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Diversity and Mechanisms of Bacterial Dehalogenation Reactions

Abstract

Halogenated aliphatic compounds occur widespread as environmental pollutants. Since many of these compounds are xenobiotics and show large differences in degradability which can be correlated to critical steps in catabolic pathways, they are suitable for studies on the evolution of dehalogenating pathways. We have investigated the degradation of 1,2-dichloroethane and 1,3-dichloropropene in detail. For both compounds, the initial step is hydrolytic dehalogenation. The 1,2-dichloroethane and 1,3-dichloropropene dehalogenases were found to belong to different groups of identical enzymes detected in bacteria isolated from various sites. Genetic analysis and adaptation experiments indicated that the 1,2-dichloroethane degradation pathway may be of recent evolutionary origin. The large-scale use of 1,3-dichloropropene in agriculture may have contributed to the distribution of genes encoding hydrolytic dehalogenases in the environment.

Introduction

The biodegradation of synthetic chlorinated chemicals that enter the environment is dependent on the capacity of microbial enzymes to recognize these xenobiotic molecules and cleave or labilize carbon-halogen bonds (Janssen *et al.*, 1994). Microbiological studies have led to the isolation of a range of organisms that degrade halogenated aliphatic compounds and use them as a carbon source for growth, and a several dehalogenating enzymes that directly act on carbon-halogen bonds have now been identified (Leisinger and Bader 1993; Janssen *et al.*, 1994; Fetzner and Lingens, 1994).

In a few cases, the carbon-halogen bond is not directly cleaved but labilized by introduction of other functional groups (Ensley, 1991). This way of dechlorination is often observed in organisms that do not utilize chlorinated compounds for growth but are only able to degrade them by fortuitous co-metabolic reactions. From a biotechnological point of view, these cometabolic reactions are less attractive for application since the degradation process does not stimulate the growth of the organisms involved. On the other hand, co-

metabolic transformation provides the only possibility for the aerobic biodegradation of some important compounds, including trichloroethylene.

This paper focusses on organisms that grow on synthetic organochlorine compounds. It is highly unlikely that chemicals such as dichloromethane, 1,2-dichloroethane, and γ-hexachlorohexane did occur on earth in biologically significant concentrations prior to the start of their industrial synthesis at around the beginning of this century. If at all produced by natural processes, this probably did not lead to concentrations in the biosphere which would give a selective advantage to organisms that can degrade them. Yet, the three compounds mentioned are known to support growth of specific pure cultures that produce the required dehalogenating enzymes. This raises the questions by which catalytic mechanisms organochlorine bonds are cleaved and how the genes encoding the enzymes that perform these reactions evolved and spread in the environment.

One possibility is that dehalogenases which convert xenobiotic organohalogens are derived from enzymes that recognized naturally produced halogenated compounds. Possibly, they adapted to xenobiotic compounds by a few additional mutations (see below). To understand this process of evolution of new activities, careful investigation of the biochemistry and genetics of catabolic pathways is necessary.

In this paper, a number of dehalogenation reactions for halogenated aliphatic compounds are reviewed, and experimental results on the distribution and evolution of dehalogenases are discussed.

Use of chlorinated aliphatic compounds for growth

Of the low-molecular weight halogenated aliphatic compounds, a number of environmentally important chemicals have been found to support growth of pure cultures. This includes methylchloride, dichloromethane, 1,2-dichloroethane, 1,3-dichloropropylene, 1-chlorobutane and other primary alkylhalides, and hexachlorocyclohexane. Chloropropanols and epichlorohydrin, several chlorocarboxylic acids and chloroethanol can also serve as a carbon source for specific bacterial cultures. Detailed rules in the sense of structure-degradability relationships are difficult to establish for haloaliphatics, but a few trends can be mentioned (Fig. 1). In many cases, degradation decreases with an increasing degree of chlorine substitution. Thus, of the chloromethanes only methylchloride and dichloromethane have been found to support growth, but not chloroform and carbon tetrachloride. Similarly, of the chloroethanes, only ethylchloride and 1,2-dichloroethane serve as a carbon source for known cultures. This rule holds only for homologous series of compounds, however. When different groups of compounds are compared, the rule does no longer hold. For example, dichloromethane and trichloroacetate, which have two and 1.5 chlorines per carbon,

