The first step in the utilization of the xenobiotic chlorinated hydrocarbon 1,2-dichloroethane by *Xanthobacter autotrophicus* is catalyzed by haloalkane dehalogenase (DhlA). The enzyme hydrolyses 1-haloalkanes to the corresponding alcohols. This allows the organism to grow also on short-chain (C₂-C₆) 1-chloro-n-alkanes. We have expressed DhlA in a strain of *Pseudomonas* that grows on long-chain alcohols and have selected 12 independent mutants that utilize 1-chloroethane. Six different mutant enzymes with improved *Kₘ* or *Vₘₐₓ* values with 1-chlorohexane were obtained. The sequences of the mutated *dhlA* genes showed that several mutants had the same 11-amino acid deletion, two mutants carried a different point mutation, and three mutants had different tandem repeats. All mutations occurred in a region encoding the N-terminal part of the cap domain of DhlA, and it is concluded that this part of the protein is involved in the evolution of activity toward xenobiotic substrates.

Some bacterial strains of the species *Xanthobacter autotrophicus* (1, 2) and *Ancylobacter aquaticus* (3) are capable of growing on the synthetic chlorinated hydrocarbon 1,2-dichloroethane. Degradation proceeds via 2-chloroethanol, chloroacet-aldehyde, and chloroacetic acid to glycolate (1). Haloalkane dehalogenase (DhlA), a 310-amino acid cytoplasmic protein, catalyzes the first hydrolytic reaction step. The gene has been cloned and sequenced (4), and identical dehalogenases were detected in different strains that grow on 1,2-dichloroethane (3).

The three-dimensional structure of DhlA was recently solved by x-ray crystallography (5, 6). DhlA is composed of a globular main domain with an α/β-hydrolase fold structure and a separate cap domain (5–7). The active site cavity is located between the two domains. X-ray crystallographic studies indicated that the enzyme catalyzes cleavage of halogenated compounds by nucleophilic displacement of the halogen by Asp¹⁴⁹ (8). It was shown that during incubations of DhlA with substrate in H₂¹⁸O incorporation of *¹⁸O* occurred in Asp¹⁴⁹ and product (9). This indicates that the covalent allyl-enzyme intermediate, formed by nucleophilic displacement of the halogen by Asp¹⁴⁹, is hydrolyzed by nucleophilic attack of a water molecule on the carbonyl carbon atom. His²⁸⁹ probably activates this water molecule by substituting a proton, with assistance of Asp³⁰⁰ (8). Two tryptophans, Trp¹⁴² and Trp¹⁷⁸, are involved in substrate and halide binding (8, 10).

The α/β-hydrolase fold domain of DhlA is structurally conserved fold shared by several hydrolytic proteins (7). The overall topology of DhlA consists of an eight-stranded β-sheet, with connecting α-helices. The nucleophilic residues, either Ser, Cys, or Asp, are positioned on a sharp bend between the fifth β-strand and the following α-helix. The positions of the other two active site residues, His²⁸⁹ and Asp³⁰⁰, are conserved as well. The proteins differ by the presence, position, and sequences of cap domains, and therefore this part of the proteins was suggested to influence substrate specificity (7).

Haloalkane dehalogenase is active with several 1-chloro-n-alkanes and α,ω-dichloro-n-alkanes, but there is hardly any activity with 1-chlorohexane or chloroalkanes of longer chain length (1). The enzyme has some activity with long-chain branched alkanes (C₆–C₁₆), suggesting that the active site can accommodate longer substrates and that differences in binding affinities or reaction rates are responsible for the lower activity with long-chain chloroalkanes.

