Chapter 7

Summary and concluding remarks

INTRODUCTION

Although a large variety of halogenated hydrocarbons that occur in the environment are of natural origin, the widespread use and release of synthetic halogenated compounds in industry and agriculture over the past 100 years has introduced many man-made xenobiotic halogenated organics into the environment. Such synthetic organohalogenes are generally more persistent than natural organohalogenes. The lack of biodegradation is often due to the inability of microorganisms to effectively metabolize compounds with chemical structures to which microorganisms have not been exposed during the course of evolution. In other words, recalcitrance generally is the result of a lack of efficient metabolic pathways. Nevertheless, many examples are available of microorganisms that have the ability to metabolize xenobiotic organohalogenes (see Chapter 1 of this thesis). This led to the idea that such microorganisms must have evolved their catabolic pathways during the past few decades, and are thus suited to study the natural assembly of catabolic routes.

We decided that 1,3-dichloropropene (DCP) and 1,2-dibromoethane (DBE) are good model compounds to investigate the process of microbial adaptation to synthetic halogenated organic compounds for the following reasons. First, these compounds are not known to be produced naturally in significant concentrations (in contrast to many halogenated aromatics). Second, they were first introduced as nematocidic soil fumigants in the 1950s and are used in large quantities since that time. Third, no pure bacterial cultures were known that could use these xenobiotic pollutants as the sole carbon and energy source. Thus, our approach was to isolate bacterial strains that could aerobically utilize these xenobiotics as growth substrate and then characterize in detail the genetics and biochemistry of the corresponding pathways to establish the unique characteristics of DCP and DBE metabolism.

In the remainder of this Chapter we summarize our results and discuss the evolution of DCP and DBE degradative pathways, with the emphasis on the recruitment of the dehalogenating enzymes involved in the detoxification of these pollutants. Since it appeared that the gene encoding the first dehalogenating enzyme in both pathways originates from a haloalkane catabolic gene cluster that is widespread among gram-positive bacteria, we will first discuss the genetic organization of this globally distributed gene cluster.
GLOBAL DISTRIBUTION OF A GENE CLUSTER ENCODING HALOALKANE CATABOLISM

The capacity to use 1-chlorobutane and similar 1-halo-n-alkanes as a carbon source is widespread among gram-positive bacteria. Such organisms can easily be isolated from contaminated soil as well as from pristine soil or sludge samples (G.J. Poelarends, J.E.T. van Hylckama Vlieg, T. Bosma, and D.B. Janssen, unpublished observations). Several research groups have isolated and characterized gram-positive haloalkane degraders from widely separated geographical locations (Table 1). Some of these organisms were initially identified as strains of the genera Arthrobacter, Corynebacterium or Acinetobacter (12, 21, 33), but we showed that on the basis of 16S rRNA gene sequence analysis all these strains should be classified as strains of Rhodococcus erythropolis or a closely related species (Chapter 5).

Table 1. List of chloroalkane-degrading gram-positive strains carrying a highly conserved haloalkane catabolic gene cluster

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Growth substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain Y2</td>
<td>UK</td>
<td>1-chlorobutane</td>
<td>20</td>
</tr>
<tr>
<td>Strain NCIMB13064</td>
<td>UK</td>
<td>1-chlorobutane</td>
<td>5</td>
</tr>
<tr>
<td>Strain m15-3</td>
<td>Japan</td>
<td>1-chlorobutane</td>
<td>32</td>
</tr>
<tr>
<td>Strain HA1</td>
<td>Switzerland</td>
<td>1-chlorohexane</td>
<td>21</td>
</tr>
<tr>
<td>Strain GJ70</td>
<td>Netherlands</td>
<td>1,6-dichlorohexane</td>
<td>12</td>
</tr>
<tr>
<td>Strain TB2</td>
<td>USA</td>
<td>1-chlorobutane</td>
<td>Chapter 5</td>
</tr>
</tbody>
</table>