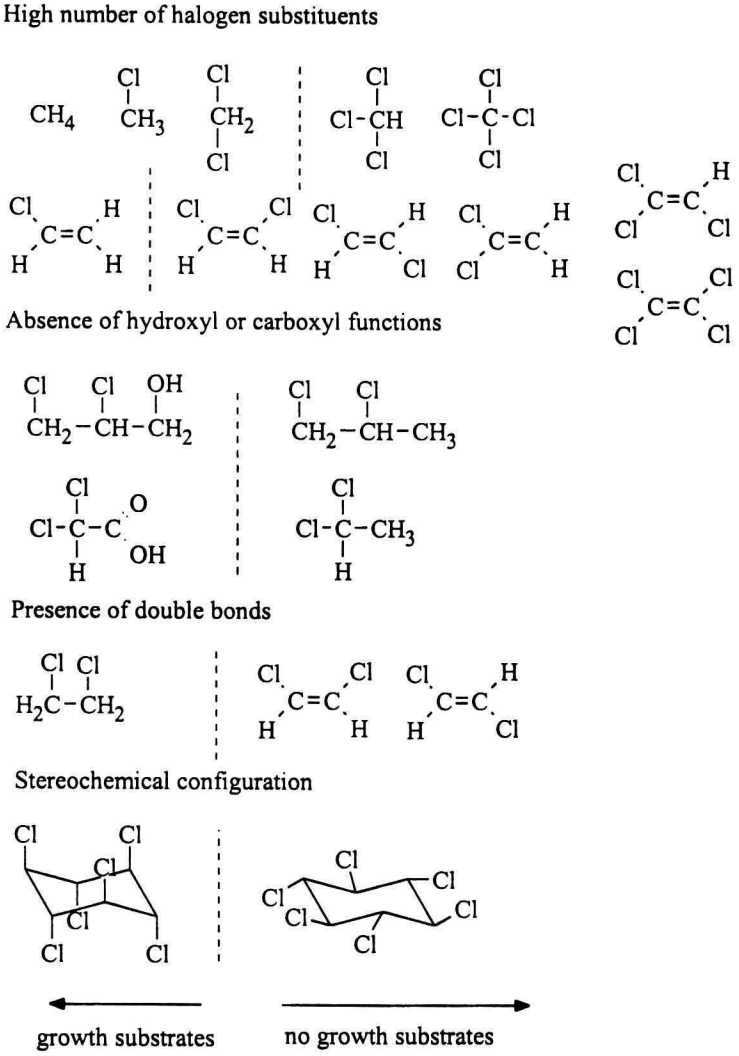

Fig. 1. Factors that limit bacterial growth on halogenated aliphatic compounds.

respectively, are easier to degrade than 1,1-dichloroethane which has only one Cl per C. A factor that increases degradability is the presence of oxo- or hydroxyl groups.

The reason for the lack of general structure-degradability relationships is twofold. First, blocks in a degradation pathway may occur at different steps, which may be catalyzed by enzymes that have completely different preferences concerning the chemical properties and substitution pattern of the substrate. Second, the specificities of dehalogenases often can not be described by simple structural rules.

This can be illustrated with the degradation of 1,2-dichloroethane as compared to 1,2-dibromoethane. The former compound may serve as a growth substrate for specific cultures of *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* that produce a haloalkane dehalogenase, the gene of which is encoded on a large catabolic plasmid (van den Wijngaard *et al.*, 1992; Tardiff *et al.*, 1991). The organisms do not grow on 1,2-dibromoethane, which instead is very toxic for them. We also have not been able to isolate 1,2-dibromoethane degraders from environmental samples that did contain chloroalkane-degrading organisms. Nevertheless, 1,2-dibromoethane is an excellent substrate for the dehalogenase that degrades dichloroethane, as it is for other haloalkane dehalogenases. The recalcitrance of dibromoethane is explained by the poor conversion and high reactivity of the intermediate bromoacetaldehyde (van der Ploeg *et al.*, 1996). Although 1,2-dibromoethane is easier to dehalogenate, it is difficult to mineralize because of a block at a later catabolic step, i.e. dehydrogenation of the aldehyde which is formed as an intermediate.

