whether an altered pathway also occurs.

ACKNOWLEDGMENTS

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REFERENCES

1. **Anders, H.-J., A. Kaetzke, P. Kämpfer, W. Ludwig, and G. Fuchs.** 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K172 and KB740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. Int. J. Syst. Bacteriol. 45:327–333.
2. **Andersson, J., and L. Eberson.** 1977. Cathodic reduction of derivatives of dibenzylidenesuccinic acid: attempted electrohydrocyclization of conjugated systems. Nouv. J. Chim. 1:413–418.
3. **Beller, H. R.** 1995. Anaerobic metabolism of toluene and other aromatic compounds by sulfate-reducing soil bacteria. Ph.D. thesis. Stanford University, Stanford, Calif.
4. **Beller, H. R., M. Reinhard, and D. Grbic-Galic.** 1992. Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 58:3192–3195.
5. **Chee-Sanford, J. C., J. W. Frost, M. R. Fries, and J. M. Tiedje.** 1996. Evidence for acetyl coenzyme A and cinnamoyl coenzyme A in the anaerobic toluene mineralization pathway in *Azoarcus tolulyticus* Tol-4. Appl. Environ. Microbiol. 62:964–973.
6. **Cook, M. P., Jr.** 1981. New ylide anions. A vinyl anion equivalent for substituted fumarate esters. Tetrahedron Lett. 22:381–384.
7. **Cooper, J. W.** 1980. Spectroscopic techniques for organic chemists, p. 278. John Wiley & Sons, New York.
8. **Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young.** 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58:496–501.
9. **Fries, M. R., J.-Z. Zhou, J. C. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802–2810.
10. **Gornowicz, G. A., and J. W. Ryan.** 1966. Thionoacyloxysilanes. J. Org. Chem. 31:3439–3440.
11. **Horning, E. C., and G. N. Walker.** 1952. Cyclizations of benzylsuccinic acids. J. Am. Chem. Soc. 74:5147–5151.
12. **Leitner, W., J. M. Brown, and H. Brunner.** 1993. Mechanistic aspects of the rhodium-catalyzed enantioselective transfer hydrogenation of α,β-unsaturated carboxylic acids using formic acid-triethylamine (5:2) as the hydrogen source. J. Am. Chem. Soc. 115:152–159.
13. **Momose, T., K.-I. Kanai, T. Nakamura, and Y. Kuni.** 1977. Synthetic studies on lignans and related compounds. V. Regiospecificity in the photocyclization of 2,3-dibenzylidenebutyrolactones. Chem. Pharm. Bull. 25:2755–2760.
14. **Montchamp, J.-L., and J. W. Frost.** 1994. Synthesis and evaluation of 3-dehydroquinate synthase transition state analogues. J. Org. Chem. 59:7596–7601.
15. **Nishiyama, H., M. Sasaki, and K. Itoh.** 1981. Stereoselective addition of readily available organocopper reagents to dimethyl acetylenedicarboxylate. Chem. Lett. 1981:905–908.
16. **Okada, T., and R. Okawara.** 1972. The reaction of triphenylphosphine with some organometal haloacetates. J. Organometal. Chem. 42:117–121.
17. **Owens, J. D., and R. M. Keddie.** 1969. The nitrogen nutrition of soil and herbage coryneform bacterium. J. Appl. Bacteriol. 32:338–347.
18. **Seyfried, B., G. Glod, R. Schocher, A. Tschech, and J. Zeyer.** 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60:4047–4052.
19. **Zhou, J.-Z., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene: description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. 45:500–506.

APPENDIX B

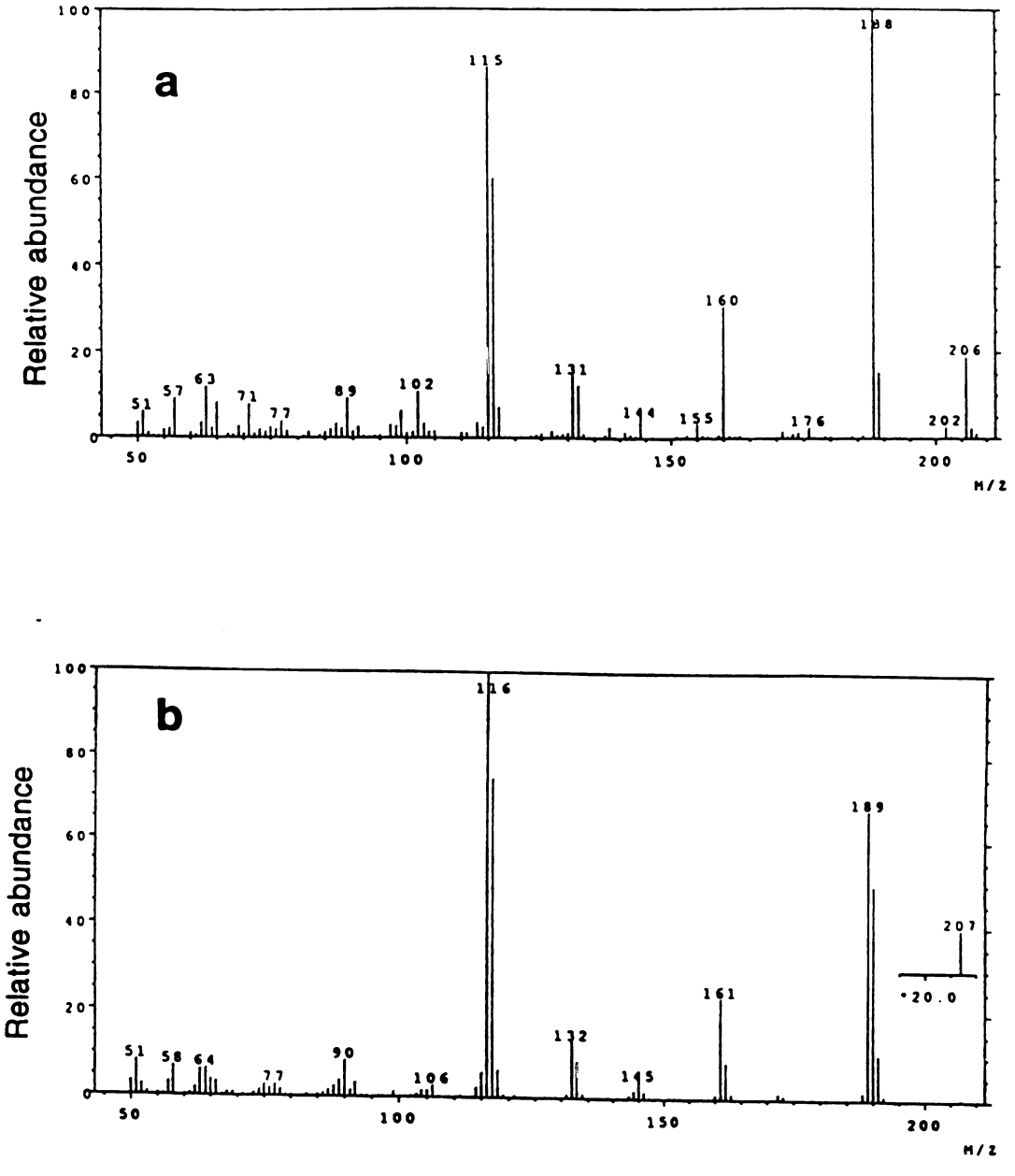


Figure B.1. Mass spectra of the natural product, *E*-phenylitaconate, produced by strain Tol-4 under anaerobic condition on ^{12}C-toluene, mass=206 (a), and ^{13}C-(methyl)-toluene, mass=207 (b).

APPENDIX C

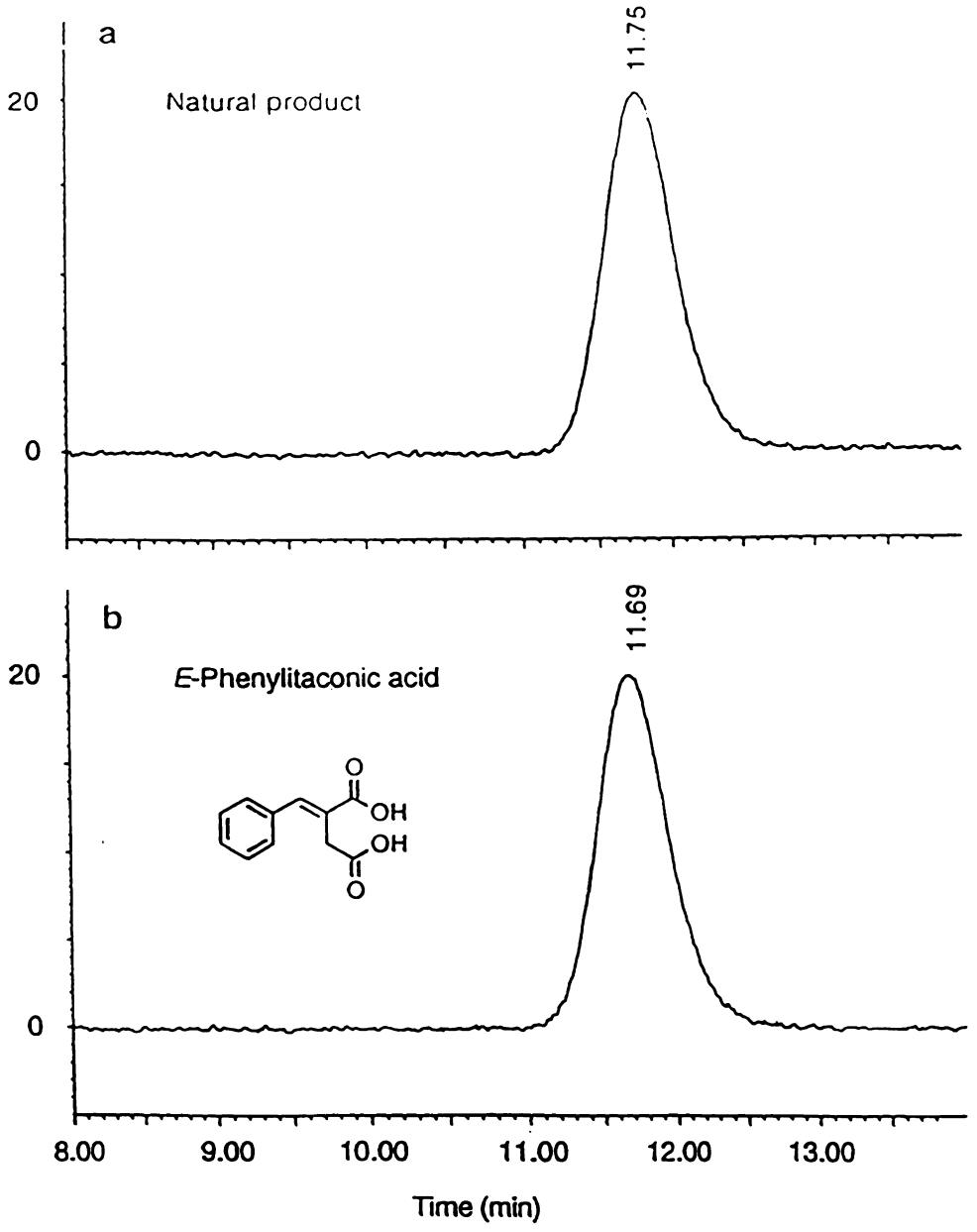
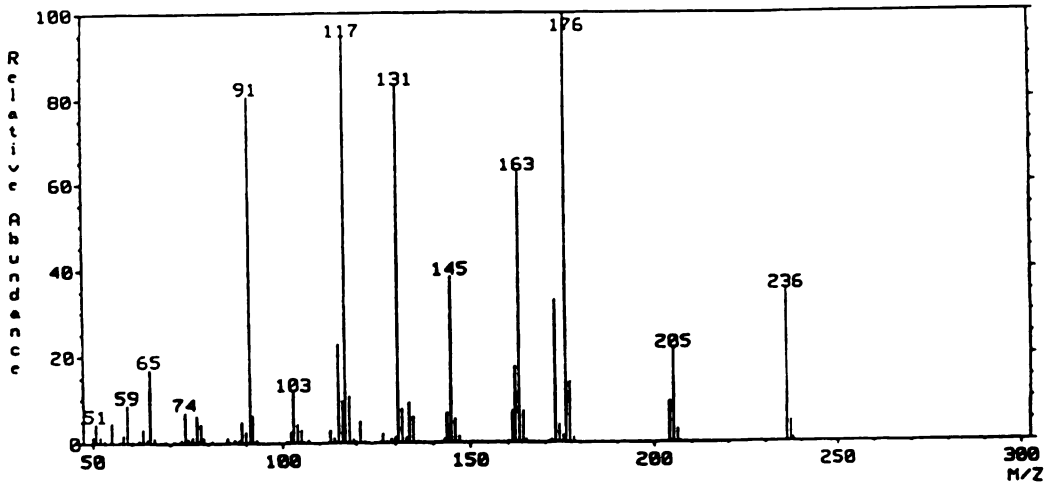
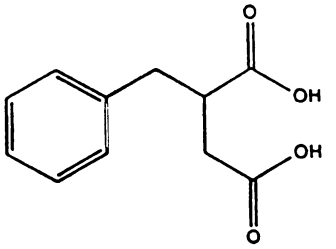


