

BIOREMEDIATION OF SYNTHETIC CHEMICALS AS A CONSEQUENCE OF GROWTH ON PETROLEUM HYDROCARBONS.

Charles C. Somerville* and Erica S. K. Becvar†
USAF Armstrong Laboratory, Environics Directorate*
and Applied Research Associates†
139 Barnes Drive, Tyndall AFB, FL 32403

INTRODUCTION

The Department of Defense uses approximately 8.5 million gallons of light distillate fuels each day (1). At this level of use, spills or leaks during transport, storage, or use can lead to a large environmental burden of fuel hydrocarbons. The Air Force alone has more than 2,500 sites that are contaminated with petroleum hydrocarbons. Fortunately, the components of light fuels are naturally occurring compounds. Because of their long term presence and wide distribution in the environment, many soil microorganisms have adapted to the degradation of fuel compounds. In fact, it is likely that all natural products can be degraded by one or more microbial species under the proper conditions (2). This does not mean that spills and leaks of light fuels are unimportant or that such contamination will always be amenable to bioremediation. However, it does mean that natural or augmented biological processes are important tools in the cleanup of fuel-contaminated sites.

Unfortunately, environmental contamination is not limited to naturally occurring chemicals. Some man-made chemicals (e.g. highly substituted chloro- and nitro-compounds) are common environmental contaminants and are known to resist biodegradation. However, the presence of petroleum hydrocarbons at contaminated sites can actually aid, directly or indirectly, in the degradation of synthetic co-contaminants. In this report, we consider the biodegradation of two types of synthetic compounds influenced by the presence of petroleum hydrocarbons in the environment. The first type includes the chlorinated ethenes: tetrachloroethylene (PCE), trichloroethylene (TCE), the isomers of dichloroethylene (DCEs), and vinyl chloride (VC). The second type includes substituted aromatic compounds such as chloro- and nitroaromatics.

CHLORINATED ETHENES

PCE and TCE have been used extensively in dry cleaning, degreasing of metals, and as solvents (3). The prevalent use of these solvents has led to widespread environmental contamination. TCE has been reported to be the major volatile organic contaminant of groundwater in the United States (4). There are a growing number of reports on the degradation of chlorinated ethenes by bacterial consortia and isolated bacterial species. The biotic degradation of chlorinated ethenes appears to occur in one of two ways: (i) cometabolic attack by non-specific oxygenases, or (ii) dehalorespiration.

Cometabolic Oxidation. Several species of bacteria produce enzymes which cometabolize PCE and/or TCE. The enzymes studied in the greatest detail are methane monooxygenase, toluene dioxygenase, and toluene monooxygenase. All three types of enzymes have relaxed substrate specificity and will fortuitously degrade chlorinated ethenes. The presence of fuel hydrocarbons in the environment can lead to the establishment of conditions favoring cometabolism of chlorinated ethenes.

In the jet fuel JP-4 *n*-alkanes constitute 32% of the mixture by weight (5). The *n*-alkanes of intermediate chain length are degraded by terminal oxidation to a carboxylic acid followed by β -oxidation. The acetyl-coenzyme A produced as a result is further metabolized to carbon dioxide by the tricarboxylic acid cycle for energy production, or converted to cellular carbon via the glyoxylate cycle. The *n*-alkanes of odd chain length result in the formation of propionyl-coenzyme A which is further metabolized to pyruvic acid with the concomitant production of hydrogen (6). The consumption of oxygen coupled with the production of carbon dioxide and hydrogen produces conditions favorable to the biological formation of methane. Methanotrophic bacteria, in turn, utilize methane via a series of oxidations to yield carbon dioxide (6). The initial oxidation of methane to methanol is catalyzed by the enzyme methane monooxygenase (MMO). Methanotrophic bacteria characterized to date produce two forms of the enzyme: a membrane-associated particulate form (pMMO) which requires copper for activity, and a soluble form (sMMO) which is expressed under copper-limited conditions (7). Both pMMO and sMMO oxidize TCE, but sMMO degrades TCE at higher rates (8) and has been demonstrated to catalyze the transformation of DCEs (9). A series of reactions leading to methane formation and TCE transformation is outlined in Fig. 1.