One of the critical steps in the biodegradation of organochlorine compounds is obviously the cleavage of the carbon-chlorine bonds. An overview of the dehalogenation reactions observed with halogenated aliphatic compounds is

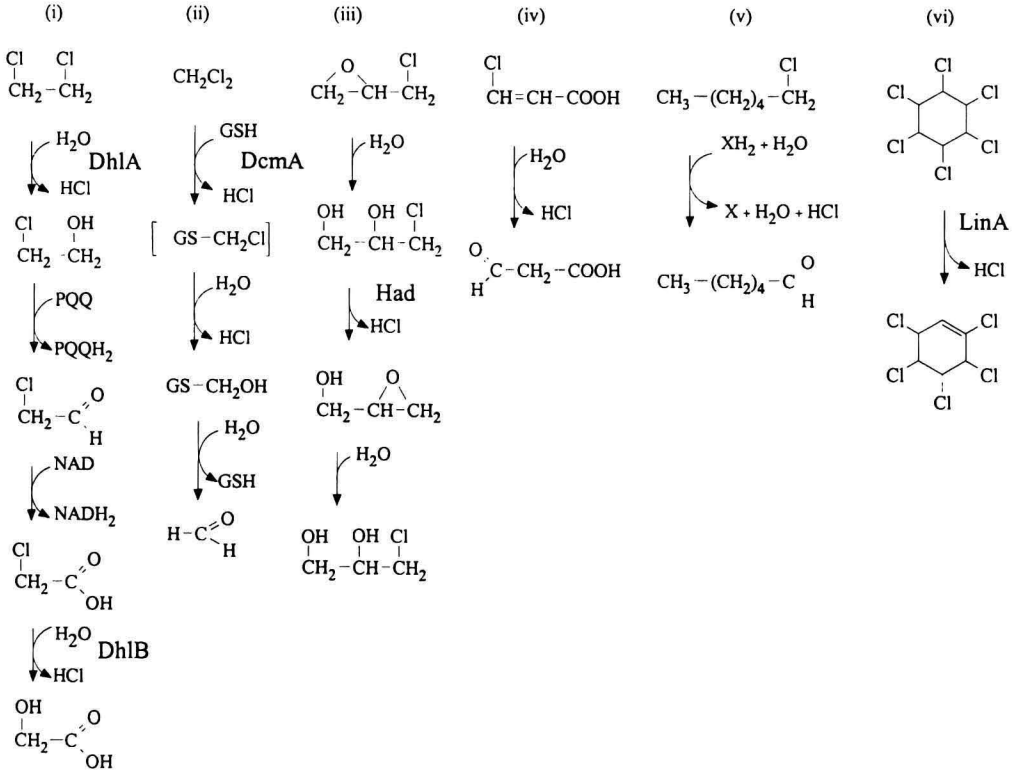

Fig. 2. Dehalogenation mechanisms for chlorinated aliphatic compounds. See text for details.

shown in Fig. 2. Briefly, six different reaction types have been investigated in some detail (Janssen *et al.*, 1994; Fetzner and Lingens, 1994; Nagata *et al.*, 1993): (i) Hydrolytic dehalogenation by dehalogenases (DhlA, DhlB), without the need for additional cofactors or oxygen; (ii) Substitution of a halogen by glutathione, catalyzed by glutathione-S-transferase (DcmA), a reaction analogous to the general detoxification mechanisms observed in mammalian systems; (iii) A lyase type of reaction (Had), proceeding by intramolecular substitution of a hydroxyl oxygen on a neighboring carbon atom carrying a halogen substituent; (iv) Substitution of a halogen on an unsaturated carbon atom, leading to an aldehyde, possibly by a hydratase type of reaction; (v) Oxidative conversion, by action of a monooxygenase in a reduced cosubstrate and oxygen-dependent reaction; (vi) Eliminative dehalogenation or dehydro-dehalogenation (LinA), as observed during the initial step of γ-hexachlorocyclohexane degradation.

