ABSTRACT

HICKS, KRISTIN ADAIR. Alternative Substrates for Estimating TCE-degrading Capabilities of Toluene-oxidizing Bacteria. (Under the direction of Michael R. Hyman.)

One of the primary impediments to the implementation of bioremediation is uncertainty about success in the field. Soils and microbial populations are heterogeneous and it is difficult to extrapolate biodegradation rates from small samples to field scale. While biodegradation rates can be estimated from microcosm studies, *in situ* methods offer a more meaningful gauge of resident microbial activity. One method used to estimate biodegradation rates in the field is the newly developed Push-Pull technique. While this technique can be conducted on site, it is normally not possible to use target pollutants as the reactive substrates. Consequently, alternative, benign reactive tracers must be used. Ideally, these alternative, reactive tracers interrogate the same enzyme systems that are responsible for the biodegradation of the target pollutant. The objective of this study was to develop a reactive tracer system that could be used to assess toluene-dependent trichloroethylene (TCE) degradation. Our approach has been to determine whether a series of pure strains of toluene-oxidizing bacteria (*Burkholderia cepacia* G4, *Pseudomonas putida* F1, *Pseudomonas putida* mt2, *Pseudomonas mendocina* KR1), each with different toluene-oxidizing enzymes systems, are capable of cometabolically oxidizing a series of eleven potential alternative substrates. These substrates include simple alkenes, alkanes, and cyclic alkanes. The kinetics (K_s and V_{max}) of the biotransformation of these compounds have been determined. While oxidation

products were observed for a number of these substrates in connection with one or more of the test organisms, isobutylene was co-oxidized by all test organisms. Oxidation of isobutylene by each organism yielded kinetics constants comparable to the corresponding kinetics of TCE degradation. The enzyme system expressed by *Burkholderia cepacia* G4 catalyzed the epoxidation of isobutylene while the remaining enzyme systems catalyzed allylic alcohol formation. Isobutylene has potential in field scale Push-Pull studies as a tool for evaluating rates of aerobic toluene-dependent TCE degradation and of differentiating the relative contributions of the TCE-degrading population. A pilot study of this alternative substrate at Edwards Air Force Base will test whether it can be used successfully to estimate *in situ* degradation of TCE. Analysis of isobutylene oxidation products in toluene-enriched ground water may offer an inexpensive and effective method of measuring the degradation of TCE at contaminated sites nationwide.

ALTERNATIVE SUBSTRATES FOR ESTIMATING TCE-DEGRADING CAPABILITIES OF TOLUENE-OXIDIZING BACTERIA

By

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the requirements for the Degree of
Master of Science

SOIL SCIENCE

Raleigh

2002

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BIOGRAPHY

Kristin Hicks was born in Charlotte, North Carolina and grew up in neighboring Gaston County. She attended the University of North Carolina at Chapel Hill where she obtained a Bachelor of Arts in English. She later moved to Raleigh, North Carolina to pursue a Master of Science in Soil Science at North Carolina State University. She plans to continue her graduate education at the University of California at Davis, where she will seek a Doctorate in Soil Science.

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INTRODUCTION

1.1. Trichloroethylene: Background and Remediation Issues. Trichloroethylene (TCE) is a chlorinated solvent widely used in degreasing operations and produced in excess of 1500 tons annually in the U.S. (USITC, 1981; SRI ,1992). The toxicity of TCE is subject to debate but is generally considered to be of low to moderate toxicity (ATSDR, 1993; RTECS, 1993) and possibly a weak carcinogen (U.S. EPA, 1998). The main concern surrounding TCE arises from the fact that it is resistant to aerobic degradation (Semprini et al., 1990, 1991; Hopkins and McCarty, 1995; Fan and Scow ,1993), exhibits limited adsorption to soil surfaces and migrates readily with groundwater (Meylan et al., 1992; Rathbun, 1998). Between 1987 and 1993, approximately 2300 tons of TCE were released to U.S. land and water (U.S. EPA, 2001). It is estimated that as much as a third of U.S. groundwater is contaminated with TCE, making it the most common organic groundwater contaminant. The EPA recognizes TCE as a Priority Pollutant with a maximum contaminant level of 0.005 mg/L (U.S. EPA, 2001). Trichloroethylene has been identified at 852 of the 1,430 National Priorities List sites (ATSDR, 1997) and the EPA estimates that cleanup will cost in excess of $45 billion over the next several decades.

Conventional remedial methods for TCE involve excavation and/or a pump-and-treat system followed by some combination of air stripping, thermal destruction, adsorption by granular activated charcoal, or biological degradation. Bioremediation is generally more cost-efficient and provides the added advantage of actual degradation (vs. relocation) of the contaminant. However, the diversity of microbial activity and of

environmental parameters between sites makes the effectiveness of bioremediation often unpredictable and necessitates lengthy feasability studies. Frequently, these studies involve the costly and laborious process of extracting soil cores under sterile conditions and performing microcosm studies in the lab. In addition, some researchers have come to question the relevance of lab-scale degradation rates to field-scale remediation plans. New techniques that could make these determinations with greater accuracy and less expense would greatly enhance the potential of bioremediation as a widespread remediation option.

1.2. The Push-Pull Test. The recently developed push-pull technique has been used successfully to measure *in situ* rates of denitrification, sulfate reduction, and methanogenesis at petroleum-contaminated sites (Istok et al., 1997). Currently, the push-pull method is under investigation as a tool for measuring *in situ* rates of microbially-mediated uranium reduction (Istok, 2000) and of *anaerobic* TCE degradation (Istok et al., 2001). In a push-pull test, the push phase consists of the injection of reactive and unreactive tracers into an existing monitoring well. In the pull phase, the test solution is extracted from the same well. The concentrations of the remaining tracers and any metabolites are measured and breakthrough curves are established for all solutes. This data is then used to predict reaction stoichiometries and *in situ* rates of degradation at the site. The push-pull method offers a number of advantages over microcosm studies. It can be used on site at existing monitoring wells and consequently explores a much larger volume of sediment and groundwater. The push-pull method is simple, inexpensive and

may be more representative of the degradative activity of the resident microbial population.

The goal of this project was to lay the groundwork for applying the push-pull method to the treatment of chlorinated aliphatic hydrocarbons (CAHs) in aerobic groundwater, specifically, on the aerobic transformation of TCE by toluene-oxidizing organisms. Since TCE is a regulated pollutant and cannot be injected into groundwater, the identification of an alternative substrate, which could serve as a surrogate for TCE, was necessary. Because TCE-degradation is carried out by a broad spectrum of microorganisms, a prepared test solution for use in the push-pull test may engage one or several microbial enzyme systems. The test solution may contain a substrate which interrogates a broad range of TCE-degrading microorganisms and gives an estimate of the *overall* activity. In addition to this "universal" substrate, the test solution may also include one or more "selective" substrates, which offer an estimate of the contribution of *specific* classes of microorganisms to the total TCE-degrading activity.

Ideally, an alternative reactive tracer should serve as either a universal or selective substrate and should satisfy a number of criteria. It should be non-toxic and water-soluble. It should be transformed by the same range of organisms and by the same enzyme systems by which TCE is transformed. Finally, it should be transformed into benign, soluble, stable, and measurable products. Once identified and the kinetics of its degradation characterized, this tracer could be used in the push-pull test to estimate TCE degradation in aerobic groundwater systems. One alternative tracer that has been used successfully to track anaerobic TCE degradation, is the fluorinated TCE analog trichlorofluoroethylene (TCFE). In an investigation using anaerobic groundwater

microcosms, TCFE demonstrated degradation pathways and kinetics comparable to those of TCE (Vancheeswaran et al., 1999). The presence of the fluorine marker offered an additional advantage by allowing differentiation between the tracer and background contaminants.

The primary objective of this study was to develop a reactive tracer system suitable for use in push-pull tests designed to estimate aerobic, toluene-dependent, TCE transformation rate. Our approach was to test the affinity of TCE-degrading, toluene-oxidizing enzyme systems for several alternative substrates. The enzyme kinetics of the alternative substrate oxidations were characterized and compared.

1.3. Biodegradation of Trichloroethylene. Biological transformation of TCE occurs both anaerobically and aerobically. Anaerobic biodegradation of TCE occurs as halorespiration, which is a metabolic process, and as reductive dechlorination, which is a cometabolic process (Vogel and McCarty, 1985; McCarty et al., 1996). In aerobic systems, which are the focus of this study, no evidence for growth-related metabolism of TCE has been observed. Current research suggests that *cometabolism* is the principle method of the transformation of TCE in aerobic environments. In contrast to metabolism, cometabolism requires the presence of a primary substrate in order to accomplish the biological transformation of the target substrate. The microorganisms involved produce enzymes targeted toward the primary substrate and obtain their carbon for growth and energy from that substrate. The enzymes produced by these microorganisms also fortuitously transform the persistent hydrocarbon, but the microbes derive no benefit from the process. With the exception of recently isolated strains from *B. cepacia* G4

(Shields and Reagin, 1992) and *R. corallinus* B-276 (Saeki, 1999), enzyme expression is induced by the presence of a specific substrate and does not occur in the absence of the substrate.

Cometabolism is usually attributed to the activity of enzymes with broad substrate specificities. Oxygenase enzymes, chiefly mono- and dioxygenases, frequently exhibit this trait and are commonly associated with cometabolic processes. Because cometabolism may be chiefly responsible for the attenuation of many recalcitrant compounds, development of effective methods of stimulating cooxidative activity could offer important advances in large-scale treatment of persistent pollutants.

A number of enzyme systems have been shown to cometabolize TCE. Various organisms that metabolize methane, ethylene, propylene, propane, butane, isoprene, ammonia, phenol, and toluene have been shown to also cometabolize TCE (Table 1.1). In particular, methane (Wilson and Wilson, 1985; Fogel et al., 1986; Little et al.,1988; Fox et al., 1990; Roberts et al., 1990; Semprini et al., 1990), phenol (Folsom et al., 1990), and toluene (Wackett and Gibson, 1988; Fan and Scow, 1993) have demonstrated their effectiveness as primary substrates in cometabolic TCE degradation, both in the lab and in the field. Both phenol and toluene have been shown to produce comparable TCE degradation rates and to exceed those of methane in field evaluations (Semprini et al., 1990; Hopkins et al., 1993; Hopkins and McCarty, 1995). More recently, a pilot study conducted at Edwards Air Force Base in California attained TCE removal rates of 97-98% from contaminated groundwater using toluene as the primary substrate (McCarty et al., 1998). The researchers who conducted this study, selected toluene over phenol in part because certain reaction products of phenol are know to pose a human health risk.

When phenol reacts with chlorine, chlorophenols, such as the probable human carcinogens pentachlorophenol and 2,4,6-trichlorophenol, are formed (U.S. EPA, IRIS). For this reason, the presence of high levels of phenol in ground water with a high chlorine content is not recommended (WHO, 1984). Because of the high rates of toluene-dependent TCE transformation, the successful use of toluene in field-scale remediation efforts and the absence of hazardous daughter products, toluene was chosen as the primary substrate in our investigation.

Table 1.1. Organisms which have demonstrated substrate-dependent TCE-transformation.

Primary Substrate	Organism	Enzyme System	Reference
Ethylene/ Propylene	Xanthobacter *Py2*	Alkene monooxygenase	Ensign 1992 Reij 1995
Propylene	*Rhodococcus corallinus* B-276	Alkene monooxygenase	Saeki 1999
Ammonia	*Nitrosomonas europaea*	Ammonia monooxygenase	Arciero 1989 Bedard 1989. Ely 1995b Hyman 1995 Rasche 1991
Phenol & 2,4 dichloro-phenoxyacetate	*Ralstonia eutropha* JMP 134	Phenol hydroxylase, 2.4-Dichlorophenol hydroxylase	Harker 1990
Phenol	*Burkholderia cepacia* G4	Toluene monooxygenase	Folsom 1990
Butane	*Pseudomonas butanavora*	Butane monooxygenase	Hamamura 1997
Propane	*Mycobacterium vaccae* JOB5	Propane monooxygenase	Wackett 1989
Isoprene	*Rhodococcus eryhtropolis*	Alkene monooxygenase	Ewers 1990
Methane	*Methylomonas trichosporium* OB3b	Particulate methane monooxygenase	DiSpirito 1992 Lontoh 1998
Methane	*Methylomonas trichosporium* OB3b	Soluble methane monooxygenase	Joergensen 1985 Koh 1993 Oldenhuis 1989,1991 Sun 1996 Tsein 1989
Methane	*Methylomonas methanica* 68-1	Soluble methane monooxygenase	Koh 1993
Toluene	***Pseudomonas putida* F1**	**Toluene dioxygenase**	Heald 1994 Leahy 1996 Sun 1996 Wackett 1988 Zylstra 1989
Toluene	***Burkholderia cepacia* G4**	**Toluene 2-monooxygenase**	Folsom 1990 Landa 1994 Leahy 1996 Shields 1991 Sun 1996
Toluene	***Pseudomonas mendocina* KR1**	**Toluene 4-monooxygenase**	Leahy 1996 Sun 1996 Winter 1989
Toluene	***Ralstonia picketti* PKO1**	**Toluene 3-monooxygenase**	Olsen 1994 Leahy 1996

1.4. Toluene Mono and Dioxygenase Systems. While utilization of toluene as a carbon source is not unique to *B. cepacia* G4, *P. putida* F1, *P. mendocina* KR1, and *R. picketti* PKO1, concomitant degradation of TCE has not been demonstrated in other wild-type microorganisms (Table 1). A number of TCE-degrading, genetically altered organisms, such as recombinant *E. coli* strains containing TCE-oxidizing genes from *P. putida* F1 (Zylstra and Gibson, 1989) and from P. mendocina KR1 (Winter, 1989), have been developed. For the purposes of this study, (i.e. *in situ* evaluation of resident microbial activity), we have excluded recombinant TCE-degraders from our discussion and have focused on characterizing the oxidative capabilities of naturally-occuring, wild-type organisms.

Enzyme-catalyzed oxidation of the toluene molecule can occur at any of the carbon-hydrogen bonding sites on the molecule with the insertion of either one or two atoms of O_2. Each organism expresses an enzyme, either a mono or dioxygenase, which preferentially targets a different site on the molecule (See Figure 1.1). Oxidation of the toluene ring primes it for further oxidative stages and eventual metabolism. In the *B. cepacia* G4 pathway, toluene is oxidized at the ortho position by toluene 2-monooxygenase (T2MO) to generate *o*-cresol. *R. picketti* PKO1 and *P. mendocina* KR1 express toluene 3-monooxygenase (T3MO) and toluene 4-monooxygenase (T4MO) respectively. Toluene 3-monooxygenase oxidizes the meta position to produce *m*-cresol while T4MO oxidizes the para position to produce *p*-cresol (Pikus, 1997). The aromatic hydrocarbon-degrading ability of *P. putida* F1 has been extensively studied and characterized. *P. putida* F1 exhibits a broad substrate range that includes toluene, as well as benzene, phenol, and ethylbenzene (Gibson et al., 1990). The toluene dioxygenase

(TDO) expressed by *P. putida* F1 oxidizes toluene at the ortho and para positions to form *cis*-toluene dihydrodiol (Gibson et al., 1970; Finette et al. 1984;Yeh et al., 1977). Toluene may also be primed for metabolism by (mono)oxidation of the methyl group to form benzyl alcohol. One organism known to carry out oxidation at this site is *Pseudomonas putida* mt2 (Assinder, 1990). Although *P. putida* mt2 can utilize toluene as a primary carbon source, current research has found no evidence that this strain cooxidizes TCE.