Characterization of the 1-chlorobutane-utilizing bacterium R. erythropolis NCIMB 13064 led to a picture of the metabolic pathway for 1-chlorobutane degradation and of the different genes which are involved in this route (5, 15, 16, Chapter 4). The first step is the conversion of 1-chlorobutane to n-butanol by an inducible hydrolytic haloalkane dehalogenase (Fig. 1A). The alcohol intermediate is oxidized in two steps to n-butyric acid by the sequential action of an alcohol dehydrogenase (AdhA) and an aldehyde dehydrogenase (AldA). Butyric acid is further metabolized by β-oxidation to acetaldehyde, which then enters the central metabolic pathway. The different genes encoding these initial metabolic steps were found in a cluster on an autotransmissible 100 kb plasmid designated pRTL1 (Fig. 2) (Chapter 4). Directly upstream of the haloalkane-degradative genes, two additional genes, dhaR and invA, were found. The dhaR gene product is a member of the TetR family of transcriptional repressor-type regulators and appears to function as a repressor for the dhaA gene. The invA gene encodes a protein that shares extensive similarity with proteins belonging to the invertase family of site-specific recombinases. InvA is most similar to the well-characterized invertases
Pin of *Escherichia coli* (50%) and Hin of *Salmonella enterica* serovar Typhimurium (48%). The Pin and Hin invertases are responsible for the inversion of a specific DNA fragment that can serve as a genetic switch that determines the expression of alternative sets of genes (18, 35), but it is not known yet whether InvA plays an analogous role in regulating expression of haloalkane catabolic genes.

Figure 1. Initial enzymatic steps in the degradation of 1-chlorobutane by *R. erythropolis* NCIMB13064 (A), 1,3-dichloropropene by *P. pavonaceae* 170 (B), and 1,2-dibromoethane by *Mycobacterium* sp. strain GP1 (C). Abbreviations: DhaA and DhaA<sub>f</sub>, haloalkane dehalogenases; AdhA, alcohol dehydrogenase; AldA, aldehyde dehydrogenase; HheB, haloalcohol dehalogenase; CaaD, *trans*-3-chloroacrylic acid dehalogenase; X, alcohol dehydrogenase cofactor; Y, aldehyde dehydrogenase cofactor.

A number of haloalkane dehalogenase-negative derivatives of strain NCIMB13064 were isolated. These spontaneous mutants showed rearrangements in both the chromosome and plasmid pRTL1 (15). Further analysis of the mutants revealed that a pRTL1-located insertion element, designated IS2112, was involved in these genome rearrangements, although no direct link between the rearrangements and IS2112 transposition could be demonstrated.
Two copies of IS2112 are present on pRTL1, but these sequences do not flank the haloalkane catabolic genes, and thus do not comprise a single composite transposon encoding haloalkane catabolism.

Until recently, nothing was known about the genes involved in haloalkane metabolism in the other five gram-positive strains listed in Table 1. The biochemical characteristics of the haloalkane dehalogenases isolated from these strains, however, closely resembled those of the haloalkane dehalogenase (DhaA) from *R. erythropolis* NCIMB13064 (6). This notion prompted us to test whether the haloalkane dehalogenase genes of these strains were similar to the *dhaA* gene of strain NCIMB13064. Remarkably, the haloalkane dehalogenase genes of these five haloalkane degraders, which were isolated from widely separated geographical locations, were all found to be completely identical to the *dhaA* gene (Chapter 5). Hybridization and sequence analysis of the genetic environment of the *dhaA* gene in each of the haloalkane degraders indicated high similarity to the haloalkane catabolic gene cluster found on plasmid pRTL1 in strain NCIMB13064 (Fig. 2) (Chapters 4 and 5). Apparently, the capacity to use haloalkanes as a carbon source has become widespread among gram-positive bacteria due to the global and recent distribution of a single catabolic gene cluster. In all strains analyzed, the gene cluster is localized on plasmids, suggesting a role for these mobile elements in gene transfer (Chapter 5).