The specificity of these dehalogenases may correlate well with the range of compounds that can support growth. Thus, the recalcitrance of compounds such as 1,1-dichloroethane, 1,1,2-trichloroethane, 1,2-dichloropropane and 1,2,3-trichloropropane, and the β- and δ-isomers of hexachlorocyclohexane may be explained by the low dehalogenase activity of the enzymes (DhlA, LinA) that are involved in the degradation of the closely related compounds or isomers (Fig. 2).

Degradation of 1,2-dichloroethane

We have studied the degradation of 1,2-dichloroethane by strains of *A. aquaticus* and *X. autotrophicus* in detail (Pries *et al.*, 1994a). The initial catabolic step is carried out by a haloalkane dehalogenase that converts 1,2-dichloroethane and a number of other chloro- and bromo-compounds. For discussing the possible evolutionary origin of these activities, the following observations are important. First, 1,2-dichloroethane is not known to be a natural compound. It does not (yet) occur on the ever growing list of naturally occurring halogenated compounds. Thus, it is unlikely that organisms that can degrade dichloroethane evolved by selective evolution before the compound was synthesized and introduced in the environment by industrial activities.

If organisms did not specifically adapt to 1,2-dichloroethane before its industrial production started, there are two possibilities for the evolutionary origin of the 1,2-dichloroethane-degrading bacteria. First, there may be organisms that already had a functional 1,2-dichloroethane-degradation pathway formed by a set of enzymes which had evolved for a different function, possibly utilization of a naturally produced organohalogen. Second, industrially emitted 1,2-dichloroethane may have been present at high concentrations in some areas, which provided a niche which stimulated rapid selection of genetically modified strains that degrade dichloroethane. Third, another synthetic compound than 1,2-di-

chloroethane may have played a role in creating selection pressure for dechlorinating activity towards dichloroethane.

We support the second possibility for different reasons. In the first place, the haloalkane dehalogenase gene and the (modified) chloroacetaldehyde dehydrogenase gene are plasmid encoded. Plasmid localization of genes that are under evolutionary pressure is also observed for other systems, for example resistance to antibiotics and heavy metals. The evolution of a catabolic plasmid for the degradation of bromoacetate was recently mimicked in the laboratory. In other strains in which a functional catabolic pathway was obtained under selection pressure, the catabolic genes are also plasmid encoded or located on transposons (van der Meer *et al.* 1992; Thomas *et al.*, 1992).

A second argument for the recent evolutionary origin is the lack of regulation of the synthesis of the 1,2-dichloroethane-degrading haloalkane dehalogenase. The gene encoding this protein is constitutively expressed from consensus *E. coli*-like promoter sequences. Although there is an open reading frame encoding a protein with sequence similarity to the tetracyclin repressor (TetR), this does not seem to be a functional repressor. The development of inducible expression for a catabolic gene requires, in addition to the catabolic enzyme itself, the evolution of a regulatory protein that can recognize and bind the substrate. This apparently has not yet occurred for the regulation of 1,2-dichloroethane degradation. Instead, the function of the repressor may have been lost and the