Since 1,2-dichloroethane is not known to occur or to be produced naturally, it is likely that an enzyme capable of hydrolyzing this compound evolved to its present form after industrial 1,2-dichloroethane production and emission started in 1922 (11, 12). No sequences that are similar to DhlA and from which the present enzyme could be derived are known, however. We decided to investigate how DhlA can evolve to convert new substrates by selecting spontaneous mutants that degrade 1-chlorohexane, an approach termed experimental enzyme evolution (13–15). The sequences and activity of the mutant enzymes that were obtained indicate that short direct repeats in the N-terminal part of the cap domain play an important role in the evolution of haloalkane dehalogenase specificity.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—*Pseudomonas* GJ31 is a chlorobenzene utilizing organism described earlier (4, 16). *Escherichia coli* JM101 was used as host strain for isolation of single strand DNA and sequencing. Plasmid pJF190 is a broad host range plasmid (4) derived from pLAFR1 (17). It contains the *dhlA* gene with its own promoter and constitutively produces DhlA in several Gram-negative bacteria (4).

**Selection of Mutants**—Selection of spontaneous mutant enzymes was achieved with the recombinant bacterial strain *Pseudomonas* GJ31(pJF20). Pseudomonas GJ31 is capable of growing on long-chain alcohols. The plasmid was introduced by triparental mating (17) and allowed the organism to grow on 1-chlorobutane by expression of the *dhlA* gene up to levels of 25% of the total cellular protein (4). Mutants of *Pseudomonas* GJ31(pJF20) that could utilize 1-chlorobutane as a growth substrate were selected (see Fig. 1) at 22°C by cultivation in 1-liter serum flasks containing 400 ml of synthetic medium (1). The medium contained 2 mM 1-chlorobutane, which did support growth of the recombinant, and 2 mM 1-chlorohexane, which is hardly hydrolyzed by the wild type enzyme and did not support growth. After 4 weeks of incubation, some cultures showed higher turbidities than a control containing no 1-chlorohexane. After three serial transfers in medium containing 2 mM 1-chlorohexane as the sole carbon source, mutants were purified on nutrient broth agar plates containing tetracycline (12 μg/ml).

**Sequencing**—The *dhlA* gene of mutant V152–153 was determined by cloning AluI–HindIII and Sau3A DNA fragments in phage M13mp18.
and M13mp19 (18), followed by dyeoxy sequencing (19). The other mutant dhlA genes were amplified by the polymerase chain reaction (20), followed by dyeoxy sequencing of the double-stranded polymerase chain reaction DNA. The primers used for sequencing are as follows (listed from 3' to 5' with the starting nucleotide number (4) given and c and n indicating coding and non-coding strand, respectively): 989 ATGATAAATGCAATTCGC (c), 1193 ACCGCAACTCTC TTCCTGCG (c), 1590 CTGACCGAAGGTACGGG (c), 1928 TTTTCTGGCCGCAAAG-TG (n), 1628 GAAAGGCGCAGATACGC (n), 1322 TGCAAGCGAAGTTCCG (n).

**Purification of Haloalkane Dehalogenase—Mutants of Pseudomonas GJ31(pPJ2O)** were grown at 30 °C in a mineral medium (1) containing 4 mM 1-chlorohexane as the sole carbon source. After 4 days, cells were harvested by centrifugation, washed in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol, and sonicated after resuspending in this buffer. Mutant haloalkane dehalogenases were purified as described before (1).

**Determination of Dehalogenase Activities**—The activities of the mutant enzymes were tested with a variety of substrates by spectrophotometric measurement of the rates of halide production (1) using purified enzymes (wild type and mutants P168S, Δ164-174, and V152-153) or crude extracts (mutants D170H, V172-174, and V145-154). The concentration of 1-chlorohexane in the assays was 3 mM, the other substrates were used at 5 mM. The dehalogenase content of cell-free extracts was determined by Coomassie Brilliant Blue staining of the protein bands of SDS-polyacrylamide gels, and subsequent peak integration of scants of the gels was taken with a desktop scanner. Accuracy was better than 10%.

