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Minireview

Bacterial degradation of xenobiotic compounds: evolution and distribution of novel enzyme activities

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Summary

Bacterial dehalogenases catalyse the cleavage of carbon-halogen bonds, which is a key step in aerobic mineralization pathways of many halogenated compounds that occur as environmental pollutants. There is a broad range of dehalogenases, which can be classified in different protein superfamilies and have fundamentally different catalytic mechanisms. Identical dehalogenases have repeatedly been detected in organisms that were isolated at different geographical locations, indicating that only a restricted number of sequences are used for a certain dehalogenation reaction in organohalogen-utilizing organisms. At the same time, massive random sequencing of environmental DNA, and microbial genome sequencing projects have shown that there is a large diversity of dehalogenase sequences that is not employed by known catabolic pathways. The corresponding proteins may have novel functions and selectivities that could be valuable for biotransformations in the future.

Introduction: degradable and recalcitrant compounds

The recalcitrance of many synthetic chemicals to biodegradation is mainly due to a lack of enzymes that can carry out critical steps in a catabolic pathway. This especially holds for low-molecular weight halogenated compounds. These xenobiotic chemicals are relatively water soluble and bioavailable, and theoretically could be converted by short metabolic routes to intermediates that support cellular growth under aerobic conditions. Yet, no organisms have been found that oxidatively degrade and use as a carbon source important environmental chemicals such as chloroform, trichloroethylene, 1,1,1-trichloroethane, 1,2-dichloropropane and 1,2,3-trichloropropene (Fig. 1).

Attempts to obtain enrichments or pure cultures that aerobically grow on these chemicals have met no success. However, some other halogenated chemicals are easily biodegradable, and cultures that utilize chloroacetate, 2-chloropropionate and 1-chlorobutane can be readily enriched from almost any soil sample (Leisinger, 1996; van Agteren et al., 1998). For still other compounds, degradative organisms can be isolated, but only after prolonged adaptation or if a suitable inoculum is used in which the catabolic activity that is searched for apparently is already enriched due to pre-exposure to halogenated chemicals in the environment. Compounds of this class of intermediate degradability include dichloromethane, 1,2-dichloroethane and the nematocides 1,2-dibromoethane and 1,3-dichloropropene (Fig. 1).

With halogenated compounds, an obvious critical step in a potential biodegradation pathway is dehalogenation. Biochemical research with organisms that grow on halogenated compounds has shown that a broad range of dehalogenases exists, both for aliphatic and aromatic compounds. The enzymes represent different reaction
types, and they may or may not use cofactors for activity. Several X-ray structures have been solved, and these suggest that most of these enzymes have evolved specifically to catalyse a dehalogenation reaction, and are not just enzymes that carry out a certain reaction with a natural compound and fortuitously also cleave carbon-halogen bonds in a xenobiotic organohalogen compound due to catalytic promiscuity. The substrate range of a dehalogenase often determines whether or not a compound is degradable, although the accumulation of toxic intermediates after an initial dehalogenation step may also be the cause of poor metabolism, especially with substrates carrying more than one halogen (van Hylckama Vlieg et al., 2000). In view of the key role of dehalogenases, several questions arise. How do new dehalogenases arise in nature? What are their catalytic mechanisms and how do they influence the range of compounds that can be converted? How are these enzymes distributed in environmental organisms?

This review addresses some of these issues, by describing examples of catalytic mechanisms of dehalogenases that were obtained from X-ray crystallography. From these mechanisms, conserved functional sequence motifs can be derived, and by using these in searches of databases, an estimate is obtained about the abundance of some key dehalogenases in bacteria. The results are compared with the situation for alkane hydroxylases, which are the key enzymes in the initial steps of alkane metabolism. Sequence information and comparison of catabolic gene clusters also shed light on the mechanisms by which new catabolic activities may evolve and become distributed.

Dehalogenase mechanisms

Five dehalogenases that are members of different protein superfamilies have been studied by X-ray crystallography. This has provided detailed insight in their catalytic mechanisms and evolutionary relationships. For several other dehalogenases, structural and mechanistic insight has been obtained by comparing their sequences with other members of the same phylogenetic family, often in combination with mutagenesis experiments. From this work, it has become clear that most dehalogenases belong to protein superfamilies that harbour both dehalogenases and proteins that carry out completely different reactions (de Jong and Dijkstra, 2003; Fig. 2).

The first example is provided by the haloalkane dehalogenases, with three solved X-ray structures (DhlA, DhaA and LinB in Table 1). These proteins possess a $\alpha/\beta$-hydrolase fold main domain, with a cap domain on top of it and the active site located in between the two domains.
Fig. 2. Common dehalogenase mechanisms for aliphatic organochlorine compounds.
A. Hydrolytic dehalogenation catalysed by an α/β-hydrolase fold type haloalkane dehalogenase (DhlA).
B. Hydrolytic dehalogenation catalysed by HAD-type haloacid dehalogenase (DhlB).
C. Dehalogenation by an SDR-type halohydrin dehalogenase (HheC).
D. Chloroacrylic acid dehalogenation by a 4-OT type chloroacrylic acid dehalogenase (CaaD).
(Verschueren et al., 1993). Their properties have recently been reviewed (Janssen, 2004). The α/β-hydrolase structural fold is also found in lipases, acetylcholinesterases, esterases, lactonases, epoxide hydrolases and others, showing that the haloalkane dehalogenases belong to a protein superfamily of which the members carry out diverse reactions, mostly with non-halogenated compounds. The key residues for identification of new haloalkane dehalogenase sequences are the catalytic pentad amino acids: a nucleophilic aspartate, a conserved histidine base close to the C-terminus, an acidic residue located in the sequence between the Asp and His, and two residues involved in binding of the halide. Of these five residues, the nucleophilic Asp, the His and one halide-binding Trp positioned next to the nucleophilic Asp are fully conserved (Fig. 2A).