Figure C.1. HPLC chromatograms of the natural product, *E*-phenylitaconate (a), and the authentic compound, *E*-phenylitaconate (b).

APPENDIX D





Benzylsuccinic acid

Figure D.1. Mass spectrum of the natural product, benzylsuccinate, produced by strain Tol-4 under anaerobic condition on toluene.

Chapter 4

CELL-FREE ANAEROBIC TOLUENE DEGRADATION ACTIVITY IN STRAIN TOL-4 AND FURTHER EVIDENCE FOR INVOLVEMENT OF ACETYL-COA

Introduction

Current reports have suggested two types of pathways for anaerobic toluene degradation: i) direct methyl group oxidation, and ii) acetyl-CoA oxidative addition. Evidence for these pathways from pure culture studies has primarily been obtained using three general approaches but none has been completely satisfactory in providing definitive proof of the major steps of anaerobic toluene degradation.

One approach taken was to search for products of toluene degradation using dense cell cultures in the presence or absence of inhibitors or trapping compounds, with both unlabeled and ^{14}C-labeled substrates (Evans et al. 1992; Frazer et al. 1992; Frazer et al. 1993; Seyfried et al. 1994; Beller 1995). Detection of accumulating or transient products during toluene metabolism in cell cultures has been difficult in most of the experiments performed without the addition of inhibitors such as iodoacetamide, an alkylating agent which acts as an inhibitor of enzymes by carboxamidomethylating, e.g., sulfhydryl groups; monofluoroacetate, a potent inhibitor of aconitase in the TCA cycle; or by addition of small amounts of putative pathway intermediates, e.g., benzoate, to trap other metabolites. The most direct evidence has been obtained when ^{14}C-labeled compounds were used.

The second approach employed toluene-induced, cell-free extracts to assay for enzymatic activities that were thought to be involved in toluene metabolism (Altenschmidt and Fuchs 1991; Biegert and Fuchs 1995; J. Champine, personal communication). The presence or absence of enzyme activities can provide indirect evidence for the pathway. The presence of benzylalcohol- and benzaldehyde dehydrogenase, and benzoate CoA ligase activities in toluene-induced K172 cells supported a pathway involving direct methyl group oxidation. Biegert and Fuchs recently reported *in vitro* toluene degradation activity in K172, where benzoate was the product (Biegert and Fuchs 1995) . They reported this activity as indicative of a toluene dehydrogenase (methylhydroxylating), however, neither benzylalcohol nor benzaldehyde were detected. The activity was O_2-sensitive and dependent on nitrate reduction and glycerol. In addition, the rate of this activity was only 5% of the *in vivo* activity. Without further knowledge of the conditions required by enzymes that could be involved in either direct methyl oxidation or oxidative addition, these types of studies are difficult to perform and interpret.

The third approach was in the use of traditional simultaneous adaptation studies which predicted that true intermediates were degraded without lag and would also inhibit the degradation of toluene. Results in these studies were indirect and sometimes difficult to interpret. One example was in the use of benzylalcohol, a substrate not utilized by several of the strains that were hypothesized to degrade toluene via direct methyl group oxidation.

Jorgensen and coworkers found in highly enriched denitrifying cultures that *o*-xylene transformation was induced in the presence of toluene and also when succinate was added (Jorgensen et al. 1995). *o*-Methylbenzaldehyde and *o*-methylbenzoate accumulated in the presence of toluene but did not accumulate in the presence of succinate. Succinate addition reportedly

induced *o*-xylene transformation in strain T1, which led Evans and coworkers to suggest that transformation products, 2-methylbenzylsuccinate and 2-methylbenzylfumarate, were formed from a succinyl-CoA attack on *o*-xylene (Evans et al. 1992). The stimulation of *o*-xylene metabolism by either toluene or succinate suggests the possibility that more than one mechanism for the initial oxidation step could be present in a culture and that this may depend on the presence of inducing substrates.

My studies on the anaerobic toluene-degradation in strain Tol-4 were done primarily by using whole cells and employing methods to detect soluble metabolites produced directly during toluene degradation, as well as by simultaneous adaptation, isotope trapping, and inhibition studies. From these results, the toluene mineralization pathway that I have proposed for Tol-4 involves an initial acetyl-CoA attack at the methyl group to form hydrocinnamoyl-CoA, followed by the formation of cinnamoyl-CoA and benzoyl-CoA via reactions analogous to β-oxidation. Cinnamoyl-CoA was proposed as the minor branch point for a second acetyl-CoA attack to form the accumulating products benzylsuccinate and *E*-phenylitaconate. The size of the CoA derivative intermediates formed during toluene degradation would prohibit them from freely diffusing across cell membranes and could explain why detection of soluble metabolites during toluene metabolism has been so difficult. The small amounts of soluble free aromatic acids detected in my experiments may have been due to cellular non-specific thioesterase activities and diffusion of these products into the culture fluids.

The following studies examined anaerobic toluene degradation using cell-free extracts of Tol-4. Specifically, I examined the assay conditions, substrates, and cofactors required to obtain toluene removal. I also assayed for metabolites, including those that could be present as CoA derivatives. In

addition, cell-extracts were tested for the presence of hydrocinnamate-, cinnamate-, benzoate- benzylsuccinate-, and *E*-phenylitaconate-ligase activities. I also show some evidence for a unique acetyl-CoA-mediated benzoate-CoA transferase activity. A method was developed to analyze ^{14}C-labeled metabolites using thin layer chromatography (TLC) coupled to autoradiography, which provided higher sensitivity for compounds which were present in amounts too low to detect by other conventional chromatography methods. With this method, I reexamined the metabolites of toluene degradation using whole cells and ^{14}C-acetate as a cosubstrate to confirm what had previously been found in my experiments with Tol-4; namely, that acetate, probably in the form of acetyl-CoA, is directly involved with anaerobic toluene degradation.

Materials and Methods

Cell extract preparation. Anaerobic cell extracts of Tol-4 were prepared by growing cells in BS-NO_3^- medium and a total of 200 µmol toluene under anaerobic conditions at 30°C as previously described (Chapter 3). Cells were centrifuged at 10,000 x *g* for 15 min at 4°C in a N_2-purged 250 ml polypropylene centrifuge bottle sealed with a septum containing screw cap. The supernatant was decanted and the cell pellet was resuspended with 100 ml sterile anaerobic phosphate buffer (25 mM, pH 7) under a steady stream of argon. The washed cells were centrifuged again at 10,000 x *g* for 15 min at 4°C, decanted, and resuspended in 3 ml of sterile anaerobic phosphate buffer and 1 mM dithiothreitol (DTT). For the CoA ligase assays, the cells were resuspended instead in 100 mM Tris-HCl (pH 8) and 1 mM DTT. The resuspended cells were transferred to an Ar-purged glass tube and sonicated

for 15 min on ice under a stream of N_2. To prepare the crude extract containing both soluble and particulate fractions, the sonicated mixture was centrifuged at 10,000 x *g* for 15 min at 4°C under N_2 to remove whole cells and then stored at -70°C for up to one week. To prepare separate soluble and particulate fractions, the sonicated mixture was centrifuged at 100,000 x *g* for 1 h at 4°C under N_2 and then stored as separate fractions at -70°C. Soluble fractions were stored up to one month. Protein concentrations in the soluble fractions were determined by using the BioRad assay (Bradford test) and protein in extracts containing particulate fractions was measured using a modified Lowry method (Stoschek 1990). Protein standards were prepared using bovine albumen.

Toluene degradation activity. *In vitro* assays were conducted in 5 ml vials with Teflon-lined butyl rubber septa closures. All solutions were prepared anaerobically. Reactant additions were made either under a steady stream of Ar gas or in an anaerobic glove box to maintain strict oxygen-free conditions. Under glove box (97% N_2 and 3% H_2) conditions, all reactants except for toluene were added to vials which were then removed from the glove box, and the headspace exchanged for Ar (5 min purge) before reaction was initiated by the addition of toluene. Headspace exchange was done to ensure a consistent atmosphere for each assay and to eliminate H_2 and contaminant organic gases that were present in the glove box. Vials were wrapped in foil and incubated on a rotary shaker at 30°C. Combinations of cell extract and reagents added in various concentrations were tested for toluene degradation activity over time and are summarized in Table 4.1. In addition to testing the combined soluble and particulate crude extract, the individual cell fractions were also tested in combination with the reactants and conditions listed in Table 4.1. Tris-HCl (100

mM, pH 8 and pH 7.5) was also used in assays in place of phosphate buffer (pH 7). The amount of protein used in the toluene degradation assays was approximately 200 µg per assay, and in the CoA ligase assays, 500 µg per assay. A final volume of 500 µl was used for each assay. Headspace analysis by GC/FID was used to determine toluene disappearance. Controls consisted of the same mixtures minus cell extract.

