Recruitment of Co-metabolic Enzymes for Environmental Detoxification of Organohalides

Lawrence P. Wackett

Department of Biochemistry and Institute for Advanced Studies in Biological Process Technology, University of Minnesota, St. Paul, Minnesota

Polyhalogenated compounds are often environmentally persistent and toxic to mammals. Microorganisms that metabolize these compounds can detoxify contaminated environments. Different biochemical mechanisms are used to metabolize polyhalogenated compounds, but few naturally occurring bacteria have this capability. A recombinant bacterium was constructed to metabolize polyhalogenated compounds to nonhalogenated end products. Seven genes were expressed in Pseudomonas putida G786 to biosynthesize cytochrome P450CAM and toluene dioxygenase. Cytochrome P450CAM catalyzed reductive dechlorinated reactions and toluene dioxygenase catalyzed oxidative dechlorination. With pentachloroethane, reductive dechlorination yielded trichloroethylene, which was further oxidized to formate and glyoxylate. The sequential action of cytochrome P450CAM and toluene dioxygenase with polyhalogenated compounds constitutes a novel engineered metabolic pathway. — Environ Health Perspect 103:Suppl 5:45–48 (1995)

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Organohalides of natural and anthropogenic origin are ubiquitous in the environment (1). They typically contain iodine, bromine, chlorine, or fluorine bonded to carbon; chlorine is most common. Environmental organochlorides are often derived from industrial sources. Many of these compounds, such as herbicides and insecticides, were intended to persist in the environment. As health problems associated with human exposure became known, environmental persistence became a detrimental characteristic. Currently, there are great pressures to minimize the use and disposal of chlorinated compounds derived from manufacturing processes (2). Still, organohalides are expected to remain important environmental toxicants for decades.

Microorganisms are largely responsible for recycling organic molecules in the environment (3). Most microorganisms fail to metabolize organohalide pollutants and this underlies the environmental persistence of these compounds. Bacteria capable of organohalide metabolism are increasingly viewed as agents with potential to detoxify natural and engineered environments. The success of biological organohalide decomposition rests largely on the ease of carbon–halogen bond cleavage for different compounds. For example, fluoro-carbons often resist microbial attack because carbon–fluorine bonds typically have bond dissociation energies in the range of 106 to 115 Kcal/mole (4). Chlorocarbons also markedly differ in their susceptibility to microbial metabolism. As a general rule, simple alkyl chlorides are metabolized more readily than alkenyl and aryl chlorides. Less chlorinated molecules are more readily metabolized than their heavily chlorinated counterparts. Unfortunately, the organochlorides that are most resistant to microbial attack, and thus the most environmentally persistent, often pose the greatest health risk to humans (5). In this context, it is important to develop new methods for bacterial detoxification of environmental organohalides.

Bacteria metabolize organohalides in several different ways (6). First, they may use organohalides as a source of carbon and energy. For example, some methylotrophic bacteria metabolize dichloromethane to assimilate intermediates supporting growth and making ATP (7). These bacteria have been isolated in Switzerland and the United States, suggesting that they are widespread. They are being employed commercially to biologically detoxify dichloromethane-containing waste streams. Second, bacteria can use organochlorides as an acceptor for electrons generated during metabolic oxidation reactions. Desulfoviridin efficiens oxidizes pyruvate coupled with a metabolic reduction of 3-chlorobenzoate to yield benzoate and chloride anion. These reactions have been linked to ATP formation, but benzoate is not assimilated to build up intracellular organic molecules (8). Last, bacteria may metabolize organohalides without accompanying ATP formation or assimilation of the carbon structure into biomolecules. This metabolism is of no apparent benefit to the organism and is often called gratuitous metabolism or co-metabolism. Co-metabolic degradation of chlorinated compounds, nevertheless, can be extremely rapid. For example, methanotrophic bacteria are estimated to oxidize approximately 10^{13} g per year of methane globally, and they oxidize trichloroethylene, a ubiquitous groundwater pollutant, nearly as rapidly as methane (9).

Little is currently known about the evolution of bacterial organohalide metabolism. It is known that four general biochemical mechanisms are involved in carbon–halogen bond cleavage (6). Evolutionary selective pressure requires
enzymes cleaving carbon–halogen bonds to feed compounds into intermediary metabolism. In nature, a gratuitous or co-metabolic dehalogenation reaction may be recruited into metabolic pathways to provide carbon, energy, or an electron sink for the cell. Under these conditions, the reaction benefits the organism and is, thus, no longer co-metabolic.

In the present laboratory studies, co-metabolic reactions were recruited to biodetoxify waters contaminated with chlorinated solvents (10). Heavily chlorinated compounds were reductively dehalogenated by bacteria that biosynthesized a nonspecific enzyme capable of mediating a direct electron transfer to carbon–halogen bonds. Less heavily chlorinated compounds were co-metabolically oxidized by a bacterial dioxygenase. In some of these reactions, the products are nonchlorinated and thus rendered completely nontoxic. The two enzymes were combined in a single organism using recombinant DNA techniques to construct a two-step metabolic pathway for biodegrading highly chlorinated compounds.