A rather common group of aliphatic dehalogenases are the haloacid dehalogenases. These have been divided in group I and group II enzymes, of which the latter are well characterized (Hisano et al., 1996; Ridder et al., 1999). They define the so-called HAD superfamily of hydrolases. The enzymes (DhiB, HAD-Ps in Table 1) have a nucleophilic aspartate close to the N-terminus and share structural similarity with the phosphatase domain found in many proteins, including eukaryotic transporter proteins. The conserved nucleophilic aspartate is involved in forming a covalent intermediate by a nucleophilic substitution, similar to what was found in haloalkane dehalogenases. For database analysis, other important catalytic residues are an arginine at a position (Nu + c. 30), which is involved in halide binding, and a lysine further downstream that acts as the catalytic base needed for cleavage of the covalent intermediate (Fig. 2B).

A third family of aliphatic dehalogenases is formed by the halohydrin dehalogenases, of which the structure has been investigated recently (de Jong et al., 2003). The catalytic mechanism of these enzymes is somewhat similar to that of members of the short-chain dehydrogenase-reductase (SDR) superfamily of proteins. Both the dehalogenases and the SDR enzymes possess a conserved catalytic triad for proton abstraction from the hydroxyl group of the substrate (van Hylckama Vlieg et al., 2001). In the case of SDR proteins, the negative charge developing on the hydroxyl oxygen is transferred via a hydride to the NAD(P)+ cofactor; instead, in the dehalogenases it is passed on to the halogen at a neighbouring carbon.

Table 1. Number of positive hits obtained with protein sequences of dehalogenases and alkane hydroxylases as queries in Blast-P searches against protein sequence databases.

<table>
<thead>
<tr>
<th>Query probe</th>
<th>GenBank GI number</th>
<th>Super family</th>
<th>Filtered by conserved pattern or residues</th>
<th>No. of identical genes found in isolates</th>
<th>No. of homologues in microbial genomes</th>
<th>No. of homologues in Archae genomes</th>
<th>No. of homologues in Sargasso Sea</th>
</tr>
</thead>
<tbody>
<tr>
<td>DcmA</td>
<td>482502</td>
<td>GST</td>
<td>126H-W*</td>
<td>11 (&gt; 96%)</td>
<td>2 (33–22%)</td>
<td>0</td>
<td>4 (30–25%)</td>
</tr>
<tr>
<td>DhiA</td>
<td>729681</td>
<td>α/β-HF</td>
<td>124D-W-G, 289H*</td>
<td>12 (100%)</td>
<td>15 (50–26%)</td>
<td>1 (30%)</td>
<td>35 (41–21%)</td>
</tr>
<tr>
<td>DhaA</td>
<td>3114657</td>
<td>α/β-HF</td>
<td>1060-W-G, 272H*</td>
<td>7 (&gt; 98%)</td>
<td>28 (53–23%)</td>
<td>1 (26%)</td>
<td>44 (49–22%)</td>
</tr>
<tr>
<td>LinB</td>
<td>9789853</td>
<td>α/β-HF</td>
<td>108D-W-G, 273F*</td>
<td>3 (&gt; 99%)</td>
<td>10 (69–25%)</td>
<td>0</td>
<td>43 (65–21%)</td>
</tr>
<tr>
<td>DhiB</td>
<td>3122178</td>
<td>HAD</td>
<td>8D, 39F, 147Kf</td>
<td>7 (49–39%)</td>
<td>39 (57–25%)</td>
<td>3 (26–24%)</td>
<td>119 (52–23%)</td>
</tr>
<tr>
<td>HAD-Ps</td>
<td>3122176</td>
<td>SDR</td>
<td>10D, 41F, 151Kf</td>
<td>12 (77–39%)</td>
<td>35 (55–23%)</td>
<td>4 (27–23%)</td>
<td>115 (50–24%)</td>
</tr>
<tr>
<td>HheA</td>
<td>15213645</td>
<td>SDR</td>
<td>135S-(7,17)-(Y-X3)-R*</td>
<td>2 (99%),</td>
<td>6 (30–21%)</td>
<td>0</td>
<td>4 (31–21%)</td>
</tr>
<tr>
<td>HheB</td>
<td>15209119</td>
<td>SDR</td>
<td>126S-(7,17)-(Y-X3)-R*</td>
<td>1 (98%)</td>
<td>16 (45–19%)</td>
<td>1 (27%)</td>
<td>10 (53–21%)</td>
</tr>
<tr>
<td>HheC</td>
<td>15213645</td>
<td>SDR</td>
<td>132S-(7,17)-(Y-X3)-R*</td>
<td>2 (100–92%),</td>
<td>6 (30–20%)</td>
<td>0</td>
<td>5 (31–21%)</td>
</tr>
<tr>
<td>CaaD</td>
<td>10637969</td>
<td>4-OT</td>
<td>9R-X-X-R*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 (35–29%)</td>
</tr>
<tr>
<td>LinA</td>
<td>51859616</td>
<td>GST</td>
<td>25D, 73H#</td>
<td>3 (&gt; 99%)</td>
<td>4 (30–27%)</td>
<td>0</td>
<td>3 (31–25%)</td>
</tr>
<tr>
<td>CbxA</td>
<td>2392484</td>
<td>ECH</td>
<td>90H, 145D#</td>
<td>6 (86–51%)</td>
<td>21 (41–26%)</td>
<td>1 (28%)</td>
<td>48 (37–27%)</td>
</tr>
<tr>
<td>AtzA</td>
<td>32455822</td>
<td>DA</td>
<td>60H-X-H, 243H, 327D*</td>
<td>4 (&gt; 98%)</td>
<td>134 (36–20%)</td>
<td>12 (31–27%)</td>
<td>20 (32–19%)</td>
</tr>
<tr>
<td>TriA</td>
<td>42558845</td>
<td>DA</td>
<td>76H-X-H, 251H, 287H*</td>
<td>2 (&gt; 99%)</td>
<td>156 (36–21%)</td>
<td>21 (26–20%)</td>
<td>32 (34–21%)</td>
</tr>
<tr>
<td>PpcA</td>
<td>22417110</td>
<td>GST</td>
<td>125-X-C*</td>
<td>1 (96%)</td>
<td>2 (37–23%)</td>
<td>0</td>
<td>49 (41–19%)</td>
</tr>
<tr>
<td>AlkB</td>
<td>113639</td>
<td>AH</td>
<td>269 N-Y-X-E-H-Y-G*</td>
<td>9 (100–77%)</td>
<td>21 (50–32%)</td>
<td>0</td>
<td>4 (82%),</td>
</tr>
<tr>
<td>AlkM</td>
<td>2623971</td>
<td>AH</td>
<td>281 N-Y-X-E-H-Y-G*</td>
<td>12 (90–62%)</td>
<td>1 (100%),</td>
<td>0</td>
<td>51 (56–28%)</td>
</tr>
</tbody>
</table>

