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Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene

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Abstract

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 aerobic cometabolism of chlorinated solvents, with a particular emphasis on the cometabolism of trichloroethylene. Monooxygenases or dioxygenases with relaxed substrate ranges initiate these transformations. The physiological role of the oxygenases is to initiate the metabolism of growth-supporting substrates (e.g., methane, propane, butane, toluene, ethylene, and ammonia). Diverse enzymes catalyze these oxidative reactions with chlorinated solvents. Synthesis of most of these enzymes is induced by the presence of the growth-supporting substrate and is largely regulated at the level of gene transcription. The genes that code for a given oxygenase are usually clustered together in a single operon and often share homology with counterparts that code for the subunits of related oxygenases in other bacteria. During cometabolism the growth-supporting and non-growth-supporting substrates can both bind to the oxygenase. 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 that are expelled from the cell, and in a few cases the cells utilize the products. The combined effects of cometabolism can have a profound influence on a cell.

Introduction

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 aerobic cometabolism as well as a discussion of the origin of this term are described in this review. Where an environmental pollutant does not support microbial growth, aerobic cometabolism offers a biological method for the removal of the pollutant from the contaminated environment. This approach to bioremediation is particularly appropriate for 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 can be addressed in part through a thorough understanding of the molecular and cellular fundamentals that make aerobic cometabolism possible. Our focus will be on the bacteria that catalyze aerobic cometabolic transformations of chlorinated aliphatic hydrocarbons (CAHs). We have placed our primary focus on trichloroethylene (TCE) as an example of a chlorinated solvent. As TCE is one of the most commonly encountered organic pollutants found in water destined for human consumption this compound has received a great deal of attention and provides a rich data set for understanding the physiological effects of cometabolism. Against this backdrop, we also include references to other CAHs. Cometabolism is not limited to bacterial systems, aerobic processes, or chlorinated solvents. Where appropriate, we will include reference to cometabolism in

other contexts. With regard to aerobic cometabolism of chlorinated solvents, we will summarize the current level of understanding of the process, identify gaps in that understanding, and where possible identify unifying themes. In doing so, we will consider those systems that 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.

We examine the definition of cometabolism and the role of individual enzymes in this process. In aerobic cometabolism of CAHs, oxygenases initiate the oxidation process. Therefore, we summarize the roles and mechanisms of oxygenases and then present in some detail the properties of those monooxygenases that have, to date, shown the greatest potential for cometabolism of chlorinated solvents. In many cases, the genes that code for the oxygenases responsible for initiating aerobic cometabolism have been identified. These genes, their regulation, and the potential for improving cometabolism through genetic engineering are also discussed. The oxidation of CAHs also presents the cell with a new set of metabolites. The result of this enzyme action on the physiology of the cell is also considered.

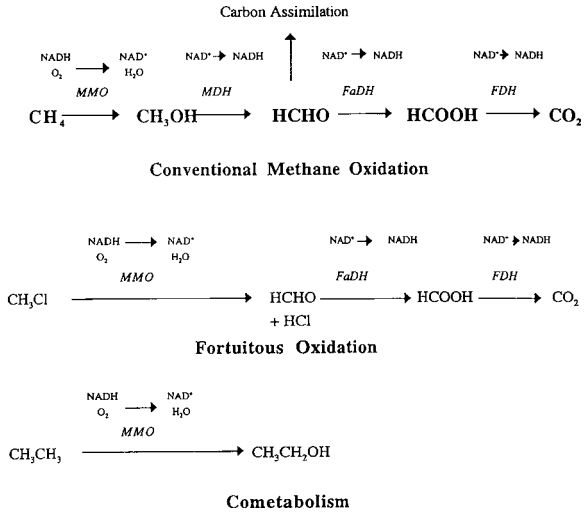
Background

Historical perspective

Cometabolic processes were first studied in the 1950s and 60s and focused on the microbial degradation of important classes of industrial chemicals including aromatics (Dagley and Pate, 1957), chlorinated organics (Jensen, 1957, 1963), pesticides (Alexander, 1967) and petroleum hydrocarbons (Foster, 1962). Collectively, these studies established that microorganisms could transform many compounds without concurrent microbial growth. Subsequent research has focused on identifying the role of cometabolism in the degradation of pollutant compounds in the environment and the mechanism and physiological basis for these reactions. These issues remain timely given 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, 1960). These studies initially revealed that the methane-utilizing bacterium *Pseudomonas* (*Methylomonas*) *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 compounds and degradation reactions beyond oxidative transformations of alkanes and closely related hydrocarbons. 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. The same principles are being applied for the remediation of chlorinated solvents some 30 years later.

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. 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 substitute 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. Compounds in the first class were oxidized without the need for additional substrates. This process was described by Stirling and Dalton 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 fortuitous oxidation and cometabolism rests on the fate



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

of the products. In fortuitous oxidation, the substrates are oxidized by methane monooxygenase (MMO) to products such as methanol (CH_3OH) and formaldehyde (HCHO), which provide energy when further oxidized by enzymes associated with the methane oxidation pathway. These products could therefore be used by the bacterium to generate the reductant necessary to support continued MMO activity. In contrast, the products of cometabolism were not subject to further oxidation at substantial rates and therefore the cells require an exogenous source of reductant to support MMO activity. These reactions are summarized in Scheme 1.

As this historical perspective illustrates, there are strong areas of overlap between cooxidation and cometabolism. First, the formal definitions of these terms were both derived principally from studies of methane-oxidizing bacteria. Second, given what is now known about the enzymology of methane oxidation, both definitions describe degradation processes that are initiated by a non-specific oxygenase, MMO. 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 these differences are reflected in the types of experiments used to originally define each process. Resting cell suspensions were used in the studies leading to Dalton and Stirling's definition of cometabolism and the focus was on identifying the sources of reductant utilized by resting cell suspensions to drive the transformation of non-growth-supporting substrates. 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 providing reductant to sustain MMO-catalyzed cometabolism. In contrast, Foster's work focused on co-oxidation reactions catalyzed by actively growing cultures where the growth substrate provides a source of carbon and energy to support further microbial growth as well as serving as an inducer of the enzyme activity required to transform the non-growth substrate.

While it is difficult to find a precise definition of cometabolism that applies to all circumstances, it nonetheless remains a useful term. In most cases, bacterial transformation of chlorinated solvents will not support growth or provide nutrients. Successful transformation of these compounds, therefore, relies on a process where the solvent is transformed as a consequence of another metabolic process. Cometabolism is a practical term to describe these processes.

Physiological types of TCE-degrading bacteria

A number of physiologically distinct microorganisms have been shown to cometabolically degrade TCE in axenic culture studies. Representative organisms, their primary growth substrates, and kinetic data with regards to TCE and the primary growth substrate are included in Table 1. The enzyme responsible for TCE oxidation in these organisms is also listed. The strongest theme to emerge from this summary is that all of the enzymes responsible for TCE transformation are mono- and dioxygenases that initiate the oxidation of various growth substrates. In fact, TCE-degrading organisms are frequently distinguished on the basis of a particular growth substrate needed to cause the expression of a TCE-degrading oxygenase. However, most of these organisms are capable of growth on many substrates, several of which may stimulate expression of the TCE-degrading oxygenase. Furthermore, 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 serves as 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 which initiates the oxidation of ammonia, ammonia monooxygenase (AMO), is therefore essentially constitutive. In contrast, there are organisms such as *Alcaligenes* (*Ralstonia*) *eutrophus* that have been shown to express two entirely different TCE degrading enzymes, a chromosomally-encoded phenol hydroxylase and plasmid-encoded 2,4-dichlorophenol hydroxylase (Harker and Kim, 1990). Furthermore, methane-oxidizers such as *Methylosinus trichosporium* OB3b can express two different forms of MMO (soluble and particulate), both of which are capable of TCE oxidation, albeit at substantially different rates.