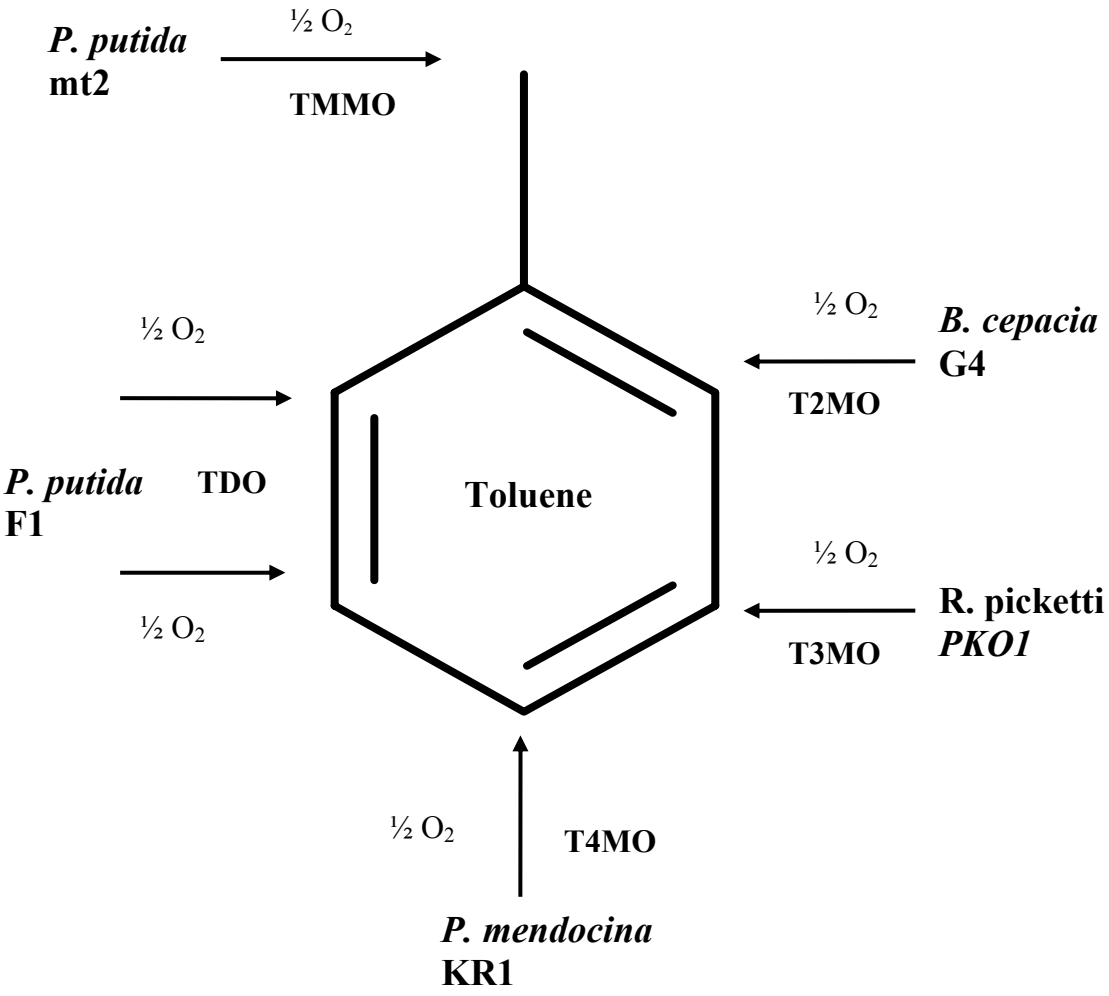

Figure 1.1. Initial oxidation sites on the toluene molecule for metabolism by five toluene-utilizing bacteria. The mono or dioxyenase enzymes produced by toluene-oxidizing organisms bind to the toluene molecule at different sites. *P. putida* mt2 oxidizes the methyl group.*P. putida* F1 produces a dioxygenase, which targets the ortho and para positions. *B. cepacia* G4, *R. picketti* PKO1, and *P. mendocina* KR1 oxidize the toluene ring at the ortho, para and meta positions respectively.

1.5. Toluene-dependent TCE-degradation. In aerobic environments, TCE can be degraded by multiple enzyme pathways. Perhaps the most thoroughly studied and understood of these pathways are those of *B. cepacia* G4 and *P. putida* F1 (Fig. 1.2). The most significant difference in the oxidative action of T2MO and TDO is formation of intermediate epoxide products by the former. The T2MO produced by *B. cepacia* G4 oxidizes the TCE molecule at the double (pi) bond to form TCE epoxide. TCE epoxide is an unstable product, which rapidly undergoes hydration to form TCE-diol (1,2-dihydroxy-TCE). Trichloroethylene diol spontaneously breaks down to form carbon monoxide, formate and glyoxylate (Miller and Guengerich, 1982; Newman and Wackett, 1997). The TDO produced by *P. putida* F1 yields glyoxlate and formate, but not carbon monoxide, as its principal products (Li and Wackett, 1992). The intermediate steps in the oxidation of TCE by TDO are not yet fully understood but two theoretical intermediates have been proposed: TCE diol (Li and Wackett, 1992) or a symmetrical iron-bridged dioxygen TCE species (Wackett, 1995).

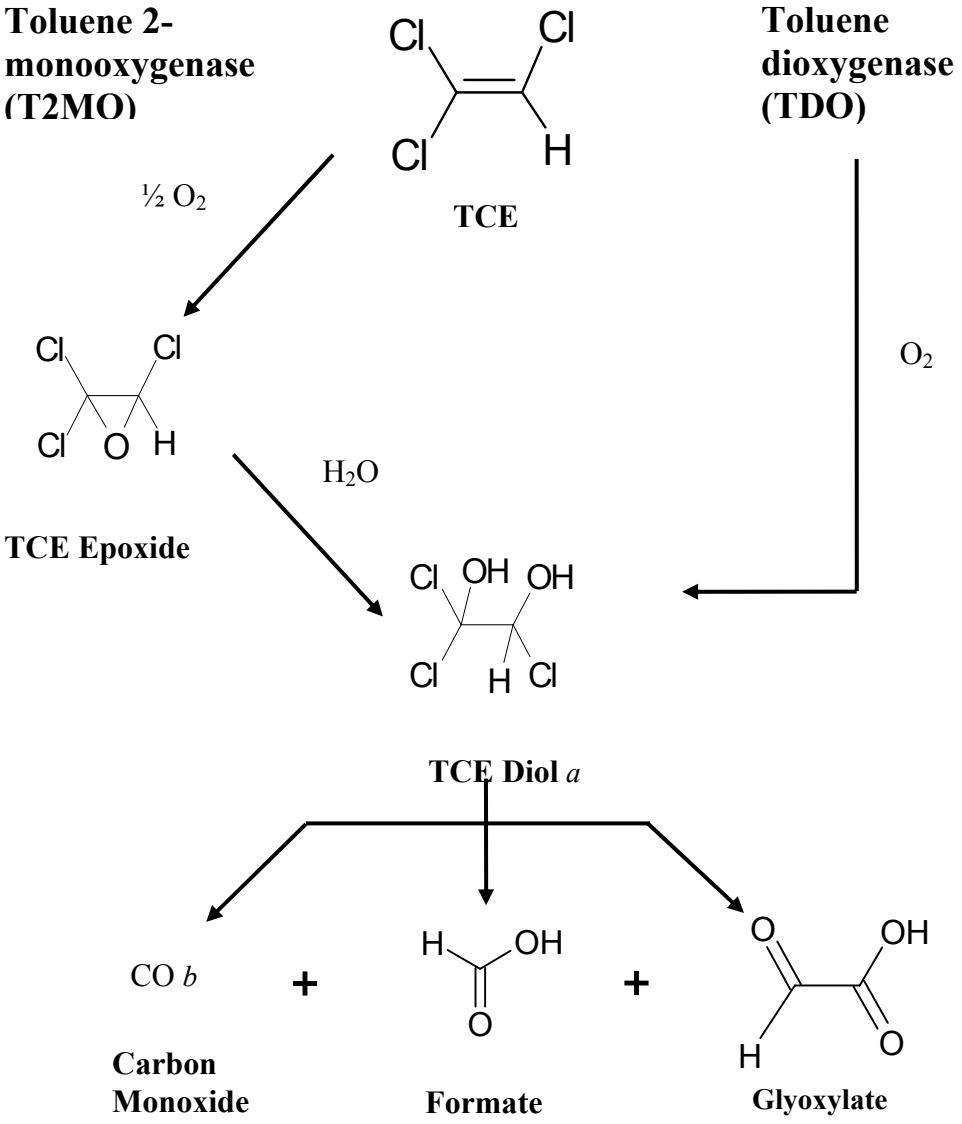

Figure 1.2. Putative pathway of TCE oxidation by toluene 2-monooxygenase (*B. cepacia* G4) and dioxygenase (*P. putida* F1). *a* TCE diol is a proposed, but not observed intermediate for dioxygenase. *b* Carbon monoxide production has been observed in the monooxygenase, but not the dioxygenase pathway.

1.6. Rationales for Alternative Substrate Selection. In this study, we selected eleven hydrocarbons and evaluated the potential of each to act as a TCE surrogate in the push-pull test. We focused our attention on a series of simple, branched or cyclic alkanes and alkenes: ethylene, propylene, 1-butene, n-butane, 1-pentene, n-pentane, isobutene, isobutane, cyclopropane, cyclopentane, and cyclohexane.

The structural similarities between TCE and the simple alkenes formed the rationale for selection of simple alkenes as potential alternative substrates for push-pull tests. We reasoned that structurally homologous tracers might lend themselves to functionally similar enzyme association. For example ethylene--a non-chlorinated alkene bears close structural similarity to TCE. Other practical advantages associated with alkenes are their relatively high solublility, low cost, and ready availability.

The cometabolic oxidation of ethylene, as well as propylene, has been observed in methane- (Hou et al., 1979) and propane-oxidizing bacteria (Hou et al. 1983) and both serve as growth substrates for the TCE-degrading organism *Xanthobacter* Py2. *Nitrosomonas europea*, an ammonia-oxidizing bacteria, also co-oxidizes ethylene (Hyman et al., 1995). Due to the broad range of bacterial enzyme systems, which transform ethylene and propylene, it has been suggested that they may be useful as universal substrates for estimating the overall CAH-transforming ability of resident microbial communities (Arp et al., 2001).

Similarly, studies of the environmental biodegradation of 1-butene indicate that it has the potential to be cooxidized by a range of microorganisms. Epoxidation of

1-butene by organisms that utilize ethylene (Hou et al., 1979), methane (Colby et al., 1977, and propane (Hou et al., 1983) have been reported. Previous studies have found that *B. cepacia* G4 is capable of oxidizing ethylene, propylene (Yeager et al. 1999), 1-butene, and 1-pentene (McClay et al., 2000) to their respective epoxides. *P. mendocina* KR1 and *R. picketti* PKO1 have recently demonstrated the ability to oxidize 1-butene and 1-pentene to epoxybutane and epoxypentane respectively (McClay et al., 2000. In addition, epoxidation of 2-butene, 1,3-butadiene, 2-pentene, 1-hexene, and octadiene by these strains has been reported (McClay et al., 2000). Due to their transformations by a range of microorganisms and enzymatic routes, the alkenes served as a logical starting point in the selection and testing of TCE surrogates. Our study sought to ascertain the activity of toluene-oxidizing organisms towards this compound class and to determine whether one or more of these alkenes could serve as a universal or selective substrate in toluene-oxidizing environments. Evidence suggests that the monooxygenases of *B. cepacia* G4, *P. mendocina* KR1, and *P. putida* mt2 will likely generate epoxides of alkene substrates whereas the dioxygenase expressed by *P. putida* F1 is expected to catalyze dioxygenation or allylic monooxygenation of alkenes (Lange and Wackett 1997).

A series of simple alkanes—n-butane, n-pentane, and isobutene--were also selected for experimentation. Numerous organisms are known to oxidize alkanes, both as growth and non-growth substrates. Earlier research has demonstrated the ability of *P. mendocina* KR1 to consume n-pentane as a primary growth substrate (McClay et al., 1995). With this exception, little attention has focused on the alkane-oxidizing capability of toluene-oxidizing bacteria. However, structural similarities between the alkane-

oxidizing enzymes and toluene-oxidizing enzymes have been reported (Arp et al., 2000). Our selection of n-butane, n-pentane, and isobutane as test substrates sought to shed light on this structural congruity and evaluate the possibility that catalytic similarities also exist between alkane and toluene-oxidizing enzymes. An oxygenase-catalzyed transformation of the simple alkanes--n-butane, n-pentane, isobutene-- would likely produce the corresponding alcohol or aldehyde.

The rationale for selecting cyclic alkanes--cyclopropane, cyclopentane, and cyclohexane--as test substrates hinged on several premises. Firstly, these cyclic alkanes are known to be resistant to mineralization and are thought to be catabolized chiefly by co-oxidation and/or the action of a bacterial consortium (Beam and Perry, 1974; Jamison et al., 1976; Perry, 1979; Walker and Colwell, 1976; Kawasaki 1980). Utilization and mineralization of the alternative substrate as a carbon source by the degrading organism or other resident flora could adversely effect its usefulness as a gauge of TCE-degradation. In addition, cyclic alkanes are saturated analogs of toluene and we reasoned that these structural similarities might enhance the likelihood of binding and oxidation by the toluene-oxidizing enzymes. The first step in co-oxidation of cyclic alkanes is often the corresponding alcohol or aldehyde. Alcohols such as cyclohexanol and cyclopentanol are easily identifiable and remain comparatively stable in the environment. Thus, the respective alcohol of a cyclic alkane would offer a number of advantages as a tracer for co-oxidative activity.

1.7. Sustainability of TCE Cometabolism. Transformation rates of TCE are often limited by three principle factors: 1) insufficient concentrations of the growth-supporting

substrate, 2) competitive inhibition due to excessive levels of growth-supporting substrate, and 3) product-associated toxicity. Current research indicates that the ratio of the growth-supporting substrate to TCE must be carefully regulated. The primary substrate must be present in high enough concentrations to provide energy for the organisms and to induce the oxygenase enzymes (Wackett and Gibson, 1988; Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1991; Mu and Scow, 1994). Conversely, excess levels of the primary substrate may inhibit TCE transformation by preferentially binding to the enzymes (Alvarez-Cohen and Speitel, 2001).

In addition to the requirement of a controlled substrate/cometabolite ratio, TCE transformation may be limited by the toxic effects of its daughter products. Trichloroethylene oxidation products may exhibit toxicity directly to microbial cells or inactivate the enzymes involved in the transformation. Whole cell studies with a range of TCE-oxidizing organisms, including those that express both toluene mono- (Yeager et al., 2001) and toluene dioxygenase (Heald and Jenkins, 1994), showed evidence of cellular damage and a marked decline in TCE transformation ability over time (Oldenhuis et al., 1991; Rasche et al., 1991; van Hylckama Vlieg et al., 1997; Wackett and Gibson, 1988). Purified enzyme studies using soluble methane monooxygenase (Fox et al. 1990), toluene dioxygenase (Li and Wackett, 1992), and toluene 2-monoooxygenase (Newman and Wackett, 1997) demonstrated that TCE oxidation results in loss of enzyme function over time. A number of models have been created to account for toxic effects associated with transformation products (Alvarez-Cohen and McCarty, 1991; Chang and Alvarez-Cohen, 1995; Ely et al., 1995).

Recent research suggests that TCE-mediated toxicity may pose a more significant problem in remediation plans than the immediate disadvantage of cell death. The effect of TCE on the competitive behavior of toluene-oxidizing bacteria was studied by Mars et al. (1997). These researchers found that TCE had a negative effect on the survivability of TCE-degrading bacteria. Populations of *P. putida* F1, *P. putida* GJ31, and *B. cepacia* G4 declined and eventually disappeared from mixed cultures containing toluene and TCE, whereas the non-TCE oxidizer, *Pseudomonas putida* mt-2, came to dominate the culture.

1.8. Modeling of Enzyme Kinetics. The rate of an enzyme reaction (V) depends on the concentrations of both substrate (S) and enzyme (E) (Equation 1.1). The overall reaction mechanism is commonly expressed as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Eq. 1.1

At the initiation of an enzyme-catalyzed reaction, the enzyme (E) binds reversibly to the substrate (S) to form an enzyme-substrate complex (ES). This first step is governed by the rates of association (k_1) of enzyme with substrate and dissociation (k_{-1}) of enzyme from substrate. The second step is characterized by the formation of a measurable product (P). This reaction is governed by the rate constant k_{cat} (or turnover number), which is the

number of catalytic events per unit time per enzyme active site.