Protein sequences (column 2) were compared by BlastP (Altschul et al., 1990; 1997) to the NCBI whole microbial database (295 proteomes, April 2005) and to the NCBI environmental Sargasso sea proteins (Venter et al., 2004). Hits with an E-score < 0.01 were considered homologues. Output lists were filtered by looking for conserved residues in a multiple alignment (#, column 4) or by using PHI-BlastP with a conserved pattern (* column 4). If uncertain, sequences in the low-similarity region were used as Blast queries against the Uniprot database to confirm their identity. Results in columns 5–8 give the number of homologues followed by the percentage range of amino acid identities. In some cases very high scores are mentioned separately. AlkB, AlkM: only hits containing motif C (see text) were counted. AlkB scores 100% in one case in column 6 because the whole genome of the original host has been sequenced.

GST, glutathione transferase; α/β-HF, α/β-hydrolase fold family; HAD, haloacid dehalogenase; SDR, short chain dehydrogenase reductase; 4-OT, 4-oxalocrotonate tautomerase; DH, dehydratase; ECH, enoyl CoA hydratase; DA, deaminase; AH, alkane hydroxylase. The abbreviations for the proteins and their functions are given in the text.

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atom, which is displaced by an intramolecular substitution mechanism, resulting in the formation of an epoxide (Fig. 2C, HheA, HheB, HheC). Thus, no covalent intermediate is formed during dechlorination catalysis by this enzyme.

Another group of recently characterized dehalogenating proteins is formed by the chloroacrylic acid dehalogenases (CaaD in Table 1), which are present in bacteria that degrade the nematocide 1,3-dichloropropene. These proteins can dehalogenate a substrate in which the halogen is bound to an sp²-hybridized carbon atom, and there are cis- and trans-specific enzymes (Poelarends and Whitman, 2004). Structures have recently been solved, and they confirmed what was expected on basis of the sequence, i.e. that the enzymes belong to the tautomerase superfamily of proteins. The members of this superfamily are characterized by a ring-like hexameric structure, which is formed by a trimer of dimers. In some members of the tautomerase superfamily, such as 4-oxalocrotonate tautomerase (4-OT), the hexamers are composed of identical peptides, while in other members, such as trans-3-CaaD, they are composed of different but similar peptide chains. Still other members of the superfamily, like cis-3-CaaD, have a trimeric quaternary structure. In these dehalogenases, the catalytic mechanism does not involve the formation of a covalent enzyme-substrate intermediate (de Jong et al., 2004). Instead, the mechanism of trans-3-CaaD is similar to that of a hydratase, with a key role for the N-terminal proline of three of the subunits, which acts as the general acid catalyst that protonates the C-2 carbon atom in trans-3-chloroacrylic acid (Fig. 2D) (Azurmendi et al., 2004). In CaaD, no clear halide-binding site has yet been detected and it is possible that these enzymes mechanistically carry out only a hydration reaction to form an unstable halohydrin intermediate that rapidly dechlorinates.

For several other aliphatic dehalogenases, no structures have been solved, but mechanistic information can be deduced from similarity to enzymes that are well characterized (Leisinger et al., 1994). For example, dichloromethane dehalogenase (DcmA) catalyses the conversion of dichloromethane to formaldehyde in a glutathione-dependent reaction. Catalysis was proposed to proceed via the formation of a reactive S-(chloromethyl)glutathione intermediate that stays bound to the enzyme and decomposes by solvolysis, which may be the rate-limiting step (Stourman et al., 2003). Site-directed mutagenesis of DcmA demonstrated the essentiality of a serine residue in the N-terminal part of the protein, a residue also shown to be critical for catalysis in θ-class glutathione transferases (Vuilleumier and Leisinger, 1996). This conserved catalytic serine may play a role in enhancing the nucleophilicity of the glutathione thiol.