Generalizations about the whole cell kinetics of TCE and growth substrate oxidation can also be drawn from the data presented in Table 1. For example, the maximal rate of TCE oxidation by most of these organisms is about 10–100 times less than the oxidation rate of the growth-supporting substrate. In contrast it is difficult to identify any patterns when comparing the half-saturation constants (K_s) of TCE to that of the growth-supporting substrates. Also, it is obvious that the measured oxidation rates and K_s for TCE can vary substantially from organism to organism, or even between studies performed with the same organism. In fact, Oldenhuis et al. reported startling differences in the kinetic properties of TCE oxidation by *M. trichosporium* OB3b depending on the growth conditions (Oldenhuis, 1991). Cells grown in batch culture exhibited a maximal TCE degradation rate of 27 nmol min^{-1} mg of $cells^{-1}$, while cells grown in continuous culture degraded TCE at 150 nmol min^{-1} mg of $cells^{-1}$. These observations underscore the importance of determining the kinetic parameters of TCE degradation case by case rather than relying on previously reported values.

Range of chlorinated solvents subject to aerobic cometabolism

Although we have focused on TCE as a representative chlorinated solvent, most of the chlorinated C_1 and C_2 hydrocarbons have been used as solvents. In general only the least halogenated members of this group of compounds can be utilized as growth-supporting carbon and energy sources by aerobic microorganisms. These compounds include chloromethane (Vannelli 1998), dichloromethane (Braus-Stromeyer, 1993; Gisi, 1998), dichloroethane (Vandenwijngaard, 1992) and vinyl chloride (Hartmans, 1985; Hartmans and Debont, 1992). However, most C_1 and C_2 chlorinated hydrocarbons are known to be cometabolically degraded by one or more of the physiologically distinct types of aerobic microorganisms listed in Table 1. The exceptions include the fully chlorinated compounds (carbon tetrachloride, and hexachloroethane) and some other highly chlorinated compounds (pentachloroethane, 1,1,2,2-tetrachloroethane). These observations are summarized in Table 2.

Review of the topic

Description of the enzymes

In this section, we review those enzymes that have shown the greatest potential to date for use in the bioremediation of chlorinated solvents via aerobic cometabolism – oxygenases. Oxygenases constitute a subset of the enzymes classified as oxidoreductases, which is one of the six major classes of enzymes. Oxygenases serve a myriad of functions in cells including biosynthesis, detoxification, and catabolism. Oxygenases catalyze the reduction of O_2 with incorporation of one (monooxygenases) or two (dioxygenases) of the O atoms into the substrate that 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 (e.g., toluene dioxygenase). Although all oxygenases have in common the need to activate O_2, the chemical mechanisms can be quite variable. The activation of O_2 to a reactive state is typically facilitated by prosthetic groups in the enzymes. Transfer of electrons from the prosthetic group in the enzyme to O_2 usually accomplishes this activation. The resulting reactive species is stabilized by coordination with the prosthetic group. In the case of monooxygenases, the activation involves the complete reduction of one of the atoms of O_2 with subsequent release of water. A remarkable variety of active site prosthetic groups have 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. The protein composition of oxygenases can be relatively simple or quite complex (e.g., four separable protein components and 6 unique

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

Growth substrate	Organism	Enzyme	TCE oxidation rate[1]	K_s for TCE (μM)	Growth substrate oxidation rate[1]	K_s for growth substrate (μM)	Reference
Ethylene/ Propylene	*Xanthobacter* Py2	Alkene monooxygenase	8.6 16–95		92 116		Ensign 1992 Reij 1995
Propylene	*Rhodococcus corallinus* B-276	Alkene monooxygenase	2.4	187	59	16	Saeki 1999
Ammonia	*Nitrosomonas europaea*	Ammonia monooxygenase	 10.9	30 9.8	 700–1700	40	Arciero 1989 Bedard 1989 Ely 1995b Hyman 1995 Rasche 1991
Phenol and 2,4-dichloro-phenoxyacetate	*Ralstonia eutropha* JMP 134	Phenol hydroxylase 2,4-Dichlorophenol hydroxylase	0.2 0.06				Harker 1990
Butane	*Pseudomonas butanavora*	Butane monooxygenase					Hamamura 1997
Propane	*Mycobacterium vaccae* JOB5	Propane monooxygenase					Wackett 1989
Methane	*Methylosinus trichosporium* OB3b	Particulate methane monooxygenase	4.1	7.9	82	8.3	DiSpirito 1992 Lontoh 1998
Methane	*Methylosinus trichosporium* OB3b	Soluble methane monooxygenase	16.6 580[2] 37.5	126 145	 726[2] 52[2]	 92 2	Joergensen 1985 Koh 1993 Oldenhuis 1989 Oldenhuis 1991 Sun 1996 Tsein 1989
Methane	*Methylomonas methanica* 68-1	Soluble methane monooxygenase	38.8	225			Koh 1993
Toluene	*Pseudomonas putida* F1	Toluene dioxygenase	8 1.8 0.5	5	 65.2		Heald 1994 Leahy 1996 Sun 1996 Wackett 1988 Zylstra 1989
Toluene	*Burkholderia cepacia* G4	Toluene 2-monooxygenase	8 10[2] 9 3	3 6 4	 140[2] 7.6	 25	Folsom 1990 Landa 1994 Leahy 1996 Shields 1991 Sun 1996
Toluene	*Pseudomonas mendocina* KR1	Toluene 4-monooxygenase	20 2.4	10	 71 2		Leahy 1996 Sun 1996 Winter 1989
Toluene	*Ralstonia pickettii* PKO1	Toluene 3-monooxygenase	2.4		11.3		Olsen 1994 Leahy 1996

[1]Oxidation rates are expressed as nmol min^{-1} mg of $protein^{-1}$.
[2]Oxidation rates were converted from reported units of nmol min^{-1} mg of dry cell $weight^{-1}$ by assuming that 50% of the dry weight of a bacterial cell is comprised of protein.