Figure 2. A comparison of the genetic environment of the haloalkane dehalogenase genes in *R. erythropolis* NCIMB13064 and *P. pavonaceae* 170. Genes are shown as boxes, and arrows indicate the direction of transcription. Identical hatching indicates identical sequences in both dehalogenase gene regions. The two deletions within the acquired genetic element in *P. pavonaceae* 170 are indicated by dotted lines. A putative transposase ORF (*tnpA*), which is located next to the acquired genetic element, is interrupted by an insertion element, identified as IS1071 (Chapter 4). The *intP* gene encodes a putative (site-specific) DNA integrase.
EVOLUTION OF THE METABOLIC PATHWAY FOR DCP DEGRADATION: RECRUITMENT OF A HALOALKANE DEHALOGENASE THAT IS UNIQUE FOR GRAM-NEGATIVE BACTERIA

DCP is the major and active ingredient of the pesticidal formulations “D-D” (Shell Oil Company) and “Telone II” (Dow Chemical Company). These synthetic products are used worldwide in large amounts for the control of plant-parasitic nematodes. Fumigants such as D-D and Telone II represent an important class of carcinogenic water pollutants because their components can easily permeate through soils into groundwater supplies (4, 29). Initial studies indicated that the environmental degradation of both DCP isomers is mainly the result of microbial action, with the exception of the rate-limiting chemical hydrolysis of DCP to 3-chloroallyl alcohol (2, 19, 26). However, no definitive information was presented regarding the bacterial strains responsible for DCP degradation. The first report concerning enrichment and isolation of DCP-utilizing organisms was published in 1995 (30). In this report, Van Elsas and coworkers described that repeated treatment of soils with DCP resulted in accelerated biodegradation of this compound. Several DCP-degrading bacterial strains, belonging to the genera Alcaligenes and Pseudomonas, were isolated from such adapted soils. One strain was characterized and initially identified as Pseudomonas cichorii (170). On the basis of 16S rRNA gene sequence analysis, this organism was reclassified as Pseudomonas pavonaceae (170) (G. J. Poelarends and J. R. Marchesi, unpublished results).

Characterization of strain 170 (Chapter 2), along with 3-chloroallyl alcohol and 3-chloroacrylic acid-degrading strains isolated by others (1, 11, 27), led to a good picture of the metabolic pathway for DCP degradation (Fig. 1B). Although strain 170 was isolated solely for its ability to utilize cis-DCP as a growth substrate, it was also found capable of utilizing trans-DCP. In the first metabolic step, the DCP isomers are hydrolyzed to their corresponding 3-chloroallyl alcohol isomers by a haloalkane dehalogenase. Further conversion of the 3-chloroallyl alcohols proceeds via oxidation to cis- and trans-3-chloroacrylic acid. Both isomeric 3-chloroacrylic acid intermediates are converted to malonic acid semialdehyde, but by two different dehalogenating enzymes, one specific for cis-3-chloroacrylic acid and the other specific for trans-3-chloroacrylic acid. Malonic acid semialdehyde is further degraded via acetaldehyde, which enters the central metabolic pathways.

The haloalkane dehalogenase that catalyzes the first step in the degradation of DCP was purified to homogeneity (Chapter 2). Its biochemical characteristics and N-terminal amino acid sequence were indistinguishable from those of the haloalkane dehalogenase (DhaA) that was found thusfar only in gram-positive bacteria such as R. erythropolis NCIMB13064 (see above). PCR amplification of the haloalkane dehalogenase gene by using primers based on the expected sequence similarity to the dhaA gene, followed by DNA sequencing, revealed a sequence identical to that of dhaA. The presence and role of the dhaA...
gene in the gram-negative strain 170 were confirmed by the isolation and characterization of haloalkane dehalogenase-negative mutants (Chapter 2).

From a detailed comparison of the genetic environment of the $dhaA$ genes in $P. pavonaceae$ 170 and $R. erythropolis$ NCIMB13064 (Fig. 2), it appears that the $dhaA$ gene of strain 170 originated from a haloalkane catabolic gene cluster that is widespread among gram-positive bacteria (Chapter 4). Most likely, a DNA segment containing the $dhaA$ gene was horizontally transferred to strain 170. During or after this process, two deletions occurred within the recruited DNA segment (Fig. 2). One of these deletions includes the DNA sequence between a 13-bp directed repeat upstream of the $dhaA$ gene, as well as one of the repeated sequences itself. The formation of this deletion may be explained by DNA strand slippage, which allows one repeated sequence to mispair with the complement of the other. The second deletion includes the genes for the alcohol dehydrogenase ($adhA$) and aldehyde dehydrogenase ($aldA$). Since $P. pavonaceae$ 170 contains two other dehydrogenases that are involved in oxidation of 3-chloroallyl alcohol to 3-chloroacrylic acid, retention of the dehydrogenase genes in the acquired genetic element should not have been required.