An interesting type of aliphatic dehalogenase is the enzyme (LinA) that is responsible for the first step in the bacterial degradation of lindane (α-hexachlorocyclohexane). In a peculiar reaction, HCl is eliminated, converting the substrate to pentachlorocyclohexene. A structure has not been solved, but a mechanism was predicted on basis of the stereochemistry of the reaction and low but significant sequence similarity to scytalone dehydratase. The reaction is proposed to involve abstraction of an axial proton, with concomitant anti-elimination of a trans-axial chloride from the adjacent carbon atom (Nagata et al., 2001; Trantirek et al., 2001). A conserved His-Asp pair was proposed to be involved in proton abstraction, but details about halogen binding and stabilization are still lacking.

If we consider aromatic compounds, it is again possible to distinguish several phylogenetically and mechanistically different dehalogenases. The carbon-halogen bond between a halogen and an arenic or vinylic carbon atom is more difficult to cleave than the one between a halogen and an sp²-hybridized carbon atom. Therefore, most haloaromatics are dehalogenated after ring cleavage. However, a nucleophilic displacement of aromatic halogens occurs in 4-chlorobenzoate degradation. This is the fifth type of dehalogenase for which a structure has been solved (Benning et al., 1998). The compound is first activated by conjugation to coenzyme A, after which a hydrolytic dehalogenase (CbzA) that belongs to the enoyl hydratase superfamily, displaces the halogen by a nucleophilic addition-elimination mechanism. No distinct halide-binding site is present in the X-ray structure, but a nucleophilic aspartate and a histidine involved in catalysis were identified (Zhang et al., 2001). Another aromatic dehalogenase is tetrachlorohydroquinone dehalogenase. This enzyme catalyses the replacement of chlorine by hydrogen in tetra- and trichlorohydroquinone during the degradation of pentachlorophenol by Sphingobium chlorophenolicum (Kiefer and Copley, 2002). The protein (PcpC in Table 1) bears sequence and mechanistic similarity to glutathione transferases.

A specific hydrolytic dehalogenase (AtzA) is also involved in the bacterial degradation of atrazine. This enzyme is related to melamine deaminase (TriA) and the AtzA and TriA proteins differ only by nine amino acid substitutions (Seffernick et al., 2001).

Origin and distribution of dehalogenase gene sequences

Analysis of the genetic organization of biodegradation pathways provides insight into the genetic processes that led to their evolution. It appears that catabolic genes for xenobiotic compounds are often associated with transposable elements and insertion sequences. They are also fre-
dehalogenase to members of an enzyme superfamily that catalyse other reactions generally does not provide information about the process of adaptation to xenobiotic compounds. The level of sequence similarity that exists between a dehalogenase and other proteins in a phylogenetic family is usually less than 50% (Table 1). Therefore, the time of divergence should be much earlier than a century ago, and the process of divergence thus cannot be related to the introduction of industrial chemicals into the environment. If the dehalogenases and other critical enzymes that occur in catabolic pathways have undergone recent mutations, there should be closely related sequences in nature that differ from the current enzymes by only a few mutations. No such primitive dehalogenase has yet been detected, with the notable exception of TriA, the enzyme that dehalogenates the herbicide atrazine (vide infra).

Another issue is the function of the pre-industrial dehalogenase or dehalogenase-like sequences from which the current catabolic systems with their activated and mobilized genes originate. The original genes may have been involved in the dehalogenation of naturally occurring halogenated compounds, of which there are many (Gribble, 1998). Such proteins may fortuitously also have been active with a xenobiotic halogenated substrate, just because of their lack of substrate specificity. Alternatively, the evolutionary precursor of a dehalogenase may have catalysed a different reaction that has some mechanistic similarity to dehalogenation, in which case the original enzyme may or may not have shown some dehalogenation activity due to catalytic promiscuity. Finally, the precursor genes for dehalogenases may have been silent or cryptic genes, with no clear function for the pre-industrial host (Hall \textit{et al}., 1983). In all cases, the gene could have become functional in a dehalogenation pathway as a result of the fortuitous ability to catalyse dehalogenation of a xenobiotic compound, possibly after acquisition of some mutations.

One way to obtain information about the evolutionary origin of dehalogenase genes is to compare the dehalogenase sequences that have been detected in different bacterial cultures. If closely related sequences are present, this would make it possible to identify sequence differences and to determine the effect of the mutations on substrate selectivity. Another approach is to search for sequences that are closely related to dehalogenases in databases of sequenced genomes. These organisms have, with some recent exceptions, no known history of degradation of halogenated compounds. Currently, the whole sequence of more than 300 bacterial genomes is available. If we find closely related sequences in these databases, they could define evolutionary ancestors of the current dehalogenases. A further potential source of new dehalogenase sequences could come from metagenome.

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libraries. Several of these libraries have been prepared, and some have been used for massive sequencing (Venter et al., 2004), whereas others have been explored for the presence of new biotransformation enzymes.