Table 2. Susceptibility of chlorinated aliphatic hydrocarbons to aerobic metabolism or cometabolism

Compound	Structure	Abbreviation	Metabolism	Cometabolism	Growth substrates
Chloromethane	CH_3Cl	CM	Y	Y	A, M
Methylene chloride	CH_2Cl_2	DCM	Y	Y	A, M
Chloroform	$CHCl_3$	CF	N	Y	A, B, M
Carbon tetrachloride	CCl_4	CT	N	N	–
Chloroethane	CH_3CH_2Cl	CA	Y	Y	A, M
1,1-Dichloroethane	CH_3CHCl_2	1,1-DCA	Y	Y	A, M
1,2-Dichloroethane	CH_2ClCH_2Cl	1,2-DCA	Y	Y	A, C, M, T
1,1,1-Trichloroethane	CCl_3CH_3	1,1,1-TCA	N	Y	A, M
1,1,2-Trichloroethane	$CHCl_2CH_2Cl$	1,1,2-TCA	N	Y	A, C
1,1,1,2-Tetrachloroethane	CCl_3CH_2Cl	1,1,2-TCA	N	Y	A
1,1,2,2-Tetrachloroethane	$CHCl_2CHCl_2$	1,1,2,2-TCA	N	N	–
Pentachloroethane	CCl_3CHCl_2	PCA	N	N	–
Hexachloroethane	CCl_3CCl_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*)	CHCl=CHCl	*cis* 1,2-DCE	N	Y	A, B, C, M, Pr, Py, T, V
1,2-Dichloroethylene (*trans*)	CHCl=CHCl	*trans* 1,2-DCE	N	Y	A, C, M, Py, T, V
Trichloroethylene	CHCl=CCl_2	TCE	N	Y	A, B, C, D, I, M, Ph, Pr, Py, T
Tetrachloroethylene	CCl_2=CCl_2	PCE	N	Y	T

Metabolism: Y, compound will serve as growth substrate; N, compound will not serve as growth substrate.
Cometabolism: Y, compound transformed through cometabolic processes; N, 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.

polypeptides). Some oxygenases are soluble while others are associated with the membrane.

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 a wide range of unwanted compounds from an organism. In other cases, the broad substrate range does not appear to 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 an energetic 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 has a faster catalytic turnover and appears to have the broader substrate range. sMMO is found only in a small subset of Type I, 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. The physical properties of these oxygenases are summarized in Table 3, while kinetic properties, where known, are listed in Table 4.

Soluble methane monooxygenase (sMMO)

Under conditions of copper limitation, a soluble methane monooxygenase is produced in certain methanotrophs (Stanley, 1983). The enzyme consists of three protein components: a regulatory protein (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 ($\alpha_2\beta_2\gamma_2$) The crystal structure (2.2 and 2.0 angstrom resolution) (Elango, 1997; Rosenzweig, 1993) confirmed

Table 3. Properties of select enzymes that oxidize chlorinated solvents

Enzyme	Protein components	Protein molecular weight	Protein structures	Subunit sizes (kDa)	Prosthetic groups	Activity[1]	References
T2MO	Hydroxylase	211,000	$\alpha_2\beta_2\gamma_2$	55; 38; 14	2[Fe · Fe]	27	Newman 1995
	Reductase	40,000	α_1	40	FAD, [2Fe2S]	141; 512	Newman 1995
	Small component	19,000	α_1	19	–	79	Newman 1995
T4MO	Hydroxylase	220,000	$\alpha_2\beta_2\gamma_2$	55; 35; 10	2[Fe · Fe]	257	Pikus 1996
	Rieske-type ferredoxin	14,000	α_1	14	[2Fe2S]	14,270	Pikus 1996
	Effector protein	11,600	α_1	11.6	–	ND	Pikus 1996
	Reductase	ND	ND	ND	ND	23,892	Pikus 1996
TDO	Dioxygenase	151,000	$\alpha_2\beta_2$	51; 22	Fe, [2Fe2S]	7.5	Subramanian 1979
	Ferredoxin	12,000	α_1	12	[2Fe2S]	17,800	Subramanian 1985
	Reductase	46,000	α_1	46	FAD	12,300	Subramanian 1981
AlkMO	Oxygenase	195,000	$\alpha_2\beta_2\gamma_2$	53; 43; 6	2[Fe · Fe]	240	Small 1997
	Reductase	35,000	α_1	35	FAD, [2Fe2S]	30,030	Small 1997
	Small protein	15,000	α_1	15	–	4,257	Small 1997
	Ferredoxin	26,000	α_2	13, 3	2[2Fe2S]	2,249	Small 1997
AMO (alkene)	Epoxygenase	95,000	$\alpha\beta$	53; 35	[Fe · Fe]	80.9	Miura 1995
	Reductase	40,000	α_1	40	FAD, [2Fe2S]	424	Miura 1995
	Coupling protein	14,000	α_1	14	–	577	Miura 1995
sMMO	Hydroxylase	245,000	$\alpha_2\beta_2\gamma_2$	61; 45; 20	2[FeFe]	1,700	Fox 1989 Lipscomb1994
	Component B	15,800	α_1	15.8	–	11,200	Lipscomb1994
	Reductase	38,400	α_1	38.4	FAD, [2Fe2S]	26,100	Lipscomb1994
pMMO	Oxygenase	99,000	$\alpha_1\beta_1\gamma_1$	47; 27; 25	Cu, Fe	11; 5	Nguyen 1994 Nguyen 1998 Zahn1996
AMO (ammonia)	Oxygenase	100,000	$\alpha_1\beta_1\gamma_1$	40; 35; 27	Cu (?), Fe (?)	ND	Ensign 1993 Klotz 1997 McTavish 1993 Zalin 1996

[1] Activity is expressed in units of nmol substrate transformed/(min × mg protein). Different preparations or different conditions were used where more than one value is indicated.

the presence of a hydroxy-bridged diiron center (the "diamond core" structure) which is similar to that found in ribonucleotide reductase (Nordlund, 1992). The α subunits of the enzyme each contain a diiron center. The reductase transfers electrons from NADH, the physiological electron donor, to the hydroxylase via FAD and an $[Fe_2S_2]$ cluster. Component B apparently serves a regulatory role. The catalytic turnover rate increases about 150 fold in the presence of Component B. The substrate range of sMMO extends well beyond the physiologically relevant methane (Green and Dalton, 1989). Alkanes are oxidized to alcohols; alkenes are oxidized to the corresponding epoxides or to enols. Several chlorinated aliphatics (including TCE), 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 chemical mechanism of sMMO has received considerable attention (e.g. (Elango, 1997; Lipscomb, 1994; Nesheim and Lipscomb, 1996; Wilkins, 1992a)). sMMO must first produce an activated O species that 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 that a single mechanism may not be used for all substrates (Keener, 1998; Wilkins, 1994).

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. Acetylene is an effective inactivator and copper selective chelators such as allylthiourea, are inhibitors (Prior and Dalton, 1985). A 27 kDa subunit becomes labeled with ^{14}C upon inactivation of the monooxygenase activity with $^{14}C_2H_2$ which suggests that this subunit contains the active site. Particulate methane monooxygenase has proven difficult to purify to homogeneity with high activity. However, this enzyme has now been purified from membranes of *M. capsulatus* (Bath) (Nguyen, 1998; Zahn and DiSpirito, 1996). The preparations contain three distinct polypeptides (45, 26, 23 kDa) and Cu.

Like it's soluble counterpart, pMMO will catalyze the oxidation of a number of alternative substrates (including TCE) (DiSpirito, 1992) although the substrate range is more limited than the soluble form of the enzyme (Burrows, 1984; Colby, 1977; Stirling, 1979). TCE is a noncompetitive inhibitor of methane oxidation in whole cells of *M. trichosporium* OB3b expressing pMMO (Lontoh, 1999). 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 the active site.

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, and distinct enzymes have been identified that catalyze each of these reactions. *Pseudomonas mendocina* KR1 toluene 4-monooxygenase (T4MO) produces *p*-cresol. *Ralstonia 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 and all, with the exception of XMO, can oxidize chlorinated solvents. To date, three of these enzymes (T2MO, T4MO, and TDO) have been purified extensively and characterized at the molecular level.