Aromatic compounds account for approximately 20% of the jet fuel JP-4 by weight (5). Mineralization of aromatic substrates is usually initiated by hydroxylation of the ring by either dioxygenase or monooxygenase reactions. Many of the enzymes which initiate degradation of aromatic compounds (e.g. toluene) are also capable of attacking TCE (Fig. 2). Therefore, the contamination of a site with fuel hydrocarbons can select for a microbial population pre-adapted to the degradation of TCE. In fact, exogenous addition of aromatic hydrocarbons has been shown to stimulate the degradation of TCE *in situ* (11-13). Fortuitous oxidation of TCE has generally not been observed in the absence of inducer compounds such as phenol or toluene. Recently, however, Leahy et al. (14) confirmed that the toluene monooxygenase of some strains is induced by TCE. Still, TCE does not serve as a source of carbon or energy for these strains, and thus can

not sustain the degrader population. Unfortunately, none of the aromatic oxygenases have been demonstrated to attack PCE or VC, and only toluene dioxygenase has activity against the DCE isomers (see Fig. 2). Another drawback of toluene/TCE cometabolism is a high demand for oxygen which is required as a co-substrate in the oxidation of both TCE and the primary substrate. Oxygen is also required as a terminal electron acceptor by the host bacterium. Oxygen limitation has been shown to inhibit TCE degradation in soil (15). The addition of nitrate to contaminated soils may overcome oxygen limitation for denitrifying strains, but some of the best studied TCE-degrading isolates (e.g. *Burkholderia cepacia* G4 and *Pseudomonas putida* F1) are not denitrifiers (14).

Toluene and methane oxygenases have been most extensively studied. However, other bacterial oxygenases, including ammonia monooxygenase (16) and propane monooxygenase (17), also degrade TCE. Propane monooxygenase is notable for its ability to degrade DCEs and VC. Chloroethene degradation, however, does not appear to be a common property of bacterial oxygenases. Wackett et al. (17) showed that nitropropane dioxygenase, cyclohexanone monooxygenase, cytochrome P-450 monooxygenases, 4-methoxybenzoate monooxygenase, and hexane monooxygenase do not attack TCE.

Dehalorespiration. In the process of dehalorespiration, chlorinated compounds serve as terminal electron acceptors for anaerobic bacteria growing on an appropriate carbon source. Dehalorespiration differs from cometabolism in that the bacteria that mediate reductive dechlorination gain energy from the process. The combination of chloroethenes with a suitable electron donor can thus support growth of strains capable of coupling dechlorination to energy production. Reductive dechlorination of halogenated aliphatic compounds was first reported in the early 1980's (18). Vogel and McCarty (19) later established the pathway for reductive dechlorination of PCE to VC under methanogenic conditions. Although [¹⁴C]PCE mineralization was demonstrated by the detection of labeled carbon dioxide, the major products of dechlorination were TCE and VC. The formation of VC represents an increase in toxicity over TCE or PCE, and seemed to indicate that reductive dechlorination would be deleterious at contaminated sites. It was later found that PCE and TCE could be dehalogenated to ethylene (ETH) under methanogenic conditions (20). However, the dehalogenation of VC was rate limiting, and in many situations VC was found to persist (21). A breakthrough was made when DiStefano et al. (21) tested a mixed culture of predominantly methanogenic bacteria for dehalogenation of PCE. At very high initial levels of PCE, methanogenesis was completely inhibited, and the culture gave a nearly complete conversion of PCE to ETH. Further study of the culture showed that hydrogen was the direct electron donor for reductive dechlorination and that the bulk of the hydrogen was produced by acetogenic bacteria growing on methanol. High levels of PCE selectively inhibited the methanogenic bacteria that would otherwise compete for available hydrogen (22). The organism responsible for dehalogenation of chloroethenes has recently been isolated and identified as a eubacterium tentatively named *Dehalococcoides ethenogenes* (23). Furthermore, Sharma and McCarty (24) isolated a facultative enterobacterium that dehalogenates PCE primarily to *cis*-1,2-DCE in the absence of oxygen, nitrate, and fermentable carbon sources. The ability to work with pure cultures represents a major advance in the effort to determine the conditions most favorable for complete dehalogenation of chloroethenes. The pathway for the dehalogenation of PCE to ETH is shown in Fig. 3.

SYNTHETIC SUBSTITUTED AROMATICS

Synthetic substituted aromatic compounds are common industrial chemicals and are often found as soil and groundwater contaminants. Microorganisms that mineralize synthetic aromatic compounds can be isolated from contaminated soil and groundwater but have not been isolated from uncontaminated sites. This suggests that a selection or adaptation process must occur after site contamination. One hypothesis is that the enzymes which attack synthetic aromatics are homologous to enzymes specific for naturally-occurring aromatic hydrocarbons. The isolation and sequencing of the genes encoding degradative enzymes have allowed us to test that hypothesis for chlorobenzene and nitrotoluene degradative pathways.