Analysis of sequence databases such as Pfam and of the literature revealed that in several cases the same dehalogenase sequence has repeatedly been detected in organisms that have been isolated from different geographical locations. Thus, identical or almost identical haloalkane dehalogenases, dichloromethane dehalogenases and atrazine chlorohydrolases have been detected in isolates from different areas (Table 1). This suggests that either these sequences have been recruited from the pre-industrial gene pool only once, after which they became distributed by horizontal transmission, or they have been repeatedly recruited from an identical pre-industrial sequence. By itself, the worldwide distribution of identical sequences is common in the bacterial world, as 100% sequence identity is normal for specific genes within different strains of the same species. However, it is currently unknown by what mechanisms a possible process of global distribution of recently evolved catabolic genes may occur.

The structural and mechanistic analysis of dehalogenases makes it possible to define sequence fingerprints that allow the identification of genes that are phylogenetically related in genomic databases. The current version of the NCBI databases lists more than 250 proteome sequences of bacteria, and 31 archaenal proteomes. When these were searched for 15 different dehalogenase sequences that have been detected in organisms isolated on halogenated compounds as a carbon source, it appeared that not a single closely related counterpart of dehalogenase genes was present in these sequenced bacterial genomes (Table 1). Even close homologues are absent, including for those sequences that have repeatedly been detected in identical form in independently isolated bacteria. For example, both dichloromethane dehalogenase and 1,2-dichloroethane dehalogenase have been detected in essentially identical form in more than 10 independently isolated bacterial cultures that originate from different geographical locations, but no close homologue is present in the sequenced microbial genomes. Similarly, Blast searches with dehalogenase sequences against the sequences of environmental DNA obtained in the Sargasso Sea sequencing project (Venter et al., 2004) again indicated that no close homologues are present in the 1.2 million new genes that were discovered in this massive DNA sequencing project (Table 1). Thus, sequences that have repeatedly been acquired by batch enrichment were not detected in the environmental DNA. Marchesi and Weightman (2003), working with haloacid dehalogenases (HAD superfamily), also described that a bias is introduced by enrichment and isolation. Both for group I dehalogenases and for group II (HAD) enzymes, the diversity and phylogenetic grouping of dehalogenase sequences obtained via pure cultures significantly deviated from what was observed by analysis of DNA directly isolated from the samples that were used for enrichment, such as activated sludge.

At the same time, it appears that more distantly related dehalogenase sequences are quite common. For example, large numbers of putative haloalkane dehalogenase and haloacid dehalogenase sequences are present in the whole-genome database and in the Sargasso Sea database (Table 1). The identification of these sequences is based on detailed insight in structure–function relationships and the fact that several homologous sequences in these protein families have been detected that indeed encode functional dehalogenases. The exact activity of the proteins encoded by putative dehalogenase sequences is difficult to predict. Jesenska and colleagues (2002) have shown that at least one active dehalogenase is encoded by the M. tuberculosis genome, but its physiological function is unclear. In the HAD superfamily of enzymes, at least eight different sequences have been found to encode functional haloacid dehalogenases, and the environmental diversity may be large (Marchesi and Weightman, 2003). In other cases, e.g. with CaaD and hexachlorocyclohexane dechlorinase (LinA), it is more difficult to predict which of the homologues do encode dehalogenating enzymes because the sequence similarity is low and the residues involved in dehalogenation have been less well identified.

**Haloalkane dehalogenase from Xanthobacter autotrophicus (DhIA)**

Haloalkane dehalogenase (DhIA) was originally discovered in X. autotrophicus GJ10, a nitrogen-fixing hydrogen bacterium that was enriched with 1,2-dichloroethane as the sole carbon source. Subsequently, identical DhIA-encoding genes have been discovered in several other strains of X. autotrophicus, isolated in the Netherlands and in Germany, and in isolates of Ancylobacter aquaticus obtained with 1,2-dichloroethane or chloroethylvinyl ether as the growth substrate (van den Wijngaard et al., 1992). Recently, a strain of Xanthobacter flavus was isolated in South Korea, and this organism also possessed an identical dehalogenase (Song et al., 2004). In fact, DhIA is still the only known hydrolytic haloalkane dehalogenase that operates in 1,2-dichloroethane degrading bacteria; no variants have been described, and at least 12 identical copies of this gene have been obtained from different environmental isolates. The question arises: where does this enzyme that has such a remarkable activity with a very stable xenobiotic compound come from? Did
structed this so-called retro genetics approach has been con-
evolved activity with 1,2-dichloroethane. All these muta-
tions influenced the region of the cap domain of haloal-
kanate dehalogenase proximal to the tryptophan that 
does not pre-exist at the time industrial production of 1,2-
dichloroethane started, or did it evolve from an ancestor 
that converted some halogenated or non-halogenated 
compound?