The Michaelis-Menten equation (Eq.1.2) is based on this model of enzyme activity and shows the hyperbolic, mathematical relationship between V and [S]. It describes the initial velocity of enzyme activity (V) as a function of molar substrate concentration [S] where both V and [S] are experimentally determined values. Initial velocities are obtained from graphs of the increase in product concentrations over time. These graphs, when linear demonstrate that the rate of product formation, which is a function of the amount of enzyme present, is directly proportional to the amount of substrate present. The initial velocity is defined as the slope of the linear relationship, i.e. product formed per unit time for a fixed substrate concentration. These velocities, when taken at different substrate concentrations and plotted versus those substrate concentrations, typically produce a hyperbolic curve.

$$V = V_{max} * [S] \ / \ K_m + [S]$$

Eq. 1.2

V = initial velocity when substrate is limited
V_{max} = maximum velocity when substrate is unlimited
K_m = Michaelis constant or half-saturation constant
(expressed as a molar concentration)
[S] = substrate concentration

The Michaelis-Menten equation introduces two other parameters: the maximum velocity (V_{max}) and the Michaelis constant (K_m). The maximum velocity is defined as the velocity under substrate-saturated conditions. At low concentrations of substrate, the curve approximates a straight line and the reaction is first order with respect to substrate (Figure 1.3). In this section of the curve, the enzyme sites are unsaturated and increasing substrate concentration corresponds with increasing product formation. At high concentrations of substrate, the enzyme sites are saturated and enzyme velocity shows no response to greater substrate concentrations. At this stage the rate is becoming independent of the substrate concentration and is approaching V_{max}. The maximum velocity is a theoretical value derived from the hyperbolic curve. In practice, V_{max} is not experimentally observed.

A more meaningful parameter in enzyme kinetics is the Michaelis constant. Also called the half-saturation or affinity constant, K_m is expressed as a molar concentration and is defined as the concentration of substrate when the velocity is one-half of V_{max}. The Michaelis constant is the substrate concentration that, in theory, fills half of the enzyme sites (half-saturation). Unlike Vmax, K_m is independent of the amount of enzyme present-- it is an intrinsic feature of the enzyme-substrate system. The Michaelis constant can be used to gauge the catalytic efficiency of an enzyme by offering a measure of the binding affinity of the enzyme. A small K_m value suggests that the enzyme binds rapidly to the substrate and thus has a high affinity for the substrate. The term K_m is applicable in enzyme kinetics studies that use purified enzymes. When whole cell assays replace purified enzymes, as in this study, the term K_s is substituted.

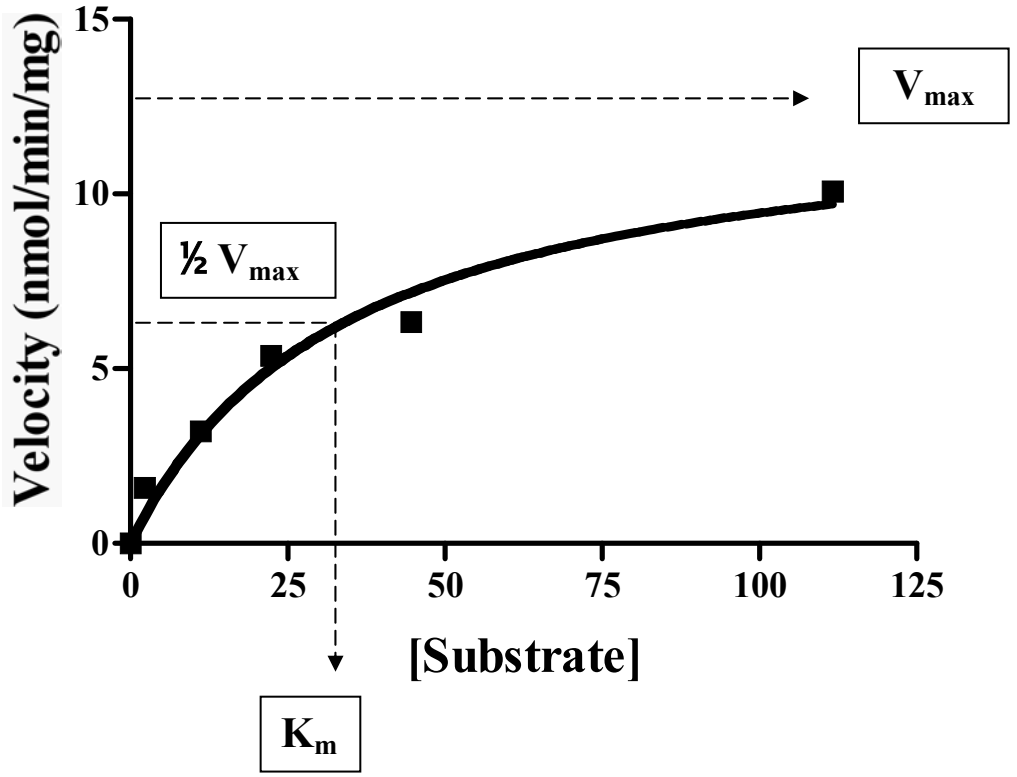

Figure 1.3. Determination of enzyme kinetics parameters based on the Michaelis-Menten equation. Experimentally-derived linear velocities are plotted versus substrate concentration and a hyperbolic curve is fitted to the data. The maximum velocity (V_{max}) and the half-saturation constant (K_m) are extrapolated from the curve.

MATERIALS AND METHODS

2.1. Organisms and Chemicals Used. The present study used three of the four known wild-type organisms capable of toluene-dependent TCE cometabolism: *B. cepacia* G4, *P. putida* F1, and *P. mendocina* KR1. *P. putida* mt2 was also selected for experimentation. *P. putida* mt2 uses toluene as a primary substrate but previous research has demonstrated no TCE-degrading activity (Mars et al., 1997). *B. cepacia* G4 was a gift from Dr. Malcolm Shields, (University of West Florida). *P. mendocina* KR1 was obtained from Amgen Inc.,Thousand Oaks, CA. *P. putida* F1 (strain # 700007) and *P. putida* mt2 (strain # 33015) were purchased from American Type Culture Collection (Manassas, VA).

Chemicals were used as either test substrates or for identification of potential products. Ethylene, ethylene oxide, propylene oxide, propylene glycol, 1-butene, 1,2-epoxybutane, 1,2-butane diol, 1-butanol, 1-pentene, 1,2-epoxypentane, 1,2-pentanediol, n-pentane, 1-pentanol, cyclopropane, cyclopentane, cyclopentanol, cyclohexane, cyclohexanol, isobutene, isobutylene oxide, methyl allyl alcohol (2-methyl-2-propen-1-ol), isobutane, isobutanol, and toluene were all 99+% purity and were purchased from Aldrich Chemical Co. (Milwaukee, WI). Propylene was obtained from Union Carbide (Danbury, CT). Ethylene glycol was obtained from Fisher Scientific (Fair Lawn, NJ). N-butane and compressed gases for gas chromatography (H_2, N_2, and air) were obtained from National Welders (Charlotte, NC).

2.2. Growth of Toluene-oxidizers. Each organism was maintained in pure culture on lactate mineral salts agar plates. The media contained per liter: 0.5 g NH_4NO_3, 0.2 g

$MgSO_4 \bullet 7H_2O$, 0.05 g $CaCl \bullet 2H_2O$, 0.01 g disodium EDTA, 0.005 g $FeCl_3$, 20 mM lactate, 15 g Bacto agar, and 10 ml of a trace elements solution (Yeager et al., 1999). The trace elements solution consisted of (per liter): 0.143 g H_3BO_3, 0.102 g $MgSO_4 \bullet 7H_2O$, 0.032 g $ZnSO_4 \bullet 7H_2O$, 0.01 g $CoCl_2 \bullet 4H_2O$, 0.008 g $CuSO_4 \bullet 5H_2O$, 0.005 g $Na_2MoO_4 \bullet 2H_2O$. After the media was autoclaved, the pH was adjusted to 7.0 by adding 1M KH_2PO_4-K_2HPO_4 (50 ml). Cells were streaked onto plates, incubated at 27°C for 48 hours in a Precision Gravity Convection oven and stored for later use. To minimize contamination, new liquid cultures were initiated from these plates each month.

Liquid batch cultures were grown in glass serum vials (125 ml) (Wheaton Science Products, Millville, NJ) containing mineral salts media (25 ml) without lactate or agar. Bottles were capped by butyl rubber stoppers and sealed with aluminum crimp caps (Wheaton Science Products, Millville, NJ). New cultures were inoculated with 1 ml of the previous culture using sterile, disposable 1 ml syringes (Becton Dickinson &Co. Franklin Lakes, NJ). Toluene (0.05% v/v) was delivered as the sole carbon source for all organisms using a gas-tight syringe (Hamilton, Reno, NV). Liquid cultures were incubated at 30°C at 150 rpm in an Innova Multi-tier Environmental Shaker (New Brunswick Scientific, Edision, NJ).

2.3. Preparation of Cells. Prior to harvesting, the optical density (OD_{600}) of each culture was determined on a Shimadzu 1601 UV-Visible Spectrophotometer. After 48 hours, culture bottles were opened and lightly shaken to replenish oxygen in the head space. Bottles were resealed and reinjected with 0.05% v/v toluene as previously described and incubated for an additional 1 hour. Liquid cultures were centrifuged for 10 minutes at

10,000 rpm and at 10° C using an IEC Centra MP4R benchtop centrifuge (International Equipment, Needham Heights, MA). The supernatant was discarded and the cell pellet was washed with 50 mM Na_2PO_4, pH 7.0 buffer (8 ml). Cells were centrifuged for an additional 10 minutes, and the cell pellet was finally resuspended in 50 mM Na_2PO_4 buffer (1 ml) and stored in an Eppendorf tube at 4°C for the duration of the experiment (<5 hours).

2.4. Analytical Methods. Identification and quantification of oxidative products were performed on a Shimadzu 8A Gas Chromatograph equipped with a flame ionization detector and fitted with two stainless steel Porapak Q (80-100 mesh) columns (6' x 1/8" and 18" x 1/8"). (Ohio Valley Specialty Chemical, Marietta, OH). Alkane and alkene transformation products (i.e. alcohols or epoxides), were separated and quantified on the short (18") and long (6') columns respectively. The detector operated at a temperature of 220° C, the injector at 200° C and the columns at 160 °C. The carrier gas was nitrogen with a flow rate of 15 ml/min. Gas chromatograph outputs were recorded by an HP 3395 Integrator (Hewlett Packard, Palo Alto, CA). All samples for gas chromatography were 2 μl liquid injections.

A portion of each cell suspension (≥ 150 μl) was frozen at 4°C for subsequent protein assays. Protein assays were conducted for each cell suspension. Protein concentration (mg/ml) was determined using the microburet assay method, as described by Gornall et al. (1949). Bovine serum albumin was used as the standard.

2.5. Substrate Oxidation Assays. Substrate oxidation tests were performed using glass serum vials (10 ml) sealed with butyl rubber stoppers and aluminum crimp caps (Wheaton Science Products, Millville, NJ). Unless otherwise stated, all reaction vials contained 50 mM Na_2PO_4buffer (900 μl) and potential substrates were added as neat compounds. Gaseous substrates were added with sterile syringes (1 ml) while liquid substrates were added with gas-tight micro syringes. Vials were incubated at 30°C in a reciprocal, shaking water bath (Precision Scientific, Winchester, VA) at 150 rpm for 10-15 minutes to allow equilibration of potential substrates between the gaseous and aqueous phases. The reactions were initiated by the addition of the concentrated cell suspension (100μl) at an average protein concentration of 2.7 mg/mL) to give a final reaction volume of 1 ml. The reaction vials were returned to the shaking water bath and assayed by gas chromatography at the indicated times. To control for non-biological substrate transformations and losses and to establish that transformations were biologically mediated, each experiment included a reaction vial containing no cells. Abiotic controls contained 1 ml of 50 mM Na_2PO_4 buffer and no cells.

2.6. Experimental strategy. Our initial approach was to add the appropriate volume of substrate—gases (1ml), liquids (5µl)—to a reaction vial, inoculate each (biotic) reaction vial with cell suspension, assay the reaction vials at 0 and 60 minutes, and note any possible transformation products. Transformation products were identified by comparing their gas chromatograph retention times to those of the commercially-available, authentic products (Fig. 2.1).

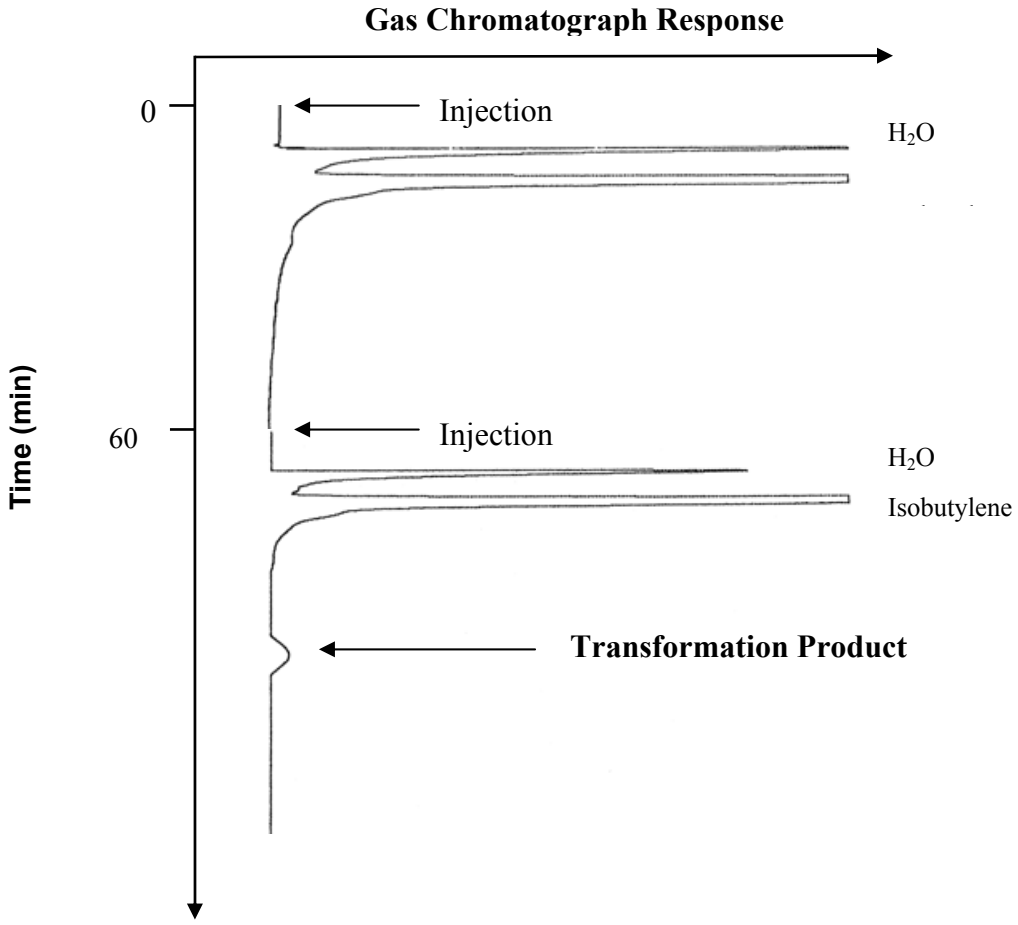

Figure 2.1. Gas chromatograph Integrator Output for a Reaction Vial Containing *B.cepacia* G4 and Isobutylene (1 ml) at Times 0 and 60 minutes. An oxidation product appeared after 60 minutes, which was subsequently identified as isobutylene oxide.