^{14}C-Labeled toluene was used in place of cold toluene in assays to analyze for ^{14}C-products from toluene degradation activity. (U-^{14}C)-Toluene (Sigma, specific activity 0.5 mCi/mmol) was added to obtain a final concentration of 0.7 mM and 122,000 dpm/500 µl. At various times, the entire 500 µl volume was purged with N_2 for 10 min and concentrated under vacuum until dryness. Samples were resuspended with 40 µl water and analyzed by thin layer chromatography (TLC) and autoradiography. A 5 µl aliquot was taken before and after purging, and after concentrating, to assay ^{14}C by liquid scintillation counting.

Dense, resting cell cultures were incubated with cold toluene and carboxy-^{14}C -acetate (Sigma, 57 mCi/mmol) in studies to analyze for products. Cells were grown anaerobically on 200 µmol toluene and concentrated to a final OD_{600}=1.5 in 3 ml BS-NO_3^- medium. Cold toluene (500 µM) and 100 µM ^{14}C-acetate (132,000 dpm/3 ml) were added and cultures were incubated at room temperature. Subsamples (0.5 ml) were removed at time intervals and treated as described above in the ^{14}C-toluene assays. Analysis of ^{14}C-labeled products was done using TLC and autoradiography.

Aromatic acid CoA ligase activities. Soluble fractions of cell extracts derived from both aerobically- and anaerobically-grown toluene cultures, and anaerobically-grown M-R2A cultures were used to assay for benzoyl-,

hydrocinnamoyl-, cinnamoyl-, benzylsuccinyl-, and *E*-phenylitaconyl-CoA ligase activities. All assays were done under anaerobic conditions in a final volume of 500 µl at room temperature. Benzoyl-CoA, hydrocinnamoyl-CoA, and cinnamoyl-CoA were identified by HPLC on the basis of comparisons to retention times of authentic compounds (see below). Assay conditions were as follows: cell extract soluble fraction (500 µg protein); Coenzyme A (1 mM); $MgCl_2$ (1 mM); and ATP (1 mM). The reaction was initiated by the addition of the aromatic acid substrate (1 mM). Subsamples (100 µl) were removed initially, after 10 min, 30 min, 1 h, and 2 h, acidified to pH 1 with 2.5 N H_2SO_4, filtered, and analyzed. Assays were done in triplicate.

Aromatic acid CoA transferase activity in the presence of acetyl-CoA. Soluble cell extracts derived from both aerobically- and anaerobically-grown toluene cultures were used to assay for aromatic acid CoA transferase activity in the presence of acetyl-CoA. Aromatic acid CoA derivatives were the products analyzed for by HPLC. The assays were done anaerobically at room temperature and contained the soluble fraction of cell extracts (500 µg protein) plus acetyl-CoA (1 mM). The reaction was initiated by the addition of benzoate, hydrocinnamate, cinnamate, benzylsuccinate, or *E*-phenylitaconate. Control assays consisted of extract-free mixtures containing acetyl-CoA and aromatic acid, and cell extract with aromatic acid minus acetyl-CoA. Assays were done in triplicate.

Chemical analyses and autoradiography. Headspace analysis was used to determine toluene concentrations using the GC/FID method described previously (Chapter 3). Soluble products were determined by HPLC analysis of filtered 20 to 100 µl samples injected onto an RP-18 (5 µm) column, and mobile phases that consisted of system #1: 0.1% H_3PO_4:methanol (60:40), 1.5 ml/min

flow rate for aromatic compounds, or system #2: 50 mM phosphate buffer (pH 5.5):isopropanol (90:10), 1.0 ml/min flow rate for separation of aromatic compounds and aromatic CoA derivatives. UV wavelengths for analyses were at 218 nm and 254 nm.

Samples (20 µl) were analyzed by thin layer chromatography (TLC) under the following conditions: TLC #1: silica gel containing a fluorescent indicator (Baxter), mobile phase toluene:ethylacetate:formic acid (5:4:1) for separation of free acids, benzylalcohol, and benzaldehyde; TLC#2: cellulose with fluorescent indicator (Kodak Co.), mobile phase n-butanol:water:acetic acid (5:3:1) for separation of free acids and CoA derivative. The range of R_f values for authentic standards obtained with TLC condition #1 were: benzoate, 0.76-0.78; hydrocinnamate, 0.74-0.76; cinnamate, 0.74-0.76; benzylsuccinate, 0.61-0.62; *E*-phenylitaconate, 0.61-0.62; benzylalcohol (which results in a smeared spot), approximately 0.64-0.66; acetate, 0.17; and acetyl-CoA, 0. Figure 4.1. shows the separation obtained by TLC. The R_f value range for benzoyl-CoA, hydrocinnamoyl-CoA, and cinnamoyl-CoA with TLC condition #2 was 0.014-0.016.

In experiments where ^{14}C-labeled substrates were used, compounds were separation by TLC, air dried and sprayed with a scintillant En^{3}Hance (NEN Research) four times, allowing plates to dry 10 min between each application. Plates were individually wrapped in plastic and exposed to X-ray film (X-OMAT, Kodak) for up to three weeks at -70°C before development.

Synthesis of CoA derivatives by *Rhodopseudomonas palustris* (Zenk et al. 1980). *Rhodopseudomonas palustris* was obtained from American Type Culture Collection (ATCC #17001). Cells were grown at 30°C on anaerobic BS medium with 1 mM benzoate, hydrocinnamate, or cinnamate as growth

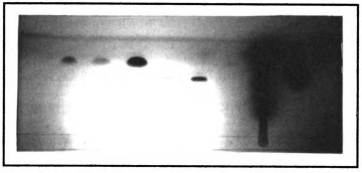


Figure 4.1. Silica gel TLC of authentic standards benzoate (a), hydrocinnamate (b), cinnamate (c), benzylsuccinate (d), *E*-phenylitaconate (e), phenylacetate (f), benzaldehyde (g), benzylalcohol (h), and acetyl-CoA (i).

substrates under a Tungsten lamp. Cells were harvested by centrifugation at10,000 x *g*, 20 min, at 4°C and resuspended in 3 ml 100 mM Tris-HCl (pH 8). The cell suspension was sonicated for 15 min on ice, centrifuged at 100,000 x *g* for 1 h at 4°C, and the soluble cell fraction was stored at -70°C until use. Benzoyl-CoA, hydrocinnamoyl-CoA, and cinnamoyl-CoA were produced using soluble fractions of the extract under the assay conditions described above for determining aromatic CoA ligase activity. The CoA derivative products from *R. palustris* were analyzed on HPLC and the fractions corresponding to the CoA derivatives were collected and concentrated under vacuum. The retention times for the CoA derivatives using HPLC system #2 were: benzoyl-CoA, 14 min; hydrocinnamoyl-CoA, 33 min, and cinnamoyl-CoA, 44 min. Portions of the concentrate were hydrolyzed at 60°C for 30 min after the addition of 10N NaOH (pH 12 final). These samples were analyzed for the hydrolysis products, the aromatic acid, and CoA, by HPLC (Figure 4.2.). The CoA derivative products were also analyzed on TLC to test for purity. Compounds were stored at -20°C. Benzylsuccinyl-CoA and *E*-phenylitaconyl-CoA were not apparently produced by *R. palustris*.

Results

Cell-free anaerobic toluene degradation activity. Anaerobic toluene degradation activity was only present when assays contained both the soluble and particulate fractions of the extract. Tris-HCl buffer (100 mM, pH 8 or pH 7.5) inhibited toluene degradation while activity did occur in phosphate buffer (25 mM, pH 7.0). Table 4.1. summarizes the substrates and assay conditions used and the loss in the amount of toluene as measured by GC after 24 h incubation at 30°C. The addition of 1 mM titanium (III) citrate generally decreased the