Insight into this issue has been obtained by inspection 
of the wild-type sequence and by experimental evolution 
methods. The N-terminal part of the cap domain of the 
dehalogenase harbours two short tandem sequence 
repeats (at the DNA level: one perfect 15 bp repeat and 
one 9 bp repeat carrying one substitution). These repeats 
occurred in a segment of the protein that was observed 
to be the target of substitutions, deletions and generation 
of new repeats when mutants were selected that pos-
sessed an enhanced activity toward 1-chlorohexane 
(Pries et al., 1994). Thus, experimental evolution leads 
to duplications and other mutations in a part of the 
sequence where the wild-type already harbours 
sequence repeats. This observation led to the hypothesis 
that the sequence repeats in the wild-type dhIA gene 
were the result of recent evolutionary events that caused 
adaptation to 1,2-dichloroethane. An insertion is detected 
in the same region of the cap domain when a sequence 
alignment is performed between DhIA and its most similar 
sequence and a regulatory gene, and on the downstream 
side an alcohol dehydrogenase and an aldehyde dehydro-
genase gene was preceded by the same invertase gene 
and a haloalkane dehalogenase, and in all cases the dehaloge-

Assuming that the short repeats present in the cap 
domain sequence of the wild-type dehalogenase were 
indeed generated recently in a pre-industrial dehaloge-
nase, one can postulate a DhIA sequence for this hypo-

thetic primitive dehalogenase (Pikkemaat and Janssen, 
2002) (Fig. 3B). The primitive dhIA sequence predicted by 
this so-called retro genetics approach has been con-
structed in vitro, an it appeared to encode a protein with 
significant activity toward bromoalkanes, but was com-
pletely inactive toward 1,2-dichloroethane. Thus, the 
ancestral dehalogenase may have been a debrominating 
enzyme. Subsequently, we created with the incremen-
tal truncation method (ITCHY) a library of derivatives of 
the primitive DhIA that carried random direct repeats in 
the same region where the wild-type sequence harboured 
repeats. Some of the repeat-carrying variants indeed had 
evolved activity with 1,2-dichloroethane. All these muta-
tions influenced the region of the cap domain of haloal-
kanate dehalogenase proximal to the tryptophan that 
donates a hydrogen bond to the leaving halide (Trp175) 
(Pikkemaat and Janssen, 2002).

These results are all in agreement with the hypothesis 
that the current 1,2-dichloroethane dehalogenase has 
evolved by a short evolutionary pathway from a pre-
existing (pre-industrial) haloalkane dehalogenase that 
was active with brominated but not with chlorinated 
compounds. Association with transmissible plasmids and 
other mobile genetic elements has facilitated the world-
wide spread of the evolved dehalogenase.

**Rhodococcus haloalkane dehalogenase (DhaA)**

Another abundant type of haloalkane dehalogenase is the 
one from *Rhodococcus erythropolis* (DhaA). Mutually 
identical copies of the *dhaA* genes have been detected in 
different organisms, of which the taxonomy has been 
rather confusing due to errors in classification. The first 
*dhaA* sequence was determined by Kulakova and col-
leagues (1997), using a strain of *Rhodococcus (Rhodo-
coccus rhodochrous)* NCIMB 13064 (that was isolated 
on 1-chlorobutane. Later sequencing of several other 
haloalkane dehalogenase genes from 1-chlorobutane-, 1-
chlorohexane- and 1,6-dichlorohexane-degrading gram-
positive organisms, some of which had been isolated 
before strain NCIMB 13064, showed that these pos-
sessed identical *dhaA* sequences. Poelarends and 
colleagues (2000b) have reclassified several organisms 
and using 16S rRNA gene sequencing they showed that 
*R. erythropolis* Y2 (England), *R. rhodochrous* NCIMB13064 (N. Ireland), *Corynebacterium* sp. strain 
m15 (Japan), *Arthrobacter* strain HA1 (Switzerland), 
strain GJ70 (the Netherlands, originally called *Acinetobacter*) 
and strain TB2 (USA) should all be classified as 
*R. erythropolis*. All these strains possessed the same 
haloalkane dehalogenase, and in all cases the dehaloge-

The reservoir of haloalkane dehalogenase-producing 
rhodococci may have been the source for catabolic path-
ways that are active with more exotic haloalkanes (Fig. 4). 
In the first place, enrichment of 1,2-dibromoethane 
degrading bacteria has yielded, after much patience, a 
culture that can slowly grow with 1,2-dibromoethane as 
sol carbon source. This *Mycobacterium* strain produces 
a haloalkane dehalogenase that is almost identical to 
DhaA, but there are three substitutions (C172F, P248S, 
Y272F), and, most remarkably, on the C-terminal side the 
enzyme is 14 amino acids longer due to an in-frame fusion 
of the 3′ end of the dehalogenase gene with 42 bases that
encode the last 14 amino acids of a halohydrin dehalogenase (hheB gene) (Poelarends et al., 1999). Thus, this chimeric dehalogenase is 307 amino acids long instead of the 293 amino acids of the standard Rhodococcus enzyme. The fusion does not seem to be necessary for the catalytic activity of the protein: 1,2-dibromoethane is an excellent substrate for both DhaA variants. Instead, it happens to be there as a fortuitous result of a gene fusion event that occurred during the evolution of the haloalkane dehalogenase gene region.

Other striking differences between the organization of the Mycobacterium GP1 dhaA region and that of the original Rhodococcus exist in the regulatory gene that represses DhaA formation in R. erythropolis when no
inducer is present (Poelarends et al., 2000a). This regulatory gene is inactivated in Mycobacterium strain GP1 by a small deletion, which is required to allow gene expression because 1,2-dibromoethane does not act as an inducer. Here, we see another sign of recent pathway evolution: constitutive expression of a structural gene due to the absence of a functional regulatory gene. Dehalogenation of a xenobiotic organohalogen compound requires a catalytic protein that can convert the compound: the dehalogenase. Regulated expression requires a second protein that recognizes and responds to the xenobiotic chemical: the regulatory protein. Constitutive expression of dehalogenase genes has been demonstrated for atrazine chlorohydrolase (AtzA), 1,2-dichloroethane dehalogenase (DhlA), L-2-haloacid dehalogenase (DhlB), trans-3-CaaD, γ-hexachlorocyclohexane dehydrochlorinase (LinA) and 1,3,4,6-tetrachloro-1,4-cyclohexadiene chlorohydrolase (LinB), suggesting that evolution is recent and has not yet led to systems for regulating gene expression. However, the haloalkane dehalogenase gene is associated with a putative integrase sequence, which is also the case in Mycobacterium sp. GP1. It is well possible that gene acquisition by integrases plays an important role during acquisition of new catabolic gene clusters for xenobiotic compounds. It is tempting to speculate that the fortuitous fusion that we see between the dhaA gene and a segment of the hheB gene in strain GP1 has something to do with the activity of an integron-like gene acquisition system.