2.7. Substrate Concentration vs. Product Studies. If an oxidation product was observed, a more detailed assay was performed to characterize the response of the enzyme system to varying concentrations of substrate. These assays were conducted in the manner previously described, except that each of the several (~7) reaction vials contained a different volume of substrate with a range of 0 to 3 ml for gases and 0 to 20 µl for liquids. In addition, abiotic controls were included for each substrate in the method described above. These experiments demonstrated the relationship between enzyme system (by peak area) and substrate concentration (by volume) and formed the basis of the later determination of K_s and V_{max} values.

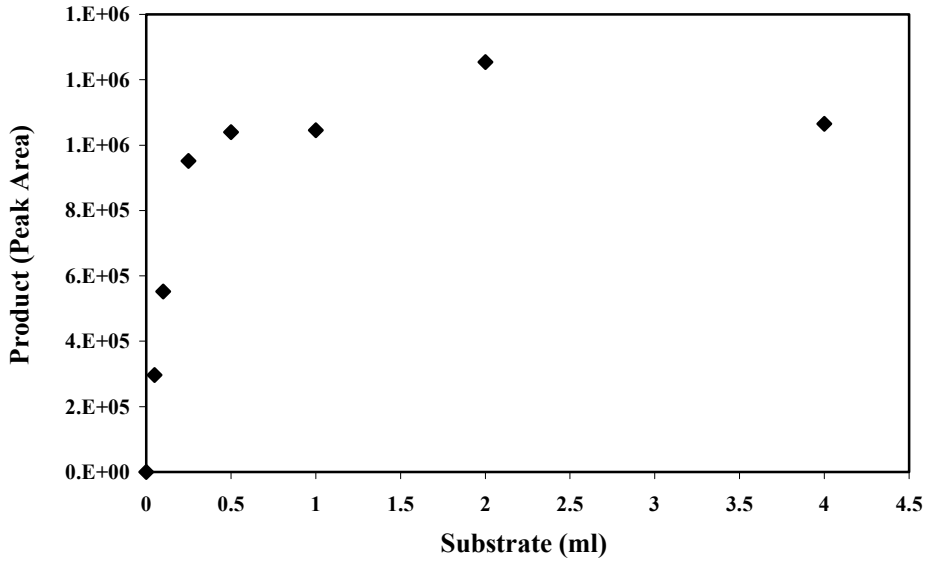

Figure 2.2. Product formation (Propylene oxide) by *B. cepacia* G4 incubated with a Test Substrate (Propylene) for one hour. The substrate is represented in terms of the volume added. The amount of transformation product is given in terms of the peak area as generated by gas chromatograph outputs.

2.8. Time vs. Activity Studies. Time course assays were conducted to establish the linearity of the transformation reaction. A single volume of each substrate was chosen from the peak of the substrate concentration-product plot. This volume was added to a reaction vial, as previously described, and samples were assayed by gas chromatography at 10-minute intervals for 90-120 minutes.

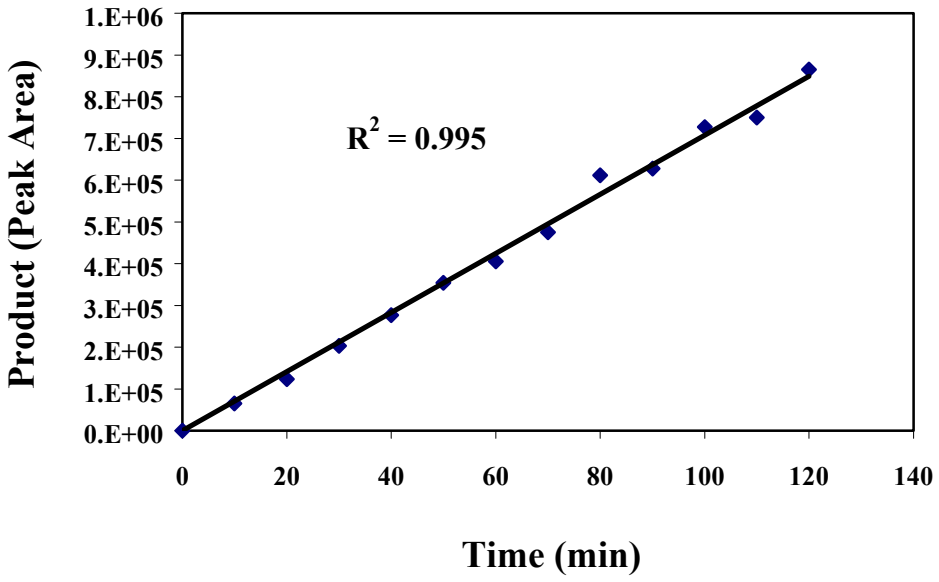

Figure 2.3. Oxidation of a Fixed Substrate Volume as a Function of Time. In this example, *B.cepacia* G4 was incubated with propylene (1 ml) and assayed every ten minutes. The amount of transformation product (propylene oxide) was recorded as a peak area by the GC integrator.

2.9. Calibration Plots and Preparation of Stock Solutions. Calibration plots were conducted using 0.1M stock solutions of the oxidation product. Stock solutions were prepared by adding the appropriate volume of sterile, deionized water and of commercial-grade hydrocarbon to a 10 ml volumetric flask. The stock solution was mixed thoroughly before use and discarded after 24 hours. A calibration plot was performed for each observed, identified product. These plots were used to quantify the peak areas of each transformation product in nanomoles. Finally, protein assays and incubation times were incorporated to give all product data in terms of nanomoles per minute per milligram of whole cell protein.

2.10. Determination of Enzyme Kinetics. The data were analyzed using Prism (GraphPad Software, Inc., San Diego, CA), a non-linear regression program. Prism uses the Marquardt method for performing nonlinear regression. A rectangular hyperbola model was fit to the data by applying the Michaelis-Menten equation for enzyme velocity as a function of substrate concentration. The half-saturation constant, initial velocity, and maximum velocity were determined for each cometabolic substrate/organism pair.

RESULTS

3.1. Oxidation of Straight-chain Alkenes by Toluene-oxidizers. For each alkene, toluene-grown cells of the test organisms were exposed to varying concentrations of substrate and the rate of product formation was determined. In support of earlier findings, our study demonstrated that *B.cepacia* G4, when grown on toluene, co-oxidizes ethylene, propylene, 1-butene, and 1-pentene to their respective epoxides and that *P. mendocina* KR1 co-oxidizes 1-butene and 1-pentene to their epoxides. Additionally, epoxidation of 1-butene and 1-pentene by *P. putida* mt2 was observed. No evidence of epoxide or diol products was seen in ethylene and propylene reactions involving *P. putida* F1, *P. mendocina* KR1, or *P. putida* mt2. No epoxide products were seen for 1-butene or 1-pentene when exposed to cells of *P. putida* F1. The presence or absence of detected products for each substrate-organism combination are summarized in Table 3.1.

Time course assays were conducted to establish the linearity of each positive reaction and to confirm that product formation remained stable over the length of the assay. Oxidation reactions that fail to accumulate product in a linear fashion may be subject to other biotic or abiotic activities. An abiotic factor, such as spontaneous product degradation, could compromise the linear transformation rate. Biotic activities that could produce such an effect include product consumption, limitation of reductant supply or substrate/product toxicity. Products that are poorly measurable and predictable would be less suitable as a gauge of TCE degradation in the push-pull test. In this study, cells were incubated with a fixed concentration of test substrate and product formation was quantified at ten-minute intervals. Product formation was linear with respect to time

for all oxidized substrates. Linear data for alkene tranformation by *B. cepacia* G4 are presented as R^2 values in Table 3.1.

Table 3.1. Summary of Enzyme Kinetics for Alkene Transformations by *B. cepacia* G4. *a* 30 minute incubation time. *b* 90 minute incubation time.

Cometabolic Substrate	Oxidation Product	Ks (μM)	Vmax (nmol/min/ mg cell protein)	R^2 Linear Rate
ethylene	ethylene oxide	5.4 *a* 37.2 *b*	34.3 *a*; 26.8 *b*	.98
propylene	propylene oxide	20.0 *a* 57.7 *b*	228.6 *a* 246.3 *b*	.99
1-butene	1,2-epoxybutane	335.2	12.7	.98
1-pentene	1,2-epoxypentane	33.6	24.2	.98

Ethylene oxidation studies involving *B. cepacia* G4 were conducted at varying concentrations and each concentration was measured at 30 and 90-minute incubation times. Initial K_s values for 30 and 90-minute incubation times were 5.4 and 37.2 μM of ethylene respectively (Figure 3.1). Corresponding V_{max} values were 34.3 and 26.8 nmol of ethylene oxide min^{-1} mg of $protein^{-1}$ (Table 3.1). A previous study of ethylene oxidation by this organism reported K_s and V_{max} values of 39.7 μM and 112.3 nmol min^{-1} mg of $protein^{-1}$ respectively (Yeager et al., 1998).

The rate of propylene oxide formation by *B. cepacia* G4 was also determined at 30 and 90-minute incubation times (Fig. 3.2). Apparent K_s and V_{max} values for propylene

at 30 minutes were 20.0 μM and 228.6 nmol min $^{-1}$ mg of protein $^{-1}$ respectively. At 90 minutes K_s and V_{max} values were 57.7 μM and 246.3 nmol min $^{-1}$ mg of protein $^{-1}$ (Table 3.1). Yeager et al. (1999) reported K_s and V_{max} values for the epoxidation of propylene of 32.3 μM and 89.2 nmol min $^{-1}$ mg of protein $^{-1}$. Our findings for propylene oxidation are comparable to those of Yeager et al. and are shown in Figure 3.2.

Differences in K_s values at 30 and 90 minutes for ethylene (5.4 and 39.7 μM) and, to a lesser degree, propylene (20.0 and 57.7 μM) may be attributable to the effect of the incubation time on the reaction velocity. Whereas V_{max} is partially controlled by the amount of enzyme present and thus susceptible to variation, K_s describes the intrinsic affinity of the enzyme system for the substrate and should be less impacted by other variables. Longer incubation times may lead to inactivation of the enzymes by epoxide products, which would alter V_{max}. However, V_{max} for ethylene and propylene experiments were consistent between incubation times, which implies that the amount of enzyme did not vary significantly over time. Also, time course assays for ethylene and propylene were both linear for 90+ minutes, which argues against enzyme inactivation.

Perhaps a more likely explanation for the effect of the incubation time on K_s lies in the sensitivity of the assay to lower substrate concentrations. The model assumes that the rate at 30 and 90 minutes occurs in the presence of the initial substrate volumes. In reality, a percentage of the initial substrate concentration has been consumed in the reaction. At low initial substrate concentrations, the percentage of the initial concentration that remains is smaller than the percentage at high concentrations and this effect is even more pronounced at longer incubation times. The assumption that the initial concentrations remain constant affects the shape of the curve, causing a distortion

in the projected V_{max} and making data taken at longer incubation times more subject to error. After evaluating the T2MO enzyme system at two incubation times (30 and 90 minutes), we decided to conduct the remaining experiments at a single incubation time of 60 minutes.

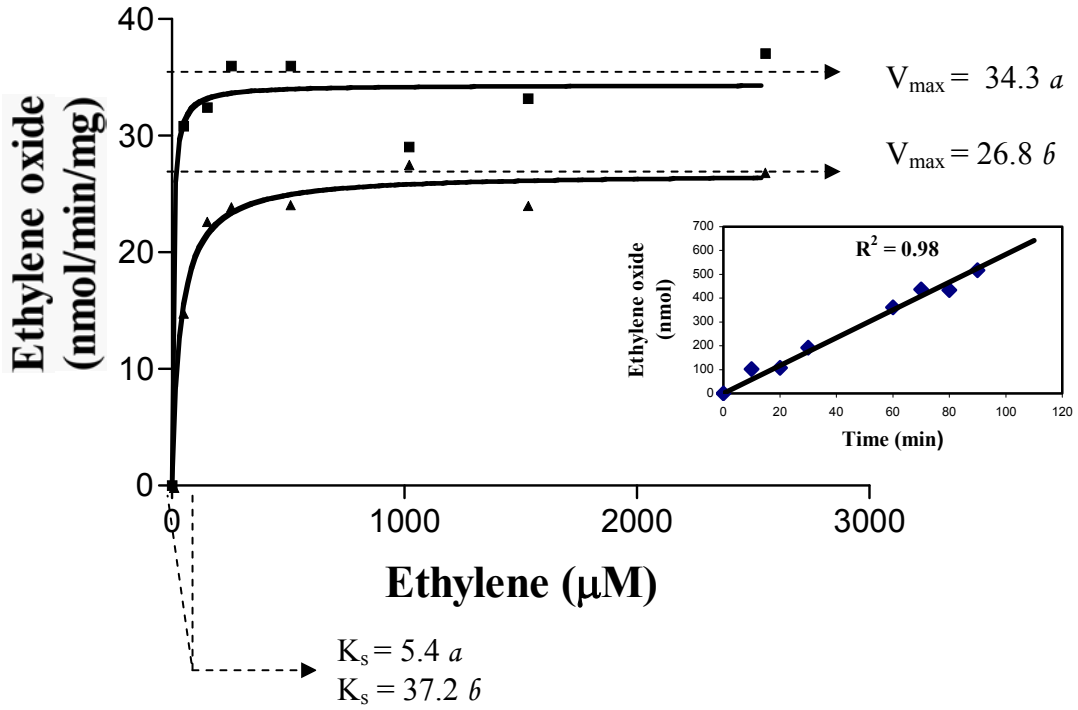

Figure 3.1. Production of ethylene oxide from ethylene by toluene-grown cells of *B. cepacia* G4. Toluene-grown cells were incubated with a range of concentrations of ethylene and samples were assayed by gas chromatography at the indicated times. The incubation times--30 minutes (■ R^2 = .96) and 90 minutes (▲ R^2 = .98)-- were incorporated into the rate of ethylene oxide production to give product formation in nmol min $^{-1}$ mg of protein $^{-1}$. A hyperbola was fitted to the data from which the kinetics constants, K_s and V_{max} were derived. Inset data shows the linear transformation rate of ethylene to ethylene oxide as determined by gas chromatography at ten minute intervals.

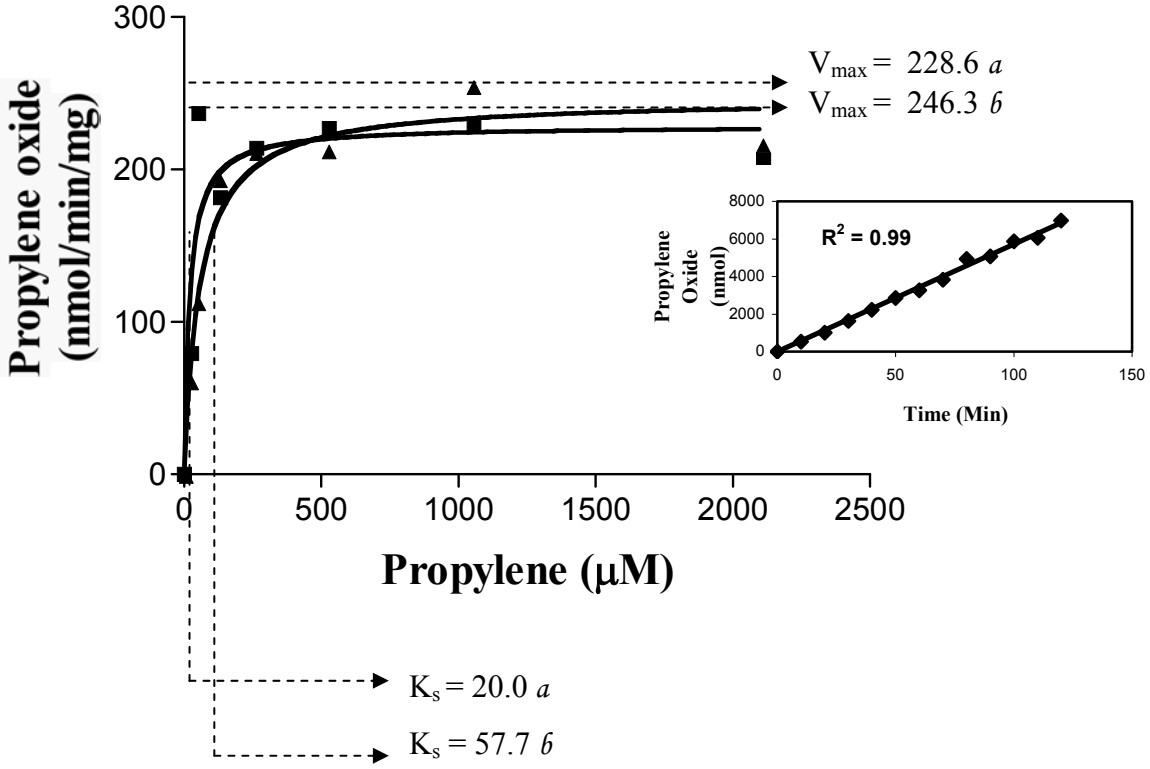

Figure 3. 2. Production of propylene oxide from propylene by toluene-grown cells of *B. cepacia* G4. Cells were incubated with a range of concentrations of propylene and samples were assayed by gas chromatography at the indicated times. The incubation times, 30 minutes (■ $R^2 = .84$) and 90 minutes (▲ $R^2 = .97$), were incorporated into the product formation rate to give values in nmol min $^{-1}$ mg of protein $^{-1}$. A hyperbola was fitted to the data from which the kinetics constants, K_s and V_{max} were derived. Inset data shows the linear transformation rate of propylene to propylene oxide as determined by gas chromatography at ten minute intervals.