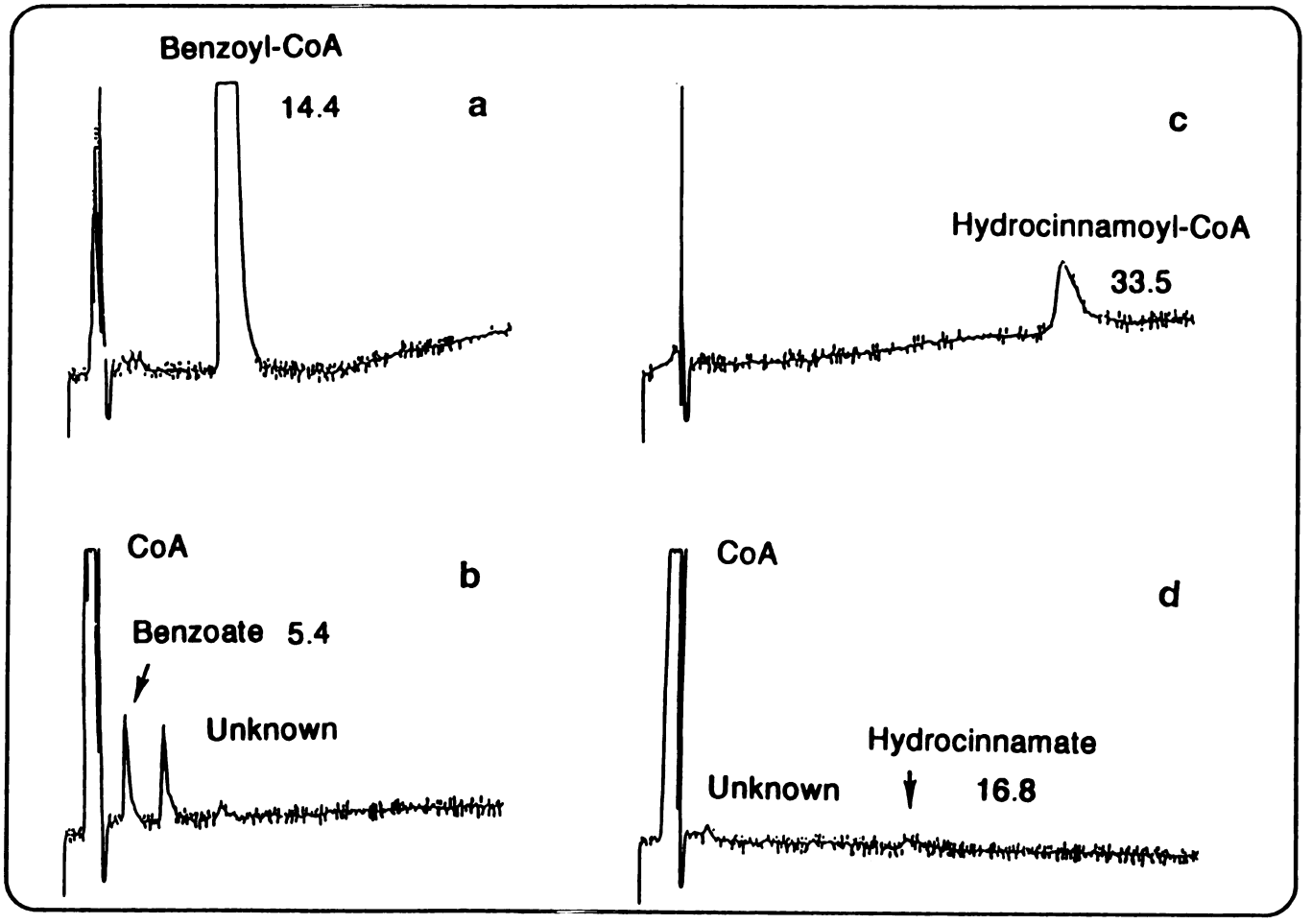


Figure 4.2. Aromatic CoA derivatives produced by *Rhodopseudomonas palutris*. Benzoyl-CoA (a) after base hydrolysis (b), hydrocinnamoyl-CoA (c) after base hydrolysis (d), and cinnamoyl-CoA (e) after base hydrolysis (f). HPLC system #2 was used. Retention times (min) of the relevant compounds are shown.

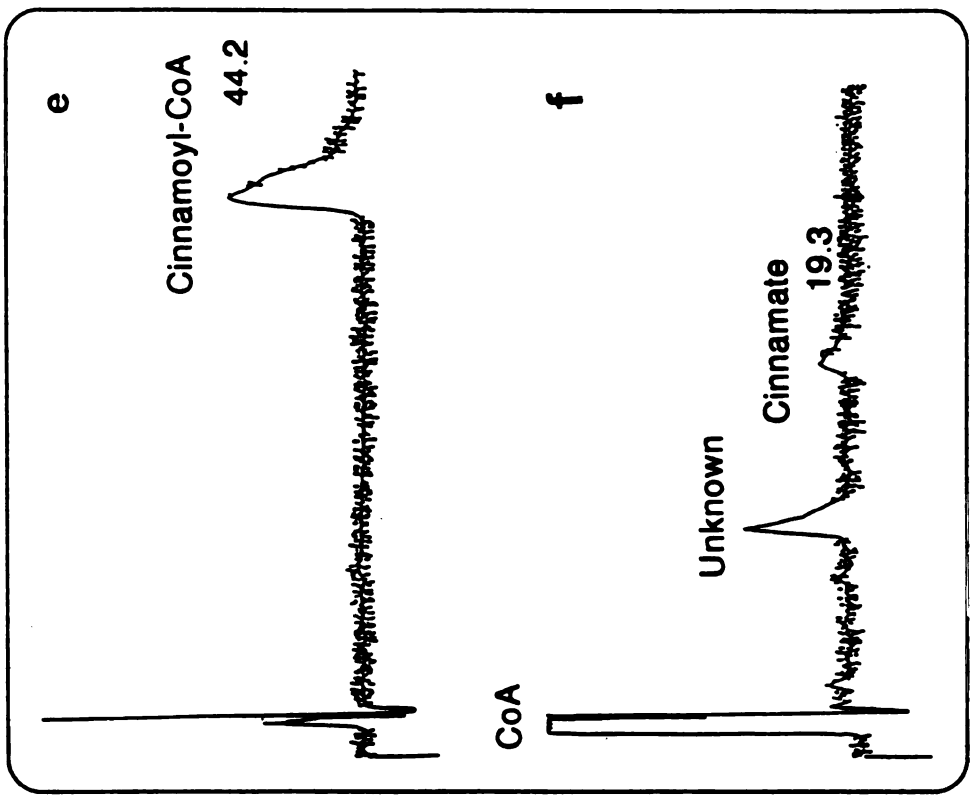


Figure 4.2. (cont.).

Table 4.1. Toluene degradation activity in cell-free extracts of Tol-4

Substrate or assay condition[a]	Reaction mixture contents																								
Soluble+particulate fractions[b] (230 µg protein)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			
25 mM PO4= pH 7	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Toluene (0.5 mM)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Acetyl-CoA (1 mM)		●				●		●			●			●	●					●	●	●	●		
CoA (1 mM)			●				●		●			●				●	●							●	
Glycerol (20%)				●															●						
NO3- (1 mM)				●															●			●			
Metals[c]					●	●	●														●				
FAD (1 mM)														●		●		●							●
NAD (1 mM)															●		●								
ATP (1 mM)																				●					
Dithionite (0.1 mM)								●	●	●															
Titanium citrate (1 mM)											●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
% Toluene loss (24 h)[d]	0-11	17-50	0-33	0-6	13	20-52	0-38	0	0	0	20-25	0-10	0-11	10-30	20-23	22-34	2-10	10-20	0-6	18	22	14	0-2	0	0-6

[a]Assays were carried out under an argon headspace in 500 µl volumes, 30°C, in the dark. All solutions were prepared anaerobically.

[b]Cell extracts were prepared with 1 mM DTT.

[c]The metals solution was from the formulation used for BS medium.

[d]Results are a compilation of separate experiments performed for each assay condition.

variability in toluene losses. Titanium citrate also increased the rate of reactionfor some assays (Figure 4.3.). For instance, in the absence of titanium citrate, toluene degradation usually did not reach the maximum extent in assays containing acetyl-CoA until 12 h to 24 h while in the presence of reductant, the maximum extent was reached at 4 h to 8 h. Dithionite completely inhibited toluene degradation. Toluene losses between 20-25% were consistently obtained under anaerobic assay conditions containing soluble plus particulate protein fractions in 25 mM phosphate buffer (pH 7), with 1 mM acetyl-CoA and 1 mM titanium citrate. Increasing the amount of protein or acetyl-CoA did not result in increased activity (data not shown). When CoA was substituted for acetyl-CoA, the rate and extent of toluene degradation was reduced by one-half (Figure 4.3.). In some assays slow toluene degradation was noted in the absence of either acetyl-CoA or CoA, with losses in toluene concentrations ranging between 0% and 11% by 24 h (Table 4.1.). The addition of FAD to extracts without acetyl-CoA or CoA resulted in 10-20% losses in toluene concentrations, but this loss occurred rapidly within the first two hours (Figure 4.3.). Assays with FAD in the presence of acetyl-CoA or CoA resulted in toluene losses up to 34%, but was not consistent. NAD^+ addition did not increase the toluene loss in the presence of acetyl-CoA. Assays containing 20% glycerol and 1 mM NO_3^- along with the addition of titanium citrate did not result in significant toluene loss. Addition of ATP or a complex metals solution also did not enhance toluene degradation activity. Control assays performed in the absence of protein showed neglible losses of toluene.

In vitro assays containing cell extract, phosphate buffer, and acetyl-CoA produced a labeled product after 24 h (Figure 4.4., lane 2). A similar result was observed in extracts incubated in the absence of acetyl-CoA (Figure 4.4., lane 7). No signal was observed in similar assays containing CoA instead of acetyl-

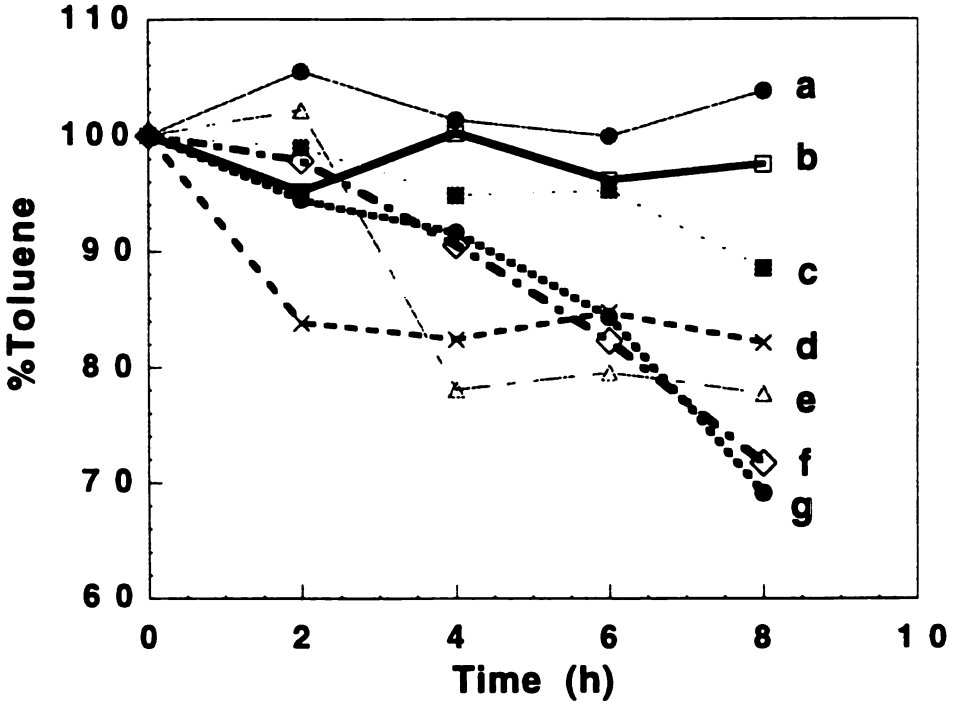


Figure 4.3. Toluene loss (%) in crude cell extracts of Tol-4 under anaerobic conditions in 25 mM phosphate (pH 7) with 1 mM titanium citrate added. Crude extract only (a), no crude extract (b), crude extract + 1 mM CoA (c), crude extract +1 mM FAD (d), crude extract + 1 mM AcCoA (e), crude extract + 1 mM AcCoA + 1 mM FAD (f), and crude extract + 1 mM CoA + 1 mM FAD (g).