From these analyses, it seems likely that horizontal transfer of a genetic element containing the $dhaA$ gene from a gram-positive to a gram-negative bacterium played a role in the recent evolution of a gram-negative strain adapted to degrade DCP. The possibility that the 1-chlorobutane-catabolic gene cluster evolved in an (unknown) gram-negative species and subsequently became widely distributed among gram-positive bacteria, and was also transferred to $P. pavonaceae$ 170, must be considered as well. DCP is lethal to a strain of $P. pavonaceae$ 170 which has lost the haloalkane dehalogenase gene (G. J. Poelarends and D. B. Janssen, unpublished results). Thus, only the complete pathway allows $P. pavonaceae$ 170 to grow in the presence of DCP.

**CONSIDERATIONS OF THE EVOLUTIONARY ORIGIN OF TRANS-3-CHLOROACRYLIC ACID DEHALOGENASE**

*Trans*-3-chloroacrylic acid dehalogenase (CaaD) is the second dehalogenating enzyme in the DCP degradation pathway in $P. pavonaceae$ 170 (Fig. 1B). It catalyzes the hydrolytic dehalogenation of *trans*-3-chloroacrylic acid to yield malonic acid semialdehyde. Mature CaaD was found to be a hexamerix protein of 50 kDa that consists of three $\alpha$-subunits of 75 amino acid residues and three $\beta$-subunits of 70 residues (CaaD is postulated to be a trimer of $\alpha\beta$-dimers) (Chapter 6). A homologue of CaaD has been found in the 3-chloroacrylic acid-degrading coryneform bacterial strain FG41 (27). So far, no natural substrate for CaaD has been identified, and hydrolytic dehalogenation of *trans*-3-chloroacrylic acid and *trans*-3-bromoacrylic acid is the only known activity for the enzyme. The precursor of *trans*-3-
chloroacrylic acid in *P. pavonaceae* 170, *trans*-DCP, is not known to be formed naturally. The industrial production of *trans*-DCP started in the 1950s (29). Therefore it is reasonable to propose that CaaD arose from an existing enzyme, catalyzing the conversion of some other substrate in soil bacteria, with a structural scaffold and catalytic groups that allow adaptation to *trans*-3-chloroacrylic acid. It is conceivable that such an enzyme happened to have some level of dehalogenase activity in the initial encounter with *trans*-3-chloroacrylic acid.

In an attempt to find the origin of CaaD by homology analysis, we discovered that CaaD has low but significant sequence identity to 4-oxalocrotonate tautomeras (4-OTs) (*Chapter 6*). 4-OTs catalyze the isomerization of 2-oxo-4-hexene-1,6-dioate to 2-oxo-3-hexene-1,6-dioate during the catabolism of catechol to metabolites in the Krebs cycle (3, 24). They are also hexameric proteins consisting of small subunits (62 to 79 amino acid residues), but, in contrast to CaaD, they are composed of identical subunits (4-OT is a trimer of homodimers). Certain aspects of the proposed mechanism of the CaaD-catalyzed reaction (*Chapter 6*) bear a strong resemblance to the mechanism of 4-OTs (Fig. 3). Both 4-OT and CaaD utilize their amino-terminal proline (Pro-1) as proton acceptor/donor, and in both proteins Arg-11 plays an important role in substrate binding. Consistent with the location of Pro-1 and Arg-11 on adjacent subunits within the same homodimer of the 4-OT molecule, the catalytically important proline of CaaD is located on the β-subunit, whereas Arg-11 is located on the α-subunit, showing that residues from both subunits contribute to the dehalogenase active site. The role of these active site residues in CaaD has been validated by site-directed mutagenesis; the substitutions βP1A/G and αR11A resulted in inactive enzymes, whereas the substitution αP1A/G had no significant influence on dehalogenase activity (*Chapter 6*). Although CaaD has a low overall identity to the known 4-OTs, the active site region around Arg-11 is highly conserved (Fig. 4A).