While ethylene and propylene oxidation by *B. cepacia* G4 fit the saturation model quite well, yielded promising K_s constants and, in the case of propylene, high maximum velocities, no accompanying evidence for the oxidation of ethylene and propylene by *P. putida* F1, *P. mendocina* KR1, or *P. putida* mt2 was obtained. The expected products for the cometabolic oxidation of ethylene and propylene are the corresponding epoxides or alcohols (glycols). None of these potential products were observed for *P. putida* F1, *P. mendocina* KR1, or *P. putida* mt2, which limits the usefulness of ethylene and propylene as estimators of *overall* TCE-degrading activity by toluene-oxidizers. Conversely, the oxidation of ethylene and propylene by *B. cepacia* G4 alone, positions these compounds as potential selective substrates in estimating the contribution of *B. cepacia* G4 to toluene-dependent TCE degradation.

Oxidation of 1-butene and 1-pentene by toluene grown cells of *B. cepacia* G4 was observed and the corresponding products identified as 1,2-epoxybutane and 1,2-epoxypentane. Prior studies reported oxidation of 1-butene and 1-pentene by *B. cepacia* G4 but yielded no kinetics data on the rate of transformation (McClay et al, 2000). In this study, oxidation of 1-butene to 1,2-epoxybutane and 1-pentene to 1,2-epoxypentane was successfully quantified in relation to substrate and a hyperbola was fitted to the experimental data. The reaction governing the transformation of 1-butene fit the Michaelis equation well (R^2 =.97), generating apparent K_s and V_{max} values of 335.2 μM and 12.7 nmol min^{-1} mg of protein $^{-1}$ respectively (Fig. 3.3). In comparison with the other alkenes that were evaluated in the present study, the oxidation of 1-butene was governed by a high K_s, indicating a relatively low affinity of the enzyme system for 1-

butene. The oxidation of 1-pentene by *B. cepacia* G4 also fit the hyperbolic saturation model (R^2 = .82) producing K_s and V_{max} values of 33.6 μM and 24.2 nmol min^{-1} mg of protein $^{-1}$ (Fig.3.4).

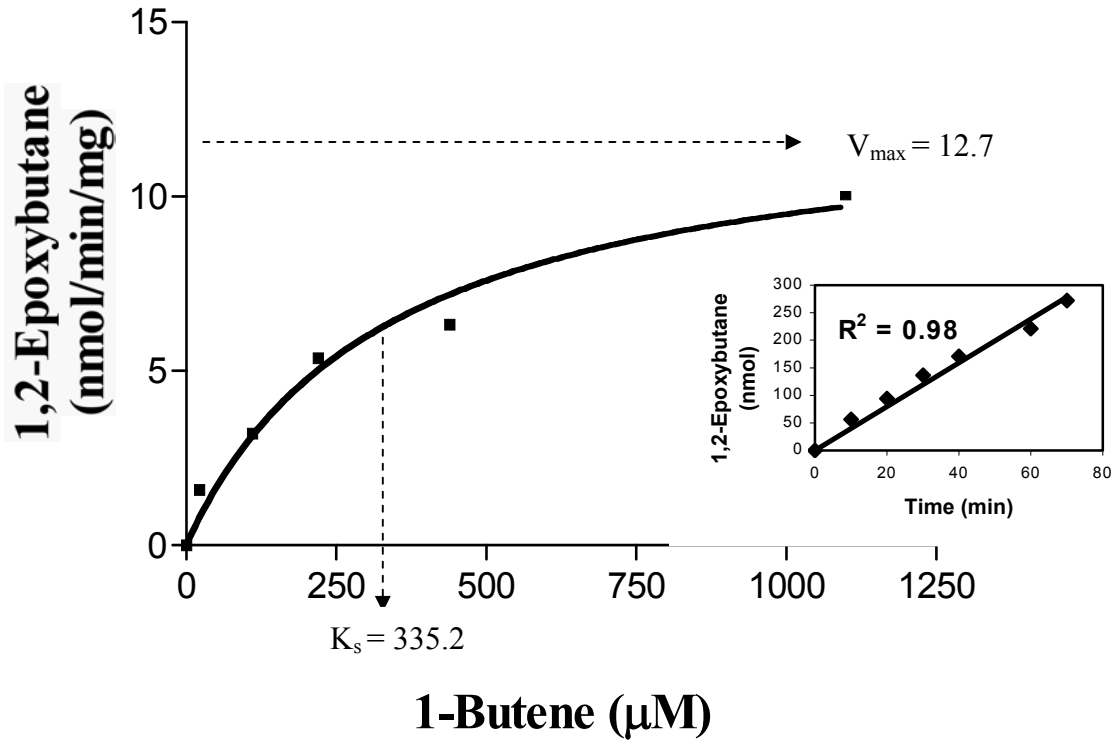

Figure 3.3. Production of 1,2-epoxybutane from 1-butene by toluene-grown cells of *B. cepacia* G4. Toluene-grown cells were incubated with a range of concentrations of 1-butene and samples were assayed by gas chromatography at 60 minutes. A hyperbola was fitted to the data from which the kinetics constants, K_s and V_{max} were derived (R^2 =.97). Inset data displays the linear transformation rate of 1-butene to 1,2-epoxybutane as determined by gas chromatography at ten-minute intervals.

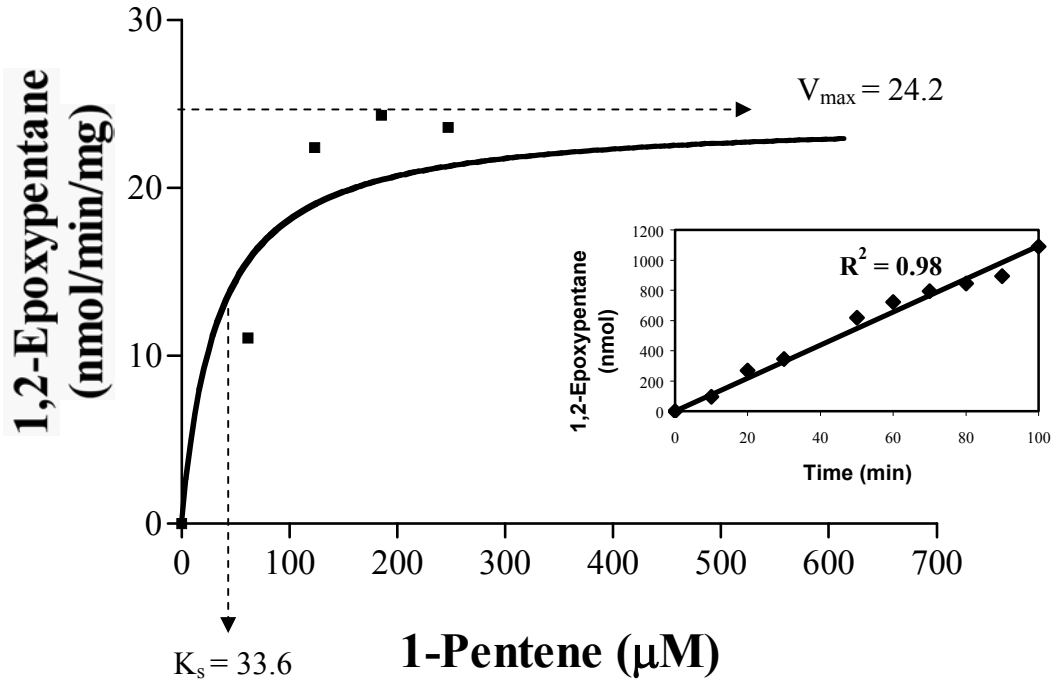

Figure 3.4. Production of 1,2-epoxypentane from 1-pentene by toluene-grown cells of *B. cepacia* G4. Toluene-grown cells were incubated with a range of concentrations of 1-pentene and samples were assayed by gas chromatography at 60 minutes. A hyperbola was fitted to the data from which the kinetics constants, K_s and V_{max} were derived (R^2 = .82). Inset data displays the linear transformation rate of 1-pentene to 1,2-epoxypentane as determined by gas chromatography at ten-minute intervals.

As with *B. cepacia* G4, the formation of epoxides of 1-butene and 1-pentene by *P. mendocina* KR1 and *P. putida* mt2 were detected. However, subsequent, unexplained declines in the observed oxidative activity prevented the characterization of the associated enzyme kinetics. No epoxide formation for 1-butene or 1-pentene by *P. putida* F1 was detected.

3.2. Oxidation of Alkanes by *B. cepacia* G4, *P. putida* F1, *P. mendocina* KR1, and *P. putida* mt2. An oxygenase-catalzyed transformation of n-butane, n-pentane, isobutane, would likely produce the corresponding alcohol or aldehyde. No evidence of oxidation of n-butane, n-pentane, or isobutane, was observed for *B. cepacia* G4. No evidence for n-butane, n-pentane, isobutane oxidation by *P. putida* F1 was noted. Since a surrogate substrate for TCE must be detectable, quantifiable and subject to co-oxidative activity by a range of toluene-oxidizers, we did not examine the activities of *P. mendocina* KR1 or *P. putida* mt2 toward n-butane, n-pentane, and isobutane and concluded that these compounds would likely be unsuitable in the push-pull test. Also, the utilization of n-pentane as a primary growth substrate by *P. mendocina* KR1 would diminish its efficacy as a measure of TCE cooxidation and make it incompatible with the project goals.

3.3. Oxidation of Cyclic Alkanes by *B. cepacia* G4, *P. putida* F1, *P, mendocina* KR1, and *P. putida* mt2. The first step in co-oxidation of cyclic alkanes is often the corresponding alcohol or aldehyde. Evidence of oxidation of cyclopropane to cyclopropanol or propionaldehyde was not seen for any of the four organisms. No

oxidation of cyclohexane was noted for *B. cepacia* G4. Oxidation of cyclohexane to cyclohexanol was observed for *P. putida* F1 but the reaction proved to be poorly linear. Also, the rate and amount of product generated was low enough to make the transformation difficult to quantify or replicate. Cyclohexane was omitted as a test substrate for *P. mendocina* KR1 and *P. putida* mt2.

Of all the alkanes examined in this study, only cyclopentane produced evidence of oxidation that was detectable and quantifiable across a span of test organisms. All four of the toluene-oxidizers under consideration demonstrated the capability of oxidizing cyclopentane to cylcopentanol. Evaluations of transformation velocity as a function of time and of concentration were conducted and the R^2 , K_s , and V_{max} values for each organism are reported in Table 3.2. The hyperbolic plots representing the substrate-velocity relationships are given in Figure 3.5 for *P. putida* F1 and *P. mendocina* KR1 and in Figure 3.6 for *B. cepacia* G4 and *P. putida* mt2.

Table 3.2. Summary of Enzyme Kinetics for Transformation of Cyclopentane to Cyclopentanol by *B. cepacia* G4, *P. putida* F1, *P. mendocina* KR1, and *P. putida* mt2.

Organism	Ks (M)	Vmax (nmol/min/mg cell protein)	R^2 Linear Rate
B. cepacia G4	0.48	482.9	.81
P. putida F1	0.0047	13.9	.99
P. mendocina KR1	0.0016	8.2	.97
P. putida mt2	0.0006	3.9	.93

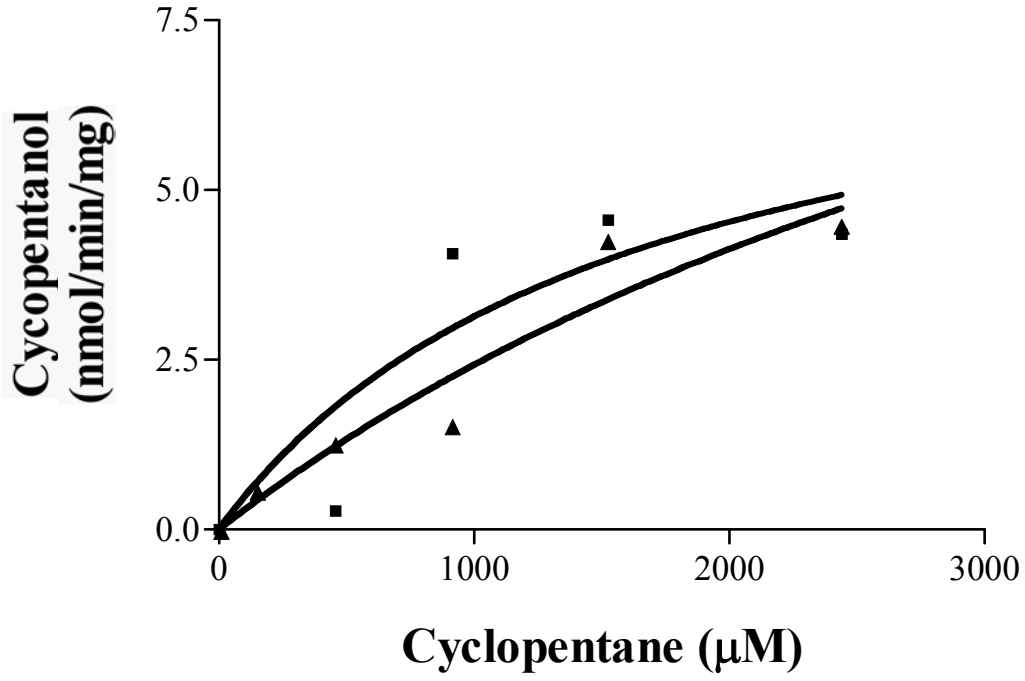

Figure 3.5. Production of cyclopentanol from cyclopentane by toluene-grown cells of *P. putida* F1 (▲) and *P. mendocina* KR1 (■). Cells were incubated with a range of concentrations of cylopentane and samples were assayed by gas chromatography at 60 minutes. The kinetics constants K_s and V_{max} were derived from a hyperbola fitted to the experimentally-determined data. Kinetics constants for *P. putida* F1 (R^2 = .93) and *P. mendocina* KR1 (R^2 = .80) exceeded the displayed axis range and are not presented in this figure.