Chlorobenzenes. *Pseudomonas* sp. strain P51 grows on 1,2-dichlorobenzene, 1,4-dichlorobenzene, and 1,2,4-trichlorobenzene as sole sources of carbon and energy (25). The metabolic pathway for chlorobenzenes in strain P51 proceeds from an initial dioxygenase attack to yield a chloro-dihydrodiol intermediate which is rearomatized by the action of a diol dehydrogenase to form a di- or trichlorocatechol. The aromatic ring is opened in the *ortho* position by a chlorocatechol dioxygenase, and the substrate is mineralized by a series of reactions analogous to the β -ketoadipate pathway (6, 25). Removal of the chloro substituents is thought to occur after ring cleavage. The striking similarities between the metabolism of chlorobenzenes and naturally occurring aromatic hydrocarbons (e.g. benzene, phenol, toluene) strongly suggests that pre-existing enzymes have become adapted to chlorinated compounds. The genes that encode the initial chlorobenzene dioxygenase from strain P51 have been isolated and sequenced (26), and that data can be used to determine the phylogeny of the enzyme.

Fig. 4 is a phylogram of large subunits of the terminal dioxygenase (ISPLs) of ten aromatic ring dioxygenases identified by their primary substrate. The phylogenetic analysis indicates a close and statistically significant relationship between chlorobenzene dioxygenase and benzene and toluene dioxygenases. The topology of the phylogram suggests that the chlorobenzene dioxygenase of strain P51 shares a recent common ancestor with benzene and toluene dioxygenases, and that their common ancestor evolved from a biphenyl dioxygenase. The data clearly support the theory that enzymes which attack chloroaromatics arise by adaptation of

enzymes specific for aromatic petroleum hydrocarbons.

Nitrotoluenes. The metabolic pathways for both 2-nitrotoluene (2-NT) and 2,4-dinitrotoluene (2,4-DNT) are initiated by dioxygenase reactions which form diol-derivatives of the parent compounds (37, 38). The degradation of these nitroaromatic compounds differs from that of the chlorobenzenes in three respects: (i) rearomatization of the ring occurs spontaneously, (ii) rearomatization is accompanied by elimination of a substituent nitro group prior to ring cleavage, and (iii) the ring is opened by a *meta* ring cleavage. Although the initial reactions differ in these details, the overall degradation of nitrotoluenes is analogous to the *meta*-cleavage pathway of naturally-occurring aromatic compounds (6). Sequence data are available for three oxygenases that attack nitrotoluenes (39-41), and can be used to establish the origin of these enzymes.

Fig. 5 is a phylogram of the ISPLs of ten aromatic ring dioxygenases including the three nitrotoluene dioxygenases. The nitrotoluene degrading enzymes form a statistically significant, exclusive group, and clearly share a recent common ancestor with enzymes that hydroxylate polyaromatic hydrocarbons. This relationship again implies that enzymes for naturally-occurring substrates have adapted to the degradation of synthetic compounds (although the chemical similarity between PAHs and nitrotoluenes is not immediately apparent). The fact that the three nitrotoluene dioxygenase sequences cluster together is interesting because the organism that degrades 2-NT was found at a site hundreds of miles away from the site where the 2,4-DNT degraders were found (37, 44). This observation suggests that enzymes which attack synthetic compounds do not arise *de novo* at each contaminated site; however, more data will have to be collected before the mechanisms of adaptation are clear.

CONCLUSIONS

The studies of chlorinated ethenes, chloroaromatics, and nitroaromatics clearly show that the presence of bacteria adapted to the degradation of fuel hydrocarbons can have a positive impact on the bioremediation of man-made chemicals. Cometabolic processes are clearly of interest; however, there are obstacles to relying on these processes for site remediation. Because cometabolism does not provide the bacterial community with carbon or energy, it is not a self-sustaining process. The cometabolism of TCE by methane or toluene oxygenases is oxygen intensive, and will therefore be of limited extent in unamended soil and groundwater. Also, the enzymes which catalyze the breakdown of chloroethenes are primarily limited to TCE degradation and will not be useful at sites where PCE or VC have accumulated.

Dehalorespiration is an exciting alternative to cometabolic oxidation of chloroethenes for two reasons. First, the biological degradation of light fuels is an oxygen-intensive process. Removal of oxygen inhibits cometabolic degradation but favors reductive dehalogenation. Second, reductive dehalogenation appears to function on a much wider range of chloroethenes than cometabolism indicating that the process could prove useful at many contaminated sites. Furthermore, the presence of DCEs and VC at sites known to have been contaminated with PCE or TCE indicates that reductive dehalogenation is already occurring in the environment (45-47).

We now know that enzymes which attack chlorobenzenes and nitrotoluenes are homologous to enzymes specific for naturally occurring compounds. Therefore, the presence of petroleum hydrocarbons at a site co-contaminated with one or more of these synthetic compounds may enrich for the presence of the genotypes required for bioremediation of the man-made chemicals. Research is now in progress to answer some of the basic questions regarding how bacteria adapt to the degradation of xenobiotic compounds.

Based on the information presented here, we recommend careful analysis of data from contaminated sites in order to take advantage of fortuitous interactions between co-contaminants. We encourage both industry and regulatory agencies to continue to look for opportunities to use *in situ* biological remediation strategies as alternatives, or supplements, to physical remediation of sites contaminated with synthetic compounds.

