



AFRL-ML-TY-TR-1998-4603

MOLECULAR AND CELLULAR FUNDAMENTALS OF AEROBIC COMETABOLISM

Daniel J. Arp, Michael R. Hyman

Department of Botany and Plant Pathology
Oregon State University
Corvallis, OR 97331

Contract No. F41624-94-D-8055-0204

October 1998

DISTRIBUTION A: Approved for public release; distribution unlimited.

**AIR FORCE RESEARCH LABORATORY
MATERIALS AND MANUFACTURING DIRECTORATE**

DISCLAIMER

Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not constitute or imply its endorsement, recommendation, or approval by the United States Air Force. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Air Force.

This report was prepared as an account of work sponsored by the United States Air Force. Neither the United States Air Force, nor any of its employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights

This document is submitted as an historical record of work performed. Limitations of the available media rendered editing impractical; therefore it is retained "as is."

REPORT DOCUMENTATION PAGE					Form Approved OMB No. 0704-0188	
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.						
PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.						
1. REPORT DATE (DD-MM-YYYY) 31-OCT-1998		2. REPORT TYPE Thesis		3. DATES COVERED (From - To) 30-JUN-1995 -- 30-SEP-1998		
4. TITLE AND SUBTITLE Molecular and Cellular Fundamentals of Aerobic Cometabolism				5a. CONTRACT NUMBER F41624-94-D-8055-0204		
				5b. GRANT NUMBER		
				5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Arp, Daniel J.; Hyman, Michael R.				5d. PROJECT NUMBER		
				5e. TASK NUMBER		
				5f. WORK UNIT NUMBER 2103W504		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Botany and Plant Pathology Oregon State University Corvallis, OR 97331				8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Air Force Research Laboratory Materials and Manufacturing Directorate Airbase Technologies Division 139 Barnes Drive, Suite 2 Tyndall Air Force Base, FL 32403-5323				10. SPONSOR/MONITOR'S ACRONYM(S) AFRL/MLQL		
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) AFRL-ML-TY-TR-1998-4603		
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution A: Approved for public release; distribution unlimited.						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT Aerobic cometabolism recognizes that microorganisms can transform non-growth supporting substrates. The term "cometabolism" was first introduced over 30 years ago and has been redefined, criticized, and used widely ever since. In this review we have examined the cometabolism of chlorinated solvents. These transformations are initiated by monooxygenases or dioxygenases with relaxed substrate ranges. The physiological role of the monooxygenases is to initiate the metabolism of growth substrates which are most often hydrocarbons. A metabolically and structurally diverse set of enzymes catalyze oxidative reactions with chlorinated solvents. Common growth-supporting substrates include methane, propane, toluene, ethylene and ammonia. The transformation of chlorinated solvents by these enzymes presents the cell with a new set of compounds. Some of these compounds are toxic to the cells, others are stable products which are expelled from the cell and in a few cases, the products are utilized by the cells. Production of most of the enzymes with potential for degradation of chlorinated solvents is induced by the presence of the growth-supporting substrate. In some cases, chlorinated aliphatics will also induce production of the enzyme. Cometabolism can have a profound influence on a cell.						
15. SUBJECT TERMS aerobic cometabolism, microorganisms						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
a. REPORT	b. ABSTRACT	c. THIS PAGE			Alison Thomas	
U	U	U	UU	46	19b. TELEPHONE NUMBER (Include area code)	

Reset

**TITLE: Molecular and Cellular Fundamentals of Aerobic
Cometabolism**

AUTHORS: Daniel J. Arp and Michael R. Hyman, Department of Botany and
Plant Pathology, Oregon State University, Corvallis, OR 97331

ABSTRACT

Aerobic cometabolism recognizes that microorganisms can transform non-growth supporting substrates. The term “cometabolism” was first introduced over 30 years ago and has been redefined, criticized, and used widely ever since. In this review we have examined the cometabolism of chlorinated solvents. These transformations are initiated by monooxygenases or dioxygenases with relaxed substrate ranges. The physiological role of the monooxygenases is to initiate the metabolism of growth substrates which are most often hydrocarbons. A metabolically and structurally diverse set of enzymes catalyze oxidative reactions with chlorinated solvents. Common growth-supporting substrates include methane, propane, toluene, ethylene, and ammonia. The transformation of chlorinated solvents by these enzymes presents the cell with a new set of compounds. Some of these compounds are toxic to the cells, others are stable products which are expelled from the cell and in a few cases, the products are utilized by the cells. Production of most of the enzymes with potential for degradation of chlorinated solvents is induced by the presence of the growth-supporting substrate. In some cases, chlorinated aliphatics will also induce production of the enzyme. Cometabolism can have a profound influence on a cell.

INTRODUCTION

Aerobic cometabolism describes the ability of microorganisms to transform non-growth supporting substrates, typically in the presence of a growth supporting substrate. The molecular underpinnings for this type of metabolism as well as a discussion of the origin of this term are

described in this review. Where the non-growth supporting substrate is an environmental pollutant, aerobic cometabolism offers a biological method for the removal of the pollutant from the contaminated environment. This approach to bioremediation is particularly appropriate to chlorinated solvents and related compounds where other methods (such as reductive dechlorination) are less likely to result in the complete mineralization of the target compound. However, exploitation of this process faces a number of challenges. These challenges will best be addressed through a thorough understanding of the fundamental molecular underpinnings which make aerobic cometabolism possible. The purpose of this review article is to consider those molecular underpinnings. Our focus will be on the bacteria that catalyze aerobic cometabolic transformations of chlorinated aliphatic hydrocarbons (CAHs). However, cometabolism is not limited to bacterial systems, aerobic processes, or chlorinated solvents. Where appropriate, we will consider cometabolism in other contexts. With regard to aerobic cometabolism of chlorinated solvents, we will summarize our current level of understanding, identify gaps in that understanding, and where possible identify unifying themes. In doing so, we will consider those systems which have received the greatest attention to date, whether as early examples of aerobic cometabolism, model systems, or systems already demonstrated to be effective in field sites.

In the Background section of this article, we will consider the definition of cometabolism and the role of individual enzymes in this process. In aerobic cometabolism, oxygenases initiate the oxidation of cometabolites. Therefore, we consider the role and mechanism of oxygenases in general. We present in some detail the properties of those monooxygenases which have, to date, shown the greatest potential for cometabolism of chlorinated solvents. Although these monooxygenases are indeed at the heart of the process of cometabolism, their action on cometabolites essentially presents the cell with a new set of metabolites. The result of this enzyme action on the physiology of the cell is also considered. In most cases, the genes which code for the enzymes responsible for initiating aerobic cometabolism have been identified. These genes, their regulation, and the potential for improving the enzymes through genetic engineering are also considered.

We have placed our primary focus on trichloroethylene (TCE) as an example of a chlorinated solvent. TCE is one of the most commonly encountered organic pollutants found in

water destined for human consumption. As a result, TCE has received a great deal of attention and provides the richest data set for understanding the physiological effects of cometabolism. Against this backdrop, we also consider other chlorinated solvents and other chlorinated or halogenated pollutants. This review, as part of a series on the topic of aerobic cometabolism, focuses on the molecular underpinnings of cometabolism. The reader is directed to the subsequent reviews in this series for other topics of interest.

BACKGROUND

Historical perspective. Cometabolic processes were first studied in the 1950's and 60's and focused on the microbial degradation of important classes of industrial chemicals including aromatics (Dagley and Patel, 1957), chlorinated organics (Jensen, 1957; Jensen, 1963), pesticides (Alexander, 1967) and petroleum hydrocarbons (Foster, 1962). Collectively, these studies established that many compounds can be transformed by microorganisms without concurrent microbial growth. Much of the research that has followed has attempted to address important issues raised by these observations including the role of cometabolism in the degradation of pollutant compounds in the environment and the mechanism and physiological basis for these reactions. These issues still represent important considerations today in view of the recent interest in applying microbial cometabolic processes to the degradation of chlorinated solvents.

The principle features of cometabolism were first embodied in the "co-oxidation" process characterized by Foster and coworkers during early studies of the substrate range of methane- and alkane-utilizing bacteria (Leadbetter and Foster, 1959; Leadbetter and Foster, 1960). These studies initially revealed that the methane-utilizing bacterium *Pseudomonas* (*Methylobacter*) *methanica* could not grow on hydrocarbons such as ethane, propane and *n*-butane but could oxidize these compounds to products which retained the same carbon number as the parent substrate (Leadbetter and Foster, 1960). Foster's group also observed similar reactions in alkane-utilizing bacteria and Foster later defined co-oxidation as "*the oxidation of non-growth hydrocarbons when present in a medium where one or more different hydrocarbons are furnished for growth*" (Foster, 1962). The term cometabolism was subsequently introduced by Alexander (Alexander, 1967) to allow the basic tenet of co-oxidation (the transformation of non-

growth supporting substrates) to be extended to classes of compound and degradation reactions beyond the oxidative transformations of hydrocarbons described by co-oxidation. As the following quotation illustrates, Alexander recognized the similarities between co-oxidation and cometabolism and the environmental significance of these processes, both as possible natural attenuation mechanisms and as facilitated approaches for the bioremediation of persistent compounds. As other chapters in this volume will demonstrate, the same principles are being applied for the remediation of chlorinated solvents some 30 years later.

“Cooxidation or cometabolism can be of considerable ecological significance and may account for the degradation of many pesticides which do not sustain microbial growth. It might even be possible to exploit cometabolism in enhancing the rate of destruction of persistent pesticides; for example, the application to soil of a biodegradable simulant for a refractory pesticide could increase the population of organisms possessing the appropriate degradative enzymes for both the simulant and the long-lived pesticide and thereby increase the rate of destruction of the pest control agent”.

In view of the similarities between co-oxidation and cometabolism it is hardly surprising that the terms have often been used synonymously. However, one of the criticisms of cometabolism is that, unlike co-oxidation, the “co”-element has been marginalized to the extent that it is meaningless. Although Horvarth (Horvarth, 1972) has been criticized for initiating this trend (Gibson, 1993; Hulbert and Krawiec, 1977) the following quotation indicates that he clearly appreciated the role of growth substrates in cometabolism but also recognized that the same transformation reactions could be observed with both resting and actively growing cells.

“Although cometabolism does imply the concomitant oxidation of a non-growth substrate during growth of a microorganism on a utilizable carbon and energy source, it also describes oxidation of non-utilizable substrates by resting cell suspensions grown at the expense of substances capable of supporting microbial growth. Therefore, usage of cometabolism refers to any oxidation of substances without utilization of the energy derived from the oxidation to support microbial growth and does not infer presence or absence of growth substrate during oxidation.”

Dalton and coworkers (Dalton and Stirling, 1982) essentially reintroduced the “co” element into cometabolism and defined the process as follows: “*the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound*”. Like Foster’s earlier work, this definition arose from a study of hydrocarbon oxidation by methane-oxidizing bacteria. Stirling and Dalton (Stirling and Dalton, 1979) demonstrated that resting cells of *Methylococcus capsulatus* (Bath) can oxidize two classes of non-growth supporting substrates. The first group of compounds were oxidized without the need for additional oxidizable substrates. This process was described as “fortuitous oxidation”. A second and much larger group of compounds were only oxidized in the presence of formaldehyde (HCHO), a normal intermediate in the methane oxidation pathway. The oxidation of these substrates was described as cometabolism. The biochemical basis for the distinction between these reactions rests on the fact that the majority of the substrates oxidized in the absence of formaldehyde are initially oxidized by the non-specific methane monooxygenase (MMO) enzyme to products such as methanol (CH₃OH) and formaldehyde (HCHO) that can be further oxidized by enzymes associated with the methane oxidation pathway (methanol dehydrogenase, MDH; formaldehyde dehydrogenase, FaDH; and formate dehydrogenase, FDH). These selected compounds could therefore be used by the bacterium to generate the reductant necessary to support continued MMO activity. In contrast, the initial MMO-catalyzed oxidation of the second group of substrates generates products that are not subject to further oxidation at substantial rates and therefore the cells required an exogenous source of reductant to support MMO activity. These reactions are summarized in Scheme 1.