Toluene 2-monooxygenase

Toluene 2-monooxygenase has been purified from *B. cepacia* G4 (Newman and Wackett, 1995) and shown to consist of three protein components (Table 3). A 40 kDa flavin and iron-sulfur containing protein oxidizes NADH with subsequent transfer of electrons to the hydroxylase component (55 kDa) of the enzyme. A small polypeptide (10.4 kDa) is required for activity, but for unknown reasons. No prosthetic groups or metals have been detected in this component. The hydroxylase component contains the site for activation of molecular O_2 and oxidation of toluene. This hydroxylase component contains iron but no acid labile sulfur. The iron content and EPR spectra associated with this protein indicate that the active site of the enzyme contains binuclear iron clusters. *o*-Cresol, the product of toluene oxidation by T2MO, is further oxidized to 3-methyl-catechol by T2MO. Purified toluene 2-monooxygenase oxidizes TCE (Newman and Wackett, 1997) and the major reaction products are formic acid, carbon monoxide and glyoxylic acid. *In vitro*, about 12% of the TCE oxidation products become covalently attached to the enzyme polypeptides and a loss of enzyme activity occurs. In intact cells of *B. cepacia* G4, TCE is a noncompetitive inhibitor of toluene oxidation (Yeager and Arp, unpubl.) but kinetic studies with 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). As with sMMO and T2MO, a multicomponent enzyme was identified. The components of T4MO were recently purified to homogeneity from a recombinant *Escherichia coli* strain which expressed the T4MO genes isolated from *P. mendocina* KR1 (Pikus, 1996). 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 activity (T2MO does not require the ferrodoxin). Thus, while similarities

in the catalytic sites are apparent, there are distinct differences in the overall structure of these enzymes. Detailed characterizations of the kinetic properties of this purified enzyme have not been 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, 1997). T4MO can catalyze the oxidation of TCE (McClay, 1995). Toluene/*o*-xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 also oxidizes a number of CAHs including TCE (Chauhan, 1998). The deduced amino acid sequence of this enzyme is similar to that of T4MO and T3MO, although the regiospecificity of ToMO is more relaxed.

Toluene dioxygenase

In addition to the attacks on toluene by monooxygenases, toluene dioxygenase (TDO) will also initiate the degradation of toluene. Three separable protein components are required to complete the reaction (Subramanian, 1981; Subramanian, 1985; Zylstra and Gibson, 1989). $\text{Reductase}_{\text{TOL}}$, a flavoprotein, catalyzes the oxidation of NADH with subsequent transfer of electrons to a small iron sulfur protein, $\text{ferredoxin}_{\text{TOL}}$. 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. TDO is a versatile enzyme which can catalyze monooxygenation, desaturation, O-dealkylation, N dealkylation, sulfoxidation and oxidative dehalogenation (Lange and Wackett, 1997). Halogenated aliphatics that serve as substrates include TCE, l,1-dichloro-1-propene, and 1,1-dichloroethene but not tetrachloroethene.

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 oxidizes a number of chlorinated aliphatics including TCE (Ensign, 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 that 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 (one for each $\alpha\beta\gamma$ unit) based on metal content and EPR spectra.

A fundamentally different alkene monooxygenase has been purified from *Rhodococcus corallinus* (*Nocardia corallina*) B-276. In contrast to the monooxygenase from *Xanthobacter* Py2, the enzyme from *R. corallinus* B-276 is composed of three components: an epoxygenase, a 40 kDa NADH reductase containing flavin and iron-sulfur prosthetic groups, and a 14 kDa coupling protein (Miura and Dalton, 1995). The epoxygenase consists of two subunits (53 and 35 kDa) and contains a binuclear iron cluster as determined by electromagnetic resonance spectral analysis (Gallagher, 1997). Even though the alkene monooxygenase from *Xanthobacter* Py2 and *R. corollinus* B-276 are structurally distinct, the substrate ranges of these two enzymes are remarkably similar. Both enzymes catalyze the oxidation of alkenes and chlorinated alkenes, but not alkanes or their chlorinated derivatives (Ensign, 1992; Saeki, 1999).

Ammonia monooxygenase

Ammonia monooxygenase (AMO) from *N. europaea* has a remarkably broad substrate range. In addition to a number of chlorinated aliphatics, including TCE, 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, 1996; Arciero, 1989; Rasche, 1991). 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 (AMOa) that becomes covalently modified upon inactivation of ammonia oxidation by acetylene (Hyman and Wood, 1985) copurifies with a second polypeptide (AMOb) of 40 kDa (McTavish, 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 (AMOc) is suggested by genetic characterizations (Klotz, 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 deduced amino acid sequences of the polypeptides indicate a number of membrane-spanning helices in each of the

proteins. AMO is likely to contain Cu (Ensign, 1993) and may also contain Fe (Zahn, 1996). In a study with *N. europaea* of several alternative substrates of AMO that 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). While a competitive inhibition pattern was observed for some co-substrates (ethylene, methane), the predominant kinetic pattern was that indicative of noncompetitive inhibition.

Summary

With regard to the enzymes identified as being of potential use in aerobic cometabolism of chlorinated solvents, one is struck by the remarkable differences in the structures of their primary substrates – 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 emerged as systems with strong potential for aerobic bioremediation of chlorinated solvents. While the prevailing model for inhibition patterns between CAHs and growth substrates during aerobic cometabolism calls for the competitive binding of these substrates, kinetic studies of TCE oxidation by whole cells of *N. europaea*, *B. cepacia* G4, and *M. trichosporium* OB3b indicate that alternative inhibition patterns may occur (Keener and Arp, 1993; Lontoh, 1999) (Yeager and Arp, unpubl.). Since detailed kinetic analyses have not been carried out for most of the oxygenases that degrade CAHs, it remains to be seen how often the competitive inhibition model is appropriate. In spite of these differences, some similarities in the properties of these enzymes can also be noted. The primary substrates are uncharged, hydrophobic molecules. All of the enzymes have relaxed substrate ranges and include TCE and other chlorinated aliphatics. Another common feature among these enzymes is that each is at the beginning of a catabolic pathway used to harvest a growth substrate. Other monooxygenases and dioxygenases that catalyze subsequent metabolic steps 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 that catalyze their transformations, there are also some common themes.

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 was eliminated (Table 4). Purified sMMO generates glyoxylate, formate, dichloroacetate, and CO as major products of TCE oxidation with trichloroacetaldehyde (chloral) as a minor component (Fox, 1990). The addition of trichloroethylene oxide to an enzyme-free incubation leads to the same products in very similar ratios indicating that this compound non-enzymatically decomposes to generate the observed products. TCE-epoxide production by sMMO has been confirmed for the purified enzyme by trapping this compound with 4-(*p*-nitrobenzyl) pyridine (Fox 1990) and for whole cells using an on-line gas chromatographic method (van Hylckama Vlieg, 1996). sMMO also generates a small quantity of chloral, a compound that is not generated from trichloroethylene oxide. Chloral is converted to 2,2,2-trichloroethanol and trichloroacetic acid by whole cells of methane-oxidizing bacteria (Newman and Wackett, 1991). Most recently, studies with the purified toluene 2-monooxygenase from *B. cepacia* G4 (Newman and Wackett, 1997) showed that the principal products of TCE oxidation by this enzyme were CO, formate, and glyoxylate. An interesting feature of the cometabolism of TCE by monooxygenases is that one of the major products is carbon monoxide. In the case of methane- and ammonia oxidizing bacteria, CO is known to be a substrate for both MMO and AMO (Bedard and Knowles, 1989). The production of carbon monoxide from TCE potentially complicates the kinetic analysis of TCE oxidation.