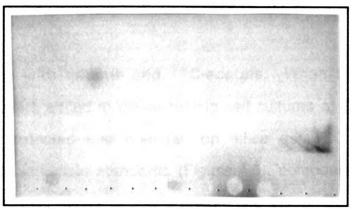


Figure 4.4. Autoradiograph of crude cell extract assays of Tol-4 incubated with ^{14}C-toluene under anaerobic conditions in 25 mM phosphate (pH 7) with 1 mM titanium citrate added, +1 mM AcCoA at 0 h (Lane 1) and 24 h (Lane 2), +1 mM CoA at 0 h (Lane 5) and 24 h (Lane 6), no AcCoA or CoA added at 24 h (Lane 7) and 0 h (Lane 8), no extract + 1 mM AcCoA at 24 h (Lane 9), no extract + 1 mM CoA at 24 h (Lane 10), no extract + 1 mM FAD at 24 h (Lane 11), and ^{14}C-acetate (Lane 12). Cell extract that was stored for over one week at -70°C prior to use + 1 mM AcCoA at 0 h (Lane 3) and 24 h (Lane 4).

CoA (Figure 4.4., lane 6). Under TLC condition #1, compounds correspondingto CoA derivatives are not mobile. An unidentified signal appeared after 24 h in a separate assay containing acetyl-CoA and extract that was stored for 3 weeks at -70°C (Figure 4.4., lane 4). This result was not reproducible. Liquid scintillation counts of samples from this experiment confirmed that radioactive, non-volatile products (not toluene) were present only in the assays perfomed in both the presence and absence of acetyl-CoA (Table 4.2.). The radioactivity in products accounts for less than 1% of the total counts added as ^{14}C-toluene. Samples analyzed under TLC condition #2, which allows separation of CoA derivatives, did not result in any ^{14}C-labeled products (data not shown).

Whole cell studies with toluene and ^{14}C-acetate. When toluene, ^{14}C-acetate, and MFA were added to dense resting cell cultures of Tol-4, labeled compounds were detected that coeluted on silica gel TLC plates with hydrocinnamate or cinnamate standards (Figure 4.5., compound a, lanes 3 through 7), and benzylsuccinate or *E*-phenylitaconate standards (Figure 4.5., compound c, lanes 3 through 7). One compound did not coelute with any of the standards used (Figure 4.5., compound b, lanes 3 through 7). None of the labeled compounds appeared to be benzylalcohol or benzaldehyde since these chemicals usually smeared when run under TLC condition #1, used in this analysis. Labeled products with similar R_f values eluted in both the presence and absence of MFA (data not shown). The radioactive signals associated with the eluting compounds were stronger in the presence of MFA which possibly indicated that a higher quantity of these compounds were produced. The labeled compounds were not detected after 1 h in the cultures containing MFA and after 2 h in those with no MFA addition. In a separate

Table 4.2. Non-volatile ^{14}C-labeled products produced from ^{14}C-toluene by cell-free extracts[a]

Assay Components	Incubation time (h)				
	0	2	4 (dpm/500 µl)	6	24
Extract+Toluene+ AcCoA	32	48	192	272	544
Extract+Toluene	32	56	nd	208	304
Toluene only	32	32	nd	32	32
Toluene+AcCoA	32	32	nd	32	37

[a]Total dpm measurements were taken using 5 µl samples which were acidified to pH 2 and purged for 2 min with N_2; results were normalized to a blank containing 20 µl phosphate buffer and liquid scintillant.

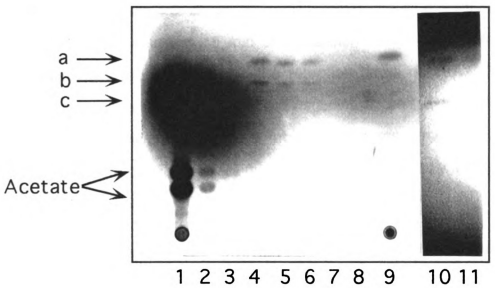


Figure 4.5. Autoradiograph of samples taken from whole cell cultures incubated with toluene, ^{14}C-acetate, and 100 μM MFA at 0 h (Lane 1), 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 45 min (Lane 6), 1 h (Lane 7), 2 h (Lane 8). Standards ^{14}C-benzoate (Lane 9), hydrocinnamate + benzylsuccinate (Lane 10), and cinnamate + *E*-phenylitaconate (Lane 11). Acetate formed two bands after acidification.

experiment, cells incubated with ^{14}C-acetate (minus toluene) did not produce the same products as cells incubated with ^{14}C-acetate and toluene.

The same samples were also analyzed by HPLC and a radioactivity detector. Figure 4.6. shows the radiochromatograms of samples taken initially, and after 20 min and 45 min of incubation. Several peaks with significant radioactivity corresponded to the retention times on HPLC for standards of hydrocinnamate, cinnamate, benzylsuccinate, and *E*-phenylitaconate. The maximum amount of soluble labeled products appeared in the cultures incubated with MFA after 20 min and was approximately 10% of the total label that was added as ^{14}C-acetate.

Aromatic CoA ligase and transferase activities. Soluble fractions of Tol-4 cell extracts were used to determine the presence of benzoate, hydrocinnamate, cinnamate, benzylsuccinate, and *E*-phenylitaconate CoA ligase activities. Samples were analyzed by HPLC and compared to standards of benzoyl-CoA, hydrocinnamoyl-CoA, and cinnamoyl-CoA produced from *R. palustris*. Benzylsuccinyl-CoA and *E*-phenylitaconyl-CoA were not apparently produced by *R. palustris* and the HPLC retention times for these compounds in assays with Tol-4 were estimated based on the relationship of retention times between benzoate, hydrocinnamate, cinnamate, and their respective CoA derivatives. Cells which were grown on toluene both aerobically and anaerobically demonstrated benzoate CoA ligase activity (Table 4.3.). Only the extracts prepared from cells grown anaerobically on toluene showed any CoA ligase activity for hydrocinnamate and cinnamate. No benzylsuccinyl-CoA and *E*-phenylitaconyl-CoA were produced for benzylsuccinate and *E*-phenylitaconate in Tol-4 extracts. Extracts prepared from cells grown on M-R2A medium did not produce any of the CoA derivative products. The production of

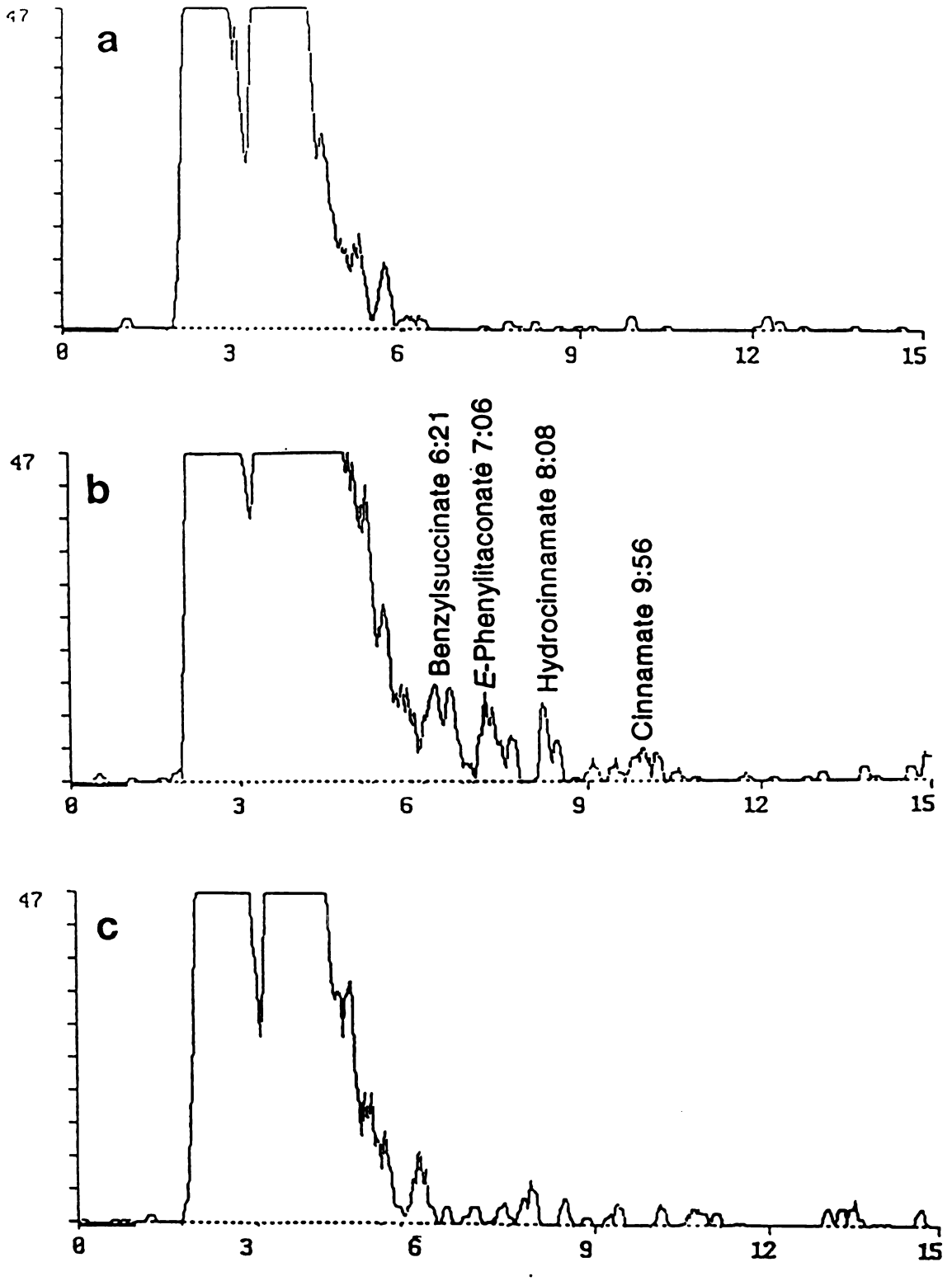


Figure 4.6. HPLC radiochromatograms of ^{14}C-labeled metabolites produced from whole cell cultures incubated with toluene, ^{14}C-acetate, and 100 μM MFA at 0 h (a), 20 min (b), and 45 min (c). HPLC system #1 was used.

Table 4.3. Aromatic acid CoA ligase and CoA transferase activities present in soluble fractions of Tol-4 extracts[a]

Substrates	CoA derivative product (μM) under indicated condition		
	Anaerobic M-R2A induced	Aerobic toluene induced	Anaerobic toluene induced
CoA ligase activity			
Benzoate + CoA	-	260	200-260
Hydrocinnamate + CoA	-	nd	40
Cinnamate + CoA	-	nd	60
Benzylsuccinate + CoA	-	nd	-
E-Phenylitaconate + CoA	-	nd	-
CoA transferase activity			
Benzoate + AcCoA	-	-	13
Hydrocinnamate + AcCoA	-	nd	-
Cinnamate + AcCoA	-	nd	-
Hydrocinnamate + AcCoA	nd	nd	-
Benzylsuccinate + AcCoA	nd	nd	-
E-Phenylitaconate + AcCoA	nd	nd	-

[a]Assays contained Tris-HCl (pH 8), 500 μl total volume under anaerobic conditions at room temperature. All assays were done in triplicate. CoA ligase assays contained 500 μg protein in soluble cell fraction, 1 mM aromatic substrate, 1 mM CoA, 1 mM Mg^{2+}, 1 mM ATP. CoA transferase assays contained 500 μg protein in soluble cell fraction, 1 mM aromatic substrate, 1 mM acetyl-CoA.
nd=not determined

benzoyl-CoA, hydrocinnamoyl-CoA, and cinnamoyl-CoA corresponded to a decrease in the respective aromatic substrates. Based on the loss of aromatic acid substrates measured, the concentrations of the CoA products were 260 μM benzoyl-CoA, 40 μM hydrocinnamoyl-CoA, and 60 μM cinnamoyl-CoA. No activity was observed in any controls lacking cell extract.