As our brief summary illustrates, there are strong areas of overlap between co-oxidation and cometabolism. First, the formal definitions of these terms were both derived principally from studies of methane-oxidizing bacteria. Second, based on what is now known about the enzymology of methane oxidation, both definitions describe degradation processes that are initiated by the non-specific MMO enzyme. Finally, both definitions recognize a role for the growth substrate in the transformation process. However, the roles of the growth substrate in these two processes are somewhat different and the key to this lies in the type of experiment used to originally define each process. For example, the studies leading to Dalton and Stirling’s

definition of cometabolism focused exclusively on identifying the sources of reductant utilized by resting cell suspensions to support non-growth supporting substrate transformation reactions. In these experiments cells were grown in the absence of a cosubstrate and MMO activity was already established in the cells by virtue of the previous growth of cells on methane. Under these circumstances the role of the growth substrate (methane) was limited to being one of several potential sources of reductant capable of sustaining MMO activity. In contrast, Foster's co-oxidation processes focused on transformation reactions catalyzed by actively-growing cultures continuously exposed to a non-growth supporting substrate. Under these circumstances the growth substrate serves two additional roles. First, it serves as a source of carbon and energy to support further microbial growth. In the industrial applications of co-oxidation envisioned by Foster (Foster, 1962), the provision of a carbon and energy source could lead to economically important increases in the rates and sustainability of non-growth substrate transformation reactions. Second, and perhaps more importantly, the growth substrate serves as an inducer of the enzyme activity required for the non-growth substrate transformation reaction to occur. Oxygenase induction and other roles for growth substrates in cometabolic processes are discussed further in later sections.

Possible contributions of cometabolism to cell metabolism. Early studies of cometabolism and co-oxidation essentially differentiated between non-growth-supporting substrates which undergo partial metabolism and growth-supporting substrates which are fully mineralized. This distinction led to the notion that cometabolic reactions are of no benefit to microorganisms because there was no assimilation of the reaction products. As noted by Hulbert and Krawiec (Hulbert and Krawiec, 1977), in practice the mass balance between substrate utilization and product excretion is often not established for cometabolic reactions. The possibility that the mass balance of cometabolism might be less than complete has raised the suggestion that cometabolism allows microorganisms to supplement their conventional carbon metabolism through assimilation of cometabolic products, albeit at a level insufficient to support growth. There is some evidence to support this from studies with autotrophic ammonia-oxidizing bacteria where it has been established that ^{14}C -label accumulates in cells during the cometabolic oxidation of $^{14}\text{CH}_4$ and ^{14}CO (Bedard and Knowles, 1989). However, these observations do not

eliminate the possibility the carbon from these substrates is either fixed conventionally via CO₂ derived from CO oxidation or through indiscriminate direct chemical fixation arising from the production of reactive formaldehyde during methane oxidation. The possibility that microorganisms use cometabolism as a source of supplementary carbon under oligotrophic conditions led Higgins and coworkers (Higgins, et al., 1980) to suggest this is was a beneficial effect that might explain why methane-oxidizing bacteria have retained such a highly non-specific enzyme like MMO. Although this argument was dismissed by Dalton and Stirling (Dalton and Stirling, 1982), the realization that a cometabolic transformation might lead to incorporation of the products into biomass without concurrent growth represents an important aspect of their discussion of cometabolism.

Although many studies of cometabolism were conducted with axenic cultures, there is also strong evidence that cometabolism represents an important mechanism for the degradation of a wide variety of compounds in mixed microbial communities. For example, cyclohexane is cometabolized to cyclohexanol by a pseudomonad, and cyclohexanol is then mineralized by a different pseudomonad (deKlerk and van der Linden, 1974). Similarly, a mixed culture containing a methane-oxidizing bacterium and heterotrophic bacteria was capable of complete mineralization of TCE while the methane-oxidizing bacterium alone did not completely mineralize the TCE (Uchiyama, et al., 1992). Alexander summarized other examples of these types of synergistic effects and has suggested they are important factors influencing the rate degradation of many recalcitrant compounds (Alexander, 1994). Some critics of cometabolism have suggested that this type of process is the only semantically valid use of the term cometabolism since the process involves both a “co” function (i.e. two microorganisms) and a true metabolism of the substrate (Wackett, 1996).

Physiological types of chlorinated solvent-degrading bacteria. Several microorganisms have been shown to cometabolically degrade TCE in studies using axenic cultures. Representative examples are included in Table 1 for a variety of growth substrates that have been used in TCE degradation studies. The enzyme that is thought to be responsible for TCE oxidation in these organisms is also listed. The organisms listed in Table 1 include a wide range of physiologically unrelated organisms. However, the strongest theme to emerge from this

summary is that all of the enzymes responsible for TCE oxidation are mono- and dioxygenases that initiate the oxidation of growth substrates. TCE-degrading organisms are frequently distinguished simply on the basis of the growth substrate needed to cause the expression of a TCE-degrading oxygenase. This process tends to disregard the fact that most of these organisms are capable of growth on many other substrates and that substantially different enzymes may be induced by the same substrate in different organisms. The diversity of toluene-oxidizing enzymes and bacteria described in Table 1 is an excellent example. There is also an interesting diversity in the inducibility of TCE-degrading activity in the bacteria listed in Table 1. For example, the ammonia-oxidizing bacterium *Nitrosomonas europaea* is restricted to the use of ammonia as a source of energy for growth and the expression of the non-specific oxygenase, ammonia monooxygenase (AMO), is therefore essentially constitutive. In contrast, there are organisms such as *Alcaligenes (Ralstonia) eutrophus* which have been shown to express two entirely different TCE degrading enzymes, a chromosomally-encoded phenol hydroxylase and plasmid-encoded 2,4-dichlorophenol hydroxylase, depending on which growth substrate is used as a growth substrate and oxygenase inducer (Harker and Kim, 1990). Furthermore, methane-oxidizers such as *Methylosinus trichosporium* OB3b can also express two different forms of MMO (soluble and particulate), both of which are capable of TCE oxidation, albeit at substantially different rates.

Range of chlorinated solvents subject to aerobic cometabolism. Although we have a focused on TCE as a representative chlorinated solvent, most of the chlorinated C₁ and C₂ hydrocarbons have been used as solvents. In general only the least halogenated members of this group of compounds can be utilized by aerobic microorganisms as carbon and energy sources that support growth. These fully metabolizable compounds include chloromethane (Vannelli, et al., 1998), dichloromethane (Braus-Stromeier, et al., 1993; Gisi, et al., 1998), dichloroethane (Vandenwijngaard, et al., 1992) and vinyl chloride (Hartmans, et al., 1985; Hartmans and Debont, 1992) (Table 1). In contrast, all of the C₁ and C₂ chlorinated hydrocarbons, other than the fully halogenated species (carbon tetrachloride, tetrachloroethylene and hexachloroethane), are known to be cometabolically degraded by one or more physiologically distinct types of aerobic microorganisms listed in Table 1. These observations are summarized in Table 2.

REVIEW OF THE TOPIC

Description of the enzymes. Cometabolism of chlorinated solvents occurs because certain enzymes have relaxed substrate ranges which include substrates other than the physiologically relevant substrate or substrates. In this section, we review those enzymes which have shown the greatest potential to date for use in the bioremediation of chlorinated solvents. All of these enzymes are oxygenases and all but one are monooxygenases. However, there are also many differences among these enzymes. Different prosthetic groups, cellular locations, substrate ranges, and protein structures are found among these enzymes. Following a brief description of oxygenases in general, we consider the methane monooxygenases, with particular emphasis on soluble methane monooxygenase. We then turn our attention to the monooxygenases and dioxygenase which oxidize toluene followed by a discussion of ammonia and alkene monooxygenases.

Oxygenases constitute a subset of the enzymes classified as oxidoreductases (which is one of the six major classes of enzymes). Oxygenases catalyze the reduction of O_2 with incorporation of one (monooxygenases) or two (dioxygenases) of the O atoms into the substrate which is being oxidized. Monooxygenases require an input of reductant which is used to reduce the second atom of O to H_2O . Dioxygenases do not necessarily require reductant as both atoms of O_2 are reduced upon incorporation into the substrate. However, some dioxygenases do use reductant to further reduce the substrate. Oxygenases serve a myriad of functions in cells including biosynthesis, detoxification, and catabolism. The protein composition of oxygenases can be relatively simple or quite complex (e.g. four separable protein components and 6 unique polypeptides). The activation of O_2 to a reactive state is typically facilitated by prosthetic groups in the enzymes. A remarkable variety of prosthetic groups has been recognized including flavin, heme, binuclear iron clusters, mononuclear iron centers, and Cu. Additional prosthetic groups including flavins and iron sulfur clusters can also be present to facilitate the transfer of electrons to the active site. Some oxygenases are soluble while others are associated with the membrane. The chemical mechanisms of oxygenases are also variable, though all have in common the need to activate O_2 . Typically, this activation is accomplished by transfer of electrons from the

prosthetic group in the enzyme to O_2 . The resulting reactive species is stabilized by coordination with the prosthetic group. In the case of monooxygenases, the activation typically involves the complete reduction of one of the atoms of O_2 with subsequent release of water. Not surprisingly given the variety of substrates and prosthetic groups, different chemical mechanisms appear to be at work with different oxygenases. While some monooxygenases are very specific for particular substrates, others have remarkably relaxed substrate ranges. In some cases, this relaxed substrate range appears to be by design. For example, in eukaryotes the broad substrate range of P450 monooxygenases is essential to their ability to assist in the removal of unwanted compounds from an organism. In other cases, the broad substrate range does not appear to provide any particular advantage or serve any particular purpose. For example, ammonia monooxygenase has a very broad substrate range which extends to several classes of compounds. However, the bacterium appears to derive benefit only from the oxidation of ammonia, the growth-supporting substrate.

Methane monooxygenase. Two forms of methane monooxygenase have been described. Soluble methane monooxygenase (sMMO) is so named because it is released into the supernatant when cells are broken. In contrast, particulate methane monooxygenase (pMMO) remains with cellular particles (e.g. membrane vesicles) following cell breakage and centrifugation. sMMO appears to have the broader substrate range and a faster catalytic turnover. sMMO is found only in Type II and Type X methanotrophs and is expressed under conditions of Cu limitation. It is not known how often the Cu-deficient conditions required for production of this enzyme actually occur in nature. pMMO, in contrast, is found in all methanotrophs. It is expressed under conditions of Cu sufficiency in Type II methanotrophs.