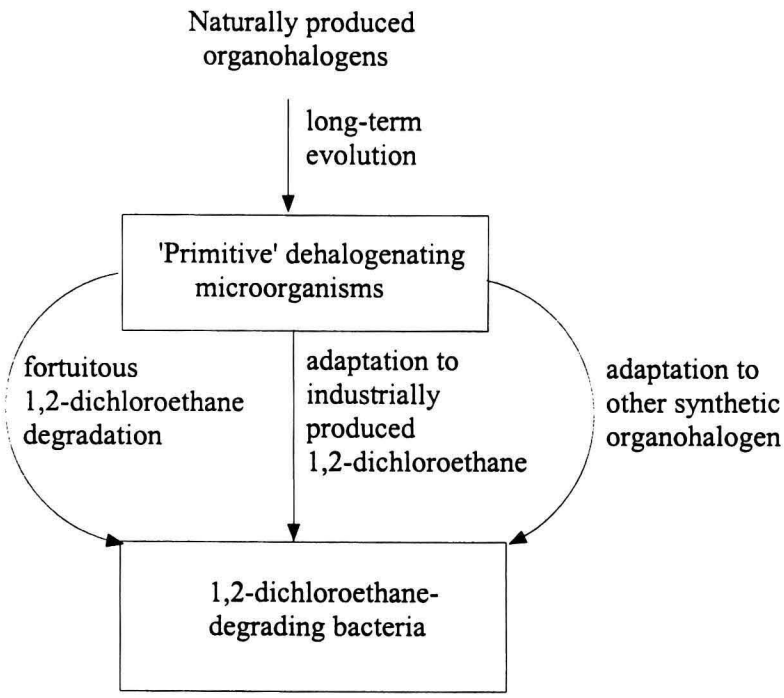

Fig. 3. Possible routes for the evolution of dehalogenating organisms.

dhlA gene is expressed from a constitutive promoter that may have been added during evolution of the pathway.

Other indications that the 1,2-dichloroethane pathway is of recent origin come from structural and biochemical studies on haloalkane dehalogenase itself, discussed below.

Haloalkane dehalogenases: diversity, evolution, and distribution

Haloalkane dehalogenases catalyze the hydrolytic cleavage of carbon-chlorine bonds in a wide range of halogenated alkanes. These enzymes play a role in the degradation of various alkylhalides. Based on the X-ray structure of the haloalkane dehalogenase from *X. autotrophicus*, and the sequence similarity with other hydrolytic enzymes, it has become clear that the haloalkane dehalogenases (Fig. 3, Table 1) belong to a group of hydrolytic proteins called α/β-hydrolase fold enzymes (Ollis *et al.*, 1992). This group also includes various other bacterial proteins involved in the biodegradation of natural and xenobiotic compounds. Details on the reaction mechanism and kinetics of haloalkane dehalogenase can be found elsewhere in this volume.

For reasons outlined above, the dehalogenase must have preexisted as an enzyme with a closely related function in preindustrial times, or it must have adapted to 1,2-dichloroethane during the last century or so from an enzyme that had a different function. We have proposed, on the basis of the sequence of mutant enzymes, that the dehalogenase has recently evolved from a more primitive dehalogenase. The sequence of the dehalogenase gene harbors sequence duplications that are indicative of recent evolutionary changes, since similar duplications arise when the gene is put under selection pressure in the laboratory (Pries *et al.*, 1994b).

Recently, we have sequenced the dehalogenase genes from a number of different 1,2-dichloroethane-degrading bacteria. Surprisingly, the haloalkane dehalogenases present in 10 independently isolated cultures obtained from different locations turned out to be identical, and they all contained the same sequence repetitions in the cap domain. In this respect, this haloalkane dehalogenase clearly distinguishes itself from the enzymes that hydrolyze chloroacetic acid, for which a large diversity has been found. At least 8 homologous genes have been sequenced, including the chloroacetate-converting dehalogenase of the dichloroethane catabolic pathway (van der Ploeg *et al.*, 1991). Chloroacetate, however, is a naturally occurring compound, and bacteria that have the capacity to degrade it are easily obtained from soil and water samples.