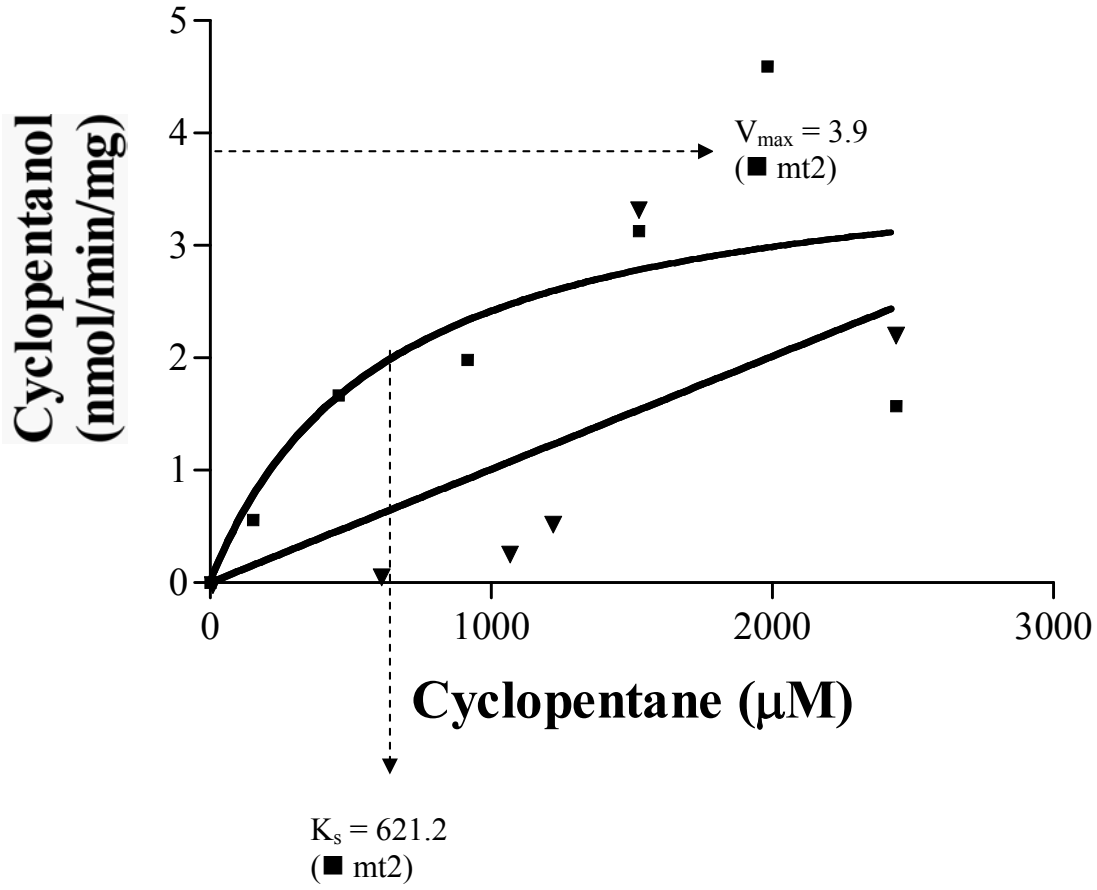

Figure 3.6. Production of cyclopentanol from cyclopentane by toluene-grown cells of *B. cepacia* G4 (▲R^2 = .50) and *P. putida* mt2 (■R^2 = .63). Cells were incubated with a range of concentrations of cylopentane and samples were assayed by gas chromatography at 60 minutes. The kinetics constants K_s and V_{max} were derived from a hyperbola fitted to the experimentally-determined data. Kinetics constants for *B. cepacia* G4 exceed the displayed axis range and are not presented in this figure.

While cyclopentane satisfied a number of the criteria for selection as a surrogate substrate, the ensuing kinetics data demonstrated its impracticality. The Michaelis constant for all organisms was large (0. 0006-0.4 M) indicating a low enzyme affinity for the substrate and, by extension, the necessity of using high concentrations of substrate in order to generate detectable levels of product. In this case, increasing the biologically available substrate is prevented by the poor aqueous solubility of cyclopentane. The major disadvantage associated with employing cyclic alkanes in the push-pull test is their low aqueous solubilities in comparison with other substrates. For a compound of low solubility to be suitable, the transformation reaction must be rapid and this condition does not appear to be satisfied by the velocity and affinity constants for cyclopentane. The experimental data also fit poorly to the hyperbolic model (R^2 .50-.93), which hinders the usefulness of the model for predicting substrate-product kinetics in field applications.

3.4. Oxidation of Isobutylene by *B. cepacia* G4, *P. putida* F1, *P, mendocina* KR1, and *P. putida* mt2. As a potential alternative substrate, isobutylene shares the advantages offered by other alkenes. It is relatively soluble, inexpensive and non-toxic. More significantly, isobutylene was the only alkene substrate shown to be oxidized by each test organism. Interestingly, two different products were observed. For assays involving *B. cepacia* G4, isobutylene oxide was the detected product. The enzyme kinetics were characterized, yielding K_s and V_{max}, of 70.6 μM and 28.4 nmol of product min^{-1} mg of protein $^{-1}$ respectively (Fig. 3.8 and Table 3.3). The transformation product seen in studies using *P. putida* F1, *P, mendocina* KR1, and *P. putida* mt2 was identified as methyl allyl alcohol (2-methyl-2-propen-1-ol). The substrate-velocity relationship was

evaluated and the kinetics of the reactions are presented in Figure 3.9 and Table 3.3. The linearity of the reaction was established for each organism and corresponding R^2 values are presented in Table 3.3.

Table 3.3. Enzyme Kinetics for Transformation of Isobutylene to Isobutylene oxide or to Methyl Allyl Alcohol by Four TCE-oxidizing Organisms.

Organism	Oxidation Product	Ks (μM)	Vmax (nmol/min/mg cell protein)	R^2 Linear Rate
B. cepacia G4	Isobutylene oxide	6.8	28.4	.95
P. putida F1	Methylallyl alcohol	70	3.3	.99
P. mendocina KR1	Methylallyl alcohol	631	2.7	.99
P. putida mt2	Methylallyl alcohol	265	1.3	.95

The formation of two divergent products points to distinct differences in the functionality and specificity of the enzyme systems. Whereas the T2MO of *B. cepacia* G4 catalyzes oxidation at the double bond, TDO, T4MO, and TMMO catalyze oxidation of the methyl group (Figure 3.7.) Only one product was identified for each enzyme system. The detection limits for the instrumentation used in these experiments were 15 nmol ml^{-1} of isobutylene oxide and 13 nmol ml^{-1} of methyl allyl alcohol. For *B. cepacia* G4, the linear transformation of isobutylene to isobutylene oxide was 5.6 nmol min^{-1}, with a maximum mass of 426 nmol $ml^{-1}$of observed product. Based on the minimum detection

limits for methyl allyl alcohol, we estimated that 97% or more of the T2MO-catalyzed oxidation of isobutylene produced isobutylene oxide. For the remaining three organisms, the highest linear transformation rate of isobutylene to methyl allyl alcohol was 0.6 nmol min^{-1} (*P. putida* F1**)** with a maximum mass of 48 nmol ml^{-1}. Based on the detection limits for isobutylene oxide, we determined that 69% or more of TDO, T4MO, and TMMO-catalyzed product of isobutylene was methyl allyl alcohol

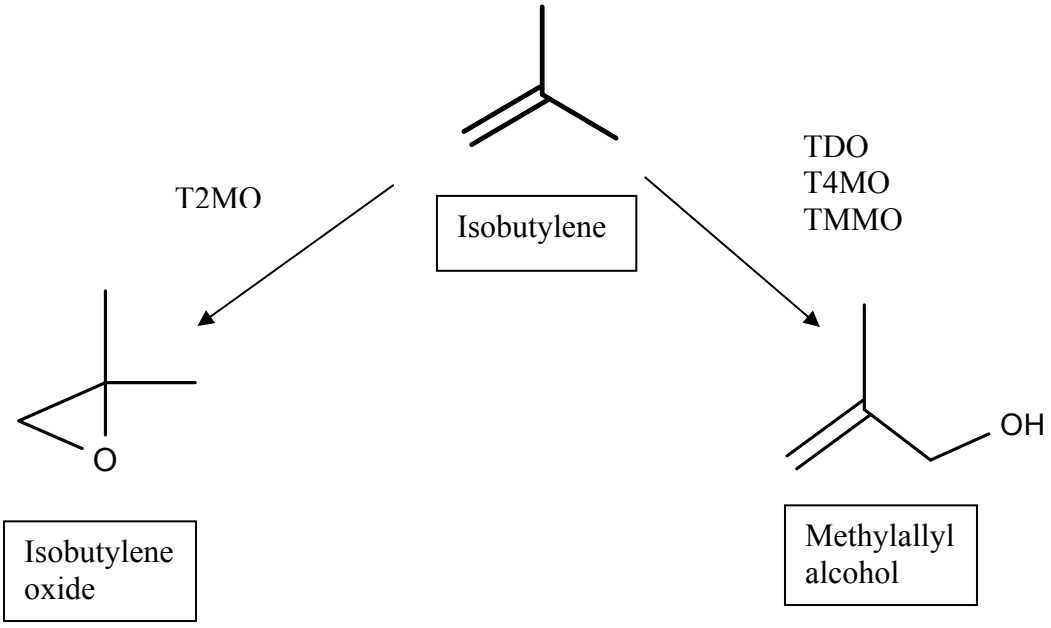

Figure 3.7. Divergent product formation by toluene-oxidizing organisms exposed to isobutylene.

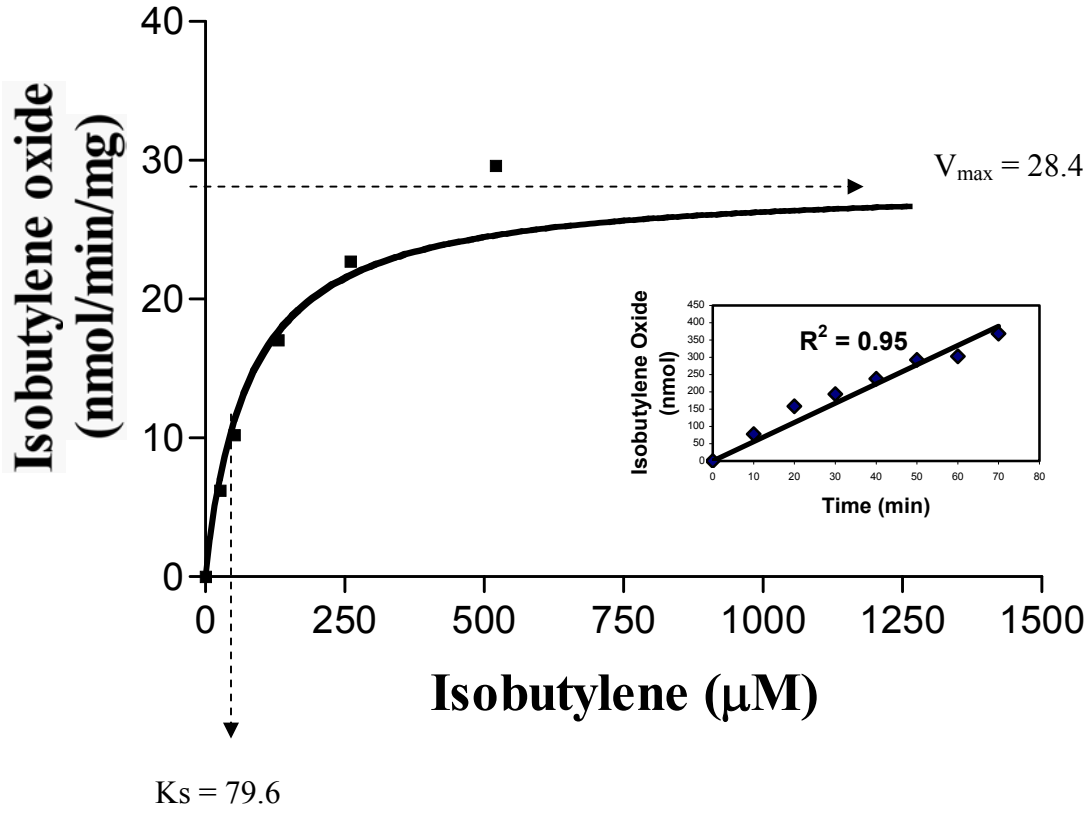

Figure 3.8. Production of isobutylene oxide from isobutylene by toluene-grown cells of *B. cepacia* G4. Toluene-grown cells were incubated with a range of concentrations of isobutylene and samples were assayed by gas chromatography at one hour. The kinetics constants K_s and V_{max} were derived from a hyperbola fitted to the experimentally-determined data (R^2= .93). Inset data displays the linear transformation rate of isobutylene oxide formation by toluene-grown cells of *B. cepacia* G4 exposed to isobutylene.

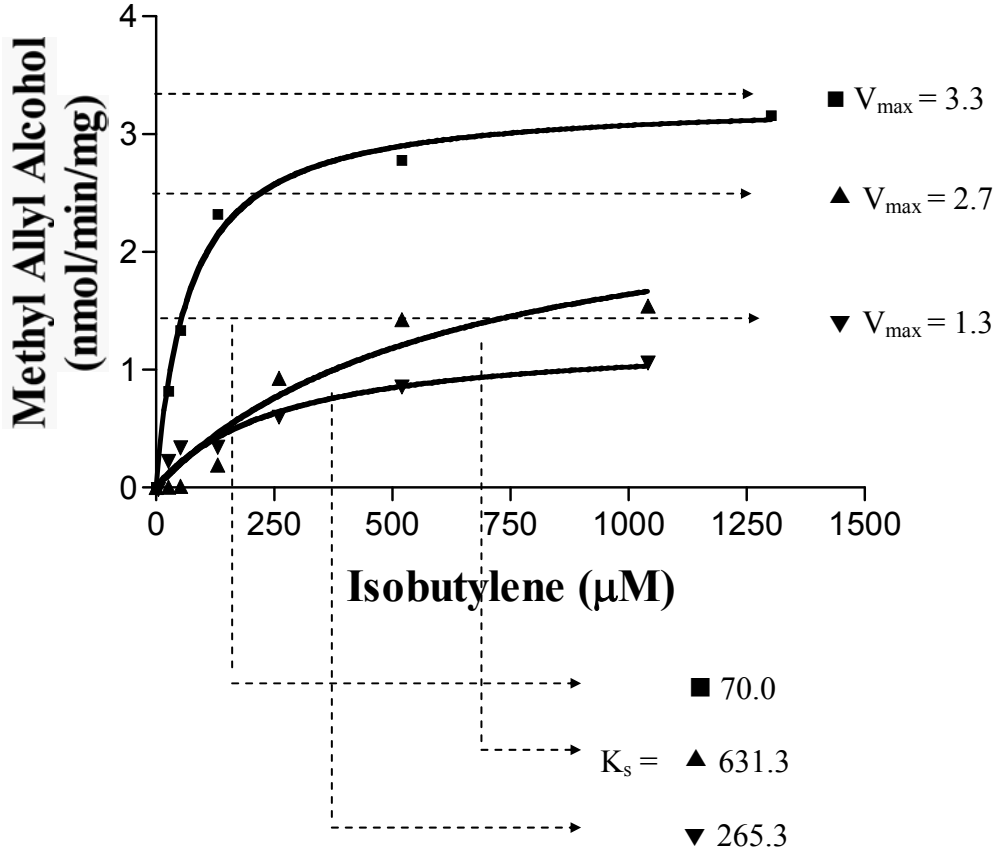

Figure 3.9. Production of methyl allyl alcohol from isobutylene by toluene-grown cells of *P. putida* F1 (■ R^2 =.99), *P. mendocina* KR1 (▲R^2 =.93) and *P. putida* mt2 (▼R^2 =.96). Toluene-grown cells were incubated with a range of concentrations of isobutylene and samples were assayed by gas chromatography after one hour. The kinetics constants K_s and V_{max} were derived from a hyperbola fitted to the experimentally-determined data.

The epoxide production exhibited by *B. cepacia* G4 appears to have a greater catalytic efficiency than the methyl group oxidation seen in the other three organisms. With a comparatively small K_s constant (indicating high enzyme affinity for isobutylene) and ~ten times the V_{max} of allylic alcohol monooxygenation, toluene-dependent epoxidation of isobutylene by the T2MO system may offer an effective means of assessing TCE-epoxidation. Formation of methyl allyl alcohol in isobutylene oxidation by *P. putida* F1 is consistent with the findings of Lange and Wackett (1997). These researchers examined the oxidation of several halogenated alkenes by purified TDO and found the primary intermediates to be allylic alcohols and diols. Although the kinetics associated with methyl allyl alcohol formation appear to be inferior to those of epoxide formation, this limitation may be outweighed by the combined contributions of three enzyme systems (TDO, T4MO, TMMO). Also, the slow transformation and low affinity exhibited by the non-TCE degrader *P. putida* mt2 could actually enhance the effectiveness of isobutylene as a gauge of TCE-degradation. Use of isobutylene as an alternative substrate in the push-pull test would offer the additional advantage of providing a simple method of differentiating the relative contributions of toluene-oxidizing enzyme systems.