Soluble Methane Monooxygenase (sMMO). Under conditions of copper limitation, a soluble methane monooxygenase is produced (Stanley, et al., 1983). NADH serves as the electron donor. The enzyme consists of three protein components: Component B, a reductase (Component C), and a hydroxylase (Component A) (Lipscomb, 1994). Methane oxidation occurs at the active site found on the hydroxylase component which consists of six polypeptides. The crystal structure (2.2 and 2.0 angstrom resolution) (Elango, et al., 1997; Rosenzweig, et al., 1993) confirmed the presence of a hydroxy-bridged diiron center (the “diamond core” structure) which is similar to that found in ribonucleotide reductase (Nordlund, et al., 1992). The α subunits

contain the diiron center. Component B serves a regulatory role and the reductase component transfers electrons from NADH to the hydroxylase via FAD and an $[\text{Fe}_2\text{S}_2]$ cluster. The substrate range of sMMO extends well beyond the physiologically relevant methane (Green and Dalton, 1989). Alkanes (up to C_7) are oxidized to alcohols, alkenes are oxidized to the corresponding epoxides or to enols. Several chlorinated aliphatics, some aromatics, ethers and heterocycles are also substrates. Acetylene (Prior and Dalton, 1985) and longer alkynes are mechanism-based inactivators (Bedard and Knowles, 1989). The genes which encode the polypeptides of the three protein components of sMMO are clustered in a similar arrangement in *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (Murrell, 1992). A gene cluster which contains *mmoXYZorfYC* encodes the polypeptides $\text{A}\alpha$, $\text{A}\beta$, B, $\text{A}\gamma$, unknown Y, and C, respectively.

The chemical mechanism of sMMO has received considerable attention (e.g. (Elango, et al., 1997; Lipscomb, 1994; Nesheim and Lipscomb, 1996; Wilkins, et al., 1992)). In general, the chemical mechanism of sMMO involves first the production of an activated O species which is a sufficiently strong oxidizing agent to attack the C-H bonds in methane. Several lines of evidence support the idea that sMMO produces an activated O species that is similar to that which is produced by P450 monooxygenases, namely, an Fe(IV)=O (oxene) species. This species would then abstract an electron from the substrate resulting in formation of a radical intermediate. Rebound of the substrate radical with the reduced O species would result in the observed product formations. It is noteworthy, especially when considering the fortuitous metabolism of compounds by oxygenases, that a single mechanism may not be used for all substrates, especially where the differences are at later stages in the reaction pathway.

Particulate Methane Monooxygenase (pMMO). pMMO is produced under conditions of Cu sufficiency in methanotrophs. Many methanotrophs are only capable of producing this form of methane monooxygenase. Like its soluble counterpart, pMMO will catalyze the cooxidation of a number of alternative substrates (including propane and butane (Colby, et al., 1977)). Acetylene is an effective inactivator and copper selective chelators such as allylthiourea, are inhibitors (Prior and Dalton, 1985). Particulate methane monooxygenase has not been purified to homogeneity with high activity. However, recently a preparation of low activity was prepared from membranes of *M. capsulatus* (Bath) (Zahn and DiSpirito, 1996). Duroquinol was used as

the electron donor. Notably, NADH will not serve as a reductant for these purified preparations (possibly because of the lack of a flavoprotein containing reductase). The preparation contained three major polypeptides (47, 27 and 25 kDa) and 2.5 Fe atoms and 14.5 Cu atoms per 99 kDa. However, most of the copper appears to be associated with a small (618 Da) cofactor which can be separated from the pMMO polypeptides by treatment with detergent. Chan and his coworkers (Nguyen, et al., 1994) reported that the MMO activity in membranes increases with the copper content of the membranes. EPR and magnetic susceptibility experiments indicated that the Cu in the membranes could exist in the Cu(II) and Cu(I) forms and that a trinuclear Cu(II) cluster was present. The 27 kDa subunit becomes labeled with ^{14}C upon inactivation of the monooxygenase activity with $^{14}\text{C}_2\text{H}_2$. The structural genes for these polypeptides were recently isolated (Semrau, et al., 1995). Three contiguous genes, *pmoC*, *pmoA*, and *pmoB* code for the three structural polypeptides. These genes are present in multiple copies.

Toluene mono- and dioxygenases. The oxidation of toluene can be initiated by insertion of O into any of the four unique C-H bonds in this molecule. However, a separate enzyme is required for each insertion. *Pseudomonas mendocina* KR1 toluene-4-monooxygenase (T4MO) produces *p*-cresol; *Pseudomonas pickettii* toluene-3-monooxygenase (T3MO) produces *m*-cresol, and *Burkholderia cepacia* G4 toluene-2-monooxygenase (T2MO) produces *o*-cresol. Xylene monooxygenase (XMO) hydroxylates the methyl carbon to form benzyl alcohol. In addition, toluene dioxygenase (TDO) can insert both atoms from O_2 into toluene to form (+)-(1S,2R)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-dihydrodiol). All five enzymes have somewhat relaxed substrate ranges which include chlorinated solvents. To date, three of these enzymes (T2MO, T4MO, TDO) have been purified extensively and characterized at the molecular level..

Toluene-2-monooxygenase. Toluene-2-monooxygenase was purified from *B. cepacia* G4 (Newman and Wackett, 1995). The enzyme consists of three protein components (Table 1). A 40 kDa flavin and iron sulfur containing protein oxidizes NADH with subsequent transfer of electrons to the hydroxylase component of the enzyme. A small (10.4 kDa polypeptide) was required for activity, but for unknown reasons. No prosthetic groups or metals were detected in this component. The third component, the hydroxylase, contained the site for activation of molecular O_2 and oxidation of toluene. This hydroxylase component contained iron but no acid labile sulfur. The iron content and EPR spectra associated with this protein indicate that the

active site of this enzyme contains binuclear iron clusters. Toluene-2-monooxygenase also catalyses the oxidation of the product of toluene oxidation, *o*-cresol, to 3-methyl-catechol. The K_m for oxidation of *o*-cresol is 0.8 μ M.

Toluene-2-monooxygenase oxidizes TCE (Newman and Wackett, 1997). The K_m for TCE was 12 μ M. The V_{max} for TCE was 37 nmol/(mg hydroxylase x min) which compares to 27 nmol toluene oxidized/(min x mg hydroxylase) and 131 nmol *o*-cresol oxidized/(min x mg hydroxylase). The major products of TCE oxidation are formic acid, carbon monoxide and glyoxylic acid. About 12% of the products were found covalently attached to the enzyme polypeptides. Enzyme activity was also lost as a result of TCE oxidation. In intact cells, toluene is an inhibitor of toluene oxidation at sufficiently high concentrations. TCE is a noncompetitive inhibitor of toluene oxidation in intact cells of *B. cepacia* G4. Kinetic patterns for the purified enzyme have not been reported.

Toluene-4-monooxygenase. Toluene-4-monooxygenase was first partially purified from *P. mendocina* KR1 (Whited and Gibson, 1991) and then components were later purified to homogeneity from a recombinant *Escherichia coli* strain which contained the T4MO genes isolated from *P. mendocina* KR1 (Pikus, et al., 1996). As with sMMO and T2MO, a multicomponent enzyme was identified. A hydroxylase component similar to that in T2MO was identified which contains a binuclear iron cluster. A small protein similar to the 10.4 kDa polypeptide of T2MO was also identified. The reductase, TMOF, was only partially purified, but functions in concert with T4MOC, a Rieske-type iron sulfur protein, to transfer electrons to the hydroxylase component. Note that a fourth protein is required for in vitro activity (T2MO does not require the ferredoxin.). Thus, while similarities in catalytic site are apparent, differences in other aspects of these enzymes are also apparent. Detailed characterizations of the kinetic properties of this purified enzyme were not presented. T4MO is highly specific with regards to hydroxylation of the ring at the para position. 96% of the product is *p*-cresol, with 2.8% *m*-cresol, 0.4% *o*-cresol and 0.8% benzyl alcohol (Pikus, et al., 1997).

Xylene monooxygenase. The enzyme which catalyzes hydroxylation of the methyl carbon of toluene to form benzyl alcohol also oxidizes xylenes at the methyl carbons. This enzyme is generally referred to as xylene monooxygenase. XMO has not been purified extensively, but is membrane-bound and appears to be related to the iron-containing monooxygenases which

oxidize alkanes (alkane hydroxylase) and desaturate fatty acids (fatty acid desaturases) (Suzuki, et al., 1991). These enzymes also share eight conserved histidine residues which may serve as ligands to the active site irons (Shanklin, et al., 1994). Toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 oxidizes a number of CAHs including TCE (Chauhan, et al., 1998).

Toluene dioxygenase. In addition to the attacks on toluene by monooxygenases, a dioxygenase, toluene dioxygenase (TDO), will also initiate the degradation of toluene. NADH is oxidized to NAD⁺ in the reaction cycle. Three separable protein components are required to complete the reaction (Subramanian, et al., 1981; Subramanian, et al., 1985; Zylstra and Gibson, 1989). Reductase_{TOL}, a flavoprotein, catalyzes the oxidation of NADH with subsequent transfer of electrons to a small iron sulfur protein, ferredoxin_{TOL}. The reduced ferredoxin subsequently transfers electrons to the dioxygenase, which is designated ISP_{TOL}. The active site of TDO contains both a Rieske-type [2Fe-2S] center and a mononuclear iron center. The genes which code for the protein components of TDO are arranged in an operon: *todC1C2BADE*, where *C1C2* code for the dioxygenase, *B* codes for the ferredoxin, *A* codes for the reductase, and *D* and *E* code for enzymes which catalyze the further metabolism of the product of TDO (Zylstra and Gibson, 1989). TDO is a versatile enzyme which can catalyze monooxygenation, desaturation, O-dealkylation, N dealkylation, sulfoxidation and oxidative dehalogenation (Lange and Wackett, 1997). Halogenated aliphatics which serve as substrates include TCE, 1,1-dichloro-1-propene, and 1,1-dichloroethene but not tetrachloroethene. TCE is oxidized at a rate of 33 nmol/(min x mg protein) by purified TDO, which compares to a rate of 225 nmol toluene oxidized/(min x mg protein) (Lange and Wackett, 1997; Li and Wackett, 1992).

Alkene monooxygenase. Alkene monooxygenase was purified from *Xanthobacter* PY2 (Small and Ensign, 1997). This enzyme initiates the oxidation of propylene and other alkenes as growth substrates. It also fortuitously oxidizes a number of chlorinated aliphatics including TCE (Ensign, et al., 1992). Interestingly, saturated alkanes (e.g. ethane) are not oxidized. Like T4MO, the enzyme consists of four separable protein components. The reductase component transfers electrons to a Rieske-type iron sulfur protein which in turn transfers electrons to the hydroxylase component. A small protein, similar to that found in T4MO, T2MO, and sMMO is also required for high activity. The hydroxylase component apparently contains two binuclear iron clusters based on metal content and EPR spectra.

Ammonia monooxygenase. Ammonia monooxygenase (AMO) has a remarkably broad substrate range. In addition to a number of chlorinated aliphatics, AMO will also catalyze the oxidation of several alkanes, alkenes, aromatics (including benzene and several derivatives, several heterocycles, and several heteroatom ring compounds), ethers, thioethers and primary amines (Arp, et al., 1996). AMO has not yet been purified to homogeneity with activity, so the detailed properties of this enzyme are not available. Nonetheless, considerable information has been obtained from studies of the enzyme in intact cells and cell extracts. AMO consists minimally of two polypeptides. A 27 kDa polypeptide becomes covalently modified upon inactivation of ammonia oxidation activity in the presence of acetylene (Hyman and Wood, 1985). Purification of this (or a similarly modified polypeptide) results in the copurification of a second polypeptide of 40 kDa (McTavish, et al., 1993). This second polypeptide does not become labeled with acetylene, but both protein and genetic evidence support a role as a component of AMO. A third polypeptide is suggested by genetic characterizations (Klotz, et al., 1997), but physical evidence for this protein is still lacking for AMO (though it has been identified for the closely related pMMO). AMO is tightly associated with the membrane fraction upon cell breakage and is only released by treatment with detergents. The primary sequence of the protein (as deduced from the nucleotide sequence of the gene) indicates a number of membrane spanning helices in each of the proteins. AMO is likely to contain Cu (Ensign, et al., 1993) and may also contain Fe (Zahn, et al., 1996).