For the determination of $K_m$, $V_{max}$, and $k_{cat}/K_m$, alcohol production was measured in 4.5-ml incubations containing 0.01-4 mM substrate in 50 mM Tris-HCl buffer, pH 8.2, and 10 milliliters of 1.2-dibromoethane dehalogenase or 100 milliliters of 1-chlorohexane or 1,2-dichloroethane dehalogenase. Samples were incubated for 15 min at 30 °C, and the amount of alcohol produced was determined on a Chrompack 438s gas chromatograph with a CP Wax 52 CB column, using an ECD detector for 2-bromoethanol and a FID detector for 2-bromoethanols and 1-hexanol. The carrier gas was nitrogen (60 KPa), and the temperature program was 3-min isothermal at 45 °C followed by an increase to 200 °C at 10 °C/min. $K_m$ and $V_{max}$ values were calculated from the rates of alcohol production by nonlinear regression analysis using the Michaelis-Menten equation and the Enzfitter program of Leatherbarrow (21).

**RESULTS**

**Isolation of 1-Chlorohexane-utilizing Mutants**—The dehalogenase-expressing strain Pseudomonas GJ31(pPJ2O) was found to be capable of growth on 1-chlorobutane, but not on 1-chlorohexane. The specific activity of purified haloalkane dehalogenase with 1-chlorobutane as sole carbon source were easily obtained with cell-free extracts that contained a mixture of 1-chlorobutane and 1-chlorohexane as sole carbon sources (Fig. 1).

Twelve independent mutants were purified and analyzed for the presence of mutations in the dhlA gene by DNA sequencing (Table I) and determining dehalogenase activities in crude extracts (Table II).

**Sequences of Mutated Dehalogenases**—From sequencing the dhlA genes of the 1-chlorohexane utilizing mutants, six different mutants were identified. Only two mutants carried a single base substitution, leading to the mutations Asp170→ His, and Pro168→ Ser. The other mutants had much larger changes, being deletions or insertions. A large in frame deletion of 33 bp, causing an 11-amino acid deletion of Phe164-Ala174, was detected in six independently selected mutants. The duplications that were found were in frame direct repeats, varying in length from 6 to 30 bp. The 9-bp duplication leading to a tandem repeat of Met152-Thr153 was found twice. The other duplications were detected only once, and caused tandem repeats of Phe172-Ala174 and Ile145→ Asp146, respectively. The 9-bp duplication in mutant V172-174 encompassed the last 9 bp of the 33-bp deletion in mutant Δ164-174 (see also Fig. 2).

The deletions and duplications caused slight changes in electrophoretic mobility of some mutant dehalogenases on SDS-polyacrylamide gels (Fig. 3). The dehalogenase carrying the 11-amino acid deletion clearly had a higher mobility, whereas mutants V172-174 and V145-154 produced dehalogenases that migrated somewhat slower than the wild type.

No plasmids could be isolated from the D170H and V145-154 mutants suggesting that the plasmid had integrated into the chromosome of Pseudomonas GJ31. These mutants still produced haloalkane dehalogenase, but the D170H mutant expressed the enzyme at a lower level than the wild type and the other mutants (Fig. 3).

**TABLE I**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutation</th>
<th>No. of mutants found</th>
</tr>
</thead>
<tbody>
<tr>
<td>D170H</td>
<td>G→C substitution (Asp170→His)</td>
<td>1</td>
</tr>
<tr>
<td>P168S</td>
<td>C→T substitution (Pro168→Ser)</td>
<td>1</td>
</tr>
<tr>
<td>Δ164-174</td>
<td>33 bp deletion (Phe164-Ala174)</td>
<td>6</td>
</tr>
<tr>
<td>V172-174</td>
<td>9 bp tandem duplication (Phe172-Ala174)</td>
<td>1</td>
</tr>
<tr>
<td>V145-154</td>
<td>6 bp tandem duplication (Met152-Thr153)</td>
<td>2</td>
</tr>
<tr>
<td>V145-154</td>
<td>30 bp tandem duplication (Ile145→Asp146)</td>
<td>1</td>
</tr>
</tbody>
</table>

**Activities of In Vivo Mutants**—All mutants produced a dehalogenase with increased activity toward 1-chlorohexane and other C4 and C3 1-chloro-n-alkanes (Table II). With all mutant enzymes, both the $K_m$ and $V_{max}$ values for 1-chlorohexane had improved, whereas these values were worse for 1,2-dichloroethane. The $K_m$ value for 1,2-dibromoethane, which is the best substrate for the wild type enzyme, was also higher with all mutant enzymes (Table III). The individual mutants showed varying activities with several other substrates of the enzyme.

