Genetic Adaptation of Bacteria to Halogenated Aliphatic Compounds

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The bacterial degradation and detoxification of chlorinated xenobiotic compounds requires the production of enzymes that are capable of recognizing and converting compounds which do not occur at significant concentrations in nature. We have studied the catabolic route of 1,2-dichloroethane as an example of a pathway for the conversion of such a synthetic compound. In strains of *Xanthobacter* and *Ancylobacter* that have been isolated on 1,2-dichloroethane, the first catabolic step is catalyzed by a hydrolytic haloalkane dehalogenase. The enzyme converts 1,2-dichloroethane to 2-chloroethanol but is also active with many other environmentally important haloalkanes such as methylchloride, methylbromide, 1,2-dibromoethane, epichlorohydrin, and 1,3-dichloropropene. Further degradation of 2-chloroethanol proceeds by oxidation to the carboxylic acid and dehalogenation to glycolate. The aldehyde dehydrogenase prevents toxicity of the reactive chloroacetaldehyde that is formed as an intermediate and is necessary for establishing a functional 2-chloroethanol degradative pathway in a strain that is not capable of growth on this compound. — Environ Health Perspect 103(Suppl 5):29–32 (1995)

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Introduction

The observed environmental persistence and toxicity of a broad range of halogenated aliphatic and aromatic compounds is due to the xenobiotic structural features of these chemicals. Chlorine substitution causes natural compounds such as methane, ethane, ethylene, propane, benzene, or biphenyl to become recalcitrant to biodegradation by microorganisms. Recent studies on the microbial degradation of a number of compounds with a low degree of chlorine substitution has led to the identification of critical metabolic steps. Recalcitrance is usually caused by a lack of transformation at some point, which leads to partial transformation with formation of dead-end products, toxic effects, or even complete inertness. If a productive metabolic route is not present, i.e., in case of co-metabolism the chemical does not stimulate growth, and adaptation at a population level does not occur.

An obvious critical step is the actual dehalogenation reaction. Microbial detoxification of chlorinated substrates requires the production of enzymes that can cleave or labilize carbon–halogen bonds. A variety of bacterial enzymes that have this capability are known (*1*). The question then arises: how did such enzymes evolve? They could be derived from enzymes that convert natural halogenated substrates, many of which are known (*2*). Another possibility is that dehalogenases have evolved from proteins that catalyze the same reaction type on nonhalogenated compounds. Thus, a key question is whether and how dehalogenases specifically recognize the halogen function, and how mutations leading to recognition of synthetic compounds occur.

In this overview, we discuss the bacterial pathway of 1,2-dichloroethane conversion and the possible evolutionary changes that have led to organisms that can use it as a growth substrate. Dichloroethane is, from a production point of view, the largest chlorinated chemical. It is used for various purposes and is a frequent groundwater contaminant.

Degradation of 1,2-Dichloroethane

The degradation of 1,2-dichloroethane, which is not known to occur naturally, was investigated with cultures of *Xanthobacter autotrophicus* and *Ancylobacter aquaticus*, which use it as a sole carbon source (*3,4*). This led to the establishment of the catabolic route shown in Figure 1. The pathway is composed of special enzymes and more general enzymes. The first step is the hydrolytic conversion of 1,2-dichloroethane to 2-chloroethanol, which is converted by two different dehydrogenases to chloroacetate. This compound is cleaved by a hydrolytic dehalogenase to glycolate, which supports growth of many bacteria. Both dehalogenases are produced constitutively, whereas the alcohol dehydrogenase and the aldehyde dehydrogenase are inducible (*3–5*). The haloalkane dehalogenase gene (*dhlA*) and the chloroacetaldehyde dehydrogenase are plasmid encoded (*6*). The haloalkane dehalogenase, the aldehyde dehydrogenase, and the chloroacetate dehalogenase are only produced by strains of *Xanthobacter* that utilize 1,2-dichloroethane (*5,6*).

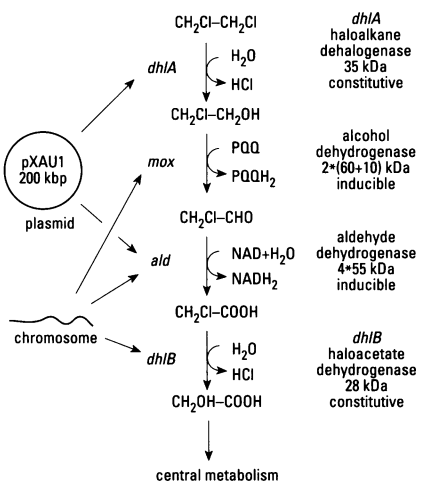

Figure 1. The catabolic pathway of 1,2-dichloroethane in *X. autotrophicus* and *A. aquaticus*.