On the basis of these structural and mechanistic similarities, we suggest that CaaD shares a common ancestry with 4-OTs. However, CaaD does not share high sequence identity to the known 4-OTs, indicating that the time of branching from a common ancestor occurred long ago. Thus, the recent relative(s) from which CaaD evolved in response to the introduction of DCP into the environment is not known. It should be noted, however, that prior to the introduction of *trans*-DCP into the environment by human activities, *trans*-3-chloroacrylic acid (or a structurally related compound) might have been formed from a natural product, for example a chlorinated aromatic compound. Consequently, the possibility that CaaD arose from a dehalogenase that is involved in the conversion of such a naturally produced organohalogen should be considered as well.
Figure 3. A comparative reaction scheme for 4-oxalocrotonate tautomases and trans-3-chloroacrylic acid dehalogenase. Primed residues come from other subunits. (A) The reaction catalyzed by 4-OTs (modified from reference 10). (B) The reaction catalyzed by CaaD. The order of protonation at C-2 and Cl⁻ departure is uncertain, so two possible reaction schemes are shown: one involving the formation of the intermediate 3-chloro-3-hydroxypropanoic acid (route 1) and the other involving the formation of a carbanion intermediate (route 2).

EVOLUTION OF THE METABOLIC PATHWAY FOR DBE DEGRADATION: A NOVEL COMBINATION OF TWO EXISTING DEHALOGENASE GENES

DBE is an effective nematocidic agent of which more than 20 million pounds were used for soil fumigation in the United States before it was banned in 1983 by the U.S. Environmental Protection Agency because of its carcinogenic properties and widespread occurrence in groundwater supplies (25). Many years after its last application, residual DBE can still be found in soil because it strongly interacts with the soil matrix and it is highly resistant to microbial degradation (22, 31). DBE can slowly leach from such contaminated soils to groundwater over extremely long periods, and due to its slow chemical conversion in aqueous milieu, it forms a continuing threat for groundwater quality (22).

Many attempts to obtain pure cultures of bacteria that can degrade DBE have been unsuccessful, although the structural analog 1,2-dichloroethane (DCE) can be utilized by various bacteria of the genera Xanthobacter and Ancylobacter (13). These bacteria degrade DCE by hydrolytic conversion to 2-chloroethanol, which is oxidized via chloroacetaldehyde to chloroacetate and finally hydrolyzed to glycolic acid. DCE-degrading bacteria thus seem to
possess all the enzymes necessary to degrade DBE, but they are not able to utilize DBE because both DBE and the expected intermediate 2-bromoethanol are toxic at concentrations in the µM range. The presence of an active alcohol dehydrogenase in the absence of a functional aldehyde dehydrogenase, which causes the accumulation of the highly reactive bromoacetaldehyde, seems to be the cause of this toxicity (28).

The first report concerning microbial utilization of 1,2-dibromoethane as a carbon source was published in 1996 (8). In this report, Freitas dos Santos and coworkers described the enrichment of a mixed bacterial culture capable of slow aerobic mineralization of DBE. We succeeded in isolating from this consortium a pure bacterial culture (Mycobacterium sp. strain GP1) that can aerobically utilize DBE as the sole carbon and energy source (Chapter 3). It was obtained by prolonged adaptation and selection in batch culture. Metabolism of DBE by strain GP1 was initially expected to proceed via a modified DCE-degradation route, in which the aldehyde dehydrogenase should be overexpressed or adapted so that no accumulation of the toxic intermediate bromoacetaldehyde would occur. Surprisingly, strain GP1 utilizes another pathway in which DBE is metabolized via the sequential action of a hydrolytic haloalkane dehalogenase and a haloalcohol dehalogenase (Fig. 1C). The latter enzyme was found to be highly active towards 2-bromoethanol and rapidly converted this intermediate to ethylene oxide. In this way, the organism circumvents the formation of the toxic intermediate bromoacetaldehyde, which was lethal when DCE-degrading bacteria were exposed to DBE. Although ethylene oxide is toxic as well, pathways for its degradation have been described (7). Complete metabolism of DBE thus employs two dehalogenating enzymes to prevent the formation of toxic brominated intermediates.