Although active, purified preparations of AMO are not available, considerable information about the enzyme has been obtained from studies with intact cells. The substrate range of AMO extends to a number of halogenated aliphatics which includes TCE (Arciero, et al., 1989; Rasche, et al., 1991). The affinity for TCE is surprisingly high; the K_m for TCE is about 12 μ M which compares to the K_m for NH_3 of about 40 μ M (K_m for NH_4^+ of 1 mM at pH 7.8) (Ely, et al., 1995 b; Hyman, et al., 1995). However, the turnover rate for TCE is low. Other chlorinated aliphatics are oxidized more rapidly, others not at all (PCE and CT are not oxidized) (Rasche, et al., 1991). In a study of several alternative substrates which did not lead to inactivation (including several nonhalogenated alkanes and alkenes), the inhibition patterns were examined with respect to ammonia oxidation (Keener and Arp, 1993). Because both ammonia and the co-substrate must compete for the activated O species it has been assumed that both

would bind at the same site thereby leading to competitive inhibition patterns. (Competitive inhibition occurs when the binding of the substrate and inhibitor are mutually exclusive and usually indicates that both compounds are binding to the same site on the enzyme.) This pattern was observed for some co-substrates (ethylene, methane). However, the predominant kinetic pattern was that indicative of noncompetitive inhibition. (Noncompetitive inhibition occurs when the inhibitor can bind either to the enzyme or to the enzyme complexed with the substrate and implies that there is a separate binding site for the inhibitor.) Although both the co-substrate and the physiological substrate must compete for the activated O formed in the enzyme's active site, the noncompetitive inhibition patterns suggest that there are separate approaches to this active site.

Summary. With regard to the enzymes involved identified as being of potential use in aerobic cometabolism of chlorinated solvents, one is struck by the remarkable differences in the structures of the physiologically relevant compounds--from NH_3 to toluene. Likewise, there are some striking differences in the structure of the enzymes and the prosthetic groups involved in catalysis. sMMO and T2MO contain binuclear iron centers, while AMO and pMMO likely contain Cu and possibly Fe in their prosthetic groups, and toluene dioxygenase contains a mononuclear Fe center. Interestingly, P450 monooxygenases, many of which are well known for their broad substrate ranges, have not yet been recognized as prosthetic groups in enzymes with potential for bioremediation of chlorinated solvents. In spite of these differences, some similarities in the properties of these enzymes can also be noted. The preferred substrates are uncharged, hydrophobic molecules. All of the enzymes have relaxed substrate ranges which include TCE and other chlorinated aliphatics. A non-chlorinated substrate which appears to be oxidized by all of these enzymes is ethylene. Universal substrates are of interest in screening populations or environments for TCE degrading potential. All of the monooxygenases which have been examined are inactivated by acetylene. Another common feature among these enzymes is that each is at the beginning of a catabolic pathway used to harvest a substrate from the environment. Other monooxygenases and dioxygenases which catalyze subsequent metabolic steps, even those involved in subsequent transformations of toluene, have not been recognized as capable of rapid rates of chlorinated aliphatic degradation. Thus, while there are

distinct differences among the growth-supporting substrates and the enzymes which catalyze these transformations, there are also some common themes.

Physiological effects of cometabolism. Oxygenase Induction, Specificity and

Abundance. The role of growth substrates as inducers of requisite degradative enzymes is a key issue which is not adequately addressed by existing definitions of either cometabolism or co-oxidation, especially when one applies these definitions to microorganisms other than methane-oxidizers. For instance, using the resting cells approach used to define cometabolism (*sensu* Dalton and Stirling) it is obviously possible to grow microorganisms on a non-inducing substrate and then observe no transformation reactions whatsoever when resting cells are incubated with a non-growth supporting substrate and any number of other transformable compounds, including the original growth substrate. Likewise, according to the growth-oriented approach used to define co-oxidation (*sensu* Foster), microbial growth could be supported by a hydrocarbon substrate that does not induce an enzyme system necessary for the oxidation of a non-growth hydrocarbon included in the growth medium. Again one would not observe a transformation reaction under these conditions. Examples of these effects are distributed throughout the literature of microbial hydrocarbon and chlorinated hydrocarbon oxidation. For example, cells of *Burkholderia cepacia* G4 grown in the presence of toluene and TCE oxidize both toluene and TCE through the activity toluene-2-monooxygenase (Landa, et al., 1994). In contrast, cells of the same organism growing in the presence of lactate and TCE do not express toluene-2-monooxygenase activity and are unreactive towards TCE. It is also interesting to note that there are several reports of the induction of oxygenase enzymes by chlorinated solvents such as TCE (Heald and Jenkins, 1994; Leahy, et al., 1996; McClay, et al., 1995) and other chlorinated ethylenes (Ensign, 1996).

As the previous sections have indicated, a consistent biochemical theme that emerged from earlier studies of the aerobic cometabolic and co-oxidative processes is that the key enzyme responsible for initiating the transformation of non-growth substrates is typically a non-specific oxygenase. The same theme has also emerged from more recent studies of the degradation of chlorinated solvents such as TCE. The lack of substrate specificity of many oxygenases may simply be a consequence of mechanisms involving the generation of reactive oxygen species. An alternative explanation is that this lack of specificity is less than fortuitous and may allow the

microorganisms that harbor these enzymes to initiate the oxidation of a diverse range of potential growth substrates through the activity of one indiscriminate enzyme. Although critics of the term “cometabolism” have noted that a lack of substrate specificity is a common feature of all enzymes to one degree or another (Wackett, 1996), it is also important to note that some of the enzymes (e.g. MMO) shown to be capable of oxidizing chlorinated solvents (e.g. TCE) are some of the most non-specific enzymes known. Irrespective of the underlying cause of the “sloppiness” of these oxygenase enzymes, for applied purposes it is obviously advantageous that these enzymes have high levels of activity in whole cells. This can be obtained either by high levels of enzyme expression or through high specific activities, or both. These conditions are most frequently met when the oxygenase serves as the initial catalyst in the growth substrate oxidation pathway; conditions where the oxygenase holds a prominent role in overall cellular metabolism.

The significance of enzyme abundance and specific activity is supported by biochemical studies of two of the most well-characterized of the TCE-degrading oxygenases. As shown in Table 4, the hydroxylase components of soluble MMO and toluene-2-monooxygenase are abundant in the cells and have specific activities and affinities towards TCE that are comparable with those of the growth substrates, methane and toluene. There are likely many undiscovered oxygenase enzymes that are capable of oxidizing chlorinated solvents. Some of these enzymes may be induced by substrates that are either expensive or more toxic than the chlorinated solvents towards which their activities might be directed. Likewise, some of these enzymes may serve peripheral roles in biosynthetic processes or secondary metabolism and the level of activity of these enzymes may be small relative to enzymes involved in the metabolism of growth-supporting substrates. Enzyme systems with such features would be unattractive for bioremediation processes where cost-effectiveness and a lack of further environmental impact are important considerations.

Physiological Aspects of Chlorinated Solvent Cometabolism. In this section we will briefly consider some of the broader physiological aspects of the cometabolism of chlorinated solvents, again with an emphasis on TCE. The following sections will first consider 1) the pathways of TCE oxidation, 2) the reversible, inhibitory effects associated with TCE oxidation,

and 3) the irreversible or toxic cellular consequences associated with chlorinated solvent cometabolism.

Pathway of TCE Oxidation: The most comprehensive studies of the pathway of TCE oxidation have involved purified enzyme studies where the possibility of further enzyme-catalyzed product transformations were eliminated. Purified sMMO generates glyoxylate, formate, and CO as major products of TCE oxidation with dichloroacetate as a minor component (Fox, et al., 1990). The addition of trichloroethylene oxide to an enzyme-free incubation leads to the same products in very similar ratios indicating this compound non-enzymatically decomposes to generate the observed products. The sMMO also generates a small quantity of trichloroacetaldehyde (chloral) and this compound is not generated when trichloroethylene oxide is added to the enzyme system. Chloral is converted to 2,2,2-trichloroethanol and trichloroacetic acid by whole cells of methane-oxidizing bacteria (Newman and Wackett, 1991). These product distributions and their accompanying kinetics of formation (Fox, et al., 1990) indicate that sMMO primarily oxidizes TCE to TCE epoxide. Epoxide production by sMMO has been confirmed for the purified enzyme by trapping this compound with 4-(p-nitrobenzyl) pyridine (Fox, et al., 1990) and for whole cells using an on-line gas chromatographic method (van Hylckama Vlieg, et al., 1996). For purified toluene dioxygenase from *P. putida* F1, the principal products generated from TCE oxidation were also glyoxylate and formate (Li and Wackett, 1992). A dioxygenation mechanism involving 1,2-dihydroxy-trichloroethane as an initial product was proposed but not confirmed for this enzyme (Li and Wackett, 1992). Notably this enzyme also catalyzes monooxygenation reactions including the oxidation of nitrotoluenes (Robertson, et al., 1992), indene (Wackett, et al., 1988), and phenol (Spain, et al., 1989). It is therefore possible that the oxidation of TCE by this dioxygenase is a reflection of its monooxygenase-like characteristics. The most recent studies of the products of TCE oxidation have been made with the purified toluene-2-monooxygenase from *B. cepacia* G4 (Newman and Wackett, 1997) where again been the principal products of TCE oxidation were CO, formate, and glyoxylate.

All of these purified enzymes are all inactivated to one degree or another as a consequence of TCE oxidation. Experiments using ^{14}C -labeled TCE established that all of the protein components of these enzymes become covalently modified with ^{14}C as a consequence of ^{14}C -TCE oxidation. While these results are consistent with the idea that enzyme inactivation is

caused by a diffusible and reactive TCE oxidation product, these experiments have not established a clear relationship between the kinetics of enzyme inactivation and radiolabeling. In addition, these experiments are biased in the sense that the purified enzyme components are the only possible targets available for a reactive intermediate to react with and this is clearly not the case in the whole cell environment. However, an important insight that has been gained from these studies is that cysteine provides protection against the inactivating effects of TCE. In the whole cell environment toluene-2-monooxygenase activity is apparently relatively insensitive to the toxic effects of TCE oxidation while sMMO and toluene dioxygenase activities are susceptible to inactivating effects both *in vivo* and *in vitro*. Perhaps the organisms that harbor these different enzymes have different steady state levels of thiol-containing species in the cytoplasm which accounts for the differing susceptibilities of these organisms to toxic effects associated with TCE oxidation. These and other issues related to the toxicity associated with chlorinated solvent degradation are explored more fully in a following section.

Inhibitory effects of cometabolism. Some physiological effects of TCE cometabolism are immediately reversed by removal of the TCE. These effects are mainly the result of the competitive interaction between growth substrates and non-growth substrates for oxidation by the same enzyme and the cumulative effects of this interaction on reductant availability and growth.