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Chloroacetate Dehalogenase

The last step in 1,2-dichloroethane degradation is catalyzed by a 28-kDa haloacetate dehalogenase that is active with chloroacetate, dichloroacetates, bromoacetate, and 2-chloropropionic acid. The enzyme is produced constitutively, but higher levels are found in the stationary phase than in the exponential phase of growth (*7*). Sequence analysis and biochemical characterization (*8*) have shown that the enzyme belongs to a class of L-chloropropionic acid dehalogenases that converts their substrates with inversion of configuration. Enzymes of this class are not uncommon in various haloacetate-degrading bacteria, and the natural occurrence of haloacetates makes it probable that no specific adaptation was needed for the acquisition of the last step of 1,2-dichloroethane conversion.

Aldehydes as Critical Intermediates

A critical step in the degradation of halogenated aliphatics is the oxidation of the aldehydes that are produced as intermediates. The 2-haloaldehydes are bifunctionally reactive and can form covalent products with amines and nucleophilic groups. A lack of aldehyde dehydrogenase activity therefore causes toxicity of 2-chloroethanol (*5*). Tardif et al. (*6*) have shown that *X. autotrophicus* GJ10 produces a plasmid-encoded aldehyde dehydrogenase for the conversion of chloroacetaldehyde. We have recently purified the enzyme and found that it is a tetrameric protein with 55-kDa subunits. Genetic studies have indicated that chromosomally encoded genes are also essential for chloroacetaldehyde metabolism (*9*). Thus, the presence of these two activities is sufficient to allow growth on chloroethanol.

The lack of a functional aldehyde dehydrogenase for bromoacetaldehyde seems to be the reason that 1,2-dichloroethane degraders do not use 1,2-dibromoethane for growth. In fact, both 1,2-dibromoethane and 2-bromoethanol are very toxic for strains of *X. autotrophicus* or *A. aquaticus*. This can be used to select mutants that lack the alcohol dehydrogenase (*10*) or the haloalkane dehalogenase (*11*).

Alcohol Dehydrogenase

The alcohol dehydrogenase responsible for 2-chloroethanol conversion is the periplasmic quinoprotein alcohol dehydrogenase that is commonly present in methylotrophic bacteria. The tetrameric enzyme is composed of 60-kDa and 10-kDa subunits. The enzyme is induced by methanol or 2-chloroethanol (*5*). There is no indication of adaptational mutations being needed for 2-chloroethanol oxidation by this enzyme.

Haloalkane Dehalogenase

The first step in 1,2-dichloroethane metabolism is catalyzed by haloalkane dehalogenase, a protein that is identical in different isolates of *X. autotrophicus* and *A. aquaticus* (*4,12*). Haloalkane dehalogenase is a soluble 35-kDa, 310-amino acid protein that hydrolytically cleaves carbon–chlorine bonds to produce alcohols. The substrate range is very broad and includes a number of environmentally important compounds (Table 1). The best substrate known is the nematocide 1,2-dibromoethane, a compound that has been used widely as a soil fumigant and that is a frequent groundwater contaminant. The V_{max} values with 1,2-dichloroethane and 1,2-dibromoethane are both about 6 U/mg protein, but the K_m for 1,2-dibromoethane is 0.008 mM, which is 100-fold lower than for 1,2-dichloroethane.

The evolutionary origin of the dehalogenase is unknown, but the three-dimensional structure that was recently solved (*13*) shows that the enzyme is composed of two domains. The main domain is similar to a number of hydrolytic enzymes that can be classified as α/β-fold hydrolases (*14*). This group of enzymes includes other bacterial proteins catalyzing hydrolytic steps in the bacterial degradation of xenobiotic substrates (Figure 2). The sequence around the active site nucleophilic residue is conserved.

The mechanism of haloalkane dehalogenase was recently investigated by X-ray crystallography and involves nucleophilic attack by Asp124, followed by ester

Table 1. Substrate range of haloalkane dehalogenase.