Mutant P168S had the highest $V_{max}$ for 1-chlorohexane and 1,2-dibromoethane whereas the $K_m$ for 1-chlorohexane was only slightly improved. The Δ164-174 deletion enzyme had lower activities than the wild type for all chlorinated compounds mentioned in Table II, except 1-chlorohexane, 1-chloropentane, and 1,6-dichlorohexane. The duplication mutant enzyme V172-174, which has changes in the same part of the protein as the Δ164-174 deletion, had similar activities, except that the specificity constant $k_{cat}/K_m$ for 1-chlorohexane was only slightly improved and the activity for 1,2-dichloroethane was less reduced.

The other two duplication mutant enzymes had the largest increase of the specificity constant for 1-chlorohexane, about 20 fold. Both $V_{max}$ and $K_m$ values were improved. They had a broad activity with long-chain (C7-C8) chloroalkanes. Summarizing, the general substrate range of the mutant enzymes thus had shifted from short chain substrates to 1-chloro-n-alkanes of longer chain length. This modification of specificity was not
Spontaneous Cap Domain Mutations in DhlA

The part of the gene that encodes the N-terminal region of the cap domain is shown, together with the amino acid sequence. Residues forming the active site cavity are encircled. The boxes above the protein sequence indicate the position of strand 6 of the β-sheet and α-helices 4 and 5. The large solid arrows mark the 15-bp direct repeat. One copy of the repeat with flanking nucleotides is deleted in the Δ164-174 mutant enzymes (dashed box), the formation of which is explained by misalignment of the 9-bp imperfect direct repeat at the ends of the deleted sequence (open arrows). Of this 9-bp repeat, the right-hand copy is again duplicated in mutant V172-174 (closed box).

The other repeats detected in the mutants are located in the N-terminal part of the sequence encoding the cap domain (closed boxes). The boxes printed in bold characters mark the position of the mutations in D170H and P168S, causing the substitutions indicated.

observed with bromoalkanes, as illustrated by the high activity of both the wild type and the mutant enzymes with 1,2-dibromoethane (Table III).

Location of Mutations—Attempts to crystallize purified mutant enzymes have failed so far, probably due to disturbance of the crystal contacts of the DhlA molecule at Glu167 and Ala169 by the mutations (6). Examination of the amino acids that are changed using the three-dimensional structure of the wild type enzyme (5) showed that none of the mutations directly affects active site residues, with the exception of the 11-amino acid deletion, in which the active site cavity forming residues Phe164 and Phe172 are lost. The other mutations thus have indirect effects.

The mutations were all located in a segment of the dhzA gene that encodes the N-terminal part of the cap domain (Fig. 2). In the mutant enzymes D170H, P168S, Δ164-174, and V172-174, the changes affect the structurally important salt bridge Asp170-Lys261 (Fig. 4). Although the Asp170 O6 is 15.6 Å away from the active site Asp139 O6, the loss of the Asp170-Lys261 salt bridge appears to change the active site cavity in such a way that improved 1-chlorohexane conversion is observed. Probably, the active site cavity residues Phe164 and Phe172 are more mobile or have a slightly different position in the mutants (Fig. 4). In the P168S enzyme, the proline which is located in a cis proline turn between helices 4 and 5, is substituted. This allows rotation around the Cα-N bond of residue 168 and a change of the cis bond in a trans bond (22), which could lead to disturbance of the Asp170-Lys261 salt bridge that is in its proximity. In the V172-174 mutant enzyme, Phe172 is preceded by an Ala instead of Gly172, which has unusual ψφ-angles for Ala in the structure of wild type DhlA and forms the N-cap of helix 5, where Ala is less favored (23, 24). This will affect the cavity geometry via Phe172 or via the salt bridge by increasing the size of the cis proline turn. In the Δ164-174 mutant enzyme, Phe164 and Phe172, which point to the cavity, are deleted, as is the salt bridge residue Asp170. In the other two mutant enzymes, V152-153 and V145-154, cavity size may be influenced, but this is less clear since the amino acids surrounding the duplication are not in contact with the active site.