The lack of evolutionary divergence between the 1,2-dichloroethane dehalogenases isolated from different organisms again indicates that the enzyme is of recent evolutionary origin, but also immediately raises the question of how these genes then were distributed to different geographic locations. One possibility is the widespread use of the chlorinated aliphatic 1,3-dichloropropene as a nematocide in agriculture. It has been suggested that a gene similar to the 1,2-dichloro-

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LinB     MSLGAKP----------FG-EKKFIEIK----GRRMAYIDEGT--GDPIL  33
DehH1    M---------------DFP---GFKNSTVTVDGVDIAYTVSG--EGPPVL  30
DhaA     MSEIGTG----------FPFDPHYVEVL----GERMHYVDVGPRDGTPVL  36
DhlA     MINAIRTPDQRFSNLDQYPFSPNYLDDLPGYPGLRAHYLDEGNSDAEDVF  50
         *                 .     . .      *    *   *   .  ..

LinB     F-QHGNPTSSYLWRNIMPHCAGLG-RLIACDLIGMGDSDKLD-PSGPERY  80
DehH1    M-LHGFPQNRAMWARVAPQLAEHHT-VVCADLRGYGDSDKPKCLPDRSNY  78
DhaA     F-LHGNPTSSYLWRNIIPHVAPSH-RCIAPDLIGMGKSDKPD-LD----Y  79
DhlA     LCLHGEPTWSYLYRKMIPVFAESGARVIAPDFFGFGKSDKP---VDEEDY  97
            ** *    .   . *  *      .  *. * * ***         *

LinB     AYAEHRDYLDALWEALDLGDRVVLVVHDWGSALGFDWARRHRERVQGIAY 130
DehH1    SFRTFAHDQLCVMRHLGF-ERFHLVGHDRGGRTGHRMALDHPEAVLSLTV 127
DhaA     FFDDHVRYLDAFIEALGLEE-VVLVIHDWGSALGFHWAKRNPERVKGIAC 128
DhlA     TFEFHRNFLLALIERLDLRN-ITLVVQDWGGFLGLTLPMADPSRFKRLII 146
          .             *.. .   ** .↑.*.  *   .  .      .

LinB     ME--AIAMPIEWADFPEQDRDLFQAFRS--------QAGEELVLQD-NVF 169
DehH1    MD-----IVPTYAMFMNTNRLVAASYWHWYFLQQPEPFPEHMIGQDPDFF 172
DhaA     ME--FIRPIPTWDEWPEFARETFQAFRTA-------DVGRELIIDQ-NAF 168
DhlA     MNACLMTDPVTQPAFSAFVTQPADGFTAW-------KY--DLVTPS-DLR 186
         *.                        ..             ...    .

LinB     VEQVLPGLILRPLSEAEMAAYREPFLAAEARRPTLSWPRQIPIAGTP-AD 218
DehH1    YETCLFGW-----GATKVSDFDQQMLNA-YRES-WRNPAMIH---GSCSD 213
DhaA     IEGALPKCVVRPLTEVEMDHYREPFLKPVDREPLWRFPNELPIAGEP-AN 217
DhlA     LDQFMKRWA-PTLTEAEASAYAAPFPDTSYQAGVRKFPKMVAQRDQACID 235
          .  .                .         .      *  .     .  .

LinB     -----VVAIARDYAGWLSESPIPKLFINAEPGALTT-GRMRDFCRTWPN- 261
DehH1    YRAAATIDLEHDSADIQRKVECPTLVFYGSKGQM---GQLFDIPAEWAKR 260
DhaA     -----IVALVEAYMNWLHQSPVPKLLFWGTPGVLIPPAEAARLAESLPN- 261
DhlA     -----ISTEAISF--WQNDWNGQTFMAIGMKDKLLGPDVMYPMKALING- 277
                                  .   .  . .