Compound	% Activity[a]	Use, relevance
1,2-Dichloroethane	100	Vinyl chloride synthesis, solvent
Chloromethane	28	Blowing agent, naturally produced
Dichloromethane	9	Solvent
Ethylchloride	24	Cooling agent
1-Chloropropane	48	–
1,3-Dichloropropane	87	–
1,2-Dichloropropane	0.6	Chemical waste
1,3-Dichloropropene	96	Nematocide
2-Chloroethylvinylether	12	Waste product
2-Chloroethylmethylether	6	Waste product
Epichlorohydrin	8	Pharmaceutical synthesis, resins
2-Chloroethanol	<1	Solvent
1,2-Dibromoethane	94	Nematocide, gasoline
Methylbromide	13	Nematocide
Dibromomethane	112	Naturally produced
Ethylbromide	24	–
2-Bromoethanol	24	–

[a]Activities are given at a substrate concentration of 5 mM. Values are expressed as the percentage of activity found with 1,2-dichloroethane, which was 6 μmole/min/mg of protein (turnover number k_{cat} 3.5 substrate molecules/enzyme molecule per second).

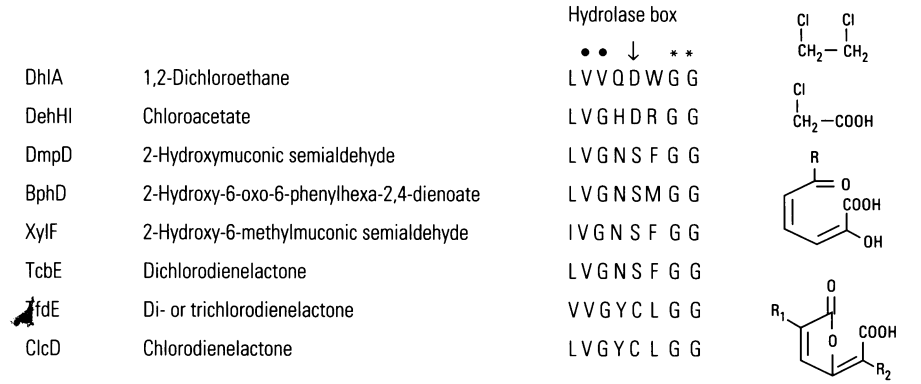

Figure 2. Sequence of a region around the active site nucleophile of several α/β-hydrolase fold enzymes involved in the bacterial degradation of xenobiotics. The enzymes are: DhlA, haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, from Janssen et al. (*21*); DehH1, haloacetate dehalogenase from *Moraxella*, from Kawasaki et al. (*17*); DmpD, 2-hydroxymuconic semialdehyde hydrolase from *Pseudomonas*, from Nordlund and Shingler (*22*); BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase from *Pseudomonas*, from Kimbara et al. (*23*); XylF, 2-hydroxymuconic semialdehyde hydrolase from *Pseudomonas putida*, from Horn et al. (*24*) ; TcbE, dienelactone hydrolase from *Pseudomonas* P51, from van der Meer et al. (*25*); TfdE, dienelactone hydrolase from *Alcaligenes eutrophicus*, from Perkins et al. (*26*); ClcD, dienelactone hydrolase from *Pseudomonas* B13, from Frantz et al. (*27*). The ↓ indicates the position of the nucleophile: *, conserved residues; •, similar residues.

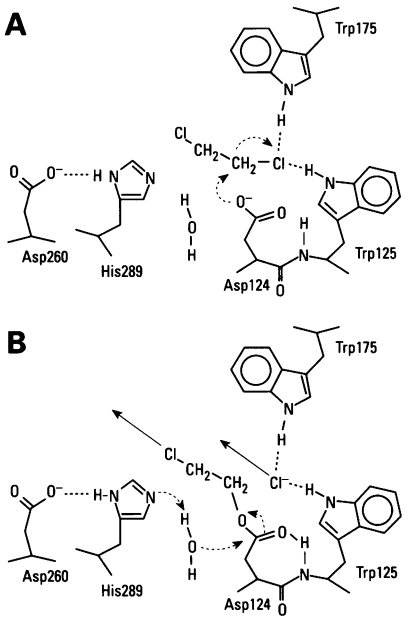

Figure 3. Proposed catalytic mechanism of haloalkane dehalogenase. (*A*) Cleavage of carbon-halogen chlorine bond by nucleophilic substitution. (*B*) Hydrolysis of the covalent intermediate.

hydrolysis (*15*) (Figure 3). The enzyme is, for several reasons, a specific dehalogenating enzyme. First, there is a specific halide binding site, which was identified by X-ray crystallography and fluorescence measurements (*16*). It is formed by two tryptophan residues. The site has a much higher affinity for bromide than for chloride, which can explain the lower K_m value of 1,2-dibromoethane compared to that for 1,2-dichloroethane.