An intrinsic feature of monooxygenases is that they require a source of reductant to reduce one atom of molecular oxygen to water while the other atom is introduced into the substrate. During the metabolism of growth-supporting substrates the reductant invested in the initial activation of the growth substrate by a monooxygenase is recouped during subsequent metabolic reactions. In many cometabolic reactions the non-growth supporting substrate is only subject to a single monooxygenase-catalyzed reaction and the products of this reaction are not further metabolized to replenish the expended reductant. In situations where a microorganism is simultaneously exposed to a growth supporting and non-growth supporting substrate the net drain of reductant caused by non-growth substrate oxidation will be dictated by the relative concentrations of the two substrates, their relative affinities for the monooxygenase and their relative oxidation rates. Under conditions where there are high growth-substrate concentrations and low non-growth-substrate concentrations the effects of non-growth substrate oxidation are

likely to be small. However, as the relative concentration of the non-growth substrate is increased this compound will progressively outcompete the growth substrate for the active site of the monooxygenase, thereby concurrently decreasing the rate of growth substrate oxidation and the rate of supply of reductant to the monooxygenase.

In short term experiments using resting cells the consequences of this competitive interaction between substrates can have unusual kinetic effects because an increase in non-growth substrate concentration can decrease rather than increase the rate of oxidation of that compound.

However, the majority of studies of the interaction between growth substrates and chlorinated solvents have tended to treat these interactions as simple competitive interactions and have determined K_s and K_i values, respectively. An interesting feature of the oxidation of TCE is that one of the major products is carbon monoxide. In the case of methane- and ammonia-oxidizing bacteria (Bedard and Knowles, 1989), CO is known to be a substrate for both MMO and AMO. The production of carbon monoxide from TCE potentially further complicates the kinetic analysis of TCE oxidation.

Toxic Effects of Chlorinated Solvent Cometabolism: The once conventional view that cometabolic reactions are inconsequential to microorganisms has been dramatically altered by the observations that chlorinated solvent oxidation often gives rise to toxic effects that limit the further oxidation of these compounds and growth-supporting substrates. The fact that this toxicity occurs is perhaps not surprising given that the oxygenase-catalyzed degradation of chlorinated solvents is an important aspect of the human toxicity of many of these compounds. The toxic effects associated with the cometabolism of chlorinated solvents have again been most intensively studied with TCE and most TCE-degraders are recognized to suffer toxic effects to one degree or another. These effects have been examined at both the whole cell and purified enzyme level.

The prevailing model used to account for the toxic effect associated with the oxidation of compounds such as TCE is that this compound behaves as a mechanism-based inactivator. This model implies that the TCE-oxidizing oxygenase enzymes become covalently modified and inactivated by reactive intermediate generated during the TCE oxidation reaction. One of the first studies characterizing the toxic effects of TCE oxidation demonstrated that the rate of TCE

degradation by *P. putida* F1 decreases rapidly with time (Wackett and Householder, 1989). The growth rates of toluene-induced *P. putida* F1 on arginine are also reduced in the presence of TCE in an exposure-dependent manner (Heald and Jenkins, 1994) although TCE exposure does not hinder growth in *P. putida* F4, a mutant lacking toluene dioxygenase activity. These observations certainly support the notion that the toxic effects of TCE arise due to TCE oxidation rather than simply due to exposure of cells to this compound. Subsequent studies have examined the question of the site of action of TCE-associated toxicity and have examined the distribution of radiolabeled ^{14}C -TCE among cellular components. Wackett and Householder (Wackett and Householder, 1989) demonstrated that ^{14}C -label becomes incorporated into a variety of macromolecules as a result of TCE oxidation by *P. putida* F1. The oxidation of ^{14}C -TCE also leads to a non-specific incorporation of radiolabel into a variety of polypeptides as a consequence of TCE oxidation by methane and ammonia-oxidizing bacteria (Oldenhuis, et al., 1991; Rasche, et al., 1991). In the case of ammonia-oxidizers it was also demonstrated that this radiolabel incorporation was prevented when cells were incubated with ^{14}C -TCE and a specific inhibitor of ammonia monooxygenase (AMO).

While toxic effects associated with TCE oxidation have been observed with several different types of TCE-degraders, the severity of these effects is highly variable. For example, studies involving *B. cepacia* G4 consistently indicate that toxicity related to TCE co-metabolism is relatively insignificant. Resting suspensions of phenol-induced *B. cepacia* G4 degraded TCE at constant rates over a 3 hour time course, following an initial lag period (Folsom, et al., 1990). Also, steady rates of TCE degradation have been observed in bioreactors containing toluene-fed *B. cepacia* G4 with TCE (Folsom and Chapman, 1991). In contrast, ammonia oxidizers appear to be very sensitive to toxic effects associated with chlorinated solvent degradation (Hyman, et al., 1995). An understanding of the mechanism of these toxic effects is of obvious importance as the toxicity can strongly influence the efficiency of any bioremediation processes designed around cometabolism. However, our understanding of the mechanism of toxicity is complicated by several observations which indicate that these effects are not strictly enzyme specific, but rather they are also host specific. For example, the toxic effects associated with TCE oxidation by toluene dioxygenase in *P. putida* F1 are lost when the enzyme is expressed in an active form in a recombinant strain of *E. coli* (Zylstra and Gibson, 1989). A similar effect of host organisms has

also been reported for TCE oxidizing activities of *P. mendocina* KR1 and recombinant *E. coli* containing genes encoding for toluene-4-monooxygenase (Winter, et al., 1989).

The chemical species responsible for the toxic effects of TCE have also been investigated. Although most mechanisms of TCE oxidation suggest TCE epoxide is the initial unstable oxidation product of TCE oxidation, the current kinetic evidence suggests that it is not directly responsible for the inactivating effects observed during TCE oxidation. Recently, it has been demonstrated that sMMO in *Methylosinus trichosporium* OB3b can oxidize the stable epoxide, *cis*-1,2-dichloroethylene oxide and that this reaction gives rise to a toxic effect (van Hylckama Vlieg, et al., 1996). This observation raises the interesting possibility that the inactivating effect of TCE is due to effects caused by the further enzymatic oxidation of trichloroethylene oxide rather than an as yet uncharacterized decomposition product of trichloroethylene oxide or a minor product of TCE oxidation. This possibility is supported by several previous studies which demonstrate monooxygenase-specific inactivation reactions caused by epoxides are quite common (Habets-Crützen, et al., 1985; Hartmans, et al., 1989). These observations suggest that toxicity in whole cells may be a function of multiple factors. For instance, glutathione is known to react rapidly with epoxides. The changes in toxicity observed when altering the host for a TCE-degrading enzyme may therefore simply reflect differences in cellular glutathione concentrations. Alternatively, the apparent resistance of cells of *B. cepacia* G4 to toxic effects associated with TCE oxidation may reflect an inability of this enzyme to further oxidize epoxides.

The significance of the toxic effects associated with chlorinated solvent degradation has been formally recognized in a variety of mathematical models (Alvarez-Cohen and McCarty, 1991; Criddle, 1993; Ely, et al., 1995 a). Toxicity has also been recognized in the concept of transformation capacities. Simply stated, the transformation capacity defines the mass of substrate that can be transformed by a given mass of cells. The relative transformation capacities of different organisms for different substrates have become important considerations in the design of cometabolic processes for chlorinated solvent degradation. While the notion of a finite cometabolic capacity is useful for some purposes it is also important to recognize the limitations of this concept. For example, transformation capacities poorly distinguish between limitations caused by truly irreversible toxic effects and more cryptic effects caused by reductant limitation.

In contrast, the transformation yield, a closely-related term, recognizes the finite transformation capabilities of microorganisms in the continuous presence of a growth substrate where the effects of reductant limitation are minimized, although not completely excluded.

Another important aspect of the toxic effects associated with chlorinated solvent degradation is that several types of microorganisms have been shown to recover from these effects. For example, even the early studies of the toxic effect of TCE co-metabolism on *P. putida* F1 demonstrated that TCE-inactivated cells are able to regain their maximal growth rate after a period of time. This recovery effect has been more extensively examined with the nitrifying bacterium *N. europaea* (Ely, et al., 1995 b; Ely, et al., 1995 a; Hyman, et al., 1995). The recovery process involved *de novo* protein synthesis rather than new cell growth and the rate of recovery was strongly dependent on the extent of inactivation. This recovery effect has also been integrated into a mathematical model which accurately describes TCE co-metabolism by this organism under quasi-steady state conditions (Ely, et al., 1995 b; Ely, et al., 1995 a).

Although there is a growing understanding of the mechanism of the toxicity associated with TCE cometabolism, the wealth of information concerning the mechanisms of toxicity of these diverse compounds in mammalian systems indicate it is wrong to assume that insights gained from studies of TCE with bacterial systems can be applied to all other chlorinated solvents which exhibit toxic effects on microorganisms. For example, it has also been demonstrated in field studies that toluene-oxidizing organisms which generate *o*-cresol as an intermediate likely suffer a toxic effect during the co-metabolism of 1,1-dichloroethylene (Hopkins and McCarty, 1995). These studies clearly suggest that organisms like *B. cepacia* G4, which utilize a toluene-2-monooxygenase to initiate toluene oxidation, are responsible for the oxidation of this compound and this further suggests that this organism is more susceptible to the toxic effects of chlorinated solvent cometabolism than studies with TCE would suggest. Our most recent studies with *N. europaea* also suggest that the notion that the oxygenase enzyme responsible for chlorinated solvent degradation is the ultimate target of toxic effects is also too simple a model. For example, we have observed that the oxidation of TCE results in an inactivation of both AMO and the other key enzyme in the ammonia oxidation pathway, hydroxylamine oxidoreductase (Fawcett, Arp, Hyman; unpublished results). Our previous studies have also demonstrated that there is a poor correlation between the kinetics of the TCE-

dependent loss of AMO activity and the incorporation of radiolabel from ^{14}C -TCE into polypeptides known to be components of AMO (Rasche, et al., 1991). These observations challenge the notion that covalent modification of proteins by products of TCE oxidation is directly relevant to the mechanism of TCE toxicity.

In longer term studies where microorganisms are cultured in the presence of both a growth substrate and a non-growth substrate the effects of the competitive interaction between the two substrates is compounded and can be expected to lead to a decrease the growth rate and growth yield of an organism. These competitive interactions have also been characterized in many instances and have been incorporated into several mathematical models of cometabolism. This effect is expected to be further exacerbated when a toxic effect is associated with the oxidation of the non-growth supporting substrate. The second of these effects has recently been studied in a fed-batch bioreactor using *B. cepacia* G4 grown on toluene under growth-limited conditions. It was observed that TCE increases the maintenance energy demand of the cells, an effect which was attributed to a toxic effect and the need to resynthesize damaged cellular components, although the identity of the affected cellular components was not established (Mars, et al., 1996).

Genetics. The genes which code for several of the enzymes involved in transformations of co-substrates have been isolated and characterized. Several of the genes which code for specific monooxygenases were mentioned above. The genes for each monooxygenase tend to be clustered within the genome. Once a gene is isolated, experiments can be designed which result in disruption of the gene in the host organism (gene knock-outs) or transfer of the gene to a new host (transformation). Such experiments can help to determine the role of the gene and its product.