The genomic region of strain GP1 that encodes the dehalogenases has been cloned and sequenced (Fig. 5) (Chapters 3 and 4; Van Hylckama Vlieg et al., unpublished data).
Information that might indicate the genetic origin of the haloalkane dehalogenase (\textit{dhaA}$_{f}$) and haloalcohol dehalogenase (\textit{hheB}) genes comes from a comparison with the sequence of the dehalogenase gene regions of \textit{R. erythropolis} NCIMB13064 (see above) and \textit{Corynebacterium} sp. strain N-1074 (34). Strain GP1 harbors a genetic element, containing \textit{dhaR}, \textit{dhaA}, and part of \textit{invA}, that is highly similar to a segment of the 1-chlorobutane catabolic gene cluster found in gram-positive bacteria such as strain NCIMB13064 (Fig. 2). The \textit{hheB} gene and its flanking regions in strain GP1 are highly similar to the \textit{hheB} gene region of the haloalcohol-degrading strain N-1074. From these findings, it appears that in strain GP1 two existing genetic regions for dehalogenation are combined, one containing the \textit{dhaA} gene (segment A in Fig. 5) and the other containing the \textit{hheB} gene (segment B in Fig. 5). The combination of these two dehalogenase genes in a single organism resulted in a productive pathway for DBE degradation. Since strain GP1 was isolated by prolonged adaptation of a mixed batch enrichment culture (\textit{Chapter 3}), it is likely that recent genetic recombination events led to the formation of this novel dehalogenase gene cluster.

\textbf{Figure 5.} Genomic region of \textit{Mycobacterium} sp. strain GP1 encoding the haloalkane (DhaA$_{f}$) and haloalcohol (HheB) dehalogenases. Segment A is identical to a segment of the haloalkane catabolic gene cluster found in gram-positive bacteria such as \textit{R. erythropolis} NCIMB13064, except for three nucleotide substitutions in \textit{dhaA} and a 12-nucleotide deletion in \textit{dhaR}. Segment B is identical to a segment of the haloalcohol dehalogenase gene region in \textit{Corynebacterium} sp. strain N-1074, except for four nucleotide substitutions in \textit{hheB}. The 42-nucleotide extension of the \textit{dhaA} ORF, which is the result of a fusion between the acquired genetic element and HheB encoding DNA, is indicated by an open box. The function of the putative ORF1 and ORF2 proteins is not known. The \textit{intM} gene encodes a putative (site-specific) DNA integrase.

At least two other genetic alterations appear to have occurred in the dehalogenase region of the GP1 genome. First, we found that the \textit{dhaA} gene is fused in frame to a segment of the \textit{hheB} gene, resulting in an extension of the \textit{dhaA} open reading frame (ORF) with 42 nucleotides (i.e. 14 amino acids at the protein level) (Fig. 5). This new ORF was accordingly named \textit{dhaA}$_{f}$ (were f stands for fusion). Since this \textit{hheB} part of \textit{dhaA}$_{f}$ is identical to the 3’ end
of the intact *hheB* gene that is located approximately 2.6 kb downstream of *dhaAf* (see Fig. 5), it seems likely that a duplication event leading to two copies of part or the whole *hheB* gene occurred prior to acquisition and insertion of the *dhaA* gene. Nevertheless, it is surprising that the recruited *dhaA* gene became fused to the *hheB* gene, which normally codes for a haloalcohol dehalogenase that could be involved in the metabolism of DBE. The 14-amino-acid extension has no influence on the activity of DhaA, however, and may just reflect the recombination events that led to acquisition of a catabolic pathway. Second, a deletion of 12 nucleotides has occurred within the *dhaR* gene, which normally encodes a repressor (DhaR) for the *dhaA* gene. Although this deletion is in frame, it inactivated the DhaR protein, leading to constitutive expression of *dhaAf* (Chapter 4). It may have been a necessity for strain GP1 to inactivate this protein since the compound used for selection (DBE) is known not to be an inducer of *dhaA* expression.