The principal products generated from TCE oxidation by toluene dioxygenase from *P. putida* F1 were glyoxylate and formate, but not CO (Li and Wackett, 1992). A dioxygenation mechanism involving 1,2-dihydroxy-trichloroethane as an initial product was proposed for this enzyme (Li and Wackett, 1992). Notably this enzyme also catalyzes monooxygenation reactions including the oxidation of nitrotoluenes (Robertson, 1992), indene (Wackett, 1988), and phenol (Spain, 1989).

Table 4. Comparison of the catalytic activity towards TCE and the growth-supporting substrates for purified enzymes known to degrade TCE

Enzyme	V_{max} for TCE[1]	K_m for TCE (μM)	V_{max} for growth substrate[1]	K_m for growth substrate (μM)	Products (%)	Reference
Soluble methane monooxygenase	682	35	1700	25	CO(53) Formate(35) Chloral(16) Glyoxylate(5) Dichloroacetate(5)	Fox 1989 Fox 1990
Toluene 2-monooxygenase	37	12	27 (toluene) 131 (*o*-cresol)	0.8 (*o*-cresol)	CO(41) Formate(21) Glyoxylate(10) Dichloroacetate(<1) Trichloroacetate(<1) Chloral(<1)	Newman 1995 Newman 1997
Toluene 4-monooxygenase				10–30		Pikus 1996
Toluene dioxygenase	33		225		Formate, Glyoxylate	Lange 1997 Li 1992

Oxidation rates are expressed as nmol min^{-1} mg of $protein^{-1}$.

Genetics

A growing number of genes that code for enzymes involved in the aerobic cometabolism of chlorinated solvents have been identified. Sequence analysis has revealed that the genes coding for any given oxygenase in a bacterium tend to cluster together within the genome. In fact, these genes are often part of a single operon and are thus under the control of the same regulatory mechanism(s). Additionally, genes coding for individual components of various oxygenases from different bacteria often share sequence similarity and can be grouped together within readily discernible gene families. Once gene sequences are determined, opportunities exist to perform detailed experiments on the role and regulation of the gene and its product. Furthermore, sequence information can be used to improve the catalytic efficiency of a given enzyme or microorganism via genetic engineering, design gene probes to monitor microbial populations, and search for potential catalytic capability, even among microorganisms that are difficult to culture.

Gene stucture and organization

Several of the enzymes capable of degrading TCE including, but not limited to sMMO, T2MO, T4MO, and AikMO share structural similarity and cofactor requirements (Table 3). These monooxygenases are all part of a proposed enzymatic family characterized by the presence of a non-heme binuclear iron catalytic center in the hydroxylase subunit (Fox, 1994; Wilkins, 1992b). This similarity is also reflected in the DNA sequences coding for these enzymes. The genes coding for each of the individual subunits of a given diiron monooxygenase often show strong homology with the genes that code for the corresponding subunits in other monooxygenases in this family. Additionally, the genes encoding the individual subunits of a given monooxygenase are usually clustered together, yet gene order and the presence or absence of ancillary open reading frames within the monooxygenase gene clusters can vary between organsims.

In *Ralstonia pickettii* PKO1, the toluene 3-monooxygenase gene cluster *tbuA1UBVA2C* encodes for the α subunit of the hydroxylase (*A1*), the γ subunit of the hydroxylase (*U*), a ferredoxin (*B*), the effector protein B (*V*), the β subunit of the hydroxylase (*A*2), and an oxidoreductase (*C*) (Byrne, 1995). The toluene 4-monooxygenase gene cluster from *P. mendocina* KR1, *tmoABCDEF*, is homologous to the *tbu* locus (Yen, 1991). By comparison, the alkene monooxygenase gene cluster from *Xanthobacter* sp. Py2, *aamABCDEF*, encodes similar polypeptides to those encoded by the operons described above. The arrangement of the gene cluster that encodes the polypeptides of the three protein components of sMMO, *mmoXYBZorfYC*, seems to be highly

conserved among group I (*Methylomonas* sp. strains KSWIII and KSPIII), group II (*M. trichosporium* OB3b and *Methylocystis* sp. strain M), and group X methanotrophs (*M. capsulatus* Bath) (Murrell, 1992, Shigematsu, 1999). The gene cluster encodes the polypeptides Aα (hydroxylase subunit), Aβ (hydroxylase subunit), B (regulatory protein), Aγ (hydroxylase subunit), unknown Y, and C (reductase), respectively. As is apparent from the above examples, natural gene shuffling has led to many variations upon a central theme, allowing for the selection of evolutionarily related gene clusters which encode diiron monooxygenases suited to the nutritional and energetic needs of different microorganisms. The relative abundance of genes encoding diiron-type monooxygenases among bacterial strains or populations that are capable of degrading TCE is apparent from the rapidly increasing number of homologous DNA sequences in databases such as GenBank.

Genes that code for other classes of enzymes capable of TCE oxidation have also been identified including, the *pmo* genes from *M. capsulatus* (Bath), the *amo* genes from *N. europaea*, and the *tod* genes from *P. putida* F1. The genes for the γ, α, and β subunits of pMMO are encoded by the *pmoCAB* gene cluster (Semrau, 1995). The homologous *amoCAB* gene cluster is thought to encode the corresponding polypeptides for AMO, although physical evidence for AMOc is still lacking (Klotz, 1997; McTavish, 1993). Interestingly, the *pmoCAB* and *amoCAB* gene clusters are present in multiple copies within the genomes of methanotrophs and ammonia oxidizing bacteria, respectively. The genes that code for the protein components of TDO are arranged in the *todC1C2BADE* operon, where *C1C2* code for the dioxygenase, *B* codes for the ferredoxin, *A* codes for the reductase, and *D* and *E* code for enzymes that catalyze the further metabolism of the product of TDO (Zylstra and Gibson, 1989).

Regulation of gene expression

For aerobic cometabolism to be effective in bioremediation of CAHs, the oxygenases that initiate the degradations must be present in abundance. The induction of oxygenase synthesis occurs when the cell responds to the presence of an inducer, which is typically a substrate for the enzyme. In the presence of an appropriate effector molecule (inducer), transcription of the structural genes for the oxygenase is up-regulated resulting in a significant increase in the synthesis of the oxygenase and/or other enzymes involved in a specific catabolic pathway. Transcriptional regulation of several oxygenase operons has been extensively studied.