When acetyl-CoA was added to cell extracts along with benzoate, a compound corresponding to benzoyl-CoA was detected (Figure 4.7.b.). This activity was not dependent upon CoA, ATP, or Mg^{2+} addition. When this product was collected, hydrolyzed with 10N NaOH and analyzed by HPLC, peaks corresponding to the retention times of benzoate and CoA appeared (data not shown). Control assays containing cell extract and benzoate only showed no activity. CoA derivative products were not observed in similar assays containing hydrocinnamate or cinnamate and acetyl-CoA (Table 4.3.).

Discussion

The results from this study indicate that anaerobic toluene degradation activity can be obtained using cell-free extracts of Tol-4. This activity is dependent on both the soluble and particulate fractions of the crude cell-free extract. A small amount of toluene degradation was measured when the particulate fraction alone was used but this was likely due to the presence of some soluble cell constituents remaining after extract preparation. A dependence on nitrate reduction for anaerobic toluene degradation was reported by Biegart and Fuchs for *in vitro* studies with strain K172 (Biegert and Fuchs 1995). This nitrate dependence suggests that toluene oxidation may be coupled to electron transport components of the cell. The *in vitro* activity of Tol-4 did not depend on the presence of nitrate, however, the requirement for a

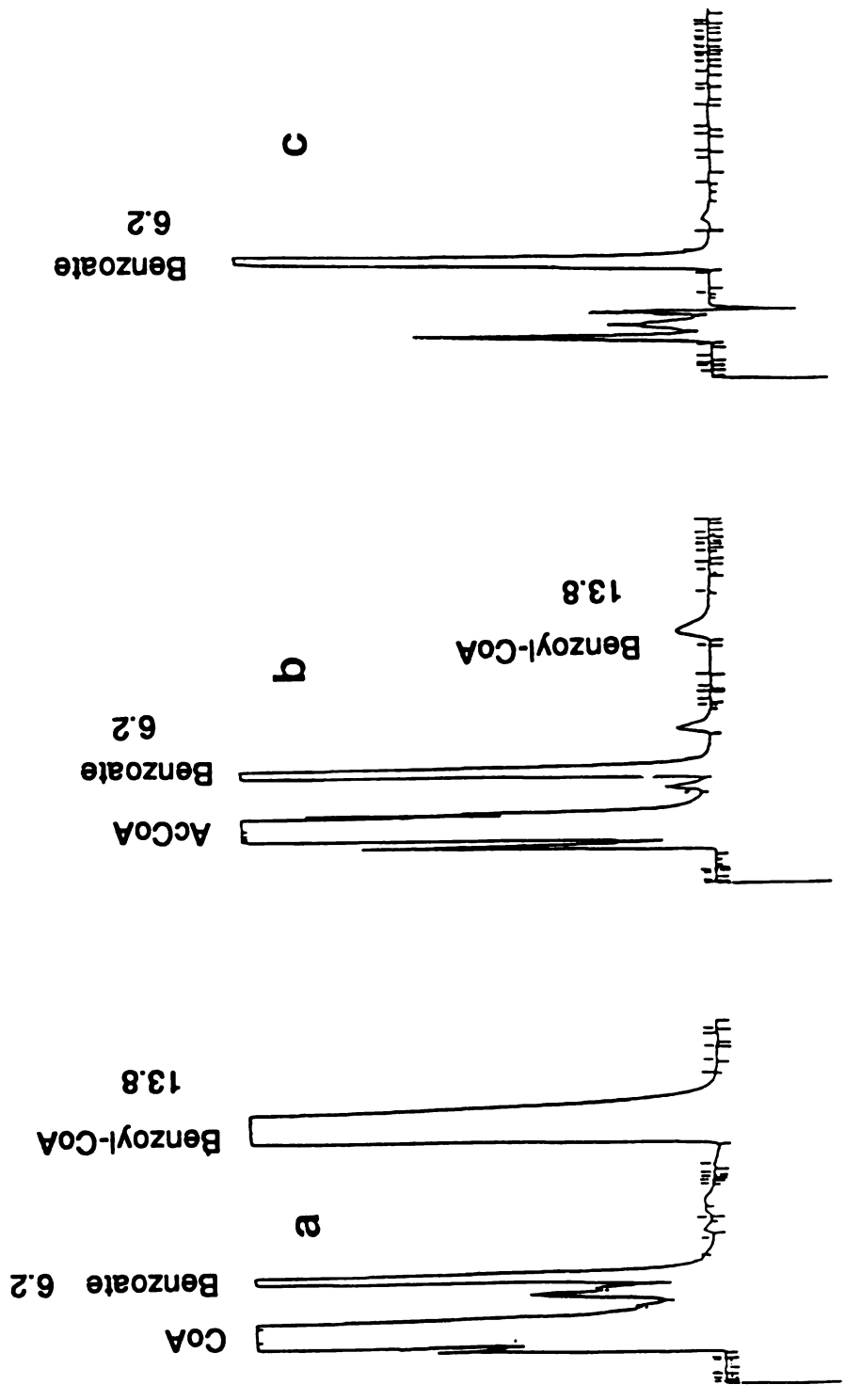


Figure 4.7. HPLC chromatograms of benzoyl-CoA production from cell extracts of Tol-4. Extract + benzoate + CoA +ATP+Mg2+ (a), extract + benzoate + acetyl-CoA (b), and extract + benzoate (c). HPLC system #2 was used. Retention times (min) for compounds are shown.

membrane-associated cell fraction might suggest that an electron transport component is involved. The mechanism of the initial step proposed for anaerobic toluene degradation by Tol-4 involves a net two electron transfer to form hydrocinnamoyl-CoA (Figure 4.8.). The complete oxidation of toluene would hence, require a mechanism of electron transfers involving some key electron acceptor(s) in the process.

The addition of titanium citrate reduced the range of variability in toluene loss compared to when no reductant was added. In addition, the rate of toluene degradation was increased when titanium citrate was added suggesting that the activity was sensitive to even trace amounts of oxygen that were probably present. Adding dithionite as a reductant completely inhibited toluene degradation. Some variability was still observed even in assays containing titanium citrate. For example, toluene degradation ranged from no loss observed to 10% and 11% in the assays which contained CoA, or performed in the absence of CoA or acetyl-CoA, respectively (Table 4.1.). This may be attributed to variations in separate extract preparations that cannot be currently specified.

The involvement of acetyl-CoA in toluene degradation has been reasoned from the detection of intermediates such as cinnamate, benzylsuccinate, and *E*-phenylitaconate. MFA inhibition of the TCA cycle caused an increase in the production of benzylsuccinate and *E*-phenylitaconate (Chapter 3). Acetyl-CoA addition to cell extracts of Tol-4 consistently resulted in a 20-25% loss of toluene. The addition of CoA instead, had variable results, and when loss of toluene did occur, the extent was only half of that which was found when acetyl-CoA was present. It is possible that a small pool of acetate may be present in the crude extract that could be used in reaction with the added CoA to form acetyl-CoA, subsequently resulting in toluene degradation.

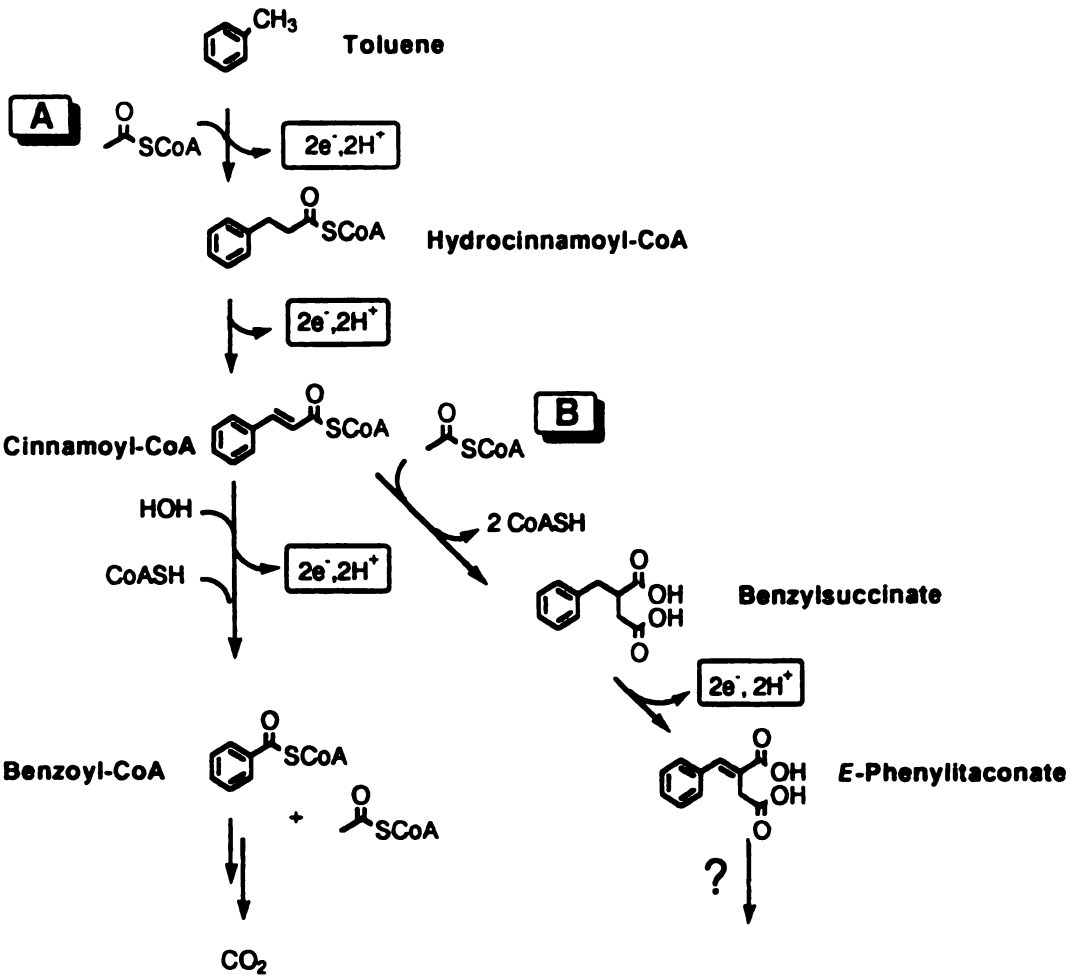


Figure 4.8. Summary of the oxidation reactions in the proposed mineralization pathway (A) and in the formation of *E*-phenylitaconate (B) from the anaerobic degradation of toluene by strain Tol-4.