DISCUSSION

Structure-Activity Relationships of Mutant Enzymes—The mutants of haloalkane dehalogenase described here show that the cap domain of DhlA acts as an activity-modifying domain that can accommodate spontaneous mutations which can be selected in vivo and lead to alterations of substrate specificity. Since all mutants showed improved kinetics for 1-chlorohexane, the substrate binding site must have been altered in these enzymes such that improved binding and/or conversion is achieved. It is known that two tryptophans in binding of the halidi moiety of the substrate (10), but other residues are probably responsible for determining the substrate specificity. Candidates for residues that interact with the R-group of the substrate are Phe156, Phe164, Phe172, Phe222, Val223, Leu261, and Leu263 (5, 6). All mutations that influence substrate range were located in the part of the cap domain that is N-terminal of Trp154. This part is mainly stabilized by a surface located salt bridge between Asp172 and Lys261 of the main domain (Fig. 4) and hydrophobic interactions of 4 buried residues (Phe164, Phe164, Phe172, and Gly172). Since buried resi-
Spontaneous Cap Domain Mutations in DhlA

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FIG. 3. SDS-polyacrylamide gel electrophoresis of crude extracts from Pseudomonas GJ31(pJP20) and its 1-chlorohexane utilizing mutants. See Table I1 for properties of the mutants. The arrow indicates the position of the haloalkane dehalogenase protein. Note the differences in mobility of mutant proteins carrying the deletion and duplications. The different lanes indicate crude extracts from: 1, 2, and 3, three independent mutants Δ164-174; 4 and 5, two independent mutants V165-16; 6, mutant P168S; 7, mutant V172-174; 8, mutant V145-154; 9, mutant D170H; 10, purified wild type haloalkane dehalogenase.

Table III

Kinetic constants of dehalogenase mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>1,2-Dibromoethane</th>
<th>1-Chlorohexane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>units/mg</td>
<td>mM</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.60</td>
<td>0.0072</td>
</tr>
<tr>
<td>D170H</td>
<td>4.4</td>
<td>0.087</td>
</tr>
<tr>
<td>P168S</td>
<td>8.1</td>
<td>0.031</td>
</tr>
<tr>
<td>Δ164-174</td>
<td>4.06</td>
<td>0.092</td>
</tr>
<tr>
<td>V172-174</td>
<td>4.5</td>
<td>0.18</td>
</tr>
<tr>
<td>V152-153</td>
<td>6.4</td>
<td>0.51</td>
</tr>
<tr>
<td>V145-154</td>
<td>6.4</td>
<td>0.51</td>
</tr>
</tbody>
</table>

dues serve as structural anchors and strongly resist translocation (25) and because of the low number of hydrogen bonds and salt bridges, structural changes will be easily accommodated by the N-terminal part of the cap domain. If the mutations influence the Asp<sup>159</sup>-Lys<sup>166</sup> salt bridge, as indicated by examination of the position and role of the residues influenced by the mutations, then this part of the cap domain could have undergone significant changes that allow improved binding or conversion of long chain chloroalkanes.