**DO DNA INTEGRASES PLAY A ROLE IN THE ACQUISITION OF DEHALOGENASE GENES?**

Acquisition of foreign DNA by horizontal gene transfer requires integration into a replicon that is stable maintained in the recipient microorganism. Interestingly, both in *P. pavonaceae* 170 and in *Mycobacterium* sp. strain GP1 a gene encoding a putative site-specific recombinase (*intP* in strain 170 and *intM* in strain GP1) is present directly upstream of the recruited DNA segment harboring the haloalkane dehalogenase gene (Fig. 2 and 5). The *intP* and *intM* gene products share significant sequence similarity with members of the integrase (Int) family of site-specific recombinases, and both harbor the conserved, catalytically important, tetrad R-H-R-Y of the Int family (Chapter 4). These putative integrase proteins probably mediated the insertion of the dehalogenase genes into the genome, although their activity remains to be established experimentally. The finding of putative integrase genes next to the dehalogenase genes further reinforces our hypothesis that these DNA segments were acquired by horizontal transmission.

Integrase-mediated gene acquisition has previously been associated with an unique class of genetic elements, called integrons (23). Integrons harbor a gene for a site-specific DNA integrase, which can mediate the incorporation of one or more foreign genes in a specific site (the recombination or core site) directly upstream of the integrase gene. Thusfar, integrons have been implicated in the acquisition of antibiotic resistance genes by various bacterial species (23) and of virulence determinants by *Vibrio cholerae* (17). Integrons harboring antibiotic resistance genes may be mobilized after association with insertion elements, transposons, or conjugative plasmids. This fascinating class of genetic elements thus forms an extremely powerful mechanism for the acquisition and distribution of genes involved in adaptation or colonization of new environments.
The acquired DNA segments in *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1 lack the specific structural characteristics of previously identified integron-inserted gene cassettes, which consist of an imperfect inverted repeat located at the 3’ end of the inserted gene (called a 59-base element) and two core sites (GTTRRRY) located at the boundaries of each inserted gene cassette (9). Nevertheless, the localization of an integrase gene directly next to an apparently recently assembled genetic element suggests an integron-like structure. The lack of high sequence similarity of IntP and IntM with known integron encoded DNA integrases may explain why the structural features of the inserted gene cassettes are not conserved in strains 170 and GP1. Thus, it will be interesting to find out whether dehalogenase genes can be propagated via integron-like elements.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The isolation of pure bacterial cultures capable of utilizing and degrading the priority pollutants DCP and DBE is important for understanding their environmental fate and makes it possible to develop bioreactors for the removal of these compounds from contaminated groundwater. Moreover, these organisms provide nice examples of the different adaptation mechanisms that are involved in the generation of new catabolic pathways, i.e. horizontal transmission of degradative genes, insertion of these genes into the genome by means of an integration system, inactivation of a repressor of a catabolic gene, and the circumvention of the formation of toxic catabolic intermediates.

The data presented in this thesis clearly show that microorganisms have assembled new metabolic pathways by the recruitment and novel association of existing dehalogenating enzymes. Questions concerning the evolutionary origin of these enzymes (and of dehalogenases in general) and the adaptive mutations that may have occurred in the initial encounter with xenobiotic organohalogen s remain to be answered. This requires insight into all potential sources of DNA encoding (ancestral forms of) dehalogenases in the environment. We envisage the following sources: (i) Organohalogen-producing organisms. Such organisms may synthesize dehalogenating enzymes to protect themselves against the toxic effects of their metabolites. (ii) Organohalogen-degrading organisms. Organisms degrading naturally produced organohalogen s may possess the ancestral forms of the dehalogenases involved in the degradation of synthetic organohalogen s. Candidates are organisms degrading abundant natural organohalogen s such as haloaromatics and their degradation products. (iii) Organisms-degrading non-halogenated structural analogs of synthetic organohalogen s. Such organisms may possess enzymes that were recruited to serve as dehalogenases in the biodegradation of xenobiotics. Insight into the evolutionary origins of dehalogenases involved in the conversion
of xenobiotics thus awaits the isolation and characterization of dehalogenase genes from these potential sources.

REFERENCES