Two clusters of *xyl* genes from the *Pseudomonas putida* TOL plasmid code for enzymes (including xylene monooxygenase and toluate 1,2-dioxygenase) that catalyze the metabolism of toluene and xylenes. Two proteins, XylS and XylR, are known to coordinate expression of the *xyl* gene clusters (Ramos, 1997). Both XylS and XylR are synthesized constitutively at low levels. In the presence of an appropriate inducer (alkylbenzoates), XylS is activated and stimulates the transcription of the lower *xyl* gene cluster from a σ^{70}-type promoter. XylR stimulates transcription of the upper *xyl* gene cluster when bound to a wide range of aromatic effector molecules. XylR, which belongs to the conserved NtrC family of regulatory proteins, has four distinct domains. The A domain interacts specifically with aromatic inducers, the B domain links the C and A domains, the C domain is involved in ATP hydrolysis (which is required for induction), and the D domain contains a helix-turn-helix DNA binding motif. Apparently, XylR binds to DNA via the D domain in a non-active form. Upon the binding of an aromatic inducer to the A domain, XylR interacts with a σ^{54}-type RNA polymerase (most likely via the C domain) at the promoter of the upper *xyl* gene cluster, thus stimulating transcription. An important feature of NtrC-like regulatory systems is that the growth substrate range of a particular bacterial strain may be limited by the specificity of the transcriptional regulator for effector molecules rather than the actual substrate specificity of the catabolic oxygenases (Ng, 1995). Furthermore, it is possible to alter the range of compounds that serve as effector molecules for XylR-type transcriptional activators by modifying the A domain of these proteins (Delgado and Ramos, 1994; Pavel, 1994).

Although the *xyl* genes do not encode enzymes capable of oxidizing TCE, similar regulatory systems are involved in the expression of genes that do code for TCE-degrading oxygenases. For example, TbuT and TouR both belong to the XylR/NtrC regulator family and positively control transcription of the genes encoding two enzymes known to degrade TCE, toluene 3-monooxygenase and toluene-*o*-xylene monooxygenase, respectively (Arenghi, 1999; Byrne and Olsen, 1996). Genes that share homology with TbuT, TouR, and XylR have been found clustered with genes encoding other monooxygenases capable of oxidizing TCE in at least five other bacteria. In contrast.

it has been suggested that transcription of the genes that encode the TCE-degrading enzyme, isopropylbenzene dioxygenase, in *Pseudomonas* sp. strain JR1 is controlled by a regulatory protein similar to XylS (Berendes, 1998).

Less is known about the regulation of the sMMO or pMMO structural genes. In *M. capsulatus* (Bath) *mmoX* is preceded by sequences homologous to known σ^{54}- and σ^{70}-type promoters (Murrell, 1992). Whereas in *M. trichosporium* OB3b, sMMO gene expression appears to be directed from a σ^{54}-type promoter directing transcription of *mmoX* and a σ^{70}-type promoter directing transcription of genes downstream of *mmoX* (Nielson, 1997). Recent evidence suggests that expression of the *pmoCAB* gene cluster in *M. trichosporium* OB3b is directed from a σ^{70}-type promoter (Gilbert, 2000). Copper plays a significant role in the regulation of sMMO and pMMO synthesis. In *M. trichosporium* OB3b and *M. capsulatus* (Bath) copper represses transcription of the *mmo* gene cluster and activates transcription of *pmo* genes (Nielson, 1996, 1997). Since sMMO has a wider substrate range and generally exhibits higher activity than pMMO, it is desirable to determine methods of expressing sMMO regardless of the copper concentration.

In each of the organisms described above, the synthesis of TCE-degrading oxygenases requires the presence of a specific inducer, which is usually a physiological substrate for the oxygenase. Recently, there have been several reports of TCE-mediated induction of oxygenases capable of TCE degradation (Heald and Jenkins, 1994; Leahy, 1996; McClay, 1995). Although TCE seems to have a direct effect on the transcription of oxygenase genes in certain microorganisms, the molecular mechanism is unknown and oxygenase activity is substantially lower in cells induced with TCE than those induced with a physiological substrate for the enzyme. However, it was recently reported that glucose- and propene-grown cells of *R. corallinus* B-276 are both capable of efficient TCE degradation, and furthermore, that the alkene monooxygenase responsible for TCE oxidation in this strain is expressed constitutively (Saeki, 1999). Additionally, a number of genetically altered strains have been developed that constitutively express TCE-degrading oxygenases. From these results it is apparent that the synthesis of TCE-degrading oxygenases need not necessarily be coupled to the presence of a physiological substrate for the enzyme. This approach could eliminate the complicating effects of growth substrate and CAH competition for the same oxygenase, which is discussed in greater detail later in this review.

In addition to the specific regulatory control exerted on oxygenase synthesis by effector molecules and their corresponding transcriptional activators, transcription of oxygenase operons is also dependent on more global control mechanisms. It has been shown that the physiological growth status of the cell and/or the presence of alternative growth substates can influence the transcription of oxygenase genes (Ampe, 1998; Ayoubi and Harker, 1998; Duetz, 1996; Sze and Shingler, 1999; Yuste, 1998). Although the molecular basis of this phenomenom is largely unknown, it has been found that synthesis of the catabolic oxygenases is reduced if a rapidly metabolizable carbon source, other than the inducer, is available. Obviously, the presence of these global control mechanisms will need to be considered when selecting a particular carbon source for biostimulation or bioaugmentation efforts.

Genetic engineering

With the genes in hand, applications of genetic engineering also become feasible. For example, new pathways can be engineered by transforming a bacterium with a gene or genes from another bacterium. Wackett et al. (Wackett, 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, an activity of which neither enzyme alone was capable. In another study, *E. coli* cells harboring dimethyl sulfide (DMS) monooxygenase genes and isopropylbenzene dioxygenase genes degraded up to 88% of the TCE in an assay, while *E. coli* cells containing the DMS monooxygenase genes or the isopropylbenzene dioxygenase genes singly degraded 50% and 75% of the TCE, respectively (Takami, 1999). Several genes are required to code for a single monooxygenase and the genes from one bacterium are often similar to corresponding genes from another bacterium. Therefore, it is sometimes possible to create new monooxygenases with mixtures of genes. Specific oxygenase genes from one bacterium are used to replace the corresponding genes in the host. For example, recombinant *E. coli* cells with high rates of TCE degradation were produced by introducing specific *tod* and *bph* genes (for biphenyl dioxygenase) (Furukawa, 1994).

Genetic engineering also offers the prospect of "improving' an enzyme for bioremediation by sub-

stituting one or more amino acids at specific sites. Improvements could include an enhanced substrate range, improved affinities for co-substrates, or increased stability of the enzymes. The feasibility of altering the substrate range of a monooxygenase was demonstrated with T4MO. *Pseudomonas mendocina* KR1 toluene 4-monooxygenase catalyzes the ring hydroxylation of toluene to *p*-cresol predominantly (Pikus, 1997). Additionally, this monooxygenase catalyzes the oxidation of *p*-xylene to 4-methyl benzyl alcohol (82%) and 2,5-dimethyl phenol (18%). Substitution of the glutamine residue at position 141 with a cysteine resulted in an isoform with an altered regiospecificity. The predominant product became 2,5-dimethylphenol (78%). However, this isoform had a k_{cat} for toluene oxidation approximately one tenth that of the wild-type enzyme.