With the genes in hand, applications of genetic engineering also become feasible. For example, new pathways can be engineered into bacteria by transforming a bacterium with a gene or genes from another bacterium. Wackett et al. (Wackett, et al., 1994) constructed a *Pseudomonas putida* strain which contained the *tod* genes (which code for toluene dioxygenase) and cytochrome P450_{cam} genes. This engineered strain could couple the activities of these two enzymes to completely dehalogenate pentachloroethane. Because each unique subunit of a

monooxygenase requires a gene, there is also the possibility to create constructs with similar genes from another bacterium replacing that gene in the host. Recombinant *E. coli* cells with high rates of TCE degradation were produced by introducing specific *tod* and *bph* genes (for biphenyl dioxygenase) (Furukawa, et al., 1994). Genetic engineering also offers the prospect of “improving” the enzyme for bioremediation by substituting one or more amino acids at specific sites with other amino acids. Improvements could include an enhanced substrate range, improved affinities for co-substrates, or increased stability of the enzymes. *Pseudomonas mendocina* KR1 toluene 4-monooxygenase catalyzes the ring hydroxylation of toluene to *p*-cresol predominantly (Pikus, et al., 1997). Additionally, this monooxygenase catalyzes the oxidation of *p*-xylene to 4-methyl benzyl alcohol predominantly. However, substitution of the phenylalanine residue at position 141 with a cysteine resulted in an isoform with an altered regiospecificity. The results demonstrate the feasibility of altering the product distribution of enzymes by genetic engineering. However, this isoform had a k_{cat} for toluene oxidation approximately one tenth that of the wild-type enzyme.

DISCUSSION

As described above, cometabolism has its genesis in the relaxed substrate range of certain enzymes. A process based on a relaxed substrate range of enzymes stands somewhat in contradiction to the notion of enzymes as catalysts with a remarkable level of selection for a particular substrate or group of related substrates. Indeed, most enzymes do have this high degree of selectivity for a substrate. But more relaxed substrate ranges are certainly not uncommon among enzymes, and especially monooxygenases which catalyze the oxidation of small hydrophobic compounds. As pointed out by Wackett (Wackett, 1996), the difference in free energy between the substrate and enzyme, both free in solution, and the enzyme substrate complex is what provides the driving force for substrate discrimination by an enzyme. In the case of small hydrophobic molecules, the amino acid residues on the enzyme which form the binding pocket are expected to be hydrophobic and must rely on Van de Waals contacts for recognition of the preferred substrates. For a similarly shaped hydrophobic molecule, the binding energy for enzyme substrate complex formation will be only slightly different from that of the preferred

substrate, thus allowing little margin for discriminating between the substrates. While this explanation rationalizes the binding of vinyl chloride to alkene monooxygenase, where ethylene is the preferred substrate, it falls short in explaining the binding of naphthalene to sMMO, where methane is the preferred substrate. The crystal structure of sMMO revealed a “hydrophobic pocket” near the active site which may serve as the binding site for co-substrates (Elango, et al., 1997; Rosenzweig, et al., 1993). But it is interesting to note that even with a crystal structure, the determinants of specificity are not readily apparent. Indeed, the binding sites for the preferred substrate and the co-substrate need not be identical. Given the fortuitous nature of the interaction of co-substrates with these enzymes, predictions about reactivity, reaction rates, and product distributions have been and are likely to continue to be difficult to make.

In laboratory settings with cultures consisting of a single bacterium, cometabolism is most associated with detrimental effects on the bacterium. Energy is directed away from growth and metabolism to be used for oxidation of co-substrates and to repair the damage caused to the cell by the products of the co-substrate oxidations. While the net impact of cometabolism on a complex ecosystem is as yet difficult to predict, the impacts on the microorganisms initiating the degradation are likely to be similar whether in laboratory culture or environmental settings. As such, bioremediation schemes must take into account the impacts of cometabolism on the cells. However, these effects are not well understood at the molecular level. Indeed, current dogma holds that the toxicity which often accompanies cometabolism is directed at least partially at the active site. While this can be the case, other cellular targets may be of even more importance. For example, disruption of the electron transport chain or membrane integrity could have more far-reaching effects on a cell than loss of a monooxygenase. Furthermore, current dogma does not draw distinctions among the different toxic effects associated with the degradation of different compounds. However, each co-substrate will produce a new set of metabolites with which the cell must interact. Certainly, the production of phosgene from chloroform is not expected to influence the cell in the same way as the production of acetaldehyde from vinyl chloride. The maintenance cost to a bacterium carrying out cometabolism will be expected to be much higher than the maintenance cost to the same bacterium not involved in cometabolism. However, these increased maintenance costs have received scant attention.

Another area requiring additional information is in the search for diagnostic substrates which are indicative of bioremediation potential. The ideal diagnostic substrate would be environmentally friendly, be transformed to easily measured products, and have broad reactivity with regard to the monooxygenases known to carry out chlorinated solvent transformations. Likewise, inactivators of specific processes are needed to help dissect the contribution of various processes to the observed reactions. The ideal inactivator would be specific for a particular subset of transformation reactions (e.g. inhibit methane oxidations but not toluene oxidations), would be environmentally friendly, and would function at low concentrations.

Genetic engineering offers considerable promise to improve the ability of bacteria to degrade chlorinated solvents. In theory, problems with competition from the physiological substrate, need for an inducer, substrate range and affinity, and other factors can be addressed through genetic engineering. In practice, such improvements are not likely to come easily. Most engineered isoforms of enzymes are either fully active (the amino acid substitution is without effect) or virtually inactive (the residue is essential for activity). Substitutions which lead to enzymes with improved properties are the exception. The prospect of using genetically engineered microorganisms at a site clearly implies the need for augmentation in which case competition between the introduced bacterium and the indigenous population becomes a concern. Regulatory restrictions on the release of genetically engineered microorganisms are also a concern. Nonetheless, with improved selection strategies (e.g. with the use of robotics to screen large numbers of samples), it may be possible to isolate engineered organisms with improved bioremediation properties.

Summary

The molecular underpinnings for cometabolism of chlorinated solvents begin with the substrate ranges of monooxygenases and dioxygenases which extend beyond the preferred or physiologically relevant substrate to chlorinated solvents. Binding of the chlorinated solvent to the enzyme can inhibit the binding of the preferred substrate, thereby depriving the cell of the benefits of the oxidation of the preferred substrate. The products of the chlorinated solvent oxidation can also have profound influences on the cell and often include toxic effects on the

cell. The oxygenases which oxidize chlorinated solvents are typically not constitutively produced in the cell. Rather, the enzyme production is induced in the presence of the growth substrate. Many of the genes which code for these oxygenases have been characterized thereby opening the prospects for genetic engineering of pathways and enzymes.

While the basic tenets of aerobic cometabolism are well understood, many of the details remain to be elucidated. For example, mechanisms of toxicity are not well understood. The competitive model for binding of growth substrate and CAH to the oxygenase is not always appropriate. Furthermore, the results are likely to be different for different oxygenases. Even for a given oxygenase, different CAHs are likely to have different effects on the enzyme and the cell. In deciding which problems to address next, it is worthwhile to look to field tests of aerobic cometabolism. What limits the efficacy of these cometabolic processes in the field and how can an enhanced understanding of the molecular underpinnings of cometabolism mitigate these limitations?

Alexander, M. 1967. *The Breakdown of Pesticides in Soils*, vol. 85. American Association for the Advancement of Science, Washington D.C.

Alexander, M. 1994. *Biodegradation and bioremediation*. Academic Press, Inc., San Diego.

Alvarez-Cohen, L. and P.L. McCarty. 1991. "A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity". *Environ. Sci. Technol.* 25:1381-1387.

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

Arp, D.J., N.G. Hommes, M.R. Hyman, L.Y. Juliette, W.K. Keener, S.A. Russell and L.A. Sayavedra-Soto. 1996. "Ammonia monooxygenase from *Nitrosomonas europaea*", p. 159-166. In M. E. Lidstrom and F. R. Tabita (ed.), *Microbial growth on C₁ compounds*. Kluwer Academic Publishers, Dodrecht/Boston/London.

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.

- Braus-Stromeyer, S.A., R. Hermann, A.M. Cook and T. Leisinger. 1993. "Dichloromethane as the sole carbon source for an acetogenic mixed culture and isolation of a fermentative, dichloromethane-degrading bacterium". *Appl. Environ. Microbiol.* 59:3790-3797.
- Chauhan, S., B. Paola and T.K. Wood. 1998. "Oxidation of trichloroethylene, 1,1-dichloroethylene, and chloroform by toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1". *Appl. Environ. Microbiol.* 64:3023-3024.
- 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, aromatic and heterocyclic compounds". *Biochem. J.* 165:395-402.
- Criddle, C.S. 1993. "The kinetics of cometabolism". *Biotechnology and Bioengineering.* 41:1048-1056.
- Dagley, S. and M.D. Patel. 1957. "Oxidation of *p*-cresol and related compounds by a *Pseudomonas*". *Biochem. J.* 66:227-233.
- Dalton, H. and D.I. Stirling. 1982. "Co- metabolism". *Phil Trans. R. Soc. Lond. B.* 297:481-496.
- deKlerk, H. and A.C. van der Linden. 1974. *Antonie van Leeuwenhoek.* 40:7-15.
- DiSpirito, A.A., J. Gullledge, 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.
- Elango, N., R. Radhakrishnan, W.A. Froland, B.J. Wallar, C.A. Earhart, J.D. Lipscomb and D.H. Ohlendorf. 1997. "Crystal structure of the hydroxylase component of methane monooxygenase from *Methylosinus trichosporium* OB3b". *Protein Science.* 6:556-568.
- Ely, R.L., M.R. Hyman, D.J. Arp, R.B. Guenther and K.J. Williamson. 1995 b. "A cometabolic kinetics model incorporating enzyme inhibition, inactivation and recovery: II Trichloroethylene degradation experiments". *Biotechnol. Bioeng.* 46:232-245.
- Ely, R.L., K.J. Williamson, R.B. Guenther, M.R. Hyman and D.J. Arp. 1995 a. "A cometabolic kinetics model incorporating enzyme inhibition, inactivation and recovery: I Model development, analysis and testing." *Biotechnol. Bioeng.* 46:218-231.
- Ensign, S.A. 1996. "Aliphatic and chlorinated alkenes and epoxides and inducers of alkene monooxygenase nad epoxidase activities in *Xanthobacter* strain Py2". *Appl. Environ. Microbiol.* 62:61-66.

- Ensign, S.A., M.R. Hyman and D.J. Arp. 1992. "Cometabolic Degradation of Chlorinated Alkenes by Alkene Monooxygenase in a Propylene-Grown *Xanthobacter* Strain". *Applied and Environmental Microbiology*. 58:3038-3046.
- Ensign, S.A., M.R. Hyman and D.J. Arp. 1993. "In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper.". *J. Bacteriol.* 175:1971-1980.
- Folsom, B.R. and P.J. Chapman. 1991. "Performance characterization of a model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia*-G4". *Appl. Environ. Microbiol.* 57:1602-1608.
- 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.* 56:12-79-1285.
- Foster, J.W. 1962. Bacterial oxidation of hydrocarbons. Academic Press Inc, New York.
- Fox, B.G., J.G. Borneman, L.P. Wackett and J.D. Lipscomb. 1990. "Haloalkene oxidation by soluble methane monooxygenase from *Methylosinus trichosporium* Ob3b: mechanistic and environmental implications". *Biochemistry*. 29:6419-6427.
- Fox, B.G., W.A. Froland, J.E. Dege and J.D. Lipscomb. 1989. "Methane monooxygenase from *Methylosinus trichosporium* OB3b. Purification and properties of a three-component system with high specific activity from a type II methanotroph". *J. Biol. Chem.* 264:10023-10033.
- Furukawa, K., J. Hirose, S. Hayashida and K. Nakamura. 1994. "Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase". *Journal of Bacteriology*. 176:2121-2123.
- Gibson, D.T. 1993. "Biodegradation, biotransformation and the Belomont". *J. Ind. Microbiol.* 12:1-12.
- Gisi, D., L. Willi, H. Traber, T. Leisinger and S. Vuilleumier. 1998. "Effects of bacterial host and dichloromethane dehalogenase on the competitiveness of methylotrophic bacteria growing with dichloromethane". *Appl. Environ. Microbiol.* 64:1194-1202.
- Green, J. and H. Dalton. 1989. "Substrate specificity of soluble methane monooxygenase. Mechanistic implications". *J. Biol. Chem.* 264:17698-17703.