The nucleophile in haloalkane dehalogenase and also that in haloacetate dehalogenase (*17*) is an Asp rather than a Ser or Cys residue as in most α/β-fold hydrolases. Nucleophilic displacement of a halogen from an alkylhalide by a Ser or Cys would produce an ether intermediate, which is much more difficult to hydrolyze than an ester. Thus, the presence of a carboxylate as a nucleophile is also directly related to the nature of the dehalogenase reaction (*18*).

The active site is located between the two domains of haloalkane dehalogenase. One of the tryptophan residues (Trp125) is located in the main domain and the other (Trp175) is located in the cap that lies on top of the main domain. Thus, both domains are specifically evolved to contribute to the catalysis of carbon–halogen bond cleavage. The enzyme clearly is not a general hydrolytic protein that fortuitously also converts chlorinated substrates.

Environmental Relevance of Haloalkane Dehalogenase

The above data suggest that haloalkane dehalogenase may have evolved from an enzyme that converts related compounds of natural origin. We have recently found that 2-chloroethylvinylether may be used as a sole carbon source by strains of *A. aquaticus* and that the haloalkane dehalogenases produced by these organisms are identical to the *X. autotrophicus* GJ10 enzyme (*4,10*). Little is known about the bacterial utilization of most of the other compounds listed in Table 1. It is conceivable, however, that the enzymes involved in the degradation of synthetic chloroaliphatics such as 1,2-dichloroethane are related to enzymes involved in the degradation of some of the compounds listed in Table 1 or to enzymes that play a role in the conversion of naturally produced halogenated compounds.

The haloalkane dehalogenase is by far not a perfect enzyme. The k_{cat} (turnover number) for 1,2-dichloroethane is 3.5 sec^{-1}, and the k_{cat}/K_m is 5100 $s^{-1}M^{-1}$. The kinetics have consequences for the K_s (Monod constant) of the organism: a high k_{cat} and a low K_m of the dehalogenase will decrease the K_s (*19*), and this will lower steady-state substrate concentrations in mixed reactors. In both natural environments and treatment systems, the production of increased levels of dehalogenase or an enzyme with higher affinity would yield reduced concentrations of toxicants.

It is conceivable that the dehalogenase can evolve further to improve its kinetics with 1,2-dichloroethane or other substrates. This is also suggested by the finding that the same enzyme is present in organisms isolated on 1,2-dichloroethane and 2-chloroethylvinylether; it seems unlikely that the same amino acid sequence yields a protein with optimal activity for two such different substrates.

Recently, we have experimentally shown that the substrate range of the enzyme can be modified to long-chain chloroalkanes by spontaneous mutations in the cap domain of the protein (*20*). The position and character of the mutations and the sequence of the wild-type enzyme suggested that similar mutations had occurred during the evolution of the original enzyme. Thus, short tandem sequence repeats that were detected in the mutants were also present in the wild type.

Evolution of a 1,2-Dichloroethane Degradative Pathway

The evolution of an organism that can grow on 1,2-dichloroethane seems to require a number of steps. First, a host organism that produces an alcohol dehydrogenase which can be used for 2-chloroethanol conversion may have acquired a gene encoding a haloacetate dehalogenase. Addition of a plasmid encoding a chloroacetaldehyde dehydrogenase and a haloalkane dehalogenase, modified to become active with the synthetic substrate 1,2-dichloroethane, allows utilization of this compound. Horizontal transmission of the plasmid to other facultative methylotrophs (*Xanthobacter* or *Ancylobacter*) that produce a haloacetate dehalogenase may have led to a variety of 1,2-dichloroethane and 2-chloroethylvinylether degrading organisms, as can now be isolated from environmental samples.

All 1,2-dichloroethane degraders isolated so far are facultative methylotrophs. An explanation for this could be that a quinoprotein alcohol dehydrogenase is needed for 2-chloroethanol conversion, but on the other hand, not all organisms isolated on 2-chloroethanol are methylotrophs. A difference between chloroethanol and 1,2-dichloroethane utilization is that in the latter case 2-chloroethanol is generated in the cell rather than taken up from the medium. It is conceivable that a periplasmic alcohol dehydrogenase helps to prevent toxic cellular levels of chloroacetaldehyde that are produced as an intermediate.

The origin of the plasmid-encoded haloalkane dehalogenase and chloroacetaldehyde dehydrogenase is unknown. It is likely that these enzymes are specifically adapted to xenobiotic substrates, i.e., they underwent mutations that were selected for as a result of the introduction in nature of synthetic chlorinated compounds. It thus remains to be determined from which proteins the present specialized enzymes have evolved and which mutations took place during their adaptation to the degradation of toxic haloalkanes.

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