The mutations all influence the binding and/or reaction rate of various substrates (Tables II and III). The higher V<sub>max</sub> for 1-chlorohexane shows that the reaction of bound substrate is faster in the mutants. Since this effect is observed with chlorinated but not with brominated substrates, formation of the covalent alkyl-enzyme intermediate must be the rate-limiting step in the wild type that is accelerated in the mutants. This may be caused by a better average positioning of the Cα of the bound substrate for nucleophilic attack by Asp<sup>159</sup> and stabilization of the reaction intermediate. The lower K<sub>m</sub> could indicate that substrate binding is improved in the mutants, but it can also be explained by a higher rate of formation of the covalent intermediate. How 1-chlorohexane is bound is unknown, but a larger or more flexible hydrophobic cavity that increases the binding energy of the larger substrate is likely. The wild type enzyme also must have considerable flexibility to allow substrate binding, since the active site cavity (5, 6) is too small for binding 1-bromohexane, which is a substrate for wild type haloalkane dehalogenase. Furthermore, the cavity is buried which requires some cap domain residues to move for substrate entrance. Large substrate-induced structural changes are observed in the mobile surface loops of lipase of Rhizomucor miehei (26), human pancreatic lipase (27), and lipoprotein lipase (28, 29), which are also α/β-hydrolase fold excursions (7).

Mechanism of in Vivo Mutagenesis—Of the six mutations found, only two appeared to be substitutions. The high frequency with which the 11-aminoc acid deletion mutant was found (6 out of 12 mutants) can be explained by a strand slippage mechanism involving the 9-bp imperfect repeat that flanks in the wild type sequence the segment that is deleted in the Δ164-174 mutant. This can be followed by excision during mismatch repair or replication (Figs. 2 and 5). Such a process has been observed in β-galactosidase of Lactobacillus bulgaricus (15) and bacteriophage T4 (30) and was also proposed for the evolutionary divergence of benzoate dioxygenases (31). A role for replication was suggested (32, 33). Plasmid pJP20 is derived from plasmid RK2, which has an unidirectional mode of replication (34). The orientation of the dhlA gene in pJP20 is such that the direction of transcription is the same as the direction of replication (4). All deletion mutants still contained the proximal copy of the 9-bp imperfect repeat encoding FSA, not FTA. This means that if the strand slippage occurred during leading strand synthesis (Fig. 5A), excision and mismatch repair occurred only in the template strand, but if strand slippage occurred during lagging strand synthesis (Fig. 5B), excision and mismatch repair took place in both strands, which seems less likely.

The occurrence of three different mutations that are direct tandem repeats of varying length, rather than insertions of random sequence, suggests a specific mechanism for their origin. Formation of direct repeats has been found to occur at sites flanked by short repeats that create DNA structures that are easily misaligned, recombined and extended (30, 35, 36). The tandem repeats in the mutated dhlA genes are not flanked by repeated sequences, however, indicating that specific sequences are not required for their generation. The results suggest that an aspecific stuttering type of process can lead to the formation of short repeats, irrespective of existing repetitions.

Evolution of Haloalkane Dehalogenase—1,2-Dichloroethane is a synthetic compound. Although traces of it may be formed by haloperoxidases (37), it is unlikely that sufficient selective pressure to generate a dehalogenase for this chlorinated hydrocarbon existed until its industrial production and emission started in 1922. DhlA could have adapted to 1,2-dichloroethane by similar modifications of the cap domain as found here in the spontaneous mutants. Our results present experimental evidence that the cap domain, which is an excursion on the α/β-hydrolase fold structure (7), is involved in the evolutionary modification of specificity, and that generation of repeats is an important mutational event during this process. The DNA coding for the N-terminal part of the cap domain of the wild type enzyme already contains two in frame direct repeats: a 15 bp perfect direct repeat encoding the sequence Val-Thr-Gln-Pro and Phe-Thr-Ala, respectively (Fig. 2). The two large repeats are in the same reading frame, but they are not tandem. It is highly unlikely that the repeats are required for the optimal catalytic performance of the enzyme. The direct repeats may well have been generated from an older dehalogenase during a
process similar to the selection of the mutations that arose during cultivation on 1-chloroethane, with a few additional fine tuning modifications leading to the present wild type DhlA. Thus, we hypothesize that the direct repeats in the DNA sequence encoding the N-terminal part of the cap domain are of recent evolutionary origin and were selected during the adaptation of an older dehalogenating enzyme to industrially produced 1,2-dichloroethane.

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REFERENCES