An alternative approach in designing improved strains for *in situ* TCE bioremediation is to introduce the appropriate catabolic genes into strains that are more "ecologically fit" for a particular target environment. For example, a TCE-degrading, rhizosphere-competent bacterial strain was constructed by inserting the T2MO genes from a derivative of *B. cepacia* G4 into *Pseudomonas fluorescens* 2-79 (Yee, 1998). The engineered strain was capable of colonizing wheat roots while constitutively degrading TCE in microcosm studies. This strategy, or rhizoremediation, is advantageous in that plant roots provide a significant surface area for bacterial colonization, they can provide a continuous supply of nutrients for microbial growth, and root growth can enhance the transport of bacteria through a soil matrix. *Deinococcus radiourans* has also received attention as an organism that is capable of degrading contaminants within a specialized niche. This bacterium is known to possess extraordinary DNA repair capabilites, and can withstand high doses of radiation. Consequently, researchers are exploring the possibility that this organism can be used to degrade aromatic and halogenated pollutants in sites co-contaminated with high levels of nuclear waste. Toluene dioxygenase from *P. putida* F1 has already been successfully expressed in *D. radiourans* (Lange, 1998). The strain effectively degraded toluene in a highly irradiating environment; however, the TCE degrading activity was low, possibly due to enzyme inactivation.

Physiological effects of cometabolism

In this section we will briefly consider some of the broader physiological aspects of the aerobic cometabolism of chlorinated solvents, again with an emphasis on TCE. The following sections will consider: (1) the possible contributions of cometabolism to cell metabolism; (2) oxygenase abundance; (3) the reversible, inhibitory effects associated with TCE oxidation; and (4) the irreversible or toxic cellular consequences associated with chlorinated solvent cometabolism.

Possible contributions of cometabolism to cell metabolism

Early studies of cometabolism differentiated between non-growth-supporting substrates, which are only partially oxidized 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 is no assimilation of the reaction products and no contribution to the energy needs of the cell. As noted by Hulbert and Krawiec (Hulbert and Krawiec, 1977), 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 possibility from studies with autotrophic ammonia-oxidizing bacteria where it has been established that ^{14}C -label accumulates in cells during the cometabolic oxidation of $^{14}CH_4$ and ^{14}CO (Bedard and Knowles, 1989). However, the carbon from these substrates could be fixed either conventionally via CO_2 derived from CO oxidation or through the nonenzymatic reaction of formaldehyde, which is produced during methane oxidation, with cellular constituents such as proteins and nucleic acids. The possibility that microorganisms use cometabolism as a source of supplementary carbon under oligotrophic conditions led Higgins and coworkers (Higgins, 1980) to suggest that this beneficial effect of cometabolism might explain why methane-oxidizing bacteria have such a non-specific 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 (De Klerk 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, 1992). Alexander summarized other examples of these types of synergistic effects and has suggested that they are important factors influencing the degradation rate 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).

Oxygenase abundance

For applied purposes it is advantageous that the degradative oxygenases have high levels of activity in whole cells, which 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 oxidation pathway of a growth substrate and when 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 best characterized of the TCE-degrading oxygenases. As shown in Table 4, the hydroxylase components of soluble MMO and toluene 2-monooxygenase have specific activities and affinities towards TCE that are comparable with those of the growth substrates, methane and toluene. Furthermore, sMMO and toluene 2-monooxygenase make up 12 and 14% of the soluble cell protein in *M. trichosporium* OB3b and *B. cepacia* G4, respectively (Fox, 1989; Newman and Wackett, 1995). The list of oxygenases that are capable of oxidizing chlorinated solvents is likely to continue to grow. However, if these enzymes are to compete with those already described, they will need to be constitutively expressed or induced by inexpensive compounds. Likewise, the enzymes will need to be abundant in the cell. Oxygenases with roles in biosynthetic processes or secondary metabolism are not likely to be as abundant as those oxygenases with roles in the metabolism of growth-supporting substrates.

Inhibitory effects of cometabolism

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 oxygenase-catalyzed reaction and the products of this reaction are not further metabolized to replenish the expended reductant. Therefore, reductant limitations can constrain the extent of cometabolic degradation.

As discussed earlier, the induction of oxygenase synthesis typically occurs when the cell responds to the presence of an inducer, which is usually a growth substrate. 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 of high growth-substrate concentrations relative to the 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. Under these conditions maximal, sustained rates of CAH cometabolism require the careful balancing of CAH and inducer/growth substrate concentrations, a difficult task *in situ*. Thus, it is desirable to isolate or develop bacterial strains that are capable of expressing CAH-degrading oxygenases in the presence of alternative growth substrates that do not compete for the transforming oxygenase (see genetics section).

Toxic effects associated with chlorinated solvent cometabolism

The once conventional view that cometabolic reactions are inconsequential to microorganisms has been dramatically altered by the observation that chlorinated solvent oxidation often gives rise to toxic effects that not only limit the further oxidation of these compounds, but also that of the growth-supporting substrates. The toxic effects associated with the cometabolism of chlorinated solvents have been most intensively studied with TCE, and most bacteria that degrade TCE suffer toxic effects to one degree or another. These effects have been examined at both the whole cell and purified enzyme level.

One of the models used to account for the toxicity associated with the oxidation of compounds such as TCE is that this compound behaves as a mechanism-based inactivator. Indeed, purified oxygenases are inactivated to some degree as a consequence of TCE oxidation (Fox, 1990; Newman and Wackett, 1997). 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 (Fox, 1990; Li and Wackett, 1992; Newman and Wackett, 1997). Additionally, cysteine provided protection against the inactivating effects of TCE in these experiments. While these results are consistent with the idea that enzyme inactivation is caused by a diffusable and reactive TCE oxidation product, they 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 macromolecular targets available for the reactive intermediates.

In whole cells, the toxic effects of TCE oxidation can extend beyond the oxygenase. 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 are also reduced in the presence of TCE in an exposure-dependent manner (Heald and Jenkins, 1994; Wackett and Householder, 1989). TCE exposure does not hinder growth of *P. putida* F4, a mutant lacking toluene dioxygenase activity. These observations certainly support the notion that the toxic effects of TCE arise from TCE oxidation rather than simply from exposure of cells to TCE. It was also reported that TCE oxidation can damage the general respiratory activity (Chu and Alvarez-Cohen, 1999) and viability (van Hylckama Vlieg, 1997) of methanotrophs. The site of action of TCE-associated toxicity has been investigated by examining 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, including proteins, DNA, RNA, and lipids, as a result of ^{14}C-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 in methane- and ammonia oxidizing bacteria (Oldenhuis, 1991; Rasche, 1991). In the case of ammonia oxidizers it was 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, several studies with *B. cepacia* G4 indicate that toxicity related to TCE cometabolism 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, 1990). Also, steady rates of TCE degradation have been observed in bioreactors containing toluene-fed *B. cepacia* G4 (Folsom and Chapman, 1991). In contrast, ammonia oxidizers appear to be very sensitive to toxic effects associated with TCE degradation (Hyman, 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, understanding of the mechanism of toxicity is complicated by several observations that indicate that these effects are not just oxygenase 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 the TCE oxidizing activities of *P. mendocina* KR1 and a recombinant *E. coli* strain containing genes from *P. mendocina* KR1 encoding toluene 4-monooxygenase (Winter, 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 predominant, initial product of TCE

oxidation, the current kinetic evidence suggests that it is not directly responsible for the inactivating effects observed during TCE oxidation. For example, Fox et al. observed an increase in the transient level of TCE epoxide in a reaction assay containing TCE, NADH, and the purified components of sMMO from *M. trichosporium* OB3b when cysteine was included (Fox, 1990). Yet, the inactivation rate of the monooxygenase decreased under these conditions. Several investigators have proposed that acyl chlorides, which are highly electrophilic compounds, generated from the hydrolysis or rearrangement of TCE epoxide are the damaging species in monooxygenase catalyzed reactions of TCE (Fox, 1990; Oldenhuis, 1991; van Hylckama Vlieg, 1997). In support of this notion, acid hydrolysis of protein fractions from *P. putida* F1 cells that had been exposed to [^{14}C]-TCE yielded compounds that had HPLC retention times corresponding to those of glyoxylic acid and formic acid (Wackett and Householder, 1989).