**Atrazine chlorohydrolase (AtzA)**

The bacterial degradation of the herbicide atrazine by Pseudomonas ADP starts with a hydrolytic dechlorination, catalysed by an enzyme called atrazine chlorohydrolase (AtzA) (Fig. 5). Identical atrazine chlorohydrolase genes have been obtained from different sources, including an Arthrobacter from China (Cai et al., 2003), strains from France (Rousseaux et al., 2001), an undescribed β-proteobacterium strain CDB21 from Japan of which the sequence was deposited by Iwasaki and colleagues (EMBL Accession number AB194097), and four other isolates from different locations including an Alcaligenes, a Ralstonia and an Agrobacterium sp. (de Souza et al., 1998). The protein belongs to the amidohydrolase superfamily, which also houses the next two enzymes of the atrazine catabolic pathway: hydroxyatrazine ethylaminohydrolase (AtzB) and N-isopropylammelide N-isopropylaminohydrolase (AtzC) (de Souza et al., 1996). Other members of the amidohydrolase superfamily are triazine deaminase, hydantoinase, melamine deaminase, cytosine deaminase and phosphotriesterase. All these (αβ)₈ barrel proteins show rather low sequence identities in pairwise comparisons, with the exception of AtzA and melamine.
(2,4,6-triamino-1,3,5-triazine) deaminase (TriA), which are 98% identical (Seffernick et al., 2001). Thus, AtzA and TriA are closely related, indicating very recent evolutionary divergence. Both atrazine and melamine are xenobiotic compounds, but melamine has been in use for a longer period of time than atrazine. Accordingly, it was proposed that TriA may have functioned as a precursor for AtzA. The proteins differ only by nine out of 475 amino acids, and using gene shuffling a number of hybrids has been obtained from which determinants of the substrate specificity could be identified (Raillard et al., 2001). It appeared that a wide range of activities could be obtained in such a library of hybrids, but leaving group specificity was mainly determined by residue 328. An Asn at this position allowed dechlorination, whereas an Asp gave an enzyme with activity mainly determined by residue 328. An Asn at this position could be identified (Raillard et al., 2001).

Dialkylamino-triazines, albeit with a relatively low catalytic rate. Dechlorination activity may thus have evolved from deamination activity.

An overlap in these two activities was indeed observed for another member of the amidohydrolase superfamily, AtzB, which is the second enzyme in the normal atrazine degradation pathway (Fig. 5). AtzB is catalytically promiscuous and can not only catalyse a deamination reaction, but also is able to catalyse the dechlorination of the substrate analogue 2-chloro-4-amino-6-hydroxy-s-triazine, which is an intermediate in an alternative atrazine degradation pathway (Fig. 5) (Boundy-Mills et al., 1997).

These observations suggest that atrazine hydrolase and melamine deaminase very recently diverged from a pre-existing hydrolase that mainly acted as a deaminase or may have had both dehalogenase and deaminase activity. It was proposed that the possibility to achieve variations in substrate range in a key enzyme by an organism is a measure of the potential to recruit new catabolic activities (Wackett, 2004). In general, catalytic promiscuity could play an important role in the evolution of new enzyme activities (Aharoni et al., 2005).

**Fig. 5.** Degradation of atrazine by AtzA and AtzB. These hydrolases both are members of the amidohydrolase superfamily. AtzA is highly similar to TriA, the enzyme that deaminates the amino analogue of atrazine called melamine. There are only nine substitutions and a short evolutionary pathway may be involved in adaptation of TriA to atrazine (Seffernick et al., 2001). AtzB catalyses the second step in atrazine degradation, which is a deamination reaction, but it can also catalyse a dechlorination reaction with the atrazine soil metabolite 2-chloro-4,6-diamino-s-triazine to yield ammelide (Boundy-Mills et al., 1997). These observations suggest that dechlorination and deamination activities are evolutionarily closely related.
of the Fe ions in the di-iron active site. The conserved histidines are situated in three motifs (HX₃₋₄H, HX₃₋₄HH and H/OX₃₋₄HH) and are here referred to as motif C. In almost all alkane hydroxylases a histidine-containing motif (NYXEHYG), referred to as motif C, was identified (Smits et al., 1999). The genes encoding alkane oxidation in P. putida GPo1 are located on the OCT-plasmid in two operons. The alkBF-GHJKL operon encodes the alkane hydroxylase (AlkB), two rubredoxins, an aldehyde dehydrogenase, an alcohol dehydrogenase, an acyl-CoA synthetase and an outer membrane protein of unknown function. The alkST operon encodes a regulator of expression and a rubredoxin reductase.