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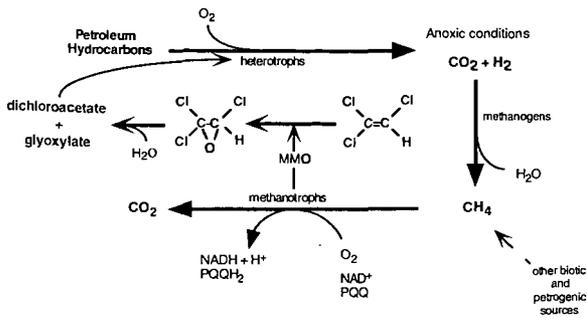


Fig. 1. A proposed scheme for the involvement of petroleum hydrocarbon degradation in cometabolism of TCE. The formation of dichloroacetic acid and glyoxylic acid was proposed by Little et al. (10). The abbreviation PQQ refers to the coenzyme methoxatin which is reduced in the oxidation of methane to carbon dioxide (6).

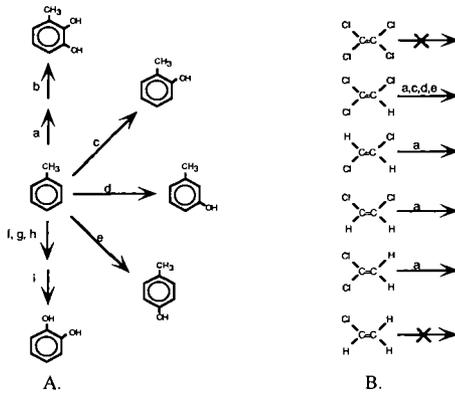


Fig. 2. (A) Enzymes involved in the degradation of toluene, and (B) their ability to cometabolize chloroethenes. Symbols: a, toluene dioxygenase; b, toluene dihydrodiol dehydrogenase; c, toluene-*ortho*-monooxygenase; d, toluene-*meta*-monooxygenase; e, toluene-*para*-monooxygenase; f, toluene oxidase; g, benzyl alcohol dehydrogenase; h, benzaldehyde dehydrogenase; i, benzoate oxidase.

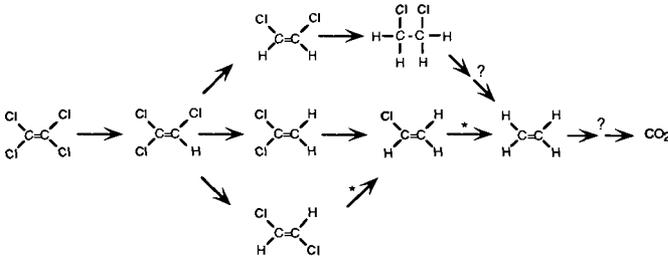


Fig. 3. Pathway for the reductive dechlorination of PCE based on the work of Vogel and McCarty (19) and Maymó-Gatell et al. (23). Question marks (?) represent transformations with unknown intermediates. Asterisks (*) indicate reactions not coupled to cell growth in *Dehalococcoides ethenogenes* (23).

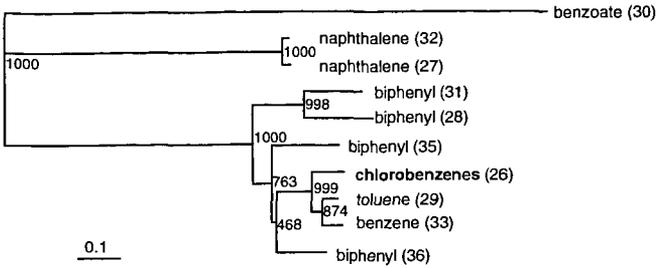


Fig. 4. Phylogram depicting the relationships between selected aromatic dioxygenases. Amino acid sequences were aligned, evolutionary distances were corrected for multiple substitutions, and bootstrap analysis was performed using the ClustalW package (34). Numbers indicate the frequency that the topology shown at each node was recovered out of 1000 bootstrap replicates.

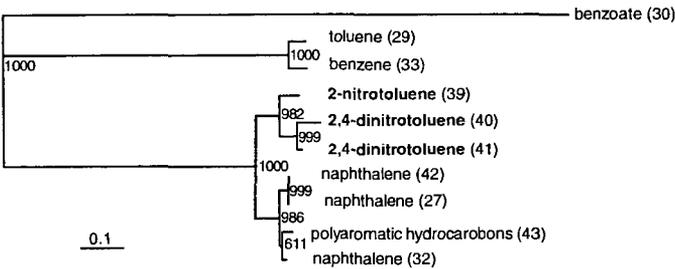


Fig. 5. Phylogram depicting the relationships between selected aromatic dioxygenases. Analyses and symbols are as described in Fig. 4.