- Habets-Crützen, A.Q. H. and J.A.M. deBont. 1985. "Inactivation of alkene oxidation by epoxides in alkene and alkane-grown bacteria". *Appl. Microbiol. Technol.* 22:428-433.
- Hamamura, N., C. Page, T. Long, L. Semprini and D.J. Arp. 1997. "Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and methane-grown *Methylosinus trichosporium* OB3b". *Appl. Environ. Microbiol.* 63:3607-3613.
- Harker, A.R. and Y. Kim. 1990. "Trichloroethylene degradation by two independent aromatic degrading pathways in *Alcaligenes eutrophus* JMP134". *Appl. Environ. Microbiol.* 56:1179-1181.
- Hartmans, S., J.A.M. de Bont and W. Harder. 1989. "Microbial metabolism of short-chain unsaturated hydrocarbons.". *FEMS Microbiol. Rev.* 62:235-264.
- Hartmans, S., J.A.M. de Bont, J. Tramper and D.C.A.M. Luyben. 1985. "Bacterial degradation of vinyl chloride". *Biotechnol. Lett.* 7:383-388.
- Hartmans, S. and J.A.M. Debont. 1992. "Aerobic Vinyl Chloride Metabolism in *Mycobacterium aurum* L1". *Applied and Environmental Microbiology.* 58:1220-1226.
- Heald, S. and R.O. Jenkins. 1994. "Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *pseudomonas putida*". *Applied and Environmental Microbiology.* 60:4634-4637.
- Higgins, I.J., D.J. Best and R.C. Hammond. 1980. "New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential.". *Nature.* 286:561-564.
- 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 primary substrates". *Environ. Sci. Technol.* 29:1628-1637.
- Horvarth, R.S. 1972. "Microbial co-metabolism and the degradation of organic compounds in nature". *Bacteriol. Rev.* 36:146-155.
- Hulbert, M.H. and S. Krawiec. 1977. "Cometabolism: A critique". *J. Theor. Biol.* 69:287-291.
- 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.

- Hyman, M.R. and P.M. Wood. 1985. "Suicidal inactivation and labelling of ammonia monooxygenase by acetylene". *Biochem. J.* 227:719-725.
- Jensen, H.L. 1957. "Decomposition of chloro-substituted aliphatic acids by soil bacteria". *Can. J. Microbiol.* 3:151-164.
- Jensen, H.L. 1963. "Carbon nutrition of some microorganisms decomposing halogen-substituted aliphatic acids". *Acta. Agric. Scand.* 13:404-412.
- Keener, W.K. and D.J. Arp. 1993. "Kinetic studies of ammonia monooxygenase inhibition in *Nitrosomonas europaea* by hydrocarbons and halogenated hydrocarbons in an optimized Whole-Cell assay". *Applied and Environmental Microbiology.* 59:2501-2510.
- Klotz, M.G., J. Alzerreca and J.M. Norton. 1997. "A gene encoding a membrane protein exists upstream of the *amoA/amoB* genes in ammonia oxidizing bacteria: a third member of the *amo* operon?". *FEMS Microbiol. Letters.* 150:65-73.
- 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". *Applied and Environmental Microbiology.* 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.
- Leadbetter, E.R. and J.W. Foster. 1959. "Oxidation products formed from gaseous alkanes by the bacterium *Pseudomonas methanica*". *Arch. Biochem. Biophys.* 82:491-492.
- Leadbetter, E.R. and J.W. Foster. 1960. "Bacterial oxidation of gaseous alkanes". *Arch. Mikrobiol.* 35:92-104.
- 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". *Biochemical and Biophysical Research Communications.* 185:443-451.
- Lipscomb, J.D. 1994. "Biochemistry of the soluble methane monooxygenase". *Annual Review of Microbiology.* 48:371-399.

- Mars, A.E., J. Houwing, J. Dolfig 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.
- 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.* 61:3479-3481.
- McTavish, H., J.A. Fuchs and A.B. Hooper. 1993. "Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*". *J. Bacteriol.* 175:2436-2444.
- Murrell, J.C. 1992. "Genetics and Molecular Biology of Methanotrophs". *FEMS Microbiology Reviews.* 88:233-248.
- Nesheim, J.C. and J.D. Lipscomb. 1996. "Large isotope effects in methane oxidation catalyzed by methane monooxygenase: Evidence for C-H bond cleavage in a reaction cycle intermediate". *Biochemistry.* 35:10240-10247.
- Newman, L.M. and L.P. Wackett. 1991. "Fate of 2,2,2-Trichloroacetaldehyde (Chloral Hydrate) Produced During Trichloroethylene Oxidation by Methanotrophs". *Applied and Environmental Microbiology.* 57:2399-2402.
- 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.
- Nguyen, H.-H.T., S.J. Elliott, J.H.-K. Yip and S.I. Chan. 1998. "The particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a novel copper-containing three-subunit enzyme: Isolation and characterization". *J. Biol. Chem.* 273:7957-7966.
- Nguyen, H.H.T., A.K. Shiemke, S.J. Jacobs, B.J. Hales, M.E. Lidstrom and S.I. Chan. 1994. "The nature of the copper ions in the membranes containing the particulate methane monooxygenase from *methylococcus capsulatus* (Bath)". *Journal of Biological Chemistry.* 269:14995-15005.

- Nordlund, P., H. Dalton and H. Eklund. 1992. "The active site structure of methane monooxygenase is closely related to the binuclear iron center of ribonucleotide reductase". *FEBS Letters*. 307:257-262.
- 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.* 55:2819-2826.
- Oldenhuis, R., R.L.J.M. Vink, D.B. Janssen and 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 pickettii* PKO1". *J. Bacteriol.* 176:3749-3756.
- Pikus, J.D., J.M. Studts, C. Achim, K.E. Kauffmann, 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.
- Prior, S.D. and H. Dalton. 1985. "Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath)". *FEMS Microbiol. Lett.* 29:105-109.
- 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". *Applied and Environmental Microbiology*. 57:2986-2994.
- Robertson, J.B., J.C. Spain, J.D. Haddock and D.T. Gibson. 1992. "Oxidation of Nitrotoluenes by Toluene Dioxygenase - Evidence for a Monooxygenase Reaction". *Applied and Environmental Microbiology*. 58:2643-2648.

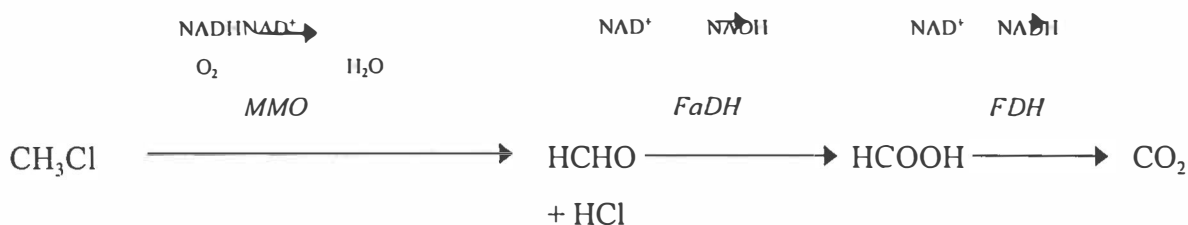
- Rosenzweig, A.C., C.A. Frederick, S.J. Lippard and P. Nordlund. 1993. "Crystal structure of a bacterial Non-Haem iron hydroxylase that catalyses the biological oxidation of methane". *Nature*. 366:537-543.
- Semrau, J.D., A. Chistoserdov, J. Lebron, A. Costello, J. Davagnino, E. Kenna, A.J. Holmes, R. Finch, J.C. Murrell and M.E. Lidstrom. 1995. "Particulate methane monooxygenase genes in methanotrophs". *J. Bacteriol.* 177:3071-3079.
- Shanklin, J., E. Whittle and B.G. Fox. 1994. "Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase. and are conserved in alkane hydroxylase and xylene monooxygenase". *Biochemistry*. 33:12787-12794.
- 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". *Applied and Environmental Microbiology*. 57:1935-1941.
- Small, F.J. and S.A. Ensign. 1997. "Alkene monooxygenase from *Xanthobacter* strain Py2". *J. Biol. Chem.* 272:24913-24920.
- Spain, J.C., G.J. Zylstra, C.K. Blake and D.T. Gibson. 1989. "Monohydroxylation of phenol and 2,5-dichlorophenol by toluene dioxygenase in *Pseudomonas putida* F1". *Appl. Environ. Microbiol.* 55:2648-2652.
- Stanley, S.H., D.J. Prior, D.J. Leak and H. Dalton. 1983. "Copper stress underlines the fundamental change in intracellular location of methane mono-oxygenase in methane utilizing organisms: studies in batch and continuous cultures". *Biotechnol. Lett.* 5:487-492.
- Stirling, D.I. and H. Dalton. 1979. "Fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath) FEMS". *Microbiol. Lett.* 5:315-318.
- Subramanian, V., T.-N. Liu, W.-K. Yeh, M. Narro and D.T. Gibson. 1981. "Purification and properties of NADH-Ferredoxin_{TOL} Reductase: A component of toluene dioxygenase from *Pseudomonas putida*". *J. Biol. Chem.* 256:2723-2730.
- Subramanian, V., T.-N. Liu, W.-K. Yeh, M. Narro and D.T. Gibson. 1979. "Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography". *Biochem. Biophys. Res. Commun.* 91:1131-1139.

- Subramanian, V., T.-N. Liu, W.K. Yeh, C.M. Serdar, L.P. Wackett and D.T. Gibson. 1985. "Purification and properties of Ferredoxin_{TOL}: A component of toluene dioxygenase from *Pseudomonas putida* F1". *J. Biol. Chem.* 260:2355-2363.
- Suzuki, M., T. Hayakawa, Shaw, J.P., M. Rekik and S. Harayama. 1991. "Primary structure of xylene monooxygenase: Similarities to and differences from the alkane hydroxylation system". *J. Bacteriol.* 173:1690-1695.
- Tsein, H.-C., G.A. Brusseau, R.S. Hanson and L.P. Wackett. 1989. "Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b". *Appl. Environ. Microbiol.* 55:3155-3161.
- Uchiyama, H., T. Nakajima, O. Yagi and T. Nakahara. 1992. "Role of Heterotrophic Bacteria in Complete Mineralization of Trichloroethylene by *Methylocystis* Sp Strain-M". *Applied and Environmental Microbiology.* 58:3067-3071.
- van Hylckama Vlieg, J.E.T., W. deKoning and D.B. Janssen. 1996. "Transformation kinetics of chlorinated ethenes by *Methylosinus trichosporium*OB3b and detection of unstable epoxides by on-line gas chromatography". *Appl. Environ. Microbiol.* 62:3304-3312.
- Vandenwijngaard, A.J., K.W.H.J. Vanderkamp, J. Vanderploeg, F. Pries, B. Kazemier and D.B. Janssen. 1992. "Degradation of 1,2-Dichloroethane by *Ancylobacter aquaticus* and Other Facultative Methylophiles". *Applied and Environmental Microbiology.* 58:976-983.
- Vannelli, T., A. Studer, M. Kertesz and T. Leisinger. 1998. "Chloromethane metabolism by *Methylobacterium* sp. Strain CM4". *Appl. Environ. Microbiol.* 64:1933-1936.
- Wackett, L.P. 1996. "Co-metabolism: Is the emperor wearing any clothes?". *Curr. Opinion Biotech.* 7:321-325.
- Wackett, L.P., G.A. Brusseau, S.R. Householder and R.S. Hanson. 1989. "Survey of microbial oxygenases: Trichloroethylene degradation by propane oxidizing bacteria". *Appl. Environ. Microbiol.* 55:2960-2964.
- 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.
- Wackett, L.P., L.D. Kwart and D.T. Gibson. 1988. "Benzylic monooxygenation catalyzed by toluene dioxygenase from *Pseudomonas putida*". *Biochemistry.* 27:1360-1367.