Table 3.4. Presence or Absence of Observed Oxidation Products for Each Substrate/Organism Combination

Alternative Substrate	***B. cepacia* G4**	***P. putida* F1**	***P. mendocina* KR1**	***P. putida* mt2**
Ethylene	YES	NO	NO	NO
Propylene	YES	NO	NO	NO
1-Butene	YES	NO	YES	YES
1-Pentene	YES	NO	YES	YES
Isobutene	YES	YES	YES	YES
n-Butane	NO	NO	undetermined	undetermined
n-Pentane	NO	NO	Growth substrate*	undetermined
Isobutane	NO	NO	undetermined	undetermined
Cyclopropane	NO	NO	NO	NO
Cyclopentane	YES	YES	YES	YES
Cyclohexane	NO	YES	undetermined	undetermined

* Previous research has demonstrated that n-pentane serves as a primary metabolic substrate for *P. mendocina* KR1. Consequently, this combination was omitted for the purposes of this study.

DISCUSSION

The objective of this study was to identify substrates that could act as surrogates for TCE in the push-pull test. Our research focused on the activity of toluene-oxidizers—an important class of TCE-degraders—towards these surrogates. We sought a substrate that would satisfy a number of criteria. The biological activity of our test organisms toward our test substrate had to be catalytically similar to that shown toward TCE and had to demonstrate an enzyme affinity and reaction efficiency comparable to that of TCE. To assess the biological criteria, we determined the affinity constants and maximum velocities for each reaction. Other practical considerations for evaluating the test substrates included cost, toxicity, and ease of identification and quantification. We chose substrates that were readily available, inexpensive and unlikely to form products that were toxic to humans. Transformation products were evaluated for their stability over time and their conformity to a common model for predicting degradation kinetics. A surrogate that was oxidized to the same end product by a range of toluene-oxidizers could act as a universal substrate in the push-pull test, gauging the total TCE-degrading potential of the resident toluene-oxidizing population. While this was our primary goal, we hoped to identify compounds that could also act as selective substrates, interrogating the enzyme systems of specific toluene-oxidizing populations.

The alkenes produced the most promising results. Ethylene and propylene exhibited potential as selective substrates for estimating T2MO activity while isobutylene could potentially serve a dual purpose as both a universal and selective substrate. Transformation products were observed and characterized for some cyclic alkanes, but

we concluded that the limited solubility of the compounds argued against the practicality of cyclic alkanes in the push-pull test. Likewise, the absence of detectable products in simple alkane experiments made this compound class inadequate for the practical purposes of the study.

Alkenes as Estimators of TCE-degrading Potential. While our results indicated that ethylene and propylene would be of limited benefit in assessing the total activity of the toluene-oxidizing fraction in the push-pull test, they may have applications as selective substrates in measuring TCE-degrading activity by organisms expressing T2MO. The enzyme kinetics associated with ethylene and propylene oxidation by *B. cepacia* G4 surpassed each of the remaining test substrates in oxidation rates and enzyme affinity, suggesting that these compounds may serve as model CAHs for the T2MO enzyme system. Documented oxidation of ethylene and propylene by TCE-degrading organisms that do not utilize toluene suggests that these compounds may also have applications for assessing TCE-degrading capabilities of broad segments of the TCE-degrading population.

Isobutylene as a Surrogate for TCE in the Push-Pull Test. At the conclusion of our study, isobutylene emerged as the most suitable candidate for employment in the push-pull test. Isobutylene was transformed by four toluene-oxidizing organisms with different TCE-degrading enzymes systems. The substrates and products are soluble, identifiable and unique enough to minimize background contaminant interference. The enzyme

kinetics associated with each substrate-organism relationship fit well to the Michaelis-Menten equation suggesting that the chosen model may be capable of effectively predicting transformation rates in the field. Oxidation rates and affinity constants corresponded well to the published data for TCE, particularly for *B. cepacia* G4 and *P. putida* F1 (Table 4.1). Maximum velocities were within the same order of magnitude for all three TCE-degraders. The affinity constants were similar for *B. cepacia* G4 and were 1-2 orders of magnitude in difference for *P. putida* F1 and *P. mendocina* KR1 respectively. Future microcosm experiments will be conducted to test isobutylene oxidation by mixed aquifer cultures and correlate its degradation to that of TCE. A range of kinetics values have been reported for TCE oxidation (Table 4.1) by these three organisms indicating that variation in the activities of these cultures is not uncommon. In order to produce a more accurate correlation between the enzyme kinetics for isobutylene oxidation and those of TCE, microcosm studies should be conducted using these two substrates in tandem. These studies will also assess abiotic losses and the possible toxicity of isobutylene epoxides to the target organisms.

Table 4.1. Comparison of Enzyme Kinetics for Cooxidation of Isobutylene and Trichloroethylene by *B. cepacia* G4, *P. putida* F1, and *P. mendocina* KR1.

Organism	V_{max} for Isobutylene [1]	V_{max} for TCE [1]	K_s for Isobutylene (μM)	K_s for TCE (μM)	Reference
B. cepacia G4	28.4	3-10	6.8	3-6	Folsom 1994; Landa 1994; Leahy 1996; Shields 1991
P. putida F1	3.3	0.5-8	70.0	5	Heald 1994; Leahy 1996; Sun 1996
P. mendocina KR1	2.7	2.4-20.0	631.3	10	Leahy 1996; Sun 1996;

[1] nmol min^{-1} mg of protein $^{-1}$

A final criterion for substrate selection is that the substrate and products have a low potential for abiotic losses. In a sediment/groundwater system, adsorption and hydrolysis are likely to be the most important abiotic fate processes. Based on its estimated K_{oc} of 450 (SRC) derived from a log K_{ow} of 2.34 (Hansch et al., 1995), isobutylene may be moderately susceptible to adsorption to sediments in the water column (SRC). However, adsorption is not expected to exceed that of TCE, which has a log K_{ow} of 2.61 (Hansch et al., 1995). Due to its lack of hydrolyzable functional groups, isobutylene is not expected to undergo hydrolysis (Lyman et al., 1990).

Adsorption and hydrolysis data were not available for isobutylene oxide. However, the environmental fate of isobutylene oxide is likely to be comparable to that of propylene oxide. With an estimated K_{oc} of 25 (SRC) based on a log K_{ow} of 0.03 (Hansch

et al., 1995), 1,2-propylene oxide is not expected to adsorb to sediments in the water column (SRC). Hydrolysis of 1,2-propylene oxide is a significant fate process in freshwater, with estimated half-lives of 11.6 days (pH 7-9) and 6.6 days (pH 5) at 25° C (Bogyo et al., 1980). Although hydrolysis of isobutylene oxide may occur, a half-life of 6-12 days is probably adequate for field studies.

Adsorption and hydrolysis data were also not available for methyl allyl alcohol. However, *allyl alcohol* has a log K_{ow} of 0.17 (Hansch and Leo 1985) and a K_{oc} of 29.5 (SRC) which suggests that adsorption to suspended sediments will be low. Both allyl alcohol and methyl allyl alcohol are water soluble and may be subject to hydrolysis. However, data suggest that biodegradation will be the most important fate processes for these compounds (SRC).

Because TCE-degradation is carried out by numerous microorganisms via multiple metabolic pathways, a prepared test solution for use in the push-pull test may interrogate one or several enzyme systems. A test solution containing a universal substrate is designed to engage a broad range of TCE-degrading microorganisms and can give an estimate of the overall activity. The test solution may also include one or more selective substrates, which offer an estimate of the contribution of specific classes of microorganisms to the total TCE-degrading activity. The divergent product formation seen in our examination of isobutylene places isobutylene in the distinctive position of potentially acting as both a universal and selective substrate in the push-pull test. The total TCE-degrading activity of the population of toluene-oxidizers could be estimated, with the added benefit of being able to differentiate T2MO-catalyzed degradation from other enzyme systems.

Challenges to Characterizing Toluene-Oxidizer Activity Toward Test Substrates. While 1-butene and 1-pentene were rejected as surrogate substrates for the push-pull test, it warrants mention that the lack of observed oxidative activity towards these compounds by *P. mendocina* KR1, *P. putida* F1, and *P. putida* mt2 was not conclusive. Epoxidation of 1-butene and 1-pentene by *P. mendocina* KR1 and *P. putida* mt2 were noted but an unexplained loss of activity prevented further characterization. A re-evaluation of the potential activity of these organisms towards 1-butene and 1-pentene may be worthwhile. No epoxide formation from 1-butene or 1-pentene was detected for *P. putida* F1. However, a study of halogenated alkene oxidation by the TDO system (Lange and Wackett, 1997) found that allylic alcohols and diols, rather than epoxides, were the principal intermediates in TDO catalyzed reactions. Conversion of 1-butene to 1-butene-3-ol by octane-grown *Pseudomonas oleovorans* has also been reported (May et al., 1975). Potential oxidation of 1-butene and 1-pentene by *P. putida* F1 should be re-examined for the presence of the respective allylic alcohol or diol .

Early in this study, propylene-oxidizing activity was employed as a benchmark for evaluating the activity of *B. cepacia* G4 in relation to1-butene, 1-pentene, cyclopentane, and isobutylene. The benchmark velocity for propylene oxidation (129 nmol min^{-1} mg of $protein^{-1}$) was determined concurrently with the characterization of the propylene saturation model (Fig. 3.2). Propylene-oxidizing activities by *B. cepacia* G4 were then recorded in tandem with the kinetics characterizations of 1-butene, 1-pentene, cyclopentane, and isobutylene (Figure 4.1).

Fluctuations in co-oxidative activity for *B. cepacia* G4 were tracked in each

experiment and throughout the study period. Reaction velocities in nmol of propylene oxide min^{-1} mg of protein $^{-1}$ were: 60.2 for 1-butene, 135.7 for 1-pentene, 70.9 for cyclopentane, and 78.6 for isobutylene. The kinetics parameters of 1-butene, 1-pentene, cyclopentane, and isobutylene by *B. cepacia* G4 should be evaluated in the context of these propylene standards. With propylene reaction velocities at approximately half that of the benchmark, the kinetics data presented for 1-butene, cyclopentane, and isobutylene may underestimate the oxidative capabilities of the T2MO system.

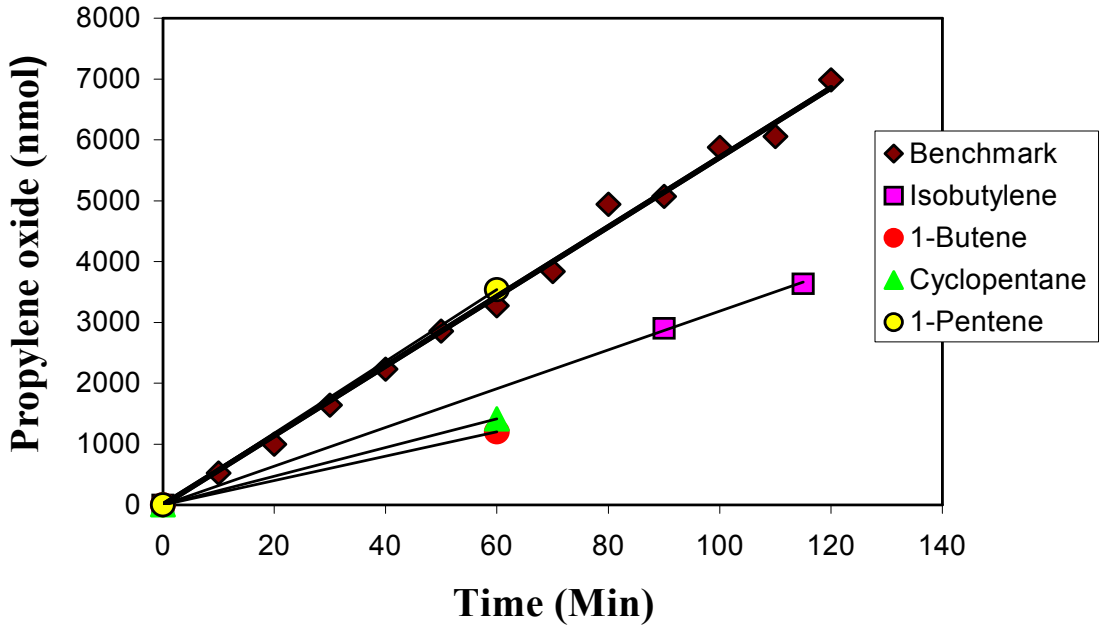

Figure 4.1. Propylene oxide formation by toluene-grown cells of *B. cepacia* recorded over the study period for each substrate experiment involving *B. cepacia* G4. The benchmark velocity represents the reaction rate obtained at the onset of the study and during the kinetics characterization of propylene. The propylene-oxidizing activity of *B. cepacia* G4 was tracked during the kinetics characterization of 1-butene, 1-pentene, cyclopentane, and isobutylene oxidation.

We were unable to determine the cause of these fluctuations but we observed that they did not correspond to loss of toluene-oxidizing activity. Cross-contamination by other toluene-oxidizers was difficult to detect due to the morphological similarities of the test organisms. Future studies should develop a method for preventing the competitive takeover of pure cultures by organisms which utilize the same substrate. We suggest that each pure culture of toluene-oxidizer be maintained on a primary growth substrate that is not shared by other toluene-oxidizers. For example, *P. mendocina* KR1 can utilize n-pentane as a growth substrate while *B. cepacia* G4, *P. putida* F1, and *P. putida* mt2 have shown no affinity for this substrate. Likewise, *P. putida* mt2 can be differentiated by growth on *p*-xylene and *B. cepacia* G4 by growth on *o*-cresol (Mars et al, 1997).

Future Investigations of Cyclic Alkane Oxidation by Toluene Utilizers. Cyclic alkanes are generally considered to be poorly degradable and their natural attenuation to be primarily mediated by cometabolic processes. As such, the oxidation of cyclopentane and cyclohexane by toluene-utilizing organisms is a novel finding of unique academic interest. Although certain characteristics of these compounds were inconsistent with the aims of the present project, the kinetics of their environmental degradation by this group of organisms may be worth exploring at a later time.

Future Investigations of Alkane Oxidation by Toluene-oxidizers. While no oxidation products were observed for cells of *B. cepacia* G4 and *P. putida* F1 exposed to n-butane, n-pentane, and isobutane, the possibility that transformations occured has not been eliminated. Rapid consumption of oxidized products would make detection of the

expected products difficult. Because ease of detection and temporal stability were important criteria for our cometabolic product, we did not explore the prospect of metabolic consumption. We feel that an effective initial strategy for future studies of alkanes would be to test for substrate depletion rather than product appearance. If substrate disappearance was observed, the possibility of product consumption could be further assessed by exposing cells to the expected products (alcohols and aldehydes) and testing for their depletion.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). 1993. *Toxicological Profile for Trichloroethylene*. U.S. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

Agency for Toxic Substances and Disease Registry (ATSDR). 1997. *ToxFAQs for Trichloroethylene*. U.S. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

Alvarez-Cohen, L. and P.L. McCarty. 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. 57: 228-235.

Alvarez-Cohen, L. and G.E. Speitel Jr. 2001. Kinetics of aerobic cometabolism of chlorinated solvents. Biodegradation. 12: 105-126.

Arciero D., T. Vannelli, M. Logan and B.B. Hooper. 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Biochem. Biophys. Res. Commun. 159: 640-643.

Arp, D.J., C.M. Yeager and M.R. Hyman. 2001. Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene. Biodegradation. 12: 81-103.

Assinder, S.J. and P.A. Williams. 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. 31:1-69.

Beam, H.W. and J.J. Perry. 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalisms. J. Gen. Microb. 82: 163-169.