LinB     QTE-ITVA--GAHFIQEDSPDEIGAAIAAFVRRLRPA              295
DehH1    CNNTTNASLPGGHFFVDQFPAETSEILLKFLAR--NG              295
DhaA     CKT-VDIG-PGLHYLQEDNPDLIGSEIA---RWLPAL              293
DhlA     CPEPLEIA-DAGHFVQEFGEQVAREALKHFAE---TE              310
                 .  . ↑.  .         .
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Fig. 4. Sequence alignment of hydrolytic dehalogenases (Table 1). LinB, tetrachorocyclohexadiene dehalogenase; DehH1, fluoroacetate dehalogenase; DhaA, chlorobutane dehalogenase; DhlA, haloalkane dehalogenase.

ethane dehalogenase gene of *X. autotrophicus* GJ10 occurs in a gram-negative organism that was selected from soil that shows a higher rate of 1,3-dichloropropene disappearance (Verhagen *et al.*, 1995), but this has not yet been confirmed by complete sequencing of the dehalogenase gene.

Recently, we have investigated in more detail the catabolic pathway and dehalogenases in a *Pseudomonas cichorii* strain that slowly utilizes 1,3-dichloropropene. The first step is hydrolysis of 1,3-dichloropropene to 3-chloroallylalcohol by a haloalkane dehalogenase. Further metabolism proceeds

Table 1. Dehalogenases of the α/β-hydrolase fold type that have been detected in bacteria that utilize halogenated aliphatics as a carbon source.

	DhlA	LinB	DehHI	DhaA
Organism	*Xanthobacter*, *Ancylobacter*, *Pseudomonas*	*Pseudomonas paucimobilis*	*Moraxella* sp. strain B	*Rhodococcus*, *Pseudomonas cichorii*
"Natural" substrate	1,2-dichloroethane	tetrachloro-cyclohexadiene	fluoroacetate	1-chlorobutane
Substrates	C-1 to C-4 1-chloro-n-alkanes, C-1 to C-12 1-bromo-n-alkanes, C-2 to C-3 α,ω-dihalo-n-alkanes	a number of mono-chlorinated alkanes, 1,2-di-bromoethane	halogenated acetates	C-2 to C-10 1-chloro-n-alkanes, C-1 to C-16 1-bromo-n-alkanes, C-2 to C-9 α,ω-dihalo-n-alkanes
No. of amino acids	310	295	294	293
Catalytic triad[1)] Halide binding	Asp-His-Asp Trp-Trp	Asp-His-Glu Trp-?	Asp-His-Asp Arg(?)-Trp(?)	Asp-His-Glu Trp-?
Molecular mass	35,143	33,050	33,307	33,248
References	Keuning *et al.*, 1985; Janssen *et al.*, 1989; Verschueren *et al.*, 1993	Nagata *et al.*, 1993	Kawasaki *et al.*, 1992	Curragh *et al.*, 1994; Kulakova *et al.*, 1997

[1)]Based on sequence similarity to haloalkane dehalogenase, in which a halide-binding residue is located immediately distal to the nucleophile. In three cases, this is a Trp. The Arg residue in DehH1 may be involved in carboxylate or fluorine binding.

via 3-chloroacrylic acid, and a dehalogenase for the *trans*-isomer of this compound is also present. The haloalkane dehalogenase was sequenced and appeared to be identical to the haloalkane dehalogenase from a *Rhodococcus* (Kulakova *et al.*, 1997). This enzyme belongs to the same group of α/β-hydrolase fold enzymes as the *X. autotrophicus* GJ10 1,2-dichloroethane dehalogenase (Janssen *et al.*, 1994), the fluoroacetate dehalogenase of *Moraxella* (Kawasaki *et al.*, 1992), and the tetrachlorocyclohexadiene hydrolase of lindane-utilizing bacteria (Nagata *et al.*, 1993) (Fig. 3).

These results are in agreement with the hypothesis that the large-scale use of the nematocide 1,3-dichloropropene has stimulated the evolution and spread of haloalkane dehalogenase producing organisms. It also suggests that during the evolution of 1,3-dichloropropene degrading bacteria, a haloalkane dehalogenase gene has been recruited by *P. cichorii* from gram-positive organisms that degrade chlorobutane or related chloroalkanes. The genetic organization of

the dichloropropene degradation genes and the exchange processes that led to the evolution of a 1,3-dichloropropene catabolic pathway are currently under study.

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