The sMMO in *Methylosinus trichosporium* OB3b can oxidize the stable epoxide, *cis*-l,2-dichloroethylene oxide and this reaction gives rise to a toxic effect (van Hylckama Vlieg, 1996). This observation raises the interesting possibility that the inactivating effect of TCE could be, in part, caused by the further enzymatic oxidation of trichloroethylene oxide. This possibility is supported by several previous studies which demonstrate monooxygenase-specific inactivation reactions caused by epoxides are quite common (Habets-Crützen, 1985; Hartmans, 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.

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, 1991a; Criddle, 1993; Ely, 1995b). Toxicity has also been recognized in the concept of transformation capacities. The transformation capacity defines the mass of substrate that can be transformed by a given mass of resting cells (Alvarez-Cohen and McCarty, 1991a). Both the toxic effects of cometabolism and reductant limitation can contribute to the measured transformation capacity. However, reductant limitation can be removed as a factor by including an electron donor (e.g., formate for methanotrophs) (Alvarez-Cohen and McCarty, 1991b; Oldenhuis, 1989). In contrast, the transformation yield gives the maximum amount of CAH (or other cosubstrate) that can be transformed per mass of growth substrate utilized.

Several types of microorganisms have been shown to recover from the toxic effects associated with chlorinated solvent degradation. For example, early studies of the toxic effect of TCE cometabolism on *P. putida* F1 demonstrated that TCE-inactivated cells are able to regain their maximal growth rate after a period of time when incubated in the absence of TCE (Wackett and Householder, 1989). Recovery from TCE-mediated toxicity was more extensively examined with the nitrifying bacterium *N. europaea* (Ely, 1995a,b; Hyman, 1995). Recovery involves *de novo* protein synthesis rather than new cell growth and the rate of recovery strongly depends on the extent of inactivation. A factor for the rate of recovery was integrated into a mathematical model that accurately describes TCE cometabolism by *N. europaea* under quasi-steady state conditions (Ely, 1995a,b).

In mammalian systems, where a wealth of information exists, the mechanism of toxicity of TCE does not serve as a universal mechanism for all CAHs. Therefore, even as our understanding of the mechanism of toxicity associated with the cometabolism of TCE by bacteria grows, we should not assume that insights gained from studies of TCE with bacterial systems can be applied to all other chlorinated solvents that exhibit toxic effects on microorganisms. For example, it has been demonstrated in field studies that toluene-oxidizing organisms likely suffer a severe toxic effect during the cometabolism of 1,1-dichloroethylene (1,1-DCE) (Hopkins and McCarty, 1995). Of 63 genetically distinct, toluene oxidizing strains isolated from this field site, 78% hybridized to a T2MO gene probe and none of the strains hybridized to probes for the other four toluene oxygenase pathways (Fries, 1997). Thus, bacteria that harbor genes similar to those which code for T2MO in *B. cepacia* G4 are more susceptible to the toxic effects of 1,1-DCE cometabolism than studies of *B. cepacia* G4 with TCE would suggest. The extreme toxicity imparted upon cells during 1,1-DCE cometabolism is not limited to toluene-oxidizing bacteria as aerobic cometabolism of 1,1-DCE often leads to rapid inactivation (Dolan and McCarty, 1995; Ensign, 1992; Oldenhuis, 1991; van Hylckama Vlieg, 1997).

Our studies with *N. europaea* also suggest that the notion that the oxygenase enzyme responsible for chlorinated solvent degradation is the primary target

of toxic effects is too simple a model. 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, 1991). More recently, we have observed that upon exposure of cells to low concentrations of TCE, the loss of ammonia oxidizing activity in intact cells is not correlated with a loss of AMO or HAO activity *in vitro* (Fawcett, Williamson, Arp and Hyman, unpublished). However, when cells are exposed to higher TCE concentrations, both AMO and HAO activities are lost *in vitro*.

Discussion

As described above, cometabolism has its genesis in the relaxed substrate range of certain enzymes. A process based on relaxed substrate ranges of enzymes stands somewhat in contradiction to the notion of enzymes as catalysts with remarkable levels of selection for a particular substrate or group of related substrates. Indeed, most enzymes do have this high degree of selectivity. But relaxed substrate ranges are certainly not uncommon among enzymes, as evidenced by the monooxygenases that 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 that form the binding pocket of the enzyme are expected to be hydrophobic and must rely on Van der Waals contacts for recognition of the substrates. For a similarly shaped hydrophobic molecule, the binding energy for enzyme-substrate complex formation with a nongrowth supporting substrate will be only slightly different from that of the growth-supporting substrate, thus allowing little margin for discriminating between the substrates. This explanation readily rationalizes the binding of vinyl chloride to alkene monooxygenase, where ethylene is the growth-supporting substrate. However, it is somewhat more difficult to imagine a binding site on sMMO for methane that can also accommodate naphthalene. The crystal structure of sMMO revealed a "hydrophobic pocket" near the active site which may serve as the binding site for cosubstrates (Elango, 1997; Rosenzweig, 1993), but even with a crystal structure, the determinants of specificity are not readily apparent. Indeed, the binding sites for the growth-supporting 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 mostly 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 by the products of 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 that often accompanies cometabolism is directed at least partially at the active site of the oxygenase. 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. Each co-substrate will produce a new set of metabolites with which the cell must interact. For example, 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. 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. The increase was attributed to a toxic effect and the need to resynthesize damaged cellular components (Mars, 1996). However, these increased maintenance costs have received scant attention.

Another area requiring additional research is in the search for diagnostic substrates that are indicative of the bioremediation potential of aerobic cometabolism at a given site. Universal substrates are of

interest in screening populations or environments for TCE degrading 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. A non-chlorinated substrate that meets many of these requirements is ethylene. 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. All of the monooxygenases that have been examined are inactivated by acetylene, though the potency varies among enzymes. Specific inactivators based on higher alkynes or other mechanism-based inactivators would be helpful in determining the role of various groups of oxygenases in the transformation of CAHs.

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, some of these improvements are not likely to come easily. Most engineered isoforms of enzymes exhibit either unaltered activity (the amino acid substitution is without effect) or are virtually inactive (the residue is essential for activity). Substitutions that 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 aerobic cometabolism of chlorinated solvents begin with the substrate ranges of monooxygenases and dioxygenases that extend beyond the physiologically relevant substrate to chlorinated solvents. Binding of the chlorinated solvent to the enzyme can inhibit the binding of the growth-supporting substrate, thereby depriving the cell of the benefits of the oxidation of the growth-supporting substrate. The products of the chlorinated solvent oxidation can also have profound influences on the cell and often include toxic effects. The oxygenases that oxidize chlorinated solvents are typically not constitutively produced in the cell. Rather, oxygenase production is induced in the presence of a particular growth substrate. Many of the genes that 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, the mechanisms of cellular damage associated with cometabolism 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. Also, many details concerning the specific and global regulatory mechanisms that govern the expression of genes coding for TCE-degrading oxygenases are as of yet unknown. 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?

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