Bedard, C. and R. Knowles. 1989. Physiology, biochemistry, and specific inhibitors of CH4. NH4+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. 53: 68-84.

Bogyo D.A., S.S. Lande and W.M. Meylan. 1980. Investigation of Selected Environmental Contaminants: Epoxides. Report to Office of Toxic Substances, US Environmental Protection Agency, Washington D.C. by Syracuse Research Corporation. EPA-560/11-80-005. pp 202. NTIS No. PB80-183197.

Chang, H.L. and L. Alvarez-Cohen. 1995. Model for the cometabolic biodegradation of chlorinated organics. Environ. Sci. Technol. 29:2357-2367.

Colby, J., D.I. Stirling and H. Dalton. 1977. The soluble methane mono-oxygenase of Methylococcus capsulatus (Bath): its ability to oxygenate n-alkanes, n-alkenes, ethers, and alicyclic, or aromatic and heterocyclic compounds. Biochem. J. 165: 395-402.

DiSpirito, A.A., J. Gulledge, A.K. Shiemke, J.C. Murrel, M.E. Lidstrom, and C.L. Krema. 1992. Trichloroethylene oxidation by the membrane-associated methane monooxygenase in Type I, Type II, and Type X, methanotrophs. Biodegradation 2: 151-164.

Ely, R.L., K.J Williamson, R.B. Guenther, M.R. Hyman. and D.J.Arp. 1995. A cometabolic kinetics model incorporating enzyme inhibition, inactivation and recovery: I Model development, analysis and testing. Biotechnol.Bioeng. 46: 218-231.

Ensign, S.A., M.R. Hyman and D.J. Arp. 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a proplylene-grown *Xanthobacter* strain. Appl.Environ.Microbiol. 58: 3038-3046.

Fan, S. and K. M. Scow. 1993. Biodegradation of trichloroethylene and toluene by indigenous microbial populations in soil. Appl. Environ. Microbiol. 59:1911-1918.

Finette, B.A., V. Subramanian, and D.T. Gibson. 1984. Isolation and characterization of *Pseudomonas putida* PpF1 mutants defective in the toluene dioxygenase enzyme system. J. Bacteriol. 160: 1003-1009.

Fogel. M.M., A.R. Tadeo, and S. Fogel. 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. 51(4): 720-724.

Folsom, B.R., P.J. Chapman, and P.H. Pritchard. 1990. Phenol and trichloroethylene degradation by Pseudomonas cepacia G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. 56: 1279-1285.

Fox, B.G., J.G. Borneman, L.P. Wackett, and J.D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: methanistic and environmental implications. Biochem. 29(27): 6419-6427.

Fries, M.R., L.J. Forney, and J.M. Tiedje. 1997. Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. Appl. Environ. Microbiol. 63:1523-1530.

Gibson, D.T., G.J. Zylstra and S. Chauhan. 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1. In: Silver S., A.M Chakrabarty., B Iglewski, S. Kaplan, editors. *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. Washington, D.C. American Society for Microbiology. P. 121-132.

Gornall, A.G., C.J. Bardawill, and M.M. David. 1949. Determination of serum proteins by means of the Biuret reaction. J.Biol.Chem. 177: 751-766.

Hamamura, N., C. Page, T. Long, L. Semprini, and D.J. Arp. 1997. Chloroform Cometabolism by Butane-Grown CF8, *Pseudomonas butanovora*, *Mycobacterium vaccae* JOB 5, and Methane-Grown *Methylosinus trichosporium* OB3b. Applied and Environmental Microbiology. 63(9), 3607-3613.

Hansch, C. and A. Leo.1985. Medchem Project. Pomona College. Claremont, CA: Issue # 26.

Hansch, C., A. Leo and D. Hoekman. 1995. Exploring QSAR: Fundamentals and Applications in Chemistry and Biology, American Chemical Society, Washington, D.C.

Heald, S. and R.O. Jenkins. 1994. Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol. 60: 4634-4637.

Hopkins, G. D., J. Munakata, L. Semprini, and P.L. McCarty. 1993. Trichloroethylene concentration effects on pilot-scale in-situ groundwater bioremediation by phenol-oxidizing microorganisms. Environ. Sci. Technol. 27:2542-2547.

Hopkins, G.D., L. Semprini, and P.L. McCarty. 1993. Microcosm and in situ field studies of enhanced biotransformations of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol. 59:2277-2285.

Hopkins, G.D., and P.L. McCarty. 1995. Field evaluation of in situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol. 29:1628-1637.

Hou C.T., R. Pate1 and A.I. Laskin. 1979. Microbial oxidation of gaseous hydrocarbons: Epoxidation of C_2 to C_4 n-alkenes by methylotrophic bacteria. Appl. Environ. Microbiol. 38: 127-134.

Hou, C.T., R. Patel, A. . Laskin, N. Barnabe, and I. Barist. 1983. Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl. Environ. Microbiol. 46:171-177.

Hyman, M.R., S.A. Russell, R.L. Ely, K.J. Williamson, and D.J. Arp. 1995. Inhibition, inactivation, and recovery of ammonia-oxidizing activity in cometabolism of trichloroethylene by *Nitrosomonas europaea*. Appl. Environ. Microbiol. 61:1480-1487.

Istok, J.D., L. Semprini and J.Field. 2001. In-situ measurement of TCE degradation using a single-well "push-pull" test. Oregon State University.

Istok, J.D., M.D. Humphrey, M.H. Schroth, M.R. Hyman, and K.T. O'Reilly. 1997. Single-Well, "Push-Pull" Test for In Situ Determination of Microbial Metabolic Activities. Ground Water. Vol. 35(4): 619-631.

Istok, J.D. 2000. In Situ Determination of Uranium Kinetics at the Bear Creek Va Center: Feasability Studies. NABIR Assessment Program, Oak Ridge, T.N. Ongoing.

Jamison, V.W., J.O. Hudson, and R.L. Raymond. 1976. pp. 187-196 in Proc. Int. Biodeg. Symp 3rd Sharpley, J.M., Kaplan, A.M., eds., Essex England.

Joergensen, L. 1985. The methane mono-oxygenase reaction system studied in vivo by membrane-inlet mass spectrometry. Biochem. J. 225(441–448).

Kawasaki, M., 1980. Experience with the test scheme under the chemical control law of Japan: An approach to structure-activity correlations. Ecotox. Environ. Safety 4: 444-54.

Koh, S., J.P. Bowman, and G. Sayler. 1993. Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph*, Methylomonas methanica* 68-1. Appl. Environ. Microbiol. 59.4:960-967.

Landa, A.S., E.M. Sipkema, J. Weijma, A.A.C.M. Beenackers, J. Dolfing, and D.B. Janssen. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. Appl. Environ. Microbiol. 60:3368-3374.

Lange C.C., and L.P. Wackett. 1997. Oxidation of aliphatic olefins by toluene dioxygenase: Enzyme rates and product identification. J.Bacteriol. 179:3858-3865.

Leahy, J.G., A.M. Byrne and R.H. Olsen. 1996. Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl. Environ. Microbiol. 62: 825-833.

Li, S.Y. and L.P. Wackett. 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Comm. 185: 443-451.

Little, C.D., A.V. Palumbo, S.E. Herbes, M.E. Lindstrom, R.L. Tyndall, and P.J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Micriobiol. 54 (4): 951-956.

Lontoh, S., A.A. DiSpirito and J.D. Semrau. 1999. Dichloromethane and trichloroethylene inhibition of methane oxidation by the membrane-associated methane monooxygenase of *Methylosinus trichosporium* OB3b Arch. Microbiol.171: 301-308.

Lyman W.J., W.J. Reehl, D.H. Rosenblatt (eds.) 1990. Handbook of Chemical Property Estimation Methods. Washington, D.C.: Amer. Chem. Soc. pp. 7-4, 7-5.

Mars, A.E., J. Houwing, J. Dolfing, and D.B. Janssen. 1996. Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. Appl. Environ. Microbiol. 62**:**886-891.

Mars, A.E., G.T. Prins, P. Wietzes, W.D. Koning, D.B. Janssen. 1998. Effect of Trichloroethylene on the competitive behavior of toluene-degrading bacteria. Appl. Environ. Microbiol. 64: 208-215.

Mars, A.E., T. Kasberg, S.R. Kaschabek, M.H. van Agteren, D.B. Janssen, and W. Reineke. 1997. Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. J. Bacteriol. 179**:**4530-4537.

Meylan, W.M., P.H. Howard, R.S. Boethling. 1992. Molecular topology/fragment contribution method for predicting soil sorption coefficients. Environ. Sci. Techno. 26: 1560-67.

McCarty, P.L. 1996. pp. 5-9 in Symp. Nat. Atten. Chlor. Org. Ground Water. USEPA/540/R-96/509.

McCarty P.L., M.N. Goltz, G.D. Hopkins, M.E. Dolan, J.P. Allan, B.T. Kawakami, and T.J. Carrothers. 1998. Full-Scale Evaluation of In Situ Cometabolic Degradation of Trichloroethylene in Groundwater through Toluene Injection, Environ. Sci. Technol. 32 (1): 88-100.

McClay, K., S.H. Streger, and R.J. Steffan. 1995. Induction of toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. Strain ENVPC5 by chlorinated solvents and alkanes. Appl. Environ. Microbiol. 61 (9): 3479-3481.

McClay, K., B.G. Fox, and R.J. Steffan. 1996. Chloroform mineralization by toluene-oxidizing bacteria. Appl. Environ. Microbiol. 62:2716-2722.

McClay, K., B.G. Fox, and R.J. Steffan. 2000. Toluene monooxygenase-catalyzed epoxidation of alkenes. Appl. Environ. Microbiol. 66 (5): 1877-1882.

Miller, R.E. and F.P. Guengerich. 1982. Oxidation of trichloroethylene by liver microsomal cytochrome P450: Evidence for chlorine migration in a transition state not involving trichloroethylene oxide. Biochemistry 21: 1090-1097.

Nelson, M.J., J.V. Kinsella and T. Montoya. 1990. *In situ* biodegradation of TCE contaminated groundwater. Environ. Prog. 9:190-196.

Newman, L.M. and L.P. Wackett. 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochem. 34: 14066-14076.

Newman, L.M. and L.P. Wackett. 1997. Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. J. Bacteriol. 179: 90-96.

Oldenhuis, R., J.Y. Oedzes, J.J. van der Waarde, and D.B. Janssen. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. 57:7-14.

Oldenhuis, R., RJLM Vink, D.B. Janssen, B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55: 2819-2826.

Olsen, R.H., J.J. Kukor, and B. Kaphammer. 1994. A Novel Toluene-3-Monooxygenase Pathway cloned from *Pseudomonas picketti* PKO1. Journal of Bacteriology. 176: 3749-3756.

Perry, J.J. 1979. Microbial cooxidation involving hydrocarbons. Microbial Rev. 43: 59-72.

Phillips, W. E. and J. J. Perry. 1974. Metabolism of *n*-butane and 2-butanone by *Mycobacterium vaccae*. J. Bacteriol. 120:987-989.

Pikus, J.D., J.M. Studts, C. Achim, K.E. Kauffman, E. Munck, R.J. Steffan, K. McClay, and B.G. Fox. 1996. Recombinant toluene 4-monooxygenase: Catalytic and Mossbauer studies of the purified diiron and rieske components of a four-protein complex. Biochemistry 35: 9106-9119.

Pikus, J.D., J.M. Studts, K. McClay, R.J. Steffan and B.G. Fox. 1997. Changes in the regiospecificity of aromatic hydroxylation produced by active site engineering in the diiron enzyme toluene 4-monooxygenase. Biochemistry 36: 9283-9289.

Rasche, M.E., M.R. Hyman, and D.J. Arp. 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*—cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. 57: 2986-2994.

Rathbun, R.E. 1998. Transport, Behavior, and Fate of Volatile Organic Compounds in Streams. USGS Professional Paper 1589, PB98-163397.

Roberts, P.V., G.D. Hopkins, D.M. Mackay, and L. Semprini. 1990. A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 1. Experimental methodology and characterization. Ground Water. 28(4): 591-604.

Saeki, H., M. Akira, K. Furuhashi, B. Averhoff and G. Gottschalk. 1999. Degradation of trichloroethylene by a linear plasmid-encoded alkene monooxygenase in *Rhodococcus corallinus* (*Nocardia corallina*) B-276. Microbiol. 145: 1721-1730.

Mu, D.Y. and K.M. Scow. 1994. Effect of trichloroethylene (TCE) and toluene concentrations on TCE and toluene biodegradation and the population density of TCE and toluene degraders in soil. Appl. Environ. Microbiol., 60 (7): 2661-2665.

Semprini. L., P.V. Roberts, G.D. Hopkins, and P.L. McCarty. 1990. A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 2. The results of biostimulation and biotransformation experiments. Ground Water 28(5): 715-727.

Semprini. L, G.D. Hopkins, P.V. Roberts, D. Grbic-Galic, and P.L. McCarty. 1991. A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 3. Studies of competitive inhibition. Ground Water 29(2): 239-250.

Shields, M.S., S.O. Montgomery, S.M. Cuskey, P.J. Chapman, and P.H. Pritchard. 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol. 57:1935-1941.

Shields, M.S. and M.J. Reagin. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. 58(12): 3977-3983.

SRI. 1992. Directory of Chemical Producers-United States of America. Menlo Park, CA: SRI International: 1032.

Sun, A.K. and T. K. Wood. 1996. Trichloroethylene degradation and mineralization by Pseudomonads and *Methylosinus trichosporium* OB3b. Appl. Microbiol. Biotech. Vol.45, p.248-256.

Syracuse Research Corporation (SRC). PhysProp Database. http://esc.syrres.com/interkow/physdemo.htm.

Tsien, H.C., G.A. Brusseau, R.S. Hanson, L.P. Wackett.. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 55: 31553161.

U.S. Department of Health and Human Services. 1993. Registry of Toxic Effects of Chemical Substances (RTECS, online database). National Toxicology Information Program, National Library of Medicine, Bethesda, MD.

U.S. Environmental Protection Agency. 1998. June 24. 63 FR 34338.

U.S. Environmental Protection Agency. 2001. Office of Ground Water and Drinking Water, Technical Factsheets, EPA 600/4-88-039 (updated).

United States International Trade Commission. 1981. Synthetic Organic Chemicals--United States Production and Sales. USITC Publications 1291 Washington, DC: United States InternationalTrade Commission, 1132.

van Hylckama Vlieg, J.E.T., W. De Koning, and D.B. Janssen. 1997. Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 63:4961-4964.

Vogel, T.M. and P.L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49: 1080-1083.

Wackett, L.P. 1995. Taken from Microbial transformation and degradation of toxic organic chemicals. Chapter 5: Bacterial co-metabolism of halogenated organic compounds. L.Y. Young and C.E. Cerniglia eds. Wiley-Liss, New York.

Wackett, L. P., and D. T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. 54:1703-1708.

Wackett, L.P. and S.R. Householder. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. 55:2723-2725.

Walker, J.D. and R.R. Colwell. 1976. Enumeration of petroleum-degrading microorganisms. Appl. Environ. Microbiol. 31(2): 198-207.

Winter, R.B., K.M. Yen and B.D. Ensley. 1989. Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. Bio/Technology 7: 282-285.

Wilson, J.T. and B.H. Wilson. 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. 49: 242-243.

World Health Organization (WHO). 1984. Guidelines for drinking water quality; Geneva.

Yeager, C.M., P.J. Bottomley, D.J. Arp and M.R. Hyman. 1999. Inactivation of Toluene 2-Monooxygenase in *Burkholderia cepacia* G4 by Alkynes. Appl. Environ. Microbiol.65(2): 632-639.

Yeager, C.M., P.J. Bottomley and D.J. Arp. 2001. Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol**.** 67 (5): 2107-2115.

Yeh, W.K., D.T. Gibson, and T.N. Liu. 1977. Toluene dioxygenase: a multicomponent enzyme system. Biochem. Biophys. Res. Comm. 78: 401-410.

Zylstra, G.J. and D.T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in Escherichia coli. J. Biol. Chem. 264.