A similar well-characterized alkane hydroxylase system is present in Acinetobacter sp. strain ADP1 (Ratajczak et al., 1998). It converts long-chain alkanes ranging from C₁₂ to C₁₆ and the sequence has the same conserved motifs for iron binding. As just mentioned, the alk genes of strain GPo1 are plasmid-localized, but in strain ADP1 the alkane hydroxylase genes are present at different locations on the chromosome. This likely correlates with the presence or absence of selection pressure in the environment from which the organisms were isolated: strain GPo1 was isolated from oil, while strain ADP1 has no record of exposure to high levels of alkanes before or during its isolation. On the other hand, both AlkB and AlkM expression are regulated, which is evolutionary more advanced that what was observed with several dehalogenases, which are often constitutively expressed.

Using the alkB gene sequence it was possible to develop probes that could be applied for detecting genes related to alkB in bacteria isolated from oil-contaminated environments or from uncontaminated soils. Many positives have been found, in some cases with a high sequence identity (> 70%) to the alkB gene (Sotsky et al., 1994; Smits et al., 1999; Vomberg and Klinner, 2000; Whyte et al., 2002a). Among these sequences is one from Alcanivorax borkumensis AP1. Thus, the alkB gene or close homologues appear to occur quite often, and nine different alkane-degrading cultures have been described to possess highly similar alkane hydroxylases (Table 1). For the P. aeruginosa PAO1 alkB variant more than 10 identical sequences were found in P. aeruginosa strains from clinical and soil samples.

Alkane hydroxylase systems that are less related to AlkB or AlkM also occur, for example in gram-positive bacteria. van Beilen and colleagues (2002) detected quite diverse alkane hydroxylases in organisms from a trickle bed reactor that degraded alkanes. Diversity was also observed by Whyte and colleagues (2002b), who studied the hydroxylase system of an alkane-degrading R. rhodochrous and a R. erythropolis strain. The organisms contain three to five distinguished alkane hydroxylase systems that all are only distantly related to AlkB, but almost identical between the two different Rhodococcus strains. There are also variations in genetic organization, and one of the Rhodococcus systems has all three alkane hydroxylase components clustered in a single putative operon.

Whole genome sequencing of bacteria revealed a significant number of putative alkane hydroxylases. Both for AlkB of P. putida GPo1 and for AlkM, hits were obtained with sequence identity percentages up to 50%. Using growth complementation assays in different hosts, the functionality of more than 18 different putative alkane hydroxylases was proven, including those from Burkholderia cepacia RR10, P. aeruginosa PAO1 and Mycobacterium tuberculosis H37Rv (van Beilen et al., 1994; 2003; Smits et al., 2002). When searching for putative alkane hydroxylase genes in environmental DNA (Venter et al., 2004), a large number of alkB and alkM homologues were again detected, including two sequences that were 82% similar to alkB. These are the closest homologues of any of the sequences tested in Table 1. Apparently, alkane hydroxylases are highly abundant in the microbial population in the Sargasso Sea. Of these alkane hydroxylases, apart from the two hits just mentioned, only a few were closely related to AlkB or AlkM or other alk homologues that have been detected in isolates. This suggests that a large number of alkane hydroxylase sequences are present in the environment. The conservation of the catalytically important motifs described above suggests that most of these sequences encode functional enzymes.

The above observations indicate that the diversity of functional alkane hydroxylases is much broader than the diversity of dehalogenases that act on xenobiotic substrates. Thus, whereas very few solutions have evolved for the incorporation of dehalogenase genes in catabolic pathways for nematocides such as 1,2-dibromoethane and 1,2-dichloroethane, several different alkane hydroxylase genes have been recruited in operons for alkane degradation. This correlates with the more widespread occurrence of alkane degraders and the easier degradability of n-alkanes as compared with many halogenated compounds.

Conclusions: accession and exploration of sequence space

One of the striking observations concerning the distribution and evolution of catabolic genes is that identical sequences have repeatedly been detected in organisms that are enriched on xenobiotic halogenated substrates as
a carbon source. This holds for dichloromethane dehalogenase (DcmA), haloalkane dehalogenases (DhIA, DhaA, LinB) and atrazine chlorohydrolase (AtzA). Probably, the number of solutions that nature has found to degrade these compounds is very small, and horizontal distribution occurs faster than generation of new pathways. Indeed, the dehalogenase genes are often associated with integrase genes, invertase genes, or insertion elements, and they are usually localized on mobile plasmids. Recent mobilization of a dehalogenase gene across the gram-border has been suggested in at least one case.

It remains unknown to what degree the current active dehalogenases differ from their proposed recent evolutionary ancestors. Only for 1,2-dichloroethane dehalogenase and atrazine chlorohydrolase short evolutionary pathways have been suggested that could describe the generation of a functional dehalogenase from a closely related precursor sequence that in pre-industrial times may have acted on a different substrate. The diversity of such specialized dehalogenases that act on xenobiotic compounds seems to be more restricted than that of enzymes converting alkanes, which are easier to degrade and for which organisms are more common.

Even though the same dehalogenase sequences are detected in organisms that are isolated in different geographical areas, and in some cases even on different substrates, they are not the most abundant dehalogenase sequences identified in whole genome sequencing projects and massive random sequencing. Thus, it appears that enrichment techniques explore a different segment of sequence space than massive sequencing of environmental DNA. The presence of large numbers of unexplored functional sequences in genomic databases suggests that the biotransformation scope of microbial systems has an enormous potential for further growth.

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