Similarly, some experiments performed in the absence of either acetyl-CoA or CoA also demonstrated smaller toluene losses. Further evidence of toluene metabolism occurring in the absence of any CoA compounds was shown by autoradiography after incubation with ^{14}C-toluene (Figure 4.4.). The assays performed with the addition of CoA did not result in the same product shown in the autoradiograph. The location of the labeled compound on TLC plates suggests that it could be a CoA thioester since these compounds are not mobile under the solvent conditions used. This result also suggests that there may have been unspecified components associated with the extract that facilitated some toluene degradation. Although it is not known what factors present in crude extracts promote some activity, these experiments provide direct evidence that acetyl-CoA has a likely role in anaerobic toluene metabolism.

Another factor investigated in this study was the possible requirement for electron carriers. FAD was selected for several reasons. Flavins are versatile redox coenzymes that, as flavoproteins, catalyze a variety of reactions on a variety of substrates (Zubay 1983). *p*-Cresol methylhydroxylase is among the enzymes that are flavin-associated. FAD is also involved in the initial step of β-oxidation, suggested to be the mechanism used by Tol-4 and T1 (Evans et al. 1992) for reactions following the formation of hydrocinnamoyl-CoA in anaerobic toluene degradation. One important feature of flavins is their ability to serve as the switch point from two-electron transfer processes, predominant in cytosolic carbon metabolism, to one-electron transfer processes, predominant in membrane-associated terminal electron transfer pathways. It was reasonable to suggest that FAD might be important as an electron carrier in toluene metabolism and could help to explain the requirement for both soluble and particulate cell fractions. The addition of FAD in the absence of acetyl-CoA or CoA did result in an initial rapid toluene removal (Figure 4.3.) however, similar

removal occurred when FAD was added in the absence of cell extract (Table 4.1.). This result suggests that there could be some abiotic toluene transformation activity associated with FAD. FAD addition along with either acetyl-CoA or CoA resulted in the greatest extent of toluene degradation in repeated experiments. This result may be due to a combination of biologically and abiotically mediated reactions. Although it does not appear that FAD is required for toluene degradation activity, these experiments do not completely eliminate the possible role of flavins. Because of the apparent abiotic reaction occurring with FAD, I chose to focus on biochemically-based toluene degrading activity. The possible role of flavins should be addressed in future studies and may prove worthwhile in the study of the electron transfer mechanism involved. NAD^+ was investigated as an electron carrier or cofactor in toluene metabolism, but in assays where NAD^+ was added with either CoA or acetyl-CoA, no significant losses of toluene resulted.

The addition of a mixed metals solution to the cell-free extract assay did not appear to enhance *in vitro* toluene degradation. The role or requirement of metals in toluene metabolism is not known, but metals may be critical. Previous experiments with Tol-4 showed that a metal supplement was required to stimulate anaerobic growth on toluene. The successful use of Co(III) as a strong chemical oxidant in reactions with aromatic compounds (Tang and Kochi 1973) and the important role of metals in biochemical reactions suggests that perhaps a mechanism involving some transition metal (maybe Co) could be involved in the redox reaction occurring in the initial step of toluene oxidation (Figure 4.8.). The metals solution used in the experiments described here contained a mixture of iron, molybdenum, cobalt, manganese, copper, and zinc. Further studies are required to determine if specific metals may promote toluene oxidation by Tol-4.

Biegart and Fuchs reported the dependence on glycerol for *in vitro* anaerobic toluene oxidation in K172 (Biegert and Fuchs 1995). Glycerol is commonly used as a protein stabilizer and might have served this function in the studies involving K172. When 20% glycerol was added in Tol-4 assays, no enhancement of toluene degradation activity was observed. The addition of ATP also did not enhance toluene degradation in cell extracts.

In addition to investigating the various cofactors and substrates required for *in vitro* toluene degradation in Tol-4, it is apparent from these studies that certain assay conditions are critical for obtaining activity. Tris-HCl (pH 8 and pH 7.5) inhibited toluene degradation while activity occurred in phosphate buffer (pH 7). It is quite possible that further adjustment of the pH and ionic strength could affect toluene degradation activity.

When samples from cell extract assays were analyzed by HPLC, no identifiable toluene degradation metabolites were observed. One problem may be due to limited sample volumes and detection limits of the instrument. Analysis by HPLC coupled to a radioactive detector to detect ^{14}C-labeled metabolites in previous studies involving Tol-4 cultures growing on ^{14}C-toluene was limited in its use because of sample size limitations and instrument sensitivity. By using ^{14}C-labeled compounds and TLC coupled with autoradiography, an extremely sensitive method for detecting compounds in low concentrations was developed. Another advantage to this method is the ability to concentrate compounds for additional analysis.

Dense cell cultures were used to reexamine the role of acetate in toluene metabolism. In previous studies (Chapter 3), the metabolism of ^{14}C-acetate and cold toluene by Tol-4 resulted in detectable quantities of compounds which coeluted with standards corresponding to cinnamate and (^{14}C)-benzylsuccinate. Label was not detected in the cinnamate product. The whole

cell experiment with ^{14}C-acetate and toluene was repeated in this study and resulted in labeled metabolites that coeluted on TLC with hydrocinnamate and cinnamate; and benzylsuccinate and *E*-phenylitaconate. Under the conditions used in this experiment, hydrocinnamate could not be reliably differentiated from cinnamate, and benzylsuccinate could not be differentiated from *E*-phenylitaconate. The R_f value for benzoate differed only slightly from the R_f for hydrocinnamate and cinnamate but it is not likely that ^{14}C-(carboxy)-benzoate would be formed from the oxidative addition of ^{14}C-acetate to unlabeled toluene. A labelled spot with an R_f different from any standard also appeared at the same time as the other metabolites. This unknown compound is likely to be aromatic because its measured R_f value was between benzoate and benzylsuccinate/*E*-phenylitaonate. Interestingly, the compound coeluting on TLC as benzylsuccinate or *E*-phenylitaconate is not detectable after 2 h in the absence of MFA, and after 1 h with MFA added. This result was reproducible. These two compounds were previously thought to accumulate and are not metabolized by Tol-4 cells (Migaud et al. 1995). The inability to metabolize benzylsuccinate and *E*-phenylitaconate was thought to be due to the lack of an uptake mechanism for these compounds. However, based on the transient presence of these compounds in the experiment described in this chapter, this may not be the case. It is possible that small amounts of these compounds are only metabolized in the presence of toluene, although no evidence supports this. The appearance of the labeled intermediates in the studies described here provide more evidence for the role of acetate, probably in the form of acetyl-CoA, in toluene metabolism. Cultures which contained MFA in this experiment also show larger amounts of labeled metabolites compared to when MFA was absent. This would be expected if MFA inhibition resulted in a larger pool of acetyl-CoA available for reaction with toluene and cinnamoyl-CoA as proposed.

Soluble fractions of extracts prepared from Tol-4 grown anaerobically on toluene demonstrated CoA ligase activities for benzoate, hydrocinnamate, and cinnamate, but not for benzylsuccinate or *E*-phenylitaconate. The presence of CoA ligase activities for benzoate, hydrocinnamate, and cinnamate are not surprising since these compounds are used as substrates for growth by Tol-4. These CoA ligases appear to be inducible in these cells. The presence of benzoyl-, hydrocinnamoyl-, and cinnamoyl-CoA ligase activities suggests that the corresponding free acids may be present during the degradation of toluene. Although the pathway I have proposed for anaerobic toluene metabolism postulates that CoA derivatives are the intermediates directly formed, small amounts of the free aromatic acids may be present due to the action of nonspecific thioesterases and the presence of these acids may serve to induce the corresponding ligases. A number of my experiments with Tol-4 have demonstrated the presence of low amounts of benzoate, cinnamate, benzylsuccinate, *E*-phenylitaconate, and possibly hydrocinnamate resulting from toluene degradation under certain conditions. Benzoate, hydrocinnamate, and cinnamate were transient in all of these experiments. There is now evidence from experiments described in this chapter that benzylsuccinate and *E*-phenylitaconate are also transient metabolites. These data suggest that these compounds are excreted, uptaken, and metabolized by Tol-4 cells. It has been shown that benzoate CoA ligase in *R. palustris* is induced by compounds other than benzoate, such as a number of aromatic compounds and some of the oxidation products after cleavage of benzoyl-CoA (Elder et al. 1992; Villemur 1995). The benzoate CoA ligase activity in Tol-4 is very high compared to hydrocinnamate CoA ligase and cinnamate CoA ligase activities, perhaps because benzoate CoA ligase in these cells is induced by several substrates. In contrast, the CoA ligases involved in hydrocinnamate and cinnamate

metabolism may be more specific in their range of inducers. The inability to detect CoA derivatives of benzylsuccinate and *E*-phenylitaconate in Tol-4 suggests that these activities are not present, which also supported previous accounts of these cells being unable to utilize these compounds. Contrary to this, the experiment involving whole cells metabolizing ^{14}C-acetate and toluene suggested that these compounds are metabolized. Perhaps mechanisms other than those involving CoA ligases are involved. Alternatively, the lack of benzylsuccinate CoA ligase and *E*-phenylitaconate CoA ligase acitivites *in vitro* may simply be due to the conditions used in the assay, although all known CoA ligase assays so far employ similar reaction conditions. Low activity for some aromatic ligases may be due to some unknown constituent in the cell extract which may inhibit the ligases involved (Dangel et al. 1991). Also, since no authentic compounds for benzylsuccinyl-CoA and *E*-phenylitaconyl-CoA were available commercially and these compounds were not apparently synthesized by *R. palustris*, I cannot completely rule out the absence of these ligase activities in Tol-4. Aerobic toluene-induced cells of Tol-4 also had high benzoate CoA ligase activity, which suggests that benzoate may be an intermediate in aerobic toluene metabolism. The induction of benzoate CoA ligase activities under both anaerobic and aerobic toluene growth suggests the possibility of shared aspects between the anaerobic and aerobic pathways for toluene degradation in Tol-4. However, it has been shown in strain KB740 that the same ligase reaction occurring under aerobic or anaerobic conditions is carried out by distinct enzymes (Altenschmidt et al. 1993).