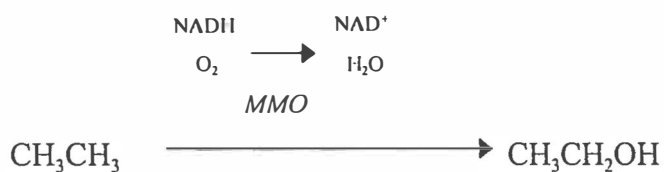
- Wackett, L.P., M.J. Sadowsky, L.M. Newman, H.G. Hur and S.Y. Li. 1994. "Metabolism of polyhalogenated compounds by a genetically engineered bacterium". *Nature*. 368:627-629.
- Whited, G.M. and D.T. Gibson. 1991. "Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* KR1". *J. Bacteriol.* 173:3010-3016.
- Wilkins, P.C., H. Dalton, I.D. Podmore, N. Deighton and M.C.R. Symons. 1992. "Biological methane activation involves the intermediacy of Carbon-Centered radicals". *European Journal of Biochemistry*. 210:67-72.
- 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.
- Zahn, J.A., D.M. Arciero, A.M. Hooper and A.A. DiSpirito. 1996. "Evidence for an iron center in the ammonia monooxygenase from *Nitrosomonas europaea*". *FEBS Letters*. 397:35-38.
- Zahn, J.A. and A. DiSpirito. 1996. "Membrane-associated methane monooxygenase from *Methylococcus capsulatus* (Bath)". *J. Bacteriol.* 178:1018-1029.
- 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.* 264:14940-14946.
- Zylstra, G.J., L.P. Wackett and D.T. Gibson. 1989. "Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes". *Appl. Environ. Microbiol.* 55:3162-3166.

$$\begin{array}{ccccccc}
 \text{NADH} \rightarrow \text{NAD}^+ & & \text{NAD}^+ & & \text{NADH} \rightarrow & & \text{NAD}^+ \\
 \text{O}_2 \longrightarrow & & \text{H}_2\text{O} & & & & \\
 \text{MMO} & & & & \text{MDH} & & \\
 \text{CH}_4 \longrightarrow & \text{CH}_3\text{OH} & \longrightarrow & \text{HCHO} & \longrightarrow & \text{HCOOH} & \longrightarrow \text{CO}_2 \\
 & & & & & & \text{FaDH} & & \text{FDH}
 \end{array}$$

Conventional Methane Oxidation



Fortuitous Oxidation



Scheme 1. Conventional methane oxidation, fortuitous oxidation, and cometabolism by methanotrophs. MMO, methane monooxygenase; MDH, methanol dehydrogenase; FaDH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

Table 1: Examples of bacteria which carry out cometabolic transformation of TCE when grown on the indicated substrates

Growth Substrate	Representative Microorganism	Oxygenase Name and Acronym	Ref
Ammonia	<i>Nitrosomonas europaea</i>	ammonia monooxygenase (AMO)	(Arciero, et al., 1989; Rasche, et al., 1991)
Butane	<i>Pseudomonas butanovorans</i>	butane monooxygenase (BMO)	(Hamamura, et al., 1997)
2,4-Dichloro phenoxyacetate	<i>Alcaligenes eutrophus</i> JMP	dichlorophenol monooxygenase	(Harker and Kim, 1990)
Ethylene/ Propylene	<i>Xanthobacter</i> Py2	alkene monooxygenase (AlkMO)	(Ensign, et al., 1992)
Methane	<i>Methylosinus trichosporium</i> OB3b	soluble methane monooxygenase (sMMO)	(Fox, et al., 1990; Oldenhuis, et al., 1989; Tsein, et al., 1989)
Methane	<i>Methylosinus trichosporium</i> OB3b	particulate methane monooxygenase (pMMO)	(DiSpirito, et al., 1992)
Propane	<i>Mycobacterium vaccae</i> JOB5	propane monooxygenase (PMO)	(Wackett, et al., 1989)
Toluene	<i>Burkholderia cepacia</i> G4	toluene-2-monooxygenase (T2MO)	(Newman and Wackett, 1997; Shields, et al., 1991)
Toluene	<i>Pseudomonas mendocina</i> KR1	toluene-3-monooxygenase (T3MO)	(Olsen, et al., 1994)
Toluene	<i>Pseudomonas picketti</i>	toluene-4-monooxygenase	(Winter, et al.,

	PK01	(T4MO)	1989)
Toluene	<i>Pseudomonas putida</i> F1	toluene dioxygenase (TDO)	(Heald and Jenkins, 1994; Zylstra, et al., 1989)

Table 12: Susceptibility of chlorinated aliphatic hydrocarbons to biotransformations

Compound	Structure	Abbreviation	Metabolism	Cometabolism	Growth Substrates
chloromethane	CH ₃ Cl	CM	Y	Y	A,M
methylene chloride	CH ₂ Cl ₂	DCM	Y	Y	A,M
chloroform	CHCl ₃	CF	N	Y	A,B,M
carbon tetrachloride	CCl ₄	CT	N	N	-
chloroethane	CH ₃ CH ₂ Cl	CA	Y	Y	A,M
1,1-dichloroethane	CH ₃ CHCl ₂	1,1-DCA	Y	Y	A,M
1,2-dichloroethane	CH ₂ ClCH ₂ Cl	1,2-DCA	Y	Y	A,C,M,T
1,1,1-trichloroethane	CCl ₃ CH ₃	1,1,1-TCA	N	Y	A,M
1,1,2-trichloroethane	CHCl ₂ CH ₂ Cl	1,1,2-TCA	N	Y	A,C
1,1,1,2-tetrachloroethane	CCl ₃ CH ₂ Cl	1,1,2-TCA	N	Y	A
1,1,2,2-tetrachloroethane	CHCl ₂ CHCl ₂	1,1,2,2-TCA	N	N	-
pentachloroethane	CCl ₃ CHCl ₂	PCA	N	N	-
hexachloroethane	CCl ₃ CCl ₃	HCA	N	N	-
vinyl chloride	CH ₂ =CHCl	VC	Y	Y	A,B,C,M,Pr,Py
1,1-dichloroethylene	CCl ₂ =CH ₂	1,1-DCE	N	Y	A,C,M,Pr,T,V

1,2-dichloroethylene (cis)	$\text{CHCl}=\text{CHCl}$	cis 1,2-DCE	N	Y	A,B,C,M,Pr, Py,T,V
1,2-dichloroethylene (trans)	$\text{CHCl}=\text{CHCl}$	trans 1,2-DCE	N	Y	A,C,M,Py,T,V
trichloroethylene	$\text{CHCl}=\text{CCl}_2$	TCE	N	Y	A,B,C,D,I,M,Ph, Pr,Py,T
tetrachloroethylene	$\text{CCl}_2=\text{CCl}_2$	PCE	N	N	-

Metabolism: Y, yes (compound will serve as growth substrate); N, no (compound will not serve as growth substrate).

Cometabolism: Y, yes (compound transformed through cometabolic processes); N, no (compound not transformed through cometabolic processes).

Growth substrates which support cometabolic transformations: A, ammonia; M, methane; B, butane; C, chloroethane; T, toluene; Pr, propane; Py, propylene; V, vinyl chloride; D, 2,4-dichlorophenoxyacetic acid; I, isoprene; Ph, phenol.

Table 3: Properties of enzymes which cooxidize chlorinated solvents

Enzyme	Protein components	Protein Molecular Weight	Protein structures	Subunit sizes (kilodaltons)	Prosthetic groups	Activity ¹	References
T2MO	Hydroxylase	211,000	$\alpha_2\beta_2\gamma_2$	55, 38, 14	2[Fe•Fe]	27	(Newman and Wackett, 1995)
	Reductase	40,000	α_1	40	FAD, [2Fe2S]	141; 512	“
	Small Component	19,000	α_2	19	-	79	“
T4MO	Hydroxylase	220,000	$\alpha_2\beta_2\gamma_2$	55; 35; 10	2[Fe•Fe]	257	(Pikus, et al., 1996)
	Rieske-type ferredoxin	14,000	α_1	14	[2Fe2S]	14,270	“
	Effector protein	11,600	α_1	11.6	-	ND	“
	Reductase	ND	ND	ND	ND	23,892	“
TDO	Dioxygenase			51, 22	Fe, [2Fe2S]	7.5	(Subramanian, et al., 1979)
	Ferredoxin	12,000	α_1	12	[2Fe2S]	17,800	(Subramanian, et al., 1985)
	Reductase	46,000	α_1	46	FAD	12,300	(Subramanian, et al., 1981)
AlkMO	Oxygenase	195,000	$\alpha_2\beta_2\gamma_2$	53; 43; 6	2[Fe•Fe]	240	(Small and Ensign, 1997)

	Reductase	35,000	α_1	35	FAD, [2Fe2S]	30,030	“
	Small Protein	15,000	α_1	15	-	4,257	“
	Ferredoxin	26,000	α_2	13,3	2[2Fe2S]	2,249	“
sMMO	Hydroxylase	245,000	$\alpha_2\beta_2\gamma_2$	61; 45; 20	2[FeFe]	1,700	(Fox, et al., 1989; Lipscomb, 1994)
	Component B	15,800	α_1	15.8	-	11,200	“
	Reductase	38,400	α_1	38.4	FAD, [2Fe2S]	26,100	“
pMMO	Oxygenase	99,000	$\alpha_1\beta_1\gamma_1$	47; 27; 25	Cu, Fe (?)	11; 5	(Nguyen, et al., 1998; Nguyen, et al., 1994; Zahn and DiSpirito, 1996)
AMO	Oxygenase	100,000	$\alpha_1\beta_1\gamma_1$	40; 35; 27	Cu (?), Fe (?)	ND	(Ensign, et al., 1993; Klotz, et al., 1997; McTavish, et al., 1993; Zahn, et al., 1996)

Table 4: Comparison of Cellular Abundance and Catalytic Activity Towards TCE and the Preferred Substrates for sMMO and T2MO

Hydroxylase Component	(% soluble cell protein)	nmol TCE oxidized/ (min x mg)	K_m for TCE (μ M)	nmol CH ₄ or toluene oxidized/ (min x mg)	K_m for growth substrate (μ M)
sMMO	12	682	35	1700	25
T2MO	14	37	12, 6 ¹	27	25 ¹

¹Kinetic values obtained with intact cells, all other values refer to the purified hydroxylase component.

References: (Fox, et al., 1990; Fox, et al., 1989; Landa, et al., 1994; Newman and Wackett, 1995; Newman and Wackett, 1997)