Both enzymes were derived from independently isolated Pseudomonas putida strains. Pseudomonas sp. are well known for their diverse catabolism of organic compounds in waters and soil. Much of this well-studied metabolism is oxidative, and oxygenases figure prominently in the oxidation of hydrocarbon natural products. In the present study, both enzyme systems are oxygenases capable of oxidizing the bicyclic terpene camphor and the aromatic hydrocarbon toluene, respectively. As is true for many catabolic oxygenases, these Pseudomonas oxygenases are nonspecific in their substrate range. This property has been exploited in the present study to co-metabolize difficult-to-degrade chlorinated organic compounds.

G786 biosynthesizes cytochrome P450\textsubscript{CAM} monooxygenase to initiate metabolism via a hydroxylation reaction (Figure 1A). Cytochrome P450\textsubscript{CAM} hydroxylates other substrates, and this underlies many of the co-metabolic oxidation reactions observed with P. putida G786 (11). Under anaerobic conditions, cytochrome P450\textsubscript{CAM} transfers electrons to the carbon–halogen bond of certain organohalides (12) to catalyze reductive dehalogenation reactions (Figure 1). This nonphysiological reductive activity emanates from the ability of the active site heme–iron cofactor to reduce carbon–halogen bonds (6). The active site cavity of cytochrome P450\textsubscript{CAM} is known from X-ray structural data to be composed largely of hydrophobic amino acid side chains (13). Thus, suitably sized hydrophobic organohalides readily partition from an aqueous environment into the heme-containing active site and then undergo reduction. With vicinally chlorinated ethanes, two electrons are transferred from NADH through the heme cofactor to the substrates. Substrate reduction and concomitant $\beta$-elimination leads to the formation of alkene products (11). For example, hexachloroethane and pentachloroethane are reduced to tetrachloroethylene and trichloroethylene, respectively.

P. putida F1 biosynthesizes toluene dioxygenase to initiate toluene metabolism by inserting two atoms of molecular oxygen into the aromatic ring (Figure 1B). Like the cytochrome P450 system, toluene dioxygenase is a three-component enzyme system that transfers electrons from NADH to a terminal oxygenase (14). Unlike cytochrome P450\textsubscript{CAM}, the ISP\textsubscript{TOL} component contains nonheme iron atoms that are

![Figure 1](image1.png)

Figure 1. (A) The protein components and reactions catalyzed by cytochrome P450\textsubscript{CAM} from P. putida G786. (B) The protein components and reactions catalyzed by toluene dioxygenase from P. putida F1.

![Figure 2](image2.png)

Figure 2. Scheme showing sequential anaerobic and aerobic metabolism of highly chlorinated compounds leading to their detoxification.

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thought to function in oxygen activation during the catalytic cycle. Although an X-ray structure for ISP<sub>TOL</sub> is not available, over 40 substrates fit into the active site and undergo oxidation (14). In 1988, trichloroethylene was identified as an oxidizable substrate for <i>P. putida</i> F1, and toluene dioxygenase was implicated in the oxidation reaction (15,16). This was confirmed by conferring onto <i>Escherichia coli</i> the ability to oxidize trichloroethylene by cloning and expression of the <i> todABC<sub>1</sub>C<sub>2</sub></i> genes known to encode for toluene dioxygenase (17). Purified toluene dioxygenase components oxidize trichloroethylene–yielding the stable nonchlorinated products formate and gly oxylate (18).

Three (<i>camABC</i>) and four (<i> todABC<sub>1</sub>C<sub>2</sub></i>) genes encode for cytochrome P450<sub>CAM</sub> and toluene dioxygenase enzyme systems, respectively. The <i>cam</i> genes were localized to a large plasmid denoted CAM and <i>rod</i> genes are on the chromosome of <i>P. putida</i> F1. Both gene clusters have been independently cloned into <i>E. coli</i> (19,20).

One goal of the present study was to express cytochrome P450<sub>CAM</sub> and toluene dioxygenase simultaneously in an attempt to biodegrade recalcitrant and toxic highly chlorinated compounds. Sequential anaerobic (reductive) and aerobic (oxidative) metabolism of organohalides can lead to their complete detoxification (Figure 2). This approach has been followed by using sequential anaerobic and aerobic bioreactors to treat chlorinated pollutants. A more elegant and practical application of this concept would involve sequential reductive and oxidative metabolism by a single organism. We chose to implement this by expressing cytochrome P450<sub>CAM</sub> and toluene dioxygenase in a single <i>Pseudomonas putida</i> strain (Figure 3). The <i>todABC<sub>1</sub>C<sub>2</sub></i> genes from plasmid pDTG601a and lacF<sub>W</sub> from pMMB24 were ligated into pKT230. The recombinant plasmid was mobilized into <i>P. putida</i> G786 by a triparental mating. The resultant recombinant organism, <i>P. putida</i> G786 (pHG-2), expressed cytochrome P450<sub>CAM</sub> and toluene dioxygenase following induction by camphor and β-isopropylthiogalactoside, respectively. <i>P. putida</i> G786 (pHG-2) metabolized pentachloroethane to formate and glyoxylate.

These data highlight the potential to recruit broad specificity catalytic enzymes and use recombinant DNA methods for constructing novel dehalogenation pathways. We have been unsuccessful in isolating wild-type bacteria expressing both camphor monoxygenase and toluene dioxygenase enzyme activities (MSP Moe Logan, LP Wackett, unpublished data). This suggests that the constructed strain contains a unique ensemble of genes that is rare or not found in nature. In this context, directed evolution, drawing on a knowledge of enzyme mechanisms and gene structure, may be exploited to provide organisms capable of metabolizing toxic and environmentally persistent organohalides.

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