All of the *in vitro* studies done so far with a variety of bacteria have reported that the formation of aromatic CoA derivatives involve ATP-dependent reactions involving CoA ligases. In my study, *in vitro* assays using soluble

fractions of Tol-4 extracts demonstrated a unique aromatic CoA transferase reaction resulting in the formation of benzoyl-CoA when benzoate was added along with acetyl-CoA. This would be the first report of an aromatic acid that can be metabolized via a CoA adduct such as acetyl-CoA that was not dependent on ATP, Mg^{2+}, or CoA. The activity was reproducible, but low compared to the activity found when benzoate was present with CoA, ATP, and Mg^{2+}. The enzyme and mechanism involved in a CoA transferase reaction would be distinct from those involved with CoA ligases and would likely be a member of a new class of enzymes. No CoA transferase activity was observed with hydrocinnamate, cinnamate, benzylsuccinate, or *E*-phenylitaconate using Tol-4 extracts.

From the results of the experiments described in this chapter, I was able to achieve several goals that address anaerobic toluene degradation in Tol-4. One goal achieved was in obtaining anaerobic toluene degradation in a cell-free system and to determine some of the conditions required for *in vitro* activity. Although the activity was low in my *in vitro* assays, the detection of metabolites indicate that toluene is metabolized in cell-free systems. Further purification of the cell extract is needed in order to determine the cell fraction(s) responsible for activity, and the exact conditions and substrates required for anaerobic toluene degradation activity to occur. Further cell-free studies would also indicate whether oxidative addition of acetyl-CoA is indeed the major route by which strain Tol-4 degrades toluene under anaerobic conditions. Another goal achieved in this study provided additional, more direct evidence, for the involvement of acetyl-CoA in anaerobic toluene oxidation, namely demonstrating specifically, the production of cinnamate and possibly hydrocinnamate from toluene metabolism. Finally, these studies reveal CoA ligase activities associated with possible intermediates of anaerobic toluene

degradation, and the presence of a potentially unique CoA transferase activityin the presene of acetyl-CoA.

Acknowledgement

I wish to thank Frank Löffler for his helpful advice and discussions.

List of References

Altenschmidt U and Fuchs G (1991) Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. 156: 152-158.

Altenschmidt U, Oswald B, Steiner E, Herrmann H and Fuchs G (1993) New aerobic benzoate oxidation pathway via benzoyl-Coenzyme A and 3-hydroxybenzoyl-Coenzyme A in a denitrifying *Pseudomonas* sp. J. Bact. 175: 4851-4858.

Beller HR (1995) Anaerobic metabolism of toluene and other aromatic compounds by sulfate-reducing soil bacteria. Ph.D. Thesis, Stanford University.

Biegert T and Fuchs G (1995). Anaerobic oxidation of toluene (analogues) to benzoate (analogues) by whole cells and by cell extracts of a denitrifying *Thauera* sp., p 25, Biospektrum Sonderausgabe Zur Frühjahrstagung, Stuttgart, Germany, der Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V. (VAAM).

Dangel W, Brackman R, Lack A, Mohamed M, Koch J, Oswald B, Seyfried B, Tschech A and Fuchs G (1991) Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch. Microbiol. 155: 256-62.

Elder DJE, Morgan P and Kelly DJ (1992) Evidence for two differentially regulated phenylpropenoyl-Coenzyme A synthetase activities in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. 98: 255-260.

Evans PJ, Ling W, Goldschmidt B, Ritter ER and Young LY (1992) Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58: 496-501.

Frazer AC, Ling W, Evans PJ and Young LY (1992) Metabolites observed during anaerobic toluene degradation by strain T1. Abstracts of the 92nd Annual Meeting of the American Society for Microbiology 1992 , p. 374.

Frazer AC, Ling W and Young LY (1993) Substrate induction and metabolite accumulation during anaerobic toluene utilization by the denitrifying strain T1. Appl. Environ. Microbiol. 59: 3157-3160.

Jorgensen C, Nielsen B, Jensen BK and Mortensen E (1995) Transformation of *o*-xylene to *o*-methylbenzoic acid by a denitrifying enrichment culture using toluene as the primary substrate. Biodegradation 6: 141-146.

Migaud ME, Chee-Sanford JC, Tiedje JM and Frost JW (1995) Benzylfumaric, benzylmaleic, *Z*- and *E*-phenylitaconic acids: synthesis, characterization and correlation with a metabolite generated by *Azoarcus tolulyticus* strain Tol-4 during anaerobic toluene degradation. Appl.Environ. Microbiol. 62: 974-978.

Seyfried B, Glod G, Schocher R, Tschech A and Zeyer J (1994) Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60: 4047-4052.

Stoschek CM (1990) Quantitation of protein. In: M. P. Deutscher (Ed) Methods in Enzymology, Vol. 182 (pp 50-68), Academic Press, Inc.

Tang R and Kochi JK (1973) Cobalt (III) trifluoroacetate: an electron transfer oxidant. J. Inorg. Nucl. Chem. 35: 3845-3856.

Villemur R (1995) Coenzyme A ligases involved in anaerobic biodegradation of aromatic compounds. Can. J. Microbiol. 41: 855-861.

Zenk MH, Ulbrich B, Busse J and Stockigt J (1980) Procedure for the enzymatic synthesis and isolation of cinnamoyl-CoA thiolesters using a bacterial system. Anal. Biochem. 101: 182-187.

Zubay, G (1983) Biochemistry. Addison-Wesley Publishing Co., Reading, MA.

Chapter 5

SUMMARY AND FUTURE CONSIDERATIONS

Summary

The ability to degrade toluene and other alkylbenzenes under anaerobic conditions is not an uncommon trait among bacteria. These bacteria are widely distributed in nature and include an emerging group of closely related denitrifers, as well as sulfate-reducers, and an iron(III)-reducer. My work involving *Azoarcus tolulyticus* strain Tol-4, along with the current knowledge involving other bacterial strains capable of anaerobic alkylbenzene degradation, suggest two major pathways for anaerobic toluene degradation: 1) oxidative addition to the methyl group via a two-carbon addition reaction involving acetyl-CoA (e.g., strains Tol-4 and T1), and 2) hydroxylation of the methyl group via water as the source of oxygen (e.g., strains K172 and T). My experiments with strain Tol-4 has provided stronger support for the two-carbon addition mechanism than had previously been published by Evans and coworkers (Evans et al. 1992). In addition, data obtained through experiments with Tol-4 suggest significant modifications to the pathway Evans and coworkers suggested with strain T1 (see Chapter 3). Among the important results are the detection of hydrocinnamate and cinnamate, the identification of *E*-phenylitaconate (and not benzylfumarate) as an accumulating metabolite, and the direct involvement of acetate (possibly as acetyl-CoA) in the anaerobic degradation of toluene. Additionally, the cell-free toluene degradation activity that I obtained provides a start towards resolving the pathway and

mechanisms of anaerobic toluene degradation at an enzymatic level.

Future considerations

1. Cell-free anaerobic toluene degradation activity. The preliminary evidence involving Tol-4 *in vitro* studies demonstrated that the presence of both the soluble- and membrane-associated fractions may be necessary for toluene degradation activity. *In vitro* toluene degradation in my studies, however, was low and indicated that establishment of more optimal conditions will be necessary in order to increase the level of activity. Electron transfer mechanisms appear to be important in the pathway I have proposed for toluene degradation and further work must be done to investigate the identity of electron carriers and necessary redox reactions that must be involved. The role of strong metal oxidants such as cobalt (III) reported in abiotic benzene and toluene oxidation reactions (Tang and Kochi 1973) suggest the hypothetical role that metals may play in the critical first (activation) step. More purified cell fractions will be required to determine if toluene degradation activity does indeed require the cell membrane. Further work to determine if specific metals are important in alkylbenzene degradation is also necessary.

2. Identification of the anaerobic toluene degradation genes. Early work involving attempts to generate transposon (Tn-5) mutants of Tol-4 that were defective in anaerobic toluene degradation were not successful. New attempts should be made in designing a more successful system of generating mutants including the use of other transposons and optimizing cell transformation frequencies. One particularly difficult problem has been the inability of Tol-4 to grow on solid media consisting of basal salts and substrates such as benzoate, hydrocinnamate, and cinnamate to facilitate phenotype screening. As an

alternative to mutagenesis techniques, some preliminary work had also been initiated to generate a Tol-4 chromosomal library for attempting to recover the initial pathway genes by complementation into benzoate-degrading denitrifying strains. The genetic approach to identifying the genes involved in anaerobic toluene degradation is promising and would be certain to further define the pathway of degradation and complement the existing work that has been done using a more biochemical approach. Identification of the genes would also be a key to future studies involving protein expression and characterization, and gene probes.

3. Anaerobic degradation of benzene and polyaromatic hydrocarbons (PAHs).

The insights into anaerobic toluene degradation indicate that the initial degradation steps for the aromatic hydrocarbon will likely involve unique biochemical reactions. The focus of researchers on the use of toluene as the substrate is primarily due to the fact that isolates have been more easily obtained with the ability to degrade this particular compound. Tol-4 is restricted to degrading only toluene among the BTEX compounds, while some other isolates possess varying alkylbenzene degradation capabilities. None of the isolates obtained so far can degrade benzene anaerobically, although this substrate and naphthalene has been shown to be degraded under denitrifying and iron(III)- and sulfate-reducing conditions (Mihelcic and Luthy 1988, Lovley et al. 1995, Lovley et al. 1996, Coates et al. 1996). Efforts are underway to obtain new isolates capable of degrading benzene anaerobically. There is also considerable interest in anaerobic degradation of lower molecular weight unsubstituted PAHs. The chemical mechanism involving Co(III) oxidation of toluene and benzene (Tang and Kochi 1973) suggests some possible analogous biochemical mechanisms for the degradation of these compounds. By understanding the nature of anaerobic

toluene degradation, we may gain clues into the degradation of other alkylbenzenes and possibly substrates such as benzene and PAHs.

List of References

Coates, JD, Anderson RT, and Lovley DR (1996) Oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. Appl. Environ. Microbiol. 62:1099-1101.

Evans PJ, Ling W, Goldschmidt B, Ritter ER and Young LY (1992) Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58: 496-501.

Lovley DR, Coates JD, Woodward JC, and Philips EP (1995) Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. 61:953958.

Lovley DR, Woodward JC, and Chapelle, FH (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Appl. Environ. Microbiol. 62:288291.

Mihelcic JR and Luthy RG (1988) Microbial degradation of acenaphthalene and napthalene under denitrification conditions in soil-water systems. Appl. Environ. Microbiol. 54:1188-1198.

Tang R and Kochi JK (1973) Cobalt (III) trifluoroacetate: and electron transfer oxidant. J. Inorg. Nucl. Chem. 